PROSPECT
Journal of Cellular Biochemistry 110:1058–1062 (2010)

Journal of Cellular Biochemistry

Recent Advances in Understanding the Mechanisms of Osteoclast Precursor Fusion

Merry Jo Oursler*

Robert and Arlene Kogod Center on Aging, Endocrine Research Unit, Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota 55905

ABSTRACT

Bone marrow macrophages fuse on the bone surface to form multinucleated osteoclasts that then organize to efficiently resorb bone. Many, if not all, of the stages of macrophage fusion involve cytoskeletal components that reorganize the cells. Recruitment may involve chemotactic responses to bone matrix protein and calcium ion gradients and/or chemokine production by bone forming osteoblasts. The roles of integrins vary, depending on the particular subunits with some interfering with fusion and others having a participatory role. RANKL is essential for fusion and many identified modulators of fusion influence RANKL signaling pathways. Tetraspanins have been implicated in fusion of macrophages and myoblasts, but differences in impacts exist between these two cell types. Macrophage recruitment to apoptotic cells prior to their engulfment is driven by the exposed phospholipids on the external surface of the apoptotic cells and there is evidence that this same identification mechanism is employed in macrophage fusion. Because loss of cadherin or ADAM family members suppresses macrophage fusion, a crucial role for these membrane glycoproteins is evident. The Ig membrane glycoprotein superfamily members CD200 and MFR/SIRPα are involved in macrophage fusion, although their influences are unresolved. Differential screenings have identified the structurally related membrane proteins DC-STAMP and OC-STAMP as required components for fusion and the contributions to fusion remain active areas of investigation. While many of the key components involved in these processes have been identified, a great deal of work remains in resolving the precise processes involved and the interactions between key contributors to multinucleated osteoclast formation. J. Cell. Biochem. 110: 1058–1062, 2010.

KEY WORDS: OSTEOCLASTS; MACROPHAGE; FUSION; MFR/SIRPα; CD200; DC-STAMP; OC-STAMP

In vertebrates, there are a limited number of cell types that fuse to form multinucleated cells. Evidently multinucleation has evolved because fusion enhances the functions of the resultant multinucleated cells. Myoblast fusion leads to multinucleated myotubes, increasing myofiber size and diameter to improve contractile strength of skeletal muscle. Macrophages fuse throughout the body to form multinucleated giant cells as a component of their defensive function, presumably to aid in foreign body engulfment. Within the bone environment, macrophages fuse on the bone surface to form multinucleated osteoclasts. Because multinucleated osteoclasts are much more efficient at bone resorption than their mononuclear counterparts, understanding the mechanisms by which fusion takes place may provide new avenues to explore in the search for therapies to control bone loss [Teitelbaum, 2000; Yagi et al., 2005]. The events involved in macrophage fusion to form osteoclasts can be sorted into a series of steps, many requiring cytoskeletal involvement. To initiate this process, precursors must be recruited and migrate to the bone surface. The cells must alter gene and protein expression to establish a fusion-competent status, enabling cell–cell recognition and attachment. Finally, the cells fuse to form an integrated functional cell prior to reorganization into active multinucleated osteoclasts. Recent discoveries have expanded our understanding of this understudied area. Although key players in this sequence of events have been identified, much remains to be resolved with regard to the precise functions and interactions of the components involved in the fusion process.

RECRUITMENT, MOVEMENT TOWARD, AND ATTACHMENT TO BONE

What draws osteoclast precursors toward the bone surface has been studied for many years. Early candidates were bone derived

Grant sponsor: National Institutes of Health; Grant numbers: R01 AR52004, P01 AG004875.
*Correspondence to: Dr. Merry Jo Oursler, Mayo Clinic, 200 First Street SW, Rochester, MN 55905.
E-mail: oursler.merryjo@mayo.edu
Received 31 March 2010; Accepted 1 April 2010 • DOI 10.1002/jcb.22640 • © 2010 Wiley-Liss, Inc.
Published online 1 June 2010 in Wiley InterScience (www.interscience.wiley.com).

non-collagenous proteins such as osteocalcin [Malone et al., 1982; Glowacki and Lian, 1987]. Osteoblasts may be involved in osteoclast precursor recruitment by secretion of chemokines such as CCL12 [Wright et al., 2005]. Chemokines secreted by osteoblasts recruit early osteoclast progenitors to migrate toward the bone surface where the osteoblasts can then stimulate differentiation through production of macrophage-colony stimulating factor (M-CSF) and receptor activator of NFκB ligand (RANKL). It is likely that calcium-sensing receptor on progenitors could also contribute to recruitment [Zaidi et al., 1991]. A Ca^{2+} gradient likely exists with highest concentrations adjacent to the bone surface, creating a stimulus for recruitment. Cytoskeletal involvement is, of course, key to the initiation of movement. Filamin A, Rho GTPase, and the actin cytoskeleton have been shown to be involved in calcium receptor signaling [Rey et al., 2005]. Filamin A, which cross-links F-actin at the leading edge of migrating cells, promotes migration and osteoclast formation [Leung et al., 2009]. The Rho GTPases Rac1 and Cdc42 interact with Filamin A and are involved in macrophage fusion [Leung et al., 2009]. Studies of mice lacking the membrane associated matrix metalloproteinase (MMP) MT1-MMP have uncovered a function of the cytoplasmic tail of this transmembrane protein that is required for osteoclast formation [Gonzalo et al., 2010]. These studies documented that MT1-MMP is required for lamellipodia formation and motility prior to membrane fusion and that this effect is independent of catalytic activity. Cells lacking MT1-MMP have decreased Rac1 activity and reduced membrane targeting of Rac1 as well as the adaptor protein p130Cas. Once precursors arrive at bone, integrin-mediated attachment is required for differentiation. Studies of the roles of different integrins in modulating osteoclast differentiation support that integrins differ in their influences. The integrin β_5 functions as a repressor of differentiation as indicated by the observation that osteoclast differentiation is accelerated in mice lacking β_5 [Lane et al., 2005]. Antibody blocking of α_9/β_1 integrin reduces differentiation and function, supporting that it may be important in promoting osteoclast differentiation [Rao et al., 2006]. In mice lacking the β_3 integrin, there is an increased number of dysfunctional osteoclasts, indicating that β_3 may function more in osteoclast function than in their formation [Faccio et al., 2003]. In contrast, over-expression of α_v, which partners with β_3 in osteoclast lineage cells, stimulated differentiation [Chin et al., 2003]. Thus it is possible that there may not be an essential requirement for the α_v/β_3 integrin pairing in differentiation, but rather α_v can act to enhance differentiation, perhaps by partnering with a different beta integrin.

ESTABLISHMENT OF A FUSION-COMPETENT STATUS

RANKL signaling is essential for osteoclast fusion and several cellular components that are involved in fusion act by promoting this pathway. The adenosine A1 receptor is a G protein-coupled receptor implicated in osteoclast differentiation [Kara et al., 2010]. Antagonism of this receptor targets TRAF6, which is required for RANKL signaling, for ubiquitination and degradation. These data support that the adenosine A1 receptor enhances RANKL-mediated signaling by protecting TRAF6 protein levels. Knockout of CCL2, also known as monocyte chemoattractant protein-1 (MCP-1), reduces osteoclast differentiation [Miyamoto et al., 2009]. CCL2 regulates expression of NFATc1, which is a RANKL target that is essential for differentiation [Miyamoto et al., 2009]. CD44 binding to hyaluronic acid, chondroitin sulfates, or osteopontin inhibits macrophage fusion [Sterling et al., 1998]. Surprising, CD44 is highly expressed in macrophages just prior to fusion [Cui et al., 2006]. As macrophages fuse, the intracellular domain of CD44 is cleaved and migrates into the nucleus, where it activates the RANKL target NFκB to promote fusion [Cui et al., 2006]. It may be that CD44 binding to its substrates alters cleavage of its intracellular domain, explaining how occupancy blocks fusion. Tetraspanins are membrane glycoproteins with four transmembrane domains and intracellular C- and N-termini that associate with integrins [Singethan and Schneider-Schaulies, 2008]. Members of this family function as scaffolding proteins that anchor multiple proteins in the same region of the cell membrane [Hemler, 2005]. Antibody blocking of the tetraspanin CD63 inhibits macrophage fusion, thus CD63 may likewise be involved in osteoclast fusion [Parthasarathy et al., 2009]. CD9 and CD81 are tetraspanins that are involved in sperm/egg fusion and myoblast fusion [Tachibana and Hemler, 1999]. They have the opposite effect in macrophages in that they function as inhibitors of fusion [Parthasarathy et al., 2009]. Matrix metalloproteinase 9 (MMP9) is required for IL-4-induced macrophage fusion, however, the influences of MMP9 in fusion are unknown [MacLauchlan et al., 2009]. Reduced CD9 and CD81 leads to increased MMP9 expression, raising the possibility that one mechanism by which CD9 and CD81 inhibit macrophage fusion is by suppression of MMP9 [Takeda et al., 2008]. Purinergic2 (P2) receptors are G-protein coupled ATP receptors that act as ATP-gated ion channels. To investigate the role of P2 receptors in fusion, sub-populations of the macrophage cell line J774 were separated on the basis of $P2x_7$ activation levels [Chiozzi et al., 1997]. These studies found that cells exhibiting increased $P2x_7$ activation fuse spontaneously whereas cells with decreased activation or unsorted cells do not fuse. Treatment with oxidized ATP blocks spontaneous fusion, indicating a role for activated P2 receptors in fusion. While blocking of $P2x_7$ inhibits macrophage fusion in vitro, mice lacking this receptor have normal bone and exhibit no deficiency in osteoclast differentiation in vitro or in vivo [Falzoni et al., 1995; Gartland et al., 2003]. Thus a convincing role for $P2x_7$ in osteoclast formation is lacking and the role, if any, of ATP receptors in osteoclast precursor fusion remains an open question.

CELL–CELL RECOGNITION AND ATTACHMENT

A diverse group of cellular components are involved in osteoclast precursor interactions prior to fusion. Cadherins are calcium dependent adhesion molecules that mediate cell–cell adhesion. Mbalaviele et al. [1995] have shown that blocking E-cadherin suppresses macrophage fusion in vitro, supporting a key role for E-cadherin in attachment of precursors prior to fusion. Bones in mice lacking the v-ATPase V_0 subunit d2 have significantly higher bone density [Lee et al., 2006]. Surprisingly, there is no alteration in

the v-ATPase activity in cells, but osteoclast numbers are significantly decreased. Because there are comparable numbers of TRAP positive mononuclear cells and fewer multinucleated cells, the data indicate that fusion of the mononuclear cells is suppressed. Mixing wildtype and knockout macrophages restores fusion rates, but cultures of knockout-only macrophages fail to fuse. In the knockout cells, a disintegrin and metalloproteinase (ADAM) 8 and 12 are decreased and restoration of these restores differentiation. ADAMs cleave extracellular parts of transmembrane proteins and are involved in cell–cell and cell–matrix adhesion [Edwards et al., 2008]. Blocking ADAM8 or Adam12, which are secreted by macrophages in culture prior to cell fusion, decreased macrophage fusion in vitro [Abe et al., 1999; Choi et al., 2001]. The observation that these are released into the culture media may explain why a mixture of wildtype and knockout cells fuses at the same rate of cultures of wildtype cells by themselves in the studies of Lee et al. above. Phosphatidylserine (PtdSer) is a phospholipid component whose localization is normally restricted to the cytosolic side of the inner membrane of the plasma membrane. During apoptosis, PtdSer is relocated to the extracellular surface of the cell and functions as a beacon to attract macrophages for ingestion of the dying cell [Fadok et al., 2001]. There is evidence that transient extracellular exposure of PtdSer is involved in macrophage fusion. CD36 is a scavenger receptor that recognizes PtdSer and is involved in IL-4-induced giant cell formation, indicating a potential role in osteoclast formation [Helming and Gordon, 2009]. The mechanism by which PtdSer relocates prior to fusion could involve ATP-gated ion channels. Brief activation of these channels causes reversible PtdSer re-localization to the extracellular surface to enable macrophage-macrophage recognition [MacKenzie et al., 2001].

FUSION

CD200 is a membrane glycoprotein member of the Ig superfamily that is broadly expressed in the macrophage lineage as well as other cells and tissues throughout the body. Unlike CD200, expression of its receptor CD200R is more restricted to myeloid lineage cells including osteoclasts [Cui et al., 2007]. Within the macrophage lineage, CD200 represses macrophage lineage commitment and differentiation [Hoek et al., 2000]. Because of this effect, CD200 expression suppresses graft rejection, auto-immunity, and cartilage-induced arthritis [Gorczynski, 2005]. During osteoclast differentiation, CD200 is up-regulated just prior to fusion and enhances RANKL signaling to promote fusion [Cui et al., 2007].

Macrophage fusion receptor/signal-regulatory protein-alpha (MFR/SIRPα) is a member of the same immunoglobulin-containing family as CD200. Monoclonal antibodies to MFR/SIRPα block macrophage fusion, indicating a role for MFR/SIRPα in promoting fusion [Vignery, 2000]. Three potential MFR/SIRPα characteristics could participate in promoting fusion. These are inhibitor influences of the intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs), ligand-dependent signaling, and association with integrins. The ITIM regions are involved in MFR/SIRPα-mediated suppression of macrophage differentiation, so MFR/SIRPα has been postulated to push progenitor cells toward osteoclast lineage instead of the macrophage lineage to enhance osteoclast differentiation [Matozaki et al., 2009]. Signal transduction is initiated by interaction between MFR/SIRPα and its receptor CD47 through recruitment of the intracellular tyrosine phosphatases SHP-1&2 [Vernon-Wilson et al., 2000]. Antibody blocking of receptor-ligand interactions reduces osteoclast formation in vitro, confirming a role for MFR/SIRPα and CD47 interactions in macrophage fusion [Lundberg et al., 2007]. Within the bones of mice lacking CD47 there are fewer osteoclasts, but the size and number of nuclei per cell does not differ from wildtype [Lundberg et al., 2007]. This suggests that CD47 may be able to promote fusion initiation, but is not needed once the process is started. Finally, fusion initiation could potentially involve the association of MFR/SIRPα with integrins [Brown and Frazier, 2001]. Recently, mice expressing a mutant MFR/SIRPα that lacks a cytoplasmic tail were examined [van Beek et al., 2009]. In this study, the mutant form of MFR/SIRPα acted as an enhancer of osteoclast actin ring formation and thus resorption, with no apparent effects on osteoclast differentiation. In these mice, there was significantly reduced cortical mass, confirming the stimulatory role of truncated MFR/SIRPα in vivo in cortical bone resorption. Loss of the cytoplasmic tail would abrogate signaling as well as eliminate any ITIM-mediated influences. The hypothesis that MFR/SIRPα promotes osteoclast differentiation through suppression of macrophage maturation seems unlikely as there should have been fewer osteoclasts in mice lacking the cytoplasmic tail of MFR/SIRPα if this were the mechanism. Because osteoclast numbers are normal in the mutant mice, non-signaling and non-ITIM-mediated functions of MFR/SIRPα may promote osteoclast fusion whereas activation of signaling may be involved in controlling osteoclast actin ring formation and function. It remains to be resolved whether truncated MFR/SIRPα retains its ability to interact with integrins. If this function remains, it is a potential mechanism by which MFR/SIRPα influences on fusion are retained in the mutant mice.

Dendritic cell-specific transmembrane protein (DC-STAMP) has been called a ''master fusigen'' for osteoclast differentiation. DNA subtraction screening of pre- and post-fusion macrophages identified DC-STAMP as being highly expressing in multinucleated osteoclasts but not mononuclear macrophages [Yagi et al., 2005]. DC-STAMP knockout mice have osteopetrosis and lack multinucleated osteoclasts, thus expression is required for differentiation. Macrophages expressing DC-STAMP can fuse with knockout macrophages whereas knockout macrophages cannot fuse with each other. Thus, DC-STAMP does not need to be expressed in both cells for these cells to fuse, but one of the partners must express the protein. In addition to potentiating RANKL signaling by enhancing NFATc1 expression, CCL2 regulates DC-STAMP expression [Miyamoto, 2006]. Using the macrophage cell line RAW264.7, studies of fusion following treatment with TNF-α, LPS, RANKL, or peptidoglycan does not lead to increased expression of DC-STAMP as the cells fuse [Hotokezaka et al., 2007]. This raises the question of whether enhanced DC-STAMP expression is an absolute requirement for fusion and whether there may be other, perhaps related, proteins that can function as DC-STAMP does in macrophage fusion. Microarray studies of RAW264.7 cells pre and post fusion revealed that the expression of a novel gene whose expression is also up-regulated during fusion [Yang et al., 2008]. This gene was named

osteoclast stimulatory transmembrane protein (OC-STAMP). OC-STAMP is highly similar in structure and caboxy terminus to DC-STAMP and is a multi-pass RANKL-induced membrane protein required for fusion of macrophages. Few studies have yet examined the relationship and possible interactions of DC-STAMP and OC-STAMP in macrophage fusion. In a recent study, Mensah et al. [2010] examined characteristics of DC-STAMP on macrophage and RAW264.6 macrophage cell line fusion. RANKL treatment of these cells caused internalization of membrane DC-STAMP in roughly half of the cells, resulting in two identifiable populations on the basis of membrane DC-STAMP levels, DC-STAMP^{hi} and DC-STAMP^{lo}. To examine this further, RAW264.7 cells were sorted into DC-STAMP^{hi} and DC-STAMP^{lo} populations. DC-STAMP^{lo} cultures efficiently fuse, DC-STAMP^{hi} cells do not fuse, and a mixture containing 10% DC-STAMP^{hi} cells and 90 percent DC-STAMP^{lo} cells fuse even better than DC-STAMP^{lo} cells alone. Increasing the ratio of DC-STAMP^{hi} to DC-STAMP^{lo} cells abrogates fusion. The more fusigenic DC-STAMP^{lo} cells exhibit higher RANKL-mediated induction of OC-STAMP than the DC-STAMP^{hi} cells. Although the DC-STAMP^{hi} cells express MFR/SIRPα, they do not express CD47, the receptor for MFR/SIRPα. It will be of interest to determine whether altering OC-STAMP and/or CD47 alters the ability of the DC-STAMP^{hi} cells to fuse. Although it is projected that DC-STAMP may be a G protein-coupled receptor, the ligand is not yet known. The observed RANKL-mediated internalization may be caused by RANKL stimulation of production of this putative ligand. The ligand may then bind to DC-STAMP to initiate pro-fusion signaling prior to ligand-dependent internalization. The above study supports that pathway activation may stimulate OC-STAMP and CD47 expression. This would lead to the pro-fusigenic phenotype of the DC-STAMP^{lo} cells. Since DC-STAMP is not internalized in the DC-STAMP^{hi} cells, it can be surmised that the lack of fusion of these cells could be due to either an inability of these cells to bind the ligand or it could be the result of an inability to respond to ligand binding. Why the mixture of DC-STAMP^{hi} and DC-STAMP^{lo} cells are better at fusing is not explained by this conjecture and remain to be resolved. The observation that cultures with equal numbers of both population do not fuse indicates that the interactions of these populations in fusion may be complex.

CONCLUSIONS

While several of the studies discussed here have focused on understanding macrophage fusion into giant cells outside of the bone environment, many of the discoveries from this area have proven to be applicable to bone environment macrophage fusion into osteoclasts. Through use of differential screenings, there have been promising developments in our understanding of the mechanisms by which macrophage fuse. The identification of DC-STAMP and, more recently, OC-STAMP have greatly accelerated investigations into the mechanisms of osteoclast formation. Much remains to be uncovered concerning the regulation and functions of these and other fusion-related proteins in osteoclast precursor fusion. The potential is that uncovering of these mechanisms may provide important new therapy targets to control excess bone loss or to accelerate bone degradation when needed.

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