Microscopic study on resorption of β -tricalcium phosphate materials by osteoclasts

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Abstract Sintered compounds prepared with β -tricalcium phosphate (β -TCP) are commonly used as biocompatible materials for bone regenerative medicine. Although implanted β -TCP is gradually replaced with new bone after resorption by osteoclasts, exactly how osteoclasts resorb β -TCP is not well understood. To elucidate this mechanism, we analyzed the structure of β -TCP discs on which mouse mature osteoclasts were cultured using scanning electron microscopy. We found that β -TCP was resorbed by mature osteoclasts on one side of each disc, as evidenced by the formation of multiple spine-like crystals at the exposed areas. Because osteoclasts

secrete acid to resorb bone minerals, we mimicked this acidification by dipping β -TCP slices into HCl solution (pH 2.0). However, no spine-like crystals appeared even though the size of each β -TCP particle was reduced. On dentin slices, osteoclasts formed clear actin rings, which are cytoskeletal structures characteristic of bone-resorbing osteoclasts. No clear actin rings were observed in osteoclasts cultured on β -TCP slices, although small actin dots were observed. Analysis by transmission electron microscopy showed that osteoclasts attached to β -TCP particles. These results suggest that osteoclasts resorb β -TCP particles independently of clear actin ring formation.

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Introduction

Osteoclasts are multinucleated giant cells that form ruffled borders and clear zones toward the bone surface and resorb bone matrix (Blair et al. 1989; Salo et al. 1997). Osteoclasts attach to extracellular proteins containing Arg-Gly-Asp sequences through $\alpha_v\beta_3$ integrins and then secrete acid and proteinases such as cathepsins from the ruffled borders (Inaoka et al. 1995; Sasaki and Watanabe 1995). The hydroxyapatite in the matrices is demineralized by acid, and bone matrix proteins including fibrous collagens are

digested by proteinases. Finally, the digested bone matrices are internalized by osteoclasts via endocytosis and released from the apical membrane via exocytosis (Salo et al. 1997).

Osteoclasts form ringed F-actin filament structures known as “actin rings,” which exactly correspond to clear zones of bone-resorbing osteoclasts (Nakamura et al. 1996a, b). Since disruption of actin rings by calcitonin and bisphosphonates results in the inhibition of osteoclast function, the actin ring may serve as a functional marker of activated osteoclasts (Murakami et al. 1995; Suzuki et al. 1996).

β -Tricalcium phosphate (TCP) is a biocompatible material that is used in surgical procedures to promote bone regeneration (Liu and Lun 2012; Sagawa et al. 2010). It is generally accepted that implanted β -TCP materials in bones are resorbed by osteoclasts and subsequently replaced with new bone matrices produced by osteoblasts (Chazono et al. 2004; Ogose et al. 2002). Resorption by osteoclasts is one of the most important characteristics of β -TCP in bone regenerative medicine. However, the precise mechanism by which osteoclasts resorb β -TCP materials has not been elucidated.

Here, we analyzed β -TCP resorption by osteoclasts using scanning electron microscopy (SEM), transmission electron microscopy (TEM), and a method to visualize actin filaments in osteoclasts. Our results suggest that osteoclasts are capable of secreting acid to localized areas of β -TCP particles without forming actin rings, although F-actin filament dot structures were observed.

Materials and methods

Animals and reagents

Six-week-old male C57BL6/J mice were purchased from Sankyo Labo Service Co., Inc. (Tokyo, Japan). Alpha-minimum essential medium (α MEM) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fetal bovine serum, antibiotics (penicillin and streptomycin), an antimycotic (amphotericin B), trypsin-EDTA, and rhodamine-conjugated phalloidin were purchased from Life Technologies Japan (Osaka, Japan). Recombinant mouse RANKL and human TGF- β were purchased from R&D

systems, Inc. (Minneapolis, MN, USA). Human M-CSF (Leukoprol[®]) was purchased from Kyowa Hakko Kirin Co., Ltd. (Tokyo, Japan).

β -TCP and dentin slices

Column-shaped blocks of β -TCP compound (Osferion[®], 60 % of porosity, 8 mm in diameter, 20 mm length) were kindly provided by Olympus Terumo Biomaterials Co. (Tokyo, Japan). The β -TCP block was cut using diamond-band saws (EXAKT Co., Norderstedt, Germany) to make β -TCP slices (8 mm in diameter, 0.4 mm thickness). Dentin slices (8 mm in diameter, 0.4 mm thickness) were prepared from ivory using diamond band saws. The β -TCP and dentin slices were sterilized with 70 % ethanol and used for cell culture experiments.

Preparation of osteoclasts

Mouse bone marrow cells were cultured for 3 days in 10 ml α MEM containing 10 % FBS, penicillin (100 U/ml), streptomycin (100 μ g/ml), amphotericin B (250 ng/ml), human M-CSF (4,000 U/ml), and human TGF- β 1 (1 ng/ml) to induce the formation of osteoclast precursors (bone marrow-derived macrophages, BMMs). The BMMs were harvested in 0.05 % trypsin-EDTA and cultured further on temperature-sensitive culture plates (RepCell[®], 100 mm diameter) with 10 ml α MEM containing 10 % FBS, antibiotics and antimycotics, human M-CSF (4,000 U/ml), and mouse RANKL (50 ng/ml) to induce mature osteoclast formation. After 10 days in culture, the mature osteoclasts were kept at 25 °C for 30 min to facilitate gentle detachment. The detached osteoclasts were collected by centrifugation at $55 \times g$ for 3 min and cultured on β -TCP or dentin slices in 24-well plates (AGC Techno Glass Co., LTD., Tokyo, Japan) with 10 ml α MEM containing 10 % FBS, antibiotics, human M-CSF (4,000 U/ml), and mouse RANKL (50 ng/ml). After 72 h, the samples were observed by microscopy.

SEM studies

The medium was removed from cultured cells, and β -TCP slices were fixed in 4 % paraformaldehyde for 30 min and in 2.5 % glutaraldehyde at 4 °C for 2 h. After washing the β -TCP slices with phosphate

buffered saline (PBS), they were incubated with 1 % osmium tetroxide. The samples were dehydrated using 50–100 % ethanol and critical point drying, and coated with platinum. The surfaces of the slices were observed using a Hitachi S-4700 Scanning Electron Microscope at 10 kV (Hitachi High-Technologies Co., Tokyo, Japan).

TEM studies

β -TCP samples were fixed with 2.5 % glutaraldehyde in 0.1 M PBS (pH 7.4) for 2 h at room temperature. The fixed specimens were embedded in 2 % agarose gel to avoid sample losses during the decalcification and preparation procedures and decalcified with 10 % EDTA. All conventional TEM specimens embedded in agarose gel were subsequently fixed in 2 % osmium tetroxide in 0.1 M PBS (pH 7.4) for 2 h on room temperature. Samples were dehydrated using a graded ethanol series and were then embedded in Epon 812. Ultrathin sections were cut on a LEICA Ultracut UCT and mounted on 300 mesh grids coated with formvar and carbon, stained with uranyl acetate and lead citrate, and examined by a Hitachi H-7600 transmission electron microscope with an accelerating voltage of 75 kV (Hitachi High-Technologies Co.).

Actin staining

Cultured cells were fixed for 30 min in 10 % formalin, washed twice with PBS, and immersed in rhodamine-conjugated phalloidin solution (0.2 U/ml) containing 0.1 % triton-X for 1 h. Rhodamine-labeled actin filaments in osteoclasts were observed under a Keyence BZ-9000 fluorescence microscope (Keyence Japan Co., LTD, Osaka, Japan).

Treatment of β -TCP slices with HCl

β -TCP slices were submerged in HCl (pH 2.0) for 24 h at room temperature. The slices were then used for SEM observations.

Ethical statement

The Ethical Board for Animal Experiments of Showa University approved all animal use protocols used in this study (approval number: 14073).

Results

Osteoclasts are capable of resorbing β -TCP particles

First, we observed osteoclast cultures grown on β -TCP slices by SEM (Fig. 1). Surrounding the osteoclasts, we found rough areas composed of β -TCP particles where osteoclasts potentially attached to the discs (Fig. 1a). Under higher magnification, the surface of each β -TCP particle appeared to have been resorbed by osteoclasts (Fig. 1b). Crystal structures resembling β -TCP particles were observed (Fig. 2), suggesting that osteoclasts had resorbed β -TCP particles.

Fig. 1 SEM images of osteoclasts cultured on β -TCP material. Osteoclasts were cultured on β -TCP discs for 72 h, after which the cells were fixed and analyzed by SEM. Magnifications: **a** $\times 3,000$; **b** $\times 5,000$; OC osteoclast. Arrows indicate resorbed β -TCP particles

Fig. 2 Magnified images of β -TCP particles resorbed by osteoclasts. Bar 1 μ m

Crystal structures do not appear on β -TCP particles following HCl treatment

Because osteoclasts traffic H^+ and Cl^- ions toward bone surface by proton pumps and Cl^- channels, we examined whether crystal structures appeared on the β -TCP particles in HCl solution. While no crystal structures were observed, the average β -TCP particle size was reduced (Fig. 3a, b). These results suggested that osteoclasts resorb β -TCP particles under the different conditions tested in this study.

Osteoclasts failed to form actin rings on β -TCP materials

We next examined if osteoclasts from actin rings on β -TCP by labeling actin filaments with rhodamine-conjugated phalloidin. On dentin slices, actin rings were formed in osteoclasts (Fig. 4a). However, osteoclasts did not form clear actin rings, although an accumulation of actin dots was found (Fig. 4b). These results suggested that osteoclasts can resorb β -TCP particles independently of actin ring formation.

Osteoclasts adhere to β -TCP particles without ruffled boarder formation

To determine whether osteoclasts develop ruffled borders to resorb β -TCP particles, we observed osteoclasts cultured on β -TCP or on dentin slices by TEM (Fig. 5a, b). On the dentin slices, osteoclasts developed ruffled borders toward dentin matrices and

Fig. 3 SEM images of β -TCP particles. **a** β -TCP particle control. **b** β -TCP particle treated with HCl (pH 2.0) for 72 h

Fig. 4 Actin filaments of osteoclasts on dentin and β -TCP. Osteoclasts were cultured on **a** dentin or **b** β -TCP for 72 h. Cells were then fixed and actin filaments were labeled with rhodamine-conjugated phalloidin. Arrows indicate actin rings, while arrowheads indicate actin dots

Fig. 5 TEM images of osteoclasts cultured on dentin or β -TCP. Osteoclasts were cultured on **a** dentin or **b** β -TCP slices for 72 h. The samples were then prepared for TEM analysis. RB, ruffled boarder; * β -TCP particle

contained abundant collagen fibers (Fig. 5a). In contrast, on β -TCP discs, osteoclasts adhered to β -TCP particles even though few ruffled borders were seen projecting toward the particles (Fig. 5b). These observations suggested that osteoclasts can secrete protons from adherent surfaces during β -TCP particle resorption without a requirement of ruffled boarder formation.

Discussion

In the present study, we clearly demonstrated that osteoclasts can resorb both dentin and β -TCP materials. These findings support a model whereby osteoclasts play critical roles in the resorption of β -TCP from implants in bone regenerative medicine (Chazono et al. 2004; Linovitz and Peppers 2002; Ogose et al. 2002). In addition, we characterized new properties of osteoclasts, showing that they can resorb small β -TCP particles (1–2 μ m in diameter) without the prior formation of actin rings and ruffled borders, which are observed on dentin. Thus, osteoclasts likely arrange their cytoskeletons in response to differing substances, such as dentin and β -TCP particles.

Numerous spine-like crystals were formed on one side of each particle where the osteoclasts attached. Because osteoclasts can directly secrete acid to β -TCP particles through their cytoplasmic membranes, osteoclasts are able to resorb small particles, such as β -TCP particles, which have a diameter of 1–2 μ m. Resorption by osteoclasts resulted in crystal formation on β -TCP particles, while submerging the β -TCP disc in HCl solution did not. Interestingly, the size of each particle treated with HCl was reduced, indicating that the particles were partially, but not completely dissolved. A limitation of this study is that we did not determine the mechanism wherein crystal formation by osteoclasts occurs; the microenvironment between the cytoplasmic membrane of osteoclasts and β -TCP particles appeared to be suitable for dissolving β -TCP crystals.

Although actin rings have been used as markers for bone-resorbing osteoclasts (Nakamura et al. 1996a, b), we found no clear actin rings but many actin dots in osteoclasts adhered to β -TCP particles. The diameters of the actin rings observed on dentin slices were $\sim$ 10–100 μ m, while those of actin dots observed on β -TCP were $\sim$ 1–10 μ m. In addition, the TEM images indicated that osteoclasts fold onto each β -TCP particle. Thus, it is possible that actin dots may form on β -TCP particles, with acids being secreted near the surfaces of the β -TCP particles. Further studies are necessary to clarify the mechanism of β -TCP resorption by osteoclasts.

In conclusion, osteoclasts were found to resorb β -TCP particles independently of actin ring formation. This finding suggests an alternative mechanism of

resorption in osteoclasts, which may be useful in understanding the resorption of implanted β -TCP materials.

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