Comparative Analysis of Cytokines Released from Deciduous and Permanent Dental Pulp Stem Cells

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Min Kyung Shin

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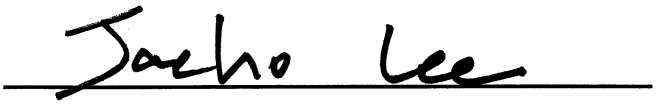
This certifies that the dissertation of Min Kyung Shin is approved.



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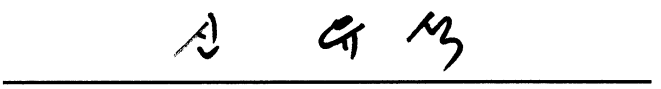

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Abstract

Comparative Analysis of Cytokines Released from Deciduous and Permanent Dental Pulp Stem Cells

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This study aimed to investigate and compare cytokine profiles secreted from stem cells from human exfoliated deciduous teeth (SHED) and dental pulp stem cells (DPSCs) to better understand the characteristics of SHED and DPSCs.

SHED-conditioned medium (CM) and DPSC-CM were extracted using seven deciduous and permanent teeth each. Cytokine membrane array was performed for each CM to quantify and compare the secretomes of 120 cytokines. Enzyme-

linked immunosorbent assay, immunocytochemistry, and immunohistochemistry analysis were performed to demonstrate cytokine membrane array analysis.

Significant differences were observed in the expression levels of 68 cytokines–27 and 41 cytokines were 1.3-fold more strongly expressed in SHED-CM and DPSC-CM, respectively. Inflammatory cytokines, proliferative cytokines and cytokines involved in immunomodulation were more strongly expressed in SHED-CM. Cytokines involved in angiogenesis were detected more strongly in DPSC-CM.

SHED and DPSCs have distinctive cytokine profiles and characteristics in terms of their stem cell regenerative potential. It might be important to consider their multipotency and paracrine effects and select the suitable stem cell type for the adequate tissue regeneration.

These observations suggest that SHED may have better cytokine profiles related to inflammatory, proliferative, osteogenic, and odontogenic potential and might enable better decisions for the choice of mesenchymal stem cells when used for therapeutic purposes.

Keywords: Cytokine membrane array, DPSCs, Paracrine effect, SHED, Tissue regeneration

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I. Introduction

Stem cell-based therapies have emerged as a potential strategy for treating damaged cells and tissues in many diseases. Regenerative medicine aims to repair or replace impaired structures and improve the biological functions of organs. Mesenchymal stem cells (MSCs) are crucial in regenerative therapies due to their self-renewal capacity and multi-lineage differentiation. Dental tissues are considered accessible sources of MSCs

and can be easily extracted and processed when a deciduous tooth is exfoliated or a permanent tooth is extracted.

Stem cells from human exfoliated deciduous teeth (SHED) and dental pulp stem cells (DPSCs) exhibit markedly greater growth potential compared to bone marrow mesenchymal stem cells (Wang et al., 2012). In particular, SHED have a high proliferative rate and are capable of differentiating into osteoblasts, neural cells, adipocytes, and odontoblasts (Miura et al., 2003). DPSCs can also differentiate into adipocytes and neural cells as well as pulp/dentin tissue and bone cell types (Shi et al., 2005).

Early studies have limited the roles of stem cells in regenerative therapy. However, in the current paradigm, the importance of biomolecules generated from stem cells beyond cell differentiation is emerging. Stem cells interact with their niche and constitute a dynamic system that determines cell and tissue fate (Scadden, 2006). Cytokines, growth factors, and chemokines secreted from stem cells act on the stem cells themselves (autocrine effect) as well as on surrounding cells (paracrine effect). Therefore, understanding the secretomes from stem cells of various origins is necessary to determine suitable cell sources for a particular regenerative therapy.

Thus far, studies on the capacity of various dental-derived stem cells have been conducted; however, studies on their paracrine effects are rare. Although the expression levels of cytokines in developing apical complex cells and DPSCs were reported (Joo et al., 2018), comparative data on the expression levels of cytokines from SHED and DPSCs

have been published insufficiently. Here, the profiles of cytokines secreted from SHED and DPSCs were investigated and compared to gain a better understanding of the cellular responses of SHED and DPSCs.

II. Materials and Methods

1. Cell culture

The Institutional Review Board of the Yonsei University Dental Hospital approved the experimental protocol (approval no. 2-2018-0012). All the subjects and their guardians have provided written informed consent. SHED were obtained from human primary teeth in a healthy state without caries infection (n=7, 3 boys and 4 girls; age, 6–11 years) and DPSCs were isolated from human permanent teeth requiring extraction for orthodontic treatment (n=7, 3 males and 4 females; age, 12–18 years) (Table 1) via enzymatic disaggregation method (Jeon et al., 2014). In brief, the teeth pulp tissues were treated with collagenase type I (3 mg/mL; Invitrogen, Carlsbad, CA, USA) and dispase (4 mg/mL; Invitrogen) for 1 h at 37℃ and then filtered through a 70 μm cell strainer. The SHED and DPSCs were cultured in a cell culture medium containing alpha-minimum essential medium (α-MEM; Invitrogen) with 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen), and 2 mM L-glutamine (Invitrogen) at 37 ℃ in 5% CO_2. The isolated SHED and DPSCs extracted from at least two or three different donors were blended at passage 2, and cells at passages 3–5 were used for further experiments.

Table 1. Information of the tooth samples

Type	Age	Gender	Tooth
Deciduous teeth	6Y 1M	M	Deciduous central incisor
	6Y 4M	M	Deciduous central incisor
	6Y 5M	F	Deciduous lateral incisor
	7Y 5M	F	Deciduous central incisor
	9Y 6M	F	Deciduous first molar
	10Y 10M	M	Deciduous first molar
	11Y 5M	F	Deciduous second molar
Permanent teeth	12Y 6M	M	First premolar
	12Y 9M	F	Second premolar
	13Y 4M	F	First premolar
	13Y 9M	F	First premolar
	18Y 2M	M	Third molar
	18Y 3M	F	First premolar
	18Y 3M	M	Third molar

2. Preparation of SHED- and DPSC-conditioned medium

SHED and DPSCs were seeded at 3×10^5 cells on a 100-mm culture dish (BD Falcon, Franklin Lakes, NJ, USA). When 80% confluency was attained, the SHED and DPSCs were washed seven times with phosphate-buffered saline (PBS; pH 7.4; Invitrogen) and cultured in a cell culture medium (described above) lacking FBS. After 48 h, the supernatant was collected and protease inhibitor cocktail (Roche Diagnostic Systems, Branchburg, NJ, USA) was added to the supernatant. Conditioned medium (CM) samples were stored at -20 ℃ until further analysis.

3. Cytokine profiles of SHED-CM and DPSC-CM and data analysis

SHED-CM and DPSC-CM were analyzed using human cytokine array C1000 (RayBiotech, Inc., Norcross, GA, USA), following the manufacturer's instructions. A pair of cytokine array membranes were blocked with blocking buffer for 30 min and then incubated with 2 mL of each CM at room temperature for 4 h. After the samples were decanted, the membranes were washed with wash buffer I thrice and wash buffer II twice at room temperature with gentle shaking. The membranes were then incubated with diluted primary antibodies at 4 ℃ overnight and washed as described above. The membranes were incubated with horseradish peroxidase-conjugated streptavidin for 30 min at room temperature. After washing, the membranes were incubated with a peroxidase substrate for 5 min at room temperature. Finally, the membranes were exposed to x-ray film (Hyper film ECL) for 1 min at room temperature. Three independent samples of SHED-CM and DPSC-CM were analyzed. Alpha MEM containing 0.1% FBS was used as the positive control, and α-MEM without FBS was used as the negative control. X-ray images were captured using an optical scanner and saved in jpeg format. Signal intensities were quantified using imaging software (Gel Quant Pro, DNR Bio-Imaging Systems Ltd., Jerusalem, Israel) to compare intensities of the same cytokine from different samples. For data normalization, the values of each cytokine (duplicate spots) measured on the same membrane were revised by subtracting the average of the negative control spot values from the intensity of each spot, including that of the positive

control spot. Using these revised values, the intensity of these positive control signals was used to normalize signal responses for data comparison. Each sample was tested in triplicates, and cytokines that exhibited statistically significance for 3 averages were selected.

4. Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was performed using a Mix and Match Custom ELISA Strip (Signosis, Inc., Santa Clara, CA, USA), following the manufacturer's instructions. 8 strips coated with different antibodies were incubated with SHED- and DPSC-CM for 1 h at room temperature with gentle shaking. After the CM samples were decanted from the 8 strips, the strips were washed with assay buffer thrice. The samples were then incubated with a diluted biotin-labeled antibody mixture for 1 h at room temperature with gentle shaking and washed with assay buffer thrice. After washing, the 8 strips were incubated with diluted streptavidin-HRP conjugate for 45 min at room temperature with gentle shaking and washed with assay buffer thrice. Next, the 8 strips were incubated with substrate for 30 min at room temperature. After incubation, stop solution was added and the absorbance was read at 450 nm within 30 min using a spectrophotometer (Benchmark Plus microplate spectrophotometer; Bio-Rad, Richmond, CA, USA). ELISA data were obtained from three independent experiments.

5. Immunocytochemistry analysis

SHED and DPSCs were cultured on coverslips (Marienfield-Superior, Lauda-Königshofen, Germany) coated with poly-L-lysin (Sigma-Aldrich). After washing with PBS (Invitrogen), the samples were fixed with 4% paraformaldehyde (Biosesang, Seongnam, Korea). The fixed cells were permeabilized with PBS containing 0.25% Triton X-100 (PBST; Bio Basic, Seoul, Korea), washed, and incubated with 5% BSA (Sigma-Aldrich) in PBST to block nonspecific antibody binding.

The cells were incubated in primary antibodies diluted in 5% BSA/PBST overnight at 4°C. The primary antibodies included antihuman interleukin-6 (IL-6; rabbit polyclonal antibody, ab6672, Abcam; 1:500), antihuman brain-derived neurotrophic factor (BDNF; rabbit monoclonal antibody, ab108319, Abcam; 1:200), antihuman placental growth factor (PLGF; rabbit polyclonal antibody, ab9542, Abcam; 1:500), and antihuman vascular endothelial growth factor D (VEGF-D; rabbit monoclonal antibody, ab155288, Abcam; 1:200). After washing, the cells were incubated with biotinylated secondary antibody (biotinylated antirabbit IgG, Vector Labs, Burlingame, CA, USA) in 5% BSA/PBST for 1 h at RT. After washing, the cells were incubated with streptavidin–HRP (Vector Labs) in 5% BSA/PBST for 30 min at RT. Color was developed using 3,3′-diaminobenzidine substrate (Abcam) and hematoxylin (Merck). Coverslips were mounted using a drop of mounting medium (Vector Labs) and stored at RT. The negative control was stained in the same manner, albeit excluding the primary antibody reaction procedure.

6. Immunohistochemistry analysis

The human deciduous and permanent teeth were fixed with 10% buffered formalin (Sigma-Aldrich, St Louis, MO, USA) for 1 day and then decalcified with 10% EDTA (pH 7.4; Fisher Scientific Co., Houston, TX, USA) for 9 weeks. The calcified teeth were embedded in paraffin, sectioned at a 3 μm thickness. The sections were deparaffinized in xylene, rehydrated, and rinsed with distilled water. For IL-6 and CXCL13 (BCA1, BLC) staining, the antigen retrieval step was not performed. The sections were immersed in 3% hydrogen peroxide for 10 min to inactivate endogenous peroxidase activity and then incubated with primary antibody overnight. The primary antibodies used included antihuman IL-6 (rabbit polyclonal antibody; ab6672, Abcam, Cambridge, UK; 1:400) and antihuman CXCL13 (rabbit polyclonal antibody; ab112521, Abcam; 1:200). The sections were subsequently incubated for 20 min with HRP-labeled polymer conjugated with secondary rabbit antibody in an EnVision+system kit (Dako, Carpinteriz, CA, USA). The color was developed using 3,3′-diaminobenzidine substrate (Dako) and counterstained with Grill's hematoxylin solution (Merck, Darmstadt, Germany).

7. Statistical analysis

All experiments were performed in triplicates. Data normality 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 (version 20.0; SPSS, Chicago, IL, USA) for ELISA analysis.

III. Results

1. Cytokine membrane array analysis

After culturing for 48 h, the SHED- and DPSC-CM were assayed using Raybiotech cytokine array for the detection of 120 human cytokines (Figure 1). Cytokine membrane array analysis revealed that 27 cytokines were expressed more strongly in SHED-CM (Table 2) and 41 cytokines were expressed 1.3-fold more strongly in DPSC-CM (Table 3).

Nine cytokines (IL-6, CNTF, CCL23, IGFBP2, IL-7, EGF, BMP6, IGFBP1, and GM-CSF) were found exclusively in SHED. The array showed elevated signals for Eotaxin1, IL-5, IFN-gamma, PARC, and IL-2 in SHED compared to that in DPSCs. CCL28 was detected exclusively in DPSCs, and β-NGF, GRO α, BTC, and HGF were relatively abundant in DPSCs. The expression levels of BLC, BDNF, and MCP-1 were 3.72-, 1.51-, and 1.31-fold higher in SHED than in DPSCs. The expression levels of β-NGF, BTC, PLGF, IGF-1, and VEGF were 6.85-, 3.6-, 2.67-, 1.54-, and 1.48-fold higher in DPSCs than in SHED.

2. Enzyme-linked immunosorbent assay (ELISA) analysis

To demonstrate the protein levels in terms of cytokine expression, ELISA was performed for some cytokines. The results revealed that the secretion of IL-6, EGF, and

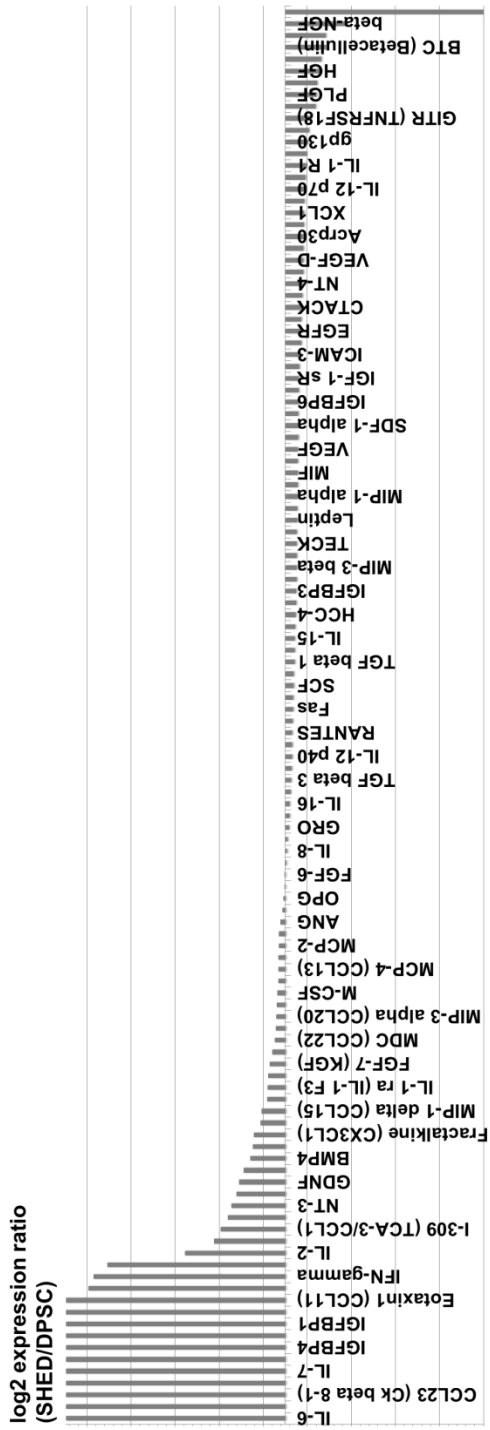

Figure 1. Cytokine membrane array analysis. Results are expressed as $\log_2$ ratio of fold-changes, $\log_2$(S/D), where S is the cytokine expression level in the SHED, D is the cytokine expression level in the DPSCs. Cytokines are ordered by fold change.

Table 2. Upregulated cytokines in stem cells from human exfoliated deciduous teeth–conditioned medium (SHED-CM) compared with dental pulp stem cells (DPSC)-CM

Cytokine	SHED	DPSC	Ratio (SHED/DPSC)	Biological function	P value
IL-6	68.24	-	-	Proliferation, Immunomodulation, Neuromodulation	0.002
CNTF	27.52	-	-	Neurogenesis	0.022
CCL23	24.94	-	-	Chemotaxis, Chemoattraction on osteoclast precursors, Angiogenesis	0.002
IGFBP2	19.54	-	-	Osteogenesis, Tooth development	0.002
IL-7	18.86	-	-	B and T cell differentiation, Inhibition of osteogenic differentiation	0.002
EGF	16.29	-	-	Osteogenic differentiation	0.022
BMP6	13.48	-	-	Osteogenic differentiation	0.002
IGFBP1	10.93	-	-	Osteogenic differentiation	0.002
GM-CSF	2.68	-	-	Inflammatory, Angiogenesis	0.022
Eotaxin1	8.07	0	2479.81	Eosinophil chemoattractant	0.022
IL-5	8.02	0.02	488.75	Inflammatory	0.003
IFN-gamma	4.06	0.01	415.42	Immunomodulation, Osteoblast differentiation	0.050
PARC	29.18	0.11	271.31	Immunosuppression	0.003
IL-2	8.15	0.34	23.68	Proinflammotory	0.087
IL-4	11.18	1.19	9.42	Anti-inflammatory	0.135
MIG	0.5	0.08	6.14	Inflammatory	0.042
NT-3	25.08	4.57	5.48	Odontoblast differentiation, Neurogenesis	0.004
GCP-2	20.77	4.48	4.63	Inflammatory	0.087
GDNF	15.2	3.54	4.29	Neurogenesis, Odontoblast differentiation	0.011
BLC (CXCL13)	17.08	4.59	3.72	B-cell chemotaxis, Odontoblast differentiation	0.000
BMP4	28.14	9.31	3.02	Odontogenesis, Osteogenic differentiation	0.000
IL-3	9.81	3.54	2.77	Inflammatory	0.008
Fractalkine (CX3CL1)	21.45	8.01	2.68	Proinflammatory, Osteoclastogenesis, Pathogenesis of periapical lesions	0.087
IL-1 ra	35.5	20.5	1.73	Proinflammatory cytokine inhibitor	0.007
BDNF	49.56	32.8	1.51	Neurogenesis	0.006
MDC (CCL22)	39.16	28.21	1.39	Inflammatory	0.004
MCP-1 (CCL2)	91.82	70.19	1.31	Odonto/Osteoclastogenesis, Angiogenesis	0.040

Table 3. Upregulated cytokines in dental pulp stem cells–conditioned medium (DPSC-CM) compared with stem cells from human exfoliated deciduous teeth (SHED)-CM

Cytokine	SHED	DPSC	Ratio (SHED/DPSC)	Biological function	P value
CCL28	0	2.89	1486.05	Proinflammatory, B and T-cell chemotaxis, Cell proliferation	0.002
beta-NGF	1.82	12.46	6.85	Neurogenesis, Odontoblast differentiation	0.001
CXCL1 (GRO α)	3.24	11.66	3.6	Angiogenesis	0.022
BTC	8.62	31.02	3.6	Cell proliferation, Neurogenesis, Angiogenesis	0.004
HGF	4.14	12.87	3.11	Angiogenesis, Mitosis, Tissue regeneration, Anti-inflammatory	0.043
PLGF	7.75	20.69	2.67	Angiogenesis	0.000
THPO	25.24	65.21	2.58	Haematopoietic cytokine	0.000
GITR	8.89	19.59	2.2	Promote effector T cell functions	0.004
CXCL11	29.38	62.28	2.12	Inflammatory	0.000
bFGF	11.15	21.82	1.96	Proliferation, Angiogenesis, Neurogenesis, Osteogenic differentiation	0.002
IL-1 R1	11.79	23.02	1.95	Immunomodulation	0.004
IL-2R alpha	7.3	13.77	1.89	Immunomodulation	0.016
IL-12 p70	8.62	15.64	1.82	Proinflammatory, Inhibit osteoclastogenesis	0.003
AREG	14.35	25.94	1.81	Epithelial cell growth, Immunomodulation, Tissue homeostasis	0.006
XCL1	46.65	83.46	1.79	Anti-apoptotic	0.000
ICAM-1	14.94	26.7	1.79	Inflammatory	0.006
Axl	14.13	24.98	1.77	Hemostasis, Inflammatory	0.005
VEGF-D	12.66	22.3	1.76	Angiogenesis, Lymphangiogenesis	0.036
CXCL5	14.35	25.17	1.76	Inflammatory	0.000
NT-4	30.16	51.37	1.7	Odontogenesis, Neurogenesis	0.016
CTACK	26.83	44.83	1.67	Inflammatory	0.027
IL-17	12.53	20.83	1.66	Inflammatory, Promotes osteoclastogenesis	0.001
EGFR	18.72	31.05	1.66	Cell proliferation, Odontogenesis	0.001
TRAIL R3	20.44	33.84	1.66	Negatively regulate TRAIL-mediated apoptosis	0.004
ICAM-3	24.03	38.72	1.61	Apoptotic cell clearance	0.008
IGF-1	17.24	26.47	1.54	Cell proliferation, Osteogenic differentiation	0.002
IGFBP6	28.98	44.05	1.52	Osteogenic differentiation	0.007

Table 3. Upregulated cytokines in dental pulp stem cells–conditioned medium (DPSC-CM) compared with stem cells from human exfoliated deciduous teeth (SHED)-CM (Cont.)

Cytokine	SHED	DPSC	Ratio (SHED/DPSC)	Biological function	P value
ANGPT2	28.31	42.75	1.51	Angiogenesis, Immunosuppression	0.003
MIP-1 beta (CCL4)	29.17	43.46	1.49	Inflammatory	0.096
VEGF	26.25	38.91	1.48	Angiogenesis, Odontogenesis, Osteogenesis, Immunosuppression	0.034
LIGHT	26.76	39.48	1.48	Inflammatory	0.045
MIF	61.3	90.25	1.47	Proinflammatory, Enhances osteoclastogenesis	0.000
AgRP	26.1	38.38	1.47	Upregulates proteasome activity	0.000
MIP-1 alpha	43.63	63.97	1.47	Inflammatory	0.000
(CCL3)	39.07	56.17	1.44	Odontogenesis	0.005
FGF-9	67.47	96.97	1.44	T-cell development, Thymocyte and macrophage chemotaxis, Osteoclastongenesis	0.037
TECK (CCL25)	18.76	26.89	1.43	Negative regulator in inflammation	0.016
MSP alpha	19.41	27.79	1.43	Immunomodulation	0.002
MIP-3 beta	36.47	51.62	1.42	Modulation of mineralizing activity of IGF-1	0.004
IGFBP3	21.22	28.39	1.34	Osteo/Odontoblast differentiation, Immunomodulation, Neurogenesis	0.046
IL-11	21.54	28.18	1.31	Odontogenesis	0.016
FGF-4	28.31	42.75	1.51	Negative regulator in inflammation	0.003

MCP-1 was significantly higher in SHED than in DPSCs (Figure 2. A-C). In contrast, β-NGF, BTC, and IGF-1 were expressed more strongly in DPSCs than in SHED (Figure 2. D-F). These results were consistent with the cytokine antibody array analysis results.

3. Immunocytochemistry analysis

IL-6, BNDF, PLGF, and VEGF-D were stained in SHED and DPSCs to identify the staining characteristics of each protein. The staining patterns of IL-6 and BDNF were stronger in SHED than in DPSCs (Figure 3. A, B, E, F), whereas the staining intensities of PLGF and VEGF-D were higher in DPSCs than in SHED (Figure 3. C, D, G, H). These results were consistent with the cytokine antibody array analysis results.

4. Immunohistochemistry analysis

Immunohistochemistry analysis was performed on IL-6 and CXCL13 (BLC); both IL-6 and CXCL13 were stained dark in the odontoblastic layer and perivascular region of SHED, demonstrating that these were highly expressed in SHED (Figure 4. A-D) Compared to that in SHED, in DPSCs, cells and tissues were not stained when IL-6 (Figure 4. E, F) antibody was used, whereas lightly stained sections were observed when the BLC antibody was used (Figure 4. G, H).

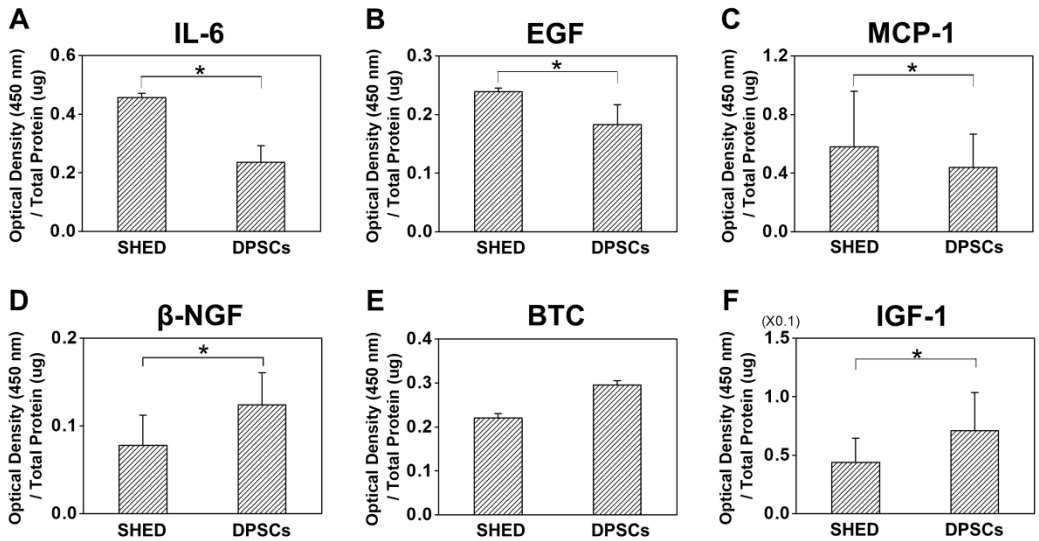

Figure 2. Validation of cytokine expression using enzyme-linked immunosorbent assay (ELISA). IL-6, EGF, MCP-1, β-NGF, BTC, and IGF-1 were used for ELISA. **(A-C)** IL-6, EGF, and MCP-1 were expressed more strongly in human exfoliated deciduous teeth–conditioned medium (SHED-CM) than in dental pulp stem cell–conditioned medium (DPSC-CM). **(D-F)** β-NGF, BTC, and IGF-1 were expressed more strongly in DPSC-CM than in SHED-CM. Statistically significant differences were observed only for β-NGF and IGF-1. Data were obtained from three independent experiments and are expressed as means ± SD (*$p<0.05$ in Mann–Whitney *U*-test).

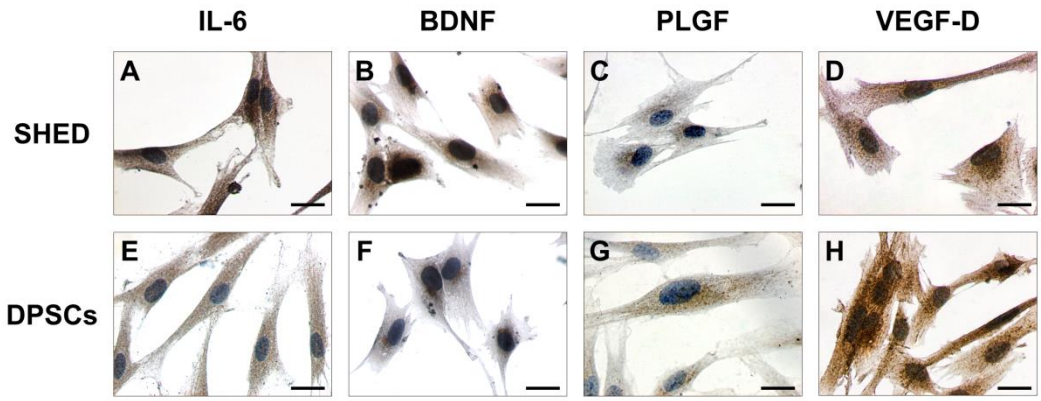

Figure 3. Immunocytochemistry findings of stem cells from human exfoliated deciduous teeth–conditioned medium (SHED-CM, A-D) and dental pulp stem cells–conditioned medium (DPSC-CM, E-H). The antibodies to IL-6 and BDNF were stained slightly more strongly in SHED-CM (A, B) than in DPSC-CM. (E, F) The antibody of PLGF and VEGF-D were clearly stained much more strongly in DPSC-CM (G, H) than in SHED-CM (C, D). Scale bars, 50 μm.

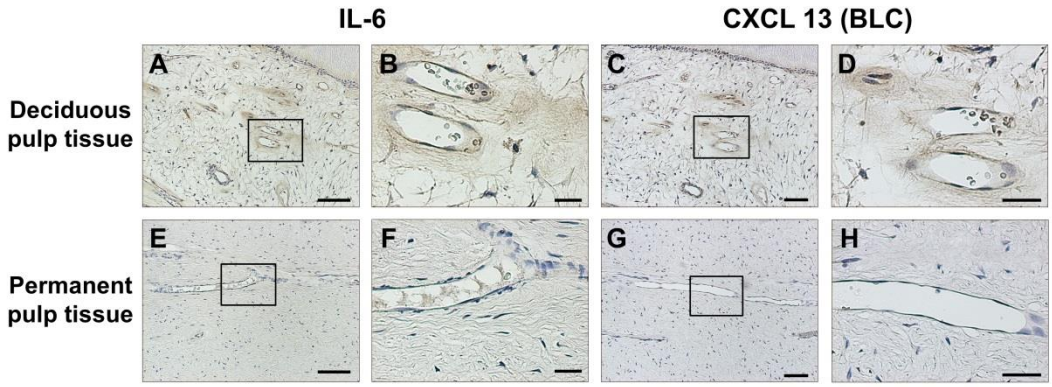

Figure 4. Immunohistochemistry analysis of deciduous pulp tissue (A-D) and permanent pulp tissue (E-H). Both IL-6 and CXCL-13 (BLC) were stained more strongly in the odontoblastic layer and perivascular region in deciduous than in permanent pulp tissue. A, C, E, G: Scale bar, 100 μm ; B, D, F, H: Scale bar, 20 μm.

IV. Discussion

In this study, cytokine expression levels were compared using a cytokine membrane array and additional ELISA. Immunocytochemistry and immunohistochemistry were employed to elucidate the paracrine effects of SHED and DPSCs. The cytokine arrays of SHED and DPSCs demonstrated that high expression levels of proinflammatory and proliferative cytokines were released in the SHED, whereas DPSCs showed relatively high expression levels of angiogenetic factors.

SHED showed higher expression of proliferative cytokines. Previous studies have shown that cells showed robust proliferation when cultured under additional IL-3 and IL-6 to the core of TPO, SCF, and FLT3-L, confirmed by a core cytokine mix of in vitro stem cell culture (Wang et al., 2017). IFN-γ, TNF-α, and IL-6 also reportedly modulate proliferation as well as both cellular and functional properties of human neural pluripotent cells in vitro (Hagman et al., 2019). In this study, IL-6 was expressed only in SHED, and IL-3 was 2.77-fold more strongly detected in SHED. Of the core cytokines, SCF and Flt3-L were expressed more strongly in DPSCs and SHED, respectively.

Proinflammatory cytokines such as IL-6 and IFN-γ were expressed more strongly in SHED than in DPSCs. IL-6 alone was the most abundantly expressed cytokine in SHED, and histological assessment of IL-6 also supported the result of cytokine array analysis. This is consistent with the gene-level findings of Wang et al., supporting fact that SHED exhibit increased proliferation (Wang et al., 2012). IL-6 is an important inflammatory

cytokine that triggers chronic inflammation and induces bone resorption (Gabay, 2006). Furthermore, there is accumulating evidence that IL-6 plays an important role as an optimal growth factor for stem cells (Brugger et al., 1993). Recent studies have reported the immunomodulatory effect of SHED in autoimmune diseases (Rossato et al., 2017). IL-6 secreted from stem cells mediates an inhibitory effect on B-cells, inhibiting B-cell proliferation, maturation, migration, and immunoglobulin and antibody production, and plays an immunomodulatory function that protects against neutrophil apoptosis (Rasmusson et al., 2007; Raffaghello et al., 2008).

IFN-γ, which was expressed 415.42-fold more strongly in SHED in this study, is also reportedly essential for immunosuppression and shows enhanced immunosuppressive activity with TNF-α (Polchert et al., 2008). In addition, it plays an important role in the induction of IL-6 levels in human MSCs (Wang et al., 2017). Enhanced paracrine actions by preconditioning stem cells with those cytokines may provide a potential strategy for immunosuppressive transplantation.

Inflammatory cytokines play positive and negative regulatory roles in neurogenesis. IL-6, the principal proinflammatory cytokine, controls neural stem cell renewal, progenitor cell division, and differentiation, and it has recently received attention as a neuromodulator (Taga and Fukuda, 2005). IL-6 increases neuronal differentiation but decreases (or does not affect) proliferation and gliogenesis (Islam et al., 2009; Oh et al., 2010). Furthermore, IL-4, IL-11, and IFN-γ increase neuronal differentiation (Borsini et al., 2015). In this study, IL-6, IFN-γ, and IL-4 were dominantly expressed in SHED,

whereas IL-11 was expressed 1.34-fold more strongly in DPSCs than in SHED. The cytokines involved in the secretion of neuroprotective factors, such as NT-3, BDNF, and GDNF, were more strongly expressed in SHED than in DPSCs. Studies have shown that more effective neural regeneration is possible in genetically overexpressed cells with neuroprotective factors or neurotransmitters and that genetically modified cells survive and exhibit enhanced functional outcomes during this extended period (Bakshi et al., 2006; Oudega et al., 2018; Descamps et al., 2018).

In the present study, of the cytokines involved in neurogenesis, the expression level of β-NGF was 6.85 times higher in DPSCs than in SHED, a finding supported by the results of the additional ELISA. Nerve growth factor (NGF) is a protein of the neurotrophin family, which includes brain-derived growth factor (BDNF), glial-cell-derived neurotrophic factor (GDNF), neurotrophic-3 (NT-3) and neurotrohpic-4 (NT-4); moreover, when compared to other types of neurotrophic factors, β-NGF is a key regulator of cell growth and neural differentiation. In previous studies, it has been reported to be capable of healing nerve injury in clinical therapy; Phyo et al. reported that increased NGF expression levels promoted reinnervation in pulp regeneration in a rat molar (Sone et al., 2020). In addition, bFGF was reported to have a paracrine effect toward increasing neural differentiation synergistically with NGF (Zhang et al., 2017), and BTC was also reported to induce neural stem cell proliferation and neurogenesis (Gómez-Gaviro et al.,2012). High expression and secretion of NGF and bFGF in DPSCs indicate that DPSCs have better neurogenic properties than SHED. NT-3, GDNF, and BDNF, which were more

strongly expressed in SHED than in DPSCs, were also reported to drive neuronal differentiation in SHED and DPSCs when treated as paracrine factors (Gonmanee et al., 2018).

The cytokines involved in angiogenesis were more strongly detected in DPSCs. GRO α and HGF were released more than 3 times higher in DPSCs than in SHED. GRO α, also known as CXCL1, is one of the key angiogenic chemokines, and HGF is a powerful pleiotropic cytokine involved in angiogenesis (Hristov et al., 2007; Matsumoto and Nakamura, 1996). PLGF and VEGF, prototypical pro-angiogenic factors, were 2.67 and 1.48 times higher, respectively, in DPSCs than in SHED. On the other hand, MCP-1, a pro-angiogenic factor, was abundantly secreted 1.31 times higher in SHED than in DPSCs.

VEGF promotes both angiogenesis and osteogenesis in bone regeneration, and its upregulation results in enhanced osteogenic differentiation and mineralization (Clark et al., 2015). Several growth factors such as TGF-β1, TGF-β3, and bFGF involved in osteogenic regeneration were more strongly detected in DPSCs than in SHED. In contrast, BMPs (BMP-4 and BMP-6) with synergistic effects in bone formation (Zhang et al., 2012) were more strongly expressed in SHED than in DPSCs. BMP, a member of the TFG-β superfamily, is a multifunctional cytokine that induces bone formation by regulating the growth and differentiation of chondroblasts and osteoblasts and functions in a wide range of biological activities as a regulator of cell proliferation and differentiation, chemotaxis, and tissue and organ morphogenesis (Kawabata et al., 1998). According to Li et al., BMP-

2, bFGF, and VEGF increased dose-dependent osteoblast proliferation, differentiation, and mineralization with only one factor; however, osteogenic differentiation was significantly promoted in a triple combined application in the rat model study (Li et al., 2014). Additionally, VEGF was reported to have immunosuppressive functions with angiopoietin 2 (Khan and Kerbel, 2018).

The secretion level of odonto/osteoclastogenesis-related cytokines was higher in SHED than in DPSCs. MCP-1 is a chemokine that recruits and activates monocytes under acute inflammation and angiogenesis. Additionally, MCP-1 has been found to stimulate chemotaxis, osteoclast differentiation and survival (Miyamoto et al., 2009), and osteoclast progenitor cell differentiation into odontoclasts (Asano et al., 2011). The increased expression level of MCP-1 may be related to the physiologic root resorption process of exfoliating deciduous teeth. In a previous study, Yoichi et al. suggested MCP-1 as a novel, potent therapeutic target for tissue repair due to its characteristics and unique immunoregulatory properties (Yamada et al., 2019). In contrast, BLC (CXCL13) was detected 3.72 times more strongly in SHED than in DPSCs. BLC was reported to induce osteogenic proliferation and differentiation in BMSCs by increasing calcium deposition into the extracellular matrix and the enhancing activity of the osteogenic marker ALP (Tian et al., 2015). Previous studies have found that IL-6 significantly induced BLC expression in human osteoblasts but not osteoclasts (Singh et al., 2009).

IGF-1, which was released more abundantly in DPSCs as a result of cytokine array and ELISA, is one of the key factors involved in bone and tooth regeneration. The IGF axis

plays a major role in the differentiation and development of dental structures. IGF-1 reportedly promotes the proliferation and osteogenic/odontogenic differentiation of dental pulp stem cells by triggering at least two pathways—Ras/Raf-1/MAPK and PI3K/PDK-1/Akt (Lv et al., 2016). In contrast, Wang et al. demonstrated that exogenous IGF-1 promoted the osteogenic differentiation and osteogenesis of SCAPs but decreased the odontogenic differentiation and dentinogenesis of SCAPs in vivo (Wang et al., 2012). While the function of IGF on osteogenesis is becoming apparent, much less information is available regarding the potential action of IGF-binding proteins (IGFBP 1-6). A direct effect of IGFBP2 in osteogenesis in a rat model was reported by Xi et al. (Xi et al., 2014); in contrast, however, IGFBP-3 was reported to inhibit osteoblast differentiation (Alkharobi et al.,2016). Conversely, the expression level of EGF was higher in SHED than in DPSCs as a result of cytokine array and ELISA. EGF has reportedly promotes cell proliferation and enhances osteogenic mineralization and has been suggested as an effective stem cell-based therapy for bone tissue engineering applicable in periodontics and oral implantology (Angel-Mosqueda et al., 2015).

Very few studies have focused on the secretomes of SHED and DPSCs, and studies on overall cytokines, chemokines, and growth factors are limited. This study demonstrates that SHED could immunologically fortify a more tolerant environment for stem cell transplantation and provide better conditions for cell proliferation and neurogenesis. In contrast, DPSCs have been suggested to be favorable for angiogenesis. However, several factors other than paracrine ones can affect stem cell behavior, and a quantitative analysis

of cytokines may not be directly related to the cell properties. The effects of cytokines on stem cells are exceedingly complex, and these effects vary not only with the type of cytokines but also with their concentration and tissue-specific location. The functions of these cytokines vary depending on the tissue site and may be even contradictory in some cases. Therefore, more specific studies on the applications of SHED and DPSCs in regenerative therapy are required.

V. Conclusion

SHED and DPSCs have distinctive cytokine profiles and are therefore expected to have different characteristics as stem cells in regenerative medicine. The expression levels of the cytokines involved in immunomodulation and odontogenic/osteogenic regeneration were higher in SHED than in DPSCs. In contrast, the expression levels of the cytokines involved in angiogenesis were higher in DPSCs than in SHED. It might be important to choose the most suitable stem cell type considering their multipotency and paracrine effects for adequate tissue regeneration.

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국문요약

유치 및 영구치 치수 유래 줄기세포에서 사이토카인 발현에 관한 비교 연구

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본 연구의 목적은 유치 및 영구치 치수 유래 줄기세포로부터 분비된 사이토카인의 프로파일을 분석하고 비교함으로써 유치 치수 줄기세포와 영구치 치수 줄기세포의 특성을 이해하는 것이다. 사람의 치아에서 획득한 유치 치수 줄기세포 조정배지와 영구치 치수 줄기세포 조정배지에서 120개의 사이토카인의 발현 정도를 측정하고 비교하였다. Cytokine membrane array의 결과를 검증하기 위하여 효소면역측정법, 조직면역화학염색법, 세포면역화학염색법을 추가로 시행하였다. 68개의 사이토카인이 통계적으로 유의할 만한 차이를 보였으며, 유치 치수 줄기세포에서 27개, 영구치 치수 줄기세포에서 41개의 사이토카인이 각각 1.3배 이상 높게 발현되었다. 염증성 사이토카인, 증식성 사이토카인, 면역조절에 관여하는 사이토카인이 유치 치수 줄기세포

에서 더 강하게 발현된 반면, 혈관신생에 관련된 사이토카인은 영구치 치수 세포에서 더 강하게 발현되었다. 유치와 영구치의 치수 줄기세포는 서로 다른 사이토카인 프로파일을 보이며 줄기세포로서 갖는 재생 능력에 차이가 있다. 각 세포의 다분화능과 주변 분비 효과를 고려하여 조직 재생에 적합한 줄기세포를 선택하는 것이 중요할 것으로 생각된다.

핵심되는 말: 사이토카인, 영구치 치수 줄기세포, 유치 치수 줄기세포, 조직 재생, 주변 분비 효과