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Trophic factors from adipose tissue-derived multi-lineage progenitor cells promote cytodifferentiation of periodontal ligament cells

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ABSTRACT

Stem and progenitor cells are currently being investigated for their applicability in cell-based therapy for periodontal tissue regeneration. We recently demonstrated that the transplantation of adipose tissue-derived multi-lineage progenitor cells (ADMPCs) enhances periodontal tissue regeneration in beagle dogs. However, the molecular mechanisms by which transplanted ADMPCs induce periodontal tissue regeneration remain to be elucidated. In this study, trophic factors released by ADMPCs were examined for their paracrine effects on human periodontal ligament cell (HPDL) function. ADMPC conditioned medium (ADMPC-CM) up-regulated osteoblastic gene expression, alkaline phosphatase activity and calcified nodule formation in HPDLs, but did not significantly affect their proliferative response. ADMPCs secreted a number of growth factors, including insulin-like growth factor binding protein 6 (IGFBP6), hepatocyte growth factor and vascular endothelial growth factor. Among these, IGFBP6 was most highly expressed. Interestingly, the positive effects of ADMPC-CM on HPDL differentiation were significantly suppressed by transfecting ADMPCs with IGFBP6 siRNA. Our results suggest that ADMPCs transplanted into a defect in periodontal tissue release trophic factors that can stimulate the differentiation of HPDLs to mineralized tissue-forming cells, such as osteoblasts and cementoblasts. IGFBP6 may play crucial roles in ADMPC-induced periodontal regeneration.

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1. Introduction

Periodontitis is a chronic inflammatory disease caused by a bacterial biofilm on the root surface. Progression of this disease leads to the destruction of periodontal tissues which support the teeth. Mechanical removal of the dental plaque allows for the control of inflammatory responses; however, this alone is not enough to induce regeneration of the damaged periodontal tissue.

Autogenous bone grafting, guided tissue regeneration (GTR) and local administration of an enamel matrix derivative (EMD), are some of the moderately effective approaches that have been used in general dental practice for the purpose of regeneration of the periodontal tissue. Furthermore, some recent studies have clinically assessed the effect of locally administered human recombinant growth factors, such as platelet-derived growth factor (PDGF) [1] and basic fibroblast growth factor (FGF-2) [2] on periodontal tissue regeneration.

Treatment strategies, such as GTR, EMD, or the local administration of growth factors, regenerate the periodontal tissue by activating the self-healing abilities of the endogenous stem cells within the periodontal tissues. The number of stem cells in the body is reported to decrease with age, likewise, the endogenous stem cell population in the periodontal tissues is believed to have decreased proliferative and differentiation ability [3]. Moreover, tissue destruction caused by severe periodontal disease results in the reduced number of endogenous stem cells. Therefore, there may be a need for “cell therapy”, wherein stem cells collected from other tissues are transplanted to the defective periodontal tissue.

Periodontal tissue regeneration therapies using bone marrow-derived mesenchymal stem cells (BMSCs) [4], periodontal

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ligament cells (PDLs) [5] and alveolar bone periosteal cells [6], have been discovered to have a regenerative effect. However, these therapies have certain limitations; PDLs and alveolar bone periosteal cells are limited in terms of the harvestable amount of tissue, while BMSCs involve an invasive harvesting procedure, and are known to show low amplification efficiency [7–9]. In contrast, mesenchymal stem cells (MSCs) from adipose tissue can be harvested less invasively and with greater ease compared to other tissues listed above. In addition, these cells have a higher self-renewal capacity and proliferative ability [8–10], and produce an abundance of humoral factors [11,12]. By transplanting adipose tissue-derived multi-lineage progenitor cells (ADMPCs) into canine experimental periodontal disease models, we have demonstrated that ADMPC induces significant periodontal tissue regeneration [13].

The cell-autonomous mechanism has been supported by many reports which indicate that MSCs directly differentiate into various cell types constituting the target tissues. In addition, recent studies have indicated that the transplanted MSCs have an important trophic effect, in which various growth factors and cytokines activate tissue regeneration at the transplant site [14,15]. These findings propose the importance of a non-cell-autonomous mechanism in addition to the cell-autonomous effects.

In this study, we investigated the effects of endogenous factors secreted by ADMPCs on the functionality of human PDLs (HPDLs), which play a central role in the induction of periodontal tissue regeneration, in order to clarify the mechanism of ADMPC-induced periodontal tissue regeneration.

2. Materials and methods

2.1. Cell culture

All human subjects provided informed consent according to a protocol that was reviewed and approved by the Institutional Review Board of the Osaka University Graduate School of Dentistry. ADMPCs were isolated from the subcutaneous adipose tissues collected from healthy volunteers. ADMPCs were prepared as described previously [16,17]. ADMPCs were seeded onto a dish coated with human fibronectin (BD Biosciences, Franklin Lakes, NJ, USA) in expansion medium (Exp-Med), which was composed of 60% Dulbecco's modified Eagle's medium (DMEM)-low glucose (Gibco Life Technologies, Carlsbad, CA, USA), 40% MCDB-201 medium (Sigma–Aldrich, St. Louis, MO, USA), 1 nM dexamethasone (Sigma–Aldrich), 100 μM L-ascorbic acid (Sigma–Aldrich), 10 mg/L insulin-transferrin-selenium solution (Gibco), 10 ng/mL epidermal growth factor (PeproTech, Rocky Hill, NJ, USA), 60 μg/mL kanamycin (Wako Pure Chemical Industries) and 5% fetal bovine serum (FBS; Sigma–Aldrich). HPDLs were commercially purchased (Lonza Biosciences, Basel, Switzerland) and were cultured in α-Modified Eagle Minimum Essential Medium (α-MEM, Sigma–Aldrich) supplemented with 60 μg/mL kanamycin and 10% FBS. ADMPCs and HPDLs were analyzed *in vitro* at passages 4–8.

2.2. Preparation of ADMPC conditioned medium (ADMPC-CM)

A million of ADMPCs were seeded on to a 100 mm human fibronectin-coated dish, and grown to sub-confluence in Exp-Med. The culture medium was subsequently changed to fresh DMEM-high glucose (DMEM-HG) supplemented with 60 μg/mL kanamycin and 10% FBS. After 3-day culture, the supernatant was collected as ADMPC-conditioned medium (ADMPC-CM). In experiments requiring the addition of ADMPC-CM, an equal amount of 10% FBS and 60 μg/mL kanamycin-containing DMEM-HG was added and used as the control.

2.3. Osteogenic differentiation

Osteogenic differentiation was induced by culturing HPDLs in 12-well plates to confluence and subsequently exposing these cells to mineralization medium, consisting of α-MEM supplemented with 10% FBS, 10 mM β-glycerophosphate, 5 μM ascorbic acid, and 60 μg/mL kanamycin with ADMPC-CM or DMEM-HG which was replaced every 3 days.

2.4. Cell count assay

HPDL proliferation was determined by calculating the total number of cells in a hemocytometer (Minato-Medical, Tokyo, Japan). HPDLs were seeded into a 6-well plate containing α-MEM supplemented with 60 μg/mL kanamycin and 10% FBS at a density of 2.5×10^4 cells/well. The culture medium was then changed to α−MEM mixed at a 1:1 ratio with ADMPC-CM or DMEM-HG 6 h after seeding. Cells were counted every day up to day 4 after seeding.

2.5. Quantitative polymerase chain reaction (PCR)

Total RNA was isolated from cells using RNA-Bee™ (TEL-TEST, Friendwood, TX, USA), according to the manufacturer protocols. Total RNA was subjected to reverse transcription, using Random Hexamer Primers (Amersham Biosciences, Milwaukee, WI, USA) and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA).

The synthesized cDNA was used as the template for real-time PCR; the reaction mixture was composed of the template cDNA, the gene-specific primers and the components of the Fast SYBR® Green Master Mix (Applied Biosystems, Waltham, MA, USA). *Alkaline phosphatase* (*ALPase*) (sense 5′-GGACCATTCCCACGTCTTCAC-3′; antisense 5′-CCTTGTAGCCAGGCCCATTG-3′), *Bone sialo protein* (*BSP*) (sense 5′-CTGGCACAGGGTATACAGGGTTAG-3′; antisense 5′-GCCTCTGTGCTGTTGGTACTGGT-3′), *Collagen type I alpha 1* (*Col1a1*) (sense 5′-CCCGGGTTTCAGAGACAACTTC-3′; antisense 5′-TCCACATGCTTTATTCCAGCAATC-3′), *IGFBP6* (sense 5′-CAGAGGAGAATCCTAAGGAGA-3′; antisense 5′-TGAGTCCAGATGTCTACGGCATGGGCC -3′), *Runt-related transcription factor 2* (*RUNX2*) (sense 5′-CACTGGCGCTGCAACAAGA-3′; antisense 5′-CATTCCGGAGCTCAGCAGAATAA -3′) and *hypoxanthine phosphoribosyltransferase* (*HPRT*) (sense 5′-GGCAGTATAATCCAAAGATGGTCAA-3′; antisense 5′-GTCAAGGGCATATCCTACAACAAAC-3′) gene specific primers were purchased from Takara-bio (Shiga, JPN).

Amplification was performed in the Step One Plus Real-time PCR System (Applied Biosystems) according to the manufacturer protocol. The relative expression was determined after normalization against *HPRT* expression.

2.6. Determination of ALPase activity

ALPase activity was measured as described previously [18]. ALPase from bovine intestinal mucosa was used as the standard; one unit (U) is defined as the amount of the enzyme that catalyzes the hydrolysis of 1 mM p-nitrophenyl phosphate per min at pH 9.8 and 37 °C. DNA concentration was determined using the modified Labarca and Paigen method [19], described in a previous study [20]; the results of this analysis were expressed as U/μg DNA.

2.7. Mineralization assay

Calcified nodules were stained with alizarin red using a method described by Dahl [21]. The density of the calcified nodules in each well was calculated using the WinRoof software program (Mitani Corporation, Fukui, Japan).

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