Negative Regulation of Osteoclastogenesis by Ectodomain Shedding of Receptor Activator of NF-κB Ligand*

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Receptor activator of NF-κB ligand (RANKL), also known as TNF-related activation-induced cytokine, osteoprotegerin ligand, and osteoclast differentiation factor, is a type II transmembrane glycoprotein of the TNF ligand family. RANKL is expressed on the plasma membrane of osteoblasts, bone marrow stromal cells, and T-lymphocytes (1–5) and exerts its activity through binding to its TNF family receptor RANK, which is expressed on monocyte-macrophage lineage osteoclast precursors (3, 4, 6). Consequently, RANK activates downstream signaling pathways such as NF-κB, p38 mitogen-activated protein kinase, c-Jun N-terminal kinase, and nuclear factor of activated T cells c1 and leads to the differentiation, activation, and survival of osteoclasts (6–9). Both RANKL- and RANK-deficient mice develop severe osteopetrosis due to a lack of osteoclasts (10, 11). Conversely, a deficiency of osteoprotegerin (OPG), a natural inhibitor of RANKL, causes severe osteoporosis due to enhanced osteoclastogenesis (12). These findings suggest a crucial role for the RANKL/RANK/OPG axis in osteoclast development.

A number of transmembrane proteins undergo proteolysis and are released from the plasma membrane, a process called ectodomain shedding. The biologic and pathologic significance of ectodomain shedding varies between substrate proteins. For example, cytokines such as TNF-α and epidermal growth factor are released from local environments and exert their activity in paracrine and endocrine signaling by ectodomain shedding (13–15). In some cases, ectodomain shedding is necessary even for the local effects of growth factors such as epidermal growth factor (16). Although membrane-bound RANKL is converted to a soluble form through ectodomain shedding (17, 18) similar to other TNF family members, such as TNF-α (13, 14) and Fas ligand (19), the RANKL sheddases involved in physiologic and pathologic bone resorption, and the role of RANKL shedding is unknown. We report that RANKL shedding is regulated by matrix metalloproteinase (MMP) 14 and a disintegrin and metalloproteinase (ADAM) 10 in bone marrow stromal cells and osteoblasts. MMP14 is mainly involved in RANKL shedding in osteoblasts, and its deficiency up-regulates osteoclastogenesis by reducing RANKL shedding in the local bone milieu.

EXPERIMENTAL PROCEDURES

Reagents—DNA polymerase, KOD plus, was purchased from TOYOBO (Osaka, Japan). Antibodies for the His-tag were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), antibodies for the V5 tag were from Invitrogen, antibodies for actin were from Sigma-Aldrich, and the antibody for RANKL was from Active Motif (Carlsbad, CA). MMP inhibitors, FR255031 and FR217840, were generous gifts from Astellas Pharma Inc. (Tokyo, Japan).

Constructs—The procedures for the construction of the tRANKL-SEAP expression vector, pcDNA3.1-RANKL-V5HisB, pcDNA3.1-mMMP14-V5HisA, pcDNA3.1-mMMP13-V5HisA, and expression vectors for human MT1, MT2, MT3, MT4, MT5, and MT6-MMP were described previously (20–26). An expression vector for mouse RANKL, pSG5-RANKL, was con-
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structured by inserting mouse RANKL cDNA into pSG5 (Stratagene, La Jolla, CA). Expression vectors for mouse MMP1, MMP2, MMP3, MMP7, MMP9, MMP11, MMP19, MMP23, MMP28, ADAM9, ADAM10, ADAM17, and ADAM19 were constructed by inserting the cDNA generated by reverse transcription-PCR of mRNA of mouse osteoblasts into pcDNA3.1-V5HisA (Invitrogen). The cDNA for ADAM10-V5His was further subcloned from pcDNA3.1- or ADAM10-V5HisA and inserted into the pSG5 vector. Small interference RNA (siRNA) plasmids for GFP, mouse ADAM10 and MMP14 were constructed using pGENE mU6 vector (iGENE Therapeutics Inc., Ibaraki, Japan) according to the manufacturer's protocol. Target sites were, for GFP, 5'-GCTACGTCCAGGAGGGAACAC-3'; for ADAM10, 5'-GGGTCTGTATTGAGAAGATA-3' and 5'-GCTGTGATTGTCAGATATCC-3'; and for MMP14, 5'-GGACTGAGATCAAGGCCAATG-3' and 5'-GGATGGGACACAGAGAACTTCG-3'. The retrovirus vector for mouse MMP14 was constructed by inserting the cDNA fragment for mouse MMP14 into the BamHI and EcoRI restriction sites of pMX-puro (kindly provided by Toshio Kita, Institute of Medical Science, The University of Tokyo). Retrovirus vectors for siGFP, siMMP14, and siADAM10 were constructed as follows; cDNA fragments for siRNA, including the mouse U6 promoter were subcloned from pGENE constructs by PCR using M13F/R primers and ligated into pcR-blunt II TOPO (Invitrogen), digested by EcoRI, and inserted to the EcoRI restriction site of pMX-puro.

Cell Culture—The human kidney cell line 293T were cultured in Dulbecco's modified eagle medium (Sigma-Aldrich) and the mouse bone marrow stromal cell line TM8B2 (generous gift from Prof. Noda, Medical Research Institute, Tokyo Medical and Dental University) were cultured with α-MEM, and both of them were supplemented with 10% FBS and 1% penicillin-streptomycin solution (Sigma-Aldrich Co.). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO2. Primary osteoblasts were harvested from the calvaria of newborn ddy (purchased from Sankyo Labo Service Co., Tokyo Japan) and C57/BL6 (MMP14-deficient mice and wild-type littermates) mice as previously described (27).

Screening of RANKL Sheddases—The alkaline phosphatase assay of the culture media and the Western blot analysis were described previously (20, 28). To detect soluble RANKL released into the culture media of TM8B2 cells, 3 ml of the culture media was incubated with 4 μl of recombinant protein G-agarose (Invitrogen) and 1 μg of recombinant OPG-Fc chimera protein (R&D Biosystems, Minneapolis, MN) for 16 h at 4 °C, then recovered by brief centrifugation. The pellets were suspended in TNE buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40), and subjected to SDS-PAGE.

Retrovirus Infection—The procedure for preparation of the retrovirus was described previously (29). Primary osteoblasts were incubated with the culture media containing retrovirus supplemented with 5 μg/ml polybrane (Sigma-Aldrich) for 16 h.

Real-time PCR—The reaction mixture for the real-time PCR was prepared using qPCR QuickGoldStar Mastermix Plus for SYBR® Green I (Nippon Gene, Tokyo, Japan), and analyzed using the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The set of primers used were 5'-CCCAAGGCAGCAACTTCA-3' and 5'-CAATTGGCAGCTGAGTTGAC-3' for mouse MMP14, 5'-TGTTGTTCTGAGCTGTTCTG-3' and 5'-TACCAGCCAGCTTCTCAGT-3' for mouse MT2-MMP, 5'-ATCATGGGCCCCATTTTATCA-3' and 5'-GGACTGAGATCAAGGCCAATG-3'. Western Blot of Primary Osteoblasts—Cells were treated with or without 10−8 M 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3), 10−6 M prostaglandin E2 (PGE2), and 10 ng/ml interleukin-1 (IL-1) for 72 h, and 10 ml of culture media were collected. Cell lysates were collected using M-PER® Mammalian Protein Extraction Reagent (Pierce) according to the manufacturer's protocol. Culture media and cell lysates were treated using the same procedure as for culture media of TM8B2.

Determination of Cleavage Sites of RANKL—pSG5-RANKL were transfected to TM8B2 cells. Seventy hours after transfection, 200 ml of the culture medium was collected, and soluble RANKL was recovered using an OPG-Fc column. Samples were subjected to SDS-PAGE, transferred to polyvinylidene membrane, and stained with Coomassie Brilliant Blue. The band was excised, and the N-terminal amino acid sequence was determined by Hokkaido System Science Co., Ltd. (Hokkaido, Japan).

ELISA—Primary osteoblasts were seeded in 48-well plates at a concentration of 1 × 105 cells/ml. If needed, cells were infected with retrovirus vectors as mentioned above. After 24 h of incubation, some cultures were treated with 10−8 M 1α,25(OH)2D3 and 10−6 M PGE2 for 72 h. The concentration of RANKL in the culture media and cell lysates (homogenized in 200 μl of TNE buffer per well) was determined by ELISA system developed using a TRANCE DuoSet ELISA development kit (R&D Biosystems).

Coculture of Primary Osteoblasts and Bone Marrow Macrophages—Bone marrow macrophages were obtained from tibias of 5–8 weeks-old C57/BL6 mice by flushing the bone marrow with α-MEM, and cultured in α-MEM supplemented with 10% FBS, 1% penicillin-streptomycin solution, and 10 ng/ml M-CSF (Wako Pure Chemicals, Osaka, Japan) for 16 h, and non-adherent cells were collected. For the coculture, primary osteoblasts were seeded in 48-well plates at a concentration of 1 × 105 cells/ml. If needed, cells were infected by a retrovirus, as described above. After 24 h incubation, macrophages were seeded on the primary osteoblasts at a concentration of 1 × 104 cells/ml and treated with 10−8 M 1α,25(OH)2D3 and 10−6 M PGE2 for 5 days. Osteoclasts were stained with tartrate-resistant acid phosphatase (3), and the cell number was counted. For separate culture, primary osteoblasts were cultured on the cell culture insert (BD Biosciences, San Jose, CA), and bone marrow macrophages were seeded on the bottom of 24-well plates.
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Osteoclast Formation Assay by Soluble RANKL Generated by MMP14—293T cells were transfected with several combinations of pcDNA3.1-V5HsA, pcDNA3.1-RANKL-V5HisB, and pcDNA-mMMP14-V5HisA using FuGENE 6 (Roche Diagnostics, Indianapolis, IN), and 48 h after transfection, culture media were collected. Bone marrow macrophages were incubated with a 1:1 mixture of the culture media and α-MEM containing 10% FBS, supplemented with M-CSF at 10 ng/ml for 4 days.

Osteoclast Formation Assay, Survival Assay, and Pit Formation Assay of Macrophages of MMP14-deficient Mice—The MMP14-deficient mice were generated and backcrossed to the C57/BL6 strain as previously described (30). Livers and spleens were harvested from newborn MMP14-deficient mice and wild-type littermates, and cells were strained using a cell strainer. The obtained cells were incubated in α-MEM supplemented with 10% FBS, 1% penicillin-streptomycin solution, and 200 ng/ml M-CSF (Wako) for 4 days in 10-cm Petri dishes, and then M-CSF-dependent macrophages were recovered by trypsinization. For the osteoclast formation assay, macrophages were seeded in 48-well plates at a concentration of 2 × 10^5 cells/ml and treated with 10 ng/ml M-CSF and 100 ng/ml RANKL (Wako) for 6 days. For the survival assay and the pit formation assay, macrophages (2 × 10^5 cells/ml) were cocultured with primary osteoblasts (1 × 10^5 cells/ml) in α-MEM containing 10% FBS, 10^{-8} M 1α,25(OH)_{2}D_3, and 10^{-6} M PGE_2 on culture dishes precoated with 0.2% collagen gel matrix (Nitta Gelatin, Osaka, Japan). When osteoclasts were formed after 7-day culture, they were released from the dishes by treatment with 0.2% collagenase in phosphate-buffered saline, and collected by centrifugation. For the survival assay, cells were cultured on 48-well plates for 4 h, and osteoclasts were isolated by removing the osteoblasts by 0.1% collagenase and 0.2% dispase in phosphate-buffered saline. Purified osteoclasts were further incubated in α-MEM containing 10% FBS for 20 h. For the pit formation assay, cells were seeded on the dentin slices (Wako) precoated with FBS, and incubated in α-MEM containing 10% FBS, 10^{-8} M 1α,25(OH)_{2}D_3, and 10^{-6} M PGE_2 for 24 h. Then the cells were removed by ultrasonication after adding 1 M NH_4OH. Resorption pits were visualized by staining with 0.5% toluidine blue and analyzed using Adobe Photoshop (Adobe, San Jose, CA).

Histology—Tibias of 2- to 3-week-old male MMP14-deficient mice, and wild-type littermates were fixed in 4% paraformaldehyde and decalcified in 10% EDTA. Paraffin-embedded samples were sectioned at 5 μm and stained with hematoxylin and eosin or tartrate-resistant acid phosphatase. Observers who were blinded to the origin of the images counted the number of osteoclasts on the trabecular bone surface and measured total length of the trabecular bone using Image J software (National Institutes of Health, Bethesda, MD).

Statistical Analysis—Statistical analyses were performed using Student’s t test for the alkaline phosphatase assay and a two-tailed unpaired Student’s t test for the real-time PCR and ELISA. A p value of <0.05 was considered to be statistically significant.

RESULTS

Screening of MMPs and ADAMs as RANKL Sheddases—We previously developed a RANKL shedding activity screening system using an expression vector encoding a fusion protein of secreted placental alkaline phosphatase (SEAP) with the C-terminal truncated form of RANKL, which contains the stalk region but lacks the TNF-like domain of RANKL, and with V5 and His×6 tags at the C terminus. Cleavage of tRANKL-SEAP can be detected as positive alkaline phosphatase activity in the supernatants. 8β, alkaline phosphatase activity of the culture media of 293T cells transfected with pcDNA3.1-tRANKL-SEAP-V5HisA and expression vectors for ADAMs or MMPs (n = 6). *, significantly different, p < 0.01; n. s., not significant.

FIGURE 1. RANKL shedding activity of MMPs and ADAMs. A, schematic representation of RANKL and tRANKL-SEAP fusion protein. tRANKL-SEAP is a fusion protein of SEAP with C-terminally truncated RANKL, which contains the stalk region but lacks the TNF-like domain of RANKL, and with V5 and His×6 tags at the C terminus. Cleavage of tRANKL-SEAP can be detected as positive alkaline phosphatase activity in the supernatants. B, alkaline phosphatase activity of the culture media of 293T cells transfected with pcDNA3.1-tRANKL-SEAP-V5HisA and expression vectors for ADAMs or MMPs (n = 6). *, significantly different, p < 0.01; n. s., not significant.

MMP14 and ADAM10 Are Major RANKL Sheddases in TMBB2 Cells—To confirm the ability of these proteinases to cleave full-length RANKL, they were overexpressed in the
mouse bone marrow stromal cell line TM8B2 together with full-length RANKL. Soluble RANKL was recovered from the culture media using an OPG-Fc recombinant protein column and subjected to Western blot analysis. Immunoblotting by anti-His shows the OPG-Fc recombinant protein used to collect soluble RANKL. B, schematic representation of the cleavage sites of RANKL. Cleavage sites were indicated by arrows. C, effect of ADAMs and MMPs on RANKL shedding in TM8B2 cells. TM8B2 cells were transfected with pcDNA3.1-RANKL-V5HisB and expression vectors for ADAMs or MMPs. Culture media were subjected to Western blot for RANKL 72 h after transfection. D, real-time PCR analysis of MT-MMP expression in TM8B2 cells and primary osteoblasts (n = 3). The y-axis represents the relative mRNA levels of the indicated genes expressed as -fold from MMP14 level. E, efficient gene knock-down by siADAM10 and siMMP14. TM8B2 cells were transfected with pcDNA3.1-V5HisA, piGENEmU6-siGFP, -siADAM10–1, -2, siMMP14–1, -2, or pcDNA3.1-mMMP14-V5HisA, and the expression of ADAM10 (endogenous) and MMP14 (overexpressed,detected by anti-V5 epitope tag antibody) was analyzed 48 h after transfection. F, Effect of siADAM10 and siMMP14 on RANKL cleavage in TM8B2 cells. TM8B2 cells were transfected with piGENEmU6-siGFP, -siADAM10–1, -2, siMMP14–1, -2, or pcDNA3.1-V5HisB, together with pcDNA3.1-RANKL-V5HisB. Seventy-two hours after transfection, culture media and cell lysates were collected and subjected to Western blot analysis. G, effects of siADAM10, siMMP14, and chemical inhibitors on RANKL shedding in TM8B2 cells. Procedures were the same as in A.
the bands disappeared. These results suggest that the upper band is produced by ADAM family proteinase(s) and the lower band by MMP(s).

ADAM9, ADAM10, and ADAM19 cleaved full-length RANKL, but only ADAM10 produced soluble RANKL with the same molecular weight as the upper band observed in TM8B2 cells (Fig. 2C). ADAM17 did not cleave full-length RANKL. MMP14, MT2, MT3, and MT5-MMP generated soluble RANKL with the same molecular weight as the lower band. MT4- and MT6-MMP did not increase the soluble RANKL detected in the culture media (data not shown). Real-time PCR showed that the MMP14 mRNA level was much higher than that of MT2-, MT3-, or MT5-MMP in TM8B2 cells and primary osteoblasts (Fig. 2D), suggesting that MMP14 is mainly involved in the production of the lower band. The mRNA level of ADAM10 was comparable to that of MMP14 in these two cells (data not shown).

To determine the role of ADAM10 and MMP14 in the constitutive shedding of RANKL in TM8B2 cells, we constructed expression vectors of siRNA for ADAM10 and MMP14 (siADAM10 and siMMP14) (Fig. 2E). We constructed two different siADAM10 and siMMP14 vectors, and all of them effectively suppressed expression of the target molecules (Fig. 2F). The upper band of soluble RANKL diminished when the RANKL construct was transfected to TM8B2 cells along with siADAM10 plasmids; the lower band diminished with siMMP14 (Fig. 2G); and only the upper band was faintly observed when both siADAM10 and siMMP14 were transduced (Fig. 2H). The faint band completely disappeared in the presence of FR217840, but not FR255031 (Fig. 2H).
results indicated that ADAM10 and MMP14 are two major RANKL sheddases in TM8B2 cells.

**MMP14 Cleaves RANKL in Primary Osteoblasts**—We next examined the RANKL sheddases in primary osteoblasts. When primary osteoblasts were stimulated with 1α,25(OH)2D3, PGE2, and IL-1, two bands appeared in the culture media with the same molecular masses (~22 and 21 kDa) as those observed in TM8B2 cells transfected with full-length RANKL without epitope tag (Fig. 3A and data not shown), but the lower band was more prominent. The expression of MMP14 was also much higher than that of MT2-, MT3-, or MT5-MMP in osteoblasts, suggesting that MMP14 is the major RANKL sheddase in primary osteoblasts (Fig. 2D). We constructed retrovirus vectors of siMMP14 that efficiently suppressed MMP14 expression in primary osteoblasts (Fig. 3B). When osteoblasts were infected with siMMP14 retrovirus, the lower band was diminished in the culture medium and membrane-bound RANKL was increased in the cell lysate (Fig. 3A). The concentration of soluble RANKL in the culture media and membrane-bound RANKL in the cell lysates was also measured by ELISA. A significant decrease in soluble RANKL and an increase in membrane-bound RANKL was observed by MMP14 knockdown (Fig. 3C), consistent with the results of Western blot analysis. Conversely, overexpression of MMP14 in primary osteoblasts increased the lower band of soluble RANKL in the culture media and reduced membrane-bound RANKL in the cell lysates (Fig. 3, D and E). To further confirm the role of MMP14 in RANKL shedding in osteoblasts, we analyzed primary osteoblasts obtained from MMP14 knock-out mice. When primary osteoblasts of MMP14-deficient mice were stimulated with 1α,25(OH)2D3, PGE2, and IL-1, only the upper band of soluble RANKL was observed in the culture medium (Fig. 3F). The amount of soluble RANKL was markedly reduced in
RANKL Shedding Negatively Regulates Osteoclastogenesis—To determine the role of MMP14 in the osteoclastogenic activity of osteoblasts, we cocultured osteoblasts infected with siMMP14 retrovirus with bone marrow macrophages. The siMMP14 retrovirus-infected osteoblasts, in which RANKL shedding activity was suppressed and membrane-bound RANKL was increased, stimulated osteoclastogenesis more effectively than control cells (Fig. 4A). Conversely, overexpression of MMP14 in primary osteoblasts suppressed osteoclastogenesis (Fig. 4B). These results suggest that membrane-bound RANKL induces osteoclastogenesis more efficiently than soluble RANKL, and the ectodomain shedding of RANKL by MMP14 negatively regulates osteoclastogenesis.

We then examined the osteoclastogenic activity of the soluble RANKL generated by MMP14. The culture media of 293T cells transfected with RANKL and MMP14 expression vectors were collected 48 h after transfection, and bone marrow macrophages were cultured in media supplemented with 10 ng/ml M-CSF (Fig. 4C). When macrophages were cultured in media recovered from the empty vector or RANKL expression vector-transfected cell cultures, mature osteoclasts did not form, even in the presence of M-CSF. In contrast, the culture media from the cells transfected with both RANKL and MMP14 expression vectors induced osteoclast formation, indicating that the soluble RANKL generated by MMP14 had osteoclastogenic activity. When primary osteoblasts overexpressing MMP14 by retrovirus vectors and bone marrow cells were cultured separately using cell culture inserts, which allow the passage of culture media but prevent cell-cell contact, and were stimulated with 1α,25(OH)2D3, PGE2, and IL-1, very few osteoclasts were formed (Fig. 4D). These results suggest that the amount of endogenous

FIGURE 5. A, coculture of bone marrow macrophages and primary osteoblasts from MMP14-deficient mice or wild-type littermates for 5 days. Bar = 200 μm. Right panel shows the number of osteoclasts formed (n = 4). *, significantly different, p < 0.01. POB, primary osteoblasts; BMM, bone marrow macrophages. B, MMP14 deficiency does not affect osteoclast differentiation. Macrophages of MMP14 knock-out mice or wild-type littermates were cultured in the presence of M-CSF and RANKL for 6 days. Scale bar = 200 μm. Right panel shows the number of osteoclasts (n = 4). n.s., not significantly different. C, MMP14 deficiency does not affect bone resorption activity or survival rate of osteoclasts. Macrophages of MMP14 knock-out mice (b, d, f, and h) or wild-type littermates (a, c, e, and g) were cocultured with wild-type primary osteoblasts on the collagen gel matrix for 7 days and subjected to the pit formation assay (a–d) and the survival assay (e–h). Scale bar = 200 μm. Graphs are the number of pit formation rate (upper), and osteoclast survival rate (lower) (n = 4). n.s., not significantly different.
soluble RANKL generated in primary osteoblasts is not sufficient to induce osteoclastogenesis in our assay system.

We finally analyzed the osteoclastogenic activity of MMP14-deficient osteoblasts. When normal bone marrow macrophages were cocultured with osteoblasts from MMP14-deficient mice, osteoclasts were formed more efficiently than in cocultures with wild-type osteoblasts (Fig. 5A). On the other hand, bone marrow macrophages from MMP14-deficient mice differentiated into mature osteoclasts with the same efficiency as wild-type cells (Fig. 5B), and their bone-resorbing activity and survival rate were comparable (Fig. 5C). Consistent with these in vitro observations, soft x-ray images of MMP14-deficient mice displayed osteoporosis (Fig. 6A). Histologic sections showed thin cortical bone, decreased cancellous bone, and increased osteoclasts on the trabecular bone surface, which agreed with a previous report (33) (Fig. 6B). These data suggested that the increased osteoclast number in MMP14-deficient mice is due to the increase in membrane-bound RANKL in osteoblasts.

**DISCUSSION**

There is accumulating evidence that protein ectodomain shedding affects a large number of cellular membrane proteins, including TNF family members, but the functional outcome of shedding depends on the particular substrate protein. For example, TNF-α is cleaved by some proteinases such as TNF-α converting enzyme and released into the circulatory system to exhibit strong systemic effects (13, 14). In contrast, Fas ligand, which is a strong apoptosis inducer, has reduced effects in its soluble form (19). RANKL is a member of the TNF family of cytokines, and recent studies indicate that RANKL is essential in skeletal homeostasis for regulating the differentiation and activation of osteoclasts. Membrane-bound RANKL is proteolytically processed to generate the soluble form, but the specific proteinases involved in RANKL shedding and its physiologic and pathologic importance are not known. Western blot analysis revealed that there are two different cleaved products in the supernatants of both TM8B2 bone marrow stromal cells and primary osteoblasts, and the lower band was the major band in primary osteoblasts (Fig. 3A). The N-terminal sequence of the upper band coincides with the constitutive cleavage site previously reported in COS7 cells, and that of the lower band coincides with the putative MMP cleavage site. The lower band was decreased in the presence of an MMP inhibitor FR255031, and both bands were diminished by treatment with FR217840, which inhibits both MMPs and ADAMs. Previous studies also demonstrated that various MMPs and ADAMs have RANKL shedding activity (17, 20, 34–36). Therefore, we first analyzed the RANKL shedding activity of several MMPs and ADAMs using the previously reported RANKL shedding assay (20). MMP7, MMP19, ADAM9, ADAM10, ADAM17, ADAM19, MMP14, MT2, MT3, MT4, MT5, and MT6-MMP had tRANKL-SEAP shedding activity. Among these proteinases, ADAM9, ADAM10, ADAM19, MMP14, MT2, MT3, and MT5-MMP cleaved full-length RANKL. Although ADAM9 and ADAM19 had RANKL shedding activity, the molecular weights of the cleaved products were different from those con-
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stitutively generated in TM8B2 cells and primary osteoblasts. Among MT-MMPs, MMP14 was predominantly expressed in TM8B2 and primary osteoblasts. MMP7 was reported to cleave RANKL in vitro or under overexpressing conditions and has an important role in prostate cancer-induced osteolysis (34). Although MMP7 had tRANKL-SEAP shedding activity, it did not cleave full-length RANKL in our assay system. Blobel and co-workers (17) demonstrated that ADAM17 (TNF-α converting enzyme) cleaves a partial fragment of RANKL protein, but not full-length RANKL (35), and we confirmed that ADAM17 did not cleave full-length RANKL. Although MMP13 deficiency leads to skeletal abnormalities (37), it did not cleave tRANKL-SEAP or full-length RANKL. These data, in combination with the results of siRNA experiments, led us to speculate that ADAM10 and MMP14 were major RANKL sheddases in TM8B2 cells and osteoblasts, although it is possible that other proteinases are involved in RANKL shedding in some pathologic conditions in which they are up-regulated. The functional difference between MMP14-cleaved RANKL and ADAM10-cleaved RANKL is currently under investigation.

In the culture media of primary osteoblasts, the lower molecular weight product was much more predominant than the higher molecular weight product, indicating that MMP14 is the major RANKL sheddase in primary osteoblasts. This was further confirmed by the results obtained from MMP14 knockdown osteoblasts and MMP14-deficient osteoblasts, in which the lower molecular weight band was almost completely diminished and the concentration of soluble RANKL was severely reduced.

Reduced MMP14 expression in primary osteoblasts by siRNA or its deficiency in MMP14 knock-out mouse osteoblasts reduced RANKL shedding and increased membrane-bound RANKL, which led to increased osteoclastogenic activity in the cells. Although soluble RANKL produced by MMP14-induced osteoclastogenesis from bone marrow macrophages, the culture medium of primary osteoblasts treated with 1α,25(OH)2D3 and PGE2 did not induce osteoclastogenesis (38), even when MMP14 was overexpressed. This was probably due to an insufficient amount of soluble RANKL, because the concentration of the soluble RANKL in the culture media was <1 ng/ml (data not shown), and the amount of recombinant soluble RANKL necessary to induce osteoclastogenesis in vitro was >10 ng/ml. Still, it is possible that when the expression of RANKL is highly up-regulated, soluble RANKL has substantial effects on general bone metabolism. Gao et al. reported that parathyroid hormone (1–34) not only increases RANKL but also decreases MMP14 production in human osteoblasts, which might be involved in the sufficient up-regulation of bone resorption by increasing membrane-bound RANKL in the cells (39).

In MMP14-deficient mice, generalized osteopenia was observed. Although this might be partially due to the reduced bone formation, as previously reported (33), the osteoclast number was much higher in these mice, possibly due to the increased membrane-bound RANKL in the osteoblasts. In fact, the serum level of soluble RANKL in MMP14 knock-out mice was undetectable, even when they were injected with 1α,25(OH)2D3 (data not shown). These phenotypes are not due to cell autonomous defects in osteoclast precursors, because there were no abnormalities in osteoclast differentiation from MMP14-deficient macrophages or bone-resorbing function of mature osteoclasts.

We were unable to determine the role of ADAM10 in vivo RANKL shedding or skeletal homeostasis, because ADAM10-deficient animals die at E9.5 due to defects in heart and central nervous system development (40). Therefore, future studies using cell-specific knock-out of ADAM10 are required.

To conclude, we identified MMP14 as a major endogenous RANKL sheddase in primary osteoblasts. RANKL shedding negatively affects local osteoclastogenesis by reducing membrane-bound RANKL in the skeletal environment.

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