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Mechanical fragmentation and transportation of calcium phosphate substrate by filopodia and lamellipodia in a mature osteoclast

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Abstract

The functions of filopodia and lamellipodia in mature osteoclasts are not well known in the process of bone resorption. We investigated the function of filopodial/lamellipodial movement in mature osteoclasts by video-enhanced contrast-differential interference contrast (VEC-DIC) microscopy. Mature osteoclasts, which were isolated from Japanese white rabbits, were cultured on calcium phosphate (CP)-coated quartz coverslips to observe filopodial/lamellipodial movement and the formation of CP-free areas precisely. Filopodia broke the CP substrate into pieces and transported them to the cell body by capturing them at the tip. Lamellipodia destroyed the CP substrate, and transported it to the cell body by capturing small particles in a mass. This study suggests two functions of filopodia and lamellipodia in mature osteoclasts, i.e., the mechanical fragmentation of the CP substrate and the transportation of the CP particles to the cell body.

Keywords: Osteoclast; Filopodia; Lamellipodia; Calcium phosphate; Video-enhanced contrast-differential interference contrast microscopy

1. Introduction

Osteoclasts are multinucleated cells whose primary function is bone resorption. In the active phase, they are polarized and form specialized cell membrane areas, a ruffled border and a sealing zone, facing the mineralized bone matrix. Acid and proteases are secreted into the resorption lacuna between the ruffled border membrane of the osteoclast and the bone surface (Baron et al., 1988; Vaananen et al., 1990). In other respects, osteoclasts are highly migrating cells with pseudopodia that are called filopodia or lamellipodia due to their morphology. Pseudopodia play a role in cell adhesion and migration (Chodniewicz and Klemke, 2004). Osteoclasts alternate between motile and resorptive phases of activity, both of which are necessary for effective bone remodeling (Kanehisa and Johan, 1988). The motile phase is characterized by lamellipodial movement, extension and retraction (Arkett et al., 1994). Except for migration, however, the other functions of filopodia and lamellipodia including participation in the resorption of bone are unknown.

Video-enhanced contrast-differential interference contrast (VEC-DIC) microscopy has provided high resolution to assess the ultrastructure of living cells (Allen et al., 1981), and has revealed physiological responses in tissues maintaining their normal structure (Terakawa et al., 1991). Recently, in order to evaluate the function of osteoclasts in the resorbing phase, cells are cultured on calcium phosphate (CP)-coated quartz coverslips in place of a thin bone slice (Kajiya et al., 2003; Lees et al., 2001). The resolution of the image in the VEC-DIC microscopy remains high quality using CP-coated quartz coverslips compared with that in the DIC microscopy with a bone slice. In this study, we analyzed the function of filopodia and lamellipodia in mature osteoclasts cultured on CP-coated quartz coverslips using VEC-DIC microscopy.

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2. Materials and methods

2.1. Materials

For culturing cells, α-minimum essential medium (α-MEM), fetal bovine serum (FBS), and antibiotics (penicillin, streptomycin) were obtained from Gibco BRL (Burlington, ON, Canada). Hepes was purchased from Sigma (St. Louis, MO, USA).

2.2. Osteoclast isolation and cell culture

All procedures involving animal treatment were approved by the Council on Animal Care at the Hamamatsu University School of Medicine. The procedure used to isolate the rabbit osteoclasts was modified from the original method developed by Tezuka et al. (1992). The femorae, tibiae, humeri, ulnae and radii were dissected from 2- to 8-day-old Japanese white rabbits (SLC, Hamamatsu, Japan). After removal of the adherent soft tissues, these bones were minced into pieces in α-MEM (pH 7.4) supplemented with 15% FBS and antibiotics (100 μg/ml penicillin, 100 μg/ml streptomycin). The cells were dissociated from the bone fragments by vigorous vortexing. After removal of the bone fragments by sedimentation under normal gravity, the cells were collected from the supernatant by centrifugation. The cells were then dispersed on CP-coated quartz coverslips (Osteologic Coverslips; Millenium Biologix, Kingston, Canada) and incubated in humidified air and 5% CO_2 at 37 °C. After 90 min, non-adherent cells were gently washed off with the culture medium. Osteoclasts were identified as cells containing more than three nuclei. In addition, after completion of the experiments, the cells were stained with tartrate-resistant acid phosphatase (TRAP) using a TRAP staining kit (Hokudo, Sapporo, Japan) to confirm the identity of osteoclasts. All experiments were performed within 4–24 h after cell isolation.

2.3. VEC-DIC microscopy and time-lapse recording

The technique was based on a VEC-DIC system similar to that described previously (Terakawa et al., 1991). After the culture medium was replaced with a medium (standard medium) containing 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES-NaOH (pH 7.3), the osteoclasts were observed under an inverted DIC microscope (TE-300, Nikon, Japan) equipped with a 40× objective lens (Plan Apo, numerical aperture = 0.95) and a 100× objective lens (Plan Apo, numerical aperture = 1.40, oil immersion). The DIC image was captured with a charge-coupled device (CCD) camera (C6489, Hamamatsu Photonics, Hamamatsu, Japan), and then digitized and processed with an image processor (ARGUS-20, Hamamatsu Photonics, Hamamatsu, Japan) for real-time enhancement of the contrast. The processed images were observed on a video monitor screen (14 in., PVM-14M4J, Sony, Tokyo, Japan) and recorded by an image acquisition and analysis system (AQUACOSMOS, Hamamatsu Photonics, Hamamatsu, Japan) at an interval of 3 s. All observations were performed at 35 °C.

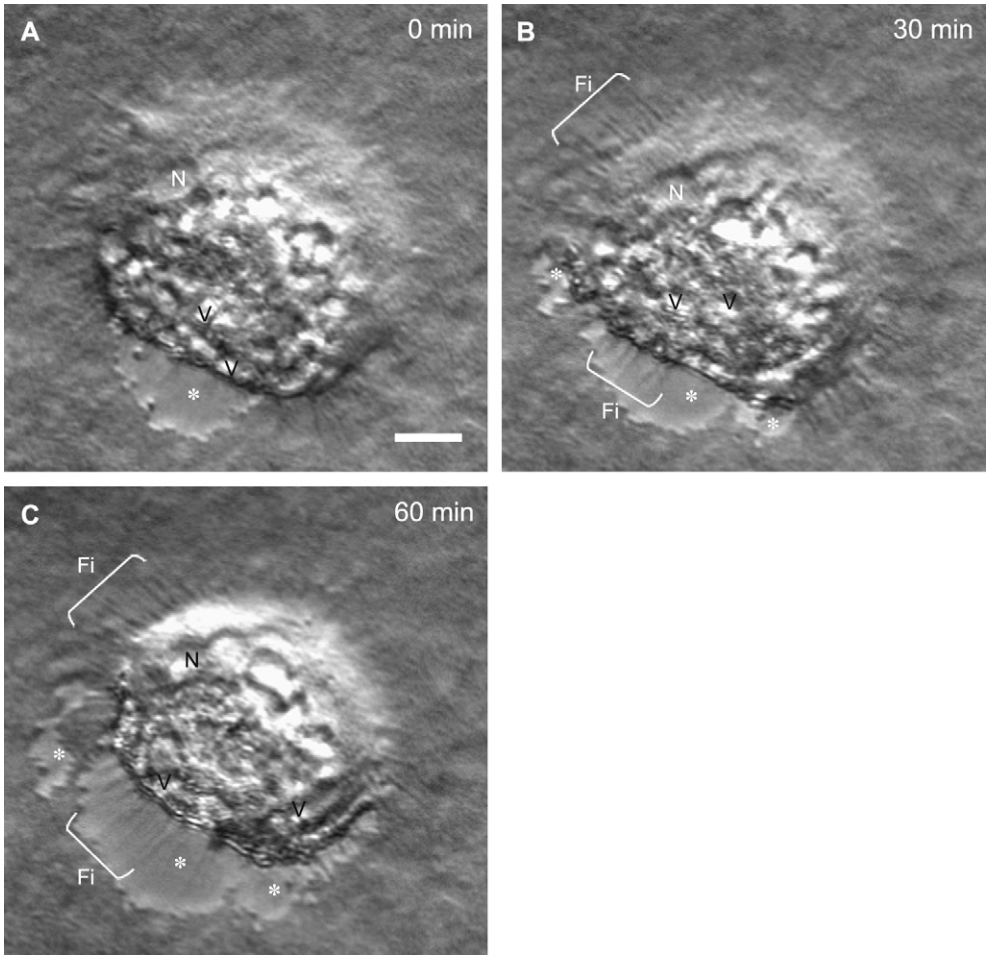

Fig. 1. Video-enhanced contrast-differential interference contrast (VEC-DIC) microscopic images of time-lapse changes in a single osteoclast with a 40× objective lens. A: The initial image for observation, B: after 30 min and C: after 60 min. The asterisks show the resorbed areas. Filopodia (Fi) revealed in the peripheral cell body. N: nuclei. V: vacuole. Scale bar = 10 μm.

2.4. Analyses of filopodial/lamellipodial movement in osteoclasts on CP substrate and measurement of CP-free areas

The cell-forming CP-free area was determined as an osteoclast with resorbing activity and was selected for observation. We observed the morphological change in three osteoclasts, especially filopodial and lamellipodial movement. Lamellipodia and filopodia were distinguished due to the morphological feature. Lamellipodia were defined as the membranous wave form with sheetlike extension of cytoplasm. On the other hand, filopodia were defined as thin fibers that radiated from the cell body. The CP-free areas were measured using photo editing software (Adobe Photoshop 6.0, San Jose, CA, USA). The edges of the CP-free areas that are easily confirmed by contrast with CP substrate were traced on the monitor, and their pixels were automatically counted. The changes in the CP-free area and the relations between the trace of CP particles and the movement of filopodia and lamellipodia were analyzed in a magnified form on the monitor. All observations were performed with an image acquisition and analysis system (AQUACOSMOS).

3. Results

3.1. VEC-DIC microscopic images of morphological changes in a single osteoclast and CP-free areas with a 40× objective lens

Fig. 1 shows the time-lapse analysis for 60 min by VEC-DIC microscope with a 40× objective lens. A CP-free area adjacent to a multinucleated single osteoclast was sharply defined (Fig. 1A). Many thin fibers, filopodia, which radiated from the cell body were visualized both on the CP substrate area and the CP-free area (Fig. 1B). Several nuclei were observed in the cell body away from the CP-free area. On the other hand, several oval-shaped vacuoles, which were found as several different-sized high-intensity spots, were found in

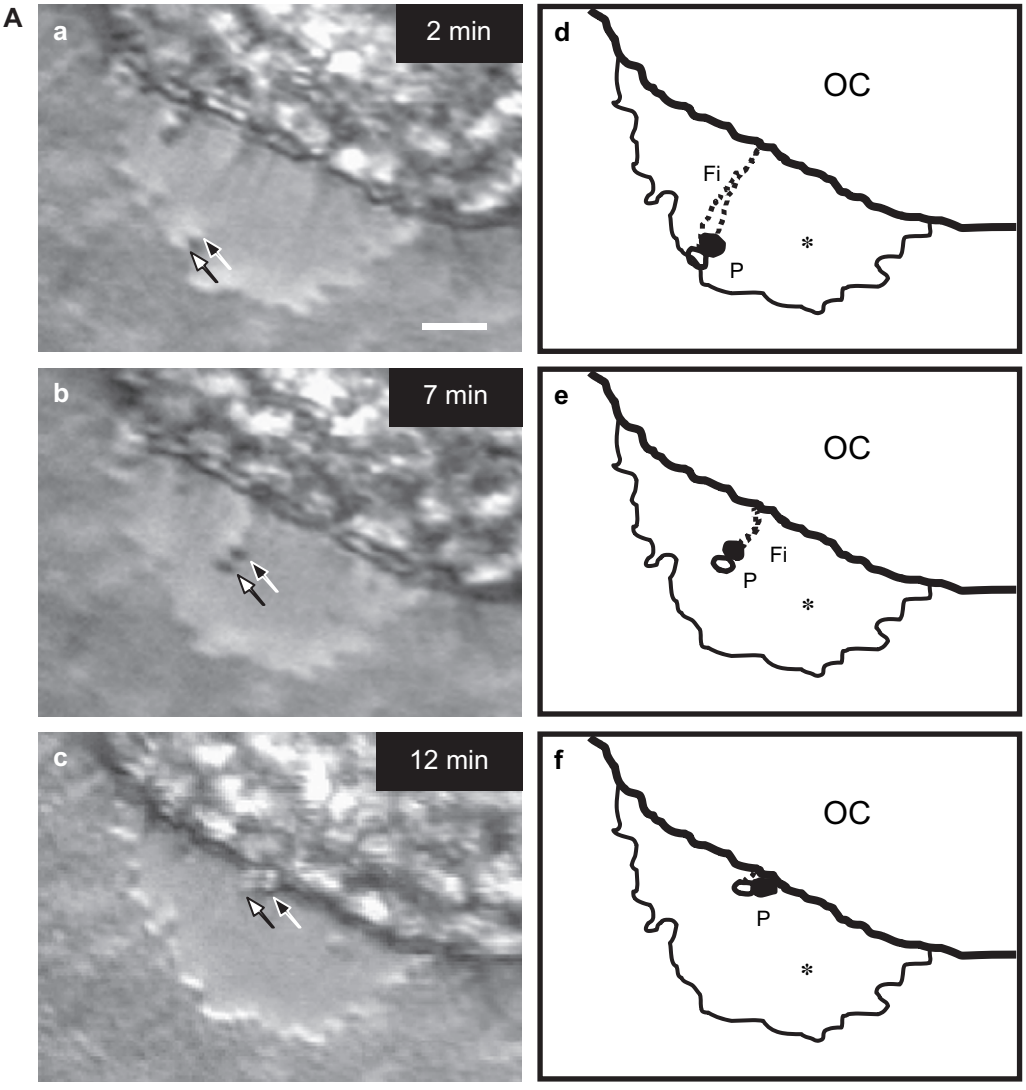

Fig. 2. Filopodia fragmented the CP substrate into small pieces, and transported the particles in three osteoclasts (A, B, and C). A-a: The image after 2 min from the beginning of observation, A-b: after 7 min and A-c: after 12 min. A-d–A-f are schematic representations of the traces of the particles of CP transported by filopodia (shown as the dotted line), and correspond to A-a–A-c, respectively. B-a: The image after 12 min 9 s from the beginning of observation, B-b: after 3 min and B-c: after 6 min. B-d–B-f are schematic representations of the traces of the particles of CP transported by filopodia (shown as the dotted line), and correspond to B-a–B-c, respectively. C-a: The image after 46 min 18 s from the beginning of observation, C-b: after 4 min and C-c: after 8 min. C-d–C-f are schematic representations of the traces of the particles of CP transported by filopodia (shown as the dotted line), and correspond to C-a–C-c, respectively. The black or white arrows show the particles of CP. The asterisk shows the CP-free areas. OC: the cell body of an osteoclast. Fi: filopodia. P: particles of CP. Scale bar = 5 μm.

the cell body near the CP-free area. After 30 min, a couple of CP-free areas appeared near the existing CP-free area (Fig. 1B). After 60 min, the cell body was contracted and the total CP-free area increased over time (Fig. 1C).

3.2. Analysis of filopodial movement in three osteoclasts

Fig. 2A shows sequential images of the magnified views of filopodial movement from 2 to 12 min after the beginning of observation in Fig. 1. Filopodia extended from the cell body, and the tips attached to the edges of the CP substrates (Fig. 2A-a). The filopodia unstuck the particles of CP substrate and transported them to the cell body by capturing them at the tip (Fig. 2A-b). The filopodia retracted until the CP particles stuck to the cell body (Fig. 2A-c). Fig. 2B, C show sequential images of the magnified views of filopodial movement in another two osteoclasts from 12 min 9 s to 18 min 9 s and 46 min 18 s to 54 min 18 s after the beginning of observation, respectively. In this transportation process, the particles moved by distances of 9.2 μm in 10 min (Fig. 2A), 11.0 μm in 6 min (Fig. 2B), and 7.0 μm in 8 min (Fig. 2C), respectively. The speed of the transportation process in Fig. 2A is nearly constant (Fig. 3A). Also, the speeds of the transportation process in Fig. 2B, C are almost constant (Fig. 3B, C). In addition, the speed of the transport process of particles of CP by lamellipodia is nearly constant alike (Fig. 3D).

3.3. Quantitative analysis of the CP-free areas under the cell body using VEC-DIC microscopic images with a 100× objective lens

The CP-free areas under the cell body were brought into focus under a 100× objective lens, leaving the cell body of the osteoclast out of focus. In Fig. 4A, the shape of the cell was

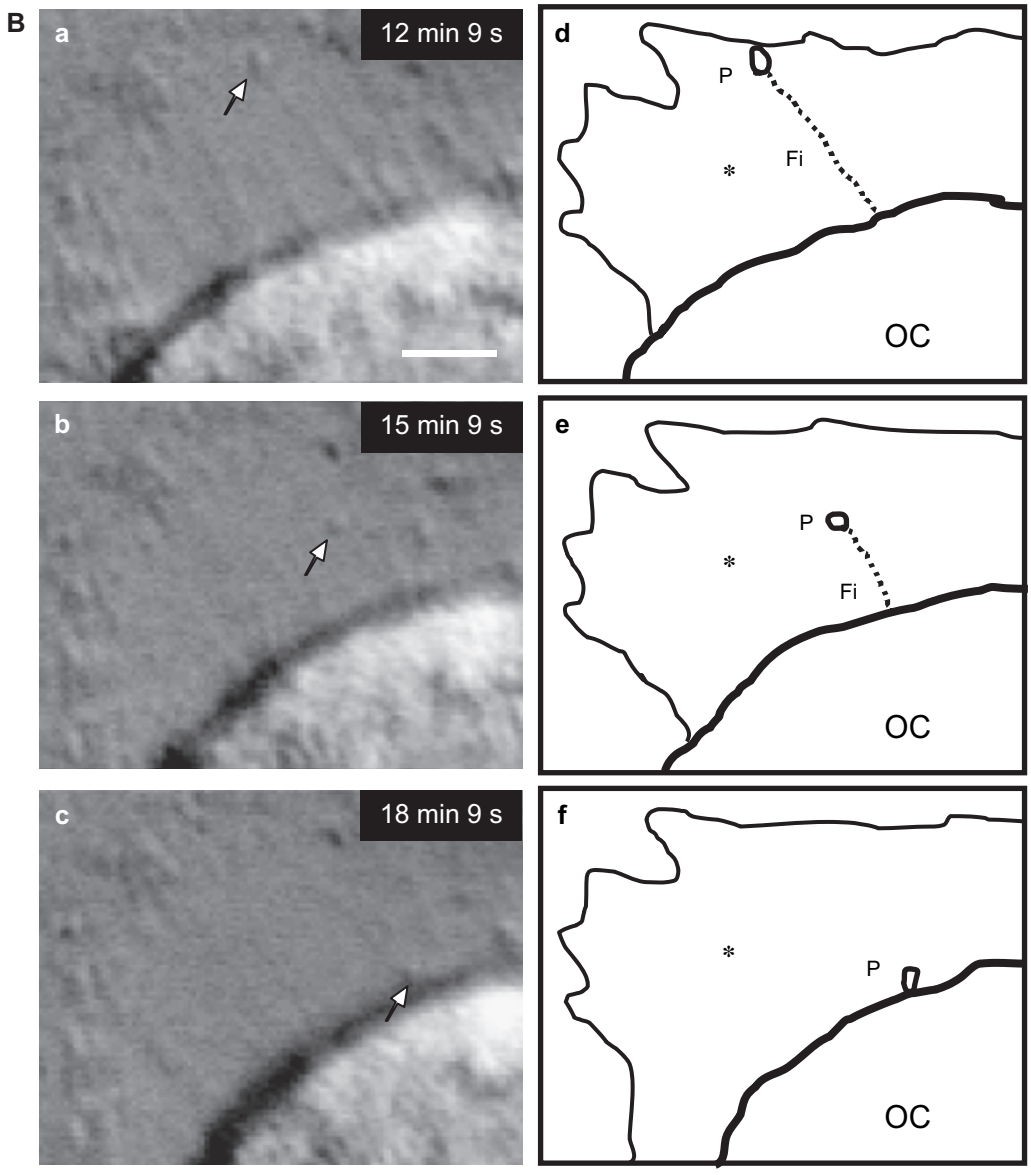

Fig. 2 (*continued*).

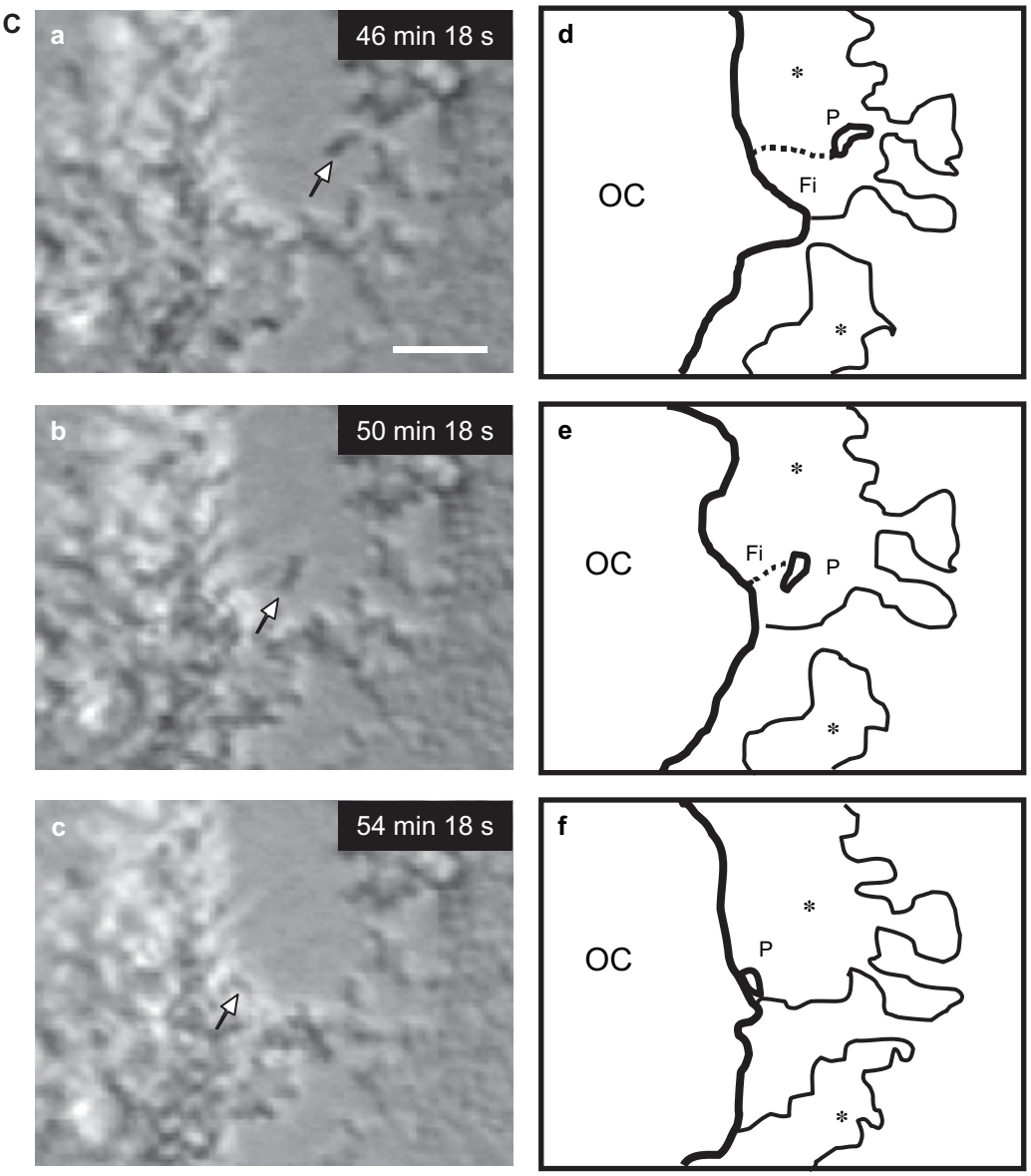

Fig. 2 (*continued*).

blurred, and the single CP-free area under the cell body, which was 195 μm^2, was clearly visualized. After 30 min (in Fig. 4B), it increased to 279 μm^2, and increased to 417 μm^2 after 60 min (in Fig. 4C). Finally, it increased to 647 μm^2 after 120 min (in Fig. 4D). Fig. 5 shows the relationship between the time course of the process in a single osteoclast and quantitative analysis of the CP-free area. The cell bodies that were not clearly visualized migrated toward the right-hand side during this process.

3.4. Analysis of lamellipodial movement in a single osteoclast

Fig. 6 shows time-lapse images starting from 66 min after the beginning of observation for 30 min, whose views were magnifications of lamellipodial movement from Fig. 4C through D. Lamellipodia spread over the CP substrate (Fig. 6A), and then the left-hand part of the lamellipodia fragmented the substrate into small particles (Fig. 6B). The CP particles were transported with the retraction of the lamellipodia to the cell body (Fig. 6C). The left-hand part of the lamellipodia retracted and the middle part of the lamellipodia started to retract with fragments of the CP substrate (Fig. 6E). In the final phase of this process, the middle and right-hand parts of the lamellipodia retracted toward the cell body to accumulate most of the CP particles and attached to the surface of the cell body (Fig. 6F). When the lamellipodia retracted completely, all the particles accumulated on the surface of the cell body (Fig. 6G).

4. Discussion

We observed the function of filopodial and lamellipodial movement in mature osteoclasts cultured on CP-coated quartz coverslips and the formation of CP-free areas by VEC-DIC

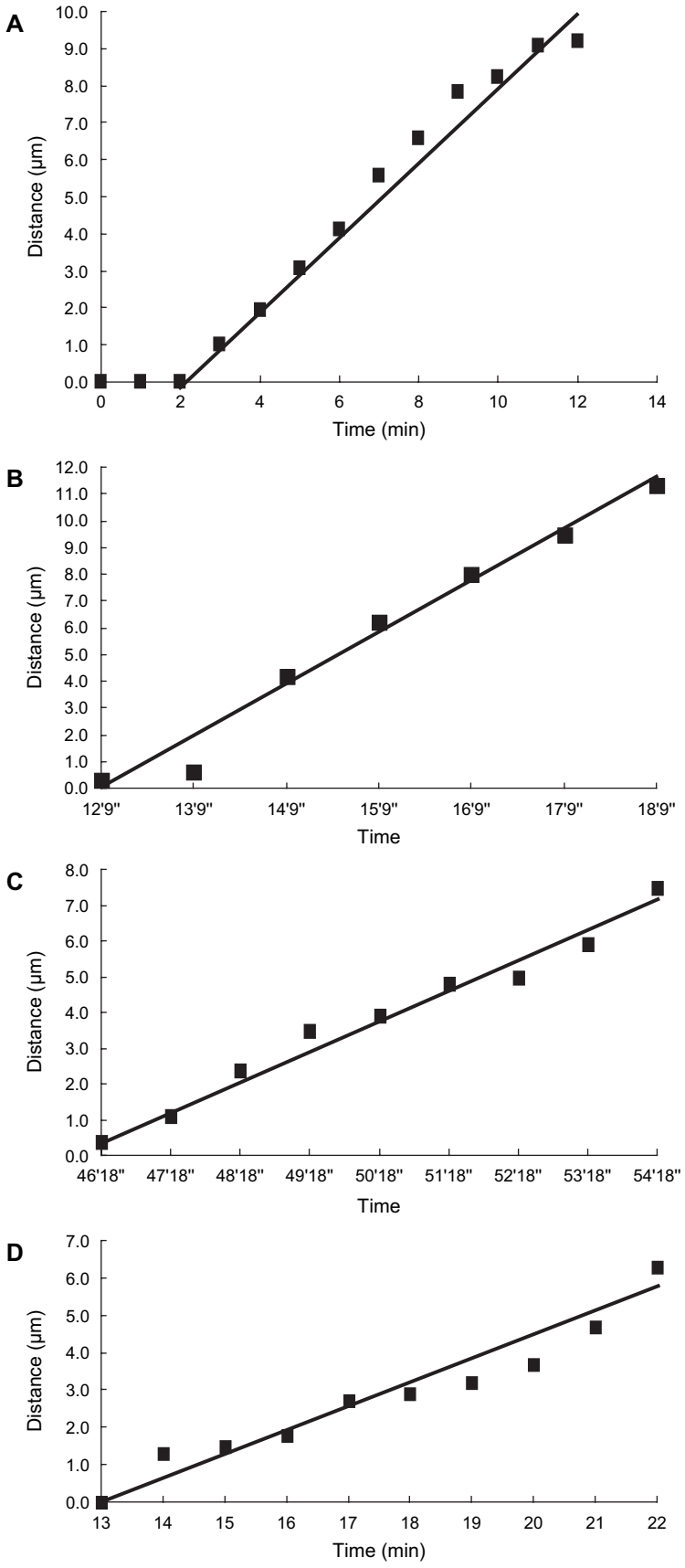

Fig. 3. The time-lapse transport process of particles of CP by filopodia (A–C) and the time-lapse transport process of particles of CP by lamellipodia (D). The horizontal axis indicates the time of observation, and the vertical axis indicates the distance from the point of the CP particles separating.

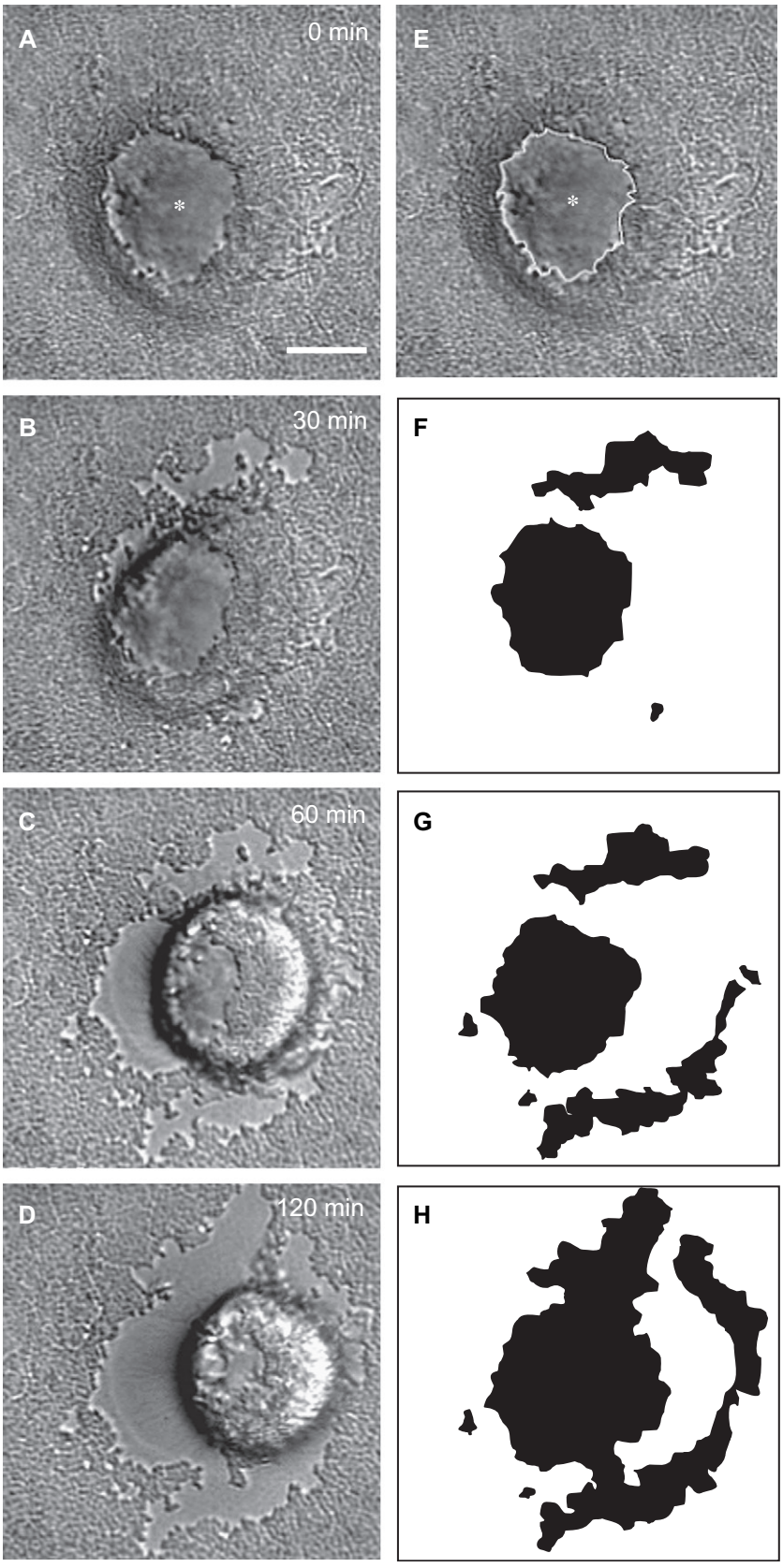

Fig. 4. VEC-DIC microscopic images of a single osteoclast and CP-free areas with a 100× objective lens, and the measurement of CP-free areas. A: The initial image for observation, B: after 30 min, C: after 60 min, and D: after 120 min. The asterisk shows the CP-free areas under the cell body (A and E). The shaded areas in F–H are the CP-free areas shown in B–D, respectively. Although the shape of the cell body is blurred, the location of the cell body can be recognized. Scale bar = 10 μm.

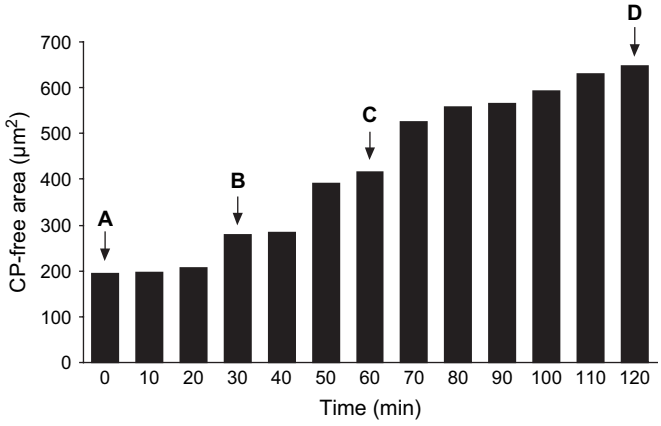

Fig. 5. The change in the CP-free area every 10 min in the image of Fig. 4 from 0 to 120 min. The CP-free area increased about 3.3 times after 120 min. A, B, C and D correspond to those in Fig. 4, respectively.

microscopy. Our study thereby showed a novel finding: mature osteoclasts have the functions of mechanical fragmentation and transportation of the CP substrate by filopodial and lamellipodial movement.

VEC-DIC microscopy provided a means of assessing dynamic changes in the morphology of the living cell, and revealed physiological responses, for example, exocytosis activities, maintaining its normal structure in the tissue (Ishihara et al., 2000; Sakurai and Terakawa, 1995; Terakawa et al., 1991). Several reports have described the behavior of osteoclasts *in vitro* on bone or on an artificial substrate, i.e., glass or plastic, using light microscopy combined with time-lapse recording (Chambers and Magnus, 1982; Kanehisa and Johan, 1988; Warshafsky et al., 1985). We used CP-coated quartz coverslips for the observation of osteoclasts with activities. Kajiya et al. reported the inhibitory mechanisms of proton extrusion by calcitonin in resorbing osteoclasts cultured on the same coverslips (Kajiya et al., 2003). Espinosa et al. observed morphological changes in osteoclasts by the oscillatory activation of a calcium-dependent potassium current using a DIC microscope, but the cells were cultured on conventional coverslips (Espinosa et al., 2002).

In this study, many vigorous vacuoles were observed in the area where the fillopodial activity seemed to be high in the osteoclast cell body using a 40× objective lens. Nuclei were clearly observed in the other area in the osteoclast cell body. Their findings suggest that the activity of mechanical fragmentation and transportation of the CP-substrate by fillopodia and lamellipodia might be polarized in the horizontal surface of the CP substrate.

Kanehisa et al. reported that the rate of migration was not constant, and that no resorption occurred during migration, as a result of an investigation on the migration and resorption of osteoclasts cultured on dentine slices using a phase-contrast microscope (Kanehisa and Johan, 1988). However, the CP-free areas increased on the CP-coated coverslip, while osteoclasts continued to migrate in this study. The mechanical fragmentation and transportation of the CP-substrate might not exactly the same function as the bone resorption by the osteoclast cultured on artificial CP-coated coverslips.

We clearly recorded filopodial movement in mature osteoclasts by VEC-DIC microscopy in this study. The observation of filopodial movement by VEC-DIC microscopy has already been reported in other cells, chromaffin cells and PC 12 cells, in reaction to electrical stimulation (Manivannan and Terakawa, 1994). In this study, filopodia fragmented the CP substrate into pieces, and they transported the particles to the cell body by capturing them at the tip in this observation. Moreover, we observed that the filopodia retracted completely until the CP particles adhered to the cell body and performed the measurements of the speed of the transportation process on three osteoclasts, which was a kinetic profile of filipodial CP transport and such kinetics might be heterogeneous varying from cell to cell. We also observed that the lamellipodia fragmented the CP substrate into small pieces and transported the pieces in a mass to the cell surface. These functions of filopodia and lamellipodia in mature osteoclasts have not been reported before. Bone degradation products are endocytosed from the ruffled border membrane, and transcytosed through the osteoclast to the basolateral membrane (Nesbitt and Horton, 1997; Salo et al., 1997). Because the ruffled border was not identified in this study using CP coated on single-layer quartz coverslips, the functions of filopodia and lamellipodia showed in this study might not be related to the bone resorption process occurred in the ruffled border which is morphologically similar to pseudopodia.

We analyzed the CP-free areas not only in the peripheral region of the cell body but also under the cell body, which were brought into focus under a 100× objective lens leaving the cell body of the osteoclast out of focus. This method allows the quantitative analysis of the activity of a living mature osteoclast. Furthermore, the morphological reaction of the osteoclast to several therapeutic agents for the osteoporosis could be evaluated in a living mature osteoclast.

The transportation by filopodia had the feature of attaching their tips to the particles and small particles of substrate being captured in a mass and transported to the cell body by retraction of the lamellipodia. In general, the motility of filopodia involves actin bundles, and the motility of lamellipodia involves the actin network (Upadhyaya and van Oudenaarden, 2003). The difference in this characteristic pattern between filopodia and lamellipodia in the mechanical fragmentation and transportation of the CP substrate observed in this study might be related to not only the method of artificially coating the CP substrate in a single layer on the coverslip but also the structure of the actin.

In conclusion, this study showed that filopodia and lamellipodia in mature osteoclasts might have the function of mechanical fragmentation and transporting the CP-substrate, in addition to the function of cell migration on the bone surface. Analysis of the CP-free areas under the cell body using VEC-DIC microscopic images might enable a quantitative evaluation of the morphological reaction of the osteoclast to several therapeutic agents for the osteoporosis.

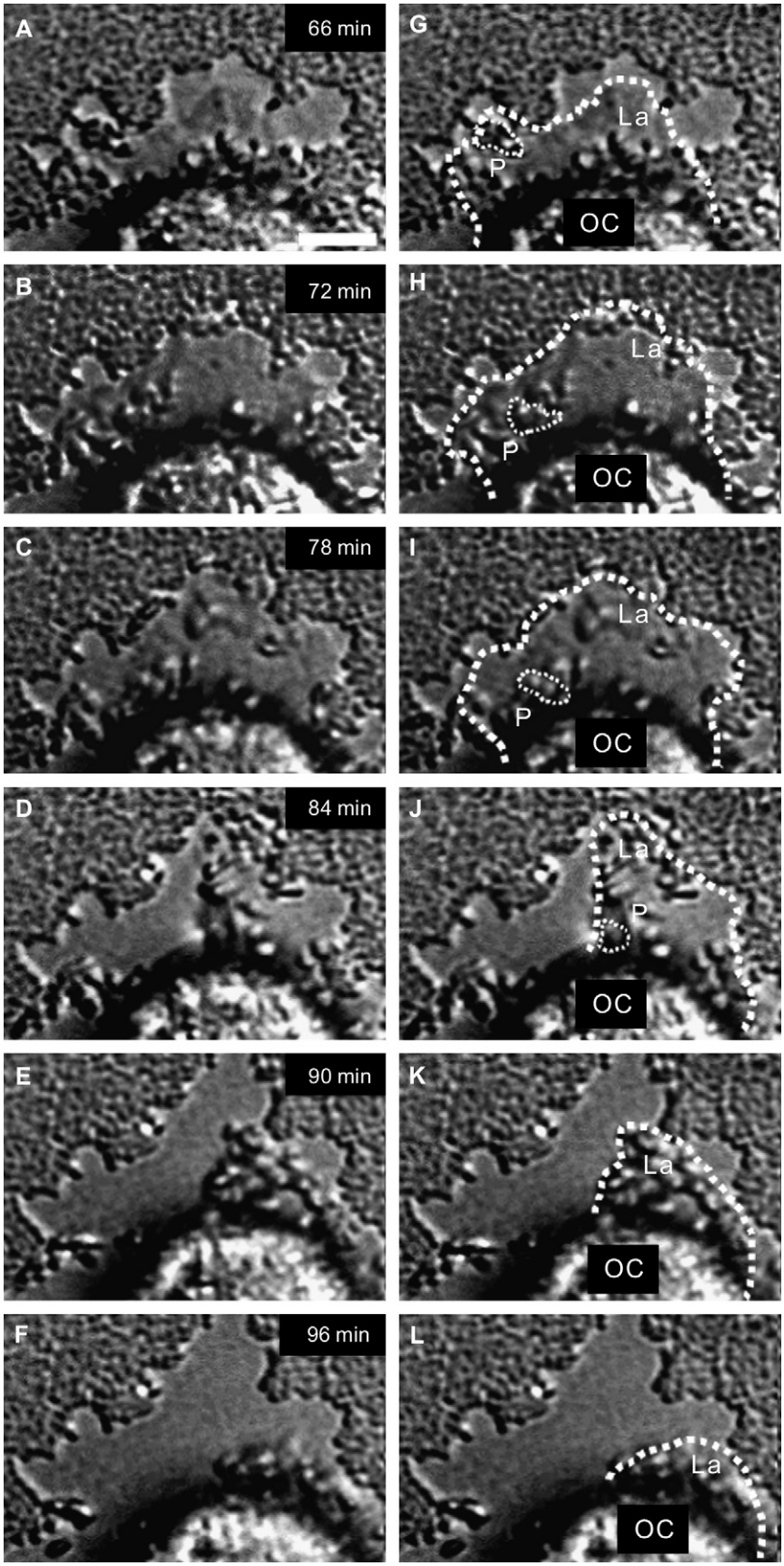

Fig. 6. Time-lapse analysis of lamellipodial movement that destroys the CP substrate, and transports the CP particles in a mass to the osteoclast cell body. A: The image after 66 min from the beginning of observation, B: after 72 min, C: after 78 min, D: after 84 min, E: after 90 min, and F: after 96 min. The lamellipodia are traced (shown as the white dotted line) in G, H, I, J, K and L, respectively. The dotted circles show the same mass of CP particles. La: lamellipodia. P: CP particles. OC: cell body of an osteoclast. Scale bar = 10 μm.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cellbi.2007.03.032.

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