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Distribution of two distinct intracellular aspartic proteinases, cathepsins D and E, in rat oral tissues as demonstrated by discriminative antibodies

Hideaki Sakai, Takashi Saku*, Yuzo Kato and Kenji Yamamoto**

Department of Pharmacology, Nagasaki University School of Dentistry, Nagasaki 852, Japan
(Chief: Prof. Yuzo Kato)

* *Department of Pathology, Niigata University School of Dentistry, Niigata 951, Japan*
(Chief: Prof. Takashi Saku)

** *Department of Pharmacology, Kyushu University Faculty of Dentistry, Fukuoka 812, Japan*
(Chief: Prof. Kenji Yamamoto)

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Abstract: The localization of two distinct intracellular aspartic proteinases, cathepsins D and E, in rat oral tissues was determined by immunoprecipitation and immunohistochemistry using antibodies specific for each enzyme. In normal gingiva and tongue, almost all the hemoglobin (Hb)-hydrolyzing activity at pH 3.8 was immunoprecipitated with the antibodies specific for cathepsin D, indicating that little or no cathepsin E activity is present in these tissues. Cathepsin E was found at high levels in the cervical lymph node, which represented about 80% of the total Hb-hydrolyzing activity. In the submandibular gland, the level of cathepsin E was much less than that of cathepsin D. Intense staining by anti-cathepsin D antibody was found in the stratum granulosum of mucosal epithelium, the fibroblasts of the periodontal ligament, the osteoblasts on the bone surface, the odontoblasts of the dental pulp and the striated duct of submandibular gland, whereas staining indicating cathepsin E antigenicity was found in the striated duct of submandibular gland and the macrophage-like cells of mesenchymal tissues. The distinct localization of cathepsins D and E in these tissues suggests their different physiological significance in these tissues.

Introduction

Mammalian cells contain two distinct intracellular aspartic proteinases, cathepsins D and E¹⁻³⁾. Cathepsin D is a typical and well-characterized lysosomal enzyme and is widely distributed in a variety of mammalian cell types¹⁾, suggesting its important role in intracellular proteolysis and protein turnover. Cathepsin D, besides its intralysosomal proteolysis, has been suggested to be involved in a variety of pathological processes such as inflammation⁴⁻⁸⁾, neoplastic transformation^{9,10)} and amyloid formation within senile plaques in Alzhei-

mer's disease¹¹⁾.

Cathepsin E is a relatively poorly characterized enzyme and has limited distribution in cells within gastrointestinal tracts, lymphoid tissues, urinary organs and blood^{2,3)}. More recently, non-lysosomal localization of cathepsin E in various cell types has been shown by various immunochemical methods^{3,8,12)}. From these studies, it has been suggested by its limited distribution and its characteristic intracellular localization that cathepsin E is associated with specialized functions of various cell types. For instance, its microvillus localization in gastric parietal cells, renal proximal tubule cells, hepatic cell borders, intestinal and tra-

cheobronchial epithelial cells suggests that it is closely related to such functions as absorption, secretion and digestion of various cellular components. Cathepsin E has also been suggested to be involved in pathological processes of gastric lesion, besides extralysosomal proteolysis¹³⁻¹⁵. However, little information is available about the distribution of cathepsins D and E in various cell types of oral tissues. Therefore, to understand the physiological significance of catalytically similar cathepsins D and E in oral tissues, it would be worthwhile to examine the distribution and levels of both cathepsins within various cell types of rat oral tissues.

Materials and Methods

Preparations of rat oral tissues for immunoprecipitation

Three normal Wistar rats (males weighing, 110~120 g, 5 weeks old) were anaesthetized with ether and sacrificed by intracardiac perfusion with isotonic saline. Alveolar gingiva derived from buccal surface, tongue, submandibular gland and cervical lymph node were collected and homogenized with a Waring blender in 5 volumes of 5 mM sodium phosphate buffer, pH 8.0, containing 0.1% Triton X-100. The soluble extracts of these tissues were prepared by ultrasonication of the homogenates followed by ultracentrifugation. More than 95% of the aspartic proteinase activity in each homogenate was recovered in the supernatant fraction under the conditions employed.

Proteinase assay

Acid proteinase activity was determined with acid-denatured hemoglobin as a substrate as basically described previously¹⁶. The enzyme solution (10~50 μ l) was mixed with 0.5 ml of 1.5% of hemoglobin in 0.1 M acetate buffer, pH 3.8. After incubation at 40°C for 40 min, the reaction was stopped by adding 0.5 ml of 5% trichloroacetic acid (TCA). The mixture was then centrifuged at 3,000 rpm for 20 min and the liberated peptide in the supernatant (0.2 ml) was measured by the Folin-Lowry reaction¹⁷. One unit of activity is defined as the amount of enzyme which gives an absorbance at 660 nm equivalent to 1 μ g of tyrosine

in 1 min.

Immunoprecipitation

Cathepsins D and E were purified to homogeneity from rat spleen as described by Yamamoto et al.^{18,19}. The antisera against each enzyme were raised in rabbits^{20,21}, and the fraction of anti-cathepsin D IgG was further prepared with ammonium precipitation and DE-52 column chromatography. Immunoprecipitation was performed by the same procedure as described by Sakai et al.³. Each tissue extract containing 12 units of acid proteinase activity was incubated with anti-cathepsin E antiserum (0~20 μ l) or anti-cathepsin D IgG (0~0.64 mg) at 37°C for 10 min and then at 4°C for 16 h. To adjust the protein components in each tube, non-immune rabbit serum or control IgG was added to the reaction mixture. After centrifugation at 18,000Xg for 45 min to remove immunoprecipitates, the enzyme activity remaining in the supernatant was measured.

Immunohistochemical methods

Specimens were obtained from normal Wistar rats (2 males and 2 females, 5 weeks old) which were killed by intracardiac perfusion with saline. Tissue blocks from tongue, submandibular gland and gingiva were fixed in 95% ethanol for 6 h at 4°C and embedded in paraffin. The specimens including bone and tooth were decalcified in phosphate-buffered saline (PBS) containing 0.13 M EDTA and 0.7% sucrose (pH 7.2) for 3 weeks before embedding. Serial sections were blocked with 5% milk protein in PBS and subsequently immunostained by a peroxidase/antiperoxidase (PAP) procedure as described by Sternberger et al.²². Completing the immunostaining involved sequential exposure of the sections to rabbit antibodies specific for each enzyme (10~50 μ g/ml), 1:100 dilution in PBS of goat anti-rabbit IgG (Cappel Labs.) and 1:100 dilution in PBS of peroxidase/antiperoxidase (Cappel Labs.). The antigenic sites were then visualized by incubating the sections in the 3,3'-diaminobenzidine H₂O₂ substrate medium for peroxidase. The sections were then counterstained with hematoxylin. Control sections were treated in the same way except that the rabbit antibodies were replaced by a non-immune rabbit IgG.

Fig. 1 Immunoprecipitation of various rat oral tissue extracts by rabbit antiserum to rat spleen cathepsin E (●) and by rabbit anti-rat spleen cathepsin D IgG (○). The extracts containing equal amounts of acidic Hb-hydrolyzing activity (12 units) were reacted with different concentrations of the antiserum or the antibody. After centrifugation, the activity remaining in the supernatant fraction was measured. Values are means of three determinations.

(a) : alveolar gingiva, (b) : tongue, (c) : submandibular gland, and (d) : cervical lymph node.

Results

Concentrations of cathepsins E and D in various rat oral tissues

When the antibodies against each enzyme were examined for the specificities, 10 μ l of the antiserum against cathepsin E and 0.32 mg of the anti-cathepsin D IgG completely immunoprecipitated 12 units of cathepsins E and D, respectively. The antiserum against cathepsin E had no effect on cathepsin D in solution, and the anti-cathepsin D IgG did not precipitate cathepsin E in solution. Therefore, for

the differential assay for measurements of each enzyme extracted from various tissues, aliquots of each of the supernatants (containing 12 units of total acid proteinase activity) were subjected to immunoprecipitation with the respective antibodies. As shown in Figure 1, more than 95% of the activity found in rat gingiva and tongue were precipitated with anti-cathepsin D IgG. Addition of the antiserum against cathepsin E in the aliquots had no effect on the activity. In submandibular gland, 33% and 4% of the activity were precipitated with the anti-cathepsin D IgG (0.64 mg) and the antiserum against cathepsin E

Fig. 2 Immunohistochemical localization of cathepsin E and cathepsin D in rat oral tissues. (a), (c) and (e) are staining for cathepsin E; (b), (d) and (f) are staining for cathepsin D. In the gingival mucosa (a and b), the epithelial cells have heavy immunostaining for cathepsin D, but not for cathepsin E. Cathepsin D was also found in the fibroblasts of periodontal ligament and in the osteoblasts located adjacent to alveolar bone (d), contrasted with no evidence for cathepsin E in this area (c). In the dental pulp (e and f), the odontoblastic cells contain a large amount of cathepsin D-positive coarse granules. Bars=100 μ m (a-d) and 50 μ m (e and f).

(20 μ l), respectively. About 60% of the activity found in this tissue was not immunoprecipitated by either antibodies or by conjugation of both antibodies, and not inhibited by pepstatin (20 μ M), leupeptin (10 mM) and E-64 (10 mM) (data not shown). In cervical

lymph node, 80% and 20% of the activity were attributed to cathepsins E and D, respectively. Knowing the specific activities of the respective enzymes purified from rat spleen (cathepsin E, 35,000 units/mg; cathepsin D, 8,200 units/mg), the concentrations of each

Fig. 3 Immunohistochemical localization of cathepsin E (a and c) and cathepsin D (b and d) in rat submandibular gland and tongue. In the submandibular gland (a and b), cathepsin E-positive cells are found mainly in the striated ducts. Cathepsin D-positive cells are also found in the striated ducts, especially in the basal area. The population and staining intensity for cathepsin D are more prominent than those for cathepsin E. In the lingual muscle (c and d), cathepsin E is present mainly in tissue fixed macrophage-like cells. Muscle fibers contain a small amount of coarse granules positive for cathepsin D. Bars=100 μ m.

enzyme in these tissues were also calculated from the amount of enzyme activity immunoprecipitated. The concentrations of cathepsins D and E (μ g/mg tissue protein each) were as

follows: tongue (D, 0.155; E, not detectable), gingiva (D, 0.337; E, not detectable), submandibular gland (D, 0.20; E, 0.006) and cervical lymph node (D, 0.22; E, 0.18).

Immunohistochemical study

In oral mucosa, cathepsin E was not evident in the mucosal epithelium (Fig. 2-a), while prickle cells in the epithelium showed a strong immunoreaction for cathepsin D. The staining was diffuse in the cytoplasm and most intensive in the stratum granulosum in the free gingiva and the sulcular epithelium of the gingiva (Fig. 2-b). Macrophage-like large cells which contained cathepsin D-positive granules were scattered in the lamina propria.

Cathepsin D was also found within osteoblasts located adjacent to alveolar bone or within the fibroblasts of periodontal ligament (Fig. 2-d). Osteocytes near the surface of alveolar bone also contained cathepsin D positive granules, but its concentration was less than that of osteoblasts. Cathepsin E cross-reactivity was not found within osteoblasts, fibroblasts and osteocytes (Fig. 2-c). The dental pulp contained a large amount of cathepsin D-positive odontoblasts (Fig. 2-f). However, the cathepsin E-positive staining was not found in the odontoblasts but weakly in a few cells in the capillary (Fig. 2-e).

In the submandibular gland, both cathepsins E and D were present in the epithelial cells of the striated duct and of the larger ducts (Fig. 3-a and 3-b). However, either cathepsins E or D was hardly found in the acinar cells. In the striated ducts, cathepsin D was accumulated in the basal area of ductal cells, whereas cathepsin E was found in the fine granules that were evenly distributed in the cells. No significant difference in the staining pattern of each cathepsin in submandibular gland was observed between male and female rats. The intercalated ducts of parotid and sublingual glands contained the positive cells for both cathepsins D and E (data not shown). In the tongue, coarse granules containing cathepsin D were detected in the perinuclear striated muscle cells, whereas the cathepsin E-positive cells were present in a few tissue fixed macrophage-like cells but not in the muscle cells (Fig. 3-c and 3-d).

Discussion

We previously reported different distributions of two antigenically distinct intracellular as-

partic proteinases, cathepsins D and E, in various rat tissues³⁰, but we did not examine their localization in the oral tissues. Here, we reported different distributions of these two enzymes in rat oral tissues. Cathepsin D was localized in almost all the cell types examined, although the concentration of the enzyme varied in each cell type. The greatest accumulation of cathepsin D in the stratum granulosum of oral epithelium suggested that the enzyme was associated with autophagic function of the cells differentiating to keratinized cells. In contrast, cathepsin E was hardly detectable in the oral epithelium either by immunoprecipitation or by immunohistochemistry. We reported previously that, although cathepsin E was greatly accumulated in the gastric and intestinal mucosa, it was hardly detectable in the squamous epithelial cells of esophagus³⁰. The expression of cathepsin E, therefore, may be closely related to specially differentiated function of epithelial cell types such as absorption and secretion, rather than functions in squamous epithelial cells.

The high levels of cathepsin D were also found in both osteoblasts and odontoblasts, suggesting the association with functions of these cells such as hard tissue formation. Poole et al.²³⁾ and Linde and Persliden²⁴⁾ reported that cathepsin D activity in the osteogenic and odontogenic regions might be related to the degradation of proteoglycans taking place simultaneously with crystal formation at the mineralization front. We could not find cathepsin E in these cell types, although its enzymatic properties, such as substrate specificity, optimum pH and susceptibility to various proteinase inhibitors, are very similar to that of cathepsin D.

Like other notable biologically active proteins, such as nerve growth factor (NGF), epidermal growth factor (EGF), renin and kallikrein³⁰⁾, both cathepsins D and E localize in the ductal cells of the submandibular gland. Cathepsin D has been recently suggested to be involved in the processing of pro-enzymes, by *in vitro* and *in vivo* studies^{26-28,31)}. From this view, cathepsin D might play a role in processing of the precursor polypeptide produced in the ductal cells of submandibular gland, besides its role in lysosomal digestion²⁵⁾. It is worth-

while to examine the changes in levels of cathepsin D by sympathetic nervous stimulation which mediate the release of secretion products from the ducts^{30,32,33}, to elucidate above possibility. Also, it has been recently suggested that cathepsin E is a possible processing enzyme for converting of big-endothelin into endothelin²⁹. Unfortunately we have at present no available information suggesting colocalization of endothelin and cathepsin E in

submandibular glands. If cathepsin E was involved in the production of any biologically active polypeptides in the ductal cells, it might be occurred in a different pathway to that of cathepsin D, knowing that the intracellular localization of cathepsin E is quite different to that of cathepsin D. Studies are under way to explore these problems hopefully related to functions of cathepsins D and E in oral tissues.

抄録：正常ラット口腔領域において、2つの細胞内アスパルティックプロテイナーゼ、カテプシンDおよびカテプシンEの局在を、免疫沈降法による活性測定と免疫組織学的手法により検索した。歯肉と舌におけるヘモグロビン水解活性(pH 3.8)は、すべてカテプシンDに対する抗体で沈降でき、カテプシンE活性はほとんど存在しなかった。一方、頸部リンパ節においてはカテプシンE活性が優位を示し、全ヘモグロビン水解活性中80%を占めていた。顎下腺においては両酵素とも存在していたが、カテプシンE量はカテプシンD量よりもはるかに少なかった。免疫染色の結果、カテプシンDは粘膜上皮の顆粒層、歯根膜の線維芽細胞、歯槽骨表面の骨芽細胞、歯髓の象牙芽細胞および顎下腺導管の線条部に多く認められた。一方、カテプシンEは顎下腺導管の線条部と間葉系組織中のマクロファージ様細胞に認められた。このようなカテプシンDとカテプシンEの異なる局在は、同じ細胞内アスパルティックプロテイナーゼに属しながらも細胞内における両酵素の機能がそれぞれ異なっていることを示唆している。

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