Parathyroid Hormone-induced Bone Resorption Does Not Occur in the Absence of Osteopontin*

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Osteopontin is an RGDS-containing protein that acts as a ligand for the α3β1 integrin, which is abundantly expressed in osteoclasts, cells responsible for bone resorption in osteopenic diseases such as osteoporosis and hyperparathyroidism. However, the role of osteopontin in the process of bone resorption has not yet been fully understood. Therefore, we investigated the direct function of osteopontin in bone resorption using an organ culture system. The amount of 45Ca released from the osteopontin-deficient bones was not significantly different from the basal release from wild type bones. However, in contrast to the parathyroid hormone (PTH) enhancement of the 45Ca release from wild type bones, PTH had no effect on 45Ca release from organ cultures of osteopontin-deficient bones. Because PTH is located upstream of receptor activator of NF-κB ligand (RANKL), that directly promotes bone resorption, we also examined the effect of RANKL. Soluble RANKL with macrophage-colony stimulating factor enhanced 45Ca release from the bones of wild type fetal mice but not from the bones of osteopontin-deficient mice. To obtain insight into the cellular mechanism underlying the phenomena observed in osteopontin-deficient bone, we investigated the number of tartrate-resistant acid phosphatase (TRAP)-positive cells in the bones subjected to PTH treatment in cultures. The number of TRAP-positive cells was increased significantly by PTH in wild type bone; however, no such PTH-induced increase in TRAP-positive cells was observed in osteopontin-deficient bones. These results indicate that the absence of osteopontin suppressed PTH-induced increase in bone resorption via preventing the increase in the number of osteoclasts in the local milieu of bone.

Osteoclastic bone resorption is a key event in the pathophysiology of osteopenic diseases such as osteoporosis and hyperparathyroidism. Osteoclastic bone resorption involves a number of sequential events, including differentiation and activation of osteoclasts. These processes are regulated both by key cytokines and hormones interacting with various cell surface receptors and by interactions of osteoclast precursors with osteoblasts/stromal cells (1–3). The α3β1 integrin is a major receptor on the osteoclast that can interact with RGD sequence-containing ligands such as osteopontin and vitronectin (4). It possibly promotes osteoclast attachment to bone and, when engaged by either immobilized or soluble ligands, stimulates signaling pathways that regulate osteoclast migration and function. Bone matrix consists of about 90% type I collagen and about 5% noncollagenous proteins. Among these noncollagenous proteins, at least five proteins, osteopontin, bone sialoprotein, thrombospondin, fibronectin, and vitronectin, contain RGD sequences that can be recognized by some integrins (5–8). These noncollagenous bone proteins are candidate ligands for the α3β1 integrin expressed on the osteoclasts. In vitro data suggest that the β3 integrin subunit is involved in the attachment of osteoclasts to osteopontin and bone sialoprotein, whereas the β1 integrin subunit is responsible for the attachment of these cells to fibronectin (9). With regard to osteoclastic bone resorption in vitro, interactions between osteopontin and/or bone sialoprotein and the α3β1 integrin (10, 11) have been proposed to play a crucial role. The signaling pathways stimulated by the integrin lead to modulation of cytoskeletal reorganization via regulatory molecules such as gelsolin (12). A recent study also indicates that the β3 integrin is important in osteoclastic bone resorption in vivo (13).

Osteopontin (14), is a mineralized matrix protein and a cytokine that has been suggested to act in several types of organs and systems, including bone (15), the immune system (16), the vascular system (17), and kidney (18). In addition, osteopontin is expressed at sites of inflammation. Osteopontin modifies cell behavior (19, 20) and alters gene expression. In mineralized tissues, osteopontin is produced by osteoblasts and osteoclasts and has been proposed to play a key role in both types of cells (21–25). In bone, osteopontin is particularly concentrated in cement lines and the lamina limitans (26). However, the role of osteopontin in bone has not yet been fully elucidated.

Recently, osteopontin-deficient mice and osteopontin/vitronectin double-null mutant mice have been created (27, 28). Although these animals show normal development, we recently found that the lack of osteopontin makes osteopontin-deficient mice resistant to ovariectomy-induced bone resorption in vivo, in an established animal model of postmenopausal osteoporosis (29). How-
ever, the mechanism through which osteopontin deficiency protects bone is not understood. Namely, whether osteopontin deficiency directly causes a reduction in bone resorption or not has yet been known. To elucidate the mechanism of osteopontin action, we investigated bone resorption in organ cultures in which osteoelastic activity was stimulated by PTH.1

MATERIALS AND METHODS

Reagents—Recombinant soluble RANKL was provided by Snow Brand Milk Products Co. (30). Recombinant murine M-CSF was purchased from R&D Systems (Minneapolis, MN). Human PTH was obtained from National Hormone & Peptide Program (Rockville, MD) and dissolved in 1 M acetic acid containing 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO). The osteopontin-deficient mice were produced as described by Rittling et al. (28).

Organ Cultures—Either wild type or osteopontin-deficient mice at the ages of 3–10 months in a background of 129/S3 × C57BL/6 F2 were mated, and 17- or 18-day pregnant (dated from time of sperm-positive vaginal smears) mice were injected with 50 μCi of 45Ca (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) as calcium chloride as described previously (31). 24 h later, the 18- or 19-day post coitum embryos were removed, and the radii and ulnae were dissected out under a binocular microscope. The cartilaginous ends of the long bones were cut off, and the shafts were rinsed in PBS with antibiotics. The sections of the cartilages were cultivated on pieces of filter membrane (Millipore) placed in a 96-well plate (Corning Glass, Corning, NY) containing 50 μl of α-modified minimum essential medium (α-MEM, Sigma-Aldrich, Steinheim, Germany) supplemented with 5% serum prepared from the mother mice of the corresponding genotype. The bones were preincubated for 1 day, and then they were subsequently cultured in fresh media for 6 days under an atmosphere of 5% CO2 at 37°C with one change of half of medium on day 3. In each experiment, bones from the right and left side of the embryos were collected. The bone from one side was used as an experimental group and treated with either PTH (10−11 M) or soluble RANKL (100 ng/ml) plus M-CSF (10 ng/ml), and the other was used as untreated control.

Bone Resorption Assay—When the culture terminated, the amount of 45Ca released from the bones into the medium was measured by counting the radioactivity in the medium. The radioactivity in the media was quantified using a scintillation counter. Although fetal bovine serum (FBS) has been used in the organ culture system, we replaced FBS with mouse serum obtained from the mothers at the time of sacrifice; this was to exclude the influence by the possible presence of osteopontin in FBS. Therefore, the radioactivities in the media used for the cultures were determined to essentially the same background levels. The bone-resorbing activity was estimated using the culture media taken on day 6 of the organ cultures according to the following formula. Typically, the amount of radioactivity in the mother mouse’s serum was less than 10% of the amount released from the bone as follows: 45Ca released into 50 μl of medium = [45Ca activity in 20 μl of sample medium × 2.5 – 45Ca activity in 2.5 μl (5%) serum sample] × 2. Values in calcium 45 counts in dpm at the 6-day period of contralateral side limb bones, which were treated with vehicle, were defined as 100%. The equation is as follows. The calcium 45 counts in dpm at the 6-day period from bone cultures treated with PTH or RANKL were divided by the calcium 45 counts released into the cultures of vehicle-treated bone specimens.

Results—The bones cultured in the presence or absence of RANKL and M-CSF for 6 days were fixed in 4% paraformaldehyde, followed by soft x-ray examination using FUJI industrial FR film (Minami-Asagiri City, Kanagawa, Japan). The distance between the anode to film was set to be 540 mm, and the exposure time, current, and voltage were 120 s, 3 mA, and 15 kV, respectively. The radiographs of the bones were subjected to densitometry, which was based on the digitalization of the image pixels whose intensity was coded on the gray scale with 256 levels (corresponding to 8 bits) using a digital image processor. The threshold was set at level 50 of the 256 gray scale. The number obtained by integration of the counts of the levels of the density scales (over 50) was divided by the area of bone to yield bone density. To make sure that the comparisons of relative bone density by digital morphometric analysis was not influenced by the differences in bone density at different anatomical sites, we have compared the entire bone of the forelimbs of the mice used for the organ cultures for this quantification. Thus, in doing this type of analysis, the x-ray picture of the whole cultured bone was taken, and the density of the cultured bone was measured. The values for that density were normalized against the size (area) of the bones.

Osteoclast Formation in Spleen Cell Culture—Spleens from 12-month-old osteopontin-deficient mice or wild type mice were used as described (32) to make a suspension of spleen cells in α-MEM containing 10% FBS (Life Technologies, Gaithersburg, MD). Briefly, spleen cells were smeared out through the opening of the capsule made at the distal end of the spleen, were subjected to pipetting, and were spun down to form a pellet. The cells were resuspended in 1 ml of ice-cold double-distilled water for less than 10 s to remove reticulocytes, immediately resuspended in 10 ml of medium supplemented with 10% FBS, and spun down. The cells were resuspended again in FBS-supplemented medium and filtered through a cell strainer before estimating the viable cell number based on trypan blue exclusion method using one volume of an aliquot of the cell suspension mixed with 3 volumes of the dye solution. Spleen cells at 3.5 × 105 cells/cm2 were plated into 6-well plates (Corning Costar Co., Cambridge, MA) and were cultured in the presence of 30 μl soluble RANKL and 10 ng/ml M-CSF. Cells were maintained (under an atmosphere of 5% CO2, at 36°C) in 4.0 ml of α-MEM/FBS for up to 11 days. Following incubation for the appropriate period, the cultures were fixed and stained histochemically for the osteoclast-associated enzyme TRAP (30). TRAP-positive multinucleated (cells containing more than three nuclei) cells were counted at 100× magnification in 40 rectangular fields of 41.6 mm2 in each of the wells. In separate cultures, spleen cells were cultured in the presence of 100 ng/ml soluble RANKL and 30 ng/ml M-CSF on dentin slices placed in a 96-well plate in the media supplemented with 5% wild type serum or osteopontin-deficient mouse serum. Some cells were subjected to RNA isolation and PCR analyses.

Immunofluorescence Examinations—for immunofluorescence examinations, spleen cells were prepared as described (32) and were cultured on glass coverslips. On the next day of the plating, the cells on the coverslips were rinsed with PBS twice and fixed for 10 min at room temperature with 4% paraformaldehyde. The cells were rinsed with PBS twice and were subjected to membrane permeabilization for 2 min with 0.1% Triton X-100. After rinsing with PBS twice, the cells were incubated for 30 min with 1% bovine serum albumin to block nonspecific antibody binding. The cells were fixed at 4°C in 4% paraformaldehyde for 10 min at room temperature with 1:200 dilution of anti-CAS or Src antibodies followed by the treatment with second antibodies conjugated with Alexa to visualize the fluorescein isothiocyanate fluorescence (33). The cells were subsequently stained for actin-fibers by using rhodamin-phallolidin (Molecular Probe Inc.) and were also stained for TRAP.

Osteoclast Number in Forelimb Bone—At the end of the culture period of PTH experiments, bones were rinsed in PBS, fixed for 7 days in 4% paraformaldehyde acid, and decalcified by 10% EDTA, pH 7.4, for 3 days. Serial sections were prepared from paraffin blocks at 7-μm thickness and were stained for TRAP (tartrate resistant acid phosphatase) activity, with Alcian blue for counter-staining. The TRAP-positive cells with more than three nuclei were counted using fifteen 7-μm-thick serial sections from each bone. Each of the fifteen sections was taken at five-section intervals from a total of 75 sections per bone. The total number of osteoclasts per bone was obtained by summing the number in all fifteen sections. For RANKL experiments, both mononuclear and multinucleated TRAP+ cells were counted.

RT-PCR—RT-PCR reactions were carried out according to the standard method using 10× PCR buffer (Takara, Japan), 1.25 mM dNTP, primer 3, primer 4, cDNA, and Taq polymerase. Based on preliminary experiments, the cycle number was optimized to be 35 cycles. PCR primers for osteopontin and GAPDH were as follows. OPNEX5, 5′-TCCAATTGAAGCGACCC-3′, OPNXUT5, 5′-GAAGGCTGCTGGTACACATGCA-3′. The bands of osteopontin were 331 bp. The GAPDH primer was purchased from system Science Co. (Hokkaido, Japan). GAPDH Sense, 5′-ACACGTCATTCGACACCATC-3′; GAPDH antisense, 5′-TCCACACCCCTGCCTGTCGTA-3′. The bands were examined after fractionation in 1% agarose gel stained with ethidium bromide.

Statistical Analysis—The statistical significance of the data was evaluated by using Fisher’s protected least significant difference or Mann-Whitney’s U test.

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1 The abbreviations used are: PTH, parathyroid hormone; RANKL, receptor activator of NF-κB ligand; M-CSF, macrophage-colony stimulating factor; PBS, phosphate-buffered saline; α-MEM, α-modified minimum essential medium; FBS, fetal bovine serum; RT, reverse transcription; PCR, polymerase chain reaction; CAS, CRK-associated substrate; TRAP, tartrate-resistant acid phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s).
RESULTS

We examined whether organ cultures of bones derived from osteopontin-deficient fetal mice differed from those from wild type mice with respect to their response to PTH, which is known to activate bone resorption by osteoclasts (31, 34). The basal levels of $^{45}$Ca release from the organ cultures of the wild type and osteopontin-deficient bones were similar. As reported previously, PTH treatment increased resorption of the wild type fetal bones by about 40% as judged by the amount of $^{45}$Ca release (Fig. 1). In contrast, $^{45}$Ca release from the osteopontin-deficient bones in organ culture was not enhanced by the treatment with PTH.

PTH binds to receptors expressed on osteoblasts and/or stromal cells and enhances expression of membrane-bound RANKL, which in turn binds to its receptor, receptor activator of NF-$\kappa$B, on osteoclasts or their precursors, to enhance osteoclast development and/or osteoclast activity (35–37). It is also known that PTH suppresses osteopontin expression in osteoblasts (38). Therefore, we examined whether absence of bone resorption resulting from osteopontin deficiency was between PTH and RANKL expression or downstream of RANKL. Soluble RANKL in combination with M-CSF promoted development of TRAP$^+$ cells in osteopontin-deficient spleen cells at levels comparable to those in wild type cells (Fig. 2). Therefore, osteopontin deficiency does not affect osteoclastogenesis induced by RANKL. To examine the effect of osteopontin deficiency on RANKL-induced bone resorption, we tested whether RANKL stimulated bone resorption by measuring $^{45}$Ca release in the organ culture assay. Treatment for 6 days with soluble RANKL in combination with M-CSF enhanced $^{45}$Ca release by about 40% in wild type bones. In contrast, treatment with soluble RANKL and M-CSF did not increase $^{45}$Ca release from the osteopontin-deficient bones (Fig. 3).

To evaluate morphologically the effect of soluble RANKL and M-CSF on bones in organ culture, soft x-ray pictures of the bones were taken and bone density was quantitatively determined. The bone density of untreated group was similar between control wild type and the control osteopontin-deficient bones. The density of wild type bones was decreased by the treatment with soluble RANKL and M-CSF. However, the density of osteopontin-deficient bones was not affected by the treatment with soluble RANKL and M-CSF (Fig. 4, A and B). These observations substantiated those obtained in $^{45}$Ca release assays.

To obtain insight into the mechanism underlying the defect in $^{45}$Ca release resulting from osteopontin-deficiency at cellular levels, we conducted a histological examination of the bones cultured for 6 days in the presence or the absence of PTH (Fig. 5A). We counted TRAP-positive cells that were observed in the forelimb bones. The number of TRAP-positive cells containing three or more nuclei was similar between wild type and osteopontin-deficient bones when cultured in the absence of PTH. In wild type bones, the number of TRAP-positive cells was higher in the bones treated with PTH than that in the control

FIG. 1. PTH-induced $^{45}$Ca release is impaired in osteopontin-deficient bones. $^{45}$Ca-labeled bones were cultured in the presence of $10^{-7}$ M PTH or vehicle for 6 days, and the levels of $^{45}$Ca in the medium were measured. Data are expressed as ratios against that in wild type control, which was treated with vehicle alone (acetic acid 0.15% and bovine serum albumin 0.1%). Columns and bars are mean ± S.E., respectively. Data represent the values obtained from a total of 28 bones ($^{\text{WT, PTH}(-)}$, 7 bones; $^{\text{WT, PTH}(+)}$, 7 bones; $^{\text{OPN-KO, PTH}(-)}$, 7 bones; $^{\text{OPN-KO, PTH}(+)}$, 7 bones). These experiments were performed three times with similar results. The significance of the data was evaluated using Wilcoxon’s paired signed-rank U test. The asterisk indicates a significant difference from the control, $p < 0.05$.

FIG. 2. Effect of RANKL on the differentiation of wild type and osteopontin-deficient spleen cells into TRAP-positive cells. The spleen cells were cultured in the presence soluble of RANKL and M-CSF. TRAP-positive multinucleated cells (with more than three nuclei) were counted. The data are expressed as mean ± S.E. Statistical significance of the data was evaluated as described under "Materials and Methods."
However, no such increase in the number of TRAP-positive cells was observed in osteopontin-deficient bones even after culture in the presence of PTH (Fig. 5B). Similarly, RANKL treatment increased TRAP-positive cells in wild type but not in osteopontin-deficient bones in organ culture (data not shown).

To estimate the effect of wild type serum, which would contain soluble osteopontin on TRAP(+) MNC formation, we cultured wild type or osteopontin-deficient spleen cells on dentin slice in the presence of soluble RANKL and M-CSF in medium. In this setting, the presence of osteopontin in wild type spleen cells cultured in the medium supplemented with wild type serum allowed RANKL-induced development of TRAP-positive multinucleated cells more efficiently than osteopontin-deficient spleen cells cultured in the medium supplemented with osteopontin-deficient mice serum (Fig. 6). These observations suggest that osteoclastic precursors exist in osteopontin-deficient bone tissue but require osteopontin either from themselves and/or from the serum to mature into TRAP-positive multinucleated cells.

**Fig. 4.** The reduction in bone density induced by RANKL/M-CSF does not occur in the absence of OPN. Forelimb bones were cultured for 6 days in the presence or absence (Control) of RANKL (30 ng/ml) and M-CSF (10 ng/ml). Quantification (A) of the bone density by image analysis was conducted by densitometry of soft x-ray photographs (B). Columns and bars are mean ± S.E., respectively. Five bones from each group were used for the measurement. The density of the bone is the sum of all pixel intensities (units) composing the spot of a defined area in the bone. The asterisk indicates a significant difference from the control, p < 0.05.

**Fig. 5.** Quantification of the TRAP(+) cell numbers in cultured bones. Multinucleated TRAP(+) cells in the bones treated with PTH or vehicle (A) were counted (B). The number of TRAP(+) cell was determined as described under “Materials and Methods.” Data are expressed as means ± S.E. Wild type (WT), control, three bones (a); WT, PTH-treated (PTH), three bones (b and c; e is the higher magnification of b) with an inset indicating a TRAP (+) osteoclast; OPN-KO, control, three bones (c); OPN-KO, PTH-treated (PTH), four bones (d). Arrows indicate multinucleated TRAP(+) cells. The asterisk indicates significant difference against the control, p < 0.05.
We further tested whether RANKL regulates the expression of osteopontin gene in spleen cells. As indicated in Fig. 7, RANKL and M-CSF treatment suppressed osteopontin gene expression. This is a specific effect, because GAPDH levels were not altered by the treatment with RANKL and M-CSF. This observation suggests the presence of a negative feedback system possibly to keep the balance of bone resorption.

Finally, we investigated the expression and localization of signaling molecules known to be involved in regulation of osteoclastic function. The expression of Src and CAS and their intracellular localization as well as actin cytoskeleton pattern were similar between the wild type and osteopontin-deficient osteoclasts developed from the spleen cells treated with RANKL and M-CSF (Fig. 8).

**DISCUSSION**

Our experiments indicate that PTH-induced bone resorption does not occur in osteopontin-deficient bones. Although previous ovariectomy experiments indicated that osteopontin-deficiency protected bone against bone resorption, the mechanism of this protection was not clear. Our organ culture data clearly indicate that bone resorption induced by PTH in the local milieu of bone does not occur in the absence of osteopontin, indicating that the protection of bone against bone resorption was via a direct action of osteopontin in bone but not an indirect systemic effect.

PTH exerts its regulatory effects on calcium homeostasis in part by stimulating the release of calcium from bone (31, 34). This PTH-stimulated bone resorption involves at least two cell types: osteoblasts and osteoclasts (34, 40). The binding of PTH to its receptors on osteoblasts initiates several events that activate osteoclasts and stimulate bone resorption (34, 40). These include enhancement of RANKL expressions, which promote both the proliferation and differentiation of osteoclast precursors and the activation of mature osteoclasts (41, 42). We therefore investigated which PTH-induced events were deficient in the absence of osteopontin. We observed that soluble RANKL with M-CSF did not increase ⁴⁵Ca release in osteopontin-deficient bones, indicating that the critical point where osteopontin is required lies downstream of RANKL action.

We further investigated the cellular basis for the mechanism of protection against bone resorption by osteopontin deficiency and found that, in parallel to the requirement for osteopontin in bone resorption induced by PTH in a bone organ culture system, the increase in osteoclast number in the bones in organ cultures in response to PTH treatment also required osteopontin. We also observed that osteopontin-deficient spleen cells or bone marrow cells could give rise to osteoclasts in vitro in the presence of vitamin D (27) or RANKL and M-CSF (Fig. 2). The osteoclasts developed from either wild type or osteopontin-deficient spleen cells by the treatment with RANKL revealed similar expression and localization of signaling and attachment molecules including Src, CAS, vinculin, and actin. These results indicate that the development of osteoclasts per se is not impaired in osteopontin-deficient bones; rather the specific signaling pathways activated by PTH and RANKL, resulting in an increase in the number of...
osteoclasts in bone, are inhibited in the absence of osteopontin in these organ cultures.

This notion was also supported by the reduction in the RANKL-induced TRAP(+)-MNC formation on the dentin slice in the osteopontin-deficient spleen cells cultured in the serum lacking osteopontin, supplemented with either wild type mouse serum or osteopontin-deficient mouse serum. Crossing combinations of the genotypes of spleen cells and those of serum may yield further information on the roles of osteopontin from the different sources. Human serum osteopontin levels range from 16 to 64 ng/ml according to Harris (39) and our semi-quantitative measurements of mouse serum have yielded similar results (data not shown).

It appears that, either as a solid phase (osteopontin coating the slide) or as a soluble protein, exogenous osteopontin can assist the formation of TRAP+ cells from osteopontin-deficient spleen cells via RANKL treatment. Therefore, endogenous production of osteopontin may not be necessary to develop TRAP+ cells at least in such in vitro situation, where osteoclastogenesis is fully activated by the presence of relatively high concentrations of RANKL and M-CSF. As mentioned above, analysis on the mechanism that requires osteopontin to convey PTH signal to induce final bone resorption revealed that osteopontin is not required in the pathway between PTH and RANKL but rather downstream of RANKL, because RANKL activation of bone resorption in the organ cultures was also impaired. Thus our current hypothesis is that PTH-induced RANKL signaling resulting in either an increase in osteoclast number and/or activation is disrupted by the absence osteopontin.

To estimate the activity of osteopontin-deficient TRAP-positive cells, we examined pit formation on hydroxypatite-coated slide glass after stimulation with 1,25(OH)2, -vitamin D3 and dexamethasone. Significant reduction (p < 0.05) was observed in the number of pits in the case of osteopontin-deficient TRAP-positive cells compared with wild type (Y. Muguruma and M. Noda, data not shown) in two out of four independent experiments. These observations suggest that osteopontin is required for the activity of osteoclasts as well. Obviously, it is possible that the effect on resorption needs a more detailed analysis on bone or dentin to better determine whether the observed change in hydroxyapatite removal represents true changes in resorption or another change in cell dynamics such as motility and phagocytic activity.

In the bone organ culture environment, which was used in our experiments, bone matrix does not contain osteopontin. Therefore, it is still possible that the absence of osteopontin in bone matrix may prevent rudimentary osteoclastic precursors derived from blood from homing and residing in bone tissue (while the bones were in the body).

Because osteopontin-deficient mice have been reported to grow without any retardation, it is possible that chondroclasts, which are responsible for the resorption of calcified cartilage matrix, may be functioning normally. Based on our observation on the defects in PTH-induced bone resorption in osteopontin-deficient bones in organ culture, it is possible that osteoclasts, which resorb bones, may be more susceptible to the osteopontin deficiency in the case of situations with accelerated bone resorption. If the growth plate chondroclasts may not be affected by the absence of osteopontin, it may provide differential properties to bone and cartilage with regard to its effects on the two resoring cells, i.e. chondroclasts and osteoclasts, respectively.

It is intriguing to compare in vivo and in vitro situations in osteopontin-deficient mice. Bone resorption appears to be normal in osteopontin-deficient mice at birth. In the organ cultures of osteopontin-deficient mice bones, PTH and RANKL signaling were not able to develop TRAP-positive multinucleated cells. It appears that certain molecules supplied from the tissues outside bones may compensate for the lack of osteopontin in in vivo situation. However, in organ cultures, such supply of compensatory molecules from tissues outside bones may not be available. Thus, as shown in the present data, osteopontin-deficient bones cannot respond to PTH and RANKL with regard to the development of TRAP-positive multinucleated cells as well as calcium 45 release. Our data also suggest that RANKL treatment suppressed osteopontin expression in spleen cell cultures, suggesting the presence of a certain negative feedback system.

In conclusion, our observations indicate that osteopontin is
directly required for bone resorption activated by PTH-RANKL axis via increasing the number of osteoclasts in the microenvironment of bone.

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