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Odontogenic Ameloblast-Associated Protein (Odam) Plays Crucial Roles in Osteoclast Differentiation via Control of Actin Ring Formation

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Purpose: In osteoclast differentiation, actin-rich membrane protrusions play a crucial role in cell adhesion. Odontogenic ameloblast-associated protein (Odam) contributes to cell adhesion by inducing actin rearrangement. Odam-mediated RhoA activity may play a significant role in multinucleation of osteoclasts. However, the precise function of Odam in osteoclast cell adhesion and differentiation remains largely unknown. Here, we identify a critical role for Odam in inducing osteoclast adhesion and differentiation.

Materials and Methods: The expression of Odam in osteoclasts was evaluated by immunohistochemistry. Primary mouse bone marrow and RAW264.7 cells were used to test the cell adhesion and actin ring formation induced by Odam.

Result: Odam was expressed in osteoclasts around alveolar bone. *Odam* transfection induced actin filament rearrangement and cell adhesion compared with the control or collagen groups. Overexpression of *Odam* promoted actin stress fiber remodeling and cell adhesion, resulting in increased osteoclast fusion.

Conclusion: These results suggest that Odam expression in primary mouse osteoclasts and RAW264.7 cells promotes their adhesion, resulting in the induction of osteoclast differentiation.

Key Words: Cell adhesion; Odam; Osteoclasts; RhoA

Introduction

Osteoclasts are large multinucleated cells derived from hematopoietic precursors of the monocyte-macrophage lineage. Osteoclast differentiation is controlled by responses to macrophage colony-

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stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL)[1,2]. The continuous adhesion of osteoclasts to mineralized bone surfaces influences their resorptive activity. With the initiation of bone resorption, mature osteoclasts polarize and undergo intense morphological changes such as formation of a dense belt-like F-actin structure surrounding the resorptive cavity, called the sealing zone or the ruffled border[3,4]. Osteoclast function depends on the dynamic regulation of the actin cytoskeleton to accomplish ordered cycles of movement and the Rho signaling pathway during bone resorption to modulate attachment[5-7]. Regulation in actin cytoskeleton assembly/disassembly allows for osteoclast migration, adhesion, and bone resorption[7]. Odontogenic ameloblast-associated protein (Odam) could regulate F-actin formation and rearrangement via the RhoA signaling pathway[8]. RhoA is important for controlling the actin cytoskeleton[9]. Because cell shape is regulated by reorganization of the actin cytoskeleton[10], we focused our investigations on changes of actin filament structures induced by Odam in the multinucleation process during osteoclastogenesis.

Odam has known functions in enamel formation, maturation, regeneration of the junctional epithelium, and tumorigegnesis[11-14]. Odam plays roles in enamel mineralization during amelogenesis[11,15,16]. In a previous paper, we found that Odam induces RhoA activity and the expression of downstream factors by interacting with Rho guanine nucleotide exchange factor 5 (Arhgef5). Odam-RhoA signaling causes actin filament rearrangement and activation of cell adhesion[8]. Although Odam plays roles in the organization of the actin ring[6,17], distinct pathways of Odam and related signaling molecules involved in actin ring formation remain unknown. Hence, in the present study, we evaluated the function of Odam with RhoA in osteoclasts.

Materials and Methods

1. Antibodies

Anti-Odam antibody was generated in rabbits by immunization with Odam peptides[16]. Alexa Fluor® 488 Phalloidin (rhodamine phalloidin) antibody was obtained from Invitrogen (Carlsbad, CA, USA). Anti-fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody was purchased from Life Technology (Grand Island, NY, USA).

2. Plasmids, Cloning, and Recombinant Protein (rOdam)

All cDNAs were constructed according to standard methods and verified by sequencing. The cDNAs of the Odam full length or deletion mutants were amplified by PCR and subcloned into Flag-tagged pcDNA3 (Invitrogen). For His-fused Odam proteins, pRSET-A (Invitrogen) was used. The Escherichia coli strain, BL21 (DE3) pLysS, was transformed with pRSET-Odam and cultured at 37°C in Luria-Bertani (LB) broth. The protein was extracted and purified from the cell lysates.

3. Tissue Preparation and Immunohistochemistry

All animal experiments were performed according to the Dental Research Institute guidelines of Seoul National University (SNU-111013-3). Mice teeth were decalcified in 10% EDTA (pH 7.4), embedded in paraffin, and processed for immunohistochemistry. Sections were incubated overnight at 4°C with primary antibodies (dilutions of 1:100 to 1:200). Secondary anti-rabbit immunoglobulin G (IgG) antibody was added to the sections for 30 minutes at room temperature, followed by a reaction with the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Signals were converted using a diaminobenzidine kit (Vector Laboratories). Nuclei were stained with hematoxylin.

4. Cell Culture and Transient Transfection

RAW264.7 macrophage cells (ATCC), which are a macrophage-like cell line derived from Balb/c mice, were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics in a 5% CO_2 atmosphere at 37°C. RAW264.7 cells were seeded on slides coated with rOdam or collagen (0.05%; Roche Molecular Biochemicals, Mannheim, Germany). For transfection, RAW264.7 cells were seeded on culture plates. Cells were transiently transfected with reporter constructs using Metafectene PRO reagent (Biontex, Planegg, Germany).

5. Fluorescence Microscopy

Cells on slides were washed with phosphate buffered saline (PBS), fixed for 10 minutes at room temperature with 4% paraformaldehyde in PBS, and permeabilized for 5 minutes in PBS containing 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). After washing and blocking, cells were incubated for 1 hour with anti-Odam (1:200) and Alexa Fluor® 488 Phalloidin antibodies in blocking buffer (PBS and 1% bovine serum albumin), followed by the addition of FITC-conjugated anti-rabbit goat IgG antibody (1:200). After washing, cells were visualized using fluorescence microscopy (AX70; Olympus Optical Co., Tokyo, Japan). Chromosomal DNA in the nucleus was stained using DAPI.

6. Adhesion Assay

The cells expressing *Odam* were seeded in 96-well plates and incubated for 4 hours. At the indicated times, plates were washed twice with PBS. Cells were fixed with 4% paraformaldehyde for 30 minutes, stained with crystal violet for 10 minutes, and permeabilized with the addition of Tween 20 for 30 minutes. Finally, we measured optical density at 595 nm. All quantitative data are presented as the mean± standard deviation. Statistical differences were analyzed using Student's t-tests (*$P<0.05$). All statistical analyses were performed using IBM SPSS Statistics software version 19.0 (IBM Co., Armonk, NY, USA).

7. Osteoclast Differentiation Assay

Bone marrow cells and primary osteoblasts (1×10^5 cells/well) were co-cultured in 12-well tissue culture plates for 5 days in α-MEM complete medium (Gibco BRL, Carlsbad, NY, USA) with RANKL and M-CSF (R&D Systems, Minneapolis, MN, USA). The cultures were replenished with fresh medium containing the same supplements every 2 days until the appearance of osteoclasts. At the end of the culture period, cells were fixed in 10% formalin for 10 minutes, permeabilized with 0.1% Triton X-100, and then stained for tartrate-resistant acid phosphatase activity with the leukocyte acid phosphatase assay kit (Sigma-Aldrich).

Result

1. Expression of Odam in Developing Alveolar Bone

Odam is expressed in ameloblasts, odontoblasts,

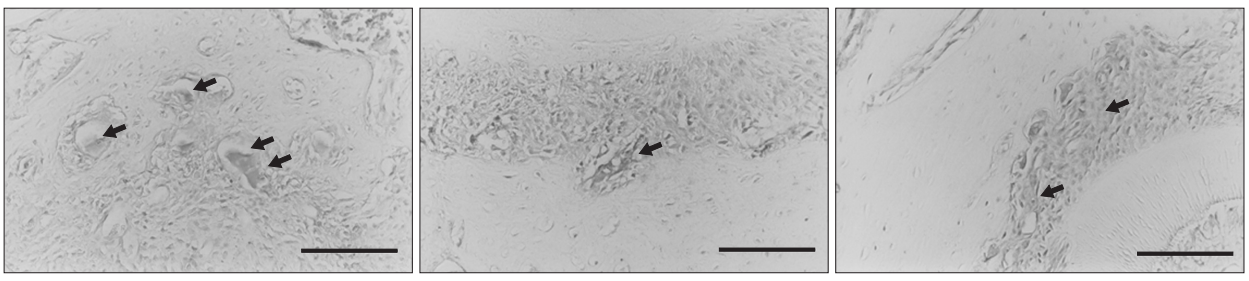

Fig. 1. The expression of Odam was detected in multinucleated osteoclasts (arrows) by immunohistochemistry in developing mouse alveolar bone at postnatal day 20. Scale bars=100 μm.

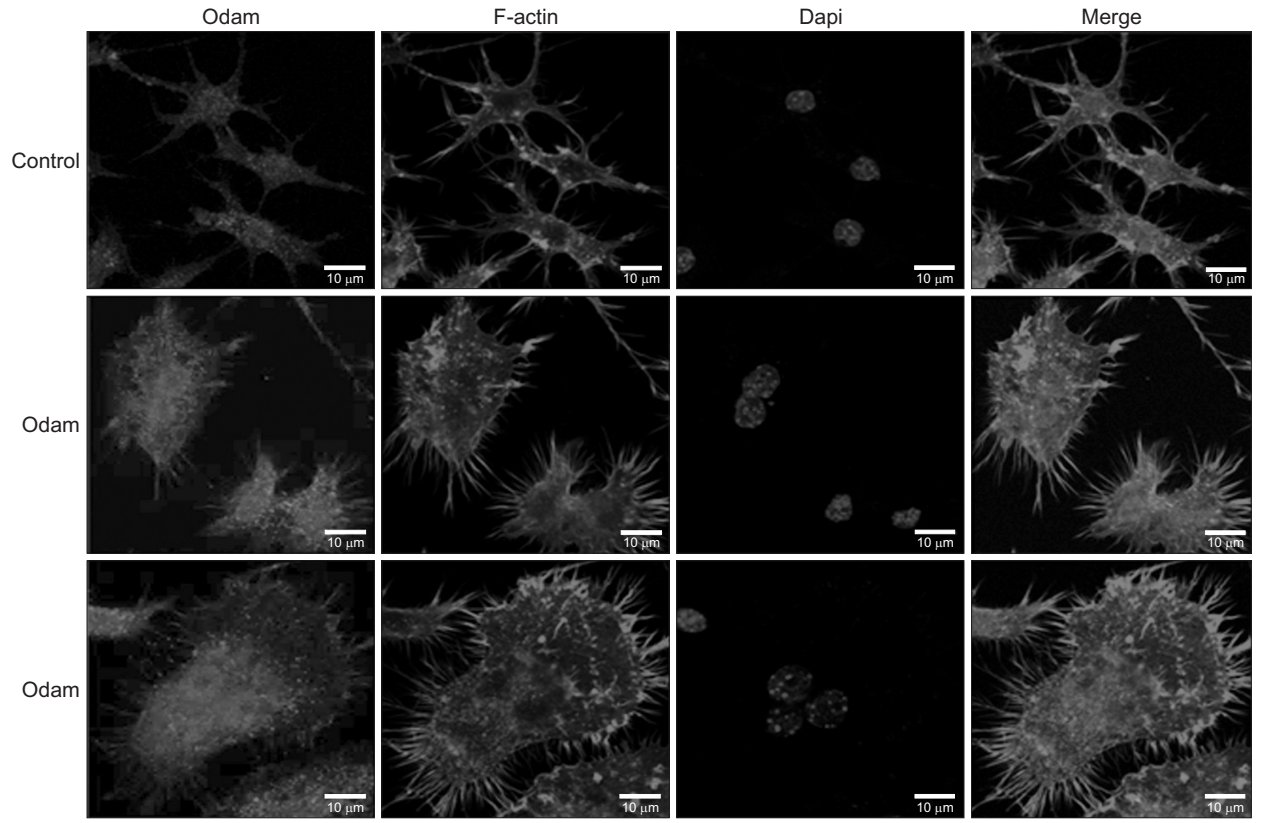

Fig. 2. Odam induced actin rearrangement in osteoclasts. RAW 264.7 cells, which were transfected with *Odam* for 24 hours, were examined by immunofluorescence staining of Odam and F-actin. Scale bars=10 μm.

osteoblasts, and various cancer cells[14]. We further investigated Odam expression in osteoclasts by immunohistochemistry. Multinucleated osteoclasts were seen adjacent to the alveolar bone surface. Odam expression was strongly detected in osteoclasts from alveolar bone (Fig. 1).

2. Odam-Induced Cytoskeleton Reorganization in Osteoclasts

Our previous paper shows that Odam induces actin filament rearrangement and enhances cell adhesion[8]. Here, we addressed whether increasing concentrations of Odam induce sealing ring formation in osteoclasts. Osteoclasts transfected with *Odam* showed sealing ring formation when stained with rhodamine phalloidin. When *Odam* was transfected in RAW264.7 cells, Odam induced changes of cell shape, actin rearrangement, and actin ring formation compared with normal cells (Fig. 2).

3. The Induction of Cell Adhesion by Odam Signaling

Actin rearrangement results in changes in cell shape, adhesion, motility, and differentiation[18]. The actin cytoskeleton is continuously reorganized through the RhoA signaling pathway during osteoclastogenesis[5-7]. We investigated whether Odam could affect the adhesion of osteoclasts to the substrate through an adhesion assay. When osteoclast cells were cultured on rOdam-coated dishes, sealing ring formation with multinuclear cells was either induced or observed in osteoclasts transduced with Odam (Fig. 3). These results suggest that Odam signaling promoted cell adhesion with actin filament rearrangement at the cell periphery of osteoclasts.

4. Role of Odam during Actin Ring Formation and Osteoclastogenesis In Vitro

To investigate the effect of Odam expression

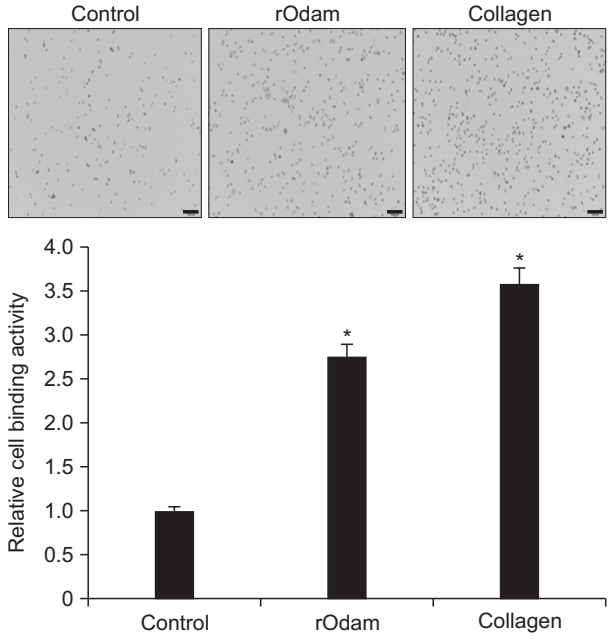

Fig. 3. Adhesion of RAW 264.7 cells to rOdam- or collagen-coated slides (methyl green staining, ×100). Binding values are based on the absorbance of adherent cells. Data are presented as the mean±standard deviation of triplicate experiments. Asterisks denote values significantly different from control and collagen-coated group (P<0.05).

on osteoclast differentiation of bone marrow stromal cells, we incubated cells in osteoclastic differentiation media with RANKL and M-CSF for 5 days. Odam localized in the actin ring (Fig. 4A). We next examined the possibility that Odam activates osteoclast differentiation by directly initiating the actin-ring formation. In the presence of M-CSF and RANKL, Odam induced osteoclast differentiation of bone marrow cells in a dose-dependent manner (Fig. 4B). These results demonstrate that Odam may be involved in the formation and change of the actin ring in osteoclasts.

Discussion

Resorption entails a complicated interaction among inflammatory cells, resorbing cells, and hard tissue structures. Injuries of bone, dentin, and cementum bring about changes of these tissues with resultant formation of multinucleated giant cells, osteoclast cells. These osteoclast cells are responsible

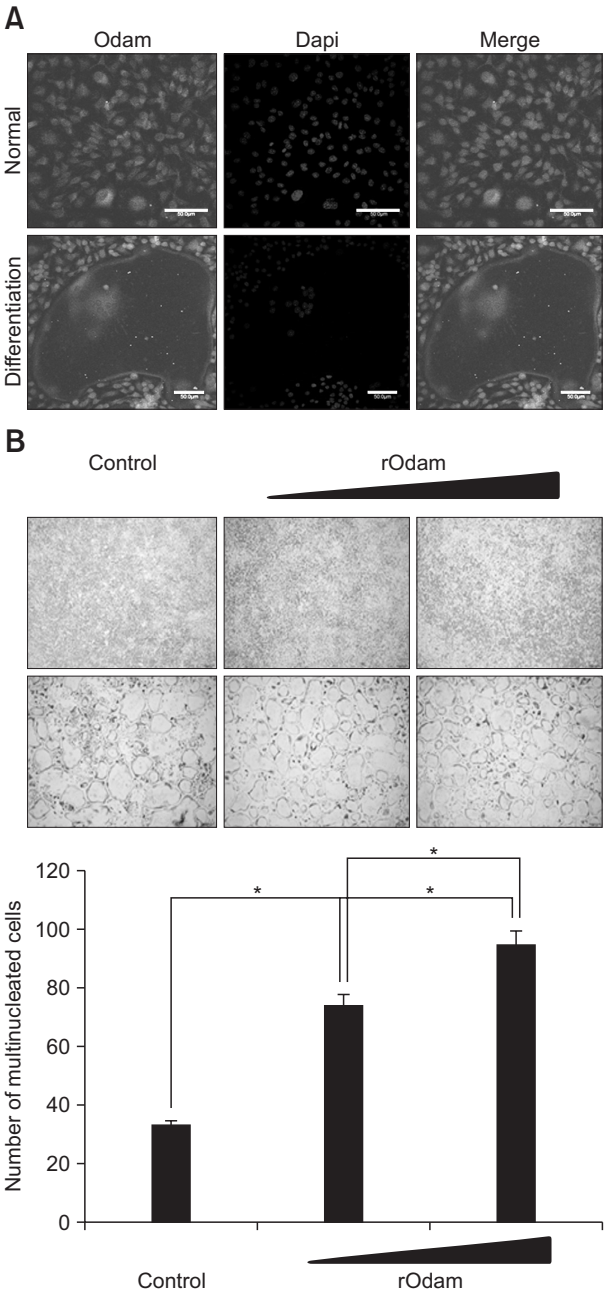

Fig. 4. Effects of Odam on osteoclast differentiation. (A) Normal and differentiated osteoclasts were stained with anti-Odam antibody. Scale bars=50 μm. (B) Bone marrow cells treated with rOdam were cultured in the presence of macrophage colony-stimulating factor (10 ng/ml) with or without nuclear factor kappa B ligand (100 ng/ml) for 4 days, fixed, and stained for tartrate-resistant acid phosphatase (scale bars=20 μm). Multinucleated cells were counted. Data are presented as mean±standard deviation of triplicate experiments. Asterisks denote values significantly different from the control group (P<0.05).

for all hard tissue resorptive processes[19]. The molecular biology underlying bone and tooth resorption is an active area of investigation[20]. In this paper, we show for the first time that Odam is a regulator of osteoclast differentiation. Furthermore, we present a novel osteogenesis mechanism by Odam.

Osteoclasts have a unique cytoskeleton, which is required for bone resorption, and a multistep process including migration, polarization, and adhesion. Upon contacting bone, osteoclasts form ruffled borders and actin rings[21]. The appearance of osteoclasts is the requisite first step in orthodontic tooth movement because orthodontic treatment induces site-specific bone resorption and formation of cytokines released from periodontal ligament (PDL) cells[22,23]. Osteoclast precursors migrate to the periodontal space via the blood circulation and differentiate into mature osteoclasts, which resorb alveolar bone under the stimulation of PDL-derived cytokines[24]. RANKL, which is a significant component of osteoclastogenesis, is expressed by odontoblasts, pulp, PDL fibroblasts, and cementoblasts[25,26]. Therefore, PDL cells likely play a pivotal role in osteoclast formation during orthodontic tooth movement. However, there is limited evidence for a role of actin regulatory proteins in osteoclast development near the PDL[20]. In this study, we show that Odam regulates F-actin rearrangement and thus cell adhesion[8], which suggests that it participates in bone resorption. We tested this hypothesis by analyzing the differentiation and function of osteoclasts that overexpressed Odam. Thereby, we identified Odam as an important regulator of osteoclast function.

Epithelial cell rests of Malassez (ERM) may play an important functional role in preserving normal periodontal cellular elements, inducing acellular cementum formation, maintaining periodontal membrane space, and preventing ankylosis[27]. ERM have been reported to express extracellular matrix proteins, growth factors, and cytokeratins, suggesting that these cells may also contribute to periodontal regeneration[28]. The disruption of the extracellular matrix and the inflammatory response of the PDL by gingivectomy and tooth movement may contribute to the activation of ERM. Odam expression is induced in activated ERM[29,30]. However, the mechanism of activation for Odam expression in ERM is not known. A significant increase in fibronectin expression is found in human PDL with inflammation and after orthodontic tooth movement compared with the normal condition[31-34]. ERM may also be associated with cementoblast development. During the early stage of cementum repair, ERM express Odam near resorption lacunae[29,30,35]. Our previous results demonstrate that fibronectin mediates integrin-Odam-Arhgef5-RhoA signaling, causing epithelial cell attachment to the matrix through actin reorganization[8]. Odam-mediated actin reorganization was also seen in the podosome of osteoclasts, which function on mineralized resorption lacunae. Thus, our current hypothesis is that Odam expression in ERM is induced by fibronectin during periodontal repair and mediates ERM adhesion to resorption lacunae to differentiate into cementoblasts.

Conclusion

In this study, Odam was investigated in osteoclasts *in vivo* and *in vitro*. Odam-mediated signaling resulted in actin filament rearrangement in osteoclasts. Odam played a critical role in inducing osteoclast adhesion and differentiation. These results suggest that Odam expression maintains cell adhesion, resulting in the induction of differentiation into osteoclasts.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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