CHAPTER 7

RANKL/RANK as Key Factors for Osteoclast Development and Bone Loss in Arthropathies

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Abstract

Osteoporosis or rheumatoid arthritis are bone diseases affecting hundreds of millions of people worldwide and thus pose a tremendous burden to health care. Ground-breaking discoveries made in basic science over the last decade shed light on the molecular mechanisms of bone metabolism and bone turnover. Thereby, it became possible over the past years to devise new and promising strategies for treating such diseases. In particular, three molecules, the receptor activator of NF-κB (RANK), its ligand RANKL and the decoy receptor of RANKL, osteoprotegerin (OPG), have been a major focus of scientists and pharmaceutical companies alike, since experiments using mice in which these genes have been inactivated unanimously established their pivotal role as central regulators of osteoclast function. RANK(L) signaling not only activates a variety of downstream signaling pathways required for osteoclast development, but crosstalk with other signaling pathways also fine-tunes bone homeostasis both in normal physiology and disease. Consequently, novel drugs specifically targeting RANK-RANKL and their signaling pathways in osteoclasts are expected to revolutionize the treatment of various bone diseases, such as cancer metastases, osteoporosis, or arthropathies.

Introduction

For all its rigidity, bone is constantly remodeled throughout adult life. Bone remodeling involves resorption by osteoclasts and the synthesis of new bone matrix by osteoblasts. If anything disturbs this intricate balance between resorption and synthesis of bone, skeletal abnormalities, such as osteoporosis or osteopetrosis, develop and become a severe burden to patients.[1-4] Osteoporosis is a disease characterized by a global decline in bone mineral density and structural deterioration of bone tissue, subsequently leading to bone fragility and an increased susceptibility to fractures especially of the hip, spine and wrist. It is estimated that in the US 10 million people already have osteoporosis, while 34 million are predicted to be osteopenic, dramatically increasing their risk for osteoporosis. Osteoporosis manifests predominantly in older people around 50 and older, with a strong gender preference for women (80% of those affected by osteoporosis) and accounts for more than 1.5 million fractures annually. By contrast, osteopetrosis, or abnormally increased bone density, is way less prevalent in our population and occurs mainly as a result of rare hereditary disorders.[5]

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The landmark discoveries of three molecules, the receptor activator of NF-κB (RANK),[6] its ligand RANKL[6-9] and the decoy receptor for RANKL, osteoprotegerin (OPG),[10-12] had a huge impact on how we think about osteoclast function and as a consequence of osteoporosis and other bone diseases. It needs to be stressed that although various calciotropic hormones and cytokines, such as PTHrP, Vitamin D3, IL-1b, or TNF-a, have all been shown to affect osteoclastogenesis at distinct stages of development,[2] only RANK(L) has proven to be absolutely required for osteoclast development in vivo as evidenced by the complete absence of osteoclasts in RANKL and RANK knockout mice.[13-15]

Osteoblasts express RANKL and binding to its cognate receptor RANK on osteoclastic precursors is crucial for the development and activation of mature osteoclasts from hematopoietic progenitor cells.[2] OPG also binds RANKL, thereby preventing RANKL binding to RANK and thus inhibits RANK signaling and bone turnover by osteoclasts.[2] Since in patients suffering from bone diseases such as osteoporosis, metastases to bone, or rheumatoid arthritis all show an increased activity of osteoclasts, it appears that the RANKL-RANK-OPG axis is the most relevant therapeutic target for osteoclast-regulated bone diseases. Here we discuss the importance of the RANKL-RANK-OPG axis in bone metabolism and review various signaling pathways known to be activated by RANK signaling in osteoclasts. This knowledge is of utmost importance for the development of novel therapeutic approaches to treat diseases of the bone that affect millions of people.

Basic Characteristics of the RANKL-RANK-OPG Axis

RANKL

RANKL (also known as osteoprotegerin ligand OPGL, osteoclast differentiation factor ODF, TNFSF11, TRANCE, CD254) was independently cloned by four groups.[6-9] RANKL is a member of the tumor necrosis factor (ligand) superfamily of cytokines. Structurally, TNF family cytokines are Type II transmembrane proteins, each containing a membrane-anchoring domain, a connecting stalk and a receptor-binding ectodomain. Human and murine RANKL encode glycoproteins of 317 and 316 amino acids, respectively and are highly similar. The crystal structure of the extracellular, biologically active domain of murine RANKL has recently been solved and revealed that functional RANKL protein self-assembles into stable, noncovalently associated trimers, which is in line with all TNF family cytokines examined to date.[16,17] RANKL is most highly expressed in skeletal and lymphoid tissues that are active in mediating the immune response, but RANKL mRNA expression can also be detected in keratinocytes of the skin, mammary gland heart, skeletal muscle, lung, spleen, thymus, stomach, placenta, thyroid gland and brain.[6,8,9,18-21] Importantly, not only is RANKL biologically active in its membrane-bound form (40-45 kDa), but also in a soluble form (31 kDa) which is derived either from proteolytical cleavage or alternative splicing. Analysis of Rankl mRNA expression revealed two alternative transcripts, encoding a membrane-bound isoform of 287 amino acids with a shorter intracellular domain and a soluble version of 199 amino acids lacking both the transmembrane and intracellular domain of canonical RANKL.[22] Biochemical studies showed direct interactions of alternatively spliced RANKL isoforms and homo- or heterotrimerization of isoforms. Although all isoforms are functional in in vitro osteoclastogenesis assays, there seems to be an inhibitory effect of the shortest isoform when co-expressed in cells with the other isoforms in that it suppresses the formation of multinucleated osteoclasts by inhibiting the fusion of preosteoclasts.[23,24] These findings leave open the possibility that regulated expression of RANKL isoforms might contribute to the control of osteoclast development. Apart from transcriptional regulation, posttranslational modification of membrane-bound RANKL by proteolytic cleavage also seems to occur. Both members of the disintegrin and metalloproteases domain (ADAM) family and matrix metalloproteases (MMPs) have been shown to contribute to ectodomain shedding of RANKL.[25,26] However, the biological effects of proteolytic cleavage remain somewhat contradictory. In a murine model of prostate cancer, it has been shown that increased MMP-7 expression by osteoclasts at the tumor-bone interface can convert membrane-bound RANKL to soluble RANKL,

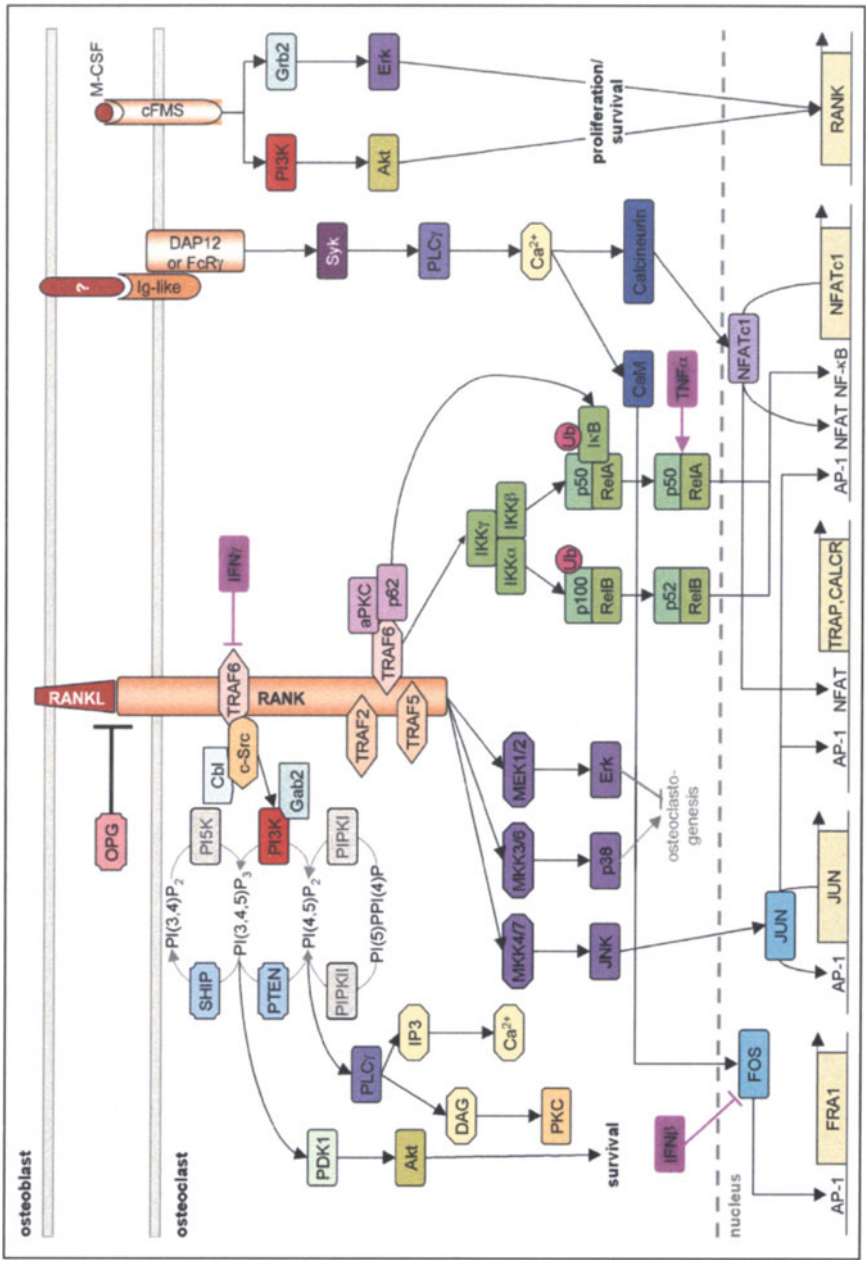

Figure 1. Legend viewed on following page.

Figure 1, viewed on previous page. Schematic diagram of RANK(L) signaling pathways that control lineage commitment and activation of osteoclasts. RANK stimulation activates various different signaling pathways, such as the MAPK, PI3K and NF-κB pathway, to control osteoclastogenesis. TRAFs and other adaptors, such as Gab2, bound to the cytoplasmic tail are key mediators of RANK(L) signaling. Both the canonical (IKKβ) and alternative (NIK-IKKα) NF-κB pathway are activated by RANK stimulation and contribute to transcriptional regulation of target genes. MAPK pathway leads to activation of AP-1 family member which are also crucial for osteoclastogenesis. PI3K signaling links RANK stimulation to activation of Akt/PKB and Ca^{2+} signaling via PLCγ, which is crucial for calcineurin-mediated NFATc1 activation. Besides RANK activation, other costimulatory signals from the osteoblasts are also required for osteoclast differentiation. M-CSF–cFMS signaling is crucial for the proliferation and survival of osteoclastic precursor cells. The ITAM-bearing adaptors DAP12 or FcRγ are essential for RANK-mediated Ca^{2+} induction and osteoclastogenesis via PLCγ, calcineurin and NFATc1 induction, respectively. There is also positive and negative crosstalk with other signaling pathways and molecules, such as TNF-α, which acts positively on NF-κB, or interferons which negatively influence RANK signaling by promoting accelerated TRAF6 degradation in the case of IFN-γ or by interfering with RANKL-induced cFos expression in the case of IFN-β.[4]

thereby promoting osteoclast activation and thus osteolysis.[27] Another recent study reported that MMP-14 plays an important role in RANKL shedding both in vitro and in vivo.[28] However, this study suggested that membrane-bound RANKL induces osteoclastogenesis more efficiently than soluble RANKL and MMP-14-mediated ectodomain shedding of RANKL would rather negatively regulate osteoclastogenesis. This observation is corroborated by the osteoporotic phenotype of MMP-14-deficient mice which the authors contributed to increased osteoclast numbers due to the increase in membrane-bound RANKL.[28] Thus it seems that the biological outcome—be it positive or negative—of RANKL ectodomain shedding by various MMP or ADAM family members on osteoclastogenesis strongly depends on the biological context and certainly needs to be addressed further in the future.

RANK

The receptor for RANKL is RANK (receptor activator of NF-κB, also known as TNFRSF11A, OFE, ODFR, TRANCE-R, ODAR, CD265), a member of the TNF receptor superfamily. Human and mouse RANK cDNA encode Type I transmembrane glycoproteins of 616 and 625 amino acids, respectively. They are comprised of a 29 and 30 amino acid signal peptide, an extracellular domain of 183 and 184 amino acids, a transmembrane domain of 21 and 20 amino acids and a large cytoplasmic domain of 383 and 391 amino acids, respectively. Since TNF receptors commonly assemble into trimeric complexes on the cell surface prior to ligand binding, as shown for FAS, TNFR1, or TNFR2, it is inferred that RANK trimerization is a prerequisite for RANKL binding and signal transmission.[29-32] RANK mRNA is expressed with highest levels in dendritic cells, bone, skeletal muscle, thymus, liver, colon, small intestine and adrenal gland.[6,33,34] Moreover, RANK protein can be detected on the surface of dendritic cells,[6,18,34] $CD4^+$ and $CD8^+$ T-cells,[35] Langerhans cells (A. Leibbrandt, J. Penninger, unpublished)[21] and on mammary epithelial cells where expression is regulated throughout pregnancy, with highest levels at day P15.5 of pregnancy.[20,36]

OPG

The third molecule in the axis is OPG (also called TNFRSF11B, osteoprotegerin, OCIF, TR1, or FDRC1), which was initially isolated as a secreted TNF receptor family member regulating bone.[12] Human and murine OPG are 85% identical and both are synthesized as 401 amino acid precursor proteins, of which a 21 amino acid signal peptide is cleaved off to give rise to the mature peptide. Since OPG lacks a hydrophobic transmembrane-spanning domain, OPG is secreted as a soluble protein. OPG is synthesized as a monomer (55-62 kDa) and is finally secreted as a homodimeric glycoprotein of ~110 kDa.[10-12,37,38] OPG mRNA can be detected in the brain, liver, lung, heart, kidney, skeletal muscle, skin, intestines, calvaria, stomach, testis and placenta.[10-12,37,38]

RANKL-RANK-OPG Interactions and Bone Remodeling

OPG was the first protein of the triad to be identified as a factor that would potentially inhibit osteoclastogenesis.[11,12] As expected for an inhibitory factor of osteoclastogenesis, transgenic mice overexpressing OPG or mice which were treated with recombinant OPG both exhibited a marked increase in bone density and osteopetrosis, respectively.[12] Not surprisingly, mice in which OPG has been inactivated by targeted deletion developed early-onset osteoporosis.[39,40] These studies not only established for the first time the critical requirement for osteoprotegerin in the maintenance of postnatal bone mass, but also suggested that OPG might act on another TNF-related factor that would do the opposite, namely stimulating osteoclast development.This postulated positive factor was soon to be found in RANKL by expression cloning: RANKL specifically bound to OPG, enhanced differentiation of bone marrow cells into osteoclasts in vitro and activated mature osteoclasts to resorb bone both in vitro and in vivo.[8,9] In a breakthrough study, the genetic inactivation of RANKL in mice was shown to lead to osteopetrotic mice with defects in tooth eruption as a result from the complete absence of osteoclasts and unanimously proofed the essential function of RANKL in osteoclastogenesis.[14] The circle was later closed by showing that the receptor for RANKL, RANK, is also essential for osteoclast differentiation and activation induced by RANKL.[33,41] Most importantly, $RANK^{-/-}$ mice phenocopy $RANKL^{-/-}$ mice, i.e., they are osteopetrotic, have a defect in tooth eruption and lack osteoclasts.[13,15] In summary, these findings unambiguously established the essential role of RANKL-RANK interactions in positively regulating osteoclastogenesis, which is counteracted and balanced by OPG in vivo by binding to RANKL thereby serving as a decoy receptor for RANKL. Most importantly, the functions of the RANKL-RANK-OPG axis in bone remodeling—as established primarily by different mouse models—have also direct relevance to human bone diseases. For example, duplications in the signal peptide of RANK have been linked to four families with Familial expansile osteolysis or Paget disease of the bone, rare autosomal dominant bone dysplasia characterized by focal areas of increased bone remodeling. Both insertion mutations (a 18 base pair and a 27 base pair tandem duplication in exon 1 of RANK) result in reduced expression levels and increased constitutive RANK-mediated NF-κB signaling in vitro.[42] Moreover, in patients suffering from expansile skeletal hyperphosphatasia, a familial metabolic bone disease characterized by expanding hyperostotic long bones, early onset deafness, premature tooth loss and episodic hypercalcemia, an insertion in exon 1 of RANK was identified as the cause of the disease.[43,44] In patients with an osteoclast-poor form of autosomal recessive osteopetrosis (ARO), various mutations in RANKL have been identified as the cause of the disease.[45] Although these ARO patients did not respond to hematopoietic stem cell transplantation, they could form functional osteoclasts from monocyte-lineage cells upon exogenous RANKL application, suggesting that these individuals could profit from a RANKL therapy.[45] Finally, several mutations in OPG, frequently affecting the ligand binding domain, have been ascribed to Juvenile Paget disease, an autosomal recessive osteopathy characterized by rapidly remodeling woven bone, osteopenia, fractures and progressive skeletal deformity.[44,46,47]

RANK(L) Signaling Pathways

Binding of RANKL to RANK results in the activation of signaling cascades that control lineage commitment and activation of osteoclasts. Given the pleiotropic effects of RANKL and RANK in vivo, it is obviously of key interest to study RANK(L) signaling pathways to identify pathways specific for osteoclast development and to understand their crosstalk to other receptor systems (Fig. 1).

Adaptor Proteins

RANK, as member of the TNFR family, does not have any kinase motif in its cytoplasmic tail and thus needs associated proteins to transduce signals. TNFR associated factors, or TRAFs, bind to the cytoplasmic tail of TNF receptors and are important mediators of TNFR signaling.[48] The cytoplasmic domain of RANK contains binding sites for several TRAFs that cluster in distinct cytoplasmic domains of RANK: a region from amino acids 235-358 as well as amino acids 359-531

bind to TRAF6, whereas the region spanning amino acids 532-625 contains multiple binding sites for TRAFs 2, 5 and 6.[49-51] These TRAF binding domains were shown to be functionally important for NF-κB and c-Jun NH2-terminal kinase (JNK) activities in response to RANK stimulation. When the membrane-proximal TRAF6 interaction domain is deleted, RANK-mediated NF-κB signaling is completely inhibited while residual JNK activation is still possible, suggesting that interactions with TRAFs are necessary for NF-κB activation but not essential for activation of the JNK pathway.[49,50,52,53] The key importance of TRAF6 in functional RANK signaling has been substantiated in Traf6 mutant mice that exhibit bone phenotypes similar to Rankl$^{-/-}$ and Rank$^{-/-}$ mice due to a partial block in osteoclastogenesis and defective activation of mature osteoclasts.[54-57] However, there is some controversy with respect to the impact of TRAF6 on osteoclastogenesis, since one Traf6$^{-/-}$ strain still has TRAP^{+} osteoclasts,[54] while another independently generated Traf6$^{-/-}$ strain is devoid of osteoclasts.[55] Since cell-permeable peptides with the TRAF6-binding motif inhibit TRAF6 signaling and can arrest osteoclastogenesis in vitro,[58] the idea that TRAF6 is indeed essential for osteoclast differentiation is strongly supported despite the controversial phenotypes of Traf6$^{-/-}$ strains.[59] Compared to TRAF6, the contributions of TRAF2 and TRAF5 to osteoclastogenesis are minor. For instance, fetal liver derived Traf2-deficient progenitor cells show only slightly (20%) reduced multinuclear osteoclasts and activation of NF-κB and JNK by RANKL was comparable.[60] TRAF5 deficient cells also show mildly reduced osteoclastogenesis and again NF-κB and JNK activation was not apparently affected upon RANK stimulation.[60,61] Thus, TRAF6 seems to be the main adaptor molecule to link RANK signaling to NF-κB for the activation of mature osteoclasts, but other TRAFs (and possibly other molecules) seem to at least partially compensate for Traf6-deficiency during osteoclast development.

Recently, another molecular adapter was found to be important for RANK signaling. Grb2 associated binder 2 (Gab2) associates with RANK and mediates RANK-induced NF-κB, Akt and JNK activation. Genetic inactivation of Gab2 in mice results in osteopetrosis and decreased bone resorption due to defective osteoclast differentiation.[62] Importantly, the contribution of Gab2 to osteoclastogenesis is relevant not only to mice but also to humans, since siRNA-mediated inactivation of Gab2 in human peripheral blood derived progenitor cells likewise prohibited osteoclastogenesis.[62] Various receptors important for osteoclastogenesis such as integrins, c-Fms, FcRγ, RANK, or G-protein coupled receptors can act through Gab2 and Gabs can bind to a variety of signaling molecules such as PLCγ, the p85 subunit of PI3K, Grb2, or SHP2. Thus, Gab2 (and possibly the Gab2 family members Gab1 and Gab3) might integrate stimulation of various receptors in osteoclasts.

NF-κB Signaling

As indicated above, RANK stimulation triggers the activation of NF-κB, dimeric transcription factors belonging to the Rel family.[63] Mammals express five Rel (NF-κB) proteins that belong to two classes. Proteins of the first class comprise RelA (p65), c-Rel and RelB, are synthesized as mature products and do not require further proteolytic processing.[63] Conversely, proteins of the second class, NF-κB1 and NF-κB2, are first synthesized as large precursors, p105 and p100, respectively and proteolytically processed to produce the mature p50 and p52 NF-κB proteins.[63] NF-κB dimers containing RelA or c-Rel are retained in the cytoplasm through association with IκB inhibitor proteins. Upon stimulation, IκB becomes rapidly phosphorylated (and thereby marked for ubiquitin-mediated degradation) by the IKK complex, which consists of two catalytic subunits, IKKα and IKKβ and the regulatory subunit, IKKγ (NEMO).[63] In general, activation of p50:RelA and p50:c-Rel dimers is referred to as the canonical pathway which depends mainly on IKKβ activity. In an alternative pathway, the IKKα subunit is required for processing of the NF-κB2/p100 protein complexed with RelB in order to release p52:RelB dimers from inhibition required for nuclear translocation.[63] Importantly, expression of both NF-κB p50 and p52 proteins is required for osteoclast formation, as p50/p52 double knockout mice develop osteopetrosis due to a defect in osteoclast differentiation.[64,65] In vitro, the NF-κB activating kinases IKKα and IKKβ have both been implicated in RANKL-RANK signaling and osteoclastogenesis. However, the

analysis of IKKα- and IKKβ-deficient mice revealed that IKKβ, but not IKKα, is essential for osteoclastogenesis in vivo.[66] Thus, it seems that RANKL-RANK signaling exerts its downstream effects on osteoclastogenesis mainly through IKKβ and the classical NF-κB activation pathway. This is further supported by the observation that mice in which the upstream activating kinase of IKKα, NIK, has been disrupted are not osteopetrotic.[67] Taken together, RANKL-RANK seems to primarily signal through IKKβ to activate the classical NF-κB pathway to control osteoclastogenesis in vivo.

MAPK Signaling

Mitogen-activated protein kinases (MAPK) are a family of Ser/Thr protein kinases consisting of extracellular signal-regulated kinases (Erk1/2), p38-MAPKs (α/β/γ/δ), c-Jun N-terminal kinases (JNK1,2,3) and 'big' MAPKs (Erk5,7,8).[68] Several MAPKs are activated downstream of RANK and help to integrate RANK activation to a cellular response. As for p38-MAPKs, it has been shown that by inhibiting p38α and p38β with the pharmacological blocker SB203580 RANKL-induced osteoclast differentiation was abrogated.[69] These results indicate that p38 MAPK activation plays an important role in RANKL-induced osteoclast differentiation of precursor bone marrow cells.[69] In addition to p38-MAPKs, JNKs and their direct upstream kinase MKK7 have also been shown to be involved in osteoclastogenesis in cell culture. JNK1, but not JNK2, is specifically activated by RANKL and required for osteoclastogenesis in vitro.[70] Moreover, c-Jun, a component of the dimeric AP-1 transcription factors, is activated by JNK and essential for efficient osteoclastogenesis.[70] Other AP-1 family members and JNK targets, namely JunB, c-Fos and Fra, but not JunD, have also genetically been shown to control osteoclastogenesis, thereby strongly supporting the critical function of MAPK-JNK signaling in RANKL-mediated osteoclast formation.[70-73] In line with these findings, overexpression of a dominant-negative form of MKK7 impaired RANKL-mediated JNK activation and consequently RANKL-mediated osteoclast formation.[72] Finally, ERK p42/p44 phosphorylation was reported to be increased by RANK stimulation but was dispensable for RANKL-mediated osteoclast differentiation in vitro.[69] Interestingly, another report suggested that specific MEK inhibitors markedly enhanced RANKL-mediated osteoclastogenesis in vitro and moreover implied a crosstalk between the p38-MAPK and ERK pathways during RANKL-mediated osteoclastogenesis.[74] The possible involvement of other ERKs, ERK5, ERK7 and ERK8, in RANK(L) signaling and osteoclastogenesis remains to be tested.

Ca^{2+}/Calcineurin/NFAT Signaling

In search for further factors regulated by RANKL during osteoclast differentiation, the transcription factor NFATc1 was identified.[75] NFATc1 expression was shown to be dependent on the NF-κB and c-Fos pathways and ectopic expression of NFATc1 resulted in efficient osteoclast differentiation in vitro even in the absence of RANKL in the culture system.[75] Likewise, NFATc1-deficient ES cells could not be differentiated into osteoclasts.[75] Since NFAT family members require the Ca^{2+}/calmodulin-dependent Ser/Thr phosphatase calcineurin for activation and nuclear translocation, it was also intriguing to test whether RANKL-mediated NFATc1 activation would depend on Ca^{2+} signaling and calcineurin, respectively. Both chelation of Ca^{2+} ions in the medium or specific inhibition of calcineurin resulted in a block of osteoclast differentiation in the presence of RANKL in vitro.[76] Moreover, RANKL induced Ca^{2+} oscillations are required NFATc1 activation. Collectively, these results argue that RANKL-mediated Ca^{2+} oscillation is critical for the terminal differentiation of osteoclasts, whereby NFATc1 would be constantly activated through the Ca^{2+}-dependent calcineurin pathway.[75-77]

Src, PKB and PI3K Kinase Activation

The tyrosine kinase c-Src has been implicated in osteoclast function by gene targeting experiments since mice deficient in c-Src develop osteopetrosis.[78] Subsequently, c-Src was connected with RANKL signaling by showing that c-Src is pivotal for RANKL-induced activation of the anti-apoptotic Ser/Thr kinase Akt/PKB in osteoclasts and osteoclast survival.[79] c-Src and TRAF6 directly interact with each other and with RANK following receptor engagement. TRAF6 seems

to enhance the kinase activity of c-Src leading to tyrosine phosphorylation of downstream signaling molecules such as c-Cbl.[79] Moreover, PKB activation also requires PI3-kinase activity and PI3-kinase activity was also shown to be important in osteoclastogenesis using specific inhibitors in vitro.[80] The role of PDK1—which phosphorylates and activates PKB—in RANK(L) signaling has not been reported yet. PIP3 production by PI3-kinase is negatively regulated by two key 3′ and 5′ lipid phosphatases, namely PTEN and SHIP1, which remove a phosphate group from PIP3.[81] While overexpression of PTEN suppressed RANKL-mediated osteoclast differentiation and overexpression of dominant-negative PTEN constitutively induced osteoclast differentiation in vitro,[82] there is still no genetic evidence that PTEN plays an important role in osteoclastogenesis in vivo. By contrast, the importance of SHIP1 in osteoclastogenesis has been shown both in vitro and in vivo. In vitro osteoclast cultures demonstrated increased osteoclastogenesis in SHIP1-deficient cells due to hypersensitivity to RANKL and M-CSF stimulation. Most importantly, SHIP1$^{-/-}$ mice exhibit osteoporosis.[83] Thus, SHIP1 seems to be an essential negative regulator for RANK(L)-induced osteoclast differentiation.

Phospholipase C (PLC) is another phosphatidylinositol-related enzyme that catalyzes the production of the second messengers IP_3 and diacylglycerol (DAG).[84] RANKL acts through PLC to release Ca^{2+} from intracellular stores. Subsequently, the increased Ca^{2+} levels activate the essential transcription factor NFATc1 via calcineurin. Blockade of PLCγ enzymatic activity impairs early osteoclast development and function.[85] Importantly, PLCγ2$^{-/-}$ mice are osteopetrotic and PLCγ2, independent of PLCγ1, is required for RANKL-induced osteoclastogenesis by differentially regulating NFATc1, AP-1 and NF-κB.[85] Interestingly, PLCγ2 can associate with the regulatory adapter molecule GAB2 in osteoclasts, is required for GAB2 phosphorylation and appears to modulate GAB2 recruitment to RANK.[76,85] The activation of PLCγ by RANK requires Syk and ITAM-bearing molecules such as DAP12 and FcRγ.[76,86] Mice lacking DAP12 and FcRγ are severely osteopetrotic and DAP12$^{-/-}$FcRγ$^{-/-}$ double mutant bone marrow cells fail to differentiate into multinucleated osteoclasts in vitro and exhibit impaired phosphorylation of the Syk tyrosine kinase.[87] Moreover, Syk$^{-/-}$ progenitors are similarly defective in osteoclast development and bone resorption.[87] These data indicate that recruitment of Syk to phosphorylated ITAMs is critical for osteoclastogenesis.[87] Mechanistically, FcRγ associates with PIR-A and OSCAR, whereas DAP12 associates with TREM-2 and SIRPβ1 and these receptors via DAP12 and FcRγ then mediate costimulatory signals from osteoblasts, leading to enhanced Ca^{2+} signaling and NFATc1 activation in cooperation with RANK signaling.[76,86] Whether osteoblasts are indeed essential for activation of TREM-2 and SIRPβ1 is, however, not yet known.

Protein Kinase C (PKC) Signaling

Besides the calcineurin-NFATc1 pathway, protein kinase C (PKC) proteins are also activated by Ca^{2+}. Recently, mutations in the atypical PKC (aPKC) scaffolding protein p62 have been shown to be the cause of the 5q35-linked Paget's disease of bone, a genetic disorder characterized by aberrant osteoclastic activity.[88] P62, like TRAF6, becomes upregulated during RANKL-induced osteoclastogenesis and genetic inactivation of p62 in mice leads to impaired osteoclastogenesis in vitro and in vivo, as well as inhibition of IKK activation and NF-κB nuclear translocation.[89] Moreover, RANK signaling induces the formation of a ternary complex involving TRAF6, p62 and atypical PKCs. These observations demonstrate that aPKC/p62 signaling is important during osteoclastogenesis and bone remodeling. As for the aPKCs, λ/ιPKC might well be the key aPKC required for osteoclastogenesis, since PKCζ$^{-/-}$ bone marrow-derived macrophages (BMDMs) do not have osteoclastogenic defects.[89] However, in vivo confirmation has to wait until conditional knockout mice will be available to circumvent the early embryonic lethality of PKCζ mutant mice.[89]

Modulators of RANKL-Mediated Osteoclastogenesis

Although RANK(L) signaling is essential for osteoclastogenesis, crosstalks with other signaling molecules fine-tune this differentiation pathway. For example, RANKL is also expressed on activated T-cells, but T-cells also secrete a factor that negatively influences RANK signaling. This

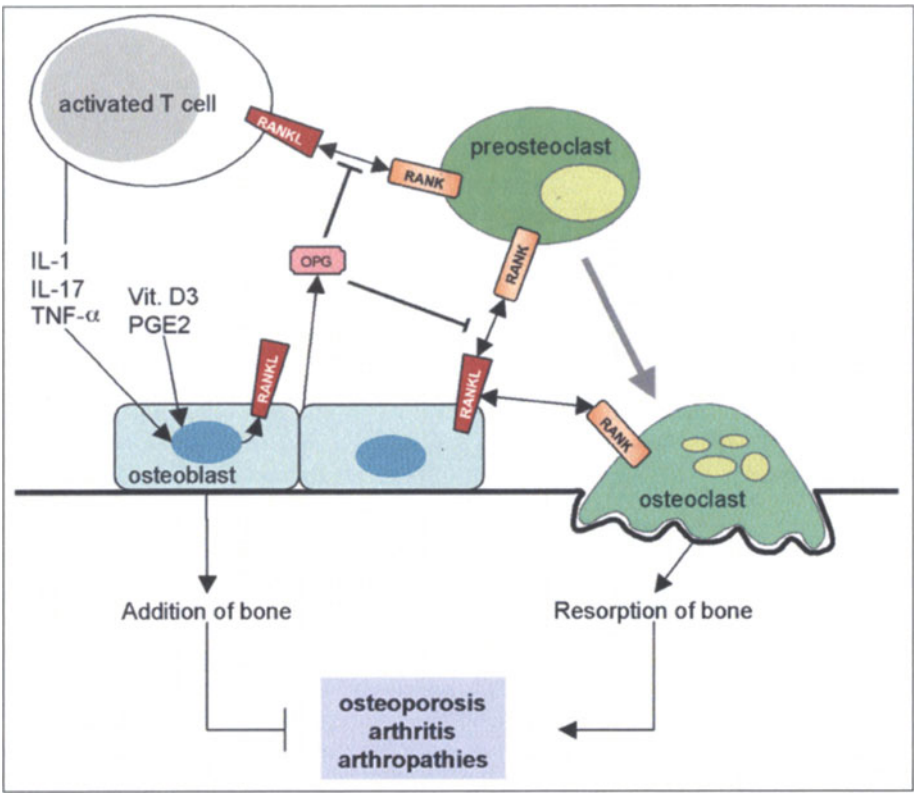

Figure 2. Activated T-cells produce inflammatory cytokines (IL-1, TNF-α, etc.) which together with calciotropic factors (Vitamin D3, PGE2, etc.) stimulate RANKL expression in osteoblasts (OBs). Activated T-cells—which directly express RANKL—and OBs induce OC differentiation from progenitors via RANKL-RANK signaling, which results in bone resorption by mature osteoclasts (OCs). The soluble decoy receptor for RANKL, OPG, blocks both pathways. Inhibition of RANKL via OPG might be useful to treat osteoporosis, crippling in arthritis, or osteopenic disorders such as Paget's disease.[2]

T-cell-derived negative factor was identified as IFN-γ.[90] IFN-γ promotes accelerated degradation of TRAF6 via the ubiquitin-proteasome pathway resulting in strong inhibition of RANKL-induced NF-κB and JNK activation.[90] Moreover, RANKL also induces IFN-β in osteoclast precursor cells and IFN-β inhibits osteoclast differentiation by interfering with the RANKL-induced expression of c-Fos, a transcription factor essential for the formation of osteoclasts.[91] This observation would suggest an autoregulatory loop in which RANKL-induced c-Fos induces its own inhibitor IFN-β. In line with these observations, IFN-$\beta^{-/-}$ mice exhibit severe osteopenia accompanied by enhanced osteoclastogenesis.[91] Sex hormones are tightly linked to bone disease. Postmenopausal women, for instance, lose estrogen production in their ovaries resulting in loss of bone mass and old-age osteoporosis. In fact, sex hormones such as estrogens and androgens suppress RANKL-induced osteoclast differentiation by down-regulating the JNK-c-Jun pathway.[92] Moreover, estrogens and androgens can control expression of the decoy receptor OPG, thereby shifting the balance of RANK(L) signaling.[92] Indeed, OPG treatment can revert increased osteoclast numbers and bone loss after ovarectomy in female rats.[12] Whether JNK-c-Jun regulation is an additional pathway for sex hormone regulated bone metabolisms in vivo needs to be demonstrated by genetic rescue experiments. Recently, the mechanism by which estrogen acts to prevent osteoporotic

bone loss has been further elucidated. When estrogen receptor α (ERα) was specifically deleted in differentiated osteoclasts in mice, $ER_{\alpha}^{\Delta Ox/\Delta Ox}$ females exhibited trabecular bone loss, similar to the osteoporotic bone phenotype in postmenopausal women.[93] Importantly, estrogen specifically induced apoptosis in trabecular bones of wild type but not $ER_{\alpha}^{\Delta Ox/\Delta Ox}$ mice by upregulation of Fas ligand expression.[93] These results suggest that estrogen regulates the life span of mature osteoclasts via the induction of the Fas/FasL system, thereby providing an explanation for the osteoprotective function of estrogen. Besides hormones, multiple cytokines affect osteoclastogenesis in different ways. For instance, IL-4 abrogates osteoclastogenesis through STAT6-dependent inhibition of NF-κB.[94] TGF family members, TGFβ1 and activin A, but not BMP-2, induce JunB expression and enhance osteoclastogenesis induced by RANKL in vitro.[95] M-CSF, a cytokine which signals through the receptor tyrosine kinase c-FMS, is crucial for the proliferation and survival of osteoclastic precursor cells as well as macrophages.[96] M-CSF mediates its effects mainly by activating ERK through GRB2 and Akt/PKB through PI3K, but also stimulates RANK expression in monocyte-macrophage precursor c-FMS+RANK- cells. M-CSF-mediated RANK induction renders these cells responsive to RANKL and induces RANKL-mediated commitment of late-stage precursor cells into osteoclasts.[76,97] In summary, while RANKL/RANK stimulation is absolutely essential for the induction of osteoclastogenesis, multiple additional receptors and signaling pathways can positively and negatively modulate RANK-mediated bone metabolism. Further analysis of these pathways and their crosstalks might provide novel strategies to control RANK(L) signaling specifically in osteoclasts.

RANK-RANKL as Therapeutic Targets for Arthropathies

Bone-related diseases, such as osteoporosis or rheumatoid arthritis, affect millions of people worldwide and pose a tremendous burden on health care. With the discovery of RANK(L) and the subsequent key experiments, i.e., genetic proof that both RANKL and RANK are absolutely essential for osteoclastogenesis and osteoclast activation in vivo, new doors have been opened for bone research and drug development. Our group was the first to demonstrate that RANKL is the key mediator of osteoclast activation and joint destruction in a rat model of arthritis:[98] inflammatory cells produce RANKL which then trigger local development and activation of osteoclasts, a finding that now has become the basis for osteoimmunology and which was reproduced in multiple laboratories using various model systems (Fig. 2).[2] Although cytokines such as IL-1 and TNF-α have also been proposed as potential therapeutic targets to control bone loss in arthritis, IL-1 and TNF-α alone or in combination are much less potent than RANKL in terms of the induction of active osteoclasts.[99] Thus, controlling RANKL and RANK at the receptor level or RANK(L) signaling seems to be most promising in the treatment of arthropathies. In particular inhibition of RANKL is a rational therapeutic strategy to develop novel drugs that block inappropriately enhanced bone resorption—which in several cases has already been shown effective in vivo, for example by using recombinant OPG,[10,12] fully human anti-RANKL blocking antibodies and RANK-Fc.[100]

Interestingly, in a mouse parathyroid hormone-regulated protein (PTHrP)-mediated bone resorption model, inhibition of RANKL using OPG causes a greater suppression of bone resorption and hyper-calcemia than that by bisphosphonates,[99] the current standard therapy for the treatment of bone loss. Thus, great hope lies in a fully human monoclonal IgG_2 antibody to human RANKL, denosumab (AMG 162), to treat postmenopausal osteoporosis or rheumatoid arthritis (RA). In a randomized, placebo-controlled, dose-ranging Phase 2 study of 412 postmenopausal women with low bone mineral density (BMD), subcutaneous application of denosumab at 3-month or 6-month intervals over a period of 12 months resulted in a sustained decrease in bone turnover and a rapid increase in BMD.[101] In an ongoing study with 227 patients with mild or moderately active RA, RANKL inhibition by denosumab also increased BMD.[102-104] In all cases, denosumab administration was appeared to be well tolerated and at least as good or superior to current standard medication, but further clinical trials are required to substantiate the indicative benefits of RANKL inhibition on suppressing bone destruction in arthritis.

Conclusions

The identification of RANKL, its receptor RANK and the decoy receptor of RANKL, OPG, has been of key importance for our understanding of osteoclast development and activation. By elucidating their exact functions and signaling pathways, it became possible over the years to devise new and promising strategies to treat bone loss in arthropathies. Although there are other potential targets such as kinases, adaptor molecules and transcription factors to interfere with osteoclastogenesis, targeting the extracellular factor RANKL and its cell surface receptor RANK are currently the most promising and advanced strategies to offer treatment to millions of patients suffering from arthropathies in the near future.

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