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Role of actin filaments in fusopod formation and osteoclastogenesis

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ABSTRACT

Cell fusion process is a critical, rate-limiting step in osteoclastogenesis but the mechanisms that regulate fusopod formation are not defined. We characterized fusopod generation in cultured pre-osteoclasts derived from cells stably transfected with a plasmid that expressed a short, actin filament binding peptide (Lifeact) fused to mEGFP that enables localization of actin filaments in living cells. Fusion was initiated at fusopods, which are cell extensions of width >2 μm and that are immunostained for myosin-X at the extension tips. Fusopods formed at the leading edge of larger migrating cells and from the tail of adjacent smaller cells, both of which migrated in the same direction. Staining for DC-STAMP was circumferential and did not localize to cell–cell fusion sites. Compared with wild-type cells, monocytes null for Rac1 exhibited 6-fold fewer fusopods and formed 4-fold fewer multinucleated osteoclasts. From time-lapse images we found that fusion was temporally related to the formation of coherent and spatially isolated bands of actin filaments that originated in cell bodies and extended into the fusopods. These bands of actin filaments were involved in cell fusion after approaching cells formed initial contacts. We conclude that the formation of fusopods is regulated by Rac1 to initiate intercellular contact during osteoclastogenesis. This step is followed by the tightly regulated assembly of bands of actin filaments in fusopods, which lead to closure of the intercellular gap and finally, cell fusion. These novel, actin-dependent processes are important for fusion processes in osteoclastogenesis.

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1. Introduction

Multinucleated osteoclasts (OCs) are specialized, short-lived cells that are essential for bone remodeling and the maintenance of normal bone architecture and function. OCs are terminally differentiated cells that form by the fusion of pre-osteoclasts (preOCs) [1]. PreOCs are tartrate-resistant acid phosphatase (TRAcP)-positive mononuclear cells derived from monocyte/macrophage-lineage precursor cells that form in bony microenvironments or in specialized cell culture conditions.

Osteoclast differentiation is thought to occur as a result of three sequential and inter-connected steps. First, cells become fusion-competent, which is initiated by a combination of the growth factor, macrophage colony stimulating factor (M-CSF; produced by monocytes, granulocytes, endothelial cells and fibroblasts) and by the receptor activator of nuclear factor kappa B ligand (RANKL [2]; produced by stromal cells, osteoblasts and T-cells). PreOCs are thought to secrete chemokines and related molecules that attract neighboring preOCs and facilitate aggregation prior to the actual fusion process [3]. Although preOCs express OC-specific markers (e.g. TRAcP) and exhibit bone-resorbing activity, bone-resorption by TRAcP-positive mononuclear cells is much slower than resorption mediated by mature OCs [4,5]. Indeed, the formation of OCs is indispensable for bone resorption since the efficiency of this process is directly proportional to the size of OCs and the number of nuclei [6–8]. The next step of OC differentiation includes the migration, aggregation and intercellular attachment of fusion-competent cells as a result of the apposition of their plasma membranes. The final step of OC differentiation is the fusion of plasma membranes of adjacent preOCs and the comingling of their cytoplasmic contents to create a new functional and unified cellular entity [9]. Immature OCs display dendritic extensions that are comprised of numerous filopodia. As these cells progressively undergo fusion events with other immature multinuclear OCs or mononuclear preOCs, they form large, mature OCs with smooth-edged plasma membranes [10].

While the differentiation of myeloid precursor cells into OCs has been examined in depth, the mechanisms that mediate the fusion of preOCs are not as well-defined. Cell fusion is thought to be a programmed process [11] that ensures temporally-appropriate OC formation. Although the migration and aggregation of preOCs are essential for the formation of multinucleated OCs, the close apposition or actual contact of adjacent preOCs does not necessarily lead to cell fusion.

Abbreviations: OC, osteoclast; preOC, pre-osteoclast; BMM, bone marrow monocyte; mEGFP, monomeric enhanced green fluorescent protein; M-CSF, macrophage colony stimulating factor; sRANKL, soluble receptor activator of nuclear factor kappa B ligand; TRAcP, tartrate-resistant acid phosphatase; DC-STAMP, dendritic cell-specific transmembrane protein

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Instead, cell–cell fusion appears to be a highly selective process that is not based on the proximity of cell membranes alone [10]. In spite of this observation, the study of preOC fusion has largely focused on the identification of putative membrane fusion receptors. The macrophage fusion receptor (also known as SIRPα), CD47 [12], CD44 [13] and E-cadherin [14] have all been implicated as fusion receptors. Recent work has focused on the dendritic cell-specific trans-membrane protein (DC-STAMP) [15–17], which is essential for the fusion of preOCs [18]. DC-STAMP-deficient mice do not form multinucleated OCs and exhibit an osteopetrotic phenotype, which is not caused by defective cell differentiation, but appears to arise solely because of a preOC fusion defect.

The critical molecules involved in the recruitment of preOCs, their transition into fusion-competent cells, the intercellular recognition systems, and their attachment and fusion have been reviewed [19]. However, attempts to define the regulatory mechanisms involved in preOC fusion that lead to OC formation have been complicated by the nature of cell fusion. The actual fusion process is brief so the molecular events that regulate fusion are visible only transiently at the outset of the fusion process [12]. Notably, cytoskeletal polymers contribute extensively to cell shape and organization [20] and the regulation of cytoskeletal proteins in the function of mature OC has been studied extensively [21]. In particular, the actin cytoskeleton plays an important role in preOC migration and polarization [22].

Actin filaments are especially important for the formation of podosomes in preOCs, which are primary adhesive structures that contribute to the sealing zones of actively resorbing cells [23–25]. In preOCs, actin-associated force generation is coordinated with podosome assembly and disassembly, which facilitate cell migration [26]. Actin filament assembly is also involved in the formation of cell extensions such as filopodia and lamellipodia, mechanosensory organelles at the leading edge that initiate and guide cell migration. The formation of actin filament-enriched cell extensions and the direct interdigitation of the plasma membranes of adjacent fusing cells have been observed in the formation of giant cells induced by IL-4 [27,28]. While limited information is available on how the actin cytoskeleton is regulated during osteoclastogenesis, especially in OC fusion [10], it is known that macrophage and syncytia fusion is disrupted by cytochalasins and latrunculin, thereby implicating actin assembly in cell fusion [29,30]. Despite recent reports that associated filopodia with the fusion of preOCs [10,31], the involvement of actin assembly in the formation of OC has not been completely defined. Accordingly, to examine the role of actin filaments in preOC fusion, we established a stable Lifeact–mEGFP-transfected RAW264.7 cell line in which recombinant soluble RANKL (sRANKL) is used to drive cell fusion. With this cell line and direct, real-time microscopic imaging of OC fusion, we found that filopodial-enriched cell extensions, designated here as fusopods, mediate preOC fusion, which leads to the formation of multinucleated OCs.

2. Materials and methods

2.1. Establishment of stable RAW264.7 cells expressing Lifeact–mEGFP

As described previously [32], pmEGFP–N1–Lifeact from Roland Wedlich-Soldner was amplified with a primer pair 5′-GCGCAGATCTATGGGTGTCGCAGATTTGATCAAGAAA-3′ and 5′-GCGCGAATTCCTATTACTTGTACAGCTCGTCCATGCCGAG-3′. The peptide sequence MGVADLIKKFESISKEE, was translated from 5′-ATGGGTGTCGCAGATTTGATCAAGAAATTCGAAAGCATCTCAAAGGAAGAA-3′. The resulting PCR product was digested with BglII and EcoRI and ligated into the corresponding sites of a retrovirus vector pMSCVpuro (Clontech). The construct (pMSCVpuro–Lifeact–mEGFP) and the packaging plasmid pVSV-G (Clontech) were co-transfected into GP-293 cells (Clontech) with FuGENE® HD Transfection Reagent (Promega, Madison, WI). The resulting viruses were transduced into RAW264.7 cells (passage 3) (ATCC, Manassas, VA; Cat. no. TIB-71; generously provided by Keying Li and Morris Manolson at the Faculty of Dentistry, University of Toronto). The newly-received cells from ATCC are designated here as passage 1 [33]. Cells were maintained in complete Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Paisley, UK; Ref. no. 11995; supplemented with 10% fetal bovine serum and 164 IU/mL of penicillin G, 50 mg/mL of gentamicin, and 0.25 mg/mL of fungizone). Individual puromycin-resistant, Lifeact–mEGFP-positive cell clones were picked by standard limiting dilution when cultured in the presence of puromycin (final concentration 7 μg/mL; Sigma-Aldrich, St. Louis, MO). A cell line (RAW264.7-H10) with high potential to differentiate into OCs was used in the experiments.

2.2. shRNA targeting Rac1 by pSIREN-RetroQ-DsRed-Express

Oligos Rac1shRNA top strand 5′-GATCCAGACAGACGTGTTCTTAATTTGTTCAAGAGACAAATTAAGAACACGTCTGTCTTTTTTTACGCGTG-3′ and bottom strand 5′-AATTCACGCGTAAAAAAAGACAGACGTGTTCTTAATTTGTCTCTTGAACAAATTAAGAACACGTCTGTCTG-3′ were annealed and ligated into pSIREN-RetroQ-DsRed-Express (Clontech, Mountain View, CA; Cat. no. 632487). The resulting vector and the packaging plasmid pVSV-G (Clontech) were co-transfected into GP-293 cells (Clontech) with FuGENE® HD Transfection Reagent. Retrovirus delivered shRNA targeting mouse Rac1 (underlined sequence) [34] was prepared for transfections and was used to knock down Rac1 in RAW264.7-H10 cells. A sense-only insert (top strand 5′-GATCCGTGCGTTGCTAGTACCAACTTCAAGAGATTTTTTACGCGTG-3′, bottom strand 5′-AATTCACGCGTAAAAAATCTCTTGAAGTTGGTACTAGCAACGCACG-3′) in the same vector containing the sense strand of the targeted luciferase (underlined sequence) was used as the negative control. DsRed-positive cell clones were established with a standard limiting dilution method.

2.3. Tartrate-resistant acid phosphatase, actin staining and osteoclast resorption

TRAcP staining was performed as described [35]. Paraformaldehyde (PFA, 4%)-fixed cells were rinsed twice with phosphate-buffered saline (PBS) and TRAcP activity was demonstrated by incubating cells in a solution of naphthol AS-BI phosphate (Sigma) and fast red TR salt (Sigma) in 0.2 M acetate buffer (pH 5.2) containing 100 mM sodium tartrate (Sigma) at 37 °C for 10 to 15 min. TRAcP stained cells were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma, Cat. no. D9542; 0.165 μM in 0.1% Triton X-100/PBS) for 10 min at room temperature (RT) in dark to determine the number of nuclei. Images were obtained with Nikon Eclipse E1000 microscope.

For staining actin filaments, cells were fixed, rinsed with PBS, incubated with 100 mM glycine in Tris-buffered saline (TBS) (pH 7.5, quenching buffer; 10 min), permeabilized in 0.1% Triton X-100 (in PBS; 3–5 min), rinsed with PBS and stained with tetramethylrhodamine (TRITC)-conjugated phalloidin (Molecular Probes, Eugene, OR) and imaged by confocal microscopy. For resorption assays, OCs formed on dentine sections of narwhal tusks were removed using 6–10% bleach (NaOCl) for 5 min. The slices were stained with 1% toluidine blue (*w*/*v*) and 1% sodium borate (*w*/*v*) for 30 s.

2.4. Apatite-coated coverslips

Apatite-coated coverslips (ACCs) were prepared as described [25,36]. Briefly, circular glass coverslips (25-mm diameter, VWR, Toronto, ON) were coated with type I collagen (1 mg/mL) and incubated for 6 days at 37 °C in 3 mL of 200 mM TBS containing alkaline phosphatase (0.13 mg/mL, Sigma), egg yolk phosvitin (0.13 mg/mL, Sigma), and the cross-linking reagent dimethyl suberimidate hydrochloride (1 mg/mL, Sigma) in 6-well plates. The cross-linked, collagen-coated glass slides were washed several times with TBS to remove unreacted chemicals and by-products, incubated for 3 h at 37 °C in 3 mL of 200 mM TBS containing alkaline phosphatase (0.13 mg/mL) and egg yolk phosvitin (0.13 mg/mL), and incubated for 20 h at 37 °C in 3 mL of 6 mM calcium

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