Journal of Dental Sciences (2015) 10, 81–87

Available online at www.sciencedirect.com

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journal homepage: www.e-jds.com

ORIGINAL ARTICLE

Osteoblasts subjected to tensile force induce osteoclastic differentiation of murine macrophages in a coculture system

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Received 8 October 2013; Final revision received 11 November 2013
Available online 4 February 2014

KEYWORDS
macrophages;
osteoblast;
osteoprotegerin;
receptor activator of nuclear factor kappa B ligand;
tensile force;
tartrate-resistant acid phosphatase

Abstract *Background/purpose:* Bone remodeling is regulated by the replacement of old bone with new bone through sequential osteoclastic resorption and osteoblastic bone formation. In this study, we compare the functional difference in osteoclastogenesis of murine macrophage when applied to the receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG) produced by osteoblasts in response to tensile stress in a coculture system.
Materials and methods: We examined the secretion of RANKL and OPG at different time points when the osteoblast cells are being cultured under tensile force. In order to confirm whether the media produced by the osteoblast cells under tensile force induce osteoclastogenesis or not, murine macrophage (RAW264.7 cells) were cocultured with osteoblasts.
Results: In both mono- and coculture systems, the two cell types cultured under tensile force and in normal environment showed no significant differences ($P > 0.05$) at any time. However, in the coculture system, the production of tartrate-resistant acid phosphatase (TRAP) in RAW264.7 cells under tensile force was found to increase significantly ($P < 0.05$; by 2.27-, 3.00-, or 3.27-fold on Day 3, Day 7, or Day 15, respectively) when compared with the corresponding TRAP activity in RAW264.7 cells in normal environment. Moreover, the results indicate that the tensile force upregulated the secretion of RANKL and inhibited OPG synthesis. Therefore, RAW264.7 cells appear to increase their production of TRAP in the media of osteoblasts under tensile force, increasing TRAP activity by nearly 2.8 times compared to that in the media of osteoblasts in a normal environment for 3 days.

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http://dx.doi.org/10.1016/j.jds.2013.11.003

Conclusion: These results suggest that osteoblasts influence the secretion of RANKL more than OPG when stimulated with osteoclastogenesis via RANKL under tensile force.

Introduction

Orthodontic tooth movement combines both physiological and pathological responses to externally applied forces. Efficient tooth movement may be accomplished by mechanical, biomechanical, or biostimulatory methods. Bone remodeling is regulated by the replacement of old bone with new bone through sequential osteoclastic resorption and osteoblastic bone formation.[1] The balance between bone resorption and formation is disturbed when a force is loaded onto a tooth, resulting in more bone formation than resorption on the tension side and more bone resorption than formation on the pressure side in the alveolar bone. This process causes the tooth to move in a specified direction.[2–4] Recently, several studies have investigated whether osteoblast cells express constitutively osteogenesis cytokines and growth factors, many of which are mechanoresponsive, such as collagen I, osteopontin, alkaline phosphatase, osteocalcin, and anti-inflammatory cytokines.[5] These cytokines are beneficial in mediating the cellular and molecular responses to orthodontic tooth movement and contribute to the remodeling of alveolar bone tissue.[6,7] Mature osteoblast-related cells produce osteoprotegerin (OPG) and the receptor activator of nuclear factor kappa B ligand (RANKL), which exert opposing effects on osteoclasts.[8,9]

Mechanical forces comprise tensile, compressive, and shear stresses. The mechanical microenvironment around a tissue affects the phenotype and function of a cell. Several studies have verified that mechanical force can change gene expression, protein secretion, and cell behavior effectively.[10–12] Accumulating evidence indicates that different mechanical forces stimulate cellular messages and results through similar mechanoreceptors and intracellular biochemical cascade signaling effectors in many types of cells.[11,12] Proinflammatory cytokines secreted by osteoblasts are upregulated immediately under tensile force and remain upregulated in the presence of force.[13] In addition, osteoclast formation and differentiation are regulated by the balance among the receptor activators RANKL, OPG, and macrophage colony-stimulating factor.[14] At least two studies have reported that osteoblasts express RANKL in response to mechanical stress,[13,15] whereas another study reports that they do not.[16] The anchylosed teeth can be moved by mechanical stress, suggesting that osteoblasts play a major role in alveolar bone resorption under tensile force.

This study investigates and compares *in vitro* coculture systems of macrophages and osteoblasts under tensile force to elucidate and distinguish between cytokine signaling conditions of these cell types and determine more representative *in vitro* cellular responses to *in vivo* models, or to provide evidence for confounding *in vitro* cell behavior.

Materials and methods

Cell culture

RAW264.7 macrophage cells and MC3T3-E1 osteoblast cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Caisson Laboratories, North Logan, UT, USA) containing 10% fetal bovine serum (FBS, GeneDireX, Las Vegas, NV, USA) and 100 U/mL penicillin/100 μg/mL streptomycin (PS, Caisson Laboratories) at 37°C in a humidified atmosphere of 5% CO_2 in air. The culture medium was changed every 3 days. Cells (5×10^4 cells/mL) were seeded on cover glasses for 24 hours, and transferred to six-well plates (GeneDireX) in a CO_2-independent medium (Gibco, Langley, OK, USA) supplemented with 10% FBS and 1% PS. Before cell experiments, the cover glasses were sterilized by immersion in 75% ethanol, followed by exposure to ultraviolet light for 6 hours. The six-well plates containing cover glasses (monoculture: MC3T3-E1 or RAW264.7 cells on cover glass; coculture: two cover glasses, one MC3T3-E1 cells and the other RAW264.7 cells) were placed in a tension incubator (Model. 3618P; Lab-Line Instrument, Thermolyne Co., IL, USA) or normal incubator. The inside of the tensile incubator was kept at −100 kPa (1 Pa = 1/100,000 kg/cm^2, equal to a negative force of 101 g/mm^2) at 37°C. A full description of the culture process implemented for each signaling condition is shown in Fig. 1.

Proliferation assays

After the various predetermined culture times, cell proliferation was evaluated using the PrestoBlue assay (Invitrogen, Grand Island, NY, USA). Briefly, at the end of the appointed time, the cover glasses were transferred to a new 24-well plate (GeneDireX) and washed with PBS three times. Each well was then filled with 400 μL solution (PrestoBlue:DMEM = 1:9) and incubated at 37°C for 30 minutes, after which the solution in each well was transferred to a new 96-well plate (GeneDireX). Plates were read using a multiwell spectrophotometer (Hitachi, Tokyo, Japan) at 570 nm, with a reference wavelength of 600 nm. The results were obtained in triplicate from three separate experiments for each test.

Enzyme-linked immunosorbent assay analysis

RANKL and OPG proteins are secreted from MC3T3-E1 cells cultured in a tensile or normal incubator for various numbers of days, and the cultured medium was collected. A RANKL enzyme-linked immunosorbent assay (ELISA) assay kit (Abcam, Cambridge, MA, USA) and an OPG ELISA assay