Multimerization of the Receptor Activator of Nuclear Factor-κB Ligand (RANKL) Isoforms and Regulation of Osteoclastogenesis*

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The receptor activator of nuclear factor-κB ligand (RANKL), a member of the tumor necrosis factor family, is a transmembrane protein, which is known as an essential initiation factor of osteoclastogenesis. Previously, we identified three RANKL isoforms. RANKL1 was identical to the originally reported RANKL. RANKL2 had a shorter intracellular domain. RANKL3 was identical to the originally reported RANKL. RANKL1 did not have the intracellular or transmembrane domains and was suggested to act as a soluble form protein. Here, we show that RANKL forms homo- or hetero-multimers. NIH3T3 cells transfected with RANKL1 or RANKL2 form mononuclear tartrate-resistant acid phosphatase-positive preosteoclasts in an in vitro osteoclastogenesis assay system. Coexpression of RANKL1 and RANKL2 induces multinucleated osteoclasts. RANKL3 has no effect on the formation of preosteoclasts or osteoclasts but significantly inhibits fusion of preosteoclasts when coexpressed with RANKL1 and RANKL2. These findings imply the presence of multiple multimeric structures of RANKL, which may regulate bone metabolism.

The receptor activator of nuclear factor-κB ligand (RANKL, also known as OPG, ODF, or TRANCE) is a type II TNF-like transmembrane protein, which plays fundamental roles for fusion of preosteoclasts by binding to the receptor, receptor activator of nuclear factor-κB (RANK) (1, 2). Another soluble form receptor-like molecule, osteoprotegerin (OPG), binds to RANKL and inhibits osteoclastogenesis by competitive inhibition of the signaling through the RANKL/RANK system (3, 4). Osteoclastogenesis is efficiently reproduced by an in vitro coculture system using macrophages and bone marrow stromal cells (5, 6). In the presence of recombinant RANKL protein, osteoclasts were formed without bone marrow stromal cells (7–9). The essential role of the RANKL/RANK system in osteoclastogenesis was confirmed in mice with a disrupted RANKL gene and mice with disruption of the RANK gene, both of which showed severe osteopetrosis (10–12).

Previously, we identified three RANKL isoforms from mouse cDNA and named them RANKL1, RANKL2, and RANKL3 (13). RANKL1 was identical to the originally reported RANKL. RANKL2 had a shorter intracellular domain. RANKL3 did not have the intracellular or transmembrane domains and was suggested to be a soluble form protein. Previous findings showed that both tumor necrosis factor (TNF)-α and TNF-β formed trimers, and the multimeric structure was essential for expressing the activities of the molecules (14–16). These findings strongly suggest that another member of this family, RANKL, also forms a multimeric structure. Recently, the crystal structure of the extracellular domain of mouse RANKL was determined, and it was shown to form a trimeric structure (17, 18). The present study revealed that the mouse RANKL isoforms interact and express different biological activities.

EXPERIMENTAL PROCEDURES

Transfection and Selection—For immunoprecipitation and confocal microscopy, the protein-coding region of cDNA for each RANKL isoform was cloned into pEGFP-N2 and pDsRed-N1 vectors (Clontech), both of which express the product of inserted cDNA as a fusion to the N terminus of fluorescent protein, EGFP and DsRed, respectively. For osteoclastogenesis assay, each RANKL isoform was cloned into selectable mammalian expression vector, pMIE GygB (13), pCNA3.1 (Invitrogen), or pEFBOSBsr (19). Transfections were performed using the TransFast transfection reagent (Promega). NIH3T3 cells transfected with pMIE GygB-RANKL were cultured in α-minimal essential medium with 10% fetal bovine serum and 250 μg/ml Hygromycin B (Calbiochem). In some clones expressing each RANKL isoform, pCNA3.1-RANKL was additionally transfected. The cells were selected and maintained in the medium supplemented with 500 μg/ml Geneticin (Sigma) in addition to the Hygromycin B. To obtain cells expressing RANKL1, RANKL2, and RANKL3, pEFBOSBsr-RANKL3 was further transfected to the cells expressing RANKL1 and RANKL2. Cells were selected and maintained in the medium supplemented with 0.2 μg/ml Blastidicin S hydrochloride (Funakoshi) in addition to the Hygromycin B. To obtain cells expressing RANKL1, RANKL2, and RANKL3, pEFBOSBsr-RANKL3 was further transfected to the cells expressing RANKL1 and RANKL2. Cells were selected and maintained in the medium supplemented with 0.2 μg/ml Blastidicin S hydrochloride (Funakoshi) in addition to the Hygromycin B and Geneticin. The expression of each RANKL isoform was analyzed by reverse transcriptase (RT)-PCR and Northern hybridization. RT-PCR was performed as described previously (13) using forward primers, 5′-CGTACGCTATTCGAGATAGTGGAGG-3′, 5′-CTGCAATTCGACAGCAATGGGCG-3′, and 5′-TGCAGAACTGGTTTGGCACAAG-3′, which recognized downstream from the transcriptional initiation site of pMIE GygB, pCNA3.1, and pEFBOSBsr, respectively, and a common reverse primer, 5′-TCACGTCCTGTCTCTCGAATTTGAGGCCC-3′, which recognized the 3′ end of the inserted mouse RANKL cDNA. Northern hybridization was performed as described previously (13).

Immunoprecipitation—RANKL-pDsRed and/or RANKL-pEGFP were transfected to NIH3T3 cells plated in 100-mm-type culture dishes. After 48 h, cells were collected and lysed with 1 ml Tris-buffered saline (pH 7.1) containing 1% digitonin, 1 mM EDTA, 0.02% NaN₃, 1 mM phenylmethylsulfonyl fluoride, 100 μM N-p-tosyl-
t-phenylalanine chloromethyl ketone, 10 μM leupeptin, and 10 μg/ml aprotinin), and the supernatants were preincubated with Sepharose 4B coupling with 100 μg of rabbit immunoglobulins and incubated with 40 μg of rabbit anti-DeRed-peptide antibody (Clontech, catalog number 8370) cross-linked to immobilized Protein G (Pierce, catalog number 45210) for 72 h. The immune complexes were washed with lysis buffer three times and eluted with 100 μl of elution buffer (1% digitonin, 50 mM glycin-HCl [pH 3.0], 150 mM NaCl, 0.02% NaN3). The eluant was adjusted to pH 7.0 with 1 M Tris-HCl (pH 9.0). Samples were separated by 10% SDS-PAGE and transferred electrophoretically onto nitrocellulose membranes (Schleicher & Schuell). Immunoblotting was performed using the rabbit anti-DeRed-peptide antibody or a mouse anti-GFP monoclonal antibody (Clontech, catalog number 8371). Immunoreactive proteins were visualized using the ECL Western blotting detection kit (Amersham Pharmacia Biotech).

**Cross-linking of Recombinant RANKL3 Protein**—Five μg of recombinant mouse RANKL3 (13) was treated with 1.2 mg/ml ethylene glycol bis(succinimidyl) succinate (EGS) at 4°C for 2 h. Part of the cross-linked samples was cleaved using trypsin and hydroxylamine hydrochloride (Pierce), pH 8.5, at 37°C for 4 h. The samples were separated by 10% SDS-PAGE and visualized by Coomassie Brilliant Blue staining.

**Confocal Microscopy**—RANKL-DsRed and/or RANKL-EGFP were transfected into NIH3T3 cells plated on 35-mm glass-bottom dishes (Iwaki Glass). After 48 h, they were viewed before or after fixation with 3.8% formaldehyde in phosphate-buffered saline using an LSM 510 laser scanning confocal microscope (Zeiss) equipped with a ×63 water immersion objective. EGFP was examined using the 488-nm line of the argon-krypton laser and a set of the 488-nm main splitter and the 505–530-nm band pass emission. DeRed was examined using the 543-nm line of the helium-neon laser and a set of the 485/543-nm main splitter HFT and the 560-nm long pass emission.

**Immunohistochemistry and Cytochemical Staining**—Mouse bone marrow macrophages were prepared from femora and tibiae of 5-week-old female ddY mice as described previously (20, 21). Using 48-well plates, the bone marrow macrophages (1.5 × 105 cells/well) were cocultured with NIH3T3 cells expressing RANKL isoform(s) (1.5 × 104 cells/well) in 0.25 ml/well of 10% fetal bovine serum, 30 ng/ml macrophage colony-stimulating factor, 1% BSA, 5% mouse bone serum, 30 μg/ml macrophage colony-stimulating factor, 1 × 10−8 M of 1α,25-dihydroxyvitamin D3, and 1 × 10−7 M of dexamethasone. After 5–10 days of culture, cells were fixed with 4% formaldehyde in phosphate-buffered saline (−) for 10 min and ice-cold methanol-acetone (1:1) for 2 min. Tartrate-resistant acid phosphatase (TRAP) was stained with 0.1 M sodium acetate buffer (pH 5.0) containing 0.1 mg/ml naphthol AS-MX phosphate (Sigma), 0.6 mg/ml fast red AL salt (Sigma), and 50 mM sodium tartrate (Wako). Statistical significance was evaluated using the Mann-Whitney U test. Animal care was in accordance with guidelines for animal welfare in Tokyo Medical and Dental University.

**In Vitro Formation of Resorption Lacunae**—Whale dentin slices were placed on 8-well plates, and cultures were performed on them (5). Ten days after the culture period, the slices were soaked in 4% gluconic acid solution for 24 h, rinsed with distilled water, and stained with Mayer’s hematoxylin. The area of resorption lacunae was quantified using the net micrometer disk in the eyepiece of a microscope, and the ratios of standard areas with resorption lacunae to the areas with and without resorption lacunae were calculated (22). The statistical significance was evaluated using the Mann-Whitney U test. Immunohistochemistry—Cells were treated with 0.1% hydrogen peroxide in 95% methanol for 5 min, rinsed with phosphate-buffered saline, and treated with 2% bovine serum albumin (Serologicals Proteins) at 4°C overnight. An antibody against calcitonin receptors (CT-Rs) (Santa Cruz Biotechnology, catalog number 8860) or the antibody preabsorbed with the blocking peptide (Santa Cruz Biotechnology) was applied on the specimens and incubated at 4°C overnight. After rinsing with phosphate-buffered saline, immunohistochemistry was performed using the ENVISION Plus system (DAKO).

**RESULTS**

**Interaction of Three RANKL Isoforms**—Constructs of RANKL1, RANKL2, and RANKL3 with a tag of EGFP at the C terminus and RANKL1, RANKL2, and RANKL3 with a tag of DeRed at the C terminus were transfected into NIH3T3 cells and served for immunoprecipitation and Western blotting. The expression of transfected RANKL1-DeRed (Fig. 1A, lanes 1–4) and RANKL2-DeRed (Fig. 1A, lane 5) was detected in whole cell lysates with the anti-DeRed antibody (Fig. 1A). The expression of RANKL3-EGFP (Fig. 1B, lanes 2) was confirmed. Treatment of NIH3T3 cells transfected with the cDNA encoding DsRed-tagged RANKL1 (lane 1), DsRed-tagged RANKL1 and EGFP-tagged RANKL2 (lane 2), NIH3T3 cells transfected with RANKL1 (lane 3), NIH3T3 cells transfected with RANKL3 (lane 4), and NIH3T3 cells transfected with RANKL1 and RANKL3 (lane 5) was detected with the anti-EGFP antibody. Immunoprecipitation of RANKL1-DeRed with the anti-DeRed antibody was confirmed using the same antibody. Immunoprecipitation of RANKL1-DeRed (Fig. 1C, lanes 1–4) and RANKL2-DeRed (Fig. 1C, lane 5) was detected among nonspecific background signals. In the specimens cotransfected with RANKL1-DeRed and RANKL1-EGFP and immunoprecipitated with the anti-DeRed antibody, coprecipitation of RANKL1-EGFP protein was detected with the anti-EGFP antibody (Fig. 1D, lane 2). Coprecipitation of RANKL2-EGFP was also detected in the specimen cotransfected with RANKL1-DeRed and RANKL2-EGFP (Fig. 1D, lane 3). In the specimens cotransfected with RANKL1-DeRed and RANKL3-EGFP in addition to RANKL2-DeRed and RANKL3-EGFP, no RANKL3-EGFP protein was detected (Fig. 1D, lanes 4 and 5).

To confirm the interaction of RANKL protein, recombinant RANKL3 protein was cross-linked with EGS and visualized by Coomassie Brilliant Blue staining. Recombinant RANKL3 treated with EGS revealed a trimer (66 kDa) and a small amount of dimer (44 kDa) (Fig. 2, lane 2), which was specifically cleaved by hydroxylamine hydrochloride to a dimer and monomer (Fig. 2, lane 3). Similar results were also obtained using bis-[2-(succinimidooxy)carbonyloxy]ethyl]sulfone (BSO-COES) as the cross-linker.

In addition, intracellular localization of three RANKL isoforms was analyzed by means of detecting the tagged fluorescence protein using a confocal microscope. Both RANKL1 and RANKL2 were scattered as rough dots over the cytoplasmic region of the cells, but RANKL3 was located as fine granules over the cells (Fig. 3, A–C). In the cells cotransfected with RANKL1-DeRed and RANKL1-EGFP, both RANKL1-DeRed (Fig. 3D) and RANKL1-EGFP (Fig. 3E) were colocalized almost identically (Fig. 3F). In the cells cotransfected with RANKL1-DeRed and RANKL2-EGFP, both RANKL1-DeRed (Fig. 3G) and RANKL2-EGFP (Fig. 3H) were colocalized, but the identity was not as complete as RANKL1-DeRed and RANKL1-EGFP (Fig. 3I). Colocalizations of RANKL1-DeRed (Fig. 3J) and RANKL3-EGFP (Fig. 3K) and of RANKL2-DeRed (Fig. 3M) and

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RANKL3-EGFP (Fig. 3N) were poorer, but in some regions of the cytoplasms of the cells, colocalized dots were detected (Fig. 3, L and O).

Functions of RANKL Isoforms on Osteoclastogenesis—We produced NIH3T3 cells expressing each RANKL isoform or different RANKL isoforms, and several clones for each transformant were used as stromal cells for in vitro osteoclastogenesis assay. In addition to macrophage colony-stimulating factor, 1α,25-dihydroxyvitamin D₃ and dexamethasone were further supplemented with the culture medium to down-regulate the expression of OPG of both NIH3T3 cells and macrophages (Fig. 4A). NIH3T3 cells expressing RANKL1 or RANKL2 induced TRAP-positive mononuclear preosteoclasts, but few multinucleated osteoclasts were formed (Fig. 4B, a and b). NIH3T3 cells expressing RANKL3 did not induce any TRAP-positive cells (Fig. 4B, c). NIH3T3 cells showing coexpression of RANKL1 and RANKL2 induced markedly expanded multinucleated osteoclasts. Most of the culture area was covered with the multinucleated osteoclasts, and few mononuclear TRAP-positive preosteoclasts were observed at 1 week after the coculture (Fig. 4B, d). NIH3T3 cells with coexpression of RANKL1 and RANKL3 and of RANKL2 and RANKL3 induced mononuclear TRAP-positive preosteoclasts, but the additional expression of RANKL3 did not change the number significantly (Fig. 4B, e and f, and 4C).

To elucidate whether the coexpression of RANKL1 and RANKL2 in the same cell is necessary to induce multinucleated osteoclasts, NIH3T3 cells expressing RANKL1 were mixed with the cells expressing RANKL2 and then used for coculture. Mixture of RANKL1-expressing cells and RANKL2-expressing cells induced mononuclear TRAP-positive preosteoclasts but did not induce multinucleated osteoclasts (Fig. 4B, g, and 4D). There is also a possibility that additional expression of the same RANKL isoform promotes the formation of multinucleated osteoclasts. To evaluate this, pcDNA3.1-RANKL1 or pcDNA3.1-RANKL2 was additionally transfected to cells showing the expression of RANKL1 or RANKL2, respectively. Additional expression of RANKL1 and additional expression of RANKL2 induced a few multinucleated osteoclasts (Fig. 4B, h, and data not shown). The expressions of RANKL1 in NIH3T3 cells used in the experiments shown in Fig. 4B, a, and 4B, h, are shown in Fig. 4E.

Previously, we reported that all the analyzed organs and a bone marrow stromal cell line ST2 expressed the three RANKL isoforms (13). Then, we further added the expression of RANKL3 in the NIH3T3 cells expressing RANKL1 and RANKL2 (Fig. 5, A and B). Additional expression of RANKL3 significantly decreased the formation of osteoclasts, and many unfused mononuclear TRAP-positive preosteoclasts were observed (Fig. 5C, a and b, and 5D).

The cocultures using NIH3T3 cells expressing RANKL1 and RANKL2 formed relatively large resorption lacunae on the dentin slices (Fig. 5C, c). On the dentin slices of the cocultures using NIH3T3 cells expressing RANKL1, RANKL2, and RANKL3, resorption lacunae were also formed, but the size was smaller (Fig. 5C, d). The area of the resorption lacunae on the dentin slices in the cocultures using NIH3T3 cells expressing RANKL1, RANKL2, and RANKL3 was significantly decreased as compared with that in the cocultures expressing RANKL1 and RANKL2 (Fig. 5E). To further analyze the effect
RANKL forms trimeric structures (17, 18, 23). On the other hand, we previously identified three isoforms of mouse RANKL cDNA. One was identical to the originally reported RANKL, another had a shorter intracellular domain, and the other lacked the intracellular and transmembrane domains, which were named RANKL1, RANKL2, and RANKL3, respectively (13). We hypothesized that these RANKL isoforms interact.

Here, we showed interactions of RANKL1 with RANKL1 or RANKL2 by immunoprecipitation using the proteins tagged with EGFP or DsRed (Fig. 1). However, RANKL3 did not precipitate with RANKL1 or RANKL2. Since RANKL3 has the complete extracellular domain of RANKL, RANKL3 may interact with RANKL isoforms as reported previously (17, 18, 23). Next, we showed that recombinant RANKL3 without the tag interacts and forms a trimer by a cross-linking study (Fig. 2). From these results, it was suggested that RANKL3 also interacts with other RANKL isoforms at the extracellular domain and forms homo- or heterotrimers, although we do not have direct evidence for multimerization of RANKL3 to RANKL1 and RANKL2. In addition, the findings suggest that the tagged proteins do not influence the multimerization of RANKL, and thus RANKL1 interacts with RANKL1 and RANKL2. Although interaction of RANKL1 with RANKL1 or RANKL2 was detected by immunoprecipitation, the interaction was suggested to be weak because the interaction was not seen when cells were lysed with the lysis buffer containing Nonidet P-40 and was only detected when one of the very mild detergents, digitonin, was used in place of Nonidet P-40. 3 Poorer colocalization of RANKL3 with RANKL1 or RANKL2 in the transfected cells (Fig. 3) suggests that the interaction of RANKL3 is still weaker than RANKL1 or RANKL2, and this might be the reason for the dissociation of RANKL3.

Localization of RANKL3 was different from those of RANKL1 and RANKL2 (Fig. 3), and the diffuse distribution of RANKL3 in the cytoplasmic region suggests that RANKL3 was not transported and secreted adequately, and accumulated in the cytoplasmic region. This might be true because in the cocultures using NIH3T3 cells expressing RANKL3, neither preosteoclasts nor osteoclasts were formed (Fig. 4B, c), although recombinant RANKL3 protein induced osteoclasts when administered to bone marrow macrophages. However, there is also a possibility that RANKL3 is secreted as a soluble form protein, but it diffuses in the culture medium, and the final concentration is not sufficient to induce preosteoclasts or osteoclasts.

Coexpression of RANKL1 and RANKL2 markedly enhanced the formation of multinucleated osteoclasts, but a mixture of RANKL1-expressing cells and RANKL2-expressing cells did not. These findings suggest that the coexpression of both RANKL1 and RANKL2 in the same cells is important for the formation of multinucleated osteoclasts. As shown in Figs. 1 and 2, RANKL isoforms interact with one another, and the coexpression is suggested to form heteromultimers of RANKL1 and RANKL2. However, overexpression of RANKL1 or RANKL2 also induced multinucleated osteoclasts. These results suggest that heteromultimers of RANKL1 and RANKL2 markedly stimulate fusion of preosteoclasts in this assay system, but they are not essential to fusion of preosteoclasts, and these results also suggest that higher expression of RANKL1 or RANKL2 is necessary for fusion than formation of preosteoclasts.

As described above, RANKL3 induced neither preosteoclasts nor osteoclasts, and the additional expression of RANKL3 to RANKL1 or RANKL2 did not change the formation of preosteoclasts (Fig. 4B, e and f, and 4C). However, the expression of

\[ \text{RANKL, a member of TNF, had been expected to form multimeric structures similar to TNF-\alpha and TNF-\beta. Studies of sedimentation equilibrium analytical ultracentrifugation and crystallography showed that the extracellular domain of RANKL forms trimeric structures (17, 18, 23).} \]

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Fig. 5. Coexpression of RANKL3 with RANKL1 and RANKL2 inhibits osteoclastogenesis. A, RT-PCR of NIH3T3 cells transfected with pMIK HygB-RANKL1, pCMV-3xHis-RANKL2, and pEF-BOS-BSR-RANKL3 (1 + 2 + 3), and NIH3T3 cells transfected with pCMV HygB-RANKL1, pCMV-3xHis-RANKL2, and pEF-BOS-BSR vector (1 + 2 + v). Lanes 1–3 show detection of RANKL1 (1), RANKL2 (2), and RANKL3 (3), respectively. B, Northern hybridization of the cells described in A and control NIH3T3 cells using the probe against mouse RANKL. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. C, cocultures of bone marrow macrophages and NIH3T3 cells expressing RANKL1 and RANKL2, which were further transfected with an additional expression vector, pEF-BOS-BSR (a), and NIH3T3 cells expressing RANKL1, RANKL2, and RANKL3 (b). Cocultures were stained to detect the TRAP activity. Formation of resorption lacunae by the coculture was shown in A (c) and in B (d). Immunohistochemistry of CT-Rs in the coculture of bone marrow macrophages and NIH3T3 cells expressing RANKL1, RANKL2, and RANKL3 is shown. The cocultures treated with anti-CT-Rs antibody (e) and the preabsorbed antibody (f) are shown. D, effect of the coexpression of RANKL3 with RANKL1 and RANKL2 on osteoclastogenesis. Osteoclasts/TRAP+ cells (%) represent the percent of multinucleated osteoclasts/total TRAP-positive cells. E, effect of the coexpression of RANKL3 with RANKL1 and RANKL2 on formation of resorption lacunae. Resorption Area (%) represents the percentage of the area of resorption lacunae/area of the dentin slice.

RANKL3 significantly decreased the percent of multinucleated osteoclasts/total TRAP-positive cells when coexpressed with RANKL1 and RANKL2. These results suggest that RANKL3 specifically inhibits fusion of preosteoclasts when coexpressed with RANKL1 and RANKL2. Considering the multimerization of RANKL isomers, the mechanism to inhibit the fusion of preosteoclasts by the expression of RANKL3 might be associated with intracellular interactions of RANKL3 with RANKL1 and/or RANKL2. As shown in Fig. 5C, e, the expression of CT-Rs was detected in preosteoclasts and osteoclasts induced by NIH3T3 cells expressing RANKL1, RANKL2, and RANKL3. These findings confirm that the expression of RANKL3 does not affect phenotypes of preosteoclasts and osteoclasts but inhibits only fusion of preosteoclasts.

The findings in the present study strongly suggest that multiple multimeric structures of RANKL are present and regulate bone metabolism. Drugs with selectable regulation of the expression of each RANKL isomorf may work as regulators of bone metabolism. Furthermore, RANKL has multiple functions throughout osteoclastogenesis: differentiation and fusion of osteoclast progenitor cells and activation and survival of osteoclasts (24–27). A part of these multiple functions may be expressed by the multiple multimeric structures of RANKL.

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Isoforms and Regulation of Osteoclastogenesis

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