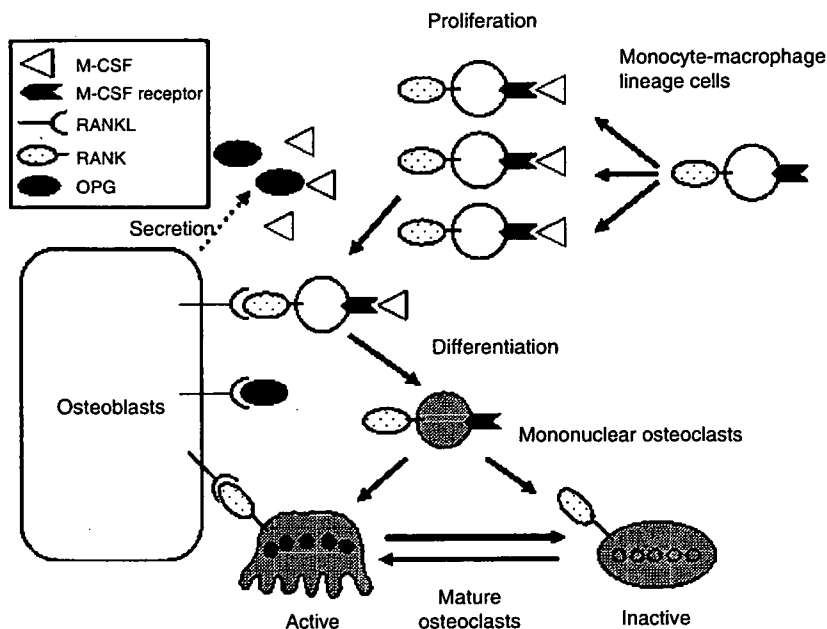


short (2–4 weeks) compared with that of the formation phase (4–6 months), and the lifespan of the osteoclast is much shorter than that of the osteoblast (9, 10). Because the recruitment of osteoclasts on the bone surface initiates the remodeling cycle in the activation phase, the process of osteoclast differentiation and activation determines the activation frequency and regulates the bone homeostasis.

Osteoclasts are specifically differentiated for bone resorption (8, 11). Osteoclasts are highly motile cells, and once attached on the bone surface, the cells become highly polarized and form a tight ring-like zone of adhesion called the sealing zone (11). The cytoplasm of the sealing zone is devoid of cellular organelles, except for numerous actin filaments surrounded by a ring containing the adhesion-related cytoskeletal molecules, vinculin and talin, the structure called 'actin ring.' The space between the cells and the bone matrix constitutes the bone-resorbing compartment. Osteoclasts synthesize several proteolytic enzymes, such as cathepsin K and matrix metalloproteinase 9, which are transported toward the apical side of the cells and secreted into the sealed compartment and play important roles in bone mineral degradation (4, 11, 12). Simultaneously, osteoclasts acidify this compartment by extruding protons via proton pumps (vacuolar type H<sup>+</sup>-adenosine triphosphatase) located on the apical membrane, and the low pH (pH 3–4) of the compartment contributes to demineralization of the bone matrix. The apical membrane of the cells, the so-called ruffled border membrane, develops a characteristic structure with numerous folds (11, 13). The extensive folding of this structure is

probably due to the intense vesicular traffic associated with secretion as well as to the need for increasing the number of proton pumps via an amplification of the apical membrane. Osteoclasts show high levels of tartrate-resistant acid phosphatase (TRAP) activity, which is widely used as a specific histochemical marker of the cells.

Remarkable progress in research has occurred during the last decade on the molecular mechanism of osteoclast differentiation and activation, especially by the finding of the receptor activator of nuclear factor (NF)- $\kappa$ B ligand (RANKL). RANKL is also known as TRANCE [tumor necrosis factor (TNF)-related activation induced cytokine], osteoprotegrin (OPG) ligand, osteoclast differentiation factor (ODF), or TNF superfamily member 11 (14–17). RANKL is a type 2 (carboxy-terminus outside the cell) transmembrane protein that belongs to the TNF family cytokines, and it is produced in various tissues and abundantly expressed in bone and lymphoid tissues. Although RANKL was originally identified as a dendritic cell survival factor produced by activated T cells, subsequent studies have established a critical role of RANKL in the differentiation and activation of osteoclasts (Fig. 1). RANKL assembles into functional trimers, binds to its specific receptor RANK, and induces the differentiation of osteoclasts from their precursor cells (15, 16). RANKL also promotes the bone resorbing activity of osteoclasts and prolongs their survival (18–20). A large body of research has shown that the RANKL–RANK system is not only involved in the physiological bone development by regulating the bone remodeling cycle but also plays an important role in the pathological bone



**Fig. 1.** Schematic representation of the cytokine milieu involved in osteoclast differentiation. Macrophage colony-stimulating factor (M-CSF)-dependent macrophages are differentiated into osteoclasts in the presence of receptor activator of nuclear factor- $\kappa$ B ligand (RANKL), which binds to the specific receptor RANK. Osteoprotegrin (OPG) binds to RANKL and suppresses osteoclast differentiation by competitively inhibiting RANKL–RANK interaction. M-CSF is indispensable for both proliferation of osteoclast progenitors and their differentiation into mature osteoclasts.

destruction. RANK–RANKL signaling therefore can be a potent target of the therapeutic intervention for various bone diseases.

### Physiological role of RANKL–RANK pathways

In the murine coculture system of osteoblasts and spleen cells, osteoblasts stimulated by osteotropic factors such as  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> [ $1\alpha,25(\text{OH})_2\text{D}_3$ ], parathyroid hormone (PTH), TNF- $\alpha$ , and prostaglandins express RANKL and support osteoclast differentiation from their precursors. RANKL binds to its receptor RANK, a type I transmembrane receptor belonging to the TNF receptor superfamily, which is expressed in monocyte-macrophage lineage osteoclast precursor cells as well as in mature osteoclasts and dendritic cells. In the presence of soluble RANKL (sRANKL) and macrophage colony-stimulating factor (M-CSF), spleen cells can differentiate into mature osteoclasts in the absence of osteoblasts. Binding of RANKL to RANK induces various intracellular signaling cascades including NF- $\kappa$ B, c-Jun N-terminus kinase (JNK), p38 mitogen-activated protein tyrosine kinase (MAPK), and NF of activated T cells c1 (NFATc1) activation (5). The adapter molecule TNF receptor-associated factor 6 (TRAF6) is critical in RANK signaling, as its specific binding sites exist in the intracellular domain of RANK (21). The other important component in this signaling system is OPG, a soluble decoy receptor of RANKL named for its effects to protect against bone loss, which also belongs to the TNF receptor superfamily (22, 23). OPG specifically binds to RANKL and inhibits RANKL activity by preventing its binding to RANK (24).

The essential role of RANKL–RANK pathways in osteoclast development was further established by a series of gene ablation experiments (25). The targeted disruption of either RANKL or RANK caused severe osteopetrosis in mice, and very few osteoclasts were observed in the skeletal tissues of these knockout mice (26–29). Conversely, the targeted disruption of OPG caused osteopenia with increased numbers of activated osteoclasts in the bone tissue (30, 31).

Subsequent research has revealed that the bone-resorbing activity and apoptosis of osteoclasts are also regulated by RANKL–RANK pathways. sRANKL treatment not only elongates the survival of mature osteoclasts but also stimulates their bone-resorbing activity both *in vitro* and *in vivo*, while administration of OPG induces apoptotic cell death of mature osteoclasts *in vivo* (18–20). These *in vitro* and *in vivo* observations uncovered that the RANKL/RANK/OPG axis is a critical regulator of normal osteoclast development and therefore essential for healthy bone growth.

### Signal transduction pathways of the RANKL–RANK system

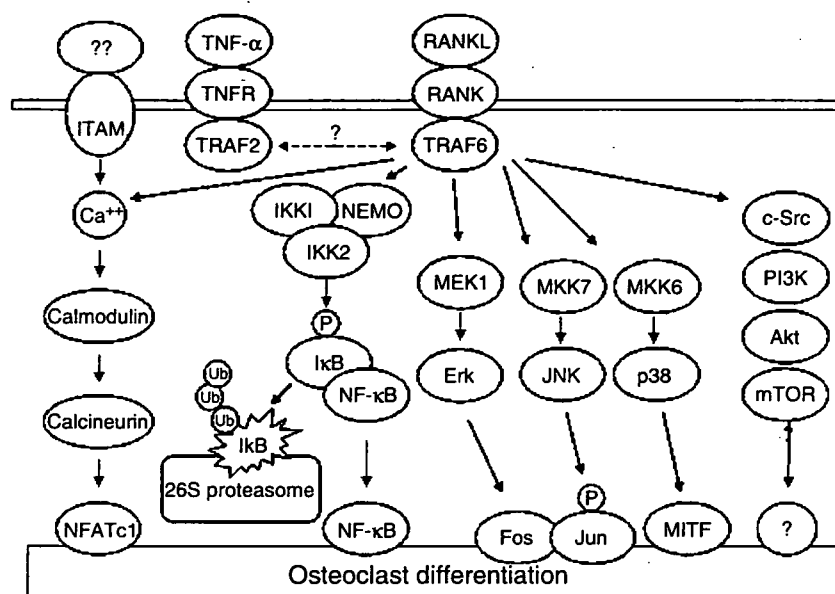
#### TRAF6

There has been remarkable progress in the study of the molecular mechanism implicated in the signal transduction pathways of RANKL/RANK system (Fig. 2). RANK is expressed on the surface of osteoclast progenitor cells and induces intracellular signals leading to osteoclastogenesis upon ligand binding or agonistic anti-RANK antibody stimulation. Like other TNF receptor superfamily members, RANK stimulation induces NF- $\kappa$ B, JNK, and p38 MAPK activation (Fig. 2).

TRAF family proteins are adapter molecules, which mediate intracellular signaling of various cytokine receptors including TNF receptor superfamily and Toll/interleukin-1 receptor (IL-1R) family members (21, 32). Seven TRAF family members have been identified, and several studies showed that RANK recruits TRAF2, TRAF3, TRAF5, and TRAF6 upon binding to its ligand (33–35). TRAF6 has the most divergent TRAF-C domain, and it is the only TRAF that is involved in the signal from members of the Toll/IL-1R family by interacting with the IL-1R-associated kinase (21). TRAF2, TRAF3, and TRAF5 bind to the membrane-distal domain in the cytoplasmic tail of RANK, whereas TRAF6 interacts with the membrane-proximal domain (36). The essential role of TRAF6 in normal bone development was established through experiments utilizing TRAF6-deficient mice, which displayed an osteopetrotic phenotype (37, 38). Very few osteoclasts were found in the skeletal tissues of the knockout mice, and Naito et al. (38) demonstrated that the spleen cells obtained from TRAF6<sup>-/-</sup> mice did not differentiate into osteoclasts, even in the presence of M-CSF and sRANKL. Interestingly, Lomaga et al. (37) found that although their knockout mice also exhibited osteopetrotic phenotypes, there were abundant dysfunctional osteoclasts. These results suggest that TRAF6 is involved not only in the differentiation of osteoclast progenitors but also in the bone-resorbing function of mature osteoclasts.

It was recently reported that there are three putative TRAF6-binding sites and two binding sites for other TRAF family members, including TRAF2, TRAF3, and TRAF5, in the cytoplasmic tail of RANK (36, 39, 40). Using retrovirus vector-mediated gene transduction system, Kadono et al. (39) and Gohda et al. (40) showed that binding sites for TRAF2, TRAF3, and TRAF5 in RANK are dispensable for osteoclast formation and that a single TRAF6-binding site is sufficient to promote osteoclastogenesis.

When M-CSF-dependent bone marrow macrophages (M-BMM $\phi$ ) were stimulated with sRANKL, rapid activation



**Fig. 2. Signaling events implicated in osteoclast differentiation.** When receptor activator of nuclear factor (NF)- $\kappa$ B ligand (RANKL) binds to RANK, multiple signaling pathways can be activated, including NF- $\kappa$ B, extracellular regulated kinase (Erk), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein tyrosine kinase, and phosphatidylinositol 3-kinase (PI3K)/Akt downstream of tumor necrosis factor (TNF) receptor-associated factor (TRAF6), and osteoclast differentiation is induced. I $\kappa$ B, inhibitor of nuclear factor- $\kappa$ B; IKK I $\kappa$ B kinase; mTOR, mammalian target of rapamycin; NFATc1, nuclear factor of activated T cells c1; Ub, ubiquitin.

of extracellular regulated kinase (Erk), JNK, and p38 as well as NF- $\kappa$ B activation was observed, while such activation was severely abrogated in M-BMM $\phi$  from TRAF6<sup>-/-</sup> mice. These results suggest a critical role of TRAF6 in the osteoclast differentiation signaling downstream of RANK. All TRAF proteins share a common stretch of amino acids (TRAF domain) at their carboxyl-terminus (21, 32). This domain can further be divided into two subregions, i.e. TRAF-C and TRAF-N domains. The TRAF-C domain is highly conserved among TRAF family members, and the more unique TRAF-N domain is predicted to form a coiled-coil structure. Two additional structures, the RING finger domain and zinc finger domain, are predicted to be shared by all TRAF family members except TRAF1. Retrovirus vector-mediated introduction of wildtype TRAF6 into TRAF6<sup>-/-</sup> M-BMM $\phi$  completely rescued the osteoclast differentiation and recovered Erk, JNK, p38, and NF- $\kappa$ B activation in response to the sRANKL treatment. Interestingly, the TRAF6 mutant without the RING finger domain and the first zinc finger domain could also rescue the osteoclast formation from TRAF6<sup>-/-</sup> cells. These cells were strongly positive for TRAP activity, and they expressed various marker molecules of osteoclasts, including matrix metalloproteinase-9, cathepsin K, and calcitonin receptors. However, when cultured on dentine slices, these cells had an impaired ability for bone resorption, while full-length TRAF6 expression fully rescued the bone-resorbing activity of the cells. These results indicate that the RING finger domain of TRAF6 is not absolutely required for the differentiation of M-BMM $\phi$  to multinucleated osteoclasts, but it is essential for the full activation of the cells. The RING finger domain was also found to be

critical for the full activation of JNK and p38 (41). These results demonstrate that the JNK and p38 activation in response to RANKL plays a critical role in osteoclast development, which is mediated by the RING finger domain of TRAF6. Further study is required to define the exact mechanism of action of TRAF6 in osteoclast development.

Several studies suggest the role of TNF- $\alpha$  receptor/TRAF2 and TRAF5 pathways in osteoclast differentiation, and TNF- $\alpha$  can substitute for RANKL in pathological conditions (42–46). However, no definite *in vivo* evidence has been proposed to confirm the significance of TNF receptor signaling pathways on physiological and/or pathological osteoclast differentiation independently of RANKL/RANK pathways. Further studies are required to establish the involvement of other TRAFs in osteoclast development.

#### NF- $\kappa$ B and activator protein-1

RANKL stimulation induces intracellular signaling pathways including JNK, NF- $\kappa$ B, p38, and NFATc1 (Fig. 2). NF- $\kappa$ B exists in the cytoplasm of resting cells but enters the nuclei in response to various stimuli, including cytokines, pathogens, and stress (47, 48). NF- $\kappa$ B activation requires sequential phosphorylation, ubiquitination, and degradation of the inhibitor of NF- $\kappa$ B (I $\kappa$ B), as well as the consequent exposure of a nuclear localization signal on NF- $\kappa$ B. The I $\kappa$ B kinase (IKK) signalosome is the protein complex that contains an inducible IKK activity and consists of IKK1 (IKK $\alpha$ ), IKK2 (IKK $\beta$ ), and NF- $\kappa$ B essential modulator (NEMO) (IKK $\gamma$ ) (47–49). Among these three components of the IKK signalosome, both IKK1 and IKK2 appear to play a critical role in I $\kappa$ B phosphorylation.

However, the studies of IKK1 and IKK2 knockout mice indicate that IKK2 is more potent for NF- $\kappa$ B activation by pro-inflammatory stimuli than IKK1 (50–53). This finding suggests that IKK2 may also play a critical role in RANKL-induced NF- $\kappa$ B activation. Mice deficient in both p50 and p52 subunits of NF- $\kappa$ B are osteopetrotic, due to the failure in osteoclast differentiation, indicating a crucial role of NF- $\kappa$ B in osteoclastogenesis (54, 55). Recently, using a gene ablation approach, Ruocco et al. (56) demonstrated that IKK1 is required for osteoclast formation *in vitro* but not *in vivo*, while IKK2 is indispensable for both *in vitro* and *in vivo* osteoclastogenesis.

JNK activation facilitates the phosphorylation of c-Jun and increases its transcriptional activity, leading to activator protein-1 (AP-1) activation. c-Fos knockout mice exhibited an osteopetrotic phenotype, which can be rescued by not only transgenic c-Fos expression but also the expression of other Fos family members such as Fra-1 (57, 58). Ikeda et al. (59) generated a transgenic mouse in which dominant negative c-Jun (DN-c-Jun) is overexpressed under the control of TRAP promoter. The mice also showed severe osteopetrotic phenotypes, and TRAP staining showed there were very few osteoclasts in the skeletal tissues of the mice. c-Jun phosphorylation by JNK1 is necessary for the efficient osteoclastogenesis in response to RANKL using JNK1<sup>-/-</sup> mice, and Srivastava et al. (60) recently reported that estrogen inhibits the osteoclast differentiation by downregulating JNK1 activation in response to RANKL. These results demonstrate that both NF- $\kappa$ B and AP-1 activation is necessary for the normal osteoclast development.

JNK is known to activate AP-1 and to be activated by the phosphorylation of Thr and Tyr residues via MAPK kinase (MKK)-4 and/or -7. MKK-7 appears to be more specific in JNK activation than MKK-4, because MKK-4 is known to activate p38 MAPK as well (61, 62). Using adenovirus vectors carrying DN IKK2 gene or DN MKK-7 gene, we successfully segregated RANKL-induced NF- $\kappa$ B activation and JNK activation. We found that the activation of NF- $\kappa$ B and JNK is independently regulated, and both of these pathways are crucial for the osteoclast development (63).

#### NFAT

Ishida et al. (64) reported that NFATc1 is induced in response to sRANKL stimulation of RAW264.7 cells and that inhibiting NFAT activity by either cyclosporine A or anti-sense oligonucleotides suppressed osteoclast differentiation *in vitro*. Takayanagi et al. (65) also demonstrated that NFATc1 is induced in osteoclast precursors by sRANKL stimulation

using DNA microarray analysis and that embryonic stem cells deficient in NFATc1 were unable to differentiate into osteoclasts *in vitro*. Matsuo et al. (66) showed that NFATc1 was not induced by sRANKL in c-Fos<sup>-/-</sup> background, and constitutively active NFAT expression at least partially restored the osteoclast-specific gene expression in c-Fos<sup>-/-</sup> precursors. Ikeda et al. (59) showed that the osteoclast differentiation induced by active NFAT introduction was abrogated by DN-c-Jun overexpression. These results suggest that AP-1 and NFATc1 act coordinately to regulate osteoclast differentiation. Although it is still obscure what molecules lie upstream of NFATc1 activation, Koga et al. (67) recently showed that intracellular calcium oscillation is an important stimuli upstream of NFATc1 activation, and molecules containing immunoreceptor tyrosine-based activation motif, such as DAP12 (DNAX-activating protein 12) and Fc receptor common  $\gamma$  chain, facilitate the calcium-mobilizing mechanism during osteoclastogenesis (Fig. 2).

#### Other transcription factors

Tondravi et al. (68) demonstrated that ETS (a winged helix-turn-helix wing motif) domain-containing transcription factor PU.1 (Sp.1) plays an essential role in osteoclast development, based on the finding that the targeted deletion of PU.1 induced osteopetrosis in mice due to the defect in osteoclast differentiation. In PU.1<sup>-/-</sup> mice, mature macrophages cannot be observed, indicating that PU.1 is important for the differentiation of monocyte-macrophage lineage cells.

Microphthalmia transcription factor (MITF) is a basic helix-loop-helix-zipper type transcription factor, which is closely related to the TFE3, TFEB, and TFEC gene products, and MITF has been identified as the responsible gene product for the murine *mi* locus, which causes microphthalmia mutation (69). Homozygous mutation (*mi/mi*) is characterized by severe osteopetrosis as well as abnormalities in melanocytes and mast cells. The osteopetrosis in *mi/mi* mice is caused by the defective differentiation of mononuclear osteoclasts into mature multinucleated osteoclasts (70). MITF binds to a specific DNA sequence called E-box as a homodimer or by heterodimerizing with other TFE family members, and it transactivates TRAP and *cathepsin K* gene (71, 72). Sato et al. (73) demonstrated that mutated MITF observed in *mi/mi* mice acts in a DN fashion, binds specifically to c-Fos and PU.1, and blocks the nuclear localization of these transcription factors, which may result in the impaired osteoclastogenesis in *mi/mi* mutant mice. Recent studies have revealed that MITF is involved in M-CSF signaling, and M-CSF induces phosphorylation of MITF and TFE3 via a conserved MAPK consensus site, thereby triggering their recruitment of the coactivator

p300 (74). McGill et al. (75) demonstrated that an anti-apoptotic molecule, Bcl-2, is a target of MITF in osteoclasts, and Bcl-2 knockout mice showed a severe osteopetrosis, indicating a critical role of MITF in osteoclast development. Steingrimsdottir et al. (74) reported that the combined loss of MITF and TFE3 caused a severe osteopetrosis in mice, although deletion of one of these genes did not induce abnormal osteoclast phenotypes, suggesting a redundant role for these two genes in osteoclast development.

### RANKL/RANK/OPG system and osteoclast apoptosis

#### Bisphosphonates

Recent studies have revealed that the rapid cell death of osteoclasts is caused by the programmed cell death. Apoptosis is a form of programmed cell death that is characterized by specific morphological and biochemical properties (77, 78). Morphologically, apoptosis is characterized by a series of structural changes in dying cells: blebbing of the plasma membrane, condensation of the cytoplasm and nucleus, and cellular fragmentation into membrane apoptotic bodies. Biochemically, apoptosis is characterized by the degradation of chromatin, initially into large fragments of 50–300 kilobases and subsequently into smaller fragments that are monomers and multimers of 200 bases (77, 79).

The first evidence that the death of osteoclasts is caused by the apoptotic process was shown by Hughes et al. (80), who reported that bisphosphonates (BPs) induced osteoclast apoptosis. BPs are pyrophosphate analogs in which the oxygen bridge between the two phosphorus atoms is replaced by a carbon with various side chains (81). They are potent inhibitors of bone resorption and are clinically applied for the treatment and prevention of osteoporosis, Paget's disease, tumor metastases in bone, and other skeletal disorders (81). They showed that risedronate, pamidronate, and clodronate strongly induced osteoclast apoptosis both *in vitro* and *in vivo* (80). They also exhibited that these BPs increased osteoclast apoptosis *in vivo* as determined morphologically in both normal mice and mice with increased bone resorption (80). Although the exact mechanism of action of BPs to induce osteoclast apoptosis is still unknown, recent studies have proposed a critical implication of the mevalonate pathways (Fig. 3). Amino-BPs (N-BPs), such as risedronate and alendronate, suppress farnesyl diphosphate synthase in the mevalonate pathways, which subsequently induces the apoptosis of osteoclasts by reducing the C-terminal prenylation of small G proteins (82). The effect of N-BPs on geranylgeranylation

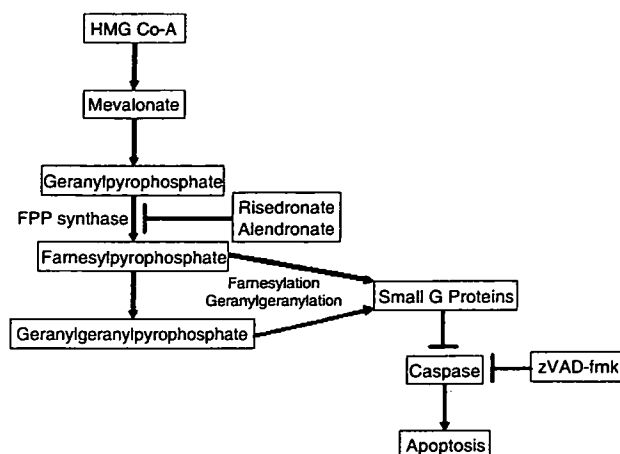


Fig. 3. Possible molecular mechanism of bisphosphonates action on osteoclast apoptosis. Amino-bisphosphonates such as risedronate and alendronate suppress farnesyl diphosphate (FPP) synthase and affect the farnesylation and the geranylgeranylation of small G proteins.

rather than farnesylation may be critical for the induction of osteoclast apoptosis, as geranylgeraniol rescues the inhibitory effects of alendronate on osteoclasts, and the action of these BPs can be mimicked by specific geranylgeranyl transferase inhibitors (83, 84). These observations suggest that the activity of small G proteins is important to maintain the integrity of osteoclasts. Recent studies also demonstrated that not only N-BPs but also non-nitrogen-containing BPs, such as clodronate, cause apoptosis in osteoclasts (83). The pro-apoptotic effect of these BPs is mediated by caspase activation, which can be blocked by an anti-apoptotic caspase inhibitor, zVAD-fmk (85). Reza and coworkers (86) further demonstrated the cleavage of mammalian sterile 20-like kinase 1 to form the active 34-kDa species in association with apoptosis induced by BPs. Interestingly, they also showed that the BP-induced inhibition of bone resorption does not require osteoclast apoptosis, because zVAD-fmk maintained the osteoclast number by suppressing osteoclast apoptosis but did not prevent the inhibition of bone resorption (87).

#### Intracellular signal transduction pathways regulating osteoclast apoptosis

Using the adenovirus vector-mediated gene transduction system, we found that the activation of Erk by transducing a constitutively active mutant of Mek1 markedly promotes the survival of osteoclasts (88). Conversely, inhibiting Erk activation by overexpressing a DN ras gene mutant rapidly induced the apoptotic cell death of the cells (88). These results, combined with the fact that the anti-apoptotic factors such as RANKL, IL-1, and M-CSF induce Erk activation in osteoclasts,

suggest that the Ras/Erk pathway plays an essential role in osteoclast survival.

The critical role of phosphatidylinositol 3-kinase (PI3K)/Akt pathways in the survival of osteoclasts has also been suggested. Xing et al. (89) reported that the transgenic overexpression of Src251, which contains SH2 and SH3 domains of chicken c-Src but lacks the kinase domain, under the control of the TRAP promoter induced osteopetrosis in mice. The mice had reduced number of osteoclasts, and osteoclasts of Src251 transgenic mice showed apoptotic phenotypes. The activity of Akt in the Src251 transgenic mouse osteoclasts was significantly reduced, and RANKL-induced Akt activation was impaired in the cells, suggesting that Akt plays a critical role in osteoclast survival and that Akt activation is at least partly mediated by c-Src (89). Using the murine osteoclast formation system, Lee et al. (90) showed that TNF- $\alpha$  prolonged the survival of osteoclasts, which was abrogated by either PI3K inhibitors or Mek/Erk inhibitors. They also revealed the involvement of Grb2 and ceramide in the TNF- $\alpha$ -induced Erk activation in osteoclasts (90). Similar effects were observed in IL-1 $\alpha$  (91). Glantschnig et al. (85) exhibited the central role of mammalian target of rapamycin (mTOR)/S6K pathways in M-CSF- and RANKL-induced osteoclast survival, which is suppressed by rapamycin treatment. We also found that the adenovirus vector-mediated overexpression of constitutively active Akt promotes the survival of osteoclasts, and small G protein Rac1 is critically involved in this process (92). These results clearly suggest the important role of PI3K/Akt pathways on osteoclast survival. However, contrary to these previous observations, Sugatani and Hruska (93) recently reported that silencing of Akt1 and/or Akt2 by small interfering RNA (siRNA) suppressed the differentiation of osteoclasts but did not affect their survival. The reason of this discrepancy remains unknown, and further investigation is required to clarify the exact role of PI3K/Akt pathways in osteoclast survival.

#### Bcl-2 family members

The apoptotic process can be divided into two different pathways, the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway (94). In the death receptor pathway, activation of a receptor belonging to TNF receptor family leads to induction of apoptosis through activation of aspartate-specific cysteine proteases (caspases-8), and the anti- and pro-apoptotic Bcl-2 family members are critically involved in the intrinsic pathway by regulating cytochrome c release from the mitochondrial intermembrane into the cytosol, which leads to the activation of caspase-9 (94). The anti-apoptotic

members include mammalian Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1, and *Caenorhabditis elegans* CED-9. So far, more than 20 members of pro-apoptotic Bcl-2 family proteins have been identified in mammals, and they can be further divided into two groups: multidomain members possessing homology in BH1-3 domains and BH3 domain-only members (95). Multidomain members include Bax, Bak, Bok, and *Drosophila melanogaster* DEBCL/DROB, and BH3-only members include mammalian Bad, Bik/Nbk, Bid, Hrk/DP5, Blk, Bim, Noxa, and *C. elegans* EGL-1 (95). BH3-only proteins fail to induce cytochrome c release and apoptosis in *bax*<sup>-/-</sup>*bak*<sup>-/-</sup> cells, suggesting that Bax-like proteins mediate death signals from various BH3-only proteins (96). Bax-like proteins are ubiquitously expressed, while BH3-only members have more tissue-specific distribution, indicating that the latter may play a role in tissue/cell-specific regulation of apoptosis.

The activity of BH3-only proteins is strictly regulated to prevent unorganized cell death. The expression of Noxa is induced by p53 or IRF-1, and DP5/Hrk is transcriptionally upregulated in the absence of growth factors or when the cells are exposed to  $\beta$ -amyloid protein. Their activity is also regulated by the post-translational modification. It is now widely recognized that Bad is phosphorylated at various sites and sequestered away from Bcl-2 family members by binding to 14-3-3 (97, 98). Many kinases including Akt, mitochondria-anchored protein kinase A (PKA), Pak1, Rsk, Raf1, and the phosphatase calcineurin have been reported to modify Bad, although physiological importance of these regulations still remains unclear.

To analyze the involvement of mitochondrial pathways in osteoclast apoptosis, we examined the depolarization of mitochondrial transmembrane potential. Depolarization of the mitochondrial transmembrane potential, chromatin condensation, and cytochrome c release from mitochondria into cytoplasm were observed in the apoptotic osteoclasts. These results show that the cytokine deprivation triggers osteoclast apoptosis through the Bcl-2-regulated pathway.

A possible role of anti-apoptotic Bcl-2 family members on osteoclast survival has been reported. Okahashi et al. (99) showed that M-CSF treatment increased Bcl-2 mRNA expression in osteoclasts, and we found that adenovirus vector-mediated overexpression of Bcl-xL markedly prolonged the survival of the cells *in vitro* (100). Roodman and coworkers (101) developed transgenic mice in which Bcl-xL together with simian virus 40 large T antigen were specifically overexpressed in osteoclasts under the control of TRAP promoter, and they successfully established an immortalized osteoclast precursor cell line from the mice. These results clearly showed that anti-apoptotic Bcl-2 family proteins play important roles

in osteoclast survival both *in vitro* and *in vivo*. The mechanism regulating Bcl-2 and Bcl-xL expression in osteoclasts has not been clarified yet, but McGill *et al.* (75) showed that Bcl-2 expression is regulated by MITF, a transcription factor essential for osteoclast and melanocyte development.

#### Pro-apoptotic BH3-only member Bim

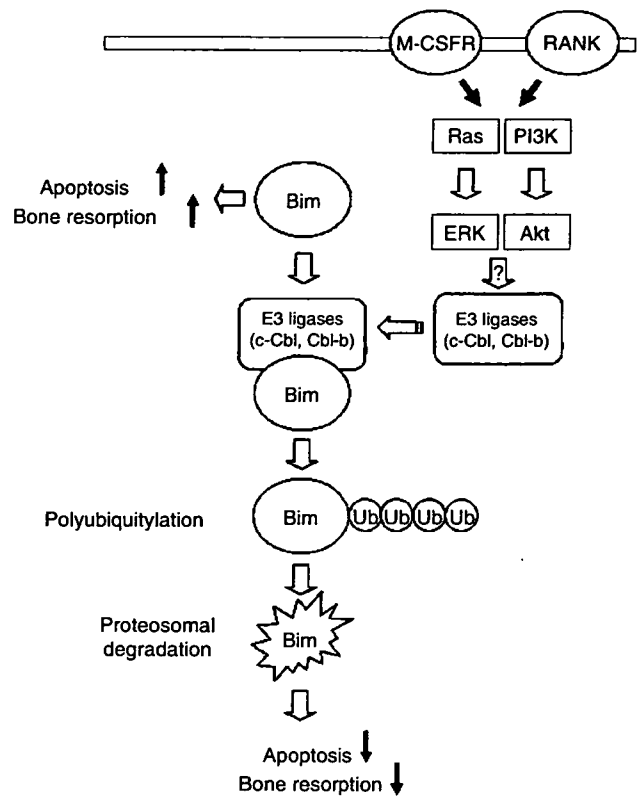
Bim, one of the BH3-only proteins, was first identified as a Bcl-2 interacting protein by screening a  $\lambda$  phage expression library constructed from a mouse thymic lymphoma (102). Bim is expressed in hematopoietic, epithelial, neuronal, and germ cells (103), and alternative splicing generates various Bim isoforms, including BimS, BimL, and BimEL. Bim has been shown to play essential roles in the apoptosis of T lymphocytes, B lymphocytes, and neurons (104, 105), and its activity is also regulated both transcriptionally and post-transcriptionally (106). The expression of Bim is downregulated at the transcriptional level by IL-3 signaling through the Raf/Erk pathways and/or PI3K/mTOR pathways in IL-3-dependent Ba/F3 cells. Transcription of Bim is also upregulated in neonatal sympathetic neurons in response to nerve growth factor deprivation.

We demonstrated a novel and unique regulation of apoptosis by ubiquitylation-dependent degradation of Bim in osteoclasts (100) (Fig. 4). In the presence of M-CSF, Bim is constitutively ubiquitylated and degraded, and the cytokine deprivation induced a rapid upregulation of Bim due to the reduced level of its ubiquitylation (100). c-Cbl is a possible candidate of E3 ubiquitin ligase of Bim in osteoclasts, because Bim ubiquitylation was reduced in c-Cbl-deficient osteoclasts (100). Bim-deficient osteoclasts exhibited a prolonged survival both *in vitro* and *in vivo*; however, their bone-resorbing activity was significantly reduced, consistent with the *in vivo* observation that Bim<sup>-/-</sup> animals showed mild osteosclerosis (100). Consistent with our observation, Sugatani and Hruska reported that silencing *bim* gene by siRNA prolonged the survival of osteoclasts (93). These results clearly demonstrate that pro-apoptotic protein Bim plays a crucial role in osteoclast apoptosis and activation (Fig. 4). Further studies are required to elucidate the regulatory mechanism of Bim in osteoclasts and the role of Bim in skeletal disorders.

#### Pathological role of RANKL/RANK/OPG system

##### Osteoporosis

Accumulating evidence has shown that RANKL is not only important for normal bone development but also plays an important role in pathological bone destruction, such as in



**Fig. 4. Regulation of ubiquitin/proteasome-dependent degradation of Bim in osteoclasts.** Cbl family E3 ligases are recruited to Bim in response to ERK and/or Akt activation, which subsequently causes polyubiquitylation and proteasome-dependent degradation of Bim. Bim is a critical regulator of apoptosis and an activator of osteoclasts.

postmenopausal osteoporosis, rheumatoid arthritis (RA), periodontal diseases, and tumor-induced osteolysis. Osteoporosis is a skeletal disorder characterized by low bone mass and loss of bone tissue integrity that causes weak and fragile bones (107). The weakness of bone strength leads to an increased risk of fragile fractures, particularly in the hip and the spine, which results in a huge social and economic problem. A hip fracture usually requires hospitalization and a major surgery and can impair a patient's quality of life and cause prolonged disability or even death. Spinal (or vertebral) fractures also have serious results, including loss of height, severe back pain, and kyphosis. Vertebral fractures also threaten human life. Cauley *et al.* (108) reported that the age-adjusted relative risk of mortality following a clinical vertebral fracture is 8.6, while that of a hip fracture is 6.7.

Although the exact etiology of osteoporosis remains unknown, it is now widely accepted that the normal balance between bone formation and bone resorption is impaired in the osteoporotic patient. The primary cause of postmenopausal osteoporosis in women is estrogen deficiency, and bone

destruction takes place at a faster rate after menopause. Recent evidence has shown that the increased remodeling frequency is a risk factor of fragile fracture independent of the change in bone mineral density (BMD), and Recker et al. (109) demonstrated that bone remodeling rates (activation frequency) were increased in postmenopausal women and osteoporotic patients. A new remodeling cycle is initiated by the novel osteoclast formation in the activation phase, and therefore, it is reasonable to speculate that the RANKL/RANK/OPG axis is critically involved in the pathogenesis of osteoporosis.

Consistent with this speculation, Xu et al. (110) reported that RANKL transcripts were highly expressed in bone tissues of ovariectomized rats. Estrogen has been demonstrated to upregulate the gene expression and protein synthesis of OPG both *in vitro* in human (111) and in rodent (112) osteoblastic cells and *in vivo* in elderly men (113). More recently, Riggs and coworkers (114) demonstrated that postmenopausal women express higher levels of RANKL on marrow stromal cells and lymphocytes than premenopausal women or postmenopausal women taking estrogen therapy. RANKL expression per cell correlated well with the bone resorption markers serum C-terminal telopeptide of type I collagen and urine N-telopeptide (NTX) of type I collagen and inversely with serum 17 $\beta$ -estradiol level (114). Taken together, RANKL/RANK/OPG pathways are critically involved in the pathogenesis of osteoporosis probably by recruiting osteoclasts and starting new remodeling cycles and therefore can be a good therapeutic target of the disease.

## RA

RA is a chronic systemic inflammatory disorder with an unknown etiology characterized by the invasive synovial hyperplasia leading to the progressive joint destruction (112). Radiographic studies have shown that the bone erosion in RA begins at the early stage of the disease and gradually (and sometimes rapidly) exacerbates (116). Bone erosion results in the severe deformity of the affected joints and impairs the normal activity and the quality of life of the patients; preventing such devastating states is one of the most challenging issues in treating RA.

Because the exact etiology and pathology of RA is unknown, most treatments of RA just relieve symptoms of the disease. Non-steroidal anti-inflammatory drugs, including cyclooxygenase 2 inhibitors, are prescribed to reduce the painful symptoms of the disease, but they usually have little effect on stopping the progression of the joint destruction. Recent studies have revealed that some disease-modifying anti-rheumatic drugs and biological agents, such as anti-

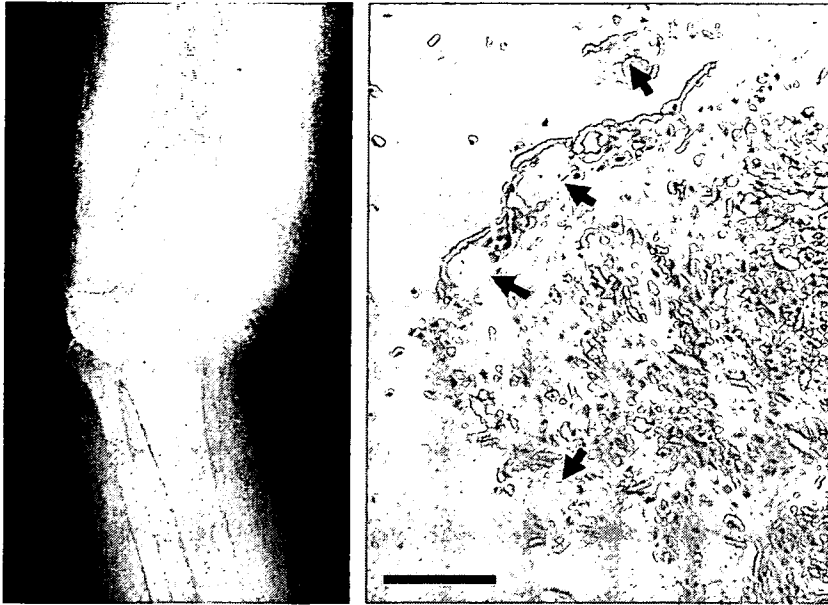
TNF- $\alpha$  antibody and IL-1 receptor antagonist, ameliorate the clinical symptoms of RA and reduce the progression of the joint destruction (117). Anti-TNF- $\alpha$  therapy using anti-TNF- $\alpha$  antibody or soluble TNF receptor fusion protein almost completely suppressed the progression of bone destruction in some RA patients (118–120).

There are still a significant proportion of non-responders to the therapies, and the bone-protective effect of these reagents is limited in most cases. Additionally, their long-term efficacy has yet to be established, and long-term use of these medicines often causes severe adverse effects. Therefore, novel therapeutic interventions specifically targeting the joint destruction of RA are greatly anticipated.

Proliferating synovium produces an elevated amount of the pro-inflammatory cytokines IL-1, IL-6, IL-17, and TNF- $\alpha$ , and matrix-degenerating enzymes matrix metalloproteinases and cathepsins, which are involved in the bone and cartilage destruction (115). Bone erosion usually begins at the interface of the cartilage and the proliferating synovium, the so-called 'bare area,' and bone-resorbing osteoclasts are observed at the erosive synovium/bone interfaces (Fig. 5). It is not long time ago that it was recognized that osteoclasts are primarily involved in the bone destruction in RA. Bromley and Woolley (121) histologically analyzed the bone-pannus interface of RA joints by light microscopy and showed the presence of acid phosphatase-positive multinucleated cells with morphologic features of osteoclasts along the surface of mineralized subchondral bone and mineralized cartilage. We (122) and Fujikawa et al. (123) established an *in vitro* osteoclast formation system using synovial cells obtained from RA synovial tissues, in which macrophage-lineage osteoclast precursor cells in synovial tissues differentiate into mature osteoclasts in the presence of supporting cells, such as synovial fibroblastic cells or osteoblastic cells.

The important role of the RANKL/RANK/OPG system in the development of bone destruction in RA has been recently established (124). We and other groups reported that RANKL is highly expressed in synovial tissues of RA patients (125–128). The cells responsible for the upregulation of RANKL are considered to be synovial fibroblastic cells and T lymphocytes (125, 129). In the coculture system of synovial fibroblasts and peripheral monocytes, 1 $\alpha$ ,25(OH) $_2$ D $_3$  treatment induced RANKL expression in synovial fibroblasts and induced osteoclast differentiation from monocytes (125). Although the detailed mechanism of upregulation of RANKL expression in the RA synovial cells remains elusive, our preliminary results showed that the treatment of synovial





**Fig. 5. Bone destruction in rheumatoid arthritis.** (Left) X-ray of the knee joint of a patient with rheumatoid arthritis. Note the medial subluxation of the joint and remarkable bone destruction in the medial tibial plateau. (Right) Proliferating synovium stimulates osteoclast differentiation, and a number of osteoclasts are observed in the pannus-bone interface (arrows) (H&E staining). Bar = 100  $\mu$ m. Courtesy of Dr Yoshiaki Kuga (Tokyo Metropolitan Bokuto Hospital, Tokyo, Japan).

fibroblasts with pro-inflammatory cytokines such as IL-1 and TNF- $\alpha$  induced RANKL expression in the cells, indicating that cytokine network in the synovial milieu plays an essential role on the RANKL induction in the cells. These results strongly suggest that RANKL/RANK pathways are primarily involved in the pathological bone destruction in RA. In spite of a remarkable success of anti-TNF- $\alpha$  therapy in treating RA patients, there exist no *in vivo* data demonstrating the role of TNF- $\alpha$  signaling pathways on osteoclast development and bone destruction independently of RANKL/RANK pathways.

#### Tumor-induced bone diseases

Some primary tumors, benign or malignant, and metastatic malignant tumors, such as breast cancer and lung cancer, invade bone tissues (130). The typical tumor-induced bone lesion is focal osteolysis, although some tumors such as prostatic cancer metastases exhibit osteogenic bone lesions. The osteolysis induced by these tumors affects the quality of life of the patients by causing pathological fractures and severe bone pain and often exacerbates the prognosis of the patients. Recent studies have shown that osteoclasts are primarily responsible for such conditions, and the involvement of RANKL/RANK/OPG system has been indicated (131). Tumor cells produce various factors, such as PTH-related protein (PTHrP), TNF- $\alpha$ , and IL-1, that can stimulate RANKL expression in osteoblastic cells or bone marrow stromal cells and induce osteoclast formation in the bone microenvironment. PTHrP is a peptide hormone leading to humeral hypercalcemia of malignancy. Other factors produced by tumor

cells, including IL-6 and macrophage inflammatory protein-1- $\alpha$ , are capable of activating osteoclasts.

Grimaud *et al.* (132) reported that RANKL/OPG ratio is increased in patients with severe osteolysis. Among various bone tumors, the molecular mechanism of bone destruction in multiple myeloma is one of the most studied. Multiple myeloma is a plasma cell malignancy that develops in the bone marrow and causes marked skeletal destruction. It has been reported that myeloma cells induce an imbalance in the OPG/RANKL system in bone environments and that this imbalance is responsible for osteolysis observed in patients (133–136). Bone marrow biopsies from patients with myeloma showed an increase of RANKL and a reduction of OPG expression by immunohistochemical analysis (137). The RANKL/RANK/OPG system is also reported to be involved in bone destruction in breast cancer cells (138, 139), prostate cancer cells (140, 141), and other metastatic bone tumors. These results suggest that the RANKL/RANK/OPG system is also important for the tumor-induced bone destruction.

#### Periodontal diseases

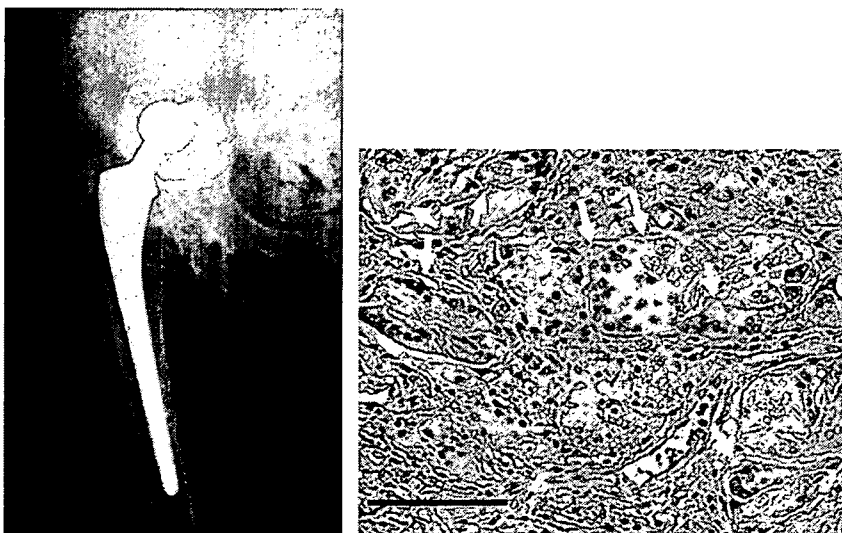
Periodontal diseases are chronic infectious inflammatory diseases characterized by a heavy leukocyte infiltration into the periodontal lesions, resulting in the secretion of a variety of cytokines, which ultimately leads to the destruction of periodontal tissues including alveolar bone (142). Cell-to-cell interaction between T cells and monocytes/osteoclast progenitor cells appears to be important for osteoclast formation in periodontal diseases (142, 143). The expression of RANKL

mRNA was shown to be upregulated in gingiva from patients with severe periodontitis (143). Teng et al. (144) reported a novel animal model of periodontitis, in which peripheral blood leukocytes from patients with localized juvenile periodontitis were transplanted into NOD/SCID mice. Oral challenge of these humanized mice with *Actinomyces actinomycetemcomitans*, a Gram-negative bacterium that causes human periodontitis, leads to the local bone resorption in alveolar bone surrounding the teeth. *In vitro* stimulation of CD4<sup>+</sup> T cells from these mice with antigens from *A. actinomycetemcomitans* leads to the expression of RANKL, and OPG treatment reduced the number of periodontal osteoclasts and diminished alveolar bone destruction after microbial challenge (144). *Porphyromonas gingivalis*, another bacterium implicated in human periodontitis, can also induce RANKL expression in lymphocytes, and this expression is associated with increased osteoclast formation in spleen cell cultures (145). These observations suggest that bacteria stimulate osteoclast formation by activating cell-to-cell interactions in the infiltrating leukocytes. Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is a strong bone-resorbing factor in periodontitis. Recent studies have revealed that Toll-like receptor (TLR) family members act as receptors for a variety of microbial ligands, and TLR4 is a signal-transducing receptor for LPS (146). The signaling cascade of TLR4 is quite similar to that of the IL-1 receptor, because both TLR4 and IL-1 receptors use common signaling molecules such as myeloid differentiation factor 88 (MyD88) and TRAF6 (146). In addition, Toll-IL-1 receptor domain-containing adapter inducing interferon- $\beta$  (TRIF)-mediated signals are involved in TLR4-induced MyD88-independent pathways (146). LPS and IL-1 stimulated osteoclast formation in

coculture of osteoblasts and hemopoietic osteoclast progenitor cells (147). Kikuchi et al. (148) showed that LPS directly induced RANKL expression in osteoblasts through PKC and Mek/Erk signals. Using MyD88-deficient mice and TRIF-deficient mice, Sato et al. (149) reported that MyD88-mediated signals but not TRIF-mediated ones are involved in RANKL expression in osteoblasts treated with LPS as well as IL-1. These results suggest that RANKL expressed by either osteoblasts or infiltrating T cells in response to bacterial infection is involved in alveolar bone destruction in periodontal diseases.

#### Periprosthetic implant loosening

Since the initial report of total joint replacement by Sir John Charnley in the 1960s (150), both total hip and total knee arthroplasty have become a successful and reliable surgical procedure to achieve natural function lost in many destructive joint diseases including osteoarthritis, RA, osteonecrosis, and very severe pathologic fractures, and more than one million replacements have been performed per year in the world (151). Although joint replacement surgeries have been extremely successful in relieving painful symptoms and improving morbidity of the patients and their long-term results are satisfactory, the periprosthetic bone loss leading to aseptic loosening of the implants is one of the most challenging complications that may result in the failure of the procedure (152, 153). Histologically, the formation of synovium-like fibrous tissues surrounding the implants, also called pseudocapsule, is frequently observed. The interface structure is rich in monocyte-macrophage lineage cells as well as fibroblastic cells, reminiscent of the inflammatory synovium observed in RA, and a number of osteoclast-like multinucleated cells can



**Fig. 6. Periprosthetic osteolysis after total hip replacement.** (Left) X-ray of the hip joint with periprosthetic osteolysis around the cemented acetabular component. (Right) Synovium-like fibrous tissues surrounding the implants contain a number of osteoclast-like multinucleated cells (arrows) (H&E staining). Bar = 100  $\mu$ m.

be observed in the bone-implant interface of the loosened joints (154, 155) (Fig. 6). Recent studies have revealed that fibroblastic cells in periprosthetic tissues are capable of inducing the differentiation of normal human peripheral blood mononuclear cells to mature osteoclasts by a mechanism that involves both RANKL and TNF- $\alpha$  (156).

#### Paget's disease and inherited bone diseases

Sporadic Paget's disease is characterized by abnormal bone remodeling, leading to excessive and aggressive bone resorption by osteoclasts, the replacement of hematopoietic bone marrow by fibrous connective tissue, and the subsequent increase in bone formation; X-ray examination shows a 'mosaic' appearance (157). There exist abundant osteoclasts in the osteolytic area, and enhanced RANKL expression is observed in the area (158). It has been reported that the excessive activity of pagetic osteoclasts may be caused by hypersensitivity of osteoclast precursors to several osteoclastogenic factors, including  $1\alpha,25(\text{OH})_2\text{D}_3$  and RANKL (158–160). A number of studies have revealed the potential involvement of measles virus nucleocapsid protein in the pathogenesis in Paget's disease, although there is still a controversy whether this phenomenon is the cause of the disease (161–167).

Several genetic disorders were found to be associated with abnormal RANKL/RANK/OPG pathways (168). Familial expansile osteolysis (FEO) is characterized by osteolytic lesions in the long bones during early adulthood. Hughes et al. (169) demonstrated a mutation in the RANK gene in members of FEO families. There was an identical tandem duplication of bases 84–101 in exon 1 of the TNFRSF11A gene, which encodes RANK, in all affected individuals. In vitro gene transfection experiments has revealed that the mutated RANK protein is not sorted to the plasma membrane normally and is intracellularly sequestered, which leads to the constitutive activation of the RANK signaling. They also identified a similar mutation in the RANK gene in one familial Paget's disease family (169). A similar shorter tandem duplication (84dup15) in the RANK gene is the cause of expansile skeletal hyperphosphatasia, an inherited disease characterized by hyperostosis, deafness, hypercalcemia, and premature tooth loss (170). Several groups also revealed the mutations in SQSTM1/p62 gene, which encodes a ubiquitin-binding protein that is involved in the IL-1, TNF- $\alpha$ , and RANK signaling pathways, in both familial and non-familial Paget's diseases (171–174).

Some patients with idiopathic hyperphosphatasia (also known as juvenile Paget's disease) have mutations in the

OPG gene (TNFRSF11B) (175). This disorder is a rare disease with an autosomal recessive inheritance pattern, and it displays various deformities of long bones and vertebral column, which increase in severity during adolescence. Deletion of the whole gene results in undetectable serum levels of OPG and marked increase in sRANKL levels (175). Cundy et al. (176) reported a 3-bp inframe deletion in exon 3 of the TNFRSF11B gene, which results in the formation of non-functional OPG protein. Chong et al. (177) reported a relationship between phenotype and genotype of the disease.

#### Vascular diseases

OPG-deficient animals developed arterial calcification of the renal arteries and the aorta, which was prevented by transgenic OPG expression from midgestation through adulthood but not by the intravenous injection of recombinant OPG after birth (30). In spite of such a protective effect of OPG on arterial calcification, clinical studies have shown that patients with coronary artery disease have significantly higher serum levels of OPG than healthy patients, and the relative risk of cardiovascular mortality was increased in patients with high OPG serum levels, which were associated with the severity of coronary artery disease and increased in elderly men and patients with diabetes mellitus (178–181). Further work is required to elucidate the role of the RANKL/RANK/OPG system in the physiological vascular integrity and the pathomechanism of cardiovascular disease.

#### Diagnostic implication of RANKL/RANK/OPG system

A number of clinical studies have been performed trying to clarify the relationship between the levels of RANKL or OPG and the pathological conditions. In spite of the potent inhibitory effect of OPG on bone resorption and the clinical observation that OPG deficiency results in the remarkable osteopenia, as shown in patients with juvenile Paget's disease, there is controversy about the relationship between serum concentration of OPG and BMD in postmenopausal women. Yano et al. (182) demonstrated a negative correlation with BMD in postmenopausal women, which could be due to the compensatory action to maintain the bone integrity by suppressing the abnormal bone resorption. Alternatively, Browner et al. (181) and Khosla et al. (113) did not find any significant relationship between serum OPG levels and BMD, and recent studies by Mezquita-Raya (183) showed that serum OPG levels are independently associated with bone mass and prevalent vertebral fractures in postmenopausal women. Serum OPG levels are significantly correlated with

age, and some age-related factors possibly regulate the expression of OPG in various tissues, which makes the interpretation of these contradictory results difficult. No definite correlation between serum levels of sRANKL and BMD has been established.

As mentioned above, there is a significant relationship between serum OPG levels and the risk of atherosclerosis and cardiovascular diseases. However, the exact mechanism and diagnostic value of these observations still remains elusive.

High RANKL serum levels and reduced OPG levels have been reported in multiple myeloma patients as compared with normal subjects (184, 185). OPG levels were decreased to a greater extent in patients with osteolytic lesions than those in patients without bone lesions. Moreover, Terpos et al. (186) demonstrated that the RANKL/OPG ratio may predict the prognosis in myeloma patients independently of other risk factors. Although these studies propose a possible diagnostic and a risk predicting value of the OPG/RANKL measurement, further study is required to establish its clinical significance.

#### Therapeutic strategies targeting RANKL/RANK/OPG system and RANK signaling

##### OPG, RANK-Fc, and anti-RANKL antibody

The crucial role of the RANKL/RANK/OPG system has been documented in various aspects of pathological bone resorption, and therefore, therapeutic strategies directly targeting this system have obtained a great deal of attention. The strategies include the usage of OPG, RANK-Fc, anti-RANKL antibody, modulators of intracellular signaling pathways of RANK, and RANKL vaccination.

OPG is a 401 amino acid protein with a short signal peptide (21 amino acids) that forms homodimers linked through a cysteine – cysteine disulfide bond at position 400 (22, 23). Unlike other members of the TNF receptor family, OPG lacks a transmembrane domain and is a secreted protein. OPG binds with the soluble and cell-bound forms of RANKL and suppresses their interaction with and therefore stimulation of RANK. Not only does OPG inhibit osteoclast formation *in vitro*, but transgenic expression of OPG in the liver induces osteopetrotic phenotypes in mice, and marked reduction in the osteoclast number was observed in the skeletal tissues of the mice (23). On the basis of these potent inhibitory actions of OPG on osteoclast differentiation and function, the therapeutic application of OPG has been extensively studied. Preclinical studies clearly showed the therapeutic effects

of OPG on pathological bone loss. Recombinant OPG administration or adenovirus vector-mediated OPG gene expression prevents ovariectomy-induced bone loss in rodents (23, 187) and ameliorates bone destruction in animal models of arthritis (129), periodontitis (144), metastatic bone destruction (188), and periprosthetic osteolysis (189). Cheng et al. (190) reported that peptidomimetics of OPG that can interfere RANKL–RANK binding suppress bone loss in ovariectomized animals. RANK-Fc fusion protein also has an inhibitory effect on RANKL action; it suppresses arthritic bone destruction (191), wear debris-induced osteolysis (192), and myeloma-induced bone diseases (193, 194).

Clinical trials were conducted in postmenopausal women to determine the anti-resorptive activity and safety of recombinant Fc-OPG fusion protein, which is more stable than natural OPG by deleting the heparin-binding domain (195). They showed that subcutaneous administration of a single dose of Fc-OPG rapidly (within 12 h of the injection) decreased bone resorption markers in these patients, and the decrease was still observed after 6 weeks of the injection. The effect of Fc-OPG on patients with multiple myeloma or breast carcinoma-related bone metastases was also investigated, and Fc-OPG caused a rapid, sustained, and dose-dependent decrease in bone resorption marker levels, which was at least comparable with the results from patients treated with pamidronate (196).

The potent therapeutic potential of anti-RANKL antibody was more recently reported. A randomized, double blind, single-dose, placebo-controlled study of a fully humanized anti-RANKL monoclonal antibody (AMG 162) was conducted to evaluate its anti-resorptive effects on postmenopausal women (197). A single subcutaneous injection of AMG 162 induced dose-dependent reduction in the serum and urinary NTX/creatinine, which was detected as early as 12 h after injection. The maximum urinary NTX/creatinine reduction was observed at 2 weeks in the 0.01, 0.03, 0.3, and 1.0-mg/kg AMG 162 group, 1 month in the 0.1-mg/kg AMG 162 group, and 3 months in the 3.0-mg/kg AMG of 162 groups (84%). Surprisingly, although the treatment effect of the antibody was reversible, a significant decrease in urine NTX was still observed after 9 months of the injection in the 3.0-mg/kg AMG 162 group. No related serious adverse events were reported. These results suggest that both Fc-OPG and anti-RANKL antibody are promising therapeutic approaches with broad application in the treatment of bone disorders.

##### RANKL vaccine

The other anti-RANKL therapeutic strategy is the vaccination approach. The vaccination method is used to induce an

antibody response against disease-related proteins by immunization with modified pathological antigen as preventive therapy to acquire disease resistance. Mouritsen et al. (198) developed a novel therapeutic vaccine approach against disease-related self-protein such as TNF- $\alpha$ . These vaccines were generated by inserting a promiscuous T helper (Th) epitope into its sequence. The promiscuous Th epitope stimulates the Th cells, and then T cells induce the antibody production by B cells. The therapeutic vaccines can thus bypass immunological tolerance and elicit polyclonal antibodies that neutralize pathological self-protein. A RANKL vaccine was developed by modifying the soluble TNF-like domain of murine RANKL to incorporate a promiscuous Th epitope (199). Immunization with RANKL vaccine resulted in a rapid and sustainable polyclonal anti-RANKL antibodies in mice, which have neutralizing activity against murine RANKL. Anti-RANKL antibody titers declined to about 10% of the maximal level in 10 weeks when boosting is discontinued. No abnormality in lymph nodes, either macroscopically or microscopically, was observed in vaccinated animals, and no apparent macroscopic abnormality was observed in any other organs of the immunized animals. We first examined the effect of RANKL vaccine on ovariectomized mice. We found that RANKL vaccine normalized high-turnover osteoporosis, and mice immunized with RANKL vaccine were resistant to bone loss in response to ovariectomy. We also examined the effect of RANKL vaccine on a mouse model of RA (SKG mouse) established by Dr Shimon Sakaguchi (Kyoto University, Japan) (200). This strain of mice spontaneously develops RA-like symptoms at 2 months of age (200). Immunization with RANKL vaccine after the onset of arthritis ameliorated not only the synovitis but also the formation of osteoclasts and the bone destruction. These results clearly demonstrate that RANKL vaccination can prevent bone loss in murine model of pathological bone destruction, establishing a therapeutic use of RANKL vaccine.

#### Therapeutic approaches targeting intracellular signaling of RANK

Remarkable advances in the understanding of intracellular signaling pathways of RANK have enabled us to develop therapeutic drugs specifically targeting downstream signaling molecules. Interferon- $\gamma$  markedly suppress the osteoclast differentiation in response to sRANKL and M-CSF stimulation, and Takayanagi et al. (201) reported that it promotes TRAF6 degradation via ubiquitin/proteasome pathways. They also showed that interferon- $\beta$  suppresses osteoclast differentiation by downregulating c-Fos induction in response to RANKL (202). Inhibitors of p38 MAPK pathways (SB203580) and

JNK pathways (SB600125) also suppress osteoclast differentiation (59, 63, 203). Nishikawa et al. (204) also reported a therapeutic effect of p38 inhibitor FR167653 on bone destruction in collagen-induced arthritis rats and its inhibitory action on osteoclast formation *in vitro*. The immunosuppressive drugs cyclosporine A and macrolide tacrolimus (FK506) inhibit osteoclast differentiation by suppressing the calcineurin phosphatase activity and therefore downregulating NFATc1 activation (59, 64, 65). Ikeda et al. (59) also exhibited that a specific peptide inhibitor of NFAT, VIVIT, suppressed osteoclast differentiation *in vitro*. Inhibitors of PI3K pathways, such as wortmannin and LY290442, and a specific inhibitor of mTOR, rapamycin, markedly suppress bone resorption by inducing apoptotic cell death of osteoclasts (85, 86, 205). NF- $\kappa$ B activation is also a possible candidate of anti-RANKL therapy. Tomita et al. (206) demonstrated that synthetic double-stranded DNA with high affinity for NF- $\kappa$ B, which works as 'decoy' cis elements to bind the transcriptional factor, suppressed the bone destruction in collagen-induced arthritis rats. Jimi et al. (207) exhibited that a cell-permeable peptide inhibitor of the IKK complex (NBD peptide), which disrupts the association of NEMO with IKK1 and IKK2, inhibited RANKL-stimulated NF- $\kappa$ B activation and osteoclastogenesis both *in vitro* and *in vivo*.

The limitation of these approaches resides in the specificity of these compounds. Because these intracellular signaling pathways are also important for other cells or tissues, the development of a sophisticated drug delivery system or cell-specific or tissue-specific inhibitors is absolutely necessary to prevent or minimize significant adverse effects. Table 1 lists the candidates for anti-RANKL therapy.

#### Possible disadvantage of anti-RANKL therapy

The concerns of therapeutic strategies targeting RANKL/RANK system are mainly related to the extraskeletal adverse effects raised by suppressing the RANKL/RANK pathway. RANKL is expressed in activated T cells, while RANK is expressed on the surface of dendritic cells, mature T cells, and hematopoietic precursors. RANKL treatment induces Bcl-xL expression in dendritic cells and promotes their survival. Therefore, anti-RANKL therapy can potentially jeopardize the function of dendritic cells, although no abnormality in the dendritic cell function has been reported in clinical studies.

Mice deficient in RANKL or RANK exhibit complete absence of lymph nodes but intact splenic architecture and Peyer's patches. Studies have revealed that the RANK signaling is essential for the lymph node formation, while IL-7 receptor  $\alpha$  is essential for Peyer's patch formation in the embryonic

**Table 1. Candidates for anti-RANKL therapy**


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OPG, OPG-Fc (23, 129, 144, 188, 189, 195, 196)
RANK-Fc (191–194)
Anti-RANKL monoclonal antibody (197)
OPG-like peptidemimetics (OP3-4) (190)
RANKL vaccine (199)
Interferon- $\beta$ , $\gamma$ (201, 202)
p38 inhibitor (SB203580, FR167653) (203, 204)
JNK inhibitor (SB600125) (59)
IKK inhibitor (NBD peptide) (209)
NF- $\kappa$ B inhibitor (NF- $\kappa$ B decoy) (206)
Calcineurin inhibitor (Cyclosporin A, FK 506) (59, 64, 65)
NFAT inhibitor (VIVIT peptide) (59)
PI3K inhibitor (wortmannin, LY290442) (85, 86, 205)

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stage. Lymphotoxin  $\beta$  signaling is indispensable for both lymph node and Peyer's patch organogenesis, and the RANK signaling regulates lymphotoxin  $\beta$  expression in lymph nodes (208). Therefore, RANKL/RANK pathways may be required for lymph node genesis in the developmental stage but not for lymph node function in adulthood.

OPG binds to TNF-related apoptosis-inducing ligand (TRAIL) with the similar affinity to RANKL (209), and therefore, OPG treatment may affect the function of TRAIL. Mice with TRAIL gene deletion were more susceptible to experimental and spontaneous tumor metastasis, and they were more sensitive to chemical carcinogens, indicating the importance of TRAIL in the host defense against transformed cells (210). Although neither the increase in the overall risk of malignancy nor the exacerbation of the metastatic bone tumors has been reported in the clinical trials of Fc-OPG or anti-RANKL antibody, these data warrant a careful observation on the patients receiving anti-RANKL therapy.

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The experience of biological agents for the treatment of RA patients displayed the potential risk of their immunogenicity, i.e. stimulation of the production of antibodies against themselves. Patients treated with mouse : human chimeric antibodies can develop human anti-mouse antibodies, which may reduce the clinical effectiveness of the drug, and patients treated with fully human antibodies can develop human anti-human antibodies. This recognition is particularly critical in case of Fc-OPG treatment, because antibodies against Fc-OPG have potential risk of cross-reacting with and neutralizing endogenous OPG. In fact, generation of anti-OPG antibodies was observed in one subject in a phase I study with an Fc-OPG fusion molecule (197).

Another potential concern about anti-RANKL therapy includes its adverse effects on mammary gland. RANKL or RANK knockout mice fail to form lobuloalveolar mammary gland structures during pregnancy and show a complete block in the formation of a lactating mammary gland (211).

## Conclusion

The accumulating knowledge on the molecular mechanism regulating osteoclast development has opened a new era of therapeutic approaches against pathological bone disorders. In spite of the overwhelming scientific evidence that the RANKL/RANK/OPG axis plays a central role in the pathological bone destruction, which makes it an ideal therapeutic target, clinical application of anti-RANKL therapy has just begun. Intense basic and clinical studies will further uncover the potential advantages and disadvantages of anti-RANKL therapy.

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## Regulation of Osteoclast Apoptosis and Motility by Small GTPase Binding Protein Rac1\*

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**ABSTRACT:** The role of Rac1 in osteoclast survival and bone-resorbing activity was examined using adenovirus vector expression systems. Rac1 is critically involved in M-CSF receptor signaling and mediates survival signaling primarily through PI3K/Akt pathways. Rac1 also plays a significant role in bone resorptive activity, probably by regulating the motility of osteoclasts.

**Introduction:** Rac1 is a member of Rho family small G-proteins, and recent studies have revealed that it mediates anti-apoptotic signals in some types of cells. Rac1 is reported to be required for the cytoskeletal organization and bone-resorbing activity of osteoclasts, but their roles in osteoclast survival and function are not fully elucidated.

**Materials and Methods:** We constructed the adenovirus vector carrying cDNA of either the *dominant negative Rac1 (Rac1<sup>DN</sup>)* or *constitutively active Rac1 (Rac1<sup>CA</sup>)* gene, and osteoclast-like cells (OCLs) generated in mouse co-culture system were infected with these viruses. To examine the role of Rac1 in osteoclast survival and function, we performed pit formation assays, survival assays, and Western blotting, including an activated-Rac1 pull-down assay using adenovirus-infected OCLs. To further clarify the mechanism of Rac1 regulation in osteoclast survival, some specific inhibitors and adenovirus vectors of signal transduction molecules were used. To quantify membrane movement before and after macrophage colony-stimulating factor (M-CSF) treatment, OCLs expressing either enhanced green fluorescent protein (EGFP) or Rac1<sup>DN</sup> were recorded with a time-lapse video microscope.

**Results:** Adenovirus vector-mediated dominant negative Rac1 (Rac1<sup>DN</sup>) expression significantly reduced pit formation, and promoted their apoptosis. M-CSF rapidly activated Rac1, and the prosurvival effect of M-CSF for OCLs was abrogated by Rac1<sup>DN</sup> overexpression. Constitutively active Rac1 enhanced OCL survival, which was completely suppressed by phosphatidylinositol 3'-kinase (PI3K) inhibitors, whereas a Mek inhibitor had only partial effect. Rac1<sup>DN</sup> also partially blocked the activation of Akt induced by the overexpressing catalytic subunit of PI3K. Using time-lapse video microscopy, we found that Rac1<sup>DN</sup> expression reduced membrane ruffling and the spreading of OCLs in response to M-CSF.

**Conclusions:** Small guanosine triphosphatase (GTPase) Rac1 is critically involved in M-CSF receptor signaling and mediates survival signaling of osteoclasts primarily by modulating PI3K/Akt pathways. Rac1 also plays a significant role in the bone resorptive activity of cells, probably by regulating the motility of osteoclasts.

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**Key words:** osteoclast, apoptosis, Rac1, Akt, macrophage colony-stimulating factor

### INTRODUCTION

OSTEOCLASTS ARE PRIMARILY responsible for bone resorption and play essential roles in maintaining skeletal homeostasis. They are terminally differentiated cells with a short life span and undergo rapid apoptosis in the

absence of trophic factors, such as macrophage colony-stimulating factor (M-CSF) and RANKL.<sup>(1,2)</sup> Although recent findings suggest that osteoclast survival is regulated through interactions of various hormones and cytokines,<sup>(3,4)</sup> the underlying molecular mechanism is not fully understood. Bisphosphonates are widely used in the management of osteoporosis and are known to suppress pathological bone resorption by directly suppressing osteoclast activity. Numerous studies have shown that one of the principle mechanisms of bisphosphonate action is to induce osteoclast apoptosis both in vitro and in vivo. The remarkable success of bisphosphonates as an anti-osteoporotic treat-

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The authors have no conflict of interest.

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