近の研究の進歩によるところが大きい．破骨細胞の分化と骨吸収とサイトカインとの関連を証明する更なる研究の発展が期待される．

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CHAPTER 7

Cells of Bone

Osteoclast Generation

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Introduction

Osteoclasts, the multinucleated giant cells that resorb bone, develop from hemopoietic cells of the monocyte–macrophage lineage. We have developed a mouse coculture system of osteoblasts/stromal cells and hemopoietic cells in which osteoclasts are formed in response to bone-resorbing factors such as 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], parathyroid hormone (PTH), prostaglandin E_2 (PGE_2), and interleukin 11 (IL-11). A series of experiments using this coculture system have established the concept that osteoblasts/stromal cells are crucially involved in osteoclast development. Cell-to-cell contact between osteoblasts/stromal cells and osteoclast progenitors was necessary for the induction of osteoclast differentiation in the coculture system. Studies on macrophage colony-stimulating factor (M-CSF, also called CSF-1)-deficient *op/op* mice have shown that M-CSF produced by osteoblasts/stromal cells is an essential factor for inducing osteoclast differentiation from monocyte–macrophage lineage cells. Subsequently, in 1998, another essential factor for osteoclastogenesis, receptor activator of nuclear factor κB ligand (RANKL) was cloned molecularly. RANKL [also known as osteoclast differentiation factor (ODF)/osteoprotegerin ligand (OPGL)/TNF-related activation-induced cytokine (TRANCE)] is a new member of the tumor necrosis factor (TNF)-ligand family, which is expressed as a membrane-associated protein in osteoblasts/stromal cells in response to many bone-resorbing factors. Osteoclast precursors that possess RANK, a TNF receptor family member, recognize RANKL through cell–cell interaction with osteoblasts/stromal cells and differentiate into osteoclasts in the presence of M-CSF. Mature osteoclasts also express RANK, and RANKL induces their bone-resorbing activity. Osteoprotegerin [OPG, also called osteoclastogenesis inhibitory factor (OCIF)] mainly produced by osteoblasts/stromal cells is a soluble decoy receptor for RANKL. OPG has been shown to function as an inhibitory factor for osteoclastogenesis *in vivo* and *in vitro*. Thus, the rapid advances in osteoclast biology have elucidated the precise mechanism by which osteoblasts/stromal cells regulate osteoclast differentiation and function. Activation of nuclear factor κB (NF-κB) and c-Jun N-terminal protein kinase (JNK) through RANK-mediated signals appears to be involved in the differentiation and activation of osteoclasts. Findings also indicate that TNFα directly induces differentiation of osteoclasts by a mechanism independent of the RANKL–RANK interaction. This chapter describes the current knowledge of the regulatory mechanisms of osteoclast differentiation induced by osteotropic hormones and cytokines.

Role of Osteoblasts/Stromal Cells in Osteoclast Differentiation and Function

Osteoblasts/Stromal Cells Regulate Osteoclast Differentiation

Development of osteoclasts proceeds within a local microenvironment of bone. This process can be reproduced *ex vivo* in a coculture of mouse calvarial osteoblasts and hemopoietic cells (Chambers *et al.*, 1993; Suda *et al.*, 1992; Takahashi *et al.*, 1988). Multinucleated cells formed in the coculture exhibit major characteristics of osteoclasts, including tartrate-resistant acid phosphatase (TRAP) activity, expression of calcitonin receptors, c-Src (p60c-src),

vitronectin receptors ($\alpha v\beta 3$), and the ability to form resorption pits on bone and dentine slices (Suda *et al.*, 1992). Some mouse stromal cell lines, such as MC3T3-G2/PA6 and ST2, are able to support osteoclastogenesis when cultured with mouse spleen cells (Udagawa *et al.*, 1989). In this coculture, osteoclasts were formed in response to various osteotropic factors, including $1\alpha,25(OH)_2D_3$, PTH, PGE_2, and IL-11. Cell-to-cell contact between osteoblasts/stromal cells and osteoclast progenitors is required to induce osteoclastogenesis. Subsequent experiments have established that the target cells of osteotropic factors for inducing osteoclast formation *in vitro* are osteoblasts/stromal cells (Table I).

IL-6 exerts its activity via a cell surface receptor that consists of two components: a ligand-binding IL-6 receptor (IL-6R) and a nonligand-binding but signal-transducing protein gp130 (Taga and Kishimoto, 1997). The genetically engineered soluble IL-6R (sIL-6R), which lacks both transmembrane and cytoplasmic domains, has been shown to mediate IL-6 signals through gp130 in response to IL-6. Neither recombinant IL-6 nor sIL-6R alone induced osteoclast formation in the coculture, but osteoclasts were formed in response to IL-6 in the presence of sIL-6R (Tamura *et al.*, 1993). This suggests that a signal(s) mediated by gp130 is involved in osteoclast development. Using transgenic mice constitutively expressing human IL-6R, it was shown that the expression of human IL-6R in osteoblasts was indispensable for inducing osteoclast recruitment (Udagawa *et al.*, 1995) (Table I). When osteoblasts obtained from human IL-6R transgenic mice were cocultured with normal spleen cells, osteoclast formation was induced in response to human IL-6 without the addition of human sIL-6R. Indeed, cytokines such as IL-11, oncostatin M, and leukemia inhibitory factor (LIF), which transduce their signals through gp130 in osteoblasts/stromal cells, induced osteoclast formation in the coculture. These results established for the first time the concept that bone-resorbing cytokines using gp130 as a common signal transducer act directly on osteoblasts/stromal cells but not on osteoclast progenitors to induce osteoclast formation.

Requirement of PTH/PTHrP receptors (PTHR1) in the osteoblast was confirmed using cocultures of osteoblasts and spleen cells obtained from PTHR1 knockout mice (Liu *et al.*, 1998). Osteoblasts obtained from PTHR1(−/−) mice failed to support osteoclast development in cocultures with normal spleen cells in response to PTH (Table I). Osteoclasts were formed in response to PTH in cocultures of spleen cells obtained from PTHR1(−/−) mice and normal calvarial osteoblasts. This suggests that the expression of PTHR1 in osteoblasts/stromal cells is critical for PTH-induced osteoclast formation *in vitro*.

PGE_2 exerts its effects through PGE receptors (EPs) that consist of four subtypes (EP1, EP2, EP3, and EP4) (Breyer and Breyer, 2000). Intracellular signaling differs among the receptor subtypes: EP1 is coupled to Ca^{2+} mobilization and EP3 inhibits adenylate cyclase activity, whereas both EP2 and EP4 stimulate adenylate cyclase activity. It was reported that 11-deoxy-PGE1 (an EP4 and EP2 agonist) stimulated osteoclast formation more effectively than butaprost (an EP2 agonist) and other EP agonists in the coculture of primary osteoblasts and bone marrow cells, suggesting that EP4 is the main receptor responsible for PGE_2-induced osteoclast formation (Sakuma *et al.*, 2000). Furthermore, the PGE_2-induced osteoclast formation was not observed in the coculture of osteoblasts from EP4(−/−) mice and spleen cells from wild-type mice, whereas osteoclasts were formed in the coculture of wild-type osteoblasts and EP4(−/−) spleen cells (Sakuma *et al.*, 2000) (Table I). These results indicate that PGE_2 enhances osteoclast formation through the EP4 subtype on osteoblasts. Li *et al.* (2000b) used cells from mice in which the EP2 receptor had been disrupted to test the role of EP2 in osteoclast formation. The response to PGE_2 for osteoclast formation was also reduced in cultures of bone marrow cells obtained from EP2(−/−) mice. In mouse calvarial organ cultures, the EP4 agonist stimulated bone resorption markedly, but its maximal stimulation was less

Table I Osteoclast Formation in Cocultures with hIL-6R Transgenic Mice or Mice Carrying the Disrupted Genes of VDR, PTHR1, or EP4

Osteotropic factor	Coculture system[a]		Osteoclast formation	Reference
	Osteoblasts	Hemopoietic cells		
hIL-6	wt	hIL-6R tg	−	Udagawa *et al.* (1995)
	hIL-6R tg	wt	+	
PTH	PTHR1(−/−)	wt	−	Liu *et al.* (1998)
	wt	PTHR1(−/−)	+	
PGE_2	EP4(−/−)	wt	−	Sakuma *et al.* (2000)
	wt	EP4(−/−)	+	
$1\alpha,25(OH)_2D_3$	VDR(−/−)	wt	−	Takeda *et al.* (1999)
	wt	VDR(−/−)	+	

[a] Osteoblasts or hemopoietic cells obtained from human IL-6R (hIL-6R) transgenic (tg) mice, VDR knockout [VDR(−/−)] mice, PTHR1 knockout [PTHR1(−/−)] mice, or EP4 knockout [EP4(−/−)] mice were cocultured with their counterpart (osteoblasts or hemopoietic cells) obtained from wild-type (wt) mice.

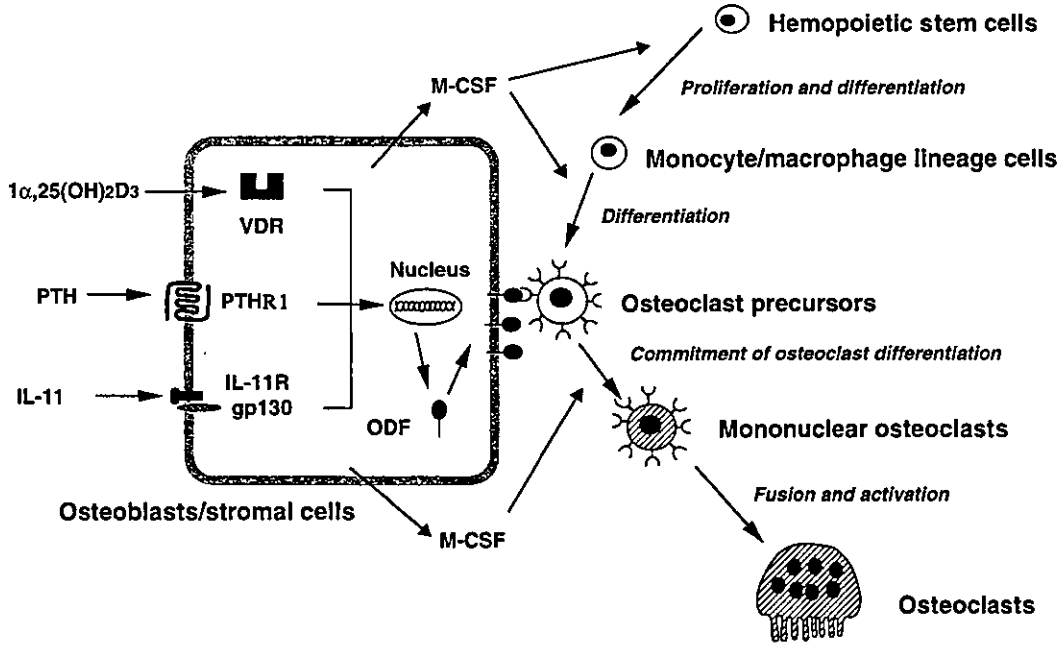


Figure 1 Hypothetical concept of osteoclast differentiation and a proposal for osteoclast differentiation factor (ODF). Osteotropic factors such as $1\alpha,25(OH)_2D_3$, PTH, and IL-11 stimulate osteoclast formation in mouse cocultures of osteoblasts/stromal cells and hemopoietic cells. The target cells of these osteotropic factors are osteoblasts/stromal cells. Three independent signaling pathways mediated by $1\alpha,25(OH)_2D_3$–VDR, PTH–PTHR1, and IL-11–IL-11R/gp130 interactions induce ODF as a membrane-associated factor in osteoblasts/stromal cells in a similar manner. Osteoclast progenitors of the monocyte–macrophage lineage recognize ODF through cell–cell interaction with osteoblasts/stromal cells and differentiate into osteoclasts. M-CSF produced by osteoblasts/stromal cells is a prerequisite for both proliferation and differentiation of osteoclast progenitors. This hypothetical concept has been proven molecularly by the discovery of the RANKL–RANK interaction.

than that induced by PGE_2 (Suzawa *et al.*, 2000). The EP2 agonist also stimulated bone resorption, but only slightly. EP1 and EP3 agonists showed no effect on bone resorption. These findings suggest that PGE_2 stimulates bone resorption by a mechanism involving cAMP production in osteoblasts/stromal cells, mediated mainly by EP4 and partially by EP2.

The other known pathway used for osteoclast formation is that stimulated by $1\alpha,25(OH)_2D_2$. Using $1\alpha,25(OH)_2D_3$ receptor (VDR) knockout mice, Takeda *et al.* (1999) clearly showed that the target cells of $1\alpha,25(OH)_2D_3$ for inducing osteoclasts in the coculture were also osteoblasts/stromal cells but not osteoclast progenitors (Table I). Spleen cells from VDR(−/−) mice differentiated into osteoclasts when cultured with normal osteoblasts in response to $1\alpha,25(OH)_2D_3$. In contrast, osteoblasts obtained from VDR(−/−) mice failed to support osteoclast development in coculture with wild-type spleen cells in response to $1\alpha,25(OH)_2D_3$. These results suggest that the signals mediated by VDR are also transduced into osteoblasts/stromal cells to induce osteoclast formation in the coculture.

Thus, the signals induced by almost all the bone-resorbing factors are transduced into osteoblasts/stromal cells to recruit osteoclasts in the coculture. Therefore, we proposed that osteoblasts/stromal cells express ODF, which is hypothesized to be a membrane-bound factor in promoting the differentiation of osteoclast progenitors into osteoclasts through a mechanism involving cell-to-cell contact (Suda *et al.*, 1992) (Fig. 1).

M-CSF Produced by Osteoblasts/Stromal Cells Is an Essential Factor for Osteoclastogenesis

Experiments with the *op/op* mouse model have established the role for M-CSF in osteoclast formation. Yoshida *et al.* (1990) demonstrated that there is an extra thymidine insertion at base pair 262 in the coding region of the M-CSF gene in *op/op* mice. This insertion generated a stop codon (TGA) 21 bp downstream, suggesting that the M-CSF gene of *op/op* mice cannot code for the functionally active M-CSF protein. In fact, administration of recombinant human M-CSF restored the impaired bone resorption of *op/op* mice *in vivo* (Felix *et al.*, 1990; Kodama *et al.*, 1991). Calvarial osteoblasts obtained from *op/op* mice could not support osteoclast formation in cocultures with normal spleen cells, even in the presence of $1\alpha,25(OH)_2D_3$ (Suda *et al.*, 1997a). The addition of M-CSF to the coculture with *op/op* osteoblastic cells induced osteoclast formation from normal spleen cells in response to $1\alpha,25(OH)_2D_3$. In contrast, spleen cells obtained from *op/op* mice were able to differentiate into osteoclasts when cocultured with normal osteoblasts. It was shown that M-CSF is involved in both proliferation of osteoclast progenitors and differentiation into osteoclasts (Felix *et al.*, 1994; Tanaka *et*

al., 1993). Begg *et al.* (1993) investigated age-related changes in osteoclast activity in *op/op* mice. Femurs of newborn *op/op* mice were infiltrated heavily with bone, and the marrow hemopoiesis was reduced significantly. However, the femoral marrow cavity of *op/op* mice enlarged progressively with the concomitant appearance of TRAP-positive osteoclasts, and by 22 weeks of age the marrow hemopoiesis was comparable to that of controls. Niida *et al.* (1999) reported that a single injection in *op/op* mice with recombinant human vascular endothelial growth factor (VEGF) induced osteoclast recruitment. These results suggest that factors other than M-CSF, including VEGF, can substitute for M-CSF to induce osteoclast formation under special occasions.

Osteoblasts/Stromal Cells Regulate Osteoclast Function

One of the major technical difficulties associated with the analysis of mature osteoclasts is their strong adherence to plastic dishes. We have developed a collagen-gel culture using mouse bone marrow cells and osteoblasts/stromal cells to obtain a cell preparation containing functionally active osteoclasts (Akatsu *et al.*, 1992; Suda *et al.*, 1997a). The purity of osteoclasts in this preparation was only 2–3%, contaminated with numerous osteoblasts. However, this crude osteoclast preparation proved to be a useful source to establish a reliable resorption pit assay on dentine slices. Therefore, osteoclasts were purified via centrifugation of the crude osteoclast preparation in a 30% Percoll solution (Jimi *et al.*, 1996b). Interestingly, these highly purified osteoclasts (purity: 50–70%) cultured for 24 h on dentine slices failed to form resorption pits. Resorptive capability of the purified osteoclasts was restored when osteoblasts/stromal cells were added to the purified osteoclast preparation. Similarly, Wesolowski *et al.* (1995) obtained highly purified mononuclear and binuclear prefusion osteoclasts using echistatin from mouse cocultures of bone marrow cells and osteoblastic MB 1.8 cells. These enriched prefusion osteoclasts failed to form resorption pits on bone slices, but their bone-resorbing activity was induced when both MB 1.8 cells and $1\alpha,25(OH)_2D_3$ were added to the prefusion osteoclast cultures. These results suggest that osteoblasts/stromal cells play an essential role not only in the stimulation of osteoclast formation, but also in the activation of mature osteoclasts to resorb bone, which is also a cell-to-cell contact-dependent process (Suda *et al.*, 1997b).

Discovery of the RANKL–RANK Interaction for Osteoclastogenesis

Discovery of OPG

OPG was cloned as a new member of the TNF receptor superfamily in an expressed sequence tag cDNA project (Simonet *et al.*, 1997). Interestingly, OPG lacked a transmembrane domain and presented as a secreted form. Hepatic expression of OPG in transgenic mice resulted in severe osteopetrosis. Osteoclastogenesis inhibitory factor (OCIF), which inhibited osteoclast formation in the coculture of osteoblasts and spleen cells, was isolated as a heparin-binding protein from the conditioned medium of human fibroblast cultures (Tsuda *et al.*, 1997). The cDNA sequence of OCIF was identical to that of OPG (Yasuda *et al.*, 1998a). Tan *et al.* (1997) also identified a new member of the TNF receptor family called TNF receptor-like molecule 1 (TR1) from a search of an expressed sequence tag database. TR1 was also found to be identical to OPG/OCIF.

OPG contains four cysteine-rich domains and two death domain homologous regions (Fig. 2). The death domain homologous regions share structural features with "death domains" of TNF type I receptor (p55) and Fas, both of which mediate apoptotic signals. Analysis of the domain-deletion mutants of OPG revealed that the cysteine-rich domains, but not the death domain homologous regions, are essential for inducing biological activity *in vitro*. When the transmembrane domain of Fas was inserted between the cysteine-rich domains and the death domain homologous regions, and the mutant protein was then expressed in the human kidney cell line 293-EBNA, apoptosis was induced in the transfected cells (Yamaguchi *et al.*, 1998). The biological significance of the death domain homologous regions in the OPG molecule, however, remains largely unknown at present.

OPG strongly inhibited osteoclast formation induced by $1\alpha,25(OH)_2D_3$, PTH, PGE_2, or IL-11 in the cocultures. Analyses of transgenic mice overexpressing OPG and animals injected with OPG have demonstrated that this factor increases bone mass by suppressing bone resorption (Simonet *et al.*, 1997; Yasuda *et al.*, 1998a). Administration of OPG to rats decreased the serum calcium concentration rapidly (Yamamoto *et al.*, 1998). The physiological role of OPG was investigated further in OPG-deficient mice (Bucay *et al.*, 1998; Mizuno *et al.*, 1998). These mutant mice were viable and fertile, but adolescent and adult OPG(−/−) mice exhibited a decrease in bone mineral density (BMD) characterized by severe trabecular and cortical bone porosity, marked thinning of parietal bones of the skull, and a high incidence of fractures. Interestingly, osteoblasts derived from OPG(−/−) mice strongly supported osteoclast formation in the coculture even in the absence of any bone-resorbing agents (Udagawa *et al.*, 2000). Bone-resorbing activity in organ cultures of fetal long bones derived from OPG(−/−) mice was also strikingly higher in the absence of bone-resorbing factors when compared to that of wild-type mice. Osteoblasts prepared from OPG(−/−) mice and wild-type mice expressed comparable levels of RANKL mRNA. These results indicate that OPG produced by osteoblasts/stromal cells functions as an important negative regulator in osteoclast differentiation and activation *in vivo* and *in vitro*.

Discovery of the RANKL–RANK Interaction

The mouse bone marrow-derived stromal cell line ST2 supports osteoclast formation in the coculture with mouse

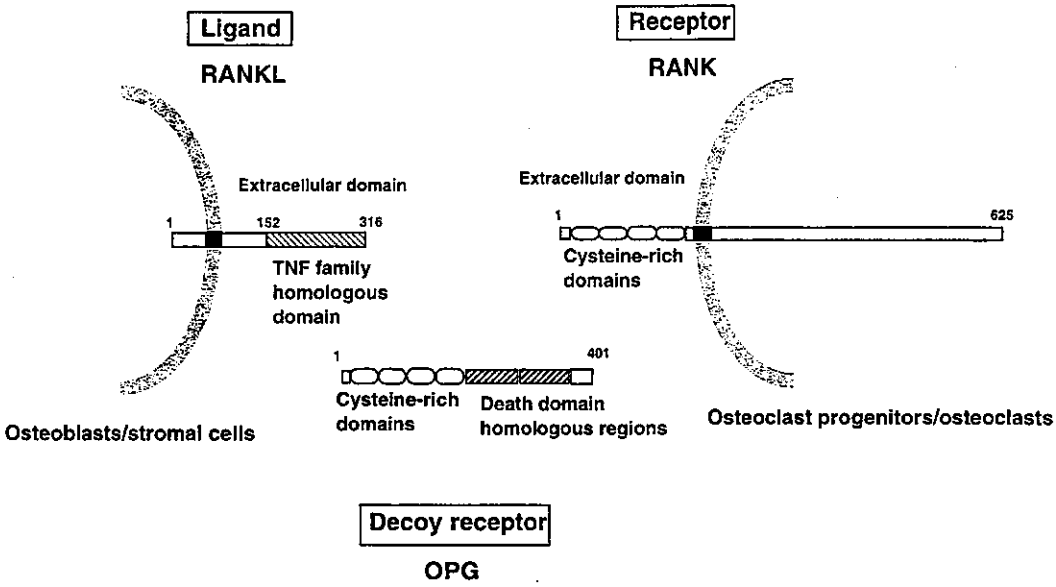


Figure 2 Diagrammatic representation of the ligand, receptor, and decoy receptor of the new TNF receptor–ligand family members essentially involved in osteoclastogenesis. Mouse RANKL is a type II transmembrane protein composed of 316 amino acid residues. The TNF homologous domain exists in Asp_{152}-Asp_{316}. RANK is a type I transmembrane protein of 625 amino acid residues. Four cysteine-rich domains exist in the extracellular region of the RANK protein. OPG, a soluble decoy receptor for RANKL, is composed of 401 amino acid residues without a transmembrane domain. OPG also contains four cysteine-rich domains and two death domain homologous regions. The cysteine-rich domains but not the death domain homologous regions of OPG are essential for inhibiting osteoclast differentiation and function.

spleen cells in the presence of $1\alpha,25(OH)_2D_3$ and dexamethasone (Udagawa *et al.*, 1989). OPG bound to a single class of high-affinity binding sites appearing on ST2 cells treated with $1\alpha,25(OH)_2D_3$ and dexamethasone (Yasuda *et al.*, 1998a). Using OPG as a probe, Yasuda *et al.* (1998b) cloned a cDNA with an open reading frame encoding 316 amino acid residues from an expression library of ST2 cells. The OPG-binding molecule was a type II transmembrane protein of the TNF ligand family (Fig. 2). Because the OPG-binding molecule satisfied major criteria of ODF, this molecule was renamed ODF. Lacey *et al.* (1998) also succeeded in the molecular cloning of the ligand for OPG (OPGL) from an expression library of the murine myelomonocytic cell line 32D. Molecular cloning of ODF/OPGL demonstrated that it was identical to TRANCE (Wong *et al.*, 1997b) and RANKL (Anderson *et al.*, 1997), which had been identified independently by other research groups in the immunology field.

TRANCE was cloned during a search for apoptosis-regulatory genes in murine T cell hybridomas (Wong *et al.*, 1997b). A recombinant soluble form of TRANCE induced activation of JNK in T lymphocytes and inhibited apoptosis of mouse and human dendritic cells. A new member of the TNF receptor family, termed "RANK," was cloned from a cDNA library of human dendritic cells (Anderson *et al.*, 1997). The mouse homologue was also isolated from a fetal mouse liver cDNA library. The mouse RANK cDNA encodes a type I transmembrane protein of 625 amino acid residues with four cycteine-rich domains in the extracellular region (Fig. 2). RANKL was cloned from a cDNA library of murine thymoma EL40.5 cells and was found to be identical to TRANCE. A soluble form of RANKL augmented the capability of dendritic cells to stimulate T cell proliferation in a mixed lymphocyte reaction and increased the survival of RANK-positive T-cells (Wong *et al.*, 1997a). The N-terminal region of RANK has a similar structure to that of OPG, a decoy receptor for RANKL (Fig. 2).

Polyclonal antibodies against the extracellular domains of RANK (anti-RANK Ab) have been shown to induce osteoclast formation in spleen cell cultures in the presence of M-CSF (Hsu *et al.*, 1999; Nakagawa *et al.*, 1998). This suggests that the clustering of RANK is required for the RANK-mediated signaling of osteoclastogenesis. In contrast, the anti-RANK antibody, which lacks the Fc fragment, (the Fab fragment), completely blocked the RANKL-mediated osteoclastogenesis (Nakagawa *et al.*, 1998). A soluble form of RANK, an extracellular domain of RANK, not only inhibited RANKL-mediated osteoclast formation, but also prevented the survival, multinucleation, and pit-forming activity of prefusion osteoclasts treated with RANKL (Jimi *et al.*, 1999a). Transgenic mice expressing a soluble RANK-Fc fusion protein showed osteopetrosis, similar to OPG transgenic mice (Hsu *et al.*, 1999). Taken together, these results suggest that RANK acts as the sole signaling receptor for RANKL in inducing differentiation and subsequent activation of osteoclasts (Fig. 2).

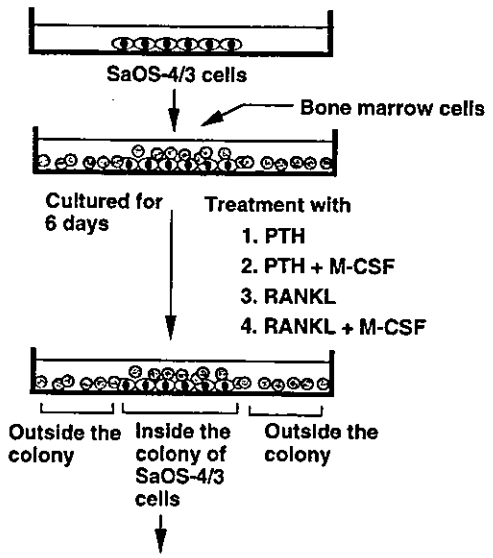
Thus, ODF, OPGL, TRANCE, and RANKL are different names for the same protein, which is important for the development and function of T cells, dendritic cells and osteoclasts. The terms "RANKL," "RANK," and "OPG" are

used in this chapter in accordance with the guideline of the American Society for Bone and Mineral Research President's Committee on Nomenclature (2000).

Role of RANKL in Osteoclast Differentiation and Function

When COS-7 cells transfected with a RANKL expression vector were fixed with paraformaldehyde and cocultured with mouse spleen cells in the presence of M-CSF, osteoclasts were formed on the fixed COS-7 cells (Yasuda *et al.*, 1998b). A genetically engineered soluble form of RANKL, together with M-CSF, induced osteoclast formation from spleen cells in the absence of osteoblasts/stromal cells, which was abolished completely by the simultaneous addition of OPG. Treatment of calvarial osteoblasts with $1\alpha,25(OH)_2D_3$, PTH, PGE_2, or IL-11 upregulated the expression of RANKL mRNA. Human osteoclasts were also formed in cultures of human peripheral blood mononuclear cells in the presence of RANKL and human M-CSF (Matsuzaki *et al.*, 1998). This suggests that the mechanism of human osteoclast formation is essentially the same as that of mouse osteoclast formation. Lum *et al.* (1999) reported that like TNFα, RANKL is synthesized as a membrane-anchored precursor and is detached from the plasma membrane to generate the soluble form of RANKL by a metalloprotease–disintegrin TNFα convertase (TACE). Soluble RANKL demonstrated a potent activity in the induction of dendritic cell survival and osteoclastogenesis. These findings suggest that the ectodomain of RANKL is released from the cells by TACE or a related metalloprotease-disintegrin and that this release is an important component of the function of RANKL in bone and immune homeostasis.

We carefully examined the mechanism of action of RANKL and M-CSF expressed by osteoblasts/stromal cells that support osteoclast formation (Itoh *et al.*, 2000b) (Fig. 3). SaOS-4/3, a subclone of the human osteosarcoma cell line SaOS-2, was established by transfecting the human PTHR1 cDNA. SaOS-4/3 cells supported human and mouse osteoclast formation in response to PTH in cocultures with human peripheral blood mononuclear cells and mouse bone marrow cells, respectively (Matsuzaki *et al.*, 1999). Osteoclast formation supported by SaOS-4/3 cells was completely inhibited by adding either OPG or antibodies against human M-CSF. This suggests that RANKL and M-CSF are both essential factors for inducing osteoclast formation in the coculture with SaOS-4/3 cells. To elucidate the functional form of both RANKL and M-CSF, SaOS-4/3 cells were spot cultured for 2 hr in the center of a culture well and then mouse bone marrow cells were plated uniformly over the well (Fig. 3). When the spot coculture was treated for 6 days with PTH together with or without M-CSF, osteoclast formation was induced exclusively inside the colony of SaOS-4/3 cells irrespective of the exogenous addition of M-CSF. Similarly, when the spot coculture was treated with RANKL, osteoclasts were formed only inside the colony of SaOS-4/3 cells, suggesting that M-CSF acts as a membrane- or matrix-associated form in the coculture. However, the concomitant treatment with RANKL and M-CSF induced osteoclast formation both inside and outside the colony of SaOS-4/3 (Fig. 3). Similar results were obtained in the spot coculture with OPG(−/−) mouse-derived osteoblasts



Treatment with	Osteoclast formation	
	Inside the colony of SaOS-4/3 cells	Outside the colony of SaOS-4/3 cells
1. PTH	+	—
2. PTH + M-CSF	+	—
3. RANKL	+	—
4. RANKL + M-CSF	+	+

Figure 3 Both M-CSF and RANKL act as membrane- or matrix-associated forms in osteoclast formation. SaOS-4/3 cells expressing recombinant human PTHR1 were spot cultured for 2 hr in the center of a culture well; subsequently, mouse bone marrow cells were plated uniformly over the well. Spot cocultures were treated for 6 days with PTH, PTH plus M-CSF, RANKL, or RANKL plus M-CSF. Cells were then fixed and stained for TRAP. The location of TRAP-positive osteoclasts in the culture well was observed under a microscope. Osteoclasts formed outside the colony of SaOS-4/3 cells were observed only when the spot cocultures were treated with both RANKL and M-CSF.

(Udagawa *et al.*, 2000). These results suggest that membrane- or matrix-associated forms of both M-CSF and RANKL are essentially involved in osteoclast formation supported by osteoblasts/stromal cells. Such a mechanism of action of RANKL and M-CSF on osteoclast progenitors may explain the reason why osteoclasts are localized in bone, despite the relatively wide distribution of RANKL (Kartsogiannis *et al.*, 1999) and M-CSF (Felix *et al.*, 1994; Wood *et al.*, 1997).

Survival, fusion, and pit-forming activity of osteoclasts are also induced by RANKL (Fig. 4). Treatment of prefusion osteoclasts with OPG suppressed their survival, fusion, and pit-forming activity induced by RANKL (Jimi *et al.*, 1999a). RANKL increased bone resorption by isolated rat authentic osteoclasts (Burgess *et al.*, 1999; Fuller *et al.*, 1998). Bone-resorbing factors, such as $1\alpha,25(OH)_2D_3$, PTH, and IL-11, enhanced pit formation by purified osteoclasts only in the presence of osteoblasts (Udagawa *et al.*, 1999). Treatment of prelabeled bone with $1\alpha,25(OH)_2D_3$, PGE_2, and PTH enhanced the release of ^{45}Ca from the bone, which was completely inhibited by the addition of OPG or anti-RANKL antibody (Tsukii *et al.*, 1998). These results suggest that osteoblasts/stromal cells are essentially involved in both differentiation and activation of osteoclasts through the expression of RANKL as a membrane-associated factor (Fig. 4).

RANKL- and RANK-Deficient Mice

The physiological role of RANKL was investigated by generating RANKL-deficient mice (Kong *et al.*, 1999b) (Table II). RANKL(−/−) mice exhibited typical osteopetrosis with total occlusion of bone marrow space within endosteal bone. RANKL(−/−) mice lacked osteoclasts but had normal osteoclast progenitors that can differentiate into functionally active osteoclasts when cocultured with normal osteoblasts/stromal cells. Osteoblasts obtained from RANKL(−/−) mice failed to support osteoclast formation in the coculture with wild-type bone marrow cells even in the presence of $1\alpha,25(OH)_2D_3$ and PGE_2. RANKL(−/−) mice exhibited defects in the early differentiation of T and B lymphocytes. In addition, RANKL(−/−) mice showed normal splenic structure and Peyer's patches, but lacked all lymph nodes. These results suggest that RANKL is an absolute requirement not only for osteoclast development, but it plays an important role in lymphocyte development and lymph node organogenesis.

The physiological role of RANK was also investigated by generating RANK-deficient mice (Dougall *et al.*, 1999) (Table II). The phenotypes of RANK(−/−) mice were essentially the same as those of RANKL(−/−) mice, except for some differences. Like RANKL-deficient mice, RANK(−/−) mice were characterized by severe osteopetrosis resulting from an apparent block in osteoclast differentiation. RANK expression was not required for the commitment, differentiation, and functional maturation of macrophages and dendritic cells from their myeloid precursors, but provided a necessary and specific signal for the differentiation of myeloid-derived osteoclasts. RANK(−/−) mice also exhibited a marked deficiency of B cells in the spleen. RANK(−/−) mice retained mucosal-associated lymphoid tissues, including Peyer's patches, but completely lacked all the other peripheral lymph nodes. These results demonstrate that RANK provides critical signals necessary for lymph node organogenesis and osteoclast differentiation.

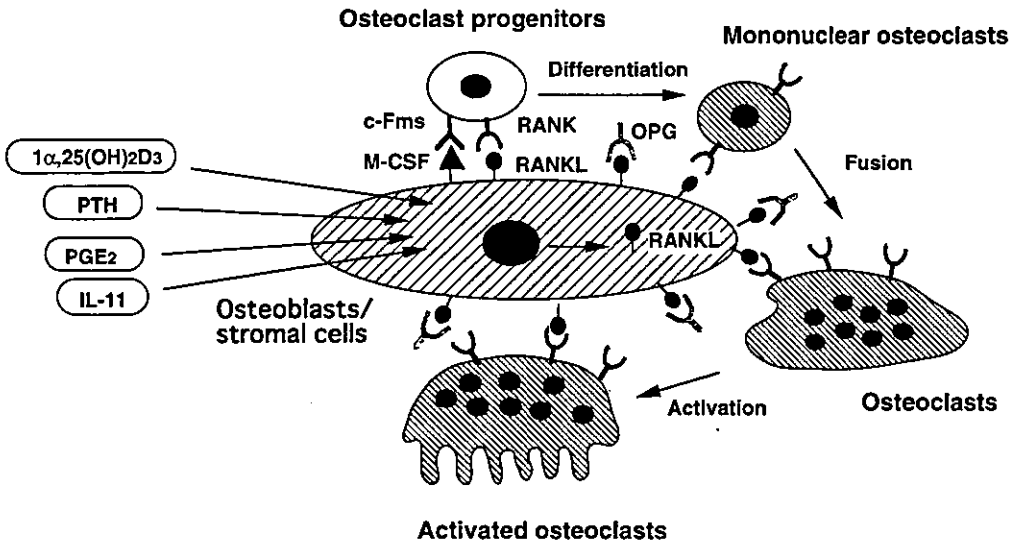


Figure 4 Schematic representation of osteoclast differentiation and function regulated by RANKL and M-CSF. Osteoclast progenitors and mature osteoclasts express RANK, the receptor for RANKL. Osteotropic factors such as $1\alpha,25(OH)_2D_3$, PTH, and IL-11 stimulate expression of RANKL in osteoblasts/stromal cells. Membrane- or matrix-associated forms of both M-CSF and RANKL expressed by osteoblasts/stromal cells are responsible for the induction of osteoclast differentiation in the coculture. RANKL also directly stimulates fusion and activation of osteoclasts. Mainly osteoblasts/stromal cells produce OPG, a soluble decoy receptor of RANKL. OPG strongly inhibits the entire differentiation, fusion, and activation processes of osteoclasts induced by RANKL.

Table II Comparison of Characteristics between RANKL-Deficient Mice and RANK-Deficient Mice

Characteristic	RANKL(−/−) mice	RANK(−/−) mice
Osteopetrosis	Severe	Severe
Osteoclasts in bone	Absence	Absence
Tooth eruption	Impaired	Impaired
Function of macrophages	Normal	Normal
Function of dendritic cells	Normal	Normal
B-cell development	Slightly impaired	Slightly impaired
T-cell development	Slightly impaired	Not impaired
Lymph node formation	Impaired	Impaired
Extramedullary hemopoiesis	Increased	Increased
Bone marrow transplantation	Not curable	Curable

Li *et al.* (2000a) further showed that RANK(−/−) mice lacked osteoclasts and had a profound defect in bone resorption and remodeling and in the development of the cartilaginous growth plates of endochondral bone. Osteopetrosis observed in these mutant mice was rescued by the transplantation of bone marrow from rag1 (recombinase activating gene 1)(−/−) mice, indicating that RANK(−/−) mice have an intrinsic defect in osteoclast lineage cells.

Osteoclastogenesis in RANK(−/−) mice was rescued by the transferring the RANK cDNA back into hematopoietic precursors. These data indicate that RANK is the intrinsic cell surface determinant that mediates RANKL effects on bone resorption.

Activating Mutations of RANK Found in Humans

Familial expansile osteolysis is a rare autosomal dominant disorder of bone characterized by focal areas of increased bone remodeling. The osteolytic lesions, which develop usually in the long bones during early adulthood, show increased osteoblast and osteoclast activity. Hughes *et al.* (2000) reported that the gene responsible for familial expansile osteolysis and familial Paget's disease of bone was mapped to the gene encoding RANK. Two mutations of heterozygous insertion were detected in the first exon of RANK in affected members of four families with familial expansile osteolysis or familial Paget's disease of bone. One mutation was a duplication of 18 bases and the other a duplication of 27 bases, both of which affected the signal peptide region (extracellular domain) of the RANK molecule. Expression of recombinant forms of the mutant RANK proteins revealed perturbations in the expression levels and lack of normal cleavage of the signal peptide. Both mutations caused an increase in RANK-mediated NF-κB signaling *in vitro*, consistent with the presence of an activating mutation. These results further confirm that RANK is involved in osteoclast differentiation and activation in humans as well.

Regulation of RANKL and OPG Expression

Osteoblasts/Stromal Cells

Treatment of calvarial osteoblasts with osteotropic factors such as $1\alpha,25(OH)_2D_3$, PTH, PGE_2 or IL-11, which stimulate osteoclast formation, up regulated the expression of RANKL mRNA. In many cases, expression of OPG mRNA is suppressed by those osteotropic factors. O'Brien *et al.* (1999) reported that the expression of dominant-negative STAT3 or dominant-negative gp130 suppressed RANKL expression in a stromal/osteoblastic cell line (UAMS-32) and osteoclast formation supporting activity stimulated by IL-6 together with soluble IL-6 receptor, oncostatin M, or IL-11 but not by PTH or $1\alpha,25(OH)_2D_3$. This suggests that the gp130/STAT3 signaling pathway induces RANKL expression in osteoblasts. The involvement of PGE receptor subtypes, EP1, EP2, EP3, and EP4, in PGE_2-induced bone resorption was examined using specific agonists for the respective EPs. Both the EP2 agonist and the EP4 agonist induced cAMP production and expression of RANKL mRNA in osteoblastic cells (Suzawa *et al.*, 2000). These results suggest that at least three signals are independently involved in RANKL expression by osteoblasts/stromal cells: VDR-mediated signals by $1\alpha,25(OH)_2D_3$; cAMP/protein kinase A (PKA)-mediated signals by PTH or PGE_2; and gp130-mediated signals by IL-11, IL-6, oncostatin M, and LIF (Fig. 5). Inverted TATA and CAAT boxes, a putative Cbfa1/Osf2/AML3 binding domain, and the repeated half-sites for VDR and the glucocorticoid receptor binding domain are found in the 5'-flanking basic promoter region of the mouse RANKL gene (Kitazawa *et al.*, 1999). Promoter analysis of the RANKL gene may elucidate the precise mechanism of the regulation of RANKL gene expression.

IL-1 stimulates osteoclast formation in the coculture of mouse primary osteoblasts and bone marrow cells, which is completely inhibited by the concomitant addition of indomethacin (Akatsu *et al.*, 1991). A positve correlation was observed between the number of osteoclasts induced by IL-1 and the amount of PGE_2 released into the culture

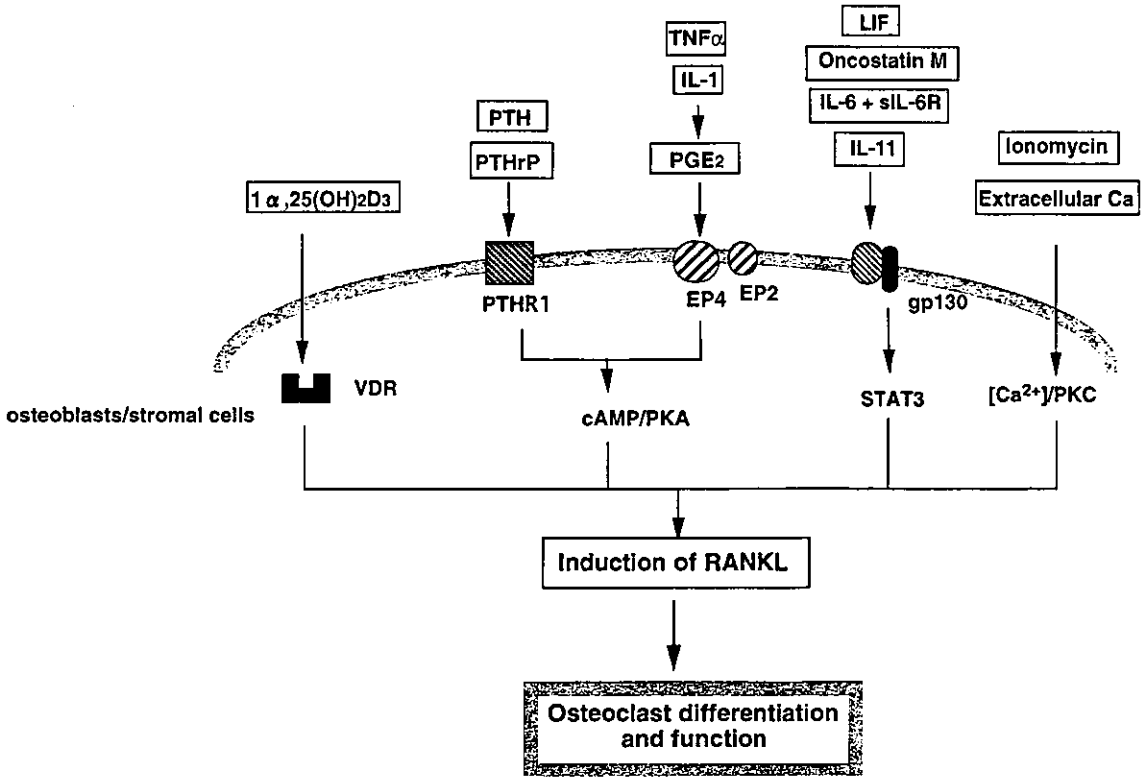


Figure 5 Signaling pathways for the induction of RANKL in osteoblasts/stromal cells. Three independent signals have been proposed to induce RANKL expression in osteoblasts/stromal cells: VDR-mediated signals by $1\alpha,25(OH)_2D_3$, cAMP/PKA-mediated signals by PTH and PGE_2, and gp130-mediated signals by IL-11, IL-6, oncostatin M, and LIF. IL-1 and TNFα also stimulate RANKL expression in osteoblasts/stromal cells through the up regulation of PGE_2 production. The calcium/PKC signal in osteoblasts/stromal cells, which is induced by iomomycin or high calcium concentrations of the culture medium, is now proposed to be the fourth signal involved in the induction of RANKL mRNA expression. RANKL expression induced by these four signals in osteoblasts/stromal cells in turn stimulates osteoclast differentiation and function.

medium. Sakuma *et al.* (2000) reported that osteoclasts were barely induced by IL-1 and TNFα in the coculture of primary osteoblasts and bone marrow cells prepared from EP4(−/−) mice, suggesting the crucial involvement of PGs and the EP4 subtype in osteoclast formation by IL-1 and TNFα (Fig. 5). In contrast, osteoclast formation induced by $1\alpha,25(OH)_2D_3$ was not impaired in the coculture of EP4(−/−) mouse-derived cells. These results suggest that PGE_2 is involved in the mechanism of IL-1- and TNFα-mediated osteoclast formation *in vitro* (Fig. 5).

Compounds, which elevate intracellular calcium, such as ionomycin, cyclopiazonic acid, and thapsigargin, also induced osteoclast formation in mouse cocultures of bone marrow cells and primary osteoblasts (Takami *et al.*, 1997). Similarly, high calcium concentrations of the culture medium induced osteoclast formation in the cocultures. Treatment of primary osteoblasts with these compounds or high medium calcium stimulated the expression of both RANKL and OPG mRNAs (Takami *et al.*, 2000). Phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C (PKC), also stimulated osteoclast formation in these cocultures and the expression of RANKL and OPG mRNAs in primary osteoblasts. PKC inhibitors, such as calphostin and staurosporin, suppressed ionomycin- and PMA-induced osteoclast formation in the coculture as well as the expression of RANKL and OPG mRNAs in primary osteoblasts. OPG strongly inhibited osteoclast formation induced by calcium-elevating compounds and PMA in the cocultures, suggesting that RANKL expression in osteoblasts is a rate-limiting step for osteoclast formation induced by calcium-elevating compounds. Forskolin , an activator of cAMP/protein kinase A (PKA) signals, also enhanced RANKL mRNA expression but, inversely, suppressed OPG mRNA expression in primary osteoblasts. Thus, calcium/PKC signals stimulate the expression of OPG mRNA whereas cAMP/PKA signals inhibit it, although both signals induce RANKL expression in osteoblasts/stromal cells in a similar manner. Therefore, the calcium/PKC signal is proposed to be the fourth signal pathway involved in the induction of RANKL mRNA expression, which in turn stimulates osteoclast formation (Fig. 5).

During embryonic bone development, osteoclasts appear just after bone mineralization takes place. Implantation of bone morphogenetic proteins (BMPs) into muscle or subcutaneous tissues induces ectopic bone formation at the site of the implantation. In this case, osteoclasts also appear just after bone tissue mineralization is initiated by BMPs. These results suggest that the physiological expression of RANKL in osteoblasts occurs in response to an endogeneous factor(s)

present in mineralized tissues. The calcium/PKC signaling system is one of the candidates that induce osteoclast formation in calcified bone. Further studies are necessary to elucidate the involvement of calcium/PKC signals in the regulation of osteoclast formation.

T LYMPHOCYTES

The RANKL–RANK interaction has been shown to regulate lymph node organogenesis, lymphocyte development, and interactions between T cells and dendritic cells in the immune system. RANKL expression in T cells is induced by antigen receptor engagement. Kong *et al.* (1999a) reported that activated T cells directly triggered osteoclastogenesis through RANKL expression. Using specific inhibitors, it was shown that the induction of RANKL by T cells depends on PKC, phosphoinositide-3 kinase, and calcineurin-mediated signaling pathways. RANKL was detected on the surface of activated T cells. Activated T cells also secreted soluble RANKL into culture medium. Both membrane-bound and soluble RANKL supported osteoclast development *in vitro*. Systemic activation of T cells *in vivo* also induced a RANKL-mediated increase in osteoclastogenesis and bone loss. In a T-cell-dependent model of rat adjuvant arthritis characterized by severe joint inflammation, treatment with OPG at the onset of the disease prevented bone and cartilage destruction but not inflammation. These results suggest that both systemic and local T-cell activation can lead to RANKL production and subsequent bone loss. Horwood *et al.* (1999) also reported that human peripheral blood-derived T cells, prepared with anti-CD3 antibody-coated magnetic beads, supported osteoclast differentiation from mouse spleen cells in the presence of Con A together with IL-1 or transforming growth factor-β (TGF-β) in the coculture. The expression of RANKL mRNA was stimulated in peripheral blood-derived T cells treated with the same factors. In synovial tissue sections with lymphoid infiltrates from patients with rheumatoid arthritis, the expression of RANKL was demonstrated in CD3-positive T cells. The ability of activated T lymphocytes to support osteoclast formation may provide a mechanism for the potentiation of osteoclast formation and bone destruction in diseases such as rheumatoid arthritis and periodontitis.

Teng *et al.* (2000) transplanted human peripheral blood lymphocytes from periodontitis patients into NOD/SCID mice. Human $CD4^{(+)}$ T cells, but not $CD8^{(+)}$ T cells or B cells, were identified as essential mediators of alveolar bone destruction in the transplanted mice. Stimulation of $CD4^{(+)}$ T cells by *Actinobacillus actinomycetemcomitans,* a well-known gram-negative anaerobic microorganism that causes human periodontitis, induced production of RANKL. *In vivo* inhibition of RANKL function with OPG diminished alveolar bone destruction and reduced the number of periodontal osteoclasts after microbial challenge. These data suggest that alveolar bone destruction observed in periodontal infections is mediated by the microorganism-triggered induction of RANKL expression on $CD4^{(+)}$ T cells.

Signal Transduction Mechanism of RANK

TRAFs as Signaling Molecules of RANK

Studies have indicated that the cytoplasmic tail of RANK interacts with TNF receptor-associated factor 1(TRAF1), TRAF2, TRAF3, TRAF5, and TRAF6 (Darnay *et al.*, 1998, 1999; Galibert *et al.*, 1998; Kim *et al.*, 1999; Wong *et al.*, 1998). Mapping of the structural requirements for TRAF/RANK interaction revealed that selective TRAF-binding sites are clustered in two distinct domains of the RANK cytoplasmic tail. In particular, TRAF6 interacts with the membrane-proximal domain of the cytoplasmic tail distinct from binding sites for TRAFs 1, 2, 3, and 5. When the proximal TRAF6 interaction domain was deleted, RANK-mediated NF-κB activation was completely inhibited and JNK activation partially inhibited (Galibert *et al.*, 1998). An N-terminal truncation mutant of TRAF6 (dominant-negative TRAF6) also inhibited RANKL-induced NF-κB activation (Darnay *et al.*, 1999). These results suggest that TRAF6 transduces a signal involved in RANK-mediated differentiation and activation of osteoclasts (Fig. 6).

Lomaga *et al.* (1999) have reported that TRAF6(−/−) mice are osteopetrotic with defects in bone resorption and tooth eruption due to impaired osteoclast function. A similar number of TRAP-positive osteoclasts were observed in bone tissues in wild-type and TRAF6(−/−) mice, but TRAP-positive osteoclasts in TRAF6(−/−) mice failed to form ruffled borders. Using *in vitro* assays, it was demonstrated that TRAF6 is crucial not only for IL-1 and CD40 signalings but also for lipopolysaccharide (LPS) signaling. Naito *et al.* (1999) reported independently that TRAF6(−/−) mice exhibited severe osteopetrosis. However, unlike the report by Lomaga *et al.* (1999), TRAF6(−/−) mice produced by Naito *et al.* (1999) were defective in osteoclast formation as well. *in vitro* experiments revealed that osteoclast precursors derived from TRAF6(−/−) mice are unable to differentiate into functional osteoclasts in response to RANKL and M-CSF. The cause of the difference between the two TRAF6(−/−) mice is not known at present, perhaps it was due to different experimental conditions, but TRAF6 is proposed to be an essential component of the RANK-mediated signaling pathway in bone metabolism and immune/inflammatory systems *in vivo* (Table III).

Takayanagi *et al.* (2000) reported that T-cell production of interferon-γ (IFN-γ) strongly suppresses osteoclastogenesis by interfering with the RANKL–RANK signaling pathway. IFNγ-induced rapid degradation of TRAF6 in osteoclast precursors, which resulted in strong inhibition of the RANKL-induced activation of the transcription factor NF-κB and JNK. These results suggest that there is cross-communication between the TNF and IFN families of cytokines, through which IFNγ provides a negative link between T-cell activation and bone resorption.

Soriano *et al.* (1991) were the first to report that the targeted disruption of the gene encoding c-Src (a member of the tyrosine kinase family) induced an osteopetrotic disorder.

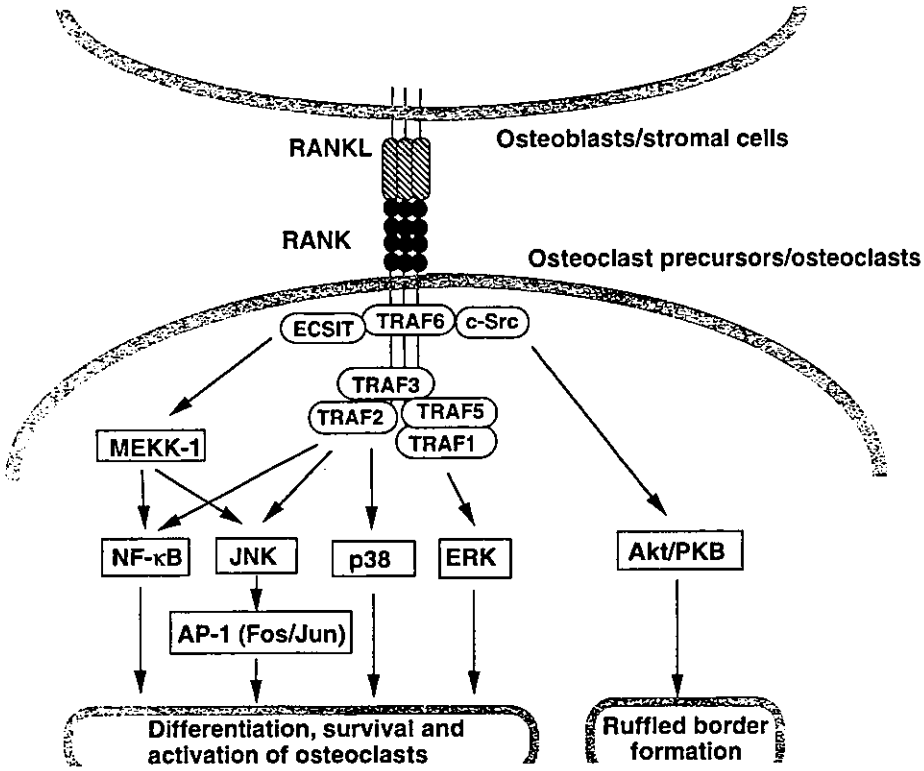


Figure 6 Signal transduction induced by the RANKL–RANK interaction in the target cell. The cytoplasmic tail of RANK interacts with TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6. The RANKL–RANK interaction induces activation of NF-κB, JNK, p38 MAP kinase, and ERK in osteoclast precursors, as well as in mature osteoclasts. In addition, RANKL activates Akt/PKB through a signaling complex involving c-Src and TRAF6. RANK-induced Akt/PKB signals appear to be involved in ruffled border formation. The adapter protein, ECSIT, may be important for TRAF6-mediated osteoclast differentiation and activation.

Local injection of PTH and IL-1 over the calvaria of c-Src-deficient mice increased the number of multinucleated cells with the morphological characteristics of osteoclasts in calvaria, but these multinucleated cells failed to develop ruffled borders (Boyce *et al.*, 1992). Using a mouse coculture system, it was shown that spleen cells obtained from c-Src-deficient mice differentiated into TRAP-positive multinucleated cells, but they did not form resorption pits on dentine slices (Lowe *et al.*, 1993). Transplantation of fetal liver cells into c-Src-deficient mice cured their osteopetrotic disorders. Indeed, osteoclasts have been shown to express high levels of c-Src (Horne *et al.*, 1992; Tanaka *et al.*, 1992). These findings suggest that c-Src expressed in osteoclasts plays a crucial role in ruffled border formation (Table III).

RANKL has been shown to activate the anti apoptotic serine/threonine kinase Akt/PKB (protein kinase B) through a signaling complex involving c-Src and TRAF6 (Wong *et al.*, 1999) (Fig. 6). A deficiency in c-Src or the addition of inhibitors of the Src family kinases blocked RANKL-mediated Akt/PKB activation in osteoclasts. The RANKL–RANK interaction triggered simultaneous binding of c-Src and TRAF6 to the intracellular domain of RANK, resulting in the enhancement of c-Src kinase activity leading to tyrosine phosphorylation of downstream signaling molecules such as c-Cbl (Tanaka *et al.*, 1996) and $p130^{Cas}$ (Nakamura *et al.*, 1998). These results suggest a mechanism by which RANKL activates Src family kinases and Akt/PKB. The results also provide evidence for the presence of cross-communication between TRAF proteins and Src family kinases. Kopp *et al.* (1999) identified a novel intermediate in the signaling pathways that bridges TRAF6 to MEKK-1 [mitogen-activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK) kinase kinase-1]. This adapter protein, named ECSIT (evolutionarily conserved signaling intermediate in Toll pathways), was shown to be specific for the Toll/IL-1 pathways and is a regulator of MEKK-1 processing. Expression of wild-type ECSIT accelerated the processing of MEKK-1 and NF-κB activation, whereas a dominant-negative fragment of ECSIT blocked both MEKK-1 processing and activation of NF-κB. These results indicate that ECSIT plays an important role for TRAF6-meditated osteoclast differentiation and function (Fig. 6).

RANK-Mediated Signals

Franzoso *et al.* (1997) and Iotsova *et al.* (1997) independently generated mice deficient in both p50 and p52 subunits

Table III Characteristics of Mice Carrying Disrupted Genes Involved in Osteoclast Differentiation and Function

Gene disrupted	Phenotype	State of bone resorption	Defective cells	References
c-src	Osteopetrosis	Osteoclasts are present, but fail to form ruffled borders	Osteoclast progenitors	Soriano *et al.* (1991), Boyce *et al.* (1992), Lowe *et al.* (1993)
c-fos	Osteopetrosis	Osteoclasts are absent	Osteoclast progenitors	Wang *et al.* (1992), Grigoriadis *et al.* (1994)
p50/p52 (NF-κB)	Osteopetrosis	Osteoclasts are absent	Osteoblasts progenitors	Franzoso *et al.* (1997), Iotsova *et al.* (1997)
rankl	Osteopetrosis	Osteoclasts are absent	Osteoblasts	Kong *et al.* (1999)
rank	Osteopetrosis	Osteoclasts are absent	Osteoclast progenitors	Dougall *et al.* (1999), Li *et al.* (2000)
opg	Osteoporosis	Osteoclastic bone resorption is enhanced	Osteoblasts	Mizuno *et al.* (1998), Bucay *et al.* (1998)
traf6 (1)	Osteopetrosis	Osteoclasts are present, but fail to form ruffled borders	Not determined	Lomaga *et al.* (1999)
traf6 (2)	Osteopetrosis	The number of osteoclasts are decreased markedly	Osteoclast progenitors	Naito *et al.* (1999)

of NF-κB (Table III). The double knockout mice developed severe osteopetrosis due to a defect in osteoclast differentiation. The osteopetrotic phenotype was rescued by bone marrow transplantation, indicating that the osteoclast progenitors are inactive in the double knockout mice. RANKL has been shown to activate NF-κB in the target cells, including osteoclast precursors and mature osteoclasts. These results suggest that RANKL-induced activation of NF-κB in osteoclast progenitors plays a crucial role in their differentiation into osteoclasts (Fig. 6).

Purified osteoclasts died spontaneously via apoptosis, whereas IL-1 promoted the survival of osteoclasts by preventing their apoptosis. Jimi *et al.* (1996a) reported that the pretreatment of purified osteoclasts with proteasome inhibitors suppressed the IL-1-induced activation of NF-κB and prevented the survival of osteoclasts supported by IL-1. When osteoclasts were pretreated with the antisense oligodeoxynucleotides to the p65 and p50 subunits of NF-κB, the expression of respective mRNAs by osteoclasts was suppressed, together with the concomitant inhibition of IL-1-induced survival of osteoclasts. These results indicate that IL-1 promotes the survival of osteoclasts through the activation of NF-κB. Miyazaki *et al.* (2000) also examined the role of mitogen-activated protein kinase and NF-κB pathways in osteoclast survival and activation, using adenovirus vectors carrying various mutants of signaling molecules. Inhibition of ERK activity by dominant-negative Ras overexpression induced the apoptosis of osteoclasts rapidly, whereas ERK activation by the introduction of constitutively active MEK (MAPK/ERK kinase) prolonged their survival remarkably. Neither inhibition nor activation of ERK affected the pit-forming activity of osteoclasts. In contrast, inhibition of the NF-κB pathway with dominant-negative IκB kinase suppressed the pit-forming activity of osteoclasts. NF-κB activation by constitutively active IκB kinase expression up regulated the pit-forming activity of osteoclasts without affecting their survival. IL-1 strongly induced both ERK and NF-κB activation. Matsumoto *et al.* (2000) found that treatment of bone marrow cells with an inhibitor of p38 MAP kinase (SB203580) suppressed osteoclast differentiation via inhibition of the RANKL-mediated signaling pathway. RAW264, a transformed mouse myeloid cell line, has been shown to differentiate into osteoclasts in response to RANKL (Hsu *et al.*, 1999). Expression of the dominant negative form of p38 MAP kinase in RAW264 cells inhibited their RANKL-induced differentiation into osteoclasts. These results indicate that activation of the p38 MAP kinase pathway also plays an important role in RANKL-induced osteoclast differentiation.

Mice lacking c-Fos have been shown to develop osteopetrosis due to an early differentiation block in the osteoclast lineage (Grigoriadis *et al.*, 1994; Wang *et al.*, 1992) (Table III). The dimeric transcription factor activator protein-1 (AP-1) is composed of mainly Fos proteins (c-Fos, FosB, Fra-1, and Fra-2) and Jun proteins (c-Jun, JunB, and JunD). RANKL activated JNK in the target cells, including purified osteoclasts and osteoclast progenitors. These results suggest that AP-1 appears to be located downstream of RANK-mediated signals. Unlike c-Fos, Fra-1 lacks transactivation domains required for oncogenesis and cellular transformation. Using a retroviral gene transfer, Matsuo *et al.* (2000) showed that all four Fos proteins, but not Jun proteins, rescued the differentiation block of c-Fos-deficient spleen cells into osteoclasts *in vitro*. Structure–function analysis demonstrated that the major carboxy-terminal transactivation domains of c-Fos and FosB are dispensable and that Fra-1 has the highest rescue activity. Moreover, a transgene expressing Fra-1 rescued the osteopetrosis of c-Fos-mutant mice *in vivo*. RANKL induced

transcription of Fra-1 expression in a c-Fos-dependent manner. These results indicate the presence of a link between RANK signaling and the expression of AP-1 proteins in inducing osteoclast differentiation (Fig. 6).

Cross-Communication between RANKL and TGF-β Superfamily Members

Bone is a major storage site for the cytokines of the TGF-β superfamily, such as TGF-β and BMPs. Osteoclastic bone resorption releases these growth factors from bone matrix. Receptors for TGF-β superfamily members are a family of transmembrane serine/threonine kinases and are classified as type I and type II receptors according to their structural and functional characteristics (Miyazono, 2000). Formation of a type I–type II receptor complex is required for the ligand-induced signals. Previous studies have shown that the extracellular domain of type I receptors is sufficient to mediate stable binding to TGF-β superfamily members and subsequent formation of a heteromeric complex with the intact type II receptors. Sells Galvin *et al.* (1999) first reported that TGF-β enhanced osteoclast differentiation in cultures of mouse bone marrow cells stimulated with RANKL and M-CSF. These results support the previous findings (1) that transgenic mice expressing TGF-β2 developed osteoporosis due to enhanced osteoclast formation (Erlebacher and Derynck, 1996) and (2) that osteoclast formation was reduced in transgenic mice expressing a truncated TGF-β type II receptor in the cytoplasmic domain (Filvaroff *et al.*, 1999). Fuller *et al.* (2000) also reported that activin A potentiated RANKL-induced osteoclast formation. Moreover, osteoclast formation induced by RANKL was abolished completely by adding soluble activin receptor type IIA or soluble TGF-β receptor type II, suggesting that activin A and TGF-β are essential factors for osteoclastogenesis. We further found that BMP-2 enhanced the differentiation of osteoclasts and the survival of osteoclasts supported by RANKL (Itoh *et al.*, 2000a). A soluble form of BMP receptor IA, which inhibits the binding of BMP-2 to BMP receptor IA, blocked RANKL-induced osteoclast formation. Thus, BMP-2 is yet another important determinant of osteoclast formation. Although the molecular mechanism by which TGF-β superfamily members potentiate the RANK-mediated signals is not known, cytokines released from bone matrix accompanying osteoclastic bone resorption appear to play an important role in RANKL-induced osteoclast formation. Further studies will elucidate the molecular mechanism of the cross-communication between TGF-β superfamily members and RANKL in osteoclast differentiation and function.

RANK Is Not the Sole Factor Responsible for Osteoclast Differentiation and Function

IL-1 stimulates not only osteoclast differentiation, but also osteoclast function through the IL-1 type 1 receptor (Jimi *et al.*, 1999b). As described earlier, purified osteoclasts placed on dentine slices failed to form resorption pits. When IL-1 or RANKL was added to the purified osteoclast cultures, resorption pits were formed on dentine slices within 24 hr (Jimi *et al.*, 1999b). Osteoclasts express IL-1 type 1 receptors, and IL-1 activated NF-κB rapidly in purified osteoclasts. The pit-forming activity of osteoclasts induced by IL-1 was inhibited completely by adding IL-1 receptor antagonist (IL-1ra) but not by OPG (Jimi *et al.*, 1999a). This suggests that IL-1 directly stimulates osteoclast function through IL-1 type 1 receptors in mature osteoclasts (Fig. 7).

Since the discovery of the RANKL–RANK signaling system, RANKL has been regarded as the sole factor responsible for inducing osteoclast differentiation. Azuma *et al.* (2000) and Kobayashi *et al.*, (2000) independently found that TNFα stimulates osteoclast differentiation in the absence of the RANKL–RANK interaction (Fig. 7). When mouse bone marrow cells were cultured with M-CSF, M-CSF-dependent bone marrow macrophages appeared within 3 days. In addition, TRAP-positive osteoclasts were formed in response to not only RANKL but also mouse TNFα, when bone marrow macrophages were cultured further for another 3 days with either ligand in the presence of M-CSF. Osteoclast formation induced by TNFα was inhibited by the addition of respective antibodies against TNF receptor type I (TNFRI, p55) and TNF receptor type II (TNFRII, p75), but not by OPG. Osteoclasts induced by TNFα formed resorption pits on dentine slices only in the presence of IL-1. These results demonstrate that TNFα stimulates osteoclast differentiation in the presence of M-CSF through a mechanism independent of the RANKL–RANK system (Fig. 7). TNFRI and TNFRII use TRAF2 as a common signal transducer in the target cells, suggesting that TRAF2-mediated signals play important roles in osteoclast differentiation. It has been reported that when osteotropic factors such as $1\alpha,25(OH)_2D_3$, PTHrP, and IL-1 were administered into RANK(−/−) mice, neither TRAP-positive cell formation nor hypercalcemia was induced (Li *et al.*, 2000a). In contrast, administration of TNFα into RANK(−/−) mice induced TRAP-positive cells near the site of injection even though the number of TRAP-positive cells induced by TNFα was not large. This suggests that TNFα somewhat induces osteoclasts in the absence of RANK-mediated signals *in vivo*. These results further strongly delineate that the RANKL–RANK interaction is not the sole pathway for inducing osteoclast differentiation *in vitro* and *in vivo*. It is, therefore, proposed that TNFα, together with IL-1, plays an important role in bone resorption in metabolic bone diseases such as rheumatoid arthritis, periodontitis, and possibly osteoporosis. Lam *et al.* (2000) also reported that a small amount of RANKL strongly enhanced osteoclast differentiation in a pure population of murine precursors in the presence of TNFα. These results suggest that RANKL-induced signals cross-communicate with TNFα-induced ones in the target cells.

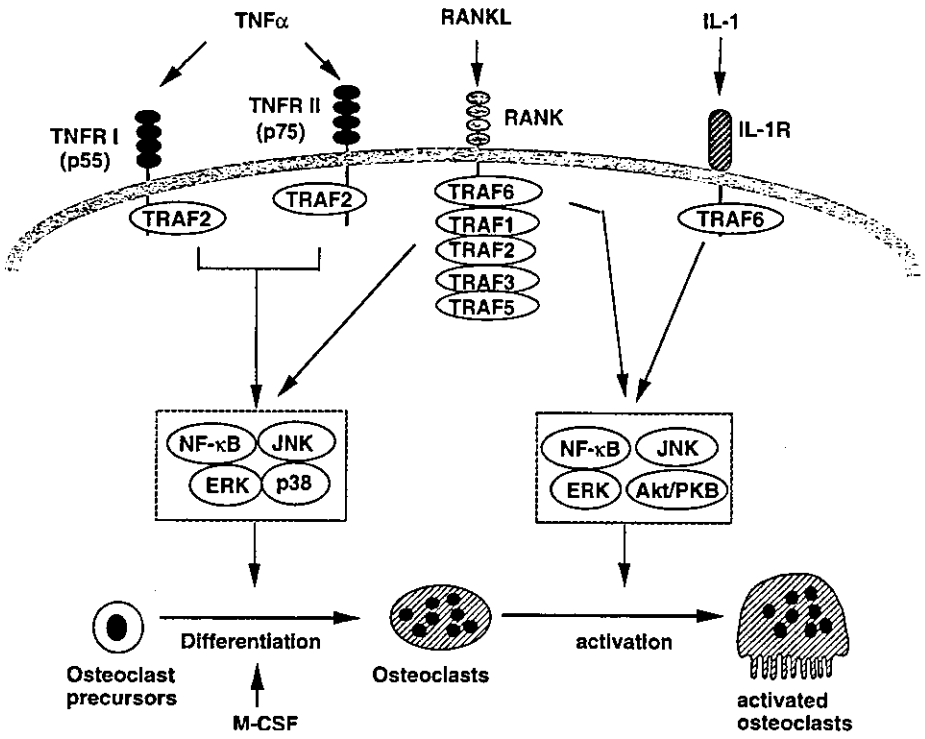


Figure 7 Schematic representation of ligand–receptor systems in osteoclast differentiation and function regulated by TNFα, RANKL, and IL-1. TNFα and RANKL stimulate osteoclast differentiation independently. Osteoclast differentiation induced by TNFα occurs via TNFRI (p55) and TNFRII (p75) expressed by osteoclast precursors. RANKL induces osteoclast differentiation through RANK-mediated signals. M-CSF is a common factor required by both TNFα- and RANKL-induced osteoclast differentiation. Activation of osteoclasts is induced by RANKL and IL-1 through RANK and IL-1 type I receptors, respectively. Common signaling cascades, such as NF-κB, JNK, p38 MAP kinase, and ERK activation, may be involved in the differentiation of osteoclasts induced by TNFα and RANKL. RANKL and IL-1 may activate osteoclast function though signals mediated by NF-κB, JNK, ERK, and Ark/PKB.

Conclusion

The discovery of the RANKL–RANK interaction now opens a wide new area in bone biology focused on the investigation of the molecular mechanism of osteoclast development and function. Osteoblasts/stromal cells, through the expression of RANKL and M-CSF, are involved throughout the osteoclast lifetime in all processes that govern their differentiation, survival, fusion, and activation. OPG produced by osteoblasts/stromal cells is an important negative regulator of osteoclast differentiation and function. Membrane- or matrix-associated forms of both M-CSF and RANKL expressed by osteoblasts/stromal cells appear to be essential for osteoclast formation. Both RANKL(−/−) mice and RANK(−/−) mice show similar features of osteopetrosis with a complete absence of osteoclasts in bone. Gain-of-function mutations of RANK have been found in patients suffering from familial expansile osteolysis and familial Paget's disease of bone. These findings confirm that the RANKL–RANK interaction is indispensable for osteoclastogenesis not only in mice but also in humans. The cytoplasmic tail of RANK interacts with the TRAF family members. TRAF2-mediated signals appear important for inducing osteoclast differentiation, and TRAF6-mediated signals are indispensable for osteoclast activation. Activation of NF-κB, JNK, and ERK, all induced by RANKL in osteoclast precursors and mature osteoclasts, may be involved in their differentiation and function. OPG strongly blocked all processes of osteoclastic bone resorption *in vivo*, suggesting that inhibiting either the RANKL–RANK interaction or RANK-mediated signals are ideal ways to prevent increased bone resorption in metabolic bone diseases such as rheumatoid arthritis, periodontitis, and osteoporosis. Studies have also shown that TNFα and IL-1 can substitute for RANKL in inducing osteoclast differentiation and function *in vitro*. These results suggest that signals other than RANK-induced ones may also play important roles in osteoclastic bone resorption under pathological conditions.

Under physiological conditions, osteoclast formation requires cell-to-cell contact with osteoblasts/stromal cells, which express RANKL as a membrane-bound factor in response to several bone-resorbing factors. In normal bone remodeling, osteoblastic bone formation occurs in a programmed precise and quantitative manner following osteoclastic bone resorption: bone formation is coupled to bone

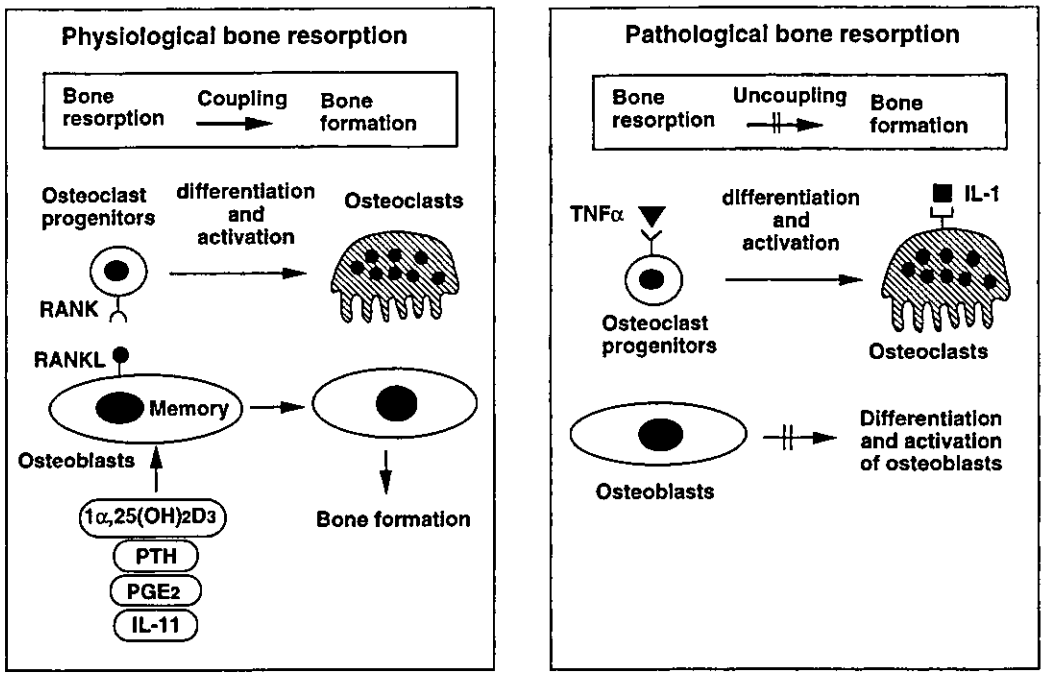


Figure 8 A hypothesis on the regulation of osteoblastic bone formation under physiological and pathological bone resorption. Under physiological conditions, osteoclast formation requires cell-to-cell contact with osteoblasts/stromal cells, which express RANKL as a membrane-associated factor in response to several bone-resorbing factors. In normal bone remodeling, osteoblastic bone formation occurs in a programmed precise and quantitative manner following osteoclastic bone resorption. It is so-called coupling between bone resorption and bone formation. In contrast, in pathological bone resorption, macrophages and/or T cells secrete inflammatory cytokines, such as TNFα and IL-1, as well as a soluble form of RANKL, which act directly on osteoclast progenitors and mature osteoclasts without cell-to-cell contact. This situation is characterized by uncoupling between bone resorption and bone formation. Cell-to-cell contact between osteoclast progenitors and osteoblasts may leave behind some memory for bone formation in osteoblasts.

resorption. In contrast, in pathological bone resorption, as in rheumatoid arthritis, macrophages and/or T cells secrete inflammatory cytokines such as TNFα and IL-1, which act directly on osteoclast progenitors and mature osteoclasts without cell-to-cell contact. This situation is characterized by uncoupling between bone resorption and bone formation. It is, therefore, noteworthy to consider that cell-to-cell contact between osteoclast progenitors and osteoblasts may leave behind in osteoblasts some memory for bone formation (Fig. 8). Further experiments are necessary to verify this hypothesis. Studies on the signal transduction of these TNF-ligand family members in osteoclast progenitors and in mature osteoclasts will provide novel approaches for the treatment of metabolic bone diseases.

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