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Significance of lysosomal proteases; cathepsins B and H in maxillary mucosa and nasal polyp with non-atopic chronic inflammation

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SUMMARY

Tissue extracts from maxillary mucosa (MM) and nasal polyp (NP) with non-atopic chronic inflammation were applied to DEAE-Sepharose, and hydrolytic activity of lysosomal proteases (cathepsins B and H) was measured by the fluorometric assay. Hydrolytic activity of cathepsins B and H in MM, of which cathepsin B was a major one, was about 3.5 times as high as that in NP. Protein composition resembled in both extracts except for several apparent protein bands with high molecular weight observed in NP. These results suggest that in MM with chronic inflammation, mucosal destruction induced by excess lysosomal granuloproteases overwhelming protease inhibitors would occur, being much related to the formation of its irreversible lesion.

INTRODUCTION

In inflammation, lysosomal proteases participate in proteolytic degradation of inflammatory products and contribute to the repair of inflammed tissue (Lesser et al., 1983). On the other hand, excess activity of these proteases against the control by protease inhibitors may lead to tissue destruction. Of all lysosomal granuloproteases, cathepsin B which is the best known of the lysosomal thiol proteases has a number of strong proteolytic function at optimal pH 6 (Barrett, 1980). Nasal polyp (NP) and maxillary mucosa (MM) in chronic sinusitis (CS) are typical representatives of irreversible mucosal lesions in nasal and paranasal mucosae. In this study, to clarify the significance of mucosal damage induced by lysosomal granuloproteases to the pathogenesis of irreversible mucosal lesion, we have analyzed the kinetics of cathepsins B and H as lysosomal granuloproteases in tissue extracts from MM and NP with non-atopic chronic inflammation. The differences of protein compositions in both tissue extracts were also analyzed.

MATERIALS AND METHODS

Maxillary mucosa (MM) was collected from 20 adult patients (12 male and 8 female) with non-atopic chronic sinusitis, who were diagnosed by clinical symptoms, rhinoscopy and radiology. Nasal polyp (NP) was also incised from 10 of the same patients (5 male and 5 female) at the same time. These collected tissues had typical histological findings; dense connective tissue in the subepithelial stroma with neutrophilic and lymphocytic infiltrations, and proliferation of submucosal glands in MM and remarkable interstitial edema with a little cellular infiltration, and proliferation of loose connective tissue in NP. Collected samples were pooled and washed with cold saline several times to remove mucous and blood as possible, and were stored at −20 °C until analysis.

All the procedures were carried out at 4 °C, otherwise mentioned.

1. *Extraction with 3M KCl*

Collected samples were mixed and minced with scissors, and dispersed into cold 3M KCl solution (10 ml per one gram of wet weight). They were vigorously stirred overnight, and were treated with ultrasonic homogenizer (Tomy Seiko Co., Tokyo) for 20 min in an ice bath. After centrifugation at 15,000 rpm for 20 min, supernatant was recovered and adjusted to pH 4.2 with 0.1 HCl.

2. *Precipitation with saturated ammonium sulfate*

Two fold volume of saturated ammonium sulfate was added slowly to the supernatant during continuous stirring. The solution was readjusted to pH 4.2 with 0.1 HCl. After settling for a few hours, precipitate was recovered by centrifugation at 5,000 rpm for 20 min. The precipitate was dispersed into minimal soluble distilled water and dialyzed against distilled water for 48 hours. It was lyophilized using Freeze Drier (Labconco, Kansas City).

3. *DEAE-Sepharose chromatography*

Lyophilized powders from both MM and NP were dissolved with 0.1M Tris-HCl buffer, pH 7.2 and were stirred for 3 hours. After centrifugation at 18,000 rpm for 20 min, protein concentration of the supernatant was adjusted to 72.5 mg/dl. It was applied to DEAE-Sepharose column (2.6 × 30 cm, Pharmacia Fine Chemicals, Uppsala) equilibrated with 0.1M Tris-HCl, pH 7.2. Tris-HCl buffer was added to the column, until the extinction at 280 nm became negligible (under 0.03). Then, a linear gradient from 0 to 0.5M NaCl in the same buffer was applied to the column. Fractions of 10 ml were collected at the flow rate of 20 ml/h.

4. *Enzyme assay for various proteases*

Hydrolytic activity of various proteases was measured by the fluorometric assay (Hamaguchi et al., 1984), using the specific MCA-peptide (Protein Research

Foundation, Osaka); Bz-Arg-MCA for trypsin (Kanaoka et al., 1977), Boc-Val-Leu-Lys-MCA for plasmin (Kato et al., 1980). For the assay of cathepsins B and H, 2 ml of 0.05M sodium phosphate buffer, pH 6.0 containing 1 mM EDTA-2Na and 2 mM cysteine-HCl was used as the assay buffer, and Z-Phe-Arg-MCA for cathepsin B and Arg-MCA for cathepsin H and aminopeptidase B were used as the substrates (Barrett, 1980). In order to exclude the interference of plasma kallikrein activity, 50 µl of soybean trypsin inhibitor (SBTI, 1 mg/ml, Sigma, St. Louis) was added to the assay buffer before incubation of enzyme sample with 50 µl of the substrates (Ohishi et al., 1979). Each enzyme activity was expressed as the relative fluoro unit (RFU) (Hamaguchi et al., 1984).

5. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*
Lyophilized powder of both extracts from MM and NP was dissolved with 0.1M Tris-HCl, pH 6.8 containing 2% SDS with or without 2-mercaptoethanol. The protein concentration was adjusted to 50 mg/dl. Fifty µl of each sample was denatured in boiling water for 5 min, then it was applied to SDS-PAGE (stacking gel; 5%, separating gel; 10%), according to the method of Laemmli (Laemmli, 1973). After electrophoresis for about 6 hours, the gel was stained with 0.1% Coomassie Brilliant Blue R-250 (Nakarai Kagaku, Kyoto).

6. *Protein concentration*
Protein concentration was determined by spectrophotometrically, using Protein Assay Kit (Bio Rad, Richmond). Bovine serum albumin was used as the standard reference.

RESULTS

1. *DEAE-Sepharose chromatography*
Elution profiles of two tissue extracts from MM and NP on DEAE-Sepharose chromatography were shown in Figure 1. Similar peak patterns of protein concentration indicated by OD 280 nm were observed in both extracts, of which a major peak was detected in the fractions eluted with Tris-HCl containing 0.3M NaCl (Figure 1a). Little plasmin activity to hydrolyze Boc-Val-Leu-Lys-MCA was detected in both extracts, and small peak of trypsin activity to hydrolyze Bz-Arg-MCA was observed near the major protein peak in both extracts. The peak values of trypsin activity in the extract from MM was about 2.2 times as high as that from NP (Figure 1b).
Two peaks of cathepsin B activity to hydrolyze Z-Phe-Arg-MCA were observed in MM; one was a major peak and was detected in the fractions eluted with Tris-HCl containing 0.27M NaCl, and the other was a minor which was detected in those eluted with Tris-HCl containing 0.4M NaCl. The peak value of cathepsin B activity in the extract from MM was about 3.5 times as high as that from NP. In NP,

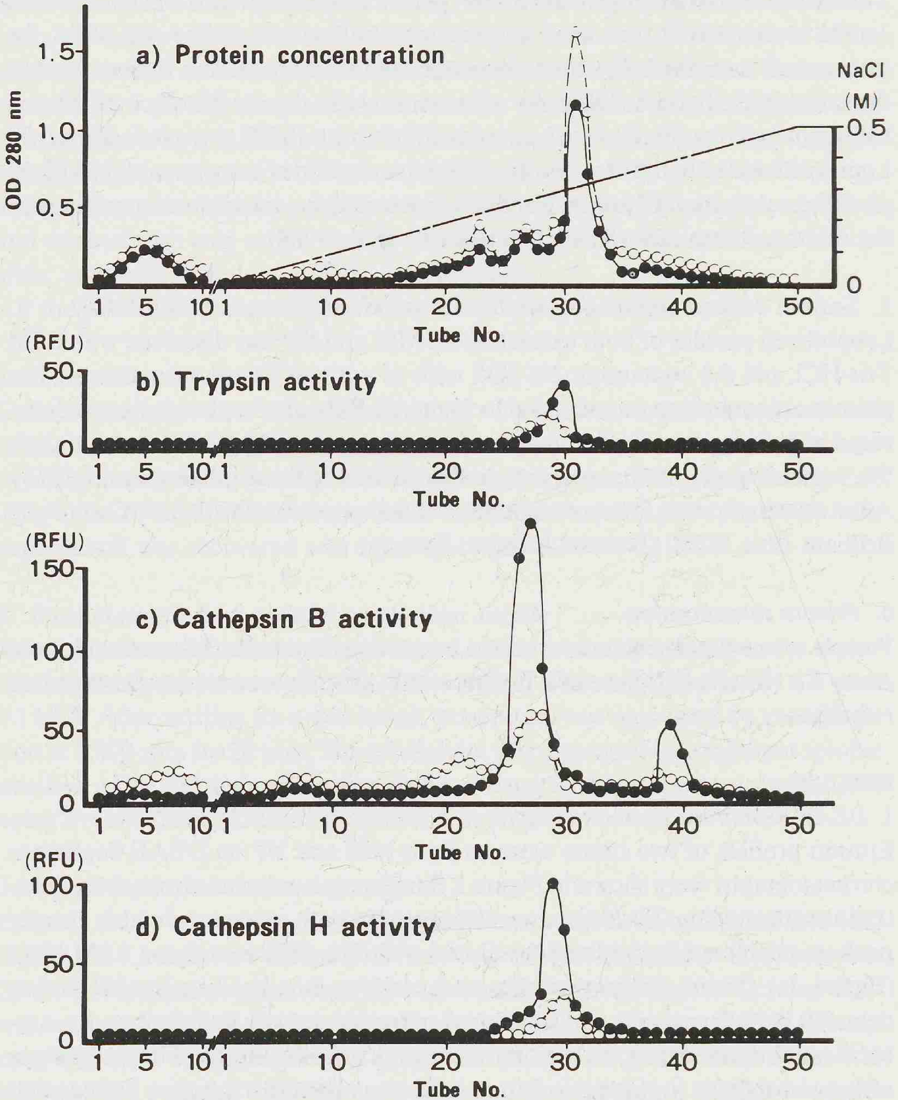

Figure 1. DEAE-Sepharose chromatography: column size, 2.6 × 30 cm, fraction size 10 ml, flow rate, 20 ml/h; samples tissue extracts from maxillary mucosa (MM) and nasal polyp (NP); starting buffer, 0.1M Tris-HCl buffer, pH 7.2; gradient, 0–0.5M NaCl in the same buffer (total 500 ml); ●—●, MM; ○—○, NP; a. protein concentration indicated by OD 280 nm; b: trypsin activity (using Bz-Arg-MCA); c. cathepsin B activity (using Z-Phe-Arg-MCA); d.cathepsin H and aminopeptidase B activity (using Arg-MCA).

double major peaks of cathepsin B activity were observed. A peak of cathepsin H to hydrolyze Arg-MCA was observed between cathepsin B activity and major protein peak. The peak value in the extract from MM was about 3 times as high as that from NP (Figure 1d).

2. *SDS-PAGE of both extracts from MM and NP*
Electrophoretic patterns of SDS-PAGE resembled in both extracts without adding 2-mercaptoethanol (Figure 2, −ME). Albumin was one of the major protein bands in both extracts, and numbers of protein bands with higher molecular weight than albumin (MW = 67,000; 67K) could be detected. Especially, in the extract from NP, larger amount of protein bands with high molecular weight over 100K could be observed than that from MM. Using 2-mercaptoethanol, almost protein bands with higher molecular weight shifted to the lower molecular weight position than albumin (Figure 2, +ME).

DISCUSSION

Cathepsins are the major lysosomal proteases which have some important roles in phagocytosis, destruction of inflammatory products and repair of inflammed tissues (Lesser et al., 1983; Liebermann et al., 1971). Large amount of cathepsins B and H, of which optimal pH are 6.0 and 6.8 respectively (Barrett, 1980), exist in granulocytes and macrophages. These cathepsins have strong collagenolytic activity (Burleigh et al., 1974). Other cathepsins such as cathepsin D, which is an acid protease and so called leukokininogenase to liberate leukokinin from kininogen, has hydrolytic activity under very acidic pH (pH 3.0) (Greenbaum, 1972). Cathepsin G, which is a chymotrypsin-like serine protease (Mounter et al., 1960), is easily inhibited by α_1-antitrypsin and α_2-macroglobulin. In this study, proteolytic activity of trypsin and plasmin was very weak in both extracts of MM and NP, because these serine proteases would be inhibited rapidly by serum protease inhibitors (Heimburger et al., 1971). Therefore, cathepsins B and H are considered to play a significant role in the mucosal inflammation.
Chronic sinusitis (CS) is one of the most prevalent nasal illnesses, and maxillary mucosa is the most affected paranasal sinus in CS. Because of complicated anatomical localization, fresh normal maxillary mucosa can not be obtained without entering into the maxillary sinus. An enough amount of maxillary mucosa was collected from 20 patients with CS who underwent radical surgery. So, comparison with healthy control was not done.
Elution position of cathepsins B and H on DEAE-Sepharose chromatography was similar in both extracts of MM and NP. The other minor peak on DEAE-Sepharose in case of using Z-Phe-Arg-MCA would be attributed to glandular kallikrein not to cathepsin B, because this peak was considerably inhibited by aprotinin (Hamaguchi et al., 1985), and had higher affinity for Pro-Phe-Arg-MCA

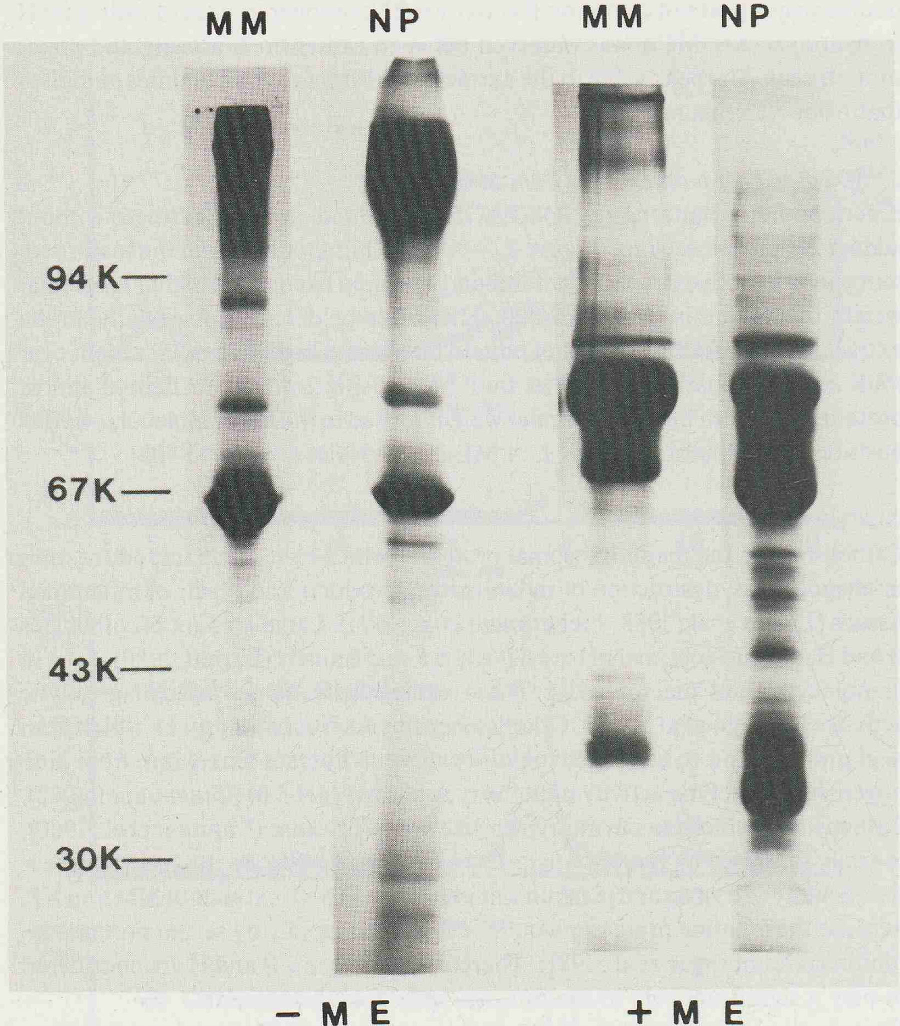

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis: samples, tissue extracts from maxillary mucosa (MM) and nasal polyp (NP); +ME, with adding 2-mercaptoethanol; –ME, without adding 2-mercaptoethanol; stacking gel, 5%; separating gel, 10%; staining, 0.1% Coomassie Brilliant Blue R-250. Molecular weight markers indicated by short lines are also applied.

(Ohishi et al., 1979; Hamaguchi et al., 1985). High molar KCl solution used as an extract solution and treatment by ultrasonic homogenization can lead to an effective extract from intra-cellular lysosomal proteases. Cathepsin B had much higher peak of activity than cathepsin H did in both extracts. The peak value of hydrolytic activity of both cathepsins in the extract from MM was about 3.5 times

as high as that from NP. These results indicate that both cathepsins B and H exist in both extracts and cathepsin B is the major one, and that in MM with chronic inflammation, larger amount of these cathepsins would be present than those in NP, proving much larger infiltration of granulocytes observed in MM than that in NP. Because of high collagenolytic activity of cathepsin B (Burleigh et al., 1974), excess lysosomal cathepsins overwhelming the control of protease inhibitors can destruct not only inflammatory products, but also normal mucosal elements; epithelium, basement membrane, etc. Severe damage to the selectivity of vascular permeability enables profuge leakage of plasma proteins into the tissue space, leading to edemato-polypous mucosal changes.

Electrophoretic patterns of SDS-PAGE indicate that protein compositions in both extracts are almost identical except for high molecular weight (HMW) protein bands in NP, and albumin is the major protein in both extracts. In both tissues, active leakage of albumin via enhanced vascular prmeability would occur. It is interesting that tissue extract from NP has considerably less activity of cathepsins B and H than that from MM, and contains larger amount of HMW proteins escaped from proteolytic degradation. HMW proteins in NP could be the remainder of inflammatory products which were left undegradates by the shortage of hydrolytic lysosomal proteases. Low activity of these cathepsins in NP would be due to the less cellular infiltration in the subepithelial stroma of NP.

All these findings suggest that in MM with chronic inflammation, mucosal destruction induced by lysosomal proteases would occur, which may be much related to the formation of its irreversible mucosal lesion. On the other hand, in NP lysosomal proteases would considerably decrease and HMW proteins escaped from proteolytic degradation are accumulated in tissue spaces.

RÉSUMÉ

Des extraits de tissu de muqueuse maxillaire (MM) et de polype nasal (PN) présentant une inflammation chronique non-atopique, furent mis dans une colonne de DEAE-Sepharose, l'activité hydrolytique des protéases lysosomiales étant mesurée au moyen de l'essai fluorométrique. L'activité hydrolytique des cathepsines B et H mesurée dans la MM fut environ 3,5 fois plus grande que celle mesurée dans le PN, la cathepsine B étant la plus importante. Les compositions de protéine se ressemblèrent dans les deux extraits de tissu, à l'exception de plusieurs bandes de protéine apparentes au poids moléculaire élevé, observées dans les extraits de tissu de PN. Ces résultats suggèrent qu'il peut se produire dans la MM présentant une inflammation chronique, une destruction de muqueuse provoquée par un excès de granuloprotéases lysosomiales écrasant les inhibiteurs de la protéase, se rattachant fort à la production de la lésion irréversible.

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