Distribution of actin filaments, non-muscle myosin, M-Ras, and extracellular signal-regulated kinase (ERK) in osteoclasts after calcitonin administration*

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Summary. Scanning electron microscopy (SEM) was employed to study the effect of calcitonin on the distribution of actin filaments in osteoclasts obtained from rat tibiae. Fluorescent microscopy was also applied to examine calcitonin-induced changes in the distribution of actin filaments, non-muscle myosin, M-Ras, and extracellular signal-regulated kinase (ERK) to clarify the role of ERK in the cytoskeleton of osteoclasts. SEM of control osteoclasts revealed a ring-like structure in the peripheral region. Labeled actin filaments and non-muscle myosin were detected in the peripheral region and exhibited a ring-like pattern. Immunoreactivity indicating M-Ras and ERK was also detected in the vicinity of the actin ring. After calcitonin treatment, many osteoclasts exhibited a retracted appearance and lacked a discernible actin ring. Numerous retraction fibers were found at the edge of calcitonin-treated osteoclasts. Actin filaments and non-muscle myosin were concentrated in the cytoplasm of calcitonin-treated osteoclasts, and exhibited a filamentous pattern. Labeled M-Ras and ERK also accumulated in the central region of

these osteoclasts. These findings suggest that actin-myosin interaction plays an essential role in the retraction of osteoclasts induced by calcitonin. ERK may play a role in this interaction by activating myosin light chain kinase, as previously observed in smooth muscle cells.

Introduction

Osteoclasts are highly polarized cells specialized for bone resorption. Their differentiation and activation are regulated by hormones and cytokines. Calcitonin is a polypeptide hormone that reduces serum calcium levels via the inhibition of osteoclastic bone resorption (Pondel, 2000; Ikegame *et al.*, 2004). *In vivo* studies have demonstrated that calcitonin causes a reduction of the ruffled border and clear zone of osteoclasts, subsequently inducing the loss of cell polarity (Kallio *et al.*, 1972; Lucht, 1973; Warshawsky *et al.*, 1980; Baron *et al.*, 1990; Ikegame *et al.*, 1994; Kuroda *et al.*, 1996; Nakamura *et al.*, 1997). *In vitro* research has also revealed that calcitonin disrupts the actin ring of osteoclasts, induces osteoclast retraction, and inhibits osteoclast motility (Chambers and Magnus, 1982; Warshawsky *et al.*, 1985; Hunter *et al.*, 1989; Lakkakorpi and Vaananen, 1990).

The calcitonin receptor is coupled with pathways of cAMP-dependent protein kinase A (PKA) (Nicholson *et al.*, 1986, 1987; Lin *et al.*, 1991; Suzuki *et al.*, 1996), intracellular calcium (Margaroli *et al.*, 1989; Zaidi *et al.*, 1990), and protein kinase C (PKC) (Holloway *et al.*, 1997). However, the precise signal transduction pathway that regulates morphological changes in osteoclasts remains controversial. It has been reported that calcitonin induces

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phosphorylation of extracellular signal-regulated kinase (ERK) in osteoclasts (Zhang *et al.*, 2002) as well as in HEK 293 cells that stably express the rabbit calcitonin receptor C1a (C1a-HEK) (Chen *et al.*, 1998). ERK is a member of the mitogen-activated protein kinases (MAPKs), which are important in cell growth, differentiation, and apoptosis (Nishida and Gotoh, 1993; Cobb and Goldsmith, 1995; Garrington and Johnson, 1999). Recently, it was suggested that ERK is involved in the survival of osteoclasts (Miyazaki *et al.*, 2000; Tanaka *et al.*, 2003). Moreover, we previously reported that ERK localizes in the clear zone of osteoclasts and plays a significant role in the maintenance of cell polarity (Nakamura *et al.*, 2003). However, it is not known whether ERK plays a role in cytoskeletal organization, which is responsible for the maintenance of cell polarity. Thus, there is a need to investigate the distribution of signaling molecules such as ERK in order to clarify the mechanism of cytoskeletal organization in osteoclasts.

In the present study, we investigated the localization of cytoskeleton and ERK-related proteins in cultured rat osteoclasts after calcitonin treatment for the purpose of clarifying the role of ERK in cytoskeletal organization. We used scanning electron microscopy (SEM) to examine changes in the distribution of actin filaments and fluorescent microscopy to examine changes in the distribution of non-muscle myosin, M-Ras, and ERK.

Materials and Methods

All animal procedures were performed in accordance with the Guidelines for Animal Experiments, Okayama University Graduate School of Medicine and Dentistry.

Preparation of osteoclasts

Osteoclasts were obtained using a modified version of the method by Chambers and Magnus (1982). Tibiae of 2-week-old Wistar rats were dissected and cleaned

of adherent soft tissue, bone marrow, and epiphyseal cartilage. The bones were minced with a scalpel in 4 ml of the α -modified minimal essential medium (α -MEM; Gibco BRL, Life Technologies Inc., Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories GmbH, Linz, Austria), penicillin G (100 U/ml; Gibco BRL), and streptomycin (100 μ g/ml; Gibco BRL). After mixing for 30 sec with a test tube mixer, 200- μ l aliquots of cell suspension were pipetted onto glass coverslips and incubated in a humidified incubator with an atmosphere of 5% CO₂ for 1 h at 37°C to induce adherence of the osteoclasts. After washing with α -MEM to remove non-adherent cells, the coverslips were further incubated in the medium (which was maintained at pH 6.8 using 1 N HCl) for 2 h. Then they were placed in the medium with or without 10 nM synthesized eel calcitonin (elcatonin; Ashahi Kasei Pharma Co., Tokyo). The specimens were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in a 0.05 M phosphate buffer (pH 7.4) at 5, 15, 30, 60, and 180 min after elcatonin administration. For observation of actin filaments using SEM, the fixation procedure was omitted.

SEM observation

Procedures used to visualize actin filaments by SEM were performed according to the method by Svitkina and Borisy (1998). After washing with PBS, specimens were incubated with an extraction buffer consisting of 1% Triton X-100, 4% polyethyleneglycol (MW 40,000) and 10 μ M phalloidin in a PEM buffer (100 mM PIPES, 1 mM MgCl₂, and 1 mM ethylenediaminetetraacetic acid) for 5 min at room temperature (RT). After washing twice with the PEM buffer, they were fixed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) for 1 h at 4°C, followed by incubation with 0.1% tannic acid for 20 min at RT. They were stained with 0.2% uranyl acetate, dehydrated with graded ethanol, and critical-dried. The specimens were coated with osmium using a Neo super fine osmium coater (Meiwa Shoji Co., Osaka), and were then observed by SEM (Hitachi, Tokyo).

Fig. 1. Scanning electron micrographs showing actin filaments in control (A, B, C) and calcitonin-treated (D, E, F) osteoclasts after 15 min. **A:** A ring-like structure (arrowheads) is visible in the peripheral region of an osteoclast. **B and C:** Actin filaments are arranged in various directions at the edge of the osteoclast. **D:** No ring-like structure is visible in a calcitonin-treated osteoclast. **E:** Bundles of actin filaments are visible in the peripheral region (arrows). **F:** Thin actin filaments are oriented inward at the edge of the calcitonin-treated osteoclasts (arrowheads). Bars: 20 μ m (A, D); 5 μ m (B, E); 1 μ m (C, F)

Fig.1. Legend on the opposite page.

Fig. 2. Fluorescent micrographs showing the localization of actin filaments and non-muscle myosin in control (A, B, C) and calcitonin-treated (D, E, F) osteoclasts after 5 min. **A:** Labeled actin filaments, visible in the peripheral region of an osteoclast, are arranged in an actin ring. **B:** Immunoreactivity indicating non-muscle myosin is visible in the peripheral region, and exhibits a ring-like pattern. Reactivity is also visible around the nuclei and cytoplasm of the osteoclasts. **C:** Merging of images A and B. Colocalization of actin filaments and non-muscle myosin is visible on the actin ring. **D:** Labeled actin filaments do not exhibit a ring-like pattern in a calcitonin-treated osteoclast. A filamentous pattern of reactivity is visible in the cytoplasm. **E:** Immunoreactivity indicating non-muscle myosin exhibits a filamentous pattern in the cytoplasm of the calcitonin-treated osteoclast. **F:** Merging of images D and E demonstrates that actin filaments and non-muscle myosin colocalize in the cytoplasm after calcitonin treatment. Bars: 20 μm (A–F)

Immunohistochemistry for non-muscle myosin, M-Ras and ERK

Specimens were permeabilized with 0.5% saponin in PBS and then immersed in PBS containing 10% bovine serum albumin for 15 min to block non-specific immunoreactions. They were then incubated with a rabbit anti-non-muscle myosin polyclonal antibody (Biomedical Technology, Inc., Stoughton, MA), goat anti-M-Ras polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or mouse pan-ERK monoclonal antibody (BD Transduction, Lexington, KY) for 12 h at 4°C. Next, they were immersed in Alexa-Fluor-488-conjugated anti-rabbit Ig G, anti-goat Ig G or anti-mouse Ig G (Molecular Probes, Inc. Eugene, OR). For visualization of the actin filaments, the specimens were incubated with Alexa-Fluor-594-conjugated phalloidin (Molecular Probes, Inc. Eugene, OR) diluted 1:200. Fluorescence of the specimens was assessed using a Nikon Microphot-FX light microscope (Nikon, Tokyo) with the appropriate filter combinations.

Results

Isolated rat osteoclasts cultured for 2 h at pH 6.8 exhibited an actin ring at a higher rate than osteoclasts cultured at pH 7.4, and rapidly retracted in response to calcitonin. The number of retracted osteoclasts increased with time after calcitonin treatment. We mainly observed osteoclasts at 5 or 15 min after treatment in order to assess the early effects of calcitonin on cytoskeleton.

Actin filaments in osteoclasts visualized by SEM

SEM observation using the lysis buffer containing phalloidin revealed the distribution of actin filaments in osteoclasts, as viewed from the top side of the cells. In control osteoclasts, a ring-like structure was seen in the peripheral region (Fig. 1A). Actin filaments at the edges of the osteoclasts were oriented in various directions, and exhibited a network pattern (Fig 1B, C). After calcitonin

Fig. 3. Fluorescent micrographs indicating the localization of actin filaments and M-Ras in control (A, B, C) and calcitonin-treated (D, E, F) osteoclasts after 15 min. **A:** Labeled actin filaments are arranged in an actin ring in the peripheral region of an osteoclast. **B:** Immunoreactivity indicating M-Ras is detected in the peripheral region and exhibits a ring-like pattern. Reactivity is also visible in the cytoplasm of the osteoclast. **C:** Merging of images A and B. The colocalization of actin filaments and M-Ras is visible on the actin ring. **D:** Labeled actin filaments have accumulated in the cytoplasm of a calcitonin-treated osteoclast. **E:** Immunoreactivity indicating M-Ras is diffusely visible in the cytoplasm of the calcitonin-treated osteoclast. **F:** Merging of images D and E. Actin filaments and M-Ras are visible in the cytoplasm after calcitonin treatment. Bars: 20 μ m (A–F)

treatment, many osteoclasts retracted and lacked any discernible ring-like structure (Fig. 1D). Bundles of actin filaments were frequently observed in the peripheral region of calcitonin-treated osteoclasts (Fig. 1E). Also, numerous retraction fibers were found at the edges of these osteoclasts (Fig. 1F). Networks of actin filaments were observed in the basal region of retraction fibers.

Immunolocalization of non-muscle myosin, M-Ras and ERK

In control osteoclasts, phalloidin staining revealed an actin ring in the peripheral region (Fig. 2A). Non-muscle myosin was localized in the vicinity of the actin ring and around nuclei (Fig. 2B). Colocalization of actin filaments and non-muscle myosin was seen in the peripheral region (Fig. 2C). At 5 and 15 min after calcitonin treatment, no actin ring was visible in the retracted osteoclasts. Labeled actin filaments were seen in the cytoplasm of osteoclasts, and exhibited a filamentous pattern (Fig. 2D). Non-muscle myosin also exhibited a filamentous pattern in the

cytoplasm of the osteoclasts. No peripheral localization of non-muscle myosin was observed (Fig. 2E). Merged images revealed that non-muscle myosin was colocalized with actin filaments (Fig. 2F). In control osteoclasts, M-Ras immunoreactivity was detected close to the actin ring and around nuclei (Fig. 3A, B, C). After calcitonin treatment, labeled M-Ras was detected throughout the cytoplasm of the retracted osteoclasts (Fig. 3D, E, F). No ring pattern of M-Ras localization was seen. As for ERK, intense ERK immunoreactivity was detected in the peripheral region of control osteoclasts. Colocalization of actin filaments and ERK was seen in the actin ring (Fig. 4A, B, C). Moderate ERK reactivity was also seen in nuclei. Additionally, a filamentous distribution of ERK was detected in the cytoplasm of the osteoclasts (Fig. 4B). After calcitonin treatment, ERK immunoreactivity was not detected in the peripheral region of the retracted osteoclasts. Labeled ERK was seen in the cytoplasm of the osteoclasts (Fig. 4D, E, F). Particularly intense ERK reactivity accumulated around nuclei (Fig. 4E, F).

Fig. 4. Fluorescent micrographs indicating the localization of actin filaments and ERK in control (**A**, **B**, **C**) and CT-treated (**D**, **E**, **F**) osteoclasts after 5 min. **A**: Labeled actin filaments are arranged in an actin ring in an osteoclast. **B**: Immunoreactivity indicating ERK is visible in the peripheral region and exhibits a ring-like pattern. Reactivity is also visible in the nucleus of the osteoclast. Labeling in the cytoplasm shows a filamentous pattern. **C**: Merging of images **A** and **B**. The colocalization of actin filaments and ERK is visible on the actin ring. **D**: Labeled actin filaments are diffusely visible in the cytoplasm of a calcitonin-treated osteoclast. **E**: Immunoreactivity indicating ERK has accumulated around the nucleus of the calcitonin-treated osteoclast. Labeling is also visible in the cytoplasm. **F**: Merging of images **D** and **E**. Actin filaments and ERK are visible in the cytoplasm after calcitonin treatment. Bars: 20 μm (**A**–**F**)

Discussion

After culturing for 2 h at pH 6.8, we obtained a high number of osteoclasts that possessed an actin ring and responded to calcitonin. In the culture at pH 7.4, few actin rings were seen in the osteoclasts. Thus, acidic conditions may be required for osteoclasts to acquire cell-matrix attachments and maintain cell polarity. *In vivo* osteoclasts may exist under slightly acidic conditions because they transport H^+ into Howship's lacunae via H^+ -ATPase (Blair *et al.*, 1989; Nakamura *et al.*, 1994). Although it is unknown why osteoclasts under acidic conditions frequently exhibit an actin ring, the present method is useful for a morphological study of osteoclasts because freshly isolated osteoclasts possess characteristics very similar to those of osteoclasts under physiological conditions.

We also succeeded in visualizing actin filaments in osteoclasts by SEM using a lysis buffer containing phalloidin. In our SEM studies, a ring-like structure was evident in the peripheral region of control osteoclasts, while it was not seen in calcitonin-treated osteoclasts. Because

calcitonin causes disruption of the actin ring, this evidence suggests that the ring-like structure revealed by SEM is closely related to the actin ring.

Next, we examined the effects of calcitonin on the localization of actin filaments and non-muscle myosin. In retracted osteoclasts after calcitonin treatment, non-muscle myosin was associated with actin filaments. Actin-myosin interaction is to be involved in cell contraction in muscle and non-muscle cells. The present findings indicate that actin-myosin interaction is essential for the retraction of osteoclasts induced by calcitonin.

Finally, we investigated the distribution of ERK-related proteins, M-Ras, and ERK in osteoclasts after calcitonin treatment. It has been reported that calcitonin stimulates the phosphorylation of ERK in osteoclasts and in HEK 293 cells expressing a calcitonin receptor (Chen *et al.*, 1998; Zhang *et al.*, 2002). We assessed the localization of other ERK-related proteins using anti-Raf, MEK, and phospho-ERK antibodies, but these antibodies did not reveal the immunolocalization of these proteins (data not shown). On the other hand, we found that M-Ras and ERK

were localized in the vicinity of the actin ring in osteoclasts. Moreover, calcitonin affected the distribution of M-Ras and ERK as well as actin filaments. Actin filaments, M-Ras, and ERK tended to accumulate in the central region of osteoclasts, suggesting that ERK in osteoclasts is involved in the organization of cytoskeletal elements such as actin filaments and non-muscle myosin. We previously demonstrated that ERK accumulates in the clear zone of osteoclasts — which corresponds to the actin ring — and suggested that ERK participates in the maintenance of cell polarity in osteoclasts (Nakamura *et al.*, 2003). Myosin light chain kinase (MLCK) is reported to be a downstream component of ERK (Klemke *et al.*, 1997). In addition, the activation of ERK with a subsequent phosphorylation of MLCK leads to enhanced cell migration (Nguyen *et al.*, 1999; Cho and Klemke, 2000). Phosphorylation of myosin light chains by MLCK induces a functional actin-myosin interaction involved in generating the contractile force necessary for cell retraction (Tan *et al.*, 1992). On the other hand, evidence suggests that myosin in osteoclasts is involved in the regulation of osteoclast activity because anti-myosin antibody inhibits osteoclastic bone resorption (Sato and Grasser, 1990; Krits *et al.*, 2002). Thus, the inhibition of osteoclastic resorption by calcitonin may be partly mediated by its effects on myosin function, and ERK may regulate actin-myosin interaction *via* MLCK activation.

Miyazaki *et al.* (2000) demonstrated that ERK is involved in the survival of osteoclasts by showing that an inhibition of ERK activity induced apoptosis of osteoclasts whereas ERK activation markedly prolonged their survival by preventing spontaneous apoptosis. Consistent with this report is the finding that calcitonin appears to protect osteoclasts from apoptosis and promote their survival (Selander *et al.*, 1996). These reports suggest that the phosphorylation of ERK induced by calcitonin mediates anti-apoptotic effects. In the present study, ERK immunoreactivity tended to accumulate near nuclei after calcitonin treatment, but localization of ERK in nuclei was also seen in control osteoclasts. The present findings did not allow us to conclude that ERK is involved in the anti-apoptotic effects of calcitonin. Clarification of this point requires future research.

In conclusion, the present study is the first to demonstrate that the distribution of actin filaments, non-muscle myosin, M-Ras, and ERK in osteoclasts is changed by calcitonin treatment. Morphological changes in osteoclasts induced by calcitonin appear to be a complex process involving multiple pathways, including intracellular calcium, PKC, PKA, and ERK. The activation of ERK induced by calcitonin may facilitate actin-myosin interaction, causing the retraction of osteoclasts *via* MLCK activation.

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