Interferon Gamma–treated Dental Pulp Stem Cells Promote Human Mesenchymal Stem Cell Migration *In Vitro*

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Abstract

Introduction: Chronic inflammation disrupts dental pulp regeneration by disintegrating the recruitment process of progenitors for repair. Bone marrow–derived mesenchymal stem cells (BM-MSCs) share the common features with dental pulp stem cells (DPSCs). The aim of the study was to investigate the migration of BM-MSCs toward DPSCs in response to inflammatory chemoattractants. Additionally, our studies also delineated the signaling mechanisms from BM-MSCs in mediating the proliferation and differentiation of DPSCs *in vitro*.
Methods: Human DPSCs and BM-MSCs between passages 2 and 4 were used and were grown in odontogenic differentiation medium. Mineralization was determined by alizarin red staining analysis. Migration was assessed using crystal violet staining in cells grown in Boyden chamber Transwell inserts (Corning Inc Foundation, Tewksbury, MA). The mineralization potential of DPSCs was evaluated using alkaline phosphatase activity assay. Real-time polymerase chain reaction analysis was performed to assess the gene expression profile of chemokine (C-X-C motif) ligand (Cxcl) 3, 5, 6, 10, 11, 12, 14, and 16; stromal cell-derived factor (SDF) α; vascular endothelial growth factor; and fibroblast growth factor.
Results: Interferon gamma (FN-γ) treatment significantly abrogated the differentiation potential of DPSCs as shown by using alizarin red and alkaline phosphatase activity analysis. An increase in the migration of BM-MSCs was documented when cocultured with IFN-γ–treated DPSCs. RNA expression studies showed an increase in the levels of Cxcl6 and Cxcl12 in BM-MSCs when cocultured with IFN-γ–treated DPSCs. Additionally, an up-regulation of proangiogenic factors vascular endothelial growth factor and fibroblast growth factor were observed in DPSCs exposed to IFN-γ.
Conclusions: Our findings indicate that inflamed IFN-γ–treated DPSCs release factors (presumably Cxcl6 and 12) that contribute to the homing of MSCs. This model might provide a potential research tool for studying MSC-DPSC cross talk and for future studies involving the recruitment and sustainability of progenitor stem cells sustaining the inflammatory cascade to treat pulp inflammation. *(J Endod 2015;41:1259–1264)*

Key Words

Bone marrow–derived mesenchymal stem cells, dental pulp stem cells, inflammation, interferon-γ, migration, regenerative endodontics

Inflammation and regeneration in dental pulp tissue are commonly observed as different processes; hence, they have been investigated independently. However, recent lines of evidence indicate that these processes are inter-related. The inflammatory cytokines released from the inflammatory milieu communicate with the inflammatory and stem/progenitor cells (1–4). These signaling mechanisms contribute to the mobilization and secretion of a repertoire of soluble factors with demonstrated cytoprotective and anti-inflammatory properties (5–8). Mainly, these factors signal the circulating stem/progenitor cells to migrate toward the injury site and contribute to tissue healing, which characterizes the proliferative and remodeling phases (9–11). Studies have postulated that the alterations in inflammatory signals exacerbate normal tissue healing and regeneration phases (12, 13).

Dental pulp is often submitted to damage or injury, and in most cases, dental pulp stem cells (DPSCs) deposit reparative or tertiary dentin in response to the injury (14–16). DPSCs are often referred as undifferentiated mesenchymal stem cells (MSCs) residing in the pulp. During the initial steps of inflammation, in pulp and periapical tissues, MSCs are shown to be present in inflamed tissues (17–19). The recruitment of MSCs to the injury site facilitates the reparative processes. However, prolonged exposure to inflammation impairs stem cell function and the number of MSCs as shown by Fouad and Huang (20).

MSCs have the capacity to receive signals from the inflammatory milieu through the surface markers. MSCs are documented to express receptors for a large number of cytokines such as interleukin (IL)-1, IL-4, IL-6, interferon gamma (IFN-γ), and tumor necrosis factor alpha; growth factors including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), tissuelike growth factor beta, and bone morphogenetic proteins; and chemokines (21). Conversely, incessant exposure to these cytokines potentially affects the activity of MSCs (22), leading to impairment in the immunomodulatory and anti-inflammatory roles of MSCs (23). Additionally, long-term exposure of MSCs to inflammatory mediators was shown to suppress the differentiation ability of DPSCs (20). Although these studies confirmed that inflamed MSCs disintegrate dental pulp regeneration, the signals that DPSCs emit to cross talk with MSCs and to facilitate the mobilization of MSCs to the injury site are not known. Furthermore, the mediators indeterminate for cross talk signaling remain unclear. Hence, to improve the reparative regenerative processes, it is critical that we understand the

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biological signals released by DPSCs that communicate with MSCs in response to inflammatory stimuli. Therefore, the aim of this study was to investigate cross talk signaling between DPSCs and MSCs. We hypothesized that IFN-γ–treated DPSCs release chemoattractants that facilitate the mobilization of MSCs, a process essential for dental pulp tissue regeneration.

Materials and Methods

Culture of Human DPSCs

DPSCs were obtained from healthy permanent premolars extracted during orthodontic treatment and generously donated by Dr Songtao Shi of the University of Southern California, Los Angeles (16). The single-cell suspensions were cultured in α-Minimum Essential Medium (MEM) (Life Technologies, Grand Island, NY) supplemented with 20% fetal bovine serum (Hyclone, Logan, UT), 1% antibiotic-antimycotic (Life Technologies), odontogenic medium supplemented with 100 μmol/L ascorbic acid, 2 mmol/L beta glycerophosphate, and 10 mmol/L dexamethasone. DPSCs were incubated at 37°C with 5% CO_2. DPSCs between the 3rd and 5th passages were used throughout the study.

Alizarin Red Staining

DPSCs seeded onto 12-well plates (1×10^4 cells per well) were subjected to alizarin red staining on day 14. Briefly, the cells were fixed in 4% paraformaldehyde for 20 minutes and then stained using alizarin red (Sigma-Aldrich, St Louis, MO). The phase-contrast images were then captured for analysis using the EVOS FL Cell Imaging System (Life Technologies).

5-bromo-2'-deoxyuridine Incorporation Assay

For proliferation studies, DPSCs were cultured to approximately 50% confluence in 96-well plates (BD Bioscience, San Jose, CA). At the end of the treatment period, cells were starved overnight in low-serum media followed by an 18-hour pulse with 10 μmol/L 5-bromo-2'-deoxyuridine (BrdU) in conditioned media (CM) from different time points as well as control media. After the 18-hour pulse, cells were rinsed with phosphate buffered saline and fixed in 70% ethanol with 2 mol/L HCl for 10 minutes at room temperature and then rinsed in phosphate buffered saline at least 3 times. The cell lysates were then measured at an excitation of 450 nm and emission of 595 nm using an enzyme-linked immunosorbent assay plate reader (Thermo Scientific, Bannockburn, IL). The magnitude of the absorbance for the developed color is proportional to the quantity of BrdU incorporated into DPSCs, which serves as a direct indication of cell proliferation.

Transwell Migration Assay

Cultured DPSCs were grown on the lower compartment of the 6-well plates, whereas MSCs were grown on the upper compartment of Transwell permeable support (Corning Inc Foundation, Tewksbury MA). At 3 days after the initiation of the culture conditions, the upper compartment (8-μm pore size insert) seeded with MSCs were placed onto the well by merging the bottom of the insert into the medium in the lower compartment. The cells in the Transwell plate were incubated at 37°C and 5% CO_2. Seventy-two hours after incubation, the Transwell insert was carefully removed, and the cells that did not migrate through the pores, on the upper side of the filter membranes, were gently removed with a cotton swab. Cells on the lower side of the insert filter were then quickly fixed (using 5% glutaraldehyde for 10 minutes) and stained with 1% crystal violet for 20 minutes. The time points were averaged for a total quantification from 3 independent experiments.

Reverse-transcription and Real-time Polymerase Chain Reaction Analysis

DPSCs treated with IFN-γ (500 U/mL) were washed, and total RNA was isolated with 1 mL TriZol (Life Technologies). The isolated RNA was analyzed using a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE) for ng/μL, and 260/280 readings were used to standardize the samples to 10 ng/μL with RNase-free water (Quality Biological Inc, Gaithersburg, MD); 1 μg total RNA was used to synthesize complementary DNA (cDNA) using the high-capacity cDNA reverse-transcription kit (Life Technologies). The resultant cDNA product was combined with SYBR green; the SuperScript One-Step RT-PCR System with the Platinum Taq kit; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), VEGF, FGF, and alkaline phosphatase (ALP) primers (Table 1) designed through Integrated DNA Technologies (IDT) DNA.

Statistical Analysis

Comparisons among relative expression levels of genes across the cell lineages were assessed, and the experimental values were reported as mean ± standard deviation. The crystal violet–positive cells and alizarin red nodules were manually quantified with the mean ± standard deviation reported. $P < .05$ was considered statistically significant. Statistical analysis was performed using Student's t test.

Results

IFN-γ Treatment Impairs DPSC Mineralization and Differentiation

Studies have shown that an early inflammatory reaction is a protective mechanism in pulp cells (12). However, prolonged inflammation deteriorates the mineralization and regenerative potential of pulp (24). We tested whether MSCs restore the differentiation potential of

TABLE 1. The Human Primer Sequences Used for Real-Time Polymerase Chain Reaction

Gene	Primers
GAPDH	For: GGCATCCACTGTGGTCATGAG
	Rev: TGCACCACCAACTGCTTAGC
VEGF	For: CAAAAACGAAAGCGCAAGAAA
	Rev: GCGGGCACCAACGTACAC
B-FGF	For: GGCTTCTTCCTGCGCATCCA
	Rev: GCTCTTAGCAGACATTGGAAGA
Cxcl2	For: CGCCCAAACCGAAGTCATAG
	Rev: AGACAAGCTTTCTGCCCATTCT
Cxcl3	For: TCCCCCATGGTTCAGAAAATC
	Rev: GGTGCTCCCCTTGTTCAGTATC
Cxcl5	For: GCATTTCTGTTGCTGTTCACGCTG
	Rev: CCTCCTTCTGGTTTTTCAGTTTAGC
Cxcl6	For: TGGGCCTGATCCTTGTTGCGC
	Rev: GCACCGTTTTTTGTCCATTCTTCAG
Cxcl10	For: GAACTGTACGCTGTACCTGCA
	Rev: TTGATGGCCTTCGATTCTGGA
Cxcl11	For: ATGAGTGTGAAGGGCATGGC
	Rev: TCACTGCTTTTACCCCAGGG
Cxcl12	For: TGCCAGAGCCAACGTCAAG
	Rev: CAGCCGGGCTACAATCTGAA
Cxcl14	For: CAGGTCGACATGAGGCTCCTGGCGGCCGCG
	Rev: CGGGGATCCCTATTCTTCGTAGACCCTGCG
Cxcl16	For: TCTCAAAGAATGTGGACATGC
	Rev: CAGGGGTGTGGATATCTGAA
SDF-1α	For: GGGGGAATTCCATGAACGCCAA
	Rev: GGGGTCTAGAGGGCATGGATGAAT

B-FGF, basic fibroblast growth factor; For, forward; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Rev, reverse; SDF-1α, stromal cell-derived factor-1α; VEGF, vascular endothelial growth factor.