Expression Profiles of Receptor Activator of Nuclear Factor κB Ligand, Receptor Activator of Nuclear Factor κB, and Osteoprotegerin Messenger RNA in Aged and Ovariectomized Rat Bones

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ABSTRACT

The receptor activator of nuclear factor-κB ligand (RANKL; also known as tumor necrosis factor–related activation-induced cytokine [TRANCE], osteoprotegerin ligand [OPGL], and osteoclast differentiation factor [ODF]) is a transmembrane ligand expressed in osteoblasts and bone marrow stromal cells. It binds to RANK, which is expressed in osteoclast progenitor cells, and induces osteoclastogenesis. OPG, a decoy receptor for RANKL, also binds to RANKL, and competitive binding of RANKL with RANK or OPG is thought to regulate bone metabolism. To investigate roles of the RANKL/RANK/OPG system in pathophysiological conditions, the expression of RANKL, RANK, and OPG messenger RNA (mRNA) was analyzed in bones of aged and ovariectomized rats by means of in situ hybridization. In the control 8-week-old male and sham-operated female rat bones, the expression of RANKL mRNA was detected in hypertrophic chondrocytes of the growth plate and some periosteal and endosteal mesenchymal cells. The expression of RANK mRNA was detected in osteoclast-like cells and mononuclear cells in contact with the cortical and trabecular bones. The expression of OPG mRNA was detected in proliferating chondrocytes and osteocytes. In the 2.5-year-old rat bones, the expression of RANKL, RANK, and OPG mRNA tended to decrease except for the endosteal region. In the ovariectomized rat bones, the expression of RANKL, RANK, and OPG mRNA increased, and high expression of OPG mRNA was induced in resting chondrocytes and osteocytes. These results suggest that estrogen deficiency stimulates the RANKL/RANK/OPG system and induces OPG in cells that have been thought to be less important for bone metabolism. (J Bone Miner Res 2001;16:1416–1425)

Key words: receptor activator of nuclear factor κB ligand, receptor activator of nuclear factor κB, osteoprotegerin, aging, ovariectomy

INTRODUCTION

Discovery of the receptor activator of nuclear factor κB ligand (RANKL, also called osteoprotegerin ligand [OPGL]/osteoclast differentiation factor [ODF]/tumor necrosis factor [TNF]–related activation-induced cytokine [TRANCE]) clarified the molecular mechanisms behind the initial steps of osteoclastogenesis.1–7 RANKL is a type II TNF-like transmembrane protein, which binds to the receptor activator of nuclear factor κB (NF-κB; RANK) or OPG. RANK is expressed in osteoclast progenitor cells and induces osteoclastogenesis by binding to RANKL.8–10 OPG

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is a secreted TNF receptor–related protein, which was isolated as a protein-inhibiting bone resorption. Because OPG does not have a transmembrane domain, it works as a decoy receptor and inhibits bone resorption. Osteoclastogenesis can be reproduced efficiently by coculture in vitro using bone marrow stromal cells and bone marrow macrophages or spleen cells. The essential role of bone marrow stromal cells in in vitro osteoclastogenesis is explained by the fact that RANKL is expressed in the bone marrow stromal cells on stimulation with 1α,25-dihydroxyvitamin D₃ and dexamethasone. Actually, bone marrow macrophages or spleen cells differentiated into osteoclasts without stromal cells when the soluble form of recombinant RANKL protein was added to the culture medium. The essential role of the RANKL/RANK system in osteoclastogenesis was confirmed in mice with a disrupted ogpl gene. The mice showed severe osteopetrosis and a defect of tooth eruption caused by complete lack of osteoclasts.

Bone metabolism must be regulated strictly to maintain bone structures, to heal fractures, and to supply calcium ion. We showed dynamic change in the biological activity of bone cells under physiological and pathological conditions. Physiologically, bone formation and bone resorption are synchronized nicely, with an imbalance leading to osteopetrosis or osteoporosis. A decrease in bone volume occurs in aged men. In the aged male rat bones, most osteoblasts looked flat-shaped and expressed much less type I collagen and osteocalcin messenger RNA (mRNA) than the cuboidal osteoblasts in the young rat bones. We also found that osteopontin mRNA was dominantly expressed in osteoclast progenitor-like mononuclear cells and osteoclasts rather than osteoblastic cells, and the expression in these bone resorbing cells greatly decreased in the aged rat bones. These findings indicate that the bone metabolism in aged rat bones is less active than that in young rat bones and support that osteoporosis in aged men occurs because of an imbalance between bone resorption and bone formation at low turnover of bone. Postmenopausal osteoporosis is thought to be caused by estrogen deficiency and is reproduced in an animal model by ovariectomy. Overproduction and overactivation of osteoclasts have been observed in bones of ovariectomized rats. Previously, we showed that both the expression of type I collagen and osteocalcin in osteoblasts and the expression of osteopontin in bone-resorbing cells increased in the ovariectomized rat bones. These data confirmed that postmenopausal osteoporosis was caused by an imbalance of bone resorption and bone formation under conditions of increased bone turnover. The importance of the RANKL/RANK system for osteoclastogenesis strongly suggests that the system is associated with the pathogenesis of age-dependent reduction in bone volume and postmenopausal osteoporosis.

In this study, we analyzed the expression of mRNA for RANKL, RANK, and OPG in bones of 8-week-old and 2.5-year-old male rats and sham-operated and ovariectomized female rats. Relatively constant expression levels of RANKL and RANK mRNA in the endosteal cells in the aged rat bones indicated a different regulatory mechanism for the expression of RANKL and RANK mRNA from that of bone matrix proteins, which was analyzed previously. On the other hand, the expression of RANKL, RANK, and OPG mRNA increased in the ovariectomized rat bones like the expression of bone matrix proteins, which was reported previously. These results strongly suggested that the RANKL/RANK/OPG system was closely involved in the pathogenesis of bone loss by aging and estrogen deficiency.

**MATERIALS AND METHODS**

**Animals and tissue preparation**

Eight-week-old male and 10-week-old female Wistar rats were purchased from SRL (Shizuoka, Japan). Male Wistar rats (2.5 years old) were supplied from SPF Aging Farm of Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan). The male Wistar rats from the farm, which were purchased from SRL, were kept from the time they were 4 weeks old, and mean survival of the 2.5-year-old male Wistar rats was 8.8% (Department of Laboratory Animal Science, Tokyo Metropolitan Institute of Gerontology, unpublished data, 1998). From the farm, four healthy 2.5-year-old male rats were supplied for experiments. The 8-week-old and the 2.5-year-old male rats were anesthetized with ether and killed by cardiac perfusion. The 10-week-old female Wistar rats were anesthetized and bilaterally ovariectomized or sham-operated. These animals were killed by cardiac perfusion 3 weeks after the operation. The distal portion of the femur was dissected from each animal and fixed for 24 h at 4°C in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2), which was prepared just before use. All fixed samples were decalcified with 0.5 M EDTA solution at 4°C for up to 1 month. The decalcified samples were dehydrated in ethanol, cleared in chloroform, and embedded in paraffin, and then 4-μm thick sections were made. Four animals for each group were used for the experiments.

**Preparation of RNA probes**

Complementary DNA (cDNA) fragments of mouse RANKL and RANK were cloned from mouse thymic cDNA, and cDNA fragments of mouse OPG were cloned from mouse bone marrow macrophage cDNA by reverse-transcription–polymerase chain reaction (RT-PCR) method. The RT-PCR was carried out with 40 cycles of denaturation at 98°C for 20 s and annealing and extension at 68°C for 2 minutes using LA Taq polymerase (Takara Co., Shiga, Japan) and the following primers:

- **RANKL**: RANK5, 5′-ATGAAACAAAGGCCTTTTCAGGGCCGTCGCA-3′; and RANKL3, 5′-TCAGTCTCTTGTCCTGAACCTTTGGAAAGCCC-3′
- **RANK**: RANK5, 5′-TCACCCGGGACTGAAAGCAGGTTGG-3′; and RANK3, 5′-TGGGCTCCATCAAATGCAGCCGAG-3′
- **OPG**: OPG5, 5′-GGGACAGTTTCCTGGGGGATCGTAATGC-3′; and OPG3, 5′-TGAAGCTTGTGCGAGGAACTCATC-3′

The cDNA fragments were cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA). The vectors including...
the cDNA fragment for RANKL, RANK, or OPG were linearized and transcribed using T7 RNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany) to generate uridine 5'-triphosphate (UTP)-labeled single-stranded antisense and sense RNA probes, respectively. The [35S]-labeled probes were used for hybridization at a concentration of 1 \times 10^7 \text{ cpm/ml} in 50% formamide, 10% dextran sulfate, 0.6 M NaCl, 10 mM Tris-HCl (pH 7.6), 1 mM dithiothreitol (DTT), and 0.2 \mu g/ml of yeast total RNA.

In situ hybridization

Treatment of the slides and hybridization conditions were as described previously. Briefly, after removal of the paraffin with xylene, the sections were treated with 2 \mu g/ml of proteinase K (Sigma Chemical Co., St. Louis, MO, USA) at 37°C for 15 minutes, fixed for 15 minutes with the same fixatives as described previously, treated with 0.25% acetic anhydride in 0.1 M triethanol amine-HCl (pH 8.0) for 10 minutes, dehydrated in ethanol, and air-dried. The hybridization solution was spread over the sections and the slides were incubated overnight at 60°C in plastic humidified boxes. After hybridization, the sections were washed twice in 50% formamide solution containing 2\times SSC and treated with 12.5 \mu g/ml of RNase A (Sigma Chemical Co.). The sections were then washed twice in 2\times SSC and twice in 0.2\times SSC at 60°C for 30 minutes each time, dehydrated in ethanol, and finally dipped in NTB-3 emulsion (Eastman Kodak, Rochester, NY, USA) diluted 1:1 with a 2% glycerc solution. The dipped slides were exposed at 4°C for 3 weeks. The exposed slides were developed in D-19 developer, fixed in F-5 fixative, and finally counterstained with hematoxylin and eosin.

RESULTS

The expression profiles of RANKL, RANK, and OPG mRNA were summarized in Table 1. The grading of the expression level represents total amount of the expression in each region. Profiling of the sites and cells that appear in the table were shown in Fig. 1.

The expression of RANKL mRNA

The expression levels of RANKL mRNA were compared between the femurs of 8-week-old and 2.5-year-old male rats. In the 8-week-old rat bones, RANKL mRNA was detected most prominently in a subset of periosteal cells of the metaphyseal cortical bone (Fig. 2A, arrows). Lower levels of expression were detected in a subset of endosteal cells (Fig. 2A, arrowheads). In addition, RANKL mRNA was detected in hypertrophic chondrocytes of the growth plate (Fig. 2C, arrows). In the primary spongiosa, RANKL mRNA was not detected clearly (Fig. 2C). In the 2.5-year-old rat bones, RANKL mRNA was detected in a subset of periosteal (Fig. 2B, an arrow) and endosteal cells (Fig. 2B, arrowheads) of the metaphyseal cortical bones. The expression profile in the 2.5-year-old rat bones was similar to that in the 8-week-old rat bones except for the periosteal region. In the periosteal region, cells with a positive signal were fewer than in the 8-week-old rat bones although the expression level in each cell was similar to the 8-week-old rat bones (Figs. 2A and 2B, arrows). In the 2.5-year-old rat bones, the growth plate was composed of several layers of resting and/or proliferative chondrocytes, and hypertrophic chondrocytes were not present. In this region, little RANKL mRNA was expressed (Fig. 2D).

Next, the expression of RANKL mRNA was analyzed in the femurs of sham-operated and ovariectomized female rats. Ten-week-old female rats were sham-operated or ovariectomized and killed 3 weeks after each operation. The expression of RANKL mRNA was detected in a subset of periosteal (Figs. 2E and 2F, arrows) and endosteal (Fig. 2F, arrowheads) cells of the sham-operated and ovariectomized rat bones. The expression level was similar in this region between the sham-operated and ovariectomized rat bones (Figs. 2E and 2F). In the growth plate of the sham-operated rat bones, RANKL mRNA was detected in the hypertrophic chondrocytes (Fig. 2G, arrows). In the ovariectomized rat bones, both the number of cells expressing RANKL mRNA and the expression level of RANKL mRNA in each cell increased in comparison to the sham-operated rat bones (Fig. 2H, arrows). In addition to being up-regulated in the hypertrophic chondrocytes, the expression of RANKL
mRNA was induced in a subset of cells in the primary spongiosa of the ovariectomized rat bones (Fig. 2H, arrowheads). In the primary spongiosa of the sham-operated rat bones, RANKL mRNA was not clearly detected (Fig. 2G).

The expression of RANK mRNA

In the metaphyseal cortical bone of the 8-week-old rats, high levels of expression of RANK mRNA were detected in relatively small cells (Fig. 3A, arrows) and in large cells (Fig. 3A, arrowheads) in the periosteum and endosteum. In the periosteum of the 2.5-year-old rat bones, the number of cells expressing RANK mRNA decreased in comparison to the 8-week-old rat bones (Figs. 3B and 3C). In the 2.5-year-old rat bones, RANK mRNA was detected in mononuclear cells (Fig. 3C, arrows) and multinuclear cells (Figs. 3B and 3C, arrowheads). The expression profile in the trabecular bones of each animal was similar to that in the metaphyseal cortical bones (data not shown).

In the sham-operated and ovariectomized rat bones, RANK mRNA was detected in small (Figs. 3D and 3E, arrows) and large (Figs. 3D and 3E, arrowheads) cells in the periosteum and endosteum of the metaphyseal trabecular bones. The level of expression in these regions was similar between sham-operated and ovariectomized rat bones. However, in the trabecular bones, the expression in each cell was stronger in the ovariectomized rats than in the sham-operated rats (Figs. 3F and 3G).

The expression of OPG mRNA

OPG mRNA was detected in the cartilage rather than in the bone. In the 8-week-old rat bones, relatively strong expression of OPG mRNA was detected in the proliferative chondrocytes of the articular cartilage (Fig. 4A, arrows). In addition, the mRNA also was detected in some proliferative chondrocytes of the growth plate (Fig. 4C, arrows). In the 2.5-year-old rat bones, OPG mRNA also was detected in the proliferative chondrocytes of the atrophic growth plate (Fig. 4D, arrows), but the number of cells expressing OPG mRNA and the expression level in each cell were similar to those in the 8-week-old rat bones. In the articular cartilage, the expression was undetectable (Fig. 4B). Outside of the cartilage region in the 8-week-old rats, OPG mRNA was detected in a subset of cells in the periosteum of the metaphyseal cortical bones (Fig. 4E, arrows). In this region, the mRNA also was detected in a subset of osteocytes (Fig. 4E, an arrowhead). In the 2.5-year-old rat bones, the expression was not detected clearly in the metaphyseal cortical bone region (Fig. 4F). In the trabecular bones of the 8-week-old and 2.5-year-old rats, little OPG mRNA was expressed (data not shown).

In the articular region of the sham-operated rat bones, OPG mRNA was detected in the proliferative chondrocytic cells (Fig. 5A, arrowheads). In addition, the mRNA was detected in some osteocytes in the metaphyseal and diaphyseal cortical bones (Fig. 5C, arrowheads). In the region of the growth plate, little OPG mRNA was seen in the sham-operated rats (Fig. 5E) in contrast to 8-week-old and 2.5-year-old male rats (Figs. 4C and 4D). In the sham-operated rats, the expression also was weak in the trabecular bone region (Fig. 5G). Three weeks after the ovariectomy, the expression of OPG mRNA greatly increased in some regions of the bone. Prominent expression was detected in resting chondrocytes of the articular cartilage (Fig. 5B, arrows). In this region, the mRNA also was detected in some proliferative chondrocytes (Fig. 5B, arrowheads). In the cortical and trabecular bones, both the number of osteocytes expressing OPG mRNA and the expression level of OPG mRNA in each cell increased (Fig. 5D, arrowheads). In the growth plate region, weak expression was detected in some resting chondrocytes (Fig. 5F, arrows). In addition, many cells expressing OPG mRNA were seen in the primary (Fig. 5H) and secondary spongiosa (data not shown) of the ovariectomized rat bones.

**FIG. 1.** Profiling of the sites and cells in the femur analyzed in this study. 1, cortical periosteum; 2, cortical endosteum; 3, trabecular endosteum; 4, growth plate resting chondrocyte; 5, growth plate proliferative chondrocyte; 6, growth plate hypertrophic chondrocyte; 7, articular resting chondrocyte; 8, articular proliferative chondrocyte; 9, osteocyte.
For all these experiments, sense control hybridization was performed and no specific signal was detected (data not shown).

**DISCUSSION**

In the 8-week-old rat bones, strong expression of RANKL and RANK mRNA was detected in the periosteum and endosteum of the metaphyseal cortical bones. In the 2.5-year-old rat bones, the expression of RANKL and RANK mRNA in the periosteum greatly decreased, but the expression in the endosteum was relatively stable. Metaphyseal cortical bone is the region where bone reconstruction actively occurs during bone development. Therefore, the high level of expression of RANKL and RANK mRNA in the epiphyseal cortical bone of the 8-week-old rats was thought to be induced by active reconstruction in the developing bone. In addition, the decreased expression of RANKL mRNA in the growth plate of the 2.5-year-old rat bone might be caused by a less active bone metabolism than that in the 8-week-old rat bones. Except for the regions where bone metabolism was influenced greatly by the growth of bone, the difference in the expression level of RANKL and RANK mRNA between young and old rat
bones was not great. Considering the marked decrease in the expression of mRNA for bone matrix proteins in aged rat bones, the decrease in bone resorption may be milder than that of bone formation in the aged rat bones. This may explain the pathogenesis of osteoporosis in aged men.

We previously reported that the expression of bone matrix proteins increased in ovariectomized rat bones in addition to the number and activity of osteoclasts. The data indicated that osteopenia in ovariectomized rat bones was induced by an imbalance of bone resorption and bone formation under conditions of high turnover of bone. The RANKL/RANK system, a key regulator of osteoclastogenesis, was expected to be stimulated in the ovariectomized rat bones. Actually, in the ovariectomized rat bones, an increase in the expression of RANKL mRNA was seen in the hypertrophic chondrocytes of the growth plate, and induction of the expression was seen in the cells of the primary spongiosa (Figs. 2G and 2H). An increase in the expression of RANK mRNA was seen in the cells of the trabecular bones (Figs. 3F and 3G). However, in the cortical bone region, the expression profiles of RANKL and RANK mRNA changed little between sham-operated and ovariectomized rat bones (Figs. 2E, 2F, 3D, and 3E). In the ovariectomized rats, bone volume decreased more prominently in the trabecular bones than in the cortical bones, at least up to 6 weeks after the operation. Therefore, an increase in the

**FIG. 3.** The expression of RANK mRNA in the femurs of (A) 8-week-old male, (B and C) 2.5-year-old male, (D and F) sham-operated female, and (E and G) ovariectomized rats. (A) Strong expression of RANK mRNA in the small (arrows) and large (arrowheads) cells in the metaphyseal cortical bone of the 8-week-old rat. (B and C) The expression in the osteoclast-like cells (arrowheads) and mononuclear cells (arrows) in the periosteum (B) or endosteum (C) of the metaphyseal cortical bone of the 2.5-year-old rat. (D and E) The expression in the metaphyseal cortical bones of the sham-operated (D) and ovariectomized (E) rats. Signals in the small (arrows) and large (arrowheads) cells are seen in both specimens. (F and G) The expression of RANK mRNA in cells on the surface of the trabecular bones (arrows). (G) Strong expression is seen in the ovariectomized rat bone (×300).
expression of RANKL and RANK mRNA in the trabecular bones rather than in the cortical bones was thought to explain why the increase in bone resorption caused by estrogen deficiency was more severe in the trabecular bones than in the cortical bones. The mechanism of the differential regulation of the expression of RANKL and RANK mRNA in different portions of the bone was not clear, but because the ovariectomy induced systemic hormonal change, the differential response of trabecular bone cells and cortical bone cells to estrogen deficiency might be caused by the difference of cell populations between the trabecular bone region and cortical bone region. In addition, increased expression of RANKL mRNA in the hypertrophic chondrocytes of the ovariecetomized rat bones suggests that abnormally excessive bone resorption occurs just after enchondral bone formation in the region.

Also, it was interesting that the expression level of RANK mRNA was high in osteoclastic cells and osteoclast progenitor-like monocuclear cells on the surface of the cortical and trabecular bones but not in the bone marrow cells. Our PCR analyses indicated that bone marrow macrophages express RANK mRNA (Ikeda and Utsuyama, unpublished data, 2000), and the expression was thought to be enhanced on the surface of the cortical and trabecular bones. These results suggest that the expression of RANK mRNA increases to a level high enough to induce osteoclastogenesis on the surface of the bone matrix, and this might explain why osteoclasts are formed only on the surface of the bone matrix.

OPG mRNA was shown to be expressed in cartilages in a mouse embryo, but the expression profile in mature bone tissue had not been reported. In this study, we showed that OPG mRNA was expressed in proliferative chondrocytes, osteocytes, and a subset of periosteal cells in the 8-week-old rat bones. Relatively strong expression was seen in the proliferative chondrocytes and osteocytes. OPG works as a decoy receptor for RANKL and inhibits osteoclastogenesis by competing with RANK when binding to RANKL. The expression profile of OPG mRNA was different from that of RANK mRNA. The expression of OPG mRNA in the proliferative chondrocytes may not affect osteoclastogenesis considering the fact that there is no vascularization in the cartilage, but there is a possibility that OPG is accumulated in the cartilage and protects it from resorption. Surprisingly, in the ovariecetomized rat bones, strong expression of OPG mRNA was induced in the resting chondrocytes (Fig. 5B). In human osteoblastic cells, the expression of OPG mRNA was indicated to be stimulated.
by estrogen. In addition, serum OPG was shown to increase in women with postmenopausal osteoporosis. Our data seemed to conflict with these data. In this study, we could not detect the expression of OPG mRNA in osteoblastic cells, and in vivo change in expression of OPG mRNA in osteoblasts remained unknown. The meaning of this strong induction of OPG mRNA in the resting chondrocytes and osteocytes was not clear, but our results strongly suggested that regulation of OPG expression in chondrocytes and osteocytes was different from that of osteoblasts.

The expression of OPG mRNA in the osteocytes is interesting. The increase in the number of osteocytes expressing OPG mRNA in the ovariectomized rat bones suggests a protective reaction against excess bone resorption caused by ovariectomy. The excess bone resorption exposes the osteocytes expressing OPG mRNA at the surface of the bone, which may inhibit osteoclastogenesis. In the ovariectomized rat bones, the expression of OPG mRNA also was induced in cells on the surface of the trabecular bones (Fig. 5H). In this study, we could not characterize these cells, but there was a possibility that they were identical to the osteocytes expressing OPG mRNA, which were exposed at the surface of the bone.

There were some inconsistencies in the expression between 8-week-old male rat bones and sham-operated female...
rat bones. There are three possibilities to explain these inconsistencies: difference between 8-week-old rat bones and 13-week-old rat bones, difference between nontreated rat bones and sham-operated rat bones, or difference between male rat bones and female rat bones. In the proliferative chondrocytes, OPG mRNA was detected only in the male rat bones and was not detected in the female rat bones irrespective of age and operation. Therefore, it was suggested that the difference in the expression of OPG mRNA in the proliferative chondrocytes was caused by sex difference.

In this study, we analyzed profiles of the expression of RANKL, RANK, and OPG mRNA in the 8-week-old and 2.5-year-old male rat bones and in the sham-operated and ovariectomized female rat bones. The expression of RANKL, RANK, and OPG mRNA was relatively stably maintained in the endosteal region. In addition, high expression of OPG mRNA was induced in resting chondrocytes and osteocytes in the ovariectomized rat bones.

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