Two types of bone resorption lacunae in the mouse parietal bones as revealed by scanning electron microscopy and histochemistry

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Summary. To understand the bone resorption process on the basis of the morphology of bone resorption lacunae, the inner surface of parietal bones in juvenile mice was exposed with a treatment of ultrasonic waves or NaOCl treatment and examined by scanning electron microscopy (SEM).

The bone resorption lacunae were divided into two types (I and II) according to differences in morphological features of their walls; the wall of type I lacunae was covered with loose collagen fibrils, while that of type II lacunae was smooth with almost no fibrillar structures. Collagen fibrils in type I lacunae treated with ultrasonic waves differed in appearance from those treated with NaOCl; the collagen fibrils were thin and displayed a smooth surface in type I lacunae treated with ultrasonic waves, while they were thick and showed a rough surface in those treated with NaOCl—probably because superficial uncalcified collagen fibrils were digested with the chemical. The results indicated that type I lacunae occupied 77% of all of the bone resorption lacunae treated with ultrasonic waves, but 51% of those treated with NaOCl. This finding led to the idea that type I lacunae can be subdivided into two: lacunae (Ia), covered with partially calcified fibrils as well as superficial uncalcified fibrils; and lacunae (Ib), covered only with uncalcified fibrils. The

presence of uncalcified fibrils in the bone resorption lacunae was further confirmed by backscattered electron (BSE) imaging of SEM.

Histochemistry for acid phosphatase or immuno-histochemistry for cathepsin B or carbonic anhydrase in combination with SEM revealed that type I lacunae were located under osteoclasts but type II lacunae were not. These findings indicate that type I lacunae are in the process of bone resorption by osteoclasts, while type II lacunae are in the final stage of bone resorption and free from osteoclasts. Bone resorption may thus proceed in the order of Ia, Ib, and II.

Introduction

Bone growth is determined by a balance between bone formation by osteoblasts and bone resorption by osteoclasts on the bone surface (Jee, 1988). Previous studies on the bone resorption process (Delaisse and Vaes, 1992; Vaananen and Horton, 1995; Sasaki, 1996; Salo *et al.* 1997; Teitelbaum, 2000; Vaananen *et al.*, 2000) revealed that the osteoclast has microvillous structures (*i.e.* the ruffled border) surrounded by a ring-like perimeter of cytoplasm (the clear zone) at the bottom. Since this cell tightly attaches to the bone surface with the clear zone, a closed microenvironment for bone resorption is produced on the bottom of the cell, where proton and proteolytic enzymes are secreted from the ruffled border to dissolve the mineral matrix and to degrade collagen fibrils respectively, thus forming a resorption lacuna on the bone surface.

The shapes of bone resorption lacunae have been studied mainly by scanning electron microscopy (SEM) from various viewpoints (Abe *et al.*, 1983, 1984, 1990; Ali *et al.*, 1984; Chambers *et al.*, 1984). It has been established

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that resorption lacunae differ in shape between juvenile and adult mice (Abe *et al.*, 1984, 1990); the bone resorption lacunae in juvenile mice show spoon-cut concavities, while those in adult mice show featherlike patterns as a whole. This difference in shape of the bone resorption lacunae has been attributed to the difference in bone resorption activities of the osteoclasts. However, it is still unknown how the shape of the resorption lacunae changes three-dimensionally during the formation of these lacunae by osteoclasts.

Thus, the aim of the present study was to clarify morphologically the bone resorption process. For this purpose, we examined the bone resorption lacunae in the rapidly growing calvarium—which has a great number of bone resorption lacunae on the inner surface—by SEM after the removal of the overlying cells on the bone surface. We then obtained backscattered electron (BSE) images of the same portion to confirm the presence of uncalcified collagen fibrils on the surface of the calcified collagen fibrils exposed in the bone resorption lacunae. We also investigated histochemically and immunohistochemically the distribution of osteoclasts in relation to the morphological features of bone resorption lacunae. This paper therefore discusses the two types of bone resorption lacunae which are produced during the bone resorption by osteoclasts.

Materials and Methods

Scanning electron microscopy

Twenty-eight dd-mice at one week of age were used. The animals were killed by deep anesthesia with ether, and their parietal bones were taken from the head and freed of the dura mater and periosteum with fine forceps under a binocular microscope. Bone resorption lacunae were exposed by the following various treatments.

Ultrasonication treatment

In order to observe the collagen fibrils and bone matrix at the bottom of the bone resorption lacunae under the cells covering the bone surface, parietal bones were washed three times with ultrasonic waves in 0.1% Triton-X100 in distilled water for 15 sec, fixed in 2% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.4) for 1 h, and soaked in 3.5% EDTA in distilled water overnight. They were then immersed in 1% OsO₄ in distilled water for 1 h, 2% tannic acid in distilled water at 4°C for 1 h, 1% OsO₄ for 1 h again, washed with distilled water, dehydrated through a graded series of ethanol, and transferred into isoamyl acetate.

They were dried in a critical point dryer (HCP-AUTO, Hitachi, Tokyo) with liquid CO₂, placed inner side up on an aluminum stub, coated with osmium vapor using an osmium coater (NL-OPC80A, Nippon Laser & Electronics Lab., Tokyo), and observed in a SEM (S-4500, Hitachi, Tokyo) under an accelerating voltage of 10 kV.

NaOCl treatment

Parietal bones were immersed in 5% NaOCl in distilled water for 1 min to dissolve chemically the cells and collagen fibrils. After this treatment, the specimens were processed in the same manner described above and observed by SEM.

Backscattered electron (BSE) imaging

Parietal bones either simply washed with distilled water or treated with 5% NaOCl were directly dehydrated through a graded series of ethanol, transferred into isoamyl acetate, and dried in a critical point dryer with liquid CO₂. They were placed inner side up on an aluminum stub, coated with carbon in a vacuum evaporator (JEE-4B/4C, Joel, Tokyo), and observed by SEM (S-4500, Hitachi) equipped with a microchannel plate detector (GW Electronics Inc., GA, USA) under an accelerating voltage of 5 kV.

Panorama SEM photographs

Panorama SEM photographs of bone resorption areas on the bone surface treated with ultrasonic waves were taken at random at 600–800 times magnification. Bone resorption lacunae were analysed in photographs enlarged 900–1200 times, and the areas of the bone resorption lacunae were measured from the parietal bones in 4 mice. The number of the lacunae examined was 300–350 in each bone.

A few sets of panorama SEM photographs covering large portions of bone resorption areas on the bone surface treated with NaOCl were also taken at 300–450 times magnification in each bone, and the area of the lacunae was calculated from photographs enlarged 450–675 times from parietal bones in 4 mice. The number of the lacunae examined was 381–468 in each bone.

Panorama BSE images of bone resorption areas were taken at 400–600 times magnification. Bright and dark bone resorption lacunae were distinguished, and their proportions in the parietal bones in 4 mice were obtained. The number of the lacunae examined was 371–453 in each bone.

Fig. 1. SEM photograph of the inner surface of the NaOCl-treated parietal bone. Bone formation (F) and bone resorption (R) areas are shown. The bone resorption area consists of bone resorption lacunae.

Histochemistry and immunohistochemistry

Twelve dd-mice at one week of age were killed by deep anesthesia with ether and their parietal bones taken from the head were prepared as follows.

Histochemistry for acid phosphatase by azo-dye method

The parietal bones were fixed in 10% formalin in a 0.1 M cacodylate buffer (pH 7.4) for 30 min, freed mechanically of the dura mater and periosteum, and incubated in a medium containing naphtol AS-BI phosphate by an azo-dye method using pararosanilin for acid phosphatase (Barka and Anderson, 1963). Areas where stained spots were detected on the inner surface were photographed at 40 times magnification with a light microscope.

Immunostaining for carbonic anhydrase and cathepsin B

The parietal bones were fixed in 4% paraformaldehyde for 1 h, freed of the dura mater and periosteum, and then immersed in 5% non-fat dry milk in PBS for 1 h to inhibit non-specific staining. Some specimens were incubated with a rabbit anti-carbonic anhydrase antibody (Rockland, PA, USA) at room temperature for 3 h and then immersed in Alexa Fluor 568-conjugated goat anti-rabbit IgG (Molecular Probes, OR, USA) at room temperature for 1 h. After washing 3 times with PBS, the specimens were incubated at room temperature overnight with a FITC-conjugated sheep anti-cathepsin B antibody (Biogenesis Ltd, England). The other specimens were incubated at room temperature overnight with a FITC-conjugated sheep anti-cathepsin B antibody and propidium iodide (Wako Pure Chemical Industries, Tokyo) for nuclear staining for 1–

Fig. 2. SEM photographs of the bone resorption lacunae on the ultrasonicated bone surface. **a:** Two types of bone resorption lacunae. Type I lacunae (I) have loosely arranged collagen fibrils of irregular orientation at the bottom. Type II lacunae (II) show a smooth bottom. **b, c:** Collagen fibrils in a type I bone resorption lacuna. The fibrils show a smooth surface (**b**). The tips of the fibrils are tapered (**c**).

2 min. The specimens were mounted in 90% glycerin and observed with a confocal laser scanning microscope (MRC-1024, Bio Rad Lab, Tokyo).

SEM of histochemically or immunohistochemically stained samples

The parietal bones treated after the azo-dye method for acid phosphatase or immunohistochemical method for cathepsin B were washed with a water jet to remove osteoclasts. They were treated with the ultrasonic wave as described above. The same areas photographed with a light microscope or confocal laser scanning microscope were observed by SEM at 500 times magnification.

Results

Observation of parietal bones by conventional SEM

The inner surface of ultrasonicated or NaOCl-treated parietal bones consisted of smooth and rough areas; the smooth areas were considered to be bone formation areas, while the rough areas apparently represented bone resorption areas (Fig. 1). Osteocyte lacunae and openings for vascular channels were scattered in both smooth and rough areas; the osteocyte lacunae were oval in shape, 10–20 μm in diameter, showing many small openings of bone canaliculi. Rough areas were usually found in the periphery of the parietal bone. Rough areas were also present in the center of the bone, where rough areas of various sizes were spotted irregularly. Bone resorption lacunae showing spoon-cut concavities oval,

Fig. 3. SEM photographs of the bone resorption lacunae on the NaOCl-treated bone surface. **a:** Two types of bone resorption lacunae. Type I lacunae (I) have loosely arranged collagen fibrils maintaining their original orientation. Type II lacunae (II) show a smooth bottom. A few of type II lacunae have fibrous structures in a limited part of the bottom (II*). **b, c:** Collagen fibrils in type I bone resorption lacunae. The fibrils have a rough surface (**b**). The fibrils show thick blunt ends (**c**).

Fig. 4. Proportions (% mean and S.D.) of two types of bone resorption lacunae on the inner surface of the parietal bones. **a:** Ultrasonic wave treatment. **b:** NaOCl treatment. White columns are the proportions of type I lacunae, and black columns are those of type II lacunae.

Fig. 5. BSE image of the inner surface of the water jet-treated parietal bone. Bone formation (F) and bone resorption (R) areas are shown. The arrow shows a dark opening of vascular channels.

Fig. 6. BSE images at a higher magnification of the bone resorption area on the inner surface of the parietal bones. **a:** Water jet treatment. Bone resorption lacunae appear bright or dark. The arrow shows a bright osteocytic lacuna. **b:** NaOCl treatment. Only bright bone resorption lacunae appear.

Fig. 7. Proportions (% mean and S.D.) of bright and dark bone resorption lacunae on the inner surface of the water jet-treated parietal bones by BSEM. White column is the proportion of bright lacunae, while black column is that of dark lacunae.

polygonal, or irregular in shape were accumulated in these areas, and each lacuna was bordered by ridges with neighboring resorption lacunae. The diameter of the bone resorption lacunae ranged from 8 to 30 μm , most of them being 10 to 20 μm .

According to the morphological features of the bottoms, the bone resorption lacunae were classified into two types: type I and type II. The bottom of type I lacunae showed loose collagen fibrils, while the bottom of type II lacunae appeared smooth. The structures of the bone resorption lacunae were also different depending on the preparation techniques, as will be described in detail below.

Bone resorption lacunae treated with ultrasonic waves

Type I lacunae had loosely and delicately arranged collagen fibrils which ran three-dimensionally in irregular directions (Fig. 2a). The individual fibrils had a smooth surface and diameter of 30–45 nm (Fig. 2b), and the spaces between the interlaced collagen fibrils were wide. The fibrils often protruded from the lateral wall of the bone resorption lacunae and formed tapered tips in the lacunae (Fig. 2c).

On the other hand, type II lacunae had a smooth bottom without apparent fibrous structures, although a few type II lacunae had fibrous structures in a limited part of their bottom. The type II lacunae were usually scattered among type I lacunae (Fig. 2a). No intermediate types between type I and II were found.

The proportion of the type I to type II lacunae was $77 \pm 3\%$ (mean $\pm$ S.D.) to $23 \pm 3\%$. (Fig. 4a).

Fig. 8. Light and scanning electron microscopic photographs of the inner surface of the parietal bone. **a:** Light microscopic photograph of the bone surface stained by an azo-dye method for acid phosphatase. Intensely-stained spots (arrow) indicate the location of osteoclasts. **b:** Scanning electron microscopic photograph of the same area as **a**, after the removal of osteoclasts with water jet treatment. These two photographs show that type I lacunae (I in **b**) are situated under osteoclasts.

Bone resorption lacunae treated with NaOCl

Type I lacunae had collagen fibrils with irregular orientations at the bottom of the bone lamellae (Fig. 3a). These fibrils were somewhat irregular in shape with a diameter of 60–75 nm (Fig. 3b). Fibrils protruding from the lateral wall of the lacunae were also observed in this treatment although they showed thick blunt ends (Fig. 3c).

Type II lacunae typically had a smooth bottom except for a few of type II lacunae (almost 5%) which had fibrous

Fig. 9. Double fluorescent staining for carbonic anhydrase and cathepsin B in the inner surface of the parietal bone. **a:** Carbonic anhydrase, **b:** Cathepsin B. The sites of carbonic anhydrase invariably correspond to those of cathepsin B.

structures in a limited part of their bottom (Fig. 3a).

The proportions of type I to II in the bones from 4 mice were $51 \pm 1\%$ (mean $\pm$ S.D.) to $49 \pm 1\%$ (Fig. 4b).

BSE images of bone resorption lacunae by SEM

In BSE images of the water jet-treated, undecalcified parietal bones, osteocyte lacunae appeared bright in both the bone formation and resorption areas. Most—but not all—of the bone formation areas were also bright. Collagen fibrils were observed distinctly in the bone formation areas. In contrast, bone resorption lacunae were also observed as bright areas, but dark lacunae were scattered among the bright lacunae (Fig. 5).

Fig. 10. Confocal laser and scanning electron microscopic photographs of the inner surface of the parietal bone. **a:** Confocal laser microscopic photograph of the bone surface stained for cathepsin B. Stained spots (arrow) indicate the location of osteoclasts. **b:** Scanning electron microscopic photograph of the same area as **a**. These two photographs show that type I lacunae (I in **b**) are situated under osteoclasts.

At a higher magnification, the dark lacunae were recognized as type I because they exhibited collagen fibrils interlaced at the bottom. Bright lacunae showing interlaced fibrils at the bottom were also recognized as type I (Fig. 6a). The other bright lacunae showed collagen fibrils running in parallel at their bottom; these lacunae were type I or type II. In summary, type I lacunae consisted of both dark and bright lacunae, while type II lacunae consisted of only bright lacunae. However, bone resorption lacunae after NaOCl treatment had only bright bottoms (Fig. 6b).

The proportion of the bright lacunae to the dark lacunae in bone resorption areas was $70 \pm 3.6\%$ (mean $\pm$ S.D.) to $30 \pm 3.6\%$ (mean $\pm$ S.D.) in the specimens treated with a water jet (Fig. 7).

The relationship between osteoclasts and bone resorption lacunae

In the specimens stained with the azo-dye method for acid phosphatase, the stained areas were scattered on the inner surface of the parietal bones. These areas were generally stained weakly, with some intensely-stained spots (Fig. 8a). These spots were oval, polygonal, or irregular in shape, 20–30 μm in diameter. By changing the focus of the light microscope, negative images of multinuclei were recognized in the intensely-stained spots, indicating that these spots were osteoclasts. Comparison of light microscopic photographs with SEM photographs showed that all of type I bone resorption lacunae were situated under the intensely-stained spots (Fig. 8). Double fluorescent staining for carbonic anhydrase and cathepsin B also revealed that the sites of the spots stained for carbonic anhydrase invariably corresponded to those of the spots stained for cathepsin B (Fig. 9). Immunohistochemical staining for cathepsin B revealed that the stained spots were scattered in the negative background, appearing polygonal, oval or round in shape. The stained spots were identified as osteoclasts by multinuclei stained with propidium iodide. Comparison of confocal laser scanning microscopic photographs on the SEM photograph also showed that all of type I bone resorption lacunae were situated under the osteoclasts (Fig. 10).

Discussion

The present study has revealed that bone resorption lacunae can be divided into two types according to differences in their morphological features as shown by SEM. Type I lacunae had a rough surface due to the presence of loose collagen fibrils on their walls, while type II lacunae were smooth in surface at the bottom. Furthermore, we have proven morphologically the presence of type I lacunae with calcified collagen fibrils as well as those with uncalcified fibrils.

Murrills *et al.* (1989) previously examined the number and area of bone resorption lacunae *in vitro* to determine the bone resorption activities of human osteoclasts which had been cultured on bovine cortical bone slices. Their SEM images revealed two types of bone resorption lacunae distinguished from their morphological features at the bottom: one lacuna was rich in collagen fibrils at the bottom, while the other displayed a smooth surface there. They did not discuss the significance of these two types of lacunae, however. We consider that the three-dimensional nature of the bone surface of the bone resorption lacunae

Fig. 11. Schematic drawings of the bone resorption lacunae treated with ultrasonic waves or NaOCl. There are three kinds of lacunae on the ultrasonicated bone surface. **Ia:** Lacunae with calcified fibrils covered with uncalcified fibrils. **Ib:** Lacunae with uncalcified fibrils only. **II:** Lacunae with almost no fibrillar structures. These **Ia**, **Ib**, and **II** correspond to I, II, and II respectively on the NaOCl-treated bone surface.

changes according to the stage of bone resorption process by osteoclasts. In the present study, we observed the inner surface of the parietal bone in juvenile mice by SEM and demonstrated that the two types of bone resorption lacunae observed *in vitro* by Murrills *et al.* (1989) were actually present on the bone surface of *in vivo* tissue.

It is interesting that collagen fibrils with tapering tips were observed in type I lacunae treated with ultrasonic waves, but not in those treated with NaOCl. This finding indicates that the tapering tips of collagen fibrils are dissolved with NaOCl because they are uncalcified. We therefore consider that the wall of the type I lacunae is composed of superficial decalcified and deep calcified collagen fibrils, and that the tapering tips of the uncalcified fibrils are probably produced by digestion by osteoclastic enzymes in the lacuna. Previous studies have suggested that osteoclasts form bone resorption lacunae by the secretion of proton for decalcification followed by the secretion of enzymes for degradation of the collagen fibrils. Our findings support the idea that proton first dissolves apatite crystals in the bone matrix between the collagen fibrils and then the apatite crystals covering the fibrils.

Collagen fibrils in lamellar bones are known to be arranged parallel in each lamella and change their

orientation in the successive lamellae (Jee, 1988). However, we demonstrated that collagen fibrils in type I lacunae oriented randomly in specimens treated with ultrasonic waves. It is possible that superficial decalcified fibrils in type I lacunae may become oriented more irregularly by ultrasonic wave treatment. On the other hand, Domon *et al.* (2001) observed by transmission electron microscopy that collagen fibrils under the ruffled border are arranged loosely and oriented irregularly after decalcification. This suggests that the decalcified and degraded fibrils are actually rather irregular in arrangement in the resorption lacunae.

Hasizume *et al.* (1997) examined bone surfaces by SEM using BSE at low accelerating voltages and showed the presence of bright or dark bone resorption lacunae due to differences in mineral density of the bone surface. BSE makes visible the layer just below the superficial layer, although this depends on the accelerating voltage. We have speculated on the relationships between the types of lacunae and BSE images. Type I lacunae in which deeper calcified collagen fibrils are covered with thin superficial uncalcified collagen fibrils appear bright by BSE because the beam penetrates the thin superficial layer and projects the information of the deeper calcified collagen fibrils. Type II lacunae appear bright as BSE projects the layer of calcified fibrils under the surface. On the other hand, all dark lacunae are type I lacunae because they are covered with thick superficial uncalcified collagen fibrils. This was confirmed by the findings that no dark lacunae were observed in BSE images after the NaOCl treatment that dissolves uncalcified collagen fibrils.

We also showed the difference in proportions of type I lacunae in the total resorption area between the specimens treated with ultrasonic waves and those with NaOCl; type I lacunae occupied 77% of the bone resorption lacunae on the bone treated with ultrasonic waves, while 51% were type I even after NaOCl treatment dissolved the uncalcified collagen fibrils. As described above, uncalcified collagen fibrils are easily dissolved with the NaOCl treatment. Thus, we believe that 26% of the type I lacunae in ultrasonicated bones were very slightly covered with uncalcified fibrils on the surface of smooth calcified matrix while the others were covered densely and thickly with these. The findings described above indicate that there are three kinds of bone resorption lacunae: lacunae with uncalcified fibrils covering deep calcified fibrils (Ia), lacunae with only uncalcified fibrils (Ib), and lacunae without uncalcified fibrils (II).

Our histochemical and immunohistochemical studies in combination with SEM observations further clearly demonstrated that osteoclasts were present on the type I lacunae, but not the type II lacunae. It has been confirmed that carbonic anhydrase in osteoclasts works in producing proton which dissolves the mineral matrix (Delaisse and

Vaes, 1992; Rousselle and Heymann, 2002), and that cathepsin B in osteoclasts works in degrading collagen fibrils (Delaisse and Vaes, 1992; Goto *et al.*, 1994). We therefore tried to determine whether the bone resorption lacunae under the osteoclasts with carbonic anhydrase differ in morphological features from those under the osteoclasts with cathepsin B. Our immunohistochemical findings indicate that osteoclasts in type I bone resorption lacunae have both carbonic anhydrase and cathepsin B.

In conclusion, bone resorption proceeds *in vivo* in the order of type Ia, Ib, and II lacunae. Type Ia lacunae reflect the stage of active resorption by osteoclasts, and type Ib lacunae the later stage of bone resorption. Domon *et al.* (2001) stated that mononuclear cells remove uncalcified collagen fibrils remaining in the bone resorption lacunae. If so, then mononuclear cells are possibly found in type Ib lacunae. Type II lacunae are in the final stage of bone resorption and free from osteoclasts.

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