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Novel application of stem cell-derived factors for periodontal regeneration

Takeharu Inukai, Wataru Katagiri *, Ryoko Yoshimi, Masashi Osugi, Takamasa Kawai, Hideharu Hibi, Minoru Ueda

Department of Oral and Maxillofacial Surgery, Nagoya University Graduate School of Medicine, Japan

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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.

* Corresponding author. Address: Department of Oral and Maxillofacial Surgery, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan. Fax: +81 52 744 2352.

E-mail addresses: t-inukai@med.nagoya-u.ac.jp (T. Inukai), w-kat@med.nagoya-u.ac.jp (W. Katagiri), lianzi@med.nagoya-u.ac.jp (R. Yoshimi), masashi@med.nagoya-u.ac.jp (M. Osugi), takamasa@med.nagoya-u.ac.jp (T. Kawai), hibihi@med.nagoya-u.ac.jp (H. Hibi), mueda@med.nagoya-u.ac.jp (M. Ueda).

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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