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Review

RANKL-independent modulation of osteoclastogenesis

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ABSTRACT

Osteoclasts are functional cells required for bone resorption. They are derived from hematopoietic precursors and undergo a series of differentiation and fusion steps in response to various humoral factors. Depending on the importance in osteoclastogenesis, the pathways for the differentiation of hematopoietic precursors to mature osteoclasts can be divided into two categories: canonical and the non-canonical. Receptor activator of nuclear factor κB ligand (RANKL)-induced osteoclast formation is considered as an important canonical pathway. Non-canonical pathways of osteoclastogenesis mainly involve several humoral factors that can substitute for RANKL to induce osteoclast formation. Among these factors, tumor necrosis factor (TNF)-α, interleukin (IL)-1, "homologous to lymphotoxins, exhibiting inducible expression, and competing with herpes simplex virus glycoprotein D for herpesvirus entry mediator, a receptor expressed by T lymphocytes" (LIGHT), a proliferation-inducing ligand (APRIL), and B cell-activating factor (BAFF) belong to the TNF superfamily. Other RANKL substitutes are primarily cytokines and growth factors including transforming growth factor β (TGFβ), IL-6, IL-11, nerve growth factor (NGF), insulin-like growth factor (IGF)-I, and IGF-II. In this review, we summarize the involvement of these factors in inducing osteoclastogenesis *in vitro*. Although these factors weakly induce osteoclast formation, they may play a major role in pathological bone resorption.

Contents

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1. Introduction

Osteoclasts are highly specialized multinucleated cells (MNCs) responsible for bone resorption, and they are coupled with osteoblasts to regulate bone growth and remodeling in response to a variety of physiological and pathological conditions. Bone remodeling, an essential process for skeletal metastasis, is initiated by osteoclast-mediated old bone removal followed by subsequent bone replacement by osteoblasts. The coordinated action of osteoclasts and osteoblasts is required for bone homeostasis; however, aberrant regulation favoring osteoclasts could lead to pathological bone destruction associated with disorders of inflammatory osteolysis, such as arthritis and periodontitis [1,2].

Osteoclasts are formed by the fusion of mononuclear phagocyte osteoclast precursors derived from pluripotent hematopoietic stem cells [3,4]. As a pool for osteoclast renewal and expansion, osteoclast precursors, which account for 1–2% of peripheral blood monocytes, differentiate into large, multinucleated tartrate-resistant acid phosphatase (TRAP)-positive mature cells through a sequence of differentiation stages near the bone surface [5,6]. Various molecular pathways underlying the mechanisms of osteoclast formation have been unveiled, including the crucial receptor activator of nuclear factor κB ligand (RANKL)-receptor activator of nuclear factor κB (RANK) signaling pathway and other RANKL-independent pathways.

2. RANKL-dependent osteoclast formation

RANKL-dependent osteoclast formation is considered a canonical pathway in which osteoclast differentiation and activation is triggered by the interaction between RANKL and RANK [7]. RANKL belongs to the tumor necrosis factor (TNF) receptor-ligand family and is expressed mainly by osteoblasts/stromal cells in the bone. RANKL expression is strongly increased in response to various pro-resorptive hormones such as parathyroid hormone (PTH), 1,25-dihydroxy vitamin D3 (1,25(OH)2D3), prostaglandin E2 (PGE2), and pro-inflammatory cytokines including TNF-α, interleukin (IL)-1, IL-6, and IL-17 [8–12]. RANKL exerts its osteoclastogenic effect by binding with its receptor RANK, a type I membrane protein expressed by mononuclear phagocyte osteoclast precursors. The critical role of RANKL-RANK signaling has been evidenced by the results of *in vitro* study that soluble RANKL induced osteoclast formation/bone resorption and the performance of RANKL (-/-) mice which showed osteopetrosis with absence of normal osteoclasts on the bone surface [13,14].

The RANKL-RANK pathway is inhibited by osteoprotegerin (OPG), a member of the TNF receptor superfamily, which acts as a soluble decoy receptor for RANKL and competes for binding to RANKL with RANK, thus blocking RANKL-induced osteoclastogenesis [3,15]. OPG is mainly produced by osteoblastic lineage cells and has been reported to strongly inhibit IL-1α, $25(OH)_2D_3$, PGE2, PTH, or TNF-α-induced osteoclast formation *in vitro* [15–17]. The genetic deletion of OPG in mice was found to result in severe osteoporosis with greatly increased osteoclast formation and resorptive activity [18]. As a critical negative modulator of bone resorption, OPG interacts with RANKL to induce bone remodeling by regulating the RANKL/OPG ratio [7].

RANKL/RANK binding enables RANK to recruit adaptor molecules, especially TNF-associated factor-6 (TRAF6), with its cytoplasmic tail [19]. Upon RANK/TRAF6 complex formation, downstream signaling pathways including NF-kB, activator protein-1 (AP-1), and mitogen-activated protein kinase (MAPK) are activated [20–24]. These signaling cascades initiate the expression of several osteoclastic transcriptional factors such as nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) and c-Fos, which subsequently promote the transcription of osteoclast-specific genes including TRAP, cathepsin K, and matrix metalloproteinases (MMPs), thus leading to the commitment to mature osteoclasts with potent bone resorptive activity [19,25–27].

3. RANKL-independent osteoclast formation

Increasing evidence indicates that RANKL/RANK signaling is a major pathway for osteoclast formation, which is responsible for diseases characterized by physiological bone development and pathological bone destruction, such as rheumatoid arthritis, periodontal diseases, and postmenopausal osteoporosis. Furthermore, recent studies have shown that other cytokines can substitute for RANKL to promote osteoclast differentiation and function, especially in diseases with pathological bone resorption (Fig. 1).

3.1. TNF-α

The importance of TRAF6 in RANKL/RANK-induced signaling activation suggests the potential role of TNF in osteoclast formation. In 2000, Azuma et al. and Kobayashi et al. independently found that TNF-α could stimulate osteoclast differentiation in the absence of RANKL-RANK interaction [28,29]. TNF-α directly induced the formation of TRAP-positive MNCs, which produced resorption pits on the bone *in vitro* in the presence of M-CSF even though the bone resorption activity of TNF-α-induced MNCs was lower than that of soluble RANKL-induced MNCs [29]. The induction of osteoclasts by TNF-α was completely inhibited by

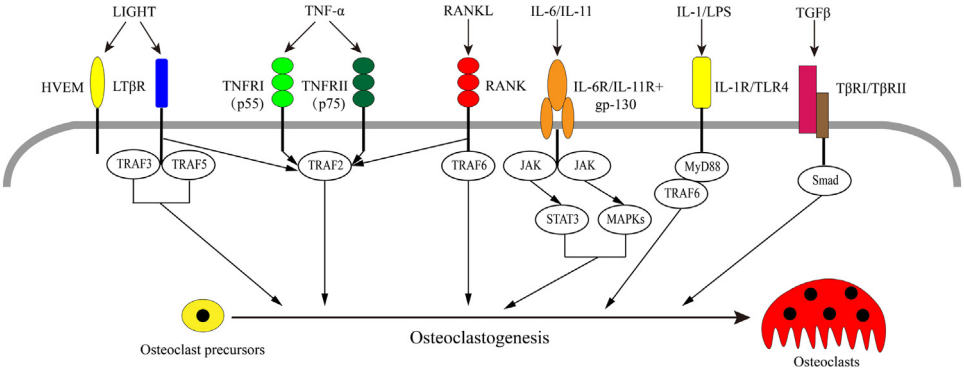

Fig. 1. Schematic diagram of RANKL-dependent and -independent modulation of osteoclastogenesis. The binding of RANKL to RANK activates TRAF6 and TRAF2. Other TNF superfamily members such as TNF-α and IL-1 also use TRAF6 or TRAF2 for signal transduction after binding to their membrane receptors. The activation of osteoclastogenesis is induced by LPS, IL-6, IL-11, TGFβ, and LIGHT through TLR4, IL-6R, IL-11R, TβRI/II, and LTβR, respectively. TRAF6-mediated signals are preferred for osteoclast differentiation.

anti-TNF receptor antibody but not OPG or the Fab fragment of anti-RANK antibody [28,29]. In combination with M-CSF, TNF-α induced mouse bone marrow cells to form TRAP-positive osteoclasts after 6 days of culture [28]. Furthermore, hematopoietic precursors from mice homozygous for a targeted deletion of RANKL, RANK, or TRAF6 have shown the capacity to differentiate into osteoclasts when they were cultured with TNF-α *in vitro* in the presence of transforming growth factor β (TGFβ) [30]. These results suggest that despite the essential role of RANK/TRAF6 in RANKL-induced signaling, it is not necessary for osteoclast differentiation induced by TNF-α.

TNF-α functions via binding with two cell membrane receptors: TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). Osteoclast formation induced by TNF-α can be inhibited by the addition of antibodies against TNFR1 or TNFR2 [28]. TRAF2 is a common signal transducer used by TNFR1 and TNFR2, which suggests that TRAF2 may be an upstream transducer of the TNF-α pathway, contributing to osteoclast induction [31]. An *in vivo* study using transgenic mice showed that TRAF2 deficiency severely impaired TNF-α-induced osteoclast formation, whereas TRAF2 overexpression induced the differentiation of osteoclast progenitors from wild-type mice into osteoclasts [32]. The administration of TNF-α to RANK-null mice has also been reported to induce TRAP-positive cells near the site of injection [33]. These results suggest that it is TRAF2 but not RANK/TRAF6 that participates in TNF-α-induced osteoclastogenesis.

The induction of osteoclast differentiation by TNF-α independent of RANKL does not indicate that TNF-α alone can function without the addition of other cytokines. Indeed, the pro-osteoclastic effect of TNF-α is relatively weak compared with that of RANKL. Besides M-CSF, a critical factor for the proliferation and early differentiation of osteoclast precursors, IL-1 and RANKL (even at a low concentration) have also been reported to highly potentiate osteoclast differentiation induced by TNF-α [28,34]. These findings suggest that the osteoclast-inductive effect of TNF-α may be synergistically potentiated by other members of the TNF superfamily.

3.2. IL-1

As an important pro-inflammatory cytokine, IL-1 induces bone destruction in a variety of diseases such as osteoporosis, rheumatoid arthritis, and periodontal diseases [35–37]. An early *in vitro* study reported that although purified osteoclasts placed on dentine slices failed to form resorption pits, the addition of IL-1 to this culture significantly increased the formation of resorption pits, and an IL-1 receptor antagonist but not OPG completely blocked the pit-forming activity of osteoclasts induced by IL-1 [38]. An *in vivo* study also confirmed the pro-resorptive properties of IL-1. The peripheral administration of an IL-1 receptor antagonist was found to decrease osteoclast formation and bone resorption in a mice model of ovariectomy-associated osteoporosis [39]. However, in another study, mice lacking a functional IL-1 receptor, IL-1 receptor type I (IL-1RI), did not lose bone mass after ovariectomy [40]. Furthermore, Fuller et al. found that IL-1 was able to substitute for RANKL to stimulate the resorptive activity of osteoclasts [41]. These results suggest that IL-1 could independently stimulate osteoclast function via binding to IL-1RI expressed on differentiated osteoclasts.

Although IL-1 can activate mature osteoclasts and enhance bone resorption, IL-1 alone cannot induce osteoclast differentiation from osteoclast precursors [36,42,43]. IL-1RI is required for the stimulatory effect of IL-1 on osteoclast function [38]. IL-1RI is expressed by osteoclasts but not osteoclast precursors, which explains why IL-1 fails to induce the differentiation of osteoclast precursors. IL-1 has been proposed to directly target osteoclast precursors to promote osteoclastic differentiation only with acceptable levels of RANKL; a similar pattern has been observed for TNF-α [34,44]. Recently, a study by Jung et al. provided a clue to the mechanism underlying the RANKL level required for the direct pro-osteoclastic differentiation effect of IL-1, which demonstrated that the overexpression of the IL-1RI receptor in bone marrow-derived macrophages (BMMs) or induction of IL-1RI by c-Fos overexpression enabled IL-1 alone to induce the formation of authentic osteoclasts via a RANKL/RANK-independent mechanism [45]. The addition of IL-1 to IL-1RI-overexpressing BMMs strongly activated NF-κB, JNK, p38, and ERK, a hallmark gene activation profile of osteoclastogenesis [45]. These results suggest that IL-1RI expression is the most critical prerequisite for osteoclasts or precursors to respond to IL-1.

3.3. IL-6 and IL-11

IL-6 is a pro-inflammatory cytokine that has been implicated in diseases associated with bone loss, such as postmenopausal osteoporosis, rheumatoid arthritis, and Paget's disease [46,47]. IL-6 can indirectly promote osteoclast formation and activation by inducing RANKL production in osteoblastic/stromal cells [48,49]. However, the direct action of IL-6 on osteoclast formation from precursors is controversial. Tamura et al. reported that simultaneous treatment with IL-6 and soluble IL-6 receptor [sIL-6R] induced osteoclast-like MNC formation; however, neither IL-6 nor sIL-6R induced MNC formation when they were added separately [50]. Similarly, Kudo et al. added IL-6 with or without sIL-6R to cultures of human peripheral blood mononuclear cells of the monocyte fraction in the presence of M-CSF and found that IL-6 alone did not induce osteoclast formation; however, when sIL-6R was added, IL-6 induced the formation of TRAP-positive MNCs with the capacity for lacunar resorption [51]. The findings of these studies suggest that the expression of membrane-bound IL-6R on osteoclast precursors is unsaturated; thus, the full realization of IL-6 biological function requires sIL-6R. Recently, IL-6 has been reported to have a negative effect rather than a positive effect on osteoclast differentiation. A study demonstrated that IL-6 could directly act on osteoclast progenitors to suppress their differentiation by inhibiting RANK signaling pathways [52]. In another study, IL-6 was shown to inhibit RANKL-induced osteoclastogenesis by diverting cells into the macrophage lineage [53]. Nevertheless, the role of sIL-6R has not been considered in both of these studies.

According to our studies, the effect of IL-6 on osteoclast formation depends on both the presence of sIL-6R and the levels of RANKL. We found that IL-6 attenuated high-level RANKL-induced osteoclast formation and bone resorptive activity regardless of whether sIL-6R was present. Conversely, a combination of IL-6 and sIL-6R enhanced low-level RANKL-induced osteoclastogenesis and osteoclast function [54]. This result indicated the existence of a bidirectional regulatory mechanism for RANKL-induced osteoclastogenesis by IL-6 in the presence of sIL-6R. In the initial stage of inflammatory osteolytic diseases such as rheumatoid arthritis and periodontitis (when RANKL levels in the local microenvironment are relatively low), the high levels of IL-6 and sIL-6R secreted by active immune cells could increase osteoclast differentiation and function in two ways: (1) indirectly acting on stromal cells or osteoblasts to stimulate RANKL production; (2) directly promoting the commitment of osteoclast precursors to mature osteoclasts. Both approaches can activate osteoclastic bone resorption and contribute to bone and articular cartilage erosion. However, in the progressive or late stage of diseases, when RANKL level is high, IL-6 and sIL-6R may serve to balance the high RANKL concentration produced in the bone microenvironment and suppress RANKL-induced osteoclast formation, thus protecting the bone against

over-resorption. In this case, IL-6/sIL-6R exerts a protective mechanism on the skeleton.

IL−11 belongs to the glycoprotein-130 (gp130) family, which shares several biological properties with IL-6 [55]. IL-11 is known to promote osteoclastogenesis and bone resorption in bone marrow cultures and has been implicated in estrogen deficiency-associated bone loss [56,57]. Previous studies on the role of IL-11 in osteoclast formation and function demonstrated that IL-11 could promote osteoclastogenesis indirectly by stimulating the osteoblast production of RANKL [25,58]. On the other hand, considering that osteoclast precursors express IL-11R [58], IL-11 may also directly target osteoclasts and/or precursors to regulate osteoclast formation. Kudo et al. reported that IL-11 induced osteoclast formation from human peripheral blood mononuclear cells, which was not inhibited by OPG or RANK-Fc but blocked by an antibody to gp130, indicating that this process of osteoclastogenesis is independent of the RANKL signaling mechanism [51,55]. In addition, IL-11 has been demonstrated to act synergistically with other osteolytic factors including $1,25(OH)_2D_3$, PTH, IL-1, and TNF-α to enhance osteoclast formation [58].

3.4. LPS

Lipopolysaccharide (LPS), a component of the outer membranes of all Gram-negative bacteria, has been proposed to be a potent stimulator of bone resorption in inflammatory bone diseases such as periodontitis, bacterial arthritis, and osteomyelitis [59]. LPS stimulate the production of cytokines including TNF-α by fibroblasts, macrophages, and other cells, which can induce osteoclast formation and activation [6]. Moreover, LPS has been reported to stimulate the survival and fusion of osteoclast progenitors independent of RANKL and induce pit-forming activity in the presence of M-CSF [60]. By using a murine macrophage cell line, RAW 264.7, Shamima et al. found that LPS induced RAW 264.7 cells to form TRAP-positive multinucleated giant cells, forming resorption pits on calcium-phosphate thin films [61]. This LPS-induced osteoclast formation was abolished by TNF-α antibody but not antibodies to M-CSF and RANKL, indicating the critical role of TNF-α in LPS-induced osteoclast formation in RAW 264.7 cells [61].

Toll-like receptor 4 (TLR4) is a crucial recognition receptor for LPS. The cytoplasmic signaling cascades of TLR4 are similar to those of IL-1 receptor as both receptors have cytoplasmic Toll/IL-1 receptor (TIR) domains [62]. TLR4 uses signaling molecules such as myeloid differentiation factor 88 (MyD88) and TRAF6 for downstream signal transduction [63]. A study reported that LPS enhanced the pit-forming activity of osteoclasts even in the presence of OPG [64]. However, the increased pit-forming ability of MyD88-deficient osteoclasts was retained when stimulated with RANKL but not LPS [65]. These results suggest that in contrast to RANKL, which requires TRAF6, LPS uses both MyD88 and TRAF6 to induce osteoclast formation.

The induction of osteoclastogenesis by LPS *in vitro* appears to be dependent on culture conditions. Odkhuu et al. reported that LPS induced osteoclast formation in RPMI-1640 medium but not α-MEM as glutathione (GSH) exclusively contained in RPMI-1640 medium prevented the cytotoxic action of LPS against osteoclast precursors by scavenging intracellular reactive oxygen species [66].

3.5. LIGHT

LIGHT (homologous to lymphotoxins, exhibiting inducible expression, and competing with herpes simplex virus glycoprotein D for herpesvirus entry mediator [HVEM], a receptor expressed by T lymphocytes) is a member of the TNF superfamily, which is expressed by activated T lymphocytes, monocytes, granulocytes, spleen cells, and immature dendritic cells. LIGHT exerts its immune modulatory effect through interaction with the signaling receptors HVEM and lymphotoxin β receptor (LTβR) as well as the soluble nonsignaling receptor decoy receptor 3 (DcR3) [67,68]. LIGHT levels were reported to significantly increase in the serum of patients with rheumatoid arthritis compared with that of normal controls [69]. An *in vitro* study also showed that LIGHT dose-dependently induced osteoclast formation from both human peripheral blood mononuclear cells and murine RAW 264.7 macrophage precursors, which was not inhibited by OPG or RANK-Fc but significantly decreased by the soluble decoy receptor for LIGHT, or DcR3 [70]. Although the molecular mechanisms underlying LIGHT-mediated osteoclastogenesis are unclear, the association of LTβR and HVEM with TRAF2 or TRAF5 may play a critical role [71].

3.6. TGFβ

TGFβ is a multifunctional growth factor abundant in the bone, which is produced mainly by osteoblasts, fibroblasts, and osteoclasts. TGFβ is embedded in the bone matrix and released during bone resorption, and it is thought to act as a coupling factor between bone resorption and bone formation [72]. Previous studies on the effects of TGFβ on osteoclast formation showed controversial results with both stimulation and inhibition being reported, which may be attributed to the different culture methods and TGFβ concentrations used in the studies [73–75]. In a co-culture of bone cells, high concentrations of TGFβ were found to inhibit osteoclast formation by suppressing RANKL and increasing OPG expression by osteoblasts [74]. However, Itonaga *et al.* reported that in the presence of M-CSF, TGFβ induced osteoclast formation from human mononuclear precursors and murine RAW 264.7 macrophages, and this induction was not inhibited by OPG, suggesting that TGFβ induced osteoclastogenesis via a RANKL-independent mechanism [76]. Similar to TNF-α-induced osteoclasts, TGFβ-induced osteoclasts are small and form small lacunar resorption pits.

3.7. APRIL, BAFF, NGF, IGF-I, and IGF-II

In 2011, Hemingway *et al.* identified five cytokines including a proliferation-inducing ligand (APRIL), B cell-activating factor (BAFF), nerve growth factor (NGF), insulin-like growth factor (IGF)-I, and IGF-II, which can substitute for RANKL to induce osteoclastogenesis [77]. APRIL and BAFF are two TNF superfamily molecules that have been associated with pathological bone resorption in multiple myeloma [78–80]. In a previous study, $TRAP^+/VNR^+$ MNCs expressing F-actin rings were generated in APRIL-treated and BAFF-treated cultures; however, these osteoclasts resulted in significantly less resorption compared with those formed in cultures treated with RANKL [77]. This finding suggests that not all the osteoclast-like cells formed in these cultures were functional.

NGF is a neuropeptide primarily involved in the regulation of growth, proliferation, and neuronal survival. The local administration of NGF is known to enhance bone remodeling and may be particularly important in promoting bone formation in fracture healing [81–83]. An *in vitro* study showed that the addition of NGF to monocyte cultures induced the formation of TRAP-positive MNCs, which produced approximately five-fold more resorption than that produced by APRIL or BAFF addition [77]. NGF in bone tissue is mainly expressed by osteoblast lineage cells, and direct neurite-osteoclast cell communication through adrenergic receptors has been reported; thus, NGF could act as a neurogenic coupling factor between osteoblasts and osteoclasts [84,85].

IGF-I and IGF-II are among the most abundant growth factors present in bone tissue. The stimulatory effects on osteoblast proliferation and matrix synthesis by IGF-I and IGF-II have been well

documented; however, their roles in osteoclastogenesis remain unclear. IGF-I may affect osteoclast formation through the regulation of RANKL expression by osteoblast/stromal cells [86]. In addition, *in vitro* studies demonstrated that IGF I and IGF-II can promote osteoclast differentiation and activation in the presence of additional growth factors [87,88]. Hemingway et al. reported that both IGF-I and IGF-II could directly support the formation of mature resorbing osteoclasts from monocyte precursors with a resorption ability that is much less than that of osteoclasts induced by RANKL or LIGHT [77]. Given that IGF-I and IGF-II receptors are expressed by resorbing osteoclasts, IGF-I and IGF-II stored in the bone matrix are released by osteoclasts and can potentially act on nearby osteoclasts to increase bone remodeling through a positive feedback loop.

4. Conclusions

A greater understanding of the control mechanisms of osteoclastogenesis has been achieved since the identification of the RANKL/RANK/OPG pathway, the activation of which is required for the physiological osteoclastic differentiation of hematopoietic precursors. However, under conditions with pathological bone resorption, such as rheumatoid arthritis, postmenopausal osteoporosis, and periodontitis, the RANKL-independent pathways of osteoclast formation are likely to be implicated. Although osteoclastogenic factors are present at low concentrations in normal tissues and are less potent than RANKL in inducing osteoclast formation, they are highly produced by local immune cells or tumor cells and can function as osteoclastogenesis modulators in osteolytic diseases. Considering the clinical applications of denosumab [89], a novel targeted RANKL inhibitor, in the treatment of osteoporosis and bone cancer, RANKL-independent osteoclast formation could represent an alternative therapeutic target for treating osteolytic bone loss.

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