Osteoclasts and Integrins

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ABSTRACT: The osteoclast is the unique bone resorptive cell that accomplishes its mission by forming an isolated acidified microenvironment between itself and the bone surface. Creation of this compartment is the first step in bone degradation and establishes that an intimate physical relationship must exist between the osteoclast and bone. Thus, identification of the mechanisms by which the osteoclast attaches to bone is essential to understanding how the cell degrades skeletal tissue. Our studies have investigated whether absence of the αvβ3 integrin modifies the ability of c-Fms to induce Rho GTPases, and the implications for formation of the osteoclast cytoskeleton.

KEYWORDS: osteoclast; integrin; bone mass; bone resorption, Rho GTPase; Rac; Vav

The osteoclast is the unique bone resorptive cell that accomplishes its mission by forming an isolated acidified microenvironment between itself and the bone surface. Creation of this compartment is the first step in bone degradation and establishes that an intimate physical relationship must exist between the osteoclast and bone.[1] Thus, identification of the mechanisms by which the osteoclast attaches to bone is essential to understanding how the cell degrades skeletal tissue.

Integrins are transmembrane αβ heterodimers that mediate cell/cell and cell/matrix recognition.[2] In the case of integrins mediating matrix attachment, their extracellular domains are liganded by extracellular proteins, while their intracellular tails interact with signaling molecules and the cytoskeleton.

A series of *in vitro* experiments established that αvβ3 is the principal osteoclast integrin. αvβ3, like all members of the αv family of integrins, recognizes the arginine-glycine-aspartic acid (RGD) motif. This ligand is present in a number of bone-residing proteins, such as osteopontin and bone sialoprotein. To determine if the αvβ3 integrin, identified *in vitro*, has physiological significance, we deleted the β3 integrin gene in mice.[3] The net result of this

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exercise is an age-dependent increase in bone mass, consistent with osteoclast dysfunction.

Because increased skeletal mass may reflect either enhanced bone formation or arrested osteoclast function, we turned our attention to the osteoclast in these mutant animals. Thus, we generated osteoclasts from wild-type, heterozygous, and β3 integrin-deficient bone marrow macrophages. Osteoclasts derived from wild-type and heterozygous mice appear identical as they are well spread, multi-nucleated, and express the osteoclast characteristic enzyme, TRAP.[3] On the other hand, osteoclasts generated from bone marrow macrophages of β3 integrin-deficient mice, have a crenated appearance indicative of a cytoskeletal abnormality. The appearance of the actin cytoskeleton of bona fide osteoclasts, isolated directly from bones of both β3 knockout and heterozygous animals, confirms this hypothesis. Specifically, osteoclastic bone resorption requires the formation of an actin ring-like structure, known as the "sealing zone," which isolates the resorptive microenvironment from the general extracellular space.[4] While such ring-like structures are apparent in osteoclasts isolated from heterozygous mice, β3 integrin-deficient osteoclasts fail to form actin rings.[3] These cytoskeletal abnormalities are functionally manifest by the inability of β3 integrin–/– osteoclasts to excavate normal resorptive lacunae when placed on dentin slices.

Cytoskeletal organization, in general, is mediated by the Rho family of GT-Pases, which include RhoA, Rac, and CDC42.[5] We, therefore, asked if the cytoskeletal abnormalities present in β3-deficient osteoclasts reflect insufficient Rho GTPase activation. Because Rho GTPases, in osteoclasts, are activated by the occupancy of the macrophage colony-stimulating factor (M-CSF) receptor, c-Fms, we addressed this issue in the context of c-Fms activation.[6] Specifically, we determined if the absence of the αvβ3 integrin modifies the ability of c-Fms to induce Rho GTPases. We find that in wild-type pre-osteoclasts, 15 minutes exposure to M-CSF leads to substantial activation of RhoA by transiting it from its GDP to its GTP bound form. In contrast, there is no evident activation of RhoA in the absence of the β3 integrin. Similarly, Rac is activated within 5 minutes of exposure to M-CSF in authentic pre-osteoclasts but not in those lacking αvβ3.

Activation of Rho GTPases such as Rac, is under the influence of guanine nucleotide exchange factors (GEFs). Specifically, GEFs transit small GTPase from their GDP to the GTP bound form eventuating in cytoskeletal organization. Because the Vav family of GEFs activates Rac, we asked if Vavs play a role in αvβ3 mediated stimulation of this Rho GTPase.[7]

Vav is known to exist in three isoforms, namely Vav1, Vav2, and Vav3. In fact, Vav1 is the dominant isoform in T-lymphocytes. On the other hand, Vav3, which is virtually undetectable in T cells, predominates in osteoclasts and is expressed, constitutively, in marrow macrophages as they differentiate into these bone-resorbing polykaryons under the influence of RANK ligand and

M-CSF. In contrast, Vav1 is present in moderate amounts and Vav2, in only small quantities, in osteoclasts.

The abundance of Vav3 in the bone-resorbing cells raises the possibility that this GEF is activated by αvβ3 occupancy. To address this issue, we placed wild-type pre-osteoclasts in suspension or on osteopontin. Tyrosine phosphorylation of Vav3 is markedly enhanced in cells on the αvβ3 ligand indicating that activation of the integrin leads to activation of Vav3. In keeping with this observation, Vav3 knockout osteoclasts fail to form normal sealing zones and similar to those lacking the β3 integrin, fibrillar actin is distributed diffusely throughout the cell. This is also manifest by the inability of the cells to spread effectively when derived from mice lacking Vav3 or both Vav1 and Vav3. Furthermore, whereas resorption lacunae generated by Vav1-deficient osteoclasts are relatively normal, those cells derived from Vav3 and Vav1,3 null mice are incapable of degrading mineralized matrix, *in vitro*. Extending these observations to the *in vivo* state, radiographic analysis documents a substantial increase in bone density in the Vav3–/– and Vav1,3–/– animals. This observation is in keeping with histomorphometric analysis, which reveals that the absence of Vav3 and/or Vav1 and Vav3 eventuates in a 2.5- to 3-fold increase in trabecular bone mass. Furthermore, whereas parathyroid hormone (PTH) administration to wild-type mice leads to an approximate doubling of serum pyridinoline cross-link content, a global indicator of bone resorption, the absence of Vav3 and Vav1 and Vav 3, completely blunts PTH-stimulated skeletal degradation.

The data presented, thus far, document that osteoclastic bone resorption is deficient in the absence of Vav3. On the other hand, they do not establish whether this defect in osteoclast function reflects a primary osteoclast abnormality or is a manifestation of dysfunctional osteoblasts producing insufficient quantities of bone resorptive cytokines such as RANK ligand (RANKL). To address this issue, we asked if Vav3 osteoclasts are capable of responding to the osteoclastogenic cytokine, with the rationale that a primary defect in these cells would result in their failure to respond to osteoblast-produced RANKL. The significance of this question is buttressed by our finding that osteoblasts, like osteoclasts, express abundant Vav3 and Vav1. The appearance of the RANKL-induced bone loss in Vav3-deficient mice, despite stimulated osteoclastogenesis, establishes that their subnormal bone resorption reflects a primary defect in the osteoclast, and not osteoblast, function. In keeping with this posture, bone formation rates remain unchanged in the absence of Vav3.

Having established that the αvβ3 integrin activates Vav3 and Vav3-deficient osteoclasts are defective, prompted us to determine if Vav3 modulates the osteoclast cytoskeleton by impacting integrin-induced Rho GTPases. Whereas M-CSF activates Rac in a Vav3-dependent manner, such is not the case regarding RhoA. Therefore, both the αvβ3 integrin and M-CSF occupancy of c-Fms partner to phosphorylate Vav3, which in turn activates Rac, leading to organization in the osteoclast cytoskeleton.

The c-Src-deficient mouse demonstrates the surprising phenotype in that osteopetrosis, due to dysfunctional osteoclasts, is its principal abnormality.[8] Indeed, c-Src-deficient osteoclasts appear reminiscent of those lacking the β3 integrin as cytoskeletal abnormalities are extant in both.[3,9] This observation suggests that c-Src and αvβ3 enjoy a common signaling pathway. In fact, c-Src associates with αvβ3 in the osteoclast and does so in the context of the tyrosine kinase, Syk. In this regard, Syk–/– osteoclasts also demonstrate the phenotype of cytoskeletal disorganization common to both c-Src and αvβ3 null cells.[10] Thus, Syk-deficient osteoclasts also fail to form actin rings.

Because Syk associates with a β3 integrin in the context of the platelet,[11] we asked if the same obtains in the osteoclast. Syk does recognize the β3 integrin in osteoclasts and does so independently of c-Src. Once Syk and Src bind to the β3 integrin cytoplasmic domain, c-Src phosphorylates, and thus activates Syk, which, in turn, induces Vav3 and ultimately reorganizes the actin cytoskeleton. Finally, the common signaling pathway of Syk and Vav3 is documented by the crossing of haplo-insufficient mice.[7] Thus, Syk/Vav3 compound heterozygous animals have a trabecular bone mass, which is greater than that of the Vav3 knockout mouse, and their osteoclasts fail to normally resorb dentin *in vitro*.

Because the αvβ3 integrin is an essential component of optimal osteoclast function, it presents as a candidate anti-resorptive therapeutic target for treatment of osteopenic diseases such as postmenopausal osteoporosis.[12] In fact, small molecule inhibitors of the integrin are bone-sparing in oophorectomized rats. Most importantly, the same holds in osteoporotic women as such compounds are presently in clinical trials.[13]

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