Laboratory Investigations

The Integrin $\alpha_v\beta_3$ and CD44 Regulate the Actions of Osteopontin on Osteoclast Motility

M. A. Chellaiah, 1 K. A. Hruska 2

1Department of Oral & Craniofacial Biological Sciences, University of Maryland, Baltimore, MD 21201, USA
2Department of Pediatrics, Washington University School of Medicine, St. Louis, MO 63110, USA

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Abstract. In the studies reported here we demonstrate that osteopontin is secreted from the basolateral surfaces of osteoclasts where it binds to the $\alpha_v\beta_3$-integrin, suggesting that it may be an autocrine factor. Osteopontin stimulation of osteoclasts produced changes in cell shape by causing disruption of peripheral podosome structures and formation of actin filaments at the leading edge of the migrating osteoclasts. The latter was part of the assumption of a motile phenotype prior to cells reforming peripheral ring type podosome containing clear zones. It is well established in our laboratory as well as in others that osteopontin stimulated osteoclast motility and bone resorption. The effect of osteopontin was mimicked by RGD containing peptides and blocked by a $\alpha_v\beta_3$ antibody, demonstrating that signals generated by integrin ligation contributed to the actions of osteopontin. In addition, the migratory effects of osteopontin on osteoclasts were also mediated through CD44 receptors since blocking antibodies to CD44 blocked stimulation of motility. Our data strongly suggest that osteopontin is an osteoclast autocrine motility factor binding to $\alpha_v\beta_3$ and CD44 during stimulation of osteoclast migration.

Key words: Osteoclasts — Osteopontin — Integrin $\alpha_v\beta_3$ — CD44 — Motility

Osteoclasts are multinucleated giant cells with bone-resorbing activity. Bone resorption appears to proceed by the intricate coordination of the processes of attachment to bone, polarized secretion of acid and proteases, and active motility of osteoclasts along the bone surface [1–3]. As osteoclasts crawl over bone surfaces they require rapid attachment and release from the extracellular matrix [4–6]. The adhesion structures, podosomes, are uniquely designed for this purpose. After osteoclasts attach to the bone surface, podosomes develop highly polarized cytoplasmic organization in their ruffled borders and clear zones. The clear zone, which surrounds the ruffled border, seals the extracellular resorbing compartment in which bone resorption takes place [7]. Actin rings in the clear zone represent specialized mechanisms of adherence to bone surfaces during resorption.

The adhesion of osteoclasts through podosomes involves interaction of cell surface receptors, integrins, especially $\alpha_v\beta_3$, with extracellular bone matrix proteins. Osteoclast $\alpha_v\beta_3$ recognizes several bone matrix proteins containing the Arg-Gly-Asp (RGD) sequence, osteopontin (OPN), bone sialoprotein (BSP), and vitronectin (VN) [8–10]. Osteopontin, an RGD-containing bone matrix protein [11–13] plays a key role in anchoring osteoclasts to bone surface. A large quantity of osteopontin secretion by osteoclasts [14] suggests additional roles for OPN besides its function as an anchorage protein. We have demonstrated that OPN, as a soluble protein, stimulates signal transduction in osteoclasts, including activation of c-src and phosphatidylinositol 3-hydroxyl kinase (PI3-kinase) [15, 16]. OPN binding to integrin $\alpha_v\beta_3$ in osteoclasts stimulates gelsolin-associated phosphatidylinositol kinases, leading to increased levels of gelsolin-associated polyphosphoinositides such as PtdIns 4,5-P2, PtdIns 3,4-P2 and PtdIns 3,4,5-P3; uncapping of actin barbed ends, stimulation of actin polymerization, and an increase in F-actin levels [15]. OPN binding to the $\alpha_v\beta_3$ integrin of osteoclast podosomes stimulated cytoskeletal reorganization and bone resorption by activating a heteromultimeric signaling complex, which includes gelsolin, src, and PI3-kinase [17, 18].

The interaction of OPN with the $\alpha_v\beta_3$ integrin through its RGD domain has been widely studied in cell adhesion [8–11] but the OPN domains involved in chemotaxis are less well defined. They may also involve the RGD sequences since cell movement involves an organized process of detachment from the matrix and reattachment [19]. According to the report by Weber et al.
Rhodamine phalloidin coverslips were mounted on a mounting solution (Vector Laboratories, Inc., Burlingham, CA). Rhodamine phalloidin coverslips were immersed in 47.5% ethanol containing 5 mM EGTA for 20 min at 37°C. The coverslips were then stained with rhodamine phalloidin (1:20 dilution) for 15 min at room temperature and rinsed with several changes of PBS-EGTA. The coverslips were then stained with rhodamine phalloidin and permeabilized with 0.1% Triton X-100 for 1 min. The cells were washed and incubated with antibodies to αvβ3 and OPN (1:100 dilution) for 2 hours, washed, and counter-stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG. Actin staining of the cells stained for αvβ3 was performed using rhodamine phalloidin (1:20 dilution) as described above. The cells were washed and mounted on a slide in a mounting solution (Vector laboratories, Inc., Burlingham, CA) and viewed on a Zeiss LSM 410 confocal laser-scanning microscope (Thornwood, NY), and photomicrographs were developed [22]. CY2 and CY3 images were recorded using the 488 nm and 568 argon excitation lines respectively. CY2 and CY3-labeled proteins were imaged with different backgrounds [23]. The background was removed and a pseudocolor (red) was assigned to the cell by the reflected light (Figs. 3, 4, 5). Images were stored in TIF image format and processed by the Adobe Photoshop software program (Adobe Systems, Inc., Mountain View, CA).

**Cell Migration Assays.** Phagokinesis assays were performed in 6-well tissue culture dishes as described [23]. Osteoclasts were gently rinsed and seeded at a low density (1 x 10³ cells per well). Some cells were treated with antibodies to the αvβ3 integrin (LM609) for 30 min prior to plating the cells on the colloidal gold surface. Once the cells were attached to the wells, substrates such as OPN or GRGDS were added to a final concentration of 10 μg/ml or 50 μg/ml, respectively. The cells migrated on this substrate, and phagocytized the gold particle to produce a white track free of the particle in a time-dependent manner. The migrating cells were visible as a black body [24]. The motility of the cell is evaluated by measuring the areas free of gold particles represented as areas moved in μm². The areas were measured by using a gridded reticle in the eyepiece of a Nikon microscope using a 10X objective. (20–25 Tracks per experiment) were measured and three separate osteoclast preparations were used. PBS-treated cells served as control. Statistical comparisons were performed by analysis of variance (ANOVA) with the Bonferroni corrections (Instat for IBM, version 2.0; Graphpad software) as described below.

**Materials and Methods**

Rhodamine phalloidin was obtained from Molecular Probes (Eugene, OR). Anti-CD44 antibodies to the standard CD44 (monoclonal and polyclonal) were purchased from BioSource International Inc. (Camarillo, CA) and Santa Cruz Biotechnology (Santacruz, CA). Transwell chamber was purchased from Corning Inc. Costar (Corning, NY). CY2 or CY3 conjugated anti-mouse or rabbit antibodies were purchased from Jackson Immunoresearch laboratories, Inc. (Westgrove, PA). GRGDS peptide was purchased from Gibco-BRL (Gaithersberg, MD). All the other chemicals including hyaluronic acid were purchased from Sigma Chemicals (St. Louis, MO).

**Preparation of Osteoclasts**

Avian osteoclast precursors were prepared as previously described [15]. Briefly, osteoclast precursors were isolated from bone marrow of egg-laying hens, maintained on Ca²⁺-deficient diets. Partially purified preparations of mononuclear cells were recovered from the interface of Ficoll/Hypaque gradients as described. Nonadherent cells were separated from the adherent population after 18–24 hours in culture. The nonadherent cells were sedimented, resuspended in fresh media (5 x 10⁶ cells/ml) and cultured in the presence of cytosine arabinoside (5 μg/ml). Multinucleated osteoclast precursor cells formed between 3 and 6 days in culture. The multinucleated cell preparations were uniformly TRAP-positive, and resorbed bone by forming resorption pits on dentine slices.

**Actin Staining.** Osteoclasts were rinsed briefly with PBS containing 5 mM EGTA (PBS-EGTA) and fixed in 4% (v/v) paraformaldehyde in PBS-EGTA for 20 min at 37°C. Coverslips were immersed in 47.5% ethanol containing 5 mM EGTA for 15 min at room temperature and rinsed with several changes of PBS-EGTA. The coverslips were then stained with 1:20 dilution of rhodamine phalloidin in PBS-EGTA for 30 min at 37°C [17]. After rinsing several times with PBS-EGTA, coverslips were mounted on a mounting solution (Vector Laboratories, Inc., Burlingham, CA). Rhodamine phalloidin images were recorded on a Zeiss LSM 410 confocal laser-scanning microscope (Thornwood, NY). Confocal images were processed by using the Adobe Photoshop software program (Adobe Systems, Inc., Mountain View, CA).

**Immunostaining.** Osteoclasts were cultured on glass coverslips or whale dentine slices. After 4 days in culture, cells were incubated with antibodies to αvβ3 and OPN as described previously [17]. Briefly, cells were fixed with 3% paraformaldehyde and permeabilized with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM CaCl₂ containing 0.1% Triton X-100 for 1 min. The cells were washed and incubated with antibodies to αvβ3 and OPN (1:100 dilution) for 2 hours, washed, and counter-stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG. Actin staining of the cells stained for αvβ3 was performed using rhodamine phalloidin (1:20 dilution) as described above. The cells were washed and mounted on a slide in a mounting solution (Vector laboratories, Inc., Burlingham, CA) and viewed on a Zeiss LSM 410 confocal laser-scanning microscope (Thornwood, NY), and photomicrographs were developed [22]. CY2 and CY3 images were recorded using the 488 nm and 568 argon excitation lines respectively. CY2 and CY3-labeled proteins were imaged with different backgrounds [23]. The background was removed and a pseudocolor (red) was assigned to the cell by the reflected light (Figs. 3, 4, 5). Images were stored in TIF image format and processed by the Adobe Photoshop software program (Adobe Systems, Inc., Mountain View, CA).

**Transwell Migration Assay.** Transwell migration chambers (Costar; 8-μm pore size) were used to assay chemotactic migration. Undersides of the membranes were coated with vitrogen 100 (collagen type 1) (30 mg/ml) at room temperature for 2 hours as directed by the manufacturer’s instructions. Osteoclasts isolated as described above were pelleted and resuspended in αMEM medium containing 1% serum and 2% BSA (5 x 10³ cells/ml). Cells were added to the upper chamber in the above-mentioned medium (100 μl) and the indicated substrates (Fig. 7) were added to the lower chamber in αMEM medium containing 1% serum and 2% BSA (600 μl). The concentrations of the substrates are as follows: OPN or VN 25 μg/ml; GRGDS 50 μg/ml; or hyaluronic acid 100 μg/ml. Some cells were pretreated with antibodies to the αvβ3 integrin (LM609) or OPN or CD44 to a final concentration of 50 μg/ml for 30 min prior to plating cells. Cell migration was allowed to proceed at 37°C in a standard tissue culture incubator for 12–14 hours. Cells that migrated to the undersides were stained, visualized, and counted as described [23]. Data are presented as the number of cells migrated (mean ± SEM) and all assays were performed in quadruplicates. Statistical significance was calculated as mentioned below.