Receptor activator of NF-κB ligand arrests bone growth and promotes cortical bone resorption in growing rats

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McHugh, Nansie A., Haydee M. Vercesi, Robert W. Egan, and John A. Hey. Receptor activator of NF-κB ligand arrests bone growth and promotes cortical bone resorption in growing rats. J Appl Physiol 95: 672–676, 2003. First published April 11, 2003; 10.1152/japplphysiol.00056.2003.—Receptor activator of NF-κB ligand (RANKL), produced by osteoblastic lineage cells and activated T cells, is an essential factor for osteoclast differentiation, activation, and survival. Therefore, RANKL is a focal point of therapies targeting bone diseases where there is an imbalance of bone metabolism in favor of bone resorption. The present study assesses the effects of exogenous RANKL on growing bone. RANKL (100 μg·kg⁻¹·day⁻¹ for 7 days) administered to Sprague-Dawley weanling rats caused major deficits in growth, appearance, and bone mineral densities (BMD). Urinary deoxypyridinoline crosslinks, a measure of bone turnover, were higher in the RANKL-treated rats (P = 0.031), and the bone mineral content was lower (P < 0.001). The final BMD in the RANKL-treated rats was lower (P = 0.039) than in the control rats (19 ± 7 vs. 38 ± 5 mg/cm³). Moreover, calculated cortical bone density in each bone slice (total BMD – trabecular BMD) indicated there was only 5% cortical bone remaining in RANKL-treated rats. We conclude that therapies targeting RANKL are likely to have effects on cortical as well as trabecular bone density.

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Materials and Methods

General. This study was conducted under a protocol approved by the Animal Care and Use Committee of Schering-Plough Research Institute. Sprague-Dawley weanling (21 days old) rats weighing 44 ± 2 g were purchased from Charles River Laboratories (Wilmington, MA). All rats were housed in a temperature- and humidity-controlled room on a 12:12-h light-dark cycle. Rats were given free access to standard rat chow (Harlan Teklad Labdiet, Madison, WI) and reverse-osmosis water. Two studies were conducted by using the same protocol. An initial study compared effects of RANKL treatment [10 μg/day sc of 462-TR, a chimeric protein consisting of the CD33 signal peptide plus 6 histidine residues fused to the amino-terminus of the mouse RANKL (amino acids 72–316); R&D Systems, Minneapolis, MN] vs. vehicle-treated rats (control, PBS given subcutaneously). On the basis of the lack of effects observed in the first study, a second study was conducted by using a supraphysiological dose of newly available RANKL [462-TEC; 100 μg/day sc, mouse RANKL (amino acids 158–317 expressed in Escherichia coli); R&D Systems]. Each group included eight rats.

Protocol. On day 1, the rats were anesthetized with isoflurane (“to effect”, IsoFlo, Abbott Laboratories, Chicago, IL), and a depolarizing dose of newly available RANKL [462-TEC; 100 μg/day sc, mouse RANKL (amino acids 158–317 expressed in Escherichia coli); R&D Systems]. Each group included eight rats.

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and baseline three-dimensional bone mineral density (BMD) measurements were taken by pQCT (Stratec XCT Research SA, Pforzheim, Germany). The rats were given their supplements/vehicle daily on days 1–7. On the day 8, the rats were anesthetized with pentobarbital sodium (50 mg/kg ip, Nembutal, Abbott Laboratories), and the final BMD measurements were taken. The rats were then euthanized by exsanguination, and the scanned tibia was harvested for bone ash content.

pQCT measurement and analysis. The settings for pQCT scanning were research SA collimation at a voxel size of 0.1 mm³; therefore, the slice width was 0.1 mm. The voxel is equivalent to a pixel with volume (3 dimensional). This small voxel size minimizes partial volume effect errors (i.e., including voxels that are not completely filled with bone). Comparable placement of slices was ensured by measuring a slice in the metaphysis 2 mm from the reference line that was placed at the proximal edge of the growth plate. This placement results in the most consistent baseline measurements (14). All bone slices were analyzed with the same parameters by using Stratec Software (Stratec Medizintechnik). An automatic ContourMode 1 was used to define the outer edge of the cortical bone, and PeelMode 20 (an adaptation of PeelMode 2 that determines the threshold to be used by evaluating the BMD at a predefined percentage of total bone) was used to define the inner edge of the cortical bone and the beginning of the trabecular bone. The region of interest for the final scans of the RANKL-treated rats had to be manually drawn due to the lack of cortical bone. For determining trabecular BMD, the percent option was used with trabecular area defined at 30% with a threshold of 280 mg/cm³.

Biochemical markers. In the second study, the rats were placed in metabolic cages on day 7 for a 24-h urine collection. Urine samples were processed [Beckman Synchron Clinical Analyzer (CX3delta), Fullerton, CA] for Ca²⁺ concentration and creatinine; aliquots of the urine were used to measure deoxypyridinoline (Dpd) crosslinks via ELISA (Quidel, Mountain View, CA). Dpd values are reported as nanomoles per millimole of creatinine.

Bone ash content. The scanned tibia was harvested and cleaned of tissue. The bone was vacuum dried overnight, weighed, then placed in a furnace at 950°C, and reduced to its mineral content. Ash data are reported as a percentage of total bone dry weight.

Statistics. Statistical analysis of all data were assessed by using ANOVA with Dunnett’s test for treatments with a control (SigmaStat, SPSS Software, Chicago, IL).

RESULTS

Initial study. The baseline measurements of BMD data as measured by pQCT were not significantly different between groups (176 ± 6 mg/cm³ in the control group vs. 172 ± 3 mg/cm³ in the RANKL-treated group). Rats that were treated with the lower dose of RANKL (462-TR, 10 μg/day) showed no differences in their final BMD or bone ash content measurements compared with the vehicle-treated control rats (Fig. 1A). For both RANKL-treated and control rats, final trabecular BMD measurements were significantly higher than their respective baseline, a result expected in growing rats. Bone ash content data also showed no significant differences between the two groups (Fig. 1B).

Second study. In the high-dose study, the control group’s final trabecular BMD measurements were also significantly higher than their respective baseline; however, RANKL-treated (462-TEC, 100 μg/day) rats exhibited major differences in growth, appearance, and bone ash content compared with the other members of their group and control rats. Although the RANKL-treated group showed a small, nonsignificant increase in final trabecular BMD values compared with their baseline measurements, this was likely due to the fact that all the bone was now the consistency of trabecular bone. The change from baseline BMD measurements between the two groups was significantly different in trabecular bone measurements (Fig. 2A; \( p = 0.039 \)). Furthermore, when the difference between the total bone density less the trabecular bone density was examined, the

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Fig. 1. Lower dose study that shows the baseline and change from baseline (growth) bone mineral density (BMD) in receptor activator of NF-κB ligand (RANKL)-treated (10 μg/day) and control rats (A) and the bone ash weight (bone mineral content; B). Both BMD and bone ash weight showed the typical growth pattern of the weanling rat, and there were no significant differences between treated and untreated groups. Values are means ± SE.
RANKL-treated group was devoid of cortical bone (Fig. 2B; \(P < 0.001\)). Bone ash content data confirm these findings showing a decrement in percentage of bone ash to total bone content (Fig. 3; \(P < 0.001\)). The pQCT scan images were dramatically different after 1 wk of treatment. We observed no distinct cortical rim in the pQCT image of the RANKL-treated rats and the entire slice appeared to be trabecular bone (Fig. 4). Moreover, we had to hand-draw the region of interest for these slices because the automated computerized tomography software uses the cortical edge density as a reference point for determining the region of interest. Furthermore, after the tibia for bone ash content was removed, most of the RANKL-treated bones could not be removed without fracture. The RANKL-treated group had significantly higher urinary Dpd values than the untreated controls (Fig. 5; \(P < 0.01\)); however, their urinary and serum Ca\(^{2+}\) levels were not significantly different. There was a trend toward a lower urinary Ca\(^{2+}\) levels in the RANKL-treated group suggesting a compensatory mechanism to conserve calcium.

**DISCUSSION**

Recent studies show that various cytokines and humoral factors such as parathyroid hormone, PGE\(_2\), vitamin D\(_3\), and IL-6 that regulate osteoclast function in normal bone remodeling and pathophysiological bone diseases do so by modifying the expression of RANKL (8, 11, 17). We presently sought to investigate the role of RANKL in the developing mammal at a time when basal bone mass is being established, and it is assumed that the balance is tipped in favor of bone formation. This model was first developed by Schenk et al. (16) to test antiresorptive compounds such as bisphosphonates. We have previously employed this model to evaluate antiresorptive compounds by pQCT quantification within 1 wk (14).

In the present study, we found that a 10 \(\mu\)g/day dose of RANKL for 7 days elicited no observable changes in bone mineral content or BMD. We had attributed the lack of effect to the dose of RANKL that was used; however, recently Takayanagi et al. (18) reported that RANKL can limit its own osteoclastogenic effect by stimulating production of IFN-\(\gamma\) by osteoclast precursors. Consequently, the lower dose may not have generated serum levels high enough to elicit pathological effects. In a second attempt to show proof of principle for the role of RANKL in the growing rat, we used a newly developed protein that was assured to be effective. We chose a dose that was 10 times that used in the first study, a supraphysiological dose to define the maximal effect on the growing rat. We found that a dose of 100 \(\mu\)g·kg\(^{-1}\)·day\(^{-1}\) exerted a significant resorptive action on cortical bone by pQCT scanning techniques, suggesting that RANKL may play a large role in the developing bone.

Clinical studies reporting the effects of osteoporosis on bone primarily show the effects on trabecular bone, which, due to its porous composition and architecture,
is commonly first to manifest the effects of increased or decreased osteoclastic activity. Meanwhile, cortical bone comprises 85% of the total body bone content. Remodeling of the cortical bone occurs by endosteal resorption, and the decline in cortical bone mass normally occurs later than trabecular bone loss (23). For example, the vertebra is composed largely of trabecular bone, and, therefore, the incidence of vertebral fracture occurs earlier in life (~60 yr of age) (23). Conversely, long bones such as the femur have a large cortical component, and, therefore, the incidence of fracture in the femur and femoral neck occur more at an age of ≥70 yr (23). Osteoporosis studies are conducted on adult humans that have achieved peak bone mass, and the diagnosis of osteoporosis is determined by using dual-energy X-ray absorptiometry, a two-dimensional scan (22). In humans, the most common form of adult-onset osteoporosis is found in the postmenopausal woman and the early progression of the disease can be observed in the trabecular compartment with the loss of some trabeculae entirely leaving the remaining trabeculae with wide separations and not connected (12). Cortical bone loss occurs through enlargement of the subendocortical spaces and deeper erosion due to increased osteoclastic activity (12). Present therapies addressing this form of osteoporosis have been relatively successful in maintaining bone by inhibition of resorption, and emerging therapies are aimed at replacing bone loss.

In this study, we found that, in the developing bone, RANKL not only had effects on trabecular bone but also elicited profound effects on cortical bone. Cortical bone was virtually remodeled to the density and architecture of trabecular bone or never formed. These data have serious implications for osteopenic diseases or conditions of the juvenile bone. Osteoporotic diseases that compromise bone growth through the RANK/RANKL pathway may also compromise peak cortical bone mass. These include early nutritional deficiencies (Ca²⁺, vitamin K, vitamin D, and anorexia nervosa) or may be secondary to therapeutic steroid use. Peak bone mass has been determined to be an important factor in osteoporotic fracture risk assessment (1). It has been well established that women develop osteoporosis earlier and more severely than men who have higher initial BMD (12). Moreover, perimenopausal women who are small in stature and slight of build are predisposed to develop osteoporosis because their initial bone mass is less than their sturdier peers. This risk factor is not specific to the female gender because men who do not achieve bone masses comparable to their peers are also predisposed to the early onset of osteoporosis. For example, Van Pottelbergh et al. (21) found that men with idiopathic osteoporosis showed no indications of accelerated bone loss compared with age-matched controls. However, by measuring the BMD of three generations within the families of these men with idiopathic osteoporosis, the authors found that there was a deficit in bone acquisition. Therefore, they suggest that these men were genetically predisposed to lower peak bone masses. Therefore, if peak bone mass is not achieved during early development, initial bone mass values

Fig. 4. Representative peripheral quantitative computed tomography scan images of a control rat (A) and a RANKL-treated rat (B). The normal cortical bone is absent in the bone scans from rats treated with RANKL (100 μg·kg⁻¹·day⁻¹).

Fig. 5. Deoxypyridinoline (Dpd) cross-links, a urinary marker of bone resorption, were significantly increased in the RANKL-treated rats (100 μg·kg⁻¹·day⁻¹) compared with control rats. Data are means ± SE. *Significant difference compared with control rats, P = 0.031.
will be lower despite gender or hormonal issues, and it can, therefore, be predicted that the onset of osteoporosis will also be earlier.

The biomechanical properties and strength of bones are determined by the composition of the bone, BMD, and the cross-sectional architecture. The size and shape of the cortical bone, the composition and architecture of trabecular bone, as well as the ratio of cortical to trabecular bone all play a role in determining the strength or fragility of bone (19, 20). Simply stated, the biomechanical strength of the bone is determined by the ability of its structure to withstand the stresses demanded of it. As people age, bones generally become more brittle at a time when they are more prone to falls (24). Normal young bone is more flexible and will deform due to stresses before breaking; however, the juvenile bone is subject to additional stresses due to sports-related falls and injuries (13). The decrease in cortical bone that we observed would affect the cortical-to-trabecular bone ratios and would, therefore, potentially have serious life-long consequences. Much attention has been given to the changes in trabecular bone with osteoporosis; however, cortical bone is an important factor in determining bone strength as shown by Crabtree et al. (5) in their intracapsular hip fracture studies. The fragility of the bone is dependent as much on the amount of bone tissue as on the material properties and architecture.

In conclusion, this study has shown that the RANK/RANKL pathway potentially plays a major role in the development of peak cortical bone mass as well as its role in normal bone turnover. Further studies are warranted to determine whether lower doses affect the bone strength of juvenile bone by decreasing cortical wall thickness. Furthermore, the mouse RANKL was able to activate the rat RANK receptor to affect bone resorption and will be a useful tool in evaluating this relationship.

REFERENCES