SHORT COMMUNICATION

Immunohistochemical and immunocytochemical studies of the localization of leucine aminopeptidase in the osteoclast

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Introduction

The osteoclast is the main effector cell in bone resorption¹⁾. Its participation in the catabolism of bone matrix proteins is not fully understood.

Extracellular degradation of the bone matrix at the cell surface of osteoclasts is needed for resorption of such bone²⁻⁴⁾. Several acidic proteolytic enzymes, collagenase⁵⁻⁷⁾, cathepsin^{8,9)}, β -glucuronidase¹⁰⁾ and dipeptidylpeptidase¹¹⁾, act in the extracellular catabolism of bone matrix proteins, but most of them originate from non-osteoclastic bone cells^{5,7,9)}.

Leucine aminopeptidase (LAP) has been demonstrated in osteoclasts by enzymatic histochemistry^{12,13)}. LAP is an N-terminal exopeptidase that can hydrolyze most kinds of peptide bonds except for prolyl residues¹⁴⁾, so it is likely that LAP is involved in the osteoclastic catabolism of bone matrix protein. These earlier studies^{12,13)} suggested that LAP in the osteoclasts was to be found in the lysosomes, but details of the ultrastructural location are not known. LAP could be a marker for osteoclast activity¹²⁾, but it is difficult to demonstrate osteoclastic LAP in fixed, decalcified tissues by conventional enzymatic histochemistry, because it is labile.

The purpose of this study was to develop an immunohistochemical method to identify LAP in osteoclasts in fixed, decalcified bone tissue, and to determine the ultrastructural localization of LAP in osteoclasts by the im-

muno-gold method.

Materials and Methods

Anti-LAP

LAP (EC 3.4.11.1) from swine kidneys was purchased from Worthington Biochemicals (U.S.A.). The purity of the enzyme was tested by native PAGE; only a single band was found with Coomassie Brilliant Blue stain. Anti-LAP was obtained from rabbits by repeated subcutaneous injections of LAP conjugated with Freund's complete adjuvant. Specificity of the antiserum was evaluated by an immunoblot assay. The properties of the anti-LAP will be reported elsewhere.

Immunohistochemistry

Young adult rats were anesthetized with ether and perfused intravascularly with 4 % paraformaldehyde (PFA) (cacodylate buffer, pH 7.2), periodate lysine paraformaldehyde (PLP), or Zamboni fixative. Mandibular bones were extracted and fixed in one of the same fixatives at 4°C overnight. Calvarial bones were removed from newborn mice, and fixed in buffered 4 % PFA at 4°C overnight. After being washed in the buffer, the specimens were decalcified in Plank-Rychlo's medium or in buffered 8 % EDTA at 4°C, and embedded in paraffin.

Prepared mandibular bones of puppies and an adult monkey were also studied by immunohistochemistry. The mandibular bones from puppies were fixed in 4 % PFA and decalcified in Plank-Rychlo's medium or

EDTA at room temperature. The monkey mandibular bone was fixed in 4 % PFA and decalcified in EDTA.

Paraffin sections were cut from every specimen. The paraffin wax was removed, and the sections were treated with Streefkerk's methanol-hydrogen peroxide solutions¹⁵⁾ and incubated in anti-LAP diluted 1 : 200 and then anti-rabbit IgG conjugated with horse radish peroxidase (HRP). In control incubations, pre-absorbed anti-LAP or 1 % bovine serum albumin (BSA) was used. The reaction product was made visible by use of the diaminobenzidine (DAB) reaction.

Immunocytochemistry

The calvarial bones were extracted from anesthetized newborn mice and fixed in a fixative that contained 3 % PFA and 0.5 % glutaraldehyde (cacodylate buffer, pH 7.2) at 4°C overnight. After being washed in the same buffer, the specimens were embedded in Lewicryl K4M resin (Polysciences, U.S.A.) at -20°C without decalcification. Ultrathin sections were cut and mounted on membrane-coated copper grids. The sections on the grids were then incubated in anti-LAP diluted 1 : 20 followed by protein A-gold complex. The protein A-gold complex was prepared by the method of Slot and Gauze¹⁶⁾. Control incubation was done with 1 % BSA or pre-absorbed anti-LAP followed by protein A-gold.

Results

Polynuclear cells with an irregular contour on the wavy surface of the alveolar bone of

the rat, mouse, dog, and monkey showed an intensely positive DAB reaction after treatment with anti-LAP (Fig. A, B, D). From the specific localization and cytological features, the DAB-positive cells seemed to be osteoclasts. Besides osteoclasts, fibroblasts and osteoblasts were also stained weakly. The control incubation showed no DAB reaction of the osteoclasts (Fig. C). When the reactivity in rat mandibular bones was evaluated by different preparative conditions, PFA fixation followed by Plank-Rychlo's decalcification at 4°C gave the highest reactivity. PFA fixation followed by EDTA enhanced the background reaction.

Immuno-gold labelling of undecalcified osteoclasts embedded in Lewicryl resin showed dense precipitation of gold particles on the amorphous compartment of cytoplasm and on the mitochondria (Fig. E). The nucleus, Golgi apparatus, membrane systems, and bone matrix near the osteoclasts also had a few gold particles, indicating non-specific deposition. Osteoclasts with an irregular contour (activated osteoclasts) tended to have more gold particles than smooth-surfaced ones (resting osteoclasts), although a morphometric study was not done. The gold precipitation was reduced by control incubations (Fig. F).

Discussion

The LAP molecule differs in different animal species, but the immunological reactivity of anti-LAP against LAP is fairly interspecific¹⁴⁾. In our study, the osteoclasts of the rat, mouse, dog, and the monkey had specific

Figure legends

Fig. A, B : Osteoclasts in the rat mandibular bone stained by the immunoperoxidase method after incubation in anti-LAP. The tissue was prepared by fixation with buffered 4 % PFA and by decalcification in Plank-Rychlo's medium at 4°C. Counter-staining was done with hematoxylin. Ab, alveolar bone; Pl, periodontal ligament; D, dentin. A : $\times 150$, B : $\times 600$.

Fig. C : Control stain of the rat mandibular bone fixed in PFA and decalcified in Plank-Rychlo's medium. The first incubation was made with BSA instead of anti-LAP. No DAB-positive reaction can be seen in the osteoclast (arrowhead). $\times 600$.

Fig. D : Osteoclasts in the dog alveolar bone stained by the immunoperoxidase method using anti-LAP. Counter-staining was done with hematoxylin. Osteoclasts are stained. $\times 300$.

Fig. E : Mouse osteoclast labelled with protein A-gold complex after incubation in anti-LAP. Gold particles can be seen on mitochondria (M) and on cytoplasmic matrix. Lysosome (Ly) and vacuoles possess fewer particles. $\times 20,900$.

Fig. F : Control labelling of mouse osteoclast after incubation in pre-absorbed anti-LAP. Fewer gold particles are seen on the mitochondria (M) and cytoplasmic matrix. $\times 19,500$.

affinity to anti-swine LAP. The immunohistochemical reaction was confirmed by control incubations.

From the disposition of particles after enzymatic histochemical reaction, it has been suggested that LAP is in the lysosomes^{12,13}. However, the optimum pH for LAP action is alkaline¹⁴, so it seems improbable that LAP acts within the lysosome, where the environment is acidic. Furthermore, because the bone matrix next to the ruffled border of osteoclasts is acidic during bone resorption^{3,17}, it is unlikely that LAP is released from the osteoclasts. Thus, the question arises as to where LAP is in the osteoclasts.

In this study, the mitochondria and the cytoplasmic matrix were the main compartments labelled by the immuno-gold method. Lewicryl resin is a suitable embedding material for the preservation of soluble substances in tissues¹⁸. Control incubations diminished or reduced precipitation of the immuno-gold particles. Therefore, it is possible that the immuno-gold labelling reflects well the localization of LAP. Mitochondria are a major component of osteoclasts like lysosomes, and earlier investigators^{12,13} might have taken the mitochondrial reaction of LAP to be a lysosomal one. If so, the immunocytochemical label in the cytoplasmic matrix in this study may reflect dislocation of LAP during tissue preparation, because the enzymatic histochemical reaction of LAP in osteoclasts is definitely particulate^{12,13}. However, it is possible that LAP is synthesized or processed in the mitochondria and then transferred toward the cytoplasmic matrix to catabolize bone matrix proteins taken into the osteoclast.

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