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Osteoclasts Resorb Protein-free Mineral (Osteologic™ Discs) Efficiently in the Absence of Osteopontin

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Abstract. *Osteopontin (OPN) is both a matrix protein in mineralized tissues and a cytokine, and it has a pivotal role in osteoclast-mediated bone resorption. Here, using a proprietary hydroxyapatite substitute for bone mineral (Osteologic™ discs), we investigated the requirement for OPN in mineral resorption. Resorption pits formed in the Osteologic™ discs, revealed by staining with silver nitrite (Von Kossa stain), were analyzed using the NIH Image J program, which can determine the number of pits formed per unit area, their average size, and the fractional area resorbed. After a preincubation of bone marrow cells from $OPN^{-/-}$ and $OPN^{+/+}$ mice with M-CSF to allow the multiplication of osteoclast precursors on cell culture plastic, osteoclast formation on both Osteologic™ discs and standard cell culture plates was induced with soluble receptor activator of NFÎ B ligand, sRANKL. We did not detect a dramatic difference in osteoclast formation between $OPN^{+/+}$ and $OPN^{-/-}$ cells, as judged by staining for tartrate-resistant acid phosphatase in osteoclasts formed on cell culture plastic, nor was there a significant difference in the ability of the osteoclasts to form resorption pits in the Osteologic™ discs. Additionally, none of six different anti-OPN monoclonal antibodies had a significant and reproducible effect on the formation or subsequent functioning of the $OPN^{+/+}$ osteoclasts. These studies suggest that, in contrast to what has been found for normal bone, the efficiency of dissolution of a ceramic, protein-free (excepting protein adsorbed from the culture medium) hydroxyapatite/tri-calcium phosphate substrate by osteoclasts is not substantially enhanced by endogenous or exogenous OPN.*

Osteopontin (OPN), a component of mineralized extracellular matrix, is a phosphorylated sialic acid-rich glycoprotein with an apparent molecular weight (on SDS-PAGE) ranging from 55-70 kDa (1). Found in all body fluids, it is also a cytokine that can bind various integrins and CD44 variants (2-4). Research has revealed important roles for OPN in immune cell function, cancer metastasis, and disease states associated with inflammation, pathological tissue calcification, and bone remodeling (5-8). Post-translational modifications of OPN (phosphorylation, glycosylation, sulfation) have been implicated in osteoclast-mediated bone resorption but not in the adhesion of osteoclasts to the bone (1, 9, 10). An intracellular form of OPN that co-localizes with CD44 has also been identified and implicated in osteoclast formation and function (11).

Osteoclasts are bone-resorbing cells that originate from the hematopoietic stem cells that are also the source of macrophage/monocytes. The characteristics of a mature functional osteoclast are multinucleation, production of tartrate-resistant acid phosphatase (TRAP), formation of ruffled borders and sealing zones, and the ability to resorb bone (12). Maturation of pre-osteoclasts into osteoclasts requires the presence of two cytokines, macrophage colony-stimulating factor (M-CSF) and Receptor Activator of Nuclear Factor Î B ligand (RANKL). M-CSF binds to its receptor c-Fms on osteoclast precursors, thus promoting their proliferation and survival (13, 14). Binding of RANKL to the receptor activator of nuclear factor Î B (RANK) receptor present on the M-CSF-expanded osteoclast precursors provides a signal for osteoclast differentiation and subsequent maturation *in vitro* (15, 16).

Abbreviations: OPN, osteopontin; M-CSF, macrophage colony stimulating factor; NFÎ B, nuclear factor kappa B; sRANKL, soluble receptor activator of NFÎ B ligand; KO, knockout; WT, wild-type; ERM, ezrin radixin moesin.

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Bone resorption involves the migration of osteoclast precursors to a bone surface where they fuse to form mature bone-resorbing osteoclasts (17). Osteoclast adhesion to bone is mediated in part by integrins interacting with bone matrix proteins such as collagen, bone sialoprotein and osteopontin in an arginine-glycine-aspartate (RGD)-dependent manner (18). The interaction of the integrins with bone proteins also generates various signals important for activation of the osteoclast (19). Each osteoclast creates a sequestered extracellular microenvironment (resorption lacuna) between the osteoclast and the bone surface delimited by a sealing zone, whose formation depends on signals emanating from the $\alpha_v\beta_3$ integrin (20). The sealing zones are rich in F-actin and surround the bone-facing surface of the polarized osteoclast, which is characterized by the formation of ruffled borders and focal adhesion-like structures known as podosomes. Hydrogen ions and proteases (acid-resistant cathepsins and matrix metalloproteinases) are secreted into this extracellular space to solubilize the mineral and to degrade collagen and other bone proteins. The resulting hydrolysis (*in vitro* on substrates such as dentine slices or Osteologic™ discs) forms pits by a process assumed to be equivalent to what occurs during bone resorption *in vivo*.

OPN is necessary for osteoclast motility and normal bone remodeling (7, 21). In addition to being found in the lamina limitans and cement lines of existing bone (22), it is also produced by osteoclasts and deposited into resorption pits (23). The RGD sequence in OPN mediates osteoclast attachment and spreading *via* a_vb_3, and possibly other, integrins (24, 25). Unexpectedly, studies of OPN-deficient mice revealed that the bones and teeth developed apparently normally in the absence of OPN (26, 27). However, subsequent studies on ovariectomized $OPN^{-/-}$ mice revealed reduced bone resorption in comparison with ovariectomized $OPN^{+/+}$ mice (28). $OPN^{-/-}$ mice also exhibit impaired bone resorption upon treatment with IL-1, an inflammatory cytokine known to strongly stimulate OPN expression and bone resorption in normal animals (29). Ectopic bone implantation experiments using $OPN^{+/+}$ and $OPN^{-/-}$ host mice showed that the presence of OPN in both the implanted bone disc and the host mouse was required for efficient resorption (30). Another study revealed a diminished response of OPN-deficient bones to parathyroid hormone (or RANKL plus M-CSF), a finding which was attributed to a defect in the recruitment of osteoclasts to OPN-deficient bones; however, the formation of osteoclasts was normal (31). It has also been reported that both a polyclonal anti-OPN antiserum and an RGD-containing peptide could reduce 1,25-$(OH)_2$D3 -induced osteoclastogenesis in mouse bone marrow cells cultured on plastic for 7 days (32). The RGD sequence in OPN is known to be involved in osteoclast attachment to the bone matrix and the subsequent cytoskeletal reorganization that occurs (9).

Thus, considerable evidence suggests that, in the absence of OPN, there is a defect in osteoclast-mediated bone resorption. Here we show, contrary to our initial expectation, that in a protein-free hydroxyapatite/tri-calcium phosphate model for bone mineral, OPN is not required for resorption of the mineral by osteoclasts. This result provides an intriguing insight into the phenomenon of bone mineral resorption and the role of OPN in that process.

Materials and Methods

Materials. Osteologic discs were obtained from B. D. Biosciences (Chicago, IL, USA); sRANKL was provided by Dr. Morris Manolson (University of Toronto, Canada); M-CSF was a gift of Dr. Richard Stanley (Albert Einstein College of Medicine); fetal calf serum was obtained from Hyclone Inc. (Logan, UT, USA); αMinimal Essential Medium (αMEM) was obtained from Gibco/BRL/Life Technologies (Grand Island, NY, USA); and penicillin, streptomycin, glutamine, and fungizone were procured from Invitrogen Lifescience, (CA, USA). Other chemicals were obtained from Sigma (St. Louis, MS, USA). Anti-OPN monoclonal antibodies and recombinant murine GST-OPN (made in *E. coli*) were purified at Rutgers University; bovine milk OPN was purified at the University of Aarhus, Denmark.

Primary bone marrow culture. Osteoclasts were derived from bone marrow of the femora, tibiae and humeri of 8 to 10-week-old wild-type and OPN-deficient mice following a procedure similar to that described by Okada *et al.* (33). Mice were maintained in the Nelson Laboratory animal facility at Rutgers University, that is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and is under the care of a board-certified veterinarian. The research with these mice was approved by the Rutgers Animal Care and Facilities Committee, protocol number 97:031. Briefly, mice were sacrificed by cervical dislocation and long bones (femora, tibiae, humeri) from wild-type and OPN-deficient mice were excised, dissected free of adherent tissue, and washed in phosphate-buffered saline (PBS). The marrow was flushed from the bones with serum-free DMEM. The marrow cells were collected by centrifugation (50xg at room temperature) and incubated with 5 ml of ice-cold 0.83% ammonium chloride in 10 mM Tris-HCl (pH 7.4) for 20 min to hemolyze the red blood cells (11). The cells were collected again by centrifugation (50xg at room temperature) and resuspended in αMEM with: 10% heat-inactivated FCS (HIFCS), 50 U/ml penicillin, and 0.5 μg/ml streptomycin, 1% glutamine, 1.25 μg/ml fungizone, and 3000 U/ml of recombinant human M-CSF-1. The cells were counted, plated on 100-mm bacteriological plates (Becton Dickinson, NJ, USA) (two per mouse) and cultured for 4-7 days at 37˚C in a humidified 5% CO_2 atmosphere. The medium was changed every 3 days to permit proliferation of adherent osteoclast precursors.

Osteoclastogenesis. After 4-7 days, the osteoclast precursor cells were trypsinized and replated in the wells of a 24-well cell culture dish and on Osteologic™ discs (also in the wells of a 24-well dish) at $1x10^6$ cells/well in αMEM-HIFCS-CSF-1 supplemented additionally with 80 ng/μl of sRANKL to promote cell fusion and osteoclast differentiation (at 37˚C in a humidified 5% CO_2 atmosphere). The medium was changed every 2 days over 7-14

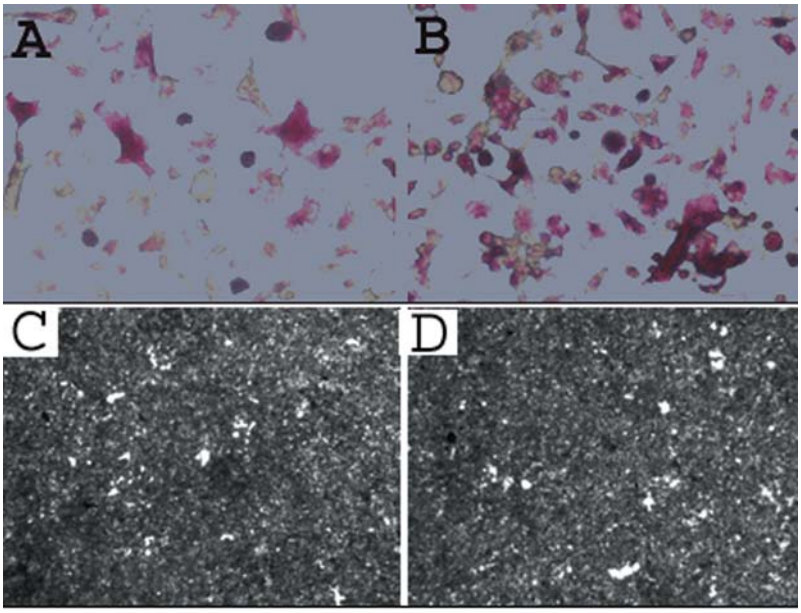

Figure 1. *Osteoclast formation and mineral resorption by $OPN^{+/+}$ and $OPN^{-/-}$ osteoclasts. Panels A and B show TRAP-stained osteoclasts derived from $OPN^{+/+}$ and $OPN^{-/-}$ marrow cells (original magnification, 40X). Panels C and D show portions of an Osteologic™ disc after incubation with osteoclasts for 1 week. The discs were stained with silver nitrite and photographed at a magnification of 40X. Below each figure are the results of the analysis of this image by the Image J program with the threshold set from 200 to 250.*

days. During this time, large multinucleated osteoclast-like cells could be seen developing by light microscopy.

Tartrate-resistant acid phosphatase (TRAP) staining. TRAP is a marker enzyme for mature functional osteoclasts. Following removal of the medium from the wells of the 24-well cell culture plates, the cells were washed twice with 1 ml of PBS. The cells were then fixed with 10% glutaraldehyde for 15 min at 37˚C and then stained for TRAP using 0.01 mg/ml of Naphthol AS-MX phosphate and 0.3 mg/ml Fast Red Violet in 0.1M sodium acetate buffer, pH 5.0. The percentage of TRAP-positive cells was determined using a standard light microscope at 40X magnification.

Von Kossa staining and image analysis of the osteologic™ discs. Cell culture medium was removed from the wells containing the Osteologic™ discs, and the wells were washed twice with 1 ml of distilled water. Then, 1 ml of bleach (Chlorox, 6% sodium hypochlorite and 5.2% sodium chloride) was added to each well, pipetted up and down to dislodge the cells and incubated for 5 min at room temperature. The wells were then washed with 2 ml of distilled water three times. The discs were examined by light microscopy (10X) and if cells were observed, bleach was added again and the process repeated to ensure that all the cells were dislodged. Once the cells were removed, the discs were stained with fresh 5% silver nitrite for 30 min. Each well was then washed four times with 1 ml distilled water to remove the silver nitrite and avoid brown-to-black precipitates from forming on the edge of the well. Each well was developed for 30 sec with 5% sodium carbonate prepared fresh in 25% formalin. The wells were again rinsed three times with distilled water and fixed with 5% sodium thiosulfate for 2 min. The discs were washed twice more with water and then viewed under the light microscope (10X). A well incubated without cells was used as a negative control. Subsequently, the discs were photographed using a digital camera (Nikon Diaphot 3000) at 40X magnification and the percentage resorption was calculated using the Image J analysis software available at the NIH website (http://rsb.info.nih.gov/ij). A threshold (200-255 pixels) was chosen for all the images so that only pits in which all the mineral had been resorbed appeared transparent. The software was then used to calculate the number of resorption pits, their average size and the percent area fraction resorbed.

Results

Impact of OPN-deficiency on osteoclast formation. After a period of M-CSF-induced proliferation of osteoclast precursors, the cells were plated either in the wells of a standard 24-well cell culture plate or on Osteologic™ discs in the wells of a 24-well dish. BD BioSciences (Bedford, MA, USA) describes the Osteologic™ disc as consisting of a bi-phasic bioceramic composite of calcium phosphates on a transparent quartz disc. The two phases are hydroxyapatite and alpha-tri-calcium phosphate in close molecular proximity to each other in a nano-

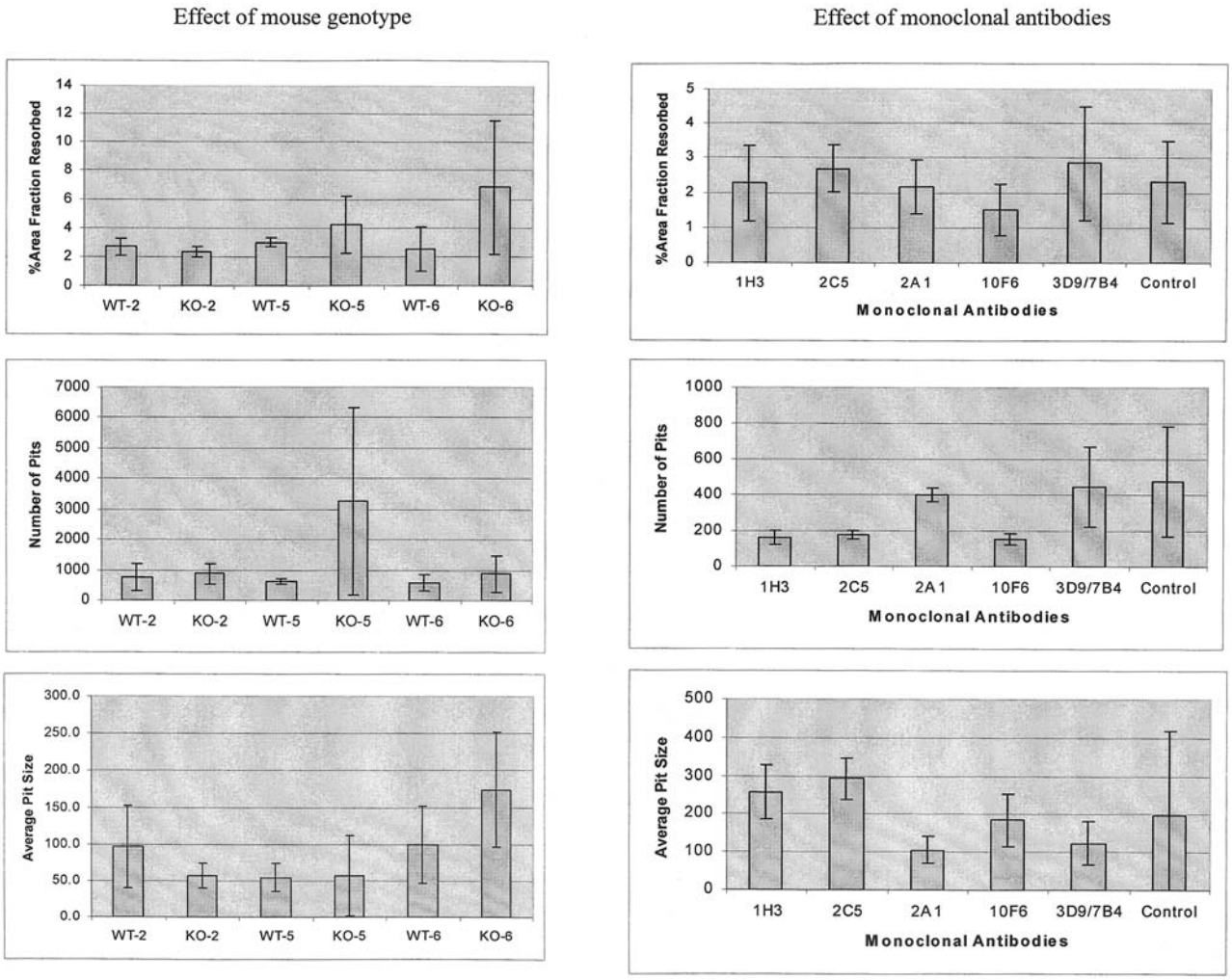

Figure 2. *Summary of the data obtained from an analysis of osteoclast action on Osteologic™ discs. The left three panels show, from top to bottom, the area fraction resorbed, the number of pits, and the average size of the pits. Results from three experiments (#2, #5, #6) comparing wild-type (WT, OPN$^{+/+}$) and knockout (KO, OPN$^{-/-}$) cells are shown. The right three panels also show, from top to bottom the area fraction resorbed, the number of pits, and the average size of the pits. This is an example of one of three experiments that could be analyzed for the consequences of allowing osteoclasts to form and then to function in the presence of 10 μg/ml of six different purified anti-OPN monoclonal antibodies. (The monoclonal antibodies 3D9 and 7B4 recognize the same epitope, Kowalski et al., unpublished data). The error bars (n=3 or 4) show the apparent standard deviations of the values obtained in these experiments. Analysis of the data, which were not normally distributed, for all three experiments by the non-parametric Mann Whitney Rank sum test established that the monoclonal antibodies did not have a significant effect on any of the parameters measured.*

crystalline subunit. After a further 8-15 days of incubation, the cells on the plastic cell culture wells were fixed and stained for TRAP activity to assess osteoclast formation; in parallel, the cells on the Osteologic™ discs were removed and the discs processed to assess osteoclast action. Figure 1 A and B show osteoclasts derived from OPN$^{+/+}$ and OPN$^{-/-}$ mice, respectively. The TRAP-positive osteoclasts derived from OPN$^{+/+}$ and OPN$^{-/-}$ mice did not appear to be very different in size or number, but no effort was made to quantify these parameters accurately since it was not a focus of this study.

Impact of OPN-deficiency on osteoclast function assessed on Osteologic™ discs. To test the resorption capability of osteoclasts derived from marrow cells of OPN$^{+/+}$ and OPN$^{-/-}$ mice, osteoclasts were cultured on Osteologic™ discs as described in the Methods section. Figure 1 C and D show typical light microscopic views of silver-stained Osteologic™ discs; also shown below each image are the analyses of these two figures by the Image J program with a threshold of 200. At this threshold, only areas where the mineral was completely resorbed are counted. A comparison of bone

resorption by osteoclasts derived from $OPN^{+/+}$ and from $OPN^{-/-}$ mouse bone marrow cells is shown in Figure 2 (left) for three separate experiments. The percent area fraction resorbed, the average size of the resorption pits, and the total number of pits formed were similar regardless of the genotype of the cells. (Fifty p^2 is equivalent to 440 μ^2.) Addition of exogenous OPN to the OPN-deficient cells did not have a consistently significant effect on the various parameters of osteoclast action (34). The two forms of OPN used in this study were bovine milk OPN, which is a highly phosphorylated form of OPN, and the unphosphorylated recombinant mouse GST-OPN made in *E. coli*.

Effect of monoclonal anti-OPN antibodies on the formation and function of osteoclasts derived from wild-type mice. Although the above results suggest that OPN is not required for efficient resorption of the mineral substrate, one cannot exclude the possibility that in the absence of OPN another protein compensated for its absence. To address that issue, the action of monoclonal antibodies to OPN on $OPN^{+/+}$ osteoclast formation and function was analyzed. Osteoclasts, stained for TRAP activity, appeared to form in a similar fashion on cell culture plastic regardless of the presence or absence of any of the monoclonal antibodies (34). Three separate experiments were performed to assess the effect of the monoclonal anti-OPN antibodies on the function (resorption of the mineral substrate in Osteologic™ discs) of osteoclasts derived from wild-type bone marrow cells. Figure 2 (right) shows the results of one of the experiments. Although in a few cases one or another of the monoclonals did appear to have a significant and compensating effect on pit size and number, detailed non-parametric analysis (the Mann Whitney Rank sum test) of all the data confirmed a lack of significance.

Discussion

Details of the mechanism of action of OPN in bone resorption remain elusive. It is clear, from the astute studies of Noda and his students, that bone remodeling is defective in the absence of OPN; there are deficits in both osteoclast and osteoblast function (7, 35). Complicating the elucidation of its primary role(s) in bone are the facts that it is produced by osteoblasts, osteocytes and osteoclasts, and it has both intracellular and extracellular roles. Intracellularly, it is found just inside the plasma membrane in a complex with ERM (ezrin, radixin, moesin) proteins, possibly the cytoplasmic domain of CD44, and cytoskeletal proteins (11). Extracellularly, it acts both as a matrix protein, enabling cell attachment to mineralized tissue and as a soluble cytokine, activating signal transduction pathways through various integrins and CD44 variants. OPN expression is increased in osteoblasts and osteocytes subjected to stress (3, 36-38), and it has been suggested that OPN is involved in sensing fluid flow by osteocytes (39).

Because OPN is required for the efficient resorption of bone, we anticipated that OPN would enhance the resorption of the mineral presented on the surface of the Osteologic™ discs. That it did not could be explained by the absence of bone proteins in the mineral, possibly Type I collagen. Kaartinen and colleagues have shown that OPN, especially when cross-linked by tissue transglutaminase, can bind to collagen Type I (40). We suggest that Type I collagen present in normal bone mineral mediates OPN enhancement of mineral resorption by osteoclasts.

Elegant studies in the laboratories of Sodek and Hruska have shown that OPN is required for normal CD44 expression and osteoclast migration. Suzuki *et al.* (11) described an intracellular form of OPN (iOPN) in osteoclasts that was present in lamellar structures and cell processes and appeared to be associated with the cytoplasmic domain of CD44 at the cell periphery and the base of filopodia. They noted that OPN-deficient osteoclasts were smaller, contained fewer nuclei and cell processes, exhibited less perimembranous CD44 staining, and were impaired in both their chemotactic response to M-CSF and their resorptive activity. Chellaiah *et al.* (21) reported that osteoclasts lacking OPN were hypomotile, exhibited reduced phagokinesis and chemotaxis, and were defective in their ability to form resorption pits on dentine; addition of exogenous recombinant OPN partially alleviated the defects. Their evidence suggested that OPN is an essential osteoclast motility factor, secreted at the baso-lateral surface (and also the ruffled border and clear zone) acting *via* $\alpha v\beta 3$ to stimulate a c-Src, phosphatidylinositol 3-kinase, and Rho-dependent signaling pathway that increases expression of CD44.

The formation on plastic of cells staining positive for TRAP in this work was qualitatively similar regardless of the genotype ($OPN^{+/+}$ or OPN-/-) of the cells or whether osteoclast formation was induced in the presence or absence of one of the anti-OPN monoclonal antibodies. There was considerable variability in the number and size of the pits formed on the Osteologic™ discs as well as the area resorbed. Despite this, the three experiments comparing $OPN^{+/+}$ and $OPN^{-/-}$ osteoclasts indicated that, if OPN does enhance the resorption of the mineral on the Osteologic™ disc, it is only to a minor extent. In contrast, Suzuki *et al.* (11) did find that the $OPN^{-/-}$ were less efficient than $OPN^{+/+}$ osteoclasts at resorbing the mineral on Osteologic™ discs. One difference between their experiments and ours is that in their experiments the discs had been precoated with BSA, BSP or OPN.

The mineral in the Osteologic™ discs differs from the mineral in bone and dentine in that it lacks an integral protein component, notably Type I collagen. OPN may

bind to bone mineral, but not to the protein-free mineral on Osteologic™ discs, in such a way as to enhance mineral resorption. Mineral associated with collagen probably interacts with osteoclasts in a different way than it does when not associated with collagen. Perhaps the formation of tight sealing zones on bone depends on both the mineral and protein components; possibly the formation of sealing zones may be partially defective in the absence of collagen. Gene expression in osteoclasts bound to the mineral on the Osteologic™ discs may differ from gene expression in osteoclasts bound to normal bone, which is rich in Type I collagen. Type I collagen has been reported to increase the abundance of the mRNAs encoding both alkaline phosphatase and OPN in UMR106-06 rat osteosarcoma cells (41).

In conclusion, these experiments show that the resorption by osteoclasts of protein-free mineral (excluding proteins deposited on the surface from the serum-containing medium or by adherent cells) is not enhanced by OPN.

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