Catabolic Effects of Continuous Human PTH (1–38) *in Vivo* Is Associated with Sustained Stimulation of RANKL and Inhibition of Osteoprotegerin and Gene-Associated Bone Formation

YANFEI L. MA, RICK L. CAIN, DAVID L. HALLADAY, XUHAO YANG, QINGQANG ZENG, REBECCA R. MILES, SRINIVASAN CHANDRASEKHAR, T. JOHN MARTIN, AND JUDE E. ONYIA

Gene Regulation, Bone and Inflammation Research Division (Y.L.M., R.L.C., D.L.H., X.Y., Q.Z., S.C., T.J.M., J.E.O.), Lilly Research Laboratories, Eli Lilly & Co., Indianapolis, Indiana 46285; and St. Vincent's Institute of Medical Research (T.J.M.), Fitzroy, Victoria 3065, Australia

Continuous infusion of PTH *in vivo* results in active bone resorption. To investigate the molecular basis of the catabolic effect of PTH *in vivo*, we evaluated the role of OPG and RANKL, which are known to influence osteoclast formation and function. Weanling rats fed a calcium-free diet were parathyroidectomized and infused with PTH via an Alzet pump to examine: 1) the changes of serum-ionized calcium and osteoclast number, 2) the expression of OPG/RANKL mRNA and protein, and 3) the expression of osteoblast phenotype bone formation-associated genes such as osteoblast specific transcription factor, osteocalcin, bone sialoprotein, and type I collagen. PTH (1–38) (0.01–20 μg/100 g) continuous infusion for 1–24 h resulted in a dose-dependent increase in serum-ionized calcium in parathyroidectomized rats and a corresponding dose-dependent increase in osteoclast number, indicating an increased bone resorption. At 20 μg/100 g PTH dose level, serum-ionized calcium was 2.1-fold of the vehicle control and not different from the Sham-parathyroidectomized rats, and osteoclast number was 3-fold of the vehicle control and 1.7-fold of the Sham-parathyroidectomized rats. In the distal femur, RANKL mRNA expression was increased (27-fold) and OPG mRNA expression was decreased (4.6-fold). The changes in RANKL and OPG mRNA levels were rapid (as early as 1 h), dose dependent, and sustained over a 24-h period that was examined. Immunohistochemical evaluation of bone sections confirmed that OPG level was reduced in proximal tibial metaphysis upon PTH infusion. Circulating OPG protein level was also decreased by 32% when compared with the parathyroidectomized control. The expression of genes that mark the osteoblast phenotype was significantly decreased [osteoblast specific transcription factor (2.3-fold), osteocalcin (3-fold), bone sialoprotein (2.8-fold), and type I collagen (5-fold)]. These results suggest that the catabolic effect of PTH infusion *in vivo* in this well-established resorption model is associated with a reciprocal expression of OPG/RANKL and a co-ordinate decrease in the expression of bone formation-related genes. We propose that the rapid and sustained increase in RANKL and decrease in OPG initiate maintain and favor the cascade of events in the differentiation/recruitment and activation of osteoclasts. (*Endocrinology* 142: 4047–4054, 2001)

THE DISCOVERY OF PTH was made 100 yr ago, yet the *in vivo* mechanisms of action of PTH are still not well understood. PTH has complex effects on bone, depending on the mode of administration. When given intermittently, PTH increases bone *in vivo* by increasing the number and activity of osteoblasts, and continuous infusion of PTH decreases bone mass by stimulating a net increase in bone resorption (1–12). The molecular events that mediate these different biological responses in bone are unknown. It has been shown that a single dose of PTH in rats triggers an anabolic process that induces a greater level of the expression of early response genes and cytokines as well as genes associated with the differentiated osteoblast phenotype (11–21). When given by continuous infusion, the catabolic effect of PTH is explained by its ability to stimulate bone resorption by promoting an increase in the number of actively resorbing osteoclasts. However, there are limited *in vivo* data on the early

molecular targets that initiate and mediate the catabolic response to PTH.

The recent discoveries of members of the TNF receptor and TNF ligand families (OPG and RANKL) as powerful influences on osteoclast formation and activity have generated great interest in their role and regulation by calciotropic agents such as PTH. OPG is a TNF receptor family member that inhibits osteoclast formation at a late stage of its development (22–27). Overexpression of OPG in transgenic mice resulted in osteopetrosis because of failure of osteoclast formation. Targeted deletion of the OPG gene resulted in severe, early-onset osteopenia (28). Furthermore, treatment of ovariectomized rats with OPG prevented bone loss (23). This same molecule was identified independently and called osteoclastogenesis inhibitory factor (22, 29). The ligand for OPG has been identified as osteoclast differentiation factor, also known as RANKL, or OPG ligand, a member of the TNF ligand family (24, 30–41). It is expressed on the osteoblast/stromal cell surface and promotes osteoclast formation in the presence of macrophage colony–stimulating factor and without any accompanying stromal/osteoblastic cells (24, 40). *In*

Abbreviations: BSP, Bone sialoprotein; cbfa-1, osteoblast specific transcription factor; COL1A1, type I collagen; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; hPTH, continuous human PTH; OC, osteocalcin; PX, parathyroidectomized.

vitro studies show that the mRNA levels of both RANKL and OPG in osteoblasts/stromal cells are influenced by factors such as cytokines that increase bone resorption (42–47). However, it is not yet clear that OPG and RANKL expression are regulated *in vivo* by relevant bone active factors to stimulate bone resorption.

In the present study, we examined the role of OPG and RANKL in bone resorption induced by continuous infusion of PTH 1–38 in parathyroidectomized (PX) rats. PX animals were used to eliminate the confounding effects of endogenous PTH, and it is a well-established bone resorption model. We propose that the catabolic effects of PTH and the net decrease in bone mass after PTH infusion occur via rapid but sustained regulation of OPG and RANKL and a decrease in the osteoblast phenotype and bone formation.

Materials and Methods

Animals and experimental protocols

Weanling, virus-antibody-free, Sprague Dawley female rats (Harlan, Indianapolis, IN) weighing 60–70 g were used for all the studies. For continuous PTH infusion studies, animals were PX by vendor and delivered to our facility 2 to 3 d after surgery. They were maintained on a 12-h light/dark cycle at 22 C with *ad lib* access to tap water. To minimize the gut and kidney effects on serum calcium, rats were fed a calcium-free diet containing 0.02% Ca, 0.3% P (TD 99171, Teklad, Madison, WI) during the experimental period. Two experiments were conducted to examine the dose and time responses. Study 1, synthetic human PTH 1–38 (Zenaca Inc., Wilmington, DE) 0.01 to 20 μg/100 g/6 h was given by sc infusion via an Alzet pump (no. 20011, Durect Corp., Palo Alto, CA) to the rats with at least four rats in each group for 6 h. Study 2, PTH 1–38 20 μg/100 g/6 h was given to the rats as indicated above for 1, 3, 6, and 24 h. For acute PTH treatment, a single normal serum calcium, anabolic dose of PTH 1–38 8 μg/100 g sc injection was given to the same age intact rats, and the bones were collected at the same time course as indicated above (47). PTH was prepared in a vehicle of acidified saline containing 2% heat-inactivated rat sera. Serum was collected under isoflurane anesthesia at indicated time points for serum ionized calcium (Ciba-Corning, Inc., 634 Ca2+/pH analyzer, Chiron Diagnostics Corp., East Walpole, MA) and circulating OPG analyses. The animals were then killed by cervical vertebra dislocation. Bones were collected for histology and mRNA analyses. All studies were approved by Eli Lilly & Co. Animal Care Committees.

Serum-circulating OPG

Serum OPG level was detected by "Sandwich" ELISA with rabbit polyclonal anti-hOPG IgG. Briefly, 96-well plates (DYNEX Tech., IMMULON 4HBX, Chantilly, VA) were coated with 0.5 μg/well of purified rabbit anti-OPG antibody (IgG) diluted in 100 μl of carbonate/bicarbonate buffer (Sigma, St. Louis, MO C-3041), 4 C overnight, and blocked with 200 μl/well of blocker casein in PBS (Pierce Chemical Co., Rockford, IL) for 1 h at room temperature. The plate was incubated with 100 μl of serum diluted in 1% BSA-TPBS for 1 h and three washes with TPBS. To each well was added 100 μl of diluted biotinylated anti-OPG IgG for 1 h and followed by 100 μl/well of Streptoavidin-HRP conjugate (Zymed Laboratories, Inc., San Francisco, CA) for 30 min with TPBS washing. ELAST ELISA amplification system (NEN Life Science Products, Boston, MA) was applied before color development according to the manufacturer's instruction. Color was developed with 100 μl/well of 3,3',5,5'-tetramethylbenzidine substrate for 15 min and stopped with 100 μl/well of 1 N phosphoric acid with reading OD at A_{450nm}.

Quantitation of osteoclast number

Proximal tibiae were fixed in 10% buffered formalin for 2 days, decalcified in decalcifier II (Surgipath, Richmond, IL) for 3 days, and processed for embedding in paraffin. Longitudinal cut, 5-μm-thick, decalcified sections were stained by 0.1% Toluidine blue (Sigma). Oste-

oclast number was measured on the entire marrow region within the cortical shell between 0.67 and 2 mm distal to the growth plate metaphyseal junction under 20$\times$ magnification. Trabecular bone surface was measured by using a digitizing image analyzing system named Osteomeasure (OsteoMetrics, Inc., Atlanta, GA). The osteoclast numbers were then normalized to trabecular bone surface (48).

Isolation of poly A+RNA and Northern blotting

The mRNA expression was analyzed by Northern blotting. At autopsy, femora were resected and all connective tissue, including periosteum, completely removed. The distal epiphysis, including the growth plate, was removed and a subjacent 3-mm-wide band of the metaphyseal trabecular primary spongiosa was frozen in liquid nitrogen. Bone samples were pooled into treated or control groups for each indicated time point. Total RNA was extracted from bone by homogenization in Ultraspec-II using an LS 10–35 Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) as recommended by the manufacturer. Poly A+ RNA was isolated from total RNA using Oligotex (QIAGEN, Santa Clarita, CA) according to the manufacturer's protocol and quantitated by spectrophotometry. The absorbance at 260 nm was determined and the 260/280-nm absorbance ratio was calculated to ensure the absence of protein contamination. Samples of poly A+ RNA (2 μg) were denatured in 0.04 M 3-(*N*-morpholine) propanesulfonic acid, pH 7.0, 10 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde, and 50% formamide at 60 C for 10 min, and size fractionated by electrophoresis through 1% agarose gels in 2.1 M formaldehyde and 1$\times$ 3-[*N*-morpholino]propanesulfonic and transferred to nylon membranes (Brightstar-Plus, Ambion, Inc., Austin, TX). The membranes were air dried and the RNA samples cross-linked to the nylon membrane by UV irradiation in a Stratalinker (Stratagene, La Jolla, CA). Migration of 28S and 18S ribosomal RNAs were determined by ethidium bromide staining. DNA probes were labeled by the random primer method (Life Technologies, Inc., Grand Island, NY) using $\propto^{32}$P-dCTP. Prehybridization and hybridization were carried out at 48 C in NorthernMax buffers (Ambion, Inc.). After hybridization, membranes were washed for 30 min at room temperature in buffer containing 2$\times$ sodium citrate and 0.1% SDS, then 30 min at 48 C in 0.2$\times$ sodium citrate and exposed to Biomax MS x-ray film (Kodak, Rochester, NY) at -70 C. Autoradiograms were quantitated by scanning laser densitometry (2400 Gel Scan XL, LKB, Piscataway, NJ). Labeled bands were quantitated as densitometric units and the data were expressed as percent change *vs.* untreated control samples. The experiments were repeated two to four times for each time point to confirm findings.

Radiolabeled probes

The mouse RANKL cDNA was PCR cloned from cDNA derived from BALC stromal osteoblasts using the following primer pair, 5'atc aga aga cag cac tca ct 3' and 5'atc tag gac atc cat gct aat gtt c 3' as published (42). Mouse cDNA probe for osteoblast specific transcription factor (cbfa-1) was obtained from Dr. Gerard Karsenty (Houston, TX). The other cDNA probes were cloned using PCR and specific primer pairs for the respective genes as published previously (14, 47, 49, 50). The cloned cDNAs were confirmed by restriction enzyme mapping and/or sequencing.

OPG immunohistochemistry

Five-μm-thick proximal tibial sections were prepared as described for osteoclast number measurement. Anti-hOPG serum used for immunohistochemistry was generated by immunization of a rabbit with purified monomeric human OPG. Immunohistochemical stains were performed using the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). Tissue sections were deparaffinized in two successive 10-min washes of xylene, followed by two washes in ethanol, and a brief wash in PBS containing 0.1% Tween 20, pH 7.4. Sections were then immersed in 0.3% hydrogen peroxide in absolute methanol for 30 min to quench endogenous peroxidase activity. Before the addition of the primary antibody, nonspecific tissue binding was blocked by incubating the tissue section for 30 min at room temperature by 10% normal goat serum in PBS for 30 min. The primary antibodies were applied and incubated overnight at 4 C in a humid environment. The sections were then washed three times in 0.1% Tween 20. The secondary biotinylated antibody and
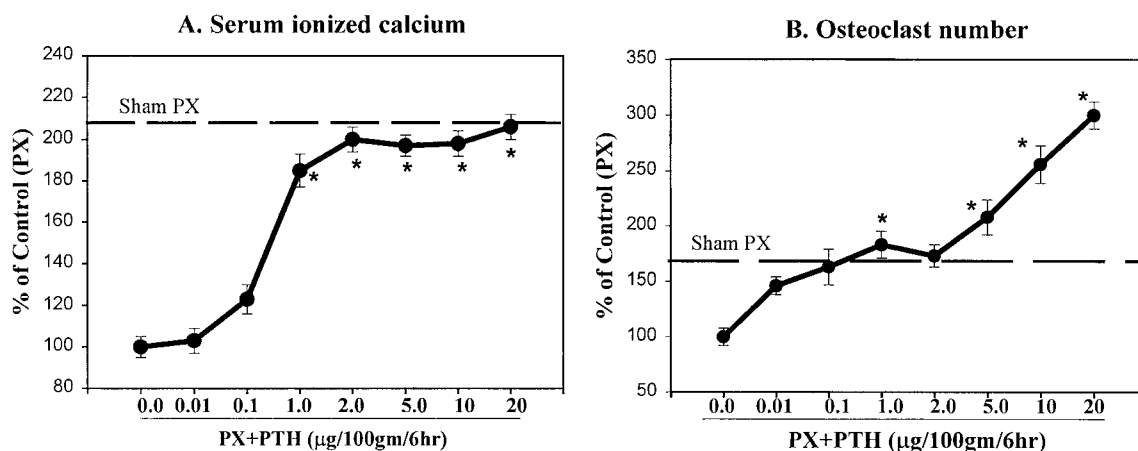
FIG. 1. PTH dose effects on serum ionized calcium (A) and osteoclast number of the proximal tibial metaphyses (B) in PX rats. The hPTH (1–38) at indicated doses was given sc infusion via an Alzet pump for 6 h. Data were presented as percent change of the vehicle-treated PX control. PTH infusion resulted in a dose response increase in serum-ionized calcium and osteoclast numbers. *, $P < 0.05$ *vs.* vehicle control. Data were presented as mean percent change of control ± SEM.

the Streptavidin-HRP conjugate complex were applied in a humidified chamber for 60 and 30 min, respectively. After washing in buffer, the chromogen diaminobenzidine was applied for 5 min followed by a counterstain with Mayer's hematoxylin. Negative controls included substituting the primary antisera with preimmune sera from the same species and omitting the primary antibody. All controls revealed the expected negative results.

Statistical analysis

Serum calcium, osteoclast number, and serum OPG levels were presented as mean ± SEM. Raw data of group differences were assessed by ANOVA using Fisher's protected least significant difference for which the significance level was $P < 0.05$.

Results

Effect of continuous human PTH (hPTH) infusion on bone resorption

To establish and validate the catabolic effects of PTH, PX rats were treated with either hPTH1–38(0.01–20 µg/100 g per 6 h) or vehicle by continuous infusion via Alzet pump for 6 h. The resorptive effects of PTH were monitored by measurement of serum calcium (Fig. 1A) and by quantitation of osteoclast number (Fig. 1B). A 6-h PTH infusion resulted in a dose-dependent increase in serum-ionized calcium level in PX rats (Fig. 1A). At PTH 20 µg/100 g per 6-h dose, serum ionized calcium was 205% of the vehicle-treated PX rats (0.65 ± 0.02 mmol/liter *vs.* 1.33 ± 0.03 mmol/liter) and was no different from sham rats (1.36 ± 0.03 mmol/liter *vs.* 1.33 ± 0.03 mmol/liter). The increase in serum calcium was correlated with a corresponding dose-dependent increase in osteoclast number, which was up to 300% of the vehicle-treated PX rats and 175% of the sham rats at a 20-µg dose, indicating increased bone resorption (Fig. 1B).

Effect of hPTH infusion on the expression of mRNA for OPG and RANKL: time and dose responses

Because OPG and RANKL have been reported to influence both osteoclast differentiation and function, we evaluated whether their expression was altered during bone resorption induced by PTH infusion. First, we examined the expression of OPG and RANKL after 6 h of continuous infusion of hPTH
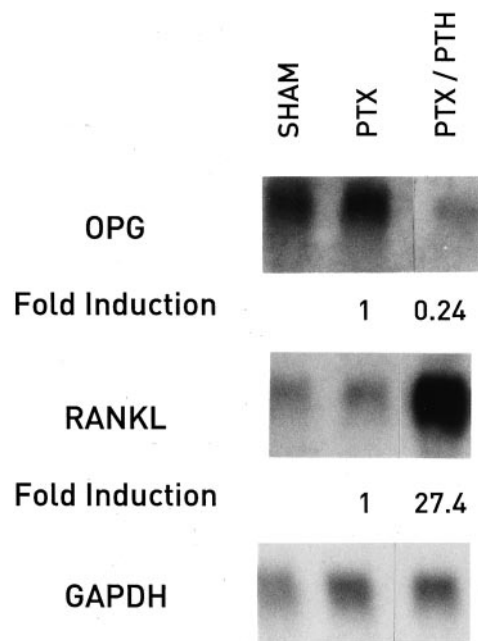

FIG. 2. Northern analysis showing the effect of continuous PTH on OPG and RANKL expression. PolyA+RNA was isolated immediately from the distal femur after 6 h of PTH infusion and analyzed for OPG and RANKL expression (pooled, n = 4/group). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was rehybridized as a control for RNA quantification. PTH 20 µg/100 g per 6-h infusion resulted in a 3-fold decrease in OPG mRNA and a 27-fold increase in RANKL mRNA.

1–38 (20 µg). This dose and time point (6 h) was chosen in this initial experiment because at this dose and time point, there is significant increase in bone resorption (Fig. 1). As shown in Fig. 2, in the controls (sham and PX vehicle treated), both OPG and RANKL mRNA was detectable with OPG more readily than RANKL. Six-hour PTH infusion resulted in a 3-fold decrease in OPG mRNA and a 27-fold increase in RANKL mRNA. To determine the effects of lower doses of PTH, we next examined the expression of OPG and RANKL after 6 h of continuous infusion of lower doses (0.1–10 µg) of hPTH 1–38. As shown in the autogram in Fig. 3A and plotted

in Fig. 3B, PTH infusion resulted in a dose-dependent decrease in OPG mRNA (optimal 3-fold) and reciprocal increase in RANKL (5-fold at 10 μg of PTH). The effects on both OPG and RANKL were detectable and reproducible with infusion as low as 1 μg of PTH. To further ascertain the kinetics of this effect on both OPG and RANKL, we examined the expression of mRNA for both OPG and RANKL at 0, 1, 3, 6, and 24 h after the start of hPTH1–38 (20 μg) infusion. Treatment of animals with hPTH 1–38 (20 μg/100 g per 6 h, or 3.3 μg/100 g per hour) caused a rapid and sustained decrease in OPG and reciprocal increase in RANKL mRNA.

The inhibition of OPG expression was evident within 1–6 h and was sustained up to 24 h (7.5-fold). The stimulation of RANKL was detected at 3 h, optimal at 6 h, and still elevated at 24 h (5.5-fold) (Fig. 4). We next compared this effect of continuous PTH to acute exposure of PTH. In this experiment, intact (non-PX) animals were given a single normal calcium dose sc as previously described (2, 12, 47). In contrast to the effects of continuous PTH infusion, acute exposure to PTH 1–38 resulted in a rapid but transient change in OPG and RANKL expression. The decrease in OPG mRNA (3.4-fold) and increase of RANKL mRNA (3.8-fold) were observed at 1 h, and they both recovered to near the control levels by 3 h
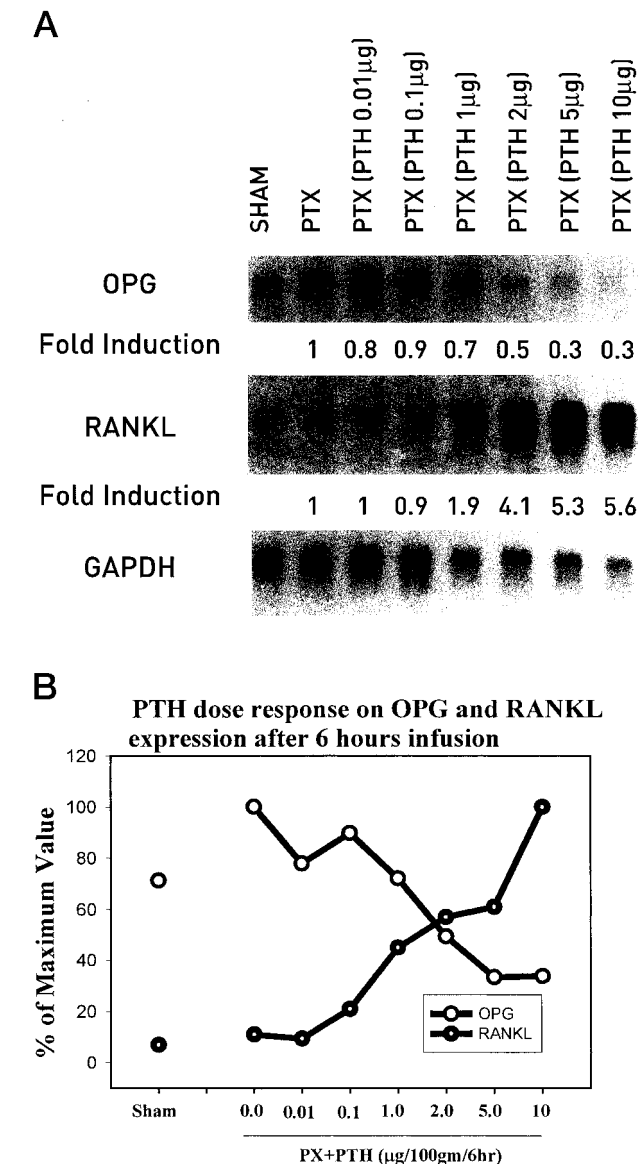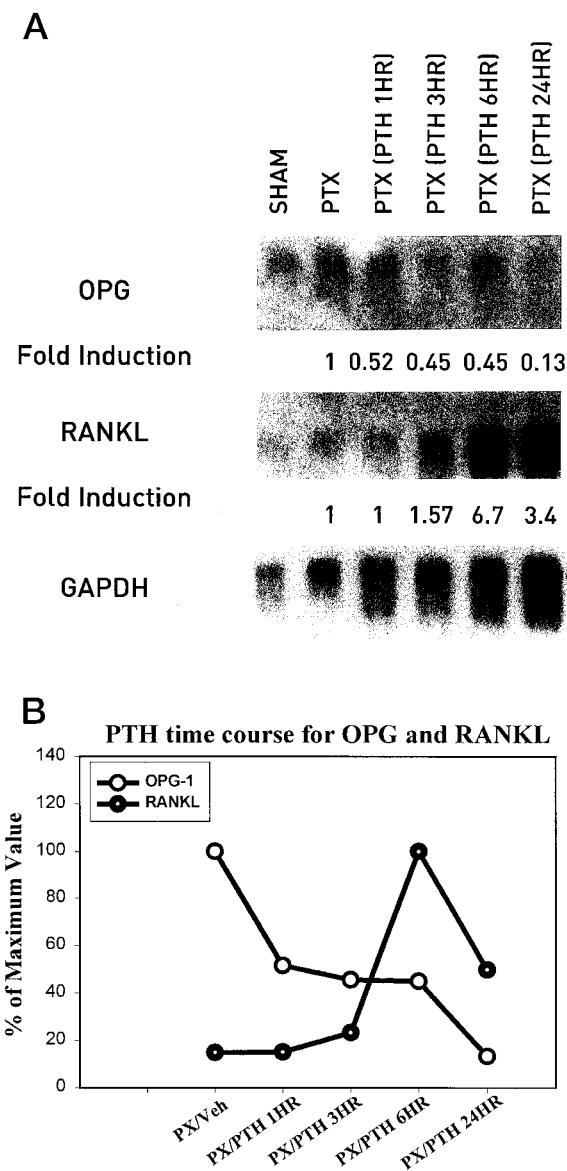

FIG. 3. Northern analysis showing a dose-dependent effect of continuous PTH on OPG and RANKL expression. PolyA+RNA was isolated immediately from the distal femur after 6 h of PTH infusion and analyzed for OPG and RANKL expression (pooled, n = 4/group). GAPDH was rehybridized as a control for RNA quantification. 3A, An actually representative Northern analysis demonstrated that hPTH (1–38) dose dependently down-regulated OPG and up-regulated RANKL mRNA expression in the distal femur metaphyses of PX rats. B, Data were expressed as percent of maximal value.

FIG. 4. Kinetics of continuous hPTH (1–38) on OPG and RANKL mRNA expression. After the indicated times of infusion with hPTH (1–38) 20 μg/100 g per 6-h administration via an Alzet pump, polyA+RNA was isolated immediately from the bone and analyzed for OPG and RANKL expression by Northern blot hybridization (pooled, n = 4/group). A, An actually representative Northern analysis. B, Data were expressed as percent of maximal value.

(Fig. 5). These results were similar and reproducible in multiple experiments.

Expression and regulation of OPG protein

To confirm actual changes in OPG protein, we analyzed: 1) OPG expression levels in serum using OPG ELISA and 2) OPG protein in bone by immunohistochemistry. The results indicated that serum OPG levels decreased in a dose-dependent manner upon PTH infusion. The effect was maximal at the PTH 10-μg dose level with about 40% inhibition (Fig. 6). In bone, OPG protein was detected predominantly in preosteoblasts, mature osteoblasts, and some newly formed bone matrix in the primary and secondary metaphyseal spongiosa. As we have noted previously, many areas of specific immunohistochemistry staining for OPG were seen in bone matrix (Fig. 7). In diaphyseal bone sections, OPG staining was less
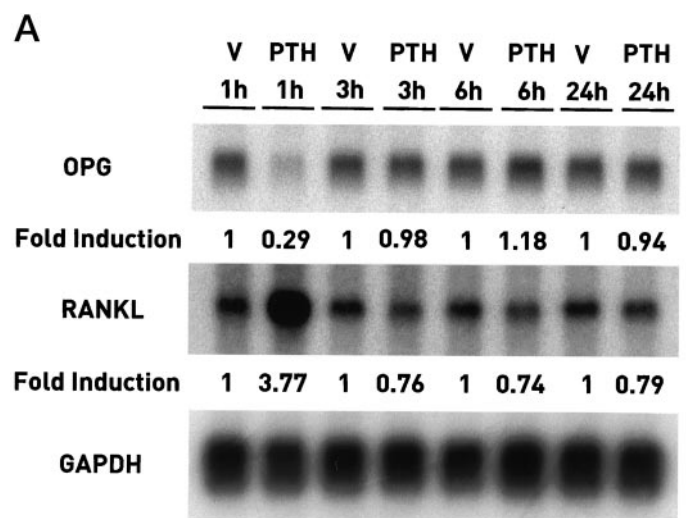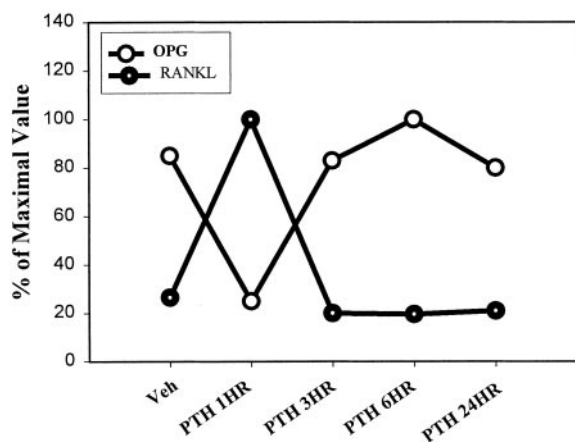
FIG. 5. Kinetics of acute hPTH (1–38) on OPG and RANKL mRNA expression. A single dose of hPTH (1–38) 8 μg/100 g was given sc to intact rats; polyA+RNA was isolated at indicated times from the bone and analyzed for OPG and RANKL expression by Northern blot hybridization (pooled, n = 4/group). A, An actually representative Northern analysis. B, Data were expressed as percent of maximal value.
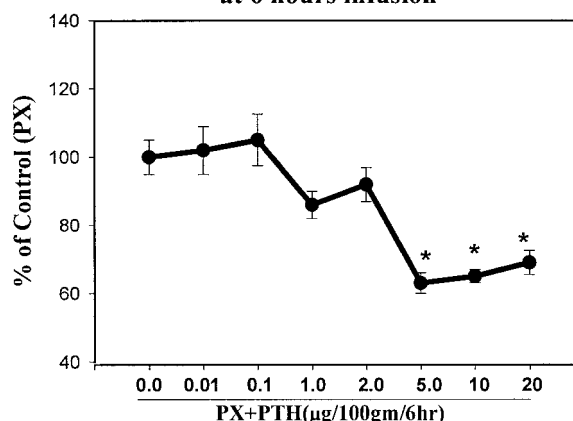

FIG. 6. Effects of PTH infusion on serum levels of OPG protein in PX rats. Parathyroidectomized rats were infused via an Alzet pump with hPTH (1–38) as indicated doses for 6 h (PX/PTH, n = 4). Serum OPG was examined by ELISA assay. PTH infusion dose dependently decreased circulating OPG when compared with vehicle-treated PX rats. *, $P < 0.05$ *vs.* vehicle control. Data were presented as percent change of control ± SEM.

intense than that seen in the metaphysis and was localized only to endosteal osteoblasts and occasional marrow cells (data not shown). No staining was observed in tissue sections in which the primary antibody was omitted. Treatment with PTH infusion resulted in weaker staining and in loss of matrix staining, indicating PTH infusion decreased the OPG expression in bone (Fig. 7).

Effect of hPTH infusion on the expression of mRNA for the genes associated with osteoblast-phenotype in rat femur

To further explore the molecular basis of the resorptive effects of PTH infusion, we also examined the expression of genes associated with the osteoblast-phenotype [such as the cbfa-1, osteocalcin (OC), bone sialoprotein (BSP), and type I collagen (COL1A1)] whose expressions typically increase under conditions in which PTH is anabolic. After 6 h of PTH infusion (20 μg/100 g) as described in the experiment in Fig. 1, mRNA expression was analyzed. As shown in Fig. 8, the expressions of genes that mark the osteoblast phenotype and bone formation were drastically decreased [cbfa-1 (2.3-fold), OC (3-fold), BSP (2.8-fold), and COL1A1 (5-fold)]. This result is consistent with a decrease in osteoblast activity and bone formation after continuous PTH infusion.

Discussion

Continuous infusion of PTH *in vivo* leads to increased bone resorption resulting in a net loss in bone mass. It has long been believed that at a cellular level, this resorptive effect results from direct actions on the cells of the stromal-osteoblastic lineage that in turn elaborate signals that promote the cascade of events leading to increased osteoclast differentiation and activity. However, the molecular mechanisms that underlie this phenomenon have not been well understood. The recent discoveries of RANKL, RANK, and OPG and the role of these molecules in promoting osteoclast differentiation suggest that these molecules may be the targets for PTH.
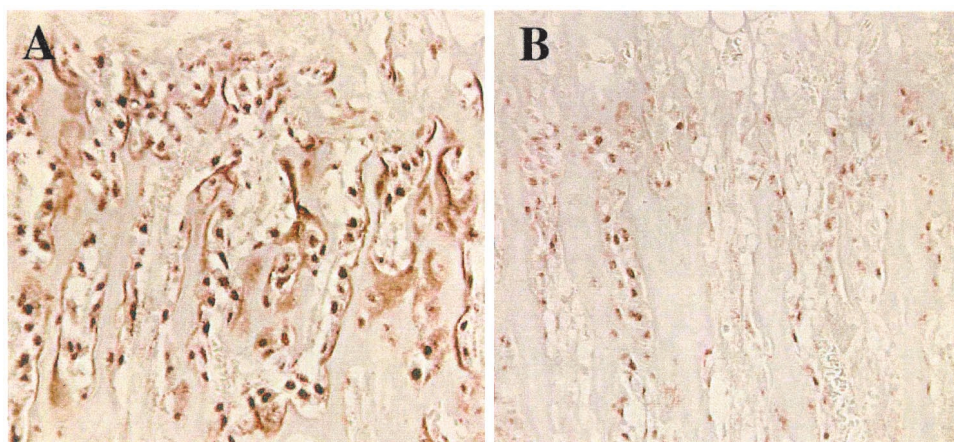
FIG. 7. Effects of PTH infusion on proximal tibiae OPG protein expression in PX rats detected by immunohistochemistry. Bones were counterstained with Mayer's hematoxylin after incubated with rabbit antirat OPG primary antibody. A, OPG was detected on the nuclear and cytoplasm of preosteoblasts, mature osteoblasts, and some newly formed bone matrix (*arrow*). B, PTH infusion down-regulated OPG expression when compared with the vehicle-treated rats. Original magnification ×225.

A direct demonstration of the role of local osteoblast-derived RANKL and OPG in PTH action has been confined to *in vitro* studies. To confirm the role of these molecules and to characterize molecular mediators in the physiology of normal bone resorption triggered by PTH, studies that examine the expression and regulation of these molecules *in vivo* are needed.

In the present study, we demonstrate that the expression of RANKL and OPG are rapidly and robustly altered in a well-validated *in vivo* model of PTH-induced bone resorption. Our results indicate that resorptive effects of PTH were marked by an increase in osteoclast number and serum calcium levels. Infusion of PTH induced a rapid and sustained increase in RANKL mRNA and decrease in both OPG mRNA and protein in the osteoblasts. The changes in RANKL and OPG were dose and time dependent and preceded peak increases in bone resorption. Additionally, a decreased expression of genes associated with the bone formation-osteoblast phenotype was found. Similar findings have been reported in several *in vitro* systems (42, 51, 52). Our results provide further evidence that the regulation of these molecules by calciotropic agents such as PTH may be an important regulatory mechanism linking osteoblast and osteoclast. The immunohistochemical and Northern blot studies presented here and elsewhere clearly establish that osteoblasts produce OPG and RANKL and that both are regulated *in vivo* upon PTH infusion. The increase in RANKL and decrease in OPG confirmed that the true balance of RANKL and OPG is altered to favor bone resorption. Further, OPG levels were also decreased in serum, highlighting the extent and magnitude of PTH effects to favor bone resorption. This is consistent with an important regulatory role for these molecules in the initiation and maintenance of the catabolic effect of PTH in normal physiology of osteoclast differentiation.

Our results show that the expression and regulation of these molecules in bone fulfill their proposed role as local regulators of bone resorption. As shown by immunohistochemistry, OPG was expressed predominantly in osteoblasts and their precursors. These are the target cells regulated by calciotropic hormones to elicit a resorptive response. The
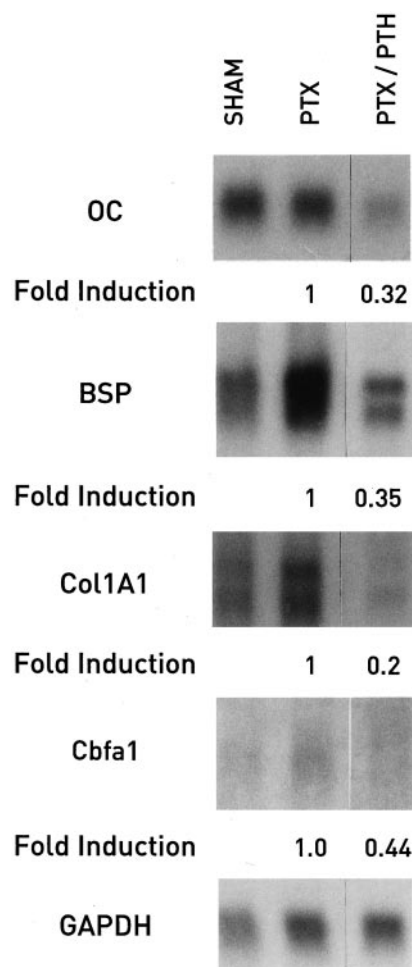

FIG. 8. Effects of hPTH (1–38) infusion on expression levels of osteoblast phenotype-associated genes in the distal femur metaphysis of PX rats. The hPTH (1–38) 20 μg/100 g per 6 h was infused via an Alzet pump for 6 h, and polyA+RNA was isolated immediately from the distal femur and analyzed for OC, BSP, Col1A1, and cbfa-1 expression by Northern blot hybridization (pooled, n = 4/group). The hPTH (1–38) down-regulated OC, BSP, Col1A1, and cbfa1.

consistent findings of OPG in the bone matrix might reflect its availability from this site to exercise control over osteoclast formation. In the control state (untreated) there was a strong constitutive expression of OPG and relatively much lower RANKL mRNA. This balance favoring increased OPG is consistent with low basal resorption in normal physiology in the mature animals. The rapid and sustained alteration in this balance after continuous PTH infusion to create an increased RANKL/OPG ratio clearly would favor increased resorption and decreased bone mass. It was also noted that OPG was lost from the matrix under this condition. Perhaps this sustained resorptive signal is required to ensure a catabolic effect of PTH infusion. In support of this premise, acute exposure to a single anabolic normal calcemia dose of PTH triggered a rapid but transient alteration in the RANKL/OPG ratio (47). The decrease in OPG mRNA and increase in RANKL mRNA was detected in 1 h but recovered to control levels by 3 h.

It is interesting to note that a continuous PTH infusion also resulted in down-regulation of various genes that are associated with the osteoblast bone formation phenotype *in vivo*, such as cbfa-1, OC, BSP, and COL1A1. The observation was in contrast with the findings of a single injection or intermittent administration of PTH, which resulted in an increased bone formation and up-regulation of these genes (11, 13–15, 21; Onyia *et al.,* unpublished data). On the basis of the response to these two different regimens of PTH, we propose a role for OPG and RANKL in both anabolic and catabolic effects of PTH in rat bone. The differential responsiveness can be explained by the differences in the magnitude and duration of resorptive signaling. Continuous PTH results in enhanced resorption and decreased bone mineral density via pronounced and sustained increases in osteoclast formation and activity. On the other hand, PTH effects on formation may require a subtle and/or transient increase in osteoclast formation and activity, perhaps to prepare the bone surface for the deposition of new matrix (44, 47).

In conclusion, these results provide *in vivo* evidence that OPG and RANKL are important regulators of bone homeostasis in PTH action. Our results demonstrate that PTH concurrently inhibits production of the antiresorptive cytokine receptor OPG and stimulates the levels of the bone-resorbing cytokine RANKL. In continuous infusion of PTH, the changes in OPG and RANKL were rapid and sustained and preceded peak effects on bone resorption. We also demonstrated that the resorptive effect of continuous PTH is associated with decreased expression of osteoblast bone-formation genes. Taken together, these findings provide a potential molecular explanation for PTH effects on bone resorption.

Acknowledgments

References

1. **Jerome CP, Johnson CS, Vafai HT, et al.** 1999 Effect of treatment for 6 months with human parathyroid hormone (1–34) peptide in ovariectomized Cynomolgus monkeys (*Macaca fascicularis*). Bone 25:301–309
2. **Dempster DW, Cosman F, Parisien M, Shen V, Lindsay R** 1993 Anabolic actions of parathyroid hormone on bone. Endocr Rev 14:690–708
3. **Hock JM, Fonseca GJ, Raisz LG** 1989 Human parathyroid hormone-(1–34) increases bone mass in ovariectomized and orchidectomized rats. Endocrinology 122:2899–2904
4. **Hock JM, Fonseca J, Gunness-Hey M, Kemp BE, Martin TJ** 1989 Comparison of the anabolic effects of synthetic parathyroid hormone-related protein (PTHrP) 1–34 and PTH 1–34 on bone in rats. Endocrinology 125:2022–2027
5. **Hock JM, Gera I** 1992 Effects of continuous and intermittent administration and inhibition of resorption on the anabolic response of bone to parathyroid hormone. J Bone Miner Res 7:65–72
6. **Hock JM, Hummert JR, Boyce R, Fonseca J, Raisz LG** 1989 Resorption is not essential for stimulation of bone growth by hPTH-(1–34) in rats *in vivo*. J Bone Miner Res 4:449–457
7. **Oxlund H, Ejersted C, Andreassen TT, Torring O, Nilsson MHL** 1993 Parathyroid hormone (1–34) and (1–84) stimulate cortical bone formation both from periosteum and endosteum. Calcif Tissue Int 53:394–399
8. **Reeve J, Meunier PJ, Parsons JA, et al.** 1980 Anabolic effect of human parathyroid hormone fragment on trabecular bone in involutional osteoporosis: a multicenter trial. BMJ 280:1340–1344
9. **Riggs BL, Melton LJ, III** 1992 The prevention and treatment of osteoporosis. N Engl J Med 327:620–627
10. **Slovik DM, Neer RM, Potts JTJ** 1981 Short-term effects of synthetic human parathyroid hormone-(1–34) administration on bone mineral metabolism in osteoporotic patients. J Clin Invest 68:1261–1271
11. **Hock JM, Onyia JE, Miller B, et al.** 1994 Anabolic PTH targets proliferating cells of the primary spongiosa in young rats, and increases the number differentiating into osteoblasts. J Bone Miner Res 9:S412 (abstr)
12. **Schmidt IU, Dobnig H, Turner RT** 1995 Intermittent parathyroid hormone treatment increases osteoblast number, steady state messenger ribonucleic acid levels for osteocalcin, and bone formation in tibial metaphysis of hypophysectomized female rats. Endocrinology 136:5127–5133
13. **Hock JM, Onyia JE, Bidwell J** 1995 Comparisons of *in vivo* and *in vitro* models of the response of osteoblasts to hormonal regulation with aging. Calcif Tissue Int 56:S44–S47
14. **Onyia JE, Bidwell J, Herring J, Hulman J, Hock JM** 1995 *In vivo,* human parathyroid hormone fragment (hPTH 1–34) transiently stimulates immediate early response gene expression but not proliferation in trabecular bone cells of young rats. Bone 17:479–484
15. **Onyia JE, Miller B, Hulman J, et al.** 1997 Proliferating cells in the primary spongiosa express osteoblastic phenotype *in vitro*. Bone 20:93–100
16. **Lee K, Deeds JD, Chiba S, Un-No M, Bond AT, Segre GV** 1994 Parathyroid hormone induces sequential *c-fos* expression in bone cells *in vivo: in situ* localization of its receptor and *c-fos* messenger ribonucleic acids. Endocrinology 134:441–450
17. **Liang JD, Hock JM, Sandusky GE, Santerre RF, Onyia JE** 1999 Immunohistochemical localization of selected early response genes expressed in trabecular bone of young rats given hPTH1–34. Calcif Tissue Int 65:369–373
18. **Miles RR, Sluka JP, Santerre RF, et al.** 2000 Dynamic regulation of RGS2 in bone: potential new insights into parathyroid hormone signaling mechanism. Endocrinology 141:28–36
19. **Pollock JH, Blaha MJ, Lavish SA, Stevenson S, Greenfield EM** 1996 *In vivo* demonstration that parathyroid hormone and parathyroid hormone-related protein stimulate expression by osteoblasts of interleukin-6 and leukemia inhibitory factor. J Bone Miner Res 11:754–759
20. **Takeda N, Tsuboyama T, Kasai R, et al.** 1999 Expression of the *c-fos* gene induced by parathyroid hormone in the bones of SAMP6 mice, a murine model for senile osteoporosis. Mech Ageing Dev 108:87–97
21. **McClelland P, Onyia JE, Miles R, et al.** 1998 Intermittent administration of parathyroid hormone (1–34) stimulates matrix metalloproteinase-9 (MMP-9) expression in rat long bone. J Cell Biochem 70:391–401
22. **Tsuda E, Goto M, Mochizuki S, et al.** 1997 Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. Biochem Biophys Res Commun 234:137–142
23. **Simonet WS, Lacey DL, Dunstan CR, et al.** 1997 Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 89:309–319
24. **Yasuda H, Shima N, Nakagawa N, et al.** 1998 Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 95:3597–3602
25. **Yasuda H, Shima N, Nakagawa N, et al.** 1998 Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. Endocrinology 139:1329–1337
26. **Morinaga T, Nakagawa N, Yasuda H, Tsuda E, Higashio K** 1998 Cloning and characterization of the gene encoding human osteoprotegerin/osteoclastogenesis-inhibitory factor. Eur J Biochem 254:685–691
27. **Shalhoub V, Faust J, Boyle WJ, et al.** 1999 Osteoprotegerin and osteoprote-

gerin ligand effects on osteoclast formation from human peripheral blood mononuclear cell precursors. J Cell Biochem 72:251–261

28. **Bucay N, Sarosi I, Dunstan CR, et al.** 1998 osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev 12:1260–1268

29. **Mizuno A, Amizuka N, Irie K, et al.** 1998 Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin. Biochem Biophys Res Commun 247:610–615

30. **Suda T, Takahashi N, Martin TJ** 1992 Modulation of osteoclast differentiation. Endocr Rev 13:66–88

31. **Suda T, Udagawa N, Nakamura I, Miyaura C, Takahashi N** 1995 Modulation of osteoclast differentiation by local factors. Bone 17:87S–91S

32. **Martin TJ, Udagawa N** 1998 Hormonal regulation of osteoclast function. Trends Endocrinol Metab 9:6–12

33. **Matsuzaki K, Udagawa N, Takahashi N, et al.** 1998 Osteoclast differentiation factor (ODF) induces osteoclast-like cell formation in human peripheral blood mononuclear cell cultures. Biochem Biophys Res Commun 246:199–204

34. **Tsukii K, Shima N, Mochizuki S, et al.** 1998 Osteoclast differentiation factor mediates an essential signal for bone resorption induced by 1 alpha,25-dihydroxyvitamin D3, prostaglandin E2, or parathyroid hormone in the microenvironment of bone. Biophys Res Commun 246:337–341

35. **Lacey DL, Timms E, Tan HL, et al.** 1998 Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93:165–176

36. **Wong BR, Josien R, Lee SY, et al.** 1997 TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. J Exp Med 186:2075–2080

37. **Anderson DM, Maraskovsky E, Billingsley WL, et al.** 1997 A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature 390:175–179

38. **Wong BR, Rho J, Arron J, et al.** 1997 TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. J Biol Chem 272:25190–25194

39. **Kodaira K, Kodaira K, Mizuno A, et al.** 1999 Cloning and characterization of the gene encoding mouse osteoclast differentiation factor. Gene 230:121–127

40. **Takahashi N, Udagawa N, Suda T** 1999 A new member of tumor necrosis factor ligand family, ODF/OPGL/TRANCE/RANKL, regulates osteoclast differentiation and function. Biochem Biophys Res Commun 256:449–455

41. **Kong YY, Yoshida H, Sarosi I, et al.** 1999 OPGL is a key regulator of oste-

oclastogenesis, lymphocyte development and lymph-node organogenesis. Nature 397:315–323

42. **Horwood NJ, Elliott J, Martin TJ, Gillespie MT** 1998 Osteotrophic agents regulate the expression of osteoclast differentiation factor and osteoprotegerin in osteoblastic stromal cells. Endocrinology 139:4743–4746

43. **Tsukii K, Shima N, Mochizuki S, et al.** 1998 Osteoclast differentiation factor mediates an essential signal for bone resorption induced by 1 alpha,25-dihydroxyvitamin D3, prostaglandin E2, or parathyroid hormone in the microenvironment of bone. Biochem Biophys Res Commun 246:337–341

44. **Delmas PD, Vergnaud P, Arlot ME, Pastoureau P, Meunier PJ, Nilssen MHL** 1995 The anabolic effect of human PTH (1–34) on bone formation is blunted when resorption is inhibited by bisphosphonate tiludronate—is activated resorption a prerequisite for the *in vivo* effect of PTH on formation in remodeling system? Bone 16:603–610

45. **Kanzawa M, Sugimoto T, Kanatani M, Chihara K** 2000 Involvement of osteoprotegerin/osteoclastogenesis inhibitory factor in the stimulation of osteoclast formation by parathyroid hormone in mouse bone cells. Eur J Endocrinol 142:661–664

46. **Dunstan CR** 2000 Osteoprotegerin and osteoprotegerin ligand mediate the local regulation of bone resorption. Endocrinologist 10:18–26

47. **Onyia JE, Miles RR, Yang Y, et al.** 2000 In vivo demonstration that parathyroid hormone (hPTH 1–38) inhibits the expression of osteoprotegerin (OPG) in bone with the kinetics of an immediate early gene. J Bone Miner Res 15:863–870

48. **Parfitt A, Drezner MK, Glorieux FH, et al.** 1987 Bone histomorphometry: standardization of nomenclature, symbols and units. J Bone Miner Res 2:595–610

49. **Alvarez M, Long H, Onyia J, Hock J, Xu W, Bidwell J** 1997 Rat osteoblast and osteosarcoma nuclear matrix proteins bind with sequence specificity to the rat type I collagen promoter. Endocrinology 138:482–489

50. **Boguslawski G, Hale LV, Yu XP, et al.** 2000 Activation of osteocalcin transcription involves interaction of protein kinase A- and protein kinase C-dependent pathways. J Biol Chem 275:999–1006

51. **Lee SK, Lorenzo JA** 1999 Parathyroid hormone stimulates TRANCE and inhibits osteoprotegerin messenger ribonucleic acid expression in murine bone marrow cultures: correlation with osteoclast-like cell formation. Endocrinology 140:3552–3561

52. **Murakami T, Yamamoto M, Ono K, et al.** 1998 Transforming growth factor-beta1 increases mRNA levels of osteoclastogenesis inhibitory factor in osteoblastic/stromal cells and inhibits the survival of murine osteoclast-like cells. Biochem Biophys Res Commun 252:747–752