CELLULAR ORIGIN OF CATHEPSIN B IN CARRAGEENIN-INDUCED GRANULOMA TISSUES IN RATS*

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Cathepsin B isolated from carrageenin-induced granuloma tissue was separated into two peaks (one large and one small) on CM-cellulose column chromatography. The cathepsin B activities from the peritoneal macrophages and polymorphonuclear leukocytes were eluted at the positions identical to those of the larger (P-I) and the smaller (P-II) peaks, respectively. Similarly, the cathepsin B activities in the P-I and P-II fractions from granuloma tissue corresponded to those from macrophages and leukocytes, respectively, in isoelectric focusing in polyacrylamide gel. Both the cathepsin B activities were completely inhibited by iodoacetamide and leupeptin, but phenylmethanesulfonyl fluoride and *o*-phenanthroline were ineffective. The molecular weight of the cathepsin B of the cellular origin and granuloma tissue was approximately 24,000 by gel filtration. The pI value of cathepsin B in the P-I and P-II fractions from granuloma tissue was 5.09 and 5.18, respectively. These results strongly suggest that two types of cathepsin B in granuloma tissue may be different in cellular origin and may be isozymes.

Keywords—lysosomal enzyme; cathepsin B; carrageenin-induced inflammation; granuloma tissue; macrophage; polymorphonuclear leukocyte; isoelectric focusing in polyacrylamide gel

INTRODUCTION

Cathepsin B is known as a lysosomal thiol proteinase present in various tissues.¹⁾ The enzyme is capable of cleaving non-helical portion of collagen molecule as well as non-collagen proteins at acid pH *in vitro*. Thus, cathepsin B has been thought to participate in the intralysosomal degradation of proteins.²⁾

Based on the findings that cathepsin B activity from carrageenin-induced granuloma tissues in rats was separated into two peaks on CM-cellulose column chromatography and one of the two cathepsin B activities increased with the time during the period of resorption process of the granuloma tissue, we have suggested the role of cathepsin B in the active resorption of the granuloma tissue.³⁾

The carrageenin-induced granuloma tissue is mainly composed of fibroblasts, macrophages and leukocytes.^{4,5)} The present study was undertaken to determine what type of the cells in the granuloma tissue produced the cathepsin B which increased in the resorption stage and to compare chromatographic and electrophoretic behaviors of cathepsin B from granuloma tissue with those from peritoneal macrophages and polymorphonuclear leukocytes, both of which are lysosome-rich cells.

MATERIALS AND METHODS

Materials—Phenylmethanesulfonyl fluoride, soybean trypsin inhibitor (SBTI, type 1-S), ovalbumin (from turkey, grade VI), cytochrome c (horse heart, type III) and Coomassie Brilliant

* The abbreviations used are: BAPA, α -N-benzoyl-DL-arginine-*p*-nitroanilide; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

Blue G 250 were purchased from Sigma Chemicals Co., pharmalyte (pH 4–6.5) and Sephadex G-75 were from Pharmacia Fine Chemicals, leupeptin and pepstatin were from Peptide Institute, Osaka, *o*-phenanthroline, casein (from milk) and soluble starch were from Wako Pure Chemical Industries, Ltd., pI-Marker (kit A) for a standard in isoelectric focusing was from Oriental Yeast Co., Ltd., Japan, α -N-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPA) was from Fluka AG, Buchs SG, Dulbecco's modified Eagle's medium (DMEM) was from Flow Laboratories, bactopeptone was from Difco Laboratories, calf serum was from Gibco Laboratories, Seakem 202 carrageenin was from Marine Colloid Inc., Springfield, N.J., U.S.A., and all other reagents used were of analytical grade.

Animal — Male rats of Donryu strain, weighing 150–180 g were used for the induction of granuloma tissue, and those from 200 to 500 g were used for the collection of peritoneal exudate cells throughout the experiments.

Induction of Granuloma Tissue — Granuloma tissue was induced by subcutaneous injection of a 2% solution of carrageenin according to the procedure described previously.⁶⁾ Granuloma tissue was removed immediately after sacrifice of rats bearing 8- or 17-day-old granuloma tissue and stored at -40°C until required.

Collection of Cells — Macrophages were collected by peritoneal lavage which was performed 3 times with phosphate-buffered saline (PBS) 3 days after intraperitoneal injection of 5% (w/v) bactopeptone–5% soluble starch solution (40 ml/kg body wt.). The cells collected were washed 5 times with PBS and centrifuged at $300\times g$ for 5 min at 4°C . The cell suspension (2.4×10^7 cells/10 ml/culture dish, 100 mm culture dish, Corning) in DMEM containing 10% heat-inactivated (56°C , 30 min) calf serum was incubated for 3–6 h at 37°C under 5% CO_2 in air. The non-adherent cells were removed by washing 3 times with PBS. The adherent cells were recovered with a rubber policeman few minutes after adding about 5 ml of PBS (pH 7.4) containing 0.2% bovine serum albumin and 0.1% EDTA by the method of

Weiss *et al.*⁷⁾ at room temperature. The cells were obtained by centrifugation at $300\times g$ for 5 min and were used as macrophages. In some experiments, glass culture flask (bottle type) was also used for the collection of macrophages. Peritoneal exudate cells (6×10^6 cells/ml of DMEM containing 10% heat-inactivated calf serum) were incubated for 2 h. The adherent cells were recovered with a rubber policeman after removal of the non-adherent cells by washing with PBS.

Peritoneal polymorphonuclear leukocytes were collected by the method of Reed and Tepperman⁸⁾ with modifications. The peritoneal exudate cells were recovered by peritoneal lavage twice with PBS 16 h after intraperitoneal injection of 1% (w/v) casein in Ca^{2+} , Mg^{2+} -free Krebs Ringer bicarbonate solution (40 ml/rat). The cells collected were washed 5 times with PBS. The last pellet was used as leukocytes. The macrophages and leukocytes were stored at -40°C until required.

Macrophages and polymorphonuclear leukocytes were determined morphologically under Giemsa stain. Viability of the cells was determined by dye exclusion of 0.1% trypan blue in PBS. Proportions of macrophages from the adherent cells was 90% and viability was 65–70% when cultured in the Corning culture dish. On the other hand, proportions and viability of macrophages from glass adherent cells was 58–65% and above 95%, respectively. Proportions and viability of polymorphonuclear leukocytes were higher than 90% and 95%, respectively.

Extraction and Partial Purification of Cathepsin B — Cathepsin B was directly extracted from the thawed cells and granuloma tissues without a step of autolysis.^{3,9)} Subsequent operations for extraction and partial purification of cathepsin B were performed at $1-4^{\circ}\text{C}$. The frozen-stored granuloma tissues were thawed and minced. The minced granuloma tissue was added to 3 vol (v/w) of 0.1% (v/v) Triton X-100 solution containing 3% NaCl, 1 mM EDTA and 0.5 mM dithiothreitol and homogenized with a Vir-Tis homogenizer. The frozen-stored cells were

thawed. Macrophages or leukocytes were added to 10 ml of the above Triton solution and homogenized with a glass homogenizer. Each of the homogenates was adjusted to pH 3.5 with 2N and 0.1 N HCl, stirred for 2 h and centrifuged at $100,000 \times g$ for 60 min. The supernatant was fractionated with ammonium sulfate. The protein fraction precipitating between 20% and 70% saturation with ammonium sulfate was collected and dissolved in a small volume of 20 mM acetate buffer (pH 5) containing 1 mM EDTA and 0.5 mM dithiothreitol and exhaustively dialyzed against the buffer overnight. After centrifuging at $15,000 \times g$ for 1 h, subsequent supernatant was subjected to CM-cellulose column chromatography in 20 mM acetate buffer (pH 5) containing 1 mM EDTA and 0.5 mM dithiothreitol. Elution was achieved on a column (1×10.5 cm) with a linear gradient of NaCl from 0–70 mM over a total volume of 200 ml. Flow rate was 60 ml/h. Aliquots of each 3 ml (in some experiments, 3.5 ml) were assayed for cathepsin B activity.

Gel Filtration —Sephadex G-75 gel chromatography was carried out on a column (3.6×84 cm for macrophage, 3.2×84 cm for leukocyte) in 0.15 M sodium acetate buffer (pH 4.6) containing 0.1 M NaCl, 1 mM EDTA and 0.5 mM dithiothreitol. Flow rate was 11 ml/h. Aliquots of each 5 ml were assayed for cathepsin B activity.

Assay for Cathepsin B —Cathepsin B activity was measured by the method described previously¹⁰ which was a modification of the method of Barrett.¹¹ Briefly, samples were incubated with BAPA as substrate for 30 min at 40°C. *p*-Nitroaniline released during the incubation was extracted with toluene and *p*-dimethylaminobenzaldehyde reagent was added to the extract. The absorbance of the chromogen formed was read at 470 nm. A unit of enzyme activity is defined as the amount of enzyme releasing 1 μ mol of *p*-nitroaniline in 1 min at 40°C.

Isoelectric Focusing in Polyacrylamide Gel —Gels for isoelectric focusing were prepared in glass tubes ($0.6 \text{ cm} \times 10 \text{ cm}$) by using 2% pharmalyte with 5% (w/v) acrylamide, 0.15% methylenebisacrylamide. Ammonium persulfate (0.015

%, w/v) was used for polymerization. The upper (cathode) buffer was 0.2 M L-histidine and the lower (anode) buffer was 0.04 M DL-glutamic acid. Gel's running and sample application were mainly followed by the procedure of McCroskery *et al.*¹² Gels were prerun for 30 min at 40°C (1 mA/tube). The sample (400 μ l) was introduced in 30% (w/v) sucrose under a layer of 0.2 ml of 5% sucrose for the protection from the denaturation of the enzyme and gels were run for 1 h (1 mA/tube). The gels were stained with Coomassie Brilliant Blue G250 by the method of Blakesley and Boezi¹³ after electrofocusing. For the detection of cathepsin B activity in gels, the gels without staining were sliced into small pieces (2.2 mm in length) and the sliced gels were incubated in the assay system described above at 37°C for 15 h instead of incubating at 40°C for 30 min. The released *p*-nitroaniline was measured and the activity was expressed as relative activity (the highest value as 100%).

RESULTS AND DISCUSSION

Cathepsin B in granuloma tissue was directly extracted from the tissue homogenate without being subjected to the step of autolysis such as described by authors^{3,9} and partially purified by CM-cellulose column chromatography.

The ammonium sulfate-fractionated cathepsin B activity was separated into two peaks (P-I and P-II) on the CM-cellulose column chromatography (Fig. 1, A). These results were consistent with those of previous experiments in which cathepsin B was partially purified after autolysis of the homogenate of granuloma tissue.³⁾

Extraction and partial purification of cathepsin B of macrophages and leukocytes were done with a similar manner of granuloma tissues. Each cathepsin B activity from macrophages and polymorphonuclear leukocytes showed a single peak accompanied with a small shoulder (Fig. 1, B and C). The cathepsin B activity (the main peak) from the macrophages was eluted at a different position compared with that from the leukocytes; the enzyme activities from macrophages and leukocytes were eluted at the positions corres-

ponding to the P-I and P-II, respectively, from the granuloma tissues (Fig. 1). These results obtained by CM-cellulose column chromatography were further confirmed by isoelectric focusing in polyacrylamide gel; the peaks of cathepsin B activities of the P-I and P-II from granuloma tissue corresponded to those from macrophages and polymorphonuclear leukocytes, respectively (Fig. 2). pI value of cathepsin B in the P-I and P-II was 5.09 and 5.18, respectively, and that of leukocytes was 5.18 (Fig. 3).

Each main fraction of cathepsin B activity from the cells was subjected to gel filtration (Sephadex G-75). Both enzymes had the same

molecular weight of 24,000 (Fig. 4) which was in good agreement with that of cathepsin B reported previously.³⁾ In addition, it was found by SDS polyacrylamide gel electrophoresis that the molecular weight of cathepsin B in both P-I and P-II from granuloma tissue was approximately 24,000 (data not shown). As shown in Fig. 5, optimal pH of cathepsin B in both P-I and P-II was similar (approximately pH 6.3).

BAPA, a substrate for cathepsin B, is also hydrolyzed by trypsin-like enzymes (serine proteinases). Therefore, inhibitor studies were conducted to clarify as to whether the BAPA-hydrolyzing activity on CM-cellulose column chromatography was attributed to cathepsin B or other enzymes, or both. The results are summarized in Table I. Iodoacetamide (a thiol proteinase inhibitor) and leupeptin (a considerably

FIG. 1. CM-cellulose Column Chromatograms of the 20–70% satd.-(NH₄)₂SO₄ Fractions from (A) 8-day-old Granuloma Tissue (13 g), (B) Macrophages (2.7×10^8 cells) and (C) Leukocytes (1.1×10^{10} cells)

Macrophages were obtained from culture dish-adherent cells. Arrows indicate the start of linear gradient elution. Experimental conditions are described in the text.

FIG. 2. Isoelectric Focusing in Polyacrylamide Gel of the Cathepsin B Fractions isolated by CM-cellulose Column Chromatography (Fig. 1)

(A); P-I (x-x) and P-II (●-●) from 8-day-old granuloma tissue, and (B); the main fractions from macrophages (x-x) and leukocytes (●-●) in Fig. 1.

specific inhibitor of cathepsin B) completely inhibited the BAPA-hydrolyzing activities of macrophages, polymorphonuclear leukocytes and granuloma tissues (P-I and P-II), whereas phenylmethanesulfonyl fluoride (a serine proteinase inhibitor) and *o*-phenanthroline (a metallo-proteinase inhibitor) were ineffective. Pepstatin (a carboxy proteinase inhibitor) slightly inhibited the BAPA-hydrolyzing activities of leukocytes. These results indicate that these BAPA-hydrolyzing activities on CM-cellulose

FIG. 3. Plot of *pI* of Protein against Distance Migrated after Isoelectric Focusing in Polyacrylamide Gel

P-I and P-II fractions from 8-day-old granuloma tissue and the main fractions from macrophages and polymorphonuclear leukocytes were isolated by CM-cellulose column chromatography (Fig. 1). The main fraction from cells were concentrated 20-fold before application. The sample from macrophages gave no band owing to a low protein content.

The *pI* values taken for the reference proteins were: cytochrome *c* (cyt. *c*, 5.6); bovine serum albumin (BSA, 4.9); soybean trypsin inhibitor (SBTI, 4.5).

FIG. 4. Plot of Logarithm of Molecular Weight against Elution Volume (V_e)/Bed Volume (V_t) obtained by Sephadex G-75 Gel Filtration

The sample applied to Sephadex G-75 column were the cathepsin B fractions isolated by CM-cellulose column chromatography (Fig. 1); the main peaks from macrophages and polymorphonuclear leukocytes. Both the cathepsin B had the same molecular weight (approximately 24,000).

The molecular weights taken for the reference proteins were: a, bovine serum albumin (68,000); b, ovalbumin (45,000); c, soybean trypsin inhibitor (21,500); and d, cytochrome *c* (13,400).

FIG. 5. Effect of pH on the Cathepsin B Activity in P-I (○—○) and P-II (●—●) Isolated by CM-cellulose Column Chromatography of the 20–70% satd.-(NH_4) $_2$ SO $_4$ Fraction from 8-day-old Granuloma Tissue (Fig. 1)

Each point is the mean of two determinations.

TABLE I. *Effect of Various Inhibitors on Partially Purified Cathepsin B from Carrageenin-induced Granuloma Tissues, Macrophages and Polymorphonuclear Leukocytes*

Inhibitors	Concn (mM)	Percent inhibition ^{a)}			
		Granuloma tissue		Macrophage	Leukocyte
		P-I	P-II		
Iodoacetamide	15	99.8	99.7	100	100
Leupeptin	0.01	99.9	100	100	100
Pepstatin	0.01	0	3.3	0	24.1
Phenylmethanesulfonyl fluoride	1	0	8.6	0.5	7.4
<i>o</i> -Phenanthroline	1	0	0	0	3.6

a) Assays were determined in duplicate and mean per cent inhibition was described. Cathepsin B of granuloma tissues was purified by CM-cellulose column chromatography (Fig. 1) and cathepsin B of macrophages and leukocytes was purified by gel filtration (Sephadex G-75) of the main peak of CM-cellulose column chromatography (Fig. 1).

column chromatography are attributed to cathepsin B.

Based on the histochemical studies employing fluorescent antibody against cathepsin B in tissue sections in 10-day-old talcum granuloma, Sylvén and Snellman¹⁴⁾ suggested that fibroblasts as well as macrophages had granules containing cathepsin B. Cells obtained by trypsinization of 10- to 20-day-old granuloma tissue are macrophage like cells (70%) and fibroblast like cells (30%).¹⁵⁾ Although the cell proportion is known to vary in the stages of the development of granuloma tissue and the nature of cathepsin B from fibroblast is unknown, the present studies suggest that cathepsin B in P-I originates, at least in part, from macrophages in granuloma tissue. Furthermore, the present studies also suggest that macrophages may be important in the stage of active resorption of granuloma tissue, because cathepsin B activity in P-I increased in 17-day-old granuloma tissue (data not shown and ref. 3), where tissue resorption was probably occurring. This speculation is consistent with the view of Bonney *et al.*¹⁶⁾ that mononuclear phagocytes from carrageenin-induced granuloma tissue are activated macrophages based on their phagocytosis, low 5'-nucleotidase activity and some characteristics, and consistent with the view of macrophage as effec-

tor cell¹⁷⁾ secreting various proteinases¹⁸⁻²⁰⁾ when the cell is activated by various stimuli.

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