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Receptor Activator of NF-κB (RANK) Ligand Induces Ectodomain Shedding of RANK in Murine RAW264.7 Macrophages

Akihiro Hakozaki,* Masaki Yoda,* Takahide Tohmonda,* Mitsuru Furukawa,* Tomohiro Hikata,* Shinichi Uchikawa,* Hironari Takaishi,* Morio Matsumoto,* Kazuhiro Chiba,* Keisuke Horiuchi,*† and Yoshiaki Toyama*

Osteoclastogenesis is a highly sophisticated process that involves a variety of membrane-bound proteins expressed in osteoblasts and osteoclast precursors. Over the past several years, proteolytic cleavage and release of the ectodomain of membrane-bound proteins, also referred to as ectodomain shedding, has emerged as an important posttranslational regulatory mechanism for modifying the function of cell surface proteins. In line with this notion, several membrane-bound molecules involved in osteoclastogenesis, including CSF-1R and receptor activator of NF-κB ligand (RANKL), are proteolytically cleaved and released from the cell surface. In this study, we investigated whether receptor activator of NF-κB (RANK), one of the most essential molecules in osteoclastogenesis, undergoes ectodomain shedding. The results showed that RANK is released in the form of a soluble monomeric protein and that TNF-α-converting enzyme is involved in this activity. We also identified potential cleavage sites in the juxtamembrane domain of RANK and found that rRANKL induces RANK shedding in a macrophage-like cell line RAW264.7 via TNFR-associated factor 6 and MAPK pathways. Furthermore, we found that RANKL-induced osteoclastogenesis is accelerated in TNF-α-converting enzyme-deficient osteoclast precursors. These observations suggest the potential involvement of ectodomain shedding in the regulation of RANK functions and may provide novel insights into the mechanisms of osteoclastogenesis. The Journal of Immunology, 2010, 184: 2442–2448.

Receptor activator of NF-κB (RANK) is one of the most crucial molecules involved in the differentiation, survival, and activation of osteoclasts (1–4). RANK is a type 1 transmembrane protein and is expressed predominantly in immune cells. Association with its cognate ligand, RANK ligand (RANKL), expressed on osteoblasts and stromal cells, is the key event in osteoclast development. As shown in studies on genetically engineered animals, the absence of either of these two molecules or inhibition of the RANKL–RANK association with an endogenous decoy receptor (osteoprotegerin) results in defective osteoclastogenesis in vivo, which is highlighted by the severe loss of osteoclasts and an osteopetrosis-like phenotype (5–7). Because RANKL

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Abbreviations used in this paper: AP, alkaline phosphatase; BMM, bone marrow macrophage; HA, hemagglutinin; mEF, mouse embryonic fibroblast; RANK, receptor activator of NF-κB; RANKL, alkaline phosphatase-tagged RANK expression vector; RANKHA-Myc, HA-epitope and Myc/His-epitope dually tagged receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; sRANKHA-Myc, HA-epitope and Myc/His-epitope dually tagged soluble receptor activator of NF-κB; SS, signaling sequence; TACE, TNF-α-converting enzyme; TM, transmembrane domain; TRAF6, TNFR-associated factor 6; TRAP, tartrate-acid resistant alkaline phosphatase; Wt, wild-type.
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osteoclast precursors lacking TACE. These observations reveal a previously unknown contribution of ectodomain shedding to the functions of RANK and may provide novel insights into the mechanisms involved in osteoclastogenesis.

Materials and Methods

Cell lines and reagents

TACE-deficient and wild-type mouse embryonic fibroblasts (mEFs) derived from E13.5 embryos were immortalized, as previously described (18, 19). RAW264.7 cells and COS-7 cells were obtained from the RIKEN cell bank. The anti-hemagglutinin (HA) mAbs were from Sigma-Aldrich (HA-7; St. Louis, MO) and Roche Diagnostics (3F10; Indianapolis, IN). Anti-Myc polyclonal Ab was purchased from Medical and Biological Laboratories (Nagoya, Japan). Anti-Myc mAbs were from Abgent (9E10; San Diego, CA) and Medical and Biological Laboratories (PL-14). TNF-associated factor 6 (TRAF6) inhibitory peptide was from Ingenex (San Diego, CA). The fluorochrome-conjugated Abs and streptavidin used in immunostaining were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-human placental alkaline phosphatase Ab was from Sigma-Aldrich (8B6), U0126, SP600125, SB202190, and GM6001 were from Calbiochem (San Diego, CA). All other reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

Generation of RANK expression vectors

A cloning vector containing murine RANK cDNA was generously provided by Dr. Akira Kudo (Tokyo Institute of Technology) and was used as a PCR template to generate epitope-tagged RANK expression vectors. HA-epitope and Myc/His epitope-dually tagged RANK (RANKHA-Myc) was generated by inserting an HA-epitope sequence after the signal peptide sequence of the RANK cDNA and by cloning it into the pDNA4/Myc-His expression vector (Invitrogen, Carlsbad, CA). Alkaline phosphatase (AP)-tagged RANK expression vector (RANKAP) was generated by cloning RANK cDNA (Lys171-Ala242) into pAPtag5 (Genhunter, Nashville, TN) (see Fig. 1A for the schema of the constructs). Cell-expression of RANKHA-Myc and RANKAP was confirmed by Western blot and immunostaining (Fig. 1A and data not shown). RANKAP vector was further used as a PCR template to generate cleaved and released RANK expression vectors (Fig. 1B). Mutations in the juxtamembrane region of RANK were introduced by a PCR-based method using a KOD plus Mutagenesis kit (Toyobo, Tokyo, Japan), according to the manufacturer’s instructions.

Cell culture, transfection, and shedding assay

COS-7 cells and mEFs were grown in DMEM supplemented with 5% FCS and antibiotics. RAW264.7 cells were grown in α-MEM supplemented with 10% FCS and antibiotics. Bone marrow cells collected from the tibiae and femurs of 6–10-wk-old Tace/LysM−/− mice (Tace/LysM−/−) were analyzed by liquid chromatography MALDI time-of-flight mass spectrometry (BIFLEX3, Bruker, Billerica, MA).

Immunoprecipitation and Western blot analysis

Cells were lysed with 1% Triton-X 100/PBS containing a protease inhibitor mixture and 10 mM 1,10-phenanthroline (Sigma-Aldrich). The lysates were cleared by centrifugation and incubated with anti-Myc polyclonal Ab for 12 h, and then with protein G-coupled Sepharose beads for 1 h at 4°C with rotary agitation. After separating the lysates by 10% SDS-PAGE, Western blot analysis was performed using anti-HA and anti-Myc mAbs.

Flow cytometry analysis

COS-7 cells transiently expressing RANKHA-Myc were incubated at 37°C for 1 h with growth medium in the presence or absence of PMA and/or GM6001. The cells were washed twice with ice-cold PBS, trypsinized, and resuspended in ice-cold 5% BSA/PBS. Subsequently, the cells were incubated with anti-HA Ab at 4°C for 30 min and then washed with ice-cold 5% BSA/PBS. The Abs on the cell surface were detected with a fluorochrome-conjugated secondary Ab. RAW264.7 cells and BMMs were incubated with Opti-MEM containing PMA and/or GM6001 at 37°C for 1 h. The cells were collected using cell dissociation solution (Sigma-Aldrich) and incubated with ice-cold BSA/PBS containing 2 mM EDTA and anti-mouse CD16/32 Ab (clone, 93; BioLegend, San Diego, CA) for 15 min to block nonspecific binding of IgG to the Fc receptor. RAW264.7 cells were further incubated with biotin-labeled anti-mouse RANK Ab (R12-31, BioLegend), and BMMs were incubated with anti-mouse RANK Ab and anti-CD11b Ab (M1/70, BioLegend). The biotin-labeled anti-mouse RANK Ab was detected by allophycocyanin-streptavidin (BioLegend). Biotin-labeled rat IgG2aa was used as an isotype control. Fluorochrome-labeled Abs were analyzed by a laser flow cytometer (FACSCalibur system, BD Biosciences, San Jose, CA) and by FlowJo software (Tree Star, Ashland, OR).

Generation of recombinant soluble RANK

HA-epitope and Myc/His epitope-dually tagged soluble RANK (sRANKHA-Myc) expression vector was generated by cloning RANKHA-Myc cDNA (Met1-Ser198) into pcDNA4.0 (Fig. 7A, left panel). The construct was introduced into COS-7 cells by using FuGENE HD, and the cells were cultured for 2 d. At the end of the incubation, the cells were lysed in lysis buffer, and sRANKHA-Myc in the lysates was collected using a c-Myc-tagged protein purification kit (Medical and Biological Laboratories), as instructed by the manufacturer. Expression of sRANKHA-Myc in COS-7 cells was confirmed by Western blot (Fig. 7A, right panel).

Osteoclastogenesis assay

BMMs were plated on 48-well plates at 1 × 105 cells/well and incubated with 30 ng/ml CSF-1 and 50 ng/ml rRANKL in the presence or absence of sRANKHA-Myc (∼50 ng/ml) for 5 d. The cells were stained for tartrate-resistant acid phosphatase (TRAP), and the number of osteoclasts (defined as TRAP-positive multinucleated cells with more than three nuclei) was counted under the microscope.

Real-time RT-PCR assay

Total RNA was extracted using an RNasy Mini Kit (Qiagen, Valencia, CA) and was reverse-transcribed by RevertAid ACE (Toyobo). PCR amplification and quantification were done using SYBR Premix ExTaq II (Takara Shuzo, Shiga, Japan) and the LightCycler Quick System (Roche Diagnostics). Relative mRNA expression levels were obtained by normalizing to β-actin expression. Results are representative of at least three individual experiments.

Statistical analysis

The Student t test for two samples, assuming equal variances, was used to calculate the p values. The p values <0.05 were considered statistically significant. All data are presented as mean ± SD.

Results

The extracellular domain of RANK can be cleaved to become a soluble protein

We first investigated whether RANK could be proteolytically cleaved and released into the supernatant by conducting cell-based assays. To facilitate the detection of cleaved RANK, we generated an HA-epitope (inserted between the signaling sequence and extracellular domain) and Myc/His epitope (added to the C terminus of the intracellular domain) dually tagged RANK construct (Fig. 1A) and introduced it into COS-7 cells. As shown in Fig. 1B, an ∼85-kDa protein was detected in the lysate from RANKHA-Myc-transfected COS-7 cells.
FIGURE 1. A, Schema of HA- and Myc/His-tagged RANK (RANKHA-Myc) and AP-tagged RANK (RANKAP) constructs. SS, signaling sequence; TM, transmembrane domain. B, Western blot analysis of RANKHA-Myc and RANKAP expressed in COS-7 cells.

COS-7 cells. Cell surface expression of the RANKHA-Myc protein introduced into COS-7 cells was confirmed by immunostaining (data not shown). We next examined the supernatant from RANKHA-Myc-transfected COS-7 cells for the presence of cleaved soluble RANK. The supernatants were concentrated with ConA-Sepharose lectin beads and subjected to Western blot analysis with anti-HA Ab, which recognizes the extracellular domain of RANKHA-Myc. Under unchallenged conditions, almost no protein was found, but an ∼25-kDa band was detected in the supernatant from the cells stimulated with PMA (Fig. 2A). Moreover, the intensity of the band was suppressed by the addition of a broad metalloprotease inhibitor, GM6001, indicating that the proteolytic activity is metalloprotease dependent. Accordingly, when the cell surface expression level of RANKHA-Myc in COS-7 cells was investigated by flow cytometry, a significant decrease was observed in the cells treated with PMA, whereas treatment with GM6001 abolished the effects of PMA stimulation (Fig. 2B). To confirm these observations, we attempted to determine whether the cleaved stub of RANKHA-Myc remaining in the cells could be detected in the cell lysates. The lysates from the cells incubated with or without PMA were immunoprecipitated with rabbit anti-Myc sera and probed with murine anti-HA Ab and then with murine anti-Myc Ab. As expected, full-length RANK was found when the sample was analyzed with anti-HA Ab, whereas when the membrane was washed and reprobed with anti-Myc Ab, which recognizes the cytoplasmic end of RANKHA-Myc, an ∼60-kDa band, in addition to the full-length RANK, was obtained (Fig. 2C). Identical results were obtained with a murine anti-Myc Ab from a different clone (data not shown). Taken together, these observations suggest that RANKHA-Myc that has been transfected into COS-7 cells can be proteolytically processed and released into the supernatant and that the PMA-stimulated proteolytic activity is metalloprotease dependent.

Cleaved soluble RANK is present in the form of a monomer protein

It was shown that RANK forms a trimer without ligand binding and that the self-association of RANK is mediated through its cytoplasmic tail (22). Based on these observations, we hypothesized that, upon cleavage, the extracellular domain of RANK is released in the supernatant as a monomer protein. To test this hypothesis, we analyzed the cell lysates and supernatants of COS-7 cells transfected with RANKHA-Myc by Western blot with or without the addition of a reducing agent, DTT. Under nonreducing conditions (without the addition of DTT), as much as half of the RANKHA-Myc in the cell lysate appeared in the form of a >200-kDa protein, presumably representing a RANK trimer, whereas, under reducing conditions, the >200-kDa band disappeared, and only the 85-kDa band was detected (Fig. 3A). In contrast, the protein species detected in the supernatant were insensitive to DTT and seemed to be identical under nonreducing and reducing conditions (Fig. 3B). These results suggest that RANK cannot form a trimer after it is released from the cell surface and that the cleaved extracellular domain is present in the supernatant in the form of a monomer.

TACE mediates cleavage of RANK

Because the combination of a response to short-term PMA stimulation and the sensitivity to metalloprotease inhibitors is a hallmark of TACE activity (23), we next attempted to determine whether TACE was involved in the ectodomain shedding of RANK. To facilitate the transfection and the detection of cleaved RANK in the supernatant, we used the AP reporter system (20) and generated a truncated RANK construct with an AP module added to its N terminus (RANKAP, Fig. 1A). We first investigated whether this construct could reproduce the shedding properties of RANKHA-Myc by introducing RANKAP into wild-type mEFs and measuring AP activity in the lysates and supernatants by in-gel staining, as

FIGURE 2. Cleavage of RANK in COS-7 cells. A, The extracellular domain of RANKHA-Myc is cleaved and released into the supernatant. COS-7 cells transfected with RANKHA-Myc were incubated with PMA (25 ng/ml) and/or GM6001 (10 μM) for 1 h. Supernatants were concentrated and analyzed by Western blot with anti-HA Ab. A protein weighing ∼25 kDa was detected in PMA-treated cells (arrowhead). B, The same cells as in A were treated with PMA and/or GM6001 and then incubated with anti-HA Ab. The bound Abs were detected with fluorochrome conjugated secondary Ab and evaluated by flow cytometry. The means of the values obtained in three independent experiments are shown. *p < 0.05. C, Membrane-bound stubs of RANK were detected in RANKHA-Myc-transfected COS-7 cells. COS-7 cells transiently expressing RANKHA-Myc were stimulated with PMA, and cell lysates were immunoprecipitated with anti-Myc Ab. The immunoprecipitates were blotted with anti-HA Ab (which recognizes the extracellular domain of RANKHA-Myc) and anti-Myc Ab (which recognizes the cytoplasmic domain of RANKHA-Myc). White arrowhead, full-length RANKHA-Myc; black arrowhead, membrane-bound stub; *, nonspecific bands; (−), nontransfected COS-7 cells.
RANK is cleaved in the stalk region

To identify the potential RANK cleavage site(s) in more detail and determine whether TACE directly cleaves RANK, we synthesized a peptide covering the juxtamembrane domain of RANK (Ser194-Tyr211) and incubated it with rTACE. Based on the results of the mass spectrometry analysis, we concluded that rTACE directly cleaved the peptide and deduced two potential cleavage sites in the juxtamembrane domain of RANK: a major site between Thr200-Leu201 and a minor site between Met199-Thr200 (Fig. 5A and data not shown). We next generated several mutant RANK<sup>AP</sup> constructs (Fig. 5A, Mut1–6) and investigated how the mutations in the putative cleavage sites affected the cleavage efficiency. Cell surface expression of the mutants (Mut1–6) was confirmed by immunostaining and confocal microscopic analysis (data not shown). As shown in Fig. 5B, when Met199-Thr200-Leu201 was replaced with Ile-Ser-Pro (Mut1) or with Ile-Thr-Pro (Mut5), there was a significant decrease in the AP activity in the supernatant. Although cleavage of these mutants was still increased by PMA stimulation, overall shedding efficiency was significantly diminished. In contrast, the shedding efficiency of Mut2–4 was comparable to that of the wild-type control. The deletion mutant (Mut6), in which Met199-Thr200-Leu201 had been removed, was as resistant to shedding as Mut1 and Mut5. Taken together, these observations suggest that, although RANK shedding by TACE is not absolutely dependent on the Met199-Thr200-Leu201 sequence in the juxtamembrane domain, it is required for efficient cleavage.

RANK shedding in RAW264.7 cells is upregulated by rRANKL

To extend our findings, we next investigated whether RANK is cleaved in a macrophage-like cell line (RAW264.7) that expresses endogenous RANK and differentiates into multinucleated osteoclasts in response to RANKL stimulation. Expression of TACE in this cell line was confirmed by Western blot (data not shown). RAW264.7 cells transfected with RANK<sup>AP</sup> were incubated with PMA and/or GM6001, and the supernatant was analyzed for AP activity by colorimetry, as described in Materials and Methods. As

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**FIGURE 5.** A, Schema of the synthesized peptide used in the peptide-cleavage assay and the putative cleavage sites (major site, black arrowhead; minor site, white arrowhead) deduced from the results of the mass spectrometry analysis of the cleaved peptides. B, Evaluation of the shedding efficiency of RANK<sup>AP</sup> mutants (Mut1–6). The mutant constructs were introduced into COS-7 cells, and the AP activity in the supernatants and lysates was visualized by in-gel staining. Asterisks indicate substituted amino acids.
shown in Fig. 6A, the results were comparable to those observed in wild-type mEFs (Fig. 4A), indicating that RANK is similarly processed in RAW264.7 cells. Based on an analogy to the induction of the shedding of vascular endothelial growth factor receptor by its ligand, vascular endothelial growth factor-A (24), we next investigated whether RANKL could trigger the cleavage of RANK. As shown in Fig. 6A, RANK shedding was upregulated by rRANKL in RAW264.7 cells to a level comparable to that induced by PMA, and the increased activity was abolished by the addition of GM6001. It has been well established that following ligand binding, RANK activates various signaling pathways essential for osteoclast development and that the initial step in the signaling involves binding of TRAF6 to the cytoplasmic domain of RANK. To explore whether RANKL-induced RANK shedding was TRAF6 dependent, we used a TRAF6 inhibitory peptide, which functions as a TRAF6 decoy by binding to the TRAF6-binding motif of RANK (25), and examined how it would affect RANKL-induced shedding activity. When RANKAP-transfected RAW264.7 cells were incubated with soluble RANKL (100 ng/ml) in the presence of the inhibitory peptide (inh. pep., 0–30 μM), C, TRAF6 inhibitory peptide (30 μM) abolished RANKL-induced shedding activity but had little effect on PMA-stimulated RANK shedding. D, Evaluation of the effects of the MAPK inhibitors U0126 (5 μM), SB202190 (20 μM), and SP600125 (20 μM) on the RANKL-induced RANK shedding activity. **p < 0.05. E, RAW264.7 cells were incubated with PMA (10 nM, left panel) or RANKL (100 ng/ml, right panel) in the presence or absence of GM6001 (25 μM), and the cell surface expression levels of RANK were analyzed by flow cytometry. The shaded areas represent isotype Ab.

RANKL-induced shedding was sensitive to U0126 (MEK inhibitor) and SB202190 (p38 inhibitor) but not to SP600125 (JNK inhibitor), indicating that RANKL-induced shedding activity is TRAF6 and MAPK dependent (Fig. 6D). To further confirm the observation that RANK shedding can be induced by RANKL stimulation, we examined the cell surface expression of endogenous RANK by flow cytometry. As shown in Fig. 6E, incubation with PMA or RANKL significantly decreased the cell surface expression of RANK in RAW264.7 cells, and this activity was abolished by the addition of GM6001.

RANK shedding functions as a negative regulator in RANKL-induced osteoclastogenesis in vitro

To gain insight on the physiological relevance of RANK shedding, we generated recombinant soluble RANK composed of the extracellular region of RANK N terminus to the putative cleavage site (Met1-Ser198, Fig. 7A), and examined how the cleaved extracellular domain of RANK would affect osteoclastogenesis in vitro. RAW264.7 cells were incubated with rRANKL in the presence or absence of sRANKHA-Myc for 5 d, and the number of TRAP-positive multinucleated cells was evaluated. As shown in Fig. 7B, the addition of sRANKHA-Myc almost completely abolished RANKL-induced osteoclastogenesis, indicating that cleaved soluble RANK functions as an antagonist to RANKL in a similar manner to osteoprotegerin, an endogenous decoy receptor for RANKL.
described in Materials and Methods (Wt) mice or bar, 250 ng/ml RANKL (50 ng/ml) in the absence (2) were concentrated, and the sRANK HA-Myc was detected by Western blot right panel using anti-Myc Ab (right panel) or presence of sRANK HA-Myc. The number of TRAP-positive multinucleated cells was counted on day 5. Scale bar, 250 μm; original magnification ×40. BMMs derived from wild-type (Wt) mice or Tace/LysM mice were incubated with RANKL and CSF-1 as described in Materials and Methods. The expression levels of NFATc1 and β-actin were evaluated at the designated time points (C), and the number of TRAP-positive multinucleated cells was counted on day 4 (D). *p < 0.05.

Furthermore, we found that TACE-deficient BMMs derived from Tace/LysM mice exhibited increased levels of NFATc1, a transcription factor essential for osteoclastogenesis, after RANKL stimulation (Fig. 7C) and that RANKL-induced osteoclastogenesis is accelerated compared with the wild-type control (Fig. 7D). These observations indicate that RANK shedding functions as a negative regulator for osteoclastogenesis, presumably via the production of soluble RANK and by decreasing cell surface RANK availability in osteoclast precursors.

Discussion

Several membrane-bound molecules involved in osteoclast development, including RANKL (12), CSF-1R (16), TNF-α (26), and the membrane-bound isoform of CSF-1 (15), are proteolytically cleaved to become soluble. The current study shows for the first time that RANK, a molecule essential for osteoclastogenesis, also undergoes ectodomain shedding and is released as a monomeric protein. We also found by means of cell-based assays, using TACE-deficient cells, that TACE is involved in RANK shedding activity and that this activity can be upregulated by RANKL in macrophage-like cell line RAW264.7. These results indicate a potential involvement of ectodomain shedding of RANK in the regulation of osteoclastogenesis.

Consistent with the results of a previous study (22), RANKHA-Myc introduced into COS-7 cells self-assembled in the absence of RANKL (Fig. 3A), whereas the cleaved extracellular domain of RANK released in the supernatant was in the form of a monomeric protein, not as a trimer, indicating that the cytoplasmic domain or juxtamembrane domain is required for the self-association of RANK and that once cleaved from the cell surface, RANK is no longer able to form a trimer. In contrast, it is possible that RANK is resistant to ectodomain shedding when it is in the trimeric conformation and that only the unassociated monomer proteins are cleaved from the cell surface.

The consequences of ectodomain shedding of receptors can be complex in some cases. Ectodomain shedding of receptors can result in downregulation of receptor availability on the cell surface and can simultaneously give rise to soluble receptors, which have a potential to function as decoy receptors and to interfere with ligand–receptor association. Moreover, as in the case of CD44 and Notch (27–29), ectodomain shedding of receptors may be necessary to trigger intramembranous cleavage by presenilin and the release of the cytoplasmic domain into the cytoplasm. For example, it is well established that cleaved TNFR1 and TNFR2 are capable of binding to TNF-α and suppressing TNF-α–TNFR signaling and that dysregulation of the shedding of these receptors results in aberrant immunoreactions (30–32). Similarly, recombinant soluble RANK (Glu30-Pro213) and RANK-Fc were demonstrated to inhibit RANKL-induced osteoclastogenesis in vitro and to prevent the formation of metastatic bone lesions in xenograft tumor models (33–36). We also generated soluble RANK composed of the extracellular domain N terminus to the putative cleavage sites and observed a similar inhibitory effect on RANKL-induced osteoclastogenesis in vitro. These observations suggest that the cleaved extracellular domain of RANK is biologically active and potentially functions as a decoy receptor that blocks RANKL binding in a manner similar to that of osteoprotegerin. Because osteoprotegerin can bind not only to RANK but also to the TNF-related apoptosis-inducing ligand (TRAIL/Apo2 ligand) (37), soluble RANK may function as a more specific inhibitor of RANK.

Based on the observations that cell surface expression of RANK decreases following RANKL stimulation, it is tempting to hypothesize a negative feedback loop in which RANKL induces RANK shedding and, thereby, downregulates RANKL–RANK signaling during osteoclast development. In accordance, we found that TACE-deficient BMMs form multinucleated cells more rapidly than did the control BMMs and that the expression of NFATc1 is upregulated in TACE-deficient BMMs, indicating that RANK shedding negatively regulates osteoclastogenesis. However, in contrast, no overt bone defects were found in Tace/LysM mice, in which TACE is inactivated in monocytes and macrophages (19), at least under unchallenged conditions (data not shown). Thus, other enzyme(s) may compensate for the lack of TACE in the cleavage of RANK, or the regulation of cell surface RANK availability may also be regulated by different mechanisms that do not involve ectodomain shedding (e.g., via endocytosis). Furthermore, it is also possible that the lack of TACE leads to an increase in the amount of other cell surface proteins, which may function in an antiosteoclastogenic manner, and offsets the effects of enhanced RANK signaling. Further research, including a generation of uncleavable mutant RANK knock-in mice, is needed to elucidate the significance of RANK shedding and its impact on osteoclastogenesis.

In conclusion, the current study demonstrated that RANK is subjected to ectodomain shedding by TACE and identified potential cleavage sites in the juxtamembrane domain of RANK. The cleavage of RANK should decrease its availability on osteoclasts and their precursors and simultaneously generate soluble decoy receptors that may inhibit the RANKL–RANK association. Moreover, the observation that RANKL–RANK signaling induces RANK shedding suggests a possible negative-feedback mechanism regulating the cell surface availability of RANK on osteoclasts and their precursors. Therefore, upregulation of RANK shedding via TACE...
activation may be beneficial in suppressing overt osteoclastogenesis under pathological conditions, including rheumatoid arthritis, bone metastasis, and osteoporosis.

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Disclosures
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