Comparative analysis of secretomes from deciduous and permanent periodontal ligament cells

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Comparative analysis of secretomes from deciduous and permanent periodontal ligament cells

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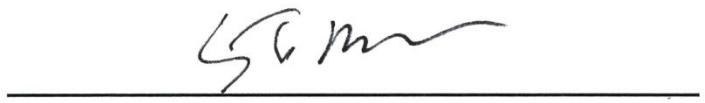
Thesis Supervisor: Song, Je Seon



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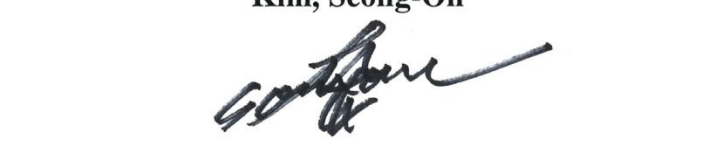

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감사의 글

박사 논문을 마무리 하며 이 논문이 끝날 수 있도록 물심 양면으로 도와주시고 인도해주신 분들께 깊은 감사의 마음을 전하고 싶습니다.

첫 지도교수님이시자 제가 앞으로 살아감에 있어 소아치과 의사의 귀감이 되어주신 손흥규 교수님, 또 지도교수직을 이어받아 제 연구를 실제적으로 이끌어주시고 훌륭한 멘토가 되어주신 송제선 교수님, 제가 무사히 수련받고 학위를 수여할 수 있도록 도와주신 소아치과의 존경하는 모든 교수님들께 감사드립니다. 또한 제 연구와 논문을 감수해주시고 보다 좋은 결과물을 낼 수 있도록 아낌없는 조언을 해 주신 김성오 교수님, 문석준 교수님, 박정철 교수님, 조성원 교수님께 깊은 감사를 전합니다.

연구에 대해 서툴고 배울게 많았던 저의 수많은 질문에도 불구하고 자기 일처럼 도와준 전미정 연구원님도 너무 고맙습니다. 물리적으로 멀리 있어 미처 챙길 수 없는 일들을 불평 없이 도와준 소아치과 의국 후배 주기훈 수련의도 수고 많았습니다.

그리고 지금까지 항상 옆에서 묵묵히 저의 모든 필요를 채워주시고 공부를 지속할 수 있도록 깊은 배려와 기도로 응원해 주신 사랑하는 부모님께 감사를 드립니다. 또한 응당 함께 해야 할 결혼 준비를 멀리 떨어져 있는데다 학위준비로 정신 없는 저로 인해 혼자 짊어져 고생한 지금의 제 아내가 된 은영이에게 특별히 감사하다고 말하고 싶습니다.

부족한 제가 이 논문을 쓸 수 있었던 것은 이 모든 분들의 아낌없는 도움이 있었기에 가능했었습니다. 감사합니다.

2015년 1월

김기림 드림

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Abstract

Comparative analysis of secretomes from deciduous and permanent periodontal ligament cells

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The aims of the present study were to identify and compare the secretomes of human deciduous and permanent periodontal ligament (PDL) cells.

Secreted proteins were isolated and identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Antibodies against Tudor domain containing 7 (TDRD7) and Vasorin (VASN) were used for western blot. In addition, we used the cytokine

membrane array analysis and enzyme-linked immunosorbent assay (ELISA) was performed for some cytokine (VEGF, EGF, PDGF-BB, SCF, and FGF beta).

Collagen type VI, alpha I, collagen type I, alpha I, collagen type III, alpha I, cullin 7, and nuclear factor I/X (CCAAT-binding transcription factor) related to cell growth and/or maintenance were identified in deciduous PDL group. But vasorin (VASN), IGF binding protein 4, and S100 calcium binding protein A9 related to cell communication and signal transduction were identified in permanent PDL group. The secretomes that were upregulated in deciduous PDL tissues were involved in inflammatory or immune reactions (IL-1 alpha, IL-1 beta, IL-2, I-309, IL-12 p40/70, IL-4, MMP1).

Proteins related to cell growth and/or maintenance, cell communication; signal transduction, inflammatory or immune reactions were slightly different between permanent PDL and deciduous PDL groups. These results show that humoral factors released from deciduous and permanent PDL cells have different characteristics.

Although the findings in our study are not sufficient to explain difference between permanent and deciduous PDL clearly, our study provides clues to develop our understanding of the molecular basis.

Keywords: secretome, cytokine, humoral factor, periodontal ligament cell, deciduous and permanent teeth

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I. INTRODUCTION

Human have two dentitions, deciduous and permanent. The deciduous and permanent teeth exhibit distinctive developmental processes, morphologies, histological characteristics and life cycles. In addition, the proliferation activities and differentiation patterns differ between the cells isolated from the two types of teeth (Govindasamy et al., 2010; Miura et al., 2003; Nakamura et al., 2009).

Since deciduous teeth are resorbed and exfoliated in association with the eruption of successive permanent teeth, the periodontal tissues of the former are more easily resorbed than those of the latter (Davies et al., 2001; Harokopakis-Hajishengallis, 2007). To explain this at the molecular level, some investigators reported that the periodontal tissues of deciduous teeth contain more bone sialoprotein and osteopontin with the Arg-Gly-Asp (RGD) sequence, to which odontoclasts bind.(Bosshardt et al., 2005; Lee et al., 2004) Others reported that periodontal ligament (PDL) cells obtained from areas being resorbed in deciduous teeth express more of the receptor activator of the nuclear factor κ-light-chain-enhancer of activated B cells ligand (RANKL) gene, which is known to be associated with osteoclastogenesis.(Fukushima et al., 2003; Lossdorfer et al., 2002) In addition, extracellular-matrix-degrading enzymes such as collagenase (Alexander and Swerdloff, 1980), metalloproteinases (Linsuwanont et al., 2002; Wu et al., 1999), and mucopolysaccharidase (Alexander and Swerdloff, 1979) were found to be upregulated upon the resorption of deciduous periodontium. However, these findings are not sufficient to explain the differences in the periodontium of deciduous and permanent teeth.

Besides, humoral factors of adjacent tissues have been focused recently as playing an important role in regulating grafted stem cells and characterizing differentiation process. Mesenchymal stem cells (MSCs) possess remarkable self-renewal ability and are able to differentiate into various cell lineages. MSCs can also enhance tissue repair and angiogenesis through a paracrine mechanism (Wang et al., 2011). In the experimental study, the results suggest that MSCs produce a broad repertoire of trophic factors with

tissue-regenerative activities (Ando et al., 2014). Previous study shows that PDL cells were also suggested to engage in differentiation and proliferation of adjacent tissues, such as MSCs (Mizuno et al., 2008) or the human alveolar bone–derived stromal cells (hABCs) (Park et al., 2012), by paracrine mechanism. However, in comparison with MSCs, the reports analyzing these humoral factors from PDL cells are insufficient. Especially, little is known about the secretomic signatures of deciduous PDL cells and the difference with that of permanent PDL cells. Identifying the key factors of various secretomes and their functions in secretome-mediated repair will contribute to the development of regenerative therapies.

Considering these precious studies, it is a task of great significance to investigate the secretomes of two kinds of dentition, primary and permanent. The aims of the present study were to identify and compare the secretomes of human deciduous and permanent periodontal ligament in order to enhance our understanding of the molecular basis

II. Materials and Methods

1. Cell Culture

Permanent teeth periodontal ligament cells (P-PDL) were obtained from the permanent teeth (n=7 from 3males and 4 females, aged 7-18 years) and deciduous teeth periodontal ligament cells (D-PDL) were obtained from the deciduous teeth (n=7 from 3males and 4 females, aged 2-11 years) under approved guidelines set by the Institutional Review Board of the Dental hospital, Yonsei University (approval no. 2-2014-0030). P-PDL and D-PDL cells were isolated by outgrowth (Song et al., 2012). Briefly, P-PDL and D-PDL tissue was cut into three fragments that were subsequently placed onto a 60-mm culture dish (BD Falcon, Lincoln Park, NJ, USA), and covered with a cover glass to allow the cells to grow out. P-PDL and D-PDL cells were cultured in a cell culture medium comprising alpha-minimum essential medium (α-MEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and 2 mM L-glutamine (Invitrogen) at 37℃ in 5% CO_2. The isolated P-PDL and D-PDL cells originated from at least two or three different donors were blended at passage 2, and cells at passages 3-5 were used for further experiments.

2. Preparation of P-PDL and D-PDL-conditioned medium (P-PDL-CM and D-PDL-CM)

PDL and DPDL cells were seeded at $3X10^5$ cells on 100-mm culture dish (BD Falcon). When reaching 80% confluence, P-PDL and D-PDL cells were washed seven times with

PBS (pH 7.4; Invitrogen), and cultured in a cell culture medium descriebed above without FBS. After 48 hours, supernatant was collected and protease inhibitor cocktail (Roche Diagnostic Systems, Branchburg, NJ, USA) was added into supernatant. After filtration using an Amicon stirred cell (Merck Millipore, Darmstadt, Germany), 500 ml of harvested conditioned media (CM) were concentrated using Amicon ultra-15 centrifugal filter unit with ultracel-3 membrane (nominal molecular weight limit (NMWL) 3kDa; Merck Millipore) and vivaspin® 500 centrifugal concentrator (molecular weight cut off (MWCO) 5000; Sartorius, Bohemia, NY, USA) according to the manufacturer's instructions. CM samples were dialyzed against PBS at 4ºC and used for further analysis. Protein concentration of CM was determined with the Bradford assay using the Bradford Dye Reagent (Bio-Rad, Hercules, CA, USA).

3. Separation by one-dimensional polyacrylamide gel electrophoresis

One-dimensional polyacrylamide gel electrophoresis (1DE) was performed using a 12% polyacrylamide gel. 12% polyacrylamide gel was prepared from 40% Acrylamide/BisTM (29:1) (Amresco, Solon, OH, USA), Sodium Dodecyl Sulfate (SDS) (Affymetrix, Santa Clara, CA, USA), Ammonium persulfate (APS) (Sigma-Aldrich, St Louis, MO, USA), Tris (Amresco), Glycine (Amresco), and N,N,N',N'-Tetramethylethylenediamine (TEMED) (Sigma-Aldrich, St Louis, MO, USA). Gels were run using a Mini-Protean Tetra cell system (Bio-Rad) and stained with Coomassie PRO solution (ProteomeTech Inc., Seoul, Korea). PageRuler Broad Range Unstained Protein Ladder (Thermo, Pittsburgh, PA, USA) was used for 1DE. After staining, gels were

washed with distilled water and scanned using ChemiDoc XRS+ (Bio-Rad). Gel images were processed using Image Lab software (Bio-Rad). Secreted protein sample lanes from CM were cut and processed using in-gel digestion procedures after polyacrylamide gel electrophoresis. 1DE was performed for three independent experiments.

4. In-gel protein digestion

Protein spots of interest were excised and digested in-gel with sequencing grade, modified trypsin (Promega, Madison, WI) as previously described.(Bahk et al., 2004) In brief, each protein spot was excised from the gel, placed in a polypropylene (Eppendorf, Hamburg, Germany) tube, and washed 4-5 times (until the gel was clear) with 150㎕ of 1:1 acetonitrile (ACN)/25 mM ammonium bicarbonate (ABC), pH 7.8. The gel slices were dehydrated by 100% ACN, and then dried in a Speedvac concentrator. After reduction (incubation at 56℃ for 45 minutes with 10 mM DTT in 100 mM ABC) and alkylation (incubation at room temperature for 30 minutes at dark place with 55 mM iodoacetamide in 100 mM ABC), the gel slices were dried by 100% ACN and the Speedvac. And then samples were rehydrated in 30㎕ of 25 mM ABC, containing 20 ng of trypsin. After incubation at 37℃ for 20 hours, the liquid was transferred to a new tube. Tryptic peptides remaining in the gel matrix were extracted for 40 minutes at 30℃ with 20㎕ of 50% (v/v) aqueous acetonitrile containing 0.1% (v/v) formic acid. The combined supernatants were evaporated in the Speedvac and dissolved in 8㎕ of 5 % (v/v) aqueous acetonitrile solution containing 0.1% (v/v) formic acid for mass spectrometric analysis.

5. Identification of proteins by liquid chromatography-coupled electrospray ionization mass spectrometry

The resulting tryptic peptides were separated and analyzed using reversed phase capillary HPLC directly coupled to a Finnigan LCQ ion-trap mass spectrometer (LC-MS/MS) (Zuo et al, 2001, with a slight modification) (Zuo et al., 2001). Both of a 0.1 x 20 mm trapping and a 0.075 x 130mm resolving column were packed with Vydac 218MS low trifluoroactic acid C18 beads (5㎛ in size, 300Å in pore size; Vydac, Hesperia, CA, USA) and placed in-line. Following the peptides were bound to the trapping column for 10 min at with 5% (v/v) aqueous acetonitrile containing 0.1% (v/v) formic acid, then the bound peptides were eluted with a 50 minutes gradient of 5– 80% (v/v) acetonitrile containing 0.1% (v/v) formic acid at a flow rate of 0.2 μl/min. For tandem mass spectrometry, a full mass scan range mode was m/z = 450– 2000Da. After determination of the charge states of an ion on zoom scans, product ion spectra were acquired in MS/MS mode with relative collision energy of 55%.

The individual spectra from MS/MS were processed using the TurboSEQUEST software (Thermo Quest, San Jose, CA). The generated peak list files were used to query either MSDB database or NCBI using the MASCOT program (http://www.matrixscience.com). Modifications of methionine and cysteine (Carbamidomethyl (C), Deamidated (NQ), Oxidation (M)), peptide mass tolerance at 2 Da, MS/MS ion mass tolerance at 1 Da, allowance of missed cleavage at 1, and charge states (+1, +2, and +3) were taken into account. Only significant hits as defined by MASCOT probability analysis were considered initially. LC-MS/MS data were obtained for three independent experiments

and data were accumulated from three independent experiments. P-PDL and D-PDL secreted proteins with individual ion scores more than 20 (cut-off level was 20) were listed in table 1 and table 2.

6. Western blot Analysis

A protein amount of 20 μg from each sample was used for western blot analysis. 1DE was performed using a 8% polyacrylamide gel. Gels were run using a Mini-Protean Tetra cell system (Bio-Rad). Xpert 2 prestained protein marker (GenDEPOT, Barker, TX, USA) was used for Western blot After 1DE, blots were transferred to nitrocellulose membranes using TE77 ECL SemiDry Transfer Unit (Amersham biosciences, Piscataway, NJ, USA). Membranes were stained with Ponceau S (Siama-Aldrich) to verify loading equivalency. Membranes were incubated with antibodies against tudor domain-containing protein 7 (TDRD7) diluted 1:5000 (rabbit polyclonal antibody; Ab103866, Abcam, Cambridge, UK) and vasorin (VASN) diluted 1:5000 (rabbit polyclonal, SLITL2; Ab156868, Abcam) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:3000 (#AP307P, Merck Millipore). Blots were developed with ECL (Pierce, Rockford, IL, USA) and visualized by using ChemiDoc XRS+ (Bio-Rad). Western blot data were obtained for three independent experiments.

7. Cytokine Profiles of PDL-CM and DPDL-CM and Data Analysis

Conditioned medium of PDL and DPDL (48 hours of culture) were analyzed using human cytokine array C5 (RayBiotech, Inc., Norcross, GA, USA), according to the

manufacturer's instruction, as described in 2008 by Ohshima et al (Ohshima et al., 2008). A pair of cytokine array membranes were blocked with blocking buffer for 30 minutes and then incubated with 2ml of each conditioned medium at room temperature for 4hours. After samples were decanted from the container, the membranes were washed with wash buffer I three times and wash buffer II twice at room temperature with gentle shaking. Then membranes were incubated with diluted primary antibodies at 4℃ overnight and washed as described above. Membranes were incubated with horseradish peroxidase-conjugated streptavidin for 30 minutes at room temperature. After washing, membranes were incubated with a peroxidase substrate for 5 minutes at room temperature. Finally, membranes were exposed to x-ray film (Hyper film ECL) for 5 to 15 minutes at room temperature. Three independent samples of PDL –CM and DPDL-CM were analyzed. Alpha MEM containing 0.1% FBS was used for positive control and alpha MEM without FBS was used for negative control. The images on x-ray films were captured by an optical scanner and saved as jpeg images. The signal intensities were quantified with image software (Gel quant pro, DNR Bio-Imaging Systems Ltd., Jerusalem, Israel) to compare intensities of the same cytokine from different samples. To prepare for normalization of the results, the measured v7alues of each cytokine (duplicate spots) on the same membrane were revised by subtracting the average of negative control spot values from the intensity of each spot, including positive control spot. Using these revised values, a percentage of the value of a cytokine spot versus the average of the positive control spot value on the same membrane was calculated as the relative expression lever (REL) of the cytokines as described in 2008 by Ohshima et al (Ohshima

et al., 2008). : REL (%) = (signal intensity of sample – mean intensity of negative control) × 100 (%)/(mean intensity of positive control – mean intensity of negative control).

8. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) assay was performed using human growth factor ELISA strip II according to the manufacturer's instructions. In brief, 8-strip coated with different antibodies was incubated with PDL-CM and DPDL-CM for 1 hour at room temperature with gentle shaking. After the CM samples were decanted from the 8-strip, and 8-strip was washed with assay buffer three times. Then samples were incubated with diluted biotin-labeled antibody mixture for 1 hour at room temperature with gentle shaking and washed with assay buffer three times. After washing, 8-strip was incubated with diluted streptavidin-HRP conjugate for 45 minutes at room temperature with gentle shaking and washed with assay buffer three times. And, 8-strip was incubated with substrate for 5 to 30 minutes at room temperature. After incubating, stop solution was added and measured the absorbance at 450 nm within 30 minutes using a spectrophotometer (Benchmark Plus microplate spectrophotometer, Bio-Rad). ELISA data were obtained from three independent experiments.

9. Statistical Analysis

All experiments were performed in triplicate. The normality of the data was evaluated using the Shapiro-Wilk test ($p<0.05$). The Mann-Whitney U test ($p<0.05$) was performed for all experiments using SPSS software (20.0 SPSS, Chicago, IL, USA)

III. Results

1. 1DE and LC-MS/MS analysis of human P-PDL and D-PDL secreted protein

1DE analysis was used to compare different protein band size between P-PDL and D-PDL. The band pattern was nearly similar with slightly different size and wideness (Fig. 1A). These bands were cut and trypsinized and used for LC-MS/MS analysis. The results of LC-MS/MS analysis indicated that 112 proteins were identified in P-PDL, 23 proteins were identified in D-PDL, and 17 proteins were identified in both samples (Fig 1B). P-PDL and D-PDL secreted proteins with individual ion scores more than 20 and number of repetition more than 2 were listed in tables 1 and 2. To translate the data into a more meaningful biological context and to characterize more thoroughly the sets of functionally related proteins, the differentially identified data sets were organized into molecular function and biological process terms (Figure 2 and Table 1-3). Fig 2A shows molecular function analysis of P-PDL and D-PDL and Fig 2B shows biological process analysis of P-PDL and D-PDL. Proteins related to cell growth and/or maintenance, cell communication; signal transduction were slightly different between P-PDL and D-PDL. Collagen type VI, alpha I, collagen type I, alpha I, collagen type III, alpha I, cullin 7, and nuclear factor I/X (CCAAT-binding transcription factor) related to cell growth and/or maintenance were identified in D-PDL (Table 2, cullin 7 and nuclear factor I/X (CCAAT-binding transcription factor) that number of repetition was 1 were not shown in table 2.). But vasorin (VASN), IGF binding protein 4, and S100 calcium binding protein A9

related to cell communication; signal transduction were identified in P-PDL (Table 1). Proteins related with catalytic activity indicated similar proportions in P-PDL and D-PDL but percentage of enzyme inhibitor activity protein was slightly higher in P-PDL than D-PDL. Tudor domain containing 7, catalytic activity protein, was identified in D-PDL, and cathepsin B and glucose-6-phosphate isomerase, catalytic activity protein, were identified in P-PDL (Tables 1-2). Plasminogen activator inhibitor, follistatin like 1, and alpha2-HS-glycoprotein related with enzyme inhibitor activity were identified in P-PDL (Table 1). Transporter activity proteins such as lactotransferrin were identified in P-PDL (Table 1).

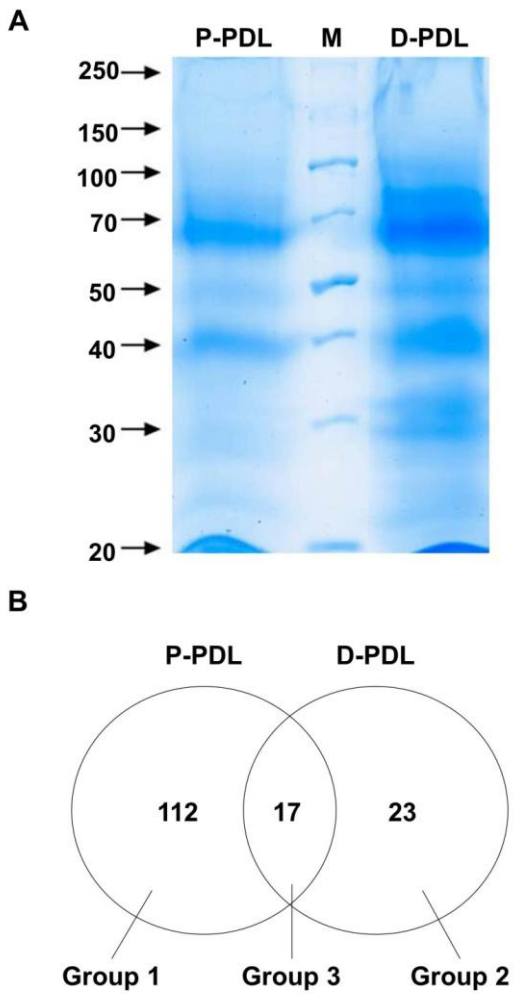

Figure 1. 1DE and LC-MS/MS analysis of human permanent teeth periodontal ligament cell (P-PDL) and human deciduous teeth periodontal ligament cell (D-PDL) secreted protein. (A) P-PDL and D-PDL secreted protein were separated by SDS PAGE and stained with Coomassie blue dye. (B) Venn diagram of overlapping proteins of P-PDL and D-PDL and the number of individual proteins identified with liquid chromatography-coupled electrospray ionization mass spectrometry (LC-MS/MS) analysis.

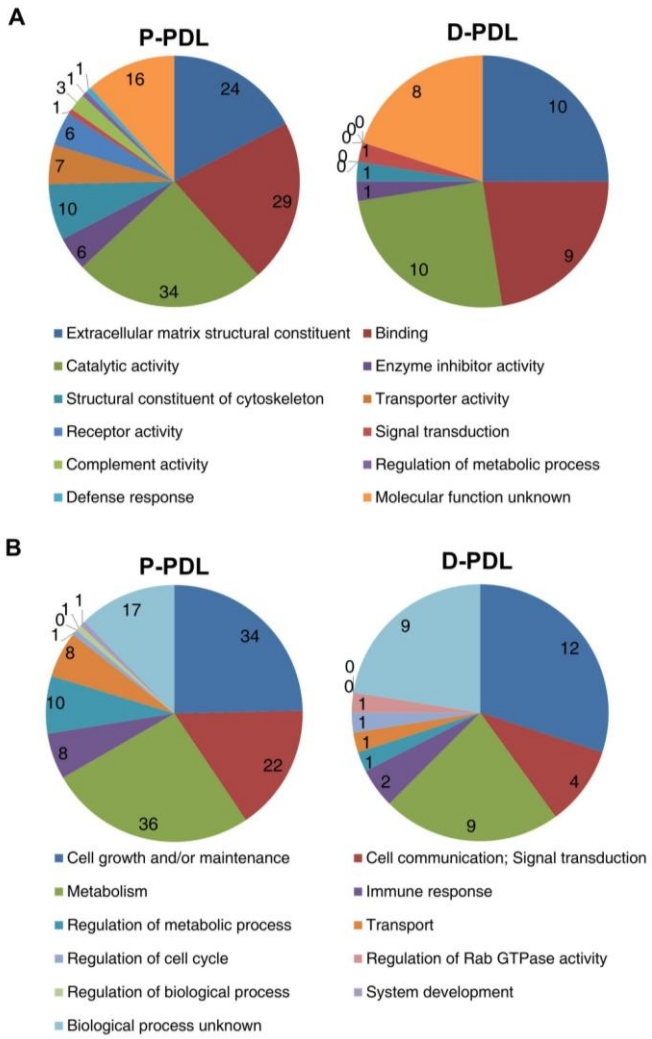

Figure 2. Molecular function and biological process analysis of LC-MS/MS. (A) Molecular function analysis of P-PDL and D-PDL. Molecular function was identified with 12 categories. (B) Biological process analysis of P-PDL and D-PDL. Biological process was analyzed with 11 categories. Molecular function and biological process term were represented as different color wedges in the pie charts, with the number of proteins per group shown in the pie charts.

Table 1. List of P-PDL secreted proteins in LC-MS/MS analysis. Proteins with individual ion score more than 20 and number of repetition more than 2 were listed.

Protein name	NCBI BLAST	Score	Mass	Number of repetition	Molecular function	Biological process
Collagen type VI, alpha 1	gi|87196339	3892	108462	3	Extracellular matrix structural constituent	Cell growth and/or maintenance
Collagen type I, alpha 2	gi|30102	2206	41496	3	Extracellular matrix structural constituent	Cell growth and/or maintenance
Osteonection	gi|2624793	1292	27054	2	Calcium ion binding	Cell growth and/or maintenance
Collagen type I, alpha 1	gi|180392	1205	98495	3	Extracellular matrix structural constituent	Cell growth and/or maintenance
Quiescin Q6 sulfhydryl oxidase 1	gi|133250075	1045	82526	3	Thiol oxidase activity	Regulation of cell cycle
Lumican	gi|642534	846	38375	3	Extracellular matrix structural constituent	Cell growth and/or maintenance
Tissue inhibitor of metalloproteinase 1	gi|31189	753	23182	2	Extracellular matrix structural constituent	Cell growth and/or maintenance
Matrix metalloprotease 2	gi|180671	581	72196	2	Metallopeptidase activity	Protein metabolism
IGF binding protein 7	gi|221045552	461	28769	2	Cell adhesion molecule activity	Cell communication ; Signal transduction
Mac 2 binding protein	gi|5031863	397	65289	3	Extracellular matrix structural constituent	Immune response
Plasminogen activator inhibitor	gi|189578	389	45045	2	Protease inhibitor activity	Protein metabolism
Vasorin	gi|154893339	381	64139	3	Receptor activity	Cell communication; signal transduction
Albumin	gi|119626083	293	58614	3	Extracellular matrix structural constituent	Transport
Extracellular matrix protein 1	gi|194379312	260	59925	2	Extracellular matrix structural constituent	Cell growth and/or maintenance
Follistatin like 1	gi|5901956	241	34963	2	Enzyme inhibitor activity	Metabolism
Keratin 10	gi|28317	237	59492	2	Structural constituent of cytoskeleton	Cell growth and/or maintenance
Collagen type V, alpha 1	gi|189520	194	183499	2	Extracellular matrix structural	Cell growth and/or maintenance

					constituent	
Cathepsin B	gi|181192	180	37783	2	Catalytic activity	Metabolism
IGF binding protein 6	gi|183894	149	25177	2	Cell adhesion molecule activity	Cell communication ; Signal transduction
CD248 molecule, endosialin	gi|9966885	135	80807	2	Receptor activity	Biological process unknown
Fibulin 1	gi|22761800	132	70531	2	Extracellular matrix structural constituent	Cell growth and/or maintenance
IGF binding protein 4	gi|695254	123	27923	2	Binding	Cell communication; signal transduction
Actin, beta	gi|28336	91	41786	2	Extracellular matrix structural constituent	Cell growth and/or maintenance
Collagen type VI, alpha 3	gi|55743098	82	343457	2	Extracellular matrix structural constituent	Cell growth and/or maintenance
Complement component 1, s subcomponent	gi|4502495	76	76635	2	Complement activity	Immune response
S100 Calcium binding protein A9	gi|4506773	72	13234	2	Binding	Cell communication; signal transduction
Lactotransferrin	gi|34412	57	78029	2	Transporter activity	Transport
Keratin 16	gi|186685	55	50668	2	Structural constituent of cytoskeleton	Cell growth and/or maintenance
Glucose-6-phosphate isomerase	gi|189238	54	63149	2	Catalytic activity	Metabolism

Table 2. List of D-PDL secreted proteins in LC-MS/MS analysis. Proteins with individual ion score more than 20 and number of repetition more than 2 were listed

.Protein name	NCBI BLAST	Score	Mass	Number of repetition	Molecular function	Biological process
Matrix metalloprotease 2	gi|5822007	125	70872	2	Metallopeptidase activity	Protein metabolism
Collagen type I, alpha 1	gi|119615036	122	84688	3	Extracellular matrix structural constituent	Cell growth and/or maintenance
Osteonection	gi|2624793	99	27054	2	Calcium ion binding	Cell growth and/or maintenance
-	gi|225477	85	249203	2	Molecular function unknown	Biological process unkown
Albumin	gi|28592	80	69321	3	Extracellular matrix structural constituent	Transport
Quiescin Q6 sulfhydryl oxidase 1	gi|1203965	74	86341	2	Thiol oxidase activity	Regulation of cell cycle
Collagen type VI, alpha 1	gi|30032	55	28565	2	Extracellular matrix structural constituent	Cell growth and/or maintenance
Tudor domain containing 7	gi|194378012	33	47478	3	Catalytic activity	Regulation of Rab GTPase activity

2. Western blot analysis

To confirm the expression of important proteins identified in LC-MS/MS analysis, western blot analysis was performed. Vasorin (VASN), cell communication; signal transduction protein, was identified in P-PDL and tudor domain containing 7 (TDRD7), catalytic activity, was identified in D-PDL. And two important proteins were differently expressed between P-PDL and D-PDL. TDRD7 expression was stronger in D-PDL than P-PDL, but VASN expression was stronger in P-PDL than D-PDL (Fig. 3).

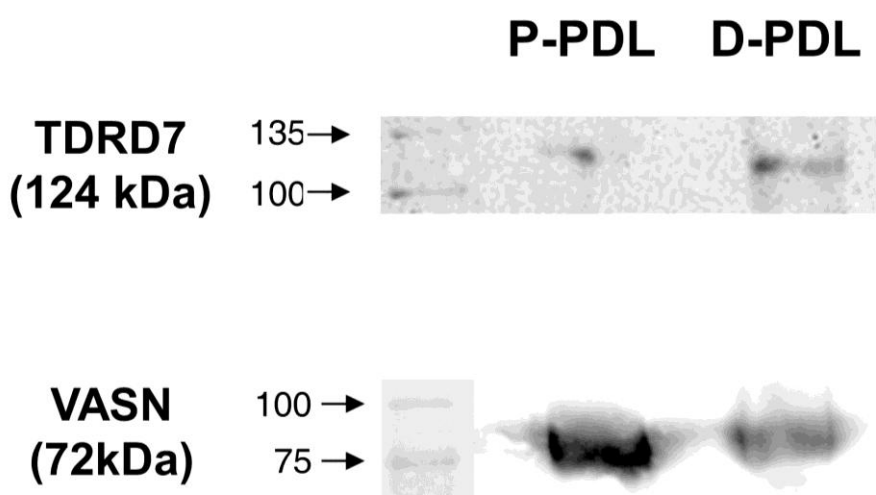

Figure 3. Protein expression using Western blot analysis. TDRD7 was expressed in D-PDL than P-PDL and VASN was expressed in P-PDL than D-PDL. Molecular weight of TDRD7 is 124 kDa and that of VASN is 72 kDa.

3. Cytokine membrane array analysis

To identify the cytokine or growth factor that was undetected in LC-MS/MS, we used the cytokine membrane array analysis. Expression ratio was shown in the number that D-PDL divided into P-PDL (Table 3). Fractalkine, IL-1 beta, IL-2, TGF beta 3, I-309, FGF7, ENA-78, IL-12 p40/70, IL-4, IL-15, FGF6, IL-5, TGF beta 1, SDF-1, IL-1 alpha, G-CSF, and IL-16 were higher expressed in D-PDL than P-PDL. And IGFBP2, Eotaxin1, ANG, Flt-3 ligand, IGF-1, GRO alpha, MIP-3 alpha, and MDC expressions were higher in P-PDL than D-PDL. But expression ratios were not significantly differences between two types of CM (Mann-Whitney U test). These cytokines were grouped with biological process terms to check the meaningful biological context. In biological process grouping, there was a similar pattern of cytokine and growth factors except immune response. Cytokines related with immune response, such as IL-1 beta, IL-2, I-309, IL-12 p40/70, and IL-4, were slightly more expressed in D-PDL than P-PDL (Fig. 4 and Table 3).

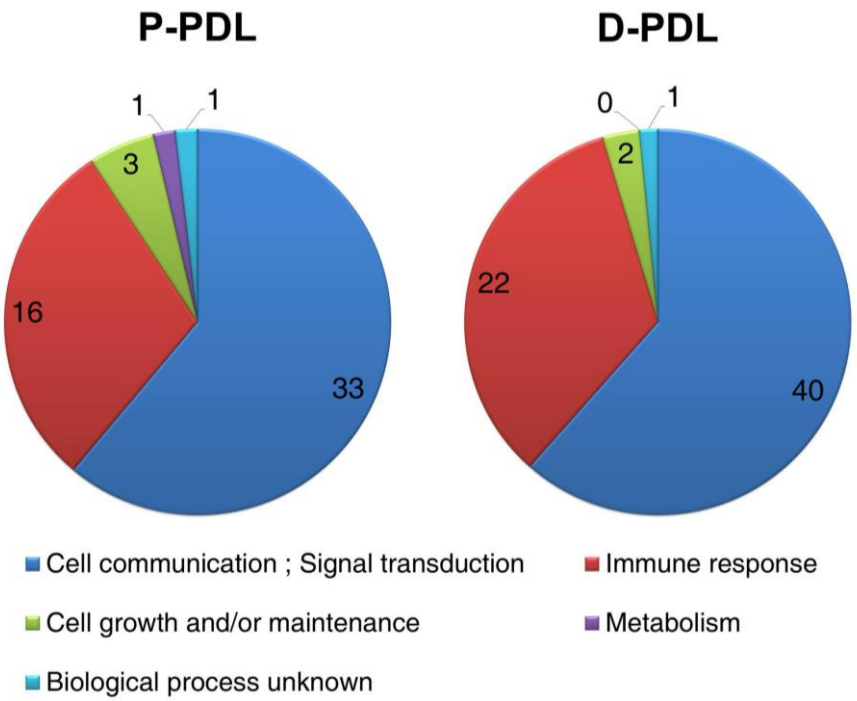

Figure 4. Biological process analysis of cytokine membrane array. Biological process was divided with 5 categories. Among these categories, cytokine expression related to immune response was slightly higher in D-PDL than P-PDL and the other categories were similar expressions of cytokines. Biological process terms were represented as different color wedges in the pie charts, with the number of cytokines per group shown in the pie charts.

Table 3. List of cytokines in cytokine membrane array

Name of cytokine	Biological Process	P-PDL	D-PDL	D-PDL/P-PDL
Fractalkine / CX3CL1	Cell communication; Signal transduction	0.00	3.36	-
IL-1 beta	Immune response	0.39	13.84	35.26
IL-2	Immune response	0.30	9.93	32.86
TGF beta 3	Cell communication; Signal transduction	0.08	1.28	17.08
I-309	Immune response	1.33	14.69	11.04
FGF7	Cell communication; Signal transduction	1.69	6.53	3.85
ENA-78(CXCL5)	Cell communication; Signal transduction	0.63	2.21	3.50
IL-12 p40/70	Immune response	0.90	2.79	3.11
IL-4	Immune response	0.71	2.19	3.09
IL-15	Immune response	1.76	4.81	2.74
FGF6	Cell communication; Signal transduction	1.74	4.71	2.71
IL-5	Immune response	0.67	1.75	2.60
TGF beta 1	Cell communication; Signal transduction	1.69	4.35	2.58
SDF-1(CXCL12)	Cell communication; Signal transduction	0.45	1.10	2.44
IL-1 alpha	Immune response	6.83	15.46	2.26
G-CSF	Immune response	0.55	1.15	2.08
IL-16 / IL16	Cell communication; Signal transduction	6.67	13.64	2.04
Eotaxin3 / CCL26	Cell communication; Signal transduction	1.32	2.54	1.92
IL-3	Immune response	5.42	9.67	1.78
SCF	Cell communication; Signal transduction	2.36	4.10	1.74
PDGF-BB / PDGFB	Cell communication; Signal transduction	3.95	6.33	1.60
IL-6	Immune response	22.41	30.18	1.35
PLGF	Cell communication; Signal transduction	27.45	36.17	1.32
MCP-4	Cell communication; Signal transduction	1.24	1.60	1.29

Table 3. (Continued)

Name of cytokine	Biological Process	P-PDL	D-PDL	D-PDL/P-PDL
TGF beta 2	Cell communication; Signal transduction	64.90	83.26	1.28
VEGF / VEGFA	Cell communication; Signal transduction	5.36	6.74	1.26
IGFBP4	Cell communication; Signal transduction	3.23	3.90	1.21
FGF9	Cell communication; Signal transduction	5.52	6.66	1.21
MIF	Cell communication; Signal transduction	5.67	6.81	1.20
CCL23	Cell communication; Signal transduction	4.85	5.74	1.18
THPO	Cell communication; Signal transduction	2.03	2.33	1.15
MIG	Immune response	0.34	0.38	1.14
LEP / Leptin	Cell communication; Signal transduction	7.84	8.89	1.13
MIP-1 beta	Immune response	73.84	82.86	1.12
BDNF	Cell growth and/or maintenance	14.30	15.94	1.11
OPN(SPP1)	Cell communication; Signal transduction	20.10	21.98	1.09
TIMP-2	Biological_process unknown	45.07	49.01	1.09
IFN gamma	Immune response	0.45	0.47	1.05
FGF4	Cell communication; Signal transduction	2.61	2.75	1.05
OPG	Cell communication; Signal transduction	71.00	74.75	1.05
TNF alpha	Cell communication; Signal transduction	9.27	9.49	1.02
IL-8	Immune response	9.42	9.63	1.02
IGFBP3	Cell communication; Signal transduction	18.82	18.91	1.01
MCP-2	Immune response	3.35	3.36	1.00
OSM	Cell growth and/or maintenance	32.56	32.34	0.99
LIF	Cell communication; Signal transduction	29.59	29.38	0.99
M-CSF	Immune response	20.77	20.56	0.99

Table 3. (Continued)

Name of cytokine	Biological Process	P-PDL	D-PDL	D-PDL/P-PDL
MIP-1 delta	Chemosensory behavior ; Cytokine and chemokine mediated signaling pathway	6.85	6.78	0.99
IP-10 / CXCL10	Cell communication; Signal transduction	38.75	37.82	0.98
PARC	Cell communication; Signal transduction	6.24	6.07	0.97
NT-4	Cell communication; Signal transduction	4.61	4.41	0.96
TIMP-1	Cell communication; Signal transduction	57.82	54.44	0.94
RANTES	Immune response	28.66	26.92	0.94
IGFBP1	Cell communication; Signal transduction	14.63	13.65	0.93
IL-13	Immune response	1.83	1.70	0.93
BLC / CXCL13	Cell communication; Signal transduction	2.43	2.19	0.90
LIGHT / TNFSF14	Apoptosis ; Signal transduction	5.99	5.34	0.89
HGF	Cell communication; Signal transduction	29.19	25.54	0.88
NAP-2	Cell communication; Signal transduction	39.83	34.71	0.87
GDNF	Cell communication; Signal transduction	32.40	28.16	0.87
GM-CSF	Immune response	1.51	1.31	0.87
Eotaxin2 / CCL24	Cell communication; Signal transduction	15.14	13.08	0.86
MCP-1	Immune response	77.91	67.31	0.86
TNF beta	Cell proliferation ; Cell differentiation ; Signal transduction ; Hemopoiesis	15.24	13.15	0.86
IL-10	Immune response	24.94	21.31	0.85
NT-3	Cell communication; Signal transduction	11.70	9.74	0.83
TARC	Immune response	20.33	15.39	0.76
MCP-3	Cell communication; Signal transduction	1.77	1.21	0.69
EGF	Cell communication; Signal transduction	4.56	3.06	0.67
IL-7	Immune response	1.53	0.88	0.57

Table 3. (Continued)

Name of cytokine	Biological Process	P-PDL	D-PDL	D-PDL/P-PDL
GCP-2 / CXCL6	Immune response	4.49	2.54	0.57
GRO	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	10.78	5.86	0.54
IGFBP2	Cell communication; Signal transduction	13.88	6.24	0.45
Eotaxin1 / CCL11	Cell communication; Signal transduction	3.30	1.27	0.39
ANG	Cell growth and/or maintenance	34.42	13.06	0.38
Flt-3 Ligand / FLT3LG	Cell communication; Signal transduction	3.12	1.15	0.37
IGF-1	Cell communication; Signal transduction	3.31	1.04	0.31
GRO alpha	Immune response	4.22	1.11	0.26
MIP-3 alpha	Immune response	3.18	0.47	0.15
MDC	Cell communication; Signal transduction	8.04	1.06	0.13

4. ELISA analysis

To validate the cytokine membrane array, we used ELISA for some cytokine; VEGF, EGF, PDGF-BB, SCF, and FGF beta. In cytokine membrane array results, expression of VEGF was slightly higher in D-PDL and the other cytokine expressions were similar between two types of CM (Fig. 5). But there were not significantly differences between P-PDL and D-PDL (Mann Whitney U test).

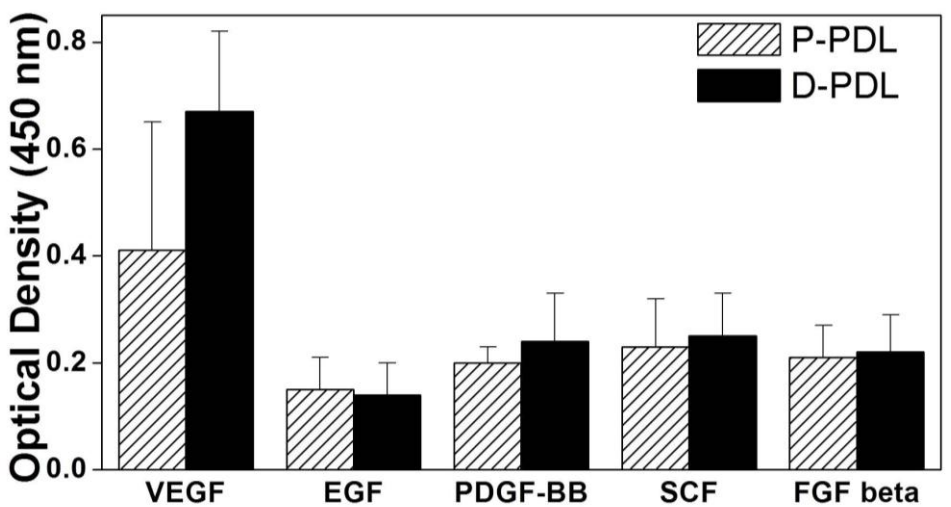

Figure 5. Validation of cytokine expression by ELISA. VEGF, EGF, PDGF-BB, and FGF were used for ELISA. Data were obtained from three independent experiments and the data are mean and standard deviation values. The optical densities of each cytokines did not differ significantly between P-PDL and D-PDL (Mann-Whitney U test)

IV. DISCUSSION

Serum-free conditioned medium (CM) derived from PDL cells was analyzed to investigate the secretomes and our observations demonstrated that a variety of active soluble factors are secreted by PDL cells. There were several remarkable differences between human deciduous and permanent PDL groups

In the present study, the secretomes that were upregulated in deciduous PDL tissues were involved in inflammatory or immune reactions leading to tissue degradation, extracellular-matrix-degrading enzymes. Interleukin 1, alpha (IL-1a) is involved in the immune response, inflammatory processes, and apoptosis (Nesic et al., 2001; Parker et al., 1989), and can enhance the biosynthesis of prostaglandin (Romero et al., 1989) and some kinds of MMPs (Shi et al., 2004). In addition, it up-regulates RANKL and down-regulates OPG in PDL cells (Fukushima et al., 2005), and positively affects the survival and differentiation of osteoclasts or odontoclasts and consecutive bone or tooth resorption (Lee et al., 2002; Tani-Ishii et al., 1999; Zhang et al., 2003). IL-1b stimulates collagenase production in the periodontal ligament and induces connective tissue degradation in periodontitis (Ohshima et al., 1994; Ohshima et al., 1995). IL-2 is necessary for the growth, proliferation, and differentiation of thymic-derived lymphocytes (T cells) to become 'effector' T cells. IL-2 is normally produced by T cells during an immune response (Liao et al., 2011). Stromal cell-derived factor 1 (SDF-1) are small cytokines that belong to the chemokine family and strongly chemotactic for lymphocytes (De La Luz Sierra et al., 2004). Matrix metalloproteinase (MMP) is

associated with turnover of PDL matix and differentiation of osteoclast (Kanzaki et al., 2001; Takahashi et al., 2003). In the previous study by Wu et al., compared with PDL cells from the permanent dentition, primary PDL cells generally produced a greater amount of collagenase but similar amounts of the inhibitor of matrix metalloproteinase (TIMP) (Wu et al., 1999). It is similar to our results. In LC-MS/MS analysis of this present study, whereas TIMP-1 was identified in both groups, P-PDL and D-PDL, MMP1 was identified only in D-PDL group. In consideration of the above, deciduous PDL tissues appear to be prone to resorption because of the relatively strong expressions of secretomes associated with tissue destruction.

In addition, LC-MS/MS results of the present study showed that Tudor domain containing 7 (TDRD-7) was identified only in D-PDL and western blot analysis coinciding these results showed that TDRD-7 expression was stronger in D-PDL than P-PDL. In the previous study, Tiejun Zhang et al. showed that PHF20, tudor domaincontaining protein, promotes NF-κB transcriptional activity (Zhang et al., 2013). NF-κB is a member of a family of transcription factors that control the expression of critical genes that regulate immune responses (Ghosh and Hayden, 2008; Pasparakis, 2009; Vallabhapurapu and Karin, 2009). Furthermore, NF-κB is involved in osteoclast and possibly in odontoclast formation (Wise et al., 2002). These findings give rise to the possibility that tudor domaincontaining protein functions as a positive effector in especially primary dentition that causes constitutive NF-κB activation, as in the context of physiologic or pathological conditions such as inflammation-linked root resorption. However, these findings are not sufficient and their precise functions in periodontal tissues remain to be elucidated.

In our present study, the secretomes involved in cell growth and/or maintenance were also upregulated in deciduous PDL tissues, and this finding concurs with the above previous studies. It was reported that the PDL cells from deciduous teeth also have a higher proliferation rate (Ji et al., 2013; Silverio et al., 2010). The results of cytokine membrane array showed that fibroblast growth factor (FGF) 6, 7 were expressed stronger in D-PDL than P-PDL. FGF family members are involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair (Zhang et al., 2006). Especially, FGF-6 may be considered a regulator of bone metabolism as shown by its activity on both osteoblasts and osteoclasts (Bosetti et al., 2010). Further investigation is required to determine their precise functions in periodontal tissues.

The secretomes that were upregulated in permanent PDL tissues were involved in cell communication such as Vasorin (VASN) and Decorin, Insulin-like growth factor 1 (IGF-1), Macrophage-derived chemokine (MDC). Particularly, LC-MS/MS results showed that Vasorin and Decorin were identified only in P-PDL and western blot analysis coinciding these results showed that Vasorin expression was stronger in P-PDL than D-PDL. Vasorin is a typical type I membrane protein, containing tandem arrays of a characteristic leucine-rich repeat motif, an epidermal growth factor-like motif, and a fibronectin type III-like motif at the extracellular domain (Klein et al., 1996; Tashiro et al., 1993). Decorin was found to share a significant homology with vasorin and interact directly with transforming growth factor-b (TGF-b) (Iozzo, 1999; Yamaguchi et al., 1990). In the previous study, Yuichi Ikeda et al. showed that that vasorin directly binds to TGF-b and

negatively modulates TGF-b (Ikeda et al., 2004). It was reported that TGF-b1 plays an important role as promoting osteoclastogenesis by enhancing the expression of genes such as MCP-1, CSF-1, IL-1a (Que and Wise, 1998; Wise, 2009; Wise and Lin, 1994). Considering the functional role of TGF-b in these studies, it is reasonably assumed that up-regulating of vasorin in permanent PDL tissue significantly inhibits resorption. Further investigations will be needed to clarify these issues. Insulin-like growth factor 1 (IGF-1) was upregulated in permanent PDL tissues. IGF-1 is a hormone similar in molecular structure to insulin. IGF-1 stimulates systemic body growth, and has growth-promoting effects on almost every cell in the body. In addition to the insulin-like effects, IGF-1 can also regulate cell growth and development (Zhang et al., 2008). MDC was also upregulated in permanent PDL tissues. MDC, also named C-C motif chemokine 22 (CCL22), may play a role in the trafficking of activated/effector T-lymphocytes to inflammatory sites and chemotactic for monocytes, dendritic cells and natural killer cells (Cronshaw et al., 2004). Angiogenin (ANG) were also upregulated in permanent PDL tissues. ANG is a key protein implicated in angiogenesis and interacts with endothelial and smooth muscle cells resulting in cell migration, invasion, proliferation and formation of tubular structures (Gao and Xu, 2008). The precise function of these cytokines in PDL tissues is in need of clarification.

Osteoprotegerin (OPG) is a secreted glycoprotein that is a member of the tumor necrosis factor (TNF) receptor superfamily (Simonet et al., 1997; Tsuda et al., 1997; Yasuda et al., 1998). It appears that OPG is a receptor for RANKL and that its binding to RANKL inhibits cell-to-cell signaling between stromal cells and osteoclast precursors,

such that osteoclasts are not formed (Yasuda et al., 1998; Yasuda et al., 1999). Fukushima et al. reported that PDL cells from deciduous teeth or permanent teeth, were found to express OPG and not RANKL cells under non-resorbing conditions. On the contrary, human PDL cells around the roots of resorbing decidous teeth express enhanced levels of RANKL and as a results, ratio of RANKL/OPG was changed (Fukushima et al., 2003). The present study showed that OPG was similar expression in P-PDL and D-PDL in cytokine membrane array analysis and concurred with the previous report. Monocyte chemoattractant protein-1 (MCP-1) was also similar expression in P-PDL and D-PDL in cytokine membrane array analysis. This factor was known to be major player in the pro-inflammatory immune responses. Especially, MCP-1 is prime candidate for recruiting the osteoclast precursors (mononuclear cells) into the dental follicle (Wise et al., 2002). The previous study reported that PDL cells play an important role in maintaining the homeostasis by dual regulation through RANKL stimulation and OPG inhibition (Kanzaki et al., 2001). Considering functions of OPG and MCP-1 in our study, PDL cells might also regulate osteoclastogenesis by opposing mechanisms and more research is needed.

The results of our study showed that Granulocyte-macrophage colony-stimulating factor (GM-CSF) and Glial cell-derived neurotrophic factor (GDNF) were detected similarly in P-PDL and D-PDL in cytokine membrane array analysis. GM-CSF was another inflammatory mediator and may be involved in the regulation of antigen-presenting cells, especially dendritic cells, in the periodontium (Pastore et al., 1996). GDNF is neurotrophic factor believed to be involved in tooth morphogenesis (Nosrat et

al., 2002). GDNF and its receptors were reported to be related to the maturation of the periodontal Ruffini endings (Igarashi et al., 2007).

Several of the expressed cytokines in our study, ANG, GDNF, IGFBP-2 and MCP-1 were also found in previous study that investigate the cytokine profile of PDL fibroblasts (Ohshima et al., 2008). However, in contrast with our study, some cytokines, VEGF, GM-CSF, IL-6 and IL-8 were not detected in that study.

On the other hand, human mesenchymal stem cells (hMSCs) are useful for regeneration of inflamed or injured tissues. In the previous study, the molecules identified in MSCs secretome clearly show the ability of MSCs not only to support hematopoiesis, but also supply autocrine/paracrine factors that influence angiogenesis, osteogenesis and chondrogenesis and immune regulation. Stem cell-based regenerative dentistry has emerged as a promising alternative for the current treatment options. With the clinically issues of treatment using stem-cell, cytokines also has emerged as the key factors regulating and characterizing stem cells. Compared the result of our study, there are comparatively small differences with the list of chemokines, cytokines and growth factors identified in the MSCs secretome (Skalnikova et al., 2011; Wang et al., 2011). In terms of chemokines, while IL-8, MCP-1, MIP-1 beta, MIP-3, ENA -78, GCP-2, IP-10, SDF-1 and PARC were identified in both the previous and our study, CCL5, CXCL1 and SDF-4 were identified only in the previous study. In terms of regulation of inflammation (pro-inflammatory or anti-inflammatory), while IL-1 alpha, IL-2, IL-4, IL-10, IL-12, IL-13, IFN gamma, TNF alpha, TGF beta and MIF were identified in both studies, LMIF was identified only in the previous study. In terms of growh factors and growth factor-

binding proteins, while G-CSF, M-CSF, GM-CSF, IGF-1, FGF-4, FGF-7, FGF-9, VEGF, HGF, SCF and IGFBP 1-6 were identified in both studies, PIGF, CTGF and IGFBP 7 were identified only in the previous study. more studies are warranted to investigate the differences of secretome profile between MSCs and PDL cells.

Although the findings in our study are not sufficient to explain difference between permanent and deciduous PDL clearly, this reports about differences of cytokine profiles between deciduous and permanent PDL tissues aids our understanding of histological and functional differences between them at the molecular level.

V. CONCLUSION

Our study showed that humoral factors released from deciduous and permanent PDL cells have different characteristics. The secretomes that were upregulated in deciduous PDL tissues were involved in inflammatory or immune reactions, cell growth and/or maintenance. On the other hand, the secretomes that were upregulated in permanent PDL tissues were involved in cell communication. Because the investigated secretome types have not been well characterized, our conclusions are partially speculative. However, the main focus of this study was to identify and compare the cytokine profile of deciduous and permanent PDL. Each PDL groups showed the different cytokine profile, and there were some differences in respect of biological process and molecular function, too. Although the findings in our study are not sufficient to explain difference between permanent and deciduous PDL clearly, our study provides clues to develop effective therapeutic interventions for clinically challenging.

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국문요약

유치 및 영구치 치주인대세포에서 분비되는 단백질체의 비교 분석 연구

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인간의 유치와 영구치는 다른 발달 과정, 형태학적 및 조직학적 특징, 생애 주기를 보인다. 또한, 유치 및 영구치의 치주인대세포에서 분비되는 체액성 인자들도 다른 특징을 보이는 듯 하다. 그럼에도 불구하고 이러한 차이에 대한 기존의 연구는 거의 없는 편이다. 본 연구의 목적은 인간의 유치 및 영구치 치주인대세포의 분비 단백질체를 확인하고 서로간에 비교하여 이에 대한 분자수준의 이해도를 높이기 위함이다. 연구에 사용된 치주인대세포는 유치 및 영구치에서 얻었으며, 여기서 분비된 단백질체를 별도로 분리시켰다. 이렇게 분리된 분비단백질체를 LC-MS/MS 분석을 이용하여 알아보았으며, 그 결과를 확인하고자 western blot 분석이 추가로 시행되었다. 더하여, LC-MS/MS에서 검출되지 못한 cytokine 또는 성장인자를 알아보기 위하여 cytokine

membrane array 분석을 시행하였다. 이렇게 얻어진 결과 중 특정 cytokine은 ELISA 분석을 통해 좀 더 면밀히 검증하였다. 이렇게 얻어진 cytokine들은 보다 의미있는 분석을 위해 생물학적 과정 및 기능에 따라 몇 가지 그룹으로 분류되었으며, 유치와 영구치간 비교에서 대체로 비슷한 양상을 보였으나 몇몇 그룹은 주목할만한 차이가 있었다. 유치의 경우 염증과 면역 관련 및 세포 성장과 유지에 관련된 분비단백질체가 높게 측정되었다. 반면에, 영구치의 경우 세포 신호교신 및 신호 전달 과정과 연관된 분비단백질체가 높게 측정되었다. 본 연구 결과는 유치와 영구치 치주인대세포의 체액성 인자 간에 차이점을 보여주었으며, 나아가 비정상적 치근 흡수 치료 및 줄기세포를 이용한 재생치의학을 다룸에 있어 유치와 영구치가 다른 작용 및 반응을 할 수 있음을 제시한다.

핵심되는 말: 분비단백질체, cytokine, 체액성 인자, 치주인대, 유치와 영구치