ORIGINAL ARTICLE

Conditioned Medium from Periodontal Ligament Stem Cells Enhances Periodontal Regeneration

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Periodontal disease is one of the most common infectious diseases in adults and is characterized by the destruction of tooth-supporting tissues. Mesenchymal stem cells (MSCs) comprise the mesoderm-originating stem cell population, which has been studied and used for cell therapy. However, because of the lower rate of cell survival after MSC transplantation in various disease models, paracrine functions of MSCs have been receiving increased attention as a regenerative mechanism. The aim of this study was to investigate the regenerative potential of transplanted conditioned medium (CM) obtained from cultured periodontal ligament stem cells (PDLSCs), the adult stem cell population in tooth-supporting tissues, using a rat periodontal defect model. Cell-free CM was collected from PDLSCs and fibroblasts, using ultrafiltration and transplanted into surgically created periodontal defects. Protein content of CM was examined by antibody arrays. Formation of new periodontal tissues was analyzed using microcomputed tomography and histological sections. PDLSC-CM transplantation enhanced periodontal tissue regeneration in a concentration-dependent manner, whereas fibroblast-CM did not show any regenerative function. Proteomic analysis revealed that extracellular matrix proteins, enzymes, angiogenic factors, growth factors and cytokines were contained in PDLSC-CM. Furthermore, PDLSC-CM transplantation resulted in the decreased mRNA level of tumor necrosis factor- α (TNF- α) in healing periodontal tissues. In addition, we found that PDLSC-CM suppressed the mRNA level of TNF- α in the monocyte/macrophage cell line, RAW cells, stimulated with IFN- γ . Our findings suggested that PDLSC-CM enhanced periodontal regeneration by suppressing the inflammatory response through TNF- α production, and transplantation of PDLSC-CM could be a novel approach for periodontal regenerative therapy.

Keywords: mesenchymal stem cells, dental and periodontal, wound healing

Introduction

MESENCHYMAL STEM CELLS (MSCs) are the cell population isolated from bone marrow aspirates, which are characterized by the plastic-adherent and fibroblast-like clonogenic capacities.^{1,2} It has been considered that MSCs are stem cells of mesoderm tissues, because they are able to differentiate into mesenchymal lineages *in vitro*, such as osteoblasts, adipocytes, and chondrocytes, and have the capacity to generate ectopic bones and cartilages *in vivo*.^{3–5}

Recently, it has been revealed that MSC-like cells can be isolated from various tissues, such as adipose tissue, placenta, umbilical cord, dental pulp, and periodontal ligament (PDL).^{6–10}

These cells were regarded as somatic stem cells, which play a functional role in promoting wound healing and maintaining homeostasis. Moreover, taking advantage of the multi-differentiation potential of MSCs, expanded MSCs are used for cell-based regenerative therapies¹¹ and a number of human clinical trials for a wide variety of diseases are now underway (<https://clinicaltrials.gov>).

Although transplanted MSCs were expected to generate new tissues through their engraftment, proliferation, and differentiation, it has been revealed that transplanted MSCs have a relatively short life and the engraftment of MSCs is limited in the recipient sites.¹² Increasing numbers of reports have recently identified that paracrine factors from MSCs

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were strongly associated with tissue regeneration and wound healing upon MSC transplantation.^{13–15} Paracrine effects of MSCs include immunomodulation, antiapoptosis, angiogenesis, support to growth of cells, and chemoattraction properties. Transplantation of conditioned medium (CM), which contains the paracrine factors, has been reported to enhance wound healing in animal models.^{16,17}

Periodontitis is a bacterially induced chronic inflammatory disease that is characterized by the destruction of the periodontium, including the cementum, PDL, and alveolar bone.^{18,19} Over the past several decades, a number of approaches have been attempted to regenerate periodontal tissues; however, complete biological regeneration of lost periodontal structures has not been achieved.^{20–22}

The PDL is a thin ligament-like tissue, connecting the tooth to the alveolar bone socket, and contains the stem/progenitor cell populations for periodontal wound healing and homeostasis.^{23,24} In 2004, the MSC population from PDL, named PDL stem cells (PDLSCs), were identified and shown to generate tooth-specific attachments, a cementum/PDL-like complex, in immunocompromised mice.¹⁰ Following the discovery of PDLSCs, transplantation of PDLSCs has enhanced periodontal regeneration in animal models and human clinical trials are currently underway.^{25,26} However, there are some problems associated with the use of PDLSCs such as limitations in stem cell number, vulnerable condition of stem cells due to donor quality,²⁷ and the risk of and tumorigenesis.²⁸

Based on the previous information that paracrine factors from MSCs enhance wound healing and the transplantation of PDLSCs induces periodontal regeneration, we hypothesized that the transplantation of paracrine factors from PDLSCs could induce periodontal regeneration and be a novel regenerative therapy for periodontal disease. The purpose of this study is to evaluate the regenerative potential of PDLSC-CM using a rat periodontal defect model.

Materials and Methods

Cell culture

PDL tissues were collected from 12 teeth, which were premolars or third molars extracted from 11 healthy donors aged 12–29 years at the Tokyo Medical and Dental University (TMDU). This study protocol was approved by the ethics committee for clinical research at TMDU (#723) and informed consent was obtained from all volunteers.

PDL tissues were minced using surgical knife (Feather Safety Razor, Osaka, Japan) in 500 μ L of minimal essential medium alpha (α -MEM; Thermo Fisher Scientific, Waltham, MA) containing collagenase type I (3 mg/mL; Wako Pure Chemicals, Osaka, Japan) and dispase (4 mg/mL; Thermo Fisher Scientific) (digestion solution). The digest solution (9.5 mL) was added to the solution containing minced PDL tissues and incubated at 37°C for 60 min in a water bath with continuous agitation.

After enzyme digestion, the reaction solution was centrifuged (5 min, 1000 rpm), and 10 mL MSC growth medium (α -MEM containing 15% fetal bovine serum [FBS, Thermo Fisher Scientific], GlutaMAX [Thermo Fisher Scientific], and antibiotic–antimycotic [Thermo Fisher Scientific]) was added. The cell suspension was passed through a cell strainer (pore size; 70 μ m) (BD Falcon, Bedford, MA) to eliminate debris. The culture medium was changed every 3 days, and colony-

forming PDLSCs were harvested and passed at 37°C, 5% CO₂. It has been confirmed that PDLSCs, isolated with this method, showed MSC-like characteristics such as cell surface marker expression (CD105⁺, CD90⁺, CD44⁺, CD146⁺, CD166⁺, CD45⁺, CD31⁺, and CD34⁺) and trilineage differentiation ability (i.e., osteoblasts, adipocytes, and chondrocytes).^{29,30} The differentiation potential into osteoblasts and adipocytes was confirmed for all PDLSC lines, before the experiment in this study. PDLSCs were not pooled and CM was prepared from each PDLSC line used for experiment.

Normal human dermal fibroblasts (NHDF) were purchased (Lonza, Walkersville, MD) and cultured in Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific) containing 10% FBS. PDLSCs and fibroblasts at passage 2–6 were used for all experiments. The murine macrophage RAW 264.7 cell line was kindly provided by Dr. K. Nakahama (Tokyo Medical and Dental University, Japan) and cultured in α -MEM containing 10% FBS.

For the stimulation of murine macrophage RAW 264.7 cells, 1×10^5 cells were seeded in 24-well plates for 18 h. The culture medium was then changed to PDLSC-CM, Fibroblast-CM, or Control-CM (300 μ L, concentrated once with ultrafiltration, ~27-fold concentrated) containing 1% FBS and cells were cultured for 24 h. The cells were exposed to IFN- γ (2 ng/mL, R&D systems, Minneapolis, MN) for 6 h and total RNA was extracted for the following assays.

Preparation of CM

PDLSCs and NHDFs were cultured in 10-cm tissue culture dish and when they attained ~70–80% confluency, they were washed with phosphate-buffered saline thrice and refreshed with 10 mL of serum-free DMEM. Culture supernatants were collected after 48 h of incubation. Collected supernatants were centrifuged (1000 rpm, 5 min at 4°C), and filtered through a 0.2 μ m pore size filter (Kurabo, Osaka, Japan) to remove cell debris. Then, the CM were concentrated using ultrafiltration with a cutoff of 10 kDa (Millipore, Billerica, MA) and stored at 4°C. We designated non- (original), once- (17- to 31-fold), and twice- (450-fold) concentrated CM as PDLSC-low, PDLSC-moderate, and PDLSC-high, respectively. CM from NHDFs were concentrated once using ultrafiltration (27-fold) with a cutoff of 10 kDa and used for the experiment as Fibroblast-CM. Control conditioned media (Control-CM) were made by collecting the culture media (serum-free DMEM) from the culture dish without cells after 48 h of incubation.

Transplantation of PDLSC-CM in a rat periodontal defect model

All study protocols were approved by the Animal Care Ethics Committee of TMDU (#0140284A, 0150199A, 0160192A, 0170345A). We made the surgical periodontal defects modifying the method reported by King *et al.* and Padial-Molina *et al.* to create clinically relevant periodontal defect model.^{31,32} Male Sprague-Dawley rats (Slc/SD; age 8 weeks, $n=50$) were anesthetized with isoflurane (Abbott Laboratories, Queenborough, United Kingdom) and pentobarbital sodium (Kyoritsu Seiyaku, Tokyo, Japan).

An extraoral incision was made at the right of the mandible and the buccal plate was exposed (Fig. 1A). The buccal bone, PDL, cementum, and dentin from the mandibular first molar roots to second molar mesial root were

FIG. 1. Creation of rat periodontal defect and CM transplantation. (A, D) Microscopic view (A) and micro-CT image (D) of the buccal area of the mandibular first molar before creating the periodontal defect. *Black dot line* represents the configuration of the defect on buccal bone; AB, alveolar bone; ML, masseter ligament. (B, E) Microscopic view (B) and micro-CT image (E) of the surgically created periodontal defect. The defect was generated around the buccal area in the mandibular first molar. Root surfaces from the medial corner of the first molar to second molar were denuded. The defect size was 2 mm in height and 3 mm in width. DR, distal root; CR, central root; MR, mesial root. (C) Microscopic view of CM transplantation. CM were transplanted into the defect with a collagen scaffold, and the transplanted site was covered with fibrin glue to stabilize the scaffold and CM. CM, conditioned medium; micro-CT, microcomputed tomography. Color images available online at www.liebertpub.com/tea

carefully removed to create a periodontal defect by rotatory instruments (Fig. 1B). The dimensions of the periodontal defect in the buccal area were as follows: 2 mm in height and 3 mm in width. PDLSC-CM, Fibroblast-CM, or Control-CM (10 μ L) was transplanted into the defect with a collagen sponge (Koken, Tokyo, Japan). The transplanted site was covered with fibrin glue (BOLHEAL, Teijin Pharma. Ltd., Tokyo, Japan) (Fig. 1C), and the masseter and skin were sutured with 7-0 or 5-0 silk (Mani, Tochigi, Japan). The decision of treatment in each experiment was made randomly by blinded third person after completion of periodontal defect creation.

Microcomputed tomography analysis

Healing of the periodontal defect was examined at 4 weeks posttransplantation using microcomputed tomography (micro-CT) scans (inspeXio SMX-100CT; Shimadzu Co, Kyoto, Japan). Three-dimensional images were constructed from the scan data using VG Studio MAX 2.0 (Volume Graphics, Heidelberg, Germany). To evaluate periodontal tissue healing, we measured the exposed root surface area up to the line connecting the mesial and distal points of the cement–enamel junction of the mandibular first molar using BZ-analyzer software (Keyence, Osaka, Japan).

Histological analysis

Four weeks after transplantation, the mandibular blocks of the rats were removed and fixed in 4% paraformaldehyde phosphate buffer for 1 day. The mandibular blocks were decalcified in 10% EDTA at 4°C for 6 weeks and embedded in paraffin. Lingo-buccal sections of 5 μ m thickness were obtained. Hematoxylin-eosin and Azan staining were performed for microscopic observation of the sections. Images

of histological sections were obtained using a microscope (BZ8000, Keyence).

Antibody array of CM

Antibody array of PDLSC-CM was performed using the Proteome Profiler Human Angiogenesis Array Kit (R&D Systems), Human Cytokine Array Panel A Kit (R&D Systems), and RayBio C-Series Human Growth Factor Antibody Array C1 (Raybiotech, Inc., Norcross, GA), according to the manufacturer's protocol.

RNA isolation and reverse transcription polymerase chain reaction

We euthanized six animals from both Control-CM and PDLSC-CM group 5 days after the transplantation and collected the soft healing tissues, which filled the created periodontal defects. Total RNA was extracted using the ReliaPrep™ RNA Tissue Miniprep System (Promega, Madison, WI). For cultured cells, total RNA was extracted using the RNeasy Mini kit (QIAGEN, Venlo, Netherlands). cDNA was synthesized with 0.2–1 μ g RNA using the First Strand cDNA synthesis kit (Roche Diagnostics, Rotkreuz, Switzerland). Reverse transcription polymerase chain reaction (RT-PCR) was performed according to the product protocol of LightCycler FastStart DNA Master SYBR Green I (Roche) using specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and interleukin-10 (IL-10) as shown in Table 1.

Statistical analysis

Data are expressed as the mean $\pm$ standard deviation. Statistical significance was determined by Student's *t*-test or

TABLE 1. PRIMER SEQUENCES FOR REAL-TIME PCR

Gene	Primer sequence (5' → 3')
rGAPDH	(F) ATGATTCTACCCACGGCAAG (R) CTGGAAGATGGTGATGGGTT
rTNF- α	(F) ACGTCGTAGCAAACCACCAA (R) GCAGCCTTGTCCTTGAAGA
rIL-1 β	(F) CACCTCTCAAGCAGAGCACAG (R) GGGTTCCATGGTGAAGTCAAC
rIL-6	(F) TAGTCCTTCCTACCCCAACTTCC (R) TTGGTCCTTAGCCACTCCTTC
rIL-10	(F) CAGACCCACATGCTCCGAGA (R) CAAGGCTTGGAACCCAAGTA
mGAPDH	(F) ACCACAGTCCATGCCATCAC (R) TCCACCACCCTGTTGCTGTA
mTNF- α	(F) TTCTGTCTACTGAAGTTCGGGG TGATCGGTCC (R) GTATGAGATAGCAAATCGGC TGACGGTGTGGG

Dunnett's test, or Tukey's honestly significant difference test. Differences with $p < 0.05$ were considered significant.

Results

Periodontal regeneration by transplantation of PDLSC-CM

We first examined whether transplantation of PDLSC-CM could induce periodontal regeneration. We prepared three PDLSC-CM with different concentrations (original: PDLSC-low, 17- to 29-fold concentrated: PDLSC-moderate, and 450-fold: PDLSC-high) and transplanted them into the rat periodontal defects. Periodontal defects were surgically created as mentioned in Materials and Methods section and showed in Figure 1A–E.

Figure 2A shows the representative 3D reconstructed micro-CT images of periodontal defects 4 weeks after transplantation of CM. A higher alveolar bone level around the distal root of the first mandibular molar was observed in PDLSC-moderate and PDLSC-high groups than in the Control-CM and PDLSC-low groups. Figure 2B shows the results of quantification of the exposed root area using CT images. Decreased exposed root surface area was observed in the PDLSC-moderate and PDLSC-high groups, compared with the Control-CM group ($p < 0.05$), whereas there was no significant difference between the PDLSC-low and Control-CM groups (Fig. 2B). We observed no differences in exposed root surface area among the Control-CM groups with different concentration ratios ($\times 1$, moderate, and high) (data not shown). These results indicated that PDLSC-CM, concentrated at moderate to high ratios, enhanced periodontal regeneration.

Next, to examine the specificity of the regenerative effect of PDLSC-CM in periodontal tissues, we compared the periodontal regeneration after treatment with CM from fibroblasts and PDLSCs in a rat periodontal defect model. As shown in Figure 3A, the PDLSC-CM group demonstrated prominent new tissue formation especially around the mesial and distal roots of the first molar, whereas the Fibroblast-CM group showed less tissue formation, which was comparable to the Control-CM group (Fig. 3A). Quantification of the exposed root area demonstrated that PDLSC-CM enhanced periodontal tissue healing compared with Control-CM and Fibroblast-CM, indicating the unique ability of PDLSC-CM to enhance periodontal regeneration (Fig. 3B) ($p < 0.05$).

We investigated the structures of newly formed tissues in histological sections. As shown in Figure 4A, spontaneous periodontal tissue healing was observed even in the Control-CM group. Regardless of the treatment, new periodontal tissue formation was observed as an extension of the residual periodontal tissues from the bottom of the defects. No

FIG. 2. The effect of PDLSC-CM transplantation on periodontal regeneration. (A) Representative micro-CT images of periodontal defects at 4 weeks posttransplantation in Control-CM group ($n = 5$), PDLSC-low group ($n = 4$), PDLSC-moderate group ($n = 6$), and PDLSC-high group ($n = 5$). Yellow dot lines represent the edge of surgically created periodontal defect. (B) Quantification of exposed root surface area on micro-CT images at 4 weeks posttransplantation. Exposed root surface area was reduced in PDLSC-high group and PDLSC-moderate group compared with Control-CM group. * $p < 0.05$ (vs. Control-CM), Dunnett's test; PDLSC, periodontal ligament stem cell. Color images available online at www.liebertpub.com/tea

FIG. 3. The effect of PDLSC-CM and fibroblast-CM transplantation on periodontal regeneration. **(A)** Representative micro-CT images of the periodontal defects at 4 weeks after transplantation. The mesial and distal roots of PDLSC-CM group were widely covered by newly formed bone, whereas bone height stayed at lower level in the Control-CM and Fibroblast-CM groups. *Yellow dot lines* represent the edge of surgically created periodontal defect area. **(B)** Quantification of the exposed root surface area at 4 weeks after transplantation. The exposed root surface areas of the first mandibular molar were measured. PDLSC-CM group significantly reduced the exposed root surface area compared with the other groups. PDLSC-CM, 28–31-fold concentration; Fibroblast-CM, 27-fold concentration. $*p < 0.05$, Tukey's HSD test, $n = 6$ for each group. HSD, honestly significant difference. Color images available online at www.liebertpub.com/tea

direct bone to tooth attachment, ankylosis, was observed in any of the sections analyzed. We observed less new formation of periodontal tissues in the Fibroblast-CM and PDLSC-low groups, which was comparable to that of the Control-CM group. More new periodontal tissue formation was observed in the defects filled with PDLSC-moderate and PDLSC-high CM. These results suggested that transplantation of moderate and high concentration ratio PDLSC-CM induced new tissue formation in periodontal defects with histologically proper structures.

Figure 4B and C demonstrate the periodontal tissues around the middle third of created defects. In PDLSC-moderate and PDLSC-high sections, prominent bone formation was found, whereas the space was filled with dense fibrous connective tissues in Control-CM, Fibroblast-CM, and PDLSC-low sections. Collagen bundles connecting the root and bone in the PDL space were observed in all groups, although no obvious anatomical differences could be seen in newly formed periodontal tissues (Fig. 4D).

Significant differences were found in the area of the bone crest. Abundant marrow cavity and honeycomb structure of trabecular bone were evident in Control-CM, Fibroblast-CM, and PDLSC-low groups (Fig. 4E). Osteoblast-like cells were seen aligned along the bone surface and small islet-like bone clusters were only found in Control-CM, Fibroblast-CM, and PDLSC-low group sections (Fig. 4E). On the other hand, in PDLSC-moderate and PDLSC-high groups, crestal bone was more united and entrapment of osteocytes was more, in comparison with Control-CM, Fibroblast-CM, and PDLSC-low groups (Fig. 4F). However, even in PDLSC-moderate and PDLSC-high groups, obvious compact bone

structure and periosteum were not observed, suggesting that the bone formation was still in the process of regeneration.

Antibody array of PDLSC-CM

Since the volume of regenerated periodontal tissue was dependent on the concentration ratio of PDLSC-CM, it was conceivable that protein content may be responsible for the regenerative function of PDLSC-CM. We next estimated the contents of PDLSC-CM using three kinds of antibody arrays, including angiogenesis-related proteins, growth factors, and cytokines.

Antibody arrays revealed that various proangiogenic factors, such as tissue inhibitor of metalloproteinase 1, urokinase-type plasminogen activator, and vascular endothelial growth factor, and growth factors, such as insulin-like growth factor binding protein 6 (IGFBP6), IGFBP2, and platelet-derived growth factor receptor β , were detected in PDLSC-CM (Fig. 5A, B). Cytokines, such as serine protease inhibitor E1 (Serpin E1) and monocyte chemotactic protein-1, were detected in PDLSC-CM, even though the numbers of detected proteins were lower than those detected using the two other protein arrays (Fig. 5C). These results revealed that PDLSC-CM contained a mixture of various angiogenesis-related factors, growth factors, and several cytokines.

We further analyzed the protein contents of CM by liquid chromatography–tandem mass spectrometry (LC/MS/MS) (Supplementary Data and Supplementary Tables; Supplementary Data are available online at www.liebertpub.com/tea). LS/MS/MS detected a total of 99 proteins in five PDLSC-CM. The top proteins found at higher peptide hits were extracellular

FIG. 4. Histological images of periodontal tissues at 4 weeks after CM transplantation. **(A)** Hematoxylin-eosin (H&E) staining of lingual-buccal section at the distal root. Higher bone levels were observed in PDLSC-moderate and PDLSC-high groups compared with other groups. The *black dot lines* represent the *bottom* of the defect. NB, new alveolar bone; *Arrowheads*, bone crest; Scale bar=300 μ m. **(B, C)** High magnification images of *middle third* of periodontal defect (the *black boxes* in **A**) with H&E (**B**) and Azan staining (**C**). NB, new alveolar bone; PDL, periodontal ligament; C, cementum; D, dentin; *Asterisk*, connective tissue filling periodontal defect; Scale bar=100 μ m. **(D)** Higher magnification images of newly formed periodontal tissues in all groups. Collagen bundles, which bridged tooth root and alveolar bone, were evident in periodontal space of all sections. NB, new alveolar bone; C, cementum; D, dentin; *Arrow*, collagen bundle in PDL; Scale bar=100 μ m. **(E)** Close images of bone tissues around bone crest. Bone tissue was immature in Control-CM, Fibroblast-CM, and PDLSC-low group, compared with PDLSC-moderate and PDLSC-high group. NB, new alveolar bone; *Arrows*, osteoblast-like cells; *Asterisk*, small islet-like bone cluster. Scale bar=100 μ m. **(F)** Higher magnification images of bone crest area of PDLSC-moderate and PDLSC-high group sections (the *black boxes* in **E**). Many osteocyte-like cells were found embedded in trabeculae. *Arrowheads*, osteocyte-like cells; Scale bar=100 μ m. Color images available online at www.liebertpub.com/tea

FIG. 5. Antibody array of PDLSC-CM. Protein levels in PDLSC-CM determined by antibody-based protein array of angiogenesis-related proteins (A), growth factors (B), and cytokines (C). PDLSC-CM were obtained from three donors (age; 12–28 years old) and concentrated once using ultrafiltration. Color images available online at www.liebertpub.com/tea

FIG. 6. Expression of inflammation-related genes in rat periodontal tissues. Total RNA was extracted from periodontal wounded site 5 days after transplantation and the mRNA levels of TNF- α (A), IL-6 (B), IL-1 β (C), COX-2 (D), and IL-10 (E) were determined by reverse transcription polymerase chain reaction. TNF- α expression was significantly decreased in the PDLSC-CM group in the transplanted site compared with the Control-CM group. * $p < 0.05$, Student's t -test, $n = 6$ for each group. IL-1 β , interleukin-1 β ; IL-6, interleukin-6; IL-10, interleukin-10; TNF- α , tumor necrosis factor- α .

matrix proteins, including collagen and fibronectin. LC/MS/MS also revealed that PDLSC-CM is composed of a mixture of a wide variety of proteins, including matrix proteins, enzymes, growth factors, cytokines, and angiogenic factors.

Inflammation levels in healing tissues after PDLSC-CM transplantation

Inflammation affects wound healing and regeneration, and MSCs possess anti-inflammatory properties through paracrine mechanisms. Therefore, we investigated whether transplantation of PDLSC-CM would affect the inflammatory response at the wounded periodontal site. We found that the gene expression level of TNF- α in the PDLSC-CM group was significantly decreased compared with the Control-CM group (Fig. 6A). Moreover, IL-6, IL-1 β , and COX-2, another inflammation-related molecule, tended to decrease following PDLSC-CM transplantation compared with the Control-CM group, although this effect was not statistically significant (Fig. 6B–D). These results suggest the close relationship between PDLSC-CM transplantation, reduced inflammation in healing tissues, and periodontal regeneration.

PDLSC-CM inhibits IFN- γ -induced TNF- α expression in RAW 264.7 cell

We next examined whether PDLSC-CM could inhibit TNF- α gene expression in monocytes, the main cellular source of TNF- α , *in vitro*. PDLSC-CM significantly suppressed mRNA levels of TNF- α in RAW cells stimulated with IFN- γ , compared with Control-CM and Fibroblast-CM

(Fig. 7). Therefore, it was suggested that PDLSC-CM exerted anti-inflammatory effects by suppressing the gene expression of TNF- α during periodontal wound healing.

Discussion

The purpose of this study was to examine whether transplantation of PDLSC-CM enhances periodontal regeneration. We demonstrated that transplantation of PDLSC-CM

FIG. 7. PDLSC-CM inhibited IFN- γ -induced TNF- α expression in RAW 264.7 cell. Messenger RNA levels of TNF- α in RAW cells. PDLSC-CM significantly suppressed mRNA levels of TNF- α in IFN- γ -treated RAW cells compared with Control-CM and Fibroblast-CM. * $p < 0.05$, Tukey's HSD test.

into rat mandibular periodontal defect enhanced periodontal regeneration depending on the concentration ratio of CM (Figs. 2 and 3).

Periodontal regeneration using MSC-CM has been reported previously.^{33,34} Inukai *et al.* implanted CM from human bone marrow-derived MSCs (BMMSCs) into canine one-wall intrabony defects and observed periodontal regeneration. In addition, Kawai *et al.* showed that implantation of BMMSC-CM enhanced periodontal regeneration in a rat maxillary periodontal defect model. There are two main differences between our study and the aforementioned studies: the source of MSCs and the concentration of CM. The previous researchers used BMMSCs as the source of CM and did not concentrate BMMSC-CM in their respective studies. It is widely accepted that PDLSCs possess similar properties to BMMSCs, such as trilineage differentiation capacity, immunomodulatory function, and antiapoptotic effects.^{35,36} However, PDLSCs have been suggested to possess certain additional functions, including the capacity to form a cementum-PDL complex, a tooth-specific structure.^{10,37} Tsumanuma *et al.* demonstrated that transplantation of PDL cells induced more cementum-PDL formation compared with transplantation of BMMSCs³⁸ and suggested that PDLSCs are the more favorable cell type for periodontal regeneration. Based on this, we hypothesized a regenerative effect of PDLSC-CM and showed that its transplantation enhanced new periodontal tissue formation in this study. Mrozik *et al.* identified the differential protein expression pattern between PDLSCs and BMMSCs by proteomic approaches³⁹ and suggested that the differential protein expression in these MSC populations could affect the high turnover of periodontal tissues. Thus, the function of CM from BMMSCs and PDLSCs may be different in periodontal wound healing after their transplantation. However, further detailed examinations are needed to compare the regenerative potentials of PDLSC-CM and BMMSC-CM.

We demonstrated that periodontal regeneration tended to be dependent on the concentration ratio of PDLSC-CM. Although we were not able to identify which protein(s) contributed to our findings, we showed that PDLSC-CM contained a wide variety of proteins by proteomic analysis and it is possible that an aggregate effect of these proteins in PDLSC-CM resulted in periodontal regeneration. Chen *et al.* demonstrated that BMMSC-CM ameliorated intestinal damage after radiation-induced intestinal injury.⁴⁰ They showed that neutralization of IGF-1 due to BMMSC-CM partially suppressed the beneficial effects of BMMSC-CM and recombinant IGF-1 treatment did not completely recover the injury. These results suggested that the mechanism of the therapeutic effect of MSC-CM is complicated and may be mediated through multiple factors. Further studies are needed to identify the responsible factor(s) driving PDLSC-CM-induced periodontal regeneration.

Our histological results demonstrated that the amount of regenerated tissues and the maturation of bone at the bone crest area were different among the study groups. In Control-CM, Fibroblast-CM, and PDLSC-low, where lesser periodontal regeneration was found, the bone tissue at bone crest still showed a honeycomb-like structure, indicating the immature stage of sponge bone formation. In contrast, in PDLSC-moderate and PDLSC-high, where larger periodontal regeneration was seen, more trabeculae formation and embedment

of osteocytes were found, suggesting the progression of bone maturation. These findings imply the fact that PDLSC-CM (moderate and high) transplantation may accelerate the periodontal wound healing and result in the enhanced periodontal tissue regeneration. However, our follow-up results were limited to 4 weeks post-CM transplantation, and further studies with different time points are needed for the clarification of the regeneration mechanism by PDLSC-CM.

We demonstrated that PDLSC-CM significantly decreased the mRNA level of TNF- α in periodontal tissues and in a macrophage cell line *in vitro*. Our results indicated that PDLSC-CM reduced the production of TNF- α in periodontal tissues. TNF- α is an important inflammatory cytokine in wound healing and it has been reported that MSC-CM promoted wound healing by downregulating the expression of TNF- α in an acute liver injury model.⁴¹ Moreover, Liu *et al.* reported that transplantation of MSCs overexpressing IGFBP-5 in swine periodontal defects suppressed the expression of TNF- α and enhanced cementum regeneration.⁴² These results indicate the relationship between inhibition of TNF- α and periodontal regeneration and support our findings that PDLSC-CM enhanced periodontal regeneration by suppressing the inflammatory response through TNF- α production. However, further detailed studies are needed to examine whether TNF- α inhibition is the main mechanism regulating PDLSC-CM-elicited periodontal regeneration.

To the best of our knowledge, this is the first study to demonstrate that PDLSC-CM transplantation can enhance periodontal regeneration *in vivo*. Although MSC transplantation for periodontal regeneration has recently been widely investigated, it is believed that PDLSC-CM transplantation has key advantages, which compensate for its shortcomings. In particular, PDLSC-CM transplantation carries a lower risk of tumorigenesis compared with MSC transplantation. Unlike cultured cells, CM are easy to preserve and handle during surgical procedures in clinical settings. Moreover, the hurdles associated with allo- or xenotransplantation are lower for PDLSC-CM compared with cultured PDLSCs. Katagiri *et al.* have recently reported that the BMMSC-CM transplantation did not exhibit any systemic and local complications in human study.⁴³ However, further studies with focus on the safety of CM transplantation are needed.

In summary, we demonstrated that transplantation of PDLSC-CM enhanced periodontal regeneration in a rat periodontal defect model. Our findings strongly suggest that transplantation of PDLSC-CM is a promising regenerative therapy approach for periodontal disease.

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Disclosure Statement

No competing financial interests exist.

References

1. Friedenstein. Osteogenic Stem Cells in Bone Marrow Bone and Mineral Research. The Netherlands: Elsevier Science Publishers, 1990.

2. Bianco, P., Robey, P.G., and Simmons, P.J. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* **2**, 313, 2008.
3. Caplan, A.I. Mesenchymal stem cells. *J Orthop Res* **9**, 641, 1991.
4. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. *Science (New York, NY)* **284**, 143, 1999.
5. Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., and Horwitz, E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8**, 315, 2006.
6. Zuk, P.A., Zhu, M., Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., Alfonso, Z.C., Fraser, J.K., Benhaim, P., and Hedrick, M.H. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**, 4279, 2002.
7. Gronthos, S., Mankani, M., Brahimi, J., Robey, P.G., and Shi, S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci U S A* **97**, 13625, 2000.
8. Fukuchi, Y., Nakajima, H., Sugiyama, D., Hirose, I., Kitamura, T., and Tsuji, K. Human placenta-derived cells have mesenchymal stem/progenitor cell potential. *Stem Cells* **22**, 649, 2004.
9. Bieback, K., Kern, S., Kluter, H., and Eichler, H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. *Stem Cells* **22**, 625, 2004.
10. Seo, B.-M., Miura, M., Gronthos, S., Mark Bartold, P., Batouli, S., Brahimi, J., Young, M., Gehron Robey, P., Wang, C.Y., and Shi, S. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* **364**, 149, 2004.
11. Mundra, V., Gerling, I.C., and Mahato, R.I. Mesenchymal stem cell-based therapy. *Mol Pharm* **10**, 77, 2013.
12. von Bahr, L., Batsis, I., Moll, G., Hagg, M., Szakos, A., Sundberg, B., Uzunel, M., Ringden, O., and Le Blanc, K. Analysis of tissues following mesenchymal stromal cell therapy in humans indicates limited long-term engraftment and no ectopic tissue formation. *Stem Cells* **30**, 1575, 2012.
13. Meirelles Lda, S., Fontes, A.M., Covas, D.T., and Caplan, A.I. Mechanisms involved in the therapeutic properties of mesenchymal stem cells. *Cytokine Growth Factor Rev* **20**, 419, 2009.
14. Kinnaird, T., Stabile, E., Burnett, M.S., Shou, M., Lee, C.W., Barr, S., Fuchs, S., and Epstein, S.E. Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation* **109**, 1543, 2004.
15. Gneccchi, M., He, H., Noiseux, N., Liang, O.D., Zhang, L., Morello, F., Mu, H., Melo, L.G., Pratt, R.E., Ingwall, J.S., and Dzau, V.J. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *FASEB J* **20**, 661, 2006.
16. Chen, L., Tredget, E.E., Wu, P.Y., and Wu, Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS One* **3**, e1886, 2008.
17. Watanabe, S., Arimura, Y., Nagaishi, K., Isshiki, H., Onodera, K., Nasuno, M., Yamashita, K., Idogawa, M., Naishiro, Y., Murata, M., Adachi, Y., Fujimiya, M., Imai, K., and Shinomura, Y. Conditioned mesenchymal stem cells produce pleiotropic gut trophic factors. *J Gastroenterol* **49**, 270, 2014.
18. Pihlstrom, B.L., Michalowicz, B.S., and Johnson, N.W. Periodontal diseases. *Lancet* **366**, 1809, 2005.
19. Darveau, R.P. Periodontitis: a polymicrobial disruption of host homeostasis. *Nat Rev Microbiol* **8**, 481, 2010.
20. Bosshardt, D.D., and Sculean, A. Does periodontal tissue regeneration really work? *Periodontology 2000* **51**, 208, 2009.
21. Reynolds, M.A., Kao, R.T., Camargo, P.M., Caton, J.G., Clem, D.S., Fiorellini, J.P., Geisinger, M.L., Mills, M.P., Nares, S., and Nevins, M.L. Periodontal regeneration—intrabony defects: a consensus report from the AAP Regeneration Workshop. *J Periodontol* **86**, S105, 2015.
22. Bartold, P.M., Gronthos, S., Ivanovski, S., Fisher, A., and Huttmacher, D.W. Tissue engineered periodontal products. *J Periodontol Res* **51**, 1, 2016.
23. McCulloch, C.A., and Bordin, S. Role of fibroblast subpopulations in periodontal physiology and pathology. *J Periodontol Res* **26**, 144, 1991.
24. Melcher, A.H. On the repair potential of periodontal tissues. *J Periodontol* **47**, 256, 1976.
25. Bright, R., Hynes, K., Gronthos, S., and Bartold, P.M. Periodontal ligament-derived cells for periodontal regeneration in animal models: a systematic review. *J Periodontol Res* **50**, 160, 2015.
26. Chen, F.M., Gao, L.N., Tian, B.M., Zhang, X.Y., Zhang, Y.J., Dong, G.Y., Lu, H., Chu, Q., Xu, J., Yu, Y., Wu, R.X., Yin, Y., Shi, S., and Jin, Y. Treatment of periodontal intrabony defects using autologous periodontal ligament stem cells: a randomized clinical trial. *Stem Cell Res Ther* **7**, 33, 2016.
27. Zheng, W., Wang, S., Ma, D., Tang, L., Duan, Y., and Jin, Y. Loss of proliferation and differentiation capacity of aged human periodontal ligament stem cells and rejuvenation by exposure to the young extrinsic environment. *Tissue Eng Part A* **15**, 2363, 2009.
28. Wang, L., Zhao, Y., Liu, Y., Akiyama, K., Chen, C., Qu, C., Jin, Y., and Shi, S. IFN-gamma and TNF-alpha synergistically induce mesenchymal stem cell impairment and tumorigenesis via NFkappaB signaling. *Stem Cells* **31**, 1383, 2013.
29. Iwasaki, K., Komaki, M., Yokoyama, N., Tanaka, Y., Taki, A., Kimura, Y., Takeda, M., Oda, S., Izumi, Y., and Morita, I. Periodontal ligament stem cells possess the characteristics of pericytes. *J Periodontol* **84**, 1425, 2013.
30. Iwasaki, K., Komaki, M., Yokoyama, N., Tanaka, Y., Taki, A., Honda, I., Kimura, Y., Takeda, M., Akazawa, K., Oda, S., Izumi, Y., and Morita, I. Periodontal regeneration using periodontal ligament stem cell-transferred amnion. *Tissue Eng Part A* **20**, 693, 2014.
31. King, G.N., King, N., Cruchley, A.T., Wozney, J.M., and Hughes, F.J. Recombinant human bone morphogenetic protein-2 promotes wound healing in rat periodontal fenestration defects. *J Dent Res* **76**, 1460, 1997.
32. Padial-Molina, M., Rodriguez, J.C., Volk, S.L., and Rios, H.F. Standardized in vivo model for studying novel regenerative approaches for multitissue bone-ligament interfaces. *Nat Protoc* **10**, 1038, 2015.
33. Inukai, T., Katagiri, W., Yoshimi, R., Osugi, M., Kawai, T., Hibi, H., and Ueda, M. Novel application of stem cell-derived factors for periodontal regeneration. *Biochem Biophys Res Commun* **430**, 763, 2013.
34. Kawai, T., Katagiri, W., Osugi, M., Sugimura, Y., Hibi, H., and Ueda, M. Secretomes from bone marrow-derived

- mesenchymal stromal cells enhance periodontal tissue regeneration. *Cytotherapy* **17**, 369, 2015.
35. Gay, I.C., Chen S. Fau, and MacDougall, M. Isolation and characterization of multipotent human periodontal ligament stem cells. *Orthod Craniofac Res* **10**, 149–160, 2007.
36. Wada, N., Menicanin, D., Shi, S., Bartold, P.M., and Gronthos, S. Immunomodulatory properties of human periodontal ligament stem cells. *J Cell Physiol* **219**, 667, 2009.
37. Menicanin, D., Mrozik, K.M., Wada, N., Marino, V., Shi, S., Bartold, P.M., and Gronthos, S. Periodontal-ligament-derived stem cells exhibit the capacity for long-term survival, self-renewal, and regeneration of multiple tissue types in vivo. *Stem Cells Dev* **23**, 1001, 2014.
38. Tsumanuma, Y., Iwata, T., Washio, K., Yoshida, T., Yamada, A., Takagi, R., Ohno, T., Lin, K., Yamato, M., Ishikawa, I., Okano, T., and Izumi, Y. Comparison of different tissue-derived stem cell sheets for periodontal regeneration in a canine 1-wall defect model. *Biomaterials* **32**, 5819, 2011.
39. Mrozik, K.M., Zilm, P.S., Bagley, C.J., Hack, S., Hoffmann, P., Gronthos, S., and Bartold, P.M. Proteomic characterization of mesenchymal stem cell-like populations derived from ovine periodontal ligament, dental pulp, and bone marrow: analysis of differentially expressed proteins. *Stem Cells Dev* **19**, 1485, 2010.
40. Chen, H., Min, X.H., Wang, Q.Y., Leung, F.W., Shi, L., Zhou, Y., Yu, T., Wang, C.M., An, G., Sha, W.H., and Chen, Q.K. Pre-activation of mesenchymal stem cells with TNF- α , IL-1 β and nitric oxide enhances its paracrine effects on radiation-induced intestinal injury. *Sci Rep* **5**, 8718, 2015.
41. van Poll, D., Parekkadan, B., Cho, C.H., Berthiaume, F., Nahmias, Y., Tilles, A.W., and Yarmush, M.L. Mesenchymal stem cell-derived molecules directly modulate hepatocellular death and regeneration in vitro and in vivo. *Hepatology* **47**, 1634, 2008.
42. Liu, D., Wang, Y., Jia, Z., Wang, L., Wang, J., Yang, D., Song, J., Wang, S., and Fan, Z. Demethylation of IGFBP5 by histone demethylase KDM6B promotes mesenchymal stem cell-mediated periodontal tissue regeneration by enhancing osteogenic differentiation and anti-inflammation potentials. *Stem Cells* **33**, 2523, 2015.
43. Katagiri, W., Osugi, M., Kawai, T., and Hibi, H. First-in-human study and clinical case reports of the alveolar bone regeneration with the secretome from human mesenchymal stem cells. *Head Face Med* **12**, 5, 2016.

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