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Novel application of stem cell-derived factors for periodontal regeneration

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ABSTRACT

The effect of conditioned medium from cultured mesenchymal stem cells (MSC-CM) on periodontal regeneration was evaluated. *In vitro*, MSC-CM stimulated migration and proliferation of dog MSCs (dMSCs) and dog periodontal ligament cells (dPDLCs). Cytokines such as insulin-like growth factor, vascular endothelial growth factor, transforming growth factor-β1, and hepatocyte growth factor were detected in MSC-CM. *In vivo*, one-wall critical-size, intrabony periodontal defects were surgically created in the mandible of dogs. Dogs with these defects were divided into three groups that received MSC-CM, PBS, or no implants. Absorbable atelo-collagen sponges (TERUPLUG®) were used as a scaffold material. Based on radiographic and histological observation 4 weeks after transplantation, the defect sites in the MSC-CM group displayed significantly greater alveolar bone and cementum regeneration than the other groups. These findings suggest that MSC-CM enhanced periodontal regeneration due to multiple cytokines contained in MSC-CM.

1. Introduction

Periodontitis is an infection or inflammation that causes destruction of the periodontal tissues, including gingiva, root cementum, alveolar bone, and the periodontal ligament (PDL). A goal of periodontal regenerative therapy is to return the tissues to their original condition and restore the form and function of the lost structures [1].

Many experimental and clinical studies about periodontal tissue engineering and regenerative medicine have been published. Historically, various regenerative methods and materials, including guided tissue regeneration [2,3], enamel matrix protein derivative (Emdogain®) [4], and osteoinductive agents and biomaterials, have been used in clinical practice for periodontal regeneration [5,6]. Cell therapy is expected to become the next-generation method. The concepts of tissue engineering and regenerative medicine involve the regeneration of tissues using a combination of cells, scaffolds, and signaling molecules [7]. Mesenchymal stem cells (MSCs) are well known to secrete a variety of growth factors and cytokines [8]. Recent studies have indicated that the paracrine effects of the growth factors and cytokines secreted from implanted MSCs may promote tissue regeneration *in vivo* [9,10]. Conditioned medium from cultured mesenchymal stem cells (MSC-CM) has been reported to have multiple positive functions in tissue regeneration [10,11]. We previously reported that bone marrow-derived MSC-CM has a very high potential for bone regeneration that is mediated by the cooperative effects of cytokines such as insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), and transforming growth factor-β1 (TGF-β1), which regulate several events of osteogenesis, including angiogenesis, cell migration, proliferation, and osteoblast differentiation [12]. Based on these findings, we hypothesized that transplantation of MSC-CM may play an important role in periodontal tissue regeneration and overcome limitations of existing therapies. The purpose of this study was to evaluate the effect of MSC-CM on periodontal regeneration.

2. Materials and methods

2.1. Cell isolation, cultivation, and preparation of MSC-CM

Dog MSCs (dMSCs) and dog periodontal ligament cells (dPDLCs) were isolated from five hybrid dogs (age 18–36 months, weight 15–25 kg) and expanded in accordance with published techniques

Abbreviations: MSC, mesenchymal stem cells; dPDLCs, dog periodontal ligament cells; IGF-1, insulin-like growth factor-1; VEGF, vascular endothelial growth factor; TGF-β1, transforming growth factor-β1; HGF, hepatocyte growth factor; FGF-2, fibroblast growth factor-2; PDGF-BB, platelet-derived growth factor-BB; BMP-2, bone morphogenetic protein-2; SDF-1, stromal cell-derived factor-1; PDL, periodontal ligament; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay.

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[13,14]. Human MSCs (hMSCs) were purchased from Lonza and cultured according to the manufacturer's instructions. All cells in this experiment were cultured at 37 °C with 5% CO_2 and 95% air in a humidified incubator. dMSCs (2nd-4th passage), dPDLCs (2nd-3th passage), and hMSCs (3rd-9th passage) were used for the experiments.

At approximately 70% confluency, the conditioned medium of hMSCs was refreshed with serum-free Dulbecco's Modified Eagle Medium (DMEM) and cultured for an additional 48 h. MSC-CM was collected and stored at 4 °C or −80 °C before use in the following experiments.

2.2. Migration and proliferation of dMSCs and dPDLCs

Transwell dishes with 8.0-μm pore filters (BD BioCoat™ Control Inserts; Becton Dickinson and Co., Franklin Lakes, NJ) were used for the migration assays. dMSCs (5×10^5 cells/cm^2) or dPDLCs (5×10^5 cells/cm^2) were seeded into the upper chamber, and MSC-CM was added to the lower chamber. Cell migration was observed in the presence of 30% FBS or serum-free DMEM, which served as positive and negative controls, respectively. After 48 h of culture, the upper side of the filters was carefully rinsed with PBS, and the remaining cells on the upper surface of the filters were mechanically removed with a cotton-wool swab. Transwell filters were stained with hematoxylin, cut with a scalpel, and mounted onto glass slides, with the lower surface facing upward. The proliferation rate of approximately 70% confluent dMSCs and dPDLCs was assessed with bromodeoxyuridine (BrdU) incorporation for 24 h, using a Zymed BrdU staining kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Both the number of migrated dMSCs and dPDLCs and the percentages of BrdU-positive cells were counted in five randomly selected fields using a light microscope (CK40; Olympus, Tokyo, Japan) at 200× magnification.

2.3. Enzyme-linked immunosorbent assay (ELISA)

To evaluate the cytokines in MSC-CM, ELISA for IGF-1, VEGF, fibroblast growth factor-2 (FGF-2), TGF-β1, hepatocyte growth factor (HGF), platelet-derived growth factor-BB (PDGF-BB), bone morphogenetic protein-2 (BMP-2), and stromal cell-derived factor-1 (SDF-1) in MSC-CM were performed. The concentrations of these factors were measured using a Human Quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.4. Dog one-wall intrabony defect model

All animal experiments were approved by the Nagoya University animal experiment committee. After a period of acclimatization of 30 days, five hybrid dogs were operated on under general anesthesia by intravenous injection of pentobarbital (Somnopentyl®; Kyoritsu Seiyaku, Tokyo, Japan) (20 mg/kg body weight), and under local anesthesia with 2% lidocaine (with 1:80,000 epinephrine, ORA® Inj. Dental Cartridge; Showa Yakuhin Kako, Tokyo, Japan). Before the experimental surgery, the mandibular first and third or fourth premolars were extracted, and the extraction sites were allowed to heal for 8 weeks. For the experimental surgery, buccal and lingual mucoperiosteal flaps were elevated, and critical-size, box-type, one-wall intrabony defects (width, 4 mm; height, 5 mm) were created at the distal aspect of the second, and the mesial aspect of the fourth premolars in the right and left jaw quadrants [15]. Following root planing to remove the root cementum, a reference notch indicating a 5-mm distance from the cement-enamel junction to the bottom of the defect was made with a burr into the root surface at the base of the defects. With no differences in bone regeneration in the various grafted areas in terms of bone healing, two defects were created and implanted with two materials at random sites. An absorbable atelo-collagen sponge (TERUPLUG®; OLYMPUS TERUMO BIOMATERIALS, Tokyo, Japan) was used as a scaffold and contained 300 μl MSC-CM or PBS. The dogs with defects were randomly divided into three groups ($n = 6$ each) and implanted with graft materials: MSC-CM plus scaffold, PBS plus scaffold, or no implant/scaffold. The mucogingival flaps were advanced, adapted, and completely closed. Post-surgical management involved antibiotics (Azithromycin, 250 mg; Pfizer, Tokyo, Japan) daily for 3 days, a soft diet, and topical application of 2% chlorhexidine (Hibitane concentrate; Dainippon Sumitomo Pharma, Osaka, Japan) twice a week. After 4 weeks, the dogs were given general anesthesia and sacrificed by exsanguination after injection of heparin sodium (400 U/kg).

2.5. Radiographic and histological analyses

Standardized radiographic images of the defect sites were obtained with an X-ray apparatus (Dent navi Hands; Yoshida Co., Ltd., Tokyo, Japan) and dental X-ray films (BW-100; Hanshin Technical Laboratory, Nishinomiya, Japan) immediately, and 4 weeks after, transplantation. Dental X-ray films were placed parallel to the tooth axis, and radiographic images of the defect site were taken in the buccolingual direction. The defect sites were dissected and fixed in 10% neutral-buffered formalin (Wako, Japan) 4 weeks after transplantation. The specimens were decalcified in Plank-Rychro solution (Wako) for 8 weeks, routinely processed into 5-μm-thick paraffin-embedded sections, stained with hematoxylin and eosin, and observed under a light microscope (Olympus). Histometric parameters were quantified using a computer-based image analysis system (ImageJ 1.44; National Institutes of Health). The following parameters were analyzed:

(1) Cementum regeneration height: distance from the root surface notch to the coronal extension of newly formed cementum on the root surface.
(2) Bone regeneration height: distance from the root surface notch to the coronal extension of newly formed bone along the root surface.
(3) Bone regeneration area: area of new alveolar bone formed coronally from the apical extension of the root surface notch.

2.6. Statistical analysis

Summary statistics (mean ± SD) based on animal means for the experimental treatments were calculated using the three central sections from each defect. Statistical differences were evaluated with Tukey's HSD (Honestly Significant Difference) test (IBM SPSS statistics 19). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of MSC-CM on dMSC migration and proliferation

The numbers of migrated dMSCs in DMEM (−), 30% FBS, and MSC-CM were 4.47 ± 3.10, 54.1 ± 13.03, and 43.87 ± 13.03, respectively (Fig. 1A, left). The numbers of migrated dPDLCs in DMEM (−), 30% FBS, and MSC-CM were 4.38 ± 1.19, 40.75 ± 5.70, and 33.0 ± 6.0, respectively (Fig. 1A, right). The percentages of BrdU-positive dMSCs cultured in DMEM (−), 30% FBS, and MSC-CM were 39.41 ± 6.76%, 69.74 ± 4.97%, and 55.31 ± 8.39%, respectively (Fig. 1B, left). The percentages of BrdU-positive dPDLCs cultured in DMEM (−), 30% FBS, and MSC-CM were 30.23 ± 7.99%, 65.49 ± 6.58%, and 61.1 ± 4.69%, respectively (Fig. 1B, right). These

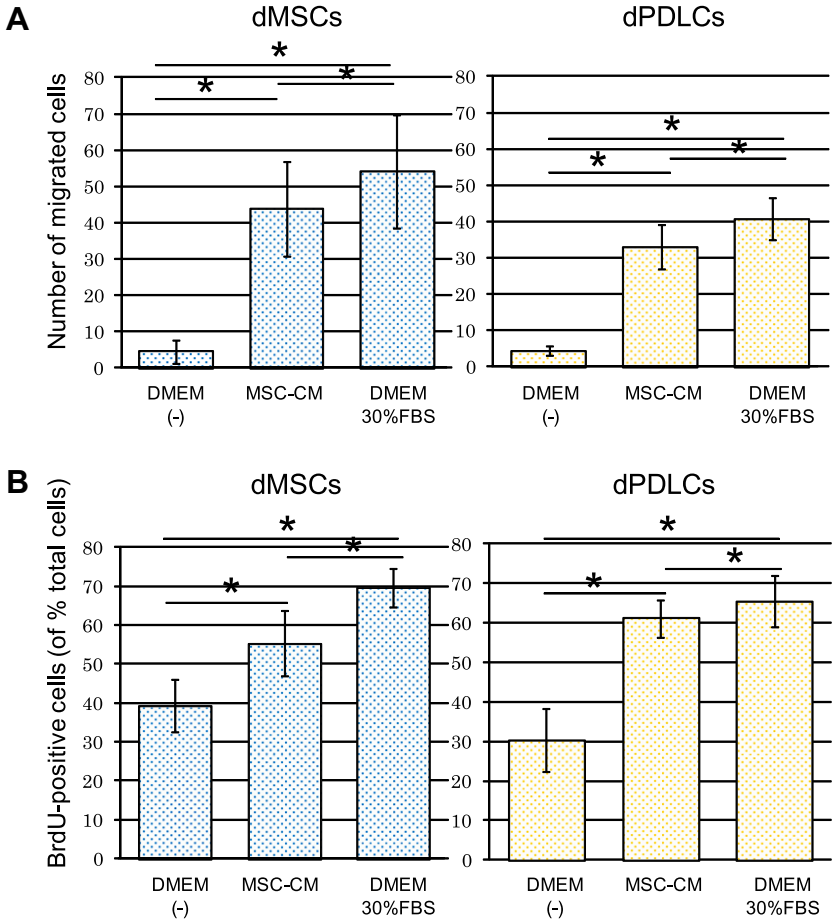

Fig. 1. (A) Transwell migration assay. The migration of dMSCs and dPDLCs cultured in MSC-CM were enhanced compared to that of dMSCs cultured in DMEM (−). (B) BrdU assay. The proliferation of dMSCs and dPDLCs cultured in MSC-CM was also enhanced compared to culturing in DMEM (−). Cells cultured in DMEM + 30%FBS were used as a positive control for both (A) and (B). Asterisks indicate a significant difference between the indicated groups ($p < 0.05$).

differences were statistically significant ($p < 0.05$), indicating that MSC-CM enhanced migration and proliferation of dMSCs and dPDLCs similar to serum-containing medium, compared with serum-free medium.

3.2. Growth factors present in MSC-CM

The concentrations of IGF-1, VEGF, TGF-β1, HGF, FGF-2, PDGF-BB, BMP-2, and SDF-1 in MSC-CM were analyzed using ELISA. These factors were not detected in DMEM (−) or 30% FBS. However, in MSC-CM, the concentrations of IGF-1, VEGF, TGF-β1, and HGF were 1515.6 ± 211.8 pg/ml, 465.8 ± 108.8 pg/ml, 339.8 ± 14.4 pg/ml, and 20.3 ± 7.9 pg/ml, respectively. Other factors were not detected in MSC-CM.

3.3. Radiographic and histological analyses

Clinical healing was generally uneventful. Representative photomicrographs of the experimental sites are shown in Fig. 2. The results from the histometric analysis are shown in Fig. 3. The cementum regeneration height, the bone regeneration height, and the bone regeneration area of the MSC-CM group were 3.01 ± 0.16 mm, 3.19 ± 0.51 mm, and 4.89 ± 1.08 mm^2, respectively. A large amount of new lamellar and woven bone formation was observed in the MSC-CM group. Thick-layered and cellular cementum on the root surface was also frequently observed in the MSC-CM group.

On the other hand, less newly regenerated bone and cementum compared to the MSC-CM group was observed in the PBS group. Dense collagen fibers were observed frequently in the PBS group. Newly regenerated bone and cementum were not apparent in the no implant group. Furthermore, there was minimal inflammatory cell infiltration in the MSC-CM group compared to the other groups.

4. Discussion

To regenerate periodontal tissue destroyed by periodontitis, several events are required, including angiogenesis, cell migration, proliferation, and differentiation into osteoblasts and cementblasts on the dental root and alveolar bone surfaces facing the region of the periodontal tissue defect, followed by regeneration of the alveolar bone and cementum. Historically, periodontal therapies have been developed, including conventional periodontal therapies such as gingival flap surgery that cleans root surfaces and improves periodontal form. Therefore, the disease process is arrested, and periodontal tissues are repaired [3]. Guided tissue regeneration is a surgical procedure that utilizes a barrier membrane that is placed under the gingiva and over the remaining bone to prevent epithelial down-growth and fibroblast trans-growth into the wound space, thereby maintaining a space for true periodontal tissue regeneration [2,3]. Autogenous bone grafting is considered to be an effective method [16], but it requires a wider surgical area. Moreover, tooth-debilitating occurrence of root resorption and ankylosis after grafting of autogenous bone have also been re-

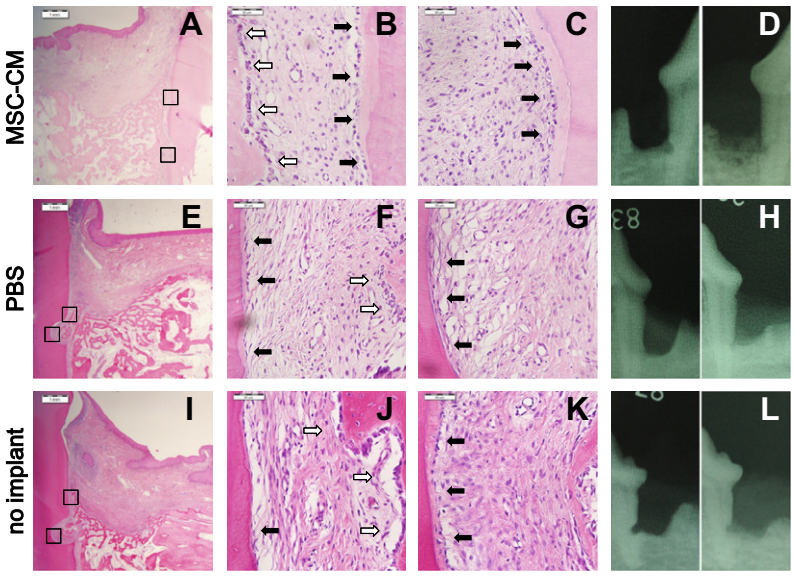

Fig. 2. Representative photomicrographs and radiographic images from sites receiving experimental materials. (A, E and I) overview of periodontal defect site. Original magnification × 12.5, scale bar = 1 mm. (B, C, F, G, J and K) Higher magnification of the boxed areas. Original magnification × 100, scale bar = 50 μm. (D, H and L) Radiographic images were taken at 0 (left) and 4 weeks (right) after surgery. White arrows: osteoblasts. Black arrows: newly regenerated cellular cementum. Photomicrographs: hematoxylin and eosin staining.

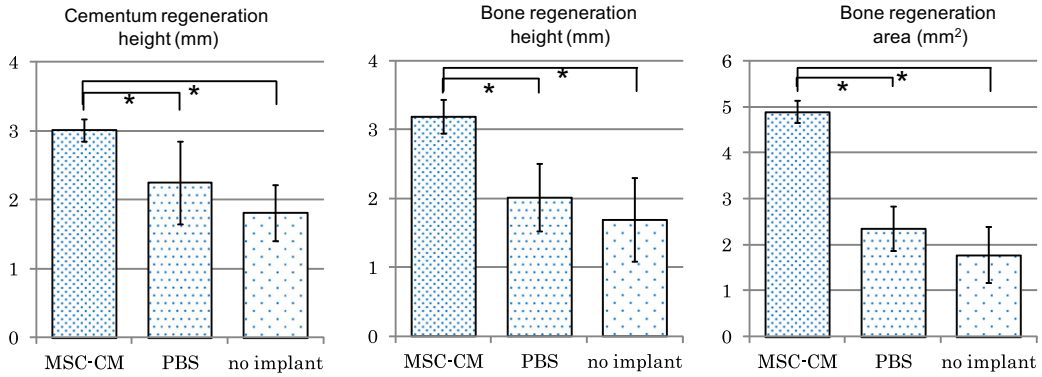

Fig. 3. Histometric analysis of periodontal regeneration following surgical implantation of MSC-CM/TERUPLUG® in dog one-wall intrabony defects (means ± SD in mm or mm²). *Statistical difference ($p < 0.05$), $n = 5$.

ported [17]. Although allografts, xenografts, and osteoinductive biomaterials (beta-tricalcium phosphate, hydroxyapatite) are available, these materials also have poor osteoinductivity and provide a risk of infection and immunological rejection [5,6]. On the other hand, enamel matrix protein derivative (Emdogain®) has shown favorable clinical results for reducing intrabony periodontal defects [4].

Cytokines enhance periodontal regeneration by stimulating the proliferation of mesenchymal cells in the periodontal tissue and differentiation into osteoblasts/cementblasts [18]. PDGF-BB and BMP-2 are already on the market [19]. The efficacy of local application of recombinant human FGF-2 [20] has been investigated in a clinical study. The significant difference in rate of increase in alveolar bone height suggests that FGF-2 therapy can be efficacious in regenerating periodontal tissue. Application of TGF-β [21], BMP-7 [22], brain-derived neurotrophic factor [23], and growth and differentiation factor-5 [24–26] has been reported *in vitro* and *in vivo*. These factors enhance cellular activation of osteogenesis [27]. However, application of a single growth factor has limited bone regeneration ability, and outcomes are not always predictable. In addition, application of these growth factors unfortunately requires superphysiological doses [28] and may induce a severe inflammatory response [29]. Therefore, a combination of several different factors will likely be better for optimizing bone regeneration [30–33].

Recently, many experimental and clinical studies of cell therapies for periodontal regeneration have been conducted. Grafting human cultured periosteum sheets for periodontal defects was reported [34]. We previously used an injectable tissue-engineered bone that is a mixture of hMSCs and platelet-rich plasma as bone graft materials [35]. However, the field is hampered by problems such as the need for large capital investment, expensive cell culture, complicated safety and quality management, and the oncogenic risk of grafted cells.

Implanted cells do not survive for a long time [36–40]. On the other hand, implanted MSCs secrete a variety of cytokines [8], and several cytokines are present in MSC-CM [41]. These findings suggest that the paracrine effects of multiple cytokines secreted from implanted MSCs may promote tissue regeneration [9–11]. We previously reported that MSC-CM has a very high potential for bone regeneration that is mediated by the cooperative effects of cytokines such as IGF-1, VEGF, TGF-β1, and HGF. These cytokines

regulate several events of osteogenesis, including angiogenesis, cell migration, proliferation, and osteoblast differentiation [12]. IGF-1 induces osteoblast proliferation and migration [42,43] and enhances periodontal regeneration by stimulating PDL cells through the PI3K pathway [44]. VEGF is thought to be the main regulator of angiogenesis. VEGF also enhances survival and differentiation of endothelial cells, and as a result, it contributes to osteogenesis [45]. TGF-β1 increases bone formation by recruiting osteoprogenitor cells and stimulating their proliferation and differentiation into osteocytes [46]. TGF-β1 also stimulates PDL regeneration and repair [47] and is expressed during the development of the alveolar bone, PDL, and cementum [48]. HGF is a potent angiogenic molecule, and this activity is mediated primarily through direct actions on vascular endothelial cells [49]. Based on these findings, we predicted that cooperative effects between these cytokines and other unknown factors towards angiogenesis and osteogenesis would mediate periodontal regeneration induced by MSC-CM after endogenous cell mobilization.

TERUPLUG®, which we use as a scaffold, is an absorbable atelocollagen sponge that is highly biocompatible [50]. It is easy to infiltrate MSC-CM into TERUPLUG and adapt it to the bone defect shape. The product is in a sponge block configuration. Also, it serves as a scaffold for osteoblast migration.

Compared with a previous study also using the one-wall intrabony periodontal defect model [23–25], the histological results in the present study at 4 weeks after treatment were equivalent to the results from the other study at 8 weeks after treatment. These results support the hypothesis that MSC-CM enhances periodontal regeneration.

This novel regenerative technique based on a unique concept that utilizes endogenous stem cells without cell transplantation may represent an alternative solution for periodontal regeneration, overcoming the limitations of existing therapies.

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