Published in final edited form as:
J Bone Miner Res. 2013 April ; 28(4): 711–722. doi:10.1002/jbmr.1885.

Advances in osteoclast biology reveal potential new drug targets and new roles for osteoclasts

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Abstract

Osteoclasts are multinucleated myeloid lineage cells formed in response to M-CSF and RANKL by fusion of bone marrow-derived precursors that circulate in the blood and are attracted to sites of bone resorption in response to factors, such as sphingosine-1 phosphate signaling. Major advances in understanding of the molecular mechanisms regulating osteoclast functions have been made in the past 20 years mainly from mouse and human genetic studies. These have revealed that osteoclasts express and respond to pro- and anti-inflammatory cytokines. Some of these cytokine activate NF-κB and NFATc1 signaling to induce osteoclast formation and activity and also regulate communication with neighboring cells through signaling proteins, including ephrins and semaphorins. Osteoclasts also positively and negatively regulate immune responses and osteoblastic bone formation. These advances have led to development of new inhibitors of bone resorption that are in clinical use or in clinical trials; and more should follow, based on these advances. This paper reviews current understanding of how bone resorption is regulated both positively and negatively in normal and pathologic states.

Keywords

Osteoclast; bone resorption; osteoclastogenic cytokine; apoptosis; osteoblast; NF-κB; RANKL; NFATc1

Introduction

Osteoclastic bone resorption coupled with bone formation helps to maintain skeletal integrity and mineral homeostasis, but it also is responsible for localized or generalized bone loss, which can weaken the skeleton and increase the risk of fracture (1, 2). Osteoclasts (OCs) are specialized myeloid lineage cells that function most effectively as multinucleated cells. Osteoclast precursors (OCPs) arise in the bone marrow and, like other leukocytes, circulate in the blood from where they are attracted to bone remodeling units (BRUs) in response to chemokines, cytokines and other factors elaborated at sites either destined for or already undergoing resorption (3, 4).

Bone loss and fractures can be prevented by anti-resorptive drugs (5). However, atypical femoral fractures have been linked to long-term treatment of osteoporosis with bisphosphonates, the most widely prescribed anti-resorptive drugs (6), and osteonecrosis of jaw bones has been reported in some patients given intravenous bisphosphonates (6) or Denosumab, a human monoclonal antibody to receptor activator of NF-κB ligand

Disclosure. The author declares no conflict of interest.

(RANKL), the major osteoclastogenic cytokine (7). These adverse events have resulted in increasing reluctance of patients with osteoporosis to take bisphosphonates long-term and point to the need for new anti-resorptive therapies designed to target osteoclasts.

Morphologic studies of human bone biopsy samples in the 1960s, 70s and 80s revealed how bone remodeling maintains skeletal integrity and is disrupted in common diseases (2). Mouse and human genetic studies since the 1980s have identified numerous signaling pathways regulating OC formation and function (1, 8) as well as some unanticipated functions of osteoclasts to regulate osteoblastic and other cells (9). These studies should lead to development of new drugs targeted specifically to these signaling pathways, which are reviewed in this Review.

(i) Regulation of osteoclast formation

(a) Cytokines and transcription factors—OCs form by cytoplasmic, but not nuclear, fusion of precursors derived from myeloid progenitor cells that give rise also to macrophages and dendritic cells (9, 10). The progenitor cells differentiate into osteoclast precursors (OCPs) in response to macrophage-colony stimulating factor (M-CSF) and RANKL, but expression of several transcription factors, including PU.1, and a heterodimeric complex of microphthalmia-associated transcription factor (MITF) and Tfe3 (11), is required earlier in myeloid progenitors to promote their differentiation (12). For example, PU.1 and MITF promote expression of c-fms (the M-CSF receptor) (12), and mice deficient in these develop osteopetrosis (13), a condition characterized by radiographically dense long bones in which trabecular bone formed during endochondral ossification is not removed due to failure of OC formation or activity.

M-CSF is an essential osteoclastogenic cytokine expressed by osteoblast lineage cells. It promotes expression of RANK on OCP cell membranes, leaving the RANK+ cells primed to respond to RANKL (14). It mediates OCP proliferation, differentiation and survival through extracellular signal-regulated kinase (ERK)/growth factor receptor bound protein 2 (Grb-2) and Akt/phosphoinositide 3-kinase (PI3K) signaling (14). M-CSF also signals through a complex comprised of phosphorylated DNAX-activating protein 12 (DAP12) and the non-receptor tyrosine kinase, Syk (14), which is also activated by co-stimulatory signaling. Thus, M-CSF has important roles in all aspects of osteoclastic bone resorption.

(b) RANKL/RANK and downstream signaling—RANKL is a member of the tumor necrosis factor (TNF) superfamily of proteins and is expressed by osteoblast lineage and other cell types, including T and B lymphocytes (15). In the absence of essential molecules that signal downstream of RANK, such as NF-κB and c-Fos, increased numbers of CD11b+ OCPs accumulate (as in RANK-/- mice (16) and NF-κBp50/p52 double knockout (dKO) mice (17)) or precursors are diverted down the macrophage lineage (as in c-Fos-/- mice) (18). Thus, treatment of patients with anti-RANKL drugs could lead to accumulation of OCPs, which could differentiate into OCs after therapy is discontinued. Such a mechanism could perhaps account for the increase in serum bone resorption markers reported in some clinical trials following cessation of Denosumab (19), but the precise mechanism remains to be determined.

During endochondral ossification, growth plate chondrocytes express RANKL, RANK and osteoprotegerin (OPG) (20). $1,25,(OH)_2D_3$, bone morphogenetic protein 2 (BMP2) and Wnt/β-catenin signaling (20-22) regulate RANKL expression by these cells to attract OCPs to growth plates and facilitate rapid removal of newly formed bone, thus preventing osteopetrosis (13). Hypertrophic chondrocytes are the major source of RANKL during endochondral ossification, not osteoblastic cells, as had been thought previously, and

osteocytes in bone are the major source of RANKL during bone remodeling and in response to mechanical stress (23-25).

Unlike c-fms, RANK lacks intrinsic kinase activity to phosphorylate and activate downstream signaling molecules. Rank recruits TRAFs, particularly TRAFs 1, 2, 3, 5 and 6, which function as adapter proteins to recruit protein kinases (26, 27). Of these, only TRAF 6 appears to have essential functions in osteoclastic cells (26, 27). RANK/TRAF6 signaling activates four main pathways to induce OC formation: (NF-κB; c-Jun N-terminal kinase (JNK)/activator protein-1 (AP-1); c-myc; and calcineurin/NFATc1); and two others to mediate osteoclast activation (Src and MKK6/p38/MITF) and survival (Src and ERK), (26-28), which will be discussed later. TRAF 2 positively and TRAF3 negatively regulates OC formation (see below).

NF-κB is a family of transcription factors, which includes the signaling proteins, RelA, p50, Rel B, p52 and c-Rel, that induce expression of genes involved in normal and aberrant immune responses, cell division, differentiation and movement, and carcinogenesis through canonical and non-canonical pathways (29, 30). Requirement of NF-κB in osteoclastogenesis was discovered unexpectedly when NF-κB p50/p52 dKO mice failed to thrive at weaning due to absence of tooth eruption associated with osteopetrosis because the mice did not form OCs (31, 32). The defect in OC formation in NF-κB dKO OCPs is rescued by either c-Fos or NFATc1 retroviral constructs (33), indicating that they act downstream of NF-κB.

NF-κB appears to cooperate with NFATc2 (which is not required for OC formation) to induce expression of NFATc1, with NF-κB p50 and p65 being recruited to the NFATc1 promoter within 1 hour of treatment of OCPs with RANKL, resulting in transient auto-amplification of NFATc1 expression (34). It remains to be determined what the precise role of this transient auto-amplification of NFATc1 is and which genes are activated in response to it, but it may be down-regulation of constitutively active repressors of RANK signaling (35) (see later).

In the canonical pathway, RANKL binding to RANK leads quickly to formation of a complex on the intracellular cytoplasmic portion of RANK that contains a number of proteins, including TRAF6 and TAK1 (TGFβ-activated kinase-1), which induce activation of IKK$\tilde{\kappa}$ (also called NF-κB essential modulator (NEMO)). This leads to phosphorylation and subsequent activation of IKKβ, which phosphorylates IκB, an inhibitory NF-κB family protein that holds p65 and p50 heterodimers in an inactive state in the cytoplasm. IκB consequently undergoes rapid degradation by the 26S proteasome resulting in release of p65 and p50 and their translocation to nuclei where they prevent apoptosis of OCPs, thus allowing them to continue differentiating (36, 37). Mice with deletion of IKKβ in OC lineage cells have impaired OC formation and osteopetrosis (36). Interestingly, a constitutively active IKKβ (IKKβ-SS/EE) expressed in OCPs induces their differentiation into OCs in the absence of RANK or RANKL treatment (38), further emphasizing the importance of NF-κB signaling in OC formation.

Activation of the non-canonical pathway occurs more slowly, typically within 3-4 hours of RANKL treatment through the activity of NF-κB-inducing kinase (NIK). This leads to processing of the precursor molecule, p100, to p52, which typically signals in association with RelB. In unstimulated cells, newly synthesized NIK gets bound by TRAF3, leading to NIK proteasomal degradation (39). NF-κB activation by RANKL recruits a TRAF/cIAP E3 ligase complex to RANK leading to cIAP1/2 activation by TRAF2. This targets TRAF3 for ubiquitination and degradation allowing NIK to accumulate and activate IKKα, which

phosphorylates p100 and leads to its proteasomal processing to p52 and subsequent nuclear translocation of RelB/p52 heterodimers (29, 36).

NIK, IKKα and p100 do not have a required function for basal OC formation (29, 36). However, they do appear to have regulatory roles in RANKL- or TNF-enhanced OC formation in pathologic states. For example, intra-tibial injection of murine melanoma cells caused localized osteolysis in WT, but not in NIK-/- mice (36). In contrast, TNF-transgenic (TNF-Tg) mice crossed with p100-/- mice developed earlier and more severe joint inflammation and bone erosion than TNF-Tg mice, indicating that p100 limits TNF-induced OC formation and inflammation (40). These studies suggest that strategies to inhibit NIK or increase p100 could reduce bone loss in inflammatory and metastatic bone disease. Pre-clinical studies with a peptide that inhibits NF-κB signaling by binding to NEMO reduced osteoclastogenesis and bone erosion in inflammatory arthritis (41). However, to date there have been no clinical studies reported with this agent.

(c) NFATc1 and Co-Stimulatory Signaling—NFAT transcription factors regulate immune responses as well as cardiovascular, muscle, and neuronal and other cell functions (42). NFATc1 is activated in OCPs by being dephosphorylated by calcineurin, a phosphatase, which is activated by calcium-calmodulin signaling (34, 43) mediated by phospholipase Cγ (PLCγ), which plays a key role by releasing calcium from stores within the cytoplasm (34, 44). NFATc1 is also activated through PLCγ by co-stimulatory signaling, which is initiated by ligand binding to immunoglobulin-like receptors, such as TREM-2 (triggering receptor expressed in myeloid cells-2) and OSCAR (osteoclast-associated receptor) (34). These receptors are expressed on OCPs and they recruit adaptor molecules, such as Fc receptor common γ subunit (FcRγ) and DAP12 leading to phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) within these adaptor proteins and activation of downstream signaling. NFATc1 is involved in all aspects of osteoclast formation and activation and seems like a prime target for anti-osteoclast therapy. Indeed the immunosuppressive agents and calcineurin/NFATc1 inhibitors, FK506 and cyclosporine-A, prevent bone loss in inflammatory arthritis because they reduce the inflammation and associated bone resorption (45). However, NFATc1 also positively regulates expression of osterix, a key osteoblastogenic transcription factor, and the net effect of these inhibitors in normal mice is bone loss (45).

The ligands for most co-stimulatory receptors remain unidentified, but OSCAR is activated in OCPs by portions of exposed collagen fibers in resorption lacunae (46). Activation of NFATc1 through RANK and OSCAR in turn induces increased OSCAR expression on OCPs in a positive feedback loop (47). Expression of OSCAR and RANKL is increased in the synovium of joints of patients with RA (48). Thus, co-stimulatory signaling likely enhances OC formation and bone resorption mediated by RANKL through this and other mechanisms in rheumatoid arthritis (RA).

(d) T and B Lymphocytes and Osteoimmunology—The recognition that RANKL is expressed not only by osteoblastic cells, but also by T and B cells and synoviocytes in inflammatory bone diseases and that RANK signaling is involved in immune responses, lymph node formation and B cell maturation (27, 44) spawned the new field of osteoimmunology (49). However, the contributions of T and B cells to the increased osteoclastogenesis in inflammatory bone disease are complex. For example, although T helper (Th) cells express RANKL, T regulatory cells (Tregs) inhibit OC formation through cytotoxic T lymphocyte antigen 4 (50, 51) and production of IL-4 and IL-10 (35) and Th1 cells express INFγ, which inhibits OC formation. Both T cell types are present in inflamed joints of RA patients and the effects of T cells overall appear to be inhibitory (52). Th17 cells are the major subset of RANKL-expressing Th cells in inflamed synovium of RA

patients (53). In inflamed synovium, they (54) and mast cells (55) express IL-17, which induces OC formation mainly by increasing RANKL expression by synovial fibroblasts (52), although one study has reported direct induction of OC formation by IL-17 from human monocytes (56). Th17 cells are also inflammatory and cause increased expression of TNF, IL-1 and IL-6 by synovial fibroblasts, which in turn increase their expression of RANKL (52). Interestingly, adoptive co-transfer of a subset of $CD11b^{-/lo}Ly6C^{hi}$ OCPs with CD4+ T cells from arthritic mice markedly decreased the severity of arthritis in Rag2-/- recipient mice, suggesting that these sub-populations of OCPs and T cells can be anti-inflammatory (57). There are also conflicting data about B cell expression of RANKL (43, 51, 58). Further study will be required to explain how these complex positive and negative functions of immune cells lead overall to increased bone resorption in inflammatory bone disease, but they point to additional mechanisms to limit bone resorption.

T cells have been implicated also in ovariectomy (Ovx)-induced the bone loss in mice, but these findings also are somewhat controversial (15). For example, T cell-deficient nude mice appear to be protected from bone loss after Ovx in some, but not all studies (15). Estrogen inhibits differentiation of Th17 cells, but the role of IL-17 in Ovx-induced bone loss is unclear because there are conflicting findings of the effects of Ovx on bone loss in IL-17 receptor-deficient mice (15). Estrogen also increases Treg numbers; but it also regulates T cell production of TNF by inhibiting expression of IL-7, which promotes OC formation. In contrast, estrogen deficiency expands the pool of TNF-producing T cells, while transgenic mice over-expressing Tregs are protected against Ovx-induced bone loss (15, 59). Some of the discrepancies among these studies may be due to differences in the strains of mice used, in study design, or to the positive effects of one set of T cells being negated by those of another set, as appears to occur in RA.

A further twist to the role of T cells in Ovx-induced bone loss is that OCs can function as antigen presenting cells and thus can behave as immune cells to activate T cells (10). For example, they express Fc receptor common γ subunit (FcRγ), major histocompatibility complex (MHC) molecules, CD40, and CD80 (60), just like dendritic cells (60) and express a wide range of cytokines. Therefore, OCs could participate in Ovx-induced T cell proliferation and activation along with or in place of dendritic cells. This positive role could be negated, however, because OCs can also inhibit T cell proliferation and suppress T cell production of TNF and IFNγ (61). These positive and negative effects of immune cells, cytokines, estrogen, and estrogen deficiency emphasize the fact that even in pathologic conditions there are mechanisms to limit excessive tissue destruction.

(e) RANKL/RANK mutations cause osteopetrosis in humans—Kindreds with RANKL or RANK deletion mutations have marked osteopetrosis and appear to lack palpable lymph nodes (62, 63). However, obvious immunodeficiencies have not been reported in any of them, suggesting that they may have compensatory mechanisms that maintain normal immune responses. Interestingly, mice deficient in RANK specifically in B cells have normal B cell development (64). This may be important for patients being treated with anti-RANKL drugs, like Denosumab, because these findings in mice suggest that they might not interfere with B cell maturation. To date, no significant increase in infections or other signs of impaired immune responses have been reported in patients in clinical trials of Denosumab (19).

Activating mutations in the *RANK* gene are responsible for a number of rare bone diseases, including familial expansile osteolysis, and expansile skeletal hyperphosphatasia (65) in which there is increased localized, rather than generalized OC formation and bone resorption. This focal involvement has similarities to adult Paget's disease, many cases of

which have mutations in genes encoding molecules that signal downstream of RANK (66), but it is not known why skeletal involvement is not diffuse in these diseases.

(ii) Recruitment of osteoclast precursors to remodeling sites

OCPs circulate in the blood from where they are attracted to BRUs (4). Morphologic studies have identified a thin canopy of connective tissue covering resorption sites and small vessels pass through it into BRUs, bringing cells and nutrients (67). Resorbing OCs pass the products of resorption through their cytoplasm within lysosomes into the extracellular space within BRUs and from there these products enter efferent vessels and the circulation (68).

(a) Chemokine attraction of OCPS—Stroma-derived factor-1 (SDF-1) is a chemokine, which mediates leukocyte migration, and its local concentration can determine the location of cells. For example, in TNF-mediated inflammatory arthritis, TNF inhibits SDF-1 production by marrow cells leading to mobilization of OCPs from the marrow (69) and to increased numbers of them in the bloodstream (70) from where they can be attracted to inflamed joints by high SDF-1 concentrations.

OCPs are attracted to the bloodstream by sphingosine-1 phosphate (S1P), a bioactive sphingolipid with numerous functions, including regulation of cell motility, proliferation and survival (71). S1P is secreted from red blood cells and platelets resulting in higher concentrations in serum than in the marrow. OCPs express S1P receptors (S1PRs) 1 and 2, signaling through which tends to have opposite effects. For example, S1PR1 signaling chemo-attracts OCPs from the marrow to the blood, while S1PR2 signaling appears to chemo-repel them back to the marrow (71). FTY720, an S1PR1, but not R2 agonist, prevented ovariectomy-induced bone resorption in mice, while more OCPs were attached to bone surfaces in S1PR1-/- mice, associated with increased OC formation and bone resorption (71).

These findings raise the possibility that low serum S1P levels or mutations in S1P receptors could be associated with increased bone resorption and osteoporosis in some patients. S1P levels are increased in the synovial fluid of patients with rheumatoid arthritis, and FTY720 significantly reduced joint destruction and inflammation in mice with inflammatory arthritis (71). Collectively, these findings suggest that drugs that can promote OCP migration to the bloodstream or prevent them from leaving it could reduce bone loss in common bone diseases. However, S1P has complex roles in inflammation and cytokine expression with positive and negative regulatory functions, which may make use of agonists challenging (71).

(iii) Regulation of osteoclast activation

Inactive, quiescent bone surfaces are covered by bone lining cells, the cytoplasm of which must be retracted exposing matrix proteins such as vitronectin before bone resorption can begin (72). Lining cells communicate with osteocytes within the bone and with osteoblastic cells in the marrow through their extensive dendritic processes, which could mediate signaling that leads to the cytoplasmic retraction (73), but the mechanisms involved remain unknown. OCs form a tight sealing zone with the exposed bone surface using actin filament-rich podosomes that are surrounded by adhesion, signaling, and adaptor molecules, protein tyrosine kinases, and actin-associated molecules, such as vinculin, talin and paxillin, which are involved in multiple facets of cell movement in normal and pathologic conditions (74).

(a) Integrin- and cytokine-mediated OC attachment to matrix and activation—OCs attach to the bone surface mainly through the vitronectin receptor, $\alpha V\beta 3$ integrin (75), in association with kindlin-3 (76), a member of a family of proteins that are recruited to

integrin adhesion sites in platelets and leucocytes. Kindlin-3 activates β3 integrin during the early events in OC activation. Humans with kindlin-3 gene mutations and kindlin-3-/- mice are osteopetrotic because their OCs do not form podosomes (77).

Integrin binding to bone matrix proteins, such as osteopontin and bone sialoprotein, activates αVβ3 and recruits Src tyrosine kinase by standard outside-in signaling (78). Src phosphorylates Syk, which recruits the co-stimulatory ITAM protein, Dap12, and Slp76, and these function as an adaptor protein complex for Vav3, a guanine nucleotide exchange protein that activates the small GTPase Rho family members, Cdc42 and Rac (78). αVβ3 and c-fms interact physically, and by inside-out signaling through αVβ3 cause a structural change in αVβ3, which is required for its activation (78). Interestingly, β3 integrin -/- mice have only mild osteopetrosis, perhaps because other integrins can substitute for it in OCs (78). RANK is also physically linked to αVβ3 by Src, forming a complex, which activates Syk, Slp-76, Vav3, and Rac, and in this respect is similar to αVβ3/Src interaction (78). Because the OC is the only cell that forms a ruffled membrane to resorb bone, it is possible that components of this activation step could be targets of future anti-resorptive drugs.

(b) Osteoclast ruffled border formation and bone resorption—Inside sealing zones formed by podosomes, the OC surface area facing the bone is increased significantly by the ruffled border membrane, which is formed by accumulation of cytoplasmic lysosomal secretory vesicle fusion with the cytoplasmic membrane and requires expression of Src (79). Vesicle fusion is promoted by the small GTPase Rab7 and synaptotagmin VII, a calcium-sensing molecule, and by proteins involved in autophagy and extracellular protein secretion, including Atg5, Atg7, Atg4B, and LC3 (80, 81). Accordingly, synaptotagmin $VII^{-/-}$ osteoclasts have severely defective ruffled border formation (82). H^+ and Cl^- ions pass through the ruffled border and form HCl to dissolve the mineral component of bone, and proteolytic enzymes, particularly cathepsin K, are secreted to degrade the matrix (75). H^+ ions are secreted through the V-type H^+ ATP6i proton pump complex, whereas Cl^- ions pass through a chloride channel encoded by *ClCN7*.

Src phosphorylates proteins involved in OC activation, including Syk, Pyk2, cortactin, and c-Cbl, which has ubiquitin ligase activity (83). It also mediates RANKL-induced survival signaling in vitro (84), but src-/- OCs have normal survival in vivo (79, 83), perhaps because other Src family members substitute for it. Src is over-expressed in many cancers in which it plays positive roles in proliferation, invasion and metastasis and thus is a therapeutic target in both OCs and tumor cells in metastatic bone disease (83). Small molecular inhibitors of Src have been developed, and of these saracatamib currently is being investigated in metastatic prostate cancer with some promising results as an adjuvant to standard chemotherapy (83). To date no Src inhibitors have been studied in osteoporosis clinical trials.

(c) Osteoclast precursor fusion—High OC nuclear numbers correlate with more aggressive resorption, as is seen in Paget's disease and giant cell tumor of bone. OCP fusion is regulated by dendritic cell-specific transmembrane protein (DC-STAMP), Atp6v0d2, OC-STAMP, and CD9 (85). Atp6v0d2 is a subunit of V-ATPase, a component of the V-type H^+ ATP6i proton pump complex, which also is involved in OCP-mediated inhibition of osteoblast precursor formation (86), one of a number of unanticipated roles for OCPs and OCs in the regulation of bone formation (9).

NFATc1 and c-Fos play major roles in OCP fusion and activation and in conjunction with MITF and PU.1 variously regulate expression of a number of genes, including, DC-STAMP, OC-STAMP, OSCAR, tartrate-resistant acid phosphatase, cathepsin K, V-ATPase-d2 and the calcitonin receptor (12, 87, 88). Vitamin E (α-tocopherol) also regulates OCP fusion by

inducing DC-STAMP expression through activation of mitogen-activated protein kinase 14 and MITF (89). Importantly, administration of α-tocopherol to rats at doses taken by some humans as dietary supplements increased OC numbers in the animals and reduced bone mass, suggesting that excessive Vitamin E consumption could adversely affect bone health (89).

(d) Osteoclast-rich osteopetrosis in humans due to defects in genes regulating OC functions—Most cases osteopetrosis in humans result from mutations in genes involved in matrix demineralization and dissolution. These include: T-cell immune regulator 1 (TCIRG1), which encodes the α3 subunit of the H^+ ATPase involved in proton generation; carbonic anhydrase II, which catalyzes hydration of CO_2 to H_2CO_3 to provide a source of H^+; the chloride channel 7 (ClCN7), through which chloride ions pass; Pleckstrin homology domain-containing family M member 1, which encodes a vesicle-associated protein linked to small GTPase signaling; and cathepsin K (13, 90, 91). Humans with cathepsin K mutations develop an osteochondrodysplasia, called pycnodysostosis, the features of which include osteopetrosis, dwarfism and defects of the craniofacial bones. In contrast, cathepsin K-/- mice have osteopetrosis, but no other bone defects (5), suggesting a more complex role for the gene in humans and raising the possibility that cathepsin K inhibitors could have adverse effects on non-stable fracture healing, in which some features of endochondral ossification are recapitulated.

Drug candidates have been developed to inhibit the proton pump and cathepsin K in OCs, but most of these have failed because of adverse effects in clinical trials. However, odanacatib, a cathepsin K inhibitor, showed promise in phase 2 clinical trials for the treatment of postmenopausal osteoporosis (92, 93). It inhibits bone resorption, but does not appear to inhibit bone formation to the same extent as other anti-resorptive drugs by mechanisms that remain to be explained, but which could involve osteoclast-stimulated bone formation (94).

(v) Negative regulation of osteoclast formation

(a) Osteoprotegerin (OPG) is the major negative regulator of bone resorption (26, 27). OPG is secreted by osteoblastic and other cell types and binds to RANKL as a decoy receptor thus preventing its interaction with RANK. Most of the factors, including growth factors and cytokines, that up-regulate expression of RANKL also increase OPG expression, typically to a lesser extent, and thus the net effect is increased bone resorption (95). Other cytokines, such as, IL-4 and IL-13, which are produced by Th2 lymphocytes, enhance OPG and inhibit RANKL expression in osteoblastic cells and therefore suppress osteoclastogenesis (35). As will be seen later, there are multiple additional mechanisms whereby cytokine signaling can limit OC formation and activation.

Interestingly, some of the signaling that regulates osteoblast formation also regulates OPG expression. For example, Wnt/β-catenin canonical signaling, which is required for osteoblast formation, positively regulates OPG expression in osteoblasts (96), while Wnt 5a-induced non-canonical signaling in osteoblasts positively regulates OC formation through receptor tyrosine kinase-like orphan receptor (Ror) proteins expressed in OCPs (97). This is a potentially important target for therapeutic intervention because a soluble form of Ror2 acted as a decoy receptor of Wnt5a and prevented bone destruction in mouse models of arthritis (97). In addition, Jagged1/Notch1 signaling, which regulates MSC numbers and osteoblast differentiation, alters the OPG/RANKL expression ratio in stromal cells to inhibit OC formation (98).

These findings are consistent with a model in which immature osteoblastic cells interact with osteoclastic cells through Wnt 5a near the cutting edges of BRUs to promote OC

formation, while more mature osteoblastic cells express OPG through Wnt canonical and Notch signaling to inhibit osteoclastogenesis and promote OC apoptosis through OPG near the reversal site in BRUs where osteoblastic cells can differentiate into matrix-forming osteoblasts.

Loss-of-function mutations of *TNFRSF11B,* the gene encoding OPG, occur in humans and account for most cases of juvenile Paget's disease (99). The mutation results in OPG deficiency and unopposed RANKL-induced bone resorption with osteoporosis, long bone and vertebral deformities during childhood; the phenotype is similar to that seen in OPG-/- mice (100).

(b) RANKL-mediated inhibition—Some inhibitory mechanisms are actually mediated by RANKL signaling itself within OCPs. For example, activation of c-Fos also induces expression by OCPs of interferon-β (INF-β), which binds to the INF-α/β receptor on OCPs leading to inhibition of OCP differentiation by post-transcriptional reduction in c-Fos protein (101). Mice deficient in INF-β or in a component of the INF-α/β receptor have severe osteopenia due to increased OC formation and activity, emphasizing how important this mechanism is (101). INF-β is used to prevent disease flares in multiple sclerosis with significant efficacy. Although some studies have reported beneficial effects on bone mineral density, patient numbers have been low, and this warrants further study.

(c) Ephrin/Eph and semaphorin/neuropilin/plexin signaling (and osteoclast regulation of osteoblasts)—Ephrins and semaphorins are widely expressed molecules that control communication between cells, including neurons and axons during nervous system development, and endothelial cells and lymphocytes during immune responses and angiogenesis (102-104). These molecules are also expressed in bone and regulate interactions between and functions of osteoclastic and osteoblastic cells (105-107). For example, RANKL-induced c-Fos/NFATc1 signaling increases expression of the ligand, ephrinB2, on the surface of OCPs. Reverse signaling through this ligand when it binds directly to the Eph4 receptor on osteoblastic cells down-regulates c-Fos and NFATc1 expression to limit OC formation; forward signaling through Eph4 stimulates osteoblast precursor differentiation by inhibiting the small GTPase, RhoA (105). Decreased ephrinA1 and EphA1 expression was identified in bones of patients with metastatic of prostate cancer (108) and giant cell tumor of bone (109) by mRNA microarray analysis implicating reduced Ephrin-Eph signaling in osteolytic bone disease.

Semaphorins (Semas) are expressed widely as secreted and membrane-associated proteins; the latter signal through plexins and the former through neuropilins (Nrps). Sema3A is secreted by osteoblasts and OCs, and its binding to Nrp1 on OCPs inhibits RANKL-induced OC formation by inhibiting ITAM and RhoA signaling (110). It also binds to Nrp1 on mesenchymal precursors to stimulate osteoblast and inhibit adipocyte differentiation through canonical Wnt/β-catenin signaling. Accordingly, Sema3A and Nrp1-/- mice have osteoporosis with reduced bone formation. Importantly, treatment of mice with Sema3A inhibited bone resorption and increased bone formation in normal mice and enhanced bone regeneration in a mouse cortical bone defect model (110). Sema4D is membrane-bound and binds to plexin1 on target cells. It is expressed by osteoclasts and inhibits osteoblast differentiation and function by activating RhoA-ROCK, which inhibits insulin-like growth factor-1 signaling (111). Consistent with these findings, sema4D-/- and plexin1-/- mice have high bone mass due to increased bone formation (111). Sema6d is membrane-bound, and by binding to plexin-A1 on OCPs induces OC formation through Trem-2/DAP12/PLCγ-induced NFAT activation as well as podosome formation through Rac-GTP generation in OCs. Accordingly, plexin1-/- mice have marked osteopetrosis, but normal osteoblast function (107). Sema7A is expressed by osteoclasts and osteoblasts and induces monocyte

production of free radicals, IL-6, and TNF, suggesting that it may play a role in inflammatory bone disease (107). It promotes OC formation and OCP fusion as well as osteoblast migration in vitro, but full understanding of its role in bone awaits generation of Sem7a-/- mice.

These studies and other studies (9) have revealed complex interactions between osteoclastic and osteoblast cells and that OCs and OCPs have important positive and negative regulatory roles in normal and pathologic bone remodeling that had not been anticipated a decade ago. Further studies will be required to determine exactly where and when these interactions take place in remodeling units to initiate and subsequently stop both resorption and formation, but they suggest that drugs could be developed to enhance or restrict some of these interactions to increase bone mass.

(d) Constitutively-expressed transcriptional repressors of RANK signaling— There are also constitutive mechanisms to inhibit basal OC formation. For example, in the absence of RANKL stimulation, Bcl6 is recruited to the NFATc1, cathepsin K, and DC-STAMP promoters to inhibit osteoclastogenesis. In contrast, RANKL induces removal of Bcl6 from these promoters and its replacement by NFATc1, to mediate osteoclastogenesis (112). Bcl6 is one of a group of constitutively-expressed transcriptional repressors in OCPs, including interferon regulatory factor-8 (IRF-8), Eos, and v-maf musculoaponeurotic fibrosarcoma oncogene family protein B (35). Their expression is down-regulated by RANK signaling. They are also direct targets of B lymphocyte–induced maturation protein 1 (Blimp-1), deletion of which in OCs results in osteopetrosis due to up-regulation of Bcl6 and impaired osteoclastogenesis (112). In contrast, Bcl6-/- mice have increased OC formation and severe osteoporosis. Thus, RANKL/RANK activation of NFATc1 in OCPs not only promotes osteoclastogenesis directly, but it also facilitates it indirectly by repressing expression of negative regulators.

(d) Negative regulation by pro-inflammatory cytokines—TNF- and RANKL-induced translocation of NF-κB RelA/p50 complexes to nuclei induces increased expression of p100, which is efficiently processed to p52 by NIK in response to RANKL, but not to TNF (40). Thus, in conditions, such as RA in which TNF expression is increased, excess p100 protein becomes available to bind to RelB and/or RelA and in this way p100 can inhibit NF-κB signaling and limit OC formation (40). This accumulation of p100 is accompanied by an increase in TRAF3 in the cells and this in part explains why TNF only modestly increases OC formation in WT mice and does not induce OC formation when given to either RANKL-/- or RANK-/- mice (40). However, when these RANKL-/- or RANK-/- mice are also deficient in p100, TNF induces OC formation in vivo (40). TNF also limits OC formation in RA by inducing expression of IRF-8 and the Notch-induced DNA binding molecule, recombinant recognition sequence binding protein at the Jκ site (35) in OCPs and by promoting secretion by OCPS and OCs of TSG-6 (TNF-stimulated gene 6), which acts synergistically with OPG to inhibit OC activity by an autocrine mechanism (113). Although T cells express RANKL in rheumatoid joints to potentially increase bone resorption, they also secrete INFγ, which degrades TRAF6 in OCPs and in this way T cells can also limit OC formation (114).

During immune responses anti-inflammatory cytokines, including IL-10, are elaborated to help resolve inflammation, and some of these can inhibit OC formation. For example, IL-10 inhibits expression of c-Fos, c-Jun, TREM-2 and NFATc1 in OCPs (35). IL-4 inhibits bone resorption by several mechanisms in addition to its effects on OPG expression. For example, it suppresses expression of RANK, NFATc1 and c-Fos as well as NF-κB, MAPK, and calcium signaling during osteoclastogenesis. In combination with GM-CSF, IL-4 cleaves c-fms from the surface of OCPs to suppress osteoclastogenesis (35).

During co-stimulatory signaling, ITAM-bearing proteins typically partner with proteins containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) (35), some of which promote and others inhibit immune responses and osteoclastogenesis. For example, Ly49Q promotes osteoclastogenesis by competing with the murine paired Ig-like receptor, PIR-B, for association with the SH2 domain-containing tyrosine phosphatase 1 (SHP-1) (115). In contrast, the human inhibitory immunoglobulin-like receptor, LILRB, and PIR-B recruit SHP-1 to negatively regulate osteoclastogenesis (115). An ITIM is present in the cytoplasmic tail of DC-STAMP whose surface expression declines during osteoclastogenesis. DC-STAMP is phosphorylated on its tyrosine residues and physically interacts with SHP-1 and CD16, which is an ITAM-associated protein (116) and by binding to this complex, DC-STAMP might actually limit OC formation (116). Finally, toll-like receptor signaling is activated in monocytes by microbial products at sites of inflammation leading to enhanced immune responses, but in OCPs it leads to inhibition of their differentiation by causing the cells to shed the extracellular domain of c-fms (35). These advances in understanding of how bone and immune cells interact could lead to development of new therapies to inhibit both inflammation and osteoclastogenesis, but given the complexity involved, this is likely to be challenging.

(vi) Osteoclast Apoptosis

(a) Promotion of osteoclast apoptosis—One of the earliest reports of OC apoptosis was in response to estrogen and was mediated by increased expression of TGFβ by bone marrow cells (117). Estrogen also induces OC apoptosis by increasing Fas-ligand expression in OCs (118), and estrogen receptor alpha signaling also inhibits expression of genes regulating OC activity, without affecting OCP proliferation or fusion (119). Thus, estrogen can inhibit bone resorption by limiting OC life span and activity. Interestingly, TGFβ appears also to support OC survival directly through TAK1/MEK/AKT-mediated activation of NF-κB in OCs (120). In addition to its expression by marrow stromal cells, TGFβ is also released from bone matrix during resorption and is activated by the acid environment under the ruffled border. Thus, these opposite effects of TGFβ on OC survival may reflect the site and source of its production.

Bisphosphonates also induce OC apoptosis in vitro and in vivo (121), in part by inhibiting the activity of enzymes in the mevalonate pathway (122) and promoting caspase cleavage of Mammalian Sterile 20-like (Mst) kinase 1, which is a pro-apoptotic substrate for the apoptosis effector enzyme, caspase 3 (123). However, some amino-bisphosphonates appear to inhibit bone resorption without inducing OC apoptosis (124), consistent with their differing affinities for the binding sites of target enzymes (125). Despite proven efficacy in many pathologic settings, bisphosphonates have only modest anti-resorptive activity in patients with rheumatoid arthritis (126, 127), which may be related to high TNF levels in their joints and blood and to glucocorticosteroid therapy. For example, TNF attenuates bisphosphonate-induced apoptosis by up-regulating Bcl- X_L expression in OCPs and OCs (128), and glucocorticosteroids can inhibit OC apoptosis (129), although the mechanism remains to be determined. Denosumab (130) and raloxifene (131) induce OC apoptosis, but other anti-resorptive drugs, including calcitonin (68) and the cathepsin K inhibitors, odanacatib and ONO-5334, which are in phase 3 clinical trials, do not (94).

OCPs are recruited continuously to the cutting edges of resorption lacunae to maintain a population of relatively young resorbing OCs at this site, while older OCs undergo apoptosis predominantly in reversal sites of resorption lacunae (132) where high extracellular calcium concentrations resulting from bone resorption (133) and OPG released by osteoblastic cells can induce OC apoptosis. However, OPG can also bind to and inhibit TNF-related apoptosis-induced ligand (TRAIL), which induces OC apoptosis. OPG appears to reduce

apoptosis of human OCs in vitro by inhibiting this mechanism (134), but further studies are required to determine if this mechanism has a functional role in vivo.

(b) Prevention of osteoclast apoptosis—An early effect of RANKL signaling in OCP differentiation is up-regulation of JNK signaling, which rather surprisingly was found to induce apoptosis of NF-κB p65-deficient OCPs by activating Bid and caspase 3 (37). These findings indicate that p65 plays an important role to prevent OCP apoptosis, and other studies in this paper show that p65 is not required for expression of genes that regulate osteoclastogenesis. Enhanced OC survival is an important component of bone resorption and is increased by cytokines, including M-CSF, RANKL, TNF, IL-1, and VEGF-A, which prevent OC apoptosis by up-regulating Rho family small G-protein Ras/Rac1/Erk and PI3 kinase/mTOR/S6K signaling (135). Withdrawal of these cytokines rapidly induces OC apoptosis due to reduced expression of the anti-apoptotic protein, Bcl-2 (136). M-CSF prevents OC apoptosis by a number of mechanisms, including: activating MITF, which increases Bcl-2 expression (135-137); increasing the proteasomal degradation of Bim by c-Cbl, an ubiquitin ligase; and up-regulating expression of Bcl-X_L(138), which inhibits cleavage of procaspase-9; and inhibiting the activity of caspases 3 and 9, which initiate apoptosis. Deletion of Bcl-X_L in OCs resulted in increased OC apoptosis, but surprisingly the mice had increased, rather than decreased bone resorption. This was associated with increased c-Src activity and expression of vitronectin and fibronectin by OCs, resulting in enhanced integrin-mediated activation of the cells (139) and suggesting that Bcl-X_L also inhibits OC resorptive activity. Bim is a pro-apoptotic Bcl-2 family member whose expression is down-regulated by IL-3 signaling through the Raf/Erk and/or PI3K/mTOR pathways. Bim-/- mice have decreased OC activity, despite increased OC survival (136). Thus, although in general, enhanced OC survival is associated with increased bone resorption and vice versa, these two activities can be uncoupled.

Acknowledgments

The author acknowledges grant support from the NIH (NIAMS), grant number AR43510-17 and thanks Nora Plumeri for secretarial assistance.

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