Inhibitory effect of zinc ions in zinc-containing β-tricalcium phosphate on function of matured osteoclasts

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Statement of Purpose: Reducing stability of bone-implant interface due to aseptic bone loss at the interface is a major problem for orthopedic implants for a long term. The long-term objective of this study is to develop calcium phosphate biomaterials incorporating optimum levels of magnesium (Mg), zinc (Zn) and fluoride (F). These ions have been associated with bone formation, biomineralization and osteoporosis therapy. The results of the previous study suggested that osteoclastic bone resorption at the bone-implant interface was inhibited by Zn in the ZnTCP316. The purpose of the present study is to clarify whether ZnTCP modulates resorption activity of mature osteoclasts or not. In this point of view, we examined formation of actin rings, number of apoptosis cells, expression of marker enzymes and the resorption function of purified mature osteoclasts on ZnTCP in vitro.

Methods:

Materials TCP and ZnTCP disks with nearly identical physical properties were prepared by the sintering, at 1100 ˚C for 1h, of TCP and ZnTCP powders. The zinc contents of the desks were 0 (TCP), 0.316 (ZnTCP 316) and 0.633 (ZnTCP 633) wt %. *Isolation of osteoclasts* Osteoclasts were isolated from the tibias, femurs, humeri, ulnas, and radii of 10-day-old Japanese white rabbits according to the method established by Kakudo et al. with minor modifications.

Cell attachment and culture The isolated osteoclasts were seeded on the TCP and ZnTCP disks or on ivory slices previously placed in each well of a 12-well plate and cultured for 2 h at 37 °C to allow the osteoclasts to attach to the substrata. After cultivation for 2 h, 2 ml of α-MEM with FBS and L-gultamine supplemented with macrophage colony-stimulation factor (M-CSF) and TRANCE were added to the culture. The osteoclasts were further cultured for 6 and 24 h at 37 °C.

Assessment of osteoclast apoptosis Detection of osteoclast apoptosis was carried out using an in situ cell death detection kit (Roche Diagnostics).

Actin ring staining After cell culture, the cells were fixed with 4% paraformaldehyde. Actin filaments were stained with rhodamin-conjugated phalloidin.

Assessment of resorbing activity The cells were stripped by ultrasonication in 0.25M NH_4OH and the disks were dehydrated in graded ethanols, processed with critical point drying methods, and coated with platinum in a cold spatter coaster. The samples were examined by color laser microscopy (VK-9500, KEYENCE) to measure the morphology of resorption pits. For measurement, we selected 30 pits / disk at random. The depth and volume of each pit were measured by using an image analysis system linked to the laser microscope

RNA extraction and RT-PCR Expression of the following genes were analyzed by RT-PCR: carbonic anhydraseII (CAII), cathepsin K/OC2, TRAP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Results: ZnTCP induced a much number of apoptotic osteoclasts than TCP. After cultivation for 24 h, the rate of apoptotic osteoclasts on ZnTCP316 (7.6±2.9%) was significantly higher than TCP (1.2±0.4%). Osteoclasts cultured on ZnTCP less formed the actin rings than those cultured on TCP. After cultivation on ZnTCP633 for 24 h, 77.2% of osteoclasts showed ringed structure of podosomes composed of F-actin and other cytoskeletal proteins although 83% of osteoclasts on TCP showed the actin rings. ZnTCP633 significantly reduced the actin ring formation on osteoclasts compared to TCP. Total number of osteoclasts in the ZnTCP633 group became lower than that in the TCP group after 24 h although there is no significant difference after 6 h among the ZnTCP and TCP groups. The expressions of CAⅡ and cathepsin K/OC2 were significantly decreased in the ZnTCP633 group. The depth and volume of resorbed pits decreased significantly with an increase in Zn content of ZnTCP. Slight (less than 4%) in Zn, P and Ca concentrations of culture medium were observed among the TCP, ZnTCP316 and ZnTCP633 groups.

Discussion and Conclusions: ZnTCP directly suppressed the activity of mature osteoclasts attached to the ZnTCP through an increase in apoptosis, a reduction in actin ring formation, and down-regulation in expression of CAII and cathepthin K, without significant changes in expression of TRAP. These results supported the previous results that the bone resorption by osteoclasts at the bone-implant interface was inhibited by Zn in the composite ceramic consisting of hydroxyapatite and ZnTCP316. No significant increase in Zn concentration of the medium was observed. Taken together these findings, we hypothesize that resorbing osteoclasts that attached to ZnTCP locally accumulates zinc ions within the space defined by the clear zone, which in turn leads to down-regulation of CAII expression, actin ring disruption and apoptosis induction. Bone substitutes or a coating layer that contain ZnTCP would be promising with a property to counteract bone resorption at the interface for treating osteoporotic patients.