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PERSPECTIVES

The Osteoclast Cytoskeleton: How Does It Work?

Steven L. Teitelbaum and Wei Zou

Washington University School of Medicine, St. Louis, Missouri, USA

Abstract

The capacity of the osteoclast to resorb bone is distinctive, as is the cell's appearance. Both characteristics reflect cytoskeletal organization that yields structures such as the sealing zone and ruffled border. The unique nature of these organelles and their dependence upon contact with bone have been appreciated for some time but insights into the mechanisms by which they are generated come from more recent studies. These insights include the role of integrins, particularly $\alpha v\beta 3$, in cytoskeletal organization and the canonical signaling pathway they activate. Investigators now appreciate that the sealing zone isolates the resorptive microenvironment from the general extracellular space, permitting secretion of matrix-degrading molecules on the bone surface. Thus, the osteoclast is a secretory cell that depends upon polarization of exocytic vesicles to the bone-apposed plasma membrane into which they insert under the aegis of vesicle/membrane fusion proteins. This process focally expands and convolutes the plasmalemma included within the sealing zone, eventuating in formation of the ruffled border. Many of these events are now better understood and are the focus of this *Perspective*. *IBMS BoneKEy*. 2011 February;8(2):74-83.

Isolating the Resorptive Microenvironment

Osteoclasts are polykaryons and members of the macrophage lineage with the unique capacity to degrade the inorganic and organic matrices of bone. If excessive, the bone resorptive activity of osteoclasts causes osteoporosis. Conversely, the osteoclast initiates remodeling that likely removes structurally compromised bone, thereby maintaining mechanical integrity (1). Skeletal health, therefore, requires optimal osteoclast function.

Resorption is initiated by attachment of osteoclasts to bone. They then develop a compartment between their plasma membrane and the bone surface into which the cells transport matrix-degrading molecules including H^+ and Cl^-, which, in concert, demineralize the target bone, and cathepsin K, which degrades the exposed collagen fibers and associated proteins. To isolate this resorptive microenvironment from the general extracellular space, osteoclasts reorganize their cytoskeleton to generate an encompassing, actin-rich, gasket-like, sealing zone. A single osteoclast, being a large cell, enjoys multiple contacts with bone and therefore generates numerous sealing zones and resorptive microenvironments.

When most other cells attach to matrix they generate focal adhesions. These stable structures contain integrins and signaling and cytoskeletal molecules that, upon contact with matrix, promote formation of actin stress fibers. Consistent with the lack of actin stress fibers in mammalian osteoclasts, they lack focal adhesions but in their place develop podosomes (2;3). These punctuate structures contain an F-actin core and a peripheral "cloud" of a loose network of radial actin cables (4-7). In contrast to focal adhesions, podosomes are transient but mediate substrate adhesion and thus formation of the resorptive microenvironment.

Most past studies of osteoclast podosomes utilized cells resident on plastic or glass. In these circumstances, the punctuate structures initially appear in clusters but ultimately coalesce, first into an

intracytoplasmic actin ring and then a peripheral actin belt (4). The physiological relevance of this observation was challenged by the absence of apparent podosomes or an actin belt in non-stressed osteoclasts on bone (4). While the relevance of the peripheral belt is not established, it is clear that, like the actin ring, the sealing zone of bone-residing osteoclasts also consists of podosome-containing structural units (8).

Microtubules Are Important

Depending upon their state of acetylation, microtubules in osteoclasts are transient or polymerized and relatively stable (9-11). Unlike actin ring formation by glass-residing cells, sealing zone generation in bone-resorbing osteoclasts is characterized by microtubule acetylation, again illustrating the influence of substrate on the cell's cytoskeleton (10).

The histone deacetylase HDAC6 depolymerizes tubulin, thereby destabilizing microtubules. Cbl proteins compete with the deacetylase for tubulin binding and thus promote polymerization (12). While destabilizing microtubules in other cells (13), RhoA appears to activate HDAC6 in glass-generated osteoclasts, suggesting the GTPase negatively regulates the cell. When on bone, however, osteoclasts in which RhoA is inhibited lose their apical-basal polarity and, consequently, are incapable of optimal resorption (6). Furthermore, RhoA promotes actin ring and podosome formation and osteoclast motility (6;14). When osteoclasts contact bone, RhoA is activated and localizes to the cytoskeleton (15;16), The fact that RhoA activation is diminished, in osteoclasts lacking $\alpha v\beta 3$, establishes that the GTPase is regulated by the integrin (3). Because of the conflicting phenotypes of osteoclasts on plastic or bone, the impact of active RhoA on stability of their microtubules remains unknown.

The Ruffled Border Is King

The ruffled border is the morphological *sine qua non* of the resorbing osteoclast as only its presence assures that the cell is degrading bone and its absence indicates the cell is not doing so. Reflecting multiple contacts with bone and attendant sealing zones, ruffled borders are also numerous in a given osteoclast.

This complex enfolding of the plasma membrane, unique to the osteoclast, abuts and extends into the resorptive space. It is surrounded by the sealing zone and is the venue by which the cell secretes matrix-degrading molecules on the bone surface (17). It is therefore the resorptive organelle and is absent or deranged in many forms of osteopetrosis. As osteoclast resorption alternates with migration, the ruffled border is a transient structure.

The ruffled border, which forms only upon contact with mineralized substrate, is initiated by transport of cathepsin K – and/or H^+ATPase – and Cl^- channel-bearing vesicles to the bone-apposed plasma membrane (17), likely under the aegis of GTPases such as Rabs 7, 9 and 3 (18-22). While unproven, studies in chicken osteoclasts raise the possibility that ruffled border-forming vesicles may polarize to the resorptive surface via microtubules (23). The polarized vesicles fuse with the bone-apposed plasma membrane, to increase its complexity, via a process mirroring exocytosis (17). Similar to that occurring in the context of neurotransmitter exocytosis, vesicle/plasmalemma fusion is regulated by v- (vesicular) and t- (target) SNAREs (soluble N-ethylmaleimide-sensitive fusion protein (NSF) attachment protein (SNAP) receptors) (24).

Ruffled border formation requires a synaptotagmin (Syt) linking the vesicle and target plasma membrane. Fifteen Syt isoforms have been identified in mammalian cells but Syt VII generates ruffled borders and is essential for bone degradation (17). Syt VII also enables osteoblasts to secrete bone matrix proteins and as such, both resorption and formation are repressed in Syt VII-deficient mice.

Autophagy is a cellular degradative process by which cells recycle organelles and long-lived proteins. Autophagosomes, which are

double-membrane bound vesicles, envelop and then deliver cellular components to lysosomes for degradation. While the process promotes survival in starved or stressed cells as well as maintenance of organelle quality (25-27), recent evidence indicates it may also participate in regulated exocytosis (28). In fact, Atg5, Atg7 and LC3β autophagy proteins, representing the two ubiquitin-like conjugation systems, are important for generation of the osteoclast ruffled border and the secretory function of osteoclasts both *in vitro* and *in vivo*. Thus, osteoclasts lacking these proteins do not efficiently polarize cathepsin K to the resorptive microenvironment and are incapable of optimal bone resorption (29).

Integrin Activity Starts It All

Skeletal resorption requires osteoclast-bone recognition that is mediated by α/β heterodimers known as integrins. β1-containing heterodimers probably participate in the process but αv/β3 is the key integrin regulating skeletal degradation (30). While the αv subunit is constitutively expressed throughout osteoclastogenesis, the associated β chains alter with differentiation. Specifically, immature osteoclast precursors, in the form of bone marrow macrophages, express abundant αvβ5 and little αvβ3. As the cells commit to the osteoclast phenotype, the magnitude of expression of the two heterodimers reverses (31). Hence, αv/β3 is a relatively specific marker of osteoclastogenesis. This integrin is liganded by the amino acids Arg-Gly-Asp (RGD), a motif present in bone proteins such as osteopontin and bone sialoprotein. Small molecules mimicking this sequence suppress osteoclast activity and are candidate anti-resorptive drugs (32-34).

In keeping with its governance of osteoclast function, absence of αv/β3, globally and conditionally in osteoclasts, increases bone mass and protects against estrogen-deficient osteoporosis (35;36). Reflecting the integrin's role in cytoskeletal organization, αv/β3 deficiency yields deranged ruffled borders, failure of cell spreading and sub-optimal bone resorption (35). Consequently, β3(-/-) mice are hypocalcemic and osteoclast number is substantially increased in these animals, likely reflecting secondary hyperparathyroidism and an abundance of osteoclastogenic cytokines in the marrow (35;37;38). However, in contrast, absence of αv/β3 diminishes the abundance of the polykaryons *in vitro* (33). As differentiation, apoptosis and precursor proliferation are not compromised, a reasonable hypothesis holds that the paucity of osteoclasts, in culture, reflects cytoskeletal dysfunction, and specifically impairment of migration necessary for cell fusion.

αv/β3 signaling in osteoclasts is initiated by changing the integrin's conformation from a low to a high affinity state by outside-in or inside-out activation (3;39). Outside-in activation is characterized by integrin clustering, thereby increasing avidity and affinity. Inside-out activation is an indirect event wherein signals emanating from liganded growth factor or cytokine receptors target the integrin's intracellular region, changing its conformation and consequently, that of the extracellular domain (40). As will be discussed, the adaptor protein, talin, is essential for inside-out αv/β3 activation in osteoclasts and its absence arrests bone resorption.

Resorption is a cyclical event wherein a portion of the osteoclast migrates to a candidate bone resorptive site and forms an actin ring and ruffled border. Following matrix degradation, the cell detaches and re-initiates the cycle. Prior to bone recognition, the integrin is predominantly in a low affinity state and confined to podosomes within the sealing zone (3;41). Activated αv/β3 leaves the podosome and transits to lamellipodia that mediate motility, compromised in the absence of the integrin (3). During resorption, the heterodimer appears in the ruffled membrane (3;41).

Integrins serve as attachment molecules but their intracellular transmission of matrix-derived signals is at least as important. For example, αv/β3 substrate robustly activates ERKs in wild-type osteoclastic cells but not those lacking the integrin (3). Since

activation of these MAP kinases typically stimulates proliferation, their absence in β3 knockout osteoclasts may contribute to their reduced numbers *in vitro*.

How Does αv/β3 Do It?

In 1991, Soriano *et al*. determined that c-src deletion eventuates in severe osteopetrosis due to osteoclast dysfunction (42). Interestingly, c-src-deficient mice, like those lacking αv/β3, have increased numbers of osteoclasts that fail to organize their cytoskeleton. c-src is a tyrosine kinase and an adaptor protein and both functions are necessary for optimal cytoskeletal organization (43).

Because c-src- and αv/β3-deficient osteoclasts share qualitatively similar cytoskeletal features, the kinase presents as a mediator of integrin signaling. In fact, under the aegis of phospholipase Cγ (PLCγ2) (44), c-src binds directly to the β3 subunit in the bone resorptive cell, and we have found this to be a constitutive event (45). Others, however, propose that αv/β3 occupancy phosphorylates the focal adhesion kinase family member, Pyk2, which recruits c-src to the integrin (44;46). αv/β3-associated c-src phosphorylates c-Cbl which, in turn, inhibits c-src's activity (3;47). Regardless of the mechanism of association, c-src activation requires integrin occupancy and again, signaling via PLCγ2. Activated c-src prompts podosomal disassembly, most probably by phosphorylating cortactin (48;49). Hence, podosomes are more abundant in *c-src*(-/-) than wild-type osteoclasts and the mutant cells are less motile. In keeping with Pyk2 regulating the cell's cytoskeleton, osteoclasts lacking the kinase are unable to generate normal sealing zones on bone (11). The cytoskeletal effects of Pyk2, however, may reflect its promotion of tubulin acetylation.

Syk is another non-receptor tyrosine kinase mediating αv/β3 signaling in osteoclasts. Upon integrin occupancy it binds the β3 cytoplasmic domain close to c-src, which activates it (45). Syk is also negatively regulated by the ubiquitinating activity of c-Cbl (50;51).

The ITAM-bearing adaptors, Dap 12 and FcRγ, are expressed by osteoclasts and their combined, but not individual deletion prompts severe osteopetrosis (45;52-54). While deletion of both co-stimulatory molecules is reported to arrest osteoclastogenesis (55), we find such is not the case (56), suggesting their resorptive abnormality reflects deranged osteoclast function but not generation. The same obtains regarding osteoblast-mediated generation of osteoclasts lacking Dap12, with or without FcRγ. These mutant cells form in normal numbers but fail to organize their cytoskeleton or resorb bone. Among the most dramatic consequences of this dysfunction is the inability of *Dap12*(-/-) osteoclasts to migrate through a layer of osteoblasts, required to attach to a candidate resorptive bone surface (56;57). Thus the dominant role of ITAM proteins in the osteoclast appears to be cytoskeletal organization and not differentiation (56;58).

Syk-mediated organization of the osteoclast cytoskeleton involves Vav3. This guanine nucleotide exchange factor (GEF) is uniquely expressed in abundance in the cell and activated upon αv/β3 occupancy in a SLP-76-dependent manner (15;59). Vavs transit cytoskeleton-organizing Rho GTPases from their inactive GDP- to their active GTP-associated conformation. Thus, *Vav3*(-/-) osteoclasts are dysfunctional and the mice from which they are derived are osteopetrotic.

Vavs are Rac GEFs and it is therefore not surprising that this Rho GTPase regulates the osteoclast cytoskeleton in an αv/β3-dependent manner (60;61). The two isoforms expressed in osteoclasts, Rac1 and Rac2, are mutually compensatory (62). Effective deletion of both, however, produces severe osteopetrosis in which osteoclasts fail to organize their cytoskeleton. Absence of the related Rho family GTPase, cdc42, also causes osteopetrosis but in this circumstance the dominant mechanism is arrested osteoclast

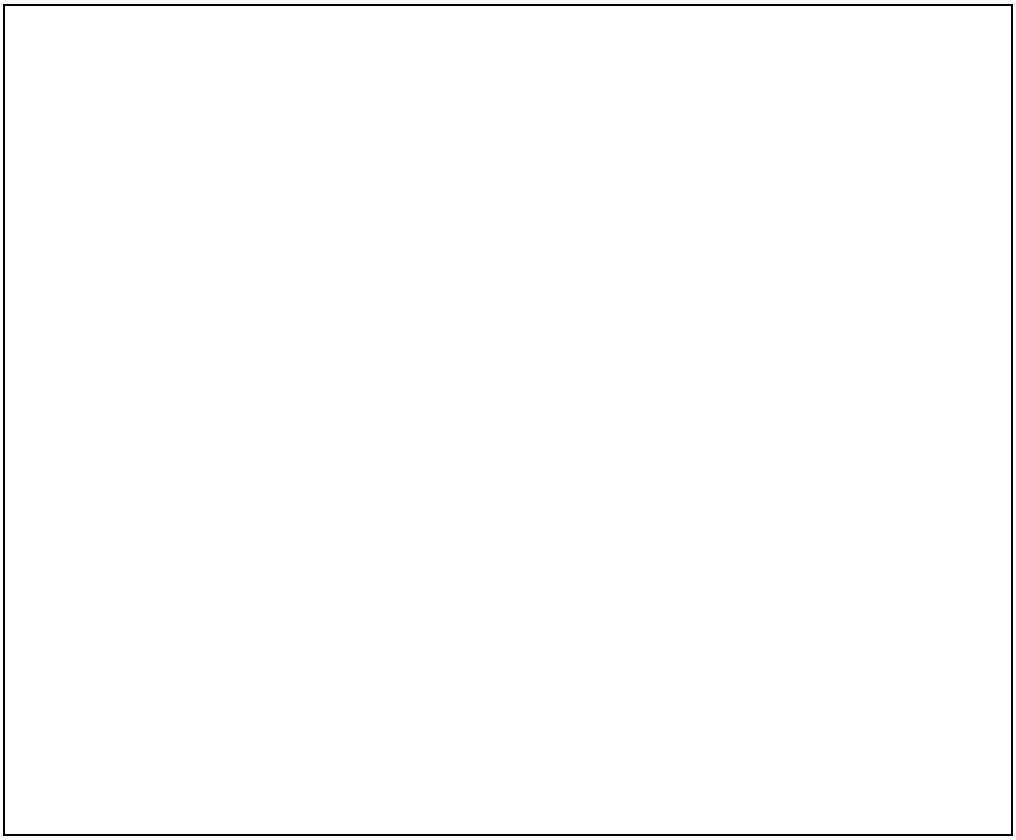

Fig. 1. Proposed mechanism organizing the cytoskeleton of resorbing osteoclasts. 1). M-CSF occupying its receptor, c-fms, stimulates inside-out αvβ3 activation by inducing talin association with the β3 cytoplasmic domain that binds c-src constitutively. 2). Clustering of the integrin by RGD ligand increases avidity as well as affinity by outside-in activation. The liganded integrin activates c-src as evidenced by Y416 phosphorylation. Activated c-src tyrosine phosphorylates ITAM proteins that recruit Syk to the integrin by binding Syk-SH2 domains. c-src activates β3-associated Syk that phosphorylates Vav3 in the context of SLP-76. Vav3 then shuttles Rac-GDP to its activated GTP-associated state. 3). Rac-GTP prompts association of lysosome-derived secretory vesicles with microtubules (MTs) that deliver them to the bone-apposed plasma membrane into which they insert under the influence of Syt VII and LC3. Rac-GTP and MTs also promote sealing zone (SZ) formation. Secretory vesicle fusion focally expands the plasma membrane forming the ruffled border and eventuating in discharge of cathepsin K (CTK) and HCl into the resorptive microenvironment.

recruitment due to inhibited precursor proliferation and accelerated apoptosis of the mature polykaryon (63).

M-CSF Helps αv/β3

RANK ligand (RANKL) and M-CSF are the requisite osteoclastogenic cytokines but each also promotes the resorptive activity of the mature polykaryon. In the case of M-CSF, the cytokine interacting with its receptor, c-fms, stimulates a signaling pathway remarkably similar to that induced by αv/β3, thereby organizing the cytoskeleton (37;58;64). The means by which M-CSF structures the osteoclast cytoskeleton may, therefore, be independent of the integrin, or alternatively, represent inside-out αv/β3 activation. In fact, M-CSF transits αv/β3 from its default low affinity to its high affinity conformation by inducing talin binding to the β3 cytoplasmic domain (39). Absence of talin, in osteoclast precursors, does not arrest differentiation but blocks substrate adherence and motility. The impaired function of talin-deficient osteoclasts results in a 5-fold increase in the bone mass of mutant mice. Interestingly, the osteopetrotic phenotype of mice with *talin* (-/-) osteoclasts is more severe than of those

lacking αv/β3, which likely represents arrest of compensatory integrins, particularly those bearing β1 (30;35).

Conclusion

The magnitude of bone resorption reflects osteoclast number and function of the individual cell, the latter dependent upon cytoskeletal organization. The osteoclast cytoskeleton is a unique structure whose conversion to its active state depends upon contact with mineralized matrix (Fig. 1). These extracellular signals, which polarize the resorptive machinery to the bone-cell interface, are transmitted intracellularly by integrins dominated by αv/β3. In conjunction with M-CSF-stimulated inside-out activation, a canonical signaling pathway emanates from the αv/β3 integrin. Occupancy of the heterodimer phosphorylates constitutively associated c-src which in turn targets Dap 12. The ITAM's phosphotyrosines serve to recruit Syk to the β3 cytoplasmic domain where it is also phosphorylated by c-src. Utilizing SLP-76, Syk activates Vav3, eventuating in formation of Rac-GTP and organization of the resorptive cytoskeleton. Given current concerns regarding long-acting anti-resorptive agents, such as bisphosphonates, short-acting counterparts are in demand. Delineating the molecular mechanism by which osteoclasts organize their cytoskeleton to degrade bone has provided an array of candidate therapeutic targets.

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