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Cytokine regulation of osteoclast formation and function

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Introduction

The multinucleated osteoclast is the major, probably exclusive, cell responsible for bone resorption. Three sets of scientific advances over the last five years have greatly increased our knowledge of how the osteoclast develops and functions. Perhaps the single most important finding was that a novel TNF superfamily member, Receptor Activator of Nuclear factor kappa B (RANK) ligand (hereafter RANKL) is the key osteoclastogenic cytokine. This observation has allowed many researchers to express the functional external domain of this protein, which, when used with macrophage colony stimulating Factor (M-CSF), allows large numbers of functional osteoclasts to be generated from their myeloid precursors. In consequence, it is now possible to produce sufficient cells to perform a wide range of biochemical and cell biological studies. The second breakthrough has come about from the major advances accompanying the explosion in genetic information. Both the cloning of genes responsible for osteoclast dysfunction in man and the generation of mice lacking a single gene as a result of targeted deletion have allowed researchers to provide unequivocal evidence for the role of specific proteins in osteoclast biology. Finally, workers in the field of bone biology have incorporated into their thinking and research major findings in a broad range of disciplines. The goal of this review is to integrate these three themes, focusing finally on the osteoclast cytoskeleton, a subject currently under-researched. Additionally, I will highlight other areas where knowledge is sparse.

Osteoclast origin, formation and function

It is now clear that osteoclasts derive from cells of the monocyte/macrophage lineage. The earliest proof involved the cure of malignant osteopetrosis in a young girl by bone marrow transplantation from her brother. Use of the Y chromosome as a marker resulted in the conclusion that the osteoclast of hematological origin[1]. Later studies, using mice lacking transcription factors expressed early during monocyte differentiation, confirmed and extended the original observation, leading to unequivocal support for the fact that the precursor is an undifferentiated monocyte[2] Independent confirmation was provided by the op/op mouse, which carries a naturally occurring mutation in the gene coding for M-CSF and thus has neither macrophages nor osteoclasts. Confirming that it is lack of M-CSF that is responsible for the phenotype, it is rescued by injection of the cytokine[3]. While it was long understood that M-CSF provides signals that stimulate survival and proliferation of precursors, it was the cloning of RANKL that solved the major question as to the nature of the molecule responsible for inducing differentiation[4]. Consistent with this result, deletion of the genes coding for either RANKL or its receptor RANK results in severe osteopetrosis[5,6]. Some of the important signaling intermediates downstream of c-fms, the receptor for M-CSF, and RANK include the mitogen-activated protein kinases ERK1 and ERK2, JNK and p38, the transcription factors c-fos, fra-1 and NFAT1c, p50 and p52, two members of the NFkB family and the phosphatidylinositol 3 kinase (PI-3K)/Akt pathway. Similarly, c-src and TRAF6, a member of the TNF Receptor Associated Factor group, couple activation of c-fms and RANK to differentiation, activation and survival of osteoclasts[2,7].

In addition to M-CSF and RANKL, the only factors required for basal osteoclastogenesis, several other cytokines also play an important role in osteoclast biology. Key among these are stromal- and/or T-cell derived TNF and interleukin (IL)-1, secretion of which is suppressed by estrogen and enhanced by a range of osteoclastogenic cytokines or the inflammatory state[8]. Since osteoclasts and their precursors express receptors for both these inflammatory proteins, they stimulate formation and function of the bone-resorbing cell.

Bone resorption

Once formed, the osteoclast degrades the combined organ-

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ic and inorganic matrix by relatively well-understood mechanisms. Following attachment to bone via the integrin avβ3[9], the cell polarizes, generating a bone-apposed ruffled border, the unique functional organelle of the osteoclast[10]. This membrane arises by trafficking towards bone of acidifying vesicles containing both a membrane-bound vacuolar H+ ATPase and a specific soluble cargo, cathepsin K. Generation of protons under the action of carbonic anhydrase II is followed by secretion into the sub-cellular space as hydrochloric acid (the anion follows through a coupled chloride channel). The low pH (~4.5) environment dissolves hydroxyapatite, while cathepsin K, an acidic collagenase that is secreted during vesicle insertion, degrades the organic component[11], largely type 1 collagen. In contrast to other aspects of the biology of the cell, little is known about the mechanisms of trafficking and vesicle insertion.

Osteoclast death

The osteoclast has a relatively short life-span, estimated at about two weeks, and so specific endogenous mechanisms for inducing apotosis must exist. Despite the potential for suppressing bone resorption by acceleration of these pathways, little is known about the detailed events that lead to cell death. While caspases clearly play a role, as with other cell types, the upstream activating signals are largely a matter of conjecture, but probably include events mediated by integrins. Two important papers demonstrate that deletion of the genes coding for either the phosphatidylinositol lipid 3-phosphatase SHIP or Bcl2, a member of the BAD family known to regulate cell death, leads to decreased osteoclast apoptosis[12,13].

The osteoclast cytoskeleton

Organization and re-modeling of the osteoclast cytoskeleton are key components of its function. Staining for fibrous actin in mammalian osteoclasts on bone or dentin reveals the existence of a unique pattern, all the material being present in a peripheral ring. Higher power examination, plus co-staining for integrins and other actin-associated proteins, unmasks a complex picture, namely the presence of podosomes, structures that are related to, but distinct from, the focal adhesions present in most other cell types[14]. In focal adhesions actin is seen as the triangular termini of long fibrous transcellular structures, while in podosomes the protein exists as short bundles perpendicular to the cell surface. Furthermore, while focal adhesion kinase is a key element of focal adhesions, it is the related Pyk2 kinase that is present in podosomes[15]. Finally, the location of specific proteins, identified by confocal microscopy, is different for the two structures. Whereas the integrin avβ3, talin, vinculin and paxillin co-localize with actin in focal adhesions, the same proteins, while still in the podosome complex, are found as a separate rosette surrounding the actin bundles. While neither the biochemical basis underlying these distinct patterns of localization, nor the functional consequences of these differences is not fully understood, it is clear that podosomes are far more dynamic than focal adhesions, reflecting the motile nature of the osteoclast.

Turning to the role of the actin ring, its formation in osteoclasts is required for generation of the sealing zone, which defines the resorptive space beneath the cell and hence is used as a marker of its polarization. In many cell types, formation or dissolution of actin rings is required for events as diverse as intracellular trafficking of vesicles and cell motility, with the latter regulating many important cellular processes, ranging from morphogenesis to immunity and wound repair. Once again, whereas the biochemical events leading to actin remodeling in other cell types is the subject of intense research (see below) there has been less progress in this area in the osteoclast. The studies have involved strategies that block one of several steps on the cycle of actin polymerization. Thus, either treatment of osteoclasts with wortmannin or the small molecule LY292003, both of which inhibit PI-3K, results in complete loss of actin rings and bone resorption[16,17]. This result indicates that PI-3K has an ongoing role in maintaining the integrity of podosomes and hence the capacity of the cell to resorb bone. Similarly, introduction of exoenzyme C3[18], a molecule that blunts Rho activity (see below) or dominant negative forms of Rho itself[19], both suppress bone resorption. Further work in this area has pioneered by several groups. Hruska has focused on the role of osteopontin, a ligand for avβ3, in stimulating PI-3K activity and hence formation of phosphatidylinositol 3,4,5 trisphosphate. This membrane-associated lipid plays a role in formation of a podosomal signaling complex, containing gelsolin, which also acts as an actin capping protein (see below), c-src and the integrin avβ3[20]. Interestingly, the same group finds that a protein tyrosine phosphatase, PTP-PEST, is also phosphorylated and recruited to podosomes following ligation of avβ3[20]. In summary, their data provide insights into early events involved in actin assembly. Based on the earlier findings of Baron in which his group demonstrated that cbl interacts with c-src and Pyk2 to mediate osteoclast mobility[21], Duong et al. showed that the kinase activity of Pyk2 is not required for the initiation of osteoclast spreading and bone resorption. However, the protein must be phosphorylated on specific tyrosine residues if it is to be functional[22]. This result suggests that other proteins must bind Pyk2, probably via SH-2 dependent interactions, but the identity of these molecules is not known. Finally, a report that brings together much of the earlier studies shows that the formation of actin rings in osteoclasts requires interactions between Pyk2 and gelsolin, resulting in regulation of the actin capping protein[23].

There is convincing evidence that the major cytokines involved in the formation and activation of osteoclasts also control formation of actin rings. Thus, both RANKL and IL-1, sharing a common signaling pathway involving activation of TRAF6, enhance osteoclast polarization[24-26]. TNF has similar effects, but little is known about the molecular mechanisms underlying this finding[27]. Finally, exposure of mature osteoclasts to M-CSF stimulates both rapid (< 5 mins) membrane

ruffling and a slower (1-3 hr) generation of actin rings (Faccio et al. and Zhao et al., unpublished data). Again, information on the mechanisms is limited, but c-src and PI-3K activity have been implicated[17,28].

Molecular mechanisms supporting dynamic alterations in organization of actin filaments[29]

The mode by which cells migrate and alter their cytoskeleton requires that actin filaments undergo both disassembly and regeneration. This process can be either spontaneous or regulated by extracellular signals, with little known about the former aspect. In contrast, a number of the molecules that control expansion of actin filaments are now known, as are the biochemical signals responsible for regulation of the assembly process.

A wide range of external signals can stimulate the two key steps in a cascade leading to actin polymerization, namely activation of one or more members of a group of small GTPases and enhancement of the availability of the lipid, phosphatidylinositol 4,5 bisphosphate. Together, these two sets of molecules control the function of proteins in the WASp/Scar family, leading in turn to production of a functional Arp2/3 complex, the immediate arbiter of actin filament generation. Arp2/3 binds both existing actin filaments and monomers of the protein, the latter present as an ATP-bound form in association with profilin, leading to branching of the filament at an angle of about 70^{o}. This event is repeated multiple times, with the result that a closely-meshed web of expanding actin fibers is generated. The growing ends of each new filament are capped by specific proteins, with gelsolin important in this regard. The force produced by this growing structure provides the means by which a cell membrane is moved forward. Debranching of actin polymers requires the initial ATPase activity of actin itself, at which point a member of the ADF/cofilin family binds to the newly-formed ADP-actin and stimulates its dis-assembly from the filament. The monomer then interacts with profilin, which catalyses replacement of ADP with ATP, yielding a complex primed to undergo re-insertion in a growing filament.

The above description suggests that a key step in this complex signal transduction pathway is receptor-mediated activation of WASp and N-WASp, the only members of the WASp family subject to such regulation. The small GTPase that binds WASps is cdc42, following which WASp changes conformation, thereby releasing an existing auto-inhibition. Like other small GTPases, cdc42 exists in an inactive GDP-bound form that can be converted to the active GTP-ligated species. Replacement of GDP with GTP is accomplished by guanine-nucleotide exchange factors (GEFs), a large family of over 40 proteins. cdc42 and its closely related GTPases, Rho and Rac, all three of which are important in regulating cell motility[30], are the targets of the VAV group of GEFs[31] . Earlier work had suggested that the major role of VAVs is as signal transducers coupling ligation of surface receptors on B and T cells with multiple downstream events, including B and T cell maturation, natural killer cell function and control of the actin cytoskeleton, specifically in the context of the immunological synapse[31].

VAVs and the osteoclast

Given the findings discussed above and the similarity in topology and complexity between the immunological synapse and the resorptive lacuna of the osteoclast, we examined the bone phenotype in mice lacking VAV genes as a result of targeted deletion. Our first exercise was to determine expression levels of VAVs in osteoclasts and their precursors. Surprisingly, while VAV1 and VAV2 are the dominant isoforms in T cells, VAV3 is by far the most abundant family member in both bone marrow macrophages and osteoclasts derived therefrom with M-CSF and RANKL. We next examined the long bones of mice lacking each VAV, as well as the VAV1/VAV3 double knockout. While there was no discernible difference between wild type and animals lacking VAV1 or VAV2, absence of VAV3 results in an increase in bone mass as assessed by Faxitron analysis. Lack of both VAVs 1 and 3 mildly exacerbates the phenotype. Quantitative histomorphometry confirms the X-ray findings; trabecular bone mass and, most notably, total bone volume are markedly raised. While many multinucleated cells are formed when purified precursurs lacking VAV1 and/or VAV3 are cultured with optimal amounts of M-CSF and RANKL, their morphology differs from that of precursors lacking VAV1, VAV2 or wild type controls; the cells fail to spread. Staining with Rhodamine-phalloidin reveals complete absence of the characteristic actin ring, which is replaced by accumulation of the protein in the cytosol. Consistent with this finding, osteoclast height, measured by confocal microscopy of cells on dentin, is decreased, indicating a failure to polarize. Since these results are similar to that of mice lacking the integrin avβ3, we asked if, as in the latter circumstance[32], high levels of M-CSF could rescue the phenotype, and find this not to be so. When osteoclasts are generated on an artificial bone matrix from precursors lacking one or more of the three VAVs, significant resorption is seen only in wild type, VAV1 or VAV2 null cells. Once again, VAV1/VAV3 double knockout osteoclasts are less competent in this assay than those lacking only VAV3, a result consistent with out earlier *in vivo* data. Retroviral introduction of intact VAV3, but not VAV1, into precursors lacking both proteins completely rescues the spreading and resorptive phenotypes. Neither M-CSF- nor RANKL-induced signal transduction is abnormal, as indexed by ERK phosphorylation and nuclear translocation of NFkB, respectively. Similarly, M-CSF stimulated proliferation or activation of Rac and Rho is unchanged irrespective of genotype. In contrast, following avβ3-dependent adherence to vitronectin, phosphorylation of both c-src and ERKs1/2 is markedly decreased or absent in cells lacking VAV3 or VAV1/3, respectively.

To summarize, the cytokines responsible for formation and survival of osteoclasts and their precursors have a separate, equally important role, generation and maintenance of the

actin ring, a unique cytoskeletal structure required for function of the cell. Knowledge of the molecular basis underlying these events is limited. Our studies have uncovered the surprising fact that VAVs, a family of GEFs thought to be important primarily for the function of lymphoid cells, also play key roles in the control of the osteoclast cytoskeleton and hence bone resorption.

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