

1.

Osteoclast: Origin and Differentiation

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Introduction

The skeleton is an engineering feat: it is strong but light enough to permit locomotion, rigid to allow muscles to be fixed but capable of bending without breaking, and its structure is programmed for variable loading (Figure 1.1). In addition to its structural role, the skeleton serves as a reservoir and sink for calcium, an ion that plays an important role in cell metabolism and function. This means that bone must be malleable, that its calcium stores must be accessible, and that its structure can be sampled, adapted, or fine-tuned, in a process termed remodeling. The primary step in remodeling is the resorption of bone. The multinucleated osteoclast, a highly specialized cell, is responsible for resorbing the mineral phase of the skeleton.

The osteoclast, therefore, is a basic element in skeletal physiology. Abnormalities in osteoclast development or function that severely limit its ability to resorb bone are incompatible with normal locomotion, and, if severe enough, with life. Defects in osteoclastogenesis can result in lethal neonatal disease because the bones lack a marrow space for hematopoiesis. Milder defects in osteoclastogenesis lead to crippling skeletal lesions in childhood. In adulthood, excess osteoclast action – due either to the presence of too many osteoclasts or hyperactivity of those present – is the predominant cause of most skeletal pathology. Within the past ten years, research into the origin of the osteoclast and the signals that direct its formation at a specific

hydroxyapatite surface has led to a fuller understanding of this important cell.

In this introductory chapter we set out the basic aspects of osteoclast origin and differentiation. Beginning with a description of the multinuclear cell found in bone and a brief introduction to its typing through functional characteristics, we discuss developing research from a historical viewpoint. However, attention will be focused on the current understanding of osteoclastogenesis, shown in Figure 1.2.

The Osteoclast: Lineage of the Multinucleated Macrophage

The osteoclast was recognized in 1873 as the principal multinuclear cell that digested bone [87], but its origin was not uncovered until more than a century later. Kahn and Simmons were able to separate the lineage of osteoclasts from the other key cell in remodeling, the osteoblast, with a clever experiment in 1975. They took advantage of the morphological differences between cell nuclei of quail and chicken by growing quail bone rudiments on well-vascularized portions of chorioallantoic membranes of chicken embryos. The osteoblasts appearing a week later were of quail origin, but the nuclei of the osteoclasts had a predominantly chicken morphology [75]. This was the first convincing evidence that osteoclasts were hematogenous in origin. Several years later, building on work showing that cure of osteopetrosis in mice was possible by transplantation of



Figure 1.1 The skeleton as interpreted by Vesalius.

normal bone marrow [187], the first successful bone-marrow transplantation was performed in a female human infant with malignant osteopetrosis. In the normally remodeling bone, the osteoclasts appearing *in situ* lacked Barr bodies, as would be expected in male nuclei: indeed, the bone marrow had been harvested from the patient's brother [24].

With evidence that the osteoclast arose from fusion of hematogenous cells, the next step in determining the lineage was to develop an *in vitro* process to obtain polykaryons capable of resorbing calcified bone matrix. Neither monocytes nor macrophages fit this definition, although they are able to resorb some mineral. Burger et al. [17], culturing hematopoietic tissue, showed that at two weeks the culture consisted largely of immature and mature mononuclear phagocytes, but contained no osteoclast-like cells. When, however, stripped bone rudiments were added to the hematopoietic cell culture, osteoclasts appeared in close

contact with the calcified matrix [17]. This demonstrated that recreation of the bone environment provided sufficiency for transformation of hematologic precursors into osteoclasts. The need for elements of the bone environment to allow osteoclastogenesis has become key to understanding the intricate relationship between bone osteoblasts/stromal cells and osteoclasts.

The understanding that osteoclasts arose from hematopoietic stem cells (HSC) created a need for adequate typing to ensure that multinucleated "osteoclasts" were not other cells arising from the HSC. Indeed, many characteristics of osteoclasts are shared by macrophages, including the ability to fuse with like cells and the ability to phagocytose. However, macrophages associated with bone surfaces do not have the ruffled border that is characteristic of the osteoclast, nor do they respond to calcitonin with a change in shape. Moreover, and most convincingly, macrophages are unable to rescue an osteopetrotic deficit [184]. Cell morphology, staining for functional characteristics such as tartrate resistant acid phosphatase, and the ability to respond to calcitonin, were all found to be useful for differentiating osteoclasts from macrophages. Many laboratories contributed to a more exacting definition of cell type by examining cell surface characteristics. For instance, osteoclasts lack the Fc and C3 surface receptors that typify macrophages. As pre-osteoclasts differentiate, they lose their early macrophage characteristics, decreasing expression of macrophage-specific surface antigens F4/80, Mac-1. Their ability to phagocytose is also diminished [20, 185]. In comparison with macrophage polykaryons, osteoclasts express a variable but restricted range of macrophage-associated antigens (e.g., CD13, CD15A, CD44, ICAM-1), whereas macrophage polykaryons strongly express CD18, CD14, CD31 [10]. This allows for a discrete separation of osteoclasts from macrophage multinuclear cells.

Cell typing that largely pertains to the available and developed antibodies has led to further demarcation between osteoclasts and cells of macrophage lineage [59, 60]. Currently markers used during flow cytometry delineate the extended progression from stem cell to the osteoclast phenotype. Indeed these steps constitute a road map of the differentiation program. (These are shown along the top of Figure 1.2.)

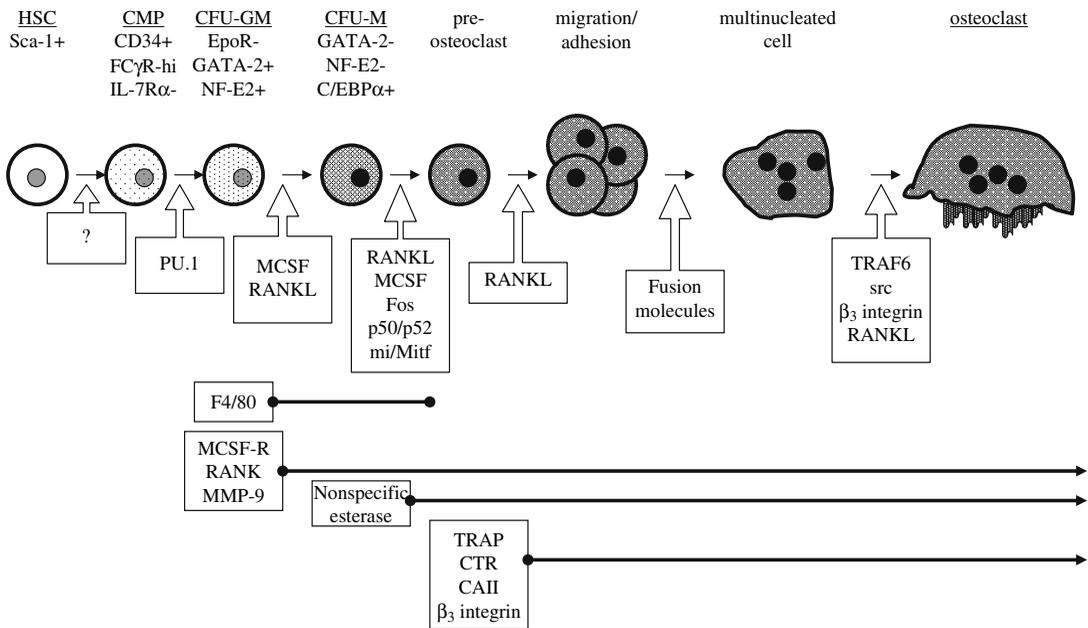


Figure 1.2 Osteoclast Differentiation. The diagram shows the progression from the hematopoietic stem cell (HSC) through the common myeloid progenitor (CMP) to the colony-forming unit for granulocytes and macrophages (CFU-GM) to the CFU for macrophages and into the osteoclast lineage. Markers that can be used to identify early stem cells are shown above the cells. Factors that stimulate progressing are shown in the arrowed boxes. The boxes on the bottom of the diagram show appearance of markers associated with osteoclast lineage.

In vitro co-culture systems that adequately simulate the bone microenvironment have been a major tool for study of progression along the osteoclast lineage. Using variations on the Dexter marrow culture [28, 29], bone biologists found that it was possible to generate osteoclasts in the presence of osteoclastogenic agents in primate [141], feline [65], and murine cultures [95, 162, 168]. Such culture systems allowed the identification of factors that enhanced osteoclast development such as 1,25-dihydroxyvitamin D [141], prostaglandins [5], and cyclic AMP [148], as well as parathyroid hormone [6]. Within the context of murine marrow cultures, other cytokines such as IL-6 and TNF have also been shown to regulate osteoclast differentiation [3, 182]. Dexter's culture [29] had opened the way to understanding the role of colony-stimulating factors (CSFs) in blood cell proliferation and differentiation. It therefore seemed logical to expect that colony-stimulating factors would also play a role in osteoclast development. Macrophage CSF (MCSF) was found to be essential for osteoclastogenesis [86] and granulocyte-macrophage-CSF (GM-CSF) was shown to

enhance osteoclast formation [95]. The effect of these CSFs gave support to the idea that macrophages and osteoclasts share a common origin. Experiments also made it possible to identify three stages in osteoclast differentiation: early differentiation, where hematopoietic stem cells proliferate within the macrophage lineage [53]; progression into the early osteoclast precursor stage with expression of calcitonin receptors and TRAP [51]; and a later stage where fusion occurs [13]. The extensive osteoclastogenic marrow culture literature adds to the conclusion that the osteoclast precursor begins as a nonadherent hematopoietic stem cell and progresses through the colony-forming unit for granulocytes and macrophages (CFU-GM) to become a CFU-macrophage (CFU-M) before entering the osteoclast lineage.

As stated above, accessory cells representing bone stromal elements are necessary for osteoclast formation. In addition, cell-cell contact is required between the myelocytic precursor and the bone stroma [163, 203]. The critical factor contributed by bone stromal cells for terminal osteoclast differentiation was identified in 1998

as RANKL, a member of the tumor necrosis factor family [97, 206]. The discovery of this factor has permitted osteoclast biology to shift into high gear, as will be described below.

Early Differentiation

The first process we will examine is the progression from the pluripotent hematopoietic stem cell to the colony-forming unit for macrophages.

Myeloid Differentiation

The pluripotent hematopoietic stem cell (HSC) in the marrow cavity awaits signals that direct its development into the myeloid lineage. This requires expression of molecules that allow response to osteoclastogenic factors. The HSC bears stem cell antigen-1 (Sca-1+), which indicates a self-renewing capacity [135, 159]. The first way station in HSC differentiation is a choice between entry into the myeloid or the lymphoid progenitor pool. Expression of the interleukin-7 receptor α -chain (IL-7R α) differentiates the common lymphoid progenitor (CLP) that reconstitutes T-cells, B-cells, and natural killer cells from cells of the myeloid lineage [88].

The immediate precursor of the myeloid progenitor population is identified by the absence of the IL-7R α . In a search for the earliest myeloid cell, Akashi and colleagues recently used cell sorting to discard IL-7R α -positive cells that have entered lymphoid lineage. They next selected an IL-7R α -negative fraction expressing the myelomonocytic Fc γ receptor [4]. In the presence of GM-CSF and a mixture of cytokines (steel factor, Flt-3 ligand, IL-11, IL-3, erythropoietin, and thrombopoietin) 80% of this fraction commits to myeloid lineage [4]. If this lineage is further separated into a fraction with high Fc γ R expression that also expresses the cell surface molecule sialomucin CD34, this fraction becomes almost exclusively myeloid. Fc γ R^{hi}CD34⁺ cells divide into three lineages that contain macrophages and/or granulocytes, i.e., CFU-M, CFU-G, and CFU-GM. In contrast the Fc γ R^{lo}CD34⁺ behave like HSCs, and the Fc γ R^{lo}CD34⁻ give rise to colonies that contain only megakaryocytes and/or erythrocytes. Thus, Akashi was able to differentiate the common

myeloid precursor, "CMP," from the common lymphoid precursor as an IL-7R α -, GATA-2+, NF-E2+, GATA-1+, GATA-3- cell [4]. It is this cell that responds to signals that generate proliferation of the CFU-GM, as distinguished from the lineage providing cells for megakaryocyte or erythrocyte lineage, or the earlier fork where the HSC enters the lymphoid pathway.

Interestingly, the absence of the Pax5 gene, a key to the B-lymphoid lineage, leads to enrichment of the myeloid lineage in transgenic Pax5^{-/-} animals [132]. The Pax5^{-/-} pro-B cells respond to M-CSF with expression of macrophage markers Mac-1 and F4/80, and, in the presence of RANKL, into osteoclasts. Although scientists were unable to force the pro-B cells into erythroid or megakaryocytic lineage, pro-B cells clearly maintain a degree of plasticity even after they are committed to a lymphoid lineage [132].

The factors responsible for herding HSC into the common myeloid progenitor lineage and then into the CFU-GM are multiple and very likely redundant. By means of targeted deletions of early lineage markers from embryonic stem cells, GATA-2 and SCL/tal-1 were shown to be important for early development [202]. The SCL/tal-1 transcription factor was first described as an abnormal translocation in T-cell leukemia and is necessary for generation of all hematopoietic cells. GATA-2, while not necessary for later expansion of osteoclast progenitors, has some role in separating CFU-M from those cells that are most responsive to RANKL. The colony-stimulating factors for macrophage and/or the granulocyte lineage cause cultured osteoclast numbers to swell in numbers by increasing the number of precursors [34, 53, 95, 160, 167, 175].

Becoming a Macrophage Progenitor

As the colony differentiates, a number of markers identify the emergence of the macrophage lineage. Macrophage colonies are readily identifiable because they express a non-specific esterase, along with the receptor for the macrophage colony-stimulating factor. The movement from the common myeloid precursor (CMP) through the CFU-GM and into the macrophage lineage is in fact, largely due to the macrophage colony-stimulating factor (MCSF). The importance of MCSF to osteoclast lineage became apparent as the result of the discovery

that the osteopetrotic (op/op) mouse, whose marble bone disease and lack of dental eruption are lethal consequences of total osteoclast deficiency, has a mutation in the gene that codes for MCSF [193, 208]. Similarly, in the toothless rat, which displays the osteopetrotic phenotype, a frameshift mutation in the MCSF gene causes a loss of function that brings about a profound deficiency of osteoclasts and macrophages [186]. In culture, MCSF is more potent than GM-CSF or interleukin-3 in stimulating osteoclast formation in primate bone marrow [137]. GM-CSF cannot rescue the op/op MCSF deficient mouse, but does support the development of osteoclast-like cells if vitamin D is present in the culture [93]. In contrast, VEGF can substitute for MCSF when administered to op/op mice and appears to be the molecule responsible for the age-related resolution of osteopetrosis seen in mice nursed through their early otherwise lethal inability to wean [131].

MCSF has importance as a growth and differentiating factor. It is also required for survival of cells of the macrophage lineage [175]. In a culture containing high levels of MCSF, monocytes will increase in number at the expense of osteoclastogenesis, probably because MCSF strongly promotes the development of mononuclear monocytes [34, 136]. Yet, in the case of estrogen deficiency, increased levels of MCSF in bone marrow may enhance osteoclastogenesis [81]. These discrepancies may be due to differences in the expression patterns of MCSF, resulting from at least two splice variants. One of these is a secreted soluble factor. The other, representing a much smaller amount of the total MCSF, ~5%, is expressed on the surface of bone stromal cells [35, 204]. Because the insoluble form of MCSF is anchored and displayed by cells within bone, this isoform may represent a local signal that regulates osteoclast development. Indeed in the op/op MCSF-deficient mouse, the membrane-bound form of MCSF can correct the osteoclast defect [205]. Perhaps the best way to summarize the literature is to state that MCSF expands the precursor pool capable of responding to osteoclastogenic signals from the bone marrow and, at the same time, enhances macrophage differentiation and survival.

The CFU-GM expresses the MCSF receptor, which is a membrane tyrosine kinase encoded by the gene *c-fms* [153]. Multiple second messengers that are activated through this membrane tyrosine kinase receptor trigger

proliferation of the CFU-M [155]. The downstream proliferative response to the MCSF ligand includes induction of G2 progression through activation of cyclin genes. It is difficult to separate the differentiative from the proliferative responses in MCSF-R-bearing cells [74, 154]. Myeloid cells become macrophage, in part, because the myeloid and B-cell-specific transcription factor PU.1 also increases. The finding that PU.1-deficient mice have a decreased number of macrophages and no osteoclasts gives support to the role played by PU.1 in macrophage recruitment [179].

The transcription factor *fos*, and perhaps other members of the AP-1 transcription factor family, play an important role in osteoclast differentiation. It has been long known that the *v-fos* oncogene induced by murine sarcoma virus causes osteosarcoma and that overexpression of the cellular homolog, *c-fos*, led to the development of bone tumors [149]. Because these lesions were in osteoprogenitor cells, not in the osteoclast lineage or in HSCs, it was surprising that mice lacking *c-fos* developed osteopetrosis, essentially an overgrowth of bone [48]. In fact, *c-fos* is not required for normal osteoprogenitor development but is required for osteoclast differentiation: *c-fos*-negative HSC progress normally into CFU-M and from there to become terminally differentiated macrophages, but osteoclasts are totally absent in this mouse. Whether the requirement for *fos* is in the stromal accessory cell (e.g., for normal expression of RANKL), or only in the osteoclast progenitor (e.g., required for response to osteoclastogenic signals) has not yet been worked out. However, overexpression of *c-fos* in osteoclast precursors can increase osteoclast differentiation [122] and RANKL does induce transcription of genes through *c-fos* activation. This suggests a link between RANKL signaling and *c-fos* activation [115]. RANKL has, in fact, been shown to induce interferon- β in osteoclast precursor cells. This interferes with RANKL-induced expression of *c-fos* and decreases osteoclastogenesis [172].

Other members of the AP-1 family are also involved: Fra-1 can rescue the *c-fos* defect in osteoclast precursors [38]; this may suggest some redundancy between AP-1 members in intracellular signaling leading to osteoclastogenesis. Overexpression of Fra-1 by itself, also, leads to increased osteoblast differentiation and thus to a progressive increase in bone mass [73].

Expression of AP-1 proteins therefore plays critical roles in osteoclast and osteoblast progenitors and is necessary for normal coupling between the remodeling cells.

CFU-M into Pre-Osteoclast: The RANKL:RANK Signal

RANKL Comes Calling

The progression of the macrophage colony-forming unit into the osteoclast lineage has been traditionally characterized by development of a panel of osteoclastic phenotypic features. These include expression of the calcitonin receptor, of enzymes such as tartrate-resistant acid phosphatase and carbonic anhydrase II, and the ability to resorb bone [12, 52, 102, 103]. Other established markers are the vitronectin receptor and the vacuolar-type proton pump [94]. Although the progression from the monocytic precursor to the osteoclast progenitor was thus clearly established, the critical factor responsible for this metamorphosis was unknown.

Experiments had shown that when the CFU-M (or its earlier precursors) collected from bone marrow or spleen was added to a culture of osteoprogenitor cells, to which any one of a handful of osteoclastogenic hormones or cytokines was added, osteoclastogenesis could proceed [112]; the osteoclastogenic factors acted synergistically [139] and furthermore appeared to target the stromal accessory cells, since the accessory cells could be pretreated with agents such as 1,25-dihydroxyvitamin D and fixed prior to culture with the hematoge-

nous precursors [35, 190]. Moreover the accessory cells actually had to be in direct contact with the osteoclast progenitor cells. Separation by a membrane prevented the process [164]. This meant that the stimulated stromal cells, even when fixed, provided a contact signal to the CFU-M. What was this signal?

Laboratories working in Japan and California discovered the answer simultaneously. TNF-related activation-induced cytokine, or TRANCE, originally described as a factor that activated signals in T-lymphocytes [197], was found to be the osteoclastogenic factor that is now known as Receptor Activator of NF κ B Ligand or RANKL [97, 206] (Figure 1.3). Discovery of RANKL involved similar strategies by both groups, using a previously identified inhibitory binding factor to screen for the osteoclastogenic factor. Yasuda's team used an osteoclast inhibitory factor from bone-cell-conditioned media that eventually was shown to be the TNF-R-like decoy receptor osteoprotegerin, to tag expression libraries encoding the ligand partner for the inhibitory protein [206]. They called their gene product "osteoclast differentiation factor" and showed that it induced osteoclast formation from spleen cells in the absence of stromal elements. Osteoclast differentiation factor expression was upregulated in stromal cells by osteoactive factors such as parathyroid hormone and 1,25-dihydroxyvitamin D. Lacey's group in California had discovered the same soluble inhibitory binding protein, named it osteoprotegerin (OPG), and showed that it prevented osteoclastogenesis *in vivo* [157]. Searching for the binding partner of OPG, they cloned "osteoprotegerin-ligand" (OPGL) from a murine expression library [97]. OPGL bound to hematopoietic progenitor cells

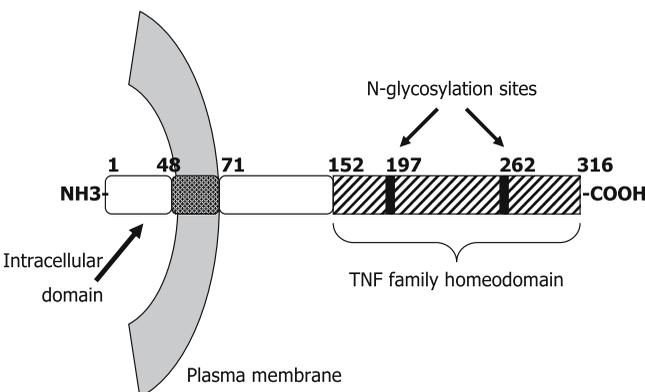


Figure 1.3 RANKL. The drawing shows the protein structure of RANKL.

and rapidly induced osteoclast genes. The final selection of the term RANKL, or receptor activator of NFκB ligand [7] as the name of the OPG binding partner in place of ODF, OPGL or the earliest manifestation, TRANCE, helped to clear the alphabet confusion.

RANKL thus was identified as a membrane-associated protein of the TNF ligand family that is expressed by stromal cells after stimulation by bone resorbing cytokines/hormones. Anderson et al. also cloned by direct expression a gene that they called RANKL (the earliest and final designation) as the ligand partner to a receptor termed RANK, that they had previously characterized [7]. The RANKL cDNA clone has an open reading frame that encodes a 316 amino acid type II transmembrane protein (Figure 1.4). A 48 amino acid intracellular amino-terminus precedes the 24-residue hydrophobic domain that passes through the plasma membrane. The carboxy-terminal residues have significant homology to members of the TNF ligand family. Wong et al. remark on similarity of the mouse TRANCE with TRAIL, FasL, and TNF, especially in those regions that form the beta strands seen in the TNF crystal structure [197]. Furthermore, there are putative N-linked glycosylation sites, all of which are highly conserved between mouse and human. Lam and colleagues successfully crystallized the ectodomain of murine RANKL [98]: RANKL

associates into a homotrimer, similarly to other TNF molecules with four unique surface loops that are necessary for full activation of RANK.

Once the identity of RANKL as the factor necessary for osteoclastogenesis was confirmed, osteoclast biology became much more transparent. First, the role of the accessory cell, and indeed the cell-cell contact necessary between the support cells and the osteoclast precursors, was found to be a function of the presence of RANKL expressed on the accessory cell membrane. Indeed, RANKL is the bone environment. When RANKL was expressed in non-bone cells osteoclastogenesis was induced [101]. Furthermore it was possible to fix these non-bone cells with paraformaldehyde prior to adding the HSC responders [206], as is also true for bone cells [35]. Interestingly, the need for a stromal cell could also be overcome by the addition of the extracellular domain of RANKL [97, 206]. MCSF, which also is made by the stromal cells, is necessary for proliferation of the progenitor pool, as well as for survival of cells of macrophage lineage; in the absence of stromal cells, a source of MCSF must also be provided [181]. Soon it was realized that “soluble RANKL,” either secreted directly by some cells [89], or cleaved from its membrane position by a metalloproteinase [110, 129], also had a role in osteoclastogenesis. Bone stromal cells have not been shown to generate secreted RANKL under

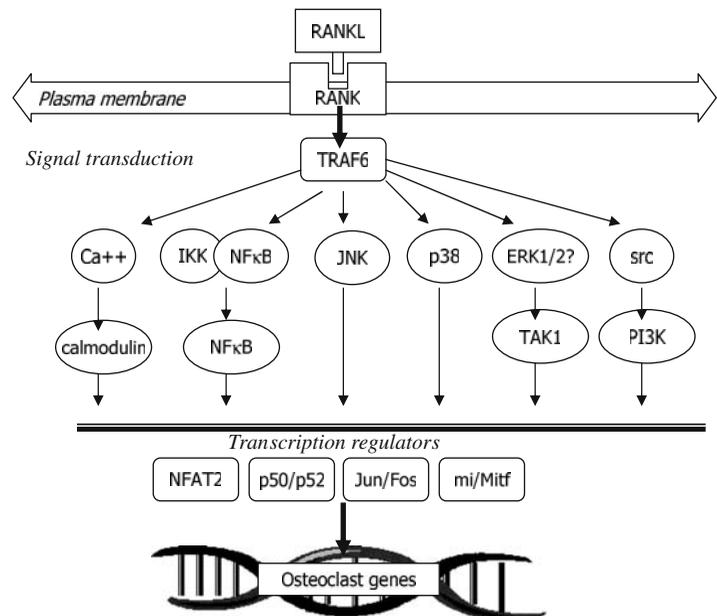


Figure 1.4 RANK signal transduction. RANK signaling is complex and involves multiple cascades. This diagram does not rule out interaction between the signals, some of which may be redundant.

known osteoclastogenic stimuli: conditioned media from stromal cells are ineffective in generating osteoclastogenesis. Spot cultures of stromal cells, in fact, generated osteoclasts only in the local area of RANKL-expressing cells. This further underscores that cell–cell interactions are involved in bone cell RANKL:RANK signaling [69].

The murine gene for RANKL exists as a single copy of five exons spanning about 40 kilobases [85]. The intracellular and transmembrane domains are encoded in the first exon, with the important ligand domain beginning in the first exon and continuing through the fifth. The organizational structure is similar to that of the other members of the TNF family, with mouse RANKL having most homology to CD40L, which also has five exons. Both molecules play a role in dendritic cell function [85]. Ablation of the RANKL gene led to some surprising phenotypic effects in the transgenic mouse [90]. The RANKL-null mouse had severe osteopetrosis due to a complete absence of osteoclasts, but it also had unexpected defects in both T and B lymphocytes and no lymph nodes. The relevance of RANKL's regulation of lymph-node organogenesis for osteoclastogenesis has not yet been worked out, but may be important in situations where osteopetrosis is accompanied by impaired lymphocyte development.

Control of RANKL Expression by Accessory Cells

Before RANKL, the response of the osteoclast-generating unit, i.e., HSC plus accessory cells, was studied as a response of one or both of these elements to agents known to increase bone resorption in the whole animal. Thus, hormones such as $1,25\text{-(OH)}_2\text{D}_3$ and PTH were known to stimulate osteoclastogenesis, as were inflammatory cytokines, such as tumor necrosis factor and many of the interleukins [163, 165]. Of the factors that increase osteoclastogenesis in culture, most, if not all, increase RANKL expression by the accessory cell. Parathyroid hormone increases RANKL expression in a dose-dependent fashion within at least 24 hours [105], as does $1,25\text{-(OH)}_2\text{D}_3$ [61, 147]. The major exception to this paradigm is stimulation of osteoclast differentiation by TNF. Although TNF stimulates RANKL expression, its primary target during stimulation of osteoclast differen-

tiation appears to be the osteoclast precursors themselves, rather than the accessory stromal cells [99, 139].

RANKL mRNA expression by stromal cells has become a reliable marker of the osteoclastogenic potential of the microenvironment or culture system. IGF-I, which stimulates long bone apposition during growth, upregulates RANKL [144]. Equally important is the fact that when a load is applied to cultured cells, RANKL expression decreases [146], whereas when weightlessness is simulated in culture, RANKL increases [76]. Disease states are also associated with changes in RANKL expression. In Paget's disease, where localized and intense bone remodeling occurs, RANKL expression is increased in marrow samples from affected, but not from unaffected, bones [120]. The expression of RANKL in the estrogen deficiency state, where increased resorption leads to decreased bone mass, was not convincingly increased in the marrow [156]. Rather, as discussed below, the response of the osteoclast precursor to RANKL signaling via RANK is diminished.

Both early bone stromal cells and osteoblasts express RANKL, and several authors have suggested that the differentiation state of the bone cell may affect the level of response to the stimulatory agent. While this certainly would hold in terms of response to PTH, where PTH-R are not expressed until after the cell is well on its terminal differentiation path [117], this is less clear for unstimulated expression of RANKL. RUNX2, an essential transcription factor for osteoblast differentiation, must be present before RANKL is expressed [42]. This suggests that the earliest stromal cells may not have the machinery to induce RANKL transcription. However, less mature osteoprogenitor cells may have an increased basal expression of RANKL [44, 178].

It should be noted that it has been difficult to measure RANKL protein in stromal cells. While soluble RANKL can be measured by a commercial ELISA, both ELISA and Western analysis of membrane-associated RANKL have been exceedingly difficult. An OPG-based pull-down assay echoing original strategies for identifying RANKL has been useful in some cases [130] but its tendency to bind other members of the TNF family limits its usefulness. Because there exist three isoforms of RANKL, one with a shorter intracellular domain, and one with no transmembrane domain at all that may repre-

sent the secreted factor [66], the assay is complicated further.

Despite problems with measurement of the RANKL protein, the endogenous mRNA for RANKL in stromal cells responds to stimulation by increasing robustly. This in turn is associated with an increase in the culture's potential to generate osteoclasts from precursor cells [147]. Attention to the RANKL promoter should therefore have led to an understanding of the mechanism by which bone stromal cells regulate RANKL expression. This has not been the case. Nearly 1,000 base pairs of murine and human RANKL promoters were cloned shortly after RANKL was identified [82]. However, RANKL expression should be tissue restricted, and the available promoter sequences are not sufficient to limit expression or to provide the expected response to agents known to increase RANKL mRNA expression [133]. Novel methods, perhaps involving study of the local chromatin structure, may be needed to understand tissue expression and its regulation.

In vivo, non-bone cells can also express RANKL, as shown by localization in white cells and the effects of RANKL in lymph nodes and dendritic cells [7, 191, 197]. T-lymphocytes secrete a soluble form of RANKL into inflamed rheumatic joints, causing osteoclastic bone destruction [89]. There is evidence that T cells with RANKL expression may be key elements in the bone loss caused by estrogen deficiency [19, 180].

RANK: RANKL Signal Transduction in the Monocytic Cell

Stimulation of the Recceptor Activator of NF κ B, or RANK, with activation of NF κ B, leads to the development of the full osteoclast phenotype, including fusion. RANK signaling also increases the net resorptive activity, and, like MCSF, promotes the survival of osteoclast cells. RANK was first identified in dendritic cells, whose immune surveillance mechanism processes and presents antigens to T cells [7]. The osteoclast precursor sequentially expresses the receptor for MCSF and then RANK [8]. RANK is highly expressed in isolated bone marrow-derived osteoclast progenitor cells, as well as in mature osteoclasts [62]. Cognate ligand binding has been shown to activate multiple downstream signaling cascades, including NF κ B, c-jun N-

terminal kinase (JNK), ERK1/2 kinase, p38 kinase, and c-src (see Figure 1.4).

The extracellular domain of RANK binds both the membrane and soluble forms of RANKL as well as other members of the TNF family. The intracellular portion of the molecule has domains that interact with the TRAF family (TNFR-associated factor) proteins. TRAF proteins serve as adaptor proteins that recruit and activate downstream transducers [62, 198]. The amino termini of TRAFs are characterized by a RING finger domain that is required for interaction with other proteins, and for the subsequent release of NF κ B from its cytoplasmic anchor. The signal cascade activated through TRAF binding is complex, but certainly involves TRAFs 2, 5, and 6 through multiple associations on the RANK intracellular domain [26]. TRAF2 knockout mice do not have obvious problems with osteoclast recruitment [128, 207], while TRAF6 knockout mice have osteopetrosis [106]. TRAF6 thus is recognized as the key adaptor to activated RANK.

Experiments using RANK constructs mutated for selective binding of various TRAF proteins have shown that while some TRAF proteins are redundant, TRAF6 was absolutely required for the formation of cytoskeletal structures and to permit the osteoclast to resorb bone [9]. Kobayashi's study of TRAF6 structure suggests that the RING finger domain of the protein, while necessary for IL-1 and LPS signal activation of NF κ B, may not be required for early osteoclast maturation [84]. Thus, while in the absence of the RING finger several osteoclast marker genes such as cathepsin K, calcitonin receptor and TRAP, are expressed, the actin-sealing machinery necessary to complete the seal of the resorption pit is not functional. The RING mutant TRAF still activates NF κ B, but perhaps does not allow association with a significant adapter protein required for late differentiation. TRAF6 also activates transforming growth factor β -activated kinase 1 (TAK1). Interestingly TAK1 mediates JNK and p38 MAP-kinases. This may explain the ability of transforming growth factor β to enhance osteoclastogenesis in the presence of RANKL [152]. To complicate issues, MAPK activates TAK1! Thus Kobayashi's finding that TRAF6 was necessary for the terminal maturation steps, as well as Wong's earlier work that showed TRAF activated c-src, a kinase necessary for the actin ring structure of the osteoclast [196], suggest that

TRAF6 is a specific transducer of later effects of RANKL.

The TRAFs have also been implicated in modulating the effects of many of the bone active cytokines. For instance, interferon-gamma strongly suppresses osteoclastogenesis by silencing RANK signal transduction. This process invokes interferon-gamma activation of a ubiquitin-proteasome system, whereby TRAF6 signals are abrogated when TRAF6 is degraded [173].

The rest of the seemingly redundant TRAFs play roles in the earlier stages of osteoclast formation [9, 26]. Because multiple signaling cascades are involved, (e.g., NF κ B, JNK, p38), one approach to disentangling the important signals is to perform gene expression profiling after RANKL stimulation of RANK. Four of more than 100 early RANKL-inducible genes have been found to be linked to induction of osteoclast maturation [67]. One important gene candidate arising from that study was NFAT2 (nuclear factor of activated T cells-2); suppression of NFAT2 with antisense interfered with osteoclast formation. NFAT2-deficient embryonic stem cells do not respond to RANKL with formation of osteoclasts. Moreover, overexpression of NFAT differentiates precursors in the absence of RANKL [171]. NFAT molecules act as co-factors with AP-1 or fos/jun proteins to bind to regulatory cis DNA elements [171]. NFATs may one day be shown to be part of the pathophysiology responsible for the osteopetrotic phenotype seen in the *fosless* mouse [48]. What are the postulated targets and partners of NFAT2? Clearly osteoclast marker genes such as TRAP, calcitonin receptor and carbonic anhydrase II have multiple sites recognized by NFAT as well as its partner AP-1 [171].

P38 MAPK is another important downstream signal of RANK, as it is involved in expression of carbonic anhydrase II and TRAP through activation of mi/Mitf [113]. As will be discussed below, estrogen may exert its effects by damping the action of the p38 MAPK.

Osteoprotegerin: A Second Binding Partner for RANKL

Osteoprotegerin (OPG) is the third element of the RANK: RANKL: OPG triumvirate. Simonet originally identified OPG as a novel member of the TNFR superfamily through sequence

homology [157]. Its importance was recognized even before its target ligand was identified, when OPG was used to identify RANKL as its binding partner in experiments using expression libraries. The binding of RANKL by this soluble decoy receptor served to prevent RANKL binding to its target on the osteoclast precursor – thus the name, osteoprotegerin (OPG), signifies that it preserves bone. Indeed, OPG is often regulated *in vivo* and *in vitro* in inverse proportion to the levels of RANKL [57].

OPG contains 401 amino acids, with a signal peptide encoding its status as a secreted molecule expressed in many tissues including liver, lung, heart, and kidney and, importantly in bone and cartilage. It contains no hydrophobic amino acids that would make for a transmembrane-spanning domain, so after secretion it is not cell-associated. The N-terminal protein has high homology with other members of the TNFR superfamily, allowing binding of RANKL [97] and of at least one other TNF family member, TRAIL [30].

Transgenic mice that overexpress OPG in the liver under the control of the human apolipoprotein E gene promoter display increases in bone density, even osteopetrosis, if the hepatic OPG output is very high [157]. Recombinant OPG can be used to simulate the phenotype of OPG-overexpressing transgenic mice. It protects rats against ovariectomy-associated bone loss. Osteoclastogenesis can be entirely inhibited if OPG is added to culture or is expressed by stromal cells and osteoblasts [183]. Conversely, OPG-deficient mice develop an early-onset osteoporosis [16]. In fact, many studies have shown that OPG regulates osteoclastogenesis both *in vivo* and *in vitro*; it does so by preventing the effects of RANKL [96, 183, 210]. Interestingly, endothelial cells also express OPG. This local OPG expression appears not only to modulate inflammatory bone states [25] but can also prevent the calcification of arteries as encountered in atherosclerosis [121]. OPG-deficient mice exhibit calcification of the large arteries [16].

The regulation of osteoprotegerin secretion from bone cells is thus of great interest. The OPG promoter has twelve RUNX2 binding elements that underwrite OPG's strong expression in osteoblasts, which express RUNX2 after differentiation. Not surprisingly then, overexpression of RUNX2 increases OPG promoter activity [177]. Transforming growth factor- β , which can

both stimulate and inhibit osteoclastogenesis, increases OPG expression [170]. Estradiol stimulates OPG expression *in vitro* [150] and may increase OPG levels *in vivo* [166]. Leptin, a hormone of lipid metabolism that modulates bone remodeling by an as yet poorly understood mechanism, also increases OPG [18]. As expected, factors that increase osteoclastogenesis and bone resorption decrease OPG secretion by bone cells. Glucocorticoids, which cause osteoporosis by stimulating bone resorption and inhibiting bone formation, inhibit OPG while also stimulating RANKL expression [56]. Parathyroid hormone also decreases OPG and increases RANKL *in vitro* [104]. It does so also *in vivo*, provided the hormone is administered continuously [111]. Insulin-like growth factor-I (IGF-I) also appears to downregulate OPG *in vitro* and *in vivo*. This may account for the remodeling and increase in bone markers seen in trials where IGF-I was given in an attempt to increase bone density [144].

There is a growing appreciation of the role of OPG in rare metabolic bone diseases. In the autosomal recessive Juvenile Paget's disease, also known as hyperostosis corticalis deformans juvenilis, a progressive osteopenic skeletal deformity arises out of intense bone remodeling. Two unrelated patients with this disease were shown to have a defect in the OPG gene, resulting in undetectable serum OPG levels [192]. Autosomal dominant familial expansile osteolysis is another rare bone disorder associated with focal areas of osteolysis that arise from a deletion of the OPG coding sequence [64].

Exceptions: Osteoclastogenesis Without RANKL:RANK

Although RANKL and MCSF are sufficient to induce osteoclast differentiation in cell culture, it would be surprising, given the complexity of most other paracrine regulatory systems, if the control of osteoclast differentiation *in vivo* involved only these two factors. The best-characterized exception to the RANKL/MCSF paradigm is stimulation of osteoclast differentiation by TNF α . Although TNF α stimulates RANKL expression by stromal cells, it can induce osteoclast differentiation in the absence of both accessory cells and exogenous RANKL [11, 83]. OPG, moreover, does not block osteo-

clast differentiation induced by TNF α [139]. Studies using either stromal cells [99] or osteoclast precursors [139] from mice lacking TNF receptors have shown that it is the osteoclast precursors rather than the accessory stromal cells that are the primary target of TNF α stimulation. However, TNF α cannot fully replace RANKL during osteoclast differentiation. Rather, the action of TNF α requires that a permissive amount of RANKL either be present in the culture or be added with the TNF α [139]. Recent studies have shown that molecules other than TNF α are synergistic with, or can replace, RANKL or MCSF in stimulating osteoclast differentiation. These molecules include TGF β [41, 152], BMP-2 [68], activin A [40], VEGF [131], ft3 ligand [100], MIP-1 α [49], and prostaglandin E2 [188]. Even this more complex view of the regulation of osteoclast differentiation is likely to be an oversimplification. For example, the effect of TGF β appears to depend on the mixture of cell types and cytokines that are present in the microenvironment in which osteoclast differentiation occurs [114]. *In vivo* osteoclast differentiation therefore must involve multiple cytokines acting in a complex regulatory network. Nonetheless, it is the balance between RANKL and OPG that must be considered the primary regulator of osteoclastogenesis.

Late Differentiation

Once the CFU-M has been exposed to RANKL and has committed to osteoclast lineage, additional differentiation steps must occur, the most striking of which is fusion of multiple committed cells into the osteoclast polykaryon. The number of osteoclast nuclei that occupy the same fused cell appears to influence the ability of the polykaryons to resorb bone. For instance, in Paget's disease, those pagetic osteoclasts responsible for site-specific increases in bone turnover have more nuclei than normal osteoclasts [91]. The increase in activity associated with a cell that contains several nuclei may be due to the increase in cytoplasm and membrane area, allowing for the formation of resorption pits in the bone area covered by the osteoclast [31]. It may also be due to the fact that the nuclei continue to be transcriptionally active [14]. Indeed, the calcitonin-mediated decrease in osteoclast resorptive activity appears due to a

decrease in transcription by the osteoclast nuclei [14].

Fusion of Mononuclear Osteoclast Precursors

Cell fusion is not a widespread phenomenon. As a rule skeletal myoblasts, syncytial trophoblasts, megakaryocytes, and cells of macrophage lineage are the only cells that fuse. It is likely that the fusion processes in these cells and in the fusion of spermatocyte with oocyte or of enveloped viruses with host cells involve similar mechanisms. Cell fusion involves many factors, including proteins that dissolve discrete areas of membrane. An example of the latter is disintegrin meltrin- α [199], a molecule that is also expressed by osteoclast precursors [2]. Other molecular factors are the receptor-ligand partners such as the HIV gp160 envelope glycoprotein and the CD4 molecule that are expressed in virus-induced cell fusion and in the osteoclast [124]. Syncytin, an aptly named protein expressed by trophoblasts, stimulates cell:cell fusion and is also expressed by retrovirus [209].

Ketoconazole, long known to inhibit cell fusion, also diminishes the fusion of osteoclasts in culture [33]. Ketoconazole inhibits HMG-CoA reductase, thus decreasing cholesterol biosynthesis and the production of N-linked oligosaccharides necessary for the fusion of myoblasts in culture [71]. Mannose is an oligosaccharide that is expressed on the outer membrane of osteoclast precursors; mannose residues are necessary for the osteoclast fusion process [92], probably the mannose receptor enhances cell-cell binding [125]. Interleukin-13 increases fusion of macrophages through upregulation of the mannose receptor [27]. This may be an important mechanism by which inflammatory cytokines upregulate pathologic bone loss. In fact, decreasing the oligosaccharide binding partners of the mannose receptor decreases bone loss. Interestingly, cholesterol depletion by means of an HMG-CoA reductase inhibitor like that used to treat hypercholesterolemia also decreases the fusion of TRAP-positive mononuclear cells [151]. The ability of bisphosphonates to inhibit cholesterol synthesis may in fact contribute to their inhibition of osteoclast differentiation, doing so by prevent-

ing the expression of membrane signals necessary to induce fusion [37].

The disintegrin eichistatin, derived from snake venom, inhibits $\alpha(v)\beta3$ integrin ($\alpha v\beta3$) by blocking RGD (arginine-glycine-asparagine) sites to which the integrin binds. Eichistatin binds to osteoclast membranes and completely inhibits the formation of multinucleated osteoclasts, even though it cannot do so when introduced in an earlier step of osteoclast differentiation [127]. Eichistatin action is partly due to inhibition of migration, a process that allows precursor cells to co-locate, and partly to detrimental effects on the fusion process itself.

The extracellular matrix, upon which the precursor cells are located, is also important in supporting fusion. This is illustrated by the role of ascorbic acid which, when present in the culture, causes the fusion of precursors to increase [138]. E-cadherin, which binds to cell adhesion molecules and is expressed in marrow mononuclear cells, is also involved in the fusion process [116].

The macrophage fusion receptor, also called SHPS-1, is another transmembrane glycoprotein that appears to have a role in macrophage fusion leading to multinucleation [50]. The absence of SHIP, a protein that blocks PI-3 kinase signaling in transgenic animals, leads to enlarged osteoclasts that may contain upwards of 100 nuclei; clearly when precursors undergo excessive fusion, excessive bone resorption results [174]. The purinergic receptor expressed on cells of macrophage lineage is another target of anti-fusogenic factors [32].

The control of osteoclast fusion thus involves multiple membrane proteins that regulate cell migration, cell-cell attachment, and intracellular signaling processes. RANKL [72] and $1,25-(OH)_2D_3$ [1] are the major determinants of cell fusion and the differentiation pathway.

MITF Involvement in Late Differentiation

Interestingly, the *mi/mi* microphthalmic mouse held a clue to late differentiation events well before any of these events were understood. Examination of osteoclasts in these osteopetrotic mice revealed that *mi/mi* osteoclasts were mononuclear and lacked ruffled

borders [176], even though they expressed other osteoclast enzymes [45, 54]. Other aspects of the *mi/mi* phenotype, such as reduced eye size and pigmentation, involve expression of a gene that encodes a basic helix-loop-helix-leucine zipper transcription factor [55], now known as microphthalmia transcription factor, or MTF.

MITF plays a role in osteoclastogenesis, by increasing the expression of TRAP through a conserved sequence (GGTCATGTGAG) that is located in the TRAP proximal promoter [108]. It also interacts with the PU.1 transcription factor [109]. MCSE, another early differentiation factor, induces phosphorylation of MTF, thereby triggering recruitment of the transcriptional co-activator, p300 [189]. Cathepsin K is downstream from these partners, as MITF upregulates expression of this important osteoclast enzyme [126].

Other Factors in Late Differentiation

Other factors that act on late differentiation continue to be discovered through screening osteoclast libraries. One such factor cloned from an immortalized murine osteoclast precursor is ADAM8, a disintegrin-like meltrin (see above). ADAM8 increases osteoclast formation during the later stage by an as yet unknown mechanisms [22].

Matrix recognition, needed to accomplish fusion or to identify bone, is a function of cell surface integrins or adhesion molecules. As noted above, the $\alpha(v)\beta3$ integrin ($\alpha v\beta3$) has been implicated in bone resorption. Mice, in whom the $\beta3$ integrin subunit was deleted, develop osteosclerosis notwithstanding an increased number of multinucleated osteoclasts [118]. These $\beta3$ subunit null osteoclasts do not adequately resorb bone due to a cytoskeletal defect leading to dysfunctions in their ability to spread and form the actin ring/ruffled borders that are necessary for sealing and bone excavation. For osteoclasts to function, therefore, the entire resorptive apparatus must be expressed. Any one of multiple osteoclast enzyme deficiencies, even if the deficiency does not affect osteoclast recruitment, can lead to osteopetrosis due to defective bone resorption. For instance, a defect in cathepsin K results in the curious disease of pycnodystosis [43], typified by osteosclerosis and short stature, and thought



Figure 1.5 Toulouse-Lautrec: pycnodystosis phenotype.

to be the phenotype exhibited by the great French painter, Toulouse-Lautrec (Figure 1.5). Another unexpected requirement is for the ubiquitous intracellular tyrosine kinase *c-src*, which is needed to form the ruffled borders that seal the resorption bay at the basal surface of the osteoclast [15, 158].

Stimulators and Repressors of Osteoclast Differentiation

Of the many regulators of osteoclast differentiation, some of the more important are discussed briefly below, in an effort to provide an overview of the large number of factors that affect osteoclastogenesis.

Hormones

Sex Steroids

Estrogen deficiency has long been known to cause a resorptive osteoporosis. While estrogen may modulate the action of T-lymphocytes and T-lymphocyte expression on RANKL [140, 191], it also dampens the RANKL-stimulated signal cascade. It does so by decreasing c-Jun expression and its phosphorylation by c-Jun N-terminal kinase [156]. Androgen deficiency also results in bone resorption and osteoporosis through the loss of androgen dampening of RANK activation of c-Jun [63].

Gonadal steroids also affect osteoclast recruitment by regulating the expression of osteoprotegerin. In ovariectomized rats, both RANKL and OPG are upregulated. This suggests that estrogen deficiency stimulates the entire RANKL system [66]. In men, serum OPG increases with age, while resorptive markers decrease [166]. In men made acutely hypogonadal, serum OPG increases. This suggests a response to an upregulated resorptive system [79]. Alternatively, estrogen appears to upregulate OPG expression by bone cells [58, 150]. These and other studies suggest that OPG is modified with respect to the bone remodeling rate [78].

Parathyroid Hormone and Vitamin D

Vitamin D is a potent stimulator of osteoclastogenesis and bone resorption. It acts by increasing MCSF [145] and RANKL [206] levels locally and decreasing OPG [61]. Parathyroid hormone, when administered continuously at high concentrations, has effects similar to those of vitamin D in terms of MCSF [190] and RANKL:OPG [61, 105], leading to increases in bone resorption. While both PTH and vitamin D alter cytokine levels, such as that of IL-6 [46, 47], it is their effect on the RANKL:OPG equilibrium that is the major factor by means of which they control osteoclast formation.

The expression of fibroblast growth factor 2, which potently induces osteoclast formation, is stimulated by PTH and may serve as a secondary mechanism to increase bone resorption. In bone marrow cultures made from the FGF-2 null mouse, PTH was less effective in stimulating osteoclast formation [134]. Interestingly, PTH stimulated RANKL to the same degree in

the FGF-2-replete and FGF-2-null cultures. The RANKL:OPG equilibrium was shifted away from resorption due to a 30-fold increase in osteoprotegerin. This result indicates that FGF-2 enhances the inhibitory effect of PTH on OPG gene expression. FGF-2 is required in PTH-induced hypercalcemia, as evidenced by the resistance of the FGF-2 null animal to PTH-induced hypercalcemia [134]. Whether this effect is entirely due to FGF-2 mediated bone remodeling, or whether FGF-2 also promotes the PTH induced increase in renal calcium excretion, is not known.

Insulin-like Growth Factor-I

Insulin-like growth factor-I (IGF-I) is secreted during puberty, inducing long bone growth [200]. Because of its anabolic effects on bone, increasing bone density [201], it was thought that IGF-I should have salutary effects in osteoporosis. Surprisingly, in clinical trials, IGF-I caused an elevation of bone markers without significant increases in bone density [39]. This may be the result of IGF-I action on osteoclastogenesis. Addition of IGF-I to osteoclast-generating systems increased the numbers of osteoclasts, as well as the pit area per osteoclast [123]. In culture IGF-I increased RANKL expression and decreased OPG [144]. It is likely that the pleiotropic effects of IGF-I in bone allow for bone remodeling, rather than pure anabolism of bone.

Calcitonin

Calcitonin, a 32-amino-acid peptide secreted from the C-cells of the thyroid, has been used in hypercalcemia of malignancy to curtail osteoclastic bone resorption. Osteoclasts express receptors for calcitonin during early differentiation and maintain these throughout their life [103]. Thus calcitonin decreases the activity of mature osteoclasts, but also inhibits early differentiation events [168].

Prostaglandins

Prostaglandins may have biphasic effects on bone remodeling, dependent on the local microenvironment. Addition of PGE₂ to murine osteoclast generating systems *in vitro* stimulates osteoclast formation [5] and increases RANKL expression [206]. PGE₂ does, however, inhibit

osteoclast formation in cultures of human cells [21]. Many of prostaglandin's variable effects may be due to differential or temporal expression of the many prostaglandin receptor types.

Secreted Factors other than Cytokines

Nitric Oxide and other Reactive Oxygen Species

Nitric oxide (NO) has effects in nearly every tissue in the body, including bone. One of the earliest reports linking NO to bone remodeling showed that inhibition of NOS, the enzyme that synthesizes NO from L-arginine, potentiates bone resorption in ovariectomized rats [77]. Since then, studies have shown that NO can slow bone loss in animals and humans [70, 194, 195]. NO alters osteoclast indices *in vitro*: osteoclastic resorption is potentiated during NOS inhibition [77]. This could result from effects on osteoclast activity, because NO induces a shape change in the osteoclasts associated with a reduction in bone resorption. Nitric oxide also decreases osteoclast recruitment and subsequent bone resorption [107]. The effect of NO on osteoclast recruitment is likely through alteration in the RANKL/OPG equilibrium as NO has been shown to both decrease RANKL and increase OPG expression in bone stromal cells [36].

Other reactive oxygen species induced by stress or as byproducts of nitric oxide are active in cells as well. Hydrogen peroxide has been shown to increase osteoclast differentiation, either directly or in combination with 1,25-dihydroxyvitamin D [161].

Factors Secreted by Osteoclasts: Annexin II and Legumain

Annexin II was identified as a candidate gene in the stimulation of osteoclast formation from a mammalian osteoclast cDNA expression library [169]. Purified recombinant AXII induced osteoclast formation in the absence of 1,25-dihydroxyvitamin D, probably acting on the proliferative stage of osteoclast precursors, and enhancing the growth of the CFU-GM [119]. Annexin II appears to stimulate the secretion of GM-CSF from T-cells and thus may have a role in inflammatory bone loss.

The osteoclast cDNA expression library [169] from which AXII was initially isolated also yielded an inhibitor of osteoclast formation found to be identical to human legumain, a cysteine endopeptidase [23]. Legumain can be detected in human marrow plasma from normal donors. When human marrow cultures are treated with an antibody to legumain, osteoclast formation is enhanced in the absence of 1,25-dihydroxyvitamin D.

Cytokines

A host of cytokines, including tumor necrosis factor and interleukins-1, -4, -6, -11, -18, have both positive and negative effects on osteoclastogenesis. These are dealt with in chapter 5.

Osteoclast-Associated Receptor Ligand

Other factors produced by osteoblasts and stromal cells have been identified through finding binding partners of discrete osteoclast receptors. One of the most interesting is the "osteoclast-associated receptor," or OSCAR, representing a novel member of the leukocyte receptor complex [80]. OSCAR expression varies from the usual immunoglobulin-like surface receptors typical of leukocytes by being expressed only in pre-osteoclasts and mature osteoclasts. The ligand for OSCAR (OSCAR-L) is expressed in osteoprogenitor cells. This is a developing area of research that may help to link the immune system genes more closely to bone remodeling.

Mechanical Factors

A major function of the skeletal structure is to provide support for locomotion. To adapt to functional loading, the skeleton has evolved a finely tuned response system that recognizes mechanical input. Unloading leads to bone resorption and loading to bone apposition and preservation. The cells of bone must thus be able to respond to unloading by initiating bone resorption. The results of bone resorption are seen in the skeletons of paraplegics and of astronauts, and quite readily in situations where unloading was generated experimentally [142, 143]. Thus, the recruitment of additional osteoclasts involved in unloading-associated resorption probably occurs via changes in the

dominant RANKL:OPG equilibrium. Experimental studies with cells subject to loading suggest that loading stromal cells decreases their expression of RANKL through a pathway that involves activation of MAP-kinases [146, 147].

Conclusion

Resorption of the calcified surface of bone involves the orchestrated action of a large variety of enzymes and disposal mechanisms. Interference with, or overactivity of, any of these processes, leads to dysregulation of bone remodeling. Thus regulation of both the formation and action of the osteoclast modulates bone development, bone growth, and bone disease.

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