

# Modulation of Osteoclast Differentiation and Function by the New Members of the Tumor Necrosis Factor Receptor and Ligand Families

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## I. Introduction

OSTEOCLASTS, which are present only in bone, are multinucleated giant cells with the capacity to resorb mineralized tissues. During the past decade, several new approaches have been developed to investigate osteoclast biology. A coculture system of mouse osteoblasts/stromal cells and hemopoietic cells for osteoclast formation has established the concept that osteoblasts/stromal cells are crucially involved in osteoclast development. Cell-to-cell contact between cells of the osteoblast lineage and hemopoietic cells is necessary for inducing differentiation of osteoclasts. It has been proposed that osteoblasts/stromal cells express osteoclast differentiation factor (ODF) or stromal osteoclast forming activity (SOFA) as a membrane-associated factor in

response to several osteotropic factors such as  $1\alpha,25$ -dihydroxyvitamin  $D_3$  [ $1\alpha,25(\text{OH})_2D_3$ ], PTH, and interleukin 11 (IL-11). Osteoclast precursors of the monocyte-macrophage lineage recognize ODF/SOFA through cell-to-cell interaction with osteoblasts/stromal cells, and then differentiate into osteoclasts. Osteoblasts/stromal cells also play an essential role in the activation of osteoclast function. We emphasize that the term "osteoblasts/stromal cells" is an operational one, used for convenience to describe those cells of the osteoblast lineage that have been shown convincingly *in vitro* to determine osteoclast formation. It is not certain *in vivo* which members of the lineage cells are responsible. *In vitro* data suggest that the osteoblast property is progressively lost with maturation of the osteoblast lineage cells, and *in vivo*, it is not at all likely that mature, synthesizing osteoblasts make any contribution to osteoclast formation. Nor are osteocytes likely to do so, but likely potential contributors are lining cells and early members of the osteoblast lineage that are situated close to the endosteal surface. Ultimately, the process of osteoclast formation is dependent on hemopoietic precursors being presented to the appropriate osteoblasts/stromal cells in an environment that provides appropriate stimulatory factors.

Recently, three laboratories independently cloned cDNAs encoding the identical proteins, giving it the names osteoprotegerin (OPG), osteoclastogenesis inhibitory factor (OCIF), and tumor necrosis factor (TNF) receptor-like molecule 1 (TR1). This protein inhibits osteoclast development *in vitro* and *in vivo*. In an attempt to adopt a uniform nomenclature for this important biological activity, we propose that the name of choice be "osteoprotegerin." OPG is a member of the TNF receptor family, but it does not have a transmembrane domain and possesses a signal sequence, suggesting that OPG functions as a secreted factor. Since OPG has the capacity to limit osteoclast formation, the ligand for this receptor was proposed to be the long-sought-after ODF/SOFA. Indeed, this hypothesis dictated the experiments carried out by the groups who subsequently identified a membrane-bound TNF-like ligand with the capacity to differentiate hematopoietic cells into functional osteoclasts. cDNA libraries from cell lines, which expressed specific binding sites for OPG, were screened by expression cloning approaches. As expected, the binding molecule of OPG was

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a membrane-associated protein of the TNF ligand family, which satisfied all the criteria of ODF/SOFA. In addition, ODF/SOFA was also able to maintain osteoclasts that had been induced by osteoblasts/stromal cells in an activated state. The discovery of this differentiation factor now opens a new era to investigate the molecular mechanism of osteoclast development and function.

This review article describes the role of osteoblasts/stromal cells in osteoclast development and function at a molecular level, especially focusing on the central role of members of the TNF receptor and ligand superfamilies. Because discoveries in this area have originated from several directions and by different research groups, nomenclature has rapidly become confusing; thus, we propose an approach to overcome this.

## II. Role of Osteoblasts/Stromal Cells in Osteoclast Differentiation and Function

### A. Origin of osteoclasts

Development of osteoclasts proceeds within the local microenvironment of bone (1–4). This process can be replicated *ex vivo* using the coculture of mouse calvarial osteoblasts and spleen cells (5–9). Multinucleated cells formed in such cocultures satisfy the major criteria of osteoclasts such as tartrate-resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts) activity, calcitonin receptors,  $p60^{c-src}$ , vitronectin receptors ( $\alpha v\beta 3$ ), and the ability to form resorption pits on bone and dentine slices. Some mouse stromal cell lines such as MC3T3-G2/PA6 and ST2 resemble calvarial osteoblasts and support osteoclastogenesis in coculture with mouse spleen cells (10).

Experiments on the osteopetrotic *op/op* mouse model have established that an osteoblast/stromal cell product, macrophage colony-stimulating factor (M-CSF, also called CSF-1), is crucial for osteoclast formation. The M-CSF gene of *op/op* mice cannot code functionally active M-CSF protein due to an extra thymidine insertion in the coding region of the M-CSF gene (11, 12). Administration of recombinant human M-CSF restored impaired bone resorption in *op/op* mice (13, 14). Calvarial osteoblasts obtained from *op/op* mice failed to support osteoclast development in cocultures with normal spleen cells, but the addition of M-CSF to cocultures induced osteoclast formation in response to  $1\alpha,25(\text{OH})_2\text{D}_3$  (15–17). These findings indicate that M-CSF produced by osteoblasts/stromal cells plays an essential role in osteoclast development.

After identification of the hemopoietic origin of osteoclasts, much attention has been focused on the cell lineages of osteoclast progenitors. Using ST2 cells as a stromal supportive cell line, it was shown that, in addition to spleen cells and bone marrow cells, peripheral blood mononuclear cells and alveolar macrophages acted as a source of osteoclast precursors (18). Chambers *et al.* (19) have established osteoclastogenic cell lines that express macrophage phenotypes from *H-2Kbts58* transgenic mice. Miyamoto *et al.* (20) have also established the macrophage-like cell line C7 from a p53-deficient mouse. These cell lines differentiated into osteoclasts when they were cocultured with stromal cells in the

presence of  $1\alpha,25(\text{OH})_2\text{D}_3$ , indicating that osteoclasts are derived from cells of the monocyte/macrophage lineage. Kurihara *et al.* (21) have shown that osteoclasts formed from highly purified populations of CFU-GM (granulocyte and macrophage colony-forming cells), the granulocyte-macrophage progenitors in human marrow cultures. Hattersley *et al.* (22) have suggested that mouse CFU-GM-derived cells also form osteoclasts in the coculture. These results indicate that osteoclast precursors are derived from cells in the monocyte-macrophage lineage, with CFU-GM as the earliest identifiable precursor.

Findings after the disruption of the *c-fos* gene and PU.1 gene in mice supported the monocyte/macrophage origin of osteoclasts. Disruption of the protooncogene *c-fos* gives rise to severe osteopetrotic disorders in bone caused by a defect in osteoclast progenitors (23, 24). Transplantation of normal bone marrow cells into *c-Fos*(–/–) mice rescued the osteopetrosis (25). In addition, the number of F4/80- and Mac-2-positive macrophages was increased in bones of *c-Fos*(–/–) mice, suggesting that the lack of the *c-fos* gene causes a lineage shift between osteoclast and macrophage differentiation (25). In cocultures of osteoblasts and spleen cells, *c-Fos*(–/–) mouse-derived spleen cells failed to differentiate into osteoclasts. PU.1 is a myeloid- and B-cell-specific transcription factor, and PU.1(–/–) mice were found to be osteopetrotic (26). The development of both osteoclasts and macrophages was arrested in PU.1(–/–) mice. The osteopetrotic disorder of PU.1(–/–) mice was cured by transplantation of normal bone marrow cells into the mice. The absence of both macrophages and osteoclasts in PU.1(–/–) mice suggests that this transcription factor regulates the initial stage of myeloid differentiation. These results further support the notion that osteoclasts are derived from cells of the monocyte/macrophage lineage. However, the mechanism by which osteoclast progenitors enter and leave the circulation is not fully understood at present.

### B. Stimulation of osteoclast differentiation by osteoblasts/stromal cells

In the coculture system, cell-to-cell contact between osteoblasts/stromal cells and hemopoietic cells was determined to be indispensable for osteoclast development (5–9, 27). Additionally, the osteoblasts/stromal cells have been identified as the target cells for osteotropic hormones and cytokines other than M-CSF to induce osteoclast development.

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 non-ligand-binding but signal-transducing protein gp130 (28). A genetically engineered soluble IL-6R (sIL-6R), which lacks transmembrane and cytoplasmic domains, was determined to mediate the IL-6 signals through gp130 in response to IL-6 (28). 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 (29). This suggests that a signal(s) mediated by gp130 is involved in osteoclast development. Cytokines such as IL-11, oncostatin M, and leukemia inhibitory factor, which transduce their signals through gp130, also induced oste-

oclast formation in coculture experiments (29–31). Additionally, in transgenic mice constitutively expressing human IL-6R, the expression of IL-6R in osteoblasts was clearly shown to be indispensable for induction of osteoclast recruitment (32). 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 adding human sIL-6R. This suggests that cytokines that use gp130 as a common signal transducer act directly on osteoblasts/stromal cells but not on osteoclast progenitors to induce osteoclast formation. Osteoclasts are present in gp130 knockout mice because of the use of other available signaling pathways as described below.

One of the major messenger pathways used to induce osteoclast formation is cAMP, with the most widely studied agonist for this pathway being PTH. The target cells of PTH in inducing osteoclasts are also osteoblasts/stromal cells but not osteoclast progenitors in the coculture. Subclones of the human osteosarcoma cell line SaOS-2 were established to overexpress human PTH/PTH-related protein (PTHrP) receptor (PTHrP1) under a heterologous promoter (33). Two cell lines, designated SaOS-2/4 and SaOS-4/3, which expressed functional recombinant PTHrP1, supported osteoclast formation in response to PTH in the coculture with mouse spleen cells, while the parent SaOS-2 cells did not (33). Confirmation of the requirement for PTHrP1 to be expressed on the osteoblast was achieved using cocultures established between osteoblasts and spleen cells from normal and PTHrP1-deficient mice (34). It was shown that osteoclasts were formed in response to PTH in cocultures of spleen cells obtained from PTHrP1(–/–) mice and normal calvarial osteoblasts (34). These results indicate that the expression of PTHrP1 in osteoblasts/stromal cells is critical for PTH-induced osteoclast formation in the coculture.

The other known pathway used for osteoclast induction is that stimulated by  $1\alpha,25(\text{OH})_2\text{D}_3$ . Kato and his colleagues (35) have succeeded in producing  $1\alpha,25(\text{OH})_2\text{D}_3$  receptor (VDR) knockout mice by targeted disruption of the gene. Osteoblasts obtained from VDR(–/–) mice failed to support osteoclast development in cocultures with normal spleen cells in response to  $1\alpha,25(\text{OH})_2\text{D}_3$  but did so in response to PTH (36). In contrast, spleen cells from VDR(–/–) mice differentiated into osteoclasts in coculture with normal osteoblasts in response to  $1\alpha,25(\text{OH})_2\text{D}_3$ . These results suggest that the signals mediated by VDR, like PTH and IL-11, are also transduced into osteoblasts/stromal cells to induce osteoclast formation in the coculture. The normal osteoclast formation in VDR(–/–) mice is readily explained by the other available pathways (PTH, cytokines).

Thus, the signals induced by all bone-resorbing factors are transduced in osteoblasts/stromal cells to induce osteoclast formation (Fig. 1). With this in mind, we have proposed the hypothesis that osteoblasts/stromal cells express ODF, which is a membrane-bound factor to promote differentiation of osteoclast progenitors into osteoclasts through a mechanism involving cell-to-cell contact (Fig. 1) (5–9). Chambers *et al.* (19) also proposed that SOFA expressed by osteoblasts/stromal cells is essentially involved in osteoclast differentiation. ODF and SOFA are the terms applied to proteins that were assumed to be identical, and reflecting the concept of contact-dependent promotion of osteoclast formation.

### C. Stimulation of osteoclast function by osteoblasts/stromal cells

One technical issue relating to the analysis of mature osteoclast function is that pertaining to their adherence to

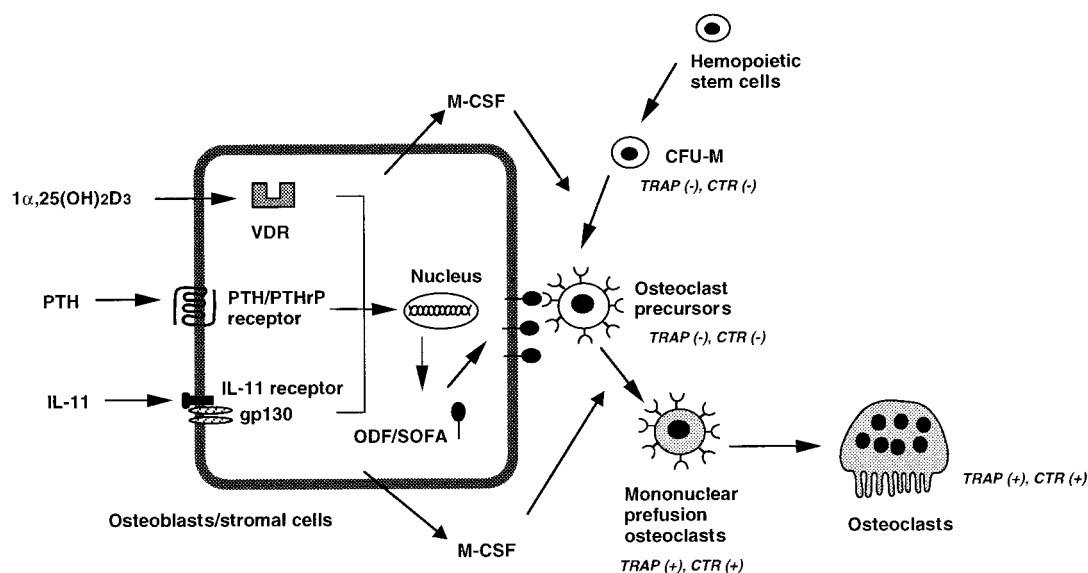


FIG. 1. A hypothetical concept of osteoclast differentiation. Osteotropic factors such as  $1\alpha,25(\text{OH})_2\text{D}_3$ , PTH, and IL-11 stimulate osteoclast formation in cocultures of osteoblasts/stromal cells and hemopoietic cells. Target cells for these factors are osteoblasts/stromal cells. Three different signaling pathways mediated by VDR, PTH/PTHrP receptor, and gp130 similarly induce ODF or stromal osteoclast forming activity (SOFA) as a membrane-associated factor in osteoblasts/stromal cells. Osteoclast progenitors of the monocyte-macrophage lineage recognize ODF/SOFA in osteoblasts/stromal cells through cell-to-cell interaction, and then differentiate into osteoclasts. M-CSF produced by osteoblasts/stromal cells is a prerequisite for both proliferation and differentiation of osteoclast progenitors.

plastic dishes. Osteoclasts formed on plastic dishes are very difficult to detach by treatment with trypsin-EDTA or bacterial collagenase or both. We have developed a collagen-gel culture of mouse bone marrow cells and osteoblasts to obtain functionally active osteoclasts (37). The purity of osteoclasts in this preparation was only approximately 2–3%, with many osteoblasts contaminating the osteoclast preparation. However, this crude osteoclast preparation proved to be a source by which to establish a reliable resorption pit assay system using dentine slices (38, 39). This procedure established a method to determine the ability of mature osteoclasts by the unique property of resorbing bone or dentine. In subsequent purification procedures, we were able to prepare highly purified osteoclasts by centrifuging the crude osteoclast preparation on a 30% Percoll solution (40). However, these highly enriched osteoclasts (purity, 50–70%) cultured for 24 h on dentine slices failed to form resorption pits. Resorptive capability of these purified osteoclasts was restored when calvarial osteoblasts were added to the enriched osteoclasts (40, 41). Some stromal cell lines such as MC3T3-E1, KS-4, MC3T3-G2/PA6, and ST2 also potentiated pit-forming activity of enriched osteoclasts. When cell-to-cell contact between osteoblasts/stromal cells and osteoclasts was prevented, osteoclasts failed to form resorption pits. Thus, osteoblasts/stromal cells appear to play an essential role not only in inducing osteoclast formation from murine precursors, but also in their capability to induce mature osteoclasts to resorb. Both are cell-to-cell contact-dependent processes. In contrast, Roodman (3) has reported that osteoclast-like cells are formed from human and murine hemopoietic progenitors in the absence of osteoblasts/stromal cells.

Wesolowski *et al.* (42) have also used echistatin (a snake venom containing RGD-sequence) to isolate highly enriched mononuclear or binuclear prefusion osteoclasts (pOCs) from cocultures of mouse bone marrow cells and osteoblasts (MB 1.8 cells). These cells expressed most of the characteristics of osteoclasts such as TRAP, calcitonin receptors, vacuolar proton ATPase, and vitronectin receptors. However, pOCs could only resorb bone when both MB 1.8 cells and  $1\alpha,25(\text{OH})_2\text{D}_3$  were present. These results further support the hypothesis that cells of the osteoblast lineage directly activate osteoclast function.

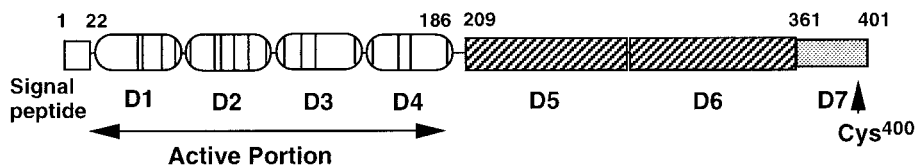
### III. New Members of the Tumor Necrosis Factor (TNF) Receptor and Ligand Families

#### A. Osteoprotegerin (OPG)

In 1997, Simonet *et al.* (43) reported the discovery of “osteoprotegerin” (OPG) that inhibited bone resorption. OPG of 401 amino acid residues was a member of the TNF receptor family, but, unlike all other members of the family, lacked a transmembrane domain and represented a secreted TNF receptor. Hepatic expression of OPG in transgenic mice resulted in an osteopetrosis. Tsuda *et al.* (44) independently isolated the same protein [termed “osteoclastogenesis inhibitory factor (OCIF)"] as a heparin-binding protein from the conditioned media of human fibroblast cultures and showed that its cDNA sequence was identical to that of OPG (45). OPG strongly inhibited osteoclast formation induced by either  $1\alpha,25(\text{OH})_2\text{D}_3$ , PTH,  $\text{PGE}_2$ , or IL-11 in the coculture. *In vivo* administration of OPG resulted in an increase in bone mineral density and bone volume associated with a decrease of active osteoclast number in normal and ovariectomized rats. Serum Ca concentration was also decreased by injecting OPG into rats (46–48). Finally, Tan *et al.* (49) also identified a new member of the TNF receptor family named “TNF receptor-like molecule 1” (TR1) from a search of an expressed sequence tag data base. TR1 was found to be identical to OPG and inhibited osteoclast formation in the coculture system, pit formation by osteoclasts, and bone resorption in organ culture of fetal mouse long bones (50). For simplicity, and to provide a uniform nomenclature system, we propose the name “osteoprotegerin” (OPG) be adopted for the molecules that include OCIF and TR1 (also see Fig. 4).

Like other members of the TNF receptor family (51), OPG contained four cysteine-rich domains (D1–D4) (Fig. 2) (43, 45). In addition, OPG had two death domain homologous regions (D5, D6) followed by a domain with a highly net positive charge (D7). D5 and D6 share structural features with “death domains” of TNF receptor p55, Fas, DR3, and TRAIL receptor, which mediate apoptotic signals. Seven structural domains (D1–D7) of human OPG have been characterized using various mutant proteins (52). The N-terminal portion of OPG containing D1–D4 was sufficient to inhibit osteoclast formation in the coculture, although the potency of the mutant protein that lacks D5–D7 was decreased to about 10% of that of the wild-type OPG. A heparin-binding site was located in D7, and the affinity for heparin did not

FIG. 2. A diagrammatic representation of functional domains of OPG. Human OPG is composed of 401 amino acid residues. Four cysteine-rich domains (D1–D4) exist in Glu<sup>22</sup>–Ser<sup>186</sup>. Vertical bars represent cysteine residues. Two death domain homologous regions (D5, D6) exist in Phe<sup>209</sup>–Val<sup>361</sup>. Cys<sup>400</sup> is responsible for dimer formation of OPG. Mouse OPG is also composed of 401 amino acid residues with 7 domains almost identical to human OPG.



D1–D4 : Cysteine-rich domains

D5, D6 : Death domain homologous regions

D7 : Heparin binding domain

Cys<sup>400</sup> : Essential for dimer formation

correlate with the osteoclastogenesis-inhibitory activity. OPG can exist as a monomer or dimer as a result of formation of a disulfide bond using Cys<sup>400</sup> present in D7. However, dimerization of OPG was not necessary for biological activity of OPG since the substitution of the Cys<sup>400</sup> to Ser did not affect the osteoclastogenic inhibitory activity of OPG. In addition, when the transmembrane domain of Fas was inserted between D4 and D5 and the mutant protein was expressed in the human kidney cell line 293-EBNA, apoptosis was induced in the transfected cells (52). Although the precise role of D5 and D6 of OPG is still not known, the death domain-homologous regions are active in mediating apoptotic signals.

The physiological roles of OPG have been studied in OPG-deficient mice produced by targeted disruption of the gene (53, 54). OPG(−/−) mice were viable and fertile, but they exhibited severe osteoporosis caused by enhanced osteoclast formation and function. Destruction of growth plates and lack of trabecular bone with an increase in the number of osteoclasts were detected in long bones of adult OPG(−/−) mice. The strength and mineral density of their bones were decreased dramatically (53, 54). Bone histomorphometric analysis showed that the osteoblast surface as well as the osteoclast surface were increased in OPG-deficient mice (54). OPG(−/−) mice also developed medial calcification of the aorta and renal arteries (54). These results indicate that OPG is a physiological regulator of osteoclast-mediated bone resorption during postnatal bone growth. It also suggests that OPG might normally prevent calcification of larger arteries.

TRAIL is a TNF-related ligand that induces apoptosis upon binding to its death domain-containing receptors, DR4 and DR5. Emery *et al.* (55) recently reported that OPG bound to TRAIL and inhibited TRAIL-induced apoptosis of Jurkat cells. They also showed that TRAIL blocked the osteoclastogenesis-inhibitory activity of OPG in the coculture. These results indicate a potential cross-regulatory mechanism involving OPG and TRAIL, which may also participate in the regulation of osteoclastic bone resorption.

### *B. Osteoclast differentiation factor (ODF) and stromal osteoclast forming activity (SOFA)*

1. *ODF*. As OPG was a member of the TNF receptor family, a likely candidate for ODF/SOFA would be a membrane-bound ligand for this receptor. Since ODF/SOFA should be expressed on the surface of ST2 cells after stimulation via  $1\alpha,25(\text{OH})_2\text{D}_3$  and dexamethasone, this cell line was assessed for the ability of OPG to bind specifically to ST2 cells treated with  $1\alpha,25(\text{OH})_2\text{D}_3$  and dexamethasone (45). Expression cloning of the ligand for OPG was performed using a cDNA library of ST2 cells. A cDNA clone with an open reading frame encoding 316 amino acid residues was isolated (56). The OPG-binding molecule was a type II transmembrane protein of the TNF ligand family (Fig. 3A). When COS-7 cells transfected with the OPG-binding molecule 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. This suggested that the OPG-binding molecule mediated cell-to-cell signals responsible for osteoclastogenesis. A genetically engineered soluble

form of the OPG-binding protein together with M-CSF induced osteoclast formation from spleen cells in the absence of osteoblasts/stromal cells, which was abolished completely by simultaneously adding OPG (Fig. 3B). Treatment of calvarial osteoblasts with the known stimulators of osteoclast formation,  $1\alpha,25(\text{OH})_2\text{D}_3$ , PTH,  $\text{PGE}_2$ , or IL-11, up-regulated expression of mRNA of this molecule (56). From these results, it was concluded that the OPG-binding molecule was ODF, which mediates an essential signal to osteoclast progenitors for their differentiation into osteoclasts. Thus, the OPG-binding molecule was called "ODF." In contrast to the stimulatory actions of  $1\alpha,25(\text{OH})_2\text{D}_3$  and dexamethasone on ODF mRNA production, OPG mRNA levels were diminished, suggesting that the regulation of OPG levels is also critical for osteoclastogenesis induced by osteotropic factors.

Treatment of <sup>45</sup>Ca-prelabeled fetal mouse long bones with a soluble form of ODF (sODF) also stimulated the release of <sup>45</sup>Ca from the bone tissues, which was completely inhibited by simultaneously adding OPG (57). Like OPG, polyclonal antibodies against ODF inhibited bone resorption in organ cultures induced by not only sODF but also by  $1\alpha,25(\text{OH})_2\text{D}_3$ , PTH,  $\text{PGE}_2$ , and IL-1 (57). These results clearly indicate that bone resorption induced by these osteotropic factors is mediated by ODF.

2. *OPGL (OPG ligand)*. Lacey *et al.* (58) also succeeded in the molecular cloning of a ligand for OPG from an expression library of the murine myelomonocytic cell line 32D. The OPG ligand (OPGL) was identical to ODF. Recombinant OPGL expressed by human fibroblasts existed in both membrane-associated and soluble forms (58). However, there is no evidence that the soluble form of OPGL (ODF) is present in the microenvironment of bone. A recombinant soluble form of OPGL (sOPGL) stimulated osteoclast development in bone marrow cultures in the presence of M-CSF, and it induced TRAP-positive colony formation supported by M-CSF in an agar culture of bone marrow cells. Pit-forming activity of osteoclasts isolated from newborn rats was also stimulated by sOPGL. When sOPGL was injected into mice twice a day for 3 days, hypercalcemia was induced although the number of osteoclasts was almost identical to those of untreated mice (58). These results indicate that OPGL stimulates not only osteoclast differentiation but also osteoclast function. Recently, Kong *et al.* (59) have succeeded in generating OPGL knockout mice. OPGL(−/−) mice exhibited typical osteoporosis with total occlusion of bone marrow space within endosteal bone. OPGL(−/−) mice lack osteoclasts but have normal osteoclast progenitors that can differentiate into functionally active osteoclasts when cocultured with normal osteoblasts/stromal cells. In addition, OPGL(−/−) mice completely lack lymph nodes and have a defect in thymocyte differentiation. These results suggest that OPGL is an absolute requirement for osteoclast development, and it plays an important role in T cell differentiation as well.

The molecular cloning of ODF/OPGL revealed that this molecule was identical to TRANCE (TNF-related activation-induced cytokine) and RANKL [receptor activator of nuclear factor (NF)- $\kappa\text{B}$  ligand], which were independently identified by other groups as a novel member of the TNF ligand family (Fig. 4).

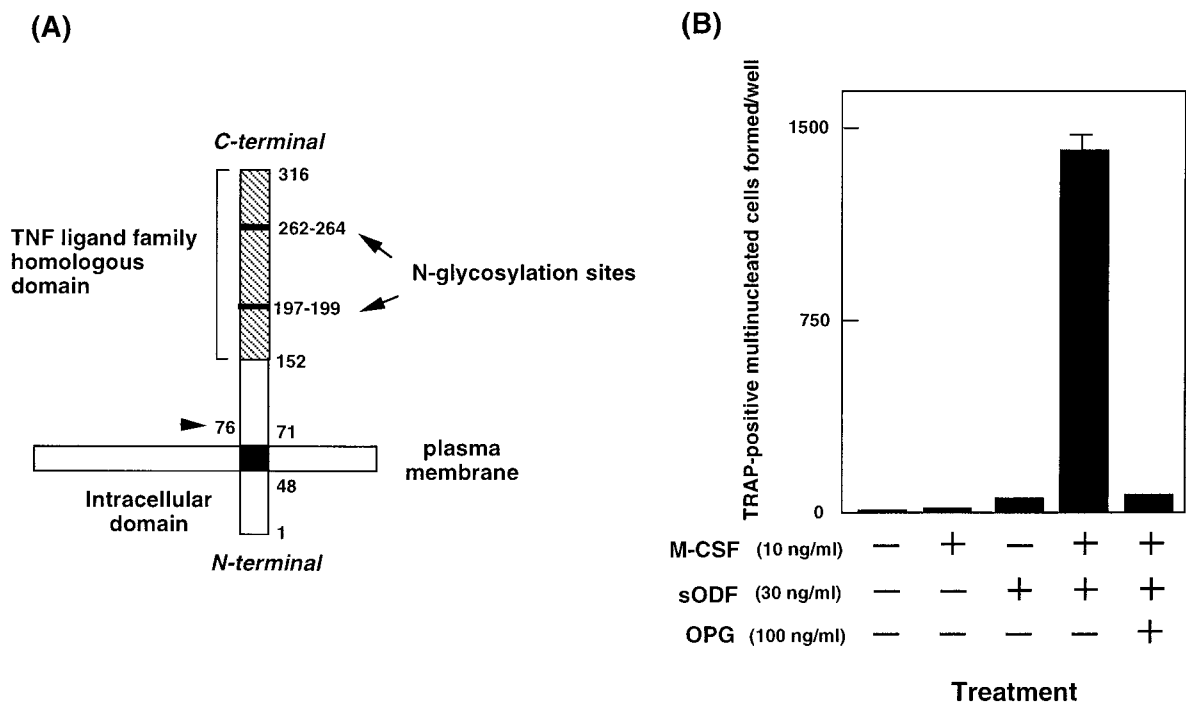


FIG. 3. A diagrammatic representation of the structure of ODF (A) and the effects of soluble ODF (sODF) on osteoclast formation in mouse spleen cell culture (B). A, Mouse ODF is composed of 316 amino acid residues. The predicted transmembrane domains exists between Ser<sup>48</sup> and Phe<sup>71</sup>. The TNF-homologous domain exists in Asp<sup>152</sup>-Asp<sup>316</sup>. There are two possible N-glycosylation sites, Asn<sup>197</sup>-Met<sup>198</sup>-Thr<sup>199</sup> and Asn<sup>262</sup>-Trp<sup>263</sup>-Ser<sup>264</sup>, in the TNF ligand family homologous domain. An arrowhead represents the N terminus (Asp<sup>76</sup>) of ODF, which is fused to the C-terminal end of thioredoxin, to prepare the soluble ODF (sODF/sRANKL) used in this study. B, Mouse spleen cells (10<sup>5</sup> cells per well) were cultured in 48-well plates in the presence or absence of M-CSF, sODF, and OPG. After culture for 6 days, the number of TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts. Data are expressed as the means  $\pm$  SD of three cultures.

3. *TRANCE*. *TRANCE* was cloned during a search for apoptosis-regulatory genes in murine T cell hybridomas (60). Northern blot analysis showed that thymus and lymph nodes expressed high levels of *TRANCE*. A recombinant soluble form of *TRANCE* induced activation of c-Jun N-terminal kinase (JNK) in T lymphocytes. The putative *TRANCE* receptor was detected on mature dendritic cells (61). Signaling by the receptor for *TRANCE* appeared to be dependent on TNF receptor-associated factor 2 (TRAF2), since JNK induction was impaired in thymocytes from transgenic mice overexpressing the dominant negative TRAF2 protein. *TRANCE* inhibited apoptosis of mouse and human dendritic cells *in vitro* with up-regulation of Bcl-xL expression (61). The increase in the survival of dendritic cells induced by *TRANCE* was accompanied by an increase in dendritic cell-mediated T cell proliferation in a mixed leukocyte reaction.

4. *RANK* (receptor activator of NF- $\kappa$ B) and its ligand (RANKL). Anderson *et al.* (62) cloned a new member of the TNF receptor family termed “RANK” from a cDNA library of human dendritic cells. The mouse homolog was also isolated from the fetal mouse liver cDNA library. The mouse RANK cDNA encoded a type I transmembrane protein of 625 amino acid residues. Like OPG, this protein bears four extracellular cysteine-rich domains. Northern blot analysis of human tissue RNAs revealed ubiquitous expression of RANK mRNA with highest levels in the skeletal muscle and thymus. RANK failed to bind other members of the TNF ligand family such

as Fas ligand, CD27 ligand, CD30 ligand, CD40 ligand, TNF $\alpha$ , or TRAIL. In searching for the binding molecule of RANK, a RANK ligand (RANKL) was cloned from a cDNA library of murine thymoma EL40.5 cells and found to be identical to *TRANCE* (62). A soluble form of RANKL augmented the ability of dendritic cells to stimulate T cell proliferation in a mixed lymphocyte reaction and increased the survival of RANK-positive T cells.

Darnay *et al.* (63) reported that TRAF2, TRAF5, and TRAF6 interacted with RANK at the C-terminal 85-amino acid tail. Furthermore, overexpression of RANK in human embryonic kidney 293 cells stimulated JNK and nuclear factor (NF)- $\kappa$ B activation. When the C-terminal tail necessary for the TRAF binding was deleted, the truncated RANK receptor was still capable of stimulating JNK activity but not NF- $\kappa$ B. This suggests that interaction with TRAFs is necessary for NF- $\kappa$ B activation but not for the activation of the JNK pathway. Wong *et al.* (64) showed that TRAF6 was also associated with the N-terminal portion of the cytoplasmic domain in addition to the C-terminal tail in the *TRANCE* receptor (RANK). Dominant negative forms of TRAF2, TRAF5, and TRAF6 inhibited *TRANCE* receptor (RANK)-mediated NF- $\kappa$ B activation in the cotransfected cells (64). These results suggest that RANKL/*TRANCE* directs differentiation and activation of osteoclasts through RANK by stimulating NF- $\kappa$ B via TRAFs. These findings appear to be contradictory to the finding that the JNK induction was impaired in thymocytes prepared from dominant negative TRAF2 transgenic mice

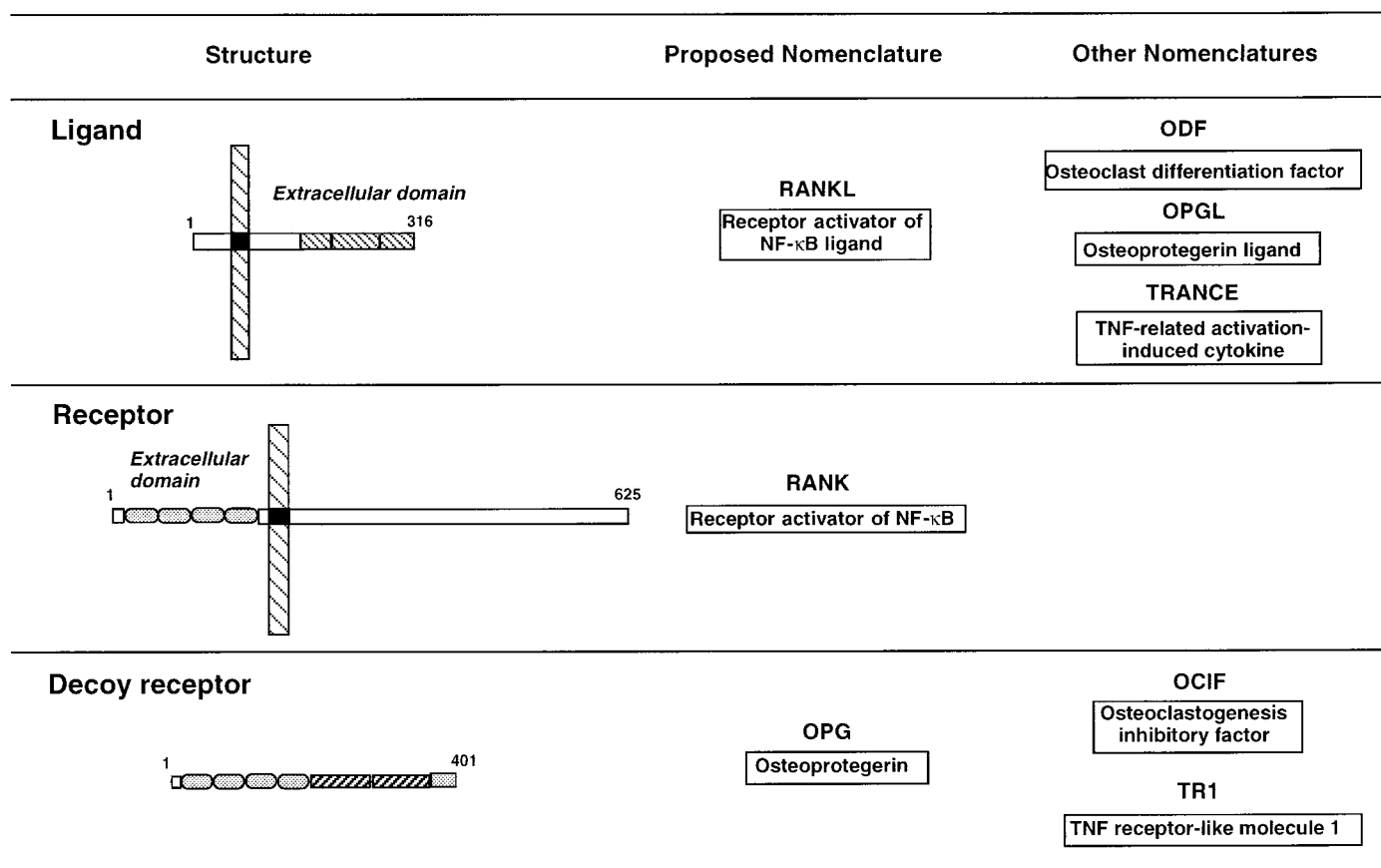


FIG. 4. A schematic representation of the ligand, receptor, and decoy receptor of the new TNF receptor-ligand family involved in osteoclast formation. Different nomenclatures for the same ligand/receptors compounds are listed. We wish to propose that RANKL, RANK, and OPG be adopted as the names of the ligand, signal transducing receptor, and soluble decoy receptor for the new TNF receptor-ligand family, respectively.

(61). Further studies are needed for elucidating the signaling pathway of RANK.

**5. Nomenclature.** The ligand, receptor, and decoy receptor of the new TNF receptor-ligand family members are schematically summarized in Fig. 4. Thus, ODF, OPGL, TRANCE, and RANKL are the same molecule important for development and function of T cells and dendritic cells as well as osteoclasts. RANK appears to be the transmembrane-signaling receptor for ODF/OPGL/TRANCE/RANKL. OCIF/OPG/TR1 is a soluble receptor for ODF/OPGL/TRANCE/RANKL and appears to function as a decoy receptor. These TNF-related ligands and receptors (membrane-bound and secreted) have a diverse range of functions and effects on cells other than osteoclasts and osteoblasts.

In the light of these other actions and their relatively wide distribution, the nomenclature of these molecules needs to be reviewed. To date there is only one report describing the putative signaling receptor, RANK, and we believe that this name should be used; it should be noted that an identical molecule that fulfills RANK's function has been proposed as the TRANCE receptor (Fig. 4) (60, 63). The ligand for RANK has been described as ODF, OPGL, TRANCE, and RANKL, and in proposing RANKL as the preferred nomenclature for this molecule we took into consideration its actions and distribution. The use of

ODF implies that its biological actions are specific to bone. While such a name might be attractive to the bone biology field, it is unlikely that ODF would be universally adopted, given its production by lymphocytes, its action on T lymphocytes and dendritic cells, and possible functions in other tissues. Use of OPGL would indicate that the molecule exerts its biological actions exclusively by binding to OPG, but OPG also binds to TRAIL (55). Furthermore, OPG appears to function as a decoy receptor and, as such, may have hitherto unrecognized TNF-related ligands. The acronym, TRANCE, derives from TNF-related activation-induced cytokine, suggesting that this molecule is expressed only after activation of cells. However, there is ample evidence for constitutive expression of this ligand, which may be stimulated further (56, 58, 62).

We have several reasons for proposing that RANKL be the preferred name: 1) this molecule is to date the only ligand identified for the membrane-bound signaling receptor, RANK; 2) it does not imply a unique tissue specificity or action; 3) it accurately describes postreceptor signaling actions, *i.e.*, activating NF- $\kappa$ B. Further, the commercial availability of the soluble ligand, sRANKL, is giving rise rapidly to wide usage of this term. On the basis of these reasons, we use the names of OPG, RANKL, and RANK subsequently in this review article (Fig. 4).

#### IV. Regulatory Mechanism in Osteoclast Development and Function

##### A. Regulatory mechanism of osteoclast differentiation by RANKL

M-CSF and RANKL are the two essential factors for inducing osteoclasts from mouse hemopoietic progenitors (56, 58). sRANKL alone had no colony-stimulating activity in a methylcellulose culture of bone marrow cells, and it did not affect the M-CSF-induced colony formation. This suggests that RANKL is not a growth factor but a differentiation factor of osteoclast progenitors. Using M-CSF, sRANKL, and OPG, the process of osteoclast differentiation was examined in more detail.

In the coculture system, the 6-day culture period can be separated into two phases: the first 4 days in which proliferation of osteoclast progenitors primarily occurs, and the final 2 days, in which their differentiation into osteoclasts is predominant (65). When hydroxyurea was added to the coculture for the first 4 days, no TRAP-positive cells appeared on day 6 even in the presence of  $1\alpha,25(\text{OH})_2\text{D}_3$ . In contrast, adding hydroxyurea to the cocultures during the final 2 days completely inhibited proliferation of osteoclast progenitors but did not affect their differentiation into osteoclasts in response to  $1\alpha,25(\text{OH})_2\text{D}_3$ . To confirm the involvement of M-CSF in osteoclast development, normal spleen cells were cocultured with osteoblasts derived from *op/op* mice (65). When M-CSF was added throughout the 6-day coculture period, osteoclasts were formed in response to  $1\alpha,25(\text{OH})_2\text{D}_3$ . However, the lack of M-CSF either for the first 4 days or for the final 2 days failed to result in osteoclast formation. These results confirm that M-CSF is indispensable for both the proliferative phase and the differentiation phase of osteoclast development (Fig. 5). Similarly, Biskobing *et al.* (66) showed that M-CSF plays important roles in proliferation and differentiation of osteoclast progenitors in mouse bone marrow cultures.

Recently, we characterized postmitotic osteoclast precursors obtained from cocultures (67). Postmitotic osteoclast precursors expressed some features of the macrophage-related phenotypes, such as Mac-1 and Mac-2, and they differentiated into osteoclasts without cell proliferation in the presence of osteoblasts. When postmitotic osteoclast precursors were further treated with sRANKL and M-CSF, they differentiated into TRAP-positive multinucleated cells

within 48 h even in the presence of hydroxyurea (68). These results also confirm that both M-CSF and RANKL are essentially involved in the differentiation phase of osteoclast precursors into osteoclasts (Fig. 5).

##### B. Regulatory mechanism of RANKL action on osteoclast function

When osteoblasts/stromal cells were removed from the coculture, osteoclasts rapidly died within 48 h by spontaneously occurring apoptosis (69). Among several cytokines and hormones examined, IL-1 and M-CSF stimulated the survival of purified osteoclasts (Fig. 5) (70). To elucidate further the regulation of fusion and function of osteoclasts by IL-1 and M-CSF, pOCs were isolated using echistatin from cocultures of mouse osteoblastic cells (MB 1.8 cells) and bone marrow cells. pOCs spontaneously died within 48 h. Both IL-1 and M-CSF potentiated survival and fusion of pOCs through their respective receptors (71). The effects of IL-1 on pOCs were inhibited by the naturally occurring inhibitor of IL-1, IL-1 receptor antagonist (IL-1ra), but not by a monoclonal antibody against M-CSF receptor (c-Fms), AFS98. The anti-c-Fms antibody (AFS98) inhibited M-CSF-induced effects on pOCs but not IL-1-induced effects. Interestingly, resorption pit-forming activity of pOCs placed on dentine slices was induced by adding IL-1 even in the absence of osteoblasts/stromal cells. M-CSF failed to induce pit formation in the pOC culture performed on dentine slices. As described above, enriched osteoclasts prepared from the coculture failed to form resorption pits. Pit-forming activity of enriched osteoclasts was markedly enhanced by adding IL-1, but not by M-CSF, even in the absence of osteoblasts/stromal cells. Thus, it is concluded that both IL-1 and M-CSF stimulate survival and fusion of pOCs, but only IL-1 treatment leads to osteoclasts that are active in resorption (Fig. 5). These results suggest that IL-1 might play a role as a real potentiator of osteoclast activation in inflammatory bone diseases.

Survival of purified osteoclasts was also enhanced by adding sRANKL (72). Treatment of purified osteoclasts with OPG suppressed the survival of osteoclasts supported by sRANKL but not that by IL-1 or M-CSF. Like IL-1 and M-CSF, sRANKL stimulated the survival and fusion of pOCs. In addition, sRANKL induced the resorbing activity of pOCs. These results indicate that both RANKL and IL-1 lead to enhanced osteoclast function even in the absence of osteo-

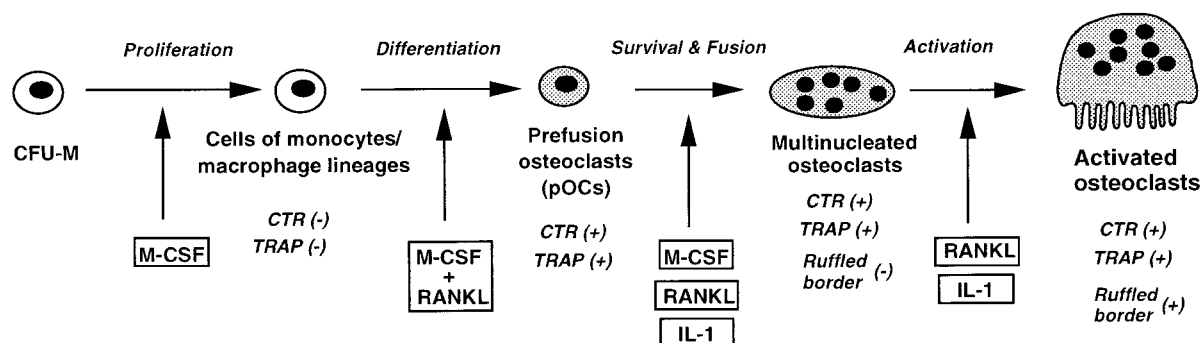


FIG. 5. The differentiation pathway of osteoclast progenitors into functionally active osteoclasts and the cytokines required for each step of the pathway.

blasts/stromal cells (Fig. 5). This notion was confirmed by the experiments using osteoclast preparations in which osteoblasts were completely absent. Bone marrow cells were cultured on collagen gel-coated dishes in the presence of sRANKL and M-CSF but in the absence of osteoblasts/stromal cells. Osteoclasts formed were collected by collagenase digestion. When these osteoclasts were placed on dentine slices, they rapidly underwent apoptosis without forming resorption pits. M-CSF, IL-1, and sRANKL all prolonged the survival of those osteoclasts, but only sRANKL and IL-1 induced the pit-forming activity. When primary osteoblasts were added to similarly prepared osteoclasts, resorption pits were formed. Bone-resorbing factors such as  $1\alpha,25(\text{OH})_2\text{D}_3$ , PTH, and IL-11 enhanced their pit formation only in the presence of osteoblasts (73). Osteoblasts prepared from *op/op* mice also induced pit-forming activity of osteoclasts, which was completely inhibited by adding OPG. These results support the hypothesis that osteoblasts/stromal cells activate osteoclast function through RANKL as a membrane-associated factor. RANKL can be replaced with IL-1 to induce survival, fusion, and activation of osteoclasts. However, IL-1 could not support differentiation of osteoclast precursors into pOCs even in the presence of M-CSF, when osteoblasts/stromal cells were absent (Fig. 5). These results also suggest that RANKL is involved in physiological bone resorption, whereas IL-1 is involved in pathological bone resorption such as rheumatoid arthritis and periodontitis. Although RANKL has been shown recently to increase resorption by isolated rat osteoclasts (74), the question of activation of mature osteoclasts is more precisely addressed by using a culture system in which no continued osteoclast formation takes place. We made use of osteoclasts generated by such a method to determine that the effects of RANKL in mature osteoclasts might result from prolongation of cell survival, as well as a signal-mediated effect on osteoclast activity. Formation and activation of osteoclasts cannot be easily distinguished *in vivo* in mammals because the single factor, RANKL, expressed by osteoblasts/stromal cells, carries out the two aspects of osteoclasts (osteoclast differentiation and function).

### C. Signals induced by IL-1 and RANKL in osteoclasts

An electrophoretic mobility shift assay revealed that IL-1 transiently activated NF- $\kappa$ B in the nuclei of purified osteoclasts, and the maximal activation occurred at 30 min after IL-1 addition (70). Osteoclasts formed in the cocultures indeed have IL-1 type I receptors (75). The degradation of I- $\kappa$ B $\alpha$ , which forms a complex with NF- $\kappa$ B and keeps the complex in the cytoplasm, coincided with the activation of NF- $\kappa$ B. The immunocytochemical study revealed that p65, a subunit of NF- $\kappa$ B, was translocated from the cytoplasm into almost all of the nuclei of the multinucleated osteoclasts. Pretreatment of osteoclasts with proteasome inhibitors or antisense oligodeoxynucleotides to p65 and p50 of NF- $\kappa$ B prevented the survival of osteoclasts supported by IL-1 (75). These results indicate that IL-1 promotes the survival of osteoclasts through NF- $\kappa$ B activation (Fig. 6). Northern blot analysis showed that osteoclasts formed in the cocultures strongly expressed RANK mRNA (72). Treatment of purified

osteoclasts with sRANKL activated NF- $\kappa$ B within 30 min, which was accompanied by the degradation of I- $\kappa$ B $\alpha$ . IL-1 and sRANKL also activated JNK within 30 min in the purified osteoclasts. These results suggest that the activation of NF- $\kappa$ B and JNK in osteoclasts by IL-1 and sRANKL results in induction of osteoclast activation (Fig. 6). Bcl-xL expression remained unchanged in the purified osteoclasts treated with RANKL, suggesting that a factor(s) other than Bcl-xL is involved in the RANKL-induced survival of osteoclasts.

Recently, Franzoso *et al.* (76) and Iotsova *et al.* (77) independently generated mice deficient in both p50 and p52 subunits of NF- $\kappa$ B. The double-knockout mice developed osteopetrosis because of a defect in osteoclast differentiation. The osteopetrotic phenotype was rescued by bone marrow transplantation, indicating that the osteoclast progenitors were impaired. Osteoclasts were totally absent, but the number of Mac-2-positive macrophages was rather increased in bone tissues from the double-knockout mice. These results suggest that p50 and p52 can be replaced with each other in dimer formation with p65 in osteoclast precursors. RANKL has been reported to activate NF- $\kappa$ B in the target cells (62), and we have also determined this in the osteoclast (72). These results suggest that the RANKL-induced activation of NF- $\kappa$ B in osteoclast progenitors also plays a crucial role in their differentiation into osteoclasts. It is also conceivable that transcription factors other than NF- $\kappa$ B are regulated by RANK-mediated signals in osteoclast precursors and mature osteoclasts.

### V. Regulation of Human Osteoclast Development

As described above, RANKL and M-CSF are two essential factors for mouse osteoclast formation. Recent findings indicate that the regulatory mechanism of human osteoclast formation is quite similar to that of mouse osteoclast formation. Fujikawa *et al.* (78) first demonstrated that UMR-106 cells and ST2 cells supported human osteoclast formation in coculture with human peripheral blood mononuclear cells in

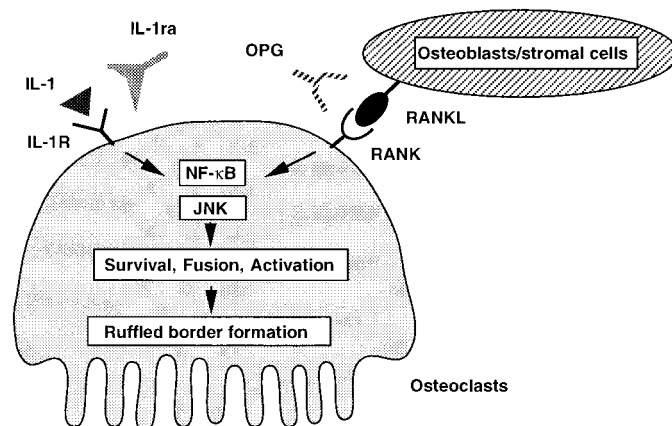


FIG. 6. Signals induced by IL-1 and RANKL in osteoclasts. Osteoclasts formed in the cocultures express IL-1 type I receptor and RANK. IL-1 and RANKL similarly induce the survival, fusion, and activation of osteoclasts in the absence of osteoblasts/stromal cells. Both IL-1 and RANKL activate NF- $\kappa$ B and JNK through their respective receptors. IL-1ra (IL-1 receptor antagonist) and OPG inhibit IL-1- and RANKL-induced signals, respectively.

the presence of  $1\alpha,25(\text{OH})_2\text{D}_3$  and dexamethasone. In their experiments, addition of human M-CSF to the coculture was essential to induce human osteoclasts because UMR-106 and ST2 cells produce rat and mouse M-CSF, respectively, which do not bind to human M-CSF receptors (c-Fms). This finding also suggested that rat and mouse RANKL can act on human cells as well. We also confirmed that the human osteoblastic cell line, SaOS-4/3, which constitutively expressed functionally active PTH receptors, supported human osteoclast formation in response to PTH and dexamethasone in coculture with human peripheral blood mononuclear cells (PBMCs) (33). Antihuman M-CSF antibody inhibited both mouse and human osteoclast formation in coculture with SaOS-4/3 cells. These results are consistent with the finding of Flanagan and her colleagues (79, 80) who demonstrated a critical role of M-CSF in human osteoclast formation as well.

Treatment of human PBMCs with mouse sRANKL and human M-CSF together with dexamethasone induced human osteoclasts (Fig. 7) (81). OPG inhibited osteoclast formation from human PBMCs that was supported either by SaOS-4/3 cells or by sRANKL plus human M-CSF. PTH induced expression of RANKL mRNA by SaOS-4/3 cells, and this was not affected by adding dexamethasone (82). These results suggest that nonadherent cells in human PBMCs produce an inhibitory factor(s) against human osteoclastogenesis, production of which is down-regulated by dexamethasone. When osteoclasts were generated from human PBMCs that had been purified on a Ficoll gradient, sRANKL and M-CSF were effective without the need for dexamethasone (83). Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been shown to be an important factor for osteoclast formation in human bone marrow cultures (3, 21). However, as in the case of mouse osteoclast

formation, GM-CSF strongly inhibited human osteoclast formation induced by sRANKL and M-CSF (Fig. 7 and Ref. 33). This suggests that GM-CSF stimulates proliferation of osteoclast progenitors but inhibits their differentiation into osteoclasts. These results also indicate that regulatory mechanisms of human osteoclast formation are essentially the same as those of mouse osteoclast formation. Regulation of human osteoclast formation and function can be deduced from the findings obtained from the mouse system.

VI. Summary and Conclusion

Osteoblasts/stromal cells are essentially involved in osteoclast differentiation and function through cell-to-cell contact (Fig. 8). Although many attempts have been made to elucidate the mechanism of the so-called “microenvironment provided by osteoblasts/stromal cells,” (5–8) it has remained an open question until OPG and its binding molecule were cloned. The serial discovery of the new members of the TNF receptor-ligand family members has confirmed the idea that osteoclast differentiation and function are regulated by osteoblasts/stromal cells. RANKL, which has also been called ODF, TRANCE, or OPGL, is a member of the TNF ligand family. Expression of RANKL mRNA in osteoblasts/stromal cells is up-regulated by osteotropic factors such as  $1\alpha,25(\text{OH})_2\text{D}_3$ , PTH, and IL-11. Osteoclast precursors express RANK, a TNF receptor family member, recognize RANKL through cell-to-cell interaction with osteoblasts/stromal cells, and differentiate into pOCs in the presence of M-CSF. RANKL is also involved in the survival and fusion of pOCs and activation of mature osteoclasts. OPG, which has also been called OCIF or TR1, is a soluble receptor for RANKL

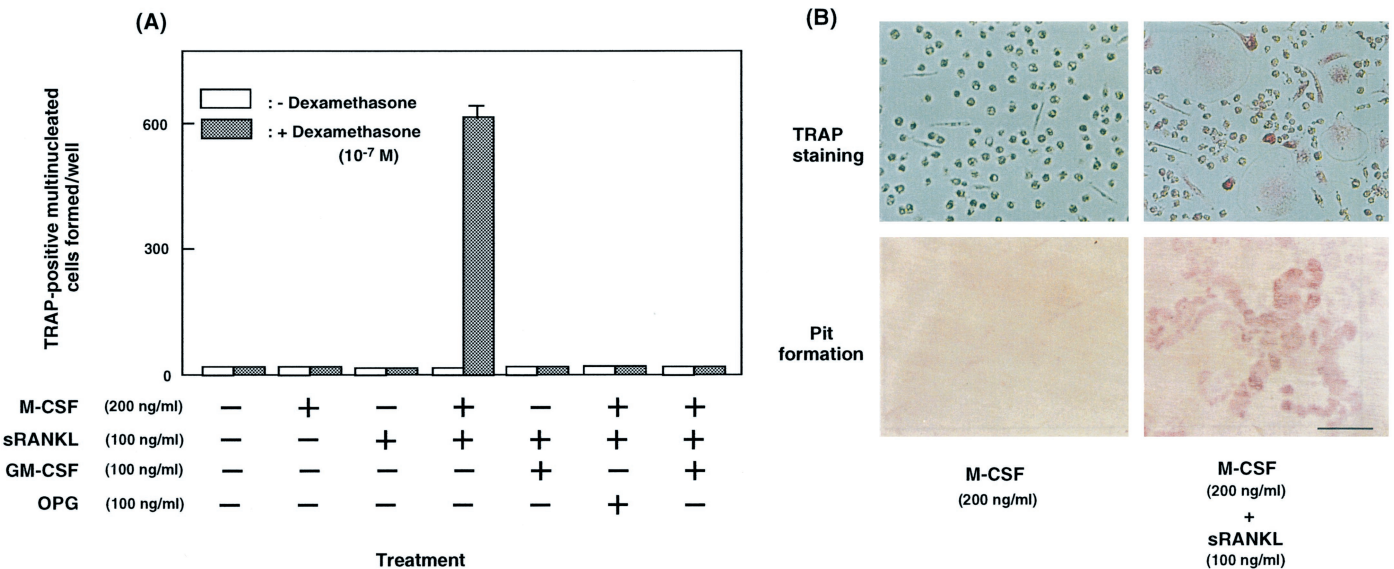


FIG. 7. RANKL, together with human M-CSF, induces human osteoclast formation. A, Human PBMCs were cultured in 48-well plates ( $4 \times 10^5$  cells per well) in the presence or absence of human M-CSF, mouse sRANKL, human OPG, human GM-CSF, and dexamethasone. After culture for 7 days, TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts. B, *Upper panels*, Human PBMCs were cultured in 48-well plates ( $4 \times 10^5$  cells per well) with or without human M-CSF and mouse sRANKL in the presence of dexamethasone ( $10^{-7}$  M). After culture for 7 days, adherent cells were stained for TRAP. *Lower panels*, Human PBMCs were cultured in 48-well plates ( $4 \times 10^5$  cells per well) in which a dentine slice had been placed. Cultures were treated with human M-CSF and mouse sRANKL in the presence of dexamethasone ( $10^{-7}$  M). After culture for 10 days, resorption pits formed on the slices were stained with Mayer's hematoxylin. Bar = 200  $\mu\text{m}$ .

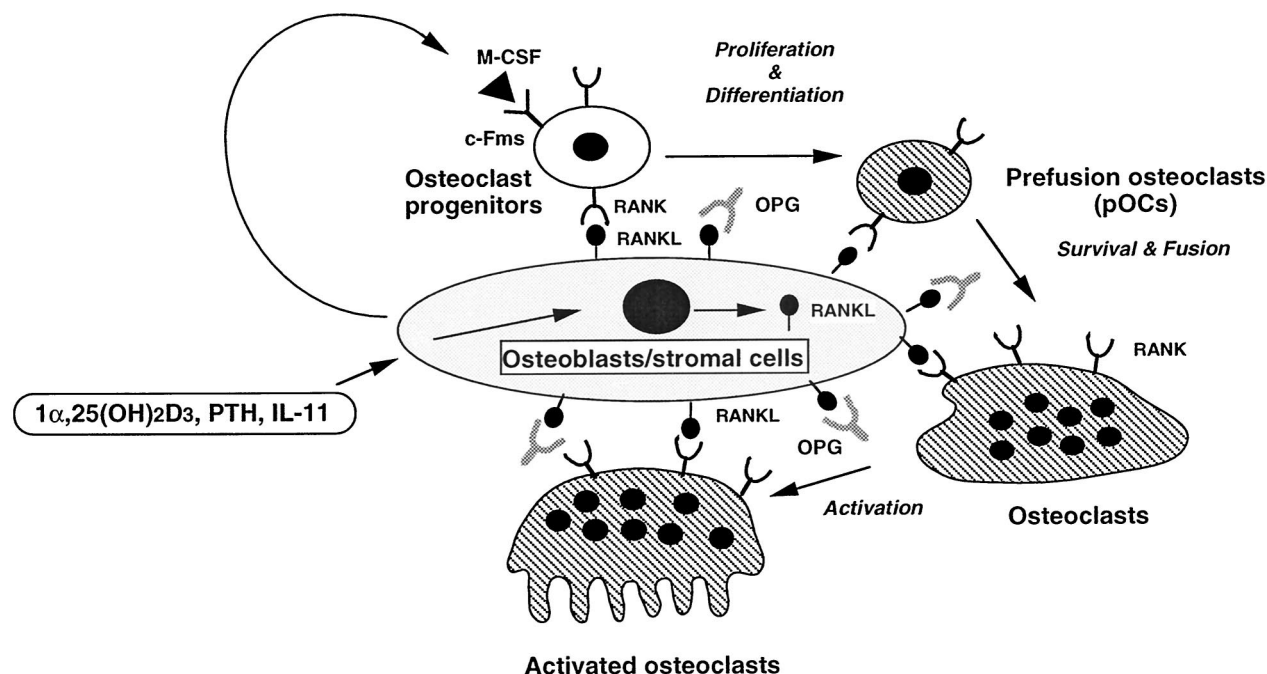


FIG. 8. A schematic representation of osteoclast differentiation and function supported by osteoblasts/stromal cells.

and acts as a decoy receptor in the RANK-RANKL signaling system (Fig. 8).

In conclusion, osteoblasts/stromal cells are involved in all of the processes of osteoclast development, such as differentiation, survival, fusion, and activation of osteoclasts (Fig. 8). Osteoblasts/stromal cells can now be replaced with RANKL and M-CSF in dealing with the whole life of osteoclasts. RANKL, RANK, and OPG are three key molecules that regulate osteoclast recruitment and function. Further studies on these key molecules will elucidate the molecular mechanism of the regulation of osteoclastic bone resorption. This line of studies will establish new ways to treat several metabolic bone diseases caused by abnormal osteoclast recruitment and functions such as osteopetrosis, osteoporosis, metastatic bone disease, Paget's disease, rheumatoid arthritis, and periodontal bone disease.

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