Phenotypic Characterization of Cementum-Derived Cells in Human

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I. Introduction

Cementum is a mineralized connective tissue that covers the roots of teeth. Its main function is to attach the periodontal ligament fibers to the tooth. Cementum is similar in structure and composition to bone. But, it differs from bone in several important respects including its microscopic organization, its lack of vascularity, and the absence of continuous remodelling.(1)

The several types of cementum with different origins, compositions, and functions that have been described make description and classification difficult. It is also perhaps the least understood of all the hard tissues. And, the principal cells of cementum are cementoblasts, which line the surface of the tooth root, and cementocytes, which are embedded in the mineralized matrix in a manner similar to osteocytes.(1-4)

Due to the critical roles of cementum in maintaining the structure of the periodontium and the high prevalence of periodontal disease, there is great interest in the physiology of cementum. Despite its clinical relevance, the goal of developing efficient, safe, and reproducible strategies to regenerate cementum has not been accomplished yet to a degree similar to that in bone.(5)

At present, only a few successful isolation and expansion of cementum-derived cells has been reported and few poorly characterized molecules have been postulated to be cementum specific.(6-10)

In this study, we report the successful isolation and in vitro expansion of cementum-derived cells in human. Futhermore, to determine the phenotypic nature of these cells, we assayed alkaline phosphatase(ALP) activity and detected the mRNA expressions of a few bone associated macromolecules using a RT-PCR.

II. Materials & Methods

1. Cell Isolation & Expansion

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Healthy human premolar teeth (patient ages ranging from 17 to 23 years) extracted for orthodontic reasons were used. Some gingival tissues were harvested by crevicular incision on lingual portion for the culture of gingival fibroblasts. Teeth and gingival tissues were kept in serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco, USA) containing 100 U/ml of penicillin and 100 μg/ml of streptomycin (Gibco, USA) processed immediately after extraction within one-hour.

1) PDL cells culture

PDL from the middle portion of the tooth root was scraped with a curet and then placed in 100mm tissue culture dishes, containing 5ml of growth medium(DMEM/F12 supplemented with 1% antibacterial-antifungal solution (Gibco, USA) and 10% fetal bovine serum). Cells were incubated in a humidified atmosphere, 95% air, 5% CO_2, at 37℃. Medium was changed every third day. When the cultures became semiconfluent, cells were passaged with trypsin-EDTA.

2) Gingival Fibroblasts culture

Gingival tissue was dissected with a surgical scalpel and epithelium was removed. Then these were incubated in the same condition of PDL cells.

3) Cementum-derived cells culture

After PDL was manually dissected from the tooth root with a surgical scalpel, each tooth was washed with DMEM/F12 medium and incubated in 4ml of medium containing 100mU/ml of collagenase P for 1h at 37℃ with rotation (25 rpm). The medium with released cells was discarded and the teeth were washed three times with fresh medium. Using a sterile surgical scalpel, cementum and a thin layer of underlying dentin were dissected and collected. To minimize the variability in the relative cementum and dentin content within a group, fragments obtained from two teeth from the same patient were always pooled and processed together. Cementum/dentin fragments were thoroughly washed with medium (five times) and then minced with scissors until small fragments were obtained. Fragments were washed with medium (five times) and then digested again with collagenase P (100mU/ml; 4ml total volume/ 0.1-0.2ml of settled bed volume of fragments) for 1h at 37℃. The medium with released material was discarded. The fragments were washed thoroughly (five times) with medium and then placed in 100mm tissue culture dishes containing 5ml of growth medium (DMEM/F12 supple- mented with 1% antibacterial-antifungal solution (Gibco, USA) and 10% fetal bovine serum). The culture were incubated in a humidified atmosphere, 95% air, 5% CO_2, at 37℃. Medium was changed every third day.

4) Cell seeding for study

Cementum-drived cells(CDC), gingival fibroblasts(GF) and PDL cells were expanded in vitro until passage 3-5 and used for the in vitro assay. And a human osteosarcoma cell line SaOs-2 obtained from Americal Type Culture Collection were used for comparison (bone-forming cells).

Cells were plated into 6-well tissue culture plates at the density of 10,000 cells/well and incubated in the differentiation medium (DMEM/F12 medium containing 50μg/ml ascorbic acid, 2.5μg/ml of insulin-transferrin-selenium, 10mM beta-glycerophosphate, and 10^{-8}M dexamethasone). Each assay was perfo- rmed in triplicate.

And each 500,000 cells were plated into 100mm tissue culture dishes and incubated in the same differentiation medium. These were used for isolation of RNA.

2. Cell morphologic analysis

Cell morphology was observed by using phase contrast microscopy and photographs were taken by photomicrography system C-35AD-4 (Olympus Japan).

3. Cell Proliferation

Cell proliferation was measured at 3 and 7 days. At each time point, media were removed from the wells. The wells were washed with Hank's balanced salt solution(HBSS) to remove any unat- tached cells and remaining media. The adherent cells were released from the substrate by incubation in 300μl of 0.25% trypsin in 4mM EDTA for 10 minutes. After digestion, the wells were washed with 300μl of HBSS for collecting retained cells. Cells in trypsin/HBSS solution were counted by the hemacytometer. After counting, the cells in the media were centrif- uged at 5000rpm for 10 minutes at 4℃. The supernatant was suctioned away and the cell pellet was prepared for alkaline phosphatase(ALPase) activity test.

4. Measurement of ALPase activity

Production of ALPase was measured spectroscopically at 7 day. Removed cells from the wells were homogenized with 200μl of double distilled water (DDW) and sonicated for 1 minute in ice. 50μl of cell lysate were mixed with 100μl of 0.1M glycin-NaOH buffer, 50μl of 15mM para-nitrophenol phosphate (pNPP), 50μl of 0.1% Triton X-100/saline, and 50μl of DDW. Aliquits were incubated for 30 minutes at 37℃. After incubation, each tube was added 1.25ml of 0.1N NaOH and placed in ice. The production of para-nitrophenol (pNP) in the presence of ALPase activity was measured by monitoring light absorbance by the solution at 405nm. The slope of absor- bance versus time plot was used to calculate the ALPase activity. All measurements were collected from at least in triplicate and expressed as mean ±standard deviation. The one way analysis of variances (ANOVA) was used to identify differences between cementum-derived cell groups (CDC A, B and D) and SaOs-2 group. The level of significance was chosen to be α=0.01.

5. Detection of mRNA by RT-PCR

Total RNA from cultured cells was isolated with the use of high pure RNA Isolation Kit (Roche Molecular Biochemicals, Manheim, Germany) according to manufacturer's instruction. RT-PCR assays were carried out with the thermal cycler (Effendorf).

2μg of total RNA were reverse transcribed into complementary DNA (cDNA) with 2 unit/μl RT (AMV reverse transcriptase, Roche Molecular Biochemicals), 2.0μl of 1X reaction buffer (100mM Tris, 500mM KCl; pH 8.3), 4.0μl of 5mM $MgCl_2$, 2.0 μl of deoxynucleotide mix primer, 1.0μl of RNAase inhibitor at 25℃ for 10 minutes for annealing and then at 42℃ for 60 minutes for reverse trans- cription resulting in cDNA synthesis. Following the 42℃ incubation, the AMV reverse transcriptase is denaturated by incubating the reaction at 99℃ for 5 minutes and then cooled down to 4℃ for 5 minutes.

The resulting single stranded DNA is amplified using the reverse transcri- bed mixture containing 250uM dNTP, 2mM $MgCl_2$, 1X volume of reaction buffer, and 0.5 unit of Tag polymerase (Roche Molecular Biochemicals, Manheim, Germany) as a template with the specific oligonucleotide primers for human which were derived from known sequences (Table 1).

PCR of the cDNA products was carried out in a final volume of 20μl containing of 2μl cDNA, 1μl of

Table 1. Nucleotide sequences of the primers used for RT-PCR

	Sequence	Expected size of PCR product
18s rRNA	(s) 5'-GCGAATTCCTGCCAGTAGCATATGCTTG-3' (as) 5'-GGAAGCTTAGAGGAGCGAGCGACCAAGG-3'	126 bp
Human OPN	(s) 5'-CCAAGTAAGTCCAACGAAAG-3' (as) 5'-GGTGATGTCCTCGTCTGTA-3'	347 bp
Human ALP	(s) 5'-ACGTGGCTAAGAATGTCATC-3' (as) 5'-CTGTAGGCGATGTCCTTA-3'	475 bp
Human COL I	(s) 5'-TATGGCGGCCAGGGCTCCGACCTG-3' (as) 5'-CCAAGGGGGCCACATCGATGATGGG-3'	325 bp
Human BSP	(s) 5'-TCAGCATTTTGGGAATGGCC-3' (as) 5'-GAGGTTGTTGTCTTCGAGGT-3'	615 bp
Human BMP2	(s) 5'-GAAACGCCTTAAGTCCAGCTGT-3' (as) 5'-CTAGCGACACCCACAACCCTC-3'	315 bp
Human OC	(s) 5'-CATGAGAGCCCTCACA-3' (as) 5'-AGAGCGACACCCTAGAC-3'	310 bp

* s : sense , as : antisense
* OPN : osteopontin, ALP : alkaline phosphatase, COL I : type I collagen
BSP : bone sialoprotein, OC : osteocalcin

20 pmol/μl of each of the forward and reverse primers, and 16μl of autoclaved DDW. PCR protocol was 30 cycles of denaturing, annealing and primer extension, which was controlled by the Thermal Cycler (Mastercycler Gradient, Eppendorf, Germany).

The mRNA level of 18s ribosomal RNA (rRNA) was analysed in the same samples as an amplication control.

III. Results

1. Cementum-derived cells cultures

Of the 5 attempts (We named each patient A-E group which included three cell type - CDC, GF, PDL) to obtain cementum cell cultures, 3 were successful. (In C & E group cementum cell culture could not be obtained)

Incipient colony formation from fragments was observed 15-23 days after plating. The cells within colonies showed typical fibroblastic morphology (Figure 1). Most of cementum-derived cell populations resembled each other during routine culturing, and extended passaging did not significantly alter their morphology. But cementumderived cell in group A exhibited nonfibroblastic morphology but a polygonal cell form. It showed markedly through extended passaging (Figure 2).

2. Cell proliferation

Cells were counted by hemacytometer at 3 and 7 days (Table 2. and Figure 3). All of cells in each group showed increased cell numbers at 7 days. Especially cementum-derived cells in group A markedly increased at 7 days.

3. ALP activity

Cementum-derived cells in all groups showed significant lower level of ALP activity than SaOs-2 cell ($p<0.01$). ALP activity of GF cells in all groups showed the lowest value among the group. PDL

Figure 1. Cementum-derived cells in Group B x40(A) x80(B), confluent x20(C) x80(D). Cementum-derived cells in Group D x40(E) x80(F). All figures show typical fibroblastic morphology and (A,B) show cementum-derived cells from fragments at the first time after plating.

Figure 2. Cementum-derived cells in Group A x20(A) x40(B). Non-fibroblastic morphology but a polygonal cell form.

Table 2. Cell Proliferation

day	cell number (104cell/well)									
	CDC A	CDC B	CDC D	SaOs-2	GF A	GF B	GF D	PDL A	PDL B	PDL D
3	3.2±0.8	1.1±0.2	1.0±0.1	1.5±0.3	1.6±0.2	1.9±0.5	2.4±0.2	1.4±0.4	1.4±0.2	1.4±0.4
7	25.5±2.2	2.1±0.1	2.3±0.3	3.3±0.7	7.9±1.5	5.7±1.6	13.0±1.5	2.6±0.8	3.2±0.6	11.0±1.0

* CDC : Cementum-Derived Cell , GF : Gingival Fibroblast,
* PDL : Periodontal Ligament cell, A,B,D : each group name

Table 3. Alkaline phosphatase activity levels

day	pmol PNP/30 min/ng protein									
	CDC A	CDC B	CDC D	SaOs-2	GF A	GF B	GF D	PDL A	PDL B	PDL D
7	4.1±0.4#	82.1±3.7#	57.7±15.9#	177.5±1.9	12.8±2.0	16.0±2.0	11.4±0.9	143.0±26.4	65.7±3.9	25.0±13.6

* CDC : Cementum-Derived Cell , GF : Gingival Fibroblast,
* PDL : Periodontal Ligament cell, A,B,D : each group name
# $p < 0.01$, as compared with Saos-2 group.

cells showed somewhat higher ALP activity than GF cells. (Table 3 and Figure 4).

4. RT-PCR

ALPase and OPN mRNA was hardly detected in all cementum-derived cells. And the expression of other bone associated protein markers in cementum-derived cells were lower than that in SaOs-2 cells (Figure 5).

Type I collagen, BMP-2 and BSP mRNA were detected in all samples. Unusually, osteocalcin mRNA was markedly detected in group A cementum-derived cells and slightly detected in periodon-

Figure 5. Alkaline phosphatase activity levels

Figure 5. mRNA expression by RT-PCR

* OPN : osteopontin, ALP : alkaline phosphatase, COL I : type I collagen BSP : bone sialoprotein, OC : osteocalcin
* CDC : Cementum-Derived Cell , GF : Gingival Fibroblast, PDL : Periodontal Ligament cell, A,B,D : each group name

tal ligament cell group.

IV. Discussion

The present study demonstrated that cementum-derived cells in human could be isolated and expanded successfully in vitro. Establishing the in vitro model systems to study cell metabolism has greatly contributed to our understanding of the physiology of numerous tissue, including bone and cartilage. However, because of the size, topography, and small number of cells, there has been limited success in cementum cell biology. At present, the paucity of available models to study human cementogenesis in general and lack of appropriate in vitro models in particular.[6,11,12]

Recently, Grzesik et al.[6,7] have established a method to isolate and expand in vitro normal

human cementum-derived cells and these cells formed mineral matrix when attached to a ceramic carrier and transplanted subcutaneously into immunodeficient mice. These mineralized matrix exhibited several features identical to cementum in situ and was significantly different from bone deposited by similarly transplanted human bone marrow stromal cells. The method for obtaining cementum-derived cells described was designed to ensure that only cells that are protected by mineralized extracellular matrix were present in the starting material at the time that the cultures were initiated.[6] Previously, a similar strategy had been successfully employed for obtaining human bone cells.[13] Cementocytes were most likely the sole source of cells in this culture system. So, cementocytes may not necessarily be terminally differentiated cells, as has been generally assumed. Furthermore, if cementocytes can be stimulated to proliferate and migrate in vivo, then they may be possible target cells for therapies aimed at cementum regeneration.[6]

Cementum-derived cells exhibit fibroblastic morphology in culture. The earliest cementoblasts originate from ectomesenchymal cells in the dental follicle surrounding developing teeth. Cementoblasts may also develop later from undifferentiated mesenchymal cells persisting in the periodontal ligament. They are morphologically similar to periodontal fibroblasts.[3,4] The various type of cementum are produced by cementoblasts or periodontal ligament cells lining the cementum surface. Some of these cells become incorporated into cementoid, which subsequently mineralizes to form cementum. The cells which are incorporated in the cementum are called cementocytes. The presence of cementocytes allows transportation of nutrients through the cementum, and contributes to the maintenance of the vitality of this mineralized tissue.[3]

Interestingly, Cementum-derived cells in Group A showed altered morphology, polygonal cell form. This may be characteristic changes through extended passaging or diversity of cementum-derived cells. More studies are needed for evaluation of cementum- derived cell line.

Whether cementum-derived cells are phenotypically unique or rather "osteoblast variants" is an important question that must be addressed in order to understand developmental mechanism and to devise therapeutic methods to enhance the formation and regeneration of cementum in post-disease situation.

The present data show that cementum- derived cells are phenotypically distinct from bone-forming cells. Cementum-derived cells exhibit low level or no alkaline phosphatase activity in this study. Cementum-derived cells in all groups showed lower level of ALP activity than SaOs-2 cells. Furthermore, ALPase mRNA was not detected in all cementum-derived cells. There are suggestions that lower ALP activity is indeed indicative of cementoblastic phenotype in vivo.[10,15]

And the expression of other osteoblastic cell markers in cementum-derived cells were lower than that in SaOs-2 cells. But, the resulting PCR product band intensities are not directly proportional to the amount of mRNA present in the cells. Hence the RT-PCR result can only be viewed as being semi-quantitative and the results obtained must be interpreted cautiously. Futhermore, the considerable heterogeneity within each group of cementum- derived cells, PDL cells and GF cells were found. The osteocalcin mRNA was markedly detected in group A cementum-derived cells and the some osteoblastic cell markers were expressed in PDL cells and GF cells group. It is likely that a small subpopulation of these cells may have the ability to express some of the bone associated proteins.[23]

However, the results of other studies are not accordance with present data. Carnes et al.[16] have obtained cells from human cementum shavings. Their cells showed alkaline phosphatase activity and were positive for osteocalcin. Bronckers et al.[17] concluded that cementoblast and cementocytes of cellular cementum procedure osteopontin and osteocalcin but not dentin sialoprotein and thus express an osteoblast-like, not an odontoblast-like, phenotype. The cells responsible for the production of acellular cemen- tum are likely cells of the PDL in close contact with the dental root surface. These fibroblast-like cells express osteopontin but not osteocalcin or dentin sialoprotein and accordingly express only a partial osteoblastic phenotype. These other studies have some limitations. The culture systems are different and the source of cementum-derived cells are unclear. Usually they used mixed cell culture.

In conclusion, cementum-derived cells in human could be isolated and expanded in vitro successfully. These cells are phenotypically distinct from bone-forming cells. This study may provide an invaluable tool to study cellular populations and molecular mechanisms of cementogenesis.

V. References

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-국문초록-

사람 백악질 유래 세포의 형질 특성에 관한 연구

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백악질 세포의 분리 및 배양방법을 확립하고, 이를 이용하여 백악질 세포의 형질특성을 알아보고자 하였다.

교정목적으로 발거된 소구치를 이용하여, 치은섬유아세포, 치주인대 세포 및 백악질 유래 세포를 분리, 배양하였다. 백악질 유래 세포 배양시에는 백악질을 절제한 후 Collagenase P를 이용하여 백악질 유래 세포 외의 다른 세포의 개재를 배제하였고, 기질을 분해하여 세포의 분리 및 배양이 용이하도록 하였다. 분리 및 배양시기의 세포의 형태를 광학현미경을 이용하여 관찰하였다.

조골세포의 특성을 가지는 SaOs-2 세포를 대조군으로 이용하여 분리 및 배양된 세포군들을 동일한 조건으로 배양 하였다. 3일 및 7일째에 세포증식도를 측정하였고 7일째에 ALPase 효소 활성도를 측정하였다.

각 세포의 형질 특성을 알아보기 위해 RT-PCR을 실시하여 조골세포 분화 표식자와 연관된 osteopontin(OPN), Alkaline phosphatase(ALP), type I collagen(COL-I), Bone sialoprotein(BSP), BMP-2 및 osteocalcin(OC)의 발현을 비교 관찰하였다.

백악질 유래 세포의 분리 및 배양을 시도한 5명의 치아 중에서 3명의 치아에서 세포군을 배양해 낼 수 있었다. 배양한 백악질 유래 세포는 섬유아세포와 유사한 형태와 증식을 보였다.

ALPase 효소 활성도 검사 결과 백악질 유래 세포는 SaOs-2 세포보다 낮은 활성도를 나타내었으며, 배양된 세포의 RT-PCR 결과 백악질 유래 세포군에서는 ALPase의 발현이 나타나지 않았고, 다른 조골세포 표식자의 발현도 낮게 나타났다. 이는 백악질 유래 세포가 조골세포 및 다른 대조군의 세포와는 다른 형질 특성을 가지고 있다는 것을 시사한다.

이상의 관찰결과로 사람의 백악질 유래 세포를 백악질의 절제 및 효소처리 방법으로 효과적인 분리 및 배양이 가능하며, 이는 향후 백악질 세포의 형질 특성 및 백악질 형성의 분자적 기전을 파악하는 중요한 연구자료로 활용 될 수 있을 것으로 사료된다.

주요어 : 백악질, 백악질 유래 세포, 분리, 배양, 형질 특성