

Research Article

Difference in Osteoclast Responses to Tricalcium Phosphate in Culture Medium Supplemented with Zinc and to Zinc-Containing Tricalcium Phosphate

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Abstract Difference in osteoclast responses (i.e., apoptosis, actin ring formation) to tricalcium phosphate (TCP) in culture medium supplemented with zinc and to zinc-containing tricalcium phosphate (ZnTCP) was investigated in this study. On the TCP ceramic, an increase in zinc ion in the culture medium within the range between 0.3 and 6.8 ppm significantly induced an increase in osteoclast apoptosis and a decrease in actin ring formation. However, even a high level of magnesium ion up to 100 ppm in the culture medium or up to 6.8 mol% in the ceramic was unlikely to influence osteoclast activity. There was almost no significant difference in osteoclast apoptosis and actin ring formation between ZnTCP with 1 mol% zinc and magnesium-containing tricalcium phosphate with 1.5 mol% magnesium ceramic which have the same solubility and the same dissolution rate. It is suggested that only an increase in zinc level outside resorption lacuna has an inhibitory effect on osteoclast resorption and that an increase in zinc level inside resorption lacuna could not influence osteoclast activity.

Keywords osteoclast; apoptosis; actin ring; tricalcium phosphate; zinc

1 Introduction

The final aim of our study is to develop zinc-containing tricalcium phosphate (ZnTCP) ceramic as bone substitutes to counteract bone resorption at the bone-implant interface and thus to prevent loosening at the bone-implant interface. Composite of ZnTCP and hydroxyapatite (HAP) had greater stability at the bone-implant interface than composite of tricalcium phosphate (TCP) and HAP in an osteopenic animal model [5].

Zinc can promote osteoblast cell proliferation and differentiation and bone formation [1,17], since zinc is involved in many metalloenzymes and proteins including

alkaline phosphatase (ALP) [8, 12]. Zinc-containing calcium phosphate led to an increase in osteoblast cell proliferation, ALP activities, and bone formation because of the release of zinc ion, compared with that without zinc [3,14].

Zinc has an inhibitory effect on bone resorption, such as the osteoclast-like cell formation derived from marrow cells and the activity of mature osteoclasts [10, 19]. Zinc ion-sensitive proton conductive pathway has been demonstrated in rabbit osteoclasts, in which zinc inhibits proton translocation across the membrane of osteoclasts [11]. ZnTCP ceramic with 0.5 and 1.0 mol% zinc suppressed resorption by mature osteoclasts in vitro compared with TCP [18].

In this study, difference in osteoclast responses (i.e., apoptosis, actin ring formation) to TCP in culture medium supplemented with zinc and to ZnTCP was investigated.

2 Materials and methods

TCP, ZnTCP, and magnesium-containing tricalcium phosphate (MgTCP) ceramic were made using pure TCP (ADVANCE Co. Ltd., Tokyo, Japan), ZnTCP with 11.8 mol% zinc, and MgTCP with 6.8 mol% magnesium powders. Pure TCP powders were mixed with either ZnTCP with 11.8 mol% zinc or MgTCP with 6.8 mol% magnesium powders, which were made by the solution method described elsewhere [4], to prepare ZnTCP with 1.0 mol% zinc and MgTCP with 1.5 mol% magnesium powders.

Osteoclasts were isolated from the tibias, femurs, humeri, ulnas, and radii of 10-day-old Japanese white rabbits [18]. Then, the isolated osteoclasts were seeded on the ceramic previously placed in each well of a 12-well plate and cultured at 37 °C for attachment. After 2 hours, 2 mL of α -MEM with 10% FBS and 5% L-glutamine, supplemented

No.	Ceramic	Culture medium	The ratio of apoptosis		The ratio of actin ring formation	
			6 hours	24 hours	6 hours	24 hours
A1	TCP	Zn0.3 ppm	5.0 ± 3.6	4.9 ± 3.1	19.2 ± 12.1	17.9 ± 13.8
A2	TCP	Zn1.3 ppm	11.5 ± 5.6	12.7 ± 13.1	16.4 ± 14.3	16.6 ± 10.2
A3	TCP	Zn6.8 ppm	18.3 ± 9.7	35.7 ± 37.0	4.8 ± 7.0	5.5 ± 6.4
B1	MgTCP1.5	Mg20 ppm	—	2.0 ± 3.1	—	12.3 ± 2.0
B2	MgTCP1.5	Mg100 ppm	—	9.1 ± 6.3	—	15.1 ± 6.5
B3	MgTCP6.8	Mg20 ppm	—	3.5 ± 3.9	—	14.1 ± 5.9
C1	MgTCP1.5	Zn1.3 ppm	10.4 ± 5.5	13.0 ± 7.8	18.7 ± 10.0	16.3 ± 6.3
C2	ZnTCP1	Zn1.3 ppm	15.1 ± 6.0	10.3 ± 5.1	15.4 ± 7.4	26.1 ± 7.4

Table 1: Osteoclast responses to TCP in culture medium supplemented with zinc and to zinc/magnesium-containing tricalcium phosphate.

with 100 ng mL⁻¹ macrophage colony-stimulation factor (M-CSF) (mouse recombinant, Calbiochem) and 50 ng mL⁻¹ TRANCE (mouse recombinant RANK ligand, TECHNE) was added to each well. In the above culture medium, certain amount of ZnCl₂ or MgCl₂ was added to obtain Zn or Mg ion containing culture medium. The osteoclasts were further cultured for 6 or 24 hours at 37 °C.

The osteoclasts were fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.1% Triton X-100 in PBS for 1 minute. Then, the osteoclasts were stained with the TUNEL method using an *in situ* cell detection kit (Roche Diagnostics, Japan), Phalloidin-Fluorescein isothiocyanate (Phalloidin-FITC, Sigma-Aldrich), and Vectashield mounting medium (Vector Lab) containing 10 μg mL⁻¹ DAPI to observe apoptosis, actin rings, and multinuclear. Before every step staining, three washes with PBS were needed. The osteoclasts on the ceramic were observed by using a fluorescence microscope (Olympus, BX51).

The numbers of multinuclear (three nuclei or higher) cells were counted as the total number of osteoclasts. The actin cytoskeletal structures of osteoclasts were classified into the closed actin ring, open actin ring, and podosome structures. The presence of closed actin ring was defined as actin ring formation. The TUNEL staining-positive osteoclasts were defined as apoptosis.

3 Results and discussion

The modulation of osteoclasts resorption can occur via two ways: apoptosis and the resorption activity [18]. The apoptosis directly implied the termination of osteoclastic resorption. The resorption activity included actin ring formation and acid and proteinase secretion in the resorption lacunae [18]. When the osteoclast changes from the nonresorbing to the resorbing stage, the initial punctuated structure (podosome) of F-actin reorganized into a dense continuous ring structure (closed actin ring) surrounding the resorption area [16]. The nonintegrity of actin ring meant the decrease in osteoclasts activity [9]. The detailed experimental design and the corresponding osteoclast responses are shown in Table 1.

The culture medium supplemented with zinc, that is extracellular zinc level outside resorption lacuna greatly inhibited osteoclastic resorption on TCP ceramic. On the TCP ceramic, an increase in zinc level (0.3, 1.3, 6.8 ppm) in the culture medium induced a significant increase in the ratio of apoptosis (ANOVA: 6 hours, $P = .00074$; 24 hours, $P = .01501$) and a significant decrease in the ratio of actin ring formation (ANOVA: 6 hours, $P = .02238$; 24 hours, $P = .02713$). The zinc ion in the previous study and in the normal body fluid is 0.3 and 1.3 ppm, respectively. The zinc ion of 6.8 ppm (1.04×10^{-4} M) was safe since zinc ion up to 10^{-4} M did not show toxicity [10]. It was reported that extracellular zinc level outside resorption lacuna inhibited the bone resorption [10,19]. The isolated rat mature osteoclasts were significantly inhibited at zinc levels of $10^{-10} \sim 10^{-14}$ M and 10^{-4} M [10]. Zinc level of $10^{-8} \sim 10^{-5}$ M lost this inhibition effects [10,19]. In the present study, the similar range of zinc level showed the similar inhibitory effects on osteoclastic resorption, regardless of the presence of TCP ceramic and different estimation method.

ZnTCP, that corresponds to extracellular zinc level inside resorption lacuna, is unlikely to influence osteoclastic resorption compared to the control. The interference of osteoclast-mediated dissolution with chemical dissolution was omitted in the previous study using ZnTCP and TCP [18], since the resorption of bone substitutes is controlled by chemical dissolution and osteoclast-mediated dissolution. MgTCP with 1.5 mol% magnesium (MgTCP1.5) ceramic, used as the control, had nearly the same chemical dissolution rate and equilibrium solubility as ZnTCP with 1 mol% zinc (ZnTCP1) ceramic [2,7]. Even a high level of magnesium ion up to 100 ppm in the culture medium was unlikely to induce an influence on apoptosis and actin ring formation. MgTCP with 6.8 mol% magnesium (MgTCP6.8) ceramic have no effect on osteoclast apoptosis and actin ring formation, compared with MgTCP1.5. There was almost no significant difference in osteoclast apoptosis and actin ring formation between ZnTCP1 and MgTCP1.5 ceramic which have the same solubility and the same dissolution rate.

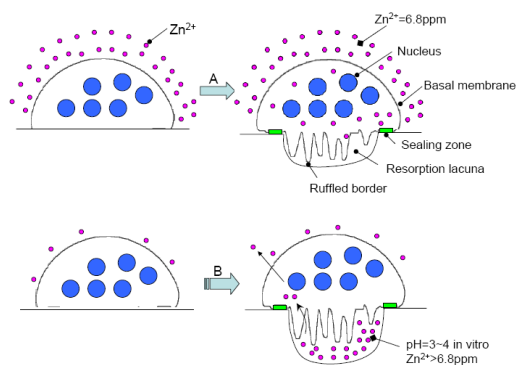


Figure 1: Scheme of the interaction between osteoclast and TCP in culture medium supplemented with zinc (A) or ZnTCP (B).

Extracellular zinc level inside resorption lacuna on ZnTCP1 ceramic should readily reach the level of 6.8 ppm that is effective for inhibiting osteoclast resorption, since ZnTCP1 ceramic contains zinc ion as high as 6300 ppm and the pH at the ruffled border is as low as $3 \sim 4$ *in vitro* [2, 13, 18].

It is indicated that only an increase in zinc level outside resorption lacuna has an inhibitory effect on osteoclast resorption and that an increase in zinc level inside resorption lacuna could not influence osteoclast activity. This result could tentatively be explained from involvement of a ubiquitous plasma membrane zinc transporter and its position on the osteoclast. The intimate contact between osteoclasts and ceramic firstly formed the sealing zone and the ruffled border [16], generated an isolated extracellular microenvironment (low pH and secretion of degrading proteinases) and thus led to the formation of resorption lacuna [15]. Osteoclasts express a ubiquitous plasma membrane zinc transporter, namely, ZIP1, which uptakes zinc ion into osteoclasts and is predominantly colocalized with actin at the sealing zone when overexpressed [6]. The high concentration zinc ion outside resorption lacuna can be uptaken by zinc transporter on the basal membrane and at the sealing zone, which inhibits the osteoclast activities and induces apoptosis (Figure 1(A)). However, the local high concentration zinc ion inside resorption lacuna cannot be uptaken by ZIP1 and must permeate out of the osteoclast to enter the culture medium (Figure 1(B)).

4 Conclusions

This study demonstrated the difference in osteoclast responses to TCP in culture medium supplemented with zinc and to ZnTCP. On TCP ceramic, the culture medium supplemented with zinc within the range between 0.3 and 6.8 ppm induced an increase in osteoclast apoptosis and a decrease in actin ring formation. ZnTCP to release comparable zinc ion inside the resorption lacuna did not influence osteoclast apoptosis and actin ring formation.

It is suggested that only an increase in zinc level outside resorption lacuna has an inhibitory effect on osteoclast resorption and that an increase in zinc level inside resorption lacuna could not influence osteoclast activity.

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