IL-6 is not required for parathyroid hormone stimulation of RANKL expression, osteoclast formation, and bone loss in mice

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O’Brien, Charles A., Robert L. Jilka, Qiang Fu, Scott Stewart, Robert S. Weinstein, and Stavros C. Manolagas. IL-6 is not required for parathyroid hormone stimulation of RANKL expression, osteoclast formation, and bone loss in mice. Am J Physiol Endocrinol Metab 289: E784–E793, 2005. First published June 14, 2005; doi:10.1152/ajpendo.00029.2005.—Continuous elevation of parathyroid hormone (PTH) increases osteoclast precursors, the number of osteoclasts on cancellous bone, and bone turnover. The essential molecular mediators of these effects are controversial, however, and both increased receptor activator of NF-κB ligand (RANKL) and IL-6 have been implicated. The goal of these studies was to determine whether continuous elevation of endogenous PTH alters IL-6 gene expression in vivo and whether IL-6 is required for PTH-induced bone loss. To accomplish this, we generated transgenic mice harboring a luciferase reporter gene under the control of IL-6 gene regulatory regions to allow accurate quantification of IL-6 gene activity in vivo. In these mice, induction of secondary hyperparathyroidism using a calcium-deficient diet did not alter IL-6-luciferase transgene expression, whereas RANKL mRNA expression was elevated in bone tissue. Moreover, secondary hyperparathyroidism induced an equivalent amount of bone loss in wild-type and IL-6-deficient mice, and PTH elevated RANKL mRNA and osteoclast formation to the same extent in bone marrow cultures derived from wild-type and IL-6-deficient mice. These results demonstrate that IL-6 is not required for the osteoclast formation and bone loss that accompanies continuous elevation of PTH.

Bone resorption; cytokine; transgene; knockout

PATHOLOGICAL BONE RESORPTION induced by parathyroid hormone (PTH) excess was demonstrated over 50 years ago by the demonstration of osseous erosions adjacent to grafts of parathyroid tissue placed directly on bone (3). Today, the molecular mediators of the erosions remain unclear and both receptor activator of NF-κB ligand (RANKL) and interleukin-6 (IL-6) are thought to be required.

RANKL is a member of the TNF family of cytokines and is essential for osteoclast formation (27). In vitro, osteoclast formation is directly proportional to the amount of available RANKL (20). PTH stimulates RANKL production in stromal/osteoblastic cells and suppresses expression of osteoprotegerin (OPG), a soluble antagonist of RANKL (28). Therefore, it has been suggested that PTH stimulates osteoclast formation in part by increasing the RANKL/OPG ratio (24, 29).

PTH also transiently stimulates IL-6 expression in vivo and in vitro (16, 42), and some studies have suggested that this cytokine is involved in PTH-stimulated osteoclast formation. Specifically, the elevation of biochemical markers of bone turnover produced by continuous infusion of PTH is blunted in IL-6-deficient mice (17) and anti-gp130 antibodies reduced in vitro osteoclast formation in response to PTH (43). Furthermore, activation of the gp130/signal transducer and activator of transcription 3 (STAT3) pathway by members of the IL-6 cytokine family can stimulate RANKL expression and osteoclast formation in vitro (37, 51). However, IL-6 alone is unable to stimulate osteoclast formation in vivo (9). In addition, blockade of gp130/STAT3 signaling in stromal/osteoblastic cells or blockade of IL-6 action abolished osteoclastogenesis induced by IL-6 but not by PTH (10, 37). Moreover, PTH stimulates RANKL expression in the absence of new protein synthesis (14). These latter in vitro studies suggest that IL-6 may not be essential for PTH-induced osteoclast formation or bone resorption.

Previous efforts to determine whether IL-6 expression is altered in osteoporotic patients or animal models of osteoporosis have yielded inconsistent results (41). This is highlighted by the observation that different laboratories have detected either a 10-fold increase (18) or no change (31) in circulating IL-6 protein in 4-wk-old ovariectomized mice. These studies have relied on measurements of IL-6 protein by immunoassay or IL-6 mRNA by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). However, measurement of IL-6 protein may be complicated by the presence of binding proteins such as soluble IL-6 receptors and complement proteins that can mask IL-6 detection (32, 33, 35). In addition, the semiquantitative RT-PCR techniques used in these studies may not have been able to detect subtle but significant differences in mRNA expression.

The goal of the studies described herein was to determine whether continuous elevation of endogenous PTH alters IL-6 gene expression in vivo and whether IL-6 is required for the increase in osteoclasts and loss of bone mineral density (BMD) associated with hyperparathyroidism. To accomplish these goals, we generated transgenic mice harboring a luciferase reporter gene under the control of IL-6 gene regulatory regions to allow accurate quantification of IL-6 gene activity in vivo. Induction of secondary hyperparathyroidism in these mice by a calcium-deficient diet did not alter IL-6-luciferase transgene expression in bone. In addition, secondary hyperparathyroidism reduced BMD and
increased osteoclasts on cancellous bone to the same extent in wild-type and IL-6-deficient mice. Nonetheless, secondary hyperparathyroidism caused an elevation of RANKL mRNA in bone as measured by quantitative RT-PCR. PTH also induced equivalent levels of RANKL mRNA and osteoclast formation in cultures of cells from wild-type and IL-6-deficient mice. These results demonstrate that elevated PTH stimulates RANKL mRNA, osteoclast formation, and bone loss independently of IL-6.

MATERIALS AND METHODS

Animals. IL-6 promoter-luciferase transgenic mice, designated IL-6-LUC, were generated with a DNA construct consisting of a SacI-SmaI fragment of the murine IL-6 gene, containing 1,796 bp of 5′-flanking region, exon 1, intron 1, and 288 bp of exon 2, inserted into the SacI-SmaI sites of pGL3-Basic (Promega, Madison, WI). The SmaI site in exon 2 of the IL-6 gene was created by site-directed mutagenesis. In addition, the translation start codon in IL-6 exon 1 was eliminated by site-directed mutagenesis so that the first AUG in the transgene mRNA encodes the first methionine of the luciferase gene. An MluI fragment containing 749 bp of the 3′-flanking region of the murine IL-6 gene was inserted downstream from the luciferase gene by creating an MluI site immediately 3′ of the luciferase stop codon by site-directed mutagenesis. The fragment containing the 3′-flanking region was amplified from murine genomic DNA using the polymerase chain reaction (PCR) and the primers 5′-CCCAA-GGCGTTCCGATATGCTAAAGCATATC-3′ and 5′-CCACCGGCTCAAGGGCGGTTAGTGTAGATC-3′ (3′-3′UTR-reverse). Transgenic mice harboring the reporter construct were produced by microinjection of a purified 4.8-kb SacI-SalI fragment into pronuclei of C57BL/6 × SJL F2 hybrids (National Institutes of Child Health and Human Development Transgenic Mouse Development Facility, Birmingham, AL). Transgenic mice were identified by PCR of the IL-6 locus, using amplification primers and conditions recommended by Jackson Laboratories. All protocols involving genetically modified mice and their wild-type littermates were approved by the Institutional Animal Care and Use Committees of the University of Arkansas for Medical Sciences and the Central Arkansas Veterans Healthcare System.

Secondary hyperparathyroidism was induced in adult female (140–170 days old) or weaning female (23 days old) mice by feeding them a calcium-deficient diet (ICN no. 9601177) for 7 days (5). Control mice received a regular diet (Harlan-Teklad no. 7004). Hyperparathyroidism was confirmed by comparing plasma PTH levels between diet by groups using an intact rodent PTH immunoassay (Immutopics, San Clemente, CA) (5). To allow dynamic bone histomorphometry, tetrazycline (30 mg/kg) was administered intraperitoneally 5 and 2 days before the mice were killed.

To compare the effect of lipo polysaccharide (LPS) on the IL-6-LUC transgene and the endogenous IL-6 gene, LPS (200 μg in 100 μl of PBS) was injected intraperitoneal into 10-wk-old male IL-6-LUC mice and total RNA was isolated from tissues after 2 or 8 h.

IL-6 and luciferase protein assays. IL-6 in culture supernatants or plasma was quantified using a mouse IL-6 immunoassay (R&D Systems, Minneapolis, MN). To quantify firefly luciferase enzymatic activity in tissues, lysates were prepared by tissue homogenization in a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) in 5 vol of lysis buffer (25 mM glycy1-glycine, pH 7.8, 1% Triton X-100, 15 mM MgSO4, 4 mM EGTA), followed by centrifugation at 12,000 g for 10 min at 4°C. Luciferase activity in lysates was determined using luciferase substrate (Promega) and light detection in a plate-reader luminometer (Packard Instruments, Meriden, CT). The protein concentration in lysates was determined by bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL). The luciferase assay was performed in triplicate and the BCA assay in duplicate. Luciferase values were divided by the protein concentration to yield relative light units. To quantify luciferase activity in cell lysates, adherent monolayers were washed once with PBS, and lysis buffer was added. After a 15-min incubation at 25°C, cells were harvested by scraping and transferred to 1.5-ml microcentrifuge tubes. The tubes were frozen at −20°C for 30 min, thawed, and centrifuged for 5 min at 12,000 g. Luciferase activity in cell lysates was determined as described above for tissue lysates.

Gene expression analysis. Total RNA was purified from cells or tissues and analyzed by Northern blot as previously described (37). Quantification of mRNA by RT-PCR was performed in pheochromocytes as previously described (38). Briefly, total RNA from vertebra L5 or cell cultures was reverse transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Murine RANKL, OPG, IL-6, or the housekeeping gene ribosomal protein S2 were subsequently amplified from the first-strand cDNA by RT-PCR by using TaqMan Universal PCR Master Mix (Applied Biosystems). The TaqMan assay numbers of the primer/probe sets used are: RANKL, Mm00444190m1; IL-6, Mm00446190m1; and OPG, Mm00435452m1. The sequences for the ribosomal protein S2 primer/probe set were described previously (38). PCR amplification and detection were carried out on an ABI Prism 7300 Sequence Detection System (Applied Biosystems) according to the manufacturer’s instructions. Gene expression was quantified using the comparative threshold cycle (CT) method by subtracting the housekeeping gene CT value from the RANKL, OPG, or IL-6 CT value, as amplification efficiencies of all primer/probe sets used were equal (data not shown).

BMD determinations. Spinal, hindlimb, and global BMD of adult animals were measured using dual-energy X-ray absorptiometry as previously described (Hologic, Bedford, MA) (23, 26, 54, 55). Initial determinations were used to allocate animals into groups with equivalent spinal BMD values. BMD measurements of weanling mice were obtained by using a PIXIplus densitometer (GE-Lunar, Madison, WI). Three sites were measured. The total body window was defined as the whole body image minus the calvarium, mandible, and teeth. Except for the first few caudal vertebrae, the tail was not included. The spine window was a rectangle depending on animal body length, reaching from just below the skull to the base of the tail. The femoral window captured the right femur. Scan acquisition time was 4 min and analysis time was 6 min. Mice were sedated before scanning with pentobarbital sodium (5 mg/ml) given at a dose of 120 μg/g body wt by intramuscular injection. One hundred seventy-two total body BMD measurements of a proprietary skeletal phantom, performed from June 2004 to April 2005, had a mean coefficient of variation of 3.1%.

Bone histomorphometry. The first through the fourth lumbar vertebrae were fixed and embedded undecalciﬁed in methyl methacrylate as previously described (55). The histomorphometric examination was done with a computer and digitizer tablet (OsteoMetrics, Decatur, GA) that was interfaced to a Zeiss Axioskop (Carl Zeiss, Thornwood, NY) with a drawing tube attachment. The identity of each specimen was concealed from the histomorphometry reader. All cancellous measurements were made using a Plan-Neoflaur x40 objective (numerical aperture 0.75). The percentage of the cancellous perimeter covered by plump, cuboidal osteoblasts lining osteoid (osteoblast perimeter) and the percentage of the cancellous perimeter bearing tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (osteoclast perimeter) were measured directly, whereas the rate of bone formation per cancellous perimeter was calculated (mineral appositional rate multiplied by mineralizing perimeter). The terminol-
ogy used was that recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (39).

Biomechanical testing. The load-bearing properties of the 5th lumbar vertebrae, or, in the experiments using weanling mice the 6th lumbar vertebrae, were measured on the day the animals were killed using a single-column material testing machine and a calibrated tension/compression load cell (model 5542; Instron, Canton, MA) as previously described (38). After preseating with <0.5 Newtons (N) of applied load, vertebrae were compressed between screw-driven loading platens using a lower-platen, miniature spherical seat that mini-

applied load, vertebrae were compressed between screw-driven load-

previously described (38). After preseating with tension/compression load cell (model 5542; Instron, Canton, MA) as

lumbar vertebrae, were measured on the day the animals were killed

pared by seeding bone marrow cells harvested as above at 1

oncy-stimulating factor; 30 ng/ml each). Cultures were maintained for

LPS injection (Fig. 1

tissues examined, with peak stimulation occurring 2 h after

change on day 10

were added for 24 h. RNA was prepared and analyzed by quantitative

RT-PCR as described above.

Statistics. To evaluate whether there were significant effects, para-

metric ANOVA models were utilized, given that the necessary as-

sumptions were satisfied. Levene’s test and the Shapiro-Wilk test

were used to test the homogeneity of variance and normality assump-

tions, respectively. Depending on the hypothesis, one-sided as well as

two-sided tests were employed. After a Bonferroni correction, P

values of <0.05 were considered significant.

RESULTS

Generation of IL-6-LUC transgenic mice. To circumvent potential problems involved with detecting changes in IL-6 gene expression in vivo, we generated mice harboring a firefly luciferase reporter gene controlled by IL-6 gene regulatory elements, designated as IL-6-LUC. Seven independent trans-

genic lines were obtained, and cells from two of the different lines were responsive to PTH, IL-1, and LPS in vitro (data not shown). The lack of response in five of the lines presumably results from the chromosomal position effect (1). The line described below was selected for the studies described herein because in calvaria cell cultures from these mice, PTH pro-

duced a fivefold increase in IL-6 protein and a sevenfold increase in luciferase activity, demonstrating that the response of the transgene to PTH in this line is similar to that of the endogenous gene (Fig. 1, A and B). IL-1 also induced both IL-6 protein and luciferase activity in these cells (Fig. 1, A and B), indicating that the transgene responds to other stimuli known to control IL-6 expression. To determine whether the transgene mimics activity of the endogenous IL-6 gene in vivo, IL-6-

LUC mice were injected with LPS, a substance known to strongly elevate IL-6 expression. Similar patterns of expression were observed for both luciferase and IL-6 mRNAs in all tissues examined, with peak stimulation occurring 2 h after LPS injection (Fig. 1C). Consistent with the mRNA results, LPS stimulated luciferase enzymatic activity in all tissues examined, with the exception of heart and brain (Fig. 1D). Because LPS strongly elevated luciferase mRNA in heart, the reason for the lack of stimulated luciferase enzyme activity in this tissue is unclear but could be due to altered translation of luciferase mRNA. LPS stimulation of luciferase activity in tissues did not vary in mice from age 3 to 15 mo (data not shown). Taken together, these results indicate that, with the exception of enzymatic activity in heart tissue, the IL-6-LUC transgene accurately mimics the response of the endogenous IL-6 gene to PTH, IL-1, and LPS.

Dietary calcium deficiency elevates PTH, stimulates osteoclast formation, and results in bone loss in IL-6-LUC transgenic mice. To determine whether continuous elevation of endogenous PTH alters IL-6 gene expression, we developed and characterized a murine model of secondary hyperparathy-

roidism induced by dietary calcium deficiency (5). We used this model of secondary hyperparathyroidism because it consistently leads to loss of bone mass, as shown in the studies described below and in previous studies (30, 40, 45, 47). Serum PTH was significantly elevated in IL-6-LUC mice that were fed a calcium-deficient diet for 7 days compared with mice fed a control diet (Fig. 2A). We (5) have shown previously that serum PTH is elevated in this model 1 day after initiating a calcium-deficient diet. After 7 days of a normal or calcium-

deficient diet, body weight was not different between the diet groups (Fig. 2B). However, secondary hyperparathyroidism caused a 13% decrease in hindlimb BMD (Fig. 2C). Consistent with this finding, vertebral compression strength was also reduced by 36% in the mice that were fed a calcium-deficient diet (Fig. 2D). Histomorphometric analyses of vertebral bone revealed increased osteoclast perimeter, osteoblast perimeter, and bone formation rate, consistent with increased bone remodeling expected in the mice with secondary hyperparathy-

roidism (Fig. 2, E-G).

Continuous elevation of endogenous PTH does not stimulate IL-6 expression. Quantification of luciferase activity in several tissues known to express both IL-6 and PTH/parathyroid hormone-related protein (PTHrP) receptor-1 revealed no change in luciferase activity in response to dietary calcium deficiency for 7 days (Fig. 3). Furthermore, in serum taken from these mice, IL-6 concentration was either below the level of detection or not significantly different between both diet groups (data not shown).

IL-6 is not required for bone loss, stimulation of RANKL mRNA expression, or increased osteoclast formation induced by elevated PTH. Although we did not detect significant changes in IL-6 gene expression, it remained possible that elevation of IL-6 expression in a tissue that we did not examine is required for PTH-induced osteoclast differentiation and bone loss. Alternatively, a tonic level of IL-6 expression may be required for PTH actions on bone. To address these issues, BMD and vertebral compression strength were compared in wild-type and IL-6-deficient mice after 7 days of dietary calcium deficiency. Dietary calcium deficiency induced significant bone loss at each skeletal site examined in both wild-type and IL-6-deficient mice (Fig. 4, A–C). Consistent with the loss of spinal BMD, loss of vertebral compression strength was similar in wild-type and IL-6-deficient mice (Fig. 4D). Finally, dietary calcium deficiency elevated the cancellous osteoblast
and osteoclast perimeters to the same extent in both wild-type and IL-6-deficient mice (Fig. 4, E and F).

A previous study found that the PTH-induced increase in markers of bone resorption observed in wild-type mice was blunted in growing IL-6-deficient mice that were continuously infused with PTH (17). To address the possibility that IL-6 is required for bone loss due to elevated endogenous PTH in growing but not adult mice we compared changes in BMD and vertebral compression strength in 3-wk-old wild-type and IL-6-deficient mice after 7 days of dietary calcium deficiency. Similarly to adult mice, dietary calcium deficiency induced significant bone loss in the spine and femur (Fig. 5, A and B), as well as loss of vertebral compression strength (Fig. 5C). In this experiment we also examined RANKL, OPG, and IL-6 mRNA expression in the fifth lumbar vertebra of each animal. Dietary calcium deficiency significantly elevated RANKL but not OPG mRNA in both wild-type and IL-6-deficient mice (Fig. 5, D and E). Furthermore, and consistent with the results obtained with our IL-6-LUC transgenic mice, endogenous IL-6 mRNA was not altered by dietary calcium deficiency in wild-type mice (Fig. 5F).

We therefore compared the ability of PTH to regulate RANKL and OPG mRNA in vitro in bone marrow cultures from wild-type and IL-6-deficient mice. Consistent with the elevation of RANKL mRNA in vivo, there was no difference in the stimulation of RANKL or suppression of OPG mRNA by PTH in bone marrow cultures from wild-type and IL-6-deficient mice (Fig. 6A). This was reflected in equivalent amounts of in vitro osteoclast formation in response to PTH in cultures from wild-type and IL-6-deficient mice (Fig. 6B).

**DISCUSSION**

The goals of the studies described herein were to determine whether continuous elevation of endogenous PTH stimulates IL-6 gene expression in vivo and whether IL-6 is required for the bone loss associated with elevated PTH. To aid these studies, we generated and characterized a transgenic mouse...
model that allows accurate quantification of IL-6 gene activity in vivo. In addition, we characterized a murine model of secondary hyperparathyroidism and used it in combination with the IL-6-LUC model to demonstrate that IL-6 gene expression is not altered by continuous elevation of PTH. We also showed that IL-6 is not required for PTH-induced osteoclastogenesis in vitro or in vivo, or for the bone loss induced by excess PTH in vivo.

We (17) have shown previously that continuous infusion of human PTH into mice for 5 days resulted in a continual increase in circulating IL-6 protein. In the same study, we found that increases in serum and urinary markers of bone resorption in response to infused PTH were blunted in IL-6-deficient mice (17). These previous studies using PTH-infused mice suggested that continuously elevated PTH stimulates bone resorption via a process that requires stimulation of IL-6 expression. Therefore, we were surprised to find in the present study that expression of the IL-6 transgene, IL-6 protein in the serum, and IL-6 mRNA in bone were not stimulated after 7 days of continuous elevation of endogenous PTH. Perhaps more striking, we found that secondary hyperparathyroidism induced significant bone loss in the absence of IL-6. Why might IL-6 be required for bone resorption when PTH is infused but not when PTH is elevated by dietary calcium deficiency? The level of PTH achieved by continuous infusion (305 pg/ml) was much higher than that achieved by dietary calcium deficiency (54 pg/ml), suggesting that there might be a threshold of PTH level below which IL-6 is not required for PTH-induced bone resorption. Consistent with this idea, recent studies have shown that PTH levels correlate with serum IL-6 levels in patients with severe, but not mild, secondary hyperparathyroidism (34).

Although the results of our present study demonstrate that IL-6 is not required for PTH-induced osteoclastogenesis or bone resorption, increased IL-6 levels may nonetheless contribute to increased bone resorption in certain conditions with continuously elevated PTH or PTHrP. Because activation of the gp130/STAT3 pathway stimulates RANKL expression in stromal/osteoblastic cells (37), even transient stimulation of IL-6 by PTH may lead to autocrine stimulation of RANKL.

Fig. 2. Dietary calcium deficiency induces secondary hyperparathyroidism and causes bone loss and decreased bone strength in mice. Eight 5-mo-old female IL-6-LUC transgenic mice were fed either a diet of normal rodent chow (open bars) or calcium-deficient chow (filled bars) for 7 days. A: serum intact PTH was measured after 7 days. B: body weight is presented as %change in body weight during the 7-day experiment. C: global, spine, and hindlimb bone mineral density (BMD) are presented as %change during the 7-day experiment. D: load-bearing properties of L5 are shown as maximum load (N). Vertebral sections (L1–L4) were used to enumerate osteoclasts (E) and osteoblasts (F) and to determine bone formation rate (G). Each value represents the mean (SD) of 4 animals. *P < 0.05 using a one-tailed t-test vs. the normal diet group.

Fig. 3. Secondary hyperparathyroidism does not stimulate IL-6 gene expression. Femurs, kidneys, liver, or spleen were harvested from IL-6-LUC mice on a normal diet (open bars) or a calcium-deficient diet (filled bars). Tissues were homogenized and luciferase activity in the lysate was normalized to protein concentration to yield RLU. Values are shown as %RLU relative to the group that was fed the normal calcium diet [mean (SD) of 4 animals].
augmenting direct PTH stimulation of RANKL. It has also been suggested that IL-6 stimulates proliferation of osteoclast precursors and thereby increases the pool of cells on which differentiation factors, such as RANKL, can act (9). However, it should be noted that there is limited evidence to suggest that IL-6 alone is capable of stimulating bone resorption in vivo. Coinjection of tumor cells expressing IL-6 and PTHrP induced bone resorption, whereas injection of IL-6-expressing cells alone did not (9). In addition, transgenic mice broadly expressing IL-6 with high circulating levels of IL-6 protein did not display increased osteoclast formation or bone resorption (25). Nonetheless, when human bone marrow cells engineered to express IL-6 were implanted into human bone in SCID mice, osteoclast surface was increased (44). When cells expressing IL-6 were injected intraperitoneally, circulating IL-6 levels were increased but osteoclast surface was not, suggesting that local expression of IL-6 in bone may be critical for influencing bone resorption (44).

Our finding that IL-6 is not required for PTH stimulation of RANKL expression and osteoclast differentiation is in accord with our earlier findings that gp130 signaling in stromal/osteoblastic cells is not required for PTH-induced osteoclast formation (37) and that new protein synthesis is not required for PTH-induced RANKL expression (14). It is possible that because PTH also stimulates IL-11 expression in vivo, this cytokine might have taken the place of IL-6 in our model of secondary hyperparathyroidism. However, transgenic mice with constitutive expression of IL-11 in bone did not display increased osteoclasts or bone resorption (49), suggesting that in vivo, IL-11 might not be osteoclastogenic and thus might not mediate the effects of PTH on bone resorption. Nonetheless, PTH stimulation of RANKL was blunted in calvaria cells from gp130-deficient mice, and these cells were not able to form mineralized nodules in vitro, suggesting that gp130 signaling influences the ability of stromal cells to support osteoclast differentiation and plays a role in osteoblast differentiation (48). However, our in vivo studies demonstrate that if gp130 signaling is required for these actions, IL-6 is not the gp130 family cytokine involved.

A previous study demonstrated that continuous PTH infusion into parathyroidectomized rats stimulated RANKL and inhibited OPG mRNA in metaphyseal bone for up to 24 h (29). A more recent study in weanling rats demonstrated that dietary calcium deficiency elevated RANKL mRNA but did not suppress OPG mRNA in cortical bone (52). Consistent with the latter study, we did not observe inhibition of OPG in our model of secondary hyperparathyroidism, but we did observe elevation of RANKL mRNA levels in whole tibia. Regardless of

Fig. 4. IL-6 is not required for bone loss associated with secondary hyperparathyroidism. Wild-type and IL-6-deficient mice were placed on normal (open bars) or calcium-deficient diets (filled bars) for 7 days. %Change from initial global (A), spinal (B), and hindlimb (C) BMD are presented [means (SD) of 16–18 mice/group]. Load-bearing properties of L5 are shown as maximum load (N) (D), and vertebral sections (L1–L4) were used to determine osteoblast (E) and osteoclast (F) perimeter. Values in D, E, and F represent means (SD) of ≥6 animals/group. *P ≤ 0.05 by ANOVA vs. normal diet group.
Fig. 5. IL-6 is not required for bone loss or elevation of vertebral receptor activator of NF-κB ligand (RANKL) mRNA associated with secondary hyperparathyroidism in growing mice. Weanling wild-type and IL-6-deficient mice were placed on normal (open bars) or calcium-deficient diet (filled bars) for 7 days. %Change in spinal (A) and femur (B) BMD are presented. Load-bearing properties of L6 are shown as maximum load (N) (C). Total RNA was isolated from the 5th lumbar vertebra, reverse transcribed, and analyzed by real-time PCR for RANKL, osteoprotegerin (OPG), IL-6, and ribosomal protein S2 mRNA. RANKL, OPG, and IL-6 values, relative to ribosomal protein S2, are presented. Values in each graph represent the mean (SD) of ≥6 animals/group. *P ≤ 0.05 by ANOVA vs. normal diet group; n.d., not determined.

Fig. 6. IL-6 is not required for PTH regulation of RANKL/OPG ratio or PTH-stimulated osteoclast formation in vitro. A: bone marrow cell cultures from wild-type or IL-6-deficient mice were treated with vehicle, 1 × 10^{-7} M PTH-(1–34), or 1 × 10^{-8} M 1,25(OH)_{2}D_{3}, for 24 h. Total RNA was isolated, reverse transcribed, and analyzed by real-time PCR for RANKL, OPG, and ribosomal protein S2. RANKL (top) and OPG (bottom) values, relative to ribosomal protein S2, are given as means (SD) of triplicate cultures. B: osteoclast formation was evaluated using bone marrow cells isolated from wild-type and IL-6-deficient mice. Cultures were incubated with vehicle, 1 × 10^{-7} M PTH-(1–34), 1 × 10^{-8} M 1,25(OH)_{2}D_{3}, or soluble RANKL (30 ng/ml) and macrophage colony-stimulating factor (M-CSF; 30 ng/ml) for 8 days and stained for tartrate-resistant acid phosphatase (TRAP). Values are mean (SD) number of TRAP-positive, multinucleated cells (MNC) from triplicate wells.
whether OPG is suppressed in all situations in which PTH is continuously elevated, RANKL was stimulated by this condition, and thus the RANKL-to-OPG ratio was elevated. As the level of available RANKL is a major determinant of the magnitude of osteoclast number, the increased RANKL expression observed in bone is likely part of the mechanism by which continuous elevation of PTH stimulates bone resorption.

An important goal of our study was to determine whether IL-6 is required for bone loss due to elevated PTH. A calcium-deficient diet was used to elevate endogenous PTH because this model of secondary hyperparathyroidism consistently leads to loss of bone mass, as measured by changes in either trabecular bone volume or BMD (30, 40, 45, 47). In contrast, elevation of PTH by continuous PTH infusion using osmotic pumps does not consistently lead to loss of bone mass in rats or mice, as some studies have demonstrated reduced trabecular bone volume (50) while others have reported no change in either trabecular bone volume (5, 11) or BMD (46).

Regardless of the method used to elevate PTH, it is important to appreciate that hyperparathyroidism is a dual hormonal abnormality. The skeletal effects are due to both increased PTH and 1,25(OH)2D3 (6). PTH elevation, whether caused by dietary calcium deficiency or infusion of exogenous PTH, stimulates renal 25-hydroxyvitamin D-1α-hydroxylase (13, 19, 47). The resultant increase in 1,25(OH)2D3 is likely to have contributed to the increase in osteoclast number that occurred in our model. Nonetheless, our results clearly demonstrate that IL-6 is not required for the bone loss in response to dietary calcium deficiency, whether this is mediated by elevated PTH alone or a combination of elevated PTH and 1,25(OH)2D3. Furthermore, the increased bone formation rate in the vertebral trabecular bone of mice that were fed the calcium-deficient diet is most likely due to increased PTH alone because elevated 1,25(OH)2D3 either does not affect or decreases trabecular bone formation rate (12, 15, 52).

We observed a greater decrease in bone compression strength relative to the decrease in BMD in both young and adult mice. It has been demonstrated that the compressive strength of bone increases with the square of the BMD (2). Thus the relationship of strength to BMD is not linear, and small changes in BMD can lead to much larger changes in strength (4, 8, 53). In addition, correlation of bone strength with BMD suggests that only 60% of bone strength is explained by BMD (53). The remaining contributors to bone strength include architecture, rate of bone turnover, properties of the collagen/mineral matrix, accumulation of microdamage, and osteocyte viability (7, 38, 53). Although we did not address the contribution of these latter factors in our study, our results are consistent with a previous study (56) in mice in which they were fed a calcium-deficient diet for 3 wk. In that study, a 12% decrease in femur ash weight (comparable to BMD) was associated with a 66% decrease in the ultimate tensile strength of the femur. In another study, rats that were fed a low-calcium diet for 3 mo lost only ~10% of their vertebral compression strength (21). The relative maintenance of bone strength in this latter study may be related to the presence of more calcium in the diet used (0.2% Ca vs. 0.01% Ca used in our study or 0.02% Ca used in the previous mouse study).

In conclusion, our results demonstrate that continuous elevation of endogenous PTH does not lead to sustained elevation of IL-6 gene activity in bone and that IL-6 is not required for bone loss due to this condition. Furthermore, our finding that continuous elevation of endogenous PTH stimulates RANKL mRNA in adult bone suggests that this cytokine, rather than IL-6, plays a dominant role in the increased osteoclasts and bone loss associated with elevated PTH.

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