M-CSF, c-Fms, and Signaling in Osteoclasts and their Precursors

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ABSTRACT: Prevention of conditions, such as osteoporosis, requires an understanding of the molecular mechanisms of bone resorption. The understanding that cells of the myeloid lineage are osteoclast precursors suggests that macrophage colony-stimulating factor (M-CSF) plays an important role in osteoclast biology. Signals generated by the binding of M-CSF to the cell-surface receptor c-Fms appear to trigger events leading to osteoclast differentiation. We have created a chimeric variant of the c-Fms receptor, which has allowed study of downstream events activated by M-CSF in a model more relevant to normal physiology than prior studies, which have relied on myeloid tissues. Our studies suggest novel regulatory signaling pathways initiated via the c-Fms receptor.

KEYWORDS: osteoporosis; osteoclast; RANKL; M-CSF; c-Fms; macrophages

INTRODUCTION

Bone mass is determined by the net activity of osteoblasts, which form bone, and osteoclasts, which degrade the matrix. An increase in the rate of skeletal degradation relative to formation results in pathological bone loss. Thus, prevention of conditions such as osteoporosis requires an understanding of the molecular mechanisms of bone resorption.

The osteoclast, the exclusive bone resorptive cell, is a member of the monocyte/macrophage family and a polykaryon that can be generated *in vitro* from myeloid precursors, with bone marrow macrophages representing the largest reservoir. Two cytokines are essential and sufficient for osteoclastogenesis, receptor activator of NF-kB ligand (RANKL)[1,2] and macrophage colony-stimulating factor (M-CSF), also called colony-stimulating factor-1 (CSF-1), with the former being the key osteoclastogenic cytokine and the latter contributing to proliferation, survival, and differentiation of early precursors.

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Each is produced by mesenchymal cells and their derivatives in the marrow environment.[3]

M-CSF was identified as a molecule that mediates the survival and proliferation of precursors of the monocyte/macrophage lineage and their differentiation into mature phagocytes.[4] The understanding that cells of the myeloid lineage are osteoclast precursors[5] suggested that M-CSF plays an important role in osteoclast biology. This hypothesis was confirmed by the observation that *op*/*op* mice, which fail to express functional M-CSF as a result of a point mutation in the *Csf1* gene, are osteopetrotic.[6,7] Furthermore, administration of soluble M-CSF to either *op*/*op* mice or to rats bearing an analogous mutation (*tl*, *toothless*) rescues their osteopetrosis.[6–8] While M-CSF is produced constitutively by a range of cells, regulated secretion of the cytokine has pathological consequences in the context of the osteoclast. Thus, absence of estrogen, the cause of postmenopausal osteoporosis, is due to enhanced bone resorption caused at least in part by increased secretion of M-CSF from marrow stromal cells in response to higher circulating levels of the inflammatory molecules IL-1 and TNF-α.[9,10]Finally, raised serum levels of parathyroid hormone stimulate osteoblasts to secrete the same cytokine.[11]

c-Fms, the cellular homologue of the feline transforming virus v-Fms, is the sole receptor for M-CSF.[12,13] The functional linkage between M-CSF and its receptor is established by the fact that mice lacking *csf1r,* the gene coding for c-Fms, exhibit the same major phenotype as the op/op mouse, namely a marked decrease in tissue macrophages and severe osteopetrosis due to a lack of osteoclasts.[14]

Binding of M-CSF to c-Fms results in dimerization and hence activation of the receptor tyrosine kinase, leading to auto-phosphorylation of the dimer in trans on selected tyrosine residues. Each phosphorylated species then acts as a binding site for SH2 or PTB domain-containing proteins, which amplify and transduce the original signal.[15] The identity of the key tyrosine residues in c-Fms, which are targets of its autokinase activity, has been the subject of considerable study. Almost all experiments exploring this issue involved the use of fibroblastic or myeloid cell lines. The fact that the myeloid lines cannot differentiate into osteoclasts raises further questions about their physiological significance. While these studies contain conflicting data regarding the importance of individual c-Fms tyrosine residues in M-CSF-dependent proliferation and/or differentiation,[4] it is generally agreed that seven tyrosine residues in the cytoplasmic tail of murine c-Fms undergo phosphorylation following activation; Y559, Y697, Y706, Y721, Y807, Y921, and Y974.

c-Fms autophosphorylation prompts downstream signals by mechanisms largely common to the RTK superfamily as a whole. In brief, the initial adaptors binding to the phosphorylated tyrosine sites associate with other kinases or adaptors.[15] Using a proteomic approach coupled to extensive purification under nondenaturing conditions, Stanley and colleagues established that in excess of 150 proteins bind to c-Fms following its activation.[16] Members of

this complex are involved in cell proliferation, survival, differentiation, and cytoskeletal reorganization, thus encompassing all known functions of the M-CSF/c-Fms pathway. While a massive macromolecular complex assembled on c-Fms in response to M-CSF suggests that the downstream signals may be complicated. Nevertheless, the available data, including our experiments using primary macrophages, indicate that PI3K and p42/44 ERK and c-Cbl, are key transducers of M-CSF signaling. Additionally, phospholipase C gamma (PLCγ) may also mediate c-Fms signals. This lipase generates diacylglycerol and releases stores of intracellular Ca^{2+}, thus activating a host of pathways.[17] Knowledge concerning the significance of the PLC pathway in mediating the response of macrophages and/or osteoclasts to M-CSF is limited[18] and further studies are required to clarify this issue.

MACROPHAGE PROLIFERATION

ERK1/2 (p42/44) and PI3K/Akt are established mediators of macrophage proliferation.[4] Binding of M-CSF to c-Fms recruits the adaptor protein complex Grb2/Sos toY697 in the cytoplasmic tail of c-Fms and Sos, acting as a guanosine exchange factor for Ras, stimulates the Ras/Raf/MEK/ERK pathway. In contrast, there is almost no activation of the ERK family members such as JNK or p38. M-CSF also robustly stimulates the PI3K/Akt pathway in macrophages. In general, PI3K/Akt regulates cell proliferation via GSK3β and the FOXO family of transcription factors.[19] In brief, GSK3β phosphorylates cyclin D1, leading to its rapid proteosomal degradation, while FOXO inhibits transcription of the same cyclin and increases the cell cycle inhibitors, p27 and p130. By phosphorylating GSK3β and FOXO, Akt suppresses their capacity to inhibit entry into the cell cycle.

The SHIP1-deficient mouse provides evidence that the PI3K/Akt stimulates preosteoclast to divide. SHIP1, as a 5″ lipid phosphatase, diminishes phosphatidylinositol 3, 4, 5 trisphosphate (PIP_3), and hence deactivates Akt. Animals lacking SHIP1 have large hyper-resorptive osteoclasts and in consequence are severely osteoporotic. Moreover, these terminally differentiated polykaryons are protected from programmed cell death, reflecting prolonged and more robust activation of the PI3K/Akt axis.[20] Given our observations on decreased apoptosis in SHIP1 null osteoclasts, we hypothesized that macrophages from the same animals would be similarly longer lived, thus contributing to the massive increase in macrophages in these mice.[21] Surprisingly, we find that the major difference from wild-type (WT) macrophages is not decreased apoptosis, but rather increased proliferation and accelerated entry into the cell cycle. Moreover, in accord with the current model,[19] PI3K mediates M-CSF-induced macrophage proliferation via a process involving suppression of p27 (Zhou *et al*., unpublished data). Overexpression studies suggest that c-Fms Y721 binds the p85 subunit of the PI3K complex, leading to activation of the catalytic moiety and hence enhanced function of Akt.[4]

Following cytokine treatment, rapid induction (via ERKs) and stabilization (via PI3K/Akt/GSK3β) of D-type cyclins, typically forms a cyclin D/cdk4 complex, which hyper-phosphorylates the pocket protein Rb.[22] Once again, consistent with present dogma, our data indicate that exposure of primary macrophages to M-CSF enhances expression of all three D cyclins and simulates Rb phosphorylation.

Receptor internalization, followed by lysosomal degradation provides an alternative mechanism by which c-Fms signaling may be suppressed. The proteosome may also participate in c-Fms degradation as M-CSF induces rapid c-Cbl phosphorylation in primary macrophages.[23] Consistent with the fact that Cbl family members are ubiquitin ligases for RTKs,[24] M-CSF treatment is also followed by multi-ubiquitination and endocytosis of c-Fms, c-Src, and c-Cbl itself, events that regulate macrophage proliferation.[25]

c-Fms SIGNALING IN AUTHENTIC OSTEOCLAST PRECURSORS

As discussed, the use of cells other than authentic members of the monocyte/macrophage family to study the M-CSF/c-Fms axis has often yields conflicting information. Because primary macrophages express native c-Fms, which is activated by M-CSF, it was not possible to express specific mutants of the receptor as a means of assessing their impact on pre-osteoclast function. To address this issue we retrovirally transduced primary macrophages, which contain endogenous c-Fms, with a chimeric receptor comprising the external domain of the erythropoietin (Epo) receptor coupled to either the WT or mutated forms of the cytosolic domain of c-Fms (FIG. 1). In this circumstance,

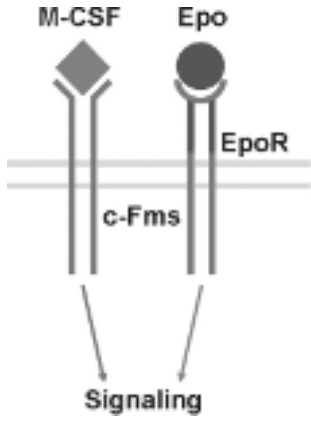

FIGURE 1. Chimeric receptor strategy for studying c-Fms signaling in primary macrophages or osteoclasts. Cells containing endogenous murine c-Fms (red line) were transduced retrovirally with a plasmid coding for the external domain of the Epo receptor (blue) linked to the transmembrane and WT cytoplasmic domains of murine c-Fms.

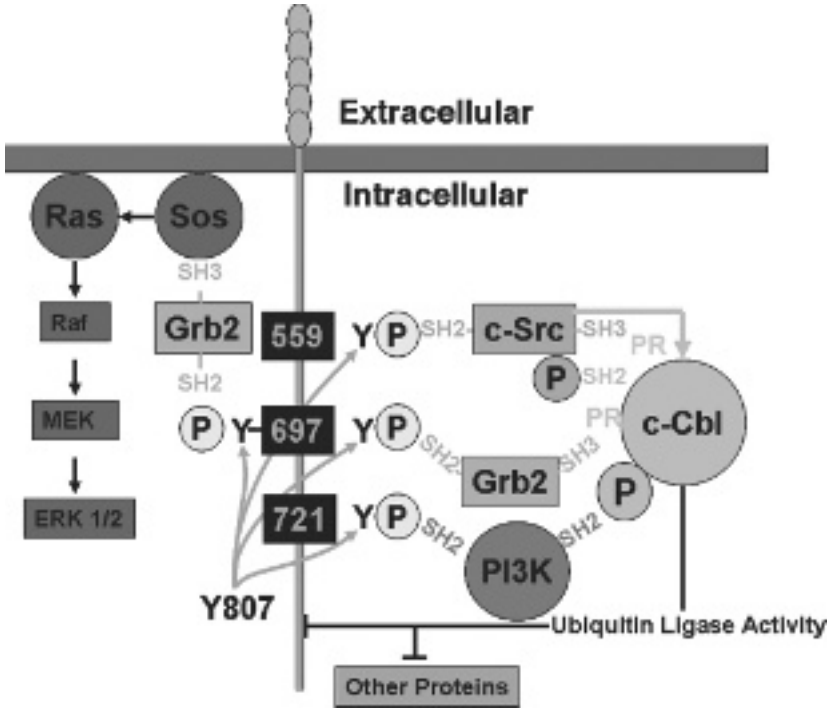

FIGURE 2. Model for major pathways leading to activation of the ERK1/2 and PI3K/Akt pathways following treatment of macrophages or osteoclasts with M-CSF. Binding of M-CSF to the extracellular domain of c-Fms stimulates phosphorylation of Y807 in the activation loop, leading to a change in its conformation and increased kinase activity of the receptor. Consequently, tyrosines 559, 697, and 721 also are phosphorylated (yellow), and become docking sites for proteins containing SH2 domains. A c-Src family kinase (in the case of the osteoclast c-Src itself) ligates position 559, resulting in autophosphorylation (position 416 in c-Src, green). c-Cbl, binding through its proline rich and SH2 domains to the SH3 and phospho-tyrosine domains of c-Src, becomes a substrate for the nonreceptor tyrosine kinase (light blue, purple). c-Cbl contains docking sites for PI3K and Grb2, each of which also interacts simultaneously with c-Fms by SH2 and/or SH3 interactions (red and orange, respectively). Shown separately for the sake of clarity, Grb2 in the same complex is constitutively bound to Sos (via a second SH3 domain in Grb2) which recruits Ras to the plasma membrane. This multimeric complex would be predicted to be highly stable and hence generate prolonged signaling, with the subsequent Raf/MEK/ERK and PI3K/PDK1/Akt cascades acting as downstream effectors. Finally, c-Cbl also acts as an E3 ubiquitin ligase for a number of proteins, including c-Fms, thereby attenuating the original signal (light blue).

M-CSF activates endogenous c-Fms and Epo the chimeric receptor. The fact that Epo, in the presence of RANKL, effectively promotes osteoclastogenesis via the chimeric receptor bearing the WT c-Fms cytoplasmic domain validates the model.[26] Furthermore, the same chimera provides proliferative stimuli

equivalent to those derived from endogenous c-Fms (data not shown). Thus, transducing specific mutants of the chimera permits structure-function analysis of the c-Fms cytoplasmic domain in the context of authentic macrophages and osteoclasts.

Using a range of single Y to F point mutants and combinations thereof, we identified the tyrosine residues in c-Fms that mediate its function. In contrast to the classical model,[4,27] we find that activation of the downstream effectors p42/44 or PI3K by chimeric receptors containing the single-point mutants Y697F or Y721F is virtually unaltered. In contrast, c-FmsY559F attenuates both Erk and Akt activity. These observations and additional studies involving M-CSF-stimulated association of adaptor proteins with its receptor suggest a novel model of c-Fms-mediated ERK and PI3K activation. Thus, we propose that binding of a src family kinase to the phosphorylated form of c-FmsY559 recruits and phosphorylates c-Cbl, which attracts and stabilizes a multi-protein complex that augments signal transduction. The complex contains Grb2/Sos and PI3K, with the former activating p42/44 and the latter the PDK1/Akt axis. As discussed previously c-Cbl is also a ubiquitin ligase,[24] in which mode it enhances receptor degradation (for further details see FIG. 2 and its legend).

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