Introduction:
Reducing stability of bone-implant interface due to aseptic bone loss at the interface is a major problem for orthopedic implants used 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, bone resorption and osteoporosis therapy. In the previous study, almost no resorption was observed in tricalcium phosphate (TCP) when Zn is partially substituted for Ca in TCP to a level of 0.316 Zn wt% (ZnTCP316). A composite ceramic consisting of hydroxyapatite and ZnTCP316 has a higher level of stability in the bone-implant interface in rabbits than a composite ceramic consisting of hydroxyapatite and TCP even though the rabbits suffered osteopenia due to immobilization for more than 24 weeks. The results 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 cultured on ZnTCP in vitro.

Materials and Methods:
Experiments were performed in accordance with the guidelines of the Ethical Committee of the University of Tsukuba, National Institute of Advanced Industrial Science and Technology and the National Institute of Health guidelines for the care and use of laboratory animals (NIH Pub. No. 85-23 Rev. 1985).

Materials
TCP and ZnTCP disks with nearly identical physical properties were prepared by the sintering, at 1100 °C for 1 h, of TCP and ZnTCP powders. The compositions of the disks were 0 (TCP), 0.316 (ZnTCP316) and 0.633 (ZnTCP633) wt %. The disks were blindly randomized before experiments so that the classes of disks were unidentifiable to the experimenters.

Isolation of osteoclasts
Osteoclasts were isolated from the tibias, femurs, humeri, ulnas, and radius 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-glutamine 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 by DAPI staining for visualization of chromatin condensation and by TUNEL method, using an in situ cell death detection kit (Roche Diagnostics). Percentages of apoptotic cells were evaluated by calculating the rate of apoptotic osteoclasts to the total number of osteoclasts.

Actin ring staining
After cell culture, the cells were fixed with 4% paraformaldehyde. Actin filaments were stained with rhodamin-conjugated phalloidin. Number of osteoclasts having the actin rings were counted and compared with the total number of osteoclasts.

Assessment of resorbing activity
The cells were stripped by ultrasonication in 0.25M NH4OH and the disks were dehydrated in graded ethanols, processed with critical point drying methods, and coated with platinum in a cold spatter coater. The samples were examined by color laser microscopy (VK-9500, KEYENCE, Japan) 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 (Fig.1).

Discussion:
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 of expression of CAII and cathepsin 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. Further studies are necessary.

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INHIBITORY EFFECT OF ZINC IONS IN ZINC-CONTAINING TRICALCIC PHOSPHATE (ZNTCP) ON RESORBERING ACTIVITY OF MATURE OSTEOCLASTS

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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).

Statistical analysis
All date are presented as the mean ± SD. Statistical analyses were performed using a functional analysis of variance (ANOVA). The post-hoc test according to Fisher’s protected least significant difference allowed the assessment of significant difference. A p-value of less than 0.05 was considered to indicate statistically significant difference.

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 but not significant differences (less than 4%) in Zn, P and Ca concentrations of culture medium were observed among the TCP, ZnTCP316 and ZnTCP633 groups.

Discussion:
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 of expression of CAII and cathepsin 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. Further studies are necessary.