An Ultrastructural Study on Bone Regeneration around True Bone Ceramic (TBC) and TBC-Collagen Compound Particles Implanted in Rat Periodontal Defects

Muhammed Ramjan Hossain*

(received for publication, March 30, 1995)

INTRODUCTION

Hydroxyapatite (HAP) is an active bioceramic material which has been used clinically as implant material into the alveolar bone defects resulting from periodontal disease. Lange et al. expressed the opinion that the general success of implantation is strongly dependent on the events which take place along the tissue implant interface. It was ascertained that the affinity of HAP to bone and the capacity of new bone formation around HAP were favorable because there was no interposition of fibrous tissue between HAP and newly formed bone. Kawaguchi et al. found that various types of cells, including osteoclast-like multinucleated cells (MNCs), were present at the interface between HAP and osseous tissue. It was reported that the appearance of MNCs is one of the striking histological features after HAP implantation. With respect to bone regeneration processes, in addition to osteoclasts, collagen free electron-dense, glycoprotein like layers were seen at the interface between the new bone and HAP at the ultrastructural level.

Some reports indicated that collagen placed into the bony defect was completely substituted by bone. Some researchers grafted the HAP-collagen compound into the bony defect and reported favorable new bone formation with no inflammatory reaction.

True Bone Ceramic (TBC) derived from sintered bovine bone is a superior bioceramic implant material. It has been widely used in plastic surgery because it possesses the properties of osteoconduction and affinity for living bone. TBC-collagen compound is also used in basic and clinical study as implant material into the alveolar bone defects resulted from periodontal diseases to determine the active bone regeneration capacity. It has been reported that the calcification ability of both TBC and TBC-collagen compound is superior than HAP. With regard to osteoconductivity, both TBC and TBC-collagen compound have the ability to regenerate detectable amounts of alveolar bone at the light microscopic level. However, to date, there has been no ultrastructural report on the mechanism of bone regeneration processes around TBC and TBC-collagen compound particles and the interfacial structure between the new bone and these implant materials.

In the present study, TBC and TBC-collagen compound were implanted into rat periodontal defects in order to investigate the mechanism of alveolar bone regeneration around these materials. In addition, various structures formed at the border zone between the implanted materials and the newly formed bone were examined at the light microscopic and ultrastructural level.

MATERIALS AND METHODS

Seventy male Wistar rats weighing approximately 250 gm were used in this study. TBC (Koken Co. Ltd., Tokyo, Japan) was prepared as follows. Bovine bone from which protein was excluded was sintered at 600°C and 1,100°C and then crushed to granular form. The granular size used in this study was 100–200 μm in diameter. TBC described above was mixed with 1% atelocollagen to make TBC-collagen compound at a ratio of 3:2 by weight (Koken Co. Ltd., Tokyo, Japan). Atelocollagen...
lagen was made by digesting telopeptides of non-soluble type I collagen of bovine dermis with pepsin.

Following intraperitoneal injection of Nembutal®, a reverse bevelled incision was made over the gingival sulcus at the palatal site of the maxillary first molar and a mucoperiosteal flap was elevated. Thereafter, three-walled alveolar bone defects (1×1×1 mm in size) were surgically formed around the mesio-palatal root of the maxillary first molar with a fissure bur #700. After irrigating the defect with sterile saline to remove bone and tooth fragments, TBC particles mixed with saline were implanted into these bony defects up to the original bone level in the TBC-group (Fig. 1). Similarly, TBC-collagen compound particles were implanted into similar defects in the TBC-collagen group. The flap was then sutured with 4–0 silk thread at the mesial site of the maxillary first molar. The animals were then sacrificed at days 1, 3, 7, 14, and 28 after implantation.

![Diagram](image)

**Fig. 1.** Schema of implantation of TBC and TBC-collagen compound particles into alveolar bone defect in rat.

For the electron microscopic study, animals were reanesthetized and perfused with half Karnovsky's fixative solution (2% paraformaldehyde and 2.5% glutaraldehyde in a 0.1 M cacodylate buffer at pH 7.4). The tissue blocks containing tooth and alveolar bone were removed and immersed overnight in the same fixative at 4°C. They were then decalcified for more than 3 months in 10% tetrasodium ethylene diamine tetraacetic acid, pH 7.4, at 4°C. After decalcification, the tissue blocks were briefly washed with the same buffer and post fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.4 for one hour. These specimens were dehydrated through a graded series of ethanol and then embedded in Epon 812.

Thick sections were cut by glass knives and stained with 0.5% toluidine blue for light microscopic observation. Ultrathin sections were cut with a diamond knife and stained with uranyl acetate followed by lead citrate. They were examined with a JEOL-100S transmission electron microscope at an accelerating voltage of 60 kV or 80 kV.

**RESULTS**

1) **Light microscopic findings:**

At day 1 after implantation, in the TBC group, a thin layer of fibrin network laid just beneath the gingival connective tissue in close contact with TBC particles. A number of polymorphonuclear leukocytes (PMNs) were infiltrated around TBC particles implanted in the defects. In addition, PMNs were also found within the canalicus of the TBC particles (Fig. 2). On the other hand, in the TBC-collagen group, a thick layer of fibrin network and a small number of PMNs were observed around the implanted TBC-collagen compound particles (Fig. 3).

At day 3, although the fibrin network was still present around TBC and TBC-collagen compound, most of the area of the defects were filled with PMNs and lymphocytes in both groups. At the periphery and near the osseous walls of the defect, fibroblast-like undifferentiated cells were observed around the implant materials. Osteoblast-like cells were sometimes visible on the surface of the wall of the osseous defect in the TBC-collagen group (Figs. 4 & 5).

At day 7, the fibrin network disappeared and the inflammatory cells markedly decreased in number in both groups. In the TBC group, at the upper portion of the defect, the TBC particles were still covered with PMNs. MNCs appeared around the TBC particles located near the osseous wall of the defect in the TBC group (Fig. 6). In the TBC-collagen group, in addition to MNCs, osteoblast-like cells and fibroblasts were found at the same position. Furthermore, new bone was formed over the old bone surface of the defect and a cement-line like structure was present between them (Fig. 7).

At day 14, inflammatory cells had disappeared in both groups. In the TBC group, from the middle to coronal part of the defect, TBC particles were encapsulated by fibrous tissue. MNCs were attached to the TBC particles adjacent to new bone, and regenerated bone enveloped the TBC particles located near the osseous wall of
Fig. 2. At day 1 after implantation in the TBC group. Numerous polymorphonuclear leukocytes are infiltrated around True Bone Ceramic particles (T) in the defect. ×66.

Fig. 3. At day 1 after implantation in the TBC-collagen group. A wide layer of fibrin network (asterisk) is seen around the implanted TBC-collagen compound (TC) at the upper portion of the defect and small number of polymorphonuclear leukocytes are infiltrated around implanted particles. ×66

Fig. 4. At day 3 after implantation in the TBC group. Undifferentiated mesenchymal cells are visible around TBC (T) near the periphery and wall of the osseous defect. ×165.

Fig. 5. At day 3 after implantation in the TBC-collagen group. Fibroblast-like cells are present around TBC-collagen compound particles (TC) near the periphery and osteoclasts-like cells (arrows) at the bony wall of the defect. ×165.
Fig. 6. At day 7 after implantation in the TBC group. Inflammatory cells had decreased in number and numerous multinucleated cells are visible around TBC particles (T) near the wall and periphery of the defect. ×165.

Fig. 7. At day 7 after implantation in the TBC-collagen group. Osteoblast-like cells and fibroblast have become close to the surface of TBC-collagen compound (TC) near the wall of the defect. A cement line-like structure (arrow) is also present between the old and new bone. ×165.

Fig. 8. At day 14 after implantation in the TBC group. TBC particles (T) are covered by new bone originating from the wall of the defect. The border zone of regenerated bone and TBC particles has a smooth outline (small arrows). A cement line-like structure is present at demarcating the old and new bone (large arrows). ×330.

Fig. 9. At day 14 after implantation in the TBC-collagen group. There is accelerated new bone formation around ceramic particles. The border zone of regenerated bone and TBC-collagen compound particles (TC) has an irregular outline (small arrows). The cement line-like structure is also present between the old and new bone (large arrows). ×165.
Fig. 10. At day 28 after implantation in the TBC group. New bone is continuously formed and the border zone between the new bone and TBC particles (T) is the same as the 14-day-old specimen. ×165

Fig. 11. At day 28 after implantation in the TBC-collagen group. A greater part of the defect is filled with new bone with irregular outline facing the ceramic particles. TC: TBC-collagen compound particles. ×330.

Fig. 12. At day 7 after implantation in the TBC group. Cellular processes are projected deep into the pore of TBC particles (T) from the multinucleated cells. Bar: 2 μm × 4,280.

Fig. 13. At day 7 after implantation in the TBC-collagen group. A resorption area is present along the attachment border between the ruffled border and TBC-collagen compound particles (TC). Bar: 2 μm × 6,420.
Fig. 14. At day 7 after implantation in the TBC group. An electron-dense granular layer (arrow) has begun to be formed. An osteoblast-like cell is visible very close to it. T: TBC particles. Bar: 2 μm × 6,420.

Fig. 15. At day 7 after implantation in the TBC-collagen group. Numerous collagen fibrils are seen closely attached to the electron-dense layer (arrow). TC: TBC-collagen compound particles. Bar: 2 μm × 4,400.

Fig. 16. At day 14 after implantation in the TBC group. The thickness of the electron-dense granular layer (arrow) is wider than the 7-day-old specimen. Surrounding the preosteoblast-like cell, there is formation of collagen fibers. T: TBC particles. Bar: 2 μm × 8,560.

Fig. 17. At day 14 after implantation in the TBC-collagen group. Preosteoblast-like cell has become attached to electron-dense, granular deposit (arrow). TC: TBC-collagen compound particles. Bar: 2 μm × 6,420.
Fig. 18. At day 14 after implantation in the TBC group. A highly electron-dense layer (arrow) is seen adjacent to implanted TBC (T) and some narrow collagen fibrils are attached to it. Bar: 2 μm ×4,280.

Fig. 19. At day 14 after implantation in the TBC-collagen group. Some vertically and obliquely arranged narrow collagen fibrils are attached to electron-dense layer with narrow thickness (arrow). TC: TBC-collagen compound particles. Bar: 2 μm ×4,280.

Fig. 20. At day 14 after implantation in the TBC group. High power magnification shows multiple layers of electron dense interfacial deposit (arrows). A few narrow layer collagen fibrils have become attached to it. T: TBC particles. Bar: 1 μm ×21,400.

Fig. 21. At day 14 after implantation in the TBC-collagen group. An electron-dense interfacial zone (arrows) is visible and has become smaller in thickness in contact with ceramic particles. TC: TBC-collagen compound particles. Bar: 1 μm ×21,400.
the defect. In the TBC-collagen group, formation of new bone surrounding the implanted particles was much more evident than that in the TBC group, and MNCs were not visible in any part of the defects. The border between newly formed bone and TBC particles had a clear contour in the TBC group (Fig. 8), whereas that in the TBC-collagen group was irregular and wide (Fig. 9). Cement line-like structures demarcated the old and new bone and the latter contained mature osteocytes and hematopoietic tissue in the TBC-collagen group.

At day 28, new bone was extensively formed around the implanted particles in the defect in both groups, but the total volume of new bone was much less in the TBC group than in the TBC-collagen group. In addition, trabeculae of new bone were much thicker in the TBC-collagen group than in the TBC group (Figs. 10 & 11). In the TBC group, the new bone had a smooth outline with the presence of MNCs near the regenerated bone, while in the TBC-collagen group, cement line-like structures were always found between new bone and TBC-collagen compound particles.

2) Electron microscopic findings:

At day 7, in the TBC group, MNCs characterized by many mitochondria and vacuoles were closely applied to the TBC surface. They were devoid of ruffled border, though they extended prominent cell processes deeply into the canalculus of the TBC particles (Fig. 12). On the other hand, in the TBC-collagen group, MNCs found near the TBC surfaces exhibited clear zone and ruffled border-like structures (Fig. 13). In both groups, preosteoblast-like cells characterized by the presence of abundant rough surfaced endoplasmic reticulum were found around the outer surface of the MNCs. They were also located near the implant particles which surface was covered by single electron-dense layer having a fine granular appearance (Figs. 14 & 15). Sometimes, only in TBC-collagen group, new bone matrix was found over these surfaces. In such a case, the electron-dense layer seemed to be connected to collagen fibrils of bone matrix which were wrapped by fibroblasts or preosteoblast-like cells (Fig. 15).

At day 14, in the TBC group, an electron-dense granular layer was seen along the TBC surface, and new collagen fibers began to be formed and were attached to it. The electron-dense granular layer was wider than the 7-day old specimen in the TBC group. Some preosteoblasts enriched with cell organelles were close to this electron-dense layer (Fig. 16). In the TBC-collagen group, in the same place as the TBC group, numerous new collagen fibrils had became attached to the electron-dense granular layer along the TBC surface. Subsequently, preosteoblast-like cell became close to these collagen fibrils and some cell processes proceeded towards them (Fig. 17).

The region where the implanted TBC was covered by new bone matrix with collagen fibers, in the TBC group, in many cases, had a highly electron-dense granular layer with wide thickness along the TBC surface. This was followed by a parallel layer of thinner collagen fibrils and subsequently by thick collagen fibrils from which bone matrix was formed (Fig. 18). While in the TBC-collagen group, at the same place as in the TBC group, the same electron-dense layer became narrower in thickness in comparison to the TBC group, and some narrow collagen fibrils with wide volume became attached to the electron-dense layer (Fig. 19).

High power magnification of the interfacial zone showed that different types of multiple-layer electron-dense deposits were visible and at its outer surface, some narrow layer of collagen fibrils was attached in the TBC group (Fig. 20). On the other hand, in the TBC-collagen group, an electron-dense layer became narrower in thickness and was in contact with the TBC surfaces (Fig. 21).

**DISCUSSION**

1) Materials and Methods

Several studies have been made on bone regeneration following artificial bone implant with the use of various experimental animals. In the present study, for the light microscopic and transmission electron microscopic study of the processes of bone regeneration around implanted TBC and TBC-collagen, three-walled bone defects having the most favorable conditions for bone regeneration were surgically created in rats having small individual difference and having periodontal tissue closely resembling that of human.

It is well known that the TBC obtained from bovine bone by sintering at 600°C and 1,100°C following the removable of lipid and protein has high biocompatibility and osteoconductivity unlike the kiel bone, a heterogenous bone material produced from bovine bone by protein removable. It has been reported that atelocollagen2) from which telopeptide occupying the large part of the
antigenecity of bovine type I collagen is removed by pepsin
digestion can elevate by itself the collagen productivity of
the wound site.\textsuperscript{21–23}

2) Process of bone regeneration around TBC
and TBC-collagen compound

In bone regeneration and bone remodeling, the old bone
is first resorbed by osteoclasts and then osteoblasts are
induced by eluted osteogenic factor. However, these
mechanisms of bone regeneration do not apply to auto-
grafts and artificial bone implants. The properties gener-
ally possessed by implant materials include ability of the
material itself to directly form new bone, osteoinductivity
of the implant material to differentiate the surrounding
undifferentiated mesenchymal-type cells into osteoblasts,
and osteoconductivity of the implant material supporting
the growth and proliferation of new bone from old bone.\textsuperscript{26}
The implant material having the ability of osteogenesis is
fresh bone marrow and the material possessing osteoind-
uctivity which has recently attracted much interest is
bone morphogenetic protein. Non-resorbable artificial
bone material such as HAP possesses osteoconductivity,
but a negative view toward osteoinductivity is predomi-
nant. With regard to TBC, a non-resorbable artificial
bone material, the possibility of elution of calcium phos-
phate from its surface has been reported as in the case of
HAP,\textsuperscript{29,33} but in view of an earlier report\textsuperscript{34} and also the
results of the present study, it is considered not to
possess osteoinductivity.

As for the mechanism of bone regeneration around TBC
and TBC-collagen, it is considered principally that the
course is similar to that of HAP. Bone regeneration
originates from the ability of osteogenesis possessed by
old bone and osteoclasts in first demineralize old bone and
the eluted osteogenic factor induces some of the osteo-
rogenitor cells of the bone marrow to form new bone
matrix with TBC.

Atelocollagen itself does not possess osteoinductivity,
but when compounded with various implant materials and
TBC, more calcification and formation of bone and
connective tissue attachment are promoted than by
atelocollagen alone.\textsuperscript{25–28,33–35} In this connection, stu-
dies reporting that it effectively serves in the attachment
or adhesion of cells to the matrix in the culture of
osteoblasts\textsuperscript{37,38} under the presence of collagen would be
of reference. It is speculated from the earlier findings
and the results of the present study that TBC compounded
atelocollagen facilitates the attachment of osteoblasts and
preosteoblasts originating from old bone to the implant
material.

3) MNCs around TBC and TBC-collagen

It is well known from the past that MNCs appear around
implanted autogenous bone and allogenic bone other than
fresh bone marrow.\textsuperscript{39–43} These MNCs are thought to be
foreign body giant cells inasmuch as MNCs are not
accompanied by clear zone and ruffled border and do not
possess tartrate-resistant acid phosphatase activity.\textsuperscript{43}
Furthermore, it has been reported that even in artificial
bone materials MNCs appear in both tricalcium phosphate
(TCP) and HAP, and that in TCP, a resorbable material,
MNCs play an important role in its resorption.\textsuperscript{44} On the
other hand, MNCs which appear around the implanted
HAP are similar to osteoclasts in ultrastructural and
cytological characteristics. Kawaguchi et al.\textsuperscript{39} have
observed that these MNCs might be involved in bone
regeneration around HAP as osteoclasts. MNCs ob-
served on the TBC-collagen surface in the present study
possess ultrastructural characteristics of osteoclasts of
showing clear zone and ruffled border and the possibility
is considered high that TBC-collagen surface is resorbed by
these MNCs. On the other hand, ruffled border which is
the characteristic of osteoclasts could not be observed in
MNCs which appeared around TBC in the present study
and in most of the cases cell processes having abundant
ruffled border-like vacuoles were found to be deeply
invaded into the pore of TBC. These characteristics of
MNCs have not been reported previously and it could not
also be ascertained from the results of the present study
whether these MNCs have resorbed the TBC surface.
However, inasmuch as the ultrastructural characteristics
of MNCs differ from those of foreign body giant cells, it is
considered that they are MNCs having osteoclast-like
function.

4) Interface between new bone and TBC and
TBC-collagen

Views differ whether an electron-dense granular layer is
present in the interface between implanted artificial bone
material and new bone. Ganeles et al.\textsuperscript{49} and Jarcho\textsuperscript{50}
have observed an electron-dense granular layer around
HAP and have speculated that it is a substance rich in
highly calcified mucopolysaccharide resembling the ce-
menting substance of bone. Stefllick et al.\textsuperscript{45}, Orly et
al. and Daculsi et al. have pointed out the possibility that it is an artificial product, as this structure could not be observed in the interface between bone and HAP in non-decalcified specimen. On the other hand, it has been ascertained that even in non-decalcified specimens fine apatite crystal differing from these are present between HAP and TCP and new bone and that there is a possibility that this is identical to the granular layer observed in decalcified specimen.

The interfacing structure closely resembles the granular layer observed in the border zone between new cementum and dentin and it has been suggested that it might be a type of binding glycoprotein in view of its histochemical characteristics. Furthermore, its similarity to lamina limitans of granular structure existing in the bone surface has also been reported and there is also a view that it is a cement line in the broad sense. A possibility has been suggested that this layer cytochemically contains osteopontin and that it is an important factor of osteogenesis.

5) Usefulness of TBC and TBC-collagen as implant materials

Though no direct comparison was made in the present study on the usefulness of TBC and TBC-collagen with HAP and TCP as other implant materials, but in the view of the results reported previously by other workers and the results of the present study, their usefulness in bone regeneration is similar to other implant materials. Furthermore, as TBC and TBC-collagen compound possess the required characteristics of implant materials having extremely high biocompatibility and osteoconductivity similar to TCP, HAP and their collagen compounds, much can be expected in their efficacy in clinical application.

CONCLUSION

In this study, the mechanism of bone regeneration around TBC and TBC-collagen compound particles in the rat periodontal defects was investigated ultrastructurally.

1) In the TBC group, MNCs covered the implant particles at day 7 after implantation and formation of new bone around the TBC particles took place at day 14. At day 28, almost half of the defect was filled with new bone. On the other hand, in the TBC-collagen group, the number of inflammatory cells were fewer in comparison to the TBC group. In this group, at day 7, MNCs also covered the implant particles and in one part, new bone began to form. At day 14, after resorption of the implant particles, formation of new bone substituted it. At day 28, a portion of the defect was filled with new bone.

2) In both the TBC and TBC-collagen groups, MNCs were enriched with vacuoles and mitochondria. In the TBC group, cytoplasmic processes penetrated deeply into the canaliculus of the implant particles. In the TBC-collagen group, typical ruffled border and clear zone structures were observed. These results suggested that MNCs are osteoclasts.

3) In the TBC group, TBC particles were covered and resorbed by MNCs. This was followed by the appearance of preosteoblasts and subsequently by the formation of new bone. However, in the TBC-collagen group, preosteoblasts sometimes became close to the implant particles without the participation of MNCs. This suggests that formation of new bone takes place directly to the TBC-collagen compound particles.

4) Regarding the interface between newly formed bone and implant particles, electron-dense, granular layers which were devoid of collagen fibrils were found in the TBC group. In the TBC-collagen group, cement line resembled resorption zone for bone repair and remodeling was observed. In this group, the thickness of the electron-dense, granular layer was smaller than in the TBC group. Many new fine collagen fibrils became attached to this layer.

It was demonstrated in this study that TBC and TBC-collagen compound may have osteoconductivity and affinity to living bone. TBC-collagen compound is a highly effective implant material for bone regeneration when compared to TBC alone.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to Professor Hiroshi Okamoto, Chairman of the Department of Endodontology and Periodontology, for his extraordinary guidance, timely decision, wholehearted cooperation and congenial attitude during my stay and study in Japan.

Sincere gratitude and appreciation are extended to Professor Hiromasa Nikai, Chairman of the Department of Oral Pathology and Professor Takashi Uchida, Chairman of the Second Department of Oral Anatomy, for their valuable suggestions and constructive criticisms during the preparation of this thesis.

Appreciation and gratitude are also extended to Associ-
ate Professor Masaharu Shirakawa for his kind advise, encouragement and help, to Assistant Professor Tetsuji Ogawa for his constant assistance and thoughtful support, to Drs. Hiroyuki Kawaguchi, Toshinobu Takemoto and Hiroshi Yoshino for their encouragement and cooperation, and to the other staff members of the Department of Endodontontology and Periodontology for their aid and cooperation during my study in this department.

REFERENCES


23) Minabe, M., Kogou, T., Kodama, T., Sugaya, A.,


47) Daculsi, G., LeGeros, R.Z. and Deudon, C.: Scanning and transmission electron microscopy, and
