Distribution of Inositol 1,4,5-Trisphosphate Receptors in Rat Osteoclasts

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Inositol 1,4,5-trisphosphate (IP₃) receptors (IP₃Rs) are Ca²⁺ channels that localize to intracellular Ca²⁺ stores such as the endoplasmic reticulum (ER). Recently, IP₃Rs were found to participate in the formation of the cytoskeleton and cellular adhesions. In this study, we examined the cellular localization of type I, II, and III IP₃Rs to assess their role in cellular adhesion in rat osteoclasts. Rat bone marrow cells were cultured in α-MEM with 10% fetal bovine serum, M-CSF, RANKL, and 1,25(OH)₂D₃ for 1 week to promote osteoclast formation. Type I, II, and III IP₃R expression in the osteoclasts was then examined by RT-PCR. Double-staining was performed using antibodies against type I, II, and III IP₃Rs and DiOC₆, an ER marker, or TRITC-phalloidin, an actin filament marker. Expression of all three IP₃Rs was detected in the newly formed osteoclasts; however, the localization of the type I and II IP₃Rs was predominantly close to nuclear, and possibly colocalized with the ER, while the type III IP₃Rs were localized to the ER and podosomes, actin-rich adhesion structures in osteoclasts. These findings suggest that type III IP₃Rs are associated with osteoclast adhesion.

Key words: inositol 1,4,5-trisphosphate receptor (IP₃R), osteoclast, podosome, cell adhesion

I. Introduction

The second messenger inositol 1,4,5-trisphosphate (IP₃) is produced primarily by the metabolism of phosphoinositol-4,5-bisphosphate by phospholipase C in response to the stimulation of G protein-coupled receptors or receptor tyrosine kinases [22]. IP₃-mediated Ca²⁺ signaling controls important cellular functions such as contraction, secretion, gene expression, and synaptic plasticity [18]. IP₃ causes the release of Ca²⁺ into the cytoplasm by binding IP₃ receptors (IP₃Rs) on intracellular Ca²⁺ stores such as the endoplasmic reticulum (ER) [27]. At least three distinct IP₃-R subtypes (types I, II, and III) [7, 31] exist, which are expressed in a tissue- and development-specific manner and form heterotetrameric channels [22]. The expression patterns of the subtypes may be responsible for the production of a cell type-specific Ca²⁺ signal. Recent studies of the individual IP₃-R subtypes in lipid bilayers have suggested functional differences [18], and IP₃ type II has been reported in the plasma membrane and karyotheca in addition to the ER, where they are closely associated with actin filaments. The IP₃Rs that are localized to the plasma membrane may function in the formation of the cytoskeleton and cellular adhesions [27].

Skeletal modeling during growth and bone remodeling in the skeleton are dependent on bone resorption, which is mediated by osteoclasts. Osteoclasts are large multinucleated, highly motile and invasive cells of hematopoietic origin. Osteoclast-mediated bone resorption is tightly regulated at multiple levels, and excessive osteoclast activity results in a progressive loss of bone mass and deterioration of bone architecture, leading to a variety of diseases [11, 15]. The resorptive activity of osteoclasts depends on their adhesion to the bone surface. When bone resorption is induced, osteoclasts migrate to the site of resorption where they become highly polarized and form four distinct membrane domains: the ruffled border, sealing zone, functional secretory domain, and basolateral domain [28]. The pri-
mary adhesive structures of osteoclasts are dot-like actin-rich structures known as podosomes [15, 24], and the dynamic changes in the cytoskeletal organization and distribution pattern of these podosomes can occur within minutes. The distribution of actin in cells helps determine cell integrity, shape, adhesion, and motility [1]. Podosomes are present not only in osteoclasts, but also in other monocyte-derived cells, such as macrophages and dendritic cells, as well as in smooth muscle cells, endothelial cells, transformed fibroblasts, and certain epithelial cells [15].

The localization of each IP3R subtype (I, II, and III) in osteoclasts is unknown. Therefore, we examined the cellular localization of each subtype and its colocalization with cytoskeletal proteins to elucidate their involvement in cellular adhesion.

II. Materials and Methods

Reagents and antibodies

Human receptor activator of nuclear factor κB ligand (RANKL) and human macrophage colony-stimulating factor (M-CSF) were obtained from Wako (Tokyo, Japan), while 1α,25-dihydroxyvitamin D3 was purchased from Funakoshi (Tokyo, Japan). Alexa Fluor 488 goat anti-rabbit IgG antibody, Alexa Fluor 568 goat anti-rabbit IgG antibody, TRITC-phallolidin, and 3,3′-dihexyloxacarbocyanine iodide (DiOC2) were purchased from Invitrogen-Molecular Probes (Tokyo, Japan). Anti-IP3R type I, II, and III rabbit polyclonal antibodies were provided by one of the authors of this paper, Dr. Akihiro Tanimura of the Health Sciences University of Hokkaido, Hokkaido, Japan [29].

Osteoclast culture

The experimental protocol in rats was reviewed and approved by the Animal Care Committee at Kyushu Dental College, Fukuoka, Japan. Twenty-four male Sprague-Dawley rats weighing 200–250 g were used. Rat bone marrow cells were isolated by a modified version of a previously published method [9, 13, 21]. Briefly, tibiae from 7-week-old Sprague-Dawley rats were dissected free of adhering tissue. Next, the ends of the bones were removed, and the marrow cavity was flushed with α-minimal essential medium (α-MEM; Gibco, Grand Island, NY) containing heat-inactivated fetal bovine serum (FBS; Wako), gentamicin (Gibco), amphotericin B (Gibco), and penicillin G (Wako) by slowly injecting the medium into one end of the bone using a sterile 22-gauge needle. The marrow cells were collected in tubes, centrifuged at 1,500g for 5 min, aspirated, and mixed with 0.83% NH4Cl and 10 mM Tris. They were centrifuged again at 1,500g for 5 min and then aspirated and covered with fresh medium. Finally, the cells were plated in 35-mm culture dishes on cover glasses (5×104 cells/dish) and in 96-well plates on a dentin slice (7×105 cells/well), and cultured for 7 days at 37°C under 5% CO2, in α-MEM containing 10% FBS, 1% gentamicin/amphotericin B/penicillin G, 10−7 M 1α,25-dihydroxyvitamin D3, 25 ng/ml M-CSF, and 20 ng/ml RANKL. Fresh medium was added every 3 days. The formation of osteoclast-like multinucleated cells was confirmed by an Olympus IMT-2 inverted phase contrast microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR)

Bone marrow cells were cultured for 1 week, then incubated with 0.01% Pronase/0.02% EDTA for 10 min at 37°C to obtain an osteoclast-rich population [6]. mRNA was then extracted using a Total RNA Extraction MiniPrep System (Viogene, Sunnyvale, CA) according to the manufacturer’s protocol. To reduce DNA contamination, the samples were treated with RNase-free DNase I (Takara Bio, Shiga, Japan) for 3 hr at 37°C. cDNA was synthesized from 2 μg of total RNA in 30 μL of reaction buffer containing 500 μM dNTPs, 20 U of ribonuclease inhibitor (Promega, Madison, WI), and 200 U of Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). Each reaction was carried out as follows: 70°C for 7 min, 45°C for 60 min, 70°C for 10 min, and 4°C. The primers used to amplify IP3R-I were 5′-TGG CAG AGA TGA TCA GGG AAA-3′ and 5′-GCT CGT TGT GTT CCC CTT CAG-3′ (GenBank J05510; 8314–8334 and 8389–8409) [2]. For IP3R-II, they were 5′-GGC ACA GCC TCT CCA TGT GGC AGG-3′ and 5′-TTC CTG TCT CGC TGA CCC CAC TGC CG-3′ (GenBank X61677; 8568–8591 and 8742–8765); for IP3R-III, 5′-CAC GGA GCT GCC ACA TTT ATG GCC GGC-3′ and 5′-TCC TCT GTC CGT GGT TCA TGA CGG-3′ (GenBank L06096; 7377–7400 and 7522–7545) [3]; for cathepsin K, 5′-CCC AGA CTC CAT CGA CTA TCG-3′ and 5′-CTG TAC CCT CTG CAC TTA GCT GCC CAC-3′ (GenBank BC078793; 418–438 and 724–747) [16]. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and a 1-min extension at 72°C for 35, or 30 cycles for the IP3Rs, cathepsin K, respectively, with a final 9-min extension at 72°C. To confirm the reproducibility of our results, each experiment was repeated at least three times. Each reaction mixture consisted of cDNA, Taq polymerase buffer, and 1 μL of each of the sense and antisense primers, for a total volume of 20 μL. The amplified products were electrophoresed in 2% agarose gels and visualized with ethidium bromide. The specificity of each primer was confirmed using a BLAST-assisted Internet search of nonredundant nucleotide sequence databases (National Library of Medicine, Bethesda, MD).

Immunocytochemistry

The bone marrow cells, thus cultured for 1 week as described above, were fixed in 4% paraformaldehyde for 10 min and then treated with 0.5% Triton X-100 for 10 min. After washing with PBS three times, the cells were blocked with 1% whole goat serum (Nichirei, Tokyo, Japan) for 30 min, washed three times in PBS, and incubated with anti-IP3R type I, II, and III rabbit IgG antibodies (1:100) overnight at 4°C. After another three washes with PBS, the samples were incubated with Alexa Fluor 568 goat anti-rabbit IgG antibody (1:40) for 2 hr at 37°C. After washing, the cells were incubated with 1 μg/mL DiOC2, an ER
marker, for 30 min at room temperature. After incubation with the primary antibodies, some samples were incubated with Alexa Fluor 488 goat anti-rabbit IgG antibody (1:40) for 2 hr at 37°C. After washing, the cells were incubated with TRITC-labeled phalloidin (1:40) for 1 hr at 37°C. The samples were examined under a confocal laser scanning microscope (Radiance 2100, Bio-Rad, Herts, UK) equipped for fluorescence and Nomarski optics.

III. Results

IP₃Rs subtype expression

IP₃R subtype expression in a rat osteoclast-rich population was examined using subtype-specific primers. Amplification was observed in all three cases, indicating that all three IP₃R subtypes are expressed at the mRNA level in cultured rat osteoclasts (Fig. 1a). To verify that the RNA was derived from osteoclasts, expression of cathepsin K, an osteoclast marker, was confirmed in each population (Fig. 1b, c). The formation of osteoclast-like multinucleated giant cells was confirmed by a phase contrast microscope (Fig. 1d).

Colocalization of the IP₃R subtypes with the ER

We next investigated the degree of colocalization between the IP₃Rs and the ER in rat osteoclasts cultured on cover glasses. IP₃R type I was predominantly observed close to the nuclear region near the ER, which was stained by DiOC₆(Fig. 2a–d). IP₃R type II was ER-localized, similar to IP₃R type I (Fig. 2e–h). The localization of IP₃R type III was similar to that of IP₃R types I and II; however, additional immunopositive reactions were observed at the periphery of the osteoclasts (Fig. 2j). To eliminate the possibility of cross-talk in double-staining between DiOC₆ and each IP₃R, we performed each IP₃R immunostaining without DiOC₆ staining (Fig. 2m, n, o). In these cases, we obtained the same results as mentioned above. Control samples treated without primary antibody showed no signal (Fig. 2p).

Colocalization of IP₃R type III with podosomes

To examine the relationship between IP₃R type III and podosomes, we used IP₃R type III immunofluorescence staining with TRITC-phalloidin staining to visualize the actin filaments in the cells. In terms of the localization of IP₃R type III, dot-like signals were observed at the periphery of the osteoclasts (Fig. 3a, c, h); the TRITC-phalloidin staining revealed substantial homology between the distribution of the type III IP₃Rs and the podosomes (Fig. 3a–d, h–k). The colocalization of the IP₃Rs and podosomes were confirmed by an X/Z reconstruction section (Fig. 3e, f, g). To eliminate the possibility of cross-talk in double-staining between TRITC-phalloidin and IP₃R type III, we performed IP₃R type III immunostaining without TRITC-phalloidin staining. Staining for IP₃R type III alone revealed dot-like immunopositive reactions at the periphery of the osteoclasts (Fig. 3l). Control staining without primary antibody produced no signal (Fig. 3m).

Double-staining for IP₃R type III and actin was also performed in osteoclasts cultured on a dentin slice. An actin band representing accumulated podosomes, which was colocalized with IP₃R type III, was observed (Fig. 4).

IV. Discussion

Our results indicate that IP₃Rs are distributed predominantly along the ER; however, type III IP₃Rs are also localized at podosomes, the osteoclast attachment structures. This study is the first paper to show an association between IP₃Rs and the attachment structures in osteoclasts.

In this study, we found that osteoclasts express three types of IP₃Rs (I, II, and III) that are colocalized with the ER around the nucleus, although type III IP₃Rs may also be found as punctate structures at the periphery. These puncta correspond to osteoclast attachment regions known as podosomes. Several previous studies have suggested that IP₃Rs are present at the plasma membrane. Moreover, type II and type III IP₃Rs, and possibly other as yet undiscovered IP₃Rs, have been suggested to contain sorting signals that are distinct from those of type III IP₃Rs, which may target them to the plasma membrane [26]. In addition, type III IP₃Rs have been detected around the nucleus and at the plasma membrane in vascular smooth muscle cells [30]. Although the localization of IP₃Rs to the plasma membrane may be cell-type dependent [29], only type III IP₃Rs are plasma membrane-localized in osteoclasts. Several studies have reported an IP₃R-induced release of Ca²⁺ from the nucleus and the localization of IP₃Rs to the nuclear membrane [9, 25]. In the present study, we were unable to determine whether the IP₃Rs were localized to the nuclear membrane.

The osteoclast cytoskeleton is characterized by the presence of specific adhesion structures called podosomes.
that contain actin microfilaments surrounded by vinculin and talin [24]. Double-staining with anti-IP₃Rs and TRITC-phalloidin revealed the colocalization of type III IP₃Rs and podosomes. IP₃R subtypes have been reported as being related to focal contact cytoskeletal proteins [27]. The colocalization of type III IP₃Rs and podosomes raises the possibility that type III IP₃Rs may be involved in Ca²⁺ signaling at focal contacts. Focal contacts, which consist of assemblies of cytoskeletal proteins, play an important role in stabilizing cell adhesion, structure, and mobility [5, 19, 20]. Numerous studies have reported that inositol phospholipids and enzymes in the inositol signaling pathway, such as phosphatidylinositol kinase, phosphatidylinositol 4-phosphate kinase, and phospholipase C, have important roles in the formation of actin stress fibers [4, 10, 12, 23]. Cellular adhesion and migration are regulated by an increase in intra-
Fig. 3. Fluorescence images showing the podosomes and type III IP₃Rs in cultured osteoclasts. (b) TRITC-phalloidin staining indicates the punctate distribution of the podosomes at the periphery of the cells. (a) The distribution of IP₃R type III was similar to that of the podosomes. (c, d) Close-up view of the area enclosed by a yellow square in (a, b). Image of IP₃R type III staining (e), image of podosome staining (f) and merged image of IP₃R type III/podosome staining (g) at the X/Z section on the yellow line in (a). Image of IP₃R type III staining (h), image of podosome staining (i), a Nomarski image (j) and merged image of IP₃R type III/podosome staining (k). (l) Staining for IP₃R type III alone. Yellow rectangle shows a close-up view of the area in the small yellow rectangle in (l). As in (a), dot-like signals were observed at the periphery of the cells. (m) Negative control. Cells were viewed under confocal laser microscopy (60×, oil). Bars=5 μm (b), 1 μm (d, g, i, inset), and 10 μm (k–m). Optical path setting: TRITC-phalloidin and IP₃R type III; KrAr Laser: 30.0, Iris: 2.0–3.0, Gain: 60.0, Offset: 0.0, Nomarski; KrAr Laser: 100.0, Gain: 100.0, Offset: 0.0.
cellular Ca\(^{2+}\) [4]. Moreover, peripheral actin accumulation is produced by potential-dependent Ca\(^{2+}\) channels [8]. The podosomes in osteoclasts contain numerous actin microfilaments; thus, type III IP\(_R\)s may induce the accumulation of actin filaments by increasing the concentration of Ca\(^{2+}\). Taken together, these findings suggest that type III IP\(_R\)s take part in cellular adhesion in osteoclasts. Additional studies using IP\(_R\) antagonists are needed to clarify the association between type III IP\(_R\)s and the formation of cellular adhesions in osteoclasts.

The formation of a complete actin ring in osteoclasts cultured on a dentin surface reveals not only the strong attachments between osteoclasts but also the high bone-resorptive activity of osteoclasts [14, 17]. We detected a relatively clear actin ring at the periphery of the osteoclasts on the dentin slice compared to those on the cover glasses, and type III IP\(_R\) immunoreactivity was observed in the region of the actin ring in the dentin-cultured osteoclasts. The colocalization of type III IP\(_R\)s and actin suggests that IP\(_R\) type III may be related to the bone-resorptive activity of osteoclasts.

In conclusion, the IP\(_R\)s in osteoclasts are predominantly ER-localized; however, type III IP\(_R\)s are also associated with attachment structures known as podosomes. Although our data strongly suggest a relationship between IP\(_R\)s and cellular attachment in osteoclasts, further study is needed to elucidate the function of IP\(_R\)s in osteoclasts.

V. References

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