Regulatory Mechanisms of RANKL Presentation to Osteoclast Precursors

Masashi Honma · Yuki Ikebuchi · Yoshiaki Kariya · Hiroshi Suzuki

Abstract It is important to understand the molecular mechanisms regulating osteoclast formation, as excess activation of osteoclasts is associated with various osteopenic disorders. Receptor activator of nuclear factor kappa B (RANKL) is a central player in osteoclastogenesis. Recent findings suggest that osteocytes are the major supplier of RANKL to osteoclast precursors. It has also been suggested that osteocyte cell death upregulates the RANKL/osteoprotegerin (OPG) ratio in viable osteocytes adjacent to apoptotic osteocytes in areas of bone microdamage, thus, contributing to localized osteoclast formation. Indeed, viable osteocytes can provide RANKL through direct interactions with osteoclast precursors at osteocyte dendritic processes. In addition, OPG tightly regulates RANKL cell surface presentation in osteocytes, which contributes to the inhibition of RANKL signaling, as well as the decoy receptor function of OPG. By contrast, the physiological role of RANKL in osteoblasts is yet to be clarified, although similar mechanisms of regulation are observed in both osteocytes and osteoblasts.

Keywords RANKL · RANK · OPG · Osteoprotegerin · Intracellular trafficking · Osteoblast · Osteocyte · Osteoclast · Bone remodeling

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Introduction

Bone quality is maintained through bone remodeling, which is a coordinated cycle of catabolic and anabolic phases [1]. An imbalance between these phases can be observed in various diseases with bone lesions. Specifically, excess bone resorption is the major cause of decreased bone mass associated with osteopenic disorders such as postmenopausal osteoporosis, rheumatoid arthritis, and bone metastasis in cancer [2]. Thus, a greater understanding of the physiological regulatory systems underlying catabolic bone remodeling is required, and may reveal novel therapeutic targets for these diseases. The catabolic phase of bone remodeling is performed by osteoclasts, which are formed on demand through the activation and fusion of osteoclast precursors [3]. Numerous studies indicate that receptor activator of nuclear factor kappa B (RANK) ligand (RANKL) is a central player in the regulation of mature osteoclast formation [4–6]. Mice that are genetically defective for RANKL have an osteopetrotic phenotype and a complete absence of mature osteoclasts [5]. Notably, denosumab, a neutralizing antibody for human RANKL, is a strong antiresorptive agent and is used clinically for the treatment of osteoporosis and related conditions [7–9].

RANK signal transduction pathways in osteoclast precursors have been elucidated in detail [10–13]. Upon binding of RANKL to RANK, the adaptor molecule, TNF receptor-associated factor 6 (TRAF6), is recruited to the RANK intracellular domain, inducing activation of nuclear factor kappa B (NF-κB) and mitogen-activated kinases, including p38 and Jun N-terminal kinase [11, 13–15]. NF-κB positively regulates the transcriptional activity of nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), which functions as a master regulator of osteoclastogenesis [10]. NFATc1 also binds to its own promoter and this auto-amplification loop is further activated by an activator protein 1 complex containing c-Fos [16]. A series of intracellular events result in the transcriptional

upregulation of osteoclast-specific proteins [10, 17–19]. Although much is known about RANKL-mediated intracellular signaling, a number of issues remain to be clarified in regard to the physiological regulation of RANKL. Here, we review recent progress in RANKL signaling and discuss future research directions.

Osteocytes are the Major Source of RANKL in Osteoclastogenesis In Vivo

Many previous studies presumed that osteoblasts were the physiological source of RANKL for osteoclast precursors, probably due to the efficient formation of mature osteoclasts when bone marrow macrophages (BMMs) are co-cultured with osteoblasts [20]. However, early studies using osteocyte-like MLO-Y4 cells indicated that osteocytes are also able to support osteoclastogenesis in coculture, raising questions about the exact physiological source of RANKL [21, 22]. Two recent reports have helped settle this issue; mature osteoclast formation was greatly reduced in genetically manipulated mice that selectively lack RANKL expression in osteocytes [23••, 24••]. Bone volume and bone mineral content were greater in adult osteocyte-specific RANKL knockout mice than in adult wild-type mice, whereas no significant difference was observed between newborn knockout and wild-type mice [23••, 24••]. These findings indicate that osteocytes are the major source of RANKL during bone remodeling [23••, 24••], raising further questions of when, where, and how osteocytes transfer RANKL to osteoclast precursors. Various studies indicate that osteocyte cell death is one of the triggers of pathophysiological osteoclastogenesis [25, 26]. Because the catabolic removal of damaged bone is indispensable for maintaining bone integrity, and this requires the presence of osteoclasts, it is likely that osteocyte apoptosis at the site of damage triggers osteoclast formation at that location [25, 26]. Indeed, mice in which osteocyte cell death was selectively induced by toxin receptor mediated cell knockout (TRECK) showed a severe osteoporotic phenotype with a marked increase in osteoclast formation [27]. Furthermore, fatigue-induced microdamage in rat ulnae resulted in upregulation of caspase-3 in osteocytes located within 100 μm of the site of damage site. Furthermore, RANKL was upregulated and osteoprotegerin (OPG) was downregulated in osteocytes located between 100 and 200 μm of the site of damage [28•]. This observation suggests that viable osteocytes adjacent to apoptotic osteocytes are the major supplier of RANKL for osteoclast precursors during osteoclastogenesis associated with bone microdamage. Estrogen withdrawal and mechanical unloading also increase osteocyte apoptosis, which may contribute to excess osteoclast activation [29]. However, the exact molecular mechanisms by which apoptotic osteocytes trigger upregulation of the RANKL/OPG ratio in adjacent osteocytes remain to be elucidated. Moreover, it is unclear whether osteoclastogenesis during physiological bone turnover is regulated in a stochastic manner or also through osteocyte apoptosis.

Viable Osteocytes Efficiently Support Osteoclastogenesis In Vitro

Osteocytes are terminally differentiated cells derived from osteoblasts that are embedded in bone matrix during bone formation [30]. Osteocytes are, therefore, completely surrounded by mineralized bone matrix; thus, dendritic processes, which extend from the cell body, are the only means of forming direct connections to other cells, including adjacent osteocytes embedded in the bone matrix or osteoblasts located at the bone surface [31]. As osteocyte-osteoclast precursor interactions cannot occur at the osteocyte cell body, conventional two-dimensional (2D) coculture systems, which are often used for osteoblast culture with BMMs, are not suitable for evaluating the osteoclastogenic ability of osteocytes in vitro. Recently, we reported a three-dimensional (3D) coculture system for osteocytes with BMMs that accurately mimics osteocyte-supported osteoclastogenesis in vitro [32•]. To avoid direct interactions between BMMs and osteocytes at osteocyte cell bodies, freshly isolated osteocytes were seeded onto a porous (3 μm) filter, and then after culturing for 8 hours, the filter was placed upside-down onto a collagen sol-gel matrix, and finally BMMs were seeded onto the filter after collagen gelation [32•]. Under these conditions, osteocytes efficiently stimulated mature osteoclast formation and their osteoclastogenic ability was not affected by inhibition of osteocyte sRANKL (the soluble form of RANKL) production [32•]. Doubling the filters or reducing the pore size both led to decreased osteoclast formation [32•]. In addition, we confirmed that osteocytes extend dendritic processes through the filter pores to the opposite side and directly interact with BMMs at the extremities of their dendrites [32•]. These observations indicate that osteocytes can provide RANKL signals to osteoclast precursors through direct interactions. One problem associated with these in vitro assays is that primary osteocytes tend to dedifferentiate into a more immature state during culture [32•]. Although less osteocyte dedifferentiation occurred in the collagen gel 3D cultures than in conventional 2D cultures, after 1 week of culture, osteocyte expression levels of RANKL and the late osteocyte markers, sclerostin (SOST) and fibroblast growth factor 23 (FGF23), were approximately half those of freshly isolated cells [32•]. We recently reported that Matrigel (BD Biosciences) slows osteocyte dedifferentiation and that a collagen matrix containing 50 % Matrigel is optimal [33]. The length and number of dendritic processes in each osteocyte were also increased by these culture conditions [33]. Furthermore, tartrate-resistant

acid phosphatase (TRAP) activity in the BMM-osteocyte co-culture system containing 50 % Matrigel was three times higher than that in the collagen-only matrix [33].

OPG Regulates RANKL Subcellular Localization in Osteocytes

Assuming that osteocytes deliver RANKL through direct interactions with osteoclast precursors, then the amount of RANKL presented at the osteocyte cell surface is a critical factor determining the magnitude of signal input to osteoclast precursors. A study using confocal laser scanning microscopy in osteocytes showed that a large proportion of RANKL expression is localized to lysosomes with limited presentation of RANKL at the cell surface [32•]. By contrast, in osteocytes derived from OPG-deficient mice, the lysosomal localization of RANKL is disrupted and RANKL presentation at the cell surface is markedly upregulated [32•]. The same study also confirmed that the exogenously introduced OPG gene restores the subcellular localization of RANKL in OPG-deficient osteocytes [32•]. Experiments using HeLa cells show that OPG forms a complex with newly synthesized RANKL at the Golgi apparatus and that this complex forms through the interaction of a cysteine-rich domain in OPG with the extracellular domain of RANKL. Furthermore, the heparin-binding domain of OPG is involved in the transport of the RANKL-OPG complex from the Golgi apparatus to lysosomes [32•]. In general, OPG is recognized as a decoy receptor for RANKL; OPG is secreted from cells and binds to the RANKL extracellular domain, preventing RANKL binding to osteoclastic RANK [32•]. However, the above observations indicate that OPG also regulates RANKL trafficking in osteocytes, restricting the cell surface presentation of RANKL [32•]. Indeed, expression of a mutant OPG protein, which retains RANKL binding to act as a decoy receptor but lacks the ability to regulate RANKL trafficking, in co-cultured OPG-deficient osteocytes did not restore osteoclast formation to wild-type levels, indicating that the osteoclastogenic ability of osteocytes is partly due to OPG's function as regulator of RANKL trafficking [32•].

RANKL Subcellular Localization is Regulated in a Similar Manner in Osteoblasts and Osteocytes

Although the function of osteocyte-expressed RANKL in physiological and pathologic conditions is highlighted herein, the role of osteoblast-expressed RANKL is less clear. It is difficult to evaluate the role of osteoblastic RANKL directly in vivo because conditional ablation of the RANKL gene in osteoblasts will lead to a simultaneous absence of RANKL

expression in osteocytes, as osteoblasts differentiate into osteocytes. Novel approaches are therefore required to enable osteoblast-specific suppression of RANKL expression. However, several hormones and cytokines strictly regulate RANKL expression in osteoblasts, implying that osteoblastic RANKL may have an important, yet to be clarified, physiological role. We have analyzed the regulatory mechanisms of RANKL subcellular localization in osteoblasts. Like in osteocytes, RANKL was predominantly localized to lysosomes, and RANKL cell surface localization was tightly restricted [34]. In addition, RANKL accumulation was observed in OPG-deficient primary osteoblasts, and cell surface presentation of RANKL was largely upregulated in the absence of OPG [35]. The behavior of RANKL localized to osteoblastic lysosomes was also analyzed. Previous reports indicate that lysosomes function as secretory organelles in some cell types [36, 37]. When osteoblastic cells were stimulated with polystyrene beads coated with recombinant RANK extracellular domain (RANK-beads), lysosomes were recruited to the cell surface near the areas contacting the RANK-beads [34, 38]. RANKL present in the recruited lysosomes was then presented to the cell surface through lysosome fusion to the plasma membrane [34, 38]. In some cell types, lysosomal organelles are not only involved in proteolysis but also mediate secretion of lysosomal content [36, 37]. With this in mind, we also confirmed that lysosomal content, specifically lysosomal enzyme α-N-acetylgalactosaminidase and preloaded fluorescent dextran, was released into culture media upon stimulation of osteoblastic cells with RANK-beads [34, 35]. In addition, treatment of osteoblastic cells with a proteasome inhibitor markedly increased RANKL expression, while treatment with a lysosome inhibitor had no effect on RANKL expression, indicating that RANKL is resistant to lysosomal protein degradation [34]. We therefore hypothesize that RANKL is stored in lysosomes and traffics to the cell surface in a stimulation-dependent manner. Furthermore, similar results were obtained in osteocytes; stimulation of osteocyte dendritic processes with RANK-beads led to the release of lysosomal contents into the culture media and translocation of lysosomal RANKL to the cell surface [32•]. It is likely that similar events occur upon the interaction of osteocyte dendritic processes with osteoclast precursors. Moreover, stimulation-dependent RANKL trafficking may increase RANKL signaling to osteoclast precursors.

Rab27a/b and Their Effectors are Involved in Lysosome-Plasma Membrane Fusion in Osteoblastic Cells

The molecular mechanisms involved in RANKL trafficking from lysosomes to the cell surface of osteoblastic cells are described in the following section. Previous studies have
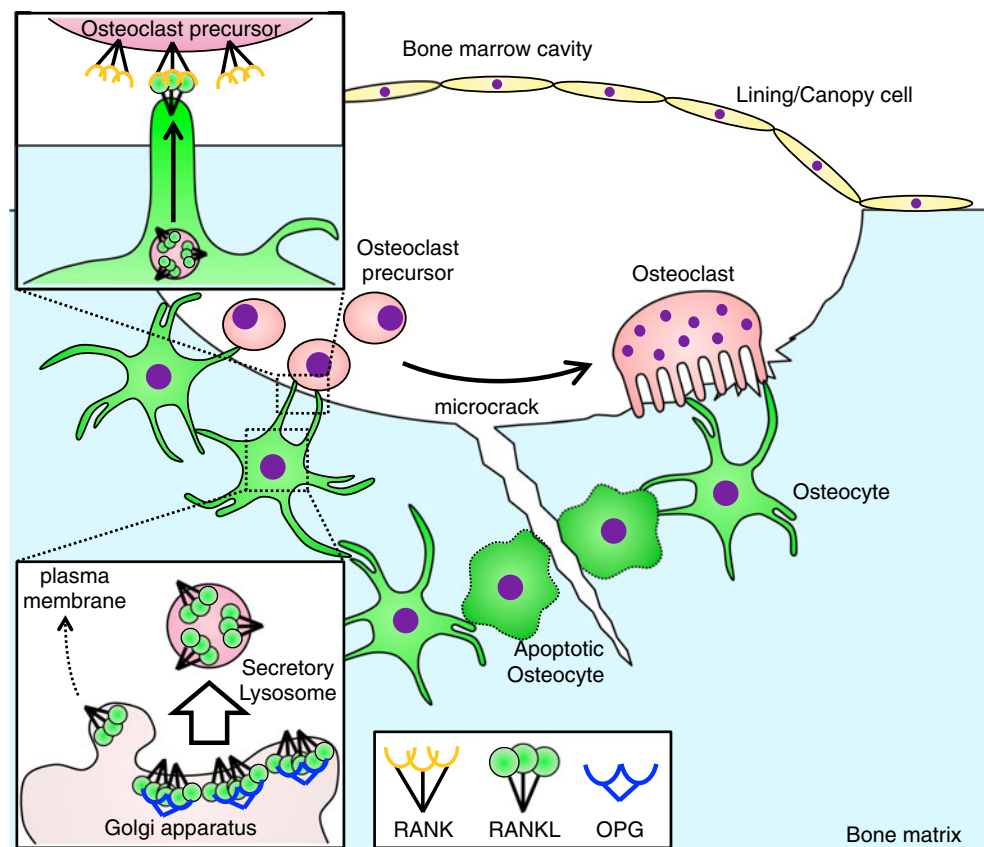
established that Rab family small GTPases are involved in secretion from various types of intracellular vesicles [36]. GTP-bound active Rab proteins bind to the surface of cargo vesicles and are involved in intracellular transport of vesicles or fusion of vesicles to the acceptor membrane, cooperating with various effector molecules in the process [36]. Several Rab family members are expressed in osteoblastic cells, and notably, siRNA-mediated knockdown of Rab27a and Rab27b resulted in decreased RANKL release upon stimulation with RANK-beads [38]. Rab27a was previously shown to be involved in secretion from lysosomal organelles in various cell types, including melanosomes in melanocytes [39, 40] and lytic granules in cytotoxic T lymphocytes [41, 42]. Rab27b, which is closely related to Rab27a, is expressed in many cell types and shares effector proteins with Rab27a [36, 43, 44]. We have also elucidated that the Rab27a/b effector proteins, Slp4-a, Slp5 and Munc13-4, are involved in RANKL release in osteoblastic cells [38]. siRNA-mediated knockdown of each effector molecule induced lysosome accumulation to regions contacting the RANK-beads, whereas cell surface presentation of RANKL was reduced, indicating that these effector molecules are involved in RANKL-associated membrane fusion [38]. Furthermore, stimulation-dependent RANKL release was suppressed in primary osteoblasts obtained from Jinx mice, which have a loss-of-function mutation in the Munc13-4 gene, supporting the above observations

[38]. Further research is required to determine the physiological role of osteoblastic RANKL and the exact function of these strict regulatory mechanisms that underlie RANKL trafficking in osteoblasts.

Conclusions

Recent studies suggest that osteocytes are the major source of RANKL in physiological and pathologic osteoclastogenesis. Osteocyte cell death associated with bone microdamage leads to upregulation of the RANKL/OPG ratio in osteocytes adjacent to the damaged site, which explains the localized formation of mature osteoclasts. Osteocytes provide RANKL to osteoclast precursors through direct interactions at the extremities of dendritic processes. In addition, OPG functions not only as a secreted decoy receptor for RANKL but also as a regulator of RANKL intracellular traffic, restricting RANKL presentation to the cell surface, which likely determines the magnitude of signal input. These hypotheses are depicted in Fig. 1. By contrast, the physiological role of RANKL in osteoblasts is unclear, although strict regulatory mechanisms for RANKL trafficking can be observed in both osteoblasts and osteocytes. The physiological significance of RANKL regulation in osteoblastic cells remains to be elucidated.

Fig. 1 Schematic illustration depicting osteoclastogenic process supported by osteocytes

Compliance with Ethics Guidelines

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Papers of particular interest, published recently, have been highlighted as:
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•• Of major importance

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