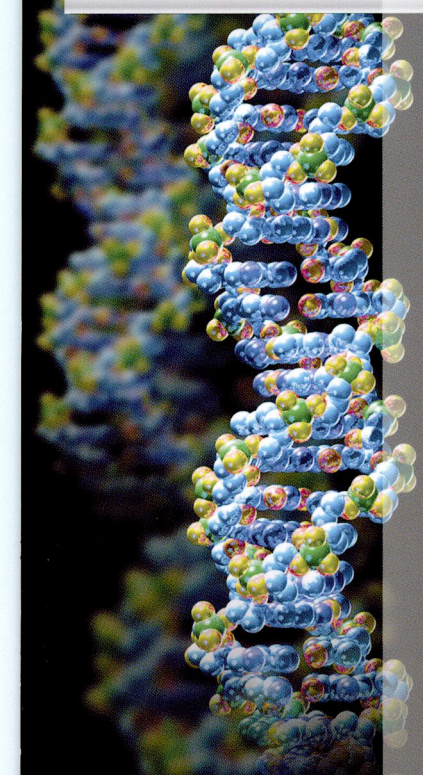
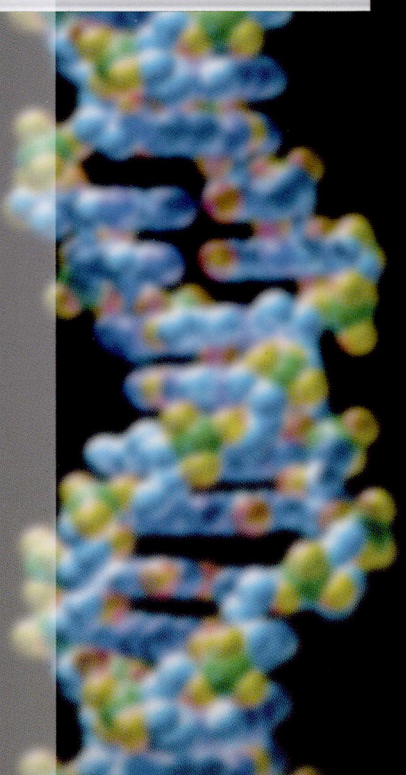
The 40th Anniversary of Hiroshima University Faculty of Dentistry

Hiroshima Conference on Education and Science in Dentistry, 2006





Hiroshima University Faculty of Dentistry

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The 40th Anniversary of Hiroshima University Faculty of Dentistry

Hiroshima Conference on Education and Science in Dentistry, 2006

Proceedings of Hiroshima Conference on Education and Science in Dentistry, 2006
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Hiroshima University Faculty of Dentistry

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PREFACE

"Hiroshima Conference on Education and Science in Dentistry" is a clear and strong intentional display that Hiroshima University Faculty of Dentistry's goal is to become the worldwide research and education center in Dentistry.

Though the education is originally social contribution itself, it is generally difficult to find the effects of the education as contribution to the society in the short term. It seems to constitute the dental education by two parts: innovation and foundation. The foundational part guarantees the educational quality, namely the medical quality in the dentistry education, and the innovative part contributes to the advance of dentistry. The innovative part becomes established knowledge and technology in the long term, and it shifts to the foundational education and results in the improvement in the medical quality. That is to say, it is considered that the advanced research is indispensable to the advanced education.

In the development of the education, both competition and coordination are important. The foundational part in the education should be raised by the coordination among Universities in the world, and it can achieve the long-term and stable contribution for the general society. On the other hand, it is possible that the innovative part is improved by international competition. The competition must be carried out in the widely opened field, because excellent results can be expected for the competition, if it is done in the wider world.

We hope that the education session of "Hiroshima Conference on Education and Science in Dentistry, 2006" greatly contributes to the coordination of the foundational part of the dental education. The research session of the conference is a source of the innovation of the education. In the research session, the many excellent lectures are carried out by worldwide distinguished researchers. It will be a great pleasure, if young students and researchers, who will contribute to the future success, can have greater stimulation for creation of new forms of knowledge in the science session.

For the first time, we invited the deans and faculty members from the universities that have "the agreement of exchanging program" with Hiroshima University. The name of "Hiroshima Conference on Education and Science in Dentistry, 2006" was chosen to represent the cosmopolitan city "Hiroshima" which has contributed in world peace. Naming of the conference is the intentional display of Hiroshima University Faculty of Dentistry for pursuit of peace through education and research.

Since 1965, many faculty and administrative staff have supported the development of Hiroshima University Faculty of Dentistry. The logo of "Hiroshima Conference on Education and Science in Dentistry" has been designed with a motif of "Liquidamber styraciflua" a tree of Hiroshima University Faculty of Dentistry. 40 years ago, the predecessors of Hiroshima University Faculty of Dentistry planted "Liquidamber styraciflua" trees in front of the building of the faculty of dentistry and prayed for its future development. These "Liquidamber styraciflua" trees have observed the development of Hiroshima University Faculty of Dentistry and at present, they are the tallest trees in the Kasumi Campus (Medical Campus). We are deeply thankful for the enthusiastic thought of the predecessors who planted "Liquidamber styraciflua" trees and we have designed the symbol of the Hiroshima conference with a motif of "Liquidamber styraciflua". We sincerely appreciate all those who understood and cooperated with our activities. Also, personally, I appreciate the strong support of the faculty and administrative staff who encouraged me to organize the present international conference of the 40th anniversary commemoration.

We desire that young students and young faculty members deeply understand the significance of this "Hiroshima Conference on Education and Science in Dentistry" and contribute to the development of Hiroshima University Faculty of Dentistry by continuous self-development.

Thank you.

December 11, 2005.

栗原英見

Hidemi Kurihara
Dean
Hiroshima University Faculty of Dentistry

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Strategic Internationalization of Hiroshima University

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INTRODUCTION

I am happy to congratulate Faculty of Dentistry in Hiroshima University on the occation of its 40th anniversary. I am very honored to be here to give a talk on the strategy in the internationalization of Hiroshima University.

It is well recognized that university reforms are now going on almost everywhere in the world. This phenomenon is an outcome of the globalization of higher education and the world-wide competitive society which emerged after the end of the cold world war period. Now that competitiveness in higher education is growing rapidly at an international level, it is important for us, Hiroshima University, to present clearly our internationalization strategy. With regard to the internationalization strategy, it is necessary to show what is our fundamental policy, what are priority items to be carried out and how we organize our system for the internationalization of Hiroshima University.

Fundamental policy for the internationalization of Hiroshima University

In January 2003, we established a long-term vision for Hiroshima University in which we presented, as our future perspective, the slogan of "a top-level comprehensive research university that is distinguished throughout the world" and at the same time we drew up the road map to achieve this future perspective. The internationalization strategy is one of the most important parts of this road map.

At Hiroshima University we have considered exchange in research and education with foreign universities as one of the important parts of our activities and we continue to maintain this stance. This stance has been highly recognized within Japan and in an evaluation conducted in 2003-2004 by an official assessment organization, the international activities of Hiroshima University were awarded a top ranking.

The number of foreign students is growing every year and has now reached about 800. The number of foreign researchers is also growing rapidly. International conferences and symposia organized by Hiroshima University have been held actively in the Hiroshima area and the exchange of researchers and professors is becoming more and more extensive. The number of inter-university agreements for cooperation between Hiroshima University and foreign universities now amount to nearly 130. Perhaps due to the increase in these cooperation agreements, courtesy visits by presidents, vice-presidents and study groups to and from these foreign universities have become more frequent. In recent years, the topics of university management and university reform are the main issues discussed by university presidents.

In establishing the future internationalization strategy of Hiroshima University, it is considered indispensable that the international activities of Hiroshima University be reviewed by external experts. For this purpose, we established the Internationalization Strategy Working Group and invited as a reviewer Mr. Robert Goddard who was serving as the Assistant Secretary General for International Relations for the Council of Australian University Presidents. As a result, the Working Group published a report entitled "Internationalization of Hiroshima University in March 2003. In this report, the internationalization objectives of Hiroshima University are presented, along with 15 specific strategies for achieving these objectives. The 15 strategies include establishing a numerical target for internationalization, establishing the Hiroshima University brand, international standardization, nurturing internationally oriented individuals, the International Network of Universities and establishing centers in foreign countries to facilitate the internationalization strategy. We consider the strategies indicated in this report to be the essential part of our perspectives for internationalization. This year(2005) we established the Internationalization-strategy Headquarters in Hiroshima University supported by the Monbusho(Ministry of Education, Culture, Sports, Science and Technology) where our strategy is succeeded.

Items with high priority in the internationalization of Hiroshima University

Although quantitative enlargement, such as the increase in the number of foreign students through the extension of international activities, is basically welcome, such quantitative enlargement must be accompanied by qualitative improvement. In order to promote qualitative improvement we need a well-organized support system for international activities and we have established an international department managed by the Vice-President for International Affairs. The Vice-President also acts as a head of the Internationalization-strategy Headquarters.

Hiroshima University has joined the International Network of Universities (INU) and is a member of the Board of Directors. INU promotes cooperation among affiliated universities through such things as student and staff exchanges and distance learning through the internet. The international cooperation of Hiroshima University with foreign universities based on inter-university agreements is expanding more and more. We

believe that it has become important to exchange ideas on university management and to promote cooperation between universities and industrial enterprises on an international scale.

In connection with this, it is appropriate to mention here that Professor Liu Limin, who is a vice-president of Capital Normal University in Beijing, China, is appointed as an advisor to Hiroshima University and Professor Bruce Johnstone, former president and currently a professor of the State University of New York, is appointed as a member of the Administrative Council of Hiroshima University. They are helping our University by giving advice on university management and international activities. Mr. Hisashi Owada, a judge of the International Court of Justice, is appointed as an academic adviser of Hiroshima University. He helps us by providing useful advice on international problems and holds lectures and forums at Hiroshima University every year.

Another important issue is to establish centers or brances of our University in foreign countries to facilitate the internationalization strategy. By establishing such centers we are able to promote research collaboration in many fields, to stimulate student exchanges and to cooperate on university management. Based on this idea, we established on October 25, 2002 the Hiroshima University Beijing Research Center at Capital Normal University. The Beijing Research Center supports research cooperation between Hiroshima University and Capital Normal University and furthermore serves as a strategic point in China for priority research on environmental policy undertaken by the Graduate School for International Development and Cooperation at Hiroshima University. It is also used for interviews for candidate students from China and will be used as a Japanese language school for prospective students. We would like to establish centers like the Beijing Research Center in other places in China and in other countries.

Our foreign students come from more than 60 countries and amount to about 800. We are also promoting active acceptance of foreign students on short-term student exchange programs. Up to now, we have several thousand foreign student graduates. There is currently no specific alumni association for foreign students, but in 2002 alumni in Korea created the first alumni association overseas. It is desirable to have such alumni association in many more countries.

Advanced research collaboration and scientific exchanges have been performed actively and will be continued all the more in the future. These research activities are taking place with Western countries as well as Asian countries. I hope that research worthy of Nobel prizes will emerge out of such research collaboration. Through the activities of the Graduate School for International Development and Cooperation and the Center for the Study of International Cooperation in Education, cooperation in education has been performed in the South-East Asia and Africa. This cooperation is useful to develop school education in developing countries and is a unique activity that is recognized as a priority program in Japan by Monbusho(Ministry of Education, Culture, Sports, Science and Technology).

CONCLUDING REMARKS

I am sure to say that, in order to promote the internationalization strategy of Hiroshima University, Faculty of Dentistry is playing a crucial role. I expect the future of Faculty of Dentistry to be very prosperous.

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To Consider e-Learning in Dental and Medical Education in Japan

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ABSTRACT

All of Japanese medical and dental schools have constructed information-technology network systems. But only about 10% of medical, dental, pharmaceutical, and health professional schools are using e-learning, and 60 % are negative for planning to introduce e-learning systems. The IT or e-learning systems in medical and dental education in Japan are seen in the on line self-learning media for dissection for gross anatomy and media for simulated training systems for clinical diagnosis, treatments, and skills and the on line problem-based learning systems constructed through the electronic health records in the university hospital to approach data of the clinical examinations and clinical images such as X-ray and MRI images. And this paper introduce the author' s experience to build up an atlas of histology, explanation movies for histology practice using images collected for broad casting TV programs for lifelong-learning of citizen of public, and e-learning constructed by students in a subject to study medical research techniques.

KEY WORDS: e-learning, medical and dental education, on line PBL, on line atlas of histology, e-learning constructed by students

INTRODUCTION

In this paper, I describes e-learning in medical and dental education in Japan on my background of the long carrier as teacher in Hokkaido University Undergraduate and Graduate School of Medicine, medical clinician for about 10 years, leader of educational reform in Hokkaido University, leader of the Broad Casting Education Committee for about 10 years, committee member to develop the space collaboration system (SCS) to use an artificial satellite for distant education by National Institute of Multimedia Education. My specialty of teaching and research is histology to learn functional structures of the human body using light and electron microscopes; my logic of thinking depends on realistic images.

The Broad Casting Education Committee we had projected 13 TV programs for 45 min each in a subject in each year from a domestic broad casting station. Large amounts of video materials and schematic figures to introduce contents of various academic fields in universities in Hokkaido were assembled in the broad casting station. Thus I have begun to utilize some of these images for teaching. Through these experiences I have involved in development of the e-learning.

The term "e-learning" is a well known in faculty of Japanese universities and the e-learning environments significantly growing in higher education in recent 5 years, but universities really using it in the curriculum are still remain small in number. This paper deals with e-learning in Japanese universities at first, then the situation in the dental and medical schools, and author's experiences in relation to e-learning.

E-LEARINING IN JAPANESE UNIVERSITIES

E-learning systems provide self-learning environments on computer-based line systems or information technology (IT) network systems for students to be able to access anytime, anywhere and any pace.

According to the recent national survey of IT use in higher education institutes in Japan (Yoshida and Taguchi, 2005), computer-based network systems had been established in 97% of Japanese universities and 98% of universities for medical, dental, pharmaceutical, and health professional education. However, medical and dental schools using the network systems are now 100%, because these schools have constructed the system for the computer based test (CBT) to examine knowledge of each student just before entering the clinical training in the hospital. CBT and objective structured clinical examination (OSCE) to evaluate clinical skills and attitude are designed depending on the standardized educational objectives in the core-curriculum established recently by the medical or dental educational organizations (2001). It has been decided that CBT and OSCE are formally begun in this year (2005) after trials for a few years in every Japanese medical and dental school. Pharmaceutical schools have completed their core-curriculum and are developing their CBT and OSCE following medical and dental education. Health professional schools for nursing, occupational therapy, physical therapy, speech therapy (corresponding therapy by speech-language pathology and audiology) remain undeveloped about CBT and OSCE, because the standardized core-curriculum has not been established in these fields.

The national survey by Yoshida and Taguchi (2005) informs the following situation of e-learning at present.

Only 10 % out of these schools are using e-learning, though nearly 100% of universities for medical professions have constructed IT network systems; 30 % of universities for medical professions are planning to introduce e-learning systems but 60% are negative for planning on e-learning systems, and it is sure that no schools

are planning courses with credits only by e-learning.

Out of universities for medical professions, 50% have set up syllabi in the curriculum in the websites and 23% are planning the setting up, about 30% placed educational materials for classroom teaching and 21% are planning it, and 15% have the place to receive for questions from students and 22% are planning it, and 20% are planning to develop special soft wears or course wears in the web sites.

About 40% of teachers and students in universities are thinking that the development of web-systems for education in their universities increases effects of teaching and motivation of students, activates communication skills among students, and widens applications. However, most of teachers complain that the construction of the system is very expensive, a certain group of teachers become increased in work load, maintenance of the systems are expensive, technical supporting members are too small in number, preparation to use the system needs a time consuming laborious work, and suitable teaching materials are very limited for enormous varieties of subjects in the curriculum of various disciplines.

Among future planning and aspects in relation to e-learning, there are ideas to put various teaching materials in the home pages, to construct the sites for discussion with students, to set video materials for classroom teaching in the websites, and to exchange information between students on the websites. There are opinions that e-learning requires to combine face to face classroom teaching and supports classroom teaching from out side by accumulations of teaching materials for the classes in the websites. They said that for development e-learning it is important to be used by every teacher, to have enough educational resources, to increase the service thorough the web and to have technical specialists to be able to respond anytime for maintenance and trouble of the systems.

From the above national survey, we can confirm that the most of Japanese higher education institutes, including universities for medical professions, have architectures of IT systems and e-learning environments and are greatly expecting important effects of varies of applications on the higher education, but only small proportions of schools are using e-learning in the curriculum. For example, many universities have constructed a certain e-learning system on the web-systems established by own staffs or of a commercial product which for each course can provide the syllabus, teaching materials, bilateral information exchange board for teacher and students, place for the guidance of repots by student' s homework, and various examination and evaluation. But only limited numbers of teachers are using this expensive system in surprisingly small numbers of courses. Most of teachers recognize that it is difficult to keep effective teaching only by e-learning for Japanese students. Face to face live teaching is essential for Japanese students who learn in undergraduate medical schools, differently medical students in USA learn in graduate medical schools after learning in undergraduate schools. For modern education in medical and dental universities in Japan, the e-learning systems is great and essential tools but complementary tools of live teaching in classrooms.

EDUCATIONAL RESOURCES IN E-LEARNING SYSTEMS IN JAPANESE MEDICAL AND DENTAL SCHOOLS

It is sure that atlases of gross anatomy, histology, pathology, X-ray film images, and MRI on web-sites of the university are useful and effective in medical and dental education. Some universities provide such educational resources in their web-sites (Kochi University, Miyazaki University and others). In such cases, the construction of such materials on the web-sites depends on teaching staffs who are talented in IT technology, because rare Japanese medical and dental universities hire specialists for IT technology for creation of the various educational materials in the web-sites and maintenance of the systems. Thus, the universities with well organized original educational materials in the web-sites are small in number.

Recently problem-based learning (PBL) is introduced in most of Japanese medical dental universities. Some of them have begun to use the e-learning system to PBL. Kanazawa Medical University introduced the electronic health record system in the university hospital in 2000 and began to use the system combining the electronic syllabus for PBL. The data-bases about various clinical cases are piled up in the electronic health record for education avoiding personal information of the patients. A student can record history and findings of the physical examinations on the electronic record after the interview with the patient and propose clinical examinations. The teacher reads the record by the student, gives the advice and demonstrates the results of the clinical examinations. Through these repeating interactions between the student and teacher on the web-site, the student can reach proper diagnosis. During this study, the student has to often visit the patient; thus the student can learn communication skills and attitude for clinics. For this system, 30 computers were set up in a room in the hospital. It is visible how many times the teacher approached to the student and how guided the student on the website. Therefore, this is used for the educational evaluation of the teacher.

Recently many university hospital have become introduced the computer managing system; the health records in this system contain large amounts of data such as results of various clinical examinations, pharmaceutical records, nursing records, medical images such as roentgenogram, CT, MRI, pictures of endoscopes, vascular images, and microgram of pathological tissue and others. They work as live materials for PBL. Thus several universities have begun to use these resources for PBL.

EXPERIENCES OF E-LEARNIG MATERIALS

In 1984, Hokkaido University produced a series of TV program about human body consisting of 13 programs 45 min each and projected them form a domestic broadcasting company. In 1984, I joined to the Broad Casting Education Committee to make TV programs and radio programs to introduce academic contents to the citizen of public. I designed the contents of each program and supervised the making videos by staffs of the broadcast-

ing company. The series consisted of the history of medicine, bones and muscles, digestive system, teeth and eating, respiratory system, endocrine system, skin, nervous system, mental system, circular system, sleep and circadian rhythm, urinary system and reproductive system. One lecturer for each program was appointed among the staffs in Hokkaido University and universities in Hokkaido. I discussed with the lecturer about details of my design, and completed the scenario with the director of the TV movie company, and supervised taking videos for the subjects, editing and forming the final complete package for broadcasting in the studio of the broadcasting company. Each video consisted of lecturer' s explanation about the contents and talking with the announcer, showing movies, pictures, and various figures. Each program was broadcasted on TV once a week for about 3 months. The audiences were 50,000 to 150,000 in Hokkaido according to the audience rating. The TV program making required nearly 3 years form the planning to broadcasting and persons above 60 or more in numbers worked for the series. This was an opportunity to learn video making for me.

After this project, I had continued to produce various series of video programs and radio programs for about 10 years in the broadcasting project of Hokkaido University. Great numbers of moving images were increasingly accumulated by the project and they appeared good as teaching materials in the university also, because the contents of each program introduced contemporary academic topics.

My specialty for teaching was histology. In 1986, a TV system including a TV microscope, video editor and 16 monitors were equipped in the histology laboratory for student' s practice to observe histological sections. In 1986, we made 19 videos for explanation today' s practice of histology; each video was made using moving images used in the TV program mentioned above, adding new histological images and narration to explain points of observation, 20 to 24 minutes in length. To complete 19 videos, it took 5 months. Every histology lab was open by watching the video.

In 1999, computers connecting with university websites were set up in the school for computer literacy education, and 20 computers among these were set up in the histology laboratory. Thus, we made an atlas for histology lab including 1000 or more light microscopic and electron microscopic images and written explanation on the home page. The 19 videos, described above, were digitalized and re-edited to set up these in the home page. It took about 3 months.

Students became to approach to the leaning materials in the home page any time from their computer or computer in the school. Students in the histology lab observed the tissue sections by microscopes, often referring the atlas. And students often repeated to watch the explanation videos when they had question during the observations. We could hear the voice of explanation from many computers in the laboratory during the microscopic observation practice. This worked just like helps by several numbers of teaching assistants. It was obvious that the atlas and videos helped learning of students. The histological images shown in the atlas and videos were from the same specimens which students used in the practice. This appeared increased motivation to learn for the students and educational effects.

From these experiences, we have learned that construction of systematic educational materials in the websites needs laborious works for long days with specialists of computer literacy, but even static or short moving images for a few minutes give great impact on students for understanding. Recently, digitalized imaging tools are easily available to set up pictures and movies in the websites. These images may become more useful if the database of images is constructed.-

E-LEARNING ENVIRONMENT THAT STUDENTS FORM IN SMALL GROUP LEARNING

We created the e-learning environment by students. In 1999, a small group learning class of 4th year students was designed to learn and understand various medical research methods, as follows. The class of 100 students is divided in to small groups of 7 or 8 students and each group learned a medical research method different from others. The group learns what is the principle of the research method, what kinds of machines and tools are used, what is the structures and handling principle of the machines or tools, what kinds of specimens are prepared for it, what kinds of data are obtained, and how to analyze and explain the results, in order in the course. Students have to record on their homepages what they learn after every class; the homepages are formed in the school website by each group in the first class. Students have first to learn from books and later they can visit a researcher for interview to know the reality of the method and can visit laboratory for inspection to get the reality of the method. Teachers set up the course homepage to show the syllabus and bulletin for communication on the website before beginning the class. Students and teachers can enter the student' s homepages from the list of the medical research methods in the course homepage.

Students have to report what they studied several times using their homepages during the series of classes. Students have to built up presentation materials in the homepages. The day of the last class is the day of presentation about the research method that they learned. They use presentation materials in their home pages. Thus, finally a homepage for various medical research methods is completed and kept in the school website.

E-learning environments are generally for individual students but the above course was characterized by face to face interaction, cooperation between students, and learning on group dynamics.

COMPUTERS AS LEARNING TOOLS FOR MEDICAL AND DENTAL EDUCATION AND LIFE-LONG LEARNING

Medical and dental education is the discipline that e-learning works most effectively. The materials for e-learning can be easily prepared because various images in medical and dental fields are digitalized in memory,

piled up in the web-sites, updating contents. However, medical and dental education can not be completed only by e-learning. Face to face teaching becomes more important as e-learning becomes more developed, because Japanese medical and dental education starts from integration of the students into college life and studies in undergraduate schools, differently from post-graduate schools which can start as adult education in United States. Development of humanity, human understanding, and ethics are important objectives for medical professionals are obtained by interactive studies among students. Any of laboratory studies such as gross anatomy dissection, histology and pathology lab, physiology lab, biochemistry lab, and practice of clinical diagnosis, and clinical clerkship can not be replaced by virtual e-learning environments, which are important as the complementary learning materials. Computers connectable with web-sites are essential tools like stethoscopes of physicians who need life-long learning.

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Towards the Education of "Universal Dentist" in the Age of Globalization

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1. Core Educational Objectives in Japan

It had been a while since graduate dilution of the quality and quantity of undergraduate clinical education in Japanese dental schools was pointed out as a major issue. Various reports full of recommendations and guidelines lacking in an appropriate evaluation system thereof did not seem to bring about expected results. The Committee on Medical and Dental Education established by the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) issued a report entitled "Measures for Improving Medical and Dental Education for the 21st Century - Restructuring Undergraduate Medical and Dental Education-" in March, 2000.

[http://www.mext.go.jp/b_menu/houdou/13/03/010330.htm (in Japanese)]
The report consisted of recommendations on the following major issues: curriculum reform; evaluation system; infrastructure for undergraduate clinical training; and promotion of faculty development. It was hallmarked by the Model Core Curriculum in undergraduate medical and dental education as well as the proposal for the Common Achievement Test.

1) Japanese Model Core Curriculum

In the past decades, simple accumulation of educational contents resulted in an overly crowded undergraduate medical and dental curricula. It was therefore crucial for educators to identify core educational contents to be learned by every single medical or dental student regardless of his/her future career choice and at the same time to allocate more time for clinical education. After including such nationally established core educational contents in mandatory courses, it was required for each medical/dental school to design elective courses corresponding to different needs of its students. In order to facilitate identification of the core educational contents, the above Committee compiled the Medical/Dental Model Core Curriculum which was an assembly of general instructional objectives and specific behavioral objectives which should be attained by all medical or dental students by the time of graduation. The time allocated for the educational objectives included in the Model Core Curriculum was supposed to occupy around 60 to 70% of the total curriculum hours. It was up to each medical or dental school to decide how to deliver the educational contents in the Model Core Curriculum; however, the report strongly recommended that they should be taught through a curriculum integrating basic and clinical subjects as much as possible rather than a traditional discipline-based one.

2) Common Achievement Test before starting undergraduate patient care

Undergraduate dental education in Japan, which used to be skill-oriented and patient-care-oriented, had been gradually focused more on the instruction of knowledge in the past decade or so. Dental educators felt it necessary to reinstate the quality and quantity of undergraduate clinical education (patient care). Though the background of the demand of improving clinical education was different between undergraduate medical and dental education, educators in both fields agreed on the necessity to enlist patients' understanding and cooperation for clinical education. The Common Achievement Test (CAT) was introduced to both undergraduate medical and dental education in order to demonstrate to patients that student physicians/dentists have acquired a certain level of knowledge, skills and attitude. Under this system, only those students who pass this examination will be able to proceed to clinical education (patient care). Since the Model Core Curriculum is expressed as a set of specific behavioral objectives (SBOs), the attainment of SBOs can be assessed by evaluators. Based on SBOs which should be attained prior to treating patients, questions in CAT are prepared. CAT comprises of Computer-Based Testing (CBT) and Objective Structured Clinical Examination (OSCE). The SBOs related to knowledge are mainly evaluated by CBT, while skills and attitude are partly evaluated by OSCE. The official implementation of CAT is scheduled for December, 2005.

2. Core Educational Objectives in Europe

In Europe, academic degrees and certificates are mutually recognized among the member nations of the European Union. Dental practitioners are included as one of the health care professionals whose basic right for free movement within the area is guaranteed. Therefore, in principle a graduate from dental school of a member country is able to move to another member country and work there as a dentist. Faced with differences in the quality and quantity of undergraduate dental education in Europe[1], the Association for Dental Education in Europe (ADEE) took the lead in the efforts geared toward more harmonization and convergence to a higher standard of dental education in Europe[2]. In September, 2004, ADEE officially adopted "Profile and Competences for the European Dentist" [http://adee.dental.tcd.ie], which indicated the profile of the graduating dentist and the core competences every graduate from dental school

in member countries must attain. This document was expected to serve as a facilitator in more harmonization and convergence of dental education in Europe.

3. Comparison between Japanese and European Core Educational Objectives

The Japanese Model Core Curriculum (JMCC) and the Profile and Competences for the European Dentist (PCED) had apparently different goals and objectives; however, they share a characteristic of defining core educational objectives all dental graduates have to fulfill. The purpose of this study was to compare mainly the contents and the expected level of achievement without getting hung up too much on details. JMCC tended to focus on the cognition level of students, which could be explained by the fact that questions in the Computer-Based Testing (CBT) in CAT were prepared based on the SBOs in JMCC. PCED was characterized by competences covering a wide range of knowledge base and those concerning students' attainment on the behavioral level. There were also some competences of importance which were not enunciated in JMCC. The results of this study were expected to offer useful information for the future revision of JMCC.

Development of Dental Curriculum Guidance in the United Kingdom

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ABSTRACT

This paper descibes the process by which the United Kingdom General Dental Council developed the second edition of curriculum guidance for undergraduate Dental degree programmes entitled "The First Five Years, a frame work for Dental Education" . It explores the national and international drivers for change and how these influenced the the content of the document.The key change is the organisation into three domains: (i) What the Dentist is able to do (ii) How the Dentist appraoches Practice (iii) The Dentist as a Professional. There was increased emphasis on IT skills, law, ethics and professionalism, integration with the education of other members of the dental team, health and safety issues, outreach teaching, the need for continuing professional development, pain and anxiety control. It looks ahead to the chalenges posed by further international developments, in particular the Bologna declaration.

Key words: Dental education, Dental curriculum

BACKGROUND

In the United Kingdom (UK) the General Dental Council (GDC) has responsibility for ensuring the quality of undergraduate dental education. This body comprises dentists elected by from those on the Dental Register and lay individuals appointed through an independent Public Appoinments Commission , funded and responsible to the government but separate from it in its day to day activity. The main role of the GDC is protection of the public by the maintaining standards of dentistal care. Part of this role is the supervision of dental eduction. As well as periodic inspections of the Dental Schools and their examinations it issues guidance on the delivery and minimum content of the degrre programmes. Their guidance was first published as "The First Five Years" in 1997 and was always intended as a minimum requirement and schools were expected to provide" added value" .

In 2000 the General Dental Council intitiated the process of updating its curriculum guidance. Previous curriculum guidance had defined the core knowledge content but now there was a need to define core skills and behavioral objectives as well.

There had been high profile problems of professionalism in medicine. This resulted in a public perception that the "professions" were arrogant, paternalistic and more concerned about professional well being than that of the public that used their services. Typified by a common public perception that they were experiencing "I am a professional I know what is best for you" . There became a need to ensure that new dental graduates were aware of their ethical and professional responsibilities and to ensure that outdated attitudes were not maintained but were replaced by an understanding of the automomy of the patient as and individual and their rights to make their own, informed, choices even where these do not accord with the professional advice. The new framework needed to ensure that professional ethics and attitudes became part of the core activities within dental education

The GDC was aware the need to meet European Union (EU) regulations on levels of qualifications.This quality assurance has to be delivered to the level expected by the Dental Directives of the EU that allow mutual recognition of qualifications and free movement and practice of dental professionals within the EU.One of the main drivers of the EU is the removal of barriers to the free movement of trade, services and individuals. Harmonisation is a key word. Part of that harmonisation process had been the definintion of common levels of qualifications and the requirement on the individual countries to map their qualifications onto this framework.. In the UK the supervision and publicfunding of degree level educatio was then through the Higher and Further Education Funding Councils (HEFC). To meet this need HEFC set up working groups to define the levels for all the degrees awarded in the UK. These groups were charged with producing documents that showed how the degree programmes met the specific level descriptors. The dental working group consisted of representatives from each of the thirteen UK dental schools and a facilitator from HEFC. This group met in parrallel with the groups for medicine and vetinerary science with some sharing of information. Traditionally these degrees are called "Batchelor of ……" that is used for all first level degrees in the UK. However these professional degrees are 5 rather than 3 years long and the groups decided that they more closely equated to the "Master" degree level because of the necessity for graduates to be capable of complex problem solving in new aspects of their work. The resulting document[2] was published in 2000 and is available on the internet.[3] The content of this document indicated that the dental schools had developed their curricula beyond those of the first five years and were starting to define skill and behavioral aspects of their courses. In particular they were beginning to define levels expectation of acheivement. There was a realisation that the potential scope and complexity of dentistry had and was continuing to increase rapidly and that stu-

dents could not be exptected to have the same level of knowledge and attainment across all the potential course content. Within the UK there was an awareness of the movement in the United States to have curricula defined by competecy with domains, and various levels of supporting competenccies. This was not well understood but the deveolpments in medical education[4] were begining to feed into detal education.[5]

On graduation UK dental students usually progress into a year of supported dental practice, with government funded salaries, day release continuing education - Vocational Training.. Feed back from the trainers indicated concerns about the level of communication skillsand some core clinical abilities of some new graduates.

Although the FFY was only three years old the GDC's education Committee decided to start on the process of updating this document in 2000 so as to guide the evolution of the dental undergraduate curriculum into the start of the 21st century.

PRESENT STATUS

Process

The GDC set up a working party with representatives from each of the UK Dental Schools. Schools were invited to nominate individuals from whom the the group was chosen. The choice being made to ensure a coverage of subject areas as well as geography. At plenary meetings of the working party ideas for areas of change in the existing document were identified. Groups of three members of the working party were charged with drafting changes or new content. These were circulated and then discussed at further plenary sessions, refined and a draft discussion document produced. This was circulated widely, to the dental schools, to specialist societies and other professional organisations including those involved in Vocational Training. Responses to this consultation were received from both individual and groups. After consideration of these responses further draft was produced and another wide consultation carried out. Further refinements were made before a final and more limited consultation. Minor changes were then made before the final document[1] was produced and published in 2002.

The Key Changes

Early on the working party identified the need for increased emphasis on IT skills, law, ethics and professionalism, integration with the education of other members of the dental team, health and safety issues, outreach teaching, the need for continuing professional development, pain and anxiety control. Familiarity with Information technology is an increasing necessity in managing a dental business and in keeping up to date and finding current information in a rapidly changing world. The necessity of understanding and working within the ethical and professional climate in which we now work is a described above as one of the drivers for change. Increasingly dentists rely on skilled support from others, such as dental nurses, hygienists and technicians to deliver effectively patient care, learning to work as the head of this team requires the dental student to learn the skills of leadership and team working. The complexity of the dental work place is increasing with legal controls, on such as pressure vessels (autoclaves), disposal of clinical waste, including mercury contamination of wastewater from removing amalgam restorations. All adding to the learning load for students and their educators. Learning ideally occurs in the circumstances in which it will be subsequently used. Few students will continue to work in dental schools most will be working in primary care and the development of outreach teaching in primary care settings is intended to facilitate the transition from student to effective primary care dentist. The first edition of FFY recognized in its title that dental education was not complete on graduation from the undergraduate course but was only the first step on a lifetime journey. The recognition of the need to continually update and extend ones professional abilities and the acquisition of the learning skills that allow this are a necessity of undergraduate dental education. A recent change in UK legislation restricts the use of general anaesthesia to hospitals. It is no longer permissible to carry out general anaesthesia in dental practices. Thus over a very short period there has been a huge reduction in dental care carried out under general anaesthesia with a resulting increased need for alternative methods of pain and anxiety control and hence the need to emphasize this in the undergraduate course.

The dental profession and many dental educators were not prepared to move to an entirely competency described curriculum. So the document describes in the familiar listing of subject areas the scope and arrangements for delivery of the curriculum. The working party developed descriptors for the different depths of learning outcome required of dental students and these are in table 1:

This enabled the expectations of student acheivements by graduation to be explained in terms that their future employers would understand. The, in my view, most useful section to the dental educator was the appendix that listed the dental domains and sub domains that for the first time explicitly took UK dental education beyond the what the graduating dentist should be able to do into how he should do it and the professional context in which it is done. These are shown in table 2. Emphasis was also placed on vertical and horizontal integrationof the curriculum and the need for early introduction to the clinical environment.

The Future

These last changes bring difficulties in mobilitiy of students between institutions. It is no longer possible to devide the dental curriculum into stand alone modules that could be recognisable subsitutes for similar modules in other institutions.

The EU Bologna Declaration 1999 was signed by higher education (HE) Ministers from 29 European nations. Its aim is to create a 'European Higher Education Area' by 2010. To achieve this it started the "Bologna Process" . The main purpose of the Bologna Process is to enhance the employability and mobility of European citizens, and to increase the international competitiveness of European Higher Education. The Declaration has six key objectives:

· Adoption of a system of easily readable and com-

parable degrees and the implementation of the Diploma Supplement in order to promote EU citizen employability and the international competitiveness of European higher education.
- Adoption of a system of two main cycles of undergraduate and graduate degrees
- Establishment of a system of credits to means of promoting student mobility
- Promotion of mobility for students and teachers.
- Promotion of European co-operation in quality assurance
- Promotion of the necessary European dimensions particularly for curricular development and inter-institutional co-operation.

Three more objectives were added at a meeting of European ministers in Prague in May 2001. These are:
- Acknowledgement of Life Long Learning
- Encourage the involvement in the development of the European Higher Education Area (EHEA)
- Promotion of the EHEA to students within Europe and elsewhere.

Copies of the declarations and all the Bologna related texts are available from the official Website of the Berlin 2003 Conference.[6]

For dental education much of the work to meet these objectives has been carried out by DENTED - the Thematic Network Project Achieving Convergence in Standards of Output of European Dental Education.. This is funded by the EU but has yet to make progress on the issue of a system of credits that would allow students to study part of their degree in one dental school and other parts in another school potentially in another country. Limited student exchanges with credit recogntion occur on a one to one inter institutional basis through "Erasmus" but the process is far from the simplicity envisaged by the ministers of education.

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Table 1.
Descriptors for the different depths of learning outcome.

Be competent at:	Students should have a sound theoretical knowledge and understanding of the subject together with an adequate clinical experience to be able to resolve clinical problems encountered, independently, or without assistance.
Have knowledge of:	Students should have a sound theoretical knowledge of the subject, but need have only a limited clinical/practical experience.
Be familiar with:	Students should have a basic understanding of the subject, but need not have direct clinical experience or be expected to carry out procedures independently.

Table 2. Domains and sub-domains.

Domain	Sub Domains
What the Dentist is able to do	Clinical Skills
	Practical Procedures
	Patient Investigation
	Patient Management
	Health Promotion and Disease Prevention
How the Dentist appraoches Practice	Communication
	Data & Information Handling Skills
	Understanding of Basic & Clinical Sciences and Underlying Principles
	Appropriate Attitudes, Ethical Understanding and Legal Responsibilities
	Appropriate Decision Making, Clinical Reasoning and Judgment
The Dentist as a Professional.	Professional Development
	Personal Development

Dental Education and Research Priorities in Kazakhstan

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Dental education is one of the important part of medical education in Kazakhstan. There are four Dental Schools in Kazakhstan. The Doctor of Dental Medicine (D.M.D.) and Doctor of Dental Surgery (D.D.S.) degrees are identical with respect to educational curriculum. Both degrees indicate that the graduate is prepared to practice as a general dentist. The curriculum for both degrees is set by the Ministry of Health of Kazakhstan. Dental school programs leading to either the D.M.D. or D.D.S. degrees are five years long, but from next year in accordance with Kazakhstani new national reforms of educational and health care systems implemented in 2005, 1 year internship will be included. It is well known that the ideal primary health care system for each country depends upon the socioeconomic development and the health status of the population. Primary health care-centered system in one of the main priorities of our new health care program. For all medical academies, including dental schools, own medical centers will be established by governmental financial support. Dental school programs will be 6 years long, including 1 year intensive clinical internship. After dental school, some dentists may choose to pursue a specialty within dentistry. This requires extra training in the form of fellowships. Some of the specialty options are orthodontistry, periodontistry, and oral or maxillofacial surgery.

Students that intend to apply for dental school must take the Integral National Admissions Test (INAT). Ministry of Education and Science establishes which scores from this exam are acceptable for admission into any dental school. The INAT covers basic natural sciences, reading comprehension in the natural and basic sciences, quantitative reasoning and perceptual ability. The INAT is normally taken several months prior to applying for dental school. A personal statement in which the applicant shares with the admissions committee their motivation for attending dental school as well as any strengths they will bring as a student is also required.

Generally, the first two years of the dental program will be spent on the biomedical sciences. These years are considered "pre-clinical" and focus on oral diagnosis and treatment. The subjects covered typically include anatomy, biochemistry, embryology, physiology, histology and pharmacology. The three clinical years of the dental programs are spent treating patients with a variety of oral diseases under the supervision and guidance of the faculty. This part of the training is usually curried out through rotations in the various clinical settings available to the medical academy. Semipalatinsk State Medical academy has own dental clinic. There are also programs that allow students to pursue the master of science (M.S.) or doctor of philosophy (Ph.D.). Students that obtain both a dental and Ph.D. degree typically pursue careers in academic dentistry.

Dental Research Priorities in Kazakhstan

The Ministry of Health of Kazakhstan and Dental School of the Semipalatinsk State Medical Academy established some research priorities for the period spanning 2002 to 2007. Those priorities were:

1. Congenital Oral and Craniofacial Diseases in the area adjacent to the Semipalatinsk Nuclear Test Site
2. Some clinical features of Dental, Oral and Craniofacial Disorders in the Semipalatinsk Region of Kazakhstan
3. Clinical Approaches to the Diagnosis, Treatment and Prevention of Dental, Oral and Craniofacial Disorders

The experts focused on oral infectious and inflammatory diseases, host-bacterial interactions, mucosal immunology. Some features of induction, development and clinical manifestation of dental and oral disorders, including various congenital diseases, caries, periodontal diseases, mucosal infections, oral cancer, and infectious diseases with oral manifestations have been studied among exposed and non-exposed population in the Semipalatinsk Region of Kazakhstan in comparison with other regions. Recent research projects also focused on the repair of hard and soft tissues, and tissue engineering, genetic research. Other topics were craniofacial anomalies and developmental biology, oral cancer research, pain research, and autoimmune disease, including the study of the oral manifestations and complications of genetic conditions and of chronic systemic diseases and disorders such as cardiovascular disease, pulmonary disease and diabetes, and the impact of oral disease on systemic health. The experts suggested that the interaction between oral health and systemic diseases are very important issue. It was noted that prevention of oral, dental and craniofacial diseases should be under special control of the Ministry of health and local dental clinics.

There are various training programs in dental research of Kazakhstan, including a Master's in Clinical Research and Ph.D. degree program. Semipalatinsk State Medical Academy in collaboration with the Dental School of Hiroshima University proposed at the first time the Ph.D. degree program, which is very helpful for Kazakhstani young specialists.

Master's programs in clinical research should be

very rigorous to solve the current deficit in well-trained clinical researchers and clinical practitioners.

Our experts together with specialists from Dental School of Hiroshima University (professor T. Okamoto) have done some joint research projects in the area adjacent to the Semipalatinsk Nuclear Test Site. Molecular-epidemiological study of oral and maxillofacial disorders among the residents of the Semipalatinsk Nuclear Test Site area was a first International study of this kind in Kazakhstan. The purpose of that study was to investigate a possible relationship between long-term radiation exposure by nuclear testing and predisposition of oral and maxillofacial disorders among residents from regions adjacent to the Semipalatinsk Nuclear Test Site (SNTS).

Screening study included an examination and assessment of oral cavity condition and dental disorders, interview on residential and health history, nationality, as well as oral hygiene, nutritional and smoking habit, review of local primary public health clinic documents, blood sampling for genetic research. The study strongly suggested that the inhabitants in exposed villages adjacent to SNTS might be considered as a population at risk for high frequency of cleft palate and tooth loss due to the radiation exposure. This study also suggests more detailed molecular research of the nature of dental disorders in the area surrounding the SNTS. Exposed inhabitants with oral and dental disorders from villages adjacent to the SNTS are really needed a specially designed rehabilitation program. Unfortunately, providers in rural primary care settings have an unacceptably small understanding of evaluation of exposed patients and what is available for their appropriate diagnosis and effective treatment.

We are planning from next year to conduct research on genetic and environmental determinants that affect oral health. Also oral care needs of the HIV/ AIDS population issues of interest to our dental school. The Ministry of Health of Kazakhstan and Ministry of Education and Science will provide scholarships for students that desire to enter fields of academic dentistry and improve the research base within the dental fields. This would also encourage all dental school students to study abroad to get Ph.D. degree in foreign universities. Partnerships and research collaboration will be established with leading foreign universities and dental schools, which will benefit to the world scientific and academic community. We trust that our research and clinical collaboration with the Dental School of Hiroshima University will be deeper and more fruitful at the nearest future.

Internationalization Strategies of Taipei Medical University

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This report is composed of three parts: (1) Introduction to Taipei Medical University, (2) Internationalization strategies of Taipei Medical University, (3) Plans and strategies of internationalization in the following three years. The first part introduces every division (including academic and administration division) of Taipei Medical University. In which, five colleges (college of medicine, college of oral medicine, college of pharmacy, college of nursing and college of public health and nutrition) are particularly described. The second part contains not only internationalization strategies and goals of our university but also outcomes of these strategies. About internationalization strategies, we focus on learning English and promoting the communication of international academic. We hope to contact and communicate with whole world hereby. Our effort is worth, now, we have contracted sixteen sister schools in America, Europe and Asia. Besides form 1999, the students of TMU began to have short-term internships abroad, the same year, TMU started to have foreign students' short-term internships in our hospitals. The last part is extension of the second part. How to stride forward to internationalization? The complete plan is necessary. In the next three years, we hope that all the students, teachers and staff have opportunities to take part in international affairs, cultivate world view and be a person with foresight.

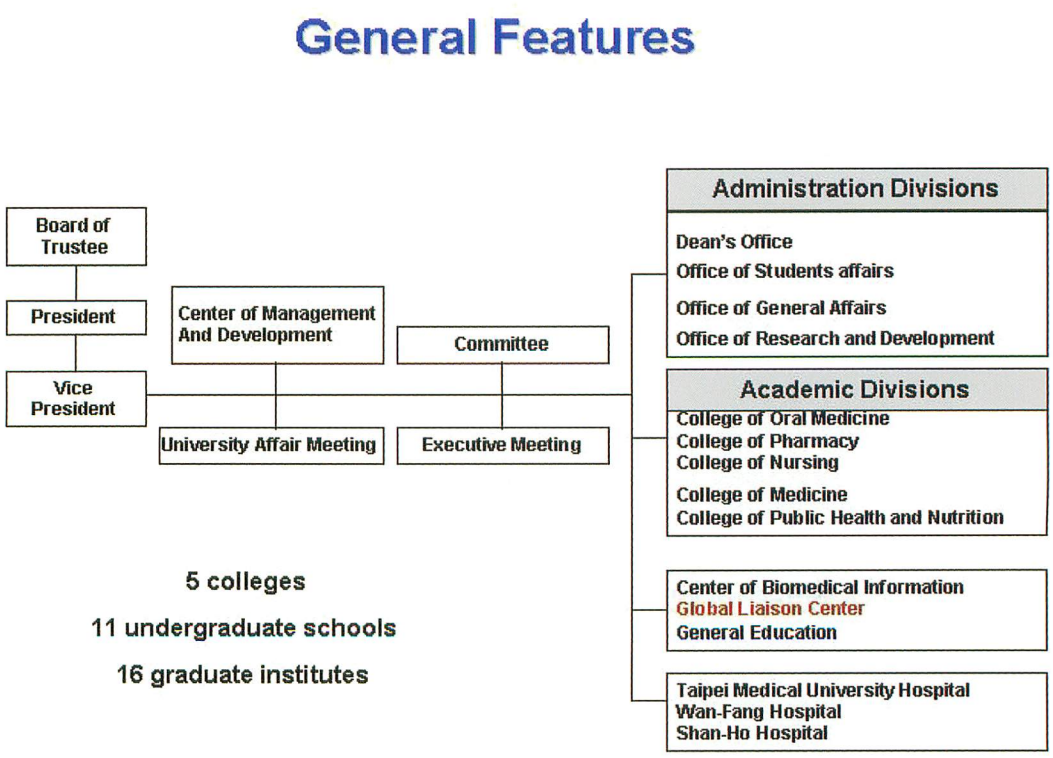
INTRODUCTION TO TAIPEI MEDICAL UNIVERSITY

Taipei Medical University (TMU), formerly known as Taipei Medical College, was founded in 1960 by Drs. Shui-Wang Hu and Cheng-Tien Hsu, and other medical specialists and ardent educators who had a dream. There are about 22,150 TMU graduates to date, and while most of our alumni serve in medical institutions and clinics, some of them have also been prominent in the fields of research, politics, and business. Taipei Medical College originally had three schools: Medicine, Dentistry, and Pharmacy. From 1990, then-president Dr. Chung-Hong Hu, embarked on a program of upgrading the school. Since then, a medical university was formed and includes five colleges, 11 schools, and 16 graduate institutes. Under the leadership of our new president, Dr. Chung-Yi Hsu, TMU has continued to improve the quality and quantity of research to accelerate its internationalization and become a world-class university by 2010.

The structure of TMU

Besides the Board of Trustees, President and Vice-President, the most important divisions of TMU are the five colleges and four offices.

The **Global Liaison Center**, which is responsible for pro-



moting international affairs, was established in 1999 for communicating and liaising with international academic institutes and sister schools. The mission and goals of this center are to:

Ⅰ Select and administer international academic cooperation;
Ⅱ Administer the affairs of exchange programs and academic cooperation between sister schools;
Ⅲ Provide receptions to scholars and students of long- and short-term exchange programs;
Ⅳ Provide information regarding international academic cooperation on the internet to students of TMU;
Ⅴ Liaise with the international industries for educational -industrial cooperation programs; and
Ⅵ Carry out other affairs concerning international communication at TMU.

TMU Today

At present, there are 5525 students who study at TMU, around 23,000 alumni, and 276 full-time administrative staff who work in the supportive environment of TMU. We also have 360 full-time and 568 part-time instructors working here, 2/3 of which have doctorates. In addition to classroom instruction, professors also enthusiastically conduct research. Over 400 research papers have been published in SCI journals.

The campus environment

TMU is located in flourishing east side of Taipei City, and is close to the world's tallest building, Taipei 101, and Taipei's World Trade Center. TMU is a bright spot in the financial district of Taipei. As a metropolitan school, TMU is easily reached by shuttle bus from the subway (the MRT) every 15 minutes. One of the most popular entertainment and shopping areas is a 15-minute walk from TMU.

There are six main buildings of TMU: Medicine and Nursing, Pharmacy, Public Health and Nutrition, Oral Medicine, TMU Hospital, and the Hall and Dormitory. The Oral Medicine building is being redone, and the new building is scheduled to be completed in 2006, and will include the most-modern equipment for teaching and research.

Taipei Medical University Hospital (TMUH)

The three missions of Taipei Medical University Hospital are health care, clinical education, and medical research. The 220-bed first medical building was established and began to serve the east side of Taipei in 1976. In 1994, the completion of the second medical building, expanded the hospital's capacity to 435 beds. More than 880 employees provide health care to approximately 360 inpatients and 2500 outpatients on a daily basis. The hospital is designed to support the concept of patient-centered care. Because of its distinguished medical quality and human-oriented service, the hospital has been accredited as a regional teaching hospital.

"Dream big and dare to try" is the motto for TMUH to achieve a further achievement — becoming a medical center. The first stone of the third medical building was laid in February 2004. In the near future (in 2006), the new 750-bed medical center will mark a milestone in TMUH's history and it will shine like a diamond in Taipei's Xinyi District.

TMU's Wan-Fang Hospital (WFH)

Taipei Medical University's Wan-Fang Hospital has been acclaimed as the most-successful public-built and private-run hospital in Taiwan since its inauguration in May 1996, when TMU began to operate Municipal Wan-Fang Hospital. TMU-WFH continues to pursue excellence in clinical services and research. In 2004,TMU-WFH was accredited as a Medical Center.

Shan-Ho Hospital

Shan-Ho Hospital, in southern Taipei, is scheduled to open in 2008. TMU is authorized by the Ministry of Health to operate it for 50 years.

Five Colleges

College of Medicine

The College of Medicine has four schools (Medicine, Medical Technology, Health Care Administration, and Respiratory Therapy) and eight graduate institutes (Medical Sciences, Cellular and Molecular Biology, Biomedical Technology, Medical Informatics, Health Care Administration, Pharmacology, Medical Research, and Medical Humanity).

The Graduate Institute of Medical Sciences, the Graduate Institute of Cellular and Molecular Biology, the Graduate Institute of Medical Informatics, and the Graduate Institute of Neuroscience offer doctoral degrees. They have become a major pillar through which Taipei Medical University promotes medical education, and are responsible for teaching, research, and services in pre-clinical, clinical, and applied medicine.

College of Oral Medicine

The College of Oral Medicine has two undergraduate schools (the School of Dentistry and the School of Hygiene) and three postgraduate institutes (the Graduate Institute of Oral Rehabilitation Sciences, the Graduate Institute of Oral Sciences, and the Graduate Institute of Biomedical Materials).

This College provides a complete educational system for dental and oral sciences, which includes undergraduate to PhD programs. The undergraduate programs provide dental professional training for dentists and dental hygienists. The master's and PhD programs provide research training in the field of oral medicine with the aim of improving dental knowledge and technologies to improve human well-being in our society. An outstanding feature is the way in which these programs integrate engineering, physics, biotechnology, and microelectronics into product-oriented R&D and clinical applications.

Besides the educational knowledge and skillful training, the College especially focuses on the ethics, morality, and humanity of our students. Its goal is to educate the best dental professionals and scientists for the new century.

The College has two teaching hospitals: Taipei Medical University Hospital and Wan-Fang Hospital. Both hospi-

tals provide clinical teaching for dental students and optimal dental care for our citizens.

College of Pharmacy

The College of Pharmacy contains the School of Pharmacy, the School of Bio-Resource Technology, the Graduate Institute of Pharmacognosy, and the Graduate Institute of Pharmacy. The main mission of the College of Pharmacy focuses on quality education, research, and professional clinical services. The 4-year BS program in the School of Pharmacy provides the basic preparation for hospital pharmacy, community pharmacy, health organizations, industry, and government. The School of Bio-Resource Technology is a continuing education program for junior college graduates and offers a 3-year BS degree. It provides basic training and skills in Chinese tradition medicine and biotechnology.

The Graduate Program offers MS and PhD degrees. It emphasizes an interdisciplinary program with a more-comprehensive approach at the chemistry/biology interface with four research clusters of drug discovery, action, delivery, and clinical evaluation.

College of Public Health and Nutrition

The College of Public Health and Nutrition, which combines public health, injury prevention and control, and nutrition and health sciences, was established in 2000. It aims to develop outstanding professionals who can promote health science, disease prevention, and the longer life of humans.

The College consists of a School of Public Health, Graduate Institute of Public Health (offering master's and PhD programs), School of Nutrition and Health Sciences, School Institute of Nutrition and Health Technology, Graduate Institute of Nutrition and Health Sciences (offering master's and PhD programs), and Graduate Institute of Injury Prevention and Control (offering a master's program). The School of Public Health focuses on both theory and technology and hopes that students acquire suitable abilities to understand social trends and analytical abilities to support medical policies. To ensue the goal of becoming internationalized, the School of Nutrition and Health Sciences is moving in the direction of strengthening the quality of the faculty and also actively introducing updated nutritional information from other countries. The Graduate Institute of Injury Prevention and Control has the goal of educating professionals to meet social needs. In addition to strengthening education and research into injury prevention and control, it contributes further information and consulting services to the Taiwanese government in health, injury prevention, and political transportation and communications.

The College has also organized a Health Consultant Board to offer consultation on health sciences, introduce the latest research results of biotechnology and medical information to general public, and apply them to promote health.

College of Nursing

The College of Nursing (CON) is devoted to educating nursing professionals and to generating programs of research and clinical scholarship that advance the science of nursing and improve the quality of health care. The CON has passed through several stages of development and revitalization. During the past 10 years, many CON nursing graduates have been recognized by the community and other healthcare institutes.

The CON mainly focuses on nursing administration, as well as cardiovascular, cancer, community, mental health, and adolescent nursing. The contents of the core program emphasize improving the quality of care, pursuing advanced nursing knowledge, and achieving cost-effective management.

In the future, the CON will continue to develop new technologies to meet the needs of society. The CON will also actively prepare nursing professionals for advanced levels, promote scientific research, and expand into new areas of nursing careers. The aims of the CON are not only to establish a doctoral program in the near future, but also to establish master's programs in cancer and hospice care and geriatric care to meet the needs of the increasing number of elderly people and to deal with dramatic changes in society.

Innovation and Incubation Center

The mission of the Innovation and Incubation Center, which opened in July 2004, is to effectively use resources of basic science and clinical medicine to carry out the cooperation between academia and industry. At present, 11 companies have moved in. There were 50 academic–industry cooperative projects in 2004.

Student Activities

There are 84 student clubs from which students can choose. The types of club vary (including sports, music, religion, literature, etc). Of course, some clubs like the Social Medical First Service Group and Medical Service Organization for Taiwanese Aborigines willingly provide humanistic care. In addition, students and teachers at TMU have formed medical group to provide medical aid in Africa and northern Thailand.

Internationalization Strategies of TMU

Internationalization is a trend of most universities and colleges. Exposure to various cultures and teaching methods can expand students' views, stimulate innovation, and motivate communication. However, we need to improve and promote many aspects of internationalization. The following is a statement of the university's internationalization strategies as well as efforts for its promotion. The statement is divided into three parts: internationalization strategies and goals; the effect of promoting internationalization; and last but not least, the plans and strategies for the next 3 years.

Internationalization Strategies

Improving student's foreign language abilities

Elective courses in foreign languages need to be strengthened in order to improve TMU students' foreign language abilities. There are many general language courses,

including Japanese (three levels and conversation), French (three levels), German (two levels), and English (writing, speaking, listening, vocabulary, English news, English conversation at the table, and English for traveling).

The GEPT (General English Proficiency Test) has been a required course at TMU since 2001. In 2003, 19 students passed the intermediate level, and 6 students passed the high-intermediate level.

Enrolling foreign students

Since March 2003, we have posted application forms on websites which foreign students can conveniently download.

Every school's and institute's homepages are now in English to provide relevant information for applicants. In addition, since January 2003, we have posted applications for admission in both English and Chinese.

We advertise for foreign students by broadcasting an "admission for foreign students" in the Central Broadcasting Station.

Promoting teaching in English

Currently, there are three subjects in two institutes which are being taught in English. The courses Molecular Biology, Advanced Molecular Biology, and A Seminar are taught in English at the Cellular and Molecular Biology Institute. Reports and term papers are required in English by Medical Informatics and A Seminar, two required courses in the Institute of Medical Informatics.

Almost all textbooks of the undergraduate and graduate schools are in English. The textbooks and teaching materials of the Respiratory Therapy School are almost all in English.

Reinforcing communication with international academics

In 2003, 45 foreign scholars were invited to give speeches or to participate in international seminars.

So far, TMU has contracted with 15 sister schools overseas.

This year, six doctoral candidates are doing research in labs of foreign universities, and their papers will be announced abroad. This is to encourage students and teachers to attend international conferences and to engage in short-term studies and research in foreign universities.

The College of Medicine, Pharmacy, School of Medicine, Institute of Medical Informatics, and Institute of Cellular and Molecular Biology have all held international conferences.

All colleges and departments are encouraged to join international organizations. The School of Medicine has joined the International Students Exchanges Organization's Standing Committee on Research Exchanges (SCORE). This committee provides medical students of every country with the chance to exchange research. The research exchange focuses on two dimensions: research into basic medicine and research into clinical medicine. The period of research is 1~2 months. In addition, professors take part in international organizations on their own behalf.

Provision of appropriate subsidies to schools, colleges, and universities

Last year, the Global Liaison Center provided US$5000 to subsidize students going abroad. This year, the same division has provided US$6000. In addition, five colleges allocate additional budgets for subsidizing student travels.

Internationalization Outcomes

We have promoted each point of the above-described strategies, and here is a description of the outcomes of our efforts.

The status of contracting sister schools

To the present, we have contracted with 16 sister schools: four in America, three in Europe (including universities in Germany and Italy), seven in Japan, and one in China.

List of sister schools

The US: University of Missouri-Kansas City, Kansas City, MO; Loma Linda University; School of Dentistry, University of California, San Francisco, CA; and School of Nursing, University of Illinois, IL.
Germany: Hamburg University of Music, Hamburg and Theatre Charite and University Medicine Serlin.
Italy: University of Florence, Florence.
Japan: Hiroshima University, Hiroshima; The Teikyo University; Health Sciences University of Hokkaido; Tokyo Medical and Dental University, Tokyo; Nihon University; Tohoku Fukushi University, Tokyo; and Tehoko University.
China: The University of Hong Kong.

Overseas internships for TMU Students

In 1999, medical students of TMU began to participate in short-term internships abroad.
In 1999, ten medical students had short-term internships in Sweden, Norway, Denmark, the Czech Republic, and Poland.
In 2000, ten medical students had short-term internships in Sweden, Germany, Austria, Poland, Norway, Japan, and Holland.
In 2001, ten medical students had short-term internships in Sweden, Germany, Norway, and Hungary.
In 2002, eleven medical students had short-term internships in Norway, Germany, Austria, the Czech Republic, and Poland.
In 2003, eight medical students had short-term internships in Germany, Portugal, Slovakia, and Holland.
In 2004, 14 medical students had short-term internships in Germany, Austria, Japan, Italy, Poland, Hungary, and Sweden.
In 2005, seven medical students had short-term internships in Denmark, Swiss, Germany, Mexico, Spain, and Czech Republic.
In 2005, ten dental students had short-term internships at Tokyo Medical and Dental University, Tokyo, Japan.

Foreign students' internships at TMU

Also, from 1999, TMU began to accept foreign students' short-term internships at Taipei Medical University Hospital and Wan-Fang Hospital.
In 1999, eight medical students from Austria, Norway, Sweden, and Germany had internships at Surgery, Internal Medicine, and Obstetrics and Gynecology.
In 2000, ten medical students from Germany, the Czech Republic, Austria,
Hungary, Poland, and Italy had internships at Surgery, Obstetrics and Gynecology, Internal Medicine, Pediatrics, Emergency Room, Psychiatry, Cardiovascular, Plastic Surgery, and General Surgery.
In 2001, ten medical students from Sweden, Germany, Poland, Hungary, and Macedonia had internships at Internal Medicine, Obstetrics and Gynecology, Pediatrics, Emergency Room, Surgery, Neurology, Otorhinolaryngology, Ophthalmology, and Dermatology.
In 2002, 11 medical students from Germany, Japan, Holland, Poland, Portugal, Slovakia, and Sweden had internships at Obstetrics and Gynecology, Internal Medicine, Emergency Room, Pediatrics, Plastic Surgery, Cardiology Surgery, Ophthalmology, General Surgery, and Cardiology.
In 2003, due to SARS, the internships of foreign students were cancelled.
In 2004, seven medical students from Germany, Japan, Italy, Spain, the Czech Republic and Holland had internships at Surgery-General, Alternative Medicine, Surgery Orthopedics, Internal Medicine-Cardiology, Obstetrics and Gynecology, and Cardiovascular Surgery.
In 2005, seven medical students from Austria, Germany, Japan, Holland, and Israel had internships at General Surgery, Internal Medicine-Cardiology, Plastic Surgery, Family Medicine Department, and Chinese Medicine.

Statistics on foreign students at TMU

In addition to the foreign students involved in the short-term internships, TMU also enrolls foreign students to study here.
In 2003, six foreign students from the US and Canada studied at TMU.
In 2004, seven foreign students from the US and Canada studied at TMU.
In 2005, 12 foreign students from the US, Canada, and Japan studied at TMU.
Most of them are medical or dental students.

Other outcomes

Thirty-four full-time teachers were engaged to teach or to do research abroad for a total of 74 times in 2004.
The expertise of these teachers is in Neurology, Cardiac Surgery, Internal Medicine, Healthcare Administration, Nursing, Ophthalmology, Cellular and Molecular Sciences, Pharmacology, and Dentistry.

The countries who invited our full-time teachers included the US, Japan, Korea, Thailand, Egypt, Australia, China, and Belgium.

The following international academic seminars were held.
On April 1-2, 2004 was the 2nd Asian Society for Mitochondrial Research and Medicine. We invited many honored guests from the US, Korea, Singapore, and Japan to give speeches.
In August 2003 was the 2nd Asia-Pacific HL7Conference on Healthcare Information Standards.
In July 2004 was the 3rd Asia-Pacific HL7Conference on Healthcare Information Standards.
In November 2004 was the International Symposium on Genomic Medicine and Molecular Diagnosis – Herbal Medicine Round Table.

Sixty-three professors took part in important international academic activities or assumed a position in an international academic organizations. For example, seven TMU professors participated in the 2nd Scientific Meeting of Asia Society for Mitochondrial Research and Medicine (ASMRM).

Many teachers are reviewers of famous journals.

PLANS AND STRATEGIES O INTERNATIONALIZATION IN THE NEXT 3 YEARS

From the second stage of our effects to promote internationalization, there is still some space to improve. The following is a list of plans and strategies for the next 3 years.

Increase partner universities: Add one university per year.

Encourage students and faculties to participate in international activities: Advertise and announce funding plans.

Establish scholarships for foreign students: Establish scholarship regulations for foreign students and a scholarship committee.

Build up a bilingual environment:

Revise the TMU homepage in English;
Complete English signs and labels on campus;
Write and print a student notebook in English;
Assist foreign students with learning Chinese;
Provide subsidies to foreign students to participate in language exchanges; and group students who are good at English as an English team to be responsible for promoting bilingual activities on campus.

Improve participation in international activities and exchange programs for students and faculty: Initiate cooperation and exchange programs with sister schools.

Improve visiting, study, clinical training, and research of students in foreign countries: Initiate cooperation and exchange programs for students in sister schools, and increase funding sources for international affairs.

Improve visiting, on-the-job training, and advanced study, teaching invited speech and services for faculty in foreign countries: Initiate cooperation and exchange programs for the staff of sister schools, and increase funding sources for international affairs.
Improve visiting, on-the-job training, and advanced study, and participation in activities and services for staff in foreign countries: Initiate cooperation and exchange programs for the staff of sister schools, and increase funding sources for international affairs.

Promote the recruitment of foreign students:

Construct a TMU English website – all departments had completed their web pages in English by February 2005;
Set up a committee and working group to enroll students – Nine representatives members from academic and administration divisions will be in this group;
Extend the scope of enrolling students – loosen constraints, advertise through alumni overseas and foreigners in Taiwan;
Build up a mailing list, cooperate with the recruiting section to send out application Information; and
Allot a part of school fees as scholarship for foreign students – foreign students can apply for two kinds of scholarship: a) scholarship of Taiwan b) scholarship allotted from school fees.

Carry out activities with sister schools:

Review the contracts of sister schools, deal with revising and extending contracts;
Confirm the contract window of sister schools and send a regular magazine of TMU;
List all sister schools that have been contacted by any academic department and then establish a contact network;
Discuss cooperative affairs with sister schools regarding teaching, research; and culture;
Invite related divisions to discuss the execution of cooperative affairs.

South-East Asia Medical Seminar:

Invite experts and scholars in Taiwan and overseas to teach and design new courses for the purpose of developing medical care in South-East Asia;
Establish a database of important medical papers on South-East Asia;
Construct a medical website to provide medical messages for the countries of South-East Asia;
Actively enroll overseas students from South-East Asia and send publications to agencies in Taiwan to promote South-East Asia.

The above is a brief introduction to the internationalization strategies of Taipei Medical University. In truth, we still need to make efforts to promote international affairs and accelerate internationalization. As a medical university, advanced technologies and skills are necessary, because we have to be responsible for the health of the Taiwanese people. So, it is very important to exchange experiences with foreign countries and learn from developed countries; this is what we are doing now. Through everyone's efforts at TMU, we expect that there will be more-outstanding outcomes over the next 3 years.

Curriculum Integration for Dental Education; A Lesson Learned from Real Experiences of Development and Implementation

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ABSTRACT

This paper presents a dental school's experience of curriculum development and the process of implementation. It includes the things that can be expected to happen or that may go beyond expectations in the development of new curriculum for the College of Dentistry (School of Dentistry), Seoul National University

In 2002, the Ministry of Education and Human Resources Development of Korea announced introduction of new school system in which provides medical and dental education after bachelor's degree. This "Professional Graduate School" system is one of the greatest challenges for Korean dental education. Now Korean dental schools chose two different dental education systems from 2005, the six year dental college system and the four year professional dental graduate school system. College of Dentistry, Seoul National University chose the new system. The school also changed curriculum. The change of dental education system is one, but not all of the reason for the curricular reform. Through the sophisticated process of problem identification and needs assessments, we chose the integrated curriculum. A careful planning and organization process were applied. The whole process of curriculum development took ten month. The evaluation is still going on. Even the curriculum intended to apply the solid principles and aims towards sound objectives; the curriculum evaluation in the present is not very encouraging. It casts lots of pending questions. Promising part of the new curriculum has the flexibility and readiness to change of environment.

Keywords: integrated curriculum, dental education, educational reform, problem based learning

BACKGROUND

January 2002, the Ministry of Education and Human Resources Development announced introduction of new school system in which provides medical and dental education after bachelor's degree (undergraduate + 4 system). This "Professional Graduate School" system allows students from a variety of academic backgrounds access to dental education. It might reflect the growing needs of renewal in dental education and general tendency of change in dental education. This is one of the greatest challenges for Korean dental education community. In the roadmap of the Ministry of Education and Human Resources, "Professional Dental Graduate Schools" will be starting from 2005. But the change is not a mandatory at the time. Dental schools can choose the new system until year 2009. 5 dental colleges (4 national schools and a private school) chose the professional school system. And the others decided to remain in the present system. Now, one private school and one national school reverse their initial decision, and total 7 out of 11 dental Schools are going to change into the professional graduate schools.

Now, Korean dental schools have two different dental education systems from 2005, the 6 year dental college system and the 4 year professional dental graduate school system, in which the only candidates with bachelor degree will be admitted. Before 2004 all Korean dental school has 2+4 system. In the 2-year pre-dental course, the curriculum includes several elementary courses for natural and social sciences and liberal arts. The 4-year undergraduate dental curriculum usually has the structure of basic dental sciences in the freshmen and sophomore and clinical courses in the junior and senior. Like many dental schools in the world, this system is adopted by most of the Korean schools with minor differences.

College of Dentistry Seoul National University chose the new system. During the change of school system, we also changed curriculum. But the graduate education isn't the only reason for the change in the curriculum of School of Dentistry, Seoul National University. For many years, the problems in our dental education have been pointed out repeatedly. The problems in the present curricula which warrant reform in the present include that;

1. The courses are heavily didactic (lectures and labs)
2. The courses focus mainly on transferring discipline-based (fragmented) knowledge, rather improving the problem-solving potential of students.
3. The lack of appropriate student evaluation process
4. The lack of linkage between the basic science courses and the clinical courses, as well as among different courses (disciplines).
5. Irrelevant arrangement of the courses in the context of whole curriculum.

All these problems have become more and more serious year by year. The rationale for the reform became prominent and was unavoidable. Its aim was to supply dentists who have basic research and clinical capacity, willingness to serve for the public interest, an ability to educate oneself, and a sense of duty.

Table 1. The changes in Korean dental education system.

Year	No. of Dental College	No. of Students	Academic Years
Before 1959	1	100	4 (dental college)
1959-2004	11	760	2 + 4 (Pre-dental 2 yr)
2005	6	420	2 + 4 (Pre-dental 2 yr)
	5	340	4 + 4 (Graduate school)
2006	5	340	2 + 4 (Pre-dental 2 yr)
	6	420	4 + 4 (Graduate school)
2009	4	240	2 + 4 (Pre-dental 2 yr)
	7	520	4 + 4 (Graduate school)

PRESENT STATUS

The reform of dental education means the re-setting the goal of dental education. It includes clarifying the basic educational goal for new dentists and detailed goal for the individual classes.

Problem Identification and Needs Assessment

For the development of new curriculum, the process should start from the review of available information from published literature, curriculum documents from other schools, and documents of the current curriculum. It also included collection of new information through the surveys of students, faculties and alumni, focus group (general practitioner, students, and faculties) meetings, visit of benchmarked schools, and consultation from experts.

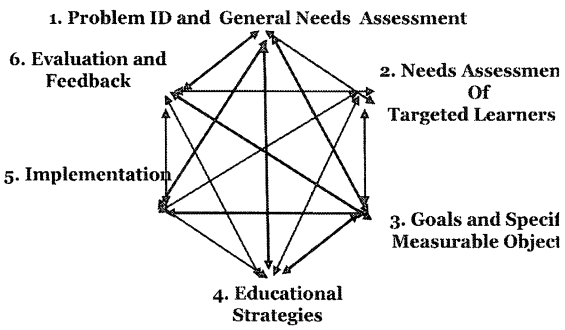

Figure 1. A process of curricular development

Through these processes, we identified problems in the curriculum and found most of them were already well-documented. These problems included;

- Basic science is weakly linked to Clinical science (Marilyn et al., 1995)
- Discipline-based or Department-based curriculum
- Too much redundancy among classes
- A few core clinical skills cannot be covered within current curriculum
- Too little time for students to consolidate concepts and develop critical thinking skills (Marilyn et al. 1995)
- Too little time to develop critical thinking skills

It became evident and agreed upon that the needs for dental curriculum reform in Seoul National University were as follows;

- To Increase the efficiency of dental education in the new millennium
- To reduce the lack of connection between disciplines
- Early exposition of the student to the clinical situation
- Increasing demands for humanism education
- Increasing demands of relevant medical education

There were also heavy demands of reinforcing the clinical education. Concentrated or intensive programs in clinical curriculum are urgent in terms of efficiency of education. Early clinical exposures, community based oral health educations, and comprehensive primary care educations were the main parts of increasing educational demands. After series of task force team meeting and workshops, we decided the directions of changes to integrated curriculum, both vertical and horizontal.

Integrated curriculum

What is an integrated curriculum? Integration does not mean only tie distantly related lectures together, but means completely different way of designing educational processes to proper positioning of the goal of dental education. Job-readiness and school-to-work curriculum model is the one of the good example for professional education. It fulfills the demands of higher levels of communication, and analytical and problem-solving skills. The applied and integrated curriculum also helps more students master the skills in an information-based society.

Integrated education is very efficient in reinforcing the connection between the basic and clinical dentistry, reducing the redundancy or repetition, enhancing the small group based educational program like problem-based learning and promoting the student-centered education. Rationale for integration is already well proven from the evidence from cognitive psychology. Learning in context is the best way considering the students' interest, motivation, and integration of theory and practice.

Integrated Curriculum is intended to acquire knowledge, skills, and attitude as a whole. The courses should be integrated horizontally (integration among courses with same subjects) and vertically (integration between basic science and clinical courses).

Integrated curriculum and goal of dentistry

Final goal of dental education is to serve community (local, global) and care for individual in the context of community & family. Art of dentistry, leadership skill of research and scholarship, life-long learning and teaching, development of professionalism, and service to community should be put together into the educational course of a dentist. This goal can be achieved through weaving of concepts of basic and clinical sciences, culture and social system

Self-directed learning and life-long learning is anoth-

er goal of curricular change. The rapid social and scientific development demands professionals with a capability to know when to change and to self-educate. The curriculum should be constructed to improve the capacity of dental students to search and analyze data by oneself and to make the right decision based on it. Professionalism is another issue to contend. The communication skills, decision-making skill, morality and sense of social duty are needed for dental health professionals.

Redefine the Mission and Goal of Education

Our mission of dental education was summarized like "the educational objectives of the undergraduate D.D.S. program are to develop individuals capable of undertaking comprehensive patient oral care, basic dental research, and social responsibilities." Principles for new curriculum development included making a new goal of education, defining the precise contents of education, integrated curriculum with core courses and elective courses.

Goals of education temporarily defined as "The goal of dental education is to supply dentists who have basic research and clinical capacity, willingness to serve for the public interest, capacity to self-educate, and a sense of duty." The goals for the whole curricula, as well as specific goals of each course, were addressed, which needed to be managed systemically. The contents of education described in the form of knowledge / skills / attitudes with promotion of self-directed learning, problem solving and decision making

Designing Issues

In the designing of the integrated curriculum, integration does not mean physical aggregation of teaching in the classroom. Careful arrangement and sequencing of specific subject to coincide with one another is essential. Inter-faculty cooperation and pedagogical ground work is necessary. In the designing an integrated curriculum, systemic connectivity based on descending organization is important.

Integration of the basic science courses and the clinical courses were the most important part to allow the conceptualization of the knowledge. Problem-based learning opportunities to the students through clinical case studies were considered to promote the capability (knowledge, skills, and attitude) as a general dentist. It was well-proven method for making self-directed and active learners, who would continuously absorb cutting-edge knowledge and solve the problems that he/she would meet as a dentist. To increase the lateral connectivity among the integrated courses, the related topic in different courses placed to teach in close temporal proximity. We defined the general principles of curriculum development which can be grouped as integration (horizontal and vertical), self-directed learning, life-long learning, professionalism and community-based education with continuous quality improvement

In the clinical education, problem-sensing and solving skill in small group or solo-practice situation was the goal for the curriculum. Early clinical exposure starting from the first semester of dental school was regarded important to develop communication skill and professional ethics.

Elective courses were included for the expanded knowledge. Externship and research projects were the choice. Elective courses included were researches, social sciences, and externships programs in a domestic and foreign dental schools and institutions. Student researches were prepared with early designation of research advisors.

For the general organization of integrated curriculum, consistent and coherent guiding principle were necessary and multi-departmental consensus group were developed for setting the contents of integrated curriculum and final goal of dental education (not from disciplinary or departmental concern)

Organization for the Curriculum management

For the successful implementation of a new curriculum, the need of administrative organization for planning, monitoring, and coordination the integrated curriculum was great. The "education committee" makes plans, controls and monitors the whole curriculum, evaluate the educational plan of each integrated course, and control the relationship among different integrated courses. The "management committees" in each integrated course take charge of setting goals, deciding the contents, operational methods and evaluation time/methods for each course. The "office of education" develops teaching methods, materials, and evaluation methods. We put these three kind of different organizations into the curriculum development and maintenance.

Evaluation of curriculum

Evaluation and quality management of curriculum are indispensable. To improve the education system, repetitive evaluation and reform processes were critical. The new education system evaluation should start upon initiation with continuous amendments.

Evaluations of education consisted of curricular evaluation and student assessment. Both were integral part of curricular development and implementation. Curricular evaluation should be practical to achieve the educational goals. Scope of evaluation of curriculum included objectives, repetitions, and omissions during the integration. It also included the effectiveness of teaching methods, appropriateness of assessment, student's achievement of affective domain (attitude) and the educational effort of faculty and staff. The integrated courses progressed as planned by comparing the education plan (teaching goal, learning goal, evaluation, and weekly progress plan) and the data collected (student's achievements, student' appraisal, and lecturer's appraisal).

For student assessment we should consider that the goal of evaluation is not grading students nor making dropouts. It is to monitor the educational progress from the point of lectures' as well as students' view, to

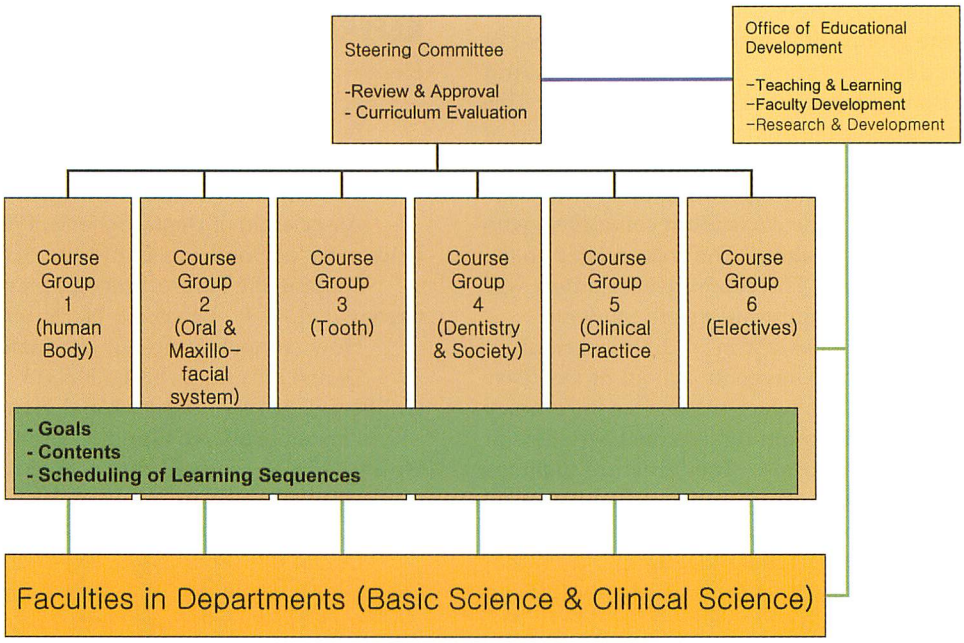

Figure 2. Organizational structure of dental education

improve the quality of education, and to assess the students' application capability of the knowledge that he/she had obtained during the course. We designed tests which will evaluate students' capacity and progress as a whole. The assessment methods included formative assessment, portfolio assessment, structured interview and OSCE.

Development process for 2004-2005 dental curriculums

The process started at 2002 April after acceptance of the new school system. Government provided research funds for study of the new education system. Careful research works started in co-operation of five dental schools who decided to change. The report was published in the early 2003. It contained lists of suggestion for admission and education in professional dental graduate schools. In April of 2003, sub-committee for development of new dental curriculum organized and had its first meeting on the direction of the curriculum change. More than 12 meetings and 3 workshops including a general faculty development workshop were held until September. The first formal report for the plan of new curriculum came out in July and three formal revisions were reported. At September 2003, the organizing and steering committee for new curriculum started and detailed curriculum writing process began. 14 scheduled meeting and several informal meeting of the committee were held until November. Final curriculum draft came out in November. Several meetings of department chairmen and education committee were necessary before the final approval in the general faculty meeting.

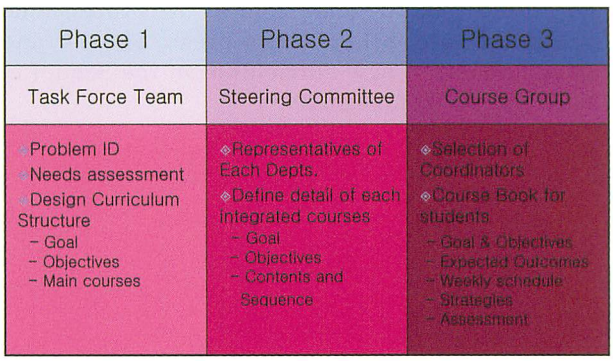

Figure 3. Schematic representation of the development process

DISCUSSION

Findings and remaining tasks includes faculties' indifference toward educational mission, change of professor teacher-centered curriculum into the student-centered one, and faculty development. Basic structure of integrated curriculum progresses from the study of the basic foundations of dentistry to applications in supervised patient management and solo performance of practice.

Leadership groups should be organized to support professional development and leadership skills through collaborative group learning activities that relate personal experience to organized studies. Students should be regarded as highly motivated for their choice of studies and responsible for their own learning. The learning process should encourage student motivation. Learning in dental school is a social process between students/teachers. It is also an individual process. A student has real co-responsibility for their own progress, and faculty should provide facilities suitable for group and individual learning.

There are varieties of methods of teaching. It includes lectures, Seminars, small group practices, lab courses (dissection, microscopy, and statistics lab) and conference. Problem based learning (PBL) is not just

method of teaching, but principle of teaching. It can be designed for the groups of 7-8 students with purpose of enhancing student activation, cooperative skills, and individual reasoning through collective reflections. This may provides theoretical learning objectives by means of "real" patient. We hope it can provide firm basis for life long learning. Planning and organizing of the new curriculum was centered to the curriculum committee, who deals with all kind of questions. The tasks related to the planning and organizing. The committee members will be pedagogic advisor, administrative staff, Students.

In conclusion, the basic aim of 2004 curriculum reform in Seoul National University School of Dentistry is an integrated curriculum which maximizes the efficacy of education through the multi-dimensional synthesis of knowledge, skill, and attitude. The structure includes horizontal integration (interdisciplinary) and vertical integration (basic-clinical science). The self-directed learning heads toward the "skill of learning" for rapidly evolving "new dentistry" through the motivation and responsibility of students.

ACKNOWLEDGEMENTS

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Airlangga University Dental Education System: Present and Future Plan

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ABSTRACT

Airlangga University Dental Education started 51 years ago on November 10th 1954. Since then many changes have been made for a better educational system. The vision of Airlangga University Faculty of Dentistry is to be an independent and accountable institution and to reach the international education standard in dentistry science and technology. Its mission is to educate and teach dentistry with basic medical and dentistry sciences in order to increase the level of community health services in general and the stomatognatic function in particular. The future aims are to do diagnostic preventative research with a predictive medicine approach, and to provide better community health services. The faculty of dentistry has four dental education degrees, which are: Doctor Dental Surgeon (DDS), dental laboratory technician, Master of Science (MS), and dental specialist (e.g. pediatric dentist, oral surgeon etc). Dental education for the DDS degree takes 5 years (10 semesters) with a total of 177 semester credit points. Now the curriculum of dental education is changing step by step to a competence based curriculum. Education and research collaboration with other faculties of dentistry is being conducted including with Hiroshima University.

Key words: Airlangga University, dental education, Indonesia

INTRODUCTION

Airlangga University was founded according to government regulation No.57/1954 and inaugurated by the president of the Republic of Indonesia on November 10th 1954. Airlangga University was presented to the city of Surabaya to express appreciation for the sacrifices and merits performed by Surabaya's youth in their independence struggle in 1945. The name of "Airlangga" for the new university was chosen in honor of an Indonesian hero who, at the beginning of the 11th century, built a national state covering the eastern part of Indonesia. Airlangga, which means "drinker of water", is the name of a king ruling over East Java from 1019 until 1042. He might have been born in Bali, because when he was born in 1001 his parents reigned over Bali representing the King of Java. After his death (1042) he was immortalized in a statue, representing Airlangga as Batara Wishnu riding the Garuda. King Airlangga used a seal representing the bird Garuda carrying an urn. It was said that urn contained "amrta" the water of eternal life. Those who after death are given amrta will be recalled to life. Those who drink amrta will live the eternal life. Now 900 years later, we continue to honor Airlangga. And therefore we expect every member of the Airlangga Community always to praise the name of Airlangga and to uphold ideals.

At the beginning of dental education in Indonesia, Surabaya city played an important role. Dental education started when Dr.Lonkhuizen - Minister of the community health department - planned to build a dental school in Surabaya. Dr.RJF Van Zaben the director of NIAS (Nederland Indische Artsen School) was asked by Dr.Lonkhuizen to lead the dental school in Surabaya. The first dental education in Surabaya started on September 28th 1928 under the name of School Tot Opleiding van Indiche Tandartsen (STOVIT 1928-1942). In the first academic year 21 students studied for 5 years including 3 years clinical education.

Under the Japanese government in 1942-1945, STOVIT was changed into Ika Daigaku Shika. The first director was Dr.Takeda and the second one was Prof. Imagawa. After The Japanese lost in World War II, the Netherland Indische Civil Administration (NICA) governed Indonesia. In 1947 Ika Daigaku Shika changed into Tandheelkundig Instituut (TI) with Dr.JM.Klinkhamer Sr as the director. A year later it changed again to Universiteir Tandheelkundig Instituut (UTI) with Facultiet voor Geneeskunde under the University of Indonesia in Surabaya. History continued, after 9 years Indonesia got independence, Airlangga University was founded by The First President of The Republic of Indonesia Soekarno on November 10th 1954. Since then the faculty of dentistry has separated from the University of Indonesia to be a part of Airlangga University. The dental education study period was 5 years long.

The dental education was changed into 6 levels in 1969, but in 1987 the curriculum was revised again to 5 years which divided into 10 semesters. Since then the dental education in faculty of dentistry Airlangga University has used a Semester Credit System. Some revisions have been made to provide a better educational system. The Comprehensive Evaluation System (CES), clinical practicum and community health service experience are the priorities of the educational system of faculty of dentistry Airlangga University. CES is the educational system that conducts the evaluation of study continuously for a semester. It does this through grades and scores for work such as personal assignments, quizzes, mid semester tests and final semester tests, so the students can be monitored continuously.

Clinical practicum and community health service experience are a learning process to understanding the health problems inside and outside campus, in order to make the students ready to provide a community health service.

Now the faculty of dentistry Airlangga University has 4 dental educational programs, which are:

1. Doctor Dental Surgeon (DDS) degree started in 1954. The aim is to educate the students to be a dentist. The education program is divided into 2 phases, which are academic education (Bachelor Dental Surgeon) and dental professional education (Doctor Dental Surgeon) with a total of 177 semester credit points for 10 semesters.
2. Dental Technician Program (Diploma Program). The first Dental Technician Educational Program in Indonesia started in 1984. The educational aim is to educate the students to be Dental Technicians, who have the ability and skill to perform and manage dental laboratory work.
3. Dental Specialist Educational Program. This program has been conducted since 1984 with 7 dental specialist programs, which are pediatric dentistry (pediatric dentist), orthodontics (orthodontist), operative dentistry, periodontics (periodontist), prosthodontics (prosthodontist), oral surgery (oral surgeon), and oral medicine.
4. Master of Science (MS) degree.

Dental education in the Faculty of Dentistry Airlangga University is more than 50 years old now. It has 1.100 students with 204 teaching staff (10 professors, 5 emeritus professors, 25 PhD degrees, 180 Master degrees, and dental specialists in their field) and 153 employees.

Dental Education Facilities

To promote the dental education process, the Faculty of Dentistry Airlangga University has some facilities, which also promote the students' activities. It has not only main lecture rooms, seminar rooms, laboratory and clinic for dental treatment, a dental library, but also an internet room for students, prayer room, cafeteria and dental suppliers.

The Faculty of Dentistry Airlangga University also has the dental hospital to serve community dental needs and to prepare the graduate to be a professional and skillful dentist. Dental technique laboratories are provided for the pre clinic students.

Community health service is conducted in community hospitals and general hospitals at several prefectures in East Java e.g. Banyuwangi, Kediri, Jember, Lumajang etc. The aim of this program is to prepare the students to be able to solve community dental problems. To promote the community dental health service we have a mobile dental unit. This facility is often used by staff members and the students to do the social work and dental health educational activity in and out of Surabaya.

In order to publish the research reports, case studies and literature studies we have a scientific magazine (Dental Journal) which is issued 4 times a year. The Dental Journal is one of few dental journals in Indonesia which has good accreditation from the Indonesian government. The contributors of the dental journal are not only from the staff members and students of Airlangga University, but also from the other dental faculties in Indonesia.

Collaboration with other institutions

The relationship and collaboration with other national and international institutions is conducted to promote the education quality process. It was started in 1959. It started when 12 academic staff members were sent to the University of California to join the USAID educational program. After that, some programs were conducted with the University Hospital in Manchester, Turner Dental School in England, Hiroshima University, Kyushu University, Kagoshima University in Japan, Erlangen & Hamburg University, University of London, New South Wales University, University of Sidney, International Course in Cosmetic & Aesthetic Dentistry in South Jordan Salt Lake City Utah USA, Hereaus Kultzer Hanau Germany and University Kebangsaan Malaysia. Recently we have had collaboration programs with the University of Malaysia and University Sains of Malaysia. In Indonesia, we also have collaboration programs with other national and private Universities and institutions, e.g. Dr. Soetomo Hospital, East Java Province government, Surabaya city government, PTPN XII (persero), Marine Hospital Surabaya and Unilever Indonesia Ltd

Dental Education System

The vision of Airlangga University Faculty of Dentistry is to be an independent and accountable institution and reach the international education standard in dentistry science and technology. Its mission is to educate and teach dentistry with basic medical and dentistry sciences in order to increase the level of community health services in general and the stomatognatic function in particular. The future aims are to do diagnostic preventative research with a predictive medicine approach, and to provide better community health services.

Dental education is being conducted using a semester credit point system. Semester credit points are used to measure the study content, the success of the students, and to measure how much effort must be made, not only by the students but also by the teaching staff. An academic year is divided in two semesters. Each semester contains 16 to 17 curricular weeks and includes lectures, working in the dental laboratory and dental clinic, public dental health services, research seminars, thesis or scientific writing, and a final semester exam.

The dental education program has two phases of programs, the academic program (147 semester credit points) and the professional program (30 semester credit points).

The academic programs are

1. General lectures (30 semester credit points): Humanities/ Philosophy, Methodology, Ethics and Medical Law (12 SKS), Basic Medical Sciences, (10 semester credit points), Behaviorism (2 semester credit points), Social Sciences (4 semester credit points), English (2 semester credit points).
2. Basic Professional Sciences (53 semester credit points) : Biomedical Sciences (32 semester credit points), Basic Dentistry Sciences (21 semester credit

points)
3. Professional Sciences (57 semester credit points): Clinical Medical Sciences (14 semester credit points), Preventive Dentistry/ Dental Public Health (7 semester credit points), Clinical Dentistry Sciences (36 semester credit points).
4. Thesis (4 semester credit points)
5. Public Work Services (3 semester credit points)

After finishing the academic programs (147 semester credit points), the students receive the Bachelor of Dental Surgeon degree.

The professional programs contain 30 semester credit points. It is a clinical learning experience and field learning experience. After finishing these programs, the students qualify for the Doctor Dental Surgeon degree.

Each student can decide by themselves how many semester credit points they can handle in a semester, after a consultation session with their academic mentor. If at the end of the semester, the student can pass with good grades, they are allowed to take extra semester credit points for the next semester. The number of semester credit points obtained by the student each semester is based on the semester grade index

Semester grade index	Number of Semester credit points
≧3,00	22-23
2,50～2,99	20-21
2,00～2,49	17-19
≦1,99	12-16

The Development of Dental Science and Technology in Airlangga University Faculty of Dentistry

The Faculty of Dentistry Airlangga University has specific goals for dental science and technology development. The developments of dental science and technology in Airlangga University Faculty of Dentistry are based on

a. The change of health paradigm from ill to healthy paradigm toward the predictive medicine era.
b. The changes of Diagnostic Symptomatic to Preventive Diagnostic, which show the diagnostic technology using hemodynamic, molecular biology, immunology, and psychoneurology. Thus Airlangga University Faculty of Dentistry develops preventive diagnostics using predictive medicine as a special program.

Preventive Diagnostic is the diagnostic technology based on not only symptomatic but also the hemodynamic study, thus it could be used to predict the future anomaly, the success of therapy (planning to prevent the anomaly or the unsuccessful therapy).

Predictive medicine is the new concept of prediction of the disease in each patient from the profile data with an accuracy of 99%. It predicts the patient's risk and pretherapeutic risk through the human genome study.

Preventive Points

The preventive point of the development of dental education and technology in Airlangga University is prioritizing the understanding, development, and application of preventive dentistry. The priorities are:

1. The development of diagnostic technology using oral fluids, dental tissue and oral tissue as preventive diagnostic materials.
2. Mastering the dento-maxillofacial growth as a basic of preventive malocclusion, and dento-maxillofacial anomaly
3. Technology development of caries vaccine, antiplaque agents, fluor application
4. Mastering the Dental Health Behavior, Dental Epidemiology as one of the basic promotive and preventive dentistry

Curative points

The curative point of the development of dental science and technology in Airlangga University is to improve the understanding of curative dentistry, and to develop and apply it, e.g. treatment of oro-maxillofacial trauma, congenital defect, neoplasm, orthognatic surgery, and osteodistraction.

1. Mastering and improving the study of bonding agent and restorative materials, laser technology for hard and soft tissue, electronic anesthesia, endodontic microsurgery, aesthetic dentistry, tissue engineering, and understanding its biological conditions
2. Mastering and improving the study of children's fear management
3. Mastering and improving the study of the Oral Health Impact Profile in order to improve the effectiveness of management of dental services
4. Mastering and improving the study of biocompatibility and material tests in order to standardize dental materials

Rehabilitative point

The rehabilitative point of dental science and technology taught at Airlangga University is to improve understanding, development and application of rehabilitative dentistry.

1. Mastering and improving the study and development of dental materials which are related to aesthetic reconstruction, stomatognatic function reconstruction, social impact reconstruction, and the safety rehabilitative appliance (the materials and the biocompatibility.
2. Mastering and improving the study and development of dental implant and tissue engineering in rehabilitative dentistry

The policy of Airlangga University Faculty of Dentistry

Airlangga University Faculty of Dentistry as the national research institution, develops and applies dental science and technology, and provides human resources. This institution must be backed up with the professionals and scientists to provide the high quality human resources. Airlangga University Faculty of Dentistry has the obligation to improve dental education, dental research, and dental public health services with up-to-date date science

and technology.

In order to realize its vision to be a dental institution with preventive diagnostic using predictive medicine, Airlangga University Faculty of Dentistry has put together a basic academic plan. That academic plan must be facilitated by the management plan, which always follows the dynamic development of dental science and technology. The harmonious combination of academic plan and management plan is the key to be a high quality dental institution.

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Dental Hygienist Education in the New Era

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HISTORICAL BACKGROUND

More than half a century has passed since the enactment of the Dental Hygienist Law in 1948. Initially, only dental prophylactic procedures were included in the service provided by dental hygienists, but dental clinical assistance and dental health guidance were added thereto in 1955 and 1989, respectively. In 1949, when dental hygienist education started, the training course was stipulated by the Law to last at least one year, but as a result of the revision of the Law in 1983, the term was prolonged to two years or more and the contents of the education also underwent a thorough revision. After this revision, the course load of the three main subjects, that is, dental clinical assistance, dental prophylactic procedures and dental health guidance, came to account for 24 % of that of the obligatory subjects. Thus, the opportunities for dental hygienists to give health guidance and take prophylactic measures against dental caries and periodontal disease on a continuous basis in dental clinics have increased; also, as a result of the revision of the dental clinical remuneration tariff, fees for guidance, superintendence, etc. provided by the dental hygienists for periodontal disease have been included. Then, as a result of the 8020 campaign (retain at least 20 teeth at 80 years of age), both home dental clinical service and dental health service for grown-ups and the elderly have spread, and the needs for dental clinical care have become diversified and sophisticated. The above-mentioned commission was set up to review the contents of the dental hygienist education including practical training. Since dental hygienists must now have the expertise and skills necessary for continuous guidance on and management of caries, periodontal disease, etc., for taking care of patients and those who require nursing care or have various diseases, and for home-visit dental health guidance and community health activities, a total of at least 2,570 hours is needed to cover the entire course. Accordingly, the commission has recommended that the course term be set to at least 3 years.

Subjects such as dental care for the elderly, dental care for the disabled, home-visiting health guidance and nursing care techniques have been added to the obligatory subjects. We think that it is necessary to educate and train people who can carry out general management in collaboration with the patients themselves, their families and healthcare and welfare personnel; our aim is to foster dental hygienists with a social welfare perspective.

Under such circumstances, it has become urgently necessary to educate and train dental hygienist apprentices properly, so that they can fall back on a wide range of expertise and sophisticated skills. Thus, four-year faculties which educate future generations of dental hygienists have been set up at Niigata University and Tokyo Medical and Dental University in fiscal 2004 and at Hiroshima University in fiscal 2005.

The change in disease incidence and the role of dental hygienists

The incidence rate of caries is continuously decreasing, whereas that of periodontal disease remains high. The fact-finding survey of dental diseases conducted in 1999 by the Ministry of Health, Labor and Welfare showed that about one third of the people surveyed between 35 and 44 years of age suffer from advanced periodontitis. Symptoms of periodontal disease were observed in more than 80 % of the people surveyed in this age range, including those with gingivitis whose symptoms were limited to the gingiva.

The tasks of dental hygienists, whose main role in the past was to assist a dentist in treating a patient in a dental clinic, have shifted from therapeutic to preventive ones; furthermore, dental hygienists have come to play a central role in the management of oral care of patients.

Large-scale epidemiologic researches in recent years have revealed that periodontal disease influence systemic diseases such as ischemic heart disease, diabetes and pneumonia as well as diseases in the teeth and periodontal tissues. Among those, diabetes is of particular importance. Diabetes is rapidly increasing in Japan and throughout the world. According to the national survey by the Ministry of Health, Labor and Welfare in 2002, there is an estimated seven million patients with diabetes and there are probably another six million people with borderline diabetes. Ischemic heart disease is the second most common cause of death in Japan.

It has been reported, for example, that the treatment of periodontal disease brought about the lowering of the highly sensitive CRP (C-reactive protein) value, which is a risk marker of ischemic heart disease (Yamazaki *et al.*, 2005), and the lowering of the HbA1c value in diabetics (Iwamoto *et al.*, 2003). Also, there were cases in which dentists suspected that their patients were suffering from diabetes because they were affected by serious periodontal disease, and eventually, their suspicion proved to be true. The above facts show that the treatment and prevention of oral diseases will bring about the promotion of systemic health and contribute to the improvement of the quality of life (QOL). At the same time, those engaging in dental clinical care should have sufficient knowledge of such systemic diseases. As stated above, a wide knowledge of medicine as well as a knowledge of dental diseases, dental therapies, dental materials and dental health has become necessary.

The ever-increasing number of elderly people and those in need of nursing care

Japan of today has become an aging society in which elderly people, 65 years of age or older account for one sixth of the population. The country is now confronting a great challenge: to provide nursing care for the bedridden people and to prevent people from becoming bedridden. Care-givers have reported that a high QOL is maintained by keeping and improving the eating function of the care receivers. Also, it has been reported that elderly persons and sickly persons are apt to suffer from aspiration pneumonia at mealtimes, especially when oral hygienic management is insufficient, and the mortality rate therefrom is high. However, if care-givers have proper knowledge of the oral physiology, they will be able to prevent aspiration pneumonia in the care receivers, who will also be able to eat food with pleasure through oral care; that is, high-quality nursing care will become possible. However, in terms of high-quality medical care, the number of the personnel engaging in consistent medical care, including nursing care, and in nursing care for sickly persons is quite insufficient. For these reasons, experts in nursing care and welfare, who have advanced professional education and training in oral hygiene, so-called social welfare counselors, are needed.

The activities of the Department of Oral Health and Welfare, Niigata University Faculty of Dentistry

As stated above, it is evident that the treatment and prevention of oral diseases and the maintenance and promotion of oral health are directly related to systemic health and QOL as well as oral health. These days, people who have enough knowledge of medical care and sufficiently understand the concept of nursing care are much needed on the nursing care scene. So, it is urgently necessary to set up educational and research institutions in the field of welfare and nursing care, with an emphasis on dentistry. This will foster those who will play a leading role on nursing care.

The Department of Oral Health and Welfare, with its quest for improving everyone's QOL based on proper eating conditions and habits, was set up to foster experts licensed in social welfare counseling and dental hygiene who can manage health care, medical care and welfare in a comprehensive manner. They must empathize with persons in need of nursing care, handicapped persons and their family members, and make the most of their sophisticated expertise regarding oral care, food intake and swallowing. They must help to create an environment in which the appropriate health care, medical care and welfare services truly needed by the persons requiring nursing care, handicapped persons, etc. can be comprehensively given. Upon graduating from the Department of Oral Health and Welfare, they will obtain the qualifications necessary to sit the national examinations for both dental hygiene and social welfare counseling.

Adoption of PBL (problem-based learning)

We have adopted an educational method called PBL for professional education; it is introduced in the second year of the course term. In the past, education was a process in which as much knowledge as possible was conveyed to students, who were then allowed to absorb it. Therefore, "lectures," which can simultaneously disseminate a great amount of knowledge to many students, were delivered, and knowledge-cramming curricula or curricula for each subject were designed. Those students who had good memorizing skills and fulfill many assigned tasks, as per the instructions of teachers, were given excellent scholastic marks. However, this concept of education is unwarrantable today, because the amount of information is now far greater than the human processing ability. Furthermore, new information is being generated very rapidly; for example, the knowledge accumulated by the time of graduation from the university will not be valid after 5 to 10 years. Accordingly, today's education must emphasize the fostering of human resources who can identify problems accurately and unaided, cope therewith and respond to change in an appropriate manner under any circumstances. This new concept of education is especially important in the medical and dental fields. Since in the fields of medicine and dentistry, knowledge and technology are progressing constantly, health care professionals should have the ability to collect and analyze new information, evaluate the effects of new diagnostic techniques or therapies, evaluate costs and risks, gain an insight into the changes in the social/disease structure, and others. The Department of Oral Health and Welfare of the Niigata University Faculty of Dentistry has drastically changed its educational policy from fostering useful people who have acquired abundant knowledge and skills by the time they graduate to fostering people with superb communication skills, problem-finding and problem-solving abilities and the ability to provide medical care based on reasonable judgments made from the perspective of lifelong learning. We decided to implement this policy change in order to foster medical care professionals who can survive in today's rapidly changing society, and we assume that this policy change also agrees with the emergent needs of society.

Now that the objectives of education, that is, the whole concept of human resource which we want to foster, have changed, we must of course adopt the most appropriate means of educating our human resources. We have introduced PBL in professional education. Under the existing circumstances, PBL is considered to be the most appropriate means of education, because it is effective in imparting deeply integrated knowledge, problem-analyzing and problem-solving skills, skills in handling human relationships, the motivation to learn continuously, etc. There are various types of PBL, but generally speaking it is a means of imparting knowledge, skills and attitude based on the learners' own responsibility, with real-world events as a start; the learner selects problems in a real-world situation and solves them by discussing them with a small group of people.

There is a wise saying by William Osler, who left his mark as a reformer of modern medical education in North America and is also considered to be the proponent of clinical clerkship: "In what may be called the natural method of teaching, the student begins with the

patient, continues with the patient, and ends his studies with the patient. This saying expresses accurately the philosophy of the PBL tutorial as well as that of clinical clerkship. In other words, according to the concept of PBL tutorial, students come to grips with a wide range of problems, including social, economic, psychological and epidemiological, instead of learning only from biomedical models. This direction agrees exactly with ours.

Practical training at social welfare facilities

Learning from the experience of apprenticeship is quite meaningful, because it reveals how students can make the most of their learning on the practical medical-care or nursing-care scene, and how they can put into action their problem-finding and problem-solving skills acquired using the PBL tutorial. The Department of Oral Health and Welfare is actively introducing practical training at special nursing homes for the elderly, etc. into its curriculum.

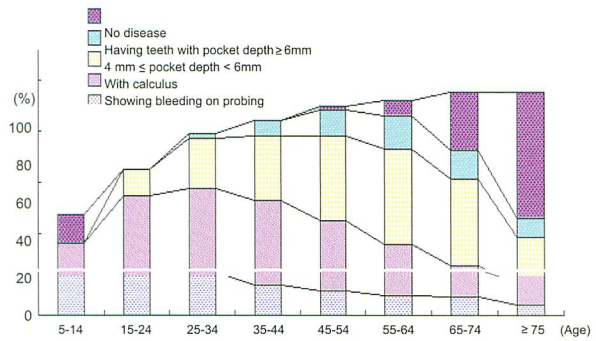

Fig. 1: Periodontal disease status in Japanese population.

Table. 1 Systemic diseases for which periodontal disease is considered to be risk

- Cardiovascular disease and atherosclerosis
- Diabetes mellitus
- Respiratory disease
- Adverse pregnancy outcome
- Infective endocarditis
- Osteoporosis

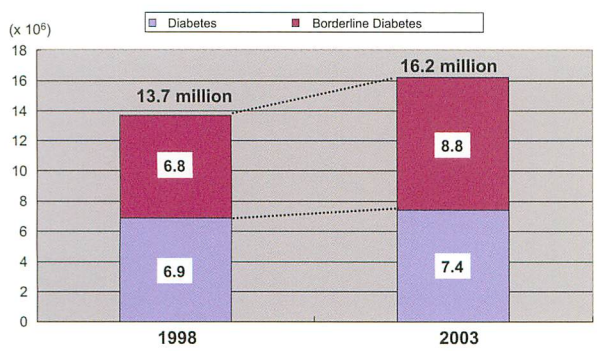

Fig. 2: Estimated diabetes patients in Japan.

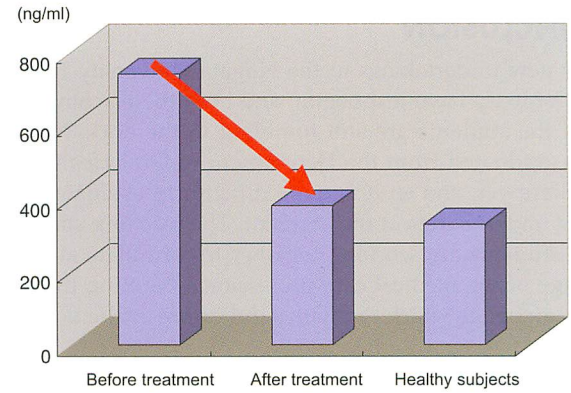

Fig. 3: Effect of periodontal treatment on serum high sensitivity C-reactive protein (hs-CRP) level. Successful periodontal treatment decreased serum level of hs-CRP. Data represent mean of 24 patients with moderate to advanced periodontitis and 21 healthy control subjects.

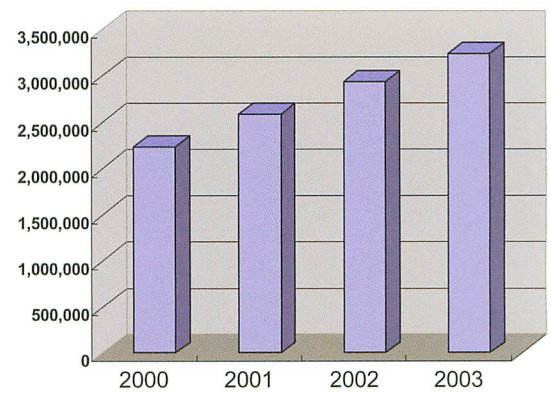

Fig. 4: The number of people needs for nursing care.

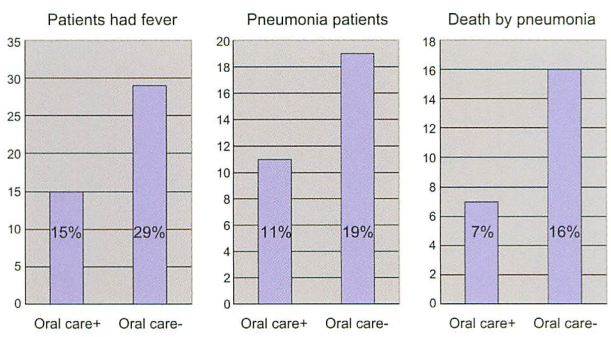

Fig. 5: Effects of oral care at nursing home. The data show that oral care can prevent aspiration pneumonia.

CONCLUSION

The new undertakings at the Niigata University Faculty of Dentistry have just begun, and both the teaching staff and the students are still fumbling in the dark. No one has graduated from the Department of Oral Health and Welfare yet, and so it is quite difficult to evaluate these new undertakings at the moment. The teaching staff and the students are working together to formulate an ideal image of the medical care professional befitting the new age, an image which can withstand future evaluation.

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Innovation of Dental Education System for Researcher, Dentist, Dental Hygienist and Dental Technician in Hiroshima University

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ABSTRACT

Hiroshima University Faculty of Dentistry's goal is to become the worldwide research and education center in Dentistry. It seems to constitute the dental education by two missions: core and characteristic. Hiroshima University Faculty of Dentistry has clarified their characteristic mission and improved dental education system to reinforce the innovative part of the education. We started two programs to cultivate researchers/educators who will be a world-wide leader of dental research and education in addition to excellent dental practitioners, the frontier dental science course and the advanced dental clinician course in 2000. The students who completed new education system gave good score to the frontier dental science. The basic clinical knowledge level of students is the same between two courses before starting clinical training. Hiroshima University Faculty of Dentistry had School for Dental Hygienists and Dental Technicians School. These two schools are integrated and reorganized to the School of Oral Health Science in 2005. Hiroshima University firstly established the School of Oral Health Science with four-year programs for dental hygienists and dental technicians in Japan. The liberal arts education is reinforced in both four-year programs. The purpose of our four-year program in oral health science is to cultivate educators in dental hygienists school, researchers in Universities, and nursing teachers who work sickbay of elementary or junior high school. The purpose of our four-year program in oral health engineering is to cultivate educators in dental technicians school and researchers in Universities or dental materials/products companies in addition to excellent dental technicians.

INTRODUCTION

Hiroshima University Faculty of Dentistry's goal is to become the worldwide research and education center in Dentistry. It seems to constitute the dental education by two missions: core and characteristic. The core mission guarantees the educational quality, namely the medical quality in the dentistry education, and the characteristic mission contributes to the advance of dentistry. Hiroshima University Faculty of Dentistry has decided characteristic mission and improved dental education system to clarify the innovative part of the education. We started the programs to cultivate researchers/educators who will be a world-wide leader of dental research and education in addition to excellent dental practitioners in 2000. Also, the education for dental co-stuff is very important to support high level dental treatment because many advanced technologies are introduced rapidly into dental treatment. Common people tend to have interests on prevention of disease, welfare and esthetic treatment because the view of health is dramatically changed in Japanese society. Hiroshima University Faculty of Dentistry had School for Dental Hygienists and Dental Technicians School. These two schools are integrated and reorganized to the School of Oral Health Science in 2005. Hiroshima University firstly established the School of Oral Health Science with four-year programs for dental hygienists and dental technicians in Japan. Here, we introduce our innovated dental education systems for researchers, dentists, dental hygienists and dental technicians.

Characteristic programs in School of Dentistry, Hiroshima University Faculty of Dentistry

Hiroshima University Faculty of Dentistry was established in 1965. At that time, dental caries and missing tooth and the shortage of dental practitioners were the biggest problem in Japan. Therefore, many dental colleges and dental schools have been newly established and many dentists have been cultivated in 1970s. Now, dental caries in childhood is almost overcome in Japan. It is generally speaking that we are in a supply-demand problem, surplus of dentist. Further, the problems on universal health insurance coverage in Japan resulted in low quality of treatment, because national insurance covers only basic and classical treatment, but does not cover high quality advanced treatments. Patients always request high-quality dental treatment. Recently, view of health has been changed dramatically in Japan; people desire to maintain their youth and beautifulness especially in aged people. These backgrounds of present dentistry and society encouraged Hiroshima University Faculty of Dentistry to reorganize undergraduate education system into two different education courses, the frontier dental science course and the advanced dental clinician course, in school of dentistry in 2000 (Table 1). Students select a course until the end of third grade and

Table. 1 Education system in Hiroshima University Faculty of Dentistry

School	Course	Number	License
Dentistry (60)	Frontier dental science	15	Dentist
	Advanced dental clinician	45	Dentist
Oral health Science (40)	Oral health science	20	Dental hygienist Nursing teacher (optional)
	Oral health engineering	20	Dental technician

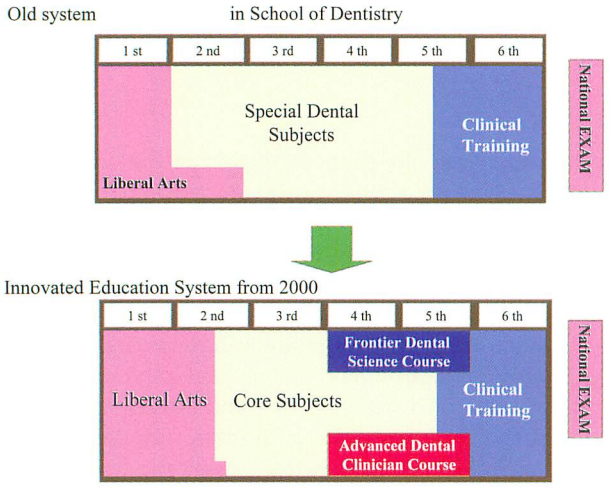

Fig. 1. Innovated Curriculum in School of Dentistry

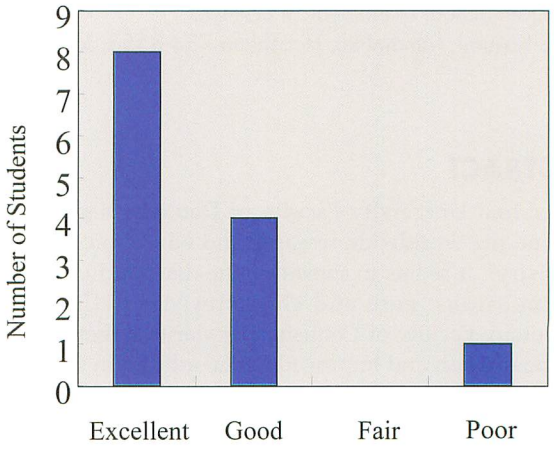

Fig. 2. Questionnaire survey on the frontier dental science course
Fig. 2. Questionnaire survey on the frontier dental science

move up to different course from the fourth grade. The curriculum for each course is composed by core curriculum and characteristic curriculum (Fig. 1). The core curriculum is common to both courses and composed by educational requirements for passing the national dentist examination. The characteristic curriculum is served for one and half year, form the first semester of the fourth grade to the first semester of the fifth grade.

Frontier dental science course

The students in the frontier dental science course (15 students), the educational curriculum was prepared to cultivate researchers/educators who will be a world-wide leader of dental research and education. The students in the frontier dental science course will basically enter to the graduate school to continue their research and complete Ph.D. program. The students select a major from basic dental sciences: oral biology, oral growth and developmental biology, bacteriology, dental and medical biochemistry, mucosal immunology, biomaterials science, oral physiology, dental pharmacology, and oral maxillofacial pathology. They learn basic concept of research, basic research technique, presentation methods, and scientific paper preparation etc. by small-group instruction. Each student has a simple research project.

Advanced dental clinician course

The students in the advanced dental clinician course (45 students), the educational curriculum was prepared to cultivate excellent dental practitioners who can give advanced dental treatments. The students in the advanced dental clinician course will have lectures and clinical training with models to learn advanced dental treatments, such as dental implant, periodontal tissue regeneration, esthetic dentistry, gene diagnosis for cancer/congenital disease, advanced orthodontics. PBL (problem based learning) and experiential training by small-group instruction are introduced in many classes. We educate capability for self-directed learning.

Evaluation of new education system

The first students entered Hiroshima University Faculty of Dentistry in 2000 completed new education courses. The new education system was evaluated by students in each course. We have done questionnaire survey on new education system. The students who took the frontier dental science course are almost satisfied the education program (Fig. 2). Probably, tight communication with students and faculty members by small-group instruction worked well to educate students in the frontier dental science course. Some students presented their research and succeeded to get awards at the student session and the general session in some dental scientific meetings. Almost all students in the frontier dental science course want to move up postgraduate school to continue their research. However, the students who took the advanced dental clinician course gave us low score. They expected to have more clinical training of advanced dental treatment with models instead of lectures. The scores of CBT* trial between the two courses are the same in average (Fig. 3). However, the scores were widely distributed in the frontier dental science students. We believe that all

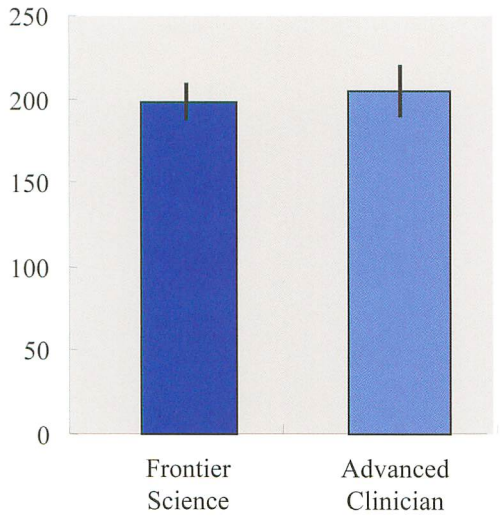

Fig. 3. CBT Trial Score

students educated by new education system pass the national dentist examination.

*CBT (the computer based test): CBT is the common nation-wide examination. The undergraduate dental students in Japan have to pass before starting their clinical training with real patients from 2006 academic year.

Education for dental hygienists and dental technicians in Hiroshima University

Hiroshima University Faculty of Dentistry had School for Dental Hygienists, established in 1976, and Dental Technicians School established in 1972. These two schools are integrated and reorganized to the School of Oral Health Science in 2005 (Table 1).

The four-year program for dental hygienists

There are three universities which have a four-year education program for dental hygienists. The four-year program for dental hygienists was firstly introduced at Tokyo Medical and Dental University and Niigata University in 2004 in Japan. In 2005, Hiroshima University firstly established the School of Oral Health Science with four-year programs for dental hygienists and dental technicians in Japan. The education for dental hygienists has been discussed to be changed because two-year program is too short to educate dental hygienists who understand and support recent progression of dental science and dental treatment. Ministry of Education, Culture, Sports, Science and Technology in Japan changed the rule of the required curriculum and the course term for dental hygienists school from two years to three years in 2005. Also, dental hygienists have not educated to have capability for research. The capability of research is most important to chart the future of dental hygienists. The knowledge about basic sciences, such as anatomy, microbiology and biochemistry, and systemic diseases are required for dental hygienists.

Furthermore, the rapid aging of Japanese society resulted in produce the needs of the oral care in senile people who generally have some systemic disease at institutions for the aged such as special elderly nursing home. At these situations, dental hygienists have to collaborate with other medical stuff, such as medical doctors, nurses, caregiver and clerks. Therefore, liberal arts education is reinforced for four-year program. The education program for taking a license for caregiver was established as an optional program at Tokyo Medical and Dental University and Niigata University.

In Hiroshima University, optional program for taking nursing teacher license is prepared in Oral Health Science Course. Hiroshima University focused on the oral health in childhood and cultivates dental hygienists capable of oral care in elementary school or junior high school. Dental caries in children had been decreased dramatically in Japan. Other dental problems, such as trauma, periodontal disease, and malocclusion have to be pay attention in childhood. Parents tend to give high quality dental care to their children because of decrease of number of children in Japan. For this optional program, pedagogy and practical training at the school are added.

The four-year program for dental technicians

The four-year program was firstly introduced at Hiroshima University as a course of the school of oral health science in 2005. Generally, dental technicians are educated with two-year program in Japan. The purpose of our four-year program is to cultivate educators in dental technicians school and researchers in Universities or dental materials/products companies. The curriculum was composed based on the results of questionnaire survey on required curriculums from the companies and on our original education policy. The capability of research is most important to chart the future of dental technicians. Furthermore, it is a different character from medical treatment that many treatment methods in dental treatment are deeply involved with engineering. We want to educate researchers who have knowledge and skill as a dental technician and potential to research and development on the basic engineering aspect of dentistry such as computer graphics, information technology, electric devices, dental treatment robot, CAD/CAM, biomaterial etc. Therefore, we have build up the tight collaboration with Faculty of Engineering in Hiroshima University. We invited a professor from Faculty of Engineering and some lectures were given by the faculty members in Faculty of Engineering. The students have simultaneously dental knowledge and skill and engineering knowledge. We expect that the students in our four-year program may become quite different from students in traditional dental technicians school.

Another purpose of this four-year program is to cultivate who can produce body epithesis based on dental technician's knowledge and skill.

From the results of questionnaire survey of the students in School of Oral Health Science, many students in the oral health engineering course do not have a clear purpose to be in future at present time. This result means there is so many choices what they can become. I believe they will innovate dental science and dental treatments from quite different aspects.

SYMPOSIUM ON SCIENCE

Special Lecture 1

Phosphate Regulates Expression of SIBLINGs and MMPs in Cementoblasts

University of Washington Professor, Dean
Martha J. Somerman

Special Lecture 3

Regulation of Hematopoietic Stem Cell and Its Interaction with Stem Cell Niche

Keio University, Assistant Professor
Fumio Arai

Session 1 Immunology and Microbiology in Mucosal Diseases

Secretory IgA Immune Responses as the Mucosal Frontline

The University of Tokyo, Professor
Hiroshi Kiyono

Antimicrobial Peptides in Human Gingival Keratinocytes

Hiroshima University, Associate Professor
Hitoshi Komatsuzawa

War and Peace at Mucosal Immune System

International Vaccine Institute, Director
Mi-Na Kweon

Session 2 Mechanisms and Assessment of Oral Functions and Sensation

Biomechanical and Clinical Assessment for Jaw Movement and the Related TMJ Loading in Patients with Temporomandibular Joint Disorders

Hiroshima University, Associate Professor
Eiji Tanaka

Taste Representations in the Mouse Brain Revealed by Genetic Tracing

Hiroshima University, Assistant Professor
Makoto Sugita

Mechanisms of Pain Sensitization and the Treatments

Hiroshima University, Professor
Toshihiro Dohi

Session 3 Signaling Molecules in Development and Cancer

In Vitro Organogenesis using Amphibian Pluripotential Cells

The University of Tokyo, Professor
Makoto Asashima

Genetic Alterations of Wnt Signal Components in Cancer Cells

Hiroshima University, Professor
Akira Kikuchi

Developmental Signaling Disorders in Craniofacial Anomalies and Cancer

Hiroshima University, Professor
Tetsuji Okamoto

Session 4 Growth Factors and Scaffolds for Tissue Regeneration

Development of Periodontal Tissue Regeneration Therapy with New Bioactive Agents
- Studies on Brain-derived Neurotrophic Factor and Ameloblastin Peptide -

Hiroshima University, Professor
Takashi Takata

Gene Therapy for Periodontal Bioengineering

University of Michigan, Associate Professor
William V. Giannobile

Present Status of Biomaterial-based Regenerative Medical Therapy

Kyoto University, Professor
Yasuhiko Tabata

Session 5 Bone Biology and Cell Therapy

Skeletal Development through the Regulation of Chondrocyte and Osteoblast Differentiation by Runx2

Nagasaki University, Professor
Toshihisa Komori

Transcriptional Regulation of Osteoblast Differentiation and Function

Tokyo Medical and Dental University, Associate Professor
Kazuhisa Nakashima

Application of Mesenchymal Stem Cells (MSC) to Regenerative Dentistry and Identification of Molecular Markers for MSC

Hiroshima University, Professor
Yukio Kato

Basic and Clinical Studies of Periodontal Tissue Regeneration by Transplantation of Own Bone Marrow Mesenchymal Stem Cells

Hiroshima University, Associate Professor
Hiroyuki Kawaguchi

Tissue - engineering of Orthopaedic Surgery

Hiroshima University, Professor
Mitsuo Ochi

Recent Advances in Tissue Engineering of Cartilage, Bone and Tendon

Shanghai Second Medical University, Professor and Chairman,
Shanghai Tissue Engineering Research Center, Director
Yilin Cao

Phosphate Regulates Expression of SIBLINGs and MMPs in Cementoblasts

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ABSTRACT

Introduction: Cementoblasts, the cells responsible for tooth root cementum formation, are especially sensitive to local phosphate and pyrophosphate during development, as evidenced by cementum phenotypes resulting from altered phosphate/pyrophosphate distribution. SIBLING family members BSP, OPN, and DMP-1 are regulated by phosphate in cementoblasts and have been shown to activate three specific matrix metalloproteinase (MMP) partners: MMP2, MMP3, and MMP9, respectively, *in vitro*. The aim of this study was to examine regulatory effects of phosphate on SIBLING and MMP expression in cementoblasts, *in vitro*.

Materials & Methods: Immortalized murine cementoblasts were treated with inorganic phosphate, *in vitro*, and effects on gene expression (by real time RT-PCR and mouse total genome microarray) were observed. Dose-response (0.1-10 mM phosphate) and time-course (1-48 hr) assays were performed. A sodium-phosphate uptake inhibitor, foscarnet, was used to better define phosphate-mediated effects on cells.

Results: Three SIBLING family members were regulated by phosphate: OPN (increased over 300% of control), DMP-1 (increased over 3,000% of control), and BSP (decreased). MMP3 was dramatically increased (4,000% of control), paralleling regulation of its partner OPN. Both MMP2 and MMP9 were slightly down-regulated. Time-course experiments indicated a response for SIBLING and MMP genes within 24 hr. Use of foscarnet demonstrated that phosphate uptake was required for observed changes in gene expression.

Discussion: These results indicate an effect of phosphate on cementoblast SIBLING and MMP expression *in vitro*. During cementum formation, phosphate may be an important regulator of cementoblast activity, including modulation of biomineralization, attachment, and matrix modification.

Key Words: cementoblasts, phosphate, SIBLING, matrix metalloproteinase, osteopontin

INTRODUCTION

The periodontium consists of the supportive tissues of the tooth, including the tooth root dentin and cementum, the periodontal ligament (PDL), and the surrounding alveolar bone. The cementum is the thin mineralized tissue covering the tooth root that plays a role in anchoring cementum to the alveolar bone via the periodontal ligament. Cementoblasts and cementum are sensitive to levels of phosphate (P_i) and pyrophosphate (PP_i), as evidenced by mutant and knock-out phenotypes with altered P_i/PP_i levels (Beertsen *et al.*, 1999; Nociti *et al.*, 2002; van den Bos *et al.*, 2005; Whyte, 1994). While this sensitivity may result in part from physicochemical interactions, there is also mounting evidence that P_i may serve as a signaling molecule (Adams and Shapiro, 2003; Beck, 2003). In cementoblasts specifically, we have shown intriguing Pi regulation of several genes involved in the processes of differentiation and mineralization, and metabolism of P_i and PP_i in the cell local environment (Foster *et al.*, In press; Rutherford *et al.*, Submitted).

The SIBLING (Small Integrin-Binding Ligand N-linked Glycoprotein) family includes noncollagenous extracellular matrix-associated proteins with several organizational features in common, as well as similar, functionally important post-translational modifications (Fisher and Fedarko, 2003; Qin *et al.*, 2004). SIBLINGs have been intimately associated with mineralized tissue formation, though continuing research has revealed additional functions and tissue localizations (Jain *et al.*, 2002; Ogbureke and Fisher, 2004).

Matrix metalloproteinases (MMPs) are a family of more than twenty endopeptidases with the capacity to degrade components of the extracellular matrix. MMPs have traditionally been regarded as being involved in activities including tissue remodeling, facilitation of cell migration, and cell response to microenvironment. Reports have additionally supported a role for MMPs in the generation of active epitopes of sequestered growth factors, adhesion molecules, cytokines, chemokines, and receptors (Sternlicht and Werb, 2001). Up-regulation of certain MMPs has also been linked to pathological conditions, including oral diseases (Sorsa *et al.*, 2004).

It has been demonstrated that three SIBLING family members partner specifically with three MMPs, and reversibly activate the proMMP forms of these enzymes without removal of the inhibitory propeptide. Bone sialoprotein (BSP) partners with MMP-2, osteopontin (OPN) with MMP-3, and dentin matrix protein-1 (DMP-1) with MMP-9 (Fedarko *et al.*, 2004; Ogbureke and Fisher, 2004). MMPs are typically inactivated by tissue inhibitors of MMPs (TIMPs), but SIBLING partners were shown to protect MMPs from this inactivation. Thus, co-expression of SIBLING and MMP partners provides a potential mechanism for localized and finely regulated MMP function, even with constitutive TIMP expression. While the SIBLING proteins can activate their MMP partners, it has been proposed that the MMPs may proteolyti-

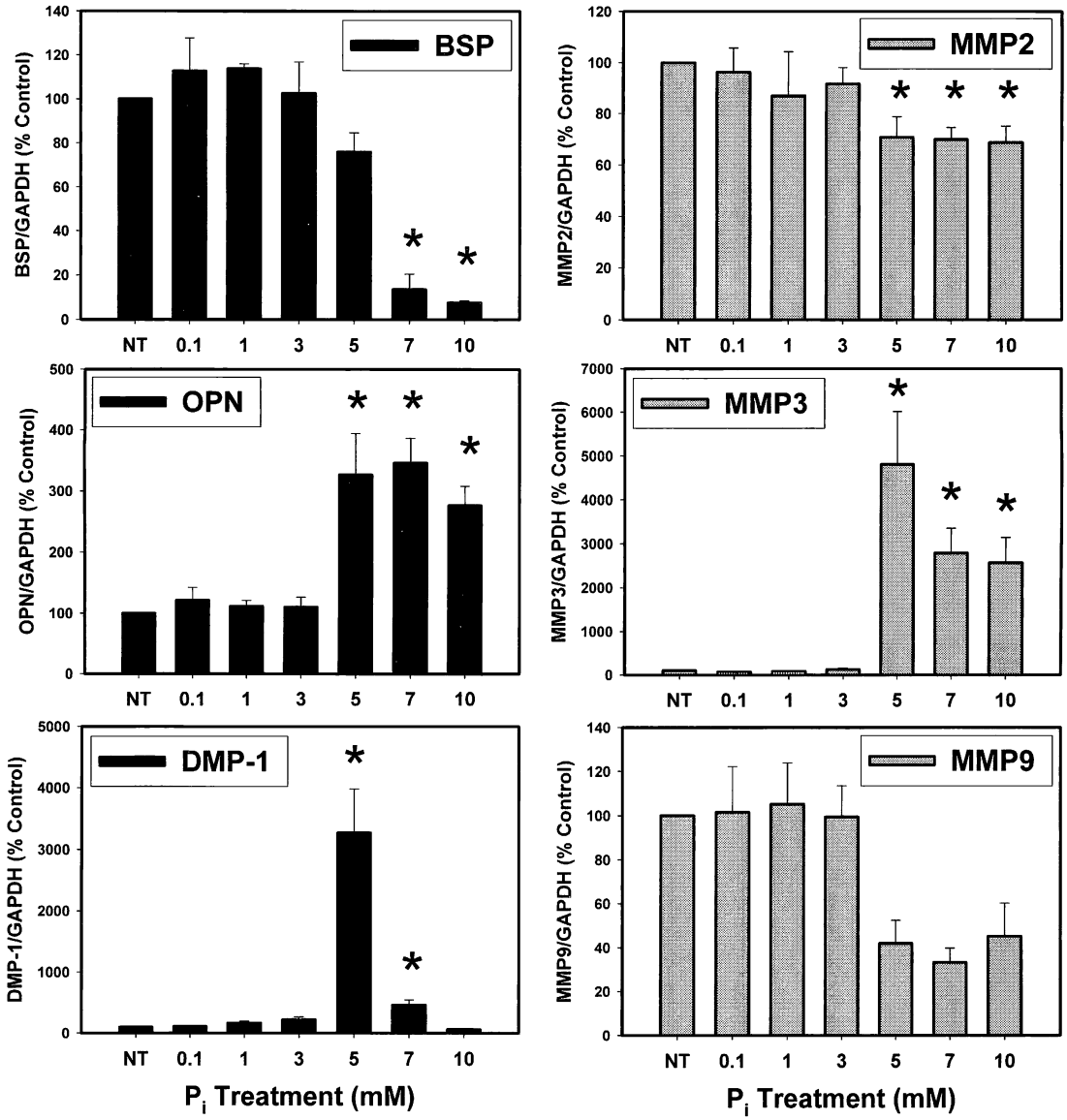

Fig 1. Phosphate regulates SIBLING and MMP genes in cementoblasts: Dose-response. Addition of 0.1-10 mM P_i to cementoblast cell cultures regulated SIBLING genes BSP, OPN, and DMP-1, and MMP genes MMP2, MMP3, and MMP9. Partners BSP and MMP2 were both decreased, OPN and MMP3 were increased, and in contrast, DMP-1 was increased though partner MMP9 was decreased, although the latter was not found to be statistically significant (see text for discussion). NT=no treatment control; bars represent SE; * is significance at $p < 0.05$.

cally process the SIBLINGs (Gao *et al.*, 2004).

The aim of this study was to examine the regulation of SIBLING proteins and MMPs by P_i in cementoblasts, *in vitro*. While we have shown previously that P_i mediates regulation of several SIBLING genes, here we additionally consider parallel effects on MMP expression. Both SIBLINGs and MMPs play critical roles during formation of mineralized tissues, and further study of the co-regulation of these factors may improve understanding of their contributions to oral mineralized tissue development, maintenance, and their potential use in regenerative therapies.

MATERIALS AND METHODS

Cell culture. Immortalized murine cementoblasts (OCCM-30) were maintained in Dulbecco' s Modified Eagle Medium (DMEM) with 10% (v/v) fetal bovine serum (FBS) and Penicillin/Streptomycin/L-Glutamine (all reagents from Invitrogen, Carlsbad, CA). Isolation and characterization of OCCM-30 cementoblasts has been previously described (D'Errico *et al.*, 2000). OCCM-30 cells were plated (2.56×10^4 cells/cm^2) and upon confluence media were changed to DMEM with 5% FBS, and treatments were added. P_i levels for dose-response were 0.1, 1, 3, 5, 7, and 10 mM, and untreated control. For time-course studies, 5 mM P_i was used. Total RNA was isolated by Trizol® reagent (Invitrogen, Carlsbad, CA) at 48 hr for dose-response experiments and at times 1, 6, 24, and 48 hr for time-course. For studies with foscarnet (phosphonoformic acid, PFA), an inhibitor of Na/P_i co-transport into the cell, cells were incubated with 3 mM

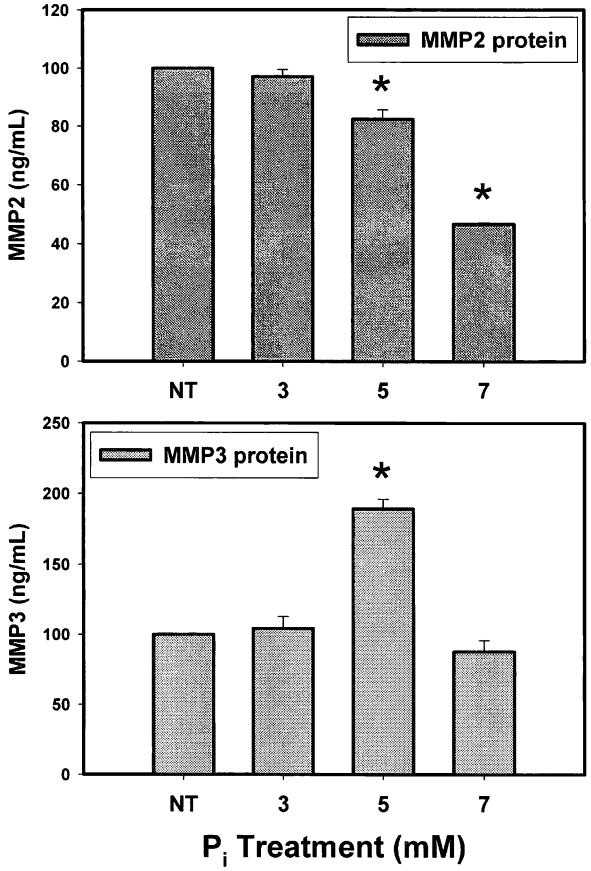

Fig 2. Phosphate regulates MMP protein levels in cementoblasts. Experiments were carried out as described under Dose-Response section, with cell media supernatant samples taken at 96 hr and analyzed by ELISA. MMP protein levels were regulated similarly to gene expression as described in Figure 1 (t=48 h), with a significant increase in MMP3 and decrease in MMP2 at 5 mM P_i. NT=no treatment control; bars represent SE; * is significance at $p < 0.05$.

PFA for 30 min prior to P_i addition, with PFA levels maintained in media until RNA isolation at 24 hr.

Real-time quantitative RT-PCR. Total RNA was DNAse treated (DNA-free™, Ambion Inc., Austin, TX), and two step RT-PCR was performed with a first-strand AMV cDNA synthesis kit and DNA Master SYBR Green I kit (Roche Diagnostics North America, Indianapolis, IN) according to manufacturer directions. A Roche Lightcycler system (Roche Diagnostics, Basel, Switzerland) was used for real time quantitative PCR and subsequent melting curve analysis of products to ensure product specificity. Primers were designed by Lightcycler probe design software and sequences checked by BLAST search. Primers (forward / reverse) designed for mouse sequences included: bone sialoprotein (BSP, 5' -GAGACGGCGATAGTTCC-3' / 5' -AGTGCCGCTAACTCAA-3'), OPN (5' -TTTACAGCCTGCACCC-3' / 5' -CTAGCAGTGACGGTCT-3'), dentin matrix protein-1 (DMP1, 5' -ATGATAACGCAATGGGT-3' / 5' -GTAATGCCTCAATGGCAC-3'), MMP2 (5' -AGATTGACGCTGTGTATG-3' / 5' -GCGATGAGCTTAGGGA-3'), MMP3 (5' - GGCTATACGAGGGCAC -3' / 5' - CAAATTCCAACTGCGAAG -3'), MMP9 (5' - CACCGAGCTATCCACT -3' / 5' -ACCTGAACCATAACGC ? 3') MMP23 (5' - GGTAACCCGAAGACGC-3' / 5' - CTCGCTGTCATCAAAGT-3'), and GAPDH (5' -ACCACAGTCCATGCCATCAC-3' / 5' -TCCACCACCCTGTTGCTGTA-3').

Relative quantification of PCR products was achieved using the LightCycler Relative Quantification Software version 1.0 (Roche Diagnostics, Basel, Switzerland) using GAPDH as a reference gene, with calibrator normalization and amplification efficiency correction.

ELISA. MMP proteins in cell culture supernatant samples were analyzed using Quantikine MMP2 and MMP3 mouse ELISA kits (R&D Systems, Minneapolis, MN). No commercially available mouse-specific MMP-9 ELISA kit was available, so MMP9 protein levels cannot be reported at this time.

Statistical analysis. All experiments were performed in triplicate (N=3) with dose-response experiments (without and with foscarnet) analyzed by one-way ANOVA (Sigmastat 3.1) and pairwise comparisons by SNK test, and time-course experiments log-transformed (ratio of treated/untreated) and a t-test applied (MS Excel). Log transformation was used to control passage-to-passage variation in basal cell gene expression. A p-value less than 0.05 was used to indicate significance.

RESULTS

Dose-response (Figures 1 and 2). Increasing the extracellular phosphate (P_i) in OCCM-30 cementoblast cell cultures regulated SIBLING and MMP genes (Figure 1). At 5 mM P_i, both OPN and DMP-1 were significantly increased (300% and 3,000% percent of untreated control, respectively), while BSP was decreased to about 80% of control. While the decrease in BSP was significant at higher P_i doses, these conditions were stressful for the cells (as indicated by cell morphology) and the doses were not used in subsequent experiments. MMP-2, partner of BSP, was also down-regulated to approximately 80% of untreated control. Like its partner OPN, MMP-3 was up-regulated by 5 mM P_i, to nearly 5,000% of control. In contrast to MMP2 and MMP3, both of which were regulated similarly to their SIBLING partners, MMP9 was seemingly decreased at higher P_i doses, but variable constitutive expression of DMP-1 mRNA in OCCM-30 cementoblast cultures confounded any determination of significance through statistical analysis.

For ELISA experiments, the same experimental design was employed, but with doses of 3, 5, and 7 mM P_i, and cell culture supernatant and cell lysates were collected at 48 hr. MMP2 and 3 protein levels corresponded with the pattern of regulation established from real-time PCR in Figure 1. MMP2 protein decreased with increasing P_i doses whereas 5 mM Pi significantly increased MMP3 protein. Though 7 mM Pi increased MMP3 mRNA (though less than the 5 mM Pi dose), this dose did

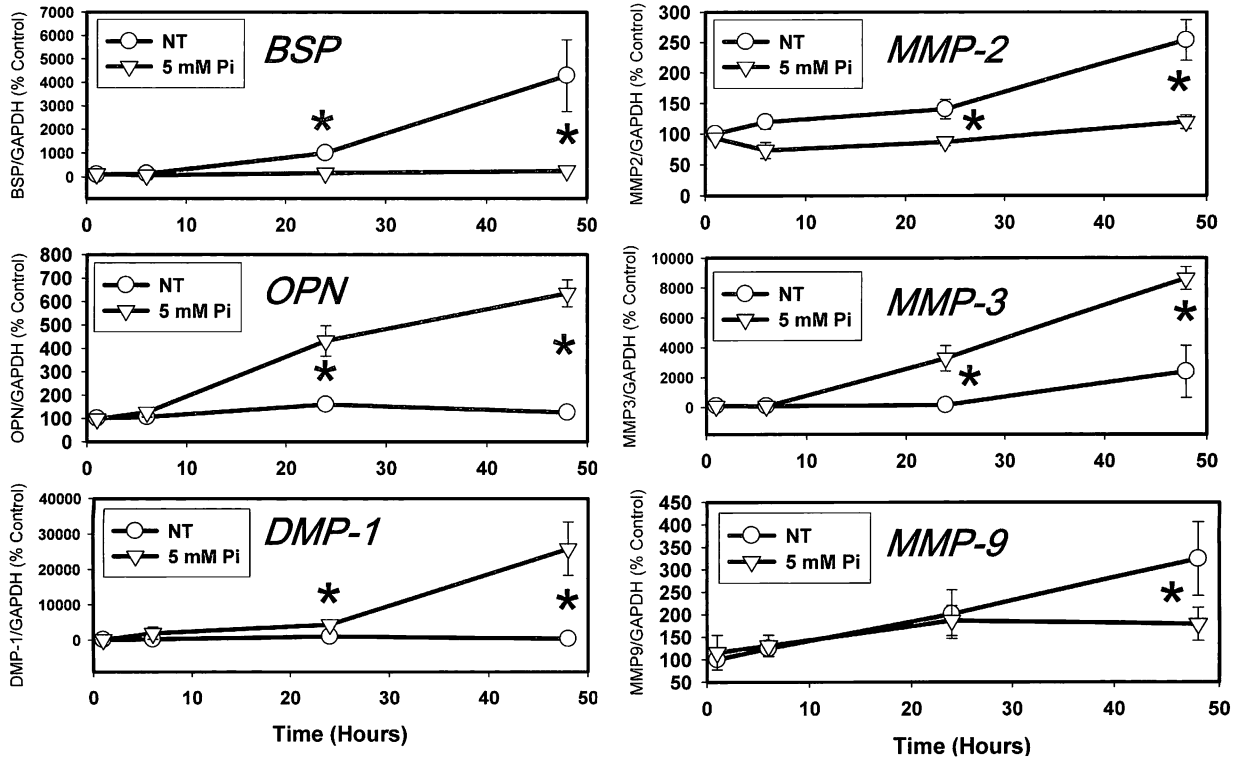

Fig 3. Phosphate regulates SIBLING and MMP genes in cementoblasts: Time-course. Addition of 5 mM P_i to cementoblast cell cultures with isolation of RNA from cementoblast cultures at 1, 6, 24, and 48 hr. Regulation of SIBLING and MMP genes was observed by t=24 hr. NT=no treatment control; bars represent SE; * is significance at $p < 0.05$.

not change MMP3 protein levels. No commercially available mouse specific MMP-9 ELISA kit was available, so MMP9 protein levels can not be reported at this time.

Time-course (Figure 3). A dose of 5 mM P_i was added to OCCM-30 cementoblast cell cultures, with RNA samples taken at times 1, 6, 24, and 48 hrs. All SIBLING genes were significantly up- or down-regulated by 24 hr. MMP2 and 3 were also regulated by 24 hr, though MMP9 was not significantly different from control until the 48 hr time. Over this time in culture, basal BSP expression (i.e., that of untreated controls) increased, while OPN and DMP-1 were at stable levels. These trends for gene expression in (untreated) cementoblasts over time in culture have been reported previously (D'Errico *et al.*, 2000) and are similar to those reported for some other mineralizing cell types of cells, such as osteoblasts (Beck *et al.*, 2000; Franceschi *et al.*, 1994). Expression of all three MMPs was observed to increase in untreated cementoblasts over 48 hr, with MMP3 increasing to about 1,000% of control (NT at time 1 hr), and MMPs 2 and 9 both increasing to about 300% of control. Thus, the three MMPs examined here increased with time in untreated OCCM-30 cementoblast cell cultures.

Blocking P_i import (Figure 4). In addition to doses of 1, 3, and 5 mM P_i, 3mM PFA (foscarnet, phosphonoformic acid) was included in OCCM-30 cementoblast cell cultures, with RNA samples taken at 24 hrs. Inclusion of PFA partially abrogated the regulation of SIBLING and MMP genes. While 5 mM Pi increased OPN mRNA to 250% of control, inclusion of PFA limited increase to 150%. Similarly, MMP3 was up-regulated to 2,000% by 5 mM P_i, but PFA limited MMP3 increase to only about 300% of control. GAPDH reference gene was not affected by PFA, and PFA regulation was similarly abrogated in other SIBLINGs/MMPs tested (data not shown). The blocking of P_i regulation of genes observed when 3 mM PFA was included may be incomplete due to less than 100% Na-P_i transporter blockage, and also due to potentially additional means of P_i entry that are not affected by PFA.

DISCUSSION

These studies demonstrate transcription-level regulation of SIBLING and MMP genes by P_i in cementoblasts, *in vitro*. SIBLING genes OPN and DMP-1 were increased, while BSP was decreased. MMP3 expression was increased, while MMP2 and 9 were decreased. Blocking P_i uptake partially abrogated regulation of SIBLING and MMP genes, indicating that P_i transportation into cells is likely necessary for gene regulation.

SIBLING family. SIBLING (Small Integrin-Binding Ligand N-linked Glycoprotein) extracellular matrix proteins play important roles in mineralized tissue formation in the tooth, as supported by studies of temporal and spatial expression of SIBLINGs during development (Baba *et al.*, 2004a; Baba *et al.*, 2004b; D'Errico *et al.*, 1997; Feng *et al.*, 2003; Hao *et al.*, 2004; MacNeil *et al.*, 1995), and reports of altered phenotypes resulting from human

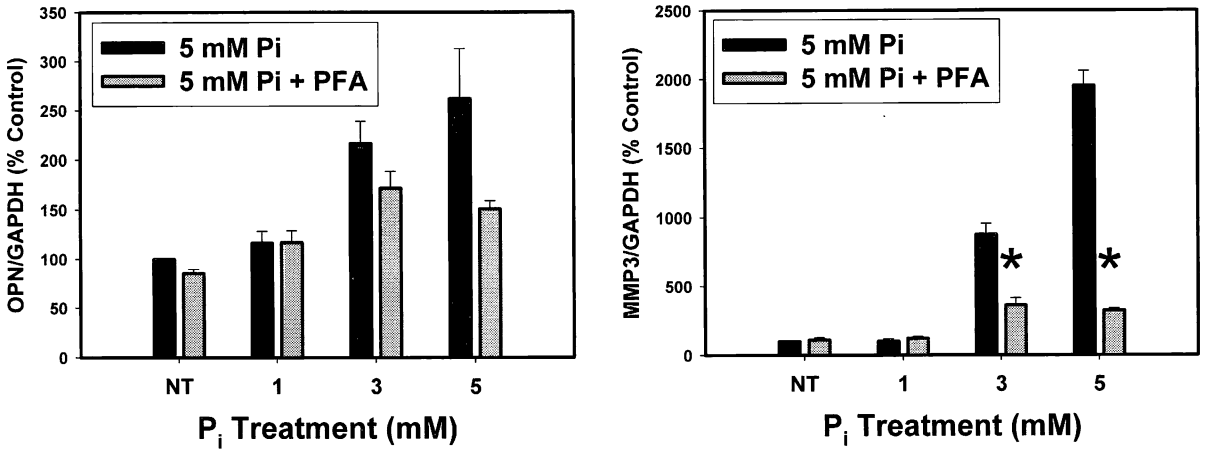

Fig 4. Blocking phosphate uptake partially abrogates regulation of SIBLING and MMP genes. A dose of 3 mM phonofosformic acid (PFA) was added to cementoblasts 30 minutes prior to and along with doses of P_i, and RNA was isolated after 24 hr. PFA blocks some P_i uptake into cells, also partially abrogating Pi-induced effects on SIBLING and MMP gene expression. This observation indicates that P_i enters the cell to regulate gene expression. NT=no treatment control; bars represent SE; * is significance at $p < 0.05$.

mutations and knock-out animals carrying SIBLING gene mutations (Dong *et al.*, 2005; Ye *et al.*, 2004). SIBLINGs have been individually characterized as nucleators, promoters, regulators, and/or inhibitors of mineralization, though this is often dependent on post-translational modifications, especially phosphorylation (Gericke *et al.*, 2005; He *et al.*, 2003; Tartaix *et al.*, 2004), but also including glycosylation, proteolytic processing, and transglutaminase cross-linking (Kaartinen *et al.*, 2005; Qin *et al.*, 2003; Qin *et al.*, 2004). Although the acidic natures of most of the SIBLING proteins (except for MEPE, not discussed here) are partly responsible for interaction with and regulation of hydroxyapatite formation, other characteristics of the SIBLING proteins relate to additional roles, including cell attachment (via $\alpha_v\beta_3$ integrin binding).

Matrix metalloproteinases. MMPs are a family of more than 20 related endopeptidases enzymes that are capable of degrading the various components of the extracellular matrix (ECM). MMPs are critical for operations including tissue development, remodeling, wound healing, and also releasing sequestered bioactive molecules from the ECM (Mott and Werb, 2004; Sternlicht and Werb, 2001; Visse and Nagase, 2003). MMPs may also contribute to a multitude of pathologies including arthritis, cancer, and oral diseases (Baker *et al.*, 2002; Sorsa *et al.*, 2004; Sternlicht and Werb, 2001). MMPs are expressed during tooth formation, and there is evidence that disruption or absence of MMPs has serious negative consequences for tooth development (Bourd-Boittin *et al.*, 2004; Bourd-Boittin *et al.*, 2005; Fanchon *et al.*, 2004; Goldberg *et al.*, 2003). MMP expression in the region of the developing tooth root has not been thoroughly characterized, but as with enamel and dentin they likely play an important role here as well. More complete characterization of MMPs in the periodontium would advance understanding of their potential roles during development of this region.

SIBLING-MMP partners. MMPs are typically secreted in a latent, inactive form (proMMP) that requires proteolytic removal of the inhibitory propeptide to activate. MMP protein activity is further regulated by the expression of tissue inhibitors of MMPs (TIMPs), a family of four endogenous protein inactivators of MMP activity that are also carefully regulated in local cell environments (Sternlicht and Werb, 2001; Visse and Nagase, 2003). Some very exciting studies have supported a role for SIBLING proteins in activating latent propetide forms of MMPs, and additionally protecting activated MMPs from TIMP inhibition or even reactivating TIMP-inhibited MMPs (Fedarko *et al.*, 2004). The specific SIBLING-MMP binding relationships are BSP-MMP2, OPN-MMP3, and DMP-1-MMP9. Additionally, these SIBLING-MMP binding pairs have been identified in several tissues including salivary glands and kidney, and in multiple types of cancer (Chaplet *et al.*, 2005; Fisher *et al.*, 2004; Karadag *et al.*, 2004). While SIBLING-MMP complexes may aid in the metastatic and osteotropic processes of some cancers, a role in normal development of teeth (and bones) can also be conceived, and in fact seems likely. There is some evidence that osteoblasts may even take part directly in bone matrix dissolution via MMPs (Parikka *et al.*, 2005). SIBLING proteins are important constituents of the ECM, MMPs are critical in matrix modeling and maturation, and both are expressed during tooth formation. Concomitant expression of SIBLING and MMP partners may be a novel mechanism for the exquisite regulation of local MMP activity during tooth development. Here we show *in vitro* that MMP2, 3, and 9 are expressed constitutively in cementoblast cell cultures, and additionally that their transcription can be regulated by inorganic phosphate (P_i).

Inorganic phosphate as a signaling molecule. Accumulating evidence supports a role for P_i as a cell signaling molecule, in addition to its role as a constituent of hydroxyapatite in teeth and bones. P_i regulates OPN expression in MC3T3-E1 pre-osteoblasts (Beck *et al.*,

2000), and much work has been done to elucidate regulatory mechanisms of OPN and other genes in osteoblasts (Beck, 2003; Beck and Knecht, 2003; Beck *et al.*, 2003; Conrads *et al.*, 2004). Pi may also serve as an apoptogen in terminally differentiated chondrocytes and osteoblasts as a mechanism for cell removal (Adams *et al.*, 2001; Adams and Shapiro, 2003). Furthermore, we have shown P_i regulation of several genes in cementoblasts related to functions of cell differentiation, mineralization, P_i/PP_i transport and metabolism, including the SIBLINGs BSP, OPN, and DMP1 studied here (Foster *et al.*, Accepted; Foster *et al.*, In press). Initial steps toward defining the mechanisms involved in P_i regulation of cementoblasts have been made and are currently being explored in greater depth. Levels of local P_i and PP_i may vary during tooth development in relation to activities such as matrix synthesis, mineralization, remodeling, resorption, and matrix maturation, and cells in the local area may be regulated in multiple ways by these processes. The importance of P_i and PP_i in formation of hydroxyapatite is well-established, but P_i may also serve as signaling molecule regulating cell behavior, for example expression of SIBLING and MMP proteins that control properties of the developing matrix. By better understanding the role of Pi in periodontal development, there is potential to advance our abilities to provide predictable therapies for periodontal regeneration, and also understand and treat genetic defects that negatively affect periodontal tissues.

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Regulation of Hematopoietic Stem Cell and Its Interaction with Stem Cell Niche

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ABSTRACT

Hematopoietic stem cells (HSCs) are responsible for Blood cell production throughout the lifetime of individuals. Interaction of HSCs with their particular microenvironments, known as stem cell niches, is critical for maintaining the stem cell properties, including self-renewal capacity and the ability of differentiation into single or multiple lineages. In the niche, the niche cells produce signaling molecules, extracellular matrix, and cell adhesion molecules, and regulate stem cell fates. Recently, long-term bone marrow (BM) repopulating (LTR) HSCs exist frequently in BM trabecular bone surface, and it was clarified that an osteoblast (OB) is a critical component for sustainment of HSCs. HSCs balance quiescence and cell division in the osteoblastic niche and also maintain the potential for long-term hematopoiesis. Especially, the quiescent state in the cell cycle is thought to be indispensable for the maintenance of hematopoietic stem cells (HSCs). We demonstrate that c-Kit$^+$Sca-1$^+$Lineage$^-$ (KSL) HSCs expressing the receptor tyrosine kinase Tie2 are quiescent and anti-apoptotic, transplantable and comprise a side-population (SP) of HSCs, which contact closely to Angiopoietin-1 (Ang-1), a ligand for Tie2, expressing osteoblasts in the BM niche. Tie2 and Ang-1 are part of a key signaling interaction between HSC and osteoblasts. Tie2 and Ang-1 are expressed in a complementary pattern, and interaction of Tie2 and Ang-1 induced integrin dependent cell adhesion of HSCs to osteoblasts and extracellular matrix. This signaling pathway regulates functional criteria of HSC in the BM niche, including quiescence, anti-cell death and tight adhesion. These observations led us to a novel model in which Ang-1 produced by osteoblasts activates Tie2 on the HSCs and promote tight adhesion of HSCs to the niche, resulting in quiescence and enhanced survival of HSCs.

Key words: Quiescence, Tie2, Angiopoietin-1, N-cadherin, ATM

Tissue stem cells are characterized by their abilities to self-renew and to produce numerous differentiated daughter cells. These two special properties enable stem cells to play a central role in maintaining tissues. The activity of tissue stem cells is crucial for supply the mature cells in normal tissue turnover. Defective functional activity or low turnover of stem cells leads exhaustion of progenitor or mature cells in tissue and is causing in disease. Unregulated and over proliferation of stem cells is the leading cause of cancer. It now clear that the stem cell niche regulates the stem cell specific property including self-renewal, multi-potentiality, and relative quiescence.

The concept of the stem cell niche was first proposed for the human hematopoietic system in the 1970s (Schofield, 1978). A similar concept has also been proposed for the epidermis, intestinal epithelium, nervous system and gonads (Fuchs et al., 2004; Li and Xie, 2005). We hypothesized that cell cycle regulation by the niche is critical for the fate of HSCs. In the niche, signaling molecules, extracellular matrix, and cell adhesion molecules produced by niche cell regulate quiescence, self-renewal, and cell fate decision of the stem cell. Up to the present date, many adult tissue stem cells and their niches, including hematopoietic system, skin epidermis, gastrointestinal epithelium, brain, and lung were identified. There observation led us the understanding of the common property of the tissue stem cells. First, stem cells are relatively quiescent or slow cycling cells in the tissue. Second, stem cells adhere to the supporting cells (niche cells) in the stem cell niche. We found that the Tie2/Ang-1 signaling pathway between HSCs and OBs contributes to quiescence of HSCs in their stem cell niche, resulting in the maintenance of self-renewal ability and protection from stresses (Arai et al., 2004)

Identification of stem cell niche and niche cells in adult BM

A stem cell niche includes three compartments: localized niche cells (supporting cells), the extracellular matrix (ECM), and soluble factors derived from niche cells (Lin, 2002) (Figure 1). A unique feature of HSCs is that they migrate toward the stem cell niche during their ontogeny. During embryogenesis, development of the hematopoietic system occurs at various anatomical sites, including para-aortic splanchnopleural mesoderm (P-Sp)/ aorta-gonad-mesonephros (AGM) region, yolk sac, fetal liver, spleen, and bone marrow. Intraembryonic hematopoietic development may be associated with the major arterial region. Histological studies have demonstrated the presence of clusters of HSCs in close association with, and often adhering to, endothelial cells on the ventral surface (floor) of the aorta (North et al., 2002). It suggests that endothelial cells play as a niche for developing HSCs. In addition, recently two groups reported that the placental labyrinth region is a source of definitive hematopoiesis and plays as a niche for HSCs during midgestation (Gakas et al., 2005; Ottersbach et al., 2005).

OBs, which derived from mesenchymal stem cell, have long been known to play a central role in skeletal

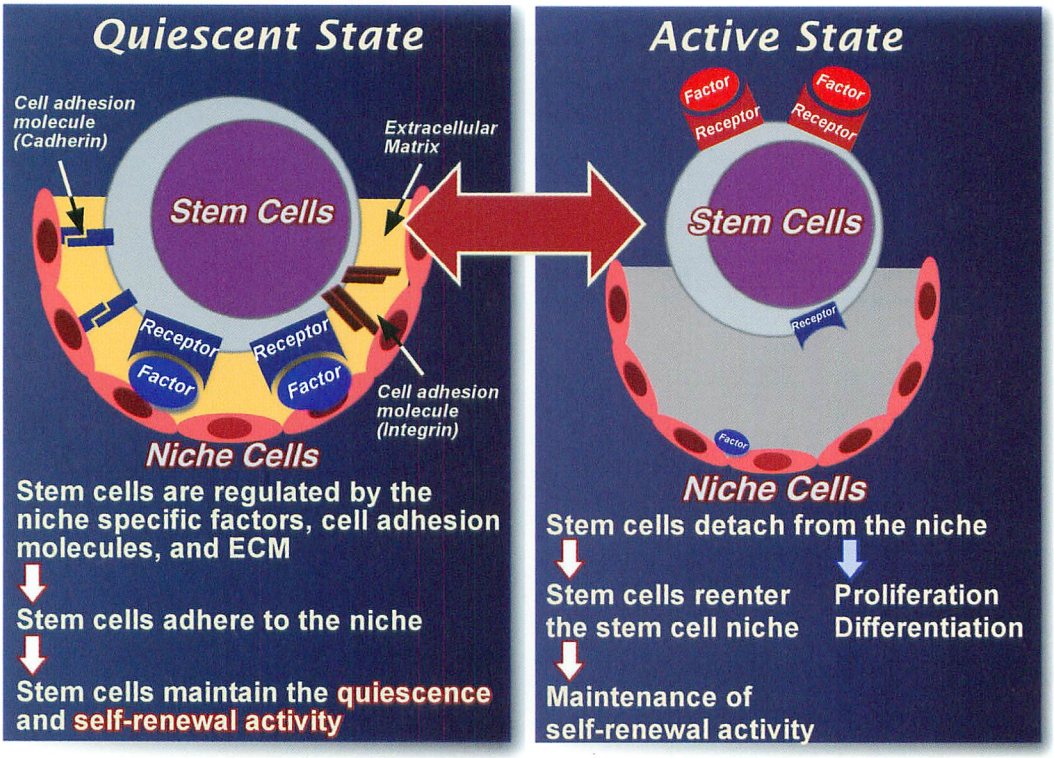

Fig 1. The model of stem cell niche
In the quiescent state, stem cell is regulated by the quiescence inducible factor and receptor complex and adheres to the niche cells, resulting in the maintenance of self-renewal activity and protection from stresses. Once the stem cells are activated by the mitogenic or differentiation factors, stem cell leaves from the niche. Detachment of the stem cell from the niche allows the differentiation and proliferation. This is in turn loss of self-renewal activity. In addition, if activated stem cell reenters in the niche, stem cell comes to quiescent state again and maintains self-renewal activity.

development. OBs also constitute part of the stromal cell support in BM. Taichman and Emerson also reported that OBs are a part of the hematopoietic microenvironment after BM cavity formation (Taichman and Emerson, 1998). Nilsson et al. reported that HSCs were significantly enriched within the endosteal region after BM transplantation (Nilsson et al., 2001).

Recently, two papers reported the identification of a HSC niche (Calvi et al., 2003; Zhang et al., 2003). These reports suggest that OBs are critical regulatory component of adult hematopoiesis. Both groups employed genetic strategies to increase the number of OB population in the trabecular region of the bone. Increasing the number of OBs causes parallel increases in the HSC population, particularly LTR-HSCs, without concomitant increases in other primitive progenitor cells. Such a specific increase in only the LTR-HSC population suggests that a specific niche is functionally enhanced. In addition, Zhang et al. (2003) showed that N-cadherin-positive spindle-shaped OBs (SNO cells) are niche cells, and N-cadherin is asymmetrically localized between HSCs and OBs in the adult BM niche. Other report has addressed the relationship between hematopoiesis and OBs. Visnjic et al. (2004) demonstrated that conditional depletion of OBs using a type I collagen promoter-herpes simplex virus thymidine kinase transgenic mice with ganciclovir treatment led reduction of BM cellularity.

Quiescent HSCs in the adult BM niche

Key features of stem cells in a niche are that they are quiescent and adhere to surrounding niche cells. The quiescent state is thought to be an indispensable property for the long-term maintenance of hematopoietic stem cells (HSCs), and is thought to be an important mechanism for protecting cells from the stress (Cheng et al., 2000). Indeed, it has been reported that HSCs are relatively quiescent when compared to transiently amplifying progenitor cells (Cheshier et al., 1999). We thought that identification of HSCs in quiescent state is viewed as a way to find the stem cell niche. For detection of quiescent HSCs in BM, we used the myelosuppressive model of treatment with 5-FU, a drug that induces apoptosis in cycling cells, and analyzed SP cells in HSCs. SP is a cell fraction weakly or non-labeled with the DNA dye, Hoechst 33342, and is able to enrich the HSCs (Goodell et al., 1996). Moreover, SP cells have been found in several tissue and species, suggesting that SP defines a general property of tissue stem cells (Zhou et al., 2001). We found that the SP cells, but not non-SP cells in HSCs are resistant to BM suppression induced by 5-Fluorouracil (5-FU), suggesting that these cells are quiescent and have anti-apoptotic properties (Figure 2).

To further characterize the cell cycle status of SP cells in the c-Kit$^+$Sca-1$^+$Lineage$^-$ (KSL) fraction, KSL cells were

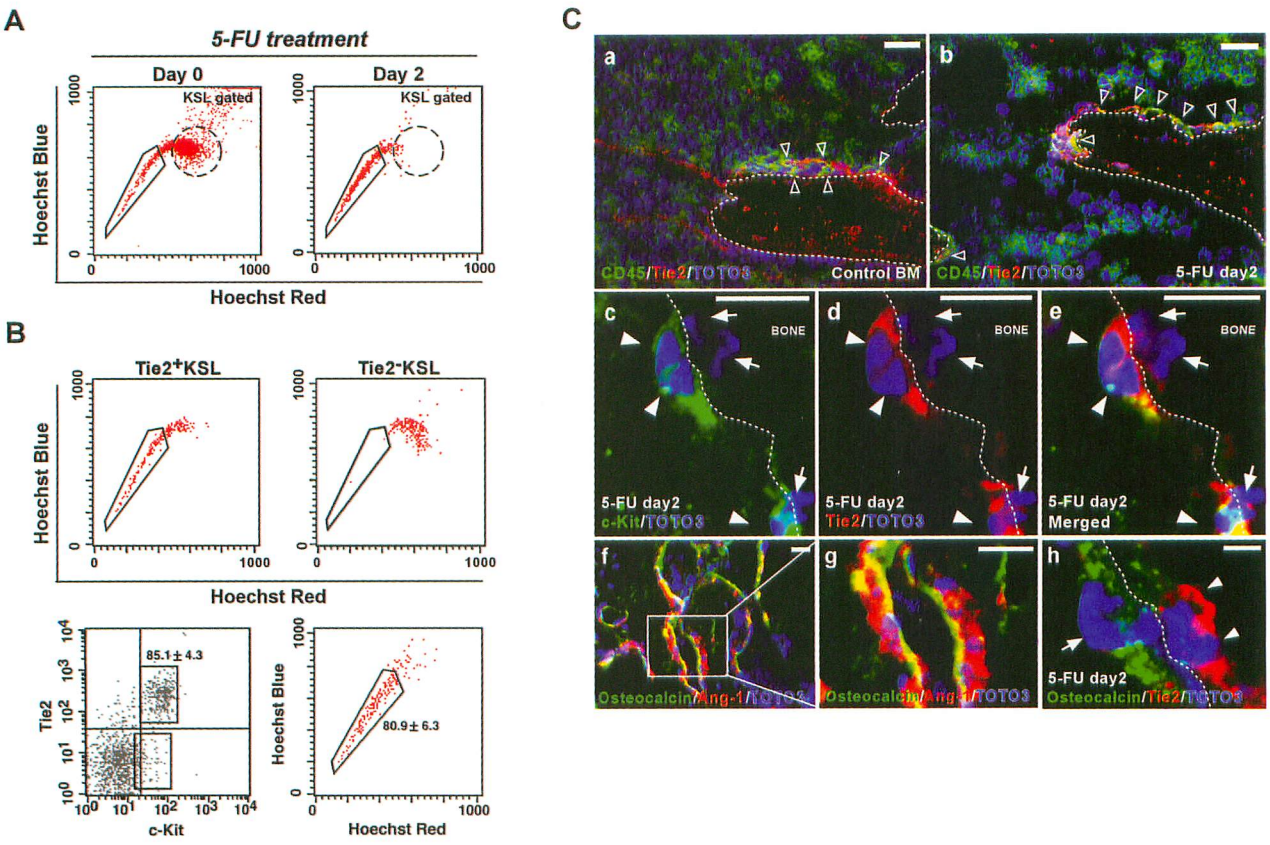

Fig 2. Identification of quiescent HSCs and their niche in adult BM.
(A) Changes in the proportion of SP and non-SP cells in KSL fraction after 5-FU treatment. In control KSL cells, 19.4 ± 0.7 % of cells were in SP, whereas after 2 days of 5-FU treatment the majority of cells in the KSL fraction were SP (70.1 ± 6.2 %), suggesting that KSL-SP cells were in quiescent state. (B) Analysis of the SP cells in $Tie2^+$ and $Tie2^-$ KSL cells. SP cells are specifically enriched in $Tie2^+$ population. (C) Immunohistochemical staining of adult BM. (a) untreated BM. (b-e) are BM section after 2 days of 5-FU treatment. Co-expression of Tie2 and CD45 (a and b). $CD45^+Tie2^+$ cells survived, adhering to the bone surface (open arrowheads). Scale bar, 25 μm. The expression of c-Kit (c) and Tie2 (d). (e) represents the merged image of (c) and (d). $Tie2^+$ cells adhered to bone surface were co-express c-Kit (arrowheads), and adhered to the OBs (arrows). Scale bar, 12.5 μm. The expression of Ang-1 and osteocalcin in BM (f and g). Ang-1 expression was detected in osteocalcin-positive OBs. (g) Higher power view of enclosed boxes in (f). $Tie2^+$ cells (arrowheads) adhered to the $osteocalcin^+$ OBs (arrows) in 5-FU treated BM (h). The dotted lines indicate the margin of the bone surface. Nuclei is labeled with TOTO3.

analyzed by Pyronin Y (PY) staining. It was previously reported that PY^- and PY^+ cells were in G0 and G1 phases of cell cycle, respectively (Huttmann et al., 2001). Expectedly, most KSL-SP cells (92.4 ± 6.7 %) were in G0. Therefore, SP is the most suitable marker for detection of quiescent HSCs. In addition, a proportional increase in the number of cells in G0 among HSCs is observed during mouse development after birth, which correlates closely with the relative increase in SP cells. To identify quiescent HSCs *in situ*, we analyzed specific markers for KSL-SP cells. We found that SP cells were specifically enriched in Tie2 receptor tyrosine kinase expressing KSL cells, and $Tie2^+$KSL cells survived after 5-FU treatment, as well as SP cells (Figure 2). Therefore we analyzed the expression of Tie2 in 5-FU treated BM, and we found that $Tie2^+$ HSCs adhered to OBs at the surface of the trabecular bone (Figure 2), in agreement with previous reports (Calvi et al., 2003; Zhang et al., 2003). Ang-1, a ligand for the Tie2 receptor is mainly produced by OBs, suggesting that Tie2 and Ang-1 are expressed complementarily in the niche (Figure 2). Taken together, our observations support the idea that the niche is a microenvironment reserved for quiescent HSCs. Hence, HSCs may transit between niche and non-niche sites and/or between quiescence and active cell cycling *in vivo*. When this balance is disrupted, such as occurs with $p21^{WAF1/Cip1}$ deficiency, HSCs cannot remain in G0 and long-term repopulating ability is lost (Cheng et al., 2000), indicating that the niche is essential for maintenance of a long-term hematopoietic system.

How does blocking cell division lead to maintenance of stem cell capacity? Every cell division must shorten telomere length in HSCs. Telomeres are composed of the tandem DNA repeats and associated proteins that cap the end of linear chromosomes. Telomeres are maintained by the reverse transcriptase, telomerase. Hematopoietic progenitors have a high replicative potential and telomerase activity to protect the ends of their chromosomes (Greenwood and Landsdrop, 2003). HSCs show telomeric shortening during replicative aging despite expression of telomerase (Greenwood and Landsdrop, 2003). Accelerated telomere erosion reduces the long-term

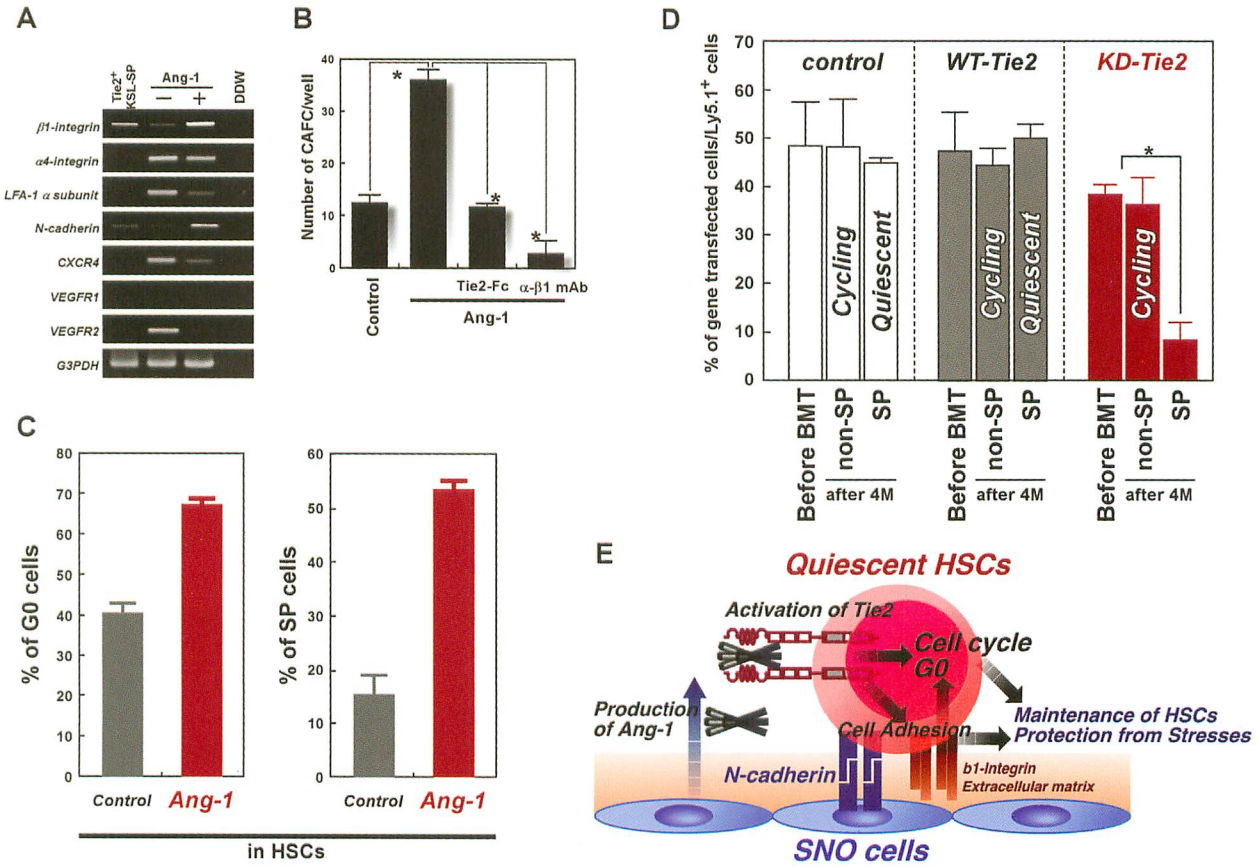

Fig 3. **Role of Tie2/Ang-1 signaling in the regulation of quiescent HSCs**
(A) Tie2+KSL-SP cells were cultured with or without Ang-1. Ang-1 induced the up-regulation of b1-integrin and N-cadherin (B) Cocultivation of Tie2+KSL-SP cells with OP9 stromal cells. Cobblestone formation was induced by the addition of Ang-1. The effect of Ang-1 was inhibited by Tie2-Fc or anti-β1-integrin antibody. (C) Overexpression of Ang-1 in hematopoietic cells. Ang-1 increased the frequency of quiescent cells and SP cells in HSCs. (D) Overexpression of WT and KD-Tie2 in HSCs. HSCs expressed KD-Tie2 failed to express the SP phenotype. (E) Model of the regulation of quiescent HSCs in the osteoblastic niche. Quiescent HSCs adhered to SNO cells in the niche. Ang-1 produced by SNO cells activates Tie2 on the HSCs. Tie2 phospholyration promotes N-cadherin and β1-integrin mediated tight adhesion of HSCs to the niche, and induced quiescence of HSCs. Adhesion and quiescence of HSCs contribute to the maintenance of self-renewal activity of HSCs and enhanced survival of HSCs.

repopulating capacity of HSCs in telomerase mutant mice (Samper et al., 2002; Allsopp et al., 2003). Recently, the important role of telomeres in human disease has been highlighted by the study of dyskeratosis congenta, in which patients exhibit stem cell dysfunction (Ruggero et al., 2003).

Regulation of quiescent hematopoietic stem cells

1) Tie2/Ang-1 signaling

What molecular mechanisms govern the cell cycle, adhesion, and survival of HSCs in the BM niche? We previously reported that Tie2/Ang-1 signaling induced adhesion of HSCs to fibronectin and collagen (Takakura et al., 1998; Sato et al., 1998), and play a critical role in the development of definitive hematopoiesis (Takakura et al., 1998). We showed that Ang-1 activate Tie2 on HSCs resulting induction of HSC adhesion and suppression cell cycle (Figure 3). Tie2/Ang-1 signaling activates β1-integrin and N-cadherin in Tie2+ HSCs. Tie2/Ang-1 signaling also promotes HSC interactions with extracellular matrix and cell components of the niche.

Cell adhesion and quiescence of HSCs act cooperatively in the inhibition of cell division. It was reported that HSCs lose their self-renewal activity after repeated cell divisions under the *in vitro* culture (Ema et al., 2000). Prevention of HSC division by Tie2/Ang-1 signaling associated with maintenance of long-term repopulating activity of Tie2+KSL-SP cells. In addition, exogenous Ang-1 dramatically increased SP cells in the KSL population indicating that increment of quiescent HSCs (Figure 3). Quiescence of cell cycle leads to cell survival from the various stresses. In hematopoietic cell, we showed that Tie2/Ang-1 signaling lead to protection of HSCs from various stresses. For instance, pre-administration of Ang-1 adenovirus or recombinant protein induced adhesion of HSCs to bone surface *in vivo*. Ang-1 treatment followed by a lethal dose of 5-FU injection or X-ray irradiation result in the protection HCS and enhancement of recovery of hematopoietic cell resulting prolonged sur-

vival of mice.

The chimeric mice composed of both normal embryonic cells and Tie receptor Tie1/Tie2-deficient cells showed that these receptors are not required for fetal hematopoiesis, including the emergence of definitive HSCs, or for their relocation to and differentiation in the FL. Although Tie receptor-deficient cells retain the capacity to home to the BM from the FL during ontogeny, they fail to be maintained in the BM microenvironment (Puri and Bernstein, 2003). Tie1-deficient cells, expressing normal levels of Tie2, contribute to hematopoiesis (Partanen et al., 1996; Rodewald et al., 1996), indicating that Tie2 is required for postnatal BM hematopoiesis but not for embryonic hematopoiesis. It is known that Tie2-null mice died at E9.5-10.5 by abnormal interactions between the endothelium and surrounding matrix and pericyte (Suri et al., 1996). We hypothesize that the defect in hematopoiesis in Tie2-null embryos is a consequence of abnormal endothelial cell development in the P-Sp region. The function of Tie2 in endothelial cells might be crucial for the maintenance of the hematopoietic microenvironment. Although Tie2 expression was detected in embryonic HSCs, Tie2 signaling may only function in quiescent HSCs of the adult BM. In addition, in developing mice BM, there were no correlations between Tie2 expression and quiescence or SP phenotype of HSCs.

Analysis of chimeric mice composed of Tie receptor-deficient donors and $Rag2^{-/-}$ hosts, which do not produce mature lymphocytes, show that Tie2/Tie1-deficient cells contribute to lymphopoiesis in the absence of competing host cells (Puri and Bernstein, 2003). We also demonstrated that kinase dead-mutant Tie2 (KD-Tie2) expressing HSCs could not maintain quiescence and failed to express the SP phenotype (Figure 3). These findings strongly suggest that Tie2 is critical for the maintenance and survival of HSCs in the adult BM and that Tie2-deficient or KD-Tie2 cells are unable to occupy the adult BM niche when competing with wild-type cells.

2) N-cadherin mediated cell-cell adhesion

We also found that $Tie2^{+}$KSL-SP cells expressed N- and VE-cadherin, and osteoblasts expressed N-, P-, and OB-cadherin. In addition, we confirmed that Ang-1 treatment upregulated the expression of N-cadherin in $Tie2^{+}$ KSL-SP cells. This suggests that an adherens junction between HSCs and osteoblasts created via N-cadherin may contribute to HSC maintenance. We analyzed the function of N-cadherin in the maintenance of the stem cell specific property, such as cell adhesion, quiescence, and LTR-activity. Homophilic interaction between HSCs and stromal cells via N-cadherin maintained long-term culture-initiating cells (LTC-ICs) and induced slow cell cycling, suggesitng that N-cadherin-mediated cell-cell adhesion between HSCs and stromal cells enhances the quiescence of HSCs and keeps HSCs in immature state in *in vitro*. We found that the overexpression of dominant-negative N-cadherin inhibited long-term reconstitution activity of HSCs. It suggests that the adhesion between HSCs and BM niche cell is indispensable for the LTR-activity. In addition, we also found that WT-N-cadherin overexpressing HSCs were enriched in the SP fraction after 6 month of BM transplantation, indicating that N-cadherin-mediated cell adhesion induced HSCs in the quiescent and kept quiescent HSCs in the niche *in vivo*.

These observations led us the novel model of the maintenance of hematopoiesis. The localization of quiescent HSCs on the bone surface is regulated by stem cell specific adhesion molecules such as N-cadherin. Once the HSCs localize to SNO cells, Ang-1 produced by SNO cells may activate Tie2 on HSCs and Tie2/Ang-1 signaling promote tight adhesion of HSCs to the niche through the up-regulation of N-cadherin. N-cadherin is a critical niche factor for the maintenance of the quiescence and self-renewal activity of HSCs.

Further studies may reveal other molecules or signaling pathways required for cell adhesion and cell cycle regulation by niche factors. Understanding factors underlying cell adhesion and cell cycle regulation in stem cells should lead to development of new strategies for regenerative medicine.

3) Regulation of reactive oxygen species (ROS)

Recently, we demonstrated that a cell cycle checkpoint molecule, ataxia telangiectasia mutated (ATM), regulates the self-renewal activity of the HSCs but not their proliferation or differentiation into progenitors (Ito et al., 2004). The ATM protein maintains genomic stability by activating a key cell cycle checkpoint in response to DNA damage, telomeric instability or oxidative stress. $Atm^{-/-}$ mice over the age of 24 weeks show progressive bone marrow failure due to a defect in HSC function associated with elevated radical oxygen species (ROS), but they do not show telomere dysfunction. Elevated ROS induces upregulation of the cyclin-dependent kinase (CDK) inhibitor $p16^{INK4A}$ and the retinoblastoma (Rb) gene in $Atm^{-/-}$ HSCs. Treatment with anti-oxidative reagents restores the reconstitutive capacity of $Atm^{-/-}$ HSCs (Figure 4). These data demonstrate that prevention of HSC senescence depends on ATM-mediated inhibition of oxidative stress. From these data, it is speculated that

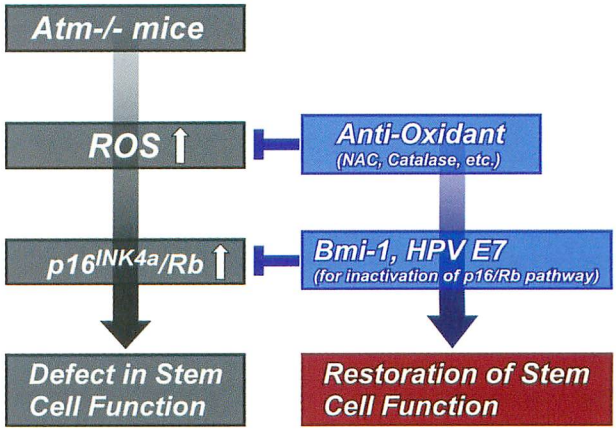

Fig 4. Role of ATM gene in the HSC functions.
Loss of ATM upregulates radical oxygen species (ROS) and $p16^{INK4a}$/Rb, resulting in the defect of stem cell function. Anti-oxidant such as N-acetyl cystein (NAC) or over-expression of Bmi-1/human papilloma virus E7 (HPV E7) restore the stem cell function.

niches or niche cells for quiescent stem cells are located in hypoxic regions of the BM, such as the trabecular zone for HSCs, where they not only keep stem cells quiescent through cell-adhesion but also protect them from ROS.

Recently, a novel concept that cancer cells include self-renewing "cancer stem cells (CSC)" has been proposed (Reya et al., 2001; Passegue et al., 2004). CSCs have the potential for self-renewal to drive tumorigenesis. There is one possibility that the cancer or leukemic stem cells also resided in the niche, and protected from anti-cancer drugs or irradiation therapies. Therefore, regulation of the passage of stem cells in and out of their niche could be a potential strategy for treatment of leukemia.

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Secretory IgA Immune Responses as the Mucosal Frontline

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ABSTRACT

The recognition that host defenses are mediated via mucosal barriers dates back several thousand years. Ingestion of *Rhus* leaves to modify the severity of reactions to poison ivy is a centuries old practice among native North Americans. A similar practice has been carried out among Asian countries as well, where lacquer craftsmen desensitized themselves via the ingestion of sumac extracts. The modern concept of local immunity, however, developed by Besredka in the early 1900s, followed by the discovery of IgA in 1953, and its isolation and characterization in 1959. Studies in the early 1960s demonstrated the presence of IgA in a unique form in milk and, shortly thereafter, in other external secretions. These studies were followed by the discovery of the secretory component and the identification of the J chain. The remarkable immunological observations were soon complemented by the identification and characterization of the gut-associated lymphoid tissue (GALT), the findings of mucosal circulation of antigen-sensitized or reactive IgA committed B cells from GALT to other mucosal surfaces such as aero-digestive tract, genital tract and glandular tissues (e.g., salivary, lacrimal and mammary glands), and the definition of immunologically unique mucosal T cells. It should be emphasized that studies of the induction of salivary IgA responses to cariogenic bacterium, *Streptococcus mutans*, have made tremendous contribution to the characterization and understanding of the immunity at mucosal surfaces. A series of immunologically important investigations in the field of caries immunity definitively played a leadership role for the establishment of principal for the mucosal immune system. In the past decades, our concept of the mucosal immune system has been expanded to include M cells, mucosal dendritic cells and macrophages, Th1/Th2 cells, regulatory T cells and other effector cell networks, and the mucosal cytokines. Finally, the biological significance of the mucosal immune system increasingly is being realized and appreciated in the context of human infections acquired via mucosal portals of entry, including classical infectious diseases as well as newly emerging infectious diseases which lead to the desire for the development of mucosal vaccine.

Dawn of the mucosal immune system

For decades, vaccine researchers have been fighting infectious diseases without much help from one of the body's major defensive weapons, **the mucosal immune system.** Most successful vaccines to date, such as the childhood measles, mumps, and rubella immunizations, have been made from body to produce serum antibodies against disease-causing organisms. Thus, most of vaccines have been given via injection for the induction of effective systemic immunity. However, the molecular and cellular understanding of the mucosal immune system allows us to consider that the use of the system such as oral and nasal immunization leads to the induction of antigen-specific immune response in the systemic compartment in addition to the mucosal surface (see below more details). It is now plausible to propose that the current injection type vaccine can be advanced to the form of mucosal vaccine.

The part of the immune system which churns out 70% of the body's antibodies has been virtually ignored, because little was known about how it works. Membranes covered with mucous line the airways, the reproductive system, and the gastrointestinal tract, and many pathogens such as the bacterium that causes cholera and the virus that causes AIDS, first encounter the body via the mucosal epithelium. The mucosae of aero-digestive and reproductive tracts have a combined surface area of at least 400 m^2 where our body is continuously exposed to the harsh outside environments and which are of course the first ports of entry of many pathogens.

During the past 40 years, we have recognized that a distinct system of immunity (manifested by production of the IgA isotype of antibodies) exists at our mucosal surfaces (1, 2). However, as early as 1927, Besredka (3) clearly suggested the presence of the mucosal immune system as he described as a local immunity by the result obtained form the experiment with oral administration of enteric bacterial vaccines. He postulated that oral vaccination with bacteria such as shigella, salmonella, and cholera resulted in local immunity in the gut that was separate from circulating immunity.

The mucosal tissues are heavily populated with cells of the immune system: it is estimated that the intestinal lining contains more lymphoid cells and produces more antibodies than any other organ and tissue in the body (4, 5). The mucosal immune system consists of specialized local inductive sites, the organized mucosa-associated lymphoid tissue (O-MALT) and wide spread effector

sites, the diffuse mucosa-associated lymphoid tissue (D-MALT) both of which are separated from mucosal surface antigens by epithelial barriers (6). Some mucosal tissues such as vagina have no local organized MALT but rely on antigen uptake and transport into lymph nodes that drain the mucosa (7).

In either case, the first step in the induction of a mucosal immune response is the transport (or uptake) of antigens across the epithelial barrier. Following antigen processing and presentation in inductive sites, post-switched antigen-specific IgA-committed B cells proliferate locally and then migrate via the bloodstream to distant mucosal and secretory tissues. There they differentiate primarily into polymeric IgA producing plasma cells. Dimeric or polymeric IgA antibodies are transported across epithelial cells into glandular and mucosal secretions via poly Ig-receptor-mediated transcytosis. In this regard, it should be emphasized that studies of the induction of salivary antibodies to antigenic components of the cariogenic bacterium, *Streptococcus mutans*, part of the efforts for the development of carries vaccine have contributed to a fuller understanding of immunity at mucosal surfaces (8).

Discovery of Mucosal IgA (Secretory IgA:S-IgA, a launch for the mucosal immunity)

The discovery by Tomasi and coworkers (9) that IgA is the major immunoglobulin found in human external secretions, and subsequent work by others that most mammalian species possess IgA as the predominant immunoglobulin in secretions, have provided the impetus for studies of the mucosal immune system in health and disease. The observation that mucosal tissues contain many localized IgA producing plasma cells, coupled with findings that local antigen exposure results in the presence of S-IgA antibodies at that site, led many investigators to assume that S-IgA responses were entirely operated by the local immune system in nature. The elegant studies of Ogra and coworkers (10) showed that immunization of the lower alimentary tract with polio vaccine resulted in the induction of S-IgA antibodies in this area. This work was one of the first to provide convincing evidence that local administration of living antigen to mucosal sites resulted in the induction of antigen-specific IgA antibody response in the same surface area.

Peyer's patches are primary inductive sites for IgA responses and existence of a common mucosal immune system

The classical studies of Craig and Cebra (11) suggested that Peyer's patches (PPs) contain IgA precursor B cells that can populate the lamina propria of the gastrointestinal tract and become IgA producing plasma cells. These studies were the first to suggest a mucosal migratory pathway involved in the IgA response. Subsequent studies have shown that oral administration of antigens leads to the production of S-IgA antibodies in the gut as well as in secretions at distant mucosal sites such as salivary and mammary glands (11). In addition, it is well known that human colostrums and milk contain antibodies to gastrointestinal bacteria, including *Escherichia coli* (12), and to the oral bacterium *Streptococcus mutans* (13). Furthermore, studies done by Robertson and Cebra (14) provided strong evidence that following antigen exposure of intestinal tissue with a Peyer's patch (PP), IgA precursor B cells migrate from the patch via lymphatics to the bloodstream and thereby reach the lamina propria of other regions of the gastrointestinal tract, where they become mature IgA synthesizing plasma cells. These elegant studies made significant contribution for the concept of the common mucosal immune system which is consisted of the inductive (e.g., PP) and effector (e.g., lamina propria region) sites.

Molecular basis of PP development

PPs occur mainly in the ileum and less frequently in the jejunum in humans. By definition, they consist of at least five aggregated lymphoid follicles but can contain up to 200 such organized structures (15). Human PP anlagen composed of $CD4^+$ dendritic cells (DCs) can be seen at 11 weeks of gestation, and discrete T- and B- cell areas occur at 19 weeks, but no germinal centers appear until shortly after birth, reflecting dependency on stimulation from the environment, a process that also induces follicular hyperplasia (16). Macroscopically visible PPs in humans increase from approximately 50 at the beginning of the last trimester to 100 at birth and 250 in the middle teens, then diminish to become approximately 100 between 70 and 95 years of age (15).

The initial steps involved in murine PP development have been studied in some detail in mice. A cluster of vascular cell adhesion molecule 1^+ (VCAM1)/intracellular adhesion molecule 1^+ (ICAM1) cells develops in the upper small intestine beginning at embryonic days 15 to 16, followed by the presence of cells expressing the IL-7 receptor (IL-7Rα) at day 17.5 (17), which appear to be the anlagen of the patch. Mice defective in IL-7Rα gene expression fail to form mature GALT (17). It now appears that IL-7-IL-7Rα triggering results in up-regulation of lymphotoxin (LT)α1β2 membrane expression by lymphoid cells, including those in developing PPs (18-21). Furthermore, mice that lack LTα or LTβ or that have been treated *in utero* with a fusion protein of LTβ receptor-immunoglobulin fail to develop PPs or systemic lymph nodes (22). In addition, alymphoplasia (aly/aly) mice with a mutation in the NFκB-inducing kinase (23), which appears to act downstream of LTα1β2-LTβ receptor signaling, also fail to develop PPs. These findings together, the initiation of tissue genesis of PP depends on the group of the mucosal organogenesis cytokine family of IL-7/IL-7R and LTα1β2-LTβR.

Unique characteristics of PP : M cells

Murine PPs contain a dome, underlying follicles (B-cell zone with germinal centers), and parafollicular regions enriched with T cells. The specialized epithelial cells covering PPs were called an FAE (follicle-associated epithelium) cell because it characterized the uniqueness of the organized lymphoid tissues in the gastrointestinal tract including the presence of antigen-sampling M cells (24). M cells were later named for its unique topical structure of microfold/membraneous with the pocket formation at the basement membrane and the entire epithelium covering mucosa-associated lymphoreticular tissue (MALT)

such as PPs is now commonly described as an FAE type (25, 26). M cells have short microvilli, small cytoplasmic vesicles and few lysosomes. Microorganisms and complex antigens can be engulfed by M cells, and the uptake and transcellular passage of luminal antigens, including proteins and small particles through the cells, has been reported (27-29). Although the biological importance of M cells is highly appreciated, the origin of the cells is still unknown. In several years ago, a method for generating M cells *in vitro* has been reported (30, 31). Co-culture of an intestinal epithelial cell (IEC) line Caco-2 with mouse PP T and B cells or with a human B cell line (Raji) transformed polarized and differentiated IEC into M cells. This observation indicates that M cells are derived from an epithelial cell-lineage under the influence of lymphocytes, especially B cells, possess signaling molecules for the induction of M cells, but the mechanisms behind M cell development still remain to be elucidated.

PPs have all the immunological elements for mucosal S-IgA response

PPs also contain all the immune-competent cells needed for the induction of antigen-specific IgA immune response, including those of antigen presenting cells (APC including dendritic cells, macrophages and intestinal epithelial cells), IgA committed B cells and Th1 and Th2 lymphocytes. After the uptake of antigens through M cells, the antigens are processed and presented by professional APCs. A major APC population in the subepithelial region of PPs is characterized as dendritic cells (DCs) (32). PP contains at least three distinct subsets of mucosal DCs characterized by the expression patterns of surface molecules including CD11b and CD8α (33, 34). The kinds of immune responses evoked by each subset also are variable, and distinct: the generation of Th2 cells for the induction of an IgA response, the induction of Th3/Tr1 cells and/or inducible CD25 Tr cells for oral tolerance and the generation of Th1 cells for humoral and cell mediated immune responses.

Mucosal dendritic cells for IgA response

It should be postulated that major population of DCs isolated from a variety of mucosal sites (PP, lamina propria, mesenteric lymph nodes and lung) have a tendency to induce T helper type 2 (Th2) responses in *in vitro* T cell priming assays and to express regulatory/suppressive cytokines such as IL-10 and possibly transforming growth factor-β (33, 35, 36). Moreover, Hachimura et al. reported that mucosal DCs and in particular the $CD11c^+CD11b^+CD8\alpha$-DC subset isolated from Peyer's patches, which preferentially polarizes antigen-specific T cells to produce Th2 cytokines and IL-10 in vitro, promote IgA production by naïve B cells, which is mediated by IL-6 and T cell help (37). These findings indicate that the inductive site contains all the necessary DC subsets for the simultaneous induction and regulation of active (e.g., S-IgA) as well as quiescent (e.g., oral tolerance) immune responses.

Mucosal T cells for IgA response

Our earlier study clearly suggested that PPs possess antigen-specific helper T cells, which support proliferation and differentiation of surface IgA positive ($sIgA^+$) B cells into IgA producing plasma cells (38). Although the study preceded discovery of Th1 and Th2 subsets, these clones would, in retrospect, have properties of Th2-type cells. Today it is obvious that $CD4^+$ Th cells and derived cytokines from mucosal inductive sites can support the IgA response (39). For example, depletion of $CD4^+$ T cell subsets *in vivo* with monoclonal antibodies or by knockout of the CD4 co-receptor gene markedly affects on the induction of mucosal IgA responses (40, 41). In addition, PP $CD4^+$ T cell subset producing TGF-β is a key element for the gene conversion of isotype switching for $sIgM^+$ B cells to $sIgA^+$ B cells in the germinal centers of distinct follicles beneath the dome area of PP (42, 43).

For the B cell terminal differentiation of $sIgA^+$ B cells to IgA plasma cells for the generation of S-IgA, IL-5 and especially IL-6, possibly in combination with other cytokines produced by Th2 cells in the mucosal effector sites, appear essential for the continued generation of IgA producing plasma cells (44).

It would be too simplistic to conclude that Th2 type cells and their derived cytokines such as IL-5 and IL-6 are the only cytokines important in the generation of S-IgA responses (45). IL-2 also synergistically augmented IgA synthesis in B cell cultures in the presence of lipopolysaccharide (LPS) and TGF-β (43). Although IFN-γ is not directly involved in the enhancement of IgA B cell responses, this cytokine has been shown to enhance the expression of poly Ig receptor (or secretory component: SC), an essential molecule for the formation and transport of S-IgA (46). B cells activated through surface immunoglobulin in the presence of IFN-γ became potent APCs for T cells (47). In summary, an optimal relationship for the cross communication between Th1 and Th2 derived cytokines is essential for the induction, regulation, and maintenance of appropriate IgA responses in mucosa-associated tissues.

PP independent IgA responses

Progress of molecular basis of PP organogenesis revealed that the progeny of mice treated with the fusion protein of LTβ receptor and Ig lack PPs but not mesenteric lymph nodes (MLNs). Thus, it allows the investigator to address the question of whether PPs are essential or not for the induction of antigen-specific IgA response in vivo. Yamamoto et al. took advantage of this unique in vivo model (22) and examined the importance of PPs for the induction of mucosal IgA antibody responses. Oral immunization of PP null mice with chicken ovalbumin (OVA) plus cholera toxin as mucosal adjuvant resulted in antigen specific mucosal IgA (and serum IgG) responses. OVA-specific $CD4^+$ T cells of the Th2 type were induced in MLN (and spleen) of PP null mice. In contrast, when TNF and LT-α double knockout mice, which lack both PPs and MLN, were orally immunized with OVA plus cholera toxin, neither mucosal IgA (nor serum IgG) anti-OVA antibodies were induced. These results clearly show that the MLN plays a crucial role than had been appreciated for the induction of mucosal IgA responses after oral immunization, suggesting that PPs are not indispensable for the induction of mucosal IgA responses in the gastrointestinal tract. In addition, the result leads

to the idea for the presence of additional antigen sampling and/inductive sites for PP-independent Ig A responses.

Mucosal IgA responses to commensal bacteria are PP independent

MacPherson et al. reported that the induction of commensal-specific IgA is independent of T helper cell activity and of the organization of lymphoid tissues such as PPs, reflecting an evolutionarily primitive form of specific immune defense (48). A related, intriguing observation is that up to 40% of IgA producing cells in murine intestinal lamina propria arise from a pool of B-1 precursors derived from the peritoneal cavity (49). They give rise to polyreactive natural S-IgA antibodies, particularly directed against polysaccharide antigens from commensal bacteria. In addition, Fagarasan et al. proposed an alternative pathway in which sIgM$^+$ B cells in the lamina propria (LP) switch to production of the IgA isotype without the need for T cell help (50). In this study, the authors speculate that dendritic cells sample antigen from the lumen and present it to B1 cells, which under the influence of cytokines derived from LP stromal cells trigger the process of isotype switching and differentiation to IgA producing plasma cells. In most recent work by MacPherson and Uhr, they characterize the active transport of live commensals by mucosal DCs from the mouse gut lumen to the intestinal mesenteric lymph nodes. The DCs carrying their commensal load do not stray beyond these lymphoid tissues, preventing a systemic infection and ensuring a commensal specific IgA response that is restricted to the gut mucosa. In this regard, Reinecker et al. (51) reported that intestinal lamina propria CD11b$^+$ DCs expressing CX3CR1 chemokine receptor were found to form transepithelial dendrites, which enable the cells to directly sample luminal antigens. Thus, CX3CR1-dependent processes, which control host interactions of specialized DCs with commensal and pathogenic bacteria, may regulate immunological tolerance, mucosal inflammation as well as humoral immune responses such as mucosal IgA antibody production.

Gut-homing lymphocyte imprinting by mucosal dendritic cells

Effector memory lymphocytes that arise in response to antigens in the alimentary tract express mostly intestinal homing receptors, particularly the integrin $\alpha4\beta7$ and CCR9, the receptor for TECK/CCL25, a chemokine expressed in the small intestine (39). In vivo studies have shown that the microenvironment in which lymphocytes encounter antigen somehow instructs them about their homing preference (52). In addition, in vitro activation of naïve T cells with DCs from PPs or MLNs, but not from peripheral lymph nodes (PLNs) or spleen, induces (instructs) effector lymphocytes with high expression of $\alpha4\beta7$ and CCR9 and the capacity to migrate to the small intestine (53, 54). The mechanism responsible for this imprinting of gut specificity was recently and elegantly shown by Iwata et al (55). They showed that T cell exposure to the vitamin A metabolite retinoic acid induced gut homing receptors and the ability to migrate to the small intestine. Importantly, many DC from PP and MLN but few from PLN or spleen expressed the prerequisite enzymes for oxidative conversion of vitamin A to retinoic acid, and inhibitors of these enzymes rendered intestinal DC incapable of inducing $\alpha4\beta7^+$T cells.

S-IgA responses in oral cavity

The oral cavity is part of the mucosal linings of the body and shows structural similarities with mucosal tissues in the gut and lungs, among other organs (56). It is also considered as the initial entry for the aero-digestive tract where all of the foreign and environmental antigens are taken up. Oral health depends on the integrity of the oral mucosa and its mucin coating, which together normally prevent the penetration of microorganisms and macromolecules that might be antigenic and/or pathogenic. The mucosa is normally protected by both immunological arms of innate and acquired defense mechanisms. Innate elements include mucins, lysozyme, lactoferin, lactoperoxidase, and various antimicrobial peptides such as histatins, β-defensins, and protease inhibitors, while the acquired mechanisms include immune cells of Th1/Th2, CTL and IgA committed B cells as well as S-IgA and other Ig isotypes. Oral epithelial cells themselves are reactive and express Toll-like receptors and produce a variety of regulatory and inflammatory cytokines upon activation (56). However, it should be noted that the usage and expression of TLR family seems to be quite unique at the mucosa where continuously exposed to abundant numbers of environmental antigens and stimuli, when compared with the systemic compartment since the latter is situated more sterile condition (57)

Although the oral cavity displays similarities with other parts of the mucosal immune system, some of its immune mechanisms differ from those found elsewhere. Oral immune system includes those that are part of the S-IgA immune system, emanating from major and minor salivary glands, and those from the systemic IgG immune system, emanating from crevicular fluid or within the gingival and mucosal tissues. Thus, oral cavity is protected by the immunological benefit of both mucosal and systemic immune systems.

The most striking difference between the oral cavity and the tissue lining the remainder of the gastrointestinal tract is the presence of the hard tissue, teeth. The junction between the teeth and mucosa allows a greater access of serum proteins (56) and immune cells to the mucosal surface than is found in other mucosae and thus presents an interface between the systemic and mucosal immune systems, either of which may influence the control and /or development of diseases in the anatomical vicinity. There is also exposure of a unique epithelium, the junctional epithelium around the necks of the teeth, to microbial challenge, and this epithelium is compromised in periodontal diseases (56).

The main sources of S-IgA into the oral cavity are major salivary glands such as parotid and submandibular glands. It has been shown that oral and/or nasal immunization is effective in the induction of antigen-specific IgA antibody producing cells in these salivary glands which account for the generation of S-IgA antibodies in saliva (8, 58). The total volume of saliva produced per day is probably between 750 and 1000 ml.

Most of the IgA in saliva is dimeric, but 5 to 10% is monomeric. In case of human, the IgA1 to IgA2 subclass ratio is about 55:45, in which the subclass ratio is similar to that seen in the intestinal LP. In whole saliva, the concentration of S-IgA is approximately 200 mg/1000 ml. Thus, S-IgA antibodies produced by the oral immune system via the use of concept of CMIS are not only a major element of mucosal immunity for the oral cavity but also contribute as a major source of the secretory form of antibodies for the rest of digestive tract as well.

Immunology of dental caries

Dental caries is one of the most common diseases of humankind. In spite of recent reductions in the rate of decay in western societies, the prevalence of caries in developed countries remains at greater than 95% of the population (56). Caries is still increasing in the developing countries with the increased consumption of refined sugars.

Dental caries is defined as the localized destruction of hard tissue by bacterial action. It requires both specific cariogenic bacteria, which are capable of producing acid, and carbohydrate in the diet, which can be metabolized by these bacteria and helps their colonization of the tooth surface (59). Dissolution of the hydroxyapatite crystals seems to precede the loss of organic components of both enamel and dentine, and thus demineralization is thought to be caused by acids resulting from the bacterial fermentation of dietary carbohydrates (59). Not all surfaces of the tooth are equally afflicted, and areas protected from cleansing such as the fissures and areas between the teeth are much more susceptible to decay.

The concept of immunity to caries depends on the demonstration that caries is a bacterial infection. Although vaccination against dental caries was attempted in the 1930s (8, 59), the real impetus for development of vaccination came with the demonstration that caries could not occur in the absence of bacteria whatever the diet, and later, that specific bacteria were needed (59).

As much as the anatomical location of tooth is an immunologically unique environment where it receives benefit of both the systemic as well as mucosal immune systems, both routes of immunizations have been examined for the generation of antigen-specific immune responses to mutans streptococci in humans, and for the protective effects in vaccination experiments in animal models (8, 59). Two different possibilities have been proposed for the mechanisms of immunological control against dental caries (59). One hypothesis, put forward primarily by British groups, is that serum IgG antibodies are mainly responsible for the protective effect, whereas American workers suggest that S-IgA in saliva inhibits adherence of *S. mutans* to tooth surfaces by using rodent caries models. However, it should be noted that these two mechanisms are not mutually exclusive. And the expansion of our knowledge in the uniqueness of oral immunity, the use of both immunological arms is most effective for the control of dental caries.

For example, Smith et al. (60) demonstrated that local immunization with formalinized whole cells of *S. mutans* resulted in an enhanced salivary IgA response and reduced caries development in both conventional and gnotobiotic rats. Furthermore, Michalek et al. (61) reported that the ingestion of formalin-treated cells of serotype g mutans streptococci has been shown to stimulate specific S-IgA antibody response in saliva and milk but not in serum of rats. Orally immunized rats developed significantly fewer carious lesions than nontreated control rats. Moreover, our studies demonstrated that nasal immunization of mice with a surface protein antigen of *S. mutans* with non-toxic mucosal adjuvant elicited salivary IgA antibody that inhibits colonization of *S. mutans* in murine oral cavity (58, 62).

Antigen-specific salivary IgA antibodies in humans can be induced by ingestion of capsules filled with *S. mutans* organisms (63), and recently nasal immunization with glucosyltransferase in humans has been shown to elicit both salivary IgA and serum IgG antibodies (64). No extended studies have been performed to see the inhibitory effect of such antibody on caries development in humans. However, artificially created *S. mutans*-specifc S-IgA antibodies by the use of transgenic plant system clearly showed that the application of the antibody influenced on the incidence of caries development (56). Although the issue for the execution of caries vaccine for the public health remains to be matter of debate, the concept of caries vaccine definitively triggered a series of outcome for the understanding of the mucosal immune system. It provided a fact that the immunological basic research initiated in the field of Dentistry contributed for the discovery and understanding of one of major arms of the host immune system, namely the mucosal immune system which is now well accepted in the scientific field of Immunology, and used for possible application of other infectious and immunological diseases threatening mankind.

SUMMARY

In this review, we described IgA comprises the body's major isotype of antibody and the bulk of the body's IgA producing cells reside in the various mucosal and exocrine sites. The production of IgA at mucosal surfaces is strictly regulated by the integrated cross communication consisting of mucosal IgA^+B cells, mucosal T cells, mucosal dendritic cells, and epithelial cells. Although it has long been recognized that a major source of IgA plasma cells at mucosal surfaces is the organized lymphoid tissue such as Peyer's patches, it was recently revealed that PP null mice can also sustain mucosal IgA responses. Mucosal dendritic cells may be specialized in inducing a non-inflammatory environment and in providing help B cells to promote IgA producing plasma cells via the activation of Th2 cells. In this regards, the induction of commensal bacteria specific IgA^+B cells is independent of T helper activity, but possibly dependent on mucosal dendritic cells expressing CX3CR1 chemokine receptor. In this context, the mesenteric lymph nodes act as inductive site for the generation of secretory IgA antibodies that prevent the microflora from reaching the systemic compartment of the host immune system and from eliciting a damaging inflammatory response.

Although it is likely that the secretory IgA responses play a crucial role for the caries immunity, the induction

and regulation of the S-IgA responses in oral cavity still remains puzzling. Mucosal dendritic cells in oral cavity such as Langerhans dendritic cells could be indispensable for the induction and regulation of Th2 cells and IgA^+B cells which mediate the humoral immune responses to cariogenic as well as periodontal pathogens. However, it still remains vague where and how the Langerhans-type dendritic cells drive T cells as well as B cells to expand and differentiate for the development of Th2-mediated IgA responses. Future studies will address more detailed molecular and cellular basis for the immune responses occurred in oral cavity.

Dedication to the late Dr. John Cebra

The year 2005 was very exciting but also very sad year for among mucosal immunologists. We had experienced scientific and social excitements of the12th International Congress of Mucosal Immunology in Boston, June in 2005. At that time John's numerous scientific contribution for the area was recognized by the first Society of Mucosal Immunology (SMI) Distinguished Science Award. During the award ceremony, we all respected his accomplishment and were glad to see his smile. Unfortunately, we recently leaned sad news from Pennsylvania that he has passed away from his long fight for the illness which he never expressed in the public. He was one of the founders and frontiers of Mucosal Immunology which we all now enjoy the scientific excitement of this fascinating field. His smile during the award ceremony still floats before our eyes. Thanks John for your scientific contribution for the establishment of Mucosal Immunology.

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Antimicrobial Peptides in Human Gingival Keratinocytes

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ABSTRACT

The innate immune system is the primary defense against bacterial infection. Epithelial cells are first defense system as not only physical barrier, but also production of several factors including antimicrobial peptides. Antimicrobial peptides produced by human are recently drawn attention due to the relevance to some diseases and also new chemotherapeutic agents. Tooth decay and periodontal disease are infectious disease caused by oral bacteria. We focused on two major antimicrobial peptides, human β-defensins (hBD) and LL37 in human gingival keratinocytes (HGK) to see the interaction of these peptides with periodontopathogenic bacteria. HGK constitutively expressed hBD1, while they inducibly expressed hBD2, hBD3 and CAP18 upon contact with periodontopathogenic bacteria. The level of their expression varied according to clinical isolates. Synthetic antimicrobial peptides of hBD1-3 and LL37 were evaluated for their antimicrobial activity to periodontopathogenic bacteria including *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia* and *Fusobacterium nucleatum*. We found these four peptides had bactericidal activity against all bacteria tested, although the degree of antibacterial activity was variable among strains and species. The antibacterial activity of hBD1 was less effective than those of other peptides. These results suggest that HGK produce several antimicrobial peptides in response to oral bacteria, although the induction ability and susceptibility to these peptides are different among species and strains.

Key word: antimicrobial peptides, defensin, periodontitis, oral bacteria, keratinocyte

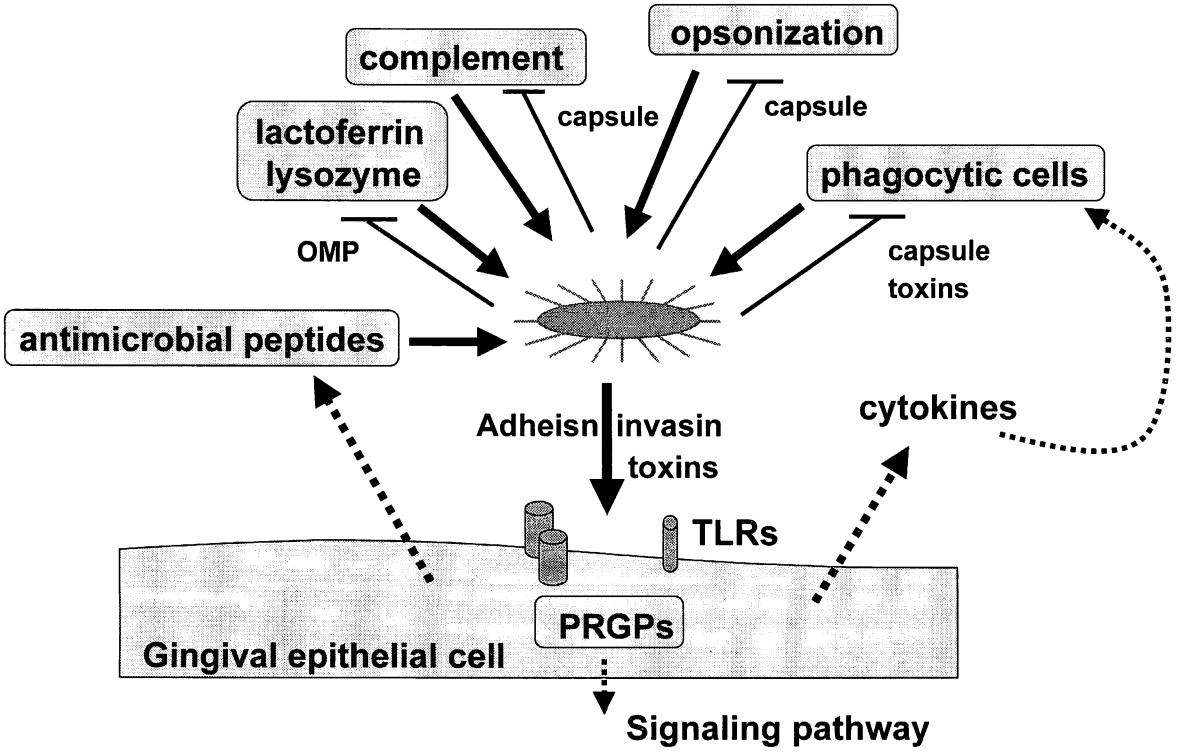

Fig. 1 Model of innate immune system against bacterial infection. Several factors involved in innate immune system attack to invaded bacteria. Also, mammalian cells respond against bacterial contact through several pattern recognition proteins (PRGP), such as TLRs, and produce several factors, such as cytokines, antimicrobial peptides. Factors resistant to innate immune system in bacteria are also indicated.

Table. 1 Human defensins and cathelicidin

α-defensins	
HNP1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP2	CYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP3	DCYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP4	VCSCRLVFCRRTELRVGNCLIGGVSFTYCCTRV
HNP5	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR
HNP6	FTCHCRR-SCYSTEYSYGTCTVMGINHRFCCL
	-C-C----C---------C---------CC
ß-defensins	
hBD1	GLGHRSDHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK
hBD2	GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP
hBD3	GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK
hBD4	EFELDRICGYGTARC-RKKCRSQEYRIGRCP-NTYACCLRKWDESLLNRTKP
	-------C------C----C---------C------CC---
cathelicidin	
LL37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES

INTRODUCTION

Environmentally, we are always exposed to many kinds of microorganisms including pathogens. The innate immune system is the primary defense against microbial infection that monitors our environmental microbial flora. Fig. 1 summarizes the factors involved in the innate immune system that modulate bacterial infection. All coordinately act for the host defense to eliminate the pathogens. Recently, anti-microbial peptides have been focused attention because low or high levels of the anti-microbial peptides *in situ* are linked to certain genetic diseases where the peptides are expected to be potential alternative to long-term use of chemotherapeutic agents. The anti-microbial peptides directly kill microorganism, and some act as chemokines. Crohn's disease (Fellermann *et al.*, 2003), atopic dermatitis (Ong *et al.*, 2002; Fellermann *et al.*, 2003), Kostmann's disease (Putsep *et al.*, 2002) and cystic fibrosis (van Wetering *et al.*, 1999; Devine *et al.*, 2003) are linked to microbial infections due to a decrease in anti-microbial peptides expressed in these patients. Whereas in the psoriasis lesion, the anti-microbial peptides are highly expressed, resulting in fewer microbial infections (Fellermann *et al.*, 2003). Furthermore, knockout mice lacking CRAMP or β-defensin-1 are highly susceptible to bacterial infections (Nizet *et al.*, 2001; Morrison *et al.*, 2002). Therefore, the antimicrobial peptides are considered to be one of the central defense systems against microbial infections.

Tooth decay (dental caries) and periodontal diseases are caused by bacterial infection. Etiologic agents have been explored for a long time, and some have been identified as causative agents such as *Streptococcus mutans* and *S. sobrinus* for caries, and *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Actinobacillus actinomycetemcomitans* for periodontitis. These bacteria, Gram-positive and Gram-negative bacteria, tend to aggregate and coexist in dental plaque. The dental plaque is the pathogenic resource for the dental caries and periodontitis. In the oral cavity, many antimicrobial agents, such as, histatin, lactofferin and lysozyme, are known to be produced and act as one of the roles for the innate immunity against bacterial infection (Tenovuo *et al.*, 1991). Gingival epithelial cells are also reported to produce antimicrobial peptides, such as β-defensins and calprotectin (Krisanaprakornkit *et al.*, 1998; Ross *et al.*, 2001; Dunsche *et al.*, 2002). Gingival epithelial cells, especially non-keratinized cells at the bottom of the periodontal pocket, are considered to produce these antimicrobial peptides in contact with bacteria in the dental plaque. Several reports concerning interaction of antimicrobial peptides with oral bacteria have been made, but detailed investigation has not been conducted so far. Therefore, an investigation for the interaction of cariogenic or periodontal bacteria to these peptides produced from human gingival keratinocytes (HGK) is of great interest to understand the potential role of innate immunity in dental diseases.

In this article, we briefly describe two major antimicrobial peptides, defensins and cathelicidin, and summarize the interaction of antimicrobial peptides with periodontopathogenic bacteria.

1 The anti-microbial peptides of human epithelial cells

Mammalian cells including humans produce several antimicrobial peptides; each playing a significant role in innate immunity. These peptides kill or deactivate bacteria, fungi, or viruses, although the spectrum of their activity is variable among the peptides. Recently, some of these peptides were shown to have potent chemokine activity. There are many anti-microbial peptides shown in various tissues and organs. In this review, we focus on two major antimicrobial peptides, β-defensins and LL37/CAP18, identified in epithelial cells.

1) β-defensins and LL37/CAP18

β-defensin:

Defensins are well known as cysteine-rich peptides classified into three types: a- (HNP), β-(hBD), and θ-defensins (Ganz *et al.*, 1985; Selsted and Quellette, 2005). a- and β-defensins are found in humans while θ-defensin is not expressed in human although an ortholog is found in humans. The defensins are cysteine-rich peptides having six cysteine residues with three disulfide bonds (Table 1). Alpha- and β-defensins differ in the location, the length of amino acid residues, and a pairing of a disulfide bond. a-defensins mainly express in neutrophils and paneth cells, while β-defensins express in epithelial cells. Four β-defensins (hBD1-4) are identified and found to be expressed in various epithelial tissues: the trachea, skin, and intestine; and in monocytes and dendritic cells (Harder *et al.*, 1997; Schroder and Harder, 1999; Garcia *et al.*, 2001; Harder *et al.*, 2001; Duits *et al.*, 2002). hBDs have three disulfide moieties of cysteine linking residues 1-5, 2-4, 3-6. *In vitro* experiments shows that hBD1 is constitutively expressed, and the production is not influenced by bacterial exposure, while other hBDs were found to be inducible when exposed to bacteria (Harder *et al.*, 2001; Liu *et al.*, 2002; Midorikawa *et al.*, 2003). Especially, hBD2, first identified from skin (Harder *et al.*, 1997), is highly responsive to bacteria, proinflammatory stimuli (IL-1β, TNFa, LPS), and phorbol myristate acetate (Schroder and Harder, 1999; Midorikawa *et al.*, 2003). Previous reports show hBD1 and hBD2 have a strong anti-bacterial activity against Gram-negative bacteria and a weak (or no) activity against Gram-positive bacteria; while hBD3 and hBD4 have a strong activity against both Gram-positive and Gram-negative bacteria (Harder *et al.*, 1997; Valore *et al.*, 1998; Garcia *et al.*, 2001; Schibli *et al.*, 2002). hBD1 is primarily found in differentiated keratinocytes suggesting the regulation of hBD1 is linked to cell differentiation (Harder *et al.*, 2004). Other hBDs are implicated in cell differentiation (Liu *et al.*, 2002; Harder *et al.*, 2004). β-defensins are shown to have variable biological activities. They include having a chemotactic factor for CCR6 dendritic cells, degranulation of mast cells, and induce production of prostaglandin D2 (Yang *et al.*, 1999; Yang *et al.*, 2002).

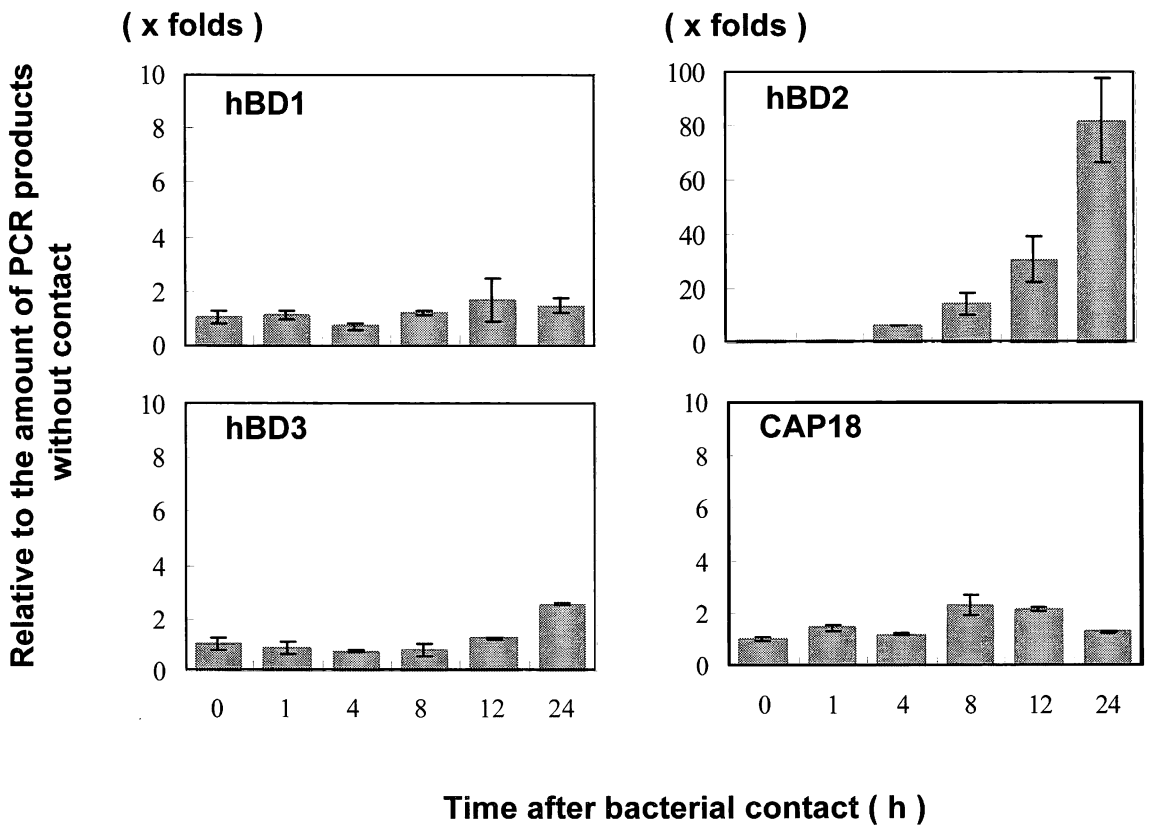

Fig. 2 Thin sections of *A. actinomycetemcomitans* Y4 exposed to antimicrobial peptides. Bacterial cells were reacted without no-peptide (1), or with 200 μg/ml of hBD3 (2a, 2b) or CAP18 (3a, 3b), respectively. Bars, 100 nm. (J Antimicrob. Chemother. 55:888-896)

Cathelicidin:

The cathelicidin family contains highly conserved regions known as cathelin (cathepsin L inhibitor) at N-terminus, and in the variable region at C-terminus that codes for the mature peptide with the antimicrobial activity. In humans, only one cathelicidin is found from human bone marrow as FALL-39 (Zaiou and Gallo, 2002), and later as LL37/hCAP18 (Zaiou and Gallo, 2002). LL37/hCAP18 is first synthesized as an 18 kDa protein, then during and after secretion, this protein is processed to the mature 5 kDa peptide (37 amino acid residues) (Ramanathan *et al.*, 2002; Zaiou and Gallo, 2002). LL37/hCAP18 is identified in neutrophils, monocytes, various epithelial cells, saliva, sweat glands and other tissues (Ramanathan *et al.*, 2002; Zaiou and Gallo, 2002). This peptide has a linear form, resembling an alpha helical structure. LL37 has a broad spectrum activity against Gram-positive and Gram-negative bacteria including antibiotic-resistant *Staphylococcus aureus, Pseudomonas aeruginosa*, and fungi (Ramanathan *et al.*, 2002; Zaiou and Gallo, 2002; Midorikawa *et al.*, 2003; Ouhara *et al.*, 2005). In addition to bactericidal activity, LL37 is able to bind to LPS, neutralize the endotoxin activity of LPS (Hirata *et al.*, 1994) and LL37 acts as a chemotactic factor for neutrophils, monocytes, T cells and mast cells (Zaiou and Gallo, 2002; Yang *et al.*, 2000).

2) Mechanism of action of antimicrobial peptides

Both defensin and LL37/CAP18 have broad antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and viruses. The anti-microbial peptides are cationic polypeptides where they electrostatically bind to negatively charged bacterial surface molecules: LPS in Gram-negative bacteria, lipoteichoic acid and teichoic acid in Gram-positive bacteria, and the anionic cell membrane (phospholipids). After attracting the peptides to the bacterial cell surface, the membrane is made permeable. This involves pore or gap formation by aggregation of the peptides in the membrane resulting in bacterial cell death.

2 Interaction of antimicrobial peptides in human gingival keratinocytes with periodontopathogenic bacteria

1) Identification of antimicrobial peptides in human gingival keratinocytes

Previously, several reports demonstrated the production of hBD2 and hBD3 in human gingival keratinocytes (Krisanaprakornkit *et al.*, 1998; Mathews *et al.*, 1999; Dale and Krisanaprakornkit, 2001). We investigated the transcript of hBD1-3 and LL37 expression in primary normal human gingival keratinocytes by RT-PCR, and found all these transcripts (data not shown), suggesting that these peptides are really expressed in gingival epithelium in vivo.

2) Expression of antimicrobial peptides and cytokines in HGEC stimulated with *periodontopathogenic* bacteria

Many experiments have demonstrated that bacteria such as *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*, induced antimicrobial peptides expression, especially hBD2, in human keratinocytes derived from various tissues, while hBD1 is constitutively expressed, and its expression does not alter by bacterial exposure (Schroder and Harder, 1999; Harder *et al.*, 2001; Midorikawa *et al.*, 2003). As for the effect of oral bacteria on the expression of antimicrobial peptides, there is one report regarding to commensal bacteria, *F. nucleatum* (Krisanaprakornkit *et al.*, 2000). Whole lysate of *F. nucleatum* induced hBD2 expression by activating MAPK pathway in HGK (Krisanaprakornkit *et al.*, 2002). We investigated the mRNA expression of antimicrobial peptides in HGEC stimulated with heat-killed *A. actinomycetemcomitans, P. gingivalis, P. intermedia, and F. nucleatum* in a time course experiment. As for *A. actinomycetemcomitans* (Fig.2), the amount of hBD2 mRNA gradually increased during the first 4 h following exposure of HGEC Y4 strain (the amount was 5 fold higher). After 8 h, the amount of hBD2 mRNA was 15 fold higher than that of 0 h, and the expression further increased up to 24 h. The amount of hBD3 mRNA did not alter up to 12 h, and increased at 2.6 fold after 24 h. The peak of the amount of CAP18 mRNA was 8 h (2 fold higher) after bacterial contact, and then gradually decreased. On the other hand, the amount of hBD1 mRNA was not affected by exposure to *A. actinomycetemcomitans* cells. Other bacteria also showed similar tendency with that of *A. actinomycetemcomitans*. The expression of hBD3 and LL37 by *F. nucleatum* or *P. intermedia* was significantly increased compared to that by *A. actinomycetemcomitans*. We further investigated the expression of 4 antimicrobial peptides in HGEC contacted with 7 clinical isolates, and found that there was difference among the strains with regard to the amount of expression of mRNA. Inducible expression of hBD2 mRNA was observed (4- to 25-fold) in all strains tested, while that of hBD3 or CAP18 mRNA was observed in some strains (hBD3: 1.5- to 6-fold, CAP18: 1- to 2-fold) for 12 h. HBD1 mRNA was not affected with bacterial contact. There was no correlation in the amount of hBD2, hBD3 and CAP18 expression in each strain.

3) Antibacterial activity of hBD1, 2, 3 and CAP18 against periodontopathogenic and cariogenic bacteria

Since HGK produce antimicrobial peptides in response to bacterial contact, it is of interest to know antibacterial activity of these peptides against oral bacteria. Several reports have shown the antibacterial activity against oral bacteria (Guthmiller *et al.*, 2001; Devine *et al.*, 2003), but total analysis using all peptides in keratinocytes have never been investigated. We investigated the susceptibilities of antimicrobial peptides including hBD1-3 and LL37 against four Gram-negative periodontopathogenic (Fig. 4). Antibacterial activity was evaluated with the method described previously. Briefly, overnight cultures of bacterial strains were harvested, washed with PBS, and suspended with 10 mM sodium phosphate buffer (NaPi) (pH 6.8). The bacterial suspension was diluted to 10^7 cells/ml with NaPi (pH6.8) and 10 μl of bacterial suspension (10^5 cells) was inoculated into 200 μl of NaPi

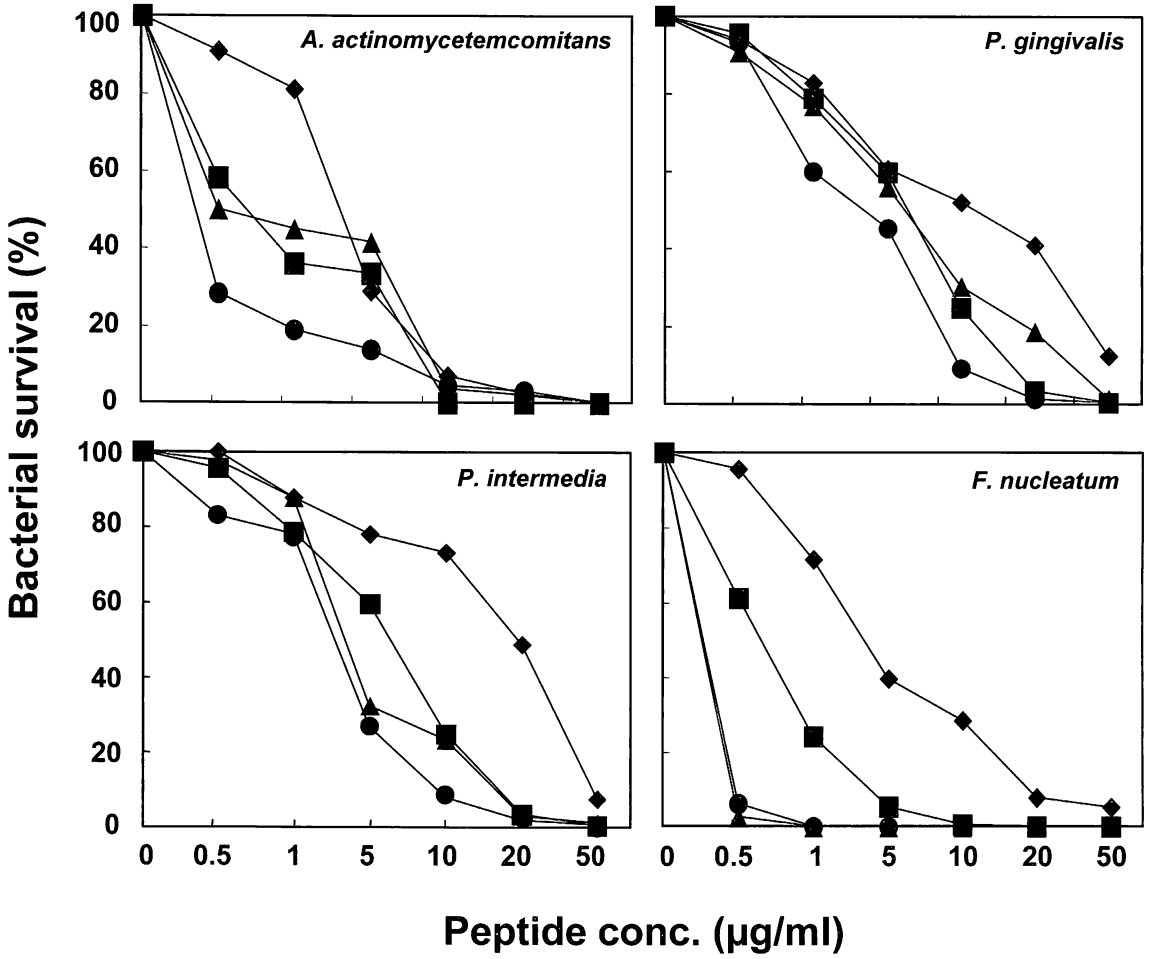

Fig. 3 Expression of β-defensins and CAP18 mRNA in HGEC stimulated with heat-inactivated bacterial cells. Overnight culture of *A. actinomycetemcomitans* Y4 was collected, and heated at 68℃ for 30 min. Bacterial cells were added to the medium cultured human gingival keratinocytes at confluency. After 12 h of bacterial exposure, total RNA was extracted from cells after contact with bacteria for various times. Real-time PCR were performed using specific primers for each antimicrobial peptide and GAPDH. The results of the real-time PCR are expressed as a ratio in comparison to the value at zero time and values represent the means S.D. of triplicate experiments.

with or without various concentrations of antibacterial peptides, and incubated anerobically for 2 h at 37℃. An appropriate dilution of the reaction mixture (100 μl) was plated on an agar plate, and then incubated at 37℃ over night. The colony forming units (cfu) were measured from the number of colonies on each plate. Antibacterial effect was estimated as the rate of cells surviving against total number of cells used.

We found antibacterial activity of all 4 peptides tested against 4 periodontopathogenic bacteria (Fig. 3). The antibacterial activity of these peptides is dose dependent. Among 4 bacterial species, *F. nucleatum* 21 had a remarkable susceptibility to hBD3 and CAP18 which had 100 % bactericidal activity in the presence of 1 μg/ml of peptides. *A. actinomycetemcomitans* Y4, *P. gingivalis* WA83 and *P. intermedia* 163 showed almost a similar susceptibility pattern to the peptides: hBD1 and hBD2 were less effective than hBD3 and CAP18. We further investigated the susceptibility of hBD3 and LL37 against forty strains of Gram-negative bacteria including 20 of *A. actinimycetemcomitans*, 7 of *P. intermedia*, 6 of *P. gingivalis* and 7 *F. nucleatum* were analyzed. Susceptibilities of all *F. nucleatum* strains to hBD3 and CAP18 were higher than those of other species. *P. intermedia* and *P. gingivalis* strains showed low susceptibilities to hBD3, while *A. actinomycetemcomitans* strains showed variable susceptibility to hBD3. As for CAP18 antimicrobial activity, these four species showed a variable response. There was no significant correlation between the susceptibility to hBD3 and CAP18 in each strain: some strains were highly susceptible to both peptides, while some were highly susceptible to either of them.

CONCLUSION

Anti-microbial peptides involved in the innate immune system are functionally active against cariogenic and periodontopathogenic bacteria. However, variable induction activity of antimicrobial peptides and variable susceptibility to the peptides among these strains imply that each strain shows variable response to innate immunity system. The ability of oral bacteria to achieve infec-

tion depends on many factors such as adhesion activity, production of cytotoxic factor(s), and counter action against the immune systems. We do not know if these different responses of each strain to these peptides affect the *in vivo* infection in the host because many factors are involved in the host defense system including the innate immune system. Previous reports indicate the importance of these peptides for protection against microbial infections. The experiments using knockout mouse lacking CRAMP or mouse β-defensin-1 revealed that these peptides were important for the protection against bacterial infection (Nizet *et al.*, 2001; Morrison *et al.*, 2002). Therefore, it suggests that variable responses to antimicrobial peptides may affect the outcome of oral bacterial infections in the host.

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War and Peace at Mucosal Immune System

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That a highly integrated and finely regulated mucosal immune system exists alongside and separate from the peripheral system might at first seem redundant and puzzling. Why should such a separated and sophisticated system be necessary when the peripheral immune system already seems to ensure immunity for the host? There can be no doubt about the sophistication and elegance of the mucosal immune system. It presents a well-tuned, two part defense, one more structured and localized, one more diffuse (McGhee and Kiyono, 1999). In the first, foreign antigens are encountered and selectively taken up into highly structured sites for the initiation of immune responses. In the second, diffuse collections of effector cells, such as B- and T lymphocytes, differentiated plasma cells, macrophages, dendritic cells, as well as eosinophils, basophils, and especially mast cells. Together, the two either produce mucosal and serum antibody responses and T cell-mediated immunity (CMI) or systemic anergy, commonly termed mucosally induced tolerance. Such a separate and sophisticated system may well have evolved as a major defense mechanism against mucosally encountered infectious agents. In the human adult, the mucosal surface is enormous (e.g., the gastrointestinal tract alone is larger than 300 m2) and so requires a significant expenditure of lymphoid cells and effector molecules for immunity. This review paper will highlight the multiple roles for lymphoreticular cells and effector molecules, including IgA, mucosal vaccine, tolerance, and inflammation.

1. ORGANIZATION OF THE MUCOSAL IMMUNE SYSTEM

The mammalian host has evolved organized secondary lymphoid tissues in the upper respiratory and gastrointestinal (GI) tract regions that facilitate antigen uptake, processing, and presentation for induction of mucosal immune responses. Collectively, these tissues are known as inductive sites. Although the gut-associated lymphoreticular tissues (GALT), e.g., Peyer's patches, are major inductive sites in all of the most common experimental mammalian systems, the degree of bronchus-associated lymphoreticular tissue (BALT) developed at airway branches for defense against intranasal/inhaled antigens differs considerably among species. In the rabbits, rats, and guinea pigs, such BALT development is significant, whereas in humans and mice it is negligible (Pabst, 1992). Instead, the major inductive tissues for intranasal/inhaled antigen in humans and mice appear to be the palatine tonsils and adenoids (nasopharyngeal tonsils), which together form a physical barrier of lymphoid tissues termed the nasopharyngeal-associated lymphoreticular tissues (NALT) (Kuper et al., 1992).

Murine Peyer's patches (PP) contain a dome, underlying follicles (B-cell zones with germinal centers), and interfollicular regions enriched with T cells (Figure 1). The surface of the dome region is covered by the specialized follicle-associated epithelium (FAE), 10% to 20% of which is composed of M cells that exhibit thin extensions

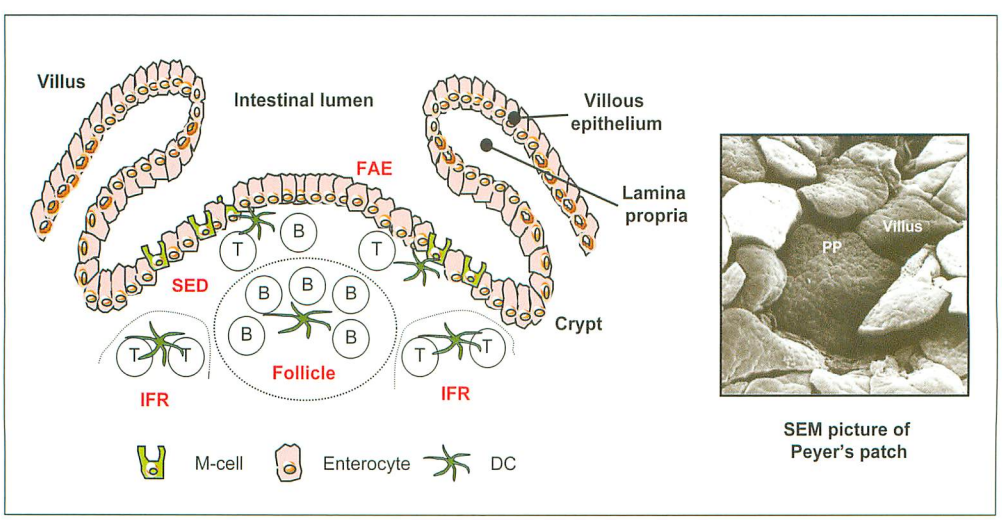

Fig. 1. Schematic representation and scanning electromicroscopy (SEM) picture of Peyer's patch (PP) on the small intestine. The follicle-associated epithelium (FAE) covers the dome of the PP. Transport across the epithelium of antigen occurs through specialized M cells. Dendritic cells (DC) are present in both the Subepithelial dome (SED) and the interfollicular region (IFR).

around lymphoreticular cells (Neutra et al., 1996). These extensions, which almost surround B and T lymphocytes and occasional macrophages and dendritic cells (DC), form an apparent pocket (Neutra et al., 1996). The M cells, which have short microvilli, small cytoplasmic vesicles, and few lysosomes, are adept at uptake and transport of lumenal antigens, including proteins and particulates such as viruses, bacteria, small parasites, and microspheres. Investigators in this field disagree on whether M cells are available to process and present antigen. Some believe that antigen uptake by M cells and transcellular passage results in delivery of intact antigen into the underlying lymphoid tissue. In addition to serving as a means of transport for lumenal antigens, the M cells also provide an entry way for pathogens.

Distinct follicles (B-cell zones) are located beneath the dome area of the PP and contain germinal centers where significant B-cell division is seen. These germinal centers, which contain the majority of $sIgA^+$ B cells (Butcher et al., 1982; Kiyono et al., 1982), are considered to be sites where frequent B-cell switches to IgA and affinity maturation occur. However, unlike immune lymph nodes and the spleen in the systemic compartment, plasma cell development does not occur. All major T-cell subsets are found in the T cell-dependent areas adjacent to follicles. The interfollicular T cells are mature, and more than 97% of these T cells use the αβ heterodimer form of the T-cell receptor (TCR). Approximately two-thirds of PP αβ TCR^+ cells are $CD4^+$ $CD8^-$ and exhibit properties of Th cells, including support for IgA induction. Approximately one-thirds of PP αβ TCR^+ cells are $CD4^-$ $CD8^+$; this cell subset contains precursors of cytotoxic T lymphocytes (CTL), whereas other $CD8^+$ T-cell subsets appear to contribute to mucosally induced tolerance. In addition, at least three DC subpopulations have been described in PP; myeloid DCs ($CD11b^+$), lymphoid DCs ($CD8\alpha^+$) and double negative DCs (Kelsall and Strober, 1996; Iwasaki and Kelsall, 2000). Myeloid DCs in the subepithelial dome (SED) appear to be immature in that they do not express maturation markers such as DEC-205. Lymphoid and double negative DCs are capable of inducing Th1 differentiation for the subsequent development of CMI, while myeloid DCs induce Th2 cells for the generation of IgA immune responses in mucosal effector sites.

After initial exposure to antigen in MALT, mucosal lymphocytes leave the inductive site and home to mucosal effector tissues. This pathway, which results in immunity as several mucous membrane sites is referred to as the Common Mucosal Immune System (CMIS). Antigen uptake by M cells occurs in PP and NALT and resulted in the initial induction of the immune response. Antigen-sensitized, precursor $sIgA^+$ B cells, $CD4^+$ Th cells, and $CD8^+$ CTLs leave via efferent lymphatics and migrate to MLNs and then into the thoracic duct to reach the bloodstream. These migrating cells enter the effector sites, where terminal differentiation, synthesis, and transport of sIgA occur. Effector sites for mucosal immune responses include the lymphoid cells in the lamina propria (i.e, lamina propria lymphocytes [LPL]) regions of the GI, the upper respiratory and reproductive tracts, as well as secretary glandular tissues such as mammary, salivary, and lacrimal glands (McGhee and Kiyono, 1999). In addition, most evidence suggests that the lymphocytes that reside in the epithelium (i.e., the intraepithelial lymphocytes [IEL]) also serve as effector cells. Effector mechanisms used to protect mucosal surfaces include CTLs, as well as effector $CD4^+$ Th cells for CMI (Th1) and for S-IgA antibody responses (Th2).

2. MUCOSAL IMMUNITY TO VACCINE

Jenner introduced vaccination 200 years ago with the use of *Vaccinia* virus to prevent smallpox. However, since this auspicious beginning, fewer than 50 vaccines have been approved for human use, and in some instances these vaccines are merely improved versions of early forms. All but three of the current vaccines are administered parenterally and as such do not induce significant mucosal immunity. Nevertheless, almost all viral and bacterial pathogens to which vaccines would be desirable invade mucosal tissues where cell-mediated and antibody-mediated immunity would be most effective. Perhaps the most important example of the importance of the mucosal immune system in vaccine development is the realization that more than 80% of current HIV infections occur through sexual transmission. Hardly less striking are the strides that would be made against respiratory infections ranging from influenza (requiring yearly vaccination) to bacterial pneumonia and the enteric infections ranging from diarrhea- inducing bacterial entheropathies (cholera, *Shigella*, and *E. coli*) to infant rotavirus infections, should research into mucosal immunity become a research priority worldwide. Furthermore, an advantage of mucosal vaccination is that this mode can induce both mucosal and systemic immune responses, which results in two layers of host protection against infectious diseases (Yuki and Kiyono, 2003).

An effectively designed mucosal vaccine must: (1) protect from physical elimination and enzymatic digestion, (2) target mucosal inductive tissues including M cells, and (3) appropriately stimulate the innate immune system to generate effective adaptive immunity. Although their functional mechanisms have not yet been fully elucidated, mucosal adjuvants can be broadly classified into two categories: those that act as immunostimulatory molecules and those that facilitate vaccine delivery vehicles (Yuki and Kiyono, 2003).The formal include adjuvants which are toxin-based, cytokine-based and innate immunity-associated. The latter contain ISCOM (immune-stimulating complexes), liposomes, live attenuated vectors, chitosan, mucosal DNA vaccine and edible vaccine. Among these mucosal adjuvants, toxin-based adjuvant, CpG motif DNA and live attenuated vectors are pathogens or pathogen-derived molecules which activate cells of the innate and acquired immune systems.

In mucosal vaccine development, it is crucial to select appropriate immunization route, and most current mucosal vaccine delivery is intended to mimic the nature encounter of mucosal inductive sites with environmental antigens and pathogens. Mucosal vaccination is usually performed by either oral (enteric) and intranasal (respiratory tract) application. In fact, many new vaccines are being tested by both routes to determine which induces

immune responses via the CMIS most effectively. In many cases, intranasal immunization is more effective and in general requires smaller vaccine doses with less adjuvant. The occasional inefficiency of oral immunization is due in part to degradation of vaccine by the acidic gastric pH and proteolytic enzymes as well as by the potential for the induction of mucosally induced tolerance. In addition, recent study provides strong evidence that transcutaneous immunization is a simple and practical innovation that may improve the vaccine delivery (Glenn, 2000).

3. MUCOSALLY IMDUCED TOLERANCE

Oral administration of a single high dose or repeated oral delivery of low doses of proteins have been shown to induce systemic unresponsiveness in the presence of mucosal IgA responses (Tomasi, 1980). These immunologically distinct responses in mucosa-associated versus systemic-associated lymphoid tissues were originally termed oral tolerance (Tomasi, 1980). More recent studies have shown that intranasal administration of proteins also induce systemic unresponsiveness (McMenamin and Holt, 1993) and has led to the more general term "mucosally induced tolerance".

This is a unique immune reaction and is characterized by the fact that experimental animals fed large quantities of protein antigen become refractory or have a diminished capability to develop an immune response when reexposed to that same antigen introduced by the systemic route (e.g., by injection). This unique response is an important natural physiologic mechanism ingested food proteins and other antigens. Furthermore, the development of mucosally induced tolerance against pollen and dust antigens could also be essential for the inhibition of allergic reactions, including lgE-mediated hypersensitivity (Li and Sampson, 2002). In addition, the induction of systemic unresponsiveness by mucosal antigen delivery offers an attractive approach to prevent autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, and encephalomyelitis (Weiner et al., 1995; Whitacre et al., 1991).

Although several possible mechanisms for the induction of mucosally induced tolerance have been proposed, the primary ones appear to be mediated by T cells involved in the generation of active suppression or clonal anergy or deletion (Chen et al., 1995; Melamed and Friedman, 1993). For example, high doses of antigen give by the oral route induced clonal deletion or anergy, characterized by the absence of T-cell proliferation and diminished IL-2 production as well as IL-2R expression. However, frequently administered low doses of antigen induced active suppression by $CD4^+$ or $CD8^+$ T cells that secreted cytokines such as TGF-β , IL-4, and IL-10 (Khoury et al., 1992). It is interesting to note that the latter scenario involves cytokines that are also known to upregulate IgA production and is thus compatible with the observation that secretory immune responses and systemic tolerance may develop concomitantly. Because tolerance can be transferred by both serum and cells from tolerized animals, it is possible that humoral antibodies (IgG and IgA? Anti-idiotype antibody? Immune complexes?), circulating undegraded antigens, or tolerogenic antigen fragments and cytokines may act synergistically to confer T-cell unresponsiveness. Because mucosally induced tolerance is specific for the antigen initially ingested or inhaled and thus does not influence the development of systemic immune responses against other antigens, its manipulation has become an increasingly attractive strategy for preventing and possibly treating illnesses associated with or resulting from the development of untoward immunologic reactions against specific antigens encountered or expressed (autoantigens) in nonmucosal tissues.

4. MUCOSAL INFLAMMATION

The gastrointestinal immune system is a major component of the mucosal barrier to foreign antigens including microbial and dietary antigens. Under normal circumstances, the mucosal immune system employs tightly regulated dynamic mucosal intra- and internets for the maintenance of an appropriate immunological balance between the host and gut environments. For example, mucosally induced tolerance is usually induced against enteric commensal bacteria and/or dietary antigens. However, in some cases, the break down of these tightly regulated mucosal immune responses led to hyperresponsiveness against these gut environmental antigens. To this end, numerous disorders could evoke in the gastrointestinal tissues, such as inflammatory bowel diseases (IBD).

Human IBD is a chronic, presumably non-infectious, inflammation limited to the large intestine (ulcerative colitis) or anywhere along the gastrointestinal tract (Crohn's disease); the former is a relatively superficial, ulcerative inflammation, while the latter is a transmural, granulomatus inflammation. The major working hypothesis concerning the pathogenesis of IBD is that the disease is due to an abnormal and uncontrolled mucosal immune response to one or more normally occurring gut constituents (Blumberg et al., 1999; Powrie, 1995). This hypothesis is based on the concept that immune homeostasis in the mucosal immune system relies on a delicate balance between the ability not to react with common ubiquitous gut constituents (food antigens and commensal bacteria flora).

A major advance in the study of IBD has been the discovery and subsequent analysis of a number of experimental animal models of mucosal inflammation that resemble IBD. As shown in Table 3, these models fall into four categories and each provides unique opportunities to discover new molecular and cellular insights into the nature of the pathogenesis of IBD. One major category consists of experimental colitis in which the mucosal inflammation develops spontaneously (Elson et al., 2000). These models offer the best possibility of defining genetic factors that lead to mucosal inflammation. Another major category involves mucosal inflammation occurring in normal animals that are exposed to an exogeneous agent such as haptenated chemicals (Elson et al., 1996; Neurath et al., 2000). A third category of experimental models involves those with particular genetic disturbances produced by either gene targeting or the introduction of a

transgene (Iijima et al., 1999; Ohta et al., 2002). These can be subgrouped into those in which a particular cytokine and cytokine receptor is involved, those in which the TCR and/or the antigen presenting complex is involved or those in which the intestinal epithelial cell layer is targeted. A fourth category of experimental inflammation is transfer model in which the disease is induced by transferring particular cell population into a neutral host lacking lymphoid tissue, for example SCID or recombination activating gene (RAG) deficient animals (Kweon et al., 2002; Powrie et al., 1994). These animal models provide useful experimental tools to further clarify the underlying molecular and cellular mechanism of initiation and development of IBD.

5. Concluding remarks

The Mucosal immune system in higher mammals, and a related form in other vertebrates, consists of an integrated network of lymphoid tissues and mucous membrane-associated cells and effector molecules that work together to achieve host protection. Major effector molecules include antibodies, largely of IgA isotype, as well as cytokines, chemokines, and their receptors, which function in synergy with innate host factors such as defensins. The goal of this system is protective immunity; however, like the systemic immune system, it is susceptible to immunologic diseases, including IgA deficiency, allergy, hypersensitivity, and inflammation. Furthermore, immunologic unreponsiveness (tolerance) is a key feature of the mucosal immune system, and deliberate or natural immunization can effectively elicit mucosally induced tolerance. The mucosal immune system is unique in that it can provide both positive and negative signals for the induction and regulation of immune responses in both mucosal and systemic compartments after mucosal antigen exposure. Intertissue communication is important for mucosal immunity, and homing of lymphocytes via adhesion molecules, which recognize mucosal addressins expressed on high endothelial venule cells, is the major pathway by which the mucosal immune system connects the diverse compartments located in the gastrointestinal, pulmonary, and genitourinary tracts, and exocrine glands. Such unique properties distinguish the mucosal from the systemic immune system. The induction of peripheral immune responses by parenteral antigen does not result in significant mucosal immunity. However, mucosal immunization, e.g., oral or nasal administration of antigen, can induce antigen-specific S-IgA and CTL responses in distant mucosa-associated TISSUES. Moreover, induction of mucosal immune responses often results in both cell-mediated and humoral responses in the systemic lymphoid compartment.

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Biomechanical and Clinical Assessment for Jaw Movement and the Related TMJ Loading in Patients with Temporomandibular Joint Disorders

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ABSTRACT

Temporomandibular joint disorders (TMD) have been demonstrated to be a multifactorial in nature. Possible explanations for the causes have been documented in the literature indicating excessive loading during jaw movement and the subsequent biomechanical imbalance in the TMJ may be assumed as an initial factor for a series of degenerative changes, resulting in condylar resorption and deformity. Therefore, an evaluation of the biomechanical environment in the TMJ would lead to a better understanding of the inducing mechanism of TMJ pain and disability, which result in proper diagnosis and available treatment planning for TMD. Recently, we developed an individual three-dimensional modeling system for the TMJ components based on the magnetic resonance (MR) image and the subsequent analysis of the TMJ loading during jaw movement. The present study was thus designed to introduce biomechanical and clinical assessment of jaw movement and the subsequent joint loading in patients with TMD. Furthermore, we would like to show a case of TMD patient treated with orthodontic approach to introduce an example of the assessment with this system.

KEY WORDS: temporomandibular joint disorders, joint loading, jaw movement, orthodontic treatment

INTRODUCTION

In the field of clinical dentistry, temporomandibular joint disorders (TMDs) are one of the major diseases as well as dental caries and periodontitis. TMDs have been defined as intraarticular morphologic abnormalities, such as different forms of disc displacement, degenerative joint disease, inflammatory arthritis, synovitis and congenital and neoplastic anomalies. Epidemiologic surveys report that 20% to 25% of the population have symptoms of TMD (Solberg *et al.*, 1979; Carlsson, 1999), although only one fifth of those with TMD symptoms require treatment (Gray *et al.*, 1995). Interestingly, it has been observed that up to 70% of patients with TMD suffer from displacement of the disc (Farrar and McCarty, 1979), which is so-called internal derangement of the temporomandibular joint (TMJ-ID).

TMJ-ID defined as an abnormal positional relationship of the disc relative to the mandibular condyle and the glenoid fossa, is accompanied with TMJ pain, clicking and/or crepitus, muscle tenderness, and limitation of mouth opening as the symptoms (Katzberg *et al.*, 1980; Westesson *et al.*, 1986). The return to prominence of the concept of TMJ-ID has contributed greatly to treatment of the TMJ dysfunction. The role of the disc in the progression of TMJ-ID is controversial. Initially, it was postulated that disc displacement preceded the onset of osteoarthritic changes in the TMJ (Nickerson and Boering, 1989). The high association of articular degradation with disc malposition, however, has led some investigators to suggest that the degenerative process predisposes to disc displacement (Dijgraaf *et al.*, 1995; Nitzan, 2001).

From a review of etiological events of TMJ disc displacement, trauma, functional overloading, joint laxity, degenerative joint disease and increased joint friction are considered to play a major role in the etiology of disc displacement (Nitzan, 2001). Therefore, an evaluation of the biomechanical environment in the TMJ would lead to a better understanding of the inducing mechanism of TMJ pain and disability, which result in proper diagnosis and available treatment planning for TMDs.

Recently, we have developed an individual three-dimensional (3-D) modeling system for the TMJ based on the magnetic resonance (MR) image and the subsequent analysis of TMJ loading during jaw movement (Tanaka *et al.*, 2001). The present study was thus designed to introduce biomechanical and clinical assessment for jaw movement and the subsequent joint loading in patients with TMDs. Furthermore, we would like to present a case of TMD patient treated with orthodontic approach to introduce the assessment by this system.

CLINICAL AND RADIOGRAPHICAL ASSESSMENT

Most of patients visit orthodontic clinic in Hiroshima University Hospital with a chief complaint of malocclusion, occlusal disturbance and facial esthetic problem. However, recently, the number of patients with a complaint of TMD increases, and 20% or more patients in our clinic have at least one symptom and/or sign of TMD such as TMJ pain, muscle pain, limited mouth opening, and unidentified complaint at the initial stage.

When the patient comes to our clinic, irrespective of presence of TMD complaint, differential diagnosis for TMD and the intraarticular pathology are conducted prior to orthodontic treatment. At first, we evaluate a his-

tory of the present illness, and conduct clinical examination. The three cardinal components in the clinical examination are range of mandibular movement, palpation tenderness, and joint sounds. After initial interview for a history of TMD and clinical examination, if the patient has any symptom and sign of TMD, radiographic examinations are performed; Panoramic radiograph and Schuller' s method as a simple radiographs, and CT as a 3-D precise radiograph. From radiographic findings, dysfunctional bone remodeling and degenerative bone deformation can be detected. If these abnormal bony changes are present, the patient is diagnosed as osteoarthritis in the TMJ (TMJ-OA). However, radiographs could not provide the spatial and structural information for the TMJ disc.

After radiographic evaluation, each patient undergoes a MR examination. Specific note is made in each case of the presence or absence and radiologic stage of TMD (Figure 1-1) (Wilkes, 1989), as well as such findings as joint effusion, articular surface irregularities, and alterations of bone marrow signal in the mandibular condyle, condylar neck, and temporal bone. Stage I indicating a simple disc displacement, is minimal early stage of TMJ derangement. Stage II and Ⅲ, intermediate stage of TMJ derangement, reveal anterior disc displacement with or without reduction but not bony remodeling and deformation. Stage IV is intermediate-late stage of TMJ derangement with abnormal bone deformation. Stage V shows severe disc and bony degeneration or remodeling such as severe sclerosis, osteophyte, flattening and erosion in the condylar cortical bone.

In order to assess gnathostomatologic functions in TMD patients, analysis of condylar movement is conducted. Jaw movement is analyzed using a six-degree-of-freedom optoelectric mandibular motion recording system (Gnathohexagraph® , JM-1000, Ono Sokki,

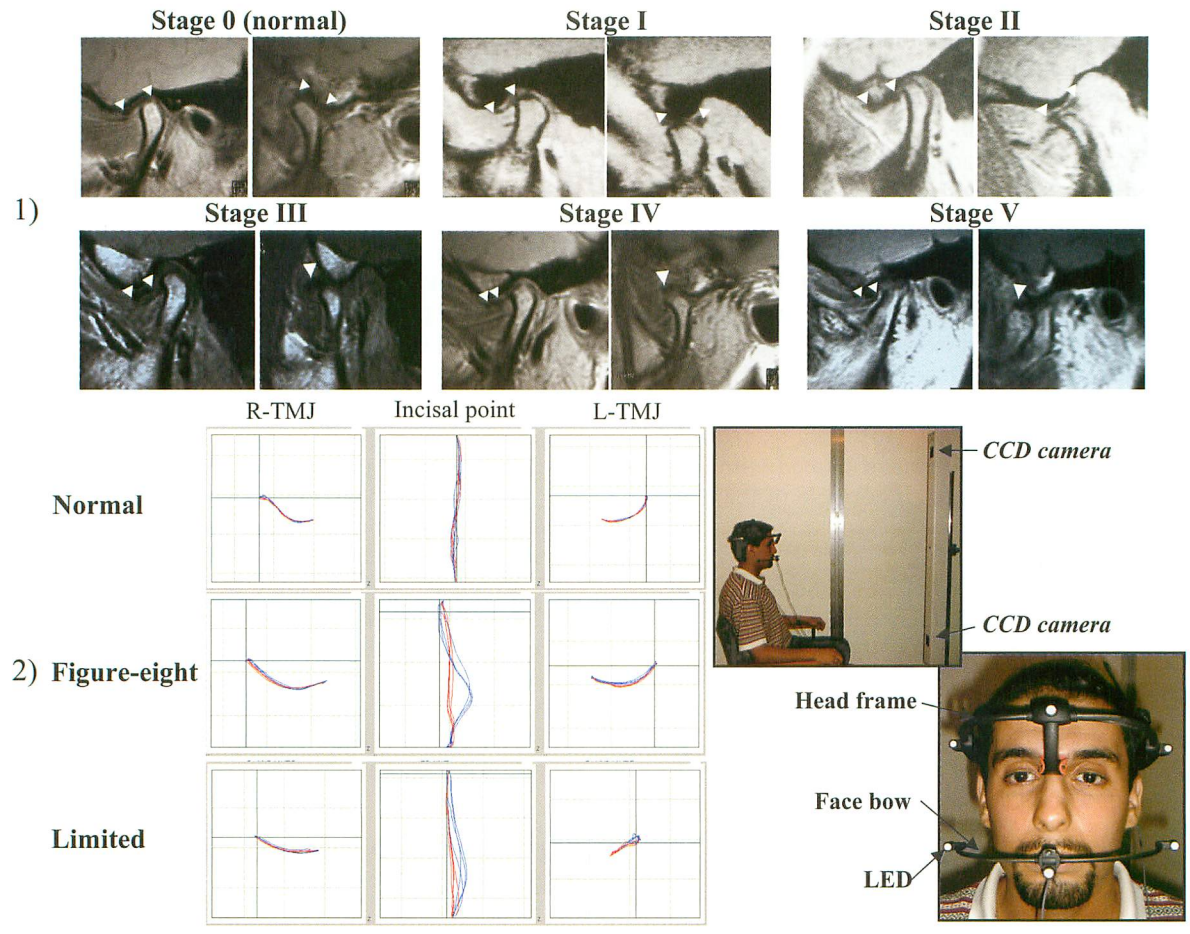

Fig. 1 MR images of the TMJ (1) and condylar trajectory during jaw-opening and -closing movements (2)
From MR images, radiographical stage of TMD was distinguished: Stage 0 indicates a normal TMJ. The white arrowheads indicate the anterior and posterior borders of the disc. Both at closing and opening positions, the disc locates between condylar and temporal bone surfaces. Stage I indicates a simple disc displacement. This is minimal early stage of TMJ derangement. Stage II is intermediate stage of TMJ derangement. MR image at the intercuspal position reveals anterior disc displacement but not bony remodeling and deformation. Stage III is also intermediate stage of TMJ derangement with disc displacement without reduction. Through mandibular movements, the disc is displaced from its normal position and on full opening. Stage IV is intermediate-late stage of TMJ derangement. The disc displaces anteriorly without reduction. The bony abnormal deformity was clearly detected. Stage V shows severe disc and bony degeneration or remodeling such as severe sclerosis, osteophyte, flattening and erosion in the condylar cortical bone.
By Gathohexagraph, condylar movements are recorded during voluntary maximum jaw-opening and -closing movement. Blue lines indicate jaw-opening movement and red lines jaw-closing movement.

Yokohama, Japan), which consists of a head frame, a face bow, light-emitting diodes (LEDs), CCD cameras, and a personal computer (Figure 1-2). The head frame with three LEDs was placed on the head parallel to the Frankfort horizontal (FH) plane of the subject, and the face bow was set to the mandible through the use of a dental clutch. The dental clutch was attached to the labial surface of the lower anterior teeth by means of cryanoacrylate adhesive. Two CCD cameras were placed in front of the subject. The position of each LED was determined three-dimensionally according to the parallax principle. The condylar points of the both sides are recorded by use of a pointer with two LEDs and calculated based on the respective three-dimensional positions of 6 LEDs attached to the head frame and face bow recorded by the pointer.

Condylar movements are recorded during voluntary maximum jaw-opening and -closing movement (Figure 1-2). Movement of the central point between the right and left central incisors is also recorded as a reference for definition of opening- and closing-phases. Sagittal condylar movement pattern (SCMP) can be categorized into six patterns: normal, figure-eight (early/intermediate/late), limited and other irregularities (Ozawa and Tanne, 1997). Limited pattern is likely to indicate closed lock (disc displacement without reduction) in stage Ⅲ, Ⅳ or Ⅴ. Figure-eight pattern was also regarded as stage I or II with reducible disc displacement in the radiographical stage of TMD although SCMP is not yet accurate enough for diagnosing a specific TMJ condition especially a condition of chronic and/or adaptive TMJ-ID (Ozawa and Tanne, 1997).

BIOMECHANICAL ASSESSMENT OF TMJ LOADING

We developed an individual 3-D modeling program for the TMJ based on MR images in order to conduct the biomechanical assessment for TMJ loading during jaw movement.

Reconstruction of 3-D TMJ model

For each patient, the MR images are taken in the axial, sagittal and coronal directions (Figure 2). The axial scans parallel to the Frankfort plane are taken in order to determine the long axis of the condyle. Based on the axial images, the sagittal plane of imaging is designed to be perpendicular to the long axis of the condyle. The coronal plane of imaging is determined to be perpendicular to the median sagittal plane and the Frankfort plane. The two sets of parallel lines in axial slice indicated the positions of the imaged sagittal and coronal planes (Figure 2). The sagittal images of the TMJ for each subject are acquired with the dentition in full intercuspal occlusion and maximum mouth opening. The coronal images were obtained and used for reconstruction of the medial and lateral portions.

The reconstruction technique used in this study has

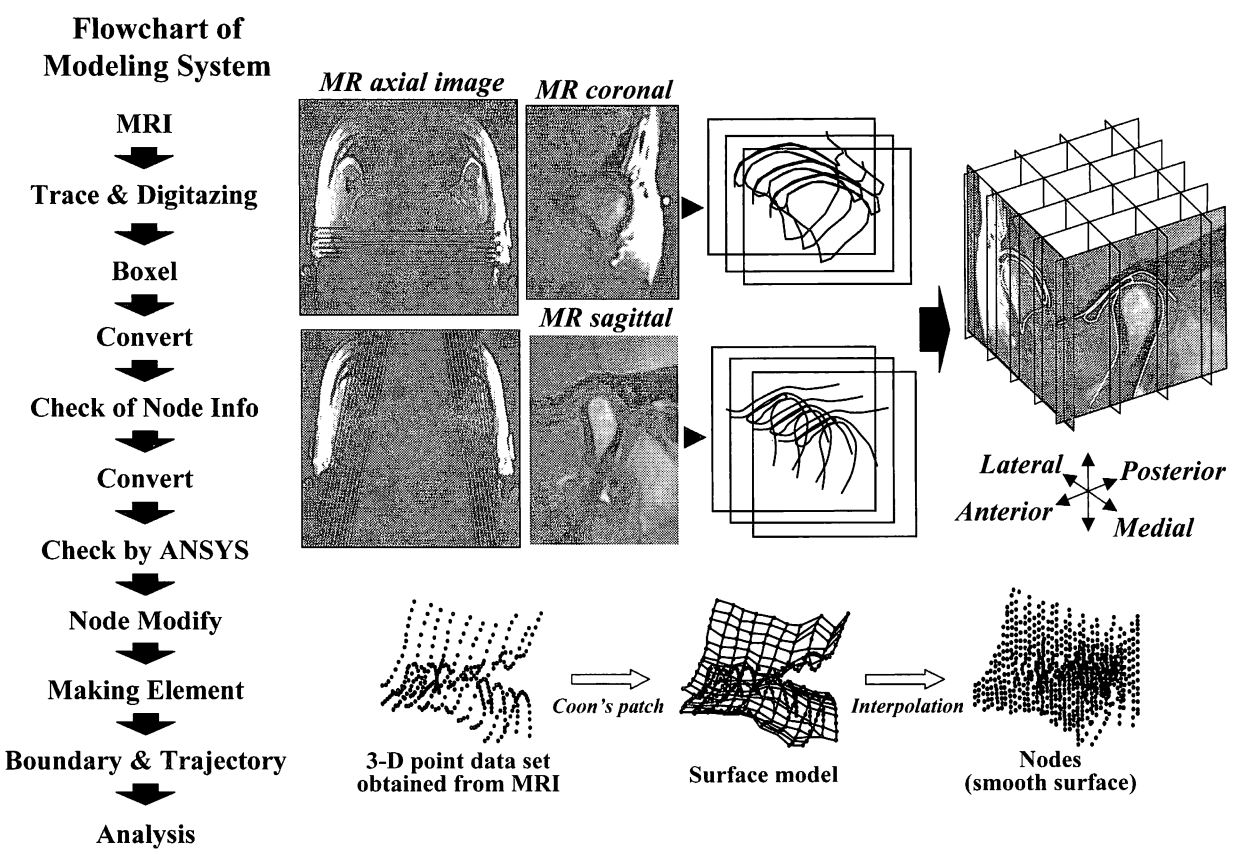

Fig. 2 Reconstruction procedure of TMJ from MR images.

already been described in detail elsewhere (Tanaka *et al.*, 2001). Briefly, from both the sagittal and coronal tracings the articular surfaces were approximated separately by using Coon' s patches (Figure 2). Both approximations were fitted to each other and averaged. The shapes of the lateral and medial end portions of the condyle and articular disc could not be traced from the sagittal slices of the MR images. Therefore, the shapes of these areas were determined only by use of the coronal slices. The upper and lower boundaries of the articular disc were shaped according to the upper and lower articular surfaces. Interface elements were placed at the bone-disc crossing so as to allow the disc to deform and to move along the articulating surfaces without penetration. Because of the detective limit of the MR images, the tissue surrounding to the articular disc was modeled as a single connective tissue mass. As a consequence, the number of nodes for each model was 380 in the condyle, 1047 in the glenoid fossa and 697 in the soft tissues including the articular disc. Finally, the TMJ model has been constructed, consisting of 2024 nodes and 8056 elements (Figure 3-1).

Stress analysis in the TMJ during jaw opening

The material properties of TMJ components used in this analysis were assumed to be linear elastic and were taken from literatures (Tanaka *et al.*, 2000, 2001). The model of the glenoid fossa was restrained for all degrees of freedom at its superior region.

TMJ receives various loading not only during clenching but also during jaw opening. Furthermore, the condylar movement during various mandibular movements especially jaw opening, produces remarkable disc motion. Then, stress analysis in the TMJ is commonly performed during jaw opening. The condylar displacement was enforced at the distal end of the condylar part as the loading condition (Chen and Xu, 1994). The condylar movements during jaw opening were recorded by use of Gnathohexagraph as described above. Thus, this set of recordings gives us the 3-D condylar pathway and its time course during function. By enforcing the calculated displacement to the distal end of the condylar model, the condyle moves incrementally along the condylar trajectory recorded by Gnathohexagraph. The stress analysis was executed on a personal computer with the finite element software, ANSYS from ANSYS Inc. (Houston, USA). In this analysis, von Mises stress distributions in the TMJ soft tissues are commonly evaluated from 0% to 100% opening with 10% increments of opening.

Figure 3-2 shows the results of stress analysis in the TMJ disc during jaw opening (del Pozo *et al.*, 2003; Tanaka *et al.*, 2004). During jaw opening, stress distributions in the asymptomatic patients showed relatively

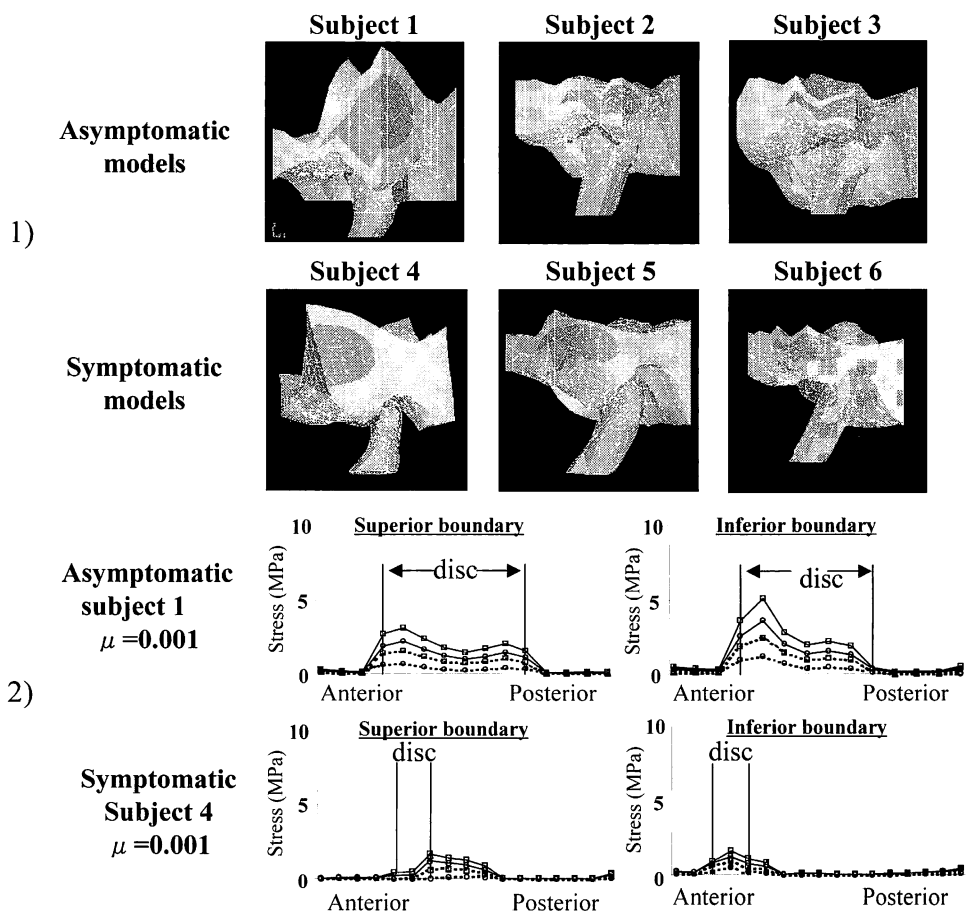

Fig. 3 Three-dimensional finite element models of TMJ (1) and stress distribution on the surfaces of the disc and retrodiscal tissue during jaw opening (2).

high von Mises stresses in the anterior and lateral portions of the disc. The stress distribution pattern on the superior boundary of the disc was similar to that on the inferior boundary. In the soft tissues, the stress level through opening was much lower than in the disc. Meanwhile, a common characteristic in the symptomatic patients was the fact that von Mises stresses were higher in the central and posterior portions of the disc and progressively increased through jaw opening. In the soft tissues stress values were higher in the retrodiscal tissue adjacent to the disc through jaw opening comparing to those in the asymptomatic subjects. The retrodiscal tissue is vessel- and nerve-rich connective tissue, and normally it is not load-bearing organ. Therefore, the TMJ pain assumes to be due to compression in the retrodiscal tissue during jaw opening.

These results provide an important clinical indication such that the best way to reduce the symptoms complained is antero-inferior repositioning of the condyle against the glenoid fossa, resulting in achievement of stress reduction in the retrodiscal tissues.

AN EXAMPLE OF BIOMECHANICAL AND CLINICAL ASSESSMENT FOR A TMD PATIENT TREATED WITH ORTHODONTIC APPROACH

The patient was a 24-year 6-month-old female who had a severe anterior open bite and crowding with a Class II molar relationship (Figure 4-1). She complained of occlusal disturbances and difficulty of lip closure because of her open bite. For TMJ signs and symptoms, she had been TMJ pain during mastication and temporal difficulty of mouth opening for at least 3 years. Her facial profile was convex with a long anterior facial height. Overjet and overbite were +3 mm and -3 mm, respectively. Maximum mouth opening without pain was 31 mm, and TMJ crepitus was detected on both sides.

From the model analysis, the arch length discrepancy was -5.1 mm on the upper and -6.6 mm on the lower arch. The cephalometric analysis indicated the features of a skeletal open bite. The mandible exhibited a backward and downward rotation. MR images showed stage Ⅲ with no reducible disc displacement in both TMJs

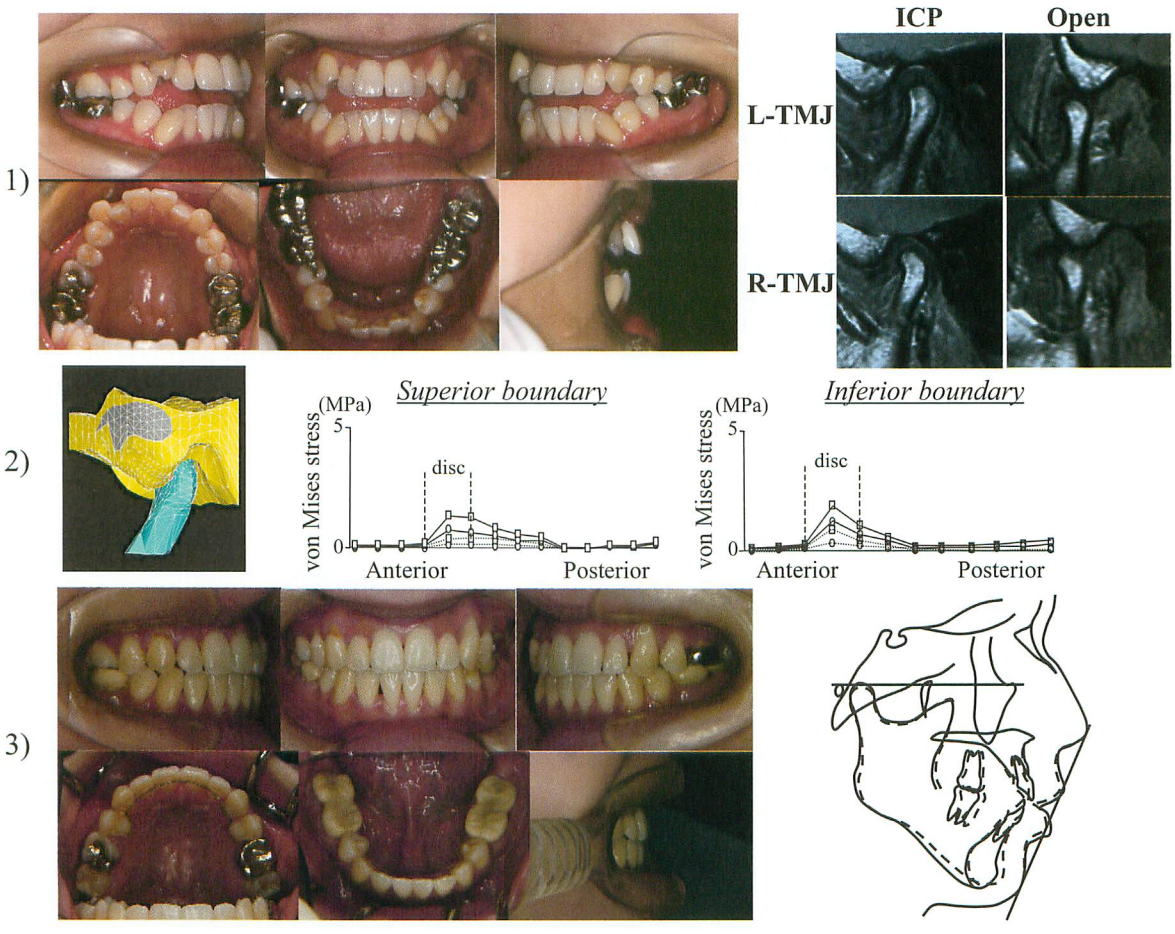

Fig. 4 Example case diagnosed by biomechanical assessment system and treated with orthodontic approach.
(1) Intraoral photos and MR images at the initial stage.
(2) Three-dimensional TMJ model and stress distribution on the surfaces of the disc and retrodiscal tissue during jaw opening.
(3) Intraoral photos after treatment and superimposition of cephalometric tracings before (solid line) and after (dotted line) treatment.

although both condyle showed no abnormal bony changes (Figure 4-2). The degree of disc displacement was very severe on the both sides, and at closed position, the posterior band of the disc had no contact with the condyle, and the retrodiscal tissue might replace the disc as a stress absorber. Then, using our 3-D modeling program, biomechanical assessment of TMJ loading during jaw opening was conducted (Figure 4-2). As the result, the relatively high stress was found at the posterior band of the disc, and the stress level progressively increased in opening (Figure 4-2). With respect to the retrodiscal tissue, the stress level was almost similar to that in the disc. This implies that the retrodiscal tissue suffered from excessive stress during mouth opening.

From these findings, this case was diagnosed as a skeletal open bite with a long lower anterior facial height and TMJ-ID. The treatment plan is 1) initial splint therapy for anteroinferior repositioning of the condyle and calmness of TMJ symptom, 2) bilateral extraction of the upper second and lower third molars and the upper and lower second premolars for correction of anterior open bite and crowding, 3) placement of multi-bracket appliances for tooth alignment, and 4) mesial movement of the upper and lower molars in order to induce a counterclockwise mandibular rotation.

After 2-year orthodontic treatment, a well-balanced facial profile and an acceptable occlusion were achieved and the multibracket appliances were removed. Overall facial balance and her profile were improved. The lower anterior facial height was decreased and the lips showed less tension in a lip closure. Acceptable occlusion was achieved, and the overbite was improved to 1.2 mm and the overjet to 1.5 mm (Figure 4-3). The molar relationships were changed to Class I in the both sides. With respect to TMD, since most of the symptom and signs were reduced or disappeared by the initial splint therapy, no recurrences were not detected. Cephalometric analysis indicated a counterclockwise rotation of the mandible, resulting in anteroinferiorly repositioning of the condyles (Figure 4-3).

CONCLUSIONS

Up to present, more than 30 patients with TMJ-ID or -OA were diagnosed by this biomechanical and clinical assessment of jaw movement and the subsequent TMJ loading, and underwent the orthodontic treatment for occlusal reconstruction. Most of the patients, but not all, recognized reduction of TMD symptoms and achieved functional and acceptable occlusion with long-term stability. Thus, it is confirmed that stress reduction in the TMJ with this therapeutic system is effective in most TMD cases.

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Taste Representations in the Mouse Brain Revealed by Genetic Tracing

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The taste sensory system is responsible for detecting various compounds that are noxious or toxic, and that provide caloric energy, while playing a critical role in the life and nutritional status of organisms. Humans can detect and discriminate between sweet, bitter, sour, salty, and umami stimuli (Fig. 1). The sense of taste also evokes responses that range from innate behavioral actions such as aversion and attraction to food sources, to the pleasure of food consumption.

Taste perception in mammals is mediated by the specialized epithelial cells (taste receptor cells), which are arranged in taste buds on the tongue. Two families of mammalian taste receptors, the T1Rs and T2Rs, have been implicated in sweet, umami, and bitter detection (Scott, 2004) (Fig. 1). The 30 members of the T2R receptor family, most of which are co-expressed in the same subset of taste cells, appear to encode bitter receptors. The T1R receptor family generates at least two heteromeric receptors. T1R2 + T1R3, and T1R1 + T1R3 function as taste receptors for sweet and umami, respectively. Although subsets of T2Rs-positive cells and T1Rs-positive cells are localized in an identical taste bud, bitter and sweet receptors are not co-expressed in the same taste receptor cells (Scott, 2004). Recently, it was reported that the signal transduction molecules, phospholipase Cβ2 (PLCβ2) and the TRPM5 ion channel, is expressed in taste receptor cells (Zhang *et al.*, 2003). Mice lacking either PLCβ2 or TRPM5 abolish sweet, amino acid, and bitter taste reception, suggesting that both receptor families converge on a common signaling pathway in the cells (Zhang *et al.*, 2003). Furthermore, the PLCβ2 transgene expressed under the control of the T2R promoter rescued the response to multiple bitter compounds, but not to sweet or umami taste (Zhang *et al.*, 2003; Mueller *et al.*, 2005), supporting the finding that bitter and sweet receptors are not co-expressed in the same taste receptor cells, and the notion that the two modalities are recognized independently. Recent reports also show that activation of a single cell-type (sweet cells or bitter cells) is sufficient to generate a behavior (attractive or aversive), arguing against that taste receptor cells respond to multiple taste modalities (Zhao *et al.*, 2003; Mueller *et al.*, 2005).

We think it is a time to start examining how sweet and bitter taste information is processed in the central nervous system, when the discovery of bitter and sweet taste receptors indicates the coding of those tastes at the periphery, and importantly, when it is found that the coding qualities of those tastes are similar because of sharing intracellular signaling pathways in taste receptor cells (Zhang *et al.*, 2003; Zhao *et al.*, 2003; Mueller *et al.*,

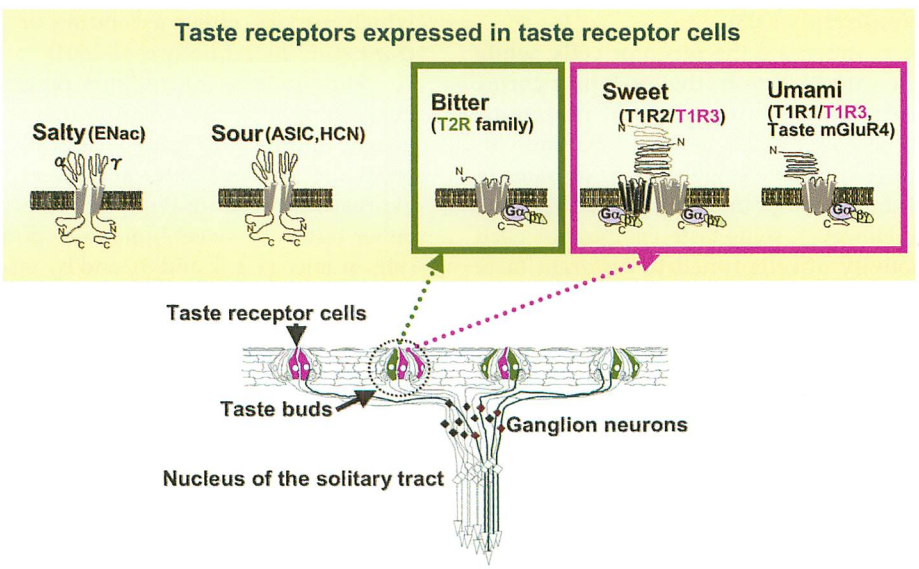

Fig. 1 Schematic representation of taste receptors expressed in taste receptor cells. The sensations of bitter and sweet/umami tastes are initiated by the interaction of sapid molecules with GPCRs such as the T2R family and T1R heterodimers in the apical membranes of taste receptor cells.

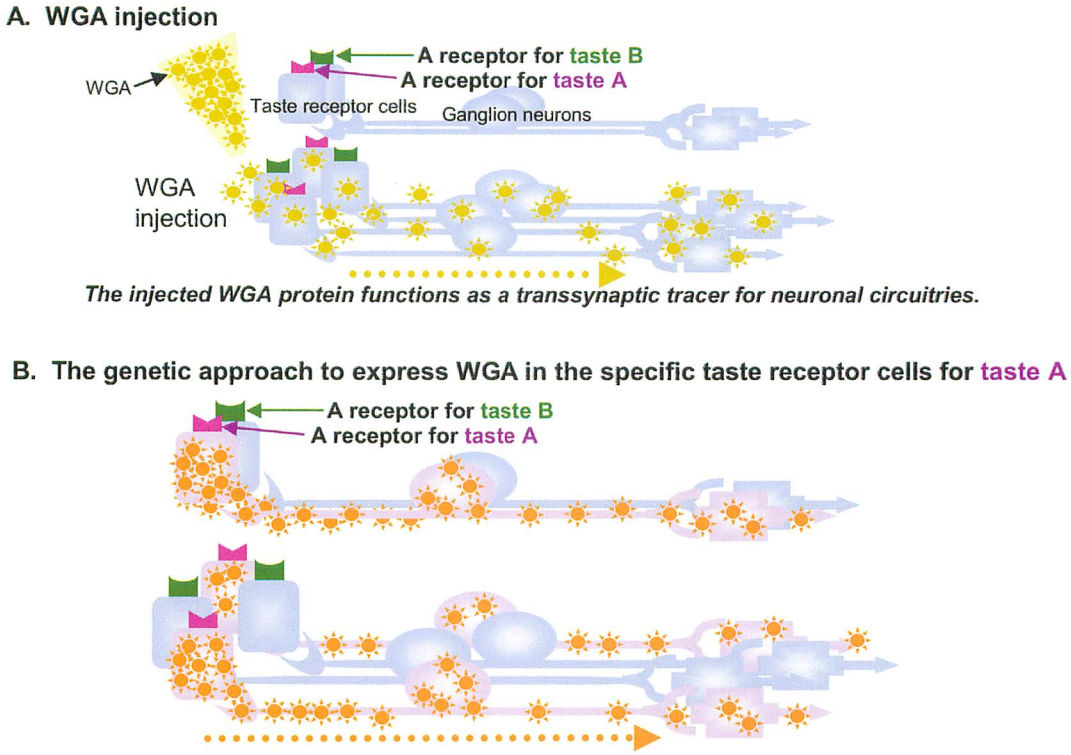

Fig. 2 The plant lectin, WGA, as a transsynaptic tracer. The previous reports indicated that the injected WGA protein functions as transsynaptic tracer for neuronal circuitries (A). However, the specific neuronal circuitries for taste A (sweet or bitter) in comparison with taste B were not clarified by using the WGA injection. How can we trace the specific neuronal circuitries for taste A? To address this issue, we expressed WGA in the specific taste receptor cells for taste A in transgenic mice (B).

2005). Animals lacking sweet and bitter reception and perception still respond sour and salty stimuli (Zhang *et al.*, 2003), importantly suggesting that sour and salty tastes are detected independently of bitter and sweet tastes although coding of sour and salty taste remains less clear (the labeled-line coding or the distinct quality of coding, such as temporal coding (Katz *et al.*, 2001; Katz *et al.*, 2002; Di Lorenzo *et al.*, 2003).

Afferent fibers innervate the receptor cells, while transmitting taste information to the gustatory cortex through synapses in the brain stem and thalamus. Previous electrophysiological data indicated that individual taste-responsive neurons change their firing rates in response to both bitter compounds and ones that taste sweet or umami. However, some taste compounds elicit the combined activity of cells tuned to different taste modalities. For example, quinine may activate not only T2Rs-expressing cells, but also the other taste receptor cells through the blockage of K^+ channels (Akabas *et al.*, 1990; Cummings *et al.*, 1992). Monosodium glutamate may evoke both salty and umami taste because of its Na^+ content (Zhao *et al.*, 2003). Many sweeteners exemplified by saccharine are likely to activate T2Rs-expressing cells. The potency of the side effects might depend on the experimental procedures. Thus, we need to carefully interpret and decode the previous reports. On the other hand, many data revealed that neurons in ganglions, the solitary tract nuclei and even in the gustatory cortex are differentially sensitive to bitter and sweet compounds (Frank, 1991; Danilova *et al.*, 1998; Lemon *et al.*, 2005; Yamamoto *et al.*, 1989), although neurons in the parabrachial nuclei and the gustatory cortex may also elicit the time-varying gustatory responses with more broadly tuned characteristics (Katz *et al.*, 2001; Nishijo *et al.*, 1997) because of interactions with other neurons within the same nucleus and with other gustatory nuclei, which provide either ascending or descending inputs (Katz *et al.*, 2002; Lundy *et al.* 2004).

How is taste information processed in the central nervous system? To address this issue, we applied a genetic approach to transsynaptically delineate the neuronal circuitries of bitter and sweet taste by selectively expressing the transsynaptic tracer, tWGA-DsRed, in either bitter- or sweet/umami-responsive taste receptor cells in mice (Fig. 2 and 3), and by visualizing the spatial distribution of tWGA-DsRed in the brain (Sugita *et al.*, 2005). The WGA proteins are well known to function as anterograde and retrograde tracers. Thus, tWGA-DsRed, which is originated from taste receptor cells and transferred to the neurons in the solitary tract nuclei, may be further transferred along their ascending pathways, or to the neurons which provide the descending inputs to the solitary tract nuclei. However, the labeling density is likely to be influenced by the efficiencies of transport and degradation of the tracer, governed by the molecular backbones which underlie anatomical and functional differences of the axonal and synaptic systems, and which may vary among neuronal types. Although the neurons,

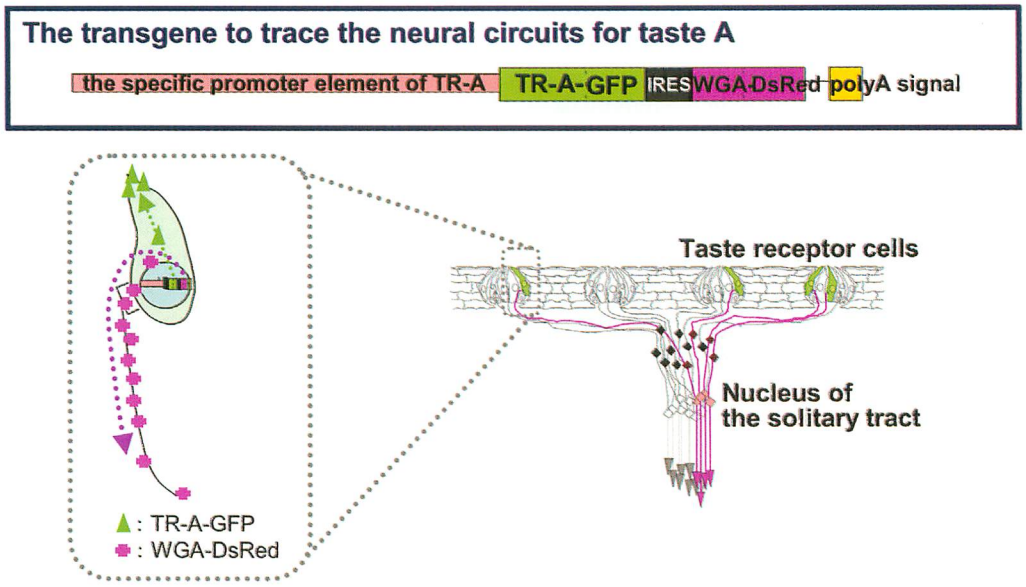

Fig. 3 The transgene to trace the specific taste neuronal circuitries. Specific taste neuronal circuitries were traced by the transgene employing the specific promoter element of a receptor for taste A (TR-A), the TR-A-GFP cDNA, the internal ribosome entry site (IRES), the WGA(truncated)-DsRed cDNA, and the polyadenylation signal. In the transgenic mice, the fusion proteins of TR-A-GFP and tWGA-DsRed were expressed in a subset of taste receptor cells, which expressed endogenous TR-A, and the WGA-DsRed fusion protein was transferred to the neurons with which they synapse, allowing us to visualize the specific transsynaptic neural pathways.

which contained only a small amount of tracer and were not detected, might also relay taste information, locations of the neurons containing a detectable amount of tWGA-DsRed revealed segregation of bitter- and sweet-inputs in the gustatory area in the brain (Sugita *et al.*, 2005), implying that the ascending input pathways are more effectively labeled than are the descending input pathways and the neuronal circuitries within the same nuclei. Comparison of the spatial distribution of tWGA-DsRed, transferred from either bitter- or sweet/umami-responsive taste receptor cells, revealed that the gustatory neurons, dispersed in the solitary tract nuclei, the parabrachial nuclei, the thalamic gustatory area, and the gustatory cortex, may be organized with sweet inputs rostral and with bitter inputs caudal, except for bitter inputs into the external lateral and external medial subdivisions of the parabrachial nuclei (Sugita *et al.*, 2005). In the amygdala and the gustatory cortex, the dispersed areas of tWGA-DsRed-labeled neurons in two strains appeared to partly overlap along the anterior-posterior axis (Sugita *et al.*, 2005). By mapping connections formed by small subsets of neurons, which process and integrate the information of bitter taste, separated from sweet taste, this genetic approach may be valuable for investigating the molecular aspects underlying the construction and refinement of taste neuronal circuitries to mediate taste discrimination, contrastive behavioral responses, emotional states and taste-associated learning. Electrophysiological characterization of tWGA-DsRed-labeled neurons under pharmacological and genetic manipulation of the descending inputs and the interactions with other neurons within the same nuclei is now of critical relevance for understanding the neuronal properties, which might be linked to taste discrimination, behavioral responses and emotional states.

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Mechanisms of Pain Sensitization and the Treatments

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ABSTRACT

Hyperalgesia and allodynia, major symptoms of neuropathic pain can results after nerve injury or chronic inflammation. Trigeminal neuropathic pain resulting from alterations in peripheral and central noxious transmission systems often produced after nerve injury by pulpectomy or tooth-extraction or from temporomandibular joint inflammation. Neuropathic also occurs in some disease state, diabetic peripheral neuropathy, post-herpetic neuropathy and trigeminal neuralgia. Allodynia is characterized by long lasting pain evoked by essentially non-painful stimuli such as just light touch and tolerance to medication with conventional analgesics.

Neuropathic pain is probably not a result of a single pathological mechanism, but the final product of an altered peripheral and central processing. Recent advances in pain research revealed that many factors derived from neurons and also non-neuronal neighboring residents participate in the initiation, development and maintenance. Among them, lipid mediators such as prostaglandins, lysophosphatidic acid and platelet-activating factor are recently found to play conspicuous roles for development of allodynia and hyperalgesia in spinal cord. Further advance by using cellular biological and molecular techniques would dig into the mechanisms underlying neuropathic pain and illustrate the new strategy and target candidate for drug development.

There are also needs for tools and methods to assess neuropathic pain, common guidelines on classification, diagnosis and management, and evidence-based approach to the treatment of neuropathic pain.

Key word: neuropathic pain, allodynia, hyperalgesia, prostaglandin, platelet-activating factor, glutamate, ATP, cyclic GMP

INTRODUCTION

Noxious stimuli to membrane of peripheral tissues produce inflammatory soup such as prostaglandins, bradykinin, histamine, serotonin, ATP, proton and NGF etc, and these mediators together activate polymodal receptors at peripheral nerve ending to evoke action potential. Primary sensory neurons mediate the transduction of the signals to central neurons, in turn, release inflammatory peptides such as substance P and CGRP from peripheral nerve ending to cause neuronal inflammation. Prostaglandins (PGs) have been recognized as the major mediators of inflammation and pain. The conversion of arachidonic acid into PGs by cyclooxgenase (COX) is the rate-limiting step. Therefore, it is believed that non-steroidal anti-inflammatory drugs (NSAIDs) produce their anti-inflammatory and analgesic effect by inhibiting COX.

Repeated noxious stimuli to peripheral tissue result in exaggerated pain sensation, hyperalgesia, one of the cardinal symptoms of persistent inflammation. This results from the increased excitability against noxious stimuli of primary afferent nociceptive nerve fibers. The different forms of sensitization can occur as the result of increased excitability of primary nociceptive afferent nerve fibers; peripheral sensitization or from alterations in the central processing of sensory stimuli, in particular, in the spinal cord dorsal horn; central sensitization.

Recent attention focusing on neuropathic pain which is often produced by peripheral nerve injury after tissue damage or surgery, injury of spinal cord, diabetic peripheral neuropathy and post-herpetic neuropathy(Fig. 1). Such patients suffer from spontaneous pain, hyperalgesia, abnormal sensation (paresthesia) and also allodynia. Allodynia, a major symptom of neuropathic pain is initiated by non-painful stimuli such as just light touch. In oral and maxillofacial region, crisis of allodynia is due to nerve injury after tooth -extraction, pulpectomy, maxillofacial surgery and temporomandibular joint inflammation. In patients of trigeminal neuralgia, it is remarkable that touch the mouth around or inside causes strong.

Neuropathic pain lasts long periods, a year or more, offers no biological advantages, impacts several millions of people around world. In addition, conventional analgesic drugs including NSAIDs and even opioids are not effective for treatment of pain, and thus the development of novel analgesics are desired.

In this content, we summarize the recent progress of two characterized pain sensitization, hyperalgesia and allodynia, from the aspect of central sensitization.

Hyperalgesia

Prostaglandins (PGs) have been recognized as the important mediators of inflammation and pain. PGE_2 plays a major role in increasing of the excitability of the peripheral ending of nociceptive nerve fibers and thereby causes primary sensitization. However, recent studies accumulate evidence which attributes to PGs a pivotal role in central pain sensitization. Peripheral inflammation leads to an increase in COX-2 expression and PGE_2 production not only in the peripheral tissue, but also in neuronal and non-neuronal cells in the spinal cord dorsal horn.

Accordingly, the analgesic action of COX inhibitors, NSAIDs which has long been thought to be primarily due to inhibition of PG synthesis in the peripheral inflamed tissue at least part of their analgesic effects arise from blockade of COX-2 in the spinal cord dorsal horn.

On the cellular and molecular mechanisms underlying PGs-mediated central pain sensitization, PGE_2 via EP2 receptor stimulation facilitates the release of exitatory neurotransmitters such as glutamate or selectively activate cation channels in the superficial laminae of the spinal cord dorsal horn, the first sites of synaptic integration in the pain pathway. However, receptor subtypes, EP1, 2 or 3 in spinal cord mediated PGE_2-induced hyperalgesia was dependent of dose of PGE_2 or type of noxious stimuli (Ito et al., 2001). PGE_2, through EP2, directly activate nonselective cation channel in deeper dorsal horn neurons, laminae III-VI, rather than those in lamina II (Baba et al., 2001). PGD_2-induced hyperalgesia was blocked by NK1 but not glutamate receptor antagonists and NOS inhibitors which blocked PGE_2-induced hyperalgesia.

The decrease of inhibitory neurotransmission as well as the increase of excitatory neurotransmission is important to exacerbate noxious transmission. Intrathecal injection of nociceptin, the opioid-like neuropeptide nociceptin/orphanin FQ (N/OFQ) induced hyperalgesia which was blocked by NK1 antagonist or exogenous administration of glycine, but not $GABA_A$ receptor agonist. Therefore, it is postulated that nociceptin produces disinhibition of the inhibitory glycinergic transmission in spinal cord and in turn stimulate the release of substance P or glutamate from the nerve endings of C fibers or increase in excitability of secondary sensory neurons, leading to hyperalgesia (Ito et al., 2001). Of particular interest findings, PGE_2 mediates specific suppression of inhibitory glycinergic synaptic transmission onto superficial dorsal horn neurons. This inhibitory effect of PGE_2 is selective onto glycinegic inhibitory neuron transmission because $GABA_A$, AMPA and NMDA receptor-mediated transmission remained unaffected and also selective to PGE_2 because $PGF_{2\alpha}$, PGD_2 or PGI_2 had no such effect (Ahmadi et al., 2001). Via this mechanism, PGE_2 may facilitate the transmission of nociceptive input through the spinal cord dorsal horn to higher brain areas where pain becomes conscious. Inhibition of glycine receptors (GlyRs) occurred via a postsynaptic mechanism involving activation of EP2 receptors and cAMP-dependent protein kinase (PKA). Harvey et al (2004) demonstrated

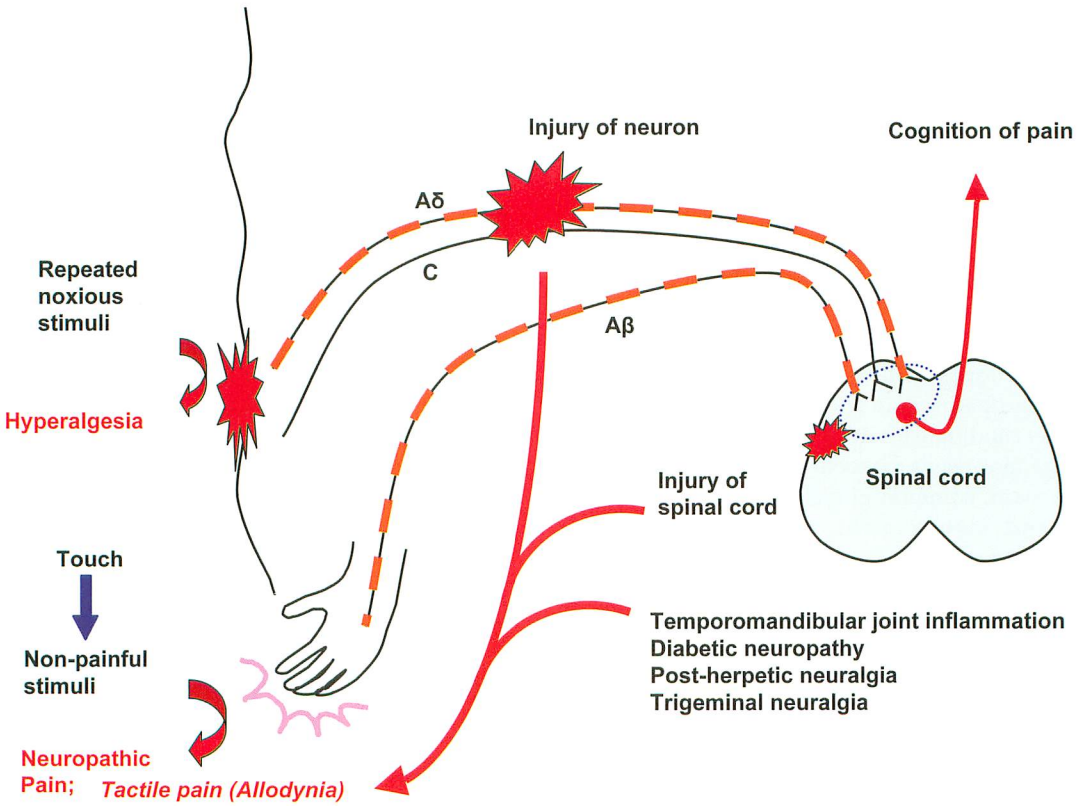

Fig. 1. Hyperalgesia and allodynia induced by nerve injury, chronic inflammation or some diseases. Repeated noxious stimuli facilitate the activation of nociceptors at the peripheral nerve endings of C or A δ sensory fibers and produce hyperalgesia through peripheral and central mechanisms. Neuropathic pain often produced by nerve injury of primary sensory neurons and spinal cord or some disease state such as temporomandibular joint inflammation, diabetes or post-herpetic infection. Allodynia is a major symptom of neuropathic pain which is evoked by essentially non-noxious stimuli such as light touch which signal is translated into spinal cord through Aβ fiber and converted into pain signals at spinal or supra spinal processing. Neuropathic pain last long periods, a year or more offers no biological advantages, impact quite lot of people around world. In addition, conventional analgesic drugs including NSAIDs are not effective for treatment of pain, and thus the development of novel analgesics is desired.

that among the GlyR subtypes, α3 subunit of GlyR could be the target molecule for PKA. GlyR α3 is distinctly expressed in the superficial laminae of the mouse dorsal horn. Mice deficient in GlyR α3 not only lack the inhibition of glycinergic transmission by PGE_2 seen in wild-type mice but also show a reduction in pain sensitization induced by spinal PGE_2 injection or peripheral inflammation. Therefore, the authors emphasized that GlyR α3 may provide a possible molecular target in pain therapy(Fig. 2).

Furthermore, the recent studies have revealed that spinal lipid metabolism is highly altered following persistent nociception and lipid such as lysophosphatidic acid (LPA) (Inoue et al., 2004) and platelet-activating factor (PAF) (Morita et al., 2005) are important messengers of hyperalgesia and tactile allodynia at spinal cord.

PAF is an alkyl-phospholipid, first described in stimulated basophiles, which has subsequently been found in various cells and organs including inflammation and immune related cells, vascular endothelial cells, spleen, ileum, heart, lung, kidney, cerebral cortex neurons (Ishi and Shimizu, 2000) and exocrine salivary gland (Dohi et al. 1991). PAF has been shown to be a potent lipid mediator, especially in platelet aggregation, allergy, endotoxin shock, reproduction, role in central nervous system (Ishi and Shimizu, 2000), secretory role in adrenal chromaffin cells (Morita et al., 1995) and salivary gland (Dohi et al., 1997). In particular, there is considerable evidence that PAF is an important mediator of the inflammatory response. PAF is released from a variety of inflammatory cells in response to various stimuli and the PAF receptor is constitutively expressed and regulates cellular functions in these cells. When administered intratracheally or into the pulmonary artery, PAF produces pulmonary vasoconstriction and edema (Voelkel et al., 1982; 1983), infiltration of PMNs and macrophages (Camussi et al.,

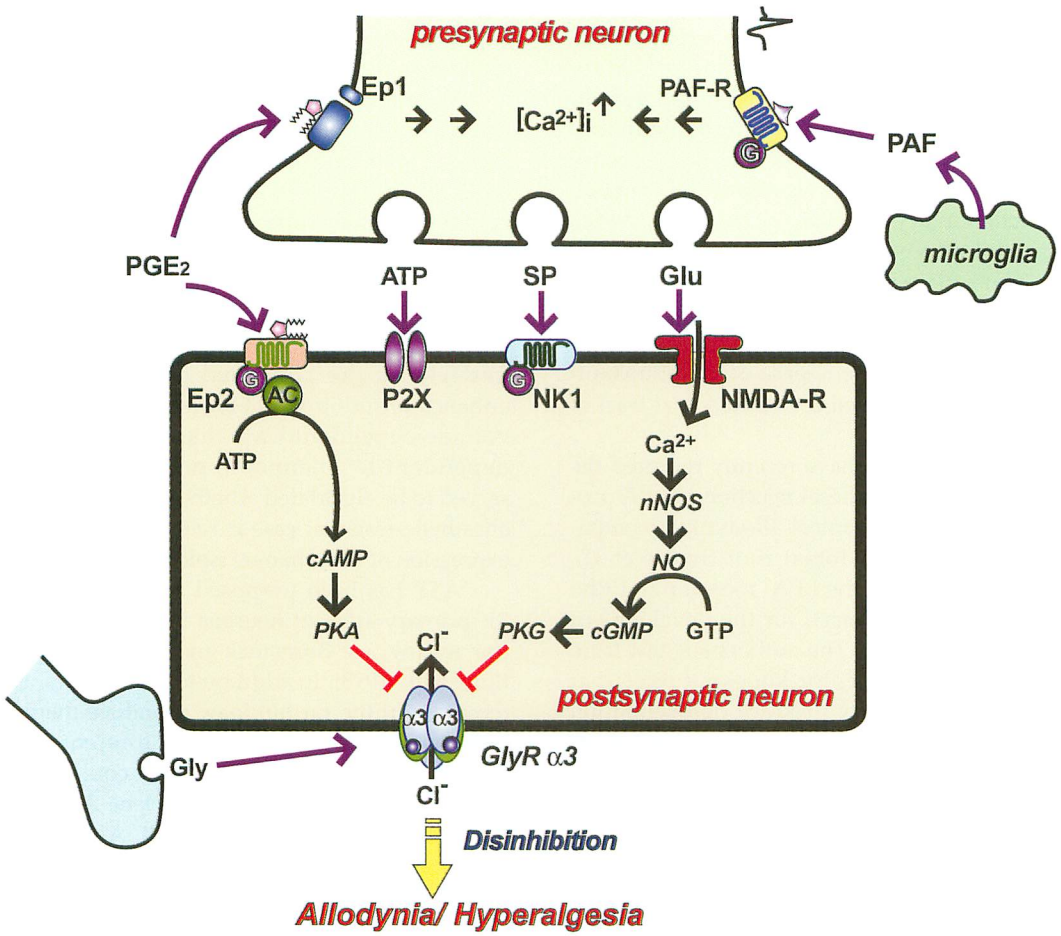

Fig. 2. Shematic model of cAMP- and cGMP-mediated regulation of nociceptive transmission at spinal cord. ATP, substance P and glutamate are the major transmitters released from primary sensory neurons at spinal cord and transduce nociceptive signals on the respective receptors at postsynaptic dorsal horn neurons. Postsynaptic PGE_2/EP1 activates adenylate cyclase which stimulates the formation of cAMP from ATP. Presynaptic PAF/PAF-R stimulates the release of ATP and glutamate, resultant activation of Ca^{2+}/nNOS/NO cascade. NO activates soluble guanylate cyclase which stimulates the formation of cGMP from GTP. Glycine (Gly) is an inhibitory neurotransmitter and activates glycine receptor α3 (GlyRα3)/Cl^- channel located at superficial dorsal horn. Gly/GlyRα3 produces hyperpolarization by stimulation of extracellular Cl^- influx and inhibits excitability of dorsal horn. cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) phosphorylate GlyRα3 and inhibit the channel activity, resultant disinhibition leading to hyperactivity of dorsal horn, producing allodynia and hyperalgesia.

1983). Although PAF is a potent proinflammatory mediator of edema formation, PAF does not seem to be involved in pain in peripheral tissue, because PAF does not cause pain when administered peripherally. However, tactile hyperalgesia and allodynia were developed by the intrathecal administration of PAF (Morita et al., 2004). PAF content in spinal cord markedly elevated by spinal cord injury and PAF receptor antagonists blocked the expression of mRNA of ILs. These evidence suggests that PAF may be a messenger of inflammation and noxious stimuli in spinal cord.

Neuropathic pain /Allodynia

Neuropathic pain is defined as a chronic pain state that develops or persist after a primary lesion or dysfunction of the peripheral or central nervous system. In such pathological conditions when nerves are damaged through surgery, compression by bone, diabetes or infection, just touch which never produce pain in normal state, become to generate pain signals, tactile allodynia, a major symptom of neuropathic pain. Although the mechanisms for generation of allodynia are far from understanding, recent progress in our understanding of neuropathic pain come from animal models of painful peripheral nerve injury and of applying various mediators directly into spinal cord. The peripheral nerve injury models include the spinal nerve ligation (SNL) model, chronic constriction injury of sciatic nerve (CCI) model, the partial sciatic nerve ligation (PSNL) model, the spared nerve injury (SNI) model, chronic constriction of the trigeminal nerve and tibial nerve transaction (TNT) model. In these models, up- or down-regulation have been observed in diverse in the release of pain transmitters and their receptors in spinal cord and spinal tract of trigeminal nerve.

Ueda and his co-workers have recently reported the interesting evidence that intrathecal injection of LPA produced hyperalgesia and mechanical allodynia accompanied with demyelination of dorsal root (Inoue et al., 2004). LPA signaling through the LPA^1 receptor and Rho A/Rho-kinase activation is crucial for the development of allodynia and hyperalgesia. The authors suggest from current study using LPA receptor knockout mice that LPA produced by nerve injury produce demyelination and initiates neuropathic pain. This mimics demyelination following partial sciatic nerve ligation (Ueda 2005).

Although prostaglandins are key mediators of peripheral pain sensitization, recent evidence demonstrated their importance of spinal pain sensitization with different cellular mechanisms from those in periphery. Peripheral injury increased expression of COX-2 and PGE_2 production in spinal cord. Intrathecal injection of PGE_2 or $PGF_{2\alpha}$ into mice generate allodynia with different characteristics as described below, while PGD_2 is inhibitory. PGE_2-induced allodynia was blocked by AMPA and NMDA receptor antagonists, morphine and inhibitors of NOS and guanylate cyclase, but not by NK1 receptor antagonist (Ito et al., 2001). They suggested that PGE_2 stimulate EP1 receptors located on pre-synaptic to release glutamate and glutamate activates AMPA and NMDA receptors on postsynaptic neuron. PGE_2- and $PGF_{2\alpha}$-induced allodynia was disappeared in mice lacking NMDA receptors containing $GluR\varepsilon1^{-/-}$ and $GluR\varepsilon4^{-/-}$, respectively. Activation of NMDA receptors containing GluRε1 subunit stimulate Ca^{2+} influx following activation of PKC, Ca^{2+}/calmodulin-dependent kinase and nNOS. NO rapidly diffuse into presynaptic nerve terminals where it modulates the excitability as a retrograde messenger (O'Dell et al., 1991). It is interesting that DL-TBOA, an inhibitor of glutamate transporters, blocked the allodynia induced by PGE_2 or $PGF_{2\alpha}$ and thus glutamate transporters may play pivotal roles in the induction and maintenance of the PGE_2-induced allodynia (Minami et al., 2001). Capsaicin,a neurotoxin when administered neonatally, causes necrotic changes of small-sized DRG neurons, a substantial loss of C fibers in peripheral nerves, and degeneration of axonic terminals in the spinal cord substantia gelatinosa. Neonatal capsaicin treatment eliminated the PGE_2- but not $PGF_{2\alpha}$-induced allodynia (Minami et al., 1991). These results suggest that capsaicin-sensitive C fibers, usually transmit noxious stimuli may participate in PGE_2-induced allodynia while $PGF_{2\alpha}$-induced allodynia could be mediated by capsaicin-insensitive, Aβ fibers which normally transmits innocuous stimuli.

Voltage-gated sodium channels (VGSCs) play a critical role in neuronal excitability. Among subtypes of pore-forming α-subunits of VGSCs (NaV1.1-1.9), some of them (NaV1.8 and NaV1.9) are specifically localized on nociceptors. Axonal transaction triggers alterations in Na^+ channel expression, turning-off of previously active Na+ channel gene (NaV1.8) and turning-on of previously silent Na^+ channel (NaV1.3) gene. This set of molecular changes have been suggested as a key molecular event underlying the abnormal processing of pain. PGs enhance tetrodotoxin (TTX)-resistant Na^+ currents and voltage-dependent Ca^{2+} channel and inhibit voltage-dependent K^+ channel in nociceptive afferents. It is served to be elucidated whether proximate cause for local anesthetic-resistant case is relevant to such heterologous expression of Na^+ channel isoforms.

ATP has been proposed as a transmitter candidate for primary afferent neurons in peripheral nerve ending (for review, see Burnstock and Wood, 1996; Ralevic and Burnstock, 1998). In addition to the important role of P2X receptors in the rat hindpaw to induce thermal hyperalgesia and nociceptive behavior, a recent study demonstrated that mechanical allodynia could be induced by plantar application of α, β-methylene ATP, an agonist of P2X receptors (Tsuda et al., 2000). A number of studies support the idea that ATP may be involved in spinal nociceptive transmission via P2X receptors (Driessen et al., 1994; Li et al., 1998). We have also demonstrated that α, β-methylene ATP by intrathecal injection elicited allodynia, and PPADS, a selective antagonist for P2X receptors, blocked allodynia induced by α, β-methylene ATP, suggesting that P2X receptors in the spinal cord is involved in the development of allodynia (Fukuhara et al., 2000).

Following traumatic spinal cord injury, numerous secondary events lead to tissue degeneration extending from the initial lesion site with severe pain. PAF antagonist treatment reduces pro-inflammatory cytokine mRNA after spinal cord injury, suggesting that PAF contributes

to secondary damage after spinal cord injury (Hostettler et al. 2002). Faden and Halt (1992) administered PAF intrathecally to examine its effects in the spinal cord and found that PAF decreased blood flow in the spinal cord, motor function or survival, suggesting the role of PAF in tissue damage after spinal cord injury. Although these events establish PAF as a signaling molecule triggering the inflammatory events (Zimmerman et al., 2002), its role in the regulation of pain is not evident because PAF cause no hyperalgesia injected intradermally in healthy volunteers, although it induced potent wheal and flare responses and subsequently erythma and cellular infiltration (Sciberras et al. 1987). We have found that intrathecal injection of PAF induced tactile allodynia and hyperalgesia. Allodynia induced by PAF was blocked by PAF receptor antagonists. PAF-induced allodynia and hyperalgesia disappeared in neonatally capsaicin-treated adult mice, while allodynia but not hyperalgesia induced by intrathecally injected αβ-methylene ATP was capsaicin-insensitive. PAF-evoked allodynia is mediated by ATP and the following NMDA and NO cascade through capsaicin-sensitive fiber, different from exogenously injected αβ-methylene ATP which is insensitive to capsaicin treatment. It has been reported that ATP is co-released with other neurotransmitter such as GABA in the spinal cords (Jo and Schlichter, 1999). We have shown that PAF stimulated ATP release from cultured DRG neuron. Gu et al demonstrated that ATP stimulate release glutamate from nerve ending of DRG neurons co-cultured with dorsal horn neurons (Gu and MacDermott, 1997). Therefore, PAF stimulates the release of ATP from DRG neurons and stimulation of P2X receptors evokes glutamate release following activation of NMDA receptors and the resultant production of NO. The subsequent cascade that NO activates soluble guanylate cyclase and cyclic GMP-dependent protein kinase (PKG) play key roles for the transduction of the noxious signaling for PAF and glutamate in spinal cord. Glycine receptors are suggested as the taaget molecules for PKG to develop allodynia, because cyclic GMP-induced allodynia disappeared in GlyR α3 knock-down mice(Fig. 2). The importance of glycinergic regulation of allodynia was suggested by the observation that nicotine through α4β2 and α7nAChR system by enhancing glycinergic neuron in spinal level exert suppression on allodynia in TNT model (Dohi et al., in press).

Recent study revealed that microglia is activated and accumulated at spinal site of peripheral nerve injury and plays an important participation in pain transduction in spinal cord (Inoue et al., 2004). Tsuda et al (2003) reported that the activation of $P2X_4$ receptors in spinal hyperactive microglia mediates tactile allodynia after nerve injury. It has been reported that PAF is released from microglia, and is also potent stimulant of microglia chemotaxis. Actually 15 minutes after the intrathecal injection of PAF, activated microglia accumulated in the superficial laminae of the mouse dorsal horn with decrement 60 min after PAF injection. PAF receptor mRNA by RT-PCR was expressed in spinal cord, DRG, cultured microglia and cultured DRG. Thus PAF may play a messenger role between microglia and neuron interaction.

Recently, it has become evident that minocyline, an antibiotics, has various biological activity, such as neuroprotective effects. Inhibitory action of minocycline on microglia activation is supposed to be one of mechanisms for neuroprotective action of minocycline. Pretreatment of minocycline did not block the initial allodynia response, however after 15 minutes facilitated the recovery from PAF-induced allodynia. Considering together with the time course of the appearance of microglia in spinal cord after injection of PAF, microglia may participate in the formation at early phase of allodynia by PAF.

Trigeminal neuropathic pain

Trigeminal neuropathic pain resulting from alterations in peripheral and central noxious transmission systems often produced after nerve injury by pulpectomy or tooth-extraction (Marbach et al., 1982; Harn and Durham, 1990; Carmichael and McGowan, 1992). If injury of branches of the trigeminal nerve occurs, sensation may be impaired, but rarely lost. Patients with such injury and pain may be more likely to report cold allodynia than patients without pain and to exhibit signs of central sensitization such as allodynia to light brushing tactile stimuli and abnormal temporal summation (Essick, 2004). It offers a most difficult challenge to therapy. In a recent development of trigeminal pain model, neuropathy is produced by loosely ligaturing the infraorbital, third branch of the trigeminal nerve of rats. Most rats of this model display signs of abnormal spontaneous pain-related behavior (Kryzhanovski et al., 1993) as well as thermal (Kryzhanovski et al., 1992; Imamura et al., 1997) and mechanical hypersensitivity (Vos et al., 1994; Benoliel at al., 2001). It has been reported in rat model with dental-injured model involving the simultaneous pulpectomy to a lower incisor and extraction of an ipsilateral upper incisor (Yonehara et al., 2002) and with loose-ligation of inferior alveolar nerves (Yonehara et al., 2003) that hypersensitivity to tactile stimulation developed on the ipsilateral side of surgery or ligation which was inhibited by MK-801 or NOS inhibitors. In such models, the number of nNOS-positive neurons increased in layers I/II of the trigeminal nucleus caudalis (SpVc) and NO production increased on the side. They suggested that NMDA receptor/NOS/NO production pathway in the SpVc may be involved in hypersensitivity to tactile stimulation developed following dental injury. Further observations that NO induced increase in excitatory amino acid (EAA) levels in the trigeminal nucleus caudalis of the rat with tactile hypersensitivity evoked by the loose-ligation of the inferior alveolar nerves suggest that NO-EAA circuit may play an important role for development and/or maintenance of neuropathic pain following dental peripheral nerve injury (Fujita et al., 2004). Phosphorylated calcium-calmodulin protein kinase IIα (pCaMKIIα) activates glutamate receptors such as AMPA receptor, and enhances its function. Ogawa et al (2005) reported that expression of pCaMKIIα increased in the trigeminal subnucleus caudalis (Vc) by inferior alveolar nerve (IAN) transaction. Intrathecal administration of KN-93, a CaMKII inhibitor, for 7 days inhibited mechano-allodynia induced by IAN, suggesting that CaMKIIα in Vc may be involved in neuropathic pain caused by IAN transaction. Increase in interleukin-6 and nerve growth factor also increased by

trigeminal nerve injury (Anderson and Rao, 2001). On the other hand, prolactin-releasing peptide (PrRP) which presents in spinal nucleus of the trigeminal nerve has potential antinociception in normal rats and antiallodynic effect in neuropathic rats (Kalliomaki et al., 2004).

Hyperalgesia and allodynia of the face often accompany migraine headache. Migraine headache is thought to result from sensitization of the spinal nuclei of the trigeminal nerve in response to sustained noxious input from distended intracranial blood vessels (Goadsby 2001).

Temporomandibular joint (TMJ) inflammation causes an increase in the excitability of the trigeminal spinal nucleus neurons. TMJ disorder patients suffer from pain against innoxious vibrotactile stimulation (Fillingim et al., 1998). This pain response occurred not only in the region of the clinical pain (face), but also on the volar forearm, where the patient reported no clinical pain. Administration of NMDA receptor antagonist, dextromethorphan attenuated the vibration-induced pain, suggesting the involvement of the NMDA receptor in this allodynia. TMJ inflammation modulates the excitability of Aβ-TRG neurons innervating the facial skin via paracrine mechanism due to substance P (SP) released TRG neuronal cell body. Such SP release may play an important role in determining the trigeminal inflammatory allodynia concerning the temprpmandibular disorder (Takeda et al, 2005a; 2005b).

Drugs

The management of neuropathic pain is still a major challenge to clinicians of its unresponsiveness to usual analgesics. Even the opioid drugs are considered to be less effective for neuropathic pain. Tricyclic antidepressants and anticonvulsant carbamazepine are clinically used in some case, although half of patients become refractory or intolerant to these medications (Swerdlow, 1984). The novel antiepileptic compound gabapentin was to exert some antinociceptive effect and introduced for treatment of some neuropathic pain. Gabapentin and pregabalin inhibit voltage-gated Ca^{2+} channels by binding to a high affinity binding site on $\alpha 2/\delta$ and this action is thought to be involved in their mechanisms of anti-neuropathic pain action. The antimigraine 5-$HT_{1B/1D}$ receptor agonists, sumatriptan, zolmitriptan and dihydroergotamine are suggested as analgesics to reduce certain types of trigeminal neuropathic pain in humans (Kayser et al., 2002). Recent studies suggest novel type of analgesics which activate K^+ channels and thus, indirectly prevents the activation of voltage-dependent NMDA channels. Flupirtine, muscle relaxant and neuroprotective agent and its analogue retigabine which open G-protein-activated inwardly rectifying K^+ channels and may prove to be effective for the treatment of neuropathic pain. Other Ca^{2+} channel blockers, NMDA-antagonists or $GABA_B$ agonist are under investigation for use of the medications.

CONCLUSION

Current research has shown that neuropathic pain may arise from not simple but a variety of physiological and pharmacologically distinct systems. A number of mediators including lipid mediators released from nerve terminals or non-synaptically from neighboring glia cells, the immune system or from some others contribute to construct the pain matrix to finally build the memory of pain. Although these make difficulty for medication, the recent advances in pain research by using electrophysiological, molecular and cellular biological techniques achieve the silver lining on the mechanisms underlying initiation, development and maintenance of pain and illustrate the new strategy and target candidate for drug development.

About 20 % of diabetic patients suffer from spontaneous pain, hyperalgesia and allodynia. Most dental treatments accompany with pain and thus would make such patients more worth. However, little attention has been paid. The lack of the assessment of patients with neuropathic pain may be a cause for such careless. Several classifications are used at present as a framework for the differential diagnosis and therapy of the neuropathy. None of them covers all aspects of etiology, anatomical distribution, symptoms, and successful treatment. Diagnostic tools to more effectively select the optimal treatment for various chronic pain states are desired. Mechanism-based and symptom-oriented classification of pain is needed to create novel targets for the development of new drugs.

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In Vitro Organogenesis using Amphibian Pluripotential Cells

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ABSTRACT

In the embryonic development of vertebrate, sequential cleavage is followed by the gastrulation as the first dynamic event of morphogenesis. Mesodermal induction is the most important event for normal body patterning such as the gastrulation, neural induction and formation of various organs. In the study to search for the mesodermal inducing factor, we reported "activin" as a strong mesoderm-inducible factor by the "animal cap assay", the *in vitro* assay system using amphibian pluripotential cell mass. We found that activin has mesodemal and endodermal inducing activity in dose-dependent manner, and then established the *in vitro* induction system for various types of tissues and organs including craniofacial cartilage from animal cap cells by the treatments with activin and other inducing factors. Embryonic transplantation method showed that the treatments of animal cap cells for the induction of pronephros, beating heart were able to induce normally functional organs *in vivo*. These *in vitro* induction methods are useful for investigation of the molecular mechanisms of organ formation and body patterning in vertebrate development.

Key words: activin, organogenesis, animal cap, mesoderm induction, organizer

INTRODUCTION

Embryonic development is the dynamic event, which consist of sequential cell division from the single cell to the various many cells and the interaction among them. The fundamental question in this phenomenon is what kind of factors and mechanisms cause these continuous processes from the simplicity to the complexity. Fertilization is the first step on the morphogenesis in early embryonic development. In amphibian, fertilized egg as a single cell develops into the blastula by the progress of cleavage, three germ layers (endoderm, mesoderm, and ectoderm) are formed, and the gastrulation and neural induction proceed simultaneously. Various tissues are differentiated on the process of axial body patterning, and many organs are formed by the interaction of various types of cells and tissues.

As a monumental study in 1924, Spemann and Mangold (Spemann et al., 1924) found the ectopic neural axis formation from the host ectoderm by the transplantation of blastoporal lip (organizer) into the blastocoel, and showed experimentally the neural induction in ectoderm by mesoderm. Nieuwkoop showed the mesoderm induction directly by the experiment using microsurgical combination of the endodermal cells with ectodermal cells (Nieuwkoop, 1969). As showed in these results, the ectodermal cells of amphibian blastula had pluripotency, and these cell mass was called "animal cap" later. Mesoderm induction was considered as a key event in early development, and the characterization of mesoderm-inducing factor became an important issue in this field. Animal cap cells were used to evaluate the inducing activity of many candidates as mesodermal inducers.

In the end of 1980s, several growth factors were identified as candidates for mesoderm inducing factors. We reported first that activin (Asashima et al., 1989; Asashima et al., 1990) could induce all types of typical mesodermal tissues including notochord (the most dorsal part of mesodermal tissues) in a concentration-dependent manner (Nakano et al., 1990; Ariizumi et al., 1991) in animal cap cell mass. Then we have established *in vitro* induction methods and the appropriate culture conditions for animal cap cells to differentiate into various tissues and organs including heart, pronephros, pancreas, cartilage, eye, neural tissues, and other mesodermal and endodermal tissues using activin and other inducing factors (Fig. 1). These methods are stable and reliable in that it is possible to induce specific tissues reproducibly by simple manipulation of animal cap cells. In this report, we describe our recent study on the mechanism of body patterning and various organogenesis based on these experimental systems using amphibian embryos in combination with molecular biological methods.

RESULTS

Induction of mesodermal and endodermal tissues by animal cap assay

Amphibian have been used as experimental animal generally in the field of developmental biology because of its rapid embryonic development and easiness to observe, to cultivate *in vitro* with simple saline solutions, and to use for microsurgical manipulation. *Xenopus laevis* belongs to anura, and it is investigated well in molecular biological studies. A fertilized egg of *Xenopus laevis* is spherical with a diameter of approximately 1.2 mm, and develops into blastula by the sequential cleavage while about 7 hr after fertilization in 20℃. The blastula of *Xenopus laevis* consists of about 10,000 cells, and has large cavity named "blastocoel" in the animal hemisphere, which is the upper hemisphere of the spherical blastula. The blasto-

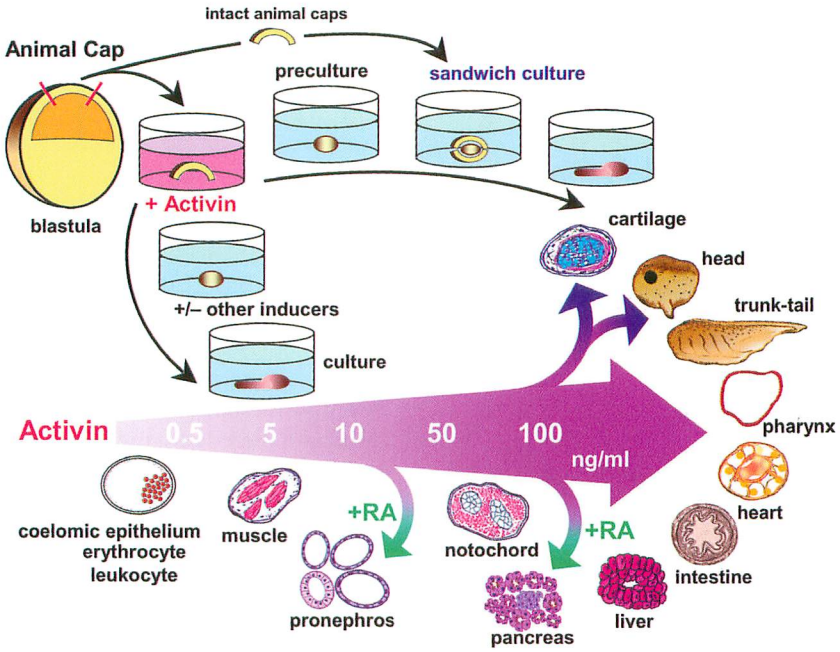

Fig. 1. *In vitro* tissue/organ induction using animal caps.
The schematic diagram of animal cap assay. The "animal cap", blastocoelic roof of amphibian blastula, is excised and cultured in saline containing inducers such as activin and retinoic acid (RA). Differentiated explants are examined by histological and immunohistological analysis, and the expression of molecular markers.

coelic roof is called "animal cap" region, consist of a few layers of presumptive ectodermal cells, which can be easily excised by the watchmakers' forceps and the sharp needles with stereoscopic microscope. Animal cap cells differentiate into ectodermal tissues such as neural tissues or epidermis in normal development of amphibian. When the animal cap cell mass is excised from the blastula and cultivated in a saline solution containing appropriate induction factors, it can be directed to differentiate into various types of tissues including mesoderm- and endoderm-derived tissues as well as ectoderm-derived tissue (Fig. 1, Animal cap assay.). An animal cap cell mass cultivated *in vitro* is also called an "explant". The explants can be cultivated stably for a week or longer, the time that is sufficient for the normal organogenesis in a tadpole of *Xenopus laevis*. Highly differentiated tissues and organs with three-dimensional structures can be formed in explants, and this fact indicate the effectiveness of the animal cap assay as a tool for the investigation of the mechanism of embryonic development and organ generation *in vitro*.

Using animal cap assay, we isolated mesoderm-inducing factor from conditioned medium of the human K-562 cell line (Nakano et al., 1990), and found it was activin (EDF) (Asashima et al., 1989). Activin treatment caused the differentiation of the animal cap cells into various mesodermal tissues in a concentration-dependent manner (Fig. 1). Untreated animal caps form atypical epidermis when it cultured for a few days. In the culture medium with 0.3-1 ng/mL activin, animal caps were differentiated into ventral mesoderm-derived cells/tissues such as blood cells, coelomic epithelium and mesenchymal cells. The animal caps cultured in 5-10 ng/mL of activin solutions showed elongation movement and muscle differentiation. Treatment with 50 ng/mL of activin induced animal cap to differentiate into notochord, the most dorsal mesoderm, and endodermal tissue that contains yolk abundantly. These results suggest that the gradient concentration of activin can regulate the differentiation of the animal cap cells into the mesodermal tissues along with dorsoventral axis (Asashima et al., 1990; Ariizumi et al., 1991). Activin could induce the tissues of endodermal organs including liver, pancreas and intestine in newt animal caps by the combined cultivation of intact animal caps and an animal cap treated with 100 ng/mL of activin (Ariizumi et al., 1999).

Animal cap can be dissociated to single cells easily by the incubation in Ca^{2+}/Mg^{2+}-free culture medium. Activin treatments with 0-0.5 ng/mL, 1-5 ng/mL and 2.5-100 ng/mL caused dissociated cells to differentiate into atypical epidermis, notochord and yolk-rich tissue respectively, when they reaggregated after the activin treatments (Kuroda et al., 1999). When the two groups of animal cap cells that treated with different concentrations of activin were cultured as a mixed aggregate, cells migrate in the aggregate and form separated clusters of the cells treated with the same concentrations of activin, within 5-10 hr after the treatments. This autonomous cell sorting in the mixed aggregate indicates that activin treatment caused the change in the adhesive properties of animal cap cells. This result suggests that there is important relation between the function of cell adhesion molecules and various tissue inductions by activin treatments using animal cap cells (Kuroda et al., 2002).

Animal caps acquire organizer-like activity by the treatment with activin

Transplantation of the newt animal cap treated with activin into the ventral side of newt gastrula caused the formation of well-organized secondary axis, as well as classical experiment of organizer transplantation (Ninomiya et al., 1998). When a newt animal cap was treated with activin 100 ng/mL for 1 hr and cultured for additional 6-12 hr or 18-24 hr ("preculture"), then sandwiched between intact animal caps for culturing, the combined animal cap cell mass (explant) differentiated into the head structure containing hindbrain with otic vesicle or forebrain with eyes, respectively. If the preculture step was omitted in this procedure, the explant differentiated into the trunk-and-tails structure including axial structures such as spinal cord, notochord and somites (Ariizumi et al., 1995) (Fig. 1). These results show that an animal cap treated with activin acquires organizer-like activity. We found actually that one of the organizer-related genes, newt homolog of chordin (*Cychd*), was expressed in both the organizer region of embryos and head-inducible animal caps treated with activin (Yokota et al., 1998).

Maxillofacial cartilage formation *in vitro*

Based on the induction method for the head-like structure from animal caps using activin, we tried to establish the procedure for inducing maxillofacial cartilage at a high rate. When *Xenopus* animal caps were treated with

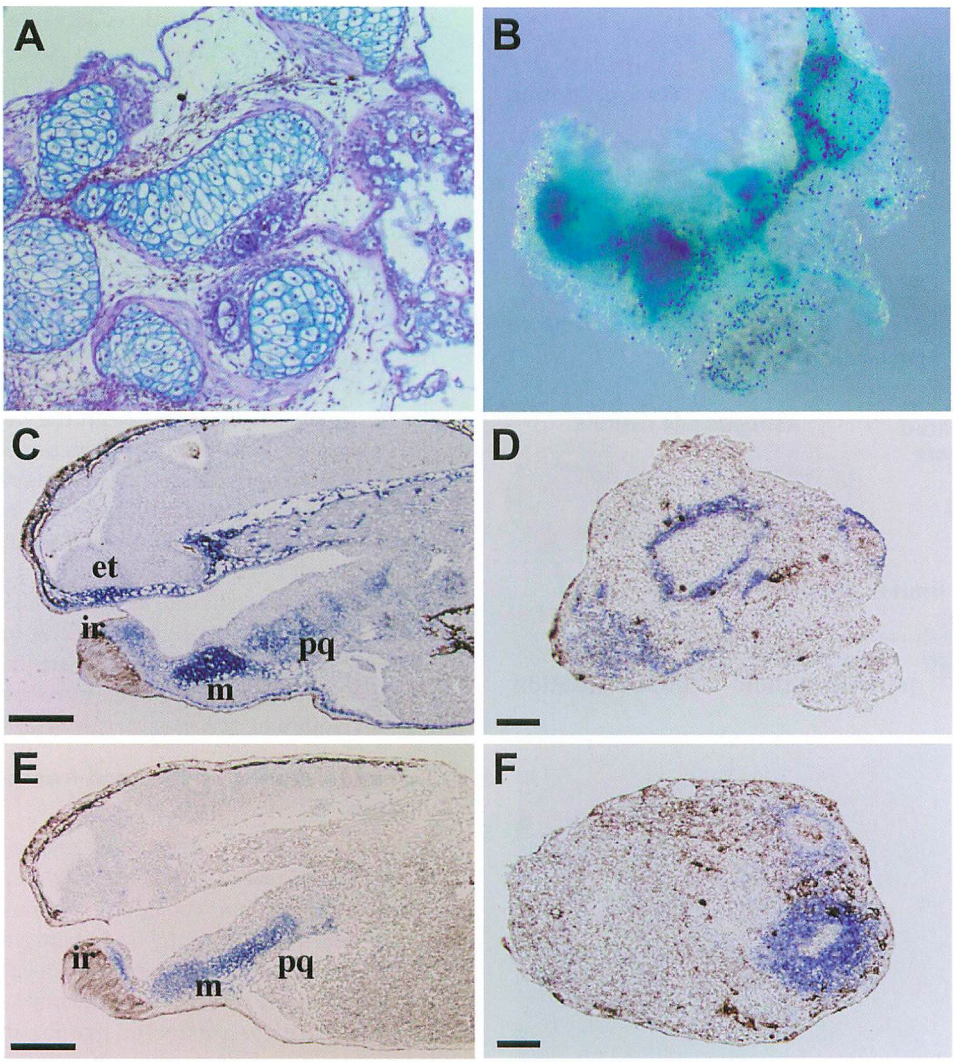

Fig. 2. *In vitro* induction of maxillofacial cartilage in animal caps.
(A) Histological section of the animal cap-derived explant differentiated into cartilage at 7-days after the treatment with activin (dissociation-reaggregation method). PAS/Alcian blue staining. (B) Whole-mount alcian blue staining of the explant treated the same condition as (A). (C,D) The expression pattern of *Collagen type II* mRNA. (C), Sagittal section in the cephalic region of the normal *Xenopus* tadpole (Stage 40). (D), Section of the explant at 4-days after the treatment. (E,F) The expression pattern of *goosecoid* mRNA. (E), Sagittal section in the cephalic region of the normal *Xenopus* tadpole (Stage 40). (F), Section of the explant at 4-days after the treatment. Abbreviations, et: ethmoid-trabecular cartilage, ir: infrarostal cartilage, m: Meckel's cartilage, pq: palatoquadrate cartilage. Scale bar: 100 μm.

activin 100 ng/mL for 1 hr, precultured for 1hr and sandwiched between untreated animal caps, formation of the chondrocytes were the most frequently observed in the explant after the 7-days culture (Fig. 2A) (Furue *et al.*, 2002). For maxillofacial cartilage induction, the method using dissociated animal cap cells were also developed as follows; dissociated animal cap cells were treated with 25 ng/mL of activin for 1hr, and mixed with untreated cells in a ratio of 1:5 to form an aggregate, then cultured for 7days as an explant (Myoishi *et al.*, 2004) (Fig. 2B). *Col*2 and *Cart*-1, the marker genes expressed in the process of cartilage differentiation, were expressed in these explants in a similar way to the normal development of maxillofacial cartilage. *Goosecoid* and *X-dll*4, the marker of cephalic ventral mesenchyme and anterior ectoderm, were also expressed in these explants (Fig. 2C-F). Ectopic tooth germ-like structures were observed in the host embryo by the transplantation of the explant made by dissociation-reaggregation method into the abdominal region of the embryo (Myoishi *et al.*, 2004). These results strongly suggest that the maxillofacial cartilage was induced in *Xenopus* animal cap explant by activin treatment.

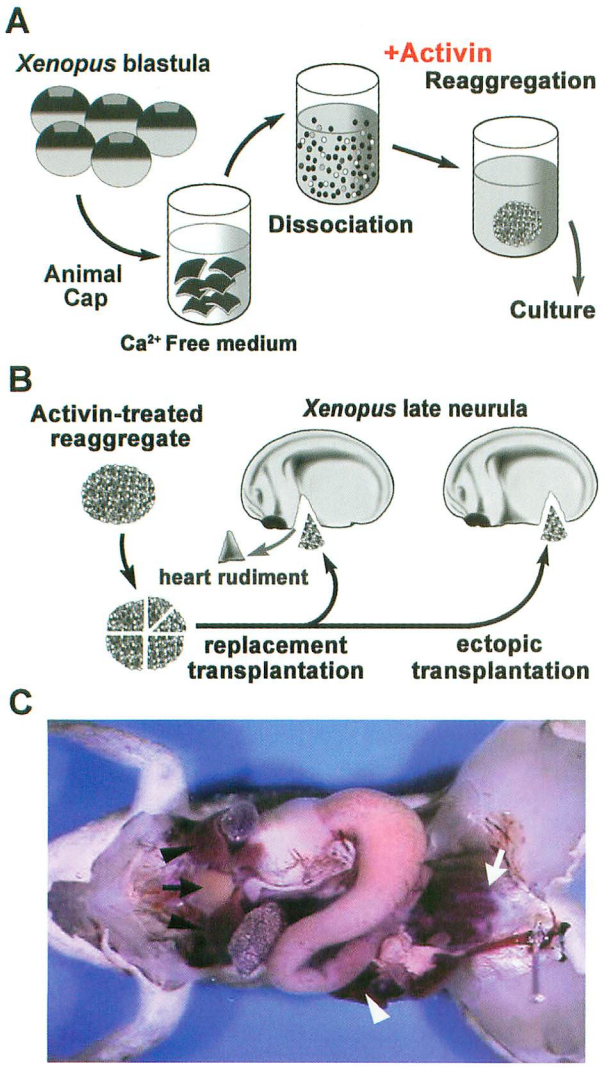

Fig. 3. *In vitro* induction of heart rudiment in animal caps. (A) The schematic diagram of the method to induce cardiac tissues in animal cap cells of *Xenopus*. (B) The schematic diagram of the transplantation experiment. (C) An 1-year-old frog with a well-developed ectopic heart that was caused by ectopically transplantation of the activin-treated reaggregate into the abdominal region at the late neurula stage. (black arrow; original heart, white arrow; ectopic heart, black arrowhead; original liver, white arrowhead; ectopic liver)

Heart formation *in vitro* and transplantation in embryo

We found the *in vitro* induction method to induce a heart-like structure in a newt animal cap. When the newt animal caps was treated with 100 ng/mL of activin, the explants differentiate into anterior endoderm, but about 20%-30% of explants differentiate into the beating heart-like structures (Ariizumi et al., 1996). These explants were anti-α-sarcomeric actin positive, and the characteristic microstructures of myocardium, Z band and intercalated discs, were also observed on electron microscopy. In our recent study, we succeed to establish the optimal induction method of beating heart using *Xenopus* animal cap cells as follows; 5 animal caps (approx. 1,000 cells total) were dissociated and treated with 100ng/mL of activin for 5 hr, then reaggregated and cultured for 3days as an explant (Fig. 3A). The expression of *XGATA-4*, the early marker for heart and anterior endoderm, was prolonged in this dissociation-reaggregation explant as compared to the intact animal cap treated with the same concentration of activin. The expression of *XNkx2.5*, the early heart-field marker, also increased in this explant. When the original heart primordium of the host embryo was replaced with this explant ("replacement transplantation"), the host embryo developed with the heart derived from donor explant, and the heart functioned normally (Fig. 3B).. When the explant was transplanted into the abdomen region of the host embryo ("ectopic transplantation"), the host embryo developed into the frog with two hearts derived from original heart primordium and donor explant (Ariizumi et al., 2003) (Fig. 3C). These results suggest that these dissociation-reaggregation methods to induce the beating heart-like structures may reproduce the induction of heart primordium in the normal development of *Xenopus*.

Kidney formation *in vitro* and transplantation in embryo

Retinoic acid (RA) is distributed endogenously in a concentration gradient along the antero-posterior axis in *Xenopus* embryo, and considered as one of the candidates for the determinants of the body patterning in embryonic development. In *Xenopus* animal cap assay, RA has the activity to affect mesoderm induction and to modify its fate to lateral or posterior property. We found the method to induce pronephros formation *in vitro* effectively in animal caps using the combination of RA and activin as inducers. By simultaneous treatment with 10 ng/mL of activin and 10^{-4} M of RA, pronephric tubules were formed in animal cap explants at a high frequency (Moriya et al., 1993) (Fig. 4A). The pronephros-specific marker genes were also expressed in these explants as well as the normal embryo (Uochi et al., 1996). Immunohistological staining and histological examination using electron microscopy revealed that these

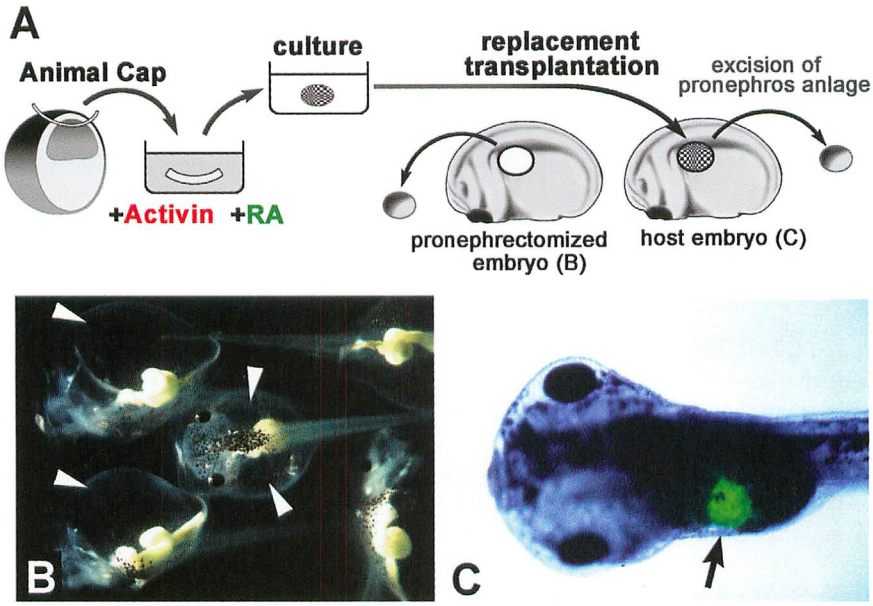

Fig. 4. In vitro induction of pronephric tubules in animal caps.
(A) The schematic diagram of the induction method of pronephros in animal caps and transplantation experiment into the late neurula of *Xenopus*. (B) Embryos from which the pronephric primordium had been excised were unable to eliminate water and showed clear edemas (arrowheads). (C) An embryo transplanted with a pronephric explant showed normal development. Green fluorescent dye previously introduced into the explant revealed that it had been incorporated into the pronephric area (arrow).

explants contained all the three components of a nephron, glomeruli, pronephric tubules, and the pronephric duct (Osafune et al., 2002). When *Xlim-1*, the essential gene for the differentiation of pronephros in normal development, was inhibited in animal cap cells, pronephros formation was suppressed in the animal caps treated with acticvin and RA. These results indicate the method for pronephric induction in animal caps may replicates well the normal kidney development in *Xenopus*. By the replacement transplantation of the presumptive pronephros region of *Xenopus* embryo with the explant treated with activin and RA, we showed that the donor explant was integrated into the host embryo, and the normally functional pronephros was formed (Chan et al., 1999) (Fig. 4A-C). We investigated the detailed expression of gene markers for pronephric development in the pronephric structure formed in the explant, and found that the timing of their expression was the same as that in normal development.

Pancreas formation *in vitro*

By the modification of the timing and the length of the treatment with activin and RA, we established the method to differentiate animal cap cells into pancreatic tissue as follows; animal cap was treated with 100 ng/mL of activin and cultured for 5 hours and then treated with 10^{-4} M RA (Moriya et al., 2000). The induction mechanism of the pancreatic tissue in this method can be explain that anterior endodermal tissue induced by high concentration of activin was posteriolized by RA, and differentiated into pancreas. This explant contained normal pancreatic structure including both exocrine regions and endocrine regions. Glucagon and insulin were also detected by immunohistological staining.

DISCUSSION

As mentioned above, we established the artificial induction methods for various tissues and organs using amphibian animal cap cells. These are simple, stable and reproducible methods for *in vitro* organogenesis, and useful tools to investigate the mechanisms of various organ formations in normal embryonic development of vertebrate. We showed these *in vitro*-induced tissues and organs are nearly the same as normal ones of the embryo from histological and molecular biological standpoints. Using the differential screening with the cDNA library constructed from these explants induced by activin and/or RA, we actually isolated many novel specific genes for organ formation and body patterning (Uochi et al., 1997; Uochi et al., 1998; Eisaki et al., 2000; Satow et al., 2002; Sogame et al., 2003; Nitta et al., 2004; Li et al., 2005), and found one of these were essential for the normal kidney development in mice (Onuma et al., 1999; Nishinakamura et al., 2001). By the improvement of these methods and detailed analysis of induction mechanisms in the explants, we are trying the application of these methods into pluripotent cell lines of mammal to contribute to regenerative medicine.

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Genetic Alterations of Wnt Signal Components in Cancer Cells

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ABSTRACT

The genetics of development and cancer have converged in the identification of intra- and extra-cellular signaling pathways that are aberrantly regulated in cancer and are also central to embryonic patterning. The Wnt signaling pathway has provided an outstanding example of this. The genes for β-catenin, APC, and Axin in the Wnt signaling pathway are often mutated in human cancers. In all such cases, the common denominator is the accumulation of cytosolic and nuclear β-catenin and the activation of transcriptional factor Tcf/Lef. The resulting gene expression profile should provide a significant clue as to cancers carrying defects in the Wnt signaling pathway. In this review, the regulation of the β-catenin stability by Axin and APC, and their genetic alterations in human cancers are described.

Key Words: Wnt; β-catenin, APC, Axin, Tcf

1. Outline of the Wnt Signaling Pathway

Wnt proteins constitute a large family of cysteine-rich secreted ligands that control development in organisms ranging from nematode worms to mammals (Wodarz and Nusse, 1998). The intracellular signaling pathway of Wnt is also conserved evolutionally and regulates cellular proliferation, morphology, motility, fate, axis formation, and organ development (Wodarz and Nusse, 1998; Polakis, 2000). Wnt regulates at least three distinct pathways; the canonical β-catenin pathway, planar cell polarity pathway, and Ca^{2+} pathway. It has been shown that abnormalities of the canonical β-catenin pathway lead to several human diseases including tumor formation and bone abnormalities. According to the most widely accepted current model of the β-catenin pathway (Fig. 1), casein kinase Iα (CKIα) and glycogen synthase kinase-3β GSK-3β) phosphorylate β-catenin in the Axin complex (Ikeda *et al.*, 1998; Kikuchi, 1999; Liu *et al.*, 2002). Phosphorylated β-catenin is ubiquitinated, resulting in the degradation of β-catenin by the proteasome (Kitagawa *et al.*, 1999). As a result, the cytoplasmic β-catenin level is low.

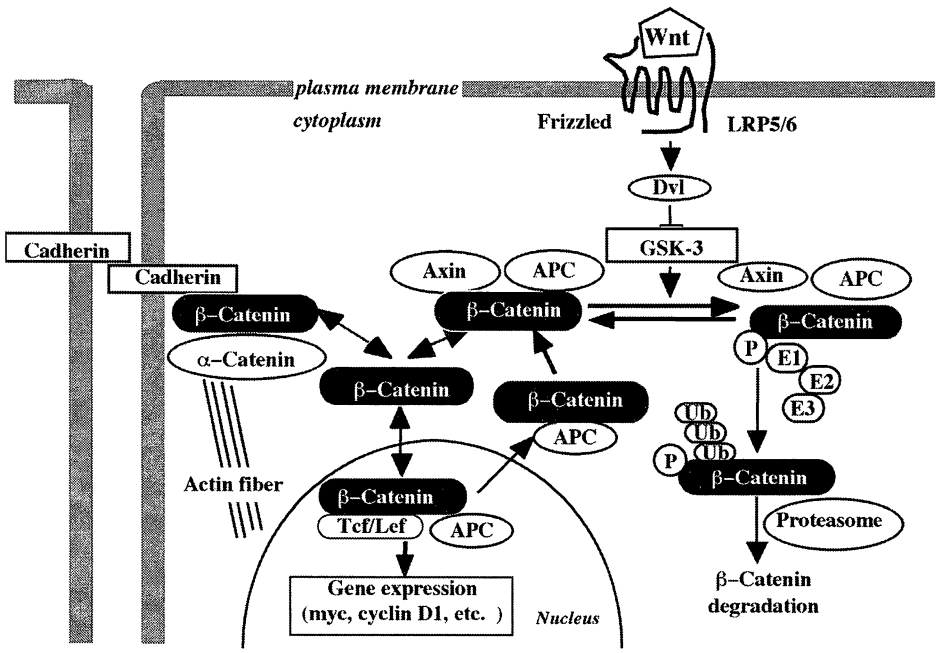

Fig. 1. Wnt signaling pathway. Wnt activates Tcf/Lef-dependent gene expression through stabilization of β-catenin. P, phosphorylation; Ub, ubiquitination; APC, adenomatous polyposis coli gene product.

When Wnt acts on its cell-surface receptor consisting of Frizzled and lipoprotein receptor-related protein 5/6 (LRP5/6), β-catenin is escaped from the degradation in the Axin complex although the mechanism is not clear (He *et al.*, 2004). Accumulated β-catenin is translocated to the nucleus, where it binds to the transcription factor T cell factor (Tcf)/lymphoid enhancer factor (Lef) and thereby stimulates the expression of various genes (Polakis, 2000; Hurlstone and Clevers, 2002). The broad impact of the Wnt signal on gene expression makes it a key element in regulation of cell survival, apoptosis, cell motility, cytoskeletal structure, and cell adhesion. Thus, Wnt increases the stability of β-catenin, thereby stimulating Tcf/Lef-mediated gene expression in the canonical β-catenin pathway.

Among various molecules involved in this pathway, alterations in the β-*catenin*, *APC*, and *Axin* genes have been frequently found in several human cancers. In these cancer cells β-catenin is abnormally accumulated and nucleus of these cancer cells and Tcf-mediated gene expression is increased. Since APC and Axin induce the degradation of β-catenin, it is conceivable that β-catenin functions as an oncogene product, while APC and Axin act as tumor suppressor gene products.

2. Regulation of degradation of β-catenin

(1) Degradation of β-catenin through phosphorylation

Cytoplasmic β-catenin is a target for the ubiquitin-proteasome pathway and the phosphorylation by GSK-3β and CKI α is required for its ubiquitination (Fig. 2). In general, degradation of proteins by the ubiquitin-proteasome pathway involves an ubiquitin-activation enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase (E3) (Ciechanover, 1998). The ubiquitin ligase is generally thought to be directly involved in substrate recognition and consists of a multiprotein complex. An F-box protein Fbw1, which is a component of the ubiquitin ligase for β-catenin, associates with β-catenin phosphorylated by GSK-3β and CKI α and stimulates ubiquitination and degradation of β-catenin (Kitagawa *et al.*, 1999; Liu *et al.*, 2002). The amino acid sequence for the phosphorylation of β-catenin is $D^{32}SGXXSXXXTXXXS^{45}$ (D, asparatate; S, serine; G, glycine; T, threonine; X, any amino acid). CKI α -dependent phosphorylation of S^{45} proceeds and the phosphorylation enhances subsequent GSK-3β-dependent phosphorylation of T^{41}, S^{37}, and S^{33}. D^{32} and G^{34} are necessary for the interaction of phosphorylated β-catenin with Fbw1. Therefore, Fbw1 directly links the phosphorylation machinery to the ubiquitination apparatus.

(2) Roles of Axin and APC as scaffold proteins in the degradation of β-catenin

Axin binds to various components of the Wnt signaling pathway (Kikuchi, 1999). APC binds to the RGS domain (Behrens *et al.*, 1998; Kishida *et al.*, 1998). GSK-3β, β-catenin, and CKI α interact with the different sites of the central region of Axin (Ikeda *et al.*, 1998; Liu *et al.*, 2002). Dvl binds to the following C-terminal region of Axin including the DIX domain (Kishida *et al.*, 1999a). In the Axin complex, CKI α and GSK-3β phosphorylate β-catenin efficiently and phosphorylated β-catenin is ubiquitinated and degraded by the proteasome (Ikeda *et al.*, 1998; Liu *et al.*, 2002). Indeed, expression of Axin induces the downregulation of β-catenin in various cell lines (Kishida *et al.*, 1998; Kishida *et al.*, 1999a).

APC also acts as a critical component for β-catenin destruction (Polakis, 2000; Fodde *et al.*, 2001). In colon

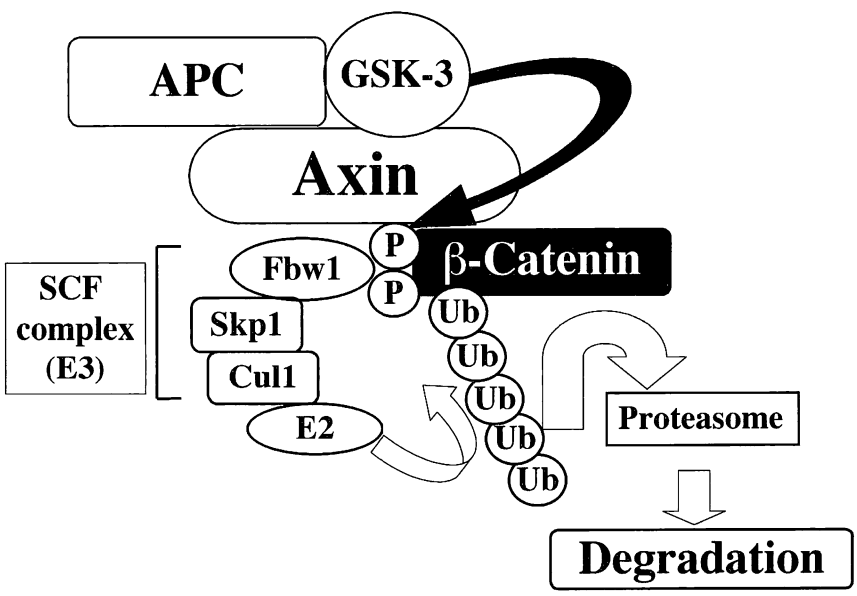

Fig. 2. Degradation of β-catenin in the Axin complex. Axin enhances GSK3-dependent phosphorylation and ubiquitination of β-catenin and accelerates degradation of β-catenin. P, phosphorylation; Ub, ubiquitination.

cancers, mutations of APC correlate with high levels of β-catenin and transcriptionally active Tcf/β-catenin complexes. Expression of wild-type APC in colorectal cancer cells reduces β-catenin levels, and the fragment of APC containing the 20-aa repeats is sufficient for the activity (Polakis, 2000; Fodde *et al.*, 2001). However, an APC fragment with either mutated β-catenin-binding sites or Axin-binding sites fails to induce degradation of β-catenin (Kawahara *et al.*, 2000). Therefore, the interaction of APC with both Axin and β-catenin is required for the ability of APC to degrade β-catenin. In the complex, GSK-3β bound to Axin efficiently phosphorylates APC, which enhances the binding of β-catenin to APC (Rubinfeld *et al.*, 1996; Ikeda *et al.*, 2000), and GSK-3β phosphorylates β-catenin bound to APC in addition to β-catenin bound to Axin (Hinoi *et al.*, 2000). It is likely that APC activates Axin in a manner that facilitates the phosphorylation of β-catenin by GSK-3β. Thus, Axin and APC form a core complex to degrade β-catenin.

3. Wnt-dependent accumulation of β-catenin

(1) Receptor internalization in response to Wnt

The initial event of the Wnt signal is that Wnt binds its receptor consisting of Frizzzled and LRP5/6 (He *et al.*, 2004). The mechanism by which Frizzled and LRP5/6 transduce the signals remains elusive. *Drosophila* genetics have shown that expression of a dominant negative form of dynamin abolishes cuticle deposition, indicating that receptor-mediated endocytosis triggers the Wnt signaling (Moline *et al.*, 1999). Wnt-5a, which is a representative ligand that does not accumulate β-catenin, induces the internalization of Frizzled4 in cooperation with Dvl and β-arrestin2 (Chen *et al.*, 2003). In this endocytosis, Frizzled4 requires PKC-dependent phosphorylation of Dvl. Since β-arrestin2 binds to clathrin, Frizzled4 could be internalized in a clathrin-mediated pathway. However, the endocytosis of receptors such as Frizzled 5 and LRP5/6 that are involved in the canonical β-catenin pathway has not yet been clarified.

(2) Stabilization of β-catenin in response to Wnt

Although the exact mechanism by which Wnt stabilizes β-catenin is unclear, several possible mechanisms have been proposed. The first one is based on the interaction of Dvl with Frat. Dvl binds to CKI ε and Axin (Kishida *et al.*, 1999b; Kishida *et al.*, 2001). CKI ε mediates Wnt-3a-dependent phosphorylation of Dvl and phosphorylated Dvl has a high affinity for Frat, which binds to and inhibits GSK-3 (Fig. 1) (Kishida *et al.*, 2001; Lee *et al.*, 2001; Hino *et al.*, 2003). Knockdown of CKI ε by RNA interference reduces the Wnt-3a-induced binding of Dvl to Frat and accumulation of β-catenin (Hino *et al.*, 2003). Therefore, when Wnt acts on the cells, Frat bound to Dvl phosphorylated by CKI ε may prevent GSK-3β bound to

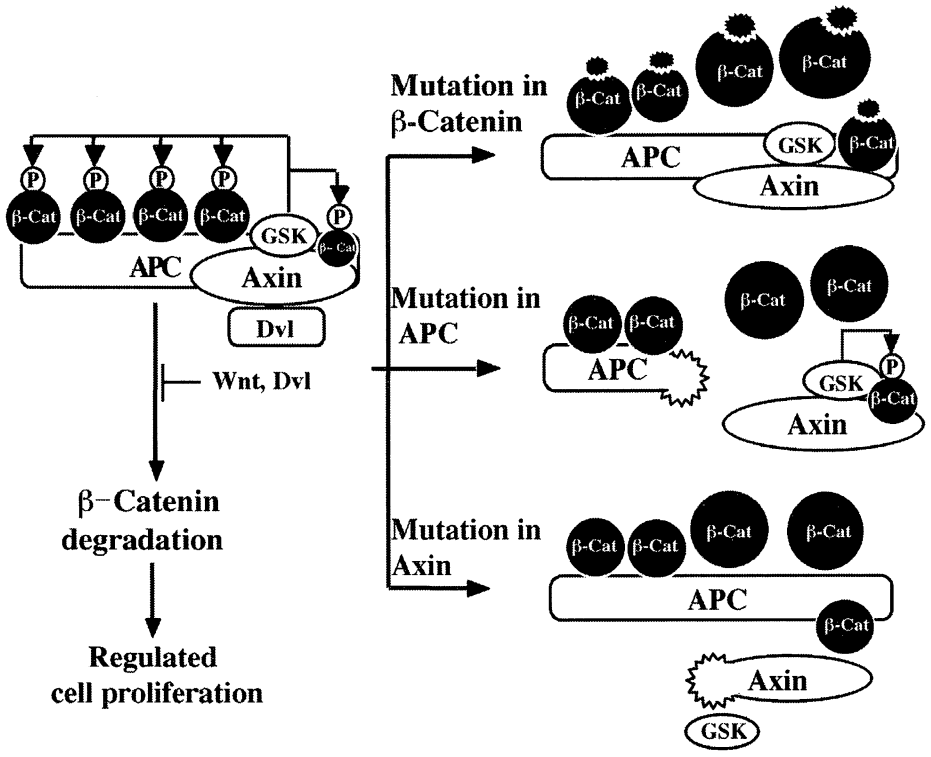

Fig. 3. Mutations of β-catenin, APC, and, Axin, and the mechanism of the abnormal accumulation of β-catenin. Wnt and Dvl suppress the degradation of β-catenin by antagonizing the functions of the Axin complex by unknown mechanisms. Mutations of β-catenin, APC, or Axin disturb the functions of the Axin complex, resulting in unregulated accumulation of β-catenin.

Axin from phosphorylating β-catenin, thereby stabilizing β-catenin. Taken together, Axin binds to positive and negative regulators of the Wnt signaling pathway and regulates the stability of β-catenin.

The second model is based on the interaction of Axin with LRP5/6. Wnt causes the translocation of Axin to the membrane and enhances the interaction between Axin and LRP5/6 (Mao *et al.*, 2001; Tamai *et al.*, 2004). The phosphorylation of LRP6 by an unknown kinase enhances their interaction, which is essential for the transmitting the signal to activate Lef and axis duplication in Xenopus embryos. Dvl and Axin have the DIX domain that is necessary for the binding of Dvl and Axin to intracellular vesicles and actin filaments (Capelluto *et al.*, 2002). Furthermore, disheveled (a fly Dvl homolog), is required for the recruitment of dAxin (a fly Axin homolog) to the plasma membranes in a manner dependent of wingless in fly cells (Cliffe *et al.*, 2003). However, how the interaction of Axin with LRP5/6 activates the β-catenin pathway is unknown.

4. Genetic alterations of β-catenin, APC, and Axin

(1) β-catenin

Mutations in the β-catenin gene that affect specific serine and threonine residues, and amino acids adjacent to them, which are essential for the targeted degradation of β-catenin, are found in a wide variety of human cancers including colon cancers, desmoid, gastric cancer, hepatocarcinoma, medulloblastoma, melanoma, ovarian cancer, pancreatic cancer, and prostate cancer (Polakis, 2000). These mutations abrogate the phosphorylation-dependent interaction of β-catenin with Fbw1, thereby stabilizing β-catenin. The β-catenin mutations occur in D^{32}, S^{33}, G^{34}, S^{37}, T^{41}, and S^{45} (Fig. 3). As described above, S^{45} is a phosphorylation site of CKI α, and S^{33}, S^{37}, and T^{41} are phosphorylation sites of GSK-3β. D^{32} and G^{34} are necessary for the interaction of β-catenin with Fbw1. Therefore, the mutated β-catenin cannot bind to Fbw1, and thereby is accumulated in the nucleus and activates Tcf/Lef. Myc and cyclin D1, which are representative of genes expressed by Tcf/Lef, are clearly relevant in tumor formation, because of their roles in proliferation, apoptosis, and cell-cycle regulation. Changes in the expression of Myc and cyclin D1 are likely to increase the overall proliferating rate. The proteins of other Tcf/Lef target genes such as matrilysin, CD44, and urokinase-type plasminogen activator receptor, seem more likely to be involved in tumor proliferation, rather than initiation.

(2) APC

Mutations in the *APC* gene are frequently identified in familial adenomatous polyposis coli (FAP) and colorectal cancers, but quite rare in other cancers (Polakis, 2000; Fodde *et al.*, 2001). Most APC mutations result in truncated proteins that lack all Axin-binding motifs and a variable number of the 20-aa repeats (Fig. 3). In FAP, germ-line mutations are scattered throughout the 5' half of the APC gene; by contrast, most somatic mutations are clustered between codons 1286 and 1513. In any case, mutations of APC relate strongly to the regulation of the stability of β-catenin. Selective pressure is directed against the presence of Axin-binding sites, because the presence of Axin-binding sites is critical to APC in the regulation of β-catenin levels. The remaining N-terminal truncated form of APC may affect cell migration by activating Asef, thereby leading to metastasis.

Importantly, the overall frequency of β-catenin mutations is quite low in colorectal cancers with APC mutations, and those with an intact APC gene contain mutations of β-catenin that alter phosphorylation sites (Polakis, 2000; Fodde *et al.*, 2001). The exclusivity of β-catenin and APC mutations in colorectal cancers is also evident from the analysis of replication error-positive tumors identified by microsatellite instability. Both the hereditary and sporadic forms of replication error-positive colorectal cancers have a relatively high frequency of β-catenin mutations, where APC mutations are relatively rare (Miyaki *et al.*, 1999).

(3) Axin

Axin was expected to be a tumor suppressor based on its ability to downregulate β-catenin, and this has been indeed verified by documentation of its biallelic inactivation in human hepatocarcinoma (Satoh *et al.*, 2000). Importantly, these mutations of Axin are found in hepatocarcinomas with intact genes for β-catenin and APC. All of the mutations generate a truncated form of Axin eliminated the β-catenin binding site (Fig. 3). Therefore, Axin is now regarded as a tumor suppressor, and constitutes the third genetic defect in the Wnt signaling pathway.

There exists an Axin homolog, termed rat Axil (Yamamoto *et al.*, 1998), mouse conductin (Behrens *et al.*, 1998), or human Axin2. The biochemical characteristics of Axil are similar to those of Axin, and Axil is able to downregulate β-catenin under conditions of overexpression. However, this redundant protein does not suppress Axin mutations in hepatocarcinoma. Therefore, Axil/conductin/Axin2 is either not functionally equivalent to Axin or not expressed at levels sufficient to compensate for its loss in hepatocarcinoma.

CONCLUSION

There are two complexes containing β-catenin, the Axin and Tcf complexes, in the cytoplasm and nucleus, respectively. Wnt may regulate the subcellular distribution of β-catenin between the cytoplasm and nucleus. In the cytoplasm, the amount of β-catenin is negatively regulated in the Axin complex. In the nucleus, gene expression induced by β-catenin is negatively regulated by inhibiting the complex formation of β-catenin, Tcf, and DNA. Mutations in *β-catenin, APC, and Axin* genes have been found in human cancers, and the mutations result in the accumulation of β-catenin. Since β-catenin functions as an oncogene, it is speculated that there are several mechanisms for protecting against abnormal cellular proliferation by inhibiting β-catenin signaling.

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Developmental Signaling Disorders in Craniofacial Anomalies and Cancer

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1. Sonic Hedgehog-Patched in craniofacial anomalies and cancer

1) Sonic Hedgehog (SHH) in craniofacial anomalies

Multiple examples of cosegregation with heterozygous sonic hedgehog gene deletions or truncations have demonstrated haploinsufficiency of SHH gene function in horoprosencphary (HPE). HPE is a complex developmental field defect of the forebrain in which the cerebral hemispheres fail to split into distinct halves. Associated craniofacial anomalies can vary widely, including cyclopia, proboscis-like nasal structure, midline cleft palate, and premaxillary agenesis.

We describe a patient with median cleft lip/madibular and identified a novel *SHH* missense mutation. Sequencing analysis revealed that the patient has a C to T mutation at nucleotide position 279 in the human *SHH* coding region resulting in a Val for Ala substitution (A43V) (Fig 1).

2) *Patched (PTCH)* mutation in nevoid basal cell carcinoma/Gorlin syndrome

Nevoid basal cell carcinoma syndrome (NBCCS)/Gorlin syndrome is a rare autosomal dominant disorder characterized by predisposition to basal cell carcinomas and several other tumors, including ovarian fibroma and medulloblastoma. Developmental defects are another prominent feature of the syndrome and include pits of the palms and soles, jaw keratocysts and other dental malformations, midline brain malformations, strabismus,

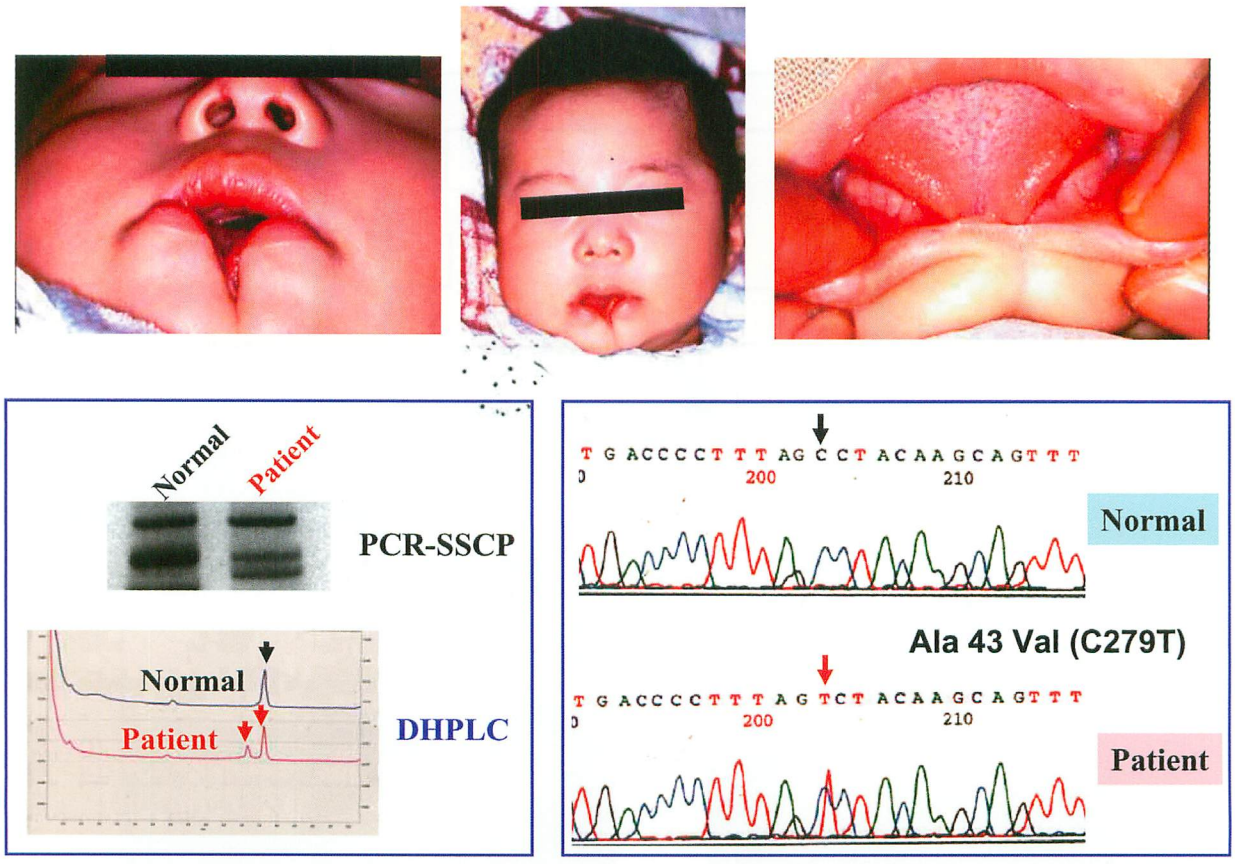

Fig. 1 Craniofacial and intra-oral phenotypes of the median cleft lip/madibular patient, PCR-SSCP, DHPLC and sequence analysis of *sonic hedgehog gene*.
Sequencing analysis revealed a C to T mutation at nucleotide position 279 in the human *SHH* coding region resulting in a Val for Ala substitution (A43V).

spine and rib abnormalities, ectopic calcifications, and macrocephaly with a characteristic coarse facies and generalized over growth. However, the syndrome is particularly noted for its extensive interfamilial as well as intrafamilial variability with respect to the manifestation and severity of the phenotype. The criteria for the diagnosis of NBCCS include presence of at least two major features of the syndrome, such as multiple basal cell carcinomas, or onset of basocellular carcinomas before the age of 20, or pits of palms and soles, jaw cysts, calcification of falx cerebri, and having a first degree relative with NBCCS.

This heritable condition has been associated with constitutional hemizygous in activation of the *PTCH* gene, which is a human homologue of ptch, Drosophila segment polarity gene. The gene appears to have a tumor suppressor role, at least in some malignancies for which the syndrome predisposes, as has been most convincingly demonstrated for basocellular carcinomas. Its protein product, located in the plasma membrane, is a part of the Shh/ PTCH signalling pathway. It has a receptor role for the Shh ligand through close connection to smoothened (smo), another membrane protein component. PTCH suppresses smo's continuous signalling into cytoplasm by binding to it. That prevents *smo* from inducing expression of several downstream genes.

We examined one Japanese NBCCS family (Fig 2) for mutations in all *PTCH* exons and introns by PCR-SSCP analysis and direct sequencing of the PCR products. As a result, we identified 3 novel *PTCH* mutations in the family. Patients (number 2, 4, 7) exhibited the three muatation including 3487insAA in exon 19, Gln 853 Lys in exon 15, and 3075+21 G→A in intron 17. Patients (number 1, 5) posessed 3487insAA in exon 19, and 3075+21 G→A in intron 17. On the other hand, Gln 853 Lys in exon 15, and 3075+21 G→A in intron 17 has been found in normal family member (number 8). It has been suggested that 3487insAA in exon 19 resulting in protein truncation due to the insertion of a couple of adenine might be a responsible cause for this Gorlin syndrome family (Table 1).

3) PTCH mutation in squamous cell carcinoma (SCC) cells

In the present study, we have analyzed tumor deoxyribonucleic acid from oral squamous cell carcinoma (OSCC) cells for *PTCH* mutations using an exon-by-exon single strand conformation polymorphism assay and direct sequencing. We found two missense mutations which affected the conserved residue in the transmembrane domains of the gene product and in the intracellular loop at the C-terminal residue implicated in regulating the smoothened molecule (Table 2). In addition, we

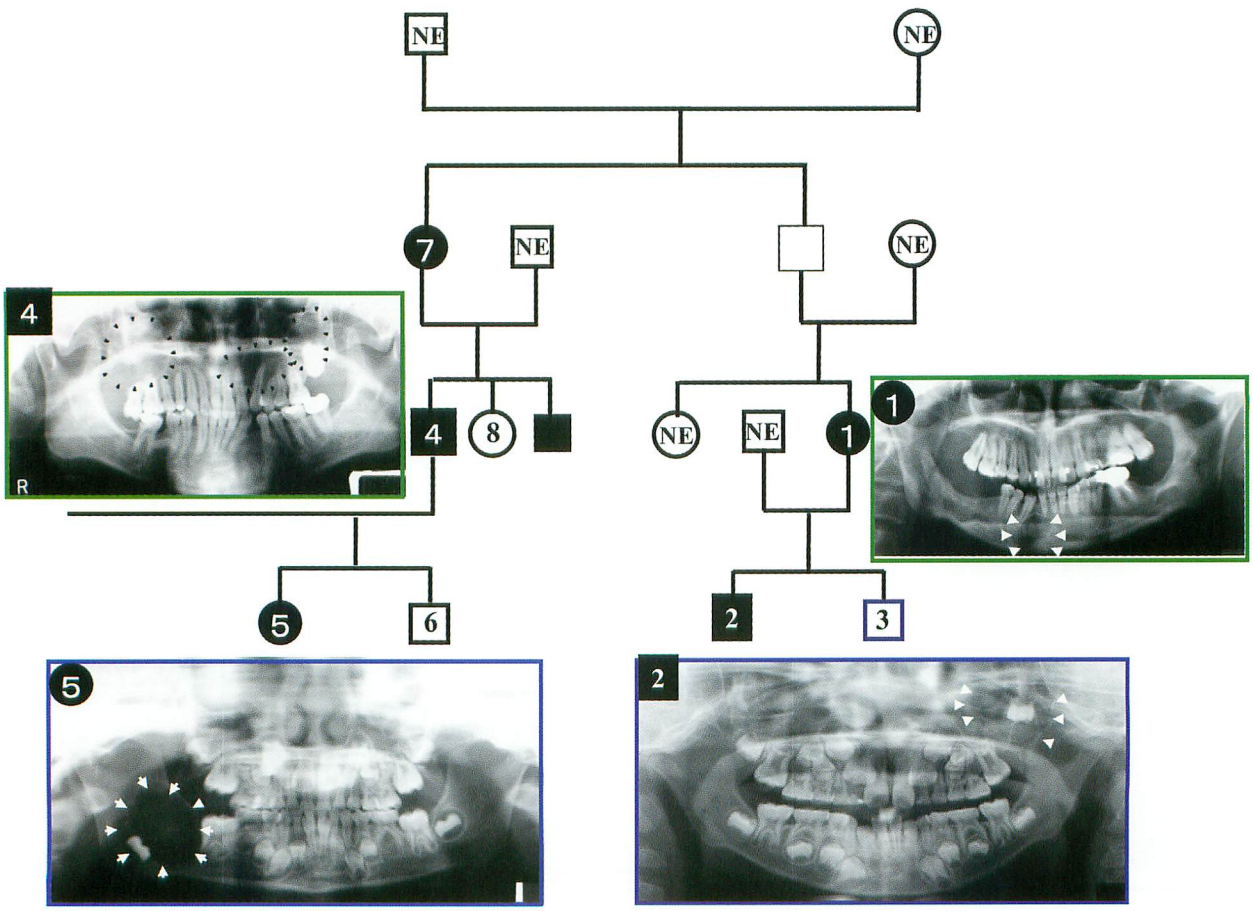

Fig. 2 Pedigree of a Gorlin syndrome family with panoramic roentogenograph exhibiting multiple jaw cysts. Number 1, 2, 4, 5, 7 were diagnosed as a Gorlin syndrome. Affected members are shown as closed symbols and nonaffected as opened symbols.

Table 1 Summary of the symptoms and *PTCH* mutations in a NBCCS/Gorlin syndrome family.

Patients	JC	P	HS	BS	CFC	AK	3487insAA (Exon 19)	Gln 853 Lys (Exon 15)	3075+21 G→A (Intron 17)
1 ●	+	+				+	+		+
2 ■	+	+					+	+	+
3 □									
4 ■	+	+	+	+	+	+	+	+	+
5 ●	+	+					+		+
6 □									
7 ●	+	+	+		+		+	+	+
8 ○								+	+

JC: Jaw Cysts
HS: Hyposeoliosis
CFC: Calcification of falx cerebri
P: Pits
BS : Bony bridging of the sella turcica
AK: Abnormal Keryotype

Table 2 Summary of PTCH mutations in SCC cells and their growth response to recombinant SHH.

Cell line	PTCH exon	Nucleotide change	Protein change	Codon	LOH	Growth Stimulation by SHH
A431	exon 23	T3944C	Leu to Pro	1315	No	No
KA	exon 12	T1682G	Met to Arg	561	No	No
KO	exon 12	T1682G	Met to Arg	561	No	No
NA		N.D	N.D			Yes
NI	exon 12	T1682G	Met to Arg	561	No	No
UE	exon 12	T1682G	Met to Arg	561	No	No

demonstrated that the N-terminal fragment of sonic hedgehog (Shh-N) stimulates the growth of normal epithelial cells, the OSCC cell line, NA, and the salivary gland adenocarcinoma cell lines, HSG and HSY, which have no detectable mutation in patched. On the other hand, Shh has no effect on human SCC cells (UE, KA, KO, NI, A431 cells) that have mutations in patched. These results strongly suggest that a Shh-patched signaling is involved in the cell growth of oral epithelial cells and in the tumorigenesis of OSCCs[1].

Furthermore, to evaluate the biological significance of patched mutations in human OSCC cells and A431 epidermoid carcinoma cells, we constructed a VSV-G pseudotyped retrovirus vector carrying the wild-type patched gene and transduced it into two human squamous cell carcinoma (SCC) cell lines, A431 and KA, that express only mutant patched mRNA. When SCC cells were transduced with Ptc virus, colony forming activity in soft agar was drastically reduced and these cells recovered anchorage independent growth when Sonic hedgehog (Shh), the ligand of Patched (Ptc), was added into the soft agar culture. Expression of exogenous patched, however, had no effect on anchorage independent growth of Ras-transformed NIH3T3 cells or SCC cell line, NA, which expresses wild-type patched mRNA (Table 3). Cyclopamine, a specific inhibitor of the Shh/Ptc/Smo signaling pathway, efficiently suppressed anchorage independent growth of A431 and KA cells. These results indicate that loss of patched function plays a major role in the acquisition of oncogenic potential in these SCCs and further that Ptc virus would be an effective reagent for suppressing tumorigenicity of such SCCs[2].

2. Angiopoietin-Tie-2 in haemangiomas

Human IMHs are benign, nonmetastatic tumours. The high postoperative recurrence of IMHs has previously been attributed to incomplete excision. This was based on their localization in deep muscle and their infiltrative growth, which make tumour resection difficult.

Table 3 Effects of PTCH virus-transfection on anchorage independent growth and growth in monolayer of SCC cells.

cells	colony formation in soft agar (%) *		growth in monolayer			
			doubling time (%) *		saturation density (%) *	
	*MOI 0.1	MOI 3	MOI 0.1	MOI 3	MOI 0.1	MOI 3
A431	35 ± 10	21 ± 3	105 ± 1	107 ± 2	95 ± 5	74 ± 8
KA	30 ± 5	25 ± 2	107 ± 3	114 ± 2	96 ± 20	88 ± 11
NA	97 ± 10	74 ± 13	101 ± 2	92 ± 2	104 ± 11	108 ± 20
NIH-ras	82 ± 7	97 ± 9	96 ± 3	110 ± 3	97 ± 19	105 ± 11

* percentage to those of control virus-transduced cells
* MOI (multiplicities of infection)

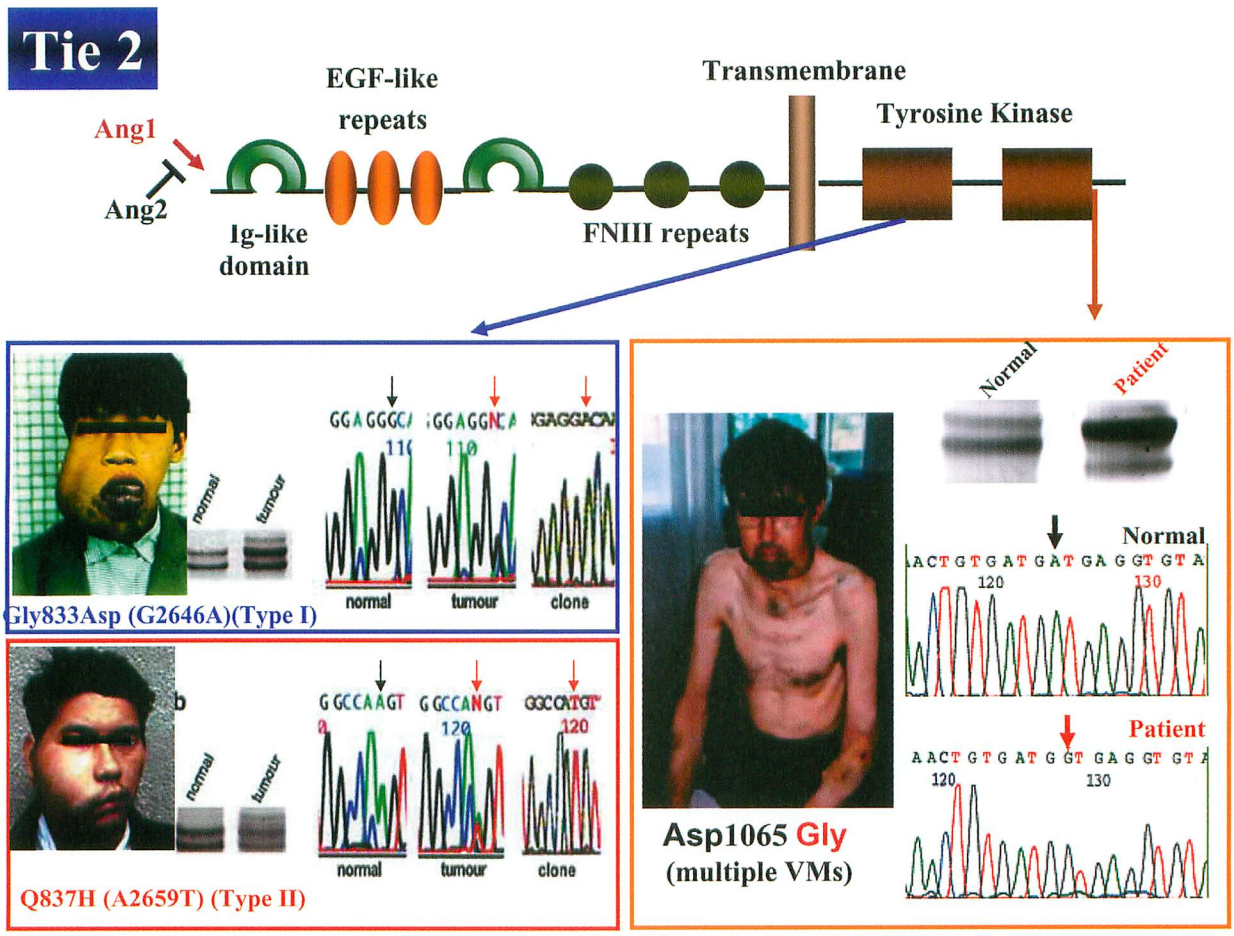

Fig. 3 Tie 2 mutations in human intramuscular haemangiomas.
The patients (left upper) with the G833D (G2646A) mutation had a large disfiguring lesion affecting one side of the face, and associated early post-operative recurrence, whereas the patient (left lower) with the Q837H (A2659T) mutation had no recurrence during postoperative follow-up after five years. The patient with multiple haemangiomas (right) has D1065G mutation in c-terminal tail of Tie 2.

Tie2, an endothelial-cell-specific receptor tyrosine kinase, collaborates with vascular endothelial growth factor (VEGF) in regulating angiogenesis and vascular maturation. Here, we report a mutation of glycine to aspartic acid at the second glycine of the GXGXXG motif of Tie2 (G833DTie2) in human intramuscular haemangiomas (IMHs) of the capillary type (Fig 3). Murine endothelial cells (ECs) overexpressing this G833DTie2 receptor

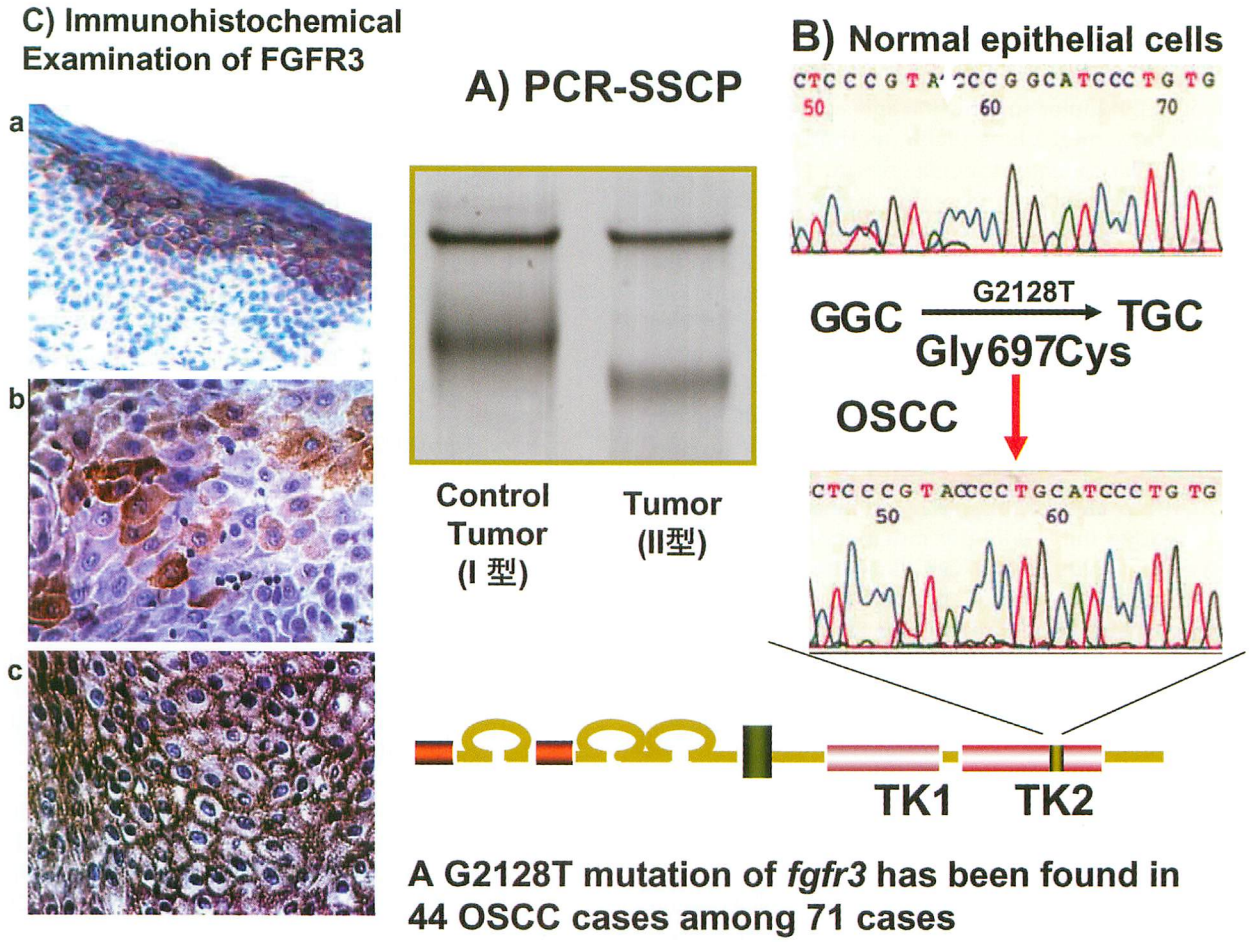

Fig. 4 Detection of mutation in exon 17 of FGFR3b gene and an immunostaining of FGFR3 protein in normal epithelia and OSCC tissues.
A) Representative PCR-SSCP analysis. PCR product from OSCC DNA (lane 2) was run alongside product from normal DNA (lane 1) isolated from non-neoplastic gingiva. *Arrow* indicates mobility shift band. B) Representative sequence analysis of exon 17 from OSCC specimen and normal control. Histogram of sequencing in the forward direction are shown. *Arrow* indicates sequence alteration. C) a, The expression of FGFR3 was observed in prickle cells in normal epithelia (X200). b, In OSCC having no G2128T mutation, the expression of FGFR3 was observed in cytoplasm and nucleus of cancer cells (X400). c, In OSCC having G2128T mutation, the strong expression of FGFR3 was seen in the cell membrane (X400).

exhibited an increase in cell proliferation at low serum concentrations and angiosarcomas developed in nude mice, whereas cells overexpressing either wild-type Tie2 or Q837H Tie2 failed to elicit these responses. Furthermore, the G833D Tie2 receptor increased VEGF expression in ECs. These characteristics could reflect clinical phenotype of early post-operative recurrence in the human G833D IMHs. These findings provide molecular mechanisms for pathogenesis of IMH. Our results and subsequent elucidation of Tie2-mutant-mediated signalling properties may be useful in terms of diagnosis of and possible therapy for various forms of IMH with Tie2 as a target. They may also extend our understanding of receptor kinase function in vascular development. The data presented here, however, demonstrate that there are significant characteristics of the G833D mutation with respect to transforming activity and VEGF expression[3].

3. FGF-FGFR in Salivary gland Adenocarcinomas and Oral Squamous cell Carcinomas

1) Growth inhibition by keratinocyte growth factor receptor/FGFR2b of human salivary adenocarcinoma cells through induction of differentiation and apoptosis

We have previously reported that normal human salivary gland-derived epithelial cells exclusively express keratinocyte growth factor receptor (KGFR). In the process of malignant transformation of human salivary gland tumors, KGFR gene expression disappeared concomitantly with the de novo expression of the fibroblast growth factor receptor 1 (FGFR1) and FGFR4 genes.

In the present study, we introduced wild-type KGFR cDNA or chimeric KGFR/FGFR1 cDNA, which encoded the extracellular domain of KGFR and the intracellular domain of FGFR1, into the HSY human salivary adenocarcinoma cell line. The KGFR tyrosine kinase suppressed the activity of FGF receptor substrate 2 (FRS2)

and inhibited the growth of HSY by inducing differentiation and apoptosis in vitro and in vivo (data not shown)[4]. Our results provided significant insight into the mechanism of KGFR tumor suppression and suggest that KGFR gene therapy might be a viable method of inhibiting human salivary adenocarcinoma growth.

2) *FGFR3b* mutation in oral squamous cell carcinomas

A G to T mutation at nucleotide position 2128 in the human FGFR3b coding region resulting in a Cys for Gly substitution (G697C) in the tyrosine kinase domain was observed in 62% (44/71) of oral squamous cell carcinomas (OSCC) examined. Immunostained FGFR3-IIIb was found in the cytoplasm of prickle cells in normal epithelia, and FGFR3b was localized in the cytoplasm and nucleus in non-FGFR3-IIIb mutant OSCC. Overexpressed FGFR3-IIIb protein on plasma membranes was noted in OSCC bearing the FGFR3-IIIb mutation. Enhanced tyrosine kinase activity of G697CFGFR3-IIIb was confirmed[5] (Fig 4). Our results indicate that G697C is an activating mutation causing constitutive ligand-independent FGFR3-IIIb signaling. This mutation may be involved in the progression of OSCC and thus the FGFR3b coding sequence may have diagnostic or prognostic value for OSCC.

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Development of Periodontal Tissue Regeneration Therapy with New Bioactive Agents

-Studies on Brain-derived Neurotrophic Factor and Ameloblastin Peptide-

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ABSTRACT

To develop the periodontal regeneration therapy using bioactive agents, effects of two potential agents, brain-derived neurotrophic factor (BDNF) and a synthetic peptide of ameloblastin on growth and differentiation of periodontal ligament cells in vitro and tissue healing in vivo. Both agents enhanced proliferation and mineralization of the periodontal ligament cells and induced periodontal tissue regeneration. We showed that BDNF and the ameloblastin peptide could be used as the promising medication to promote periodontal tissue regeneration.

KEY WORDS: periodontal tissue regeneration, brain-derived neurotrophic factor (BDNF), ameloblastin

INTRODUCTION

The goal of periodontal treatments is regeneration of periodontal tissues that have been lost by periodontal diseases. A periodontal defect after debridement is repopulated by cells from four different sources: oral epithelium, gingival connective tissue, alveolar bone, and periodontal ligament. It is known that new connective tissue attachment can be obtained only when the cells from periodontal ligament cover the denuded root surface. Based on this concept, a new regeneration therapy using a barrier membrane that guides the selective repopulation of periodontal ligament derived cells into a periodontal defect (Takata, 1994) was devised. This treatment procedure is called guided tissue regeneration (GTR) method and widely employed as a predictable regeneration modality. The clinical outcome of the GTR method is right enough for a relatively small defect of periodontal tissues, while predictability for an advanced lesion such as one-wall bony defect and through-and-through furcation involvement is poor. This limited indication of the GTR method depends on limits of the ability of spontaneous proliferation and differentiation of the cells from the remaining periodontal ligament. To overcome the limitation and promote the periodontal regeneration, biological effects of various bioactive agents on migration, growth, differentiation and matrix production of periodontal ligament derived cells have been extensively examined.

In this paper, we describe two studies aiming to develop a new periodontal regeneration therapy using a growth factor, brain-derived neurotrophic factor (BDNF), and a synthetic peptide of an enamel matrix protein, ameloblastin.

1. Periodontal Tissue Regeneration using Brain-derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF), cloned as the second member of the neurotrophin family, plays a role in the survival and differentiation of central and peripheral neurons through biding to a product of *trk*B (Barbacid, 1994.; Ebendal, 1992). It has been reported that various types of non-neural cells and tissues express BDNF. Thus, BDNF can regulate functions of non-neural cells as well as neural cells. In the present study, we examined the effect of BDNF on the expression of bone (cementum) related factors and proliferation of human periodontal ligament (HPL) cells. Subsequently, we investigated the effect of BDNF on the regeneration of periodontal tissues in experimental periodontal defects in dogs.

BDNF Increases Bone (Cementum) Related Factors at mRNA and Protein Levels and BrdU Incorporation in HPL Cells

HPL cells were obtained by explant cultures of healthy periodontal ligaments of the mid-root of premolars extracted from patients under orthodontic treatment with their informed consent. BDNF at 50 ng/ml increased mRNA levels of osteopontin (OPN), BMP-2, alkaline phosphatase (ALP) and osteocalcin (OCN) in a dose-dependent manner (Fig. 1A). BDNF also increased secreted OPN and BMP-2 levels in a dose-dependent manner up to 100 ng/ml (Fig. 1B and 1C). Adding BDNF to HPL cells resulted in an increase in procollagen type I C-terminal peptide (PIP) levels in the media (Fig. 1D). BDNF increased BrdU incorporation in HPL cells until 10 ng/ml BDNF. However, at more than 10 ng/ml BDNF the increase in the incorporation declined to the control level (Fig. 1E).

BDNF Enhances Periodontal Tissue Regeneration in Dog

Under general anesthesia with local infiltrated anesthesia, the mandibular second, third and fourth premolars at

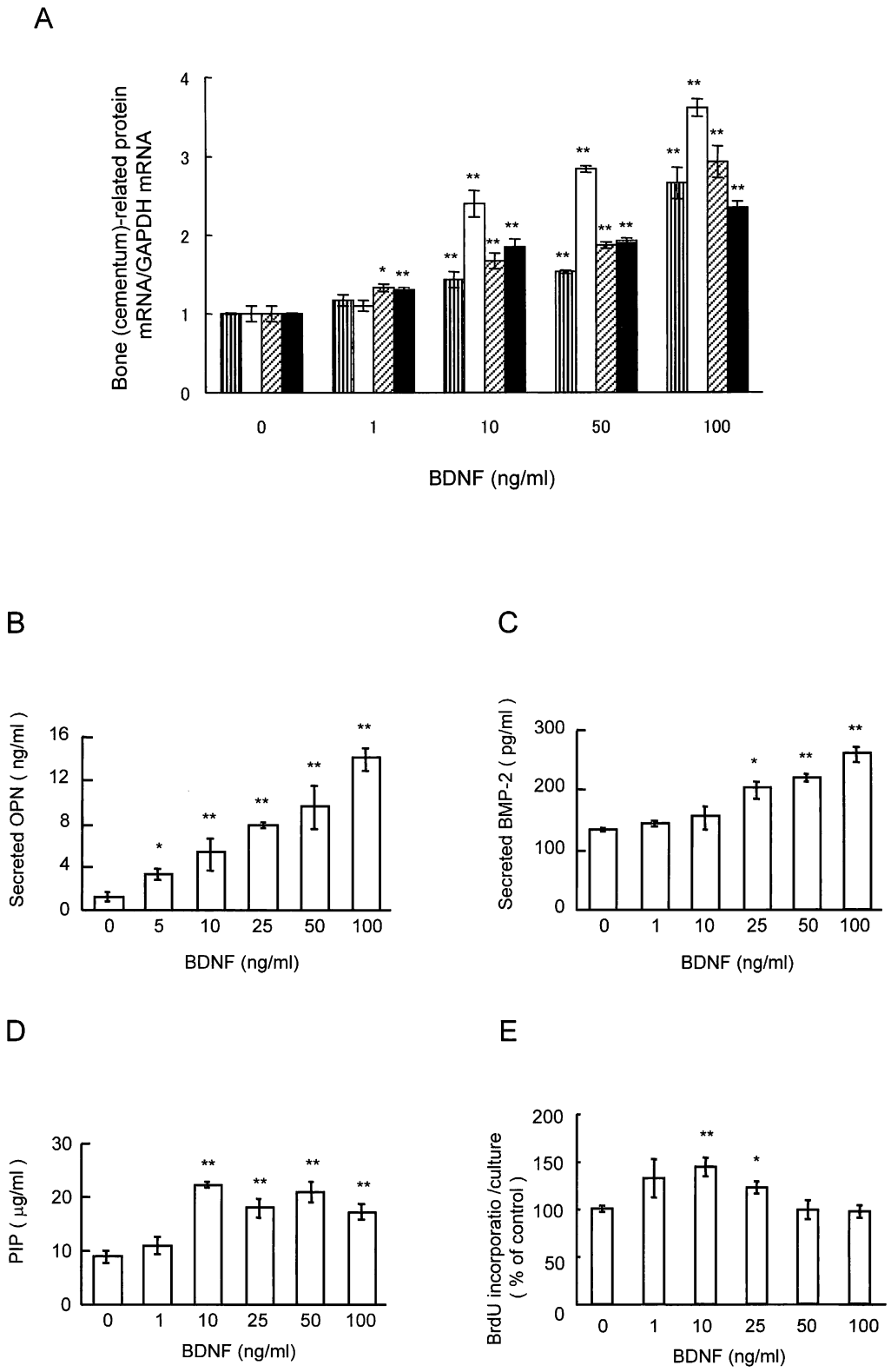

Fig. 1. A. Effects of BDNF on OPN, BMP-2, ALP and OCN mRNA expressions in HPL cells. The B-E. Effects of increasing concentrations of BDNF on the syntheses of OPN (B), BMP-2 (C), PIP (D) and DNA (E) in cultures of HPL cells. Differs significantly (*P<0.05: **P<0.01) from the control.

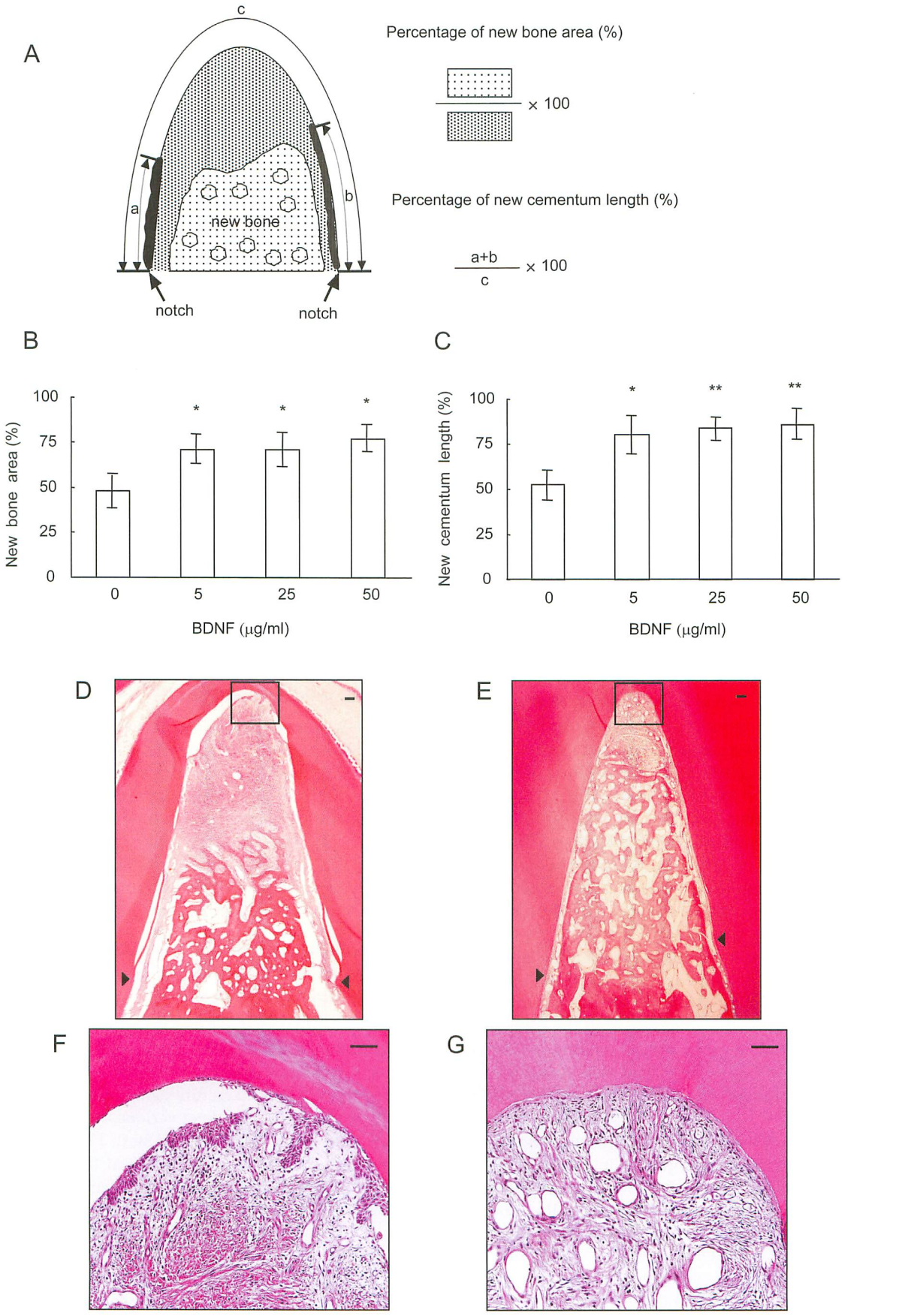

Fig. 2. A. Schematic drawing of the histometric analysis of percentages of new bone area and new cementum length. B and C. Effect of BDNF in experimentally created periodontal defects. The graphs show percentages of new bone area (B) new cementum length (C) by morphometrical analysis. Differs significantly (*P<0.05: **P<0.01) from the control. D and E. Low-power view of furcations representing the control group (D) and BDNF (5 μg/ml) group (E). F and G. Higher magnifications of the rectangular areas shown in D and E, respectively. HE staining.

the right and left sides were utilized for experimentation. Following sulcular incisions, mucoperiosteal flaps were raised and class III furcation defects were surgically created. BDNF (5, 25 and 50 μg/ml) immersed into atelocollagen sponge and applied into the defects. Six weeks after the surgery, histological sections, representing the central portion of the furcation site, were analyzed histologically and morphometrically (Fig 2A). Statistical analysis of the data was performed using ANOVA.

A greater volume of newly formed alveolar bone and a longer newly formed cementum were observed in the BDNF group at 5 μg/ml than in that of the control group (Fig 2B-E). Morphometrical analysis of new bone area showed that BDNF at 5, 25 and 50 μg/ml significantly increased bone area (Fig. 2B) and cementum length (Fig. 2C). Epithelial cells invaded the top of the furcation in the control group and newly formed cementum was not observed in this area (Fig. 2D and F). On the other hand, newly formed cementum was observed on denuded root surfaces of the furcation area and fibers were inserted into the cementum in the BDNF group (Fig. 2E and G). Furthermore, epithelial cell invasion and bone ankylosis were not observed in the BDNF sites (Fig. 2E and G). More blood capillaries were observed in the BDNF group than in the control group and the lamina in the vessels in the BDNF group was larger than in the control group (Fig. 2F and G).

DISCUSSION

BDNF increases expression of ALP, OPN, BMP-2, OCN and type I collagen and DNA synthesis in HPL cells. BDNF is known to be involved in the angiogenesis (Kim et al., 2004). The present in vivo studies showed that BDNF accelerated the formation of periodontal ligament, cementum and alveolar bone and angiogenesis in experimentally created periodontal defects in dogs. Thus, the stimulatory effects of BDNF on the periodontal tissue regeneration were found to result from enhancement of functioning of endothelial cells as well as HPL cells from the in vitro and in vivo studies.

It is noteworthy that ankylosis and epithelial down-growth were not observed in the BDNF group. Ankylosis and epithelial down-growth are not favorable repair phenomena, as they interfere with the completion of periodontal tissue regeneration. Covering the denuded root surface with newly formed cementum may be related to the prevention of ankylosis and epithelial down-growth.

Previous studies showed that fibroblasts engineered ex vivo to secrete BDNF and grafted into a partial cervical hemisection promote axon regeneration while reducing cell loss and atrophy of neurons in the Red nucleus (Liu et al., 2002). Therefore, grafting genetically modified HPL cells that express BDNF as well as BDNF into lost periodontal tissue is a possible new therapy for regenerating and innervating periodontal tissues.

The biological effect of BDNF is mediated through TrkB of high-affinity transmembrane receptor, and the low-affinity receptor of p75, which is a member of the tumor necrosis factor receptor superfamily (Barret, 2000). A truncated receptor lacking the kinase domain has been described for TrkB (Klein et al., 1990). TrkB with and without tyrosine kinase have been detected in neurons, while non-neural cells express both or either form. Our studies showed that HPL cells as well as periodontal ligament tissues expressed TrkB mRNA with and without tyrosine kinase (unpublished data). At present, the mechanism of mediating trophic effects of BDNF via TrkB domain and p75 is still unconfirmed. Therefore, additional studies will be required to clarify the significance of TrkB and p75 in periodontal tissue regeneration.

2. Periodontal Tissue Regeneration using Synthetic Ameloblastin Peptide

Enamel matrix proteins including amelogenin, enamelin, ameloblastin, enamelysin and others play important roles in odontogenesis (Simmmer et al., 2002). Based on the roles of the enamel matrix proteins in odontogenesis, enamel matrix derivatives were applied to periodontal tissue regeneration and good laboratory and clinical results have been obtained (Hammarström, 1997). Thus, it is interesting to decide which protein in EMD is most responsible for the periodontal tissue regeneration.

Ameloblastin is a minor member of enamel matrix proteins that is supposed to act as a messenger molecule for epithelio-mesenchymal interactions in the odontogenesis (Simmer et al., 2002). In the present study, therefore, we examine the effects of ameloblastin in proliferation and differentiation of HPL cells.

Ameloblastin Peptide Stimulates Cell Growth of Periodontal Ligament Cells

HPL cells were plated into a 24 well culture plate (5000 cells/well). After incubation for 24 h (day 0), the culture media were then replaced with a fresh media including the ameloblastin peptide, designed from well-conserved sequences at N-terminal of the protein, at various concentrations (1, 100 and 10000 pg/ml). The number of trypsinized cells was counted using a cell counter at 6 days. Ameloblastin peptide stimulated proliferation of HPL cells in a dose dependent manner (Fig. 3A).

Ameloblastin Peptide Enhances Alkaline Phosphatase and Mineralization Activities of Periodontal Ligament cells

The quantitative analysis of ALP activity was performed biochemically by Bessey-Lowry enzymologic method using nitrophenyl phosphate as a substrate. HPL cells were plated in 24 well culture plates and cultured DMEM with ameloblastin peptide at various concentrations for 1 week. The cells were washed with PBS and homogenized ultrasonically and aliquots of the homogenates were used for quantification of ALP activity. ALP activity was increased by the ameloblastin peptide in a dose dependent manner (Fig. 3B). Mineral nodule formation was detected by Dahl's method for calcium. Cells were placed in a 24 well plate and cultured in DMEM with ameloblastin peptide at various concentrations for 3 weeks. The medium was supplemented with 2 % FBS, 50 μg/ml ascorbic acid and 10 mM sodium β-glycerophosphate. The cells were stained with alizarin red S. The peptide increased mineralized nodule formation in a dose dependent manner (Fig. 3C). The increased ALP

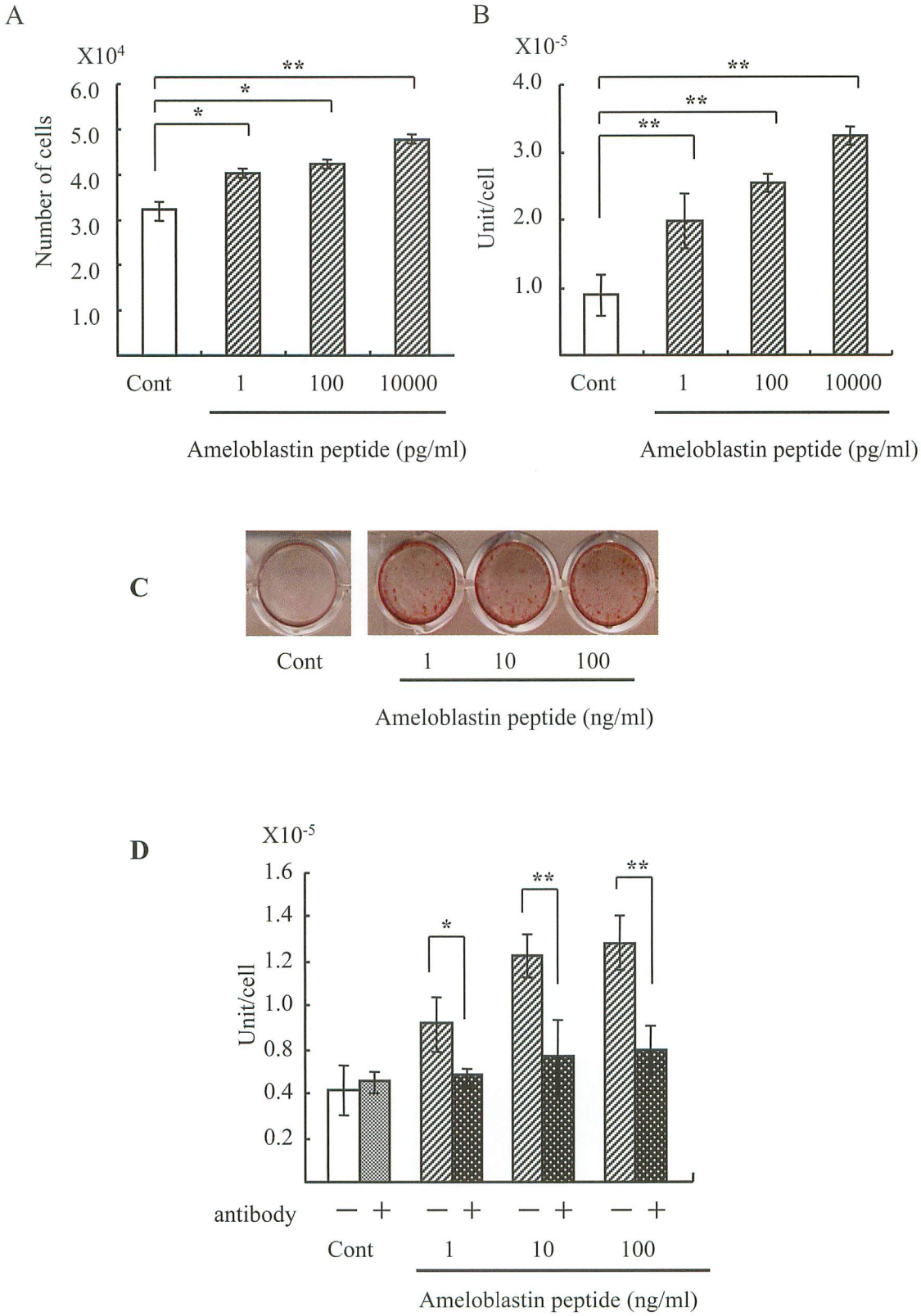

Fig. 3. A. Effect of ameloblastin peptide on proliferation of HPL cells. Cell growth of HPL cells is stimulated in a concentration dependent manner by ameloblastin peptide compared with control culture. *: $P < 0.05$, **: $P < 0.01$, B. Effects of ameloblastin peptide on ALP activity of HPL cells by biochemical method. HPL cells show higher ALP activity than control in a does dependent manner. **: $P < 0.01$, compared with control cultures. C. Alizarin red staining. The peptide increases mineralized nodule formation of in a dose dependent manner D. Effects of anti-ameloblastin antibody on ALP activity of HPL cells. The stimulation of ALP activity in HPL cells by the peptide is significantly inhibited by the anti-ameloblastin antibody. *: $P < 0.05$, **: $P < 0.01$.

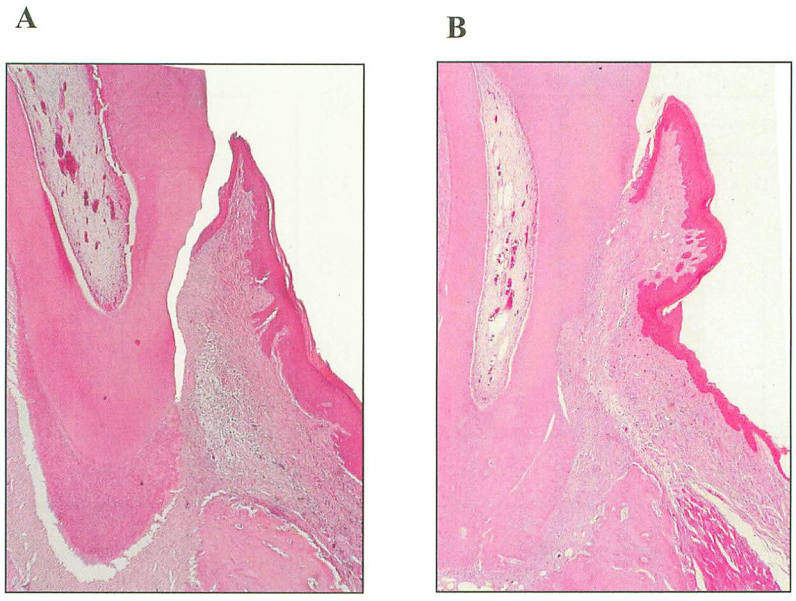

Fig. 4. Effects of the ameloblastin peptide on periodontal tissue regeneration. A. Down-growth of junctional epithelium and separation of gingival tissue from root surface are observed in the control group without the peptide application. B. In the ameloblastin peptide group (0.3mg/ml), down-growth of epithelium is not observed and new connective tissue attachment is formed.

activity induced by ameloblastin was significantly inhibited by an anti-ameloblastin antibody (Fig. 3D).

Ameloblastin Peptide Stimulates Periodontal Tissue Regeneration

Under the abdominal anesthesia, a periodontal tissue defect was experimentally prepared at the mesial root surface of the upper first molar. The defect was applied with 0.3 mg/ml ameloblastin peptide in 1 % hyaluronic acid. At 3 weeks postoperatively, the tissues were prepared for the histological analysis. In the ameloblastin peptide group, the down-growth of epithelium was not observed. And the formation of cementum-like hard tissue with insertion of collagen fiber was formed on the exposed root (Fig. 4A). On the other hand, in control and hyaluronate gel groups, junctional epithelium progressively proliferated apically along the root surface. No connective tissue formation was observed (Fig. 4B).

DISCUSSION

Ameloblastin is expressed on the dentin surface of the root immediately before cementogenesis in a spatiotemporal-specific manner and supposed to act as a messenger molecule for epithelio-mesenchymal interactions in cementogenesis (Simmer et al., 2002; Fukae et al., 2001). In this study, therefore, we examined effects of ameloblastin on cell growth and differentiation of HPL cells and evaluated clinical use of ameloblastin as a novel periodontal regeneration medication. For the study, we did not use the whole length of ameloblastin, but the synthetic peptide of N-terminal region of ameloblastin, because it is very difficult to make recombinant whole amelobastin or to retrieve large amount of ameloblastin from teeth. The region that we synthesized is genetically well conserved among animal species and expected to have important biological properties.

The ameloblastin peptide stimulated the proliferation and differentiation of HPL cells in vitro. Furthermore, the peptide enhanced formation of new connective tissue attachment in vivo experimental model. These results suggest usefulness of the peptide as a treatment agent for the periodontal tissue regeneration.

Recently, we found the possibility that EMD acts via the receptor that possesses the RTK activity on the cell membrane (Matsuda et al., 2002). In addition, since ERK activity is increased following the EMD stimulation, the involvement of a classical signal cascade, RTK-Ras-Raf-MEK-ERK, in some degree was clarified. However, to which receptor the ameloblastin peptide binds and which signal transudation systems the peptide activates must be elucidated in future for further understanding of the biological properties of the peptide and development of signaling-based medication.

SUMMARY

We showed that BDNF and synthetic ameloblastin peptide could be used as a promising medication to promote periodontal tissue regeneration.

ACKNOWLEDGMENTS

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Gene Therapy for Periodontal Bioengineering

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ABSTRACT

Although significant advancements have been achieved in periodontal therapy over the last decade, predictable regeneration of the tooth-supporting tissues is a challenge in periodontology and oral implantology. Recently, gene therapy, a new therapeutic approach for genetic and acquired diseases, has been applied for tissue bioengineering in multiple clinical situations, including the craniofacial complex, among them defects resulting from periodontal disease. The use of gene therapy vectors has enhanced the bioavailability and targeting of multiple growth and host immune factors to repair alveolar bone defects. Early pre-clinical studies utilizing both *ex vivo* and *in vivo* gene transfer strategies demonstrate the feasibility of using gene therapy for periodontal tissue engineering. This review highlights the current progress made in the field of periodontal regenerative medicine via gene targeting approaches.

Key words: gene therapy, growth factors, tissue engineering, periodontal regeneration, regenerative medicine

Tissue Engineering

Tissue engineering is an interdisciplinary field that applies the principles and methods of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function (Langer and Vacanti, 1993). It's a promising and revolutionary area that involves the most recent advances in molecular and cellular biology, polymer chemistry and physiology. An early concept of tissue engineering was first applied in the 1930's and considered the preparation *in vitro* of matrices containing viable cells which could be implanted in patients to substitute or facilitate the regeneration of damaged tissues (Bisceglie, 1933). Recently, it comprises a large variety of techniques and biomaterials stratified into substitutive, histioconductive and histioinductive approaches (Walgenbach *et al.*, 2001), which involve the use of polymeric matrices, cells and soluble regulators and allows engineering the tissues not only *in vitro* but also *in vivo*. Although significant progress has occurred in the development of organs and tissues in vitro, engineering tissues directly in vivo may be an advantage, considering that physiological environment *in vivo* facilitates the functional incorporation of the new tissue concomitantly to its formation, which is preferable for some tissues like craniofacial tissues, including alveolar bone and the periodontium (Spector, 1999; Taba *et al.*, 2005)

In Periodontics, the challenge for tissue engineering is to regenerate the tooth supporting tissues destroyed by periodontal infections. For decades, a number of different grafting biomaterials, comprising autografts, allografts, xenografts and alloplastic materials, have been developed for treatment of periodontal intrabony defects (Reynolds *et al.*, 2003). Although these biomaterials are proposed to act as scaffolds for bone regeneration, the resulting tissues are generally encased in a dense fibrous connective tissue and result in limited bone formation without improving cementum and periodontal ligament regeneration. Focusing in the regeneration of these two periodontal tissues, another principle of tissue engineering was applied in the 1980's - guided tissue regeneration (GTR) (Nyman *et al.*, 1982). GTR consists in the repopulation of the wounds by specific cells selected with the aid of a membrane used as physical barrier. GTR protects the defect and the root surface from gingival epithelial and connective tissue cells proliferation and allows the migration of periodontal ligament cells which is supposed to have greater regenerative capacity. Although attractive, this technique displays limited clinical predictability and begs for the need of a better understanding of cellular-molecular interactions in order to obtain periodontal regeneration (Murphy and Gunsolley, 2003).

Growth Factors in Periodontal Tissue Engineering

In the last decade, periodontal regeneration research has focused on soluble regulators involved in the modulation of the healing process. Mediators termed polypeptide growth factors (GFs) have been demonstrated to participate in several events required for tissue regeneration, including cellular chemotaxis, proliferation and differentiation (Anusaksathien and Giannobile, 2002). After tissue injury, wound healing is governed in part by a large number of GFs released by platelets, plasma exudates, macrophages and cells from the defect. GFs regulate the activity of cells by binding to specific cell surface receptors, that transduce signals to the cell nucleus via complex signal transduction pathways (Giannobile, 1996). In periodontia, GFs are present in the alveolar bone, cementum and periodontal ligament tissues. Several GFs have been associated with the promotion of periodontal healing, including platelet-derived-growth factor (PDGF), transforming growth factor-beta (TGF-β), basic fibroblast growth factor (FGF-2), insulin-like growth-factor-1 (IGF-

1), bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), and parathyroid hormone (PTH) (Giannobile and Somerman, 2003).

Preclinical and initial clinical studies have demonstrated encouraging results after the therapeutic use of GFs alone or combined with other GF or regenerative techniques for periodontal engineering. PDGF has been one of the most investigated and has shown positive stimulatory effects on tissue regeneration in preclinical (Giannobile *et al.*, 1994; Giannobile *et al.*, 1996; Lynch *et al.*, 1989; Lynch *et al.*, 1991; Rutherford *et al.*, 1992) and clinical studies (Camelo *et al.*, 2003; Howell *et al.*, 1997; Nevins *et al.*, 2003). Most recently, PDGF-BB has received approval by the U.S. Food and Drug Administration (FDA) for the treatment of periodontal defects (Nevins *et al.*, 2005).

FGF-2 (or bFGF) is a multifunctional GF that has been shown to enhance human periodontal ligament and endothelial cell attachment and proliferation *in vitro* (Murakami *et al.*, 1999; Terranova *et al.*, 1989). These results suggest that FGF-2 plays a role in PDL-mediated mitogenesis and angiogenesis during the early wound healing process. Preclinical studies in higher animals reveals strong potential of FGF-2 to promote the closure of furcations (Murakami *et al.*, 2003; Rossa *et al.*, 2000; Takayama *et al.*, 2001) and repair of intrabony defects (Nakahara *et al.*, 2003).

Another promising group of GFs for periodontal regeneration are the BMPs. The human genome encodes at least twenty of these multifunctional polypeptides (Reddi, 1998). Among several functions, BMPs participate in the osteoblasts production by stimulating the cellular events of mesenchymal progenitor cells. Pre-clinical investigations have shown potential regeneration of tooth-supporting alveolar bone using BMP-2 (Wikesjo *et al.*, 2003; Kinoshita *et al.*, 1997), BMP-7 (Giannobile *et al.*, 1998) and BMP-12 (Wikesjo *et al.*, 2004).

In general, the effects of topical application of different GFs in periodontal therapy have shown significant improvement in tissue regeneration, but with insufficient predictability. Although *in vitro* studies have elucidated the role of GFs in the cellular events of the different type of cells, several factors may influence the results *in vivo*. Limitations include restrict understanding of the orchestrated molecular-cellular interactions during periodontal healing, which difficult the choice of the GFs to be employed, and the short half-live of GFs after delivered in vivo. This phenomenon is presumably due to proteolytic degradation, rapid diffusion, and the solubility of the delivery vehicle in the hostile wound healing environment (Giannobile, 1996).

The development of new delivery devices has improved the efficacy of GFs in vivo. Bioabsorbable controlled-release scaffolds have been fabricated to carry GFs and release them at optimal doses in a timely manner depending on the biological demand of the target tissue (Anusaksathien *et al.*, 2006). Several studies have shown release of GFs for up to 15 days when associated with poly (lactate-co-glycolide) (PLGA) scaffolds with microspheres (Elisseeff *et al.*, 2001; Murphy *et al.*, 2000). However, even with optimal scaffolds, the local application of GFs often requires a large amount of protein to stimulate significant effects in vivo, which increases the risk of unwanted side effects (Chang *et al.*, 2003). An alternative approach using gene transfer may therefore have the advantage of transferring into specific cells with specific promoter and appropriate vectors to attain a sustained gene expression and more efficient way of GF delivery *in vivo* (Nakashima *et al.*, 2003).

Gene Therapy

The improvement in the knowledge of the genetic and cellular mechanisms of human diseases allowed the development of a new therapeutic approach for genetic and acquired diseases called gene therapy. This new clinical strategy can be defined as an introduction of specific genetic changes by homologous vectors sequences (Hendrie and Russell, 2005). Although initially designed to permanently correct a single gene in monogenetic disorders, gene therapy purposes have included modification or elimination of malignant cells, modulation of host defenses and reengineering of diseased organs or tissues. Within this approach is the potential to genetically modify the cells to express the required GFs to bone regeneration and, more specifically, periodontal regeneration (Ramseier *et al.*, 2006).

A vector is a carrier that helps to circumvent the natural barriers to DNA internalization to the cell nucleus, where it can use the cellular machinery to express the exogenous gene (Worgall, 2005). In general, they can be divided into viral and non-viral vectors. Viral vectors work as gene-delivery vehicles by replacing part of their genome with a therapeutic gene. The most commonly employed vectors are retrovirus, lentivirus, adenovirus (Ad) or adeno-associated virus (AAV).

Each of these viral vectors has characteristics that make it more or less appropriated for specific applications. Advantages and disadvantages for clinical application of each gene transfer system are presented in Table 1. In general, safety is the primary concern and it includes the risk of reversion of a non-replicative vector to a wild-type virion, tumorigenesis and immunogenic reactions.

Non-viral vectors include plasmid DNA and synthetic vectors that consist of complexes of plasmid DNA with cationic lipids and polymers, known as lipoplexes and polyplexes respectively. Although they present improved safety and are easer manufactured than viral vectors, they have low gene-transfer efficiency, and in some cases toxicity and *in vivo* instability (Table 1).

Gene Therapy Applications in Periodontology

The application of gene therapy for tissue engineering has proved to be effective and has extended to multiple areas of medicine. Tissue repair requires a transient expression of genes, initiating a cascade of events directing a self-maintained process. Adenovirus has been largely employed for this purpose as it is non-integrating and a relatively safe virus, while inducing high level of transient gene expression and transduction of several cell types. In the craniofacial area, gene therapy has been evaluated in the regenerative treatment of bony anomalies, salivary gland injuries, dental pulp healing and periodontal diseases (Ramseier *et al.*, 2006). Table 2 dis-

Table 1. Characteristics of Delivery Vectors for Gene Therapy

Vector Characteristics	Retrovirus	Adenovirus (Ad)	Adeno-Associated virus (AAV)	Lentivirus	Non-viral (plasmids/ DNA complexes)
Transduction efficiency	Low	High	High	High	Low
Genomic integration	Yes	Rare ($<10^{-3}$)	Yes	Yes	No
Gene packaging capacity	High (8kb)	High (7.5kb)	Small (4.5kb)	High (8kb)	High
Time of expression	Long-term	Transient	Long-term	Long-term	Transient
Immune response	No	Yes	Low	No	Yes (plasmid)
Insertional mutagenesis	Possible	No	Rare	Possible	No
Vector production	Easy	Easy	Difficult	Difficult	Easy
Cellular infection	Only dividing cells	Dividing/ non-dividing cells	Dividing/ non-dividing cells	Dividing/ non-dividing cells	Dividing/ non-dividing cells

Table 2: Examples Gene Therapy Approaches For Craniofacial Tissue Engineering

Tissue	Vector	Purpose	References
Craniofacial skeleton	Ad- BMP-2	Bone regeneration (craniofacial defects)	(Lindsey, 2001) (Chang *et al.*, 2003)
	Ad-BMP-2 and 9	Bone regeneration (mandible)	(Alden *et al.*, 2000)
	Ad-VGF	Angiogenesis – tissue ischemia	(Mack *et al.*, 1998)
	Ad-BMP-7	Repair of skull defects	(Krebsbach *et al.*, 2000)
	Retro-BMP-4+VEGF	Repair of skull defects	(Peng *et al.*, 2002)
Salivary glands	Ad-aquaporin-1 (Ad-AQP1)	Stimulate salivary secretion	(Delporte *et al.*, 1997) (O'Connell *et al.*, 1999) (Zheng *et al.*, 2001)
	AAV-IL10	Autoimmune epitheliitis (Sjogren's Syndrome)	(Yamano *et al.*, 1999; Yamano *et al.*, 2001)
	Liposome/plasmid complex –"gene cocktail"	Reduce levels of superoxide radicals and hydrogen peroxide (protection against irradiation damage)	(Vitolo and Baum, 2002)
Tooth pulp	Gdf11-plasmid	Stimulate dentin production (pulp capping)	(Nakashima *et al.*, 2002; Nakashima *et al.*, 2003; Nakashima *et al.*, 2004)
	Ad-BMP7	Same purpose	(Rutherford, 2001)
Temporomandibular joint (TMJ)	Ad-LacZ	TMJ articular surface repair	(Kuboki *et al.*, 1999)
Periodontal tissues	Ad-PDGF-B	Periodontal regeneration	(Jin *et al.*, 2004)
	Ad-BMP-7	Periodontal regeneration	(Jin *et al.*, 2003)
Peri-implant bone	Ad-BMP-7	Extraction socket repair at dental implant defects	(Dunn *et al.*, 2005)

plays the recent applications of gene therapy in the craniofacial complex.

In periodontics, the notable role of PDGF in the periodontal regeneration has been previously discussed in the context of bone (Lattanzi *et al.*, 2005) and other tissues (Alsberg *et al.*, 2001) highlighting the potential of PDGF gene delivering for periodontal engineering. The initial studies evaluated the ability of Ad-PDGF-A to affect cells

derived from the periodontium (Giannobile *et al.*, 2001; Zhu *et al.*, 2001). Osteoblasts, periodontal ligament fibroblasts, gingival fibroblasts and cementoblasts displayed effective expression of the PDGF-A gene for up to 7 days following gene delivery, which resulted in enhanced mitogenic and proliferative responses in these cells (Giannobile *et al.*, 2001; Zhu *et al.*, 2001). Also, dermal fibroblasts presented prolonged signaling events and downregulation of PDGFαR up to 96h after Ad-PDGF-A delivery (Chen and Giannobile, 2002).

Simulating a clinical condition, a three-dimensional ex vivo wound-healing model was constructed using human gingival fibroblasts to evaluate the effects of gene transfer by Ad-PDGF-A and Ad-PDGF-B on cell repopulation and wound fill (Anusaksathien *et al.*, 2003). The expression of PDGF genes was prolonged for up to 10 days. Ad-PDGF-B resulted in 2-fold increased rate of defect fill and 4-fold greater cell densities inside the defect than Ad-PDGF-A or control groups. Upregulation of genes associated with PDGF signaling (PI3 kinase) and fibroblast migration (integrin α-5) suggested modulation of cellular and molecular events by Ad-PDGF-B therapy (Anusaksathien *et al.*, 2003).

The first evaluation of gene therapy for periodontal regeneration in vivo utilized ex vivo gene transfer in alveolar bone wounds in rats (Jin *et al.*, 2003). Syngeneic dermal fibroblasts (SDFs) were transduced *ex vivo* with Ad-BMP-7, seeded onto gelatin carriers and then transplanted to mandibular alveolar bone defects in a rat wound repair model. The treatment stimulated periodontal wound healing including bone, periodontal ligament and cementum. However, the *ex vivo* gene transfer has the limitations of cell procurement issues and the need for an additional surgical procedure for biopsy harvest. To overcome these disadvantages, an *in vivo* viral gene delivery approach was evaluated (Jin *et al.*, 2004). A collagen matrix containing Ad-PDGF-B were applied in a similar model of bone defect in rats. Localized transgene expression was observed up to 3 weeks resulting in proliferative and regenerative effects on periodontia. A four-fold increase in bridging bone and six-fold increase in cementum repair was observed in the Ad-PDGF-B treated sites in comparison to control sites (Jin *et al.*, 2004).

Another experiment using in vivo gene delivery evaluated the bone regeneration after the treatment of large defects surrounding dental implants in rats using Ad-BMP-7 administered in a collagen matrix (Dunn *et al.*, 2005). The treatment resulted in enhancement of alveolar bone defect fill, coronal new bone formation and new bone-to-implant contact.

SUMMARY

There remains strong potential for the role of gene delivery applications to improve the bioavailability, sustainability and targeting of growth factors to periodontal and alveolar bone defects. Much of the field is in its early stages for eventual human application to the field of periodontal regenerative medicine. Critical areas for study include safety with the use of viral vectors and control of the host immune response to chronic periodontal pathogen exposure. Improvements in vector optimization, combinatorial gene delivery approaches and carrier delivery systems may soon make gene therapy for periodontal repair a clinical reality.

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Present Status of Biomaterial-based Regenerative Medical Therapy

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ABSTRACT

Recent research development of biomedical engineering including biomaterials and drug delivery system (DDS) as well as basic biology and medicine of cells relevant to regenerative phenomena has enabled cells to induce regeneration repairing of defective or injured tissues as well as substitute the biological functions of damaged organs. For successful tissue regeneration, it is undoubtedly indispensable to give cells a local environment where cells can efficiently proliferate and differentiate to result in induction of tissue regeneration. Tissue engineering is one of the biomedical technologies and methodologies to create this regeneration environment and consequently cure diseases based on the cell-induced regeneration potential of patients themselves. The regeneration repairing of tissues and organs has been realized by making use of biomaterials and DDS technology or methodology in a surgical or internally medical manner. Cell scaffolding and the controlled release system of growth factor and genes are prepared from biomaterials and given to accelerate the proliferation and differentiation of key cells for induction of tissue regeneration. This paper overviews the present status of biomaterial-based regenerative medical therapy by introducing concrete examples of tissue regeneration with cell scaffolding and DDS.

Keywords: Biomaterials, Tissue engineering, Tissue regeneration induction, Drug delivery system, Regenerative medical therapy

BACKGROUND

Tissue Engineering and Regenerative Medical Therapy

When the body tissue or organ was severely injured or largely lost or become functionally wrong, it has been clinically treated with either reconstruction surgery or organ transplantation method. Reconstruction surgery fundamentally depends on biomaterials or biomedical devices artificially prepared. However, they cannot completely substitute the biological functions even for a single tissue or organ and consequently cannot prevent progressive deterioration, either. Although it is no doubt that the surgical treatment has saved and improved the countless lives of patients, it remains problems to be resolved. One of the biggest issues for organ transplantation is the shortage of donor tissues or organs. Additionally, the permanent medication of immunosuppressive agents often causes various side-effects, while virus transfection is not completely ruled out. One promising approach to tackle the problems is to develop a new therapeutic way which allows patients to enhance their own self-healing potential for induction of body tissues and organs regeneration. To realize this therapy of regenerative medicine, in addition to the research of cell biology and medicine, it is necessary to provide cells a local environment suitable to their proliferation and differentiation for the induction of tissue regeneration. It is tissue engineering that is one of the biomedical engineering forms to build up the environment for regeneration induction. If this induction of tissue regeneration based on the tissue engineering concept is realized, it will be possible to achieve a new strategy of disease therapy on the basis of the patient natural-healing potential to induce regeneration of defective or lost tissues as well as substitute the biological functions of damaged organ. There are two approaches of tissue engineering, the surgical and internal-medicine (physical) tissue engineering. For the former, biomaterials in various forms are surgically applied to a defect of body tissue to induce tissue regeneration thereat for repairing therapy. Drug is physically applied to digest and disappear a functionally wrong tissue or organ, leading to induction of regeneration repairing at the site applied based on the natural-healing potential of the surrounding healthy tissue, which is the internal medicine approach of tissue engineering. For the technology and methodology of tissue engineering, several biomaterials are used although the role is different depending upon the purpose to be used.

Important Role of Biomaterials in Tissue Engineering

Tissue engineering for the therapy of regenerative medicine can be classified into two categories in terms of the site where regeneration or organ substitution is performed: *in vitro* and *in vivo* tissue engineering. The *in vitro* tissue engineering involves tissue reconstruction where a tissue is tried to reconstruct in the *in vitro* culture system and organ substitution, so-called bioartificial hybrid organ. However, it is quite difficult to completely reconstruct the *in vivo* event only by use of the present knowledge of biology and medicine or cell culture technologies currently available. As far as it is impossible to artificially arrange a biological environment for cell-based tissue reconstruction, it will be quite difficult to realize *in vitro* tissue engineering at present, .

Distinct from the *in vitro* tissue engineering, *in vivo* tissue engineering originally has an advantage in terms of the environment creation for tissue regeneration. It is likely that most of biological components essential to tissue

regeneration are automatically supplied by the host living body. Therefore, almost all the approaches of tissue engineering have been currently performed *in vivo*. This approach is more realistic and clinically acceptable if it works well. For *in vivo* tissue engineering, tissue regeneration is induced by using and combining three components constituting the body tissue, such as cells, the extracellular matrix (ECM) of natural scaffold for cell proliferation and differentiation, and biological signal molecules of growth factors and gene.

It is well known that the ECM is not only a physical support of cells but also has an important influence on the cell proliferation and differentiation or morphogenesis which contributes to tissue regeneration and organogenesis. For example, a large-size defect of tissue will not be naturally regenerated and repaired only by supplying cells to the defect. This is because of cells supplied readily die without their supporting environment. One promising way is to build an environment suitable to induce tissue regeneration at the defect by in advance providing a scaffold as an artificial ECM which temporarily supports cell attachment and the subsequent proliferation and differentiation. The scaffold is prepared from biomaterials. It is highly expected that self-derived cells residing around the scaffold infiltrate into the scaffold and proliferate and differentiate therein if the artificial ECM is biologically compatible to the cells. Once a new tissue is regenerated, the tissue eventually produces the intact ECM. Remaining of the cell scaffold often causes physical hindrance against tissue regeneration. Thus, since it is key for successful tissue regeneration to control the time profile of scaffold remaining at the defect, the scaffold is preferable to be biodegradable.

In case that the tissue around the defect dose not have any inherent potentials toward regeneration, the tissue regeneration cannot always be expected only by supplying the scaffold. The scaffold should be used in combining with cells or/and biological signal molecules which have a potential to accelerate tissue regeneration. However, the direct injection of growth factor in the solution form into the site to be regenerated is not generally effective. This is because the growth factor of protein is rapidly diffused away from the injected site or deactivated. To enhance the in vivo efficacy, drug delivery system (DDS) is required. Biomaterials play an important role in DDS and are used to combine growth factor for enhancement of the biological functions *in vivo*. One of the DDS techniques necessary for tissue engineering is the controlled release of growth factor at the site of action over an extended time period by incorporating the factor into an appropriate carrier. The release carrier should be degraded in the body since it is not needed any more after the growth factor release is completed. Other than the controlled release of drug, the objectives of DDS include the prolongation of drug half-life, the improvement of drug absorption, and drug targeting. When a body defect is generated, the defect space is generally occupied rapidly with the fibrous tissue produced by fibroblasts which are ubiquitously present in the body and can rapidly proliferate. This is one of the wound healing processes, but once this ingrowth of fibrous tissue into the space to be regenerated takes place, the regeneration and repairing at the space cannot be expected any more. To prevent the tissue ingrowth, a barrier membrane of biomaterials can be used. Thus, by making use of scaffold, barrier, and DDS technologies, it is of prime importance for successful tissue engineering to create an environment which allows cells to promote the proliferation and differentiation for induction of tissue regeneration.

PRESENT STATUS

Cell Scaffolding and DDS Technologies for Tissue Engineering

If the tissue to be repaired has a high activity toward regeneration, active, and immatured cells infiltrate into the matrix of biodegradable scaffold implanted from the surrounding healthy tissue, resulting in formation of a new tissue. For example, when the scaffold of collagen sponge or the combination with a biodegradable polymer sheet was applied to various tissue defects, successful regeneration of the skin dermis(Suzuki *et al.*, 1995), trachea (Okumura *et al.*, 1994), esophagus (Takimoto *et al.*, 1998), dura mater (Yamada *et al.*, 1997), and peripheral nerve (Inada *et al.*, 2004) was induced without any cells supply. However, additional means are required if the regeneration potential of tissue is very low, because of, for instance, low concentration of cells and growth factors responsible for new tissue generation. Combination of cells isolated from a blood vessel and small intestine with a biodegradable scaffold achieved the *in vivo* regeneration of the respective organs (Kaihara *et al.*, 2000; Shinoka *et al.*, 1998). Bone regeneration has been clinically tried by using autologous mesenchymal stem cells (MSC) isolated from the bone marrow together with a scaffold (Ohgushi and Caplan, 1999). Phalanges and small fingers could be regenerated by using two different scaffolds combined with periosteum, chondrocytes, and tenocytes for bone, cartilage, and tendon (ligament), respectively (Isogai *et al.*, 1999). A simpler method other than the cells combination is to supply growth factor to the site of regeneration for cell differentiation and proliferation in a controllable fashion. Based on the recent development of biology and medicine, many types of growth factors can be used while their biological functions have been elucidated. As described above, it is undoubtedly necessary for the induction of tissue regeneration with a growth factor of *in vivo* instability to make use of DDS technology, for example the controlled release of the factor. Recent researches on tissue regeneration through combination of growth factors with various carriers experimentally indicate that it is absolutely necessary for efficient combination with the carrier to allow growth factor to exert the biological activity for *in vivo* tissue regeneration. The type of growth factors to be used depends on the tissue to be regenerated and the site. Beside single use of growth factor, use of multiple growth factors and the combination with a scaffold pre-seeded with cells sometimes needs to accelerate tissue regeneration.

In place of growth factor protein itself, recently the gene encoding growth factor has been applied to promote tissue regeneration (Bonadio, 2002). For tissue engineering with gene, there are two future directions of researches and clinical therapy. The first is the conven-

tional gene therapy that various plasmid DNAs and viruses are directly injected into the body. However, the clinical application of virus is fundamentally problematic because of the immunogenicity, toxicity, and the possibility of disease induction. For the plasmid DNA, the low transfection efficiency remains unresolved. Therefore, to develop a non-viral system with high efficacy of gene transfection, DDS technologies are needed. The angiogenetic therapy of ischemic diseases (Lee and Feldman, 1998) and bone tissue regeneration (Bonadio, 2002) have been attempted by the direct injection with the plasmid DNA of growth factor genes. If such a gene is transfected into cells existing in the site of regeneration, it is no doubt that the cells transfected secrete the corresponding growth factor for a certain time period, resulting in promoted tissue regeneration. Basically this approach is also one therapeutic procedure by protein released from gene-transfected cells. The second direction is to genetically engineer cells for their enhanced biological functions. Stem cells are sometimes not powerful for cell therapy. As one trial to activate the cells, it will be a promising way to genetically engineer cells for biological activation. A DDS technology or methodology will assist to develop a system of non-viral gene transfection at the efficiency as high as that of viral system.

Successful Surgical Tissue Engineering by Controlled Release of Growth Factor

To minimize the denaturation and deactivation of protein drugs during preparation process of the release formation, polymer hydrogel may be a preferable candidate as protein release carrier because of its biosafety and its high inertness toward protein drugs. We have designed the controlled release system of growth factor from a biodegradable hydrogel. It was found that as expected, various growth factors of basic type, such as basic fibroblast growth factor (bFGF), transforming growth factor β1 (TGF-β1) or platelet-derived growth factor (PDGF), were sorbed into the hydrogel of "acidic" gelatin with an isoelectric point of 5.0 mainly due to the electrostatic interaction (Ikada and Tabata, 1998). Animal experiments revealed that the hydrogels prepared by chemical crosslinking of the acidic gelatin were degraded in the body (Tabata *et al.*, 1999b). The time profile of *in vivo* hydrogels degradation was in accordance with that of growth factor retention in the hydrogel, irrespective of the hydrogel biodegradability. The biodegradability of hydrogels can be readily regulated by their extent of crosslinking which can be controlled by changing the reaction conditions in hydrogel preparation. For this hydrogel system, the growth factor release is governed mainly by hydrogel degradation. Based on this hydrogel-degradation release mechanism, it is possible to achieve the controlled release of growth factor from any shape of hydrogels, especially even the hydrogel of injectable microsphere shape which has a large surface area/volume ratio (Tabata *et al.*, 1999a). We have succeeded in inducing the regeneration of various tissues and organs by the controlled release of various growth factors with the biological activities remaining as shown in Table 1.

bFGF was originally characterized *in vitro* as a growth factor for fibroblasts and capillary endothelial

Table 1. Regeneration induction of several body tissues and organs by making use of biodegradable hydrogels for the controlled release of bioactive growth factor.

Materials	Growth factor	Animals	Effect	Objective
Acidic gelatin (PI 5.0)	bFGF	Mouse, Rat, and Dog	Angiogenesis	Transplantation of Langerhans islands for diabetes therapy
		Rat	〃	Transplantation of hepatocytes for therapy of enzyme deficiency disease
		Rat	〃	Transplantation of renal epithelial cells
		Rat and Dog	〃	Transplantation of cardiomyocytes
		Rat and Guinea pig	〃	Promoted repairing of skin dermal layer
		Rat and Pig	〃	Treatment of cardiac infarction
		Rabbit	〃	Treatment of lower limb ischemia
		Rat, Dog, and Monkey	Osteogenesis and Angiogenesis	Repairing sternum and connective tissue
		Rat, Rabbit, and Monkey	Osteogenesis	Repairing of skull bone
		Rat, Rabbit, Dog, and Monkey	〃	Repairing of long bone
		Mouse	Adipogenesis	Repairing of breast and soft tissue reconstruction
		Mouse	Angiogenesis and activation of hair follicle tissue	Promotion of hair growth
		Dog	Periodontium repair	Repairing of periodontium
		Dog	Peripheral nerve repair	Nerve repairing
		Dog	Osteogenesis	Repairing of mandiblular bone
	TGF-β1	Rabbit and Monkey	〃	Repairing of skull, long, and mandiblular bone
		Goat	Chondrogenesis	Repairing of tracheal cartilages
	HGF	Mouse	Angiogenesis and activation of hair follicle tissue	Promotion of hair growth
		Rat、Pig	Angiogenesis and inhibition of apoptosis	Treatment of dilated cardiomyopathy
	bFGF/TGF-β1	Rabbit	Osteogenesis	Repairing of skull bone
	CTGF	Rabbit	Chondrogenesis	Repairing of articular cartilage
Basic gelatin (PI 9.0)	BMP-2	Rat, Dog, and Monkey	Osteogenesis	Repairing of skull and mandiblular bone
		Dog	Chondrogenesis	Repairing of tracheal cartilage
Collagen	TGF-β1	Rabbit	Osteogenesis	Repairing of skull bone
	VEGF	Pig	Angiogenesis	Treatment of myocardiac infarction
		Rabbit	〃	Promotion of engraftment of soft tissue grafts
		Rabbit	Osteogenesis	Osteogenesis for spinal fusion
		Mouse	Angionesis and activation of hair follicle tissue	Promotion of hair growth

bFGF:basic fibroblast growth factor TGF-β1: transforming growth factor β1 HGF: hepatocyte growth factor CTGF:connective tissue growth factor
BMP-2:bone morphogenetic protein 2 VEGF:vascular endothelial growth factor

cells and *in vivo* as a potent mitogen and chemoattractant for a wide range of cells. In addition, bFGF is reported to have a variety of biological activities (Rifkin and Moscatelli, 1989) and be effective in enhancing wound healing through induction of angiogenesis and regeneration of various tissues. Recently, the bFGF of human type has been on the Japanese market for remedy of decubitus and skin ulcer from Kaken Pharmaceutical Co. Ltd., Tokyo (Fibrast® spray). When gelatin hydrogels incorporating bFGF were subcutaneously implanted into the mouse back, significant angiogenic effect was observed around the implanted site, in marked contrast to the bFGF solution.

The technology to artificially induce *in vivo* angiogenesis is indispensable for tissue engineering. The two objectives of angiogenesis induction include the angiogenic therapy of ischemic diseases and in advance angiogenesis for cell transplantation. As the former example, when injected into the site of myocardial infarction, gelatin microspheres incorporating bFGF induced regeneration of collateral coronary arteries as well as recovered the motion of myocardium in the ischemic region (Iwakura *et al.*, 2003), whereas neither therapeutic effects were observed by the injection of bFGF solution at the same dose. The injection of this hydrogel system also showed superior angiogenic therapeutic effect for the ischemic leg model of normal and diabetic animals (Nakajima *et al.*, 2004). A human trial of angiogenic therapy for leg ischemia has been started to demonstrate the therapeutic efficacy.

It is no doubt that sufficient supply of nutrients and oxygen to cells transplanted into the body is indispensable for cell survival and the maintenance of biological functions. For successful cell transplantation, poor supply of nutrients and oxygen to the cells transplanted is the large problem comparable to the immunological rejection against the cells. It is a practically promising way to induce in advance angiogenesis throughout the transplanted site of cells by making use of angiogenic growth factors. When transplanted into the tissue where in advance angiogenesis had been induced by injection of gelatin microspheres incorporating bFGF, pancreatic islets encapsulated for immunoisolation, hepatocytes, renal cells, and cardiomyocytes showed a prolonged survival and biological functions(Ogawa *et al.*, 2001; Saito *et al.*, 2003; Sakakibara *et al.*, 2002; Sakurai *et al.*, 2004), while the grafting rate of a bioartificial dermis-epidermis skin construct cultured *in vitro* into a skin defect was enhanced.

When implanted into a bone defect of monkey skulls, the gelatin hydrogel incorporating bFGF promoted bone regeneration at the defect, whereas bFGF-free, empty gelatin hydrogels and the same dose of bFGF in the solution were not effective. How about the physiological integrity of bone tissue newly regenerated? When the bone mineral density and the orientation of hydroxyapatite of bone tissue regenerated were evaluated, they were identical with those of the surrounding healthy bone tissue(Nakano *et al.*, 2002). Bone repairing at the skull defect of rabbits by the controlled release of TGF-β1 from a gelatin hydrogel was observed, in marked contrast to free TGF-β1 even at the higher doses(Tabata *et al.*, 2000). By using another hydrogel incorporating BMP-2, we have succeeded in significantly inducing formation of bone tissue ectopically or orthotopically at the doses lower than application of BMP-2 solution while bone regeneration even in the bone defect of monkey skull at the dose as low as that necessary for the bone regeneration of rabbit and rat skull defects (Yamamoto *et al.*, 2003).

There are some cases in which combination of the controlled release of growth factor with stem cells is effective in achieving tissue regeneration. Application of the combined MSC and gelatin microspheres incorporating TGF-β1 allowed to completely close a defect of rabbit skulls by bone tissue newly formed, in marked contrast to that of either material (Tabata *et al.*, 2000). De novo adipogenesis was successfully induced only by the combinational applications of preadipocytes isolated from human fat tissues, gelatin microspheres incorporating bFGF, and a collagen sponge of scaffold (Kimura *et al.*, 2003).

We have found that a plasmid DNA could be released from a biodegradable hydrogel of cationized gelatin derivative to enhance the level of gene expression as well as prolong the time period of gene expressed (Kushibiki *et al.*, 2003; Kushibiki and Tabata, 2004). When intramuscularly injected into the ischemic leg of rats, the cationized gelatin microspheres incorporating a plasmid DNA of FGF-4 induced angiogenesis to a significantly higher extent than the plasmid DNA solution even at the dose 100 or 1000 times less than the plasmid DNA solution (Kasahara *et al.*, 2003). Another advantage of controlled release system is to induce the regeneration of blood vessels with the physiological maturation which is much higher than that of plasmid DNA solution although the mechanism is unclear at the present. Another example is an efficient strategy to genetically engineer stem cells. The microspheres incorporating plasmid DNA was effective in genetically activating cells and consequently enhancing the efficacy of cell therapy. Cationized microspheres incorporating the plasmid DNA of adrenomedulin were prepared to allow them to internalize into human endothelial progenitor cells. Intracellular controlled release of plasmid DNA enhanced the efficiently of gene transfection to the level higher than that of adenovirus transfection. The cells genetically engineered also functioned well to achieve higher therapeutic efficacy (Nagaya *et al.*, 2003).

Tissue Engineering of Internal Medicine Based on DDS Technology

Presently, there is no effective therapy for chronic fibrosis diseases, such as lung fibrosis, cirrhosis, dilated cardiomyopathy, and chronic nephritis. For these diseases, the injured site of tissue and organ is normally occupied with fibrous tissue of excessive collagen fibers and fibroblasts. It is highly possible that this tissue occupation causes the physical impairment of natural healing process at the disease site. Therefore, if such a fibrotic tissue can be digested by any method to loosen or disappear, it is expected that the disease site is regeneration repaired based on the natural healing potential of the surrounding healthy tissue. It has been demonstrated

that the injection of virus encoding a matrix metaloprotease (MMP) protein suppresses the tissue fibrosis to get better the disease symptoms (Brinckerhoff *et al.*, 2000; Iimuro *et al.*, 2003). The findings strongly indicate the possibility that the fibrous tissue can be histologically improved or repaired due to the potential to induce tissue regeneration which is naturally equipped in the surrounding healthy tissue. It is a new direction of tissue engineering that is this regeneration therapy for chronic fibrosis disease based on the natural potential of regeneration induction. This is defined as internal tissue engineering, because in this disease therapy, induction of tissue regeneration repairing is achieved by the drug treatment strategy of internal medicine. This is different from the conventional surgical tissue engineering where the regeneration repairing is induced at a tissue defect by surgically adding cells, the scaffold, and biological signal molecules or the combination. (Fig.1)

We have demonstrated that the controlled release of a MMP-1 plasmid DNA at the medulla of chronic renal sclerosis induced the histological regeneration of kidney structure, in contrast to the plasmid DNA solution (Aoyama *et al.*, 2003). When gelatin microspheres incorporating hepatocyte growth factor (HGF) was intraperitoneally injected into rats with liver cirrhosis, the liver fibrosis was histologically cured (Oe *et al.*, 2003). However, the injection of HGF solution was not effective at all and the tissue appearance was similar to that of untreated controlled group.

DISCUSSION

Without active use of stem cells with high proliferation and differentiation potentials, today, it has been possible to induce tissue regeneration only by using cell scaffolds and the controlled release system of biological active growth factors. Depending on the type of target tissue or organ and the site, it is necessary to make use of cells, their scaffold, and the barrier membrane. It is no doubt that the tissue engineering technology greatly depends on biomaterials. As for as growth factor and the related gene are used, for the tissue engineering approach, the DDS technology of growth factor and the related gene is required. From the viewpoint of disease therapy based on the natural healing potential of patients themselves, two types of tissue engineering in the surgical and internal medicine manners should be strongly carried out in future.

If a key growth factor is supplied to the target site at the right time for the appropriate period of time and at the right concentration, we believe that the living body system will be naturally directed toward the process of tissue regeneration. Once the right direction is taken, it is highly possible that the intact biological system of the body will start to function, resulting in automatic achievement of tissue regeneration. There is no doubt that whenever growth factors are used *in vivo*, their combination with DDS technology is essential. However, the present technology of controlled release does not always regulate accurately the amount and time length of growth factor release. Therefore, at present, one practical approach is to release key a growth factor necessary to increase the number of precursor, blastic or stem cells *in vivo*. It is practically impossible, however, to control cell differentiation by the release technology of growth factors currently available, since the differentiation is normally regulated by complicated network of growth factor in the time, site, or steric manner.

Regenerative medical therapy, which is a new therapy based on the natural induction potential of tissue

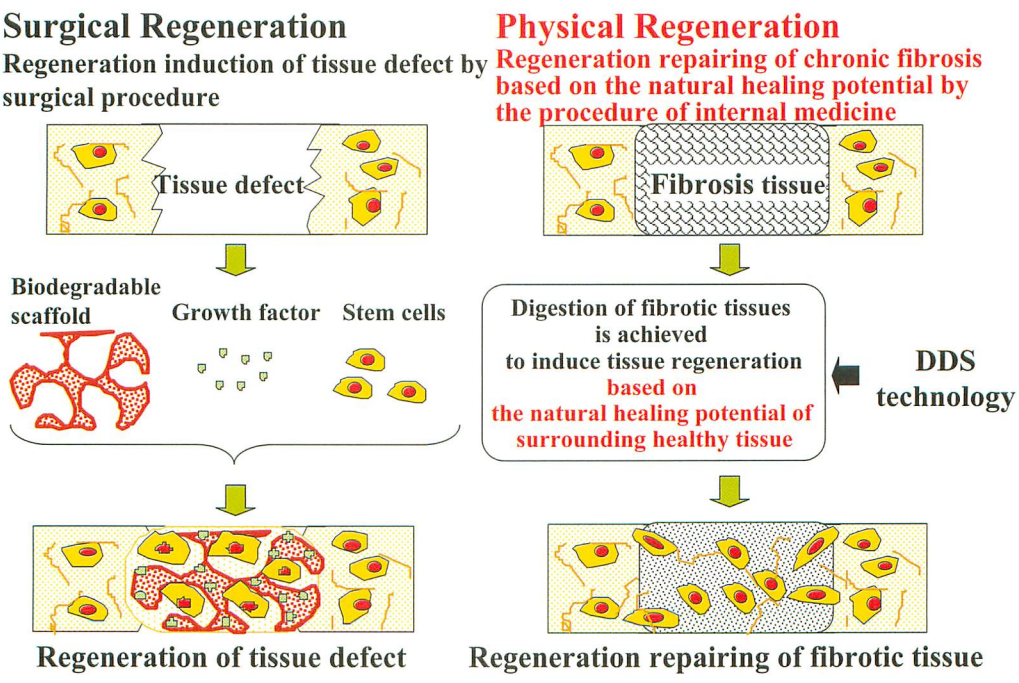

Fig. 1. Treatment of chronic fibrotic diseases by concept of (physical) tissue engineering of internal medicine.

regeneration through cells and tissue engineering, is the third therapy following reconstructive surgery and organ transplantation. To realize the therapy of regenerative medicine by use of tissue engineering technology and methodology, substantial collaborative research between material, pharmaceutical, biological, and clinical scientists is needed. Even though superior stem cells can be obtained to use by development of basic biology and medicine of cells, it is impossible to apply the cells and the related scientific results to medical therapies for patients (regeneration medicine) unless an environment suitable for cell proliferation and differentiation is created and efficiently combined with the cells to use. However, one of the large problems is the absolute shortage of biomaterial researchers of tissue engineering, such as scaffolding and DDS, aiming at tissue regeneration and the biological substitution of organ functions. Such researchers must learn medical, dental, biological, and pharmacological knowledge, in addition to material sciences. It is indispensable to educate the researchers of interdisciplinary field who have engineering background and can also understand basic biology and medicine or clinical medicine necessary for development of tissue engineering. One of the representative interdisciplinary research fields is DDS. The DDS technology is also applicable to create the non-viral vectors to prepare genetically-engineered cells for regenerative medicine. Research and development of non-viral vectors with a high efficiency of gene transfection for stem cells is required. Tissue engineering technology is not only used surgically to the tissue defect for regeneration induction thereat but also applied by making use of methodology of internal medicine to newly develop a therapeutic method for chronic fibrosis diseases.

As tissue engineering is still in its infancy, it will take a long time to become well established although a part of the research projects has been clinically started or come close to the stage of clinical applications. The regenerative medical therapy based on tissue engineering is not a science fiction, but now become real story. Increasing significance of cell scaffolding and DDS technology and methodology in future will help further help progress of tissue engineering. We will be happy if this paper stimulates readers' interest in the idea and research field of tissue engineering.

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Skeletal Development Through the Regulation of Chondrocyte and Osteoblast Differentiation by Runx2

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ABSTRACT

Runx2 is a transcription factor that belongs to Runx family (Runx1, Runx2, and Runx3). Runx2 interacts with many other transcription factors and co-regulators in the transcriptional regulation of its target genes. Cbfb is one of the co-regulators, forms heterodimers with Runx2, and is required for Runx2-dependent transcriptional regulation. Runx2 is essential for the commitment of multipotent mesenchymal cells into the osteoblastic lineage, because Runx2-deficient mice show complete lack of bone formation due to the absence of osteoblasts. Further, Runx2 inhibits adipocyte differentiation, because Runx2-deficient calvarial cells spontaneously differentiate into adipocytes. Overexpression of Runx2 in osteoblasts inhibits osteoblast maturation but decreased the expression of major bone matrix protein genes. Therefore, Runx2 triggers the gene expression of bone matrix proteins, while keeping the osteoblastic cells in an immature stage. Moreover, Runx2 strongly inhibits the transition of osteoblasts into osteocytes. Runx2 and Runx3 double knockout mice showed that Runx2 and Runx3 have redundant functions in chondrocytes, and that they are essential for chondrocyte maturation. Runx2 directly induces Ihh expression and coordinates the proliferation and differentiation of chondrocytes. Therefore, Runx2 regulates bone formation by regulating osteoblast differentiation as well as chondrocyte maturation.

Key words: Runx2, chondrocyte differentiation, osteoblast differentiation, Runx3, Cbfb

The vertebrate skeleton is composed of cartilage and bone. Bone is formed through either intramembranous or endochondral ossification. Osteoblasts directly form intramembranous bones, while chondrocytes first form a cartilaginous skeleton which is then replaced with bone by osteoblasts and osteoclasts through the process of endochondral ossification. These processes are regulated by many factors, and specific transcription factors play essential roles in the differentiation of chondrocytes and osteoblasts. Runx2 plays essential roles in chondrocyte maturation and osteoblast differentiation (Komori 2005). Runx2 belongs to Runx family (Runx1/Cbfa2/Pebp2aB, Runx2/Cbfa1/Pebp2aA and Runx3/Cbfa3/Pebp2aC). All three genes contain a runt domain, which is the DNA-binding domain and is homologous with the Drosophila pair-rule gene runt. Runx1 is essential for hematopoietic stem cell differentiation (Komori and Kishimoto, 1998). Runx3 plays important roles in the growth regulation of gastric epithelial cells and in neurogenesis, and Runx3-deficient ($Runx3^{-/-}$) mice show inflammatory bowel disease and eosinophilic lung inflammation (Li *et al.*, 2002; Inoue *et al.*, 2002; Levanon *et al.*, 2002; Brenner *et al.*, 2004; Fainaru *et al.*, 2004). Cbfb, which has no DNA binding domain, forms heterodimers with Runx proteins. Recently, we found that Cbfb is required for Runx2-dependent osteoblast differentiation and chondrocyte maturation (Yoshida *et al.*, 2002). Mutations in RUNX genes have been linked to human diseases. Aberrant translocations of RUNX1 are responsible for acute myeloid leukemia. Heterozygous mutations in the RUNX2 gene cause cleidocranial dysplasia, a pathological condition characterized by hypoplastic clavicles, opened fontanelles, supernumerary teeth and short stature. RUNX3 misfunction has been shown to be related to gastric cancer (Komori *et al.*, 2002).

Runx2 and chondrocyte differentiation

In $Runx2^{-/-}$ mice, whose entire skeleton is composed of cartilage, chondrocyte differentiation is severely disturbed throughout most of the skeleton (Inada *et al.*, 1999). Runx2 promotes chondrocyte maturation (Enomoto *et al.*, 2000; Ueta *et al.*, 2001), overexpression of dn-Runx2 inhibits chondrocyte maturation (Ueta *et al.*, 2001). Interestingly, most of the joints are fused in transgenic mice that overexpress Runx2, and the majority of chondrocytes in dn-Runx2 transgenic mice retain a marker of chondrocytes in the permanent cartilage (Ueta *et al.*, 2001). Therefore, Runx2 is required for chondrocyte maturation and is involved in determining whether chondrocytes acquire a permanent or transient phenotype (Fig. 1).

Although chondrocyte maturation is inhibited in $Runx2^{-/-}$ mice, chondrocyte maturation to the terminal stage eventually occurs in the restricted skeleton, indicating that other factors are also involved in chondrocyte maturation. All Runx genes are expressed in chondrocytes (Levanon *et al.*, 2001; Stricker *et al.*, 2002), and transgenic mice that overexpress dn-Runx2 under the control of the Col2a1 promoter/enhancer, in which the functions of all Runx proteins in chondrocytes are inhibited, show more severe inhibition of chondrocyte maturation than that seen in $Runx2^{-/-}$ mice (Uete *et al.*, 2001). These find-

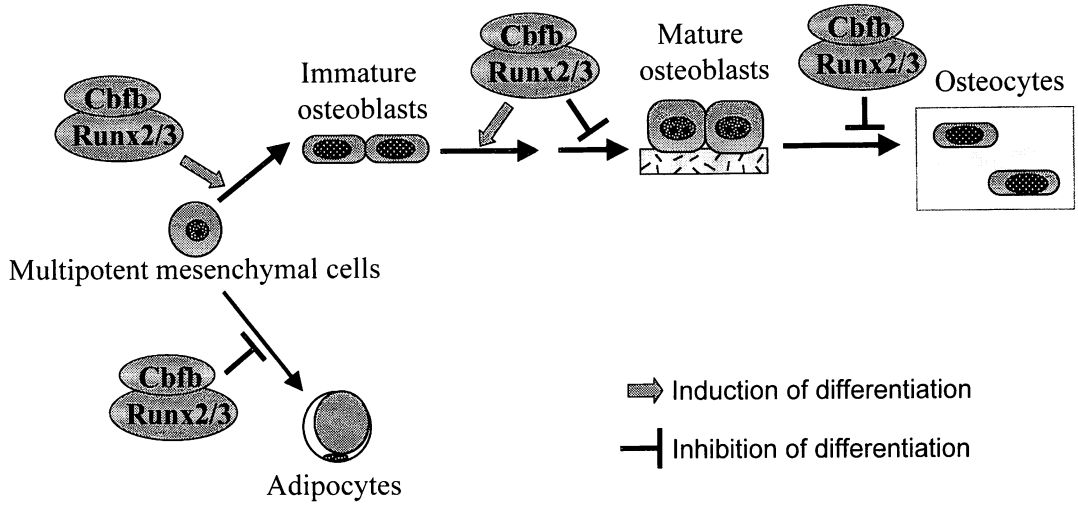

Fig. 1 Regulation of chondrocyte maturation and proliferation by Runx2 and Runx3.
Runx2 and Runx3 are essential for chondrocyte maturation and inhibit chondrocytes from acquiring the phenotype of permanent cartilage. Cbfb is required for the Runx2-dependent chondrocyte maturation. Runx2 directly induces Ihh expression, which enhances chondrocyte proliferation and inhibits chondrocyte maturation through PTHrP. With regard to Runx3, either Runx3 induces Ihh expression indirectly or additional factors are required for Ihh induction by Runx3.

ings indicate that Runx1 and/or Runx3 is involved in chondrocyte maturation. Runx1$^{-/-}$ mice are embryonic lethal at E12.5 due to the absence of hematopoiesis. Runx3$^{-/-}$ mice die after birth probably due to the abnormality in gastric epithelial cells. We tried to find the function of Runx1 and Runx3 in chondrocyte differentiation by mating Runx1$^{+/-}$ mice with Runx2$^{+/-}$ mice or by mating Runx3$^{+/-}$ mice with Runx2$^{+/-}$ mice. Runx1$^{+/-}$Runx2$^{-/-}$ mice showed a similar disturbance of chondrocyte maturation as compared with Runx2$^{-/-}$ mice, indicating that the contribution of Runx1 to chondrocyte maturation, if any, seems to be limited. In contrast, Runx2$^{-/-}$Runx3$^{+/-}$ mice showed that the chondrocyte maturation is more severely inhibited than Runx2$^{-/-}$ mice, indicating that Runx3 is also involved in chondroxyte maturation. We finally got Runx2$^{-/-}$Runx3$^{-/-}$ mice in which chondrocyte maturation in the entire skeleton was completely inhibited. It demonstrates that Runx2 and Runx3 are essential for chondrocyte maturation (Yoshida *et al.*, 2004) (Fig. 1).

Interestingly, the length of the limbs of Runx2$^{-/-}$ mice was reduced and further reduced in Runx2$^{-/-}$3$^{-/-}$ mice. The reduced chondrocyte proliferation was the cause of shortened limbs in the Runx2$^{-/-}$ mice, and the small volume of the cells in the diaphyses was the cause of the further reduction of limb length in Runx2$^{-/-}$Runx3$^{-/-}$ mice. Runx2 directly induces the expression of Ihh, which is a positive regulator of chondrocyte proliferation (Yoshida *et al.*, 2004). As Ihh is also a negative regulator of chondrocyte maturation (Vortkamp *et al.*, 1996), Runx2 and Runx3 coordinate chondrocyte maturation and proliferation and regulate limb growth through the induction of Ihh (Fig. 1). Further, Pthlh inhibits Runx2 expression through the PKA signaling pathway (Iwamoto *et al.*, 2003; Li *et al.*, 2004). As Ihh induces Pthlh expression (Vortkamp *et al.*, 1996), Runx2 expression is also regulated by a negative feedback loop (Fig. 1).

Runx2 and osteoblast differentiation

Runx2$^{-/-}$ mice completely lack bone formation due to the absence of osteoblasts, demonstrating that Runx2 is essential for osteoblast differentiation (Komori *et al.*, 1997) (Fig. 2). Runx2$^{-/-}$ calvarial cells fail to differentiate into osteoblasts both in vitro and in vivo, even in the presence of BMP-2. However, in vitro studies showed that Runx2$^{-/-}$ calvarial cells spontaneously differentiate into adipocytes, and they differentiate into chondrocytes in the presence of BMP-2. Therefore, Runx2$^{-/-}$ mesenchymal cells completely lack the ability to differentiate into osteoblasts, but retain the capacity to differentiate into adipocytes and chondrocytes (Kobayashi *et al.*, 2000). Furthermore, Runx2$^{-/-}$ chondrocytes also spontaneously differentiated into adipocytes, and introduction of Runx2 prevented adipocyte differentiation, indicating that Runx2 inhibits adipogenesis (Enomoto *et al.*, 2004).

Many recent in vitro studies demonstrated that Runx2 is a positive regulator that up-regulates the expression of or activates the promoters of genes related to bone matrix proteins including Col1a1, Col1a2, osteopontin, bone sialoprotein (BSP), osteocalcin, fibronectin, MMP13, and OPG (Komori 2002). Further, the overexpression of dominant negative (dn)-Runx2 under the control of the osteocalcin promoter, which directs reporter gene expression to mature osteoblasts, results in severe osteopenia due to drastic reductions in the expression of genes encoding the main bone matrix proteins including Col1a1, Col1a2, osteopontin, BSP, and osteocalcin (Ducy *et al.*, 1999). However, transgenic mice that overexpressed Runx2 under the control of a 2.3-kb mouse Col1a1 promoter, which specifically directs reporter gene expression to osteoblasts, showed osteopenia with multiple fractures (Liu *et al.*, 2001). Most of the osteoblasts of these mice exhibited less mature phenotypes, and the numbers of

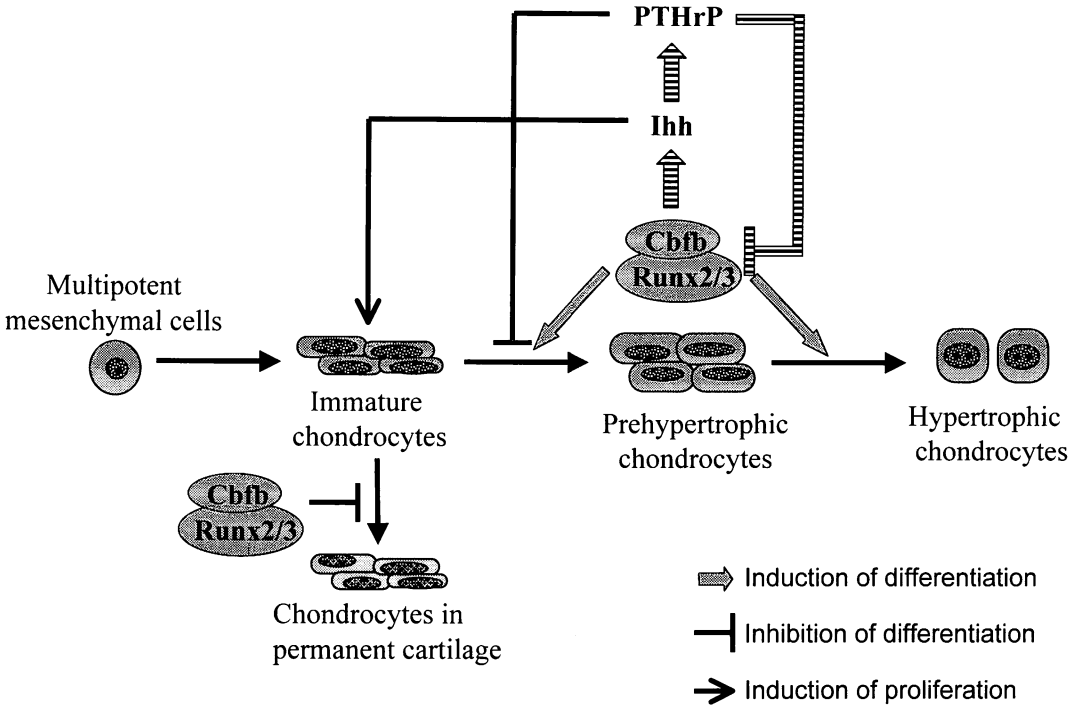

Fig. 2 Regulation of osteoblast differentiation by Runx2
Runx2 induces mesenchymal condensation, inhibits their differentiation into adipocytes, induces the expression of osteoblastic markers, and allows the mesenchymal progenitor cells to differentiate into the osteoblastic lineage. Cbfb is required for the Runx2-dependent osteoblast differentiation. However, Runx2 inhibits osteoblast differentiation at a late stage and severely inhibits the transition of osteoblasts into osteocytes.

terminally differentiated osteoblasts, which strongly express osteocalcin, and osteocytes were greatly diminished. As a result, in the osteoblasts of these mice, the expression of Col1a1, alkaline phosphatase, osteocalcin, and MMP13, all of which normally increase during osteoblast maturation, were reduced. Further, osteocytes were severely reduced in these mice. These findings indicate that Runx2 inhibits osteoblast differentiation at a late stage (Fig. 2). Our recent studies on dn-Runx2 transgenic mice with high transgene expression under the control of the same 2.3-kb mouse Col1a1 promoter showed that major bone matrix protein gene expression was not significantly affected by the suppression of Runx2 function in mature osteoblasts (manuscript in preparation). Therefore, there is a controversy in the function of Runx2 in the regulation of bone matrix protein genes. In the dn-Runx2 transgenic mice generated by Ducy *et al.* (1999), the level of transgene expression was very weak and was similar to the level of endogenous Runx2 expression, and expression of the transgene was transiently detected only at 2-4 weeks of age. To explain the drastic phenotype caused by the weak expression of dn-Runx2, they showed that dn-Runx2 binds the Runx2 binding sites at unusually strong affinity. However, we compared the affinity to the Runx2 consensus oligonucleotides between Runx2 and dn-Runx2 and found that dn-Runx2 binds Runx2 binding sites at the similar affinity with native Runx2, indicating that high expression of dn-Runx2 is required for the dominant negative effect (manuscript in preparation). These findings along with the in vitro data, indicate that Runx2 induces the expression of major bone matrix protein genes in osteoblast progenitors, allowing the cells to acquire the osteoblastic phenotype while keeping the osteoblastic cells in an immature stage (Fig. 2).

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Transcriptional Regulation of Osteoblast Differentiation and Function

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ABSTRACT

Osterix (Osx) is a zinc finger containing transcription factor that is highly specific to osteoblasts *in vivo*. To uncover physiological roles of Osx during mouse development, the *Osx* gene is disrupted by homologous recombination in ES cells. *Osx*-null mutants show a complete lack of bone formation, indicating that Osx is necessary for osteoblast differentiation and bone formation. *Runx2/Cbfa1*, the other crucial factor for bone formation is expressed in *Osx*-null mutants, whereas *Osx* is not expressed in *Runx2/Cbfa1*-null mutants, indicating that Osx acts downstream to Runx2/Cbfa1. In *Osx*-null mutants, *Runx2/Cbfa1* expressing cells express typical chondrocyte molecular markers, suggesting that chondrocytes and osteoblasts derive from common progenitor cells. In this paper, recent progress on identification of common progenitors of chondrocytes and osteoblasts during mouse embryogenesis, and on further analysis of a role of Osx in postnatal bone metabolism will be discussed.

INTRODUCTION

Bone formation occurs through two distinct developmental processes: intramembranous ossification and endochondral ossification. In intramembranous ossification, osteoblasts differentiate directly from cells in mesenchymal condensations to form a number of craniofacial bones and the medial part of the clavicle, whereas in endochondral ossification, an intermediate step is involved in which chondrocytes from cartilaginous templates to prefigure skeletal elements and play a major role in the development of long bones of the appendicular skeleton and the lateral part of the clavicle. In both cases, osteoblasts play a central role in the production of characteristic extracellular matrix molecules and in the mineralization of the bone matrix. Osteoblasts also participate in regulating the differentiation of bone-resorbing osteoclasts to establish a process called bone remodeling.

Specific transcription factors regulate the differentiation pathways of chondrocytes, osteoblasts and osteoclasts. Sox9, a transcription factor with a high-mobility group DNA-binding domain, activates chondrocyte-specific marker genes, such as *Col2a1*, *Col11a2*. Sox9 is expressed in cells in mesenchymal condensations and chondrocytes. Inactivation of *Sox9* in limb buds before mesenchymal condensations form results in the complete absence of cartilage and bone formation (1). Moreover, inactivation of *Sox9* during or after the formation of mesenchymal condensations results in a very severe chondrodysplasia, which is characterized by an almost complete absence of cartilage in the endochondral skeleton. Hence, Sox9 is required at two distinct steps in chondrogenesis.

Runx2/Cbfa1 is a member of the Runt-domain family of transcription factors, which form heterodimers with a ubiquitous partner molecule Cbfb. Runx2/Cbfa1 activates the *Osteocalcin* (*Oc*) and *Col1a1* genes *in vitro* (2). *Runx2/Cbfa1*-null mice are characterized by a block in osteoblast differentiation and the absence of both endochondral and intramembranous bone formation, indicating that Runx2/Cbfa1 has an essential role in the differentiation of mesenchymal progenitors into osteoblasts (3, 4).

We identified a second transcription factor required for osteoblast differentiation and bone formation, called Osterix (Osx) (5), that is specifically expressed in osteoblasts of all bones. Osx contains a DNA-binding domain consisting of three C2H2-type zinc finger motifs that share a high degree of sequence identity with similar motifs in Spl, Sp3, and Sp4. In addition, Osx contains a proline- and serine-rich transactivation domain and activates the *Oc* and *Col1a1* genes *in vitro*. In *Osx*-null mutant mice, neither endochondral nor intramembranous bone formation occurs (Fig.1). The mesenchymal cells in *Osx*-

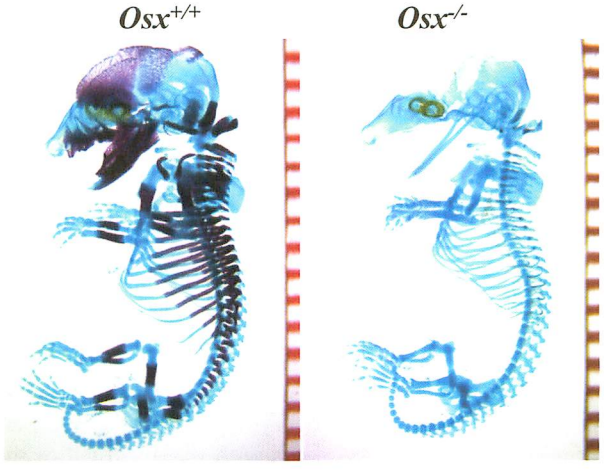

Fig. 1. Skeletal phenotype of *Osx*-mutant mice
Skeletons of E15.5 mice are stained by alcian blue followed by alizarin red.

null mutant mice do not deposit bone matrix, and cells in the periosteum and the condensed mesenchyme of membranous skeletal elements cannot differentiate into osteoblasts, although these cells express *Runx2/Cbfa1*. Arrest in osteoblast differentiation in *Osx*-null mutant mice occurs at a later step than that in *Runx2/Cbfa1*-null mice. Indeed, expression of *Osx* requires *Runx2/Cbfa1*, indicating that Osx acts downstream of Runx2/Cbfa1. *Runx2/Cbfa1*-expressing *Osx*-null cells expressed typical chondrocyte markers including *Sox9*, indicating that *Runx2/Cbfa1*-expressing *Osx*-null cells, which could not differentiate into osteoblasts, acquire a cell fate characterized by the expression of a series of genes that are typical of chondrocytes. This observation suggests that Runx2/Cbfa1-expressing cells are at least bipotential for further differentiation, and osteoblasts and chondrocytes share common progenitor cells in mouse development.

Among transcription factors involved in osteoclastogenesis, NFATc1, a transcription factor that regulates distinct developmental and metabolic events, is necessary for osteoclast differentiation (6). *NFATc1*-deficient embryonic stem cells fail to differentiate into osteoclasts in response to a key cytokine for osteoclastogenesis, receptor activator of NF-B ligand (RANKL). Ectopic expression of NFATc1 causes precursor cells to undergo differentiation without RANKL signaling, indicating that NFATc1 plays a central role in osteoclast differentiation (7). However, roles of NFAT factors in the regulation of bone metabolism remain unclear.

A bone remodeling process maintains homeostasis of the postnatal skeletal system. The bone remodeling, in which bone is constantly renewed by the balanced action of osteoblastic bone formation and osteoclastic bone resorption, is a dynamic process occuring throughout an organism's life. Since osteoblasts play a central role in the process, Osx, the critical transcription factor for bone formation, would participate in regulation of the process. However, because of the perinatal lethality of Osx-null mice, roles of Osx in postnatal bone metabolism are unclear. To understand roles of Osx in both defining osteoblast lineages and in postnatal bone metabolism, we generated mice carrying a floxed *Osx* allele to inactivate Osx in certain cell types and at certain developmental stages during mouse embryogenesis. In addition, we analysed Osx functions in mice with osteoporotic bone phenotypes after administration of an immunosuppressant FK506.

MATERIALS AND METHODS

Analysis of bone phenotype in FK506 administered mice.

4-week-old C57BL/6 male mice were treated with FK506 at a dose of 0.3 mg/kg/d for 7 d consecutively. We injected mice with calcein at an interval of 144 h and subjected them to histological, histomorphometric and microradiographic examinations. All mice were born and maintained under specific pathogen-free conditions. We performed all animal experiments with the approval of the Animal Study Committee of Tokyo Medical and Dental University, and conformed to recognized guidelines and laws.

Luciferase reporter assay.

The reporter plasmid, pGL2.3 Col1a1-luc, was constructed by inserting a 2.3 kbp fragment of the *Col1a1* promoter into a pGL3-basic vector (Promega). The Runx2-responsive *Bglap1* promoter plasmid (1050 OC-luc), and expression vectors of Osx, Runx2, and NFATc1 were described previously. The reporter plasmid containing GAL4 binding sites and the expression vector of Osx fused with the GAL4-DNA binding domain (GAL4-Osx: pSGOSX(27-428)) were described previously. We performed transfection into 293T cells and measurement of luciferase activity as described using dual-luciferase reporter assay system (Promega).

Immunoprecipitation and electrophoretic mobility shift assay.

We constructed the expression vector of FLAG-hemagglutinin-tagged NFATc1 by inserting tandem FLAG and hemagglutinin sequences into the amino terminal of NFATc1. Truncated mutants of Osx fused with the GAL4-DNA binding domain (GAL4-Osx (27-192), GAL4-Osx (192-291) and GAL4-Osx (293-428)) have been described previously. We harvested cell extracts from 293T cells 48 h after transfection using lysis buffer (20 mM Tris pH 8.0, 5 mM MgCl2, 0.1 M KCl, 10% glycerol, 0.1% Tween 20, 0.1% NP-40, 0.5 mM APMSF, 10 g/ml aprotinin) and subjected them to immunoprecipitation assay as described. For electrophoretic mobility shift assay, we harvested cell lysates from COS-7 cells 48 h after transfection, and analyzed 15 g of protein with the [γ-32PP]-ATP-labeled oligonucleotide containing a consensus Sp1 binding site.

RESULTS

Osteoblasts and chondrocytes derive from common progenitor cells.

To analyze functions of Osx, mice carrying a floxed *Osx* allele were generated (7). An Osx genomic clone was isolated from a mouse129/Sv genomic DNA library. To construct a targeting vector to introduce *loxP* sites, exon 2 was flanked by two *loxP* sites; the first was in intron 1 and the second in the 3'-flanking region (Fig. 2). An *FRT-flanked PGK-neo-bpA* cassette and an *IRES-EGFP-pA* cassette, which contained a splicing acceptor signal at the 5' end, were inserted into 3' region to the second *loxP* site. The target vector was introduced into 129SvEv AB1 ES cells, and G418-resistant ES cell clones were screened by Southern blot analysis of EcoRV-digested genomic DNA with a 3' probe external to the region of homology. Homologous recombination was verified by using a 5' probe external to the region of homology. Mouse chimeras were generated by C57BL/6 host blastocyst injection of mutant ES cell clones, and chimeras obtained were bred with C57BL/6 mice to generate floxed *Osx* heterozygous mice. The *FRT-flanked PGK-neo-bpA* cassette was removed by Flp-mediated recombination with *ACTFlpe* transgenic mice.

To delete the floxed *Osx* allele in a tissue-spscific or developmental stage- specific manner, mice carrying the Cre recombinase gene inserted into the 3'-UTR of the

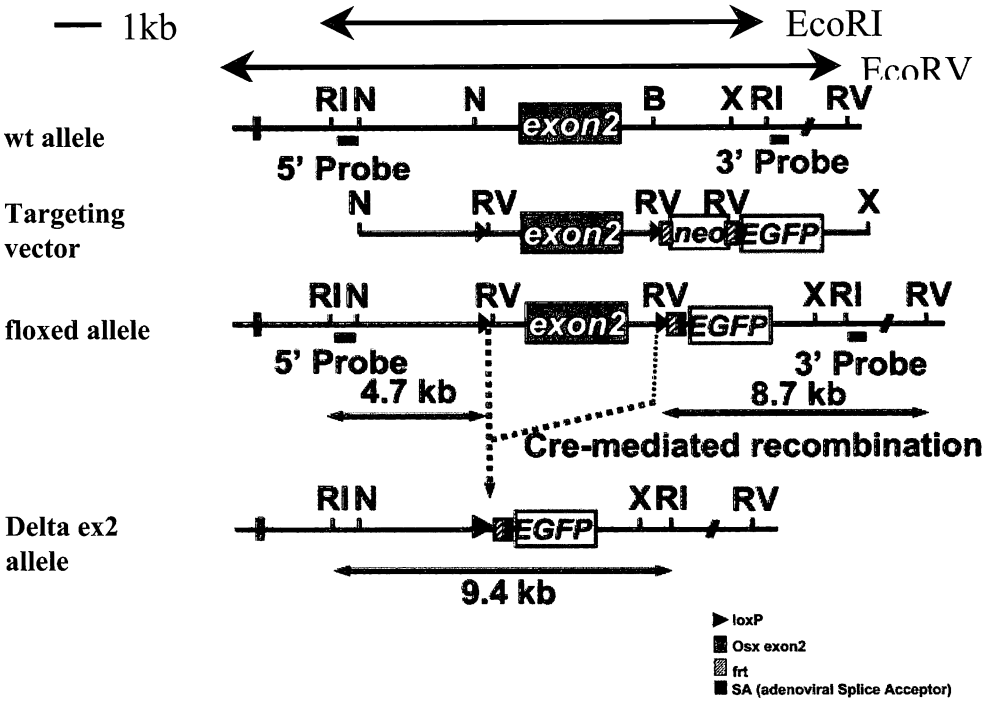

Fig. 2. Generation of a *Osx* floxed allele.
Structure of the genomic *Osx* locus, targeting vector, and mutated allele after homologous recombination. Exons are depicted as filled boxes. An *FRT-flanked PGK-neo-bpA* cassette and an *IRES-EGFP-pA* cassette are depicted as open boxes.

Sox9 were generated (7). The mice were crossed with the Rosa26 reporter (R26R) strain to determine expression pattern of the Cre recombenase genes during mice development. Expression of Cre recombinase in R26R mice activates constitutive *lacZ* gene expression providing a marker of cells that have expressed *Sox9* at any time during development. In developing limb, Sox9 expressing cells prefigured cartilage primordia and are located in perichondrium. In E17.0 limb buds, all chondrocytes as well as perichondrial, periosteal, and osteogenic cells expressed *LacZ*, suggesting that *Sox9*-expressing mesenchymal cells provide both chondrogenic and osteogenic cell lineages. We then crossed this Cre line with mice carrying the floxed *Osx* allele to inactivate the *Osx* gene in *Sox9*-expressing cells. The floxed allele was efficiently deleted in conditional *Osx* null mutant mice showing a lack of osteoblast differentiation and bone formation. Our results indicated that common progenitors expressing Sox9 defines chondrogenic and osteogenic cell lineages.

Osx regulates bone formation through interaction with NFAT.

To ask Osx functions in postnatal mice, we analysed mice treated with FK506, immunosuppressant and a calcineurin inhibitor (8). NFATc1 is a crucial transcription factor for osteoclast differentiation and calcineurin inhibitors inhibit osteoclastogenesis *in vitro* by suppressing RANKL-dependent NFATc1 induction. It is expected that the *in vivo* administration of calcineurin inhibitors would suppress osteoclastic bone resorption, resulting in an increase of bone mass. FK506-treated mice showed a significant decrease in osteoclast number and markers of osteoclastic bone resorption. However, trabecular bone volume was markedly reduced. Evaluation of osteoblastic bone formation markers revealed that both the osteoblast number and osteoblastic bone formation were severely impaired in FK506-treated mice. These results suggest that the low bone mass is caused by the suppressive effect of FK506 on osteoblastic bone formation.

To further explore the molecular mechanism by which NFAT regulates osteoblast differentiation and function, we investigated the effect of NFAT on the transcriptional activity of Osx and Runx2. A reporter plasmid driven by the *Col1a1* promoter (2.3 Col1a1-luc) is activated by Osx, and the coexpression of NFAT with Osx enhanced the activity of Osx. But coerexpression of NFAT has only a minimal effect on the activation of the *Bglap1* promoter by Runx2. To determine whether the direct interaction between NFAT and Osx has a role in the regulation of Osx-mediated transactivation, we used a fusion protein of GAL4 DNA binding domain and Osx (GAL4-Osx). A luciferase reporter plasmid containing 5x GAL4 binding sites was activated by GAL4-Osx. Cotransfection of NFATc1 with GAL4-Osx, but not GAL4, resulted in a synergistic activation of this activity, which was completely suppressed by FK506. These results show that NFAT stimulates the transcriptional activity of Osx by interaction with Osx, not by binding to the promoter. To provide further evidence that the ability of NFATc1 to enhance the Osx activity is dependent on the direct interaction between NFATc1 and Osx, we used the truncated mutant of Osx. Osx binds to NFATc1 through the amino acid residues 192-291. The mutant Osx, Osx(27-192), cannot interact with NFATc1 but still contains the transactivation domain. NFATc1 stimulated

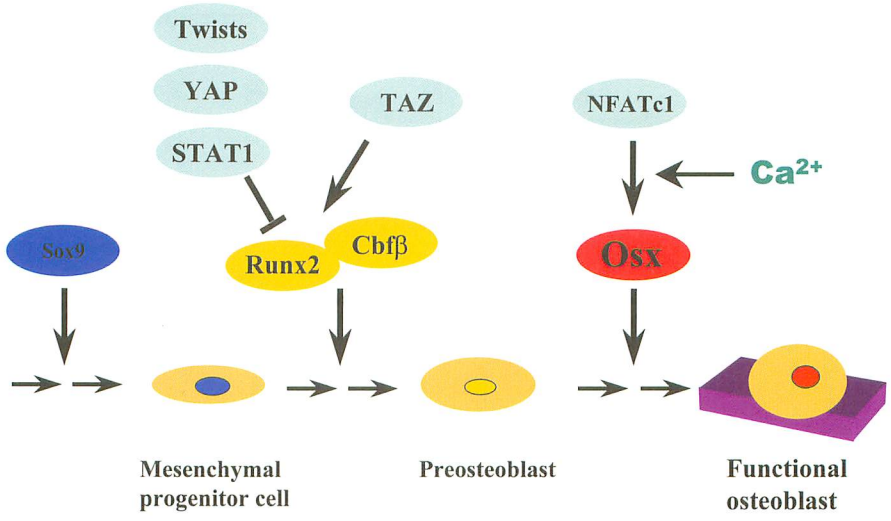

Fig. 3. Model of cellular differentiation of mesenchymal cell lineages into oateoblasts.

the transcriptional activity induced by GAL4-Osx, but had no stimulatory effect on that induced by the mutant Osx GAL4-Osx(27-192), indicating that the effect of NFATc1 on Osx activity depends on the interaction between the two proteins.

DISCUSSION

Bone formation occurs throughout life. Osteoblasts play a central role in production of bone matrix and matrix mineralization during embryogenesis and postnatal life. In the bone remodeling process regulating adulthood bone metabolism, bone formation by osteoblasts must be regulated tightly to replace the bone resorbed by the osteoclasts to maintain a constant bone mass. Thus, any qualitative or quantitative defect of osteoblasts could result in bone fragility and/or osteoporosis. These requirements underscore the need to understand the molecular basis of bone formation by the osteoblasts and to identify molecule(s) regulating this function.

Osx is necessary for osteoblast differentiation and bone formation during embryogenesis. However, the perinatal lethality of Osx-null mice remains roles of Osx in postnatal bone metabolism unclear. To understand roles of Osx in both defining osteoblast lineages and in postnatal bone metabolism, we generated mice carrying the floxed *Osx* allele to inactivate Osx. The *Osx* floxed allele was efficiently inactivated by the Cre recombinase whose expression was regulated by the *Sox9* promoter. The conditional mutant mice showed the absence of osteoblast differentiation and bone formation, indicating that osteoblasts are derived from cells that express Sox9 at any steps of cell differentation during embryogenesis (Fig. 3). This result is consistent with the results that Osx-null, Runx2-expressing cells are capable to express typical chondrocyte markers. The Osx-floxed mice will allow us to analyse roles of Osx in postnatal bone metabolism.

To demonstrate a function of Osx in differentiated osteoblasts, we took advantage of the FK506-administered mice that showed paradoxical reduction of bone mass. The results of this study demonstrate that Osx controls bone formation by differentiated osteoblasts in postnatal mice through at least its interaction with NFATc1. Runx2/*Cbfa1* is a critical transcription factor controlling osteoblast differentiation and the expression of the major osteoblast-specific and osteoblast-enriched genes. NFATc1 did not interact with Runx2/Cbfa1 nor showed cooperative activation of Runx2/Cbfa1 target promoter, suggesting that Runx2/Cbfa1 and Osx exert their effects on postnatal bones through distinct mechanisms. Since NFATc1 activated the Osx target gene in the absence of Osx, and Osx activated the Osx target gene in the absence of NFATc1, each factor exerts its unique function in osteoblast differentiation (Fig. 3).

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Application of Mesenchymal Stem Cells (MSC) to Regenerative Dentistry and Identification of Molecular Markers for MSC

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ABSTRACT

We characterized human bone marrow-derived mesenchymal stem cells (MSC) by identification of molecular markers and used these markers in clinical studies for treatment of periodontal disease: Auto-transplantation of MSC into periodontal defects enhanced regeneration of cementum, periodontal ligament and alveolar bone. Stem cell therapy could be a new frontier in dentistry.

Stem Cell Therapy May Induce Revolution of Dentistry

A newt has abundant stem cells, and its tail regenerates easily, but human tissues regenerate poorly, perhaps because human beings have fewer stem cells. If this is indeed the case, transplant of stem cells expanded ex vivo could induce regeneration of human tissues: This cell therapy would mean a broad new option for dentistry and medicine. With this optimistic view, we started studies on stem cells in 2000. At that time, the Japanese government encouraged university professors to set up venture companies on campus. Since regenerative dentistry seemed to be a promising field, I set up a venture company - Two Cells Inc. - in 2003. But which stem cells would be the most promising transplantable cells? There are many candidate stem cells, including embryonic stem cells (ES cells), hematopoietic stem cells, and mesenchymal stem cells (MSC).

Comparison of MSC and ES Cells

The human body contains 75 trillion (7.5×10^{13}) cells of ~ 200 different lineages. ES cells can differentiate into all these cells, and proliferate infinitely in vitro, so many researchers are using ES cells to explore principles of the stem-cell system and to develop stem-cell therapy. We decided to use bone marrow-derived MSC.

Bone marrow MSC can differentiate into bone, cartilage, muscle, blood vessels and nerves in culture, or by transplantation into tissue: They may be useful for treatment of periodontal diseases, osteoporosis, bone fracture, osteoarthritis, myocardiac infarction, brain infarction, and degenerative nerve diseases. In addition, MSC can differentiate into hepatocytes, which shows their remakable plasticity (Sato et al., 2005). Furthermore, MSC support hematopoiesis, and suppress graft versus host disease. In contrast, ES cells induce graft versus host disease, and may form teratomas after transplantation. The isolation of ES cells from patients is impossible because ES cells are absent in the adult body, whereas MSC can easily be isolated from patients' bone marrow. Unlike ES cells, MSC do not present ethical dilemmas, and there is no immunological rejection. Accordingly, transplantation of MSC - but not ES cells - has already been used for treatment of periodontal disease, osteoarthritis, bone diseases, skin ulcer, and myocardial infarction, etc., in Japan and other countries.

Super-expansion Method for MSC

At least 10^7 to 10^9 MSC are required for cell therapy, but adult bone marrow contains only a small number of MSC (<0.01%). The expansion of MSC in culture is therefore prerequisite for regenerative medicine, but proliferation activity of MSC has proved poor in conventional cultures. To combat this problem, we used fibroblast growth factor-2 (FGF-2) or extracellular matrix (ECM)-coated dishes.

In conventional cultures, bone marrow cells were seeded on plastic tissue culture dishes, and adherent cells were incubated with DMEM medium containing 10% fetal bovine serum. These cells underwent sequential passages, and then the cells were incubated with osteogenic-induction medium, chondrogenic-induction medium or adipogenic induction medium for 21-28 days. However, these MSC soon lost proliferation and differentiation potentials in vitro.

With our method, FGF-2 was included in the culture medium, or MSC were seeded on basement membrane-like ECM-coated dishes. FGF-2 or ECM stimulated proliferation of MSC and maintained the differentiation potential throughout many mitotic divisions (Tsutsumi et

al., 2000; Matsubara et al., 2004): After incubation with the differentiation-induction medium, almost all cells grown with FGF-2 or on ECM-coated dishes developed into osteoblasts, chondrocytes or adipocytes (Fig. 1). Using this super-expansion method, the large number of MSC required for cell therapy can thus easily be prepared from a small volume of marrow aspirates (0.5-2 ml).

In vivo stem cells bind to certain extracellular matrices or special cells - "Niche" - that stabilize their undifferentiated state and their self-renewal capacity. In addition, "Niche" may protect stem cells from apoptosis during microenvironmental changes, since stem cells need to regenerate injured tissues that have survived. Basement membrane ECM is required for proliferation and maintenance of the undifferentiated state of some stem cells, such as keratinocyte stem cells and muscle satellite cells in vivo. We found that basement membrane ECM is also useful for maintenance of self-renewal capability and the multi-lineage differentiation potential of MSC in vitro (Matsubara et al., 2004). When tissue is injured, ECM is also injured, and injured EMC often do not support self-renewal of stem cells. In such case, FGF-2 and related growth factors are released from heparan-sulfate proteoglycan of ECM and stimulate proliferation of stem cells. Either ECM or FGF is used for maintenance of stem cells, depending upon, respectively, the absence or presence of injury/inflammation. Accordingly, FGF-2 did not further increase the effect of basement membrane ECM on the proliferation of MSC (Matsubara et al., 2004). In any case, the super-expansion method is a reasonable, powerful and reliable method for the expansion of MSC, and FGF or ECM may be essential for proliferation of MSC both in vitro and in vivo.

The effect of exogenous FGF-2 on MSC proliferation was weaker with medium containing fetal bovine serum than with medium containing human serum, perhaps because fetal bovine serum contains FGF-like growth factors at higher concentrations. In medium with 10% human serum, MSC underwent proliferation for a few generations, but addition of FGF-2 markedly extended the life-span of MSC even in medium containing human serum (Matsubara et al., 2004). This is a considerable merit, since doctors prefer to use auto-serum isolated from patients.

Cell Therapy for Periodontal Disease

We examined whether auto-transplant of MSC could promote regeneration of periodontal tissues in a dog model. Bone marrow MSC were isolated from beagle dogs and expanded in vitro. The expanded MSC were mixed with 2% type I collagen at various cell concentrations and transplanted into Class III defects. Collagen gel alone was implanted into the defects as a control. After transplantation, the gums were sutured (Kawaguchi et al., 2004, 2005).

In the control group, without cells, regeneration was poor. However, in the MSC group, new formation of cementum and bone, and adequate width of periodontal ligament were observed. Furthermore, the new cementum contained Sharpey's fibers. Using GFP-labeled MSC, we confirmed that the newly formed bone and cementum, as well as the ligament, were derived from transplanted MSC (unpublished data). This transplantation of MSC revealed that differentiation into cementblasts occurs in an earlier stage than does differentiation into osteoblasts: Perhaps adhesion of MSC on the surface of denuded dentine enhanced cementblast differentiation, and the absence of a calcium-phosphate scaffold (adherent surface) in the future alveolar bone area delayed osteoblast differentiation.

On the basis of these animal studies, we commenced MSC therapy for periodontal diseases at Hiroshima University Hospital in 2004. We used individual

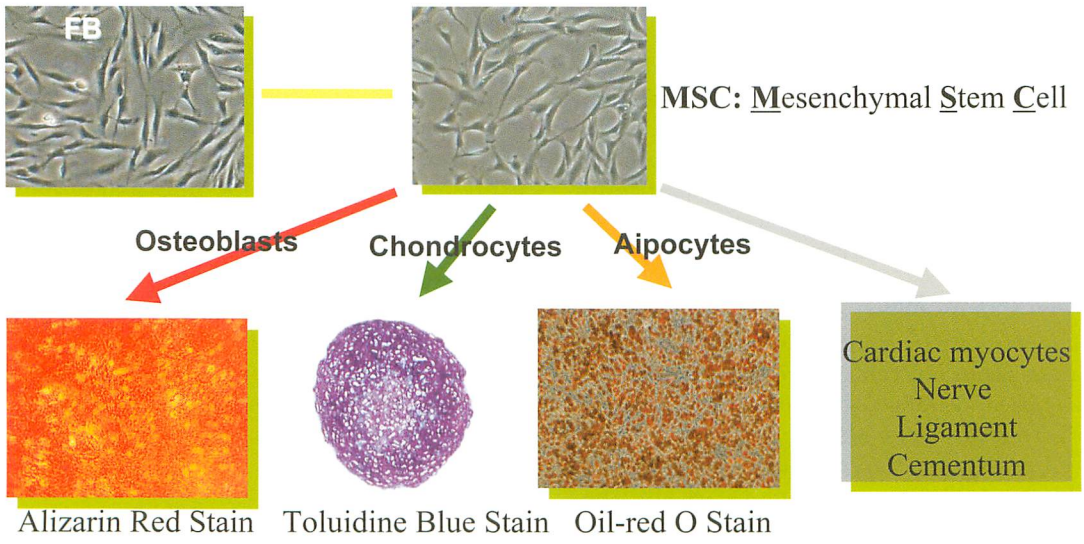

Fig. 1 Multi-lineage differentiation potentials of MSC expanded using the super-expansion method. FGF-2 was added to MSC cultures, or MSC were seeded and maintained on ECM-coated dishes. These MSC retained osteogenic, adipogenic and chondrogenic potentials throughout many mitotic divisions.

patients' serum to expand MSC in culture, because bovine serum may contain BSE prion and unknown pathogens. To isolate human serum from patients safely, we - together with stuffs of JMS Inc and Two Cells Inc - designed special bags. Two hundred ml blood was collected using a pump at a rate of 70 ml/min; the bags were centrifuged, and the serum was separated from the clot. On another day, we aspirated bone marrow from iliac bone, and the bone marrow aspirates were mixed with culture medium and patient' s own serum, and cells were seeded on tissue culture dishes. These cells were incubated in a CO_2 incubator, and we then confirmed the absence of bacteria, fungi, endotoxin and mycoplasma in the medium and the MSC layer in culture by clinical tests. MSC obtained from cultures at passage 3 were harvested and mixed with collagen gel. The gel containing MSC was transplanted to periodontal tissue defects. Comparison of bone density with X-ray 6 months before and after the MSC transplantation showed increased bone density around the tooth. We will report details of these studies in the near future.

Jaw/alveolar bone-derived MSC

Usually MSC are isolated from the ilium, but this causes considerable pain because iliac bone is covered by thick skin and muscle. To reduce pain, we isolated and expanded MSC - at a high success rate (70%) - from alveolar/jaw bone, which is covered by thin mucosa alone: These MSC had potent osteogenic potential in vitro and in vivo, although their chondrogenic and adipogenic potential was less than that of iliac MSC (Matsubara et al., 2005). Jaw MSC may be useful for treatment of oral diseases including periodontal disease, since they can easily be obtained during tooth extraction. It will be interesting to examine whether jaw MSC have greater potential for periodontal regeneration than do iliac MSC.

Adhesion of MSC to Scaffolds

MSC adhere poorly to some scaffolds, depending upon the scaffold material, and are often damaged by proteases or mechanical stimuli at site of transplantation. We found, however, that MSC - along with some other cells - that were exposed to PHA-E or ConA increased their adhesion capacity on plastic tissue culture dishes and on plates of hydroxyapatite, titanium and poly-DL-lactic-co-glycolic acid (PLGA). These cells, moreover, built up resistance to proteases and/or mechanical stimuli (Nishimura-H, et al., 2004). Thus, the lectins may have great potential in tissue engineering and cell therapy.

Molecular Markers for MSC

MSC and fibroblasts are indistinguishable in appearance: Neither marker genes nor cell surface antigens specific for MSC have been identified, so no one knows the real face of MSC. To characterize MSC at a molecular level, we compared gene expression profiles in MSC, fibroblasts, osteoblasts, and adipocytes using GeneChip: The DNA microarrays contain 54000 locations and millions of DNA strands built up in these locations. MSC were isolated from three young volunteers and expanded in vitro. These cells were not incubated, or incubated in osteogenic-induction medium, chondrogenic-induction medium or adipogenic-induction medium for 28 days.

We isolated total RNA from MSC, fibroblasts, MSC-derived osteoblasts, MSC-derived chondrocytes, and MSC-derived adipocytes. No degradation of the RNA samples was found, and cDNA was synthesized with T7 oligo-dT primers. Biotin-labeled cRNA was synthesized by in vitro transcription. Fragmented, biotin-labeled cRNA was then hybridized overnight with DNA strands for 54000 probes (48000 genes) on Affimetrix DNA chip (Human Genome, U133, +2.0). After incubation with fluorescent Strepto-avidine, the image was scanned, and the data were analyzed using a computer soft - GeneSpring. The expression level of each gene was standardized according to the GeneSpring global median normalization method: This is the method commonly used for standardization of gene expression levels.

Osteoblasts, chondrocytes and adipocytes can revert to MSC under some culture conditions, whereas fibroblasts cannot revert to MSC-like cells, and unlike MSC, fibroblasts do not have any differentiation potential. Thus, some genes expressed selectively in MSC, osteoblasts, chondrocytes and/or adipocytes - but not in fibroblasts - must be linked with the differentiation program. Using this premise, MSC-specific genes were next selected by gene filtering using GeneSpring: The criteria were more than an increase 4-times greater in MSC than in adipocytes, chondrocytes, fibroblasts, and osteoblasts. This analysis identified 88 and 127 MSC-specific gene markers, up-regulated and down-regulated, respectively.

We also identified 28 genes expressed selectively in MSC and osteoblasts (Group B), and 49 genes expressed in MSC and chondrocytes (Group C), etc. MSC-specific genes were 0.16% of total genes, and MSC/chondrocyte-specific genes were 0.09%. The number of MSC-specific genes was smaller than that of chondrocyte-sepcific genes, suggesting a low degree of transcription regulation in MSC. We also identified osteoblast-, chondrocyte-, or adipocyte-characteristic genes, as well as overlap genes expressed both in MSC and one of the differentiated cells.

Clinical Use of MSC Marker Genes

Tissue factor pathway inhibitor-2 (TFPI-2) was expressed in MSC alone, and leukemia inhibitory factor (LIF) was expressed in MSC and chondrocytes, but not in the other examined cells (data not shown). LIF and TFPI-2 were expressed in standard MSC lines and in four patients' MSC (P1-P4), but not in standard fibroblast lines (Fig. 2). In addition, we found that MMP1 and collagen type XV were expressed in fibroblasts, but not MSC. Accordingly, MMP1 and collagen type XV were not expressed in the patients' MSC (plastic adherent cells) (Fig. 3), indicating that fibroblasts were not present in the patients' MSC before transplantation (Ishii et al., 2005). As a result of this quality examination, we can confidently transplant MSC to patients' defects.

CONCLUSION

Bad habits - such as poor tooth-brushing, lack of exercise, and imbalances of calcium, sugar and fat intake - are causes of periodontal disease, osteoporosis, diabetes and

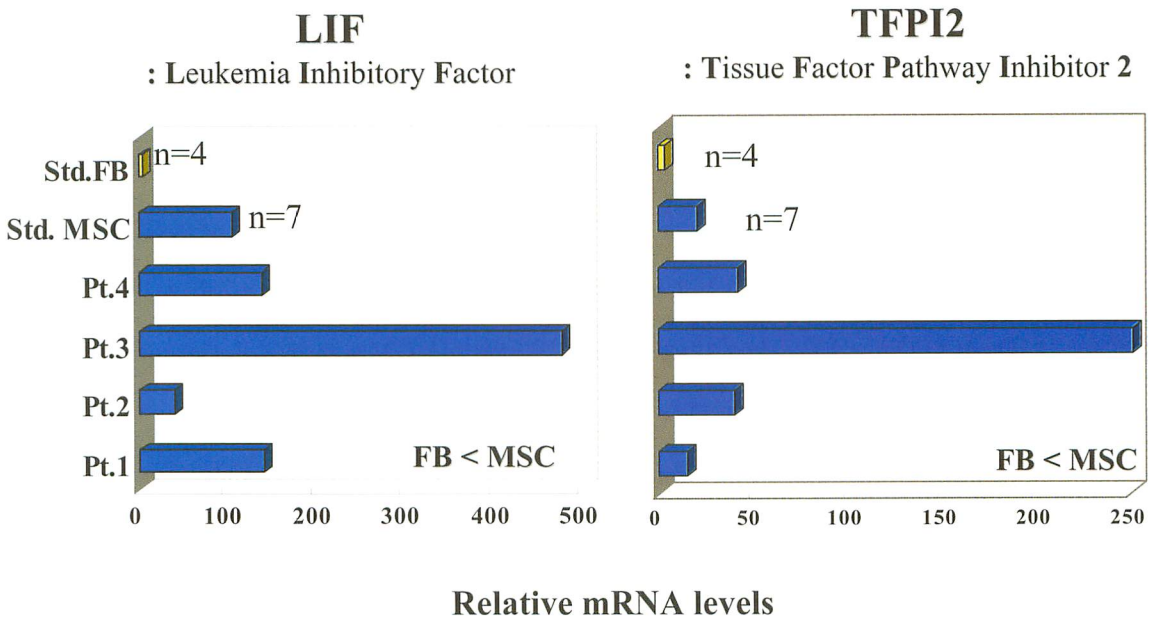

Fig. 2 The use of MSC marker genes in clinical studies. The expression levels of the genes in transplantable MSC from four patients were similar to those in standard MSC.

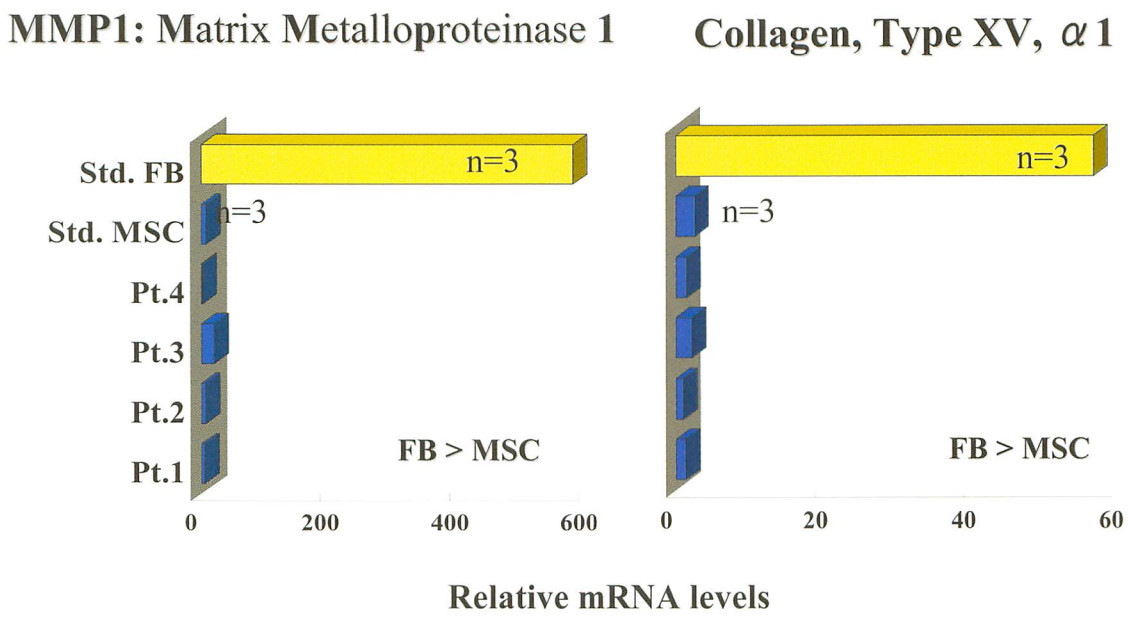

Fig. 3 MSC marker genes were used in clinical studies to confirm the absence of fibroblasts in transplantable cells.

myocardiac infarction, etc. However, age-dependent decreases in available stem cells may also have a great impact on these diseases. If this is true, the regeneration-failure syndrome should be treated with a sufficient number of stem cells. Two Cells Inc supports this business, and the application of MSC will brighten the future both dentistry and medicine.

ACKNOWLEDGMENTS

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Basic and Clinical Studies of Periodontal Tissue Regeneration by Transplantation of Own Bone Marrow Mesenchymal Stem Cells

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ABSTRACT

Use of suitable cells seeded into periodontal defects would be a powerful strategy to promote regeneration of periodontal tissue. Recent interest has focused on mesenchymal stem cells (MSCs) isolated from bone marrow, which have the potential for multilineage differentiation. Transplantation of bone marrow-derived MSCs into periodontal osseous defects would be a useful option for periodontal tissue regeneration. We have been investigating a possibility of MSCs therapy for periodontal diseases.

Our animal studies indicated that transplanted MSCs into experimentally periodontal defects survive and differentiate into appropriate periodontal cells, resulting in enhancement of periodontal tissue regeneration. Based on these scientific evidences, clinical experience entitled "periodontal tissue regeneration by transplantation of own bone marrow MSCs" has been started. Human bone marrow cells are obtained from the iliac crest and expanded in vitro at Cell and Tissue Engineering Center in Hiroshima University Hospital. MSCs are, then, isolated and mixed with Atelocollagen at final concentration of $2x10^7$ cells/ml. These MSCs in Atelocollagen are transplanted into periodontal osseous defects at the periodontal surgery.

This review article summarizes our animal studies and initial clinical experience of periodontal tissue regeneration by transplantation of own bone marrow MSCs.

Key Words: periodontal tissue regeneration, mesenchymal stem cells, transplantation, regenerative therapy, periodontal diseases

Periodontal Tissue Regeneration and MSCs

A major goal of periodontal therapy is to reconstruct healthy periodontium destroyed by periodontal diseases. Previous studies have shown that conventional regenerative therapies such as guided tissue regeneration, topical application of enamel matrix derivative or various polypeptide growth factors are useful for periodontal tissue regeneration (Cortellini *et al.*, 2005; Wang *et al.*, 2005). Since the origin of regenerative cells in these current regenerative therapies is residual periodontal tissues, the type of periodontal osseous defect is an important factor for periodontal tissue regeneration. Indications for these therapies are, therefore, limited to cases of two or three walls bony defects or class II furcation lesions. In this context, use of suitable cells seeded into periodontal defects might be a powerful strategy to promote regeneration of periodontal tissue. The cells should be nonimmunogenic, highly proliferative and easy to harvest, and have the ability to differentiate into various types of cells composing periodontal tissue.

Recent interest has focused on mesenchymal stem cells (MSCs) isolated from bone marrow, which have potential for multilineage differentiation. Bone marrow MSCs can easily be obtained repeatedly and differentiate into osteoblasts, chondrocytes, tenocytes, adipocytes, muscle cells or nerve cells *in vitro* and *in vivo* (Baksh *et al.*, 2004). Thus, transplantation of bone marrow MSCs may provide a new method for treatment of osteoporosis, arthritis, cardiac diseases, and degenerative nerve diseases. Taking all these findings into consideration, it is conceivable that MSCs might be useful for periodontal tissue regeneration. We have been investigating a possibility of MSCs therapy for periodontal diseases. Our preclinical and clinical studies of periodontal tissue regeneration with MSCs are summarized in the following text.

Animal Studies

To determine the effect of periodontal tissue regeneration with MSCs, autologous bone marrow MSCs were transplanted into periodontal defects in dogs (Kawaguchi *et al.*, 2004). Bone marrow aspirates of 1 ml were taken from the iliac crest of each animal. Cell culture was performed in accordance with the technique described by Tsutsumi et al. (Tsutsumi *et al.*, 2001). The MSCs, having been cultured for 2 weeks were suspended, mixed with Atelocollagen (2% type I collagen extracted from bovine calf skin by pepsin digestion, Koken Co., Ltd., Tokyo,

Japan) at final concentration of $2x10^6$, $5x10^6$, $1x10^7$ or $2x10^7$ cells/ml, and auto-transplanted into experimental class III defects. Atelocollagen alone was implanted into the defects as a control. In Atelocollagen, the antigenic telopeptide region has been removed by pepsin digestion. This immunogenic advantage has enabled Atelocollagen to be used in various clinical settings. Furthermore, autologous chondrocytes embedded in Atelocollagen transplanted into cartilage defects were reported to promote repair (Ochi *et al.* 2001). Accordingly, Atelocollagen was chosen as a biomaterial scaffold to hold MSCs in a temporary matrix, and to keep them suspended during transplantation and early period of regeneration. One month after transplantation, histological findings showed that the defects were regenerated with cementum, periodontal ligament and alveolar bone in MSCs-Atelocollagen transplanted groups (Figure 1). Less periodontal tissue regeneration was observed in the control group than in the MSCs-Atelocollagen groups. Morphometric analysis revealed that the percentage of new cementum length in the $5x10^6$ and $2x10^7$ cells/ml groups and the percentage of new bone area in the $2x10^7$ cells/ml group were significantly higher than that in the control group ($p<0.01$). These results revealed that transplantation of bone marrow MSCs enhanced periodontal tissue regeneration.

Next animal study was undertaken to elucidate the performance of MSCs after transplantation. Green fluorescent protein (GFP)-transduced MSCs were cultured and mixed with Atelocollagen at final concentration of $2x10^7$ cells/ml and were auto-transplanted into experimental class III defects in the dogs. One month after GFP-transduced MSCs transplantation, immunohistochemical results showed that GFP-positive cells were present in the whole area of the defects. Cementoblasts arranged along the denuded surface, osteoblasts and osteocytes of regenerated bone, and fibroblasts of the regenerated periodontal ligament were GFP-positive. These findings suggested that some transplanted MSCs survive, differentiate into periodontal tissue cells and release various kind of cytokines, all of which promote periodontal tissue regeneration.

These animal studies demonstrated the therapeutic potential of bone marrow MSCs transplantation for periodontal tissue regeneration.

Clinical Experience

Based on the results of the animal studies, since 2004, clinical study entitled "Periodontal Tissue Regeneration by Transplantation of Own Bone Marrow MSCs" has been started at Hiroshima University Hospital. A brief flow chart of this study was shown in Figure 2. By November 2005, 7 patients with periodontal osseous defects caused by periodontitis had been treated with this procedure.

Patients

The clinical study protocol and the consent form were approved by local ethics committees at both Hiroshima University School of Dentistry and Hiroshima University Hospital. We obtained written informed consent from all patients.

Patients between 20 and 65 years old were eligible for the study if they had the following characteristics: (1) they had received initial preparation of periodontal treatment and maintained good oral environment; (2) there were intrabony osseous defects caused by periodontitis. We excluded patients who met one of the following criteria: malignant diseases, severe diabetes, hepatic dysfunction, severe morbidity, or unwillingness to participate.

Bone marrow aspiration and transplantation surgery were performed at Clinic for Cell and Tissue Transplantation Therapy, Hiroshima University

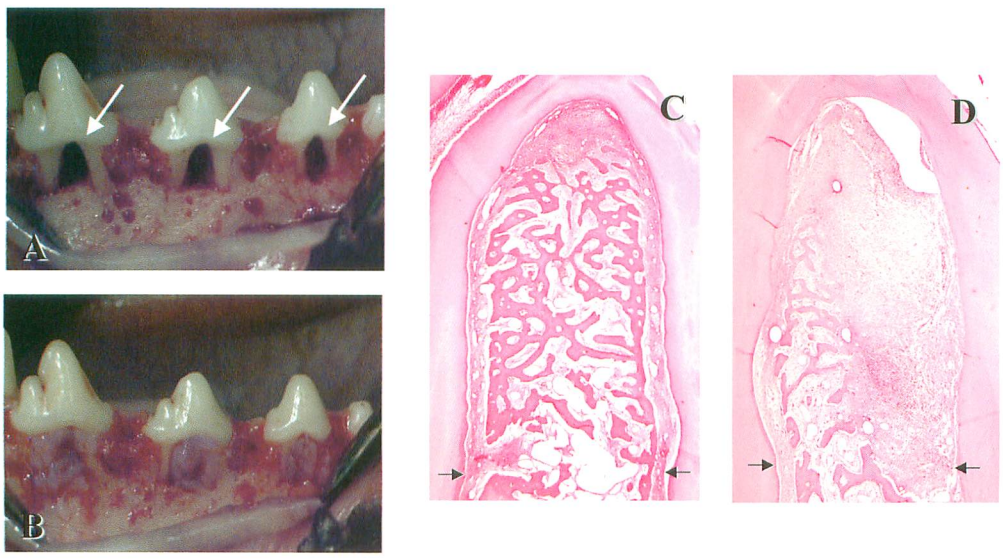

Fig.1 Periodontal regeneration by MSCs transplantation in dogs.
Surgical view of the mandibular premolar area after defect (arrows) preparation (A); after transplantation of various concentration of MSCs in Atelocollagen (B). Light micrographs of 1 month after transplantation of MSCs ($2x10^7$ cells/ml) in Atelocollagen (C) and Atelocollagen alone (D). Arrows indicate apical border of denuded root surface.

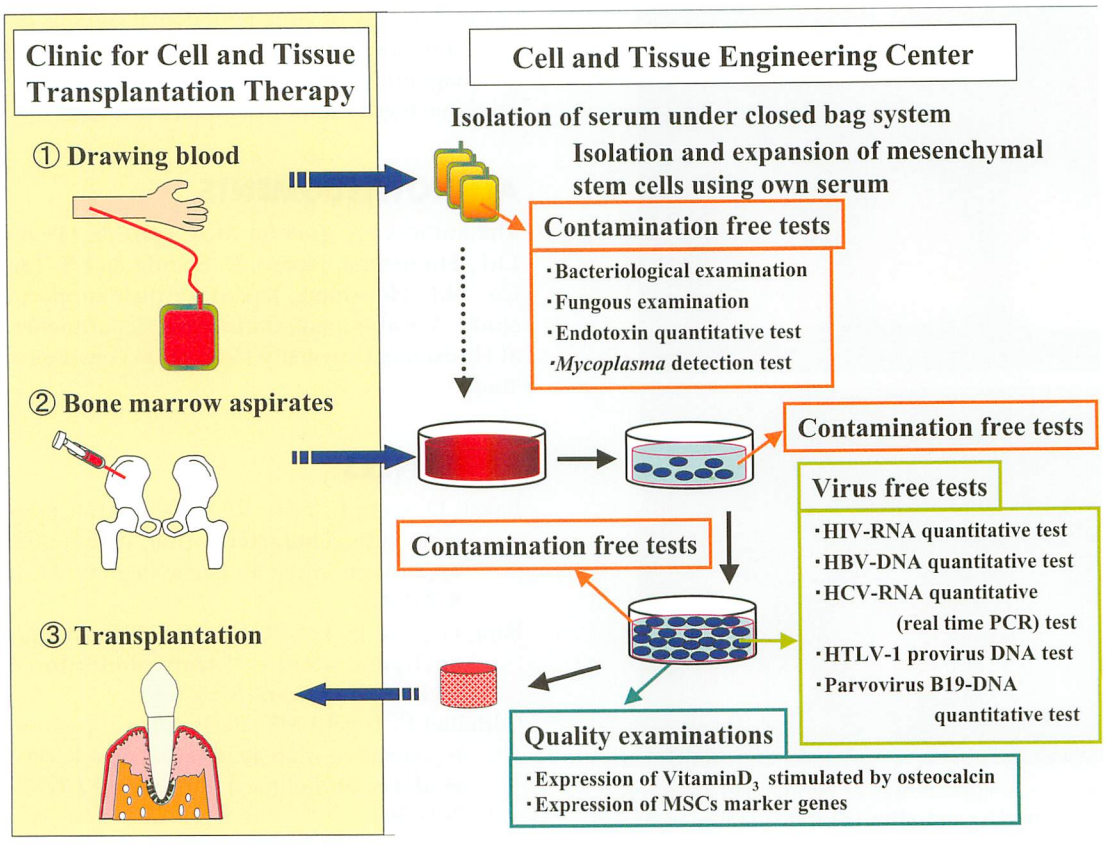

Fig.2 Flow chart of clinical study

Hospital, and isolation of MSCs and cell culture were performed at Cell and Tissue Engineering Center (CTEC), Hiroshima University Hospital.

Autologous serum for isolation and expansion of MSCs

In most previous experimental (Pittenger *et al.*, 1999, Tsutsumi *et al.*, 2001) and clinical (Vacanti *et al.*, 2001, Bang *et al.*, 2005) transplantation studies, fetal calf serum (FCS) has been used for the *in vitro* expansion of MSCs. FCS may include viral or bacterial infections and prion (bovine spongiform encephalopathy), which can cause variant Creutfeldt-Jakob disease. Other potential problems of FCS are immune or local inflammatory reactions due to contamination with bovine protein, which cannot be excluded even by several washings after expansion (Spees *et al.*, 2004). In this context, MSCs should be cultured in xeno-free environment for clinical application, suggesting that it is more appropriate to culture MSCs with autologous serum than with FCS.

We developed completely closed bag system separating autologous serum. Venous whole blood (200 ml) was drawn into special bags (JMS Co., Inc., Hiroshima, Japan). These bags were carried to CTEC and centrifuged at 2,500 *g* for 10 minutes. Approximately 90 ml of serum could be separated from the clot, corrected into other bags under closed environment, and then, stored at -20℃ until ready for use. Sixteen ml of the serum was drawn out to check the "contamination" : bacteriological and fungous examinations, endotoxin quantitative test and *mycoplasma* detection test.

Bone marrow aspiration, isolation of MSCs, and cell culture

We aspirated 15 ml of fresh marrow cells, under local anesthesia, from the posterior iliac crest of patients, and corrected to disposable syringes containing heparinized phosphate buffered saline (PBS). These syringes were carried to CTEC, centrifuged at 200 *g* for 5 minutes, and the supernatant of plasma with the fat layer was discarded. The residue, including buffy coat and red blood cells, was mixed with Dulbecco modified eagles' medium (DMEM) containing 10% of autologous serum from the patient, 50 μg/ml gentamycin and 100 μg/ml streptomycin, and seeded on 100-mm tissue culture dishes. The cells were cultured in a humidified atmosphere of 95% air with 5% CO_2 at 37℃. After 3 days of culture, non-adherent cells were removed by replacing the medium. The attached cells formed colonies within 5 to 7 days, thereafter, fibroblast growth factor-2 (3 ng/ml) was added into the culture with fresh medium. The medium change was done every 3 days. Passages were performed when cells were subconfluent.

Cells were cultured for 3 weeks, then checked for "contamination" mentioned above and "virus free tests" on cultured cell samples: human immunodeficiency virus (HIV)-RNA quantitative test, hepatitis B virus (HBV)-DNA quantitative test, hepatitis C virus (HCV)-RNA quantitative (real time PCR) test, human T-cell leukemia virus (HTLV)-1-provirus DNA test, and Parvovirus B19-DNA quantitative test. Characteristics of cultured cells were also checked (Ishii *et al.*, 2002)

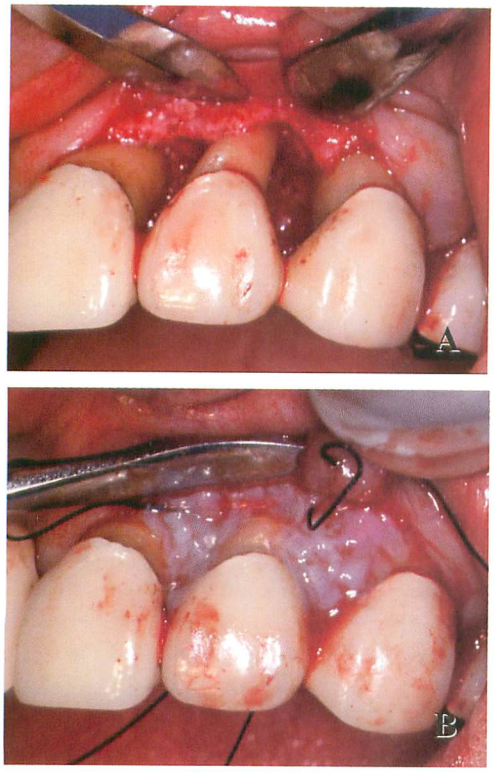

Fig.3 Clinical appearances of periodontal surgery before (A) and after (B) transplantation of MSCs in Atelocollagen.

Cell preparation for transplantation

On the day of transplantation, MSCs in cultures of the 3rd passages were harvested using trypsin- EDTA, washed with PBS prior to being embedded in 3% Atelocollagen. The final cell density was adjusted to $2x10^7$ cell/ml. The MSCs-Atelocollagen composites were further incubated at 37℃ for 30 minutes, carried to Clinic for Cell and Tissue Transplantation Therapy and then, transplanted into periodontal osseous defects at the periodontal surgery (Figure 3).

Clinical results

The results in 7 patients who were followed up for at least 6 months have shown good clinical course. None of the patients exhibited any adverse event or side effect. In a case followed up 1 year, X-ray evaluation showed complete radiographic resolution of the intrabony component of the defect. However, more prolonged follow-up and a larger number of patients are necessary to confirm the effectiveness of MSCs therapy.

Future Perspectives

Although our animal studies indicated that transplanted MSCs into periodontal defects survive and differentiate into appropriate periodontal cells, resulting in enhancement of periodontal tissue regeneration, the precise mechanism whereby MSCs differentiate into cementoblasts, osteoblasts and periodontal ligament fibroblasts is still unknown. Further basic studies (e.g., the mechanisms of MSCs differentiation in vivo and appropriate scaffold in case of large periodontal osseous defects) and the continuous clinical experience are needed to establish the magnitude of the benefit and the effects on periodontal tissue regeneration after MSCs therapy.

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Tissue-engineering of Orthopaedic Surgery

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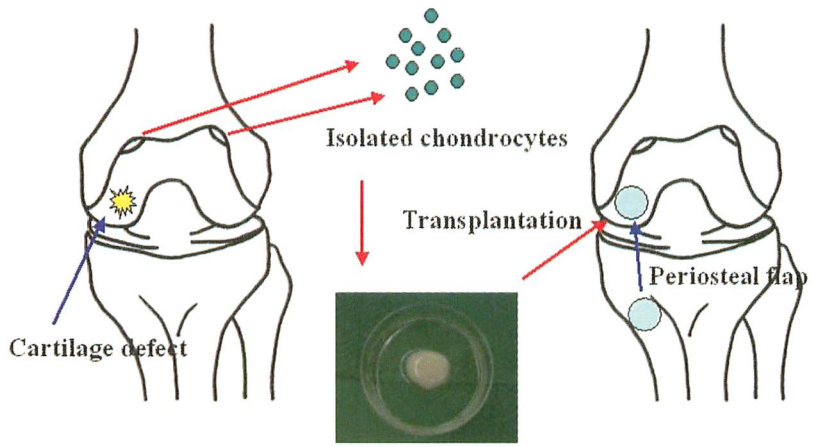
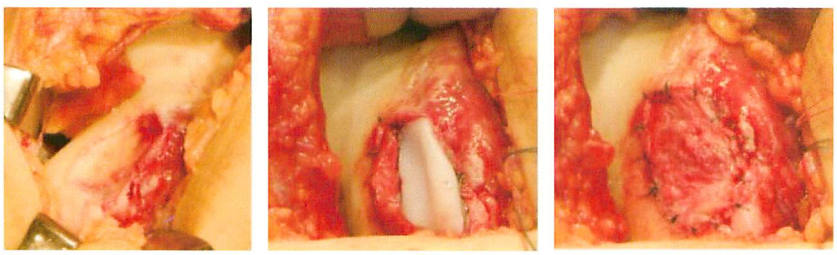
Transplantation of tissue-engineered cartilage for cartilage defect of the knee

Articular cartilage has a poor healing capacity due to its lack of vessels, nerve supply, and isolation from systemic regulation. Numerous methods have been attempted to enhance the repair of full-thickness articular cartilage defects, including abrasion arthroplasty; microfracture; transplantation of chondrocytes, perichondrium, and periosteum; and osteochondral graft. However, no known treatment has regenerated long-lasting hyaline cartilage.

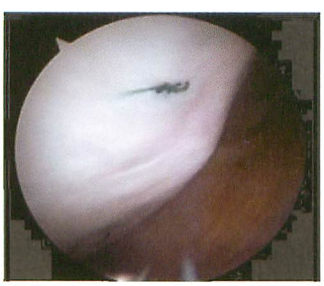
Recently, a regenerative medicine using a tissue-engineering technique for cartilage repair has been given much attention in the orthopaedic field. In 1994, Brittberg et al introduced a new cell technology in which chondrocytes expanded in monolayer culture were transplanted into the cartilage defect of the knee. As a second generation of chondrocyte transplantation, since 1996 we have been performing transplantation of tissue-engineered cartilage made ex vivo for the treatment of osteochondral defects of the joints. This signifies a concept shift from



Tissue-engineered cartilage



Cartilage defect | Transplantation | Periosteal flap



1 year after transplantation

cell transplantation to tissue transplantation made ex vivo using tissue-engineering technique.

Patients

Fifty-six knees of 56 patients (mean age: 25 y.o.) of 98 patients who had received transplantation of tissue-engineered cartilage for cartilage defects were followed up for at least 2 years. The tissue-engineered cartilage was made by cultivating autologous chondrocytes embedded in Atelocollagen gel for 3 weeks before transplantation. At 6, 12, 24 months after operation, arthroscopic, biomechanical and MRI examinations were performed. Using the Lysholm score, the clinical outcome was evaluated at the final clinical follow-up.

Results

Transplantation eliminated knee locking and reduced pain and swelling in all patients. The mean Lysholm score improved significantly. Arthroscopic assessment indicated that 50 knees had excellent or good outcomes. No problems including infection were detected, except with 11 cases of graft hypertrophy, 4 cases of partial detachment of periosteum, 1 case of partial ossification and 2 case of graft failure. Biomechanical examination also revealed that the transplants had acquired hardness similar to that of the surrounding cartilage at 12 months after operation. MRI demonstrated that the signal intensity of the grafted portion had become similar to that of normal cartilage in 77% of the cases at 24 months after operation.

Minimally invasive approach with a tissue-engineered chondral plug

Purpose

The purpose of this new approach was to evaluate the macroscopic and histological results transplanting a tissue-engineered chondral plug made of atelocollagen sponge and PLLA mesh, for the treatment of osteochondral defects.

Methods

Twelve-week-old male Japanese white rabbits were used. Fresh articular cartilage slices were taken from the humeral head, and isolated chondrocytes were embedded in atelocollagen gel which does not have antigenic portions of collagen. (2.0×10^6 cells/ml). They were seeded on the top of the atelocollagen sponge/PLLA mesh composite, and cultured for 2 weeks. The culture medium was changed every 3 days and L-ascorbic acid (50μg/ml) was added every 2 days. Culturing the composites for 2 weeks produced tissue-engineered chondral plugs. These tissue-engineered chondral plugs (4mm in diameter, 4mm in thickness) were transplanted into the osteochondral defects (4mm in diameter, 4mm in depth) in the patellar grooves of the same rabbits from which the chondrocytes had been harvested (the experimental group). In the control group, the defects were treated with the plugs without chondrocytes. The rabbits were sacrificed at 4 and 12 weeks after transplantation. The repaired tissues were evaluated macroscopically and histologically. The repaired tissue was analyzed immunohistochemically for expression of type-II collagen.

Results

Four weeks after transplantation in the experimental group, the defects were partially repaired with cartilage-like tissue with good subchondral bone formation. Twelve weeks after transplantation, the defects were repaired with hyaline cartilage-like tissue densely stained by safranin O. Well organized subchondral bone formation was also observed. In the control group, the defects were covered with only soft fibrous tissue at 4 and 12 weeks macroscopically.
Immunohistochemically, type-II collagen was detected in about 90 % of the repaired area. Histological scores in the experimental group were significantly higher than those in the control group at both 4 and 12 weeks after transplantation.

Conclusions

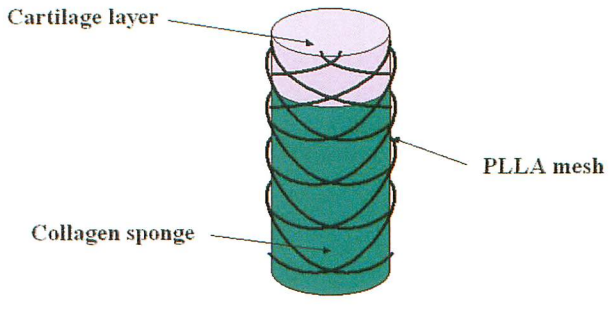
This study demonstrated that the defects treated with tissue engineered chondral plug demonstrated type-II collagen in about 90% of the repaired area.

Clinical Relevance

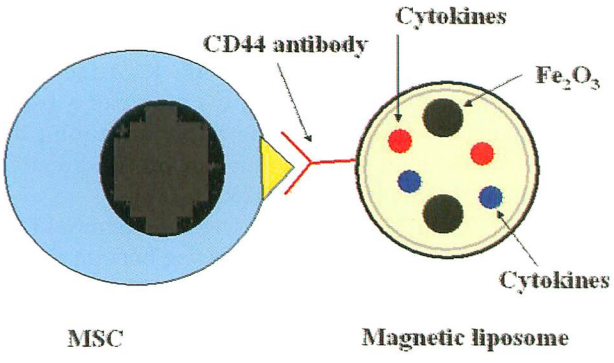
The transplantation of a tissue-engineered chondral plug will be one option for the treatment for osteochondral defects. The next step in testing our hypothesis is to evaluate the repaired tissue biomechanically and biochemically over a longer period of time.

Future direction for cartilage repair with minimally invasive tissue-engineering technique

The most optimal procedure to repair cartilage defects is not surgical treatment requiring anesthesia and hospitalization but just injection of cytokines or growth factors and cells. Our completely novel approach was to use autologous bone marrow mesenchymal stem cells attached to small-sized magnetic beads. For successful cartilage repair, it is simple to inject mesenchymal stem



Tissue-engineered chondral plug



Magnetic liposome-MSC complex

cells and effectively collect them to a specific area in the knee joint (osteochondral defect) using an external magnet force. The complex of autologous bone marrow mesenchymal stem cells and magnetic liposomes using the antibody (CD44) was created and research of the efficacy of DDS by magnetism was done as a pilot study. A full thickness cartilage defect was made in the bilateral femur condyle of a rabbit, and successfully repaired with this technique. We believe that this novel system is also effective in the treatment of brain or spinal cord injury and for malignant tumors, using natural killer cells instead of autologous bone marrow mesenchymal stem cells with our novel system.

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Recent Advances in Tissue Engineering of Cartilage, Bone and Tendon

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INTRODUCTION

Tissue engineering employs seed cells and biodegradable materials to generate various tissues in vivo or in vitro [1]. Since the initiation of tissue engineering in 1980's, the development of tissue engineering research might be artificially divided into three stages. In the first stage, many studies focused on proving the concept that various tissues could be engineered in vivo using immunodeficient animals [2,3]; in the second stages, studies are investigating the possibility of engineering tissue in large immunocompetent animals[4]; the third stage will be to explore the possibility of engineering tissue in human and its clinical application. In current stage, most investigations are still limited to the second stage, although there are few reports of clinical applications. During the past 1-2 years, tremendous progress was made in regards to the development of new seed cell source and new biomaterials for tissue engineering. However, this article focuses on reviewing the advancement in the animal studies of cartilage, bone and tendon engineering.

Advances in cartilage engineering

New seed cell source for cartilage engineering

Chondrocytes are used to be the seed cells for cartilage engineering, but seem no longer the optimal cell source due to their natures of limited expansion capacity, becoming aged and dedifferentiated during in vitro expansion. In addition, harvesting cartilage also causes donor site morbidity. In contrast, bone marrow stem cells (BMSCs) become the reliable replacement for chondrocytes due to its capabilities for chondrogenic differentiation and rapid proliferation. There were numerous reports in the past decade of *in vitro* chondrogenic induction of BMSCs, which could express chondrocyte-specific molecules such as type II collagen, aggrecan and sox9, etc. However, only few reports demonstrated that induced BMSCs actually formed cartilage tissue in vivo. Interesting, articular environment seems to play an important role in cartilage formation in vivo, because the evidence of cartilage formation by *in vivo* implanted BMSCs in most studies is related to the repair of articular cartilage by BMSCs [4,5,6]. There is no report of successful cartilage engineering using BMSCs at the location of subcutaneous tissue. A study performed in our center showed that BMSCs, which were *in vitro* chondrogenically induced to express type II collagen and aggrecan, failed to form cartilage after implantation subcutaneously in nude mice. However, implantation of mixed cells (60% BMSCs and 40% chondrocytes) could converted all BMSCs into chondrocytes and generate uniform cartilage even in the subcutaneous tissue (Zhou GD et al. unpublished data). These phenomena indicate that *in vivo* environment may be the factor more important than in vitro induction, which determines the differentiation and tissue formation of *in vivo* implanted BMSCs. However, it is still possible to fully differentiate BMSCs into chondrocytes and form cartilage *in vitro* by chondrogenic induction (Kojima K. et al. Tissue engineered trachea using sheep bone marrow-derived mesenchymal stem cells. Tissue Eng, 2002, 8:1155, O-95). Once cartilage is formed *in vitro*, the tissue is likely to maintain its structure even implanted in non-cartilage environment.

Recently, adipose tissue derived stem cells (ADSCs) also received particular attention. Similar to BMSCs, ADSCs have been demonstrated as mesenchymal stem cells with multiple differentiation potential, such as osteogenic, chondrogenic and adiogenic [7]. The advantage of ADSCs over BMSCs is that high yield of stem cells can be obtained from fat tissue. While most studies still focused on the *in vitro* induction, one study reported the successful induction of ADSCs into oesteogenic and chondrogenic cells, and these induced cells formed hyaline cartilage and bone respectively after *in vivo* implantation [8]. Thus, ADSCs may become another important cell source for cartilage engineering.

Cartilage engineering in immunocompetent animals

Repairing articular cartilage defect using tissue engineering approach was reported many years ago. While some studies focused on repairing cartilage defect only in small animal model using allogeneic chondrocyte-engineered hyaline cartilage [9], other studies repaired the osteochondral defect at knee joint in large mammals using autologous chondrocyte-engineered cartilage [10]. These studies are valuable for revealing the potential of engineered cartilage in the repair of articular cartilage defect. However, chondrocyte-based therapy may not be practical for clinical applications. Immune rejection will still be the obstacle to the use of allogeneic chondrocytes, and the use of autologous chondrocytes faces the challenge of limited cell source and donor site morbidity. In addition, repair of subchondral bone defect with chondrocyte engineered cartilage will not achieve the goal of physiological and functional repair of osteochondral defects. In contrast, mesenchymal stem cell-based therapy may potentially avoid these disadvantages.

Repair of articular cartilage with BMSCs has been reported for many years. In most cases, they were used

as cell therapy. In recent years, importance of scaffold has been better appreciated and most studies employed tissue engineering technique. Biodegradable biomaterials seeded with BMSCs were implanted to repair articular cartilage defects either in partial thickness [11], or in full thickness [5] or full thickness along with subchondral defect [4] with a success. It is important to understand the mechanism how implanted BMSCs repair the articular cartilage defect [5]. One study performed in our center showed that BMSCs that were implanted with scaffold to repair osteochondral defect in a pig model could differentiate into chondrocytes and osteogenic cells and form hyaline cartilage and subchondral bone respectively [4]. To trace the implanted BMSCs *in vivo*, the cells were first retrovirally labeled with green fluoresce protein (GFP), and then implanted *in vivo* with scaffold. Seven month later, GFP-labeled cells were detected in the lacuna of engineered cartilage and in subchondral cancellous bone, providing the direct evidence of *in vivo* BMSC differentiation (Zhou GD et al. Repair of porcine full thickness articular cartilage defect with autologous bone marrow stromal cells. 49th Annual Meeting of Orthopedic Research Society, 2002). Interestingly, there already are few reports of clinical trial of repairing articular cartilage defect using BMSC engineered cartilage, although the therapeutic effect needs to be further improved [11].

Repair of meniscus defect is another important area in orthopedic research. Although many approaches were proposed towards meniscus engineering, only few studies have reported successful generation of meniscus using tissue engineering technique. Hidaka et al. isolated meniscal cells from bovine meniscus, transfected them with VEGF gene and then seeded on polyglycolic acids (PGA) followed by *in vivo* transplantation into nude mice. Eight weeks later, a vascularized meniscus-like tissue was formed, indicating that vascularized meniscus might be possible to generate *in vivo* [12]. Our center has performed an in situ engineering of segmental meniscus in a pig model using autologous meniscal cells and PGA, which showed that a meniscus similar to normal counterpart in gross view and histology could be generated at 25 weeks post-implantation [4]. Like other types of cartilages, using BMSCs as cell source to engineer meniscus might be the future direction.

Despite the fact that most engineered cartilages were formed in the *in vivo* environment, it is noticed that *in vitro* cartilage engineering using chondrogenically induced BMSCs has become the reality. Fukumoto et al. tested *in vitro* chondrogeneis of periosteum explant. The harvested explant was *in vitro* cultured in the medium with TGF-β and IGF, and the result showed that combined use of these two growth factors could effectively induce periosteum mesenchymal stem cells into chondrocytes and form cartilage-like tissue [13]. In another study, BMSCs were embedded in a hotopolymerizing hydrogel, cultured and induced with TGF-β alone, and a cartilage-like tissue was also formed *in vitro*[14]. Comparing to TGF-β alone induction [14], chondrogenic induction with both TGF-β and IGF could achieve much better chondrogensis *in vitro* and formed the cartilage resembling native cartilage structure. Using double induction, Kojima et al. could use chondrogenically differentiated BMSCs to form a cartilage with a three-dimensional structure, such as trachea (Kojima K et al. Tissue engineered trachea using sheep bone marrow-derived mesenchymal stem cells. Tissue Eng, 2002, 8:1155, O-95). Thus, employment of autologous cartilage engineered *in vitro* by BMSCs might be another important approach to the repair of articular or meniscal cartilage defects.

Advances in bone engineering

Seed cell source for bone engineering

BMSCs remain to be the major cell source for bone engineering. As mentioned earlier, ADSCs might become the new cell source for bone repair due to their strong osteogenic ability [7]. More importantly, *in vivo* study showed that ADSCs have the same ability as BMSCs to generate bone and repair bone defect *in vivo* [15]. Osteogenically induced embryonic stem cells may become another new cell source [16] and the ability of bone formation *in vivo* by ES derived cells needs to be proved in the future study.

Bone engineering in immunocompetent animals

Engineering of flat bone is more often reported than long bone in large animal studies. Repair of calvarial defect usually serves as a good model for this type of study. While successful repair of critical sized cranial defect using engineered bone was reported in a sheep model [17], other study in a rabbit model showed that tissue engineered bone repair of calvarial defect remained less satisfactory than autologous bone [18]. The difference may reflect the variance in their choice of biomaterials and the preparation of BMSCs. Interestingly, genetic modification of BMSCs with BMP-2 gene certainly enhances the bone formation *in vivo* and is helpful for the reconstruction of large size bone defect [19] [20].

Comparing to flat bone, engineering of long bone faces a bigger challenge because the engineered bone should be able to sustain mechanical loading. It is still controversial for the choice of biomaterials that are used to repair segmental long bone defect. Slow-degradation materials like ceramics or bioglass have been widely used for many years. Some scholars also believed that use of osteoconductive and osteoinductive material alone without seeding cells is enough for long bone repair. These concepts might be subjected to changes with the development of tissue engineering. The principle of tissue engineering requires implantation of both seed cells and biomaterial and the degradation rate of implanted biomaterial must be synchronous with the pace of tissue development. A new bone forms within the period of natural bone healing time would be optimal for clinical application. The study by Petite et al. [21] has proved such a principle. They isolated BMSCs, seeded them on coral and then repaired the sheep metatarsus defect. Due to the proper degradation rate, new bones could be formed at 16 weeks. More importantly, because of complete degradation of the biomaterial, newly formed bones were able to undergo tissue remolding resulting in complete recorticalization and the formation of a medullary canal with mature lamellar cortical bone. In contrast, BMSCs loaded on ceramics will not be able to form a natural bone tissue due to the slow degradation of these materials [22]. It is also highly likely that remaining of ceramic material in

the new bone will further impede tissue remodeling.

Inspired by Petite' s report [21], our center further challenged the weight-bearing bone engineering by repairing femoral bone defect (25mm long) in a sheep model using osteogenically induced BMSCs and coral. The results showed that all animals studied had generated the segmental bones in the femoral defects within 3 months. Different from Petite' s method, osteogenic induction of BMSCs was performed before *in vivo* implantation, which may explain the high successful rate of bone regeneration [4].

Recently, Arinzeh et al. reported successful regeneration of segmental bones in a canine model using allogeneic BMSCs and hydroxyapatite-tricalcium phosphate [23]. Interestingly, use of allogeneic cells did not cause strong immune reaction either locally at the implantation site or systemically and achieved the therapeutic results similar to that of autologous BMSCs. A similar study using allogeneic BMSCs was reported in a rat model with satisfactory results. However, the authors still found that short-term treatment with immunosuppressant FK506 was needed for allogeneic cells to repair the critical bone defects to the same degree as autologous cells [24]. It is important to understand the mechanism why allogeneic BMSCs are not able to induce immune reaction after *in vivo* implantation. Is the phenomenon unique in bone engineering or generally true in other types of tissue engineering when allogeneic BMSCs are used?

Clinical application of engineered bone

Vacanti et al. performed the first clinical trial of engineered bone in thumb reconstruction using autologous osteogenic precursor cells isolated from periosteum and initially appears to have succeeded, however long-term follow-up was not reported.

Although there are less reports related to long bone, clinical applications of engineered flat bones are being tried in different countries (Schmidt RJ et al. Tissue engineered mineralized autologous 3D bone in maxillary sinus floor augmentation-A clinical and histological report. Second Annual Meeting of European Tissue Engineering Society, Genoa, 2003). Our center has started clinical trial of engineered bone since 3 years ago, mainly focused on the repair of human craniomaxillofacial bone defects using autologous BMSCs and demineralized bone matrix [25]. In this study, we have shown that implantation of osteogenically induced BMSCs were essential for stable bone formation in human being, because complete absorption was was observed with CT-scanning and histology when material alone was implanted. More importantly, implanted BMSCs could develop bone in human body and maintain stable for 18 months when a biopsy was preformed, which demonstrated the formation of cancellous bone and the expression of osteocalcin and osteopondin by the engineered human bone. In addition, engineered bones have achieved the goal of clinical repair of bone defects. However, major challenge remains, such as the size limitation and vascularization of engineered bone in human being.

Advances in tendon engineering

Comparing to cartilage and bone, less progress was achieved in tendon engineering. Tendon is a relatively simple tissue and its major function is mechanical support. For *in vivo* tendon engineering, chicken craw might be a good model because the tendon structure is similar to human hand. Cao et al. used this model for flexor tendon engineering inside the tendon sheath by using autologous tenocytes and PGA. The result demonstrated that engineered tendon at 14 weeks was similar to normal tendon in its gross view, histology and biomechanical strength, indicating that a functional tendon is possible to generate *in vivo* [26]. Other study using rabbit Achilles tendon as a model to generate tendon tissue using knitted poly-lactide-co-glycolide (PLGA) and allogeneic BMSCs and also achieved a satisfactory result in term of histology and biomechanical property [27]. Interestingly, the study also showed that implanted PLAG without cell loading also formed tissue engineering tendon similar to cell-loaded tendon at 12 weeks[27], which is different from Cao' s study that showed implanted PGA (without cell seeding) underwent complete degradation at 12 weeks [26]. This difference in the results may reflect the difference in the models they used. Achilles tendon is not surrounded by tendon sheath, thus fibroblasts from host may migrate into the scaffold and contribute to the formation of tendon tissue. It would be interesting to investigate whether implanted BMSCs will eventually differentiate into tenocytes when cell-scafford is implanted inside a tendon sheath. It is also reasonable to speculate that BMSCs can be induced to differentiate into tenocytes *in vitro* when specific chemical compound and growth factors are applied or specific transcription factor genes are transfected. Another area deserves more efforts for tendon engineering is ex vivo engineering of tendon in a bioreactor, which allows controlled introduction of biochemical and physical regulatory signals to guide cell differentiation, proliferation, and tendon tissue development [28].

CONCLUSION

Compared to *in vitro* studies of seed cells and development of biomaterial, relatively fewer papers have been published in tissue construction of bone, cartilage and tendon in animal studies during past 1-2 years. However, significant progress has been achieved in this area, which demonstrates that various types of tissues can be engineered in immunocompetent animals and can be used to repair related tissue defects. More importantly, a few successful clinical trials of engineered bone provide a promising future for clinical application of engineered tissues.

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