Identification of Matrix Mineralization-Related Genes in Human Periodontal Ligament Cells Using cDNA Microarray

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I. INTRODUCTION

The periodontal ligament (PDL) is derived from the inner layer of the dental follicle shortly root development begins[1]. Indeed, development and maturation of the PDL are dependent on the formation of root dentin and the synthesis of its investing mineralized tissue, cementum.

The PDL is a highly vascular and cellular connective tissue situated between the tooth and the alveolar bone that provide supportive, attachment, and sensory functions. The normal PDL includes osteoblasts and osteoclasts on the bone side; fibroblasts, epithelial cell rests of Malassez, macrophages, undifferentiated mesenchymal cells, neural elements,and endothelial cells in the body; and cementoblasts on the root surface. Fibroblasts are the predominant cells in the PDL. Their specialized functions are considered to be responsible for not only the formation and maintenance of the PDL, but also for the repair, remodeling and regeneration of the adjacent alveolar bone and cementum[2-4]. According to clinical and histological researches[5,6], periodontal regeneration is induced by the cells which are derived from the remaining healthy portions of the periodontal connective tissues.

Several studies have also demonstrated that the PDL cells are not homogenous but may consist of different subpopulation with unique phenotypes and distinct functional activities[7-9].

The cementum increases in thickness with age under normal physiological condition. In

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addition, deposition of newly formed cementum-like layers are observed on the hard dental tissues in pathological conditions following injury of the periodontium[10]. The alveolar bone has been to undergo continued remodeling in physiological conditions. Therefore, the continued differentiation of osteoblasts is required for the alveolar bone formation. Studies focusing on the characterization of the PDL cells in vitro indicate that these cells have osteoblasts like properties including synthesis and expression of alkaline phosphatase(ALP) and osteopontin (OPN), synthesis of 1, 25-dihydroxyvitamin D3-induced bone 'gla' protein, responsiveness to parathyroid hormone(PTH), and synthesis of dexamethasone induced PTH- mediated cAMP[11,12]. Furthermore, the PDL fibroblasts have the capacity to produce mineralized nodules in vitro under the mineralization media which include ascorbic acid, β -glycerophosphate and dexamethasone[11-13]

Therefore, cells in the PDL either have beenknown as multipotential cells or are composed of heterogeneous cell populations which have the capacities to differentiate into either cementoblasts or osteoblasts, depending on needs and conditions.

In spite of these well-known osteoblast-like propertiesof the PDL cells, very little is known about the molecules involved in the formation of the mineralized nodules in the PDL cells. cDNA microarray is one of the important tool in the gene expression analysis among many modern techniques. Therefore, in the present study, using a culture system that facilitates the formations of mineralized nodules in the human PDL cells, we analysed gene-expression profiles during the mineralization process by means of a cDNA microarray consisting of 3063 genes.

II. MATERIALS AND METHODS

1. Cell Isolation and Culture

We were received a patient written consent from patients. After, the PDL tissues were obtained from the periodontal ligament of premolar teeth extracted for orthodontic reasons. After extration the teeth were placed in biopsy media(Dulbecco's modified Eagles media [DMEM] with 10% fetal bovine serum [FBS] 500 U/ml penicillin and 500ug/ml streptomycin). Only periodontal ligament attached to the middle third of the root was removed with a curette to avoid contamination with gingival and apical tissues. The PDL tissues were cut into small pieces, rinsed with biopsy media, and placed in small culture dishes. The PDL tissues were incubated in biopsy medium at a humidified atmosphere of 95% air. The medium was replaced with culture medium (DMEM with 10% FBS 100 U/ml penicillin and 100 μg/ml streptomycin). After reaching confluence, cells were passaged with 0.25% trypsin/0.1% ethylene diaminoterraacetic acid (EDTA).

2. Mineralization Protocol

The PDL cells were seeded at an initial

density of 1 $\times 10^6$ cells in 100 mm dishes. After reaching confluence, culture dishes were divided into 2 groups and cells were cultured in DMEM containing 1) 10% FBS (control); 2) 10% FBS with mineralization supplement (50 μg/ml ascorbic acid, 10 mM β-glycerophosphate and 100 nM dexamethasone for 21 days The media for two groups were changed every 2 days. To detect the formation of mineral-like nodules, the cells were fixed and stained using the alizarin red S method[14].

3. Total and messenger RNA extraction

Total RNA was extracted from cultured cells byusing a modified acid phenol method. Briefly, the growth medium was removed and the cells were lysed with Trizol (Life Technologies). The lysate was cleared and extracted with 1/10 volume of 1-bromo-3-chloporopane. The aqueous layer was collected in a newtube and precipitated with isoporpanol. After 75% ethanol washing, the pellet was air-dried and resuspended in DEPC-treated water, and quantified by A260/A280 measurement by using UV spectrometer (DU530, Beckman, USA). For the assays of the quality, 3-5 μg of total RNA was loaded onto denaturing 1.0% formaldehyde agarose gels and electrophoresed. mRNA was isolated from the total RNA with an oligotex mRNA midi kit (Qiagen, Chatsworth, CA, USA)

4. cDNA Microarrays

The KNU Human 3K DNA chip was consisted of 3,063 cDNA clones (1741 of known genes, 1290 of novel genes, and 32 of internal control) isolated from human dermal papilla cell cDNA library. Known genes were classified according to function (Table. 1).

1) Preparations of plasmid samples

cDNA microarrays were produced by spotting PCR products representing specific genes onto a glass slide. Typically, the PCR products were purified by precipitation to remove unwanted salts, detergents, PCR primers. All insert cDNAs were amplified by PCR for 35 cycles with C6 amine-modified T7 (5'-AATTAACCCTAACTAAAGG-3') and T7 (5'-GTAATACGACTCACTATAGGGC-3') universal primer (IDT Ins., USA). For each 96 well plate to be amplified with PCR machine (MJR, USA), PCR mixtures containing 1×PCR buffer, 0.2 μM each primer, 2.5 U Taq DNA polymerase (Promega, USA), and 0.2 μmM dNTPs (Amersham Phamaciabiotech., USA) were prepared. Thermal cycling was done for 35 cycles with 94℃ for 60 seconds, 57℃ for 90 seconds, and 72℃for 120 seconds and additional extension period at 72℃ for 7 minutes. 3 μl of each PCR reaction was analyzed by electrophoresis in 1% agarose gel. The remaining 97 μl of PCR reaction was precipitated in the 96-well plates by addition of 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of pure ethanol. The 96-well plates were in-

cubated at -20℃ for overnight and the DNA was pelleted by centrifugation at 3,000 rpm at 4℃ for 1 hour. The precipitated DNA was washed once with 75% ethanol. The ethanol wash was then removed by suction and pelleted DNA was dried by air. The amino-modified DNA pellets were suspended in 3×standard saline citrate (SSC) and transferred from the 96-well plates to 384-well plates (ABgene House, UK)

2) Microarray printing

Amino-silane coated slides (CMT-GAPSTM coated slides ; Corning Inc., USA) wereused for printing microarrays. Using a robotic transfer device (Cartesian PixSYS 5500, USA), the DNA was transferred from the 384-well plates onto glass slides. A total of 3,063 cDNAs were arrayed in 1.8-cm^2 areas.

3) Post-processing of arrays

After printing amino-linked cDNA, microarrays were allowed to dry under vacuum for several days and immobilized the printed DNA. First, microarray was rehydrated by holding slides (array side down) over a bath of hot water (95~100℃) until a light vapor coating is observed across the slide, and then we snap-dry each array (DNA side up) on a 100℃ hot plate for approximately 5 to 10 seconds. Using a UV Srtaralinker (Stratagene, USA) (60 to 300 mJ), DNA was immobilized on a slides. Blocking process was done. Microarray was soaked in the succinic anhydride/sodium borate solution (5.0 g of succinic anhydride dissolved in 315 ml of n-methyl-pyrrilidinone) to reduce free aldehydes and washed in dH_2O at 95~100℃ for 2 minutes. And then slides were transferred quickly in 95% ethanol for 1 minute and dried using a centrifuge. Microarrays were stored in a desiccator at room temperature with light protection

4) Preparation of fluorescent DNA probe from mRNA

Probes were made as described[15] with several modifications. The reverse transcriptase used here was Superscript Ⅱ RNase H (Life Technologies). The Cy3 dUTP and Cy5 dUTP were purchased from Amersham. Each reverse transcripion reaction contained 4.0~6.0 ㎍ of mRNA and 1 ㎍of random hexamers. Following the reverse transcription step, samples were treated with each 1.0 ㎕ of 1.5 M sodium hydroxide and 30 mM EDTA for 10 minutes at 65℃, then neutralized by adding 468 ㎕ of TE buffer (pH 7.4). By using a Microcon 30 (Millipore, USA), the probe was purified and concentrated. Cy3 and Cy5 fluorescently labelled probes were mixed in 3×SSC, 0.1% SDS with 0.5 mg/ml poly A blocker (Amersham), and 0.5 mg/ml yeast tRNA (Life Technologies) to a final volume of 25 ㎕.

5) Microarray hybridization

For amino-silane coated slides (CMT-GAPSTM coated slides), prehybridization is required. Arrays were prehybridized in 3.5 ×SSC, 0.1% SDS, 10 mg/ml BSA in a coping jar for 20 minutes at 50℃, washed by

dipping in water and in isopropanol, and dried by using centrifuge. The prepared probes was denatrured by heating at 95-100℃ for 2 minutes and added onto a array with cover slide. The hybridization was done in a CMT-Hybridization chamber (Corning) for 20 hours in a 50℃ waterbath. Arrays were washed for 5 minutes at room temperature in low stringency wash buffer (0.1× SSC/0.1% SSC), and then twice for 5 minutes in high stringency wash buffer (0.1× SSC) and dried by using centrifuge.

6) Scanning and Image analysis

Fluorescence intensities at immobilized targets were measured by using scanarray 4000 with a laser confocal micorscope (GSI Lumonics, USA). The two fluorescent images (Cy3 and Cy5) were scanned separately from confocal microscope, and color images were formed by arbitrarily assigning differentiated cell intensity values into the red channel and control intensity into the green channel and data were analyze by using Quantarray software (version 2.0.1, GSI Lumonics). Results were also analyzed by normalization between the images to adjust for the different efficiencies in labeling and detection with the two different fluors. This was achieved by matching of the detection sensitivities to bring a set of 32 internal control genes (βactin and GAPDH) to nearly equal intensity.

For this analysis, we used a filter that included all gene exhibiting a minimum level of expression of intensify over 1,000 fluorescent units (on a scale of 0-65,535 fluorescent units) for both red and green channels for each pair of experiments.

5. Northern blot analysis

Ten μg of total RNAs were heated to 65℃ for 15 min in 50% formamide, 0.02% formaldehyde, 40 mM MOPS (3-[-N-morpholino]propanesulfonic acid), 10 mM so-

Table 1. Classification of 3063 mesenchymal cell derived genes

Classification of genes	sets of cluster
Signal transduction and communication	309
Gene/protein expression	375
Cell division/DNA synthesis	73
Cell structure/motility	145
Cell/organism defence and homeostasis	59
Metabolism	221
Unclassified	559
Novel	1,290
Internal control	32
Total	3,063

dium acetate, 1 mM EDTA, and 0.1 mg/ml ethodium bromide prior to gel electrophoresis on 1% agarose, 55% formaldehyde, 40 mM MOPS, 10 mM sodium acetate, and 1 mM EDTA. The RNA was blotted onto a Zeta-Probe Blotting Membranes in 20×SSC. The RNA was air-dried and then cross-linked by exposure to ultraviolet light. The probes were labelled with [α-P^{32}]-dCTP by Megaprime DNA labelling system kit. Prehybridization and hybridization were performed by using the Express Hyb solution. After hybridization, the membrane was washed in 2×SSC- 0.1% SDS at room temperature and then in 0.1×SSC /0.1% SDS at 55℃, and exposed to Agfa X-ray film at -70℃ with intensifying screens.

III. RESULTS

1. Morphology

Staining with alizarin red S distinguished PDL cells cultured in the media with mineralization supplements from those grown in the control media. The nodules of mineralized matrix were strongly stained with alizarin red S at day 21 (Figure 1). These data suggest that the media containing 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone are the essential supplementation for PDL cells to form matrix mineralization nodules, and that induction of these nodules in vitro requires about 3 weeks.

2. cDNA Microarray Analysis

We harvested PDL cells from each of the two different culture media on 21 day and isolated mRNAs for analysis on the cDNA microarray. After normalization of the results, we selected genes that were up- or down-regulated on 21 day in cells cultured both in the media of the mineralization supplements and in the control media.

PDL-Control PDL-Dex

Figure 1. Alizarin red S staining of calcified matrix around PDL cells cultured for 21 days without (PDL-Control) and with mineralization supplements (50ug/ml ascorbic acid, 10mM β -glycerophosphate and 100nM dexamethasone) added to the culture medium(PDL-Dex). Concentrations of each supplement and cell densities were described under Materials and Methods.

Among 3,063 genes analyzed, 35 were up-regulated more than two-fold at one or more time points in cells that developed matrix mineralization nodules, and 38 were down-regulated to less than half their normal level of expression. The selected genes and their known or suspected functions are listed in Tables 2, 3, 4, 5, 6, 7, and 8.

In accord with the morphological change we observed, several genes related to calcium-related or mineral metabolism were induced in PDL cells during osteogenesis, such as insulin-like growth factor-II(IGF-II) and insulin-like growth factor-binding protein 2(IGFBP-2). Proteogycan 1, fibulin-5, keratin 5, βactin, αsmooth muscle actin and capping protein, and cytoskeleton and extracellular matrix proteins were up-regulated during mineralization. Several genes encoding proteins related to apoptosis were expressed in the PDL cells. Dickkopf-1 (Dkk-1) and Ninteen kD interacting protein-3 (Nip3, E1B 19K/Bcl-2-binding protein) were up-regulated, and Btf (Bcl-2 associated transcriptional factor) and Tax1 binding protein 1(TAX1BP1) were down-regulated during mineralization. Also periostin and S100 calcium-binding protein A4 were down-regulated during mineralization.

3. Verification of Expression of Selected Genes

To verify the microarray results, we performed Northern blots. Probes for each tested gene were PCR products amplified from plasmid DNAs containing appropriate cDNAs. Among up-regulated genes, we selected glucocorticoid-induced leucine zipper(GILZ) (Figure 3). From the list of down-regulated genes, we selected genes encoding collagen type III α1, S100A4, and cystein knot superfamily 1/BMP

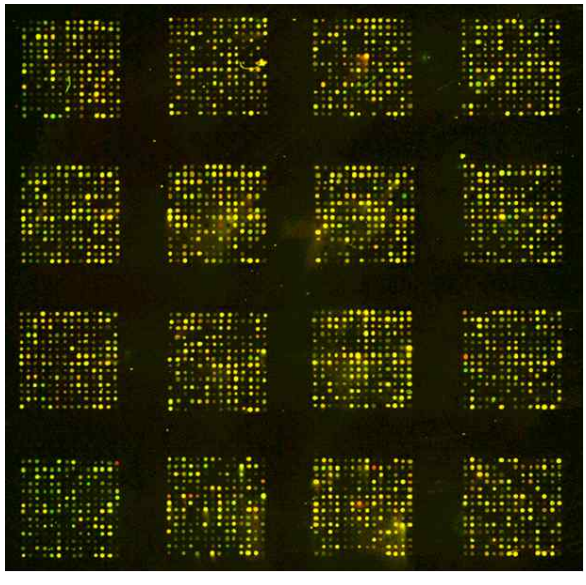

Figure 2. Image of microarray used to analyze gene expression level in PDL cells cultured with mineralization supplements and with 10% FBS. Total RNA was extracted from both cultures and each RNA sample was used as a template for synthesis of cDNA probes. The probe for PDL cells cultured with 10% FBS was labeled with Cy3(green) while the probe for PDL cells cultured with mineralization supplements was labeled with Cy5 (red). The probes were mixed and hybridized to a microarray slide. The slides were scanned in a dual-laser scanning confocal microscope (GSI Lumonics,USA) and imageswere analyzed using a Quantarray software (Version 2.0.1, GSI Lumonics). Green dots represented genes whose expression is decreased in PDL cells cultured with mineralization supplements while red dots presented genes whose expression is increased. Yellow dots indicate no change in gene expression.

Table 2. Differently expressed genes related with cell signal and communication

Gen bank	Name	Ratio
Genes up-regulated during mineralization		
NM_012242	Dickkopf-1(hdkk-1)/Wnt antagonist	4.75
	MAP	3.26
NM_000597	IGFBP 2	3.22
	IGF-II	2.89
NM_018407	Putative integral membrane transporter(LC27)	2.20
NM_006367	Adenyl cyclase-associated protein(CAP)	2.14
NM_000227	Laminin-related protein(LamA3, epiligrin alpha 3 subunit)	2.11
Genes down-regulated during mineralization		
NM_000598	IGFBP 3	0.15
	OSF-1	0.16
NM_003299	Tumor rejection antigen (gp96) 1(TRA1)	0.22
NM_013372	Cysteine knot superfamily 1/BMP antagonist 1	0.27
NM_001257	H-cadherin(calcium dependent cell adhesion protein)	0.44
NM_006209	Ectonucleotide pyrophosphatase/phosphodiesterase 2(Autotayin)	0.44
NM_006475	OSF-2(Periostin)	0.45
NM_000484	Amyloid A4 protein	0.47
NM_021137	Tumor necrosis factor, alpha-induced protein	0.50
NM_019554	S100 calcium-binding protein A4	0.50

Table 3. Differently expressed genes related with gene and protein expression.

Gen bank	Name	Ratio
Genes up-regulated during mineralization		
AF228339	Glucocorticoid-induced leucine zipper(GILZ)	8.00
NM_004052	BCL2/adenovirus E1B(19kD-interacting protein 2)	3.00
Genes down-regulated during mineralization		
NM_018834	Matrin 3 (MATR3)	0.38
NM_005180	Murine leukemia viral (bmi-1)-oncogene homolog	0.46
AF249273	Btf(BCl-2-associated transcription factor)	0.48
NM_006024.2	Tax1(human T-cell leukemia virus type I)-binding protein 1 (TAX1BP1)	0.47
NM_005098	Musculin (activated B-cell factor-1)-(MSC)	0.50

Table 4. Differently expressed genes related with cell division and DNA synthesis.

Gen bank	Name	Ratio
Genes up-regulated during mineralization		
NM_001753	Caveolin 1, caveolae protein, 22kD(CAV1)	2.40
NM_002106	Histone H2A.Z	2.18
Genes down-regulated during mineralization		
NM_003589	Cullin 4A (CUL4A)	0.50
NM_001758	Cyclin D 1 (PRAD1: parathyroid adenomatosis 1)	0.50

Table 5. Differently expressed genes related with cytoskeleton, adhesion and extracellular matrix.

Gen bank	Name	Ratio
Genes up-regulated during mineralization		
NM_002727	Proteoglycan 1, secretory granule(PRG1)	2.48
NM_006329	Fibulin-5	2.27
	Keratin 5(KRT5)	2.24
NM_001101	Actin, beta	2.16
NM_001613.1	Actin, alpha 2, smooth muscle, aorta(ACTA2)	2.09
NM_001747.1	Capping protein(actin filament),gelsolin-like	2.00
Genes down-regulated during mineralization		
NM_000090.1	Collagen, type III, alpha 1	0.28
NM_004859.1	Clathrin, heavy polypeptide (Hc) (CLTC)	0.39

Table 6. Differently expressed genes related with metabolism.

Gen bank	Name	Ratio
Genes up-regulated during mineralization		
NM-006793	Peroxiredoxin 3(PRDX3)	2.56
NM_020300	Glutathione S-transferase, microsomal	2.25
NM_001428	Enolase, alpha	2.23
NM_003945	ATPase, H+ transporting, lysosomal-(vacuolar proton pump)	2.12
NM_006169	Nicotinamide N-methyltransferase(NNMT)	2.11
NM_000291	Phosphoglycerate kinase	2.09
NM_003713	Phosphatidic acid phosphatase type 2b(PPAP2B) mRNA	2.00
Genes down-regulated during mineralization		
NM_000918	Procollagen-proline, 2-oxoglutarate 4-dioxygenase	0.36
NM_024843	Hypothetical protein FLJ23462	0.38
NM_004786	Thioredoxin isolog	0.47
NM_005313	Glucose regulated protein, 58kD(thioredoxin)	0.50

Table 7. Differently expressed genes related with unclassified genes.

Gen bank	Name	Ratio
Genes up-regulated during mineralization		
NM_024681	Hypothetical protein FLJ12242	2.43
Genes down-regulated during mineralization		
AK027115	Homosapiens cDNA : FLJ21592 fis, clone	0.32
D	KFZp434F1728	0.41
NM_006360	Dendritic cell protein(GA17)	0.41
NM_025133	Hypothetical protein FLJ12673	0.42
NM_019760	Mus musculus tumor differentially expressed 1	0.46
NM_023734	Mus musculus RIKEN cDNA(1200009H11 gene / novel)	0.47
NM_020221.1	Hypothetical protein DKFZp547I224	0.50

Table 8. Differently expressed genes related with novel genes.

Gen bank	Name	Ratio
Genes up-regulated during mineralization		
	M0594-5	3.88
	B0814-5	2.83
	K1705-5	2.55
	P1881-5	2.17
Genes down-regulated during mineralization		
	B1948-5	0.26
	K0336-5	0.36
	M3534-5	0.40
	B0620-5	0.43

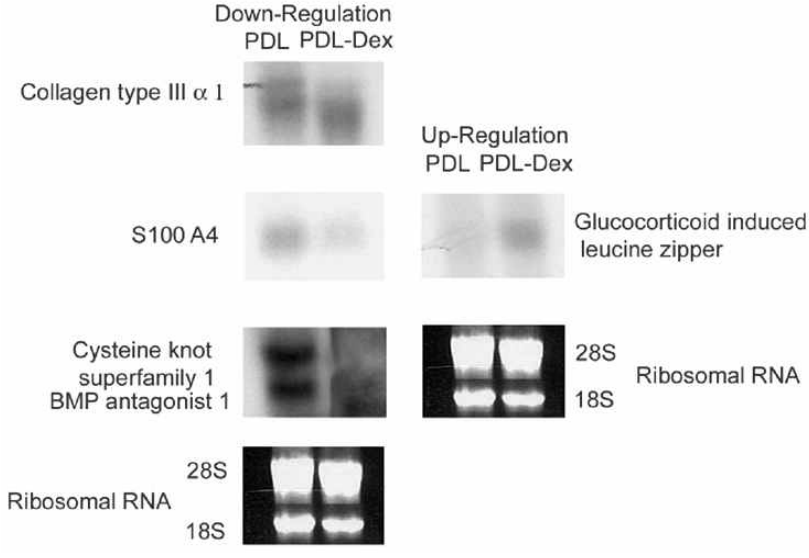

Figure 3. Northern blot analysis of gene expression during mineralization of PDL cells. To verify microarray results on selected genes, we performed Northern blot analysis.

antagonist 1 to be verified(Figure 3). A ribosomal RNA (28S and 18S) was chosen to serve as a control(Figure 3). Our Northern blot results demonstrated identical expression pattern for all selected genes as revealed by microarray analysis. The results indicated that our microarray data were highly reproducible and reliable.

IV. DISCUSSION

To identify genes involved in the mineralization of extracellular matrix during the differentiation of PDL cells, we performed cDNA microarray analysis by using PDL cells that could be induced to differentiate into osteoblastic lineages[16,17].

Among many modern techniques, one of

the most important tool in gene expression analysis is the development of DNA microarray[15,18]. DNA microarrays provide a format for the simultaneous measurement of the expression level of thousands of genes in a single hybridization asay. And it will be more efficient and resonable to have a tissue-specific DNA chip for specific area of research, as it already proved in a breast tissue DNA chip[19], and a melanocyte-specific DNA chip[20]. We have generated a dermal papilla cDNA chip consisted of 3,063 cDNA clones isolated from human hairy mesenchymal cell derived genes cDNA library. Although this chip is not specific to discover mineralization-related genes, it is adequate to analysis the characterization of mesenchymal-derived PDL cells.

In experiments using two different culture conditions, we clearly demonstrated that a medium containing mineralization supplements (50 μg/ml ascorbic acid, 10 mM β -glycerophosphate and 100 nM dexamethasone) could induce matrix mineralization, which was detected by virtue of strong staining with alizarin red S, but a medium without mineralization supplements could not (Figure 1).

Using acomputer analysis, we selected genes that showed an altered expression in the cells cultured in a medium with mineralizationsupplements compared to in the cell cultured in the media with only 10% FBS . We identified 35 genes that were up-regulated more than two-fold and 38 that were down-regulated by more than 50% during the mineralization process. Among the selected genes, several had already been noted for roles in osteogenesis. For example IGFBP2 binds prolong the half-life of the IGFs, inhibit or stimulate the growth promoting effects of the IGFs on cell culture, and alter the interaction of IGFs with their cell surface receptors[21]. IGF-II is a potent pleiotypic stimulator of both replication and differenciation of cells in the oteoblast lineage[22] and increase proliferation and alkaline phosphatase activity in the human osteosarcoma cells[23].

During the mineralization, S100 calcium-binding protein A4, a calcium-binding one with minerals in extracellular matrix, and OSF-2(periostin), a potential bone adhesion protein, were down-regulated. S100 calcium-binding proteins are small, acidic proteins which interact with target molecules and control various cellular processes such as cell cycle progression, differentiation, and cell metabolism after binding to Ca^{2+}[24]. Interestingly Strutz et al[25] have isolated one S100 calcium-binding protein known as S100A4 as a fibroblast-specific protein and demonstrated its high gene expression in the periodontal mesenchyme during early stages of tooth morphogenesis in an in situ hybridization study. S100A4 might be a useful marker for distinguishing cells of the PDL from the gingiva[26]. S100A4 has an extracellular role as a mineralization inhibitor, which is responsible for maintaining the periodontal ligament space[27]. We found that the PDL cells culture medium with 10% FBS at 21 day retained their characteristic

phenotypes during the culture period.

OSF-2(periostin)was originally identified as a secreted factor in a screen of a mouse osteoblastic library, and is thought to function in bone adhesion, based on its sequence similarity to the insect adhesion protein fasciclin-1[28,29] because it is expressed in specialized connective tissues which form and support mineralized tissues[30]. Not all of the ALP positive cells in bone express OSF-2 (periostin), only those ALP positive cells in the periosteum. OSF-2(periostin)also appears to be secreted into the surrounding extracellular matrix. Because strong expression of OSF-2(periostin)is observed in periosteal osteoblasts and not in the osteoblasts lining the trabecular bone where bone remodeling is actively taking place, it is assumed that OSF-2(periostin)is not directly but indirectly involoved in osteogenesis or in the process of bone formation or repair[30]. OSF-2(periostin) expression in osteocytes was nearly undetectable, suggesting that osteoblasts cease the production of OSF-2(periostin) during the process of differentiation. A osteocyte cell line, MLO-Y4, lacks detectable OSF-2(periostin) transcripts[31], which further supports this observation. Osteocytes are likely derived by the terminal differentiation of osteoblasts which become trapped within the forming osteoid tissue during bone formation.

We identified the genes noted for roles in apoptosis during mineralization. Apoptosis during osteogenesis has not been well studied but there are clear indications that it must play an important role in the normal bone formation and remodeling. A major fraction of osteoblasts at bone remodeling sites cannot be accounted for and probably undergo apoptosis[32]Dkk-1 and Nip3 were up-regulated, but Btf and TAX1BP1 were down-regulated during mineralization. Dkk-1 is a secreted protein that specially inhibits Wnt/βcatenin signaling by interacting with the co-receptor Lrp-6[33,34]. Dkk-1 is originally identified as a strong head inducer in Xenopus due to its potent anti-Wnt effect[35]. Dkk-1 promotes Bmp-triggered apoptosis and this might suggest that antagonism of Wnt/βcatenin signals by Dkk-1 is necessary for Bmp-triggered apotosis[36]. Nip3 is an apoptosis-inducing dimeric mitochondrial protein that can overcome Bcl-2 suppression[37]. Bcl-2 family members bear COOH-terminal transmembrane domains that allow their associtation with the outer mitochondrial membrane[38] and this mitochondrial localization is important for the suppressive function of Bcl-2[39,40]. There is growing evidence that mitochondrial function is disturbed early in the apoptotic response and may be important in mediating apoptosis[41-43]. This is often seen as the loss of mitochondrial membrane potential[41,42] and the release of cytochromec[43], which has been implicated in the activation of caspases[43-45]. Bcl-2 can suppress the release of cytochrome c from mitochondria and prevent caspase activation[44,45]. Btf is a novel death-promoting transcriptional repressor that interacts with Bcl-2-related proteins and inhibits the apoptosis[46]. TAX1BP1 is a

novel substrate for caspase family members. Thus TAX1BP1 appears to be a novel A20-binding protein which might mediate the anti-apoptotic activity of A20, and which can be processed by specific caspases[47].

Our identification of a set of genes that may be associated with the mineralization of bone matrix provides important information toward a better understanding of the capacity to differentiate PDL cells into either cementoblasts or osteoblasts, depending on needs and conditions as well as the molecular mechanism about the regeneration of periodontal tissues lost by the periodontal diseases.

V. SUMMARY

Periodontal ligament (PDL) cells have been known as multipotential cells, and as playing an important rolesin periodontal regeneration. The PDL cells are composed of heterogeneous cell populations which have the capacity to differentiate into either cementoblasts or osteoblasts, depending on needs and conditions. Therefore, PDL cells have the capacity to produce mineralized nodules in vitro in mineralization medium which include ascorbic acid, β-glycerophosphate and dexamethasone.

In spite of these well-known osteoblast like properties of PDL cells, very little is known about the molecules involved in the formation of the mineralized nodules in the PDL cells. In the present study, we analysed gene-expression profiles during the mineralization process of cultured PDL cells by means of a cDNA microarray consisting of 3063 genes.

Nodules of mineralized matrix were strongly stained with alizarin red S on the PDL cells cultured in the media with mineralization supplements.

Among 3,063 genes analyzed, 35 were up-regulated more than two-fold at one or more time points in cells that developed matrix mineralization nodules, and 38 were down-regulated to less than half their normal level of expression. In accord with the morphological change we observed, several genes related to calcium-related or mineral metabolism were induced in PDL cells during osteogenesis, such as IGF-II and IGFBP-2. Proteogycan 1, fibulin-5, keratin 5, βactin, αsmooth muscle actin and capping protein, and cytoskeleton and extracellular matrix proteins were up-regulated during mineralization. Several genes encoding proteins related to apoptosis weredifferentially expressed in PDL cells cultured in the medium containing mineralization supplements. Dkk-1 and Nip3, which are apoptosis-inducing agents, were up-regulated, and Btf and TAX1BP1, which have an anti-apoptosis activity, were down-regulated during mineralization. Also periostin and S100 calcium-binding protein A4 were down-regulated during mineralization.

VI. REFERENCES

1. Ten Cate AR, Oral histology, 4th ed.,

Mosby, St. Louis, MO, pp. 1994;56-77
2. Hou LT, and Yaeger JA: Cloning and characterization of human gingival and periodontal ligament fibroblasts, J Periodontol 1993;64:1209-1218
3. Kuru L, Parkar MH, Griffiths GS, Newman HN, and Olsen I: Flow cytometry analysis of gingival and periodontal ligament cells, J Dent Res 1998;77: 555-564
4. Lackler KP, Cochran DL, Hoang AM, Takacs V, and Oates TW: Development of an in vitro wound healing model for periodontal cells, J Periodontol 2000;71: 226-237
5. Bowers GM, Granet M, Stevens M, Emerson J, Corio R, Mellonig J, Lewis SB, Peltzman B, Romberg E, and Risom L: Histological evaluation of new attachment in humans : A preliminary report, J periodontol 1985;56:381-396
6. Pontonero R, Lindhe JM, Nyman S, Karring T, Rosenberg E, and Sanavi F: Guided tissue regeneration in degree II furcation involved mandibular molars : A clinical study, J Clin Periodontol 1988;15: 249-254
7. Carnes DL, Maeder CL, and Graves DT: Cells with osteoblastic phenotypes can be explanted from human gingiva and periodontal ligament, J Periodontol 1997;68: 701-707
8. Brandsten C, Lundmark C, Christersson C, Hammarstrom L, and Wurtz T: Expression of collagen alpha1(I) mRNA variants during tooth and bone formation in the rat, J Dent Res 1999;78:11-19
9. Chien HH, Lin WL, and Cho MI: Expression of TGF-beta isoforms and their receptors during mineralized nodule formation by rat periodontal ligament cells in vitro, J Periodontal Res 1999;34:301-309
10. Beertsen W and Everts V: Formation of acellular root cementum in relation to dental and non-dental hard tissues in the rat, J Dent Res 1990;69:1669-1673
11. Somerman MJ, Archer SY, Imm GR, and Foster RA: A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro, J Dent Res 1998;67:66-70
12. Yamaguchi M, Shimizu N, Shibata Y, and Abiko Y: Effects of different magnitudes of tension-force on alkaline phosphatase activity in periodontal ligament cells, J Dent Res 1996;75:889-894
13. Ramakrishnan PR, Lin WN, Sodek J, and Cho M-I: Synthesis of non-collagenous extracelluar matrix proteins during development of mineralized nodules by rat periodontal ligament cells in vitro, Calcif Tissue Int 1995;57:52-59
14. Dahl, LK: A simple and sensitive histochemical method for calcium, Proc. Soc. Exp. Biol. Med. 1952;80:474-479
15. Schna M, Shalon D, Heller R, Chai A, Brown PO, and Davis RW: Parallel human genome analysis: Microarray-based expression monitoring of 1000 genes, Proc. Natl Acad Sci USA 1996;93:10614 -10619
16. Bruder SP, Jaiswal N, and Haynesworth SE: Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive sub cultivation and following cryopreservation, J Cell Biochem 1997;64:278-294
17. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig, S, and Marshak DR: Multilineage potential of adult human mesenchymal stem cells, Science 1999;284:143-147

18. Brown PO, and Bostein D: Exploring the new world of the genome with DNA microarray, Nat Genet 1999;21:33-37
19. Perou CM, Jeffrey SS, Van de Rijn M, Rees CA, Eisen MB, Ross DT, Pergamenschikou A, Wolliams CF, Zhu SX, Lee JC, Lashkari D, Shalon D, Brown Po, and Botstein D: Distinctive gene expression patterns in human mammary epithelial cell and breast cancers, Pro Natl Acad Sci 1999;96:9212-9217
20. Loftus SK, Chen Y, Gooden G, Ryan JF, Birznieks G, Hilliard M, Baxevanis AD, Bittner M, Meltzer P, Trent J, and Pavan W: Informatic selection at a neural crest melanocyte cDNA set for microarray analysis, Proc. Natl Acad Sci 1999;96:9277-9288
21. Warg E, Warg J, Chin E, Zhan J, and Bondy LA: Cellular patterns of insulin-like growth factor system gene expression in murine chondrogenesis and osteogenesis, Endocrinology 1995;136:2741-2751
22. Cahalis E, McCarthy R, and Centrella M: Growth factors & cytokines in bone cell metabolism, Hnnu Rev Med 1991;42:17-24
23. Knutsen R, Honda Y, Strong DD, Sumpath TK, Boylink DJ, and Mohan S: Regulation of insulin-like growth factor system components by osteogenic protein-1 in human bone cells, Endocrinology 1995;136:857-865
24. Schafer BW, and Heizmann CW: The s100 family of EF-hand calcium-binding proteins :functions and pathology(review), Trends Biochem Sci 1996;21:134-140
25. Stratz F, Kada H, Lo CW, Danoff T, Carare RL, and Tomaszewsk JE :Identification and characterization of a fibroblast marker:FSP1, J Cell Biol 1995; 130:393-405
26. Duarte WR, Limura T, Takenaga K, Ohya K, and Ishikawa I: cDNA cloning of S100 calcium-binding proteins from bovine periodontal ligament and their expression in oral tisues, J Dent Res 1998;77:1694-1699
27. Durate WR, Limura T, Takenaga K, Ohya K, Ishikawa I, and Kasugai S: Extracellular role of S100A4 calcium-binding protein in the periodontal ligament. Biochem. Biophys Res Commun 1999;255:416-420
28. Takeshita S, Kikum R, Tezuka K, and Amann E: Osteoblast-specific factor 2: Cloning of a putative bone adhesion protein with homology with the insect protein fasciclin ?, Biochem J 1993;27:278-294
29. Sugiura T, Takamatsu H, Kudo A, and Amann E: Expression and characterization of murine osteoblast-specific factor2 (OSF-2) in a baculovirus expression system, Protein Expr Purif 1995;6:305-311
30. Horiuchi K, Amizuka N, Takeshita S, Takamatsu H, Katsuura M, Ozawa H, Toyama Y, Bonewald LF, and Kudo A: Identification and characterization of a novel protein, periostin with restricted expression to periosteum and periodontal ligament and increased expression by transforming growth factor beta, J Bone Miner Res 1993;14:1239-1249
31. Kato Y, Windle JJ, Koop BA, Mundy GR, and Bonewald LF: Establishment of an osteocyte-like cell line, MLO-Y4, J Bone Miner Res 1997;12:2014-2033
32. Tilka R, Weinstein RS, Bellido T, Parfitt AM, and Manolagas S: Osteoblast programmed cell death(Apoptosis):Modulation by growth factors and cytokines, J Bone Miner Res 1998;13:793-802
33. Bao B, Wu W, Li Y Stannek P, Glinka A, and Niehrs C: LDL-receptor-related

protein 6 is a receptor for Dickkopf proteins, Nature 2001;411:321-325
34. Zorn AM: Wnt signaling: antagonistic Dikkopfs, Curr Biol 2001;11:R592-R595
35. Glinka A, Wu W, Delius H, Monaghan AP, Blumenstock C, and Niehr C: Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction, Nature 1998;391:357-362
36. Grotewold L and Ruther U: The Wnt antagonist Dickkopf-1 is regulated by BMP signaling and c-Jun and modulates programmed cell death, EMBO J 2002;21: 966-975
37. Chen G, Ray R, Doubit D, Shi L, Cizeau J, Bleackly C, Saxena S, Gietz RD, and Greenberg AH: The E1B 19k/Bcl-2-binding protein Nip3 is a dimeric mitochondrial protein that activates apoptosis, J Exp Med 1997;12:1975-1983
38. Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, and Reed JC :Investigation of the subcellular distribution of the Bcl-2 oncoprotein : Residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes, Cancer Res 1993;53:4701-4714
39. Tanaka S, Saito K, and Reed JC: Structure-function analysis of the Bcl-2 oncoprotein. Addition of a heterologous transmembrane domain to portions of the Bcl-2 beta protein restores function as a regulator of cell survival, J Biol Chem 1993;268:10920-10926
40. Zhu W, Cowie A, Wasfy GW, Penn LZ, Leber B, and DA Andrews: Bcl-2 mutants with restricted subcellular location reveal spatially distinct pathways for apoptosis in different cell types, EMBO J 1996;15: 4130-4141
41. Zamzami N, Susin SA, Marchetti P, Hirsch T, Gomez-Monterrey I, Castedo M, and Kroemer G: Mitochondrial coordinating control of nuclear apoptosis, J Exp Med 1996;183:1533-1544
42. Marchetti P, Castedo M, Susin SA, Zamzami N, Hirsch T, Macho A, Haeffiner A, Hirsch F, Geuskens M, and Kroemer G: Mitochondrial permeability transition is a central coordinating event of apoptosis, J Exp Med 1996;184:1155-1160
43. Liu X, Kim C, Yang J, Jemmerson R, and Wang X: Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome c, Cell 1996;86: 147-157
44. Yang J, Liu XS, Bhalla K, Kim CN, Ibrado AM, Cai JY, Peng TI, Jones DP, and Wang XD: Prevention of apoptosis by Bcl-2: Release of cytochrome c from mitochondria blocked, Science 1997;275: 1129-1132
45. Kluck RM, Bossy-Wetzel E, Green DR, and Newmeyer DD : The release of cytochrome c from mitochondria: A primary site for Bcl-2 regulation of apoptosis, Science 1997;275:1132-1136
46. Kasof GM, Goyal L, and White E: Btf, a novel death-promoting Transcriptional repressor hat interacts with Bcl-2-related proteins, Mol Cell Biol 1999;19:4390-4404
47. Devalck D, Jim DY, Heyninck K, Van de Craen M, Contresras R, Fieb W, Jearg KT, and Beyaert R: The zinc finger protein A20 interacts with a novel anti-apoptotic protein which is cleared by specific caspases, Oncogen 1999;18:4182-4190

-국문초록-

cDNA microarray에 의한 치주인대세포의 광물화 결절형성에 관여하는 유전자들의 분석

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치주인대세포는 시험관적 실험에서 광물화 결절형성을 유도할 수 있으므로 광물화 결절형성에 관여하는 유전자들을 특이하게 발현할 것으로 여겨진다. 이에 본 실험은 cDNA microarray를 이용한 동시 유전자분석을 시행하여 치주인대세포의 분화에 의한 광물화 결절형성시 나타나는 유전자의 특징적 발현 양상을 알아보고자 하였다.

교정치료를 목적으로 경북대학교병원에 내원한 환자의 제일소구치를 발치하여 통상적 방법으로 치주인대세포를 분리, 배양하였고, 3세대의 치주인대세포를 사용하여 실험을 시행하였다. 치주인대세포를 100 mm 배양접시에 넣고 배양하여 매 2일 마다 배지를 교환해 주고, 10% FBS 만을 투여한 군을 대조군으로, ascorbic acid (50 ㎍/㎖), βglycerophosphate (10 mM) 및 100 nM dexamethasone을 투여한 군을 실험군으로 하였다.

배양된 치주인대세포에 ascorbic acid, βglycerophosphate, 그리고 dexamethasone을 투여한 실험군에서 21일째 광물화된 결정을 관찰할 수 있었으나 대조군에서는 관찰할 수 없었다.

3063개의 유전자를 분석한 결과 35개 유전자가 대조군에 비해 2배이상 발현이 증가하였고, 38개 유전자는 2배이상 발현이 감소하였다. 형태학적 검사에서 보여준 바와 같이 광물화 형성과정시 관여하는 IGF-II과 IGFBP2와 같은 유전자가 실험군에서 증가하였으며, 세포골격과 세포외기질 형성에 관여하는 proteogycan 1, fibulin-5, keratin 5, βactin, α-smooth muscle actin, capping protein 등도 발현이 실험군에서 증가하였다. 한편 periostin and S100 calcium-binding protein A4는 대조군에서 오히려 높게 나타나므로 이는 배양된 치주인대세포가 그 자체의 표현형을 유지하고 있음을 보여 주고 있다. 그 외 apoptosis를 유발시키는데 관여하는 Dkk-1와 Nip3는 실험군에서 높게 발현되었고, apoptosis를 억제시키는데 관여하는 Btf와 TAX1BP1는 오히려 낮게 발현됨을 알 수 있으므로 이는 실험군에서 치주인대세포가 골아세포로의 분화되었음을 나타낸다.

Key worlds: Periodontal ligament cells, cDNA Microarray, Mineralization, Apoptosis