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Original article

Interleukin-4 released from human gingival fibroblasts reduces osteoclastogenesis

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ABSTRACT

Objective: Human gingival epithelium is continuously exposed to bacteria and acts as the first line of defense in periodontal tissues. It is crucial to maintain healthy, non-inflamed gingival tissue to avoid gingivitis and periodontitis. The purpose of this study was to investigate the influence of IL-4 in human gingival fibroblasts (hGF) on the activation of osteoclasts.
Design: Two hGF samples were obtained from two healthy patients, and one was collected from a commercially available resource. The hGFs were cultured, and conditioned medium of hGF (hGF-CM) was stocked at −80 °C. The mRNA was isolated from the hGF cultures and analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) for expression of suppressive osteoclastogenetic mediators, such as interleukin (IL)-4, osteoprotegerin (OPG), IL-10, IL-27, and IL-33. The hGF-CM was used to investigate the inhibitory function of mouse macrophages supplemented with either glutathione S-transferase-Receptor activator of NF-kB ligand (GST-RANKL), human recombinant (rh)IL-4, or rhOPG but not a combination. Differentiation of osteoclasts was examined by tartrate resistant acid phosphatase (TRAP) staining and TRAP assay. The suppressive role of IL-4 was assessed by neutralizing IL-4 antibody in the TRAP assay.
Results: The hGF-CM reduced both TRAP positive staining and activity in a dose-dependent manner. IL-4 and OPG mRNA expressions were expressed in hGF-CM from three different donors but that of IL-10, IL-27, or IL-33 was not detected. In the RAW264 culture, rhIL-4 and rhOPG reduced TRAP positive staining as well as activity in a dose-dependent manner. Moreover, addition of neutralizing antibodies for IL-4 reduced the inhibitory effect of conditioned medium from gingival fibroblasts in the RAW264 culture.
Conclusion: We concluded that hGF potentially contained suppressive mediators, such as IL-4 and OPG, for osteoclastogenesis. Moreover, we confirmed that the differential inhibition of osteoclast is caused by OPG as well as IL-4 in hGF-CM.

1. Introduction

Periodontal disease is a chronic, infectious, and inflammatory disease of the periodontium (gingiva, periodontal ligament, alveolar bone, and cement) that causes resorption of the alveolar bone, ultimately leading to tooth loss (Page RC, 1976). The oral bacteria attach to the gingival cuff, causing inflammation and formation of periodontal pockets between the teeth and gingiva. Periodontal pathogens colonize in these pockets and further aggravate inflammation. The gingiva is continuously exposed to bacteria and acts as the first line of defense in periodontal tissues (Seymour, 1979). Progression of periodontal disease is influenced by the relationship between periodontal pathogens and the immune system of the host. Chewing causes mechanical irritation, resulting in accumulation of inflammatory cells in the gingival tissue and bacterial infection and leading to formation of various inflammatory cytokines (Williams, 1990). Therefore, gingival tissues may have a major influence on the resorption of alveolar bone covered by gingiva. Bone resorption caused by periodontitis is regulated by cytokines, including receptor activator of NF-kB ligand (RANKL) and osteoprotegerin (OPG) (Crotti et al., 2003). RANKL is expressed by osteoblasts, gingival fibroblasts, and activated T cells and B cells. Upon addition of conditioned media containing periodontal pathogens to gingival fibroblast culture medium, the activity of RANKL was seen to increase and the

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expression of OPG decrease (Nagasawa et al., 2002). Hence, the overall RANKL/OPG ratio increases, and this is believed to influence bone resorption (Kiviranta et al., 2005). Moreover, gingival fibroblasts produce IL-1 during inflammation, which affects bone resorption through prostaglandin E_2 (Okamatsu, Kobayashi, Nishihara, & Hasegawa, 1996). Thus, gingival fibroblasts are greatly involved in the resorption of alveolar bone.

Interleukin-4 (IL-4) is an immune-regulatory protein secreted by activated Th2 lymphocytes. It has been known to produce OPG through activation of STAT6 and inhibit the stimulation of osteoclasts. IL-4 is usually present in human skin (Bao, Alexander, Shi, Mohan, & Chan, 2014; Campbell & Kemp, 1997), synovial joints (Scola et al., 2002), and gingival fibroblasts (Jenkins, Javadi, & Borghaei, 2004). It is also present in high concentration in healthy gingiva (Bastos et al., 2009) but is rarely detected in the gingiva of chronic periodontitis (Fujihashi et al., 1993; Pradeep, Roopa, & Swati, 2008) and aggressive periodontitis (Bastos et al., 2009), both of which are associated with remarkable alveolar bone resorption. Thus, IL-4 may be involved in suppression of alveolar bone resorption.

Bone resorbing activity was investigated by using hGF-CM that hGF were extracted from healthy gingiva. Bone resorbing activity was induced by stimulation of recombinant human IL-1 in this hGF culture (Sjostrom, Hanstrom, & Lerner, 2000). In contrast, it has also been reported that hGFs may inhibit bone resorbing activity depending on OPG production (Nagasawa et al., 2002). However, the effect of IL-4 present in gingival fibroblasts on the resorption of alveolar bone is still unclear. Therefore, the aim of this study was to investigate the influence of IL-4 present in hGFs on the activation of osteoclasts.

2. Materials and methods

2.1. Ethics statement

This study obtained written approval for human gingival tissue collection and gingival fibroblast generation from the Institutional Review Board at Tsurumi University School of Dental Medicine. Written informed consent was also individually collected from all participants.

2.2. Cell culture of hGFs

Two primary hGF samples were collected from humans and one was collected from a commercially available resource. The samples obtained from patients had no clinical signs of periodontal disease and were collected from the marginal gingiva and extracted third molar and first premolar. The collected gingival tissues were washed 3 times with DMEM (Dulbecco's Modified Eagle Medium, Gibco®, Carlsbad, CA, USA) supplemented with penicillin G (167 units/mL), gentamicin (50 μg/mL), and 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA). They were then reduced into small pieces and digested with a mixture of 3 mg/mL collagenase type 1 (Sigma-Aldrich, St. Louis, MO, USA) and 4 mg/mL trypsin in supplemented medium (SM) composed of 10% fetal bovine serum (FBS), 89% DMEM (ATCC), and 1% antibiotics (167 units/mL penicillin G, 50 μg/mL gentamicin, and 0.3 μg/mL fungizone) for 1 h at 37 °C. The digested tissue was centrifuged for 5 min at 1150 rpm, and the supernatant was discarded. Thereafter, 10 mL of SM was added to the tube containing the pellet, and the cell suspension was transferred to a 75 cm^2 (T-75) polystyrene tissue culture flask (BD, Franklin Lakes, NJ, USA). Once the attached cells had reached 75%–80% confluence, they were subcultured using trypsin/EDTA for 4–6 days and spread out on T-75 flasks. The cells were plated at a density of 1.0×10^5 cells/cm^2 on 6-well plates. Our experiments were carried out with cells from the fifth to seventh passages. The commercially available hGFs were purchased from LONZA (LONZA Japan, Cyuo-ku, Tokyo, Japan). The hGFs were cryopreserved (1.0×10^6 cells/T-25 flask) in passage 19. We cultured the commercial hGFs using as P22 by the same procedure as the two human primarily hGFs described above. The cell culture medium supernatants from the primary and commercial hGFs (hGF-CM) were collected and stored at −30 °C until required for the tartrate resistant acid phosphatase (TRAP) assay. The two primary hGFs and one commercial hGF were treated for 24 h with three kinds of LPSs originating from *Porphyromonas gingivalis* (P.g), *Prevotella intermedia* (P.i), and *Escherichia coli* (*E.coli*) at a final concentration of 1 μg/mL in the cell culture medium.

2.3. Cell culture of mouse macrophages (RAW264)

The mouse macrophage cell line RAW 264 (ATCC, Manassas, VA, USA) was initially suspended in fresh SM composed of 10% FBS, 89% αlpha–MEM (Minimum Essential Medium Eagle Alpha Modification, Gibco®), and 1% antibiotics. Subsequently, the cells were plated on T-75 polystyrene tissue culture flasks and incubated at 37 °C with 5% CO_2. The medium was replaced every 2 or 3 days. The cells grew to 80% confluence, as observed by inverted phase microscopy, after which they were washed with PBS (Gibco) and removed from the culture flask after addition of 2.5 mL 0.25% trypsin (Gibco) in PBS for 5 min at 37 °C. Five mL of SM was added to inactivate the trypsin, and the cell suspension was centrifuged at 1150 rpm for 5 min. The total viable cells were counted and stored in Nitrogen tanks until required for the experiments.

2.4. Mouse macrophages (RAW264) cell culture with hGF-CM

RAW 264 cells were plated at a concentration of 5.0×10^3 cells/well in 96-well plates. Following pre-incubation for 24 h at 37 °C, 200 ng/mL of Glutathione S-transferase-Receptor activator of NF-kB ligand (GST-RANKL) was added to the seeded cell culture plates. In order to examine osteoclastogenetic functions, hGF-CM, human recombinant osteoprotegerin (rhOPG) (Santa Cruz Biotechnology Inc., Dallas, TX, USA), human recombinant IL-4 (rhIL-4) (MACS, Friedrich, Bergisch Gladbach, Germany), and/or anti-human IL-4 (R&D systems, Minneapolis, MN, USA) were added to the cell culture medium the day after plating. The individual percentages of total media hGF-CM were 0, 1, 5, 10, 20, and 25 in the RAW 264 cell culture. Individual concentrations of OPG were 20, 40, and 200 ng/mL, hIL-4 were 0.1, 0.5, 1, and 5 ng/mL, and anti-human IL-4 was 1 ng/mL in the total media.

2.5. Isolation of RNA and polymerase chain reaction (PCR)

The hGFs were plated at a density of 1.0×10^5 cells/cm^2 on 6-well plates. Total RNA was isolated from cell culture with the help of an RNA isolation kit (RNAqueos kit, Ambion, Carlsbad, CA, USA), according to the manufacturer's instructions. Thereafter, cDNA was synthesized from 3 μg of the total RNA using the reverse transcriptase kit (Ready-To-Go You-Prime First-Strand Beads, GE Healthcare, Wauwatosa, WI, USA). GAPDH, a house-keeping gene, was used as an internal control. The mRNA expression level of the hGFs was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) (Gene Amp PCR system 9700, Applied Biosystems, Foster City, CA, USA) using sequence-specific primers. The primers used were IL-4, OPG, IL-10, IL-27, and IL-33 which were designed for human use as an electronic gene database (*BLAST, National Library of Medicine*). The primer design is shown in Table 1. The expression of cDNA was assessed by RT-PCR amplification for 25–35 cycles at 55 °C–65 °C. The amplified products were analyzed by electrophoresis with 1.5% agarose gel and visualized using