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Ultrastructural analysis of apatite-degrading capability of extended invasive podosomes in resorbing osteoclasts

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ABSTRACT

Osteoclasts in culture are non-transformed cell types that spontaneously develop specific cell-adhesion devices such as podosomes. An individual podosome is a complex network of filamentous actin (F-actin) unit structure that collectively, with other proteins, self-organizes as the sealing zone. Major matrix degradation on apatite seems to proceed under the ruffled-border domain, which is an enclosed extracellular compartment tightly sealed off by this sealing zone. Presently we found that usually the top of finger-like projections of the ruffled border reached toward the plane of the apatite surface, where a shallow degradation of apatite took place. Simultaneously, we obtained several pieces of structural evidence indicating that a specific protrusion referred to as an invasive podosome (invadopodium), which was continuous with podosomes derived from the sealing zone, invaded deeply into apatite matrix and degraded it. The F-actin architecture of the invasive podosome – an active extracellular matrix-degrading, actin-rich cell protrusion – could be distinguished from that of other punctate F-actin structures including the individual podosome, sealing zone, and ruffled border projection. Invasive podosomes contained 2 different F-actin populations, *i.e.*, an interconnected meshwork and a parallel array of bundles. The morphological variability of these protrusions was apparent, having a single cylindrical to lamella-shaped cytoskeletal organization. Our present observations strongly suggest that the degradation of apatite substrate-resorbing osteoclasts appears to have been preceded by the combined appearance of ruffled border and invasive podosomes, and also occurred simultaneously with cell migration during an alternating cycle of resorption and migration.

1. Introduction

Osteoclasts are highly polarized cells that maintain their motility and degradative capacities during the bone-resorption cycle. Under culture conditions, their morphological and functional polarity would be expected to be dorsal-ventral and front-rear polarity, both being synchronized with each other. Indeed, morphological changes in the ventral membrane of osteoclasts are directly related to their functional roles such as attaching, degrading or resorbing activities. Within the interface of the ventral membrane and underlying calcified substratum, the development of degradative and adhesive structures in osteoclasts reflects their polarities. Indeed, cell adhesion to the calcified substratum is a crucial first step for degradation of the substratum. This tight attachment is established by a sealing zone, which is made of interlinked individual podosomes serving as a cytoskeletal actin-based structure. As is a well-known fact, podosomes consist of F-actin cytoskeleton and its related proteins on their ventral membrane and are highly dynamic structures to be assembled and disassembled (Marchisio et al., 1984, 1987; Zambonin-Zallone et al., 1988; Luxenburg et al., 2007; Akisaka et al., 2008).

Current understanding of bone resorption indicates that the adherence of an osteoclast to the bone surface, established by sealing off a specialized extracellular compartment into which are secreted H^+ and lysosomal enzymes through the ruffled-border membrane, is followed by lowering of the pH, dissolution of minerals, and degradation of the organic matrices of the bone (Baron et al., 1985; Blair et al., 1989; Saltel et al., 2004). Structurally, the ruffled-border surrounded by the sealing zone comprises highly convoluted membrane infoldings with a high surface area where the ruffled-border functions directly to degrade the extracellular matrix (ECM); whereas the podosome has an adhesive role as a structural unit and appears to collectively form the sealing zone, thus surrounding and isolating the ruffled-border compartment. Therefore, it is considered that the ruffled-border and sealing

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zone collectively play quite different functional roles during bone resorption.

Originally the sealing zone on osteoclasts was observed at contact sites of cell-to-extracellular matrix as rows of short protrusions termed rosettes, which contain F-actin and related proteins (Marchisio et al., 1984). In the meantime, Tarone et al. (1985) observed similar actin-based adhesion structures on Rous sarcoma virus (RSV)-transformed cells and referred to them by the term podosome, being an adhesion device. Later, these adhesion structures were shown to be nearly identical with those described in osteoclasts and in RSV-transformed cell (Marchisio et al., 1987). Independently, examining the same type of RSV-transformed cells, Chen et al. (1985) reported that contact sites similar to those seen in OCs exhibited adhesive and degradative capacities, which structures they named invadopodia after their functional capacities. Hence, it follows that the same structure was known differently as the terms podosome and invadopodium. Therefore, there appears to be some confusion between these 2 different specialized actin-based structures called podosome and invadopodium. The former is generally used for the adhesion device in non-invasive normal cells, whereas the latter applies to invasive tumors and transformed cells. It is still unclear whether certain cell types can form only podosomes or invadopodia and not both. Unfortunately, morphological uncertainties remain regarding the matrix-degrading capability of podosomes in osteoclasts. Especially, much more ultrastructural information in this regard is awaited.

Although growing evidence suggests that the podosome and invadopodium have a common derivation and can both degrade the ECM (see reviews by Linder et al., 2011; Murphy and Courtneidge, 2011), there is still much discussion as to how podosomes relate to the invadopodia of tumor cells (Artym et al., 2011). The invadopodium is a specialized actin-rich protrusion of metastatic tumor cells and transformed cells engaged in ECM degradation and invasion. Some studies have provided data at the ultrastructural level of invadopodia in macrophages (Goethem et al., 2011), invasive cancer cells (Gawden-Bone et al., 2010; Schoumacher et al., 2010; Tolde et al., 2010), and lymphocytes (Carman et al., 2007). Furthermore, there is no consensus as to whether podosomes in osteoclasts serve as adhesive or degradative structures. The possibility of the transformation of podosomes into invadopodia in osteoclasts was suggested previously (Zambonin-Zallone et al., 1988). To date, the relationship between podosome and invadopodium in osteoclasts still remains to be clarified, since a number of studies in this regard observed actin-based structures by fluorescence microscopy. The co-localization of ECM degradation with an F-actin spot is the major criterion to distinguish the invadopodium from other F-actin-based structures. Due to the limitation of the resolution of the standard fluorescence microscope, it is difficult to distinguish invasive podosomal F-actin spots from others such as ruffled-border fingers and podosomes. Only ultrastructural analysis can enable us to distinguish between these different cytoskeletal organizations and interactions of the actin cytoskeleton with the membrane surface.

To our knowledge, bi-functional roles of osteoclast podosomes have not been fully clarified yet. In this study, therefore, we have extended our previous observations (Akisaka and Yoshida, 2015) and investigated the invasive structures formed by resorbing osteoclasts seeded on apatite pellets. With the use of transmission electron microscopy (TEM) of sections prepared by freeze-substitution for thin-section images and by rotary replication for 3D images of the inner side of ventral membranes, we examined the precise structural organization of the ventral membranes and the interaction of cytoskeletons with the membrane surface of osteoclasts facing the underlying calcified apatite surface.

2. Materials and methods

2.1. Primary culture of osteoclasts

Osteoclasts were mechanically harvested from long bones of newborn rabbits as described previously (Akisaka et al., 2001). The cells were maintained in wells containing Medium 199 (GIBCO, USA) supplemented with 10% bovine serum and 100 μl of Fungizone (Sigma, USA). For experiments, the cells were plated on synthetic hydroxyapatite pellets with a porosity of 0% (HOYA Techno Co, Japan) at 37 °C and 5% CO_2 and allowed to attach it them for several hrs in the same medium. Non-adherent cells were vigorously washed away with culture medium and remaining adherent cells, mostly osteoclasts, on the apatite substrate were cultured in a petri dish for an additional 2 days.

2.2. F-actin staining

Osteoclasts cultured on apatite pellets were fixed with 4% paraformaldehyde buffered with 0.01 M phosphate-buffered saline (PBS) at room temperature for 30 min. After fixation, samples were incubated in PBS containing 0.3 mM rhodamine-conjugated phalloidin (Molecular Probes, OR, USA) for 30 min. The specimens were viewed under a confocal laser scanning microscope (Biorad MRC 2045, CA, USA).

2.3. Quick-freezing for electron microscopy

For high-quality ultrastructural analysis we employed the quick freezing cryofixation method. In contrast to routine chemical fixation, cryofixation is superior with respect to time and spatial resolution. However, a major troublesome feature is damage due to ice crystals, which are an obstacle for good cryofixation. The quality of freezing is dependent on the ice crystal size; and a poor quality of freezing is easily recognizable judging from the distortion of cell structures, whereas it difficult to select the preparation with optimal good freezing. Based on the criteria categorized as “poor” to “good” quality (Bridgman and Reese, 1984), we paid particular attention to freezing and selected “good quality” preparations for TEM observation.

The practical limitations for obtaining well-frozen samples are needed for many freezing experiments. After having been washed briefly with physiological saline, cultured OCs adhering to apatite pellets were attached to a shock-absorbing sheet of β gel (Segal Co., Japan), and then stuck onto the plunger of an Eiko MF-2 quick-freeing apparatus (Eiko Engineering, Japan). The samples were immediately pressed against a mirror-faced copper block precooled by liquid nitrogen without the use of any kinds of cryoprotectant such as glycerol or DMSO. This type of device is equipped with a magnetron with a maximum power of 500 W and a frequency of 2.45 GHz. Irradiation with microwaves for 50 ms during metal-contact quick freezing is known to reduce the sizes of ice crystals and micro-clusters of water molecules (Hanyu et al., 1991), which step protects against morphological disturbance at the EM level. Quick-frozen apatite pellets were prepared by 2 different approaches: freeze-substituted thin sectioning and freeze-dried rotary replication as described below.

2.4. Freeze-substitution for thin sectioning

Quick-frozen samples were transferred directly from liquid nitrogen into the substitution medium in a programmable Leica EM AFS automatic freeze-substitution (FS) device (Vienna, Austria). Freeze substitution was performed in anhydrous acetone containing 0.2% tannic acid at −85 °C for 12 h, and then the samples were washed with pure acetone at the same temperature. Next, they were transferred to another substitution medium, one consisting of