Immunohistochemical Localization of Vacuolar H⁺-ATPase in Osteoclasts of Rat Tibiae

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Summary. The localization of vacuolar H⁺-ATPase (V-ATPase) in osteoclasts of rat tibiae was examined immunohistochemically using antibodies to 57 kD and 72 kD subunits of V-ATPase, with confocal laser scanning microscopy and transmission electron microscopy. The two antibodies showed similar immunoreactivities in osteoclasts. We distinguished three patterns of immunoreactivity in osteoclasts: The first pattern was that immunoreactivity was diffusely detected in cytoplasm of osteoclasts; The second one was that osteoclasts showed intense immunoreactivity in their regions contacting with bone surface; and the last was that little immunoreactivity was seen in osteoclasts. Post-embedding methods with protein A-gold complex revealed that the subunits of V-ATPase were localized not only on the membranes of the ruffled borders of osteoclasts, but also around the Golgi apparatus and accumulated tubular lysosomes.

These findings suggest that: 1) immunoreactive patterns for V-ATPase in osteoclasts reflect the polarity and activity of osteoclasts; and 2) V-ATPase on ruffled borders may be transported through tubular lysosomes.

Osteoclasts are multinucleated cells that resorb bone by lysing mineral components with acids and digesting organic components through the action of hydrolytic enzymes (GÖTHLIN and ERICSSON, 1971; LUFT, 1971; BONUCCI, 1974; VAES, 1988; BARON, 1989). Carbonic anhydrase (SHIMASAKI and YAGI, 1960; GAY and MUELLER, 1974; GAY et al., 1974; ANDERSON et al., 1982) in the cytoplasm of osteoclasts and proton pump (BARON et al., 1985; AKISAKA and GAY, 1986; BLAIR et al., 1989; VÄÄNÄNEN et al., 1990; CHATTERJEE et al., 1992; LAITALA and VÄÄNÄNEN, 1993; LIN et al., 1994) on the membrane of ruffled borders have been thought to play an important role in the acidification of Howship's lacunae and activation of lysosomal enzymes. However, the intracellular localization of

V-ATPase in osteoclasts, except for the ruffled borders, has yet to be clarified.

We therefore assessed, from an immunohistochemical standpoint, the localization of V-ATPase in osteoclasts, with antibodies to subunits 57 kD and 72 kD of V-ATPase, employing a confocal laser scanning microscope and transmission electron microscope.

MATERIALS AND METHODS

Immunohistochemical study

Tibiae of male Wistar rats weighing approximately 100 g each were observed immunohistochemically. The rats were anaesthetized with nembutal and perfused through the left ventricle, first with Ringer's solution, then with 4% paraformaldehyde and 0.5% glutaraldehyde, in 0.05 M phosphate buffer (pH 7.3), for 10 min. The tibiae were dissected, immersed in the same fixative for 1 h at 4°C and decalcified in 4.13% EDTA (pH 7.3), for 10 days at 4°C. Following this, some specimens were dehydrated in graded ethanol and embedded in paraffin. After dewaxing with xylene, paraffin sections were preincubated in PBS containing 10% normal goat serum for 2 h at 4°C in order to prevent non-specific immunoreaction, then incubated with rabbit polyclonal antibody (MORIYAMA and NELSON, 1989a, b) against either the 57 kD or 72 kD subunit of V-ATPase in dilutions from 1:100 to 1:500, for 12 h at 4°C. Each of two antibodies has no cross-reactivity with another subunit. After washing with PBS, the sections were incubated with FITC-conjugated goat anti-rabbit immunoglobulins (Dakopatts, Glostrup, Denmark) diluted to 1:200 for 1 h, washed with PBS, and examined with the two-channel

detector of a confocal laser scanning microscope (Olympus, LSM-GB 200, Tokyo, Japan).

Other specimens were used for electron microscopic immunohistochemistry. After decalcification, the specimens were cut into small pieces with a razor, dehydrated with graded N, N-dimethylformamide and embedded in glycolmethacrylate (GMA, Nisshin EM Co. Ltd., Tokyo, Japan). Ultrathin sections were cut with a Porter-Blum MT-1 and mounted on nickel grids. The localization of V-ATPase was visualized by the immuno-gold method. Ultrathin GMA sections were sequentially incubated in PBS supplemented with 1% BSA for 15 min, in rabbit antibodies to V-ATPase in dilutions from 1:100 to 1:500, for 12 h at 4°C, and then with protein-A-gold complex diluted to 1:40 for 1 h. They were washed with PBS and distilled water, air-dried, then stained with uranyl acetate and lead citrate. The samples thus obtained were observed under a JEM-100CX II electron microscope (JEOL Ltd. Tokyo, Japan) at an accelerating voltage of 80 kV.

Controls

Negative control sections were incubated with normal rabbit serum in place of the primary antibodies.

RESULTS AND DISCUSSION

We were able to clearly observe the localization of immunoreactivity in osteoclasts thanks to the confocal laser scanning microscope's enabling us to get one sliced image from thick sections. In addition, we detected immunoreactivity and cell-image in green and red, respectively, by the two-channel detector. The confocal laser scanning microscope will be also useful for double staining immunohistochemistry.

The two antibodies to V-ATPase's subunits (57 kD, 72 kD) showed a similar immunoreactivity in osteoclasts. Under the confocal laser scanning microscope, osteoclasts demonstrated three types of immunoreactivity. The first type was where the immunoreactivity was diffusely detected in the cytoplasm of osteoclasts (Fig. 1a). This type was seen on osteoclasts contacting the bone surface as well as osteoclasts apart from the bone surface. The second immunoreactivity was where osteoclasts showed an intense immunoreactivity at their regions coming in contact with the bone surface. This pattern was observed only in osteoclasts attaching to the bone surface (Fig. 1b). Most osteoclasts were classified into the first or second types, although some osteoclasts showed an intermediate pattern. The third immunoreactivity was where osteoclasts showed little immunoreactivity (Fig. 1b). Scant

immunoreactivity was additionally detected in some chondroclasts attaching to calcified cartilage in the erosion zone (Fig. 1c).

No specific immunoreactivity was evident in those control sections which were incubated with normal rabbit serum.

V-ATPase, consisting of channel proteins and cytoplasmic proteins (MORIYAMA and NELSON, 1989a), was highly conserved in amino acid sequences between species (NELSON and TAIZ, 1989). Our use of antibodies to cytoplasmic subunits (57 kD and 72 kD) of V-ATPase purified from chromaffin granules of bovine adrenal glands disclosed that osteoclasts possessed V-ATPase. Our results confirm recent reports on the same subject (BLAIR et al., 1989; VÄÄNÄNEN et al., 1990) by the use of antibodies against V-ATPase from kidney or *Neurospora crassa*.

Despite some reports about the localization of V-ATPase (BLAIR et al., 1989; VÄÄNÄNEN et al., 1990; LIN et al., 1994), the intracellular localization of V-ATPase in osteoclasts has remained obscure. We used the post-embedding method to examine the immunolocalization of V-ATPase in active osteoclasts which attached to bone and had ruffled borders. The electron microscope revealed that gold particles, indicating immunoreactivity for subunits of V-ATPase, were detected around the Golgi apparatus (Fig. 2a) and accumulated tubular lysosomes (Fig. 2b). However, few were observed in the cisterns of rough endoplasmic reticulum. Numerous gold particles were detected along the cytoplasmic side of the plasma membranes of ruffled borders (Fig. 2c). These findings suggest that subunits of V-ATPase accumulated around the Golgi apparatus (MORIYAMA and NELSON, 1989b) are transported to membranes of tubular lysosomes. The relatively strong immunoreactivity seen around tubular lysosomes leads to the consideration that the ruffled borders of osteoclasts may be formed by a fusion of biological membranes of tubular lysosomes with the plasma membranes, contacting the bone surface.

If such is the case, then V-ATPase on the ruffled border of osteoclasts would transport H⁺ produced by carbonic anhydrase in cytoplasm (SHIMASAKI and YAGI, 1960; GAY and MUELLER, 1974; GAY et al., 1974; ANDERSON et al., 1982) to the bone surface. V-ATPase would be a key element in the mechanism of bone resorption by the acidification of Howship's lacunae, which causes the dissolution of mineral components in bone and the activation of lysosomal enzymes including acid phosphatase (WERGEDAL and BAYLINK, 1969; LUFT, 1971), tartrate-resistant acid phosphatase (HAMMARSTÖM et al., 1971; MINKIN, 1982; OGURO and OZAWA, 1988) or cathepsins (DELAISSE et al., 1991; RIFKIN

Fig. 1. Confocal scanning micrographs showing the localization of V-ATPase. *Bone* bone matrix, *Car* cartilage. **a.** Immunoreactivity for 57 kD subunit detected in the cytoplasm of an osteoclast (*Oc*). $\times 700$. **b.** Immunoreactivity for 72 kD subunit is detected diffusely in the cytoplasm and intensely in the regions (*arrowheads*) where the osteoclast (*Oc*) attaches to the bone surface. However, another osteoclast (*) show little immunoreactivity. $\times 700$. **c.** Some chondroclasts (*Cc*) fail to show any immunoreactivity. $\times 700$

et al., 1991; SASAKI and UENO-MATSUDA, 1993; OHSAWA et al., 1993). Osteoclasts, in which V-ATPase is localized in the ruffled border, may actively resorb bone by transporting H^+ into Howship's lacunae, as suggested by LAITALA and VÄÄNÄNEN (1993). Consequently, the three types of localization of V-ATPase in the osteoclast suggest the polarity and activity of osteoclasts.

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Fig. 2. Electron micrographs showing immunoreactivities to 72 kD subunit of V-ATPase in osteoclasts. **a.** Gold particles detected around the Golgi apparatus (*Go*). *N* nucleus. $\times 67,000$. **b.** Gold particles moderately detected around tubular lysosomes (*TL*) in an osteoclast. $\times 67,000$. **c.** Many gold particles are localized in the cytoplasmic side of the ruffled border (*RB*) in an osteoclast. *Bone* bone matrix. $\times 67,000$

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