Regulation of Osteoclast Differentiation

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ABSTRACT: The osteoclast (OCL) is derived from the cells in monocyte-macrophage lineage. The earliest identifiable OCL precursor is the granulocyte-macrophage colony-forming unit (CFU-GM), which gives rise to granulocytes, monocytes, and OCL. CFU-GM–derived cells then differentiate to committed OCL precursors, which are post-mitotic cells, and fuse to form multinucleated OCL. A variety of factors both positively and negatively regulate OCL formation and activity. These include growth factors, such as macrophage colony-simulating factor, which simulates the proliferation and prevents apoptosis of early OCL precursors, and RANK ligand (RANKL), which is the primary mediator of OCL formation. Most factors that induce OCL differentiation, such as PTHrP, IL-11, and prostaglandins, do so by inducing expression of RANKL on the surface of immature osteoblasts. Osteoprotegerin is a decoy receptor that blocks RANKL activity. In addition, OCL produce autocrine-paracrine factors that regulate OCL formation, such as IL-6, which is produced at high levels by OCL in Paget's disease and increases OCL formation. We screened human and murine OCL cDNA libraries to identify autocrine-paracrine factors that regulate OCL activity. We identified annexin-II, MIP-1α, ADAM8, eosinophil chemotactic factor, and OCL inhibitor factors 1 and 2 as factors involved in OCL formation. Most recently, we have identified the receptor for ADAM8, $\alpha_9\beta_1$ integrin, which appears to be critical for normal OCL activity. OCL differentiation is controlled by exogenous hormones and cytokines as well as autocrine-paracrine factors that positively or negatively regulate OCL proliferation and differentiation.

KEYWORDS: osteoclasts; differentiation; autocrine; paracrine; cytokines

INTRODUCTION

The osteoclast (OCL) is the primary bone-resorbing cell. It is formed from mononuclear precursors, which fuse to form multinucleated OCL.[1] Increases

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in the degree of multinucleation enhance the capacity of OCL to resorb bone[2] so that OCL, which contains greater numbers of nuclei, has an increased bone-resorbing capacity compared to OCL with fewer nuclei. OCL differentiation and activity are regulated by both systemic hormones and cytokines produced locally in the bone microenvironment. In addition, other cells in the marrow microenvironment can influence OCL formation and activity. These cells include T and B lymphocytes, marrow stromal cells, osteoblasts, and osteocytes. All of these cell types produce cytokines and chemokines that stimulate or inhibit OCL formation and activity. Studies by Walker and colleagues[3,4] clearly showed that the OCL was hematopoietic in origin. Using parabiotic experiments with mice, Walker and co-workers demonstrated that the OCL precursor was blood borne and could cure osteopetrosis in the parabiotic littermate. Further, transplantation of normal marrow, peripheral blood, or spleen cells into osteopetrotic animals have clearly shown that OCL precursors reside in hematopoietic organs. Previously, the tissue origin of the OCL was in dispute and was thought to be mesenchymal[5] but now it is clear that the OCL is hematopoietic in origin.

LINEAGE OF THE OCL

The OCL is derived from the pluripotent hematopoietic stem cell. Kurihara and co-workers[6] have shown that the multipotent hematopoietic precursor, CFU-blast, can form OCL when cultured in the appropriate cytokine milieu *in vitro*. More recently, Miyamoto and co-workers have clearly identified the different stages of OCL differentiation and the surface phenotype of these cells.[7] The pluripotent hematopoietic stem cell gives rise to a myeloid stem cell, which can further differentiate to megakaryocytes, granulocytes, monocyte-macrophages, and OCL. The earliest identifiable hematopoietic precursor that can form OCL is the granulocyte-macrophage colony-forming cell (CFU-GM).[8] A variety of studies have confirmed that CFU-GM can form OCL and that CFU-M, the more differentiated monocyte precursor, forms OCL at a much lower efficiency. Kerby and co-workers have shown, using single-cell manipulation studies with the progeny of hematopoietic stem cells, that CFU-GM rather than CFU-M form greater numbers of OCL.[9] Early OCL precursors are proliferative cells, which can increase in numbers in response to hematopoietic growth factors such as IL-3, GM-colony-stimulating factor (CSF), and M-CSF.[10] M-CSF prevents apoptosis of these precursors as well.[11] The critical role of M-CSF in OCL differentiation has been demonstrated in rodent models in which mutations in the M-CSF gene result in severe osteopetrosis.[12] The op/op mouse has a mutation in the M-CSF gene that results in a stop codon and a truncated M-CSF protein.[12] These animals develop osteopetrosis at an early age, but if maintained on an appropriate diet, survive to adult age and

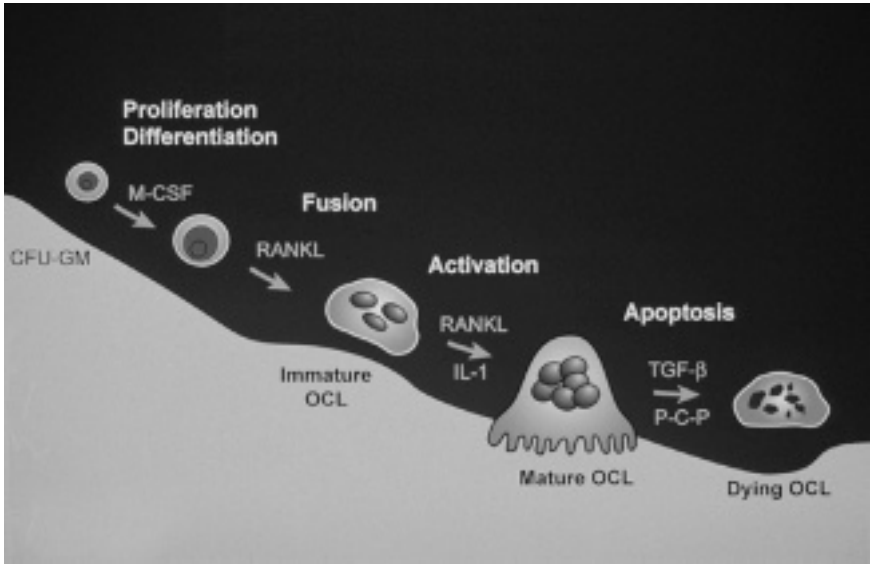

FIGURE 1. Osteoclast life cycle from proliferation and differentiation to apoptosis.

partially cure their osteopetrosis. The factors that appear to supplant M-CSF in the op/op mouse include IL-3, GM-CSF, or vascular endothelial growth factor (VEGF).[12]

The early OCL precursors proliferate and differentiate to form a post-mitotic committed OCL precursor (FIG. 1). These committed OCL precursors under the influence of RANK ligand (RANKL) or 1,25-$(OH)_2D_3$ then differentiate and fuse to form immature multinucleated OCL. These immature OCL then must be activated to form bone-resorbing OCL. Factors that can activate OCL include RANKL and IL-1.[13,14] The activated OCL survive for approximately 2 weeks in the marrow[15] and then undergo apoptosis. Factors that can enhance OCL apoptosis include bisphosphonates and TGF-β.[16,17]

KEY REGULATORS OF OCL DIFFERENTIATION

There are a number of key regulators of OCL differentiation and function. The earliest transcription factor that has been linked to OCL differentiation is PU.1. Loss of PU.1 by homologous recombination results in severe osteopetrosis in animals that lack both granulocytes and cells in the monocyte-macrophage lineage.[18] Another critical transcription factor for OCL differentiation is *c-fos*. Animals lacking *c-fos* can still develop monocyte macrophages but cannot form OCL and are osteopetrotic.[19] *c-fos* appears to regulate OCL dif-

ferentiation through induction of a second transcription factor NFAT-c1, which is critical for OCL differentiation.[20] Other genes, which have been shown to be critical for OCL function, include *c-Src* that regulates OCL attachment and the OCL cytoskeleton. Loss of *c-Src* results in formation of OCL that cannot resorb bone.[21]

RANKL AND OCL FORMATION

Both systemic and local factors can regulate OCL formation including parathyroid hormone, IL-11, prostaglandins, and 1,25-$(OH)_2D_3$. All of these factors induce OCL formation through increasing expression of RANKL on the surface of immature osteoblasts and marrow stromal cells.[22,23] RANKL is a member of the tumor necrosis factor (TNF) gene family and is predominantly expressed as a membrane-bound protein. RANKL also can be cleaved from the cell surface and expressed as a soluble protein by the action of TNF-α converting enzyme-like proteins. RANKL then binds its cognate receptor RANK on OCL precursors and induces OCL formation. RANKL has a natural occurring decoy receptor, osteoprotegerin (OPG) that can bind RANKL and block its interaction with RANK. Proof that RANKL is a critical factor in osteoclastogenesis was shown by several laboratories using the techniques of homologous recombination to delete the RANKL gene or its receptor RANK in mice.[24,25] These mice develop severe osteopetrosis. Furthermore, overexpression of RANKL or deletion of OPG resulted in severe osteoporosis, with the animals sustaining spontaneous fractures because of their markedly decreased bone mass.[26] RANKL induces OCL formation by signaling through several pathways (FIG. 2). Boyce and co-workers have clearly shown that the NF-κB pathway is critical for osteoclastogenesis.[27] Animals lacking both the p50 and p52 subunit of NF-κB develop severe osteopetrosis.[27]

The pathways that are involved in induction of RANKL in marrow stromal cells are just beginning to be identified. Both the PKA and PKC pathways and the MAP kinase pathway have all been implicated in the upregulation of RANKL by hormones and cytokines.[28,29] However, it is clear that the NF-κB signaling pathway is not required for production of RANKL, because marrow stromal cells from animals lacking both the p50 and p52 subunits of NF-κB can still produce RANKL.[28] The relative ratio of RANKL to OPG determines whether there is increased or decreased OCL formation. Normally, the levels of OPG are much higher than RANKL so there are few OCL present in normal bone. However, with inflammatory conditions, such as rheumatoid arthritis, bone metastasis, myeloma, and Paget's disease, the ratio of RANKL to OPG is increased, favoring increased osteoclastogenesis.[30–32]

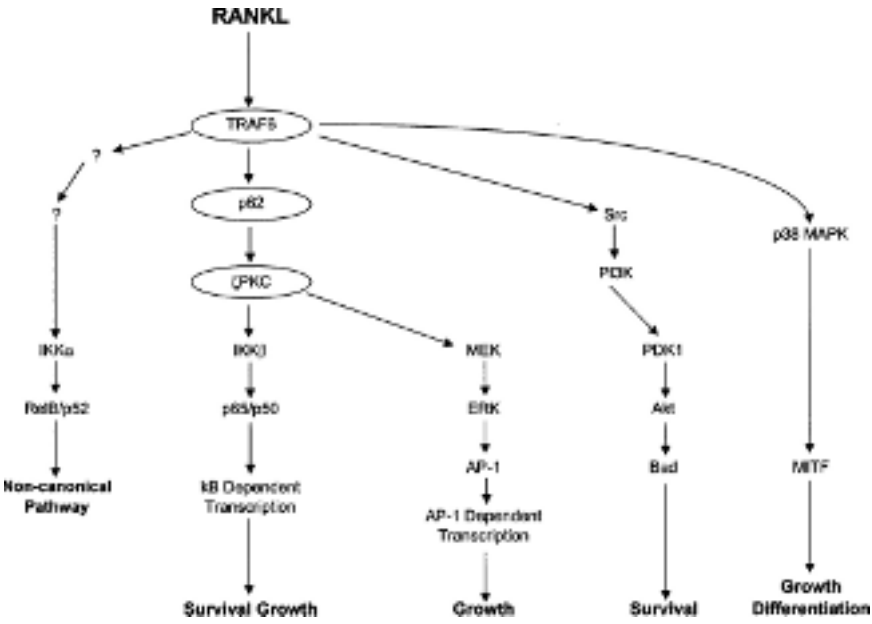

FIGURE 2. RANK signaling pathways. When RANK ligand binds RANK on OCL precursors, multiple signaling pathways are activated, which affect OCL formation and survival. These pathways include NF-κB, p38 MAP kinase, JNK, and *c-Src*.

AUTOCRINE FACTOR REGULATION OF OCL FORMATION

As noted above, both systemic and local factors can regulate OCL formation and activity. In addition, the OCL itself can produce regulatory cytokines, which stimulate and inhibit OCL formation. Initial studies in our laboratory demonstrated that the OCL was a secretory cell, which produced cytokines that simulated its own activity. In studies with giant cell tumors from bone, which are osteoclastomas, we demonstrated that these cells expressed IL-6 mRNA and protein.[33] Further, when these cells are treated with anti-IL-6, bone resorption was inhibited. The addition of IL-6 to OCL treated with anti-IL-6 restored their bone-resorbing capacity. Similarly, antisense constructs to IL-6 blocked bone resorption by OCL from giant cell tumors of bone.[34] Thus, IL-6 was the first OCL autocrine factor identified, which simulated OCL formation. We have shown that OCL from patients with Paget's disease also produced high levels of IL-6.[35] Kurihara and co-workers had previously demonstrated that IL-6 was a potent inducer of OCL formation.[36] IL-6 induces human OCL formation by acting directly on OCL precursors and does not induce RANKL expression.[37,38]

Based on these initial results, we initiated studies to identify other autocrine-paracrine factors produced by OCL that regulated OCL formation and bone

resorption. To undertake these studies, we produced large numbers of human marrow-derived OCL *in vitro*, purified the OCL, and then isolated mRNA from the highly purified OCL. An OCL cDNA expression library was established in 293 cells, and the conditioned media from these 293 cells was screened for the factors that stimulated or inhibited human or murine OCL formation in bone marrow cultures.[39] We identified nine pools that contained an OCL stimulatory or inhibitory activity. Three of these nine pools contained known factors that regulated OCL activity, including IL-6 and IL-1. The first novel autocrine-paracrine stimulator of OCL formation that we identified was Annexin-II (AX-II).[39] AX-II was previously thought to be an intracellular protein, which had no known physiologic function except as a possible calcium channel. AX-II purified from human placenta or bovine lung simulated human and murine OCL formation in a dose-dependent fashion at concentrations of 1–100 ng/mL. Furthermore, AX-II was expressed by multinucleated OCL in murine marrow cultures and in OCL from giant cell tumors of bone. Studies on the mechanism of action of AX-II demonstrated that AX-II induced OCL formation indirectly by increasing production of both GM-CSF and RANKL by marrow stromal cells.[40,41] Most recently, we have identified a putative AX-II receptor on marrow stromal cells, which binds AX-II and does not bind AX-III or AX-V. The kDa for this receptor is about 10^{-9} M, and there are about 300,000 receptors per cell.[42] The receptor specifically binds the p11 subunit of AX-II, which is heterotetramer containing two p11 and two p36 subunits.

In addition to autocrine-paracrine stimulators of OCL formation, we identified two novel inhibitors of OCL formation. One was osteoclast inhibitory peptide (OIP)-1, which is identical to the human SCA protein.[43] OIP-1 is a GPI-linked protein that can be cleaved from the cell surface to inhibit OCL formation. The mechanism of action of OIP-1 is under intensive investigation and appears to involve interferon induction.[44] The second inhibitor of osteoclastogenesis produced by OCL we identified was OIP-2, which is identical to the enzyme legumain.[44] Structure–function studies of OIP-2 showed that the C-terminal peptide, which is cleaved by autocatalysis when the protein is secreted, mediates the inhibition of osteoclastogenesis.[45] Choi and co-workers have shown that OIP-2 can block increased OCL formation and hypercalcemia *in vivo* in mice treated with parathyroid hormone-related peptide.[46]

To identify genes that were overexpressed in OCL compared to OCL precursors, which might be involved in OCL differentiation, we developed a murine OCL precursor cell line by targeting Bcl-xL and large T-antigen to the OCL lineage in transgenic mice using the tartrate acid phosphatase (TRAP) promoter.[47] Bone marrow from these mice was then cultured and an OCL precursor cell line was developed. All the cells were OCL precursors and OCL formation by these cells was 500-times greater than normal marrow. These cells responded appropriately to most osteoclastogenic factors[48] and were used to obtain large numbers of OCL precursors and OCL. Using the techniques of subtractive hybridization, we generated an OCL cDNA library enriched for genes that

were upregulated in mature OCL compared to the precursors, and detected several genes that regulated OCL formation. One of these genes was ADAM8, which is a member of the ADAM (A Disintegrin And Metalloproteinase) family of genes.[49] There are over 30 members of the ADAM gene family, and only ADAM8 mRNA is overexpressed in OCL compared to OCL precursors. Soluble ADAM8 induced formation of bone-resorbing OCL and acted at the later stages of OCL differentiation and precursor fusion. Structure-function studies showed that the disintegrin domain of ADAM8 mediated its effects on OCL formation. Recently, we have identified the receptor for ADAM8, which is $\alpha_9\beta_1$ integrin.[50] $\alpha_9\beta_1$ integrin is expressed in OCL precursors at higher levels than in mature OCL but it is predominantly expressed at late stages of OCL precursor differentiation. Neutralizing antibodies to α_9 integrin inhibits human OCL formation, and OCL from mice lacking the α_9 integrin subunit gene are abnormal. These α_9 $-/-$ OCL are small and contracted, resorb bone poorly and are similar to OCL that lack β_3 integrin. In addition, OCL from α_9 knockout mice do not form actin rings, consistent with their impaired capacity to resorb bone. Other genes which are upregulated during OCL differentiation that effect OCL formation include eosinophil chemotactic factor,[51] C3 component of complement, which was reported by Sato *et al.* to be critical for OCL formation,[52] and macrophage inflammatory peptide (MIP)-1α.[37]

CONCLUSION

OCL formation and activity are controlled by both systemic hormones, local factors, and factors produced by the OCL themselves. These factors both positively and negatively regulate OCL formation. Local factors in the bone microenvironment, such as RANKL and OPG are critical regulators of OCL formation. In addition, factors produced by OCL include IL-6, TNF-α, MIP-1α, ADAM8, AX-II, OIP-1, and OIP-2, which appear to be important regulators of OCL formation in normal and pathologic states.

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