セメント芽細胞及び歯周靭帯細胞株の樹立

（課題研究番号 11470378）

平成１１年度～平成１３年度科学研究費補助金（基盤研究(B)(2)）研究成果報告書

平成１５年３月

研究代表者　高田　隆

（広島大学歯学部教授）

はしがき

歯周靭帯には歯牙の支持や感覚，栄養などの機能に加えて，歯周組織の恒常性維持や組織再生に重要な役割が備わっている（図１）．最近，歯周靭帯由来細胞にのみ結合組織性付着形成能があることが明らかにされ，これに着目して，罹患根面に歯周靭帯細胞の増殖を誘導し結合組織性新付着獲得を目指した歯周組織再生療法（Guided Tissue Regeneration法）の開発や臨床への応用が進められつつある．GTR法はこれまでの治療法に較べて高い予知性を有する再生療法であるが，未だその適応には限界があり，より広い適応と高い予知性を備えた歯周組織再生療法の改良開発が期待されている（図１）．

また，失われた歯列の審美性ならびに機能回復に人工歯根が一般臨床にも広く応用されつつあるが，現行のインプラントシステムのいずれも，その支持様式は天然歯の歯周靭帯と異なり，歯周靭帯支持を有する理想的な次世代インプラントの開発が望まれる（図１）．我々はすでに人工歯根材料上に歯周靭帯細胞の増殖を誘導することにより人工材料上でもセメント質の添加と歯周靭帯線維の挿入が形成されることを証明しているが，歯周靭帯支持を有するインプラントシステム開発のためには，人工歯根材料上における歯周靭帯細胞の増殖分化機構の解明が必要である．

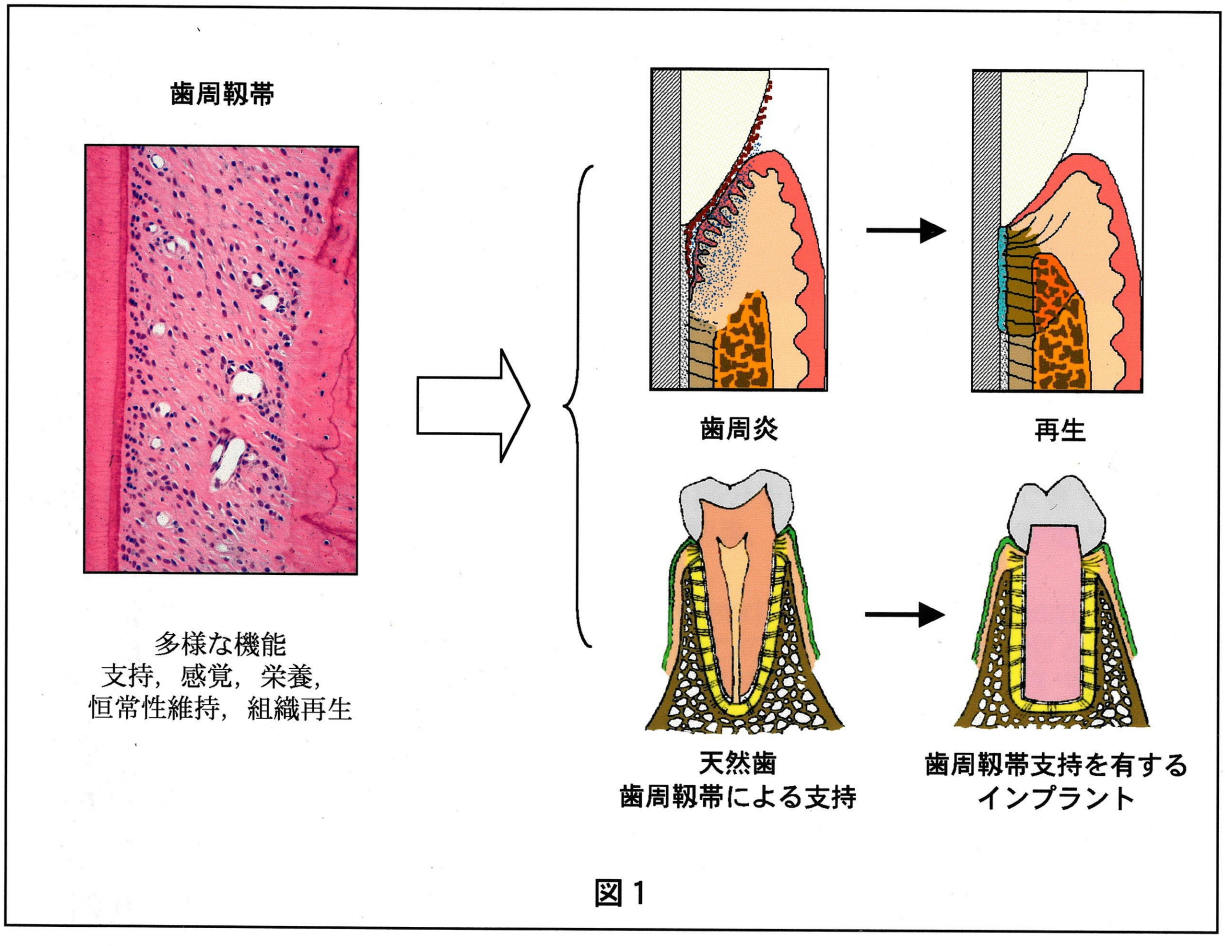


図１

そこで予知性のより高い再生療法や理想的インプラントの開発の根幹となる，歯周靭帯細胞の分化増殖制御機構の解明が必要不可欠である．しかし，歯周靭帯における細胞分化増殖機構の解明はこれまで歯周靭帯由来のheterogenousな細胞系を対象としたもので，均一な生物学的特性を有し，世界中の研究者が標準化された条件で研究可能な細胞株を用いた検討はなく，歯周靭帯における未分化な間葉細胞からセメント芽細胞に至る細胞分化や増殖の機構は未だ十分解明されていない．

一方，ヒト正常体細胞には分裂限界があり，自然不死化はめずらしく通常の培養法によって細胞株を樹立することは困難である．正常細胞が分裂可能回数を使い切る時期をmortality stage1 (M1期)と呼ぶが，通常，ヒト正常細胞では60回くらいでM1期をむかえる（図２）．DNA癌ウイルスであるSV40-TをtransfectすることによってM1期を越えさせると，さらに約20回程度分裂することが可能になるが，ほとんどの延命細胞はこの間に死滅する（M2期）（図２）．従ってげっ歯類などの細胞の不死化にしばしば用いるSV40-Tをtransfectするだけでは，ヒト組織由来細胞は不死化に至らない．

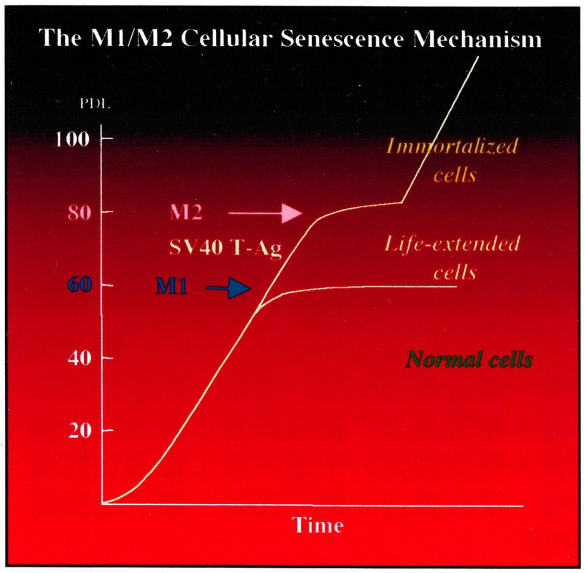


図２

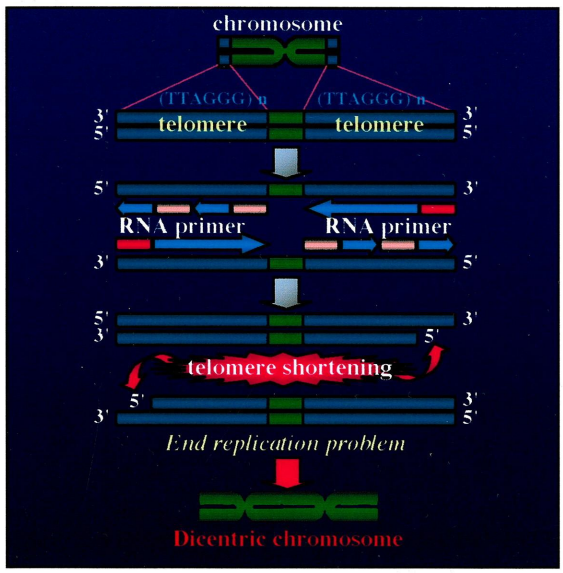


図３

ヒト正常体細胞が不死化しない理由は，染色体末端のテロメアDNAの消失にあると考えられている．DNA複製機構ではprimer担当部分を複製することができず，細胞分裂のたびにテロメアDNAの短縮が起こり，その結果，染色体DNAが不安定となって細胞増殖が不可能になると考えられている（図３）．

一方，無限増殖可能な胚細胞や癌細胞ではこのend-replication problemを解決する特殊機構が存在することが明らかとなっている．この機構はテロメラーゼと呼ばれる逆転写酵素によるもので，テロメアの3'末端を延長することができる．３'突出１本鎖DNAにテロメラーゼRNAが結合し，これを鋳型としてテロメラーゼ逆転写酵素（human teromelase transcriptase以下hTERT）によってテロメア塩基配列を連続的に合成することでテロメアを延長させる（図４）．よって，SV40-Tの導入によってＭ１期を，hTERTの導入でＭ２期を越えさせることで，ヒトの正常体細胞や良性腫瘍由来の細胞も不死化すると考えられる（図５）．

そこで，本研究では，まず歯周靭帯に存在する細胞のheterogeneityを明らかにし，ついでセメント芽細胞，前セメント芽細胞，歯周靭帯線維芽細胞，未分化間葉系細胞などの分化段階の異なる歯周靭帯構成細胞のクローニングを行ない，歯周靭帯における細胞分化や増殖の機構を解明するための細胞株をSv-40-TやhTERTの導入によって樹立することを目的とした．

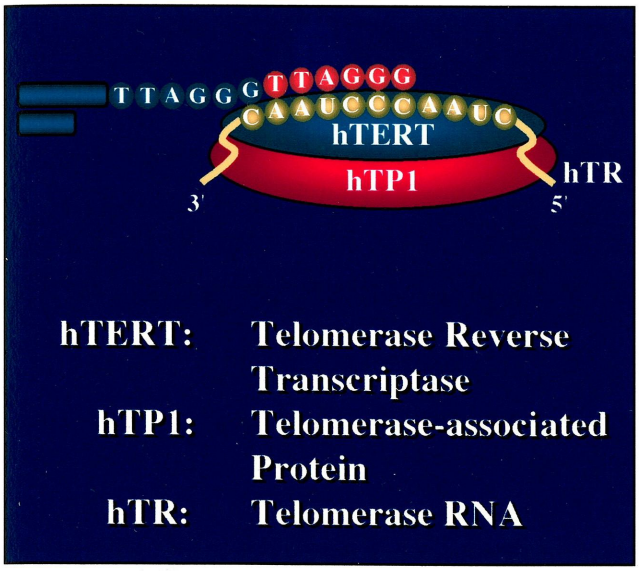


図4

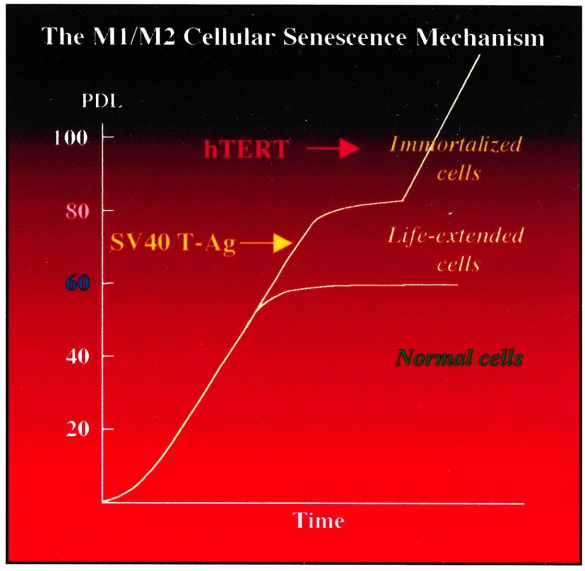


図5

研究組織

研究代表者：高田　隆（広島大学・歯学部・教　授）
研究分担者：宮内睦美（広島大学・歯学部・助教授）
研究分担者：小川郁子（広島大学・歯学部附属病院・講　師）
研究分担者：工藤保誠（広島大学・歯学部・助　手）
在外研究員出張により平成13年1月30日まで参加
研究分担者：佐藤　淳（広島大学・歯学部・助　手）
平成13年4月1日より工藤に代わって参加

研究経費

交付決定額（配分額）　（金額単位：千円）

	直接経費	間接経費	合　計
平成11年度	4,300	0	4,300
平成12年度	3,700	0	3,700
平成13年度	3,600	0	3,600
総　計	11,600	0	11,600

研究発表

1）学会誌発表

(1) Kaneda T., Miyauchi M., Takekoshi T., Kitagawa S., Kurihara H., Takata T. Characteristics of periodontal ligament regional cell subpopulations obtained by sequential enzymatic digestion of rat molar periodontal ligament. J Dent Res (submission), 2002
(2) Takata T., Kudo Y., Kitagawa S., Hiraoka M., Zhao M., Kakuo S., Ide T. Establishment of human cementifying fibroma cell lines by transfection with temperature-sensitive SV40 T-antigen gene and hTERT gene. J Dent Res 81 (special issue), 782, 2001
(3) Kudo, Y., Hiraoka, M., Kitagawa, S., Miyauchi, M., Kakuo, S., Zhao, M., Ide, T., Takata, T.: Establishment of human cementifying fibroma cell Lines by transfection with temperature-sensitive SV40 T-antigen gene and hTERT gene. Bone 30, 712-717, 2002
(4) Takata T., Hiraoka M., Sato S., Kitagawa S., Tahara H., Ide T. Characterization and immortalization of cementum lining cells. J Dent Res 81 (special issue A), 359, 2002

2) 口頭発表
(1) Takata T., Kudo Y., Kitagawa S., Hiraoka M., Zhao M., Kakuo S., Ide T. Establishment of human cementifying fibroma cell lines by transfection with temperature-sensitive SV40 T-antigen gene and hTERT gene. 79th General Session of the International Association of Dental Research, 2001, June 27-30
(2) Takata T., Hiraoka M., Sato S., Kitagawa S., Tahara H., Ide T. Characterization and immortalization of cementum lining cells. 80th General Session & Exhibition of International Association for Dental Research, 2002, March 6-9
(3) 北川尚嗣, 平岡雅恵, 佐藤　淳, 小川郁子, 宮内睦美, 高田　隆:歯周靭帯由来細胞の増殖ならびに分化に対するエナメルタンパク（エムドゲイン®）の影響-連続酵素消化法によって分取した細胞亜群を用いた検討-, 第45回春季日本歯周病学会学術大会, 2002年4月25日, 26日
(4) Hiraoka M., Kitagawa S., Sato S., Tahara H., Ide T., Miyauchi M., Takata T. Establishment of human periodontal ligament cells by infection with htert gene. 第43回日本生化学会中国・四国支部例会, 2002年, 7月19, 20日
(5) 平岡雅恵, 工藤保誠, 北川尚嗣, 斎藤彰久, 佐藤　淳, 小川郁子, 宮内睦美, 井出利憲, 高田　隆. 温度感受性SV-40T抗原およびhTERT遺伝子の導入によるヒトセメント質形成線維腫由来細胞株の樹立. 第13回日本口腔病理学会総会・学術大会, 2002年8月23, 24日
(6) 平岡雅恵, 北川尚嗣, 齊藤彰久, 佐藤　淳, 小川郁子, 宮内睦美, 田原英俊, 井出利憲, 高田　隆：hTERT遺伝子導入によるヒト歯根膜細胞株の樹立, 第39回日本口腔組織培養学会, 2002年11月9日

研究成果

1．細胞株樹立のための歯周靭帯に存在する細胞のheterogeneityに関する解析

材料と方法
1）歯周靭帯細胞の採取
（1）ヒト：矯正的便宜抜去歯あるいは埋伏智歯など健全歯周靭帯のある歯牙を収集し, 細胞採取を試みた.
（2）ラット：第３臼歯まで萌出の完了した８週齢Wistarラットから上下顎臼歯を抜歯し, 細胞採取を試みた.
（3）マウス：p53ノックアウトマウスから上下顎臼歯を抜歯し, 細胞採取を試みた.

2）歯周靭帯由来細胞培養法
（1）通常の培養法による歯周靭帯細胞mix populationの培養
抜去歯牙を培養プレート上に静置し, 歯根に付着した歯周靭帯からの outgrowthを継代した.
（2）連続酵素処理による歯周靭帯細胞regional cell subpopulationの培養
抜去歯牙をトリプシン/コラゲナーゼ混合酵素液で消化し, 歯周靭帯から遊離した細胞を経時的に採取し継代した.

3）歯周靭帯由来細胞のheterogeneity解析
（1）増殖能の解析
（2）石灰化能の解析
石灰化関連蛋白の発現や石灰化塩産生能を検索し，歯周靭帯由来培養細胞の分化の多様性を明らかにした.

4）歯周靭帯細胞subpopulationに対するエナメルタンパクの影響
歯周靭帯細胞subpopulationの特性を明らかにする一環として，セメント質形成に関与することで注目されているエナメルタンパクの歯周靭帯細胞subpopulationの増殖分化に対する影響を検討した.

結果
1．ヒトおよびラットの抜去歯に付着する歯周靭帯から，0.2%コラゲナーゼと0.25%トリプシンの混合液による連続処理によって，歯周靭帯からregional cell subpopulationを得る方法を確立した.
2．それぞれの細胞群の有する性状はoutgrowthからmixpopulationとして得られた細胞集団とは異なる性状を示した.
3．連続酵素消化法によって分画された細胞群間で，細胞増殖能や石灰化能に差があり，増殖能の高い細胞はPDL中央側に，石灰化能の高い細胞は歯根表面近くに存在することが明らかとなった.
4．歯根表面に接して高い増殖能と石灰化能を兼ね備えた細胞群が存在することが明らかにされた.

以上の結果は，論文としてまとめ，現在，Journal of Dental Researchに投稿中である（文献1参照）.

5．p53ノックアウトマウスから抜去した歯牙に付着する歯根膜からの継代培養を試みたが，明らかなoutgrowthが観察されず，以降の検討ができなかった．再度，検討を重ねようと考えたが，同マウスを独自に繁殖させることが供給元との契約上不可能であり，細胞採取の度に海外からの直接購入を余儀無くされた．そこで，再検討のための必要経費等を考慮した結果，さらなる検討は困難と考えた.

6．連続酵素消化法によって分取した歯周靭帯細胞subpopulationを用いて，歯周靭帯由来細胞の増殖ならびに分化に対するエナメルタンパクの影響を検討した結果，エナメルタンパクは検索したすべての歯周靭帯細胞subpopulationの増殖を濃度依存性に促進したが，その促進作用は１５０分＞９０分＞３０分の順で，歯面に近い歯周靭帯細胞subpopulationほど，大きい傾向を示した（図6）.
また，エナメルタンパクはすべての歯周靭帯細胞subpopulationにおいて，そのアルカリフォスファターゼの活性を増強した．とりわけ，歯面に近いセメント芽細胞を含む１５０分群で高い促進効果が認められた（図7）.
アルカリフォスファターゼの結果と良く一致して，石灰化能に対してもいずれの歯周靭帯細胞subpopulationともに，エナメルタンパクは濃度依存性に促進した．コンフルエント後２１日目では１５０分群にのみ石灰化がみられたのに対し，２８日目では９０分群にも石灰化が認められた（図8）.
さらに石灰化関連タンパクの発現でも，エナメルタンパクはまず１５０分群に，続いて９０分群にbone sialoproteinやosteocalcinの発現を誘導した（図9）.

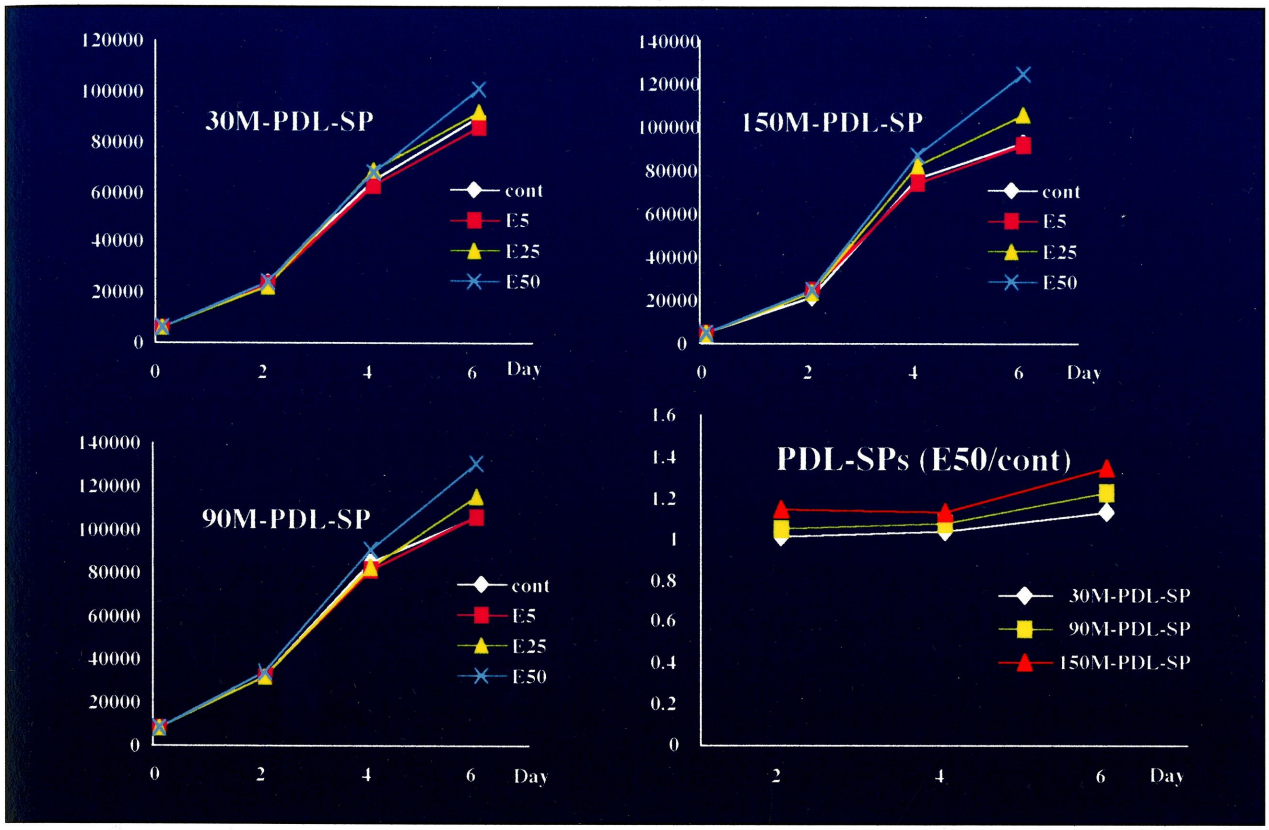

30M-PDL-SP
150M-PDL-SP
90M-PDL-SP
PDL-SPs (E50/cont)
cont
E5
E25
E50
30M-PDL-SP
90M-PDL-SP
150M-PDL-SP
Day

図 6

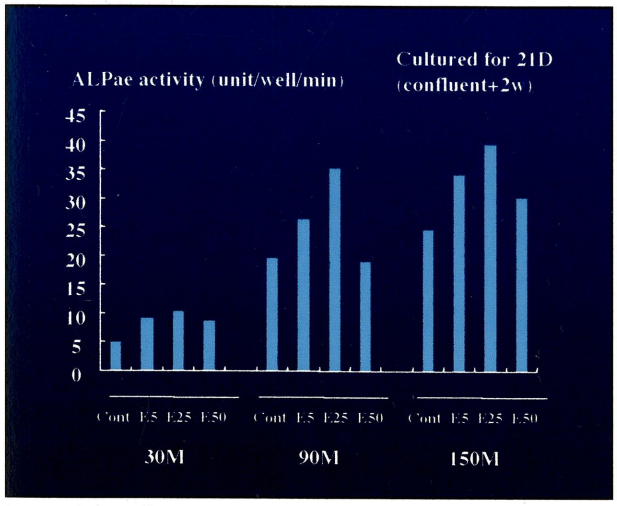

ALPae activity (unit/well/min)
Cultured for 21D
(confluent+2w)
Cont E5 E25 E50
30M
90M
150M

図 7

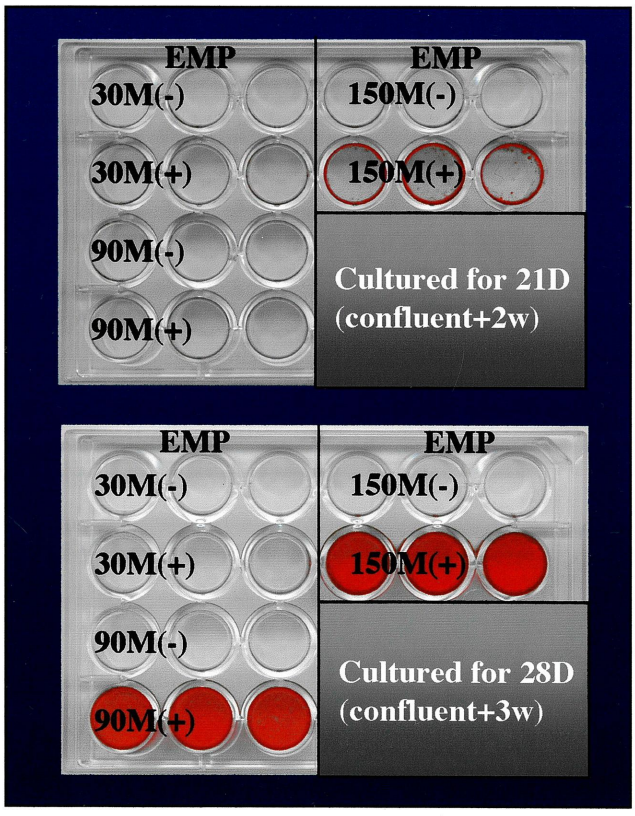

EMP
EMP
30M(-)
150M(-)
30M(+)
150M(+)
90M(-)
90M(+)
Cultured for 21D
(confluent+2w)
Cultured for 28D
(confluent+3w)

図 8

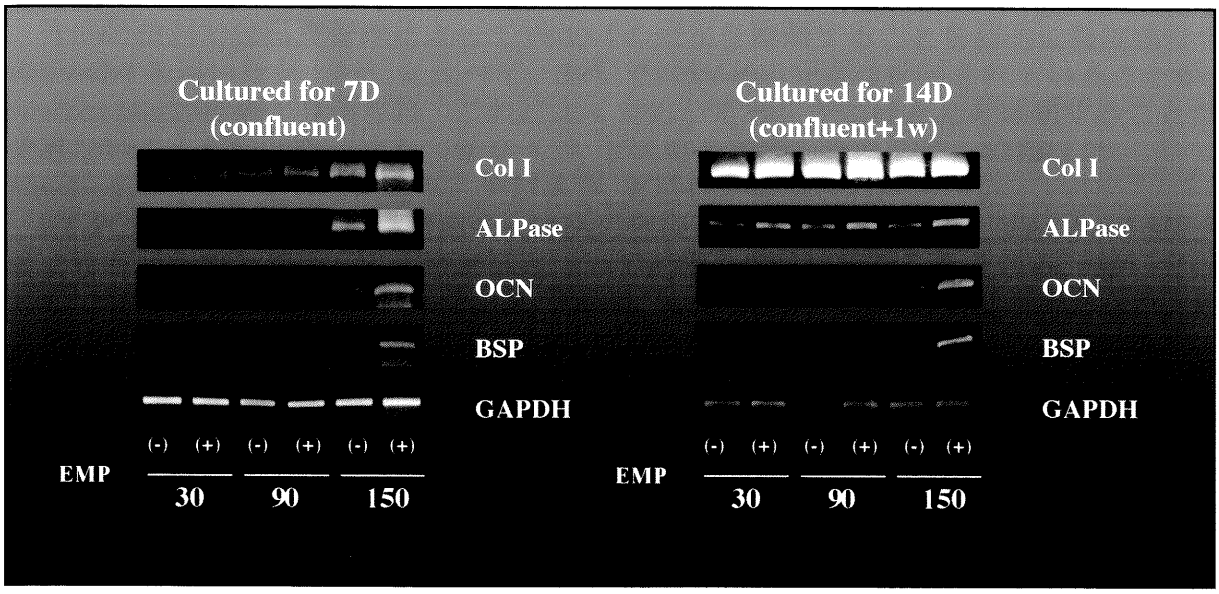


図９

２．歯周靭帯細胞のクローニング法に関する検討

材料と方法
１）ペニシリンカップ法による細胞分離の検討
２）96穴プレートを用いた限界希釈法による細胞分離の検討
３）磁気細胞分離システムによる細胞分離の検討

結果
ペニシリンカップ法ではクローンを比較的容易に得ることができたが，96穴プレートを用いた限界希釈法では，歯周靭帯細胞の増殖能が低いため十分に細胞が増殖せず，クローンを得ることができなかった．磁気細胞分離システムによる細胞分離法は細胞表面抗原に対する抗体を利用した分離法である．そこで，本研究ではアルカリフォスファターゼに対する抗体を用いて分離を試みたが，発現量は異なるものの各細胞subpopulationともに同酵素を多少なりとも発現し，効率良く細胞を分離することができなかった．従って，以降の検討には，ペニシリンキャップ法による細胞クローン分取法を用いることにした．

３．ヒトセメント質形成性線維腫からの歯周靭帯由来細胞株の樹立

ヒト歯周靭帯細胞株の樹立を目的に新鮮材料を得る機会に恵まれた歯周靭帯由来とされるヒト cementifying fibroma (以下HCF) から分離した細胞にSV40-TおよびhTERT geneを遺伝子導入し，その細胞特性を解析した．
SV40-T導入により5つのcloneを，さらにhTERTを導入して28のcloneを得ることができた．腫瘍は無細胞性セメント質に類似した石灰化物を多量に含む線維性結合組織からなっており，HCF組織断片からは紡錘形の細胞増殖が観察された．SV40-Tを導入しても分裂回数はoriginalとほとんど変わらず約４０代で増殖を停止したが，さらにhTERTを導入すると分裂回数は４００にいたり株化に成功した．

今回の検討では温度管理によってSV40-Tの発現を制御することを目的に温度感受性のSV40-Tを用いたが，33℃に比べて，39℃ではSV40の発現は抑制されていることがwestern-blotの結果より示され，これによく対応して39℃ではほとんど増殖することができないことが分った．また、テロメラ_ゼ活性を確認したところ，hTERTのtransfectionを行ったものにのみ，テロメラ_ゼ活性が確認された．

RT-PCRにより石灰化関連蛋白のmRNAの発現を，ALPase活性およびALZ red-S染色により石灰化能とその石灰化物形成について組織化学的に検討したところ，39℃では33℃で発現のみられるType I collagen，OPNに加えてBSP，OCNの発現が認められ，石灰化のためのsupplementsを添加するとALP活性や石灰化もみられた．とくにHCF–C9株では33℃でもBSPやOCNの発現が認められ，HCF-C9はcementoblastないしosteoblastとしての高い分化能を有するcell lineであることが示唆された．以上のことから，１）HCF細胞株がin vitroにおけるヒトのPDL細胞の分化や　増殖のメカニズムの研究に優れたものであること．２）SV40-TとhTERTのプラスミドによるtransfectionが良性腫瘍やヒト非腫瘍性組織から不死化細胞株を樹立するのに有用であることが示唆された．

以上の結果は，論文としてまとめ，J.ournal of Dental Research 81 (special issue), 782, 2001ならびにBone 30, 712-717, 2002に掲載された（巻末添付文献参照）．

４．ラット歯周靭帯からのセメント芽細胞，歯周靭帯線維芽細胞株に樹立

材料と方法

歯周靭帯の採取とsubpopulationの分離

８週齢ラット臼歯部歯周靭帯から，連続酵素法によって歯周靭帯subpopulationを採取し，培養した（図１０）．

SV-40Tの導入とクローンの選択

得られた歯周靭帯細胞subpopulationのうちセメント質表面をおおう細胞（セメント芽細胞）に富む分画である110分処理群に，SV-40Tを導入した．400mg/μlのG418で処理を行いペニシリンカップ法で４つのクローンを得た．細胞はsubconfluent～confluentで1:8の割合で継代を行った（図１１）．

細胞特性に関する検索

まず，細胞増殖と形態の観察を行い，次にアルカリフォスファターゼ活性や石灰化関連タンパクの発現についてRT-PCRを行った．

結果

SV-40Tを導入していないoriginalの細胞では，増殖は20代で止まったが，導入細胞では100代を越えて増殖を続けた（図１２）．細胞の形態は，population doublings 100の時もoriginalの細胞形態とほとんど違いはなかった．

いずれの細胞株も高いアルカリフォスファターゼ活性と石灰化能を示した．またType I collagen，アルカリフォスファターゼ，osteopontin，osteocalcinの明らかな発現が観察された（図１２）．

以上のことからこれらの細胞株がin vitroにおけるセメント芽細胞の増殖や分化のメカニズムを検討する際の優れた細胞株になりうるものと考えられた．

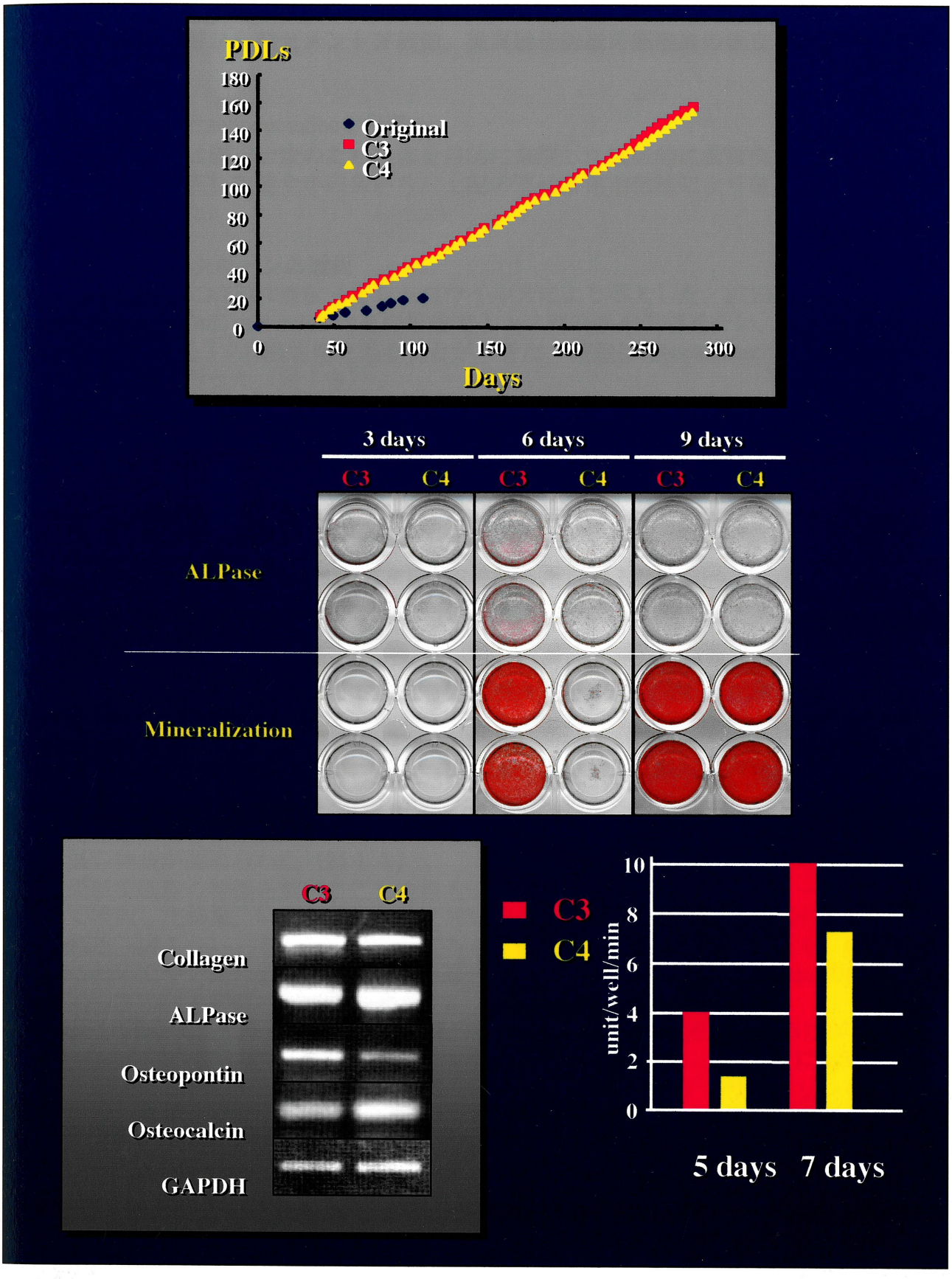

PDLs
180
160
140
120
100
80
60
40
20
0
Original
C3
C4
0
50
100
150
200
250
300
Days
3 days
6 days
9 days
C3
C4
C3
C4
C3
C4
ALPase
Mineralization
C3
C4
Collagen
ALPase
Osteopontin
Osteocalcin
GAPDH
C3
C4
unit/well/min
10
8
6
4
2
0
5 days
7 days

図１２

5．ヒト歯周靱帯からのセメント芽細胞，歯周靭帯線維芽細胞株の樹立

材料と方法

歯周靭帯の採取とsubpopulationの分離

歯周靭帯細胞は矯正治療のため抜歯を目的に来院した臨床的に歯周疾患の認められない患者から抜去した健全小臼歯から，上記の連続酵素消化法により歯周靭帯細胞を採取し，培養した．

HTERTの導入とクローンの選択

採取した細胞にhTERT遺伝子をレトロウイルスにより導入した．hTERTを導入したPDL細胞を0.3ug/mL puromycin含有の培地で１～２週間薬剤処理を行い，puromycin耐性のPDL細胞から3つの細胞株を得ることができた．細胞はsubconfluent～confluentで1:8の割合で継代を行った（図１３）．

細胞特性に関する検索

まず，細胞増殖と形態の観察を行い，次にTRAP assayによりテロメラーゼ活性の測定およびサザン解析によりテロメア長の測定を行った．

アルカリフォスファターゼ活性を組織化学的および酵素化学的に測定した．

石灰化関連タンパクであるtypeI collagenやアルカリフォスファターゼおよびbone sialoproteinのmRNAの発現についてRT-PCRを行った（図１３）．

結果

hTERTを導入していないoriginalの細胞では，増殖は60代で止まったが，hTERTを導入した細胞では２００代を越えて増殖を続けた（図１４）．

細胞の形態は，population doublings 100の時もoriginalの若いころの形態とほとんど違いはなかった（図１４）．

TRAP assayではhTERTを導入した細胞にのみはっきりとしたラダ_が観察され，高いテロメラーゼ活性を有していることが確認された（図１４）．

TRF length assayではTRAP assayの結果とよく一致してhTERTを導入した細胞にテロメア長の伸長が認められた（図１４）．

歯周靭帯細胞の特性を調べることを目的に行ったアルカリフォスファターゼ活性について検討した結果，いずれの細胞株においてもoriginalの細胞と同様の陽性反応が認められた（図１５）．Bessey-Lowry法で行った酵素化学的測定による結果も，C2，C3細胞においてやや高い値を示したが，組織化学的検索結果と一致してoriginalの細胞とほぼ同程度の活性が認められた（図１５）．

Type I collagen，アルカリフォスファターゼの発現にほとんど差はなかったが，bone sialoproteinにおいてはC2細胞に若干の発現が観察された．しかし，培地中にrhBMP-2や石灰化促進添加物であるアスコルビン酸，βグリセロリン酸，デキサメタゾンを添加して培養した場合には，どの細胞においてもbone sialoproteinの強い発現が観察された（図１６）

以上のことから（1）これらのヒトPDL細胞株がin vitroにおけるヒト歯周靭帯細胞やセメント芽細胞の増殖や分化の機構解明に有益であること．（2）hTERT geneのウイルスによるinfectionがヒトの非腫瘍性組織から不死化細胞株を樹立するのに有用なものであることが示唆された．

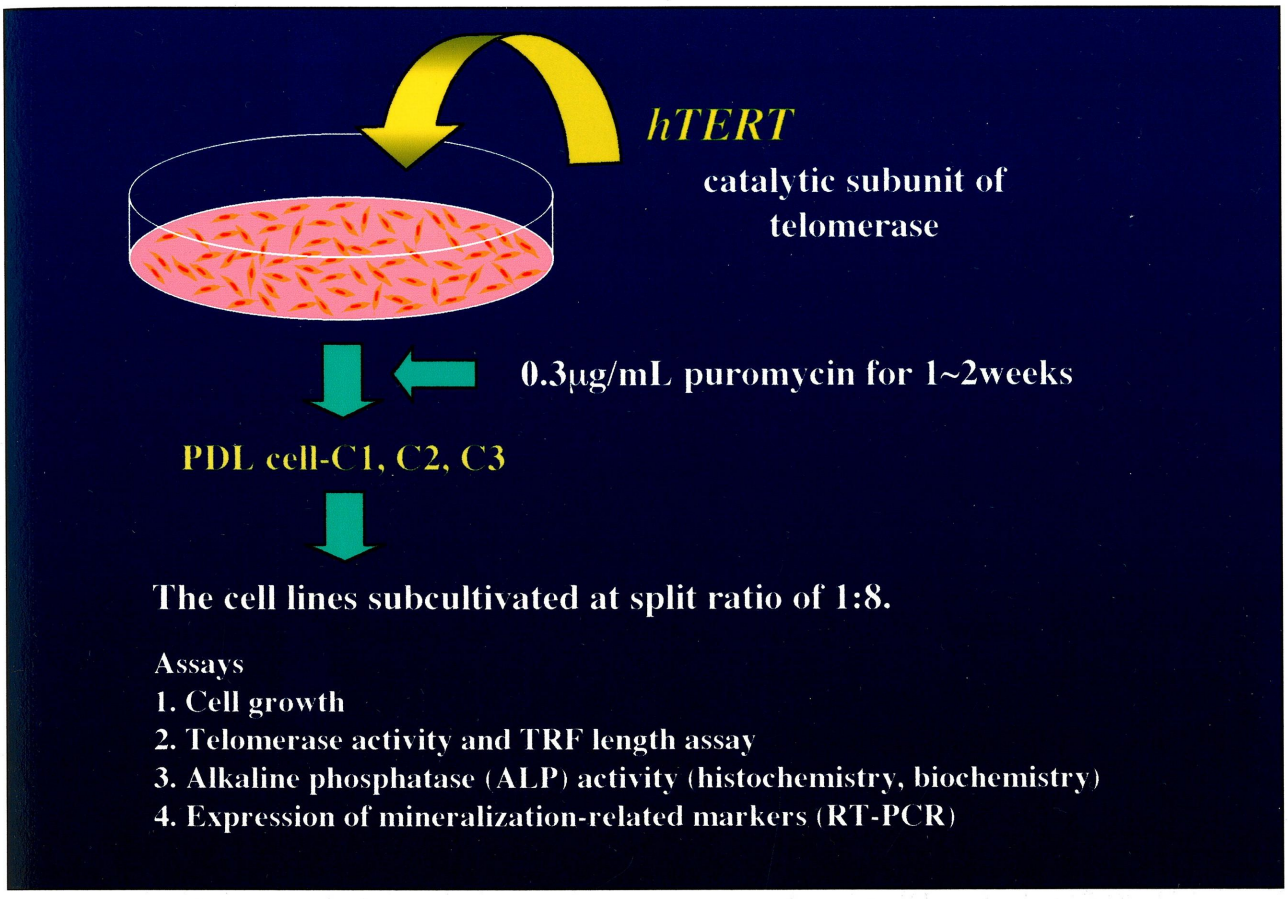

hTERT
catalytic subunit of telomerase
0.3μg/mL puromycin for 1~2weeks
PDL cell-C1, C2, C3
The cell lines subcultivated at split ratio of 1:8.
Assays
1. Cell growth
2. Telomerase activity and TRF length assay
3. Alkaline phosphatase (ALP) activity (histochemistry, biochemistry)
4. Expression of mineralization-related markers (RT-PCR)

図１３

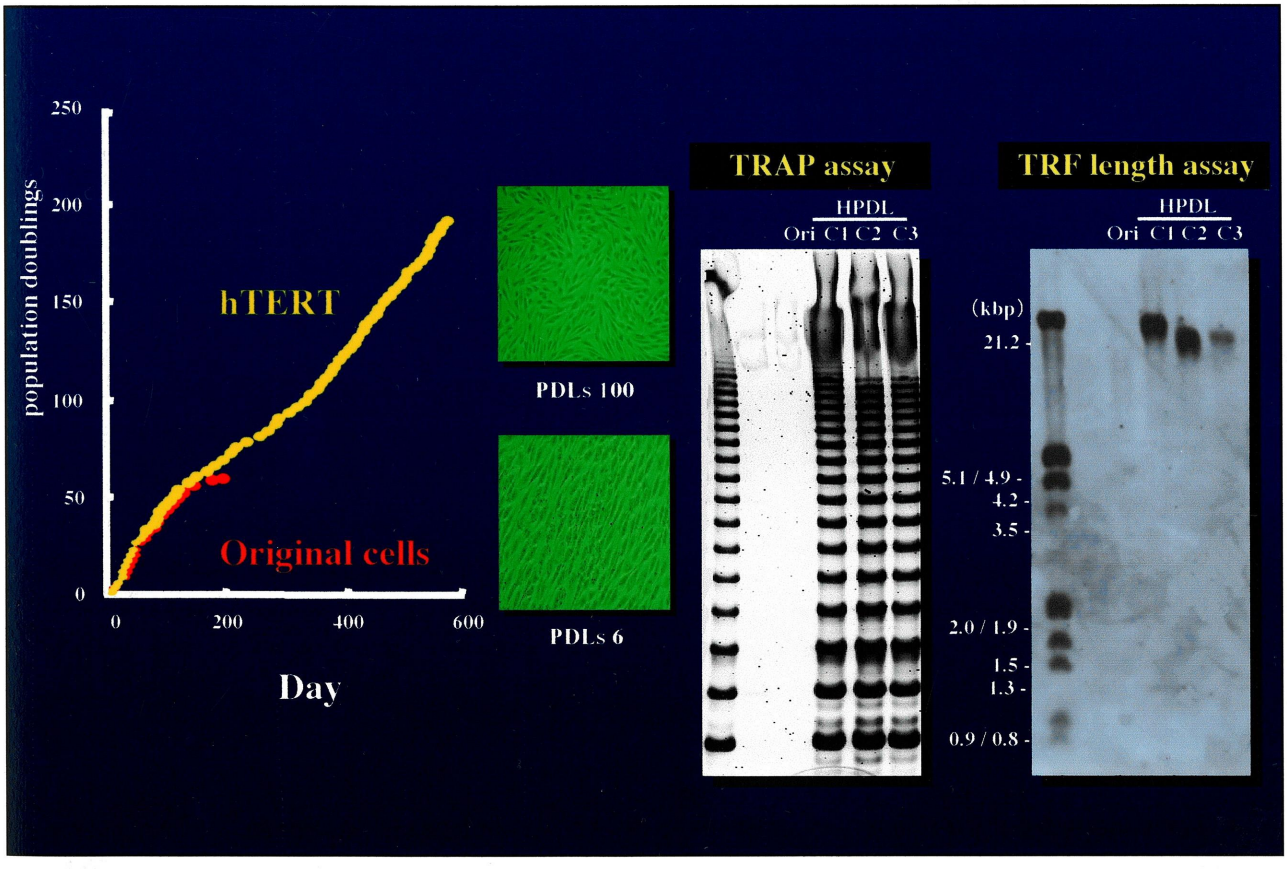

population doublings
250
200
150
100
50
0
hTERT
Original cells
0
200
400
600
Day
PDLs 100
PDLs 6
TRAP assay
HPDL
Ori C1 C2 C3
TRF length assay
HPDL
Ori C1 C2 C3
(kbp)
21.2
5.1 / 4.9
4.2
3.5
2.0 / 1.9
1.5
1.3
0.9 / 0.8

図１４

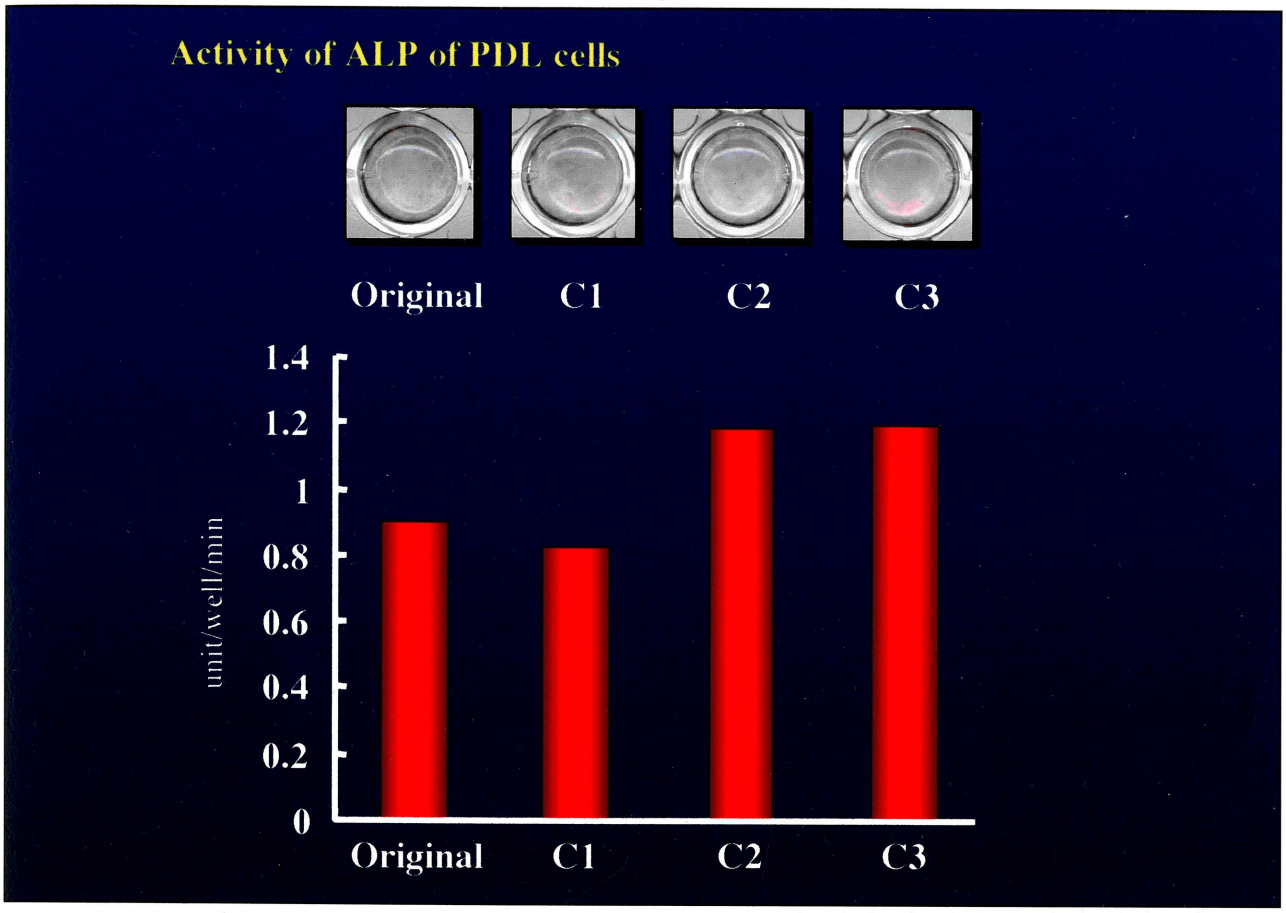

Activity of ALP of PDL cells
Original
C1
C2
C3
1.4
1.2
1
0.8
0.6
0.4
0.2
0
unit/well/min
Original
C1
C2
C3

図１５

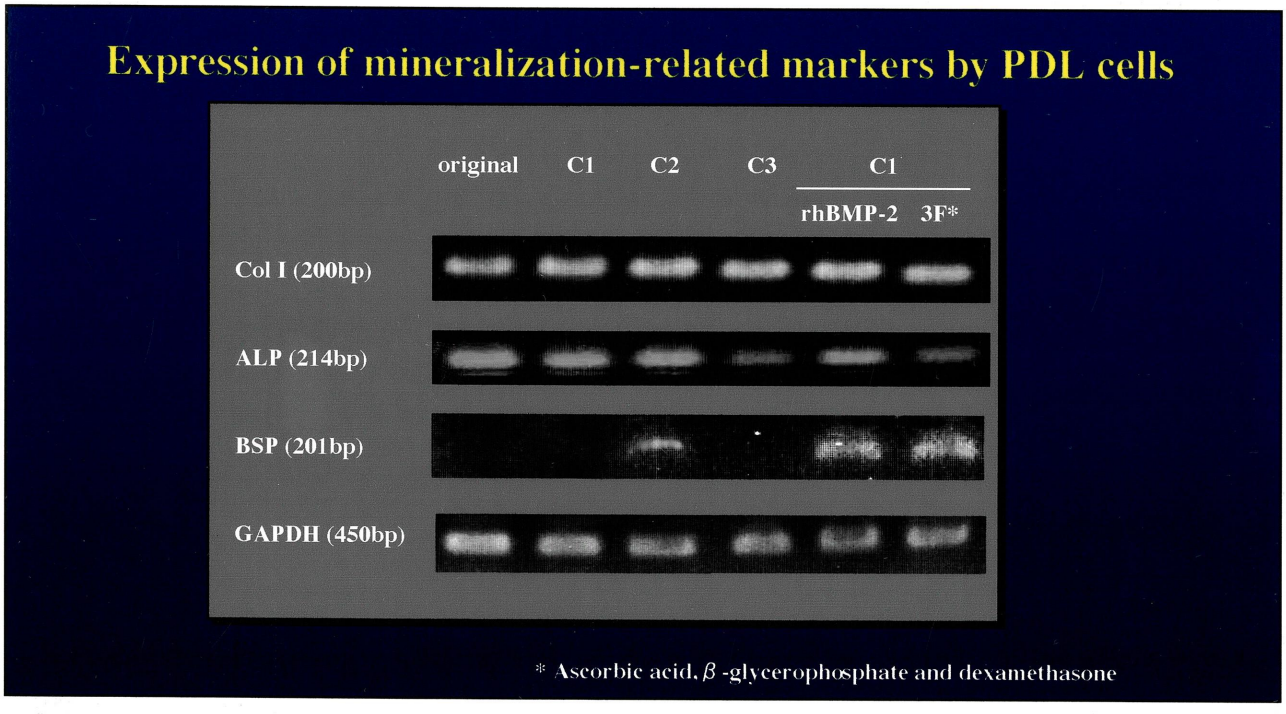

Expression of mineralization-related markers by PDL cells
original
C1
C2
C3
C1
rhBMP-2
3F*
Col I (200bp)
ALP (214bp)
BSP (201bp)
GAPDH (450bp)
* Ascorbic acid, β -glycerophosphate and dexamethasone

図１６

あとがき

本研究は歯周靭帯に存在する細胞のheterogeneityを明らかにし，ついで歯周靭帯構成細胞のクローニングを行ない，歯周靭帯における細胞分化や増殖の機構を解明するための細胞株を樹立することを目的にした．その結果，ヒトおよびラット歯周靭帯から歯周靭帯細胞およびセメント芽細胞株を樹立するとともに，歯周靭帯由来の腫瘍であるセメント質形成性線維腫からも細胞株を樹立することに成功した．本研究によってこれらの細胞をひろく他の研究者に分与供給することが可能となり，今後，標準化された細胞株での検討を通して，歯周組織再生，次世代人工歯根システムの開発，歯牙移植，再植といった歯周靭帯の重要な役割を演じるが領域の研究に大いなる発展があることを期待する．

Characteristics of Periodontal Ligament Subpopulations Obtained by Sequential Enzymatic Digestion of Rat Molar Periodontal Ligament

T. Kaneda[1,2], M. Miyauchi[1], T. Takekoshi[1], S. Kitagawa[1], H. Shiba[2], H. Kurihara[2], T. Takata[1,*]

[1]Department of Oral Pathology and [2]Department of Endodontology and Periodontology, Hiroshima University Faculty of Dentistry, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan.

RUNNING TITLE: Characteristics of PDL Subpopulations

KEY WORDS: periodontal ligament, proliferation, mineralization, sequential enzymatic digestion

Corresponding author:
*Takashi Takata, D.D.S., Ph.D
Department of Oral Pathology, Hiroshima University Faculty of Dentistry
1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan.
Tel.+81 82 257 5633; Fax +81 82 257 5619
e-mail: ttakata@hiroshima-u.ac.jp

ABSTRACT

Periodontal ligament (PDL) consists of different cell populations in various differentiation stages. In the present study, we isolated cell populations from rat molar PDL by sequential enzymatic digestion and characterized growth potential and mineralization activity of the PDL subpopulations (PDL-SP) to throw light on the mechanism of PDL remodeling and, in its turn, periodontal tissue regeneration. PDL attached to extracted rat molars were digested 2 mg/mL collagenase and 0.25 % trypsin at 37 ℃ for 30 min. Then, four consecutive digestions were performed for 20 min each in a fresh digestive solution. The solutions were centrifuged to collect released cells and 5 PDL subpopulations (30M-, 50M-, 70M-, 90M- and 110M-PDL-SP) were obtained. Light microscopic observation showed that about a half of PDL in width attached on the root surface of extracted teeth and 30M-PDL-SP was considered to contain cells mainly from middle portion of PDL. Scanning electron microscopic examination indicated that the 110M-PDL-SP was enriched by root lining cementoblastic cells. 30M-PDL-SP showed a high level of a proliferative activity. Although the growth potential of a subpopulation decreased in PDL-SP toward the root surface, 110M-PDL-SP had a high proliferative activity equivalent to that of 30M-PDL-SP. Analyses of ALP and mineralization activities showed that higher activities in PDL-SP toward the surface of roots and that 110M-PDL-SP had the highest activity of ALP and the largest number of mineralization nodules. As supposed by previous studies on cell kinetics in PDL, subpopulations with larger growth potential were generally located in the middle portion of PDL and those with higher mineralization activities toward the surface of the roots. However, the present study suggested that a possible pathway of PDL cell turnover may exist within the PDL-SP on the root surface in addition to the generally recognized pathway from the middle area of PDL to root surface.

INTRODUCTION

Periodontal ligament (PDL), a thin connective tissue between root cementum and alveolar bone, plays important roles such as supportive, sensory, nutritive and homeostatc functions (Freeman, 1994; Carranza and Ubious, 1996). Recent studies showed, furthermore, that the formative function of PDL is indispensable to consider regenerative periodontal therapies (Freeman, 1994; Carranza and Ubious, 1996).

It has been reported that PDL cells have osteoblastic characters, such as expressing high activity of alkaline phosphates (ALP) (Giannopoulou and Cimasoni, 1996; Ogata *et al.*, 1995), synthesizing cAMP at the stimulation of parathyroid hormone (Somerman *et al.*, 1990), producing mineralized nodules *in vitro* under appropriate conditions (Ramakrishnan *et al.*, 1995), and producing mineralized tissue related non-collagenous proteins such as osteopontin, osteocalcin, bone sialoprotein (Ramakrishnan *et al.*, 1995; Noutcu *et al.*, 1997). In these studies, however, PDL was examined as a tissue unit and PDL-derived cells were characterized in the lump.

McCulloch *et al.* (1983; 1987) reported that progenitor cells in marrow spaces migrate into PDL by way of vascular channels and locate perivascularly. The progenitor cells then provide daughter cells that migrate to the bone and

cementum surface and differentiate into cementoblasts or osteoblasts. Perivascular cells also considered to be the source of PDL fibroblasts. These findings suggest that PDL consists of different cell populations in various differentiation stages according to the position in PDL.

Sequential enzymatic digestion has been applied to obtained the different cell populations from bone tissue. Wong and Cohn obtained 6 subpopulations of bone cells by sequential digestion of mouse calvaria with collagenase and trypsin (Wong and Cohn, 1975). They showed that bone contains at least two types of target cells with different response to parathormone and calcitonin. Rao et al. also showed that cell populations obtained from rat calvaria by the same method had different response to parathyroid hormone or prostaglandin E1(Rao *et al.*, 1977).

In the present study, therefore, we reported the isolation of cell populations from rat molar PDL by sequential enzymatic digestion and characterized the growth potential and mineralization activity of PDL subpopulations (PDL-SPs) to throw light on the mechanism of PDL remodeling and, in its turn, periodontal tissue regeneration.

MATERIALS AND METHODS

Isolation of PDL Subpopulations and Cell Culture

The protocol of the studies was approved by the experimentation committee of the Faculty of Dentistry, Hiroshima University. After sacrificing by an overdose of chloroform, molars were extracted from 10 Lewis male rats (8-week old, 220-250 g). To avoid contamination of gingival tissues, supracrestal soft tissues attached to the cervical area of the molars were carefully curretaged before the extraction. The extracted molars with PDL were rinsed once in Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS, NISSUI PHARMACEUTICAL CO., LTD., Tokyo, Japan). Then, they were immersed in a digestion solution, 20 mL of Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, N.Y, USA) containing 2 mg/mL collagenase (Wako, Tokyo, Japan) and 0.25 % trypsin (DIFCO LABORATORIES, MI, USA), at 37 ℃ for 30 min (D'Errico et al., 1997; MacNeil *et al..*, 1998). After that, four consecutive digestions were performed for 20 min each in a fresh solution. The solutions were centrifuged to collect released PDL cells. In this way, 5 PDL-SPs were obtained and named 30M-, 50M-, 70M-, 90M- and 110M-PDL-SP, respectively. The cells in each subpopulations were then cultured in DMEM with 10 % fetal bovine serum (FBS) plus penicillin G sodium (10 unites / mL) and streptomycin sulfate (10 mg / mL) in a humidified atmosphere of 5 % CO_2 at 37 ℃. The culture medium was changed once three days and cells at the third to fifth passages were used in following studies.

Light Microscopic Observation

To examine the amount and quality of PDL attached to the root surface of extracted molars and the effect of sequential enzymatic digestion of the PDL, light microscopic observations with conventional and phase contrast microscopes were performed on some extracted teeth before and after the enzymatic digestion. For hematoxylin and eosin sections, the teeth were fixed in a 10 % formaldehyde neutral buffer solution overnight, decalcified in a 10 % EDTA solution (pH 7.4) for 2 weeks at 4 ℃ and cut into paraffin embedded 4.5-micrometer section routinely.

Scanning Electron Microscopic Observation

To observe the surface condition of digested PDL and root surface, each 3 teeth were prepared for scanning electron microscopy (SEM) at each digestion period. After gentle wash with PBS, the teeth were fixed in a 0.1 M cacodylate-buffered 2 % paraformaldehyde and 2.5 % glutaraldehyde solution (pH 7.4, 4 ℃) for 1 hour. The teeth were then post-fixed in a 0.1 M cacodylate-buffered 1 % OsO4 solution (pH 7.4, 4 ℃) for 1 hour. They were dehydrated in a graded series of ethanol, critical-point-dried, and sputter-coated with gold-palladium. The specimens were examined in a JSM-6300 scanning electron microscope (Japan Electron Co., Tokyo) at 60 kv.

Cell Growth Analysis of PDL Subpopulations

PDL cells of the subpopulations were plated in 24 well culture-plates at an initial density of $5x10^3$ cells per well and cultured in DMEM containing 10 % FBS, penicillin G sodium (10 unites / mL) and streptomycin sulfate (10 mg / mL). At day 2, 4 and 8, the cells were harvested, in triplicate wells, by incubating in 0.05 % trypsin and 0.01% EDTA, and cell number were determined by Coulter counter Z1 (Coulter Electronic Ltd., UK). Morphology of the cultured PDL cells was observed by a phase-contrast microscope.

Activity of ALPase of PDL Subpopulations

ALP activity was assayed by both histochemical and biochemical methods. Cells of each PDL-SP were plated in 24 well culture plates ($5x10^4$ cells per well) and cultured in DMEM containing 10 % FBS, penicillin G sodium (10

unites / mL) and streptomycin sulfate (10 mg / mL) for one week. Histochemical staining was made according to a modified new fuchsin method. Briefly, culture plates were washed twice with PBS and rinsed once with 0.05 M Tris-HCl (pH 7.4) for 5 min at room temperature. Then the cells were stained with 0.05 M Tris-HCl (pH 9.8) containing 10 mg/mL of sodium naphthol AS-BI phosphate salt (Sigma-Aldrich, Co., MO, USA), 4 % new fuchsin solution and 4 % sodium nitrite (Wako, Osaka, Japan) for 30 min. The cells were fixed with a 10 % formaldehyde neutral buffer solution (KATAYAMA CHEMICAL, Tokyo, Japan) for 60 min.

The quantitative analysis of ALP activity was performed by Bessey-Lowry enzymologic method using nitrophenyl phosphate as a substrate (Shiba *et al.*, 1995). The cells were washed with PBS and homogenized ultrasonically in 0.5 mL of 10 mM Tris-HCl buffer (pH 7.4) containing 25 mM $MgCl_2$. Aliquots of the homogenates were used for determination of ALPase activity and DNA content. One unite of ALP was defined as the amount of enzyme required to hydrolyze 1 nM p-nitrophenol per minute. DNA content was determined using bisbenzimidazole (Wako, Osaka, Japan) and ALP activities of PDL -SPs were expressed by unites per DNA contents.

Mineralization of PDL Cells

Mineralized nodule formation was examined by Dahl's stain for calcium (Vacca, 1985). Cells of PDL-SP were plated in 24 well-culture plates at a density of 5 $x10^4$ cells per well and cultured in DMEM supplemented with 10 % FBS, 50 mg/mL ascorbic acid, 10 mM sodium β - glycerophosphate and 10 nM dexamethasone for 3 weeks. Then the cells were fixed in a 10 % formaldehyde neutral buffer solution and stained with alizarin red S.

RESULTS

Light Microscopic Findings of PDL on Extracted Molar Roots

Compared to the normal PDL, about a half of PDL in width attached to the root surface of extracted teeth. The width of PDL decreased with the enzymatic digestion and no cells were seen on the surface of teeth after digestion for 110 min (Fig. 1a-c). There was no findings suggesting meaningful release of pulpal cells from a pulp chamber due to the enzymatic digestion even in the specimens at 110 min digestion.

Scanning Electron Microscopic Findings

Roots of the extracted teeth were covered by rough collagenous PDL tissues and exposure of cementum was not seen (Fig. 1d). With time of digestion, the surface of PDL became smoother and thickness of PDL remained on the root surface seemed to be decreased (Fig. 1d-i). Although a few layer of cells were seen on the root surface after 90 min digestion (Fig. 1h), no cells were remained on the root surface and cementum was completely exposed at 110 min digestion (Fig.1i). This result indicated that the 110M-PDL-SP was enriched by root lining cementoblastic cells.

Proliferation of PDL Subpopulations

Cultured PDL cells were spindle in shape and there was no obvious morphological difference among culture cells of PDL-SPs (data not shown). Figure 2 shows the proliferation curve of each PDL-SP. 30M-PDL-SP, which was enriched by cells from middle portion of PDL, showed a high level of a proliferative activity. Although the proliferative activity of PDL-SP decreased toward the root surface, 110M-PDL-SP, which was enriched by cells on the root surface, had high proliferative potential equivalent to that of 30M-PDL-SP.

ALP Activities of PDL Subpopulations

No or weak positive staining was seen in 30M-, 50M- and 70M-PDL-SPs (Fig. 3a-c). 90M-PDL-SP showed relatively high positivity (Fig. 3d). The most intensive staining of ALP was seen in 110M-PDL-SP (Fig. 3e). The reaction products were mainly localized on the cell membrane.

The highest ALP activity was also shown by the quantitative analysis of the enzyme (Fig.3). As a PDL-SP was derived from farther from the root surface, ALP activity of the subpopulation became less.

Mineralization

Well corresponding to he result of ALP activity, different amount of mineralization was seen among the PDL-SPs (Fig. 3). Little or no staining was seen in 30M-, 50M- and 70M-PDL-SPs (Fig. 3f-j). The most intensive staining was observed in 110M-PDL-SP (Fig. 3j) and less in 90M-PDL-SP (Fig. 3i).

DISCUSSION

Enzymatic release of PDL cells from extracted roots has been applied in some studies and cellular characteristics of the cells were studied (D'Errico *et al.*,1997; Grzesik *et al.*, 1998; MacNeil *et al.*, 1998). In those studies, however, the cells were harvested in the lump and analyzed as a tissue unit. In other words, heterogeneity of PDL has not been examined using subpopulations isolated from PDL by sequential enzymatic digestion, which has been applied to characterize heterogenous calvarial cell populations (Wong and Cohn, 1975; *Rao et al.*, 1977). Based on previous reports (D'Errico *et al.*,1997; Grzesik *et al.*, 1998; MacNeil *et al.*, 1998) and our preliminary studies, we decided on a 110 min. digestion to release all cell layers of PDL from extracted roots. Complete release of PDL cells was confirmed at the light and electron microscopic levels. It was also verified histologically that pulpal cells were not released into the digestions for the experimental period. In the present studies, we obtained 5 PDL-SPs. 30M-PDL-SP was considered to be isolated from the middle part of PDL, because about a half of the width of PDL attached to the extracted roots. Although light and electron microscopy showed a few layers of PDL cells on the root surface after 90 min digestion, those cells were completely disappeared from the root surface after 110 min digestion. We therefore regarded 110M-PDL-SP as a subpopulation enriched by lining cells on the root surface.

Studies on ALP and mineralization activities of the subpopulations showed that higher activities were seen in PDL-SP toward the root surface and that 110M-PDL-SP had by far the highest activity among the subpopulations. Groeneveld *et al.* (1993; 1995) reported that high ALPase activity is observed in the area near cementum and alveolar bones at rat maxillary molar *in vivo*. Sasaki *et al.* (1990) also showed that cementoblasts of human deciduous teeth exhibited intense ALP activity along the plasma membranes of whole cell surfaces. Our results and these findings also suggest that 110M-PDL-SP is enriched cells from the root surface: cementoblasts.

McCulloch et al.(1983; 1987), based on their radioautographic studies of mouse mandibular molar prepared from animals pulse-injected with 3H-Tdr, reported that cells migrate from endosteal spaces into the PDL and there express the phenotypes for osteoblasts or cementoblasts. They showed that, numerous cells labeling with 3H-Tdr, which seemed to be progenitor cells, were observed in the surrounding blood vessel. Well corresponding to their results, 30M-PDL-SP obtained from the middle area of PDL showed a high proliferative potential and considered to be enriched progenitor cells.

PDL-SP gradually decreased its potential of growth as it approached toward the root surface. Interestingly, however, 110M-PDL-SP had a high proliferative activity equivalent to that of 30M-PDL-SP. To confirm the high growth potential of 110M-PDL-SP, immunodetection of proliferating cell nuclear antigen in PDL and BrdU incorporation into PDL cells were examined. Positive cells for both proliferation markers were observed near the root surface as well as in the perivascular area in the middle of PDL (Fig. 4). This unexpected finding suggests that 110M-PDL-SP is composed of cells with high potentials of both mineralization and proliferation or cells with high growth potential which can differentiate to cementoblastic cells. The latter is more likely, because cultured 110M-PDL-SP showed uneven staining pattern for ALP which was most intensive around mineralized nodules. Although the origin of the cells with such a high growth potential on root surface is not decided, a potential explanation is that a part of undifferentiated cells with high proliferative activity migrated from dental follicle took up residence near the root surface after cementum formation (Ten Cate, 1994). Further studies is necessary to determine this unique cell population is existing only in PDL of the 8 week-old animals examined or lasting there to the end of their life. If such an interesting population exists ever since, the population might play great roles especially in periodontal tissue remodeling and regeneration.

Although we could not characterize PDL-SPs in alveolar bone half of PDL, the present studies showed 1. PDL-SPs obtained from the cementum half of PDL by sequential enzymatic digestion showed different activities in proliferation and mineralization. 2. PDL-SPs with higher proliferation rates were generally located in the middle portion of PDL and those with higher mineralization activities were seen toward the surface of the roots. 3. PDL–SP on the root surface showed high activities of proliferation and mineralization. It is suggested that a possible pathway of PDL cell turnover may exist within the PDL-SP on the root surface in addition to the generally recognized pathway from the middle area of PDL to root surface. To clarify the mechanism of cellular proliferation and differentiation in PDL using PDL-SPs will provide indispensable data for developing new regenerative periodontal therapies.

ACKNOWLEDGMENTS

This work was supported in part by grants 10557159 and 11470378 from the Ministry of Education, Science, Sports and Culture, Japan.

REFERENCES

Carranza FA, Ubios AM (1996). The tooth-supporting structures. In: Clinical periodontology. Carranza FA, NewmanMG, editors. Philadelphia: WB Saunders, pp.30-51.

D'Errico JA, MacNeil RL, Takata T, Berry J, Strayhorn CL, Somerman MJ (1997). Expression of bone associated markers by tooth root lining cells, *In situ* and *In vitro*. *Bone* 20:117-126.

Freeman E (1994). Periodontium. In: Oral histology: Development, structure, and function. Ten Cate AR, editor. St. Louis: Mosby, pp.276-312.

Giannopoulou C, Cimasoni G (1996). Functional characteristics of gingival and periodontal ligament fibroblasts. *J Dent Res* 75:895-902.

Groeneveld MC, Everts V, Beertsen W (1993). A quantitive enzyme histochemical analysis of the distribution of alkaline phosphatase activity in the periodontal ligament of the rat incisor. *J Dent Res* 72:1344-1350.

Groeneveld MC, Everts V, Beertsen W (1995). Alkaline phosphatase activity in the periodontal ligament and gingival of the rat molar : Its relation to cementum formation. *J Dent Res* 74:1374-1381.

Grzesik WJ, Kuzentsov SA, Uzawa K, Mankani M, Robey PG, Yamauchi M (1998). Normal human cementum-derived cells: isolation, clonal expression, and *in vitro* and *in vivo* characterrization. *J Bone Miner Res* 13:1547-1554.

MacNeil RL, D'Errico JA, Ouyang H, Berry J, Strayhorn C, Somerman MJ (1998). Isolation of murine cementoblasts: unique cells or uniquely - positioned osteoblasts? *Eur J Oral Sci* 106:350-356.

McCulloch CAG, Melcher AH (1983). Cell density and cell generation in the periodontal ligament of mice. *Am J Anat* 167:43-58.

McCulloch CAG, Nemeth E, Lowenberg B, Melcher AH (1987). Paravascular cells in endosteal spaces of alveolar bone contribute to periodontal ligament cell population. *Anat Rec* 219:233-242.

Noutcu RM, McCauley LK, Koh AJ, Somerman MJ (1997). Expression of extracellular matrix proteins in human periodontal ligament cells during mineralization *in vitro*. *J Periodontol* 68:320-327.

Ogata Y, Niisato N, Sakurai T, Furuyama S, Sugiya H (1995). Comarison of the characteristics of human gingival fibroblasts and periodontal ligament cells. *J Periodontol* 66:1025-1031.

Ramakrishnan PR, Lin WL, Sodek W-L, Cho M-Il (1995). Synthesis of noncollagenous extracellular matrix proteins during development of mineralized nodules by rat periodontal ligament cells *in vitro*. *Calcif Tissue Int* 57:52-59.

Rao LG, NG B, Brunette DM, Heersche JNM (1977). Parathyroid hormone-and prostaglandin E1-response in a selected population of bone cells after repeated subculture and storage at –80 C. *Endocrinol*;100:1233-1241.

Sasaki T, Watanabe C, Shimizu T, Debari K, Segawa K (1990). Possible role of cementoblasts in the resorbant organ of human decidous teeth during root resorption. *J Periodont Res* 25:143-7.

Shiba H, Nakamura S, Shirakawa M, Nakanishi K, Okamoto H, Satakeda H, *et al.* (1995). Effects of basic fibroblast growth factor on proliferation, the expression of osteonectin (SPARK) and alkaline phosphatase, and calcification in cultures of human pulp cells. *Dev Biol* 170:457-466.

Somerman MJ, Young MF, Foster RA, Moehring JM, Imm G, Sauk JJ (1990). Characteristics of human periodontal

ligament cells *in vitro*. *Archs oral Biol* 35:241-247.

Ten Cate AR (1994). Development of the periodontium. In: Oral histology: Development, structure, and function. Ten Cate AR, editor. St. Louis: Mosby, pp. 257-275.

Vacca LL (1985). Calcification. In: Laboratory manual of histochemistry. New York: Raven Press, pp.334-336.

Wong GL, Cohn DV (1975). Target cells in bone for parathormone and calcitonin are different ; Enrichment for each cell type by sequential digestion of mouse calvaria and selective adhesion to polymeric surface. *Porc Natl Acad Sci USA* 72:3167-3171.

Figure legends

Figure 1 Phase contrast microscopic futures of PDL before enzymatic digestion (a), after 70 min (b) and 110 min (c). The width of PDL decreases with the enzymatic digestion. No cells on the surface of teeth after 110 min digestion (c). a-c: x 100, scale bars = 100μm, Scanning electron microscopic findings of periodontal ligament before digestion (d), after digestion for 30 min (e), 50 min (f), 70 min (g), 90 min (h) and 110 min (i). A few cells on the root surface after 90 min digestion (h) are not seen after 110 min digestion (i). d-i: x 50, scale bar = 200μm

Figure 2 Proliferation of periodontal ligament subpopulations (PDL-SPs). 30M-PDL-SP shows a high level of a proliferative activity. Proliferative activity of PDL-SP decreases toward the root surface, 110M-PDL-SP , however, has high proliferative potential equivalent to that of 30M-PDL-SP.

Figure 3. Histochemical staining and quantitative measurement of alkaline phosphatase (ALP) activities and mineralization of periodontal ligament subpopulations (PDL-SPs). a: 30M-PDL-SP, b:50M-PDL-SP, c: 70M-PDL-SP, d: 90M-PDL-SP, e: 110M-PDL-SP. The most intensive staining of ALP and mineralization is seen in 110M-PDL-SP. a-e: x 100, scale bar = 50μm, f-j: x 150, scale bar = 33μm

Figure 4 Immunostaining for proliferating cell nuclear antigen in periodontal ligament (PDL) and BrdU incorporation into PDL cells. Positive cells for both markers are observed in PDL cells near the root cementum (C) as well as in the perivascular area (V) in PDL. a: x 120, scale bar = 100μm, b: x 100, scale bar = 100μm.

Figure 1

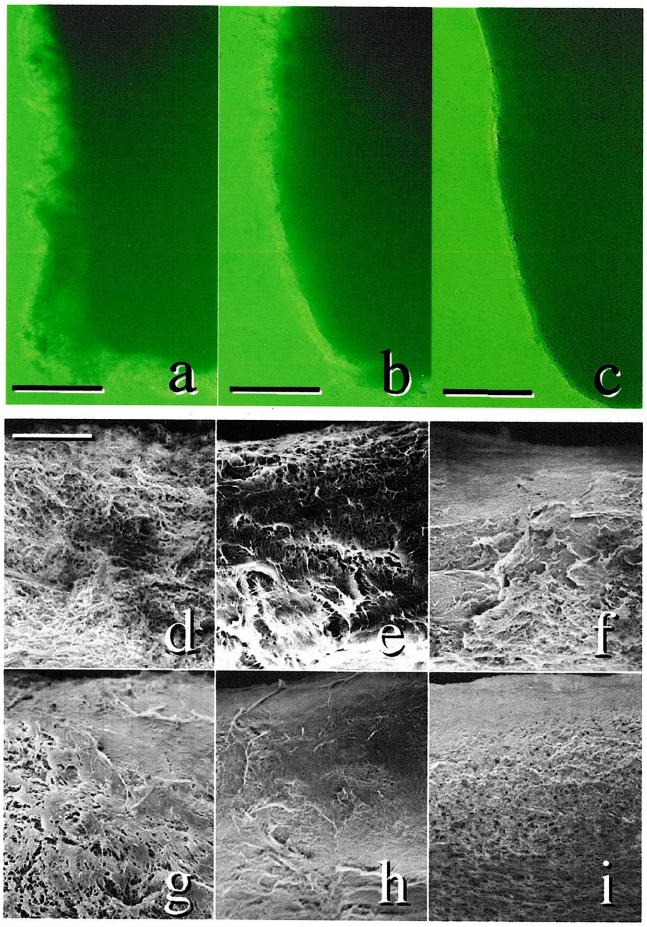
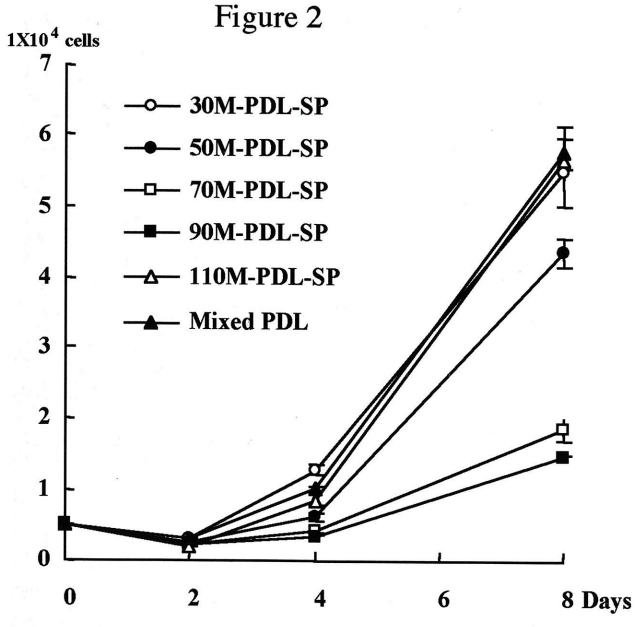


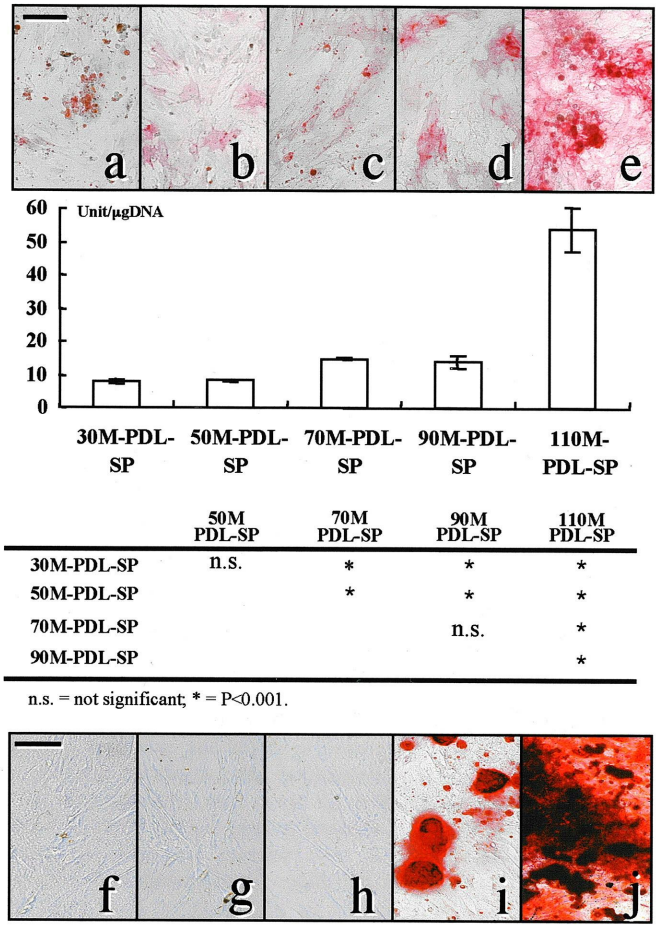
Figure 2



	50M PDL-SP	70M PDL-SP	90M PDL-SP	110M PDL-SP	Mixed PDL
30M-PDL-SP	*	*	*	n.s.	n.s.
50M-PDL-SP		*	*	*	*
70M-PDL-SP			*	*	*
90M-PDL-SP				*	*
110M-PDL-SP					n.s.

n.s. = not significant; * = P<0.001.

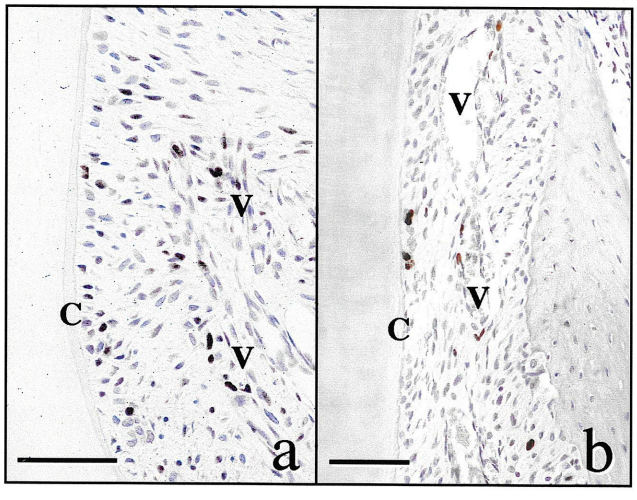
Figure 3



	50M PDL-SP	70M PDL-SP	90M PDL-SP	110M PDL-SP
30M-PDL-SP	n.s.	*	*	*
50M-PDL-SP		*	*	*
70M-PDL-SP			n.s.	*
90M-PDL-SP				*

n.s. = not significant; * = P<0.001.

Figure 4



ELSEVIER

Bone Vol. 30, No. 5
May 2002:712–717

Establishment of Human Cementifying Fibroma Cell Lines by Transfection With Temperature-sensitive Simian Virus-40 T-antigen Gene and hTERT Gene

Y. KUDO,[1] M. HIRAOKA,[1] S. KITAGAWA,[1] M. MIYAUCHI,[1] S. KAKUO,[2] M. ZHAO,[3] T. IDE,[2] and T. TAKATA[1]

[1]*Department of Oral Pathology, Faculty of Dentistry, Hiroshima University, Hiroshima, Japan*
[2]*Department of Cellular and Molecular Biology, Faculty of Medicine, Hiroshima University, Hiroshima, Japan*
[3]*Department of Periodontology, University of Michigan School of Dentistry, Ann Arbor, MI, USA*

Human cementifying fibroma (HCF) is a benign fibro-osseous neoplasm of periodontal ligament (PDL) origin containing varying amounts of mineralized material resembling cementum. In the present study, we established cell lines from HCF, which were detected in the mandible of a 54-year-old Japanese man. To obtain immortalized cell clones, we undertook transfection with temperature-sensitive simian virus-40 (SV40) T-antigen and hTERT into HCF cells. Cells transfected with SV40 T-antigen entered "crisis" state between passages 22 and 35, but activation of telomerase by transfection with hTERT in the SV40-transformed HCF cells resulted in bypass of the crisis and maintenance over passage 200. HCF cell lines decreased the expression of SV40 T-antigen and the activity of cell proliferation at a nonpermissive temperature (39°C) in comparison with that at a permissive temperature (33°C). High activities of alkaline phosphatase and mineralization and the expression of type I collagen, osteocalcin, osteopontin, and bone sialoprotein by reverse transcription-polymerase chain reaction (RT-PCR) were observed in HCF cells at 39°C. Overall, these findings suggest that: (i) HCF cell lines may represent a novel in vitro human cell model for the study of the regulatory mechanism of differentiation and proliferation of the human PDL; and (ii) transfection of plasmids encoding the temperature-sensitive SV40 T-antigen gene and hTERT gene may be useful for obtaining immortalized cell lines from benign human tumor and, probably, nonneoplastic human tissues. (Bone 30: 712–717; 2002)

Key Words: Cementifying fibroma; Periodontal ligament (PDL); Cell line; Immortalization; SV40 T-antigen; hTERT.

Introduction

Human cementifying fibroma (HCF) is a benign fibro-osseous neoplasm of the jaw, usually detected in the third and fourth decade of life, as a painless, slow-growing tumor.[25] HCF is composed of fibrous tissue containing varying amounts of mineralized material resembling cementum and/or bone.[25,38] It is generally accepted that HCF is of periodontal ligament (PDL) origin.[13,37]

Cells from nonneoplastic tissue in culture display a limited capacity to divide and reach cellular senescence. Spontaneous escape from senescence and acquisition of an indefinite lifespan is an exceptionally rare event in cultures of normal human cells.[24] Normal diploid human fibroblasts have a limited proliferative capacity and senesce after 50–80 doublings in culture.[10,17] It is generally accepted that establishment of human cell lines from nonneoplastic or benign tumor tissues is very difficult compared with rodent tissues, in which cells spontaneously immortalize with a relatively high frequency.[7,24] DNA tumor viruses such as simian virus-40 (SV40) and adenovirus are able to immortalize human cells at a very low frequency.[34] Infection with SV40 frequently results in extension of the lifespan by about 20 population doublings,[18,26,36] but most of the SV40 T-antigen-transfected clones from human tissues have failed to immortalize.[33,35]

As normal human cells gradually lose telomeric DNA with passage progression in culture, telomeric erosion is thought to limit cellular lifespan.[15] Human telomeres consist of repeats of the sequence TTAGGG/CCCTAA at chromosome ends, and these repeats are synthesized by a ribonucleoprotein enzyme, telomerase.[11] Ectopic expression of the hTERT gene, which encodes the catalytic subunit of the telomerase holoenzyme, enables lifespan extension of normal human cells.[3] It has recently been shown that ectopic expression of hTERT in combination with SV40 T-and/or H-ras results in immortalization of normal human epithelial and fibroblast cells.[14]

In the present study, by transfection with both SV40 T-antigen and the hTERT gene, we succeeded in establishing HCF cell lines, which can be a useful cell model for studying cellular differentiation and proliferation of PDL. Herein we describe the strategy for immortalization of cells of a human benign tumor with mineralization and the characteristics of established HCF cell lines.

Materials and Methods

All procedures in the present studies were performed in compliance with regulations administered by Hiroshima University.

Address for correspondence and reprints: Dr. Takashi Takata, Department of Oral Pathology, Faculty of Dentistry, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan. E-mail: ttakata@hiroshima-u.ac.jp

8756-3282/02/$22.00
PII S8756-3282(02)00689-0