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Original Article

Polarization of osteoclasts on dental implant materials is similar to that observed on bone

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ABSTRACT

Objective: Polarized osteoclasts form sealing zones, (also called clear zones) detectable as actin rings, and ruffled borders to resorb bone. They secrete protons and catabolic enzymes, including tartrate-resistant acid phosphatase (TRAP), through the ruffled borders. We previously reported that polarized osteoclasts develop areas of TRAP activity (TRAP-marks) when cultured on dentin slices [11]. In this study, we examined how osteoclasts recognize dental implant materials.
Methods: Osteoclasts obtained from murine co-cultures were cultured on implant materials such as titanium (Ti), alumina, zirconia, and sintered hydroxyapatite (sHA), in addition to dentin. Osteoclasts were also treated with reveromycin A (RM-A), which specifically acts on polarized osteoclasts and induces apoptosis. Polarization of osteoclasts cultured on implant materials was evaluated by measuring actin rings, TRAP-marks, and reveromycin A-induced apoptosis.
Results: Osteoclasts formed actin rings on all substrates examined. The formation of actin rings on Ti by osteoclasts was inhibited by the GRGDS peptide, but not by the GRGES peptide, suggesting an integrin-mediated polarization of osteoclasts on Ti. Calcitonin, an inhibitory hormone of osteoclast function, disrupted the actin rings that were preformed on Ti and sHA. Osteoclasts put TRAP-marks on sHA and dentin and formed resorption pits on dentin, but failed to form resorption pits on sHA. RM-A induced apoptosis in osteoclasts cultured on Ti and sHA; this was suppressed by calcitonin.
Conclusions: These results demonstrate that osteoclasts are able to polarize on dental implant materials similar to the polarization observed on bone.

1. Introduction

Osteoclasts are bone-resorbing cells that have a role in bone remodeling [1,2]. Osteoclasts have been shown to create polarized cytoplasmic organizations such as clear zones and ruffled borders to resorb bone [3,4]. The recognition of extracellular matrix proteins containing the Arg-Gly-Asp-(RGD) sequence by integrins is the initial step in osteoclast polarization. Osteoclasts express a large number of $\alpha_v\beta_3$ vitronectin receptors [5,6]. The clear zone serves for the attachment of osteoclasts to bone surfaces and for the isolation of resorption areas under ruffled borders from the surroundings. The clear zone consists of many distinct close contact points called podosomes and is observed as a ringed structure of F-actin dots (actin ring) [3,4]. The formation of actin rings in osteoclasts precedes the start of bone resorption. A previous study demonstrated that the disruption of actin rings suppressed osteoclast function [7].

The resorption area under the ruffled border is acidic, which favors dissolution of bone mineral. Vacuolar type H^+-ATPase (V-ATPase), which is specifically localized on the ruffled border membrane, has been shown to play a role in the secretion of protons into resorption lacunae [8]. Polarized osteoclasts also secrete catabolic enzymes including tartrate-resistant acid phosphatase (TRAP) through the ruffled borders in order to degrade the organic matrix

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of bone [9,10]. We previously investigated the relationship between positions of actin rings and resorption pits in osteoclasts, using dentin slices and osteoclasts obtained form murine co-cultures [11]. We found that pit-forming osteoclasts put TRAP activity (named TRAP-marks) on dentin slices. The position of the actin rings corresponded to that of TRAP-marks. Thus, the detection of TRAP-marks on dentin slices is a simple method to confirm the location of polarized osteoclasts.

Calcitonin (CT) is a peptide hormone that inhibits the function of osteoclasts, which generally express a large number of CT receptors [12,13]. CT has been shown to suppress bone-resorbing activity of osteoclasts by disrupting the formation of actin rings [14,15]. Reveromycin A (RM-A), which is produced by the genus *Streptomyces*, is an osteoclast-targeting antibiotic [16,17]. Woo et al. [17] demonstrated that RM-A specifically induced apoptosis in functioning osteoclasts that secreted protons, but not in non-functional osteoclasts. RM-A can be incorporated into osteoclasts under acidic conditions. RM-A-induced apoptosis can be inhibited by concanamycin A, an inhibitor of V-ATPase, or through the disruption of actin rings. Therefore, CT is able to suppress RM-A-induced apoptosis in osteoclasts by disrupting the actin rings. Osteoprotegerin is a soluble decoy receptor for receptor activator of nuclear factor kB (RANKL), a critical inducer of osteoclast differentiation and function [18]. Previous studies have reported that bone resorption was markedly upregulated in osteoprotegerin-deficient mice [19,20]. Furthermore, in vivo administration of RM-A to osteoprotegerin-deficient mice suppressed tooth movement induced by orthodontic forces [21].

Advances in biomaterial science and cell biology are intimately intertwined. Elucidating the recognition mechanism for biomaterials by host cells is an important issue for the clinical success of orthopedics and dental implants. Pertinent questions include how osteoclasts recognize implant materials, whether osteoclasts try to resorb implant materials, and whether osteoclasts can resorb hydroxyapatite (HA) coated on titanium (Ti) implants. These questions are of importance, as the success of dental implants largely depends on such vital reactions in patients. To answer these questions, we examined how osteoclasts recognized implant materials by evaluating actin rings, TRAP-marks, and RM-A-induced apoptosis in osteoclasts. The results obtained demonstrated that osteoclasts have the ability to polarize on implant materials in a manner similar to that observed on bone.

2. Materials and methods

2.1. Implant materials

Grade 2 pure Ti was purchased from Daido Steel (Nagoya, Japan). Alumina sintered compact (alumina) and yttrium-stabilized zirconia sintered compact (zirconia) were obtained from Taimei Chemicals (Shimoina, Japan) and Kikusui Chemical Industries (Nagoya, Japan), respectively. These material plates (1-mm thick) were polished using pastes that were 1 μm in diameter and cut into small pieces (5 mm × 5 mm). HA was synthesized as described previously [22]. Precipitates of HA were dry-pressed and further compacted isostatically at 200 MPa. sHA was produced by heating at 1200 °C for 2 h. Approximately 1-mm thick disks were dry cut from sHA and cut into small pieces (3-mm squares). The surface of the sHA slices was polished with pastes (1 μm in diameter). Dentin slices (4 mm × 4 mm, 0.2-mm thick) were made from an ivory block [23].

2.2. Materials for cell culture

Collagenase, calcitriol, and prostaglandin E_2 were purchased from Wako Pure Chemical (Osaka, Japan). A type I collagen gel matrix was obtained from Nitta Gelatin (Osaka, Japan). Elcatonin, a synthetic analog of eel CT, was kindly provided by Asahi Kasei (Tokyo, Japan). The Gly-Arg-Gly-Asp-Ser (GRGDS) peptide and Gly-Arg-Gly-Glu-Ser (GRGES) peptide were obtained from the Peptide Institute (Osaka, Japan) and Peptide International (Louisville, KY, USA), respectively. Reveromycin A (RM-A) was isolated and purified from the fermentation broth of *Streptomyces reveromyceticus* SN-593 strain as described previously [16]. Rhodamine-conjugated phalloidin was obtained from Molecular Probes (Eugene, OR, USA).

2.3. Preparation of osteoclasts

Six-week-old male and newborn ddY mice were obtained from Japan SLC (Shizuoka, Japan). Primary osteoblasts were obtained from calvariae of newborn mice, as described previously [23]. Bone marrow cells were obtained from the tibiae of adult mice. Osteoclasts were prepared from co-cultures of osteoblasts and bone marrow cells [23]. Primary osteoblasts (1×10^6 cells/dish) and bone marrow cells (1×10^7 cells/dish) were co-cultured in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS, USA), 10^{-8} M calcitriol, and 10^{-6} M prostaglandin E_2 in 10-cm dishes pre-coated with 0.2% type I collagen gel matrix. After being cultured for 7 days, the cells were treated with 0.2% bacterial collagenase. Cells recovered from the dish were suspended in 10 mL of α-Modified Eagle's Medium (α-MEM) containing 10% FBS and used as osteoclast preparations [23].

2.4. Identification of actin rings

Osteoclast preparations were cultured for 1 h on slices of Ti, alumina, zirconia, sHA, and dentin in 48-well culture plates (0.4 ml/well). The slices were then transferred into new 48-well culture plates and cultured in α-MEM containing 10% FBS. For certain experiments, 10^{-8} M of CT was added to some cultures. The GRGDS and GRGES peptides were suspended in phosphate-buffered saline (PBS). Osteoclasts were placed on Ti in the presence of 10% FBS with or without 1 mM GRGDS and GRGES peptides. After being cultured for 3 or 7 h, cells were processed for actin staining [14]. The number of osteoclasts having actin rings was counted.

2.5. TRAP staining

Osteoclasts on dentin, Ti, and sHA were fixed and processed for TRAP staining [23]. Cells on the slices were thoroughly removed using cotton swabs for TRAP-mark staining [11]. Slices were also incubated with a TRAP staining solution for 30 min to detect TRAP-marks.

2.6. Scanning electron microscopy

Cells were removed from the slices by using cotton swabs and the fine structure of lacunae on the surface of sHA and dentin were observed. The slices were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4), washed, air-dried, and coated with carbon by using a vacuum evaporator (JEOL JEE-420, Tokyo Japan). Surfaces of the samples were observed using SEM (JEOL JSM-6360LA, Tokyo) with accelerating voltages of 30 kV. The working distance was 10 mm and samples were imaged in the SE mode.

2.7. RM-A-induced apoptosis in osteoclasts

48-well culture plates were pre-coated with 150 μL of 0.2% type I collagen gel matrix. Osteoclasts were cultured for 12 h on plastic, collagen gel, Ti, and sHA in the presence or absence of 10^{-6} M RM-A in α-MEM containing 10% FBS. Some plates were also treated with 10^{-8} M CT. Osteoclasts were then stained for