Original Article

Distinctive cytokine profiles of stem cells from human exfoliated deciduous teeth and dental pulp stem cells

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Abstract  Background/purpose: SHED and DPSC have stem cell regenerative potential, but comparative research on their cytokine profile is rare. 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).

Materials and methods: SHED-conditioned medium (CM) and DPSC-CM were extracted using seven primary 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.

Results: 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. Cytokines involved in immunomodulation, odontogenesis, and osteogenesis 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.

Conclusion: These observations suggest that SHED may have a better cytokine profile related to inflammatory, proliferative, osteogenic, and odontogenic potential.

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Introduction

Dental tissues are considered accessible sources of MSCs and can be easily extracted and processed when a primary 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. In particular, SHED have a high proliferative rate and are capable of differentiating into osteoblasts, neural cells, adipocytes, and odontoblasts. DPSCs can also differentiate into adipocytes and neural cells as well as pulp/dentin tissue and bone cell types.

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. 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 we reported on the expression levels of cytokines in developing apical complex cells and DPSCs, comparative data on the expression levels of cytokines from SHED and DPSC have been published insufficiently. Here we investigated and compared the profiles of cytokines secreted from DPSCs and SHED to gain a better understanding of the cellular responses of DPSCs and SHED.

Materials and methods

Cell culture and preparation of DPSC— and SHED-conditioned medium

The Institutional Review Board of the Yonsei University Dental Hospital approved the experimental protocol (approval no. 2-2018-0012). All the subjects or their guardians have provided written informed consent (Table 1). 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 °C and then filtered through a 70-μm cell strainer. The isolated DPSCs and SHED extracted from at least two or three different donors were mixed at passage 2, and cells at passages 3–5 were used for cytokine array, enzyme-linked immunosorbent assay, and immunocytochemistry. DPSCs and SHED were seeded on a 100-mm culture dish and when 80% confluency was attained, the DPSCs and SHED were washed seven times with phosphate-buffered saline (pH 7.4) and cultured in a cell culture medium 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 °C until further analysis.

| Type         | Age   | Gender | Tooth                  |
|--------------|-------|--------|------------------------|
| Deciduous    | 6Y 1M | M      | Deciduous central incisor |
| teeth        | 6Y 4M | M      | Deciduous central incisor |
|              | 6Y 5M | F      | Lateral incisor        |
|              | 7Y 5M | F      | Central incisor        |
|              | 9Y 6M | F      | First molar            |
| Permanent    | 10Y 10M | M  | First molar           |
| teeth        | 11Y 5M | F      | Second molar           |
|              | 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            |

Cytokine profiles of DPSC—and SHED-CM and data analysis

DPSCs and SHED-CM were analyzed using human cytokine array C1000 (RayBiotech, Inc., Norcross, GA, USA), following the manufacturer’s instructions. The detailed experimental method was similar to the content of the previously published paper. Each sample was tested in triplicates, and cytokines that exhibited statistically significance for 3 averages were selected.

Enzyme-linked immunosorbent assay

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. In brief, 8 strips coated with different antibodies were incubated with DPSC- and SHED-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.

Immunocytochemistry analysis

DPSCs and SHED were cultured on coverslips coated with poly-L-lysine. After washing with PBS (Invitrogen), the samples were fixed with 4% paraformaldehyde. The fixed cells were stored at 4 °C until further analysis.
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, ab9942, 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.

**Immunohistochemistry analysis**

The human permanent and primary 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 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).

**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.

**Results**

**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. 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 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.

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

**Immunocytochemistry analysis**

IL-6, BDNF, 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 (Fig. 2A, B, E, F), whereas the staining intensities of PLGF and VEGF-D were higher in DPSCs than in SHED (Fig. 2C, D, G, H). These results were consistent with the cytokine antibody array analysis results.

**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 (Fig. 3A–D) Compared to that in SHED, in DPSCs, cells and tissues were not stained when IL-6 (Fig. 3E, F) antibody was used, whereas lightly stained sections were observed when the BLC antibody was used (Fig. 3G, H).

**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 angiogenic 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. IL-6, TNF-α, and IL-6 also reportedly modulate proliferation as well as both cellular and functional properties of human neural pluripotent cells in vitro. 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 only in SHED, and IL-3 was 2.77-fold more strongly detected in SHED. Of the core cytokines, SCF and Flt3-L, confirmed by a core cytokine mix of in vitro stem cell culture. 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. 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 only in SHED, and IL-3 was 2.77-fold more strongly detected in SHED.

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 that SHED exhibit increased proliferation. IL-6 is an important inflammatory cytokine that triggers chronic inflammation and induces bone resorption. Furthermore, there is accumulating evidence that IL-6 plays an important role as an optimal growth factor for stem cells. Recent studies have reported the immunomodulatory effect of SHED in autoimmune diseases. 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. 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. Furthermore, IL-4, IL-11, and IFN-γ increase neuronal differentiation. 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 proinflammatory cytokines such as IL-6 and IFN-γ were expressed more strongly in SHED than in DPSCs. 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 only in SHED, and IL-3 was 2.77-fold more strongly detected in SHED.

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

| Cytokine | SHED Ratio (SHED/DPSCs) | Biological function | P value |
|----------|--------------------------|---------------------|---------|
| IL-6     | 68.2 ± 15.7              | Proliferation, Immunomodulation, Neuromodulation | 0.002   |
| CNTF     | 27.5 ± 8.2               | Neurogenesis         | 0.022   |
| CCL23    | 24.9 ± 7.4               | Chemotaxis, Chemoattraction on osteoclast precursors, Angiogenesis | 0.002   |
| IGFBP2   | 19.5 ± 9.4               | Osteogenesis, Tooth development | 0.002   |
| IL-7     | 18.9 ± 6.0               | B and T cell differentiation, Inhibition of osteogenic differentiation | 0.002   |
| EGF      | 16.3 ± 5.4               | Osteogenic differentiation | 0.022   |
| BMP6     | 13.5 ± 5.3               | Osteogenic differentiation | 0.002   |
| IGFBP1   | 10.9 ± 6.8               | Osteogenic differentiation | 0.002   |
| GM-CSF   | 2.7 ± 2.0                | Inflammatory, Angiogenesis | 0.022   |
| Eotaxin1 | 8.1 ± 5.0                | Eosinophil chemoattractant |
| IL-5     | 8.0 ± 4.4                | Inflammatory         | 0.003   |
| IFN-gamma| 4.1 ± 2.2                | Immunomodulation, Osteoblast differentiation |
| PARC     | 29.2 ± 5.1               | Immunosuppression    | 0.003   |
| IL-2     | 8.2 ± 2.4                | Proinflammatory      | 0.087   |
| IL-4     | 11.2 ± 3.8               | Anti-inflammatory    | 0.135   |
| MIG      | 0.5 ± 0.4                | Inflammatory         | 0.042   |
| NT-3     | 25.1 ± 4.5               | Odontoblast differentation, Neurogenesis | 0.004   |
| GCP-2    | 20.8 ± 4.7               | Immunomodulatory effect of SHED in autoimmune diseases. 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.

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. Furthermore, IL-4, IL-11, and IFN-γ increase neuronal differentiation. 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.15–17.

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. Nerve growth factor (NGF) is a protein of the neurotrophin family, which is a key regulator of cell growth and neural differentiation. In previous

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

| Cytokine | SHED | DPSC | Ratio (DPSCs/SHED) | Biological function | P value |
|----------|------|------|-------------------|---------------------|---------|
| CCL28    | 0.0 ± 0.0 | 2.9 ± 2.8 | 1486.05           | Proinflammatory, B and T-cell chemotaxis, Cell proliferation | 0.002   |
| beta-NGF | 1.8 ± 2.1 | 12.5 ± 4.7 | 6.65              | Neurogenesis, Odontoblast differentiation | 0.001   |
| CXCL1 (GRO α) | 3.2 ± 5.0 | 11.7 ± 5.7 | 3.6               | Angiogenesis | 0.022   |
| BTC      | 8.6 ± 11.0 | 31.0 ± 4.9 | 3.6               | Cell proliferation, Neurogenesis, Angiogenesis | 0.004   |
| HGF      | 4.1 ± 6.1  | 12.9 ± 6.9 | 3.11              | Angiogenesis, Mitosis, Tissue regeneration, Anti-inflammatory | 0.043   |
| MMP alpha | 18.8 ± 2.5 | 26.9 ± 5.8 | 1.43              | Negative regulator in inflammation | 0.016   |
| MIP-3 beta | 19.4 ± 1.9 | 27.8 ± 4.6 | 1.43              | Immunomodulation | 0.002   |
| IGFBP3   | 36.5 ± 2.8 | 51.6 ± 6.4 | 1.42              | Modulation of mineralizing activity of IGF-1 | 0.004   |
| IL-11    | 21.2 ± 3.4 | 28.4 ± 6.9 | 1.34              | Osteo/odontoblast differentiation, Immunomodulation, Neurogenesis | 0.046   |
| FGF-4    | 21.5 ± 5.1 | 28.2 ± 2.3 | 1.31              | Odontogenesis | 0.016   |
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.\textsuperscript{18} 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.\textsuperscript{19} The cytokines involved in angiogenesis were more strongly detected in DPSCs. GRO\textsuperscript{a} and HGF were released more than 3 times higher in DPSCs than in SHED. GRO\textsuperscript{a}, also known as CXCL1, is one of the key angiogenic chemokines, and HGF is a powerful pleiotropic cytokine involved in angiogenesis.\textsuperscript{20,21} 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.\textsuperscript{22} Several growth factors such as TGF-\beta1, TGF-\beta3, and bFGF involved in osteogenic regeneration were more strongly detected in DPSCs than in SHED. In contrast, BMP-4 and BMP-6 with synergistic effects in bone formation were more strongly expressed in SHED than in DPSCs.\textsuperscript{23} 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.\textsuperscript{24}
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 and osteoclast progenitor cell differentiation into odontoclasts. The increased expression level of MCP-1 may be related to the physiologic root resorption process of exfoliating primary 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.

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. IGF-1 reportedly promotes the proliferation and osteogenic/odontogenic differentiation of dental pulp stem cells. 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. Conversely, the expression level of EGF was higher in SHED than in DPSCs as a result of cytokine array and ELISA. EGF has been suggested as an effective stem cell-based therapy for bone tissue engineering applicable in periodontics and oral implantology.

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. This study also has a limitation. The ages for collecting tooth samples are not varied, but notably, both primary and permanent teeth in healthy condition were collected. Further studies are needed on the change of cytokine expression in permanent teeth according to age. Also, more specific studies on the applications of SHED and DPSCs in regenerative therapy are required.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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