Osteoprotegerin Abrogated Cortical Porosity and Bone Marrow Fibrosis in a Mouse Model of Constitutive Activation of the PTH/PTHrP Receptor

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Intracortical porosities and marrow fibrosis are hallmarks of hyperparathyroidism and are present in bones of transgenic mice expressing constitutively active parathyroid hormone/parathyroid hormone-related protein receptors (PPR*Tg). Cortical porosity is the result of osteoclast activity; however, the etiology of marrow fibrosis is poorly understood. While osteoclast numbers and activity are regulated by osteoprotegerin (OPG), bisphosphonates suppress osteoclast activity but not osteoclast numbers. We therefore used OPG and bisphosphonates to evaluate the extent to which osteoclasts, as opposed to bone resorption, regulate marrow fibrosis in PPR*Tg mice after treatment of animals with vehicle, OPG, alendronate, or zoledronate. All three agents similarly increased trabecular bone volume in both PPR*Tg and control mice, suggesting that trabecular bone resorption was comparably suppressed by these agents. However, the number of trabecular osteoclasts was greatly decreased by OPG but not by either alendronate or zoledronate. Furthermore, intracortical porosity and marrow fibrosis were virtually abolished by OPG treatment, whereas alendronate and zoledronate only partially reduced these two parameters. The greater reductions in cortical porosity and increments in cortical bone mineral density with OPG in PPR*Tg mice were associated with greater improvements in bone strength. The differential effect of OPG versus bisphosphonates on marrow fibrosis, despite similar effects on trabecular bone volume, suggests that marrow fibrosis was related not only to bone resorption but also to the presence of

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In bone remodeling, a balance between bone formation by osteoblasts, which are cells of mesenchymal origin, and bone resorption by osteoclasts, which are cells of hematopoietic origin, allows for maintenance of skeletal and mineral homeostasis. Failure to coordinate the processes of formation and resorption results in porotic¹ or sclerotic bone.^{2–4} Parathyroid hormone (PTH) is a major regulator of bone homeostasis that positively modulates bone formation and bone resorption. Hyperparathyroidism is characterized by increased bone turnover, cortical porosity, and expansion of fibroblastoid cells in bone (marrow fibrosis).^{5–7} Cortical porosity is the result of intracortical bone resorption, which is increased with hyperparathyroidism,⁸ and also with intermittent⁹ or continuous¹⁰ PTH administration. Increased cortical porosity is associated with decreased bone strength, and may contribute to the increased fracture risk in patients with hyperparathyroidism. The mechanisms leading to the expansion of fibroblastoid cells in the bones of patients with hyperparathyroidism are not well understood. Expansion of fibroblastoid cells in trabecular areas is also observed in other conditions of high bone turnover such as Paget's disease¹¹ and fibrous dysplasia.^{12,13} Transgenic mice expressing constitutively active PTH/parathyroid hormone-related protein (PTHrP) receptors on cells of the osteoblast lineage (PPR*Tg)¹⁴ also have severe cortical porosity and expansion of fibroblastoid cells in trabecular bone areas, with increased number of osteoclasts in both trabecular and cortical areas. Of note, the marrow fibrosis

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described in conditions of high bone turnover is not related to the fibrotic process observed in some hematological diseases and typically characterized by the accumulation of silver staining positive fibers.¹⁵

Osteoclasts differentiate from precursors belonging to the monocyte/macrophage lineage. Macrophage colony stimulating factor and receptor activator of nuclear factor kappa B ligand (RANKL) are essential for osteoclast differentiation and are produced by cells of the osteoblast lineage.¹⁶ These same cells also secrete a glycoprotein called osteoprotegerin (OPG),¹⁷ a soluble member of the tumor necrosis factor receptor superfamily that acts as a decoy receptor for RANKL and prevents its interaction with the cognate receptor RANK expressed on precursor and mature osteoclasts. RANKL is essential for osteoclast formation, function and survival,¹⁸ and each of these activities are prevented by OPG. The dependence of osteoclasts on RANKL for their differentiation and survival probably explains why OPG and other RANKL inhibitors can markedly reduce osteoclast numbers.¹⁹ Bisphosphonates such as alendronate (ALN) and zoledronic acid (ZOL) are potent inhibitors of bone resorption that selectively accumulate on mineral surfaces, are subsequently internalized by osteoclasts, and impair osteoclast activity.²⁰ Despite clear evidence for antiresorptive effects of bisphosphonates in animal and human studies, bisphosphonates frequently do not reduce and can even increase osteoclast numbers.^{19,21–24} We sought to exploit the differential effects of OPG versus bisphosphonates on osteoclast numbers to determine whether osteoclasts per se, or their resorptive activity, are related to the pathophysiology of bone marrow fibrosis. This question was addressed by treating PPR*Tg mice with the RANKL inhibitor OPG, or with the bisphosphonates ALN or ZOL for 3 months. This study design also provided the opportunity to compare for the first time the effect of these agents on cortical porosity and bone strength in a murine model of high bone turnover downstream of the PTH/PTHrP receptor.

Materials and Methods

Mice and Materials

Generation of PPR* Tg mice was reported previously.¹⁴ Genotyping was performed as described.¹⁴ OPG-Fc (OPG), ALN (EMD Chemicals, Darmstadt, Germany), and ZOL (Zometa, Novartis, Cambridge, MA) were provided by Amgen Inc. OPG-Fc is a truncated form of human OPG (amino acids 22 to 194) fused to the Fc domain of human IgG1 and produced in Chinese hamster ovary cells.²⁵

In Vivo Experiments

All studies were approved by Massachusetts General Hospital's institutional animal care committee. Three-month-old male wild-type and PPR*Tg mice were subcutaneously injected with vehicle (VEH) (0.9% saline), or human OPG-Fc (3 mg/kg, three times/week), ALN (0.5 mg/kg, once/week), or ZOL (0.1 mg/kg, once/week) di-

luted in vehicle, over 3 months. Pilot studies demonstrated that these ALN and ZOL dosing regimens caused maximal to near-maximal increases in tibial bone mineral density (BMD) in young growing rats (Amgen, data on file). The human OPG-Fc dosing regimen was the minimal dose that would be predicted to overcome immune responses in immunocompetent mice. Sample numbers were wild-type (Veh:*n* = 50, OPG:*n* = 15, ALN:*n* = 25, ZOL:*n* = 10), PPR*Tg (Veh:*n* = 60, OPG:*n* = 16, ALN:*n* = 24, ZOL:*n* = 11). Mice were sacrificed by cervical dislocation at the end of each treatment period. Sera were collected to evaluate levels of biochemical markers of bone turnover. Tibiae and femurs were also collected for histology, immunohistochemistry, microCT, histomorphometric analyses, and biomechanical testing.

Histological Analysis

Left hindlimbs were fixed in 10% formalin and stored as previously described.²⁶ Hindlimbs were decalcified in 20% EDTA, and paraffin blocks were prepared by standard histological procedures. For selected samples, tartrate-resistant acid phosphatase (TRAP) staining was performed using an acid phosphatase detection kit (Sigma-Aldrich Corp., St. Louis, MO). Sample numbers were wild-type (Veh:*n* = 6, OPG:*n* = 7, ALN:*n* = 5, ZOL:*n* = 5), PPR*Tg (Veh:*n* = 6, OPG:*n* = 6, ALN:*n* = 5, ZOL:*n* = 5). Silver staining for reticular fibers was performed on selected samples using a reticulum staining kit (NewcomerSupply, Middleton, WI). Samples were wild-type (Veh:*n* = 2) and PPR*Tg (Veh:*n* = 2).

Immunohistochemistry

Tibiae of wild-type and PPR*Tg mice were fixed in 4% paraformaldehyde at 4°C for 48 hours. After decalcification with 20% EDTA at 4°C for 7 days, paraffin blocks were prepared by standard procedures. The sections were deparaffinized and antigen retrieval was performed using proteinase K (20 µg/ml, Roche, Mannheim, Germany), followed by 10 minutes treatment with 3% H₂O₂ to block endogenous peroxidase. The presence of endothelial cells were detected using anti-mouse CD31 antibody (BD Biosciences, San Jose, CA) at a dilution of 1:200 for 1 hour at room temperature, and the TSA Biotin system (Perkin-Elmer, Boston, MA) according to the manufacturer's protocol.

Histomorphometry

Right tibiae were fixed in 10% formalin, and then embedded in methylmethacrylate as previously described.²⁶ Five-micrometer sections were cut, deplasticized and stained using the trichrome method for calcified tissues. Histomorphometric analysis was done with an Osteomeasure system (Osteometrics Inc., Decatur, GA), using standard procedures²⁶ and in a blinded fashion. Trabecular bone was analyzed in the metaphyseal region of tibia 340 µm below the growth plate and the following parameters were measured: trabecular bone volume, trabecu-

lar spacing, osteoblast number, and osteoclast number. Osteoclasts were morphologically defined as multinuclear cells immediately adjacent to bony sections. Sample numbers were wild-type (Veh: $n = 7$, OPG: $n = 6$, ALN: $n = 6$, ZOL: $n = 6$), PPR*Tg (Veh: $n = 7$, OPG: $n = 6$, ALN: $n = 6$, ZOL: $n = 6$). Marrow fibrosis or stromal cell area was measured on paraffin sections. Stromal cells were identified as elongated, fibroblastoid mononuclear cells that, differently from hematopoietic cells, had a high ratio cytoplasm/nucleus and, differently from terminally differentiated osteoblasts, were not immediately adjacent to bony surfaces. Of note, area of stromal cells rather than their number was quantified as trabecular regions were often completely packed with these cells, which made challenging to count them individually. Sample numbers were wild-type (Veh: $n = 6$, OPG: $n = 7$, ALN: $n = 5$, ZOL: $n = 5$), PPR*Tg (Veh: $n = 6$, OPG: $n = 6$, ALN: $n = 5$, ZOL: $n = 5$).

MicroCT Analysis

BMD and microarchitecture were determined from right femurs using a desktop μ CT system (GE eXplore Locus SP Specimen Scanner; GE Health care, London, Ontario, Canada). Whole femurs were scanned and images were reconstructed to an isotropic voxel size of 13.2 μ m.²⁷ The three-dimensional images were reoriented relative to anatomical axes based on the femur midshaft and posterior aspect of the condyles, and analysis regions were selected within the distal femur and central femur. For the distal femur analysis, a region equivalent to 10% of the femur height was selected proximally from the growth plate within the cortex. The cancellous region was analyzed for region volumetric bone mineral density (vBMD) (no threshold), bone volume per tissue volume (BVf), trabecular spacing, and structural model index. Due to the wide range of BVf across groups, a threshold was determined for each trabecular region using an algorithm that calculated a value based on the bimodal density distribution of marrow and bone. For the cortical diaphyseal analysis, a central region equivalent to 10% of the height of the femur was selected, with contours drawn at the endocortical and periosteal surfaces. Cortical geometry and BMD were assessed using a single threshold (667 mg/ml), and cortical porosity was measured using a higher threshold (750 mg/ml).

Measurement of Biomechanical Strength of the Femur Diaphysis

Destructive three-point bend tests were performed at the right femur midshaft with a displacement rate of 6 mm/min (span length = 7 mm); maximum load, stiffness, and energy to failure data were generated (MTS 858 Mini Bionix II, FlexTest v3.5B software; MTS Systems Corp., Eden Prairie, MN). Intrinsic properties were calculated from data on whole bone extrinsic properties and micro-CT based cortical geometry. Tissues were stored frozen at -20°C and thawed to ambient temperature before testing.

Measurement of Ionized Ca^{++} , TRAP5b, and Osteocalcin

Ionized calcium was measured by the Ciba/Corning 634 Ca^{++} /pH analyzer on terminal sera. Terminal sera were evaluated for TRAP5b and osteocalcin levels using commercially available kits ($n = 8$ to 12 per group). TRAP5b in mouse serum samples was measured using solid-phase immunofixed enzyme activity assay (SBA Sciences, Turku, Finland). The mouse osteocalcin assay used a Luminex antibody-immobilized microbead platform (Linco Research - Millipore, St. Charles, MO). Both TRAP5b and osteocalcin measurements were analyzed using curve-fitting software (Softmax Pro, Molecular Devices, Sunnyvale, CA). Sample numbers were wild-type (Veh: $n = 30$, OPG: $n = 8$, ALN: $n = 12$, ZOL: $n = 10$), PPR*Tg (Veh: $n = 29$, OPG: $n = 9$, ALN: $n = 8$, ZOL: $n = 11$).

Statistics

All statistical analyses were performed in GraphPad Prism v5.01 or Microsoft Excel. The phenotypic effect of PPR transgenic overexpression was assessed using a two-tailed t -test between untreated wild-type and PPR*Tg controls. The treatment effect was examined in wild-type and PPR*Tg mice separately using one-way analysis of variance and Bonferroni post-tests. For histomorphometry analyses, one-way analysis of variance with Tukey's multiple comparison test was used, with $P < 0.05$ accepted as significant; error bars represent means $\pm$ SEM.

Results

PPR*Tg Mice Display Severe Intracortical Porosity and Marrow Fibrosis

In agreement with our previous findings in bone specimens from younger mice,¹⁴ micro-CT analysis of femurs isolated from 6-month-old PPR*Tg mice showed persistent and severe intracortical porosity and dramatic increase of trabecular bone, when compared with wild-type littermates (Figure 1, A–F). Similarly, histology demonstrated persistent and considerable expansion of a fibroblastoid population in these mice, which was also seen in younger PPR*Tg mice. This marrow fibrosis was clearly evident within intracortical pores and in the trabecular areas of bone specimens from transgenic animals (Figure 2, C–D), but not in the bones from wild-type animals (Figure 2, A–B). Of note, silver staining failed to show any accumulation of reticular fibers in the bone marrow of PPR*Tg mice (data not shown), which is consistent with the notion that “marrow fibrosis” in high bone turnover states is clearly distinct from the “marrow fibrosis” present in some hematological disease such as idiopathic myelofibrosis and typically characterized by the accumulation of reticular fibers.¹⁵

A more detailed histological characterization of the intracortical porosities and of the marrow fibrosis observed in PPR*Tg mice was done. TRAP staining showed

Figure 1. Micro-CT images of femurs of 6-month-old wild-type (WT) and PPR*Tg mice. Longitudinal images of entire femurs (**A** and **B**), and axial images of diaphysis (**C** and **E**) and of metaphysis (**D** and **F**) are shown.

that bone surfaces adjacent to the fibroblastoid population were characteristically lined by osteoclasts, in both the intracortical pores and in the trabecular areas (Figure 2, E–F). Immunostaining for CD31, a classical marker for endothelial cells, confirmed the concomitant presence of blood vessels in the same areas (Figure 2, G–H). Notably, these features, namely concomitant presence of fibroblastoid cells, blood vessels and cells of the monocyte/macrophage lineage, are also typical of the granulation tissue that forms during the wound healing process.²⁸ The biological relevance of this similarity requires further investigation.

Collectively, these findings indicate that expression of a constitutively active PTH/PTHrP receptor in cells of the osteoblast lineage led to a “trabecularization” of cortical bone that persisted with age. Moreover, they demonstrate that the histology of the intracortical porosities with the characteristic presence of fibroblastoid cells, osteoclasts, and blood vessels, was qualitatively similar to what observed in the trabecular areas of the same transgenic bones.

*OPG, but Not ALN or ZOL, Abrogated Intracortical Porosity and Bone Marrow Fibrosis in PPR*Tg Mice*

Three-month old PPR*Tg male mice and their control littermates were treated with either vehicle, OPG, or ALN or ZOL for 3 months. Femurs and tibiae were then analyzed by microCT and histomorphometry, respectively. As previously reported,¹⁴ femurs from PPR*Tg mice were significantly shorter than femurs from age- and sex-matched wild-type mice (Figure 3, A–B); to this end, we do not have a good explanation for the finding, but we believe it is secondary to modest level of expression of the transgene in chondrocytes as previously reported.¹⁴ Treatment with either OPG, ALN, or ZOL, resulted in mild osteopetrosis, as documented by the further accumulation of cartilaginous remnants in the metaphyseal regions of tibiae from both mutant and wild-type mice (Figure 3C), but did not significantly affect bone length and/or shape in either mutant or wild-type mice (Figure 3, A–C). These findings suggest that each of the three regimens reduced bone resorption without causing detectable changes in bone growth.

MicroCT analysis of cortical bone at the mid-diaphysis showed that untreated PPR*Tg mice had severe intracortical porosity and a net 50% increase in cortical thickness (Figure 4, A and F, and data not shown). Consistent with the increased cortical porosity, cortical vBMD was significantly reduced in femurs of PPR*Tg mice (Figure 4E), while cortical area was significantly increased (Figure 4D). Moreover, femurs of PPR*Tg mice were narrower than those of wild-type mice, with a significant reduction in both periosteal and endocortical perimeters (Figure 4, B–C).

OPG treatment fully rescued the intracortical porosity of PPR*Tg mice (Figure 4, A and F). ALN reduced intracortical porosity by 39%, whereas ZOL did not cause any significant change (Figure 4F). In agreement with these

Figure 2. Histology (**A–D**), TRAP staining (**E** and **F**), and immunohistochemistry (**G** and **H**) of paraffin sections of decalcified tibias. H&E staining of cortical bone (**A** and **C**) and of trabecular bone (**B** and **D**). Note the presence of fibroblastoid cells as indicated by the **arrowheads** in both the cortical and trabecular bone area of PPR*Tg mouse (magnification = original $\times 20$). **E** and **F**: TRAP staining of cortical (**E**) and trabecular bone (**F**) of PPR*Tg mouse. Sections were counterstained with H&E. **Arrows** indicate the TRAP-positive osteoclasts (magnification = original $\times 40$). **G** and **H**: Immunohistochemistry for CD31 of cortical (**G**) and trabecular bone (**H**) of PPR*Tg mice. Sections were incubated with CD31 antibody and the antibody binding was detected by DAB. The sections were counterstained with H&E. Double **arrowheads** indicate the blood vessels positive for CD31 (magnification = original $\times 40$).

Figure 3. Effect of OPG, ALN, and ZOL treatment on long bones. **A:** Longitudinal micro-CT images of femurs from 6-month-old wild-type (WT) (top panels) and PPR*Tg (bottom panels) mice. **B:** Femur lengths of wild-type and PPR*Tg mice treated with VEH, OPG, ALN, or ZOL were measured by micro-CT. $^zP < 0.05$ vs. ZOL; error bars represent SEM. **C:** H&E stained tibiae of 6-month-old wild-type (top panels) and PPR*Tg (bottom panels) mice treated with VEH, OPG, ALN, or ZOL (magnification = original $\times 4$).

findings, a significant increase in cortical vBMD was observed in OPG-treated PPR*Tg mice, whereas ZOL did not significantly affect cortical vBMD in these mice and ALN only modestly augmented it (Figure 4E). Regression analysis demonstrated a strong correlation between cortical porosity and cortical vBMD in PPR*Tg mice, independent of treatment group (Figure 4G). Although no treatment significantly altered periosteal perimeter in mutant femurs, OPG and ALN did result in significantly greater endocortical perimeter and thereby reduced cortical thickness (data not shown), consistent with a normalizing effect on the mutant phenotype. Despite these changes, no treatment in either phenotype significantly altered cross-sectional moment of inertia, a surrogate of bending strength (data not shown).

However, the altered cortical microstructure in PPR*Tg mice did result in significant reductions in bone strength parameters. Femurs from vehicle-treated PPR*Tg mice had lower peak load and energy to failure than femurs from wild-type mice (Figure 5, A and B). OPG treatment resulted in significant increases in these two parameters in PPR*Tg mice compared with vehicle controls, whereas ALN or ZOL had no significant effect (Figure 5, A and B). When peak load and energy to failure were corrected for cortical geometry, the resulting intrinsic properties ultimate strength and toughness graphs were very similar in appearance (Figure 5, C and D). Ultimate strength was positively correlated with cortical vBMD (Figure 5E) and toughness was negatively correlated with intracortical porosity (Figure 5F) in femurs from PPR*Tg mice.

The effects of OPG, ALN, and ZOL on marrow fibrosis were also evaluated. OPG treatment resulted in complete abrogation of bone marrow fibrosis in the trabecular compartment of PPR*Tg mice, as shown by both routine histology and histomorphometric analysis (Figure 6, A and B). Conversely, both ALN and ZOL treatment caused a 55% reduction in the fibroblastoid population in the trabecular area (Figure 6, A and B).

Collectively, these data indicate that OPG, differently from bisphosphonates, abrogated both the intracortical porosity and the marrow fibrosis observed in PPR*Tg mice, and improved the strength of bones from transgenic animals.

OPG, ALN, and ZOL Increase Trabecular Bone Volume Comparably in PPR*Tg Mice

In agreement with our previous findings in younger mice,¹⁴ analysis of trabecular bone by microCT showed that both the BVF and the vBMD remained dramatically increased, while trabecular spacing was reduced in 6-month-old PPR*Tg mice in comparison with wild-type littermates (Figure 7, A–D). Moreover, trabeculae from transgenic mice were more plate-like in structure compared with trabeculae from wild-type controls, as indicated by their significantly lower structural model index (Figure 7E). Histomorphometric analysis of the proximal tibia confirmed the microCT findings of trabecular bone volume and spacing at the distal femur (Figure 7, F and G).

OPG treatment dramatically augmented trabecular bone mineral density and trabecular bone volume, with concomitant decrease of trabecular spacing, in both PPR*Tg mice and wild-type littermates (Figure 7, A–G). Unlike the intracortical porosity and bone marrow fibrosis findings, ALN and ZOL also augmented trabecular bone density and volume in both PPR*Tg and wild-type mice to an extent comparable with that with OPG (Figure 7, A–G). Notably, the number of osteoblasts in the trabecular compartment of transgenic mice was not significantly affected by OPG, ALN, or ZOL treatments. Conversely, osteoblast number was positively modulated by all three treatments in the wild-type mice, though these effects were modest and at the limit of statistical significance (Figure 6C). Moreover, circulating serum levels of osteocalcin, a marker of osteoblast activity, were significantly

Figure 4. Micro-CT analysis of femoral cortices of 6-month-old wild-type (WT) and PPR^{Tg} mice treated with VEH, OPG, ALN, or ZOL. **A:** Representative two-dimensional micro-CT axial images of the femoral diaphysis. **B:** Periosteal perimeter. **C:** Endocortical perimeter. **D:** Cortical area excluding the cortical porosity. **E:** Cortical vBMD. **F:** Cortical porosity. **G:** Regression analysis of cortical porosity and cortical vBMD. $r^2 = R$ -squared value. For each measurement, central region equivalent to 10% of the height of the femur was selected distal from the midpoint. Images shown represent the median value of cortical porosity for each group. * $P < 0.01$ vs VEH; $^ZP < 0.05$ vs. ZOL; $^ΔP < 0.05$ vs. ALN. Error bars represent SEM.

decreased by each of the three drugs in both mutant and wild-type mice (Figure 6D). Dynamic parameters of bone histomorphometry could not be accurately assessed due to the dramatic increase in bone volume in the specimens from the transgenic mice and the lack of a clear separation of the fluorochrome lines, very likely secondary to reduced bone remodeling (data not shown).

Altogether, these findings suggested that, at least in bone from transgenic mice, the dramatic increase of trabecular bone volume observed with OPG, and ALN and ZOL treatments was likely secondary to comparable inhibition of trabecular bone resorption.

Effect of OPG, ALN, and ZOL on Osteoclast Number and Activity

We then analyzed osteoclast number and activity. Total osteoclast number was significantly higher in PPR^{Tg} mice compared with the wild-type mice, as shown by TRAP staining and histomorphometric analysis (Figure 8, A and B). Notably, marrow fibroblastoid cell expansion correlated with total osteoclast number in VEH-treated PPR^{Tg} mice (Figure 8D); this correlation was still present,

albeit weaker, when all three treatments were taken into consideration (Figure 8D). OPG treatment dramatically decreased osteoclast number both in PPR^{Tg} and wild-type mice (Figure 8B). Conversely, neither ALN nor ZOL treatment significantly affected osteoclast number (Figure 8B). However, circulating levels of TRAP5b were equally decreased by each of the three drugs in PPR^{Tg} mice, which suggested a similar effect of OPG, ALN, and ZOL on bone resorption (Figure 8C). The results in wild-type mice were less clear, with higher serum TRAP5b than in vehicle-treated PPR^{Tg} mice, and no effect of the antiresorptives on TRAP5b levels in wild-type animals (Figure 8C). The reasons for these differences in TRAP5b levels and response to antiresorptives in wild-type mice versus PPR^{Tg} mice are not clear. Notably, serum levels of ionized Ca^{++} were virtually identical across the different experimental groups (data not shown).

Discussion

Intracortical porosity and marrow expansion of fibroblastoid cells are hallmarks of hyperparathyroidism.^{5,6,7} Cor-

Figure 5. Effect of OPG, ALN, and ZOL treatment on bone strength at the femur diaphysis. Destructive 3-point bending tests were performed at the femur midshaft. **A:** Peak load and **(B)** energy to failure, **(C)** ultimate strength, and **(D)** toughness of femur diaphysis are shown. **E:** Regression analysis demonstrating the correlation of ultimate strength and cortical vBMD **(F)** and that of toughness and cortical porosity in PPR*Tg mice. * $P < 0.05$ vs VEH-treated PPR*Tg mice. $^Z P < 0.05$ vs ZOL-treated PPR*Tg mice. Error bars represent SEM.

tical porosity is related to cortical bone resorption, both of which are increased by recombinant RANKL²⁹ and by genetic ablation of the RANKL inhibitor OPG.³⁰ Persistently elevated serum PTH causes concentration-dependent increases in cortical porosity in rats,⁸ a finding that might be related to the ability of continuous PTH infusion to increase RANKL while decreasing OPG.³¹ Hyperparathyroid states are also associated with significant bone marrow fibrosis, the etiology of which is not well understood. Data from the current study demonstrated for the first time that recombinant OPG could fully correct both the marrow fibrosis and the cortical porosity associated with constitutive activation of PTH/PTHrP receptors in PPR*Tg mice. Unique pharmacodynamic features of OPG, compared with other antiresorptive agents, resulted in differential effects on osteoclasts in these mice that provided potential insights into the pathogenesis of marrow fibrosis.

It is important to highlight that the PPR*Tg model cannot be considered a classical model of hyperparathyroidism, due to the selective expression in a subset of cells of the osteoblast lineage of a mutant PTH/PTHrP receptor that constitutively activates only the cAMP pathway.¹⁴ Although the bone phenotype of PPR*Tg mice recapitulates classical features of a hyperparathyroid bone, such as augmented intracortical porosities and marrow fibrosis, it also lacks the defective mineralization and the presence of fibroblasts surrounding unmineralized extracellular matrix on the endocortical surface that are typical of a hyperparathyroid bone, at least in humans.³² Moreover, the modest augmentation of trabecular bone occasionally observed in humans with hyperparathyroidism³³ is not comparable with the dramatic increase of trabecular bone volume in PPR*Tg mice. These are all interesting and important differences that could be secondary, at

least in part, to the systemic action of PTH in hyperparathyroidism versus the local and selective expression of the transgene in PPR*Tg mice.

RANKL inhibition consistently results in marked reductions in osteoclast numbers, while bisphosphonates can significantly reduce bone resorption without reductions in osteoclast numbers.^{19,21–24} This difference was reflected in the current dataset, wherein OPG treatment of PPR*Tg mice reduced osteoclast numbers by 95%, while the bisphosphonates ALN and ZOL caused no significant reduction. However, serum TRAP5b, a marker of bone resorption, was similarly suppressed in PPR*Tg mice treated with OPG, ALN, or ZOL. This finding suggested that the doses of each drug used in this study probably did not cause significant differences in bone resorption at the systemic level. In support of this notion, OPG, ALN, and ZOL each caused similar increases in trabecular bone volume fraction (by micro-CT) and in trabecular bone volume (by histomorphometry).

The main histological difference observed in the trabecular compartment between PPR*Tg mice treated with these agents was virtually complete abrogation of bone marrow fibrosis (SCA/T.Ar) with OPG, versus 55% more modest reductions with ALN or ZOL. This finding implies that osteoclasts are critically important in the pathogenesis of marrow fibrosis, and it suggests that, if bone resorption is similarly affected across the three experimental groups, osteoclasts per se, in addition to osteoclast-dependent bone resorption, might contribute to the establishment or persistence of marrow fibrosis in PPR*Tg mice. Consistent with an important role of osteoclasts, stromal cell volume was positively correlated with osteoclast number in vehicle-treated PPR*Tg mice. This correlation was weaker when all treatment groups were

Figure 6. Effect of OPG, ALN, and ZOL treatment on marrow fibrosis and osteoblast number in trabecular areas of tibias. **A:** Histology of trabecular bone areas of VEH-, OPG-, ALN-, and ZOL-treated wild-type (WT) (top panels) and PPR^{Tg} (bottom panels) mice (H&E staining). **Arrows** indicate areas of fibroblastoid cells; (magnification = original $\times 20$). **B:** Measurement of stromal cell area per total area (SCA/T.Ar). **C:** Osteoblast number per bone surface (Ob/B.S.). **D:** Serum osteocalcin level. * $P < 0.05$ vs VEH; ** $P < 0.01$ vs VEH; ^ $P < 0.05$ vs ALN. Error bars represent SEM.

combined, which would be expected if the suppression of bone resorption provided some contribution to reduced marrow fibrosis. Furthermore, continuous infusion of PTH can result in extensive bone marrow fibrosis within of a week of initiating treatment, without significant increases in osteoclast number.³⁴ It is thus possible that PTH may cause marrow fibrosis with a variety of mechanisms beyond increases in osteoclast numbers, and these mechanisms might depend on dosage, modality, and duration of PTH exposure.

Bone resorption by osteoclasts releases cytokines such as transforming growth factors and fibroblast growth factors from the bone matrix, which are growth factors for cells of the osteoblast lineage.^{35,36} Pre-osteoblasts of fibroblastic origin have been implicated as a potential source of proliferative cells that account for marrow fibrosis in a rat model of hyperparathyroidism,³⁴ but the nature of signals that stimulate these cells is unknown. Blocking the release of bone-derived growth factors by suppressing bone resorption could be one of the molecular mechanisms responsible for reduced marrow fibrosis in our study. Other mechanisms, however, could be involved as well, since ALN and ZOL, which probably suppressed trabecular bone resorption as effectively as did OPG, did not reduce fibrosis as effec-

tively as OPG. It is therefore possible that osteoclasts favor the expansion of fibroblastoid cells either by direct contact with these cells, or, more likely, by secreting factors that facilitate fibroblastoid proliferation.³⁷ This hypothesis is supported by the observation that osteoclasts were still present in large numbers after administration of either ALN or ZOL, whereas these cells had been virtually eliminated by OPG treatment.

Expansion of a fibroblastoid population of cells is also observed in fibrous dysplasia, which is characterized by focal bone lesions composed primarily of stromal cells. Multinucleated osteoclasts are present in these lesions, probably as a consequence of increased release of interleukin-6 by stromal cells.³⁸ Bisphosphonates have been used clinically in patients with fibrous dysplasia but with inconsistent results. These drugs are reported to be effective against bone pain, but do not significantly improve the radiological findings.^{12,39,40} In agreement with our findings, these reports suggest that blocking bone resorption may not be sufficient for the treatment of the bone lesions in fibrous dysplasia.

The differential effect of OPG versus bisphosphonates on osteoclast numbers has been previously described,¹⁹ and there are at least two potential mechanisms for this difference. OPG prevents osteoclast differentiation,¹⁷

Figure 7. Analysis of trabecular bone in 6-month-old wild-type (WT) and PPR*Tg mice treated with VEH, OPG, ALN, or ZOL. **A–E:** Micro-CT analysis of distal femurs: **(A)** Representative three-dimensional micro-CT images of the central 0.5 mm region of trabecular bone. **B:** Trabecular volumetric bone mineral density (vBMD). **C:** Bone volume fraction (BVf). **D:** Trabecular spacing (Tb.Sp.) and **(E)** structural model index. Images shown represent median values of BVf for each group. **F** and **G:** Bone histomorphometry of trabecular bones of proximal tibiae. **F:** Bone Volume/Total Volume and **(G)** Tb.Sp. * $P < 0.05$ vs VEH; ** $P < 0.01$ vs VEH; ^ $P < 0.05$ vs ALN; ^ $P < 0.05$ vs ZOL. Error bars represent SEM.

which could have decreased the replenishment of mature osteoclasts in PPR*Tg mice. OPG also potently stimulates osteoclast apoptosis, which has been attributed to decreased osteoclast numbers in treated mice.⁴¹ The antiresorptive effects of bisphosphonates have been attributed to the inhibition of mature osteoclast activity rather than inhibition of osteoclast differentiation.⁴² Bisphosphonates have also been shown to suppress the activity of osteoclasts without causing apoptosis.⁴³ Data from the current study suggest that healthy osteoclasts in vehicle-treated PPR*Tg mice, and dysfunctional osteoclasts in bisphosphonate-treated PPR*Tg mice, could have provided pathophysiological signals that contributed to the maintenance and further stimulation of marrow fibrosis.

This was among the first studies to directly compare a RANKL inhibitor and bisphosphonates in a model of cortical porosity. Cortical porosity in PPR*Tg mice was fully corrected by OPG, but not by ALN or ZOL. The RANKL inhibitor denosumab was also shown to cause significant reductions in cortical porosity in adult ovariectomized cynomolgus monkeys, whereas alendronate did not.⁴⁴

However, other studies under different conditions have demonstrated that ALN⁴⁵ and ZOL⁴⁶ are capable of reducing cortical porosity. It is therefore important to determine under clinical conditions whether RANKL inhibitors and bisphosphonates differentially impact cortical porosity. The importance of observed differences in porosity in the current study pertains primarily to the hypothetical mechanisms by which these agents suppressed marrow fibrosis. Greater suppression of cortical porosity with OPG implies greater reductions in cortical bone resorption, which could explain greater reductions in marrow fibrosis independent of reductions in trabecular osteoclast numbers.

None of the three antiresorptive agents affected osteoblast number, as shown by histomorphometry, but each of them decreased osteoblast activity, as documented by the reduced levels of circulating osteocalcin, a marker of fully differentiated osteoblasts. Whereas it is well established that osteoblasts modulate osteoclast number and activity by secreting molecules that are essential for osteoclast differentiation from hematopoietic precursors

Figure 8. Effect of OPG, ALN, and ZOL treatment on osteoclast number and activity. **A:** TRAP staining. Representative pictures of trabecular bones of wild-type (WT) and PPR⁺Tg tibiae. Sections were counterstained with H&E. **Arrows** indicate osteoclasts (magnification = original $\times 40$). **B:** Osteoclast number per total area (Oc/T.Ar.). **C:** Serum level of TRAP5b. **D:** Correlation of Oc/TA and SCA/T.Ar. in VEH-, OPG-, ALN-, and ZOL-treated PPR⁺Tg mouse tibiae. Graphs (a) and (b) indicate the regression analysis of Oc/TA and SCA/T.Ar. in VEH-treated PPR⁺Tg group and VEH-, OPG-, ALN-, ZOL-treated PPR⁺Tg group respectively. (a) $y = 0.0869x + 1.044$, $R^2 = 0.7715$ (b) $y = 0.0354x + 2.069$, $R^2 = 0.257$. * $P < 0.05$ vs VEH; ** $P < 0.01$ vs VEH; $^{\#}P < 0.05$ vs ZOL. Error bars represent SEM.

and for their survival, a putative role for osteoclasts in controlling the osteoblast pool is less certain. In this regard, experimental evidence has been reported showing that indeed bone resorption could be, at least in part, a prerequisite for bone formation.⁴⁷ Our findings suggest that OPG treatment blunts osteoblast activity, but surprisingly does not affect osteoblast number, despite the dramatic abrogation of marrow fibrosis. More studies will be necessary to understand the biological bases of this interesting dichotomy. In any event, to this end we cannot exclude the possibility that the impaired osteoblast activity also contributed to the abrogation of the fibroblastoid population that we observe in OPG treated mutant mice.

Fracture risk is elevated in patients with primary hyperparathyroidism⁴⁸ and with fibrous dysplasia.⁴⁹ The reported twofold increase in forearm fracture risk in hyperparathyroidism patients supports our finding that elevated PTH signaling results in increased cortical fragility. The increased cortical porosity may mediate this increased fracture risk, as cortical porosity has been reported to be inversely related to the strength and toughness of human cortical bone.^{50,51} Decreased cortical density may also play a role, and BMD has been positively associated with human cortical bone strength.^{52,53,54} In PPR⁺Tg mice, the reductions in whole

femur strength were not caused by global alterations in cortical geometry, but rather by reductions in the intrinsic material properties of the bone matrix. Similar to the human data, the intrinsic property toughness was negatively correlated with cortical porosity, while ultimate strength was positively correlated with cortical BMD. The normalization of the femoral cortex by OPG resulted in significant improvements in both toughness and strength, further demonstrating the dependence of bone strength on the cortical microstructure. Although OPG increased ultimate strength to wild-type levels, it could not completely restore toughness, suggesting that the impaired toughness in bone from PPR⁺Tg is dependent on more than the alterations in porosity and BMD. The impaired bone quality in these mice may explain in part why primary hyperparathyroidism patients have a higher incidence of vertebral fracture despite preservation of cancellous bone.⁴⁸ Further examination of the bone matrix would be required to elucidate the mechanism by which bone quality is affected in PPR⁺Tg mice.

In summary, our study demonstrates that antiresorptives significantly reduced cortical porosity in mice with a hyperparathyroid skeletal phenotype secondary to constitutive activation of osteoblastic PTH/PTHrP receptors. Greater suppression of cortical porosity with OPG versus

bisphosphonates suggested that OPG caused greater reductions in intracortical bone resorption. Reductions in cortical porosity with OPG were associated with increased cortical BMD and increased femur bending strength. OPG and bisphosphonate treatments had similar effects on trabecular bone mass, suggesting similar antiresorptive effects within this compartment. Greater reductions in bone marrow fibrosis were observed with OPG versus bisphosphonates, which could be related to the unique ability of OPG to markedly reduce trabecular osteoclast numbers. We therefore hypothesize that trabecular osteoclasts, in addition to the resorptive activity of osteoclasts, might contribute to the expansion of marrow fibroblastoid cells under these conditions, and perhaps in conditions such as hyperparathyroidism and fibrous dysplasia.

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