Activation by Cathepsin G of Latent Gelatinase Secreted from Rat Polymorphonuclear Leukocytes

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Rat polymorphonuclear leukocytes secrete a latent gelatinase with a molecular weight of about 96 kilodaltons (kDa). Activation of the latent 96-kDa gelatinase by cathepsin G was studied by using sodium dodecyl sulfate-substrate polyacrylamide gel electrophoresis. Cathepsin G activated the 96-kDa gelatinase by converting it to two lower molecular-weight species of 76 and 61 kDa, which were slightly different from the gelatinase species generated by treatment with 4-aminophenylmercuric acetate, an activator of latent gelatinase.

Keywords latent gelatinase; cathepsin G; gelatinase activation; polymorphonuclear leukocyte

Introduction

In response to stimuli such as chemotactic signals, polymorphonuclear leukocytes (PMNs) predominantly secrete a latent gelatinase among various PMN neutral proteinases including collagenase, elastase and cathepsin G.[1-3] PMN active gelatinase is capable of degrading soluble type IV and type V collagens[3,4] (structural proteins of basement membrane), and enhances the degradation of insoluble collagen by collagenase.[4] Gelatinase, therefore, may participate in the passage of PMNs through basement membranes in response to a chemotactic signal and in the collagen breakdown in the extracellular matrix. Thus, the activation mechanism of latent gelatinase secreted from PMNs is important. It has been reported that PMN latent gelatinase can be activated by proteinases and by the myeloperoxidase-derived oxidant HOCl.[5-7] Murphy *et al.*[8] demonstrated that PMN latent gelatinase was activated by cathepsin G but destroyed by PMN elastase when granule extracts from human PMNs were incubated with PMN elastase and cathepsin G. These observations have prompted us to study the active species generated from PMN latent gelatinase by cathepsin G. We present here evidence suggesting that cathepsin G activates rat PMN latent 96-kilodaltons (kDa) gelatinase by converting it to two lower molecular-weight species of 76 and 61 kDa.

Materials and Methods

Preparation of PMNs Male Wistar rats, weighing 250—400 g were used for preparation of PMNs. PMNs were collected by a modification[9] of the procedure of Reed and Teppermann[10]; PMNs were collected by peritoneal lavage with phosphate-buffered saline (PBS) 16 h after intraperitoneal injection of 1% (w/v) casein (40 ml/rat) in sterilized Ca^{2+}, Mg^{2+}-free Krebs–Ringer bicarbonate solution. The PMNs were washed five times with RPMI-1640 medium. The content and viability of PMNs were both higher than 95%.

Partial Purification of Gelatinase The collected rat PMNs were suspended in RPMI-1640 medium at 10^7 cells/ml. The suspended PMNs were incubated with 3.3×10^{-8} M *N*-formyl-Met-Leu-Phe (FMLP) at 37 °C for 40 min, and centrifuged at $580 \times g$ for 20 min at 4 °C. The supernatant containing latent gelatinase secreted from PMNs was applied to a Sephadex G-150 column (3.2×85 cm) in order to separate the gelatinase from PMN elastase and cathepsin G. Latent gelatinase was eluted with 0.05 M Tris–HCl buffer (pH 7.5) containing 0.15 M NaCl, 5 mM $CaCl_2$ and 0.02% NaN_3 at a flow rate of 15 ml/h. Fractions containing latent gelatinase were pooled and used as the partially purified gelatinase, which was shown to be free from contamination by PMN elastase and cathepsin G.

Purification of PMN Elastase and Cathepsin G PMN elastase and cathepsin G were purified by a modification[11] of the method of Schmidt and Havemann.[12] All procedures were performed at 4 °C. Briefly, PMNs (10^{10} cells) were homogenized with 0.34 M sucrose, centrifuged at $700 \times g$, re-homogenized and centrifuged at $15000 \times g$. The precipitate (granule fraction) was repeatedly freeze-thawed and centrifuged at $100000 \times g$. The supernatant (granule extract) was applied to a Sephadex G-100 column and then the PMN elastase and cathepsin G fraction was applied to a CM-Sephadex C-50 column.

PMN elastase and cathepsin G were separated by CM-Sephadex C-50 column chromatography and each purified enzyme showed a single band of about 30 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Gelatinase Assay Gelatinase activity was determined by a modification[13] of the method of Sunada and Nagai,[14] using fluorescein isothiocyanate-labelled gelatin (FITC-gelatin) as a substrate; gelatinase activity was determined by measuring the amounts of 67% 1,4-dioxane-soluble degradation products of FITC-gelatin. Latent gelatinase activity was determined after activation with 0.64 mM 4-aminophenylmercuric acetate (APMA) as described previously,[13] and expressed as units. One unit of gelatinase activity is defined as the degradation of 1 μg of FITC-gelatin per min.

In addition, gelatinase activity toward gelatin was studied by using SDS-PAGE[15] to detect degradation products of the substrate.

SDS-Substrate PAGE Gelatinase species were visualized by electrophoresis on SDS-polyacrylamide gel copolymerized with 0.1% gelatin (Difco Laboratories, U.S.A.) according to a modification[13] of the method of Heussen and Dowdle.[16] Gelatinase species and their molecular weights can be determined simultaneously by SDS-substrate PAGE. For calculation of molecular weight in SDS-substrate PAGE, the following molecular-weight standards (Bio-Rad Laboratories, U.S.A.) were run simultaneously: myosin (200 kDa), β-galactosidase (116.2 kDa), phosphorylase B (92.5 kDa), bovine serum albumin (66.2 kDa) and ovalbumin (45 kDa).

Results

Latent Gelatinase Activation by Cathepsin G Rat PMN latent gelatinase was activated during SDS-substrate PAGE and gave a clear zone with a molecular weight of about 96 kDa (Fig. 1). This 96-kDa gelatinase was dose-dependently converted to lower-molecular-weight species by treatment with cathepsin G; 76-kDa and then 61-kDa species appeared when the 96-kDa species was incubated with increasing amounts of cathepsin G at 37 °C for 90 min (Fig. 1). In addition, when PMN latent gelatinase was incubated with cathepsin G, the amount of active gelatinase increased with incubation time up to 90 min (Fig. 2). This increase in active gelatinase was closely correlated with the appearance of a 76-kDa species, and subsequently of a 61-kDa species (Fig. 3). On the other hand, an activator of latent gelatinase converted the 96-kDa gelatinase to three lower-molecular-weight species (about 77, 71 and 63 kDa), but their molecular weights were slightly different from those of the gelatinase species generated by the cathepsin G treatment (Fig. 3). The results shown in Figs. 1—3 suggest that both 76- and 61-kDa species are active gelatinases

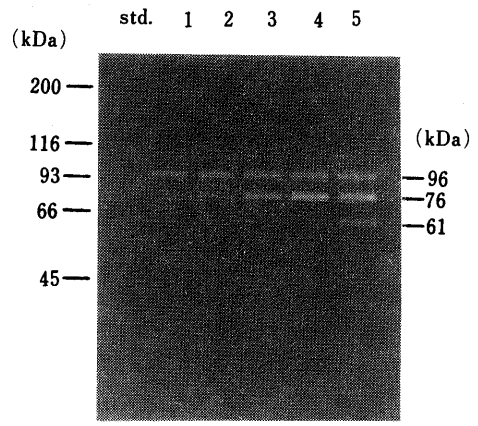

Fig. 1. Analysis of the Cathepsin G Activation of PMN Latent Gelatinase by SDS-Substrate PAGE

Partially purified rat PMN latent gelatinase (0.02 units) was incubated at 37 °C for 90 min with various amounts of purified cathepsin G [0 μg (control; lane 1), 0.039 μg (lane 2), 0.078 μg (lane 3), 0.156 μg (lane 4) and 0.390 μg (lane 5) in the final volume of 0.36 ml]. The reaction was stopped by the addition of 1 mM PMSF and the reaction mixtures were subjected to SDS-substrate PAGE. std.: molecular-weight standards.

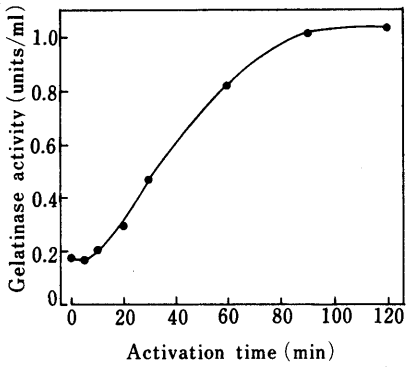

Fig. 2. Activation of PMN Latent Gelatinase by Cathepsin G

Partially purified rat PMN latent gelatinase (0.02 units) was activated with purified cathepsin G (0.62 μg) at 37 °C for various times. The activation was stopped by the addition of soybean trypsin inhibitor (20 μg) and the gelatinase activity of the reaction mixture (final volume, 0.36 ml) was determined as described under Materials and Methods.

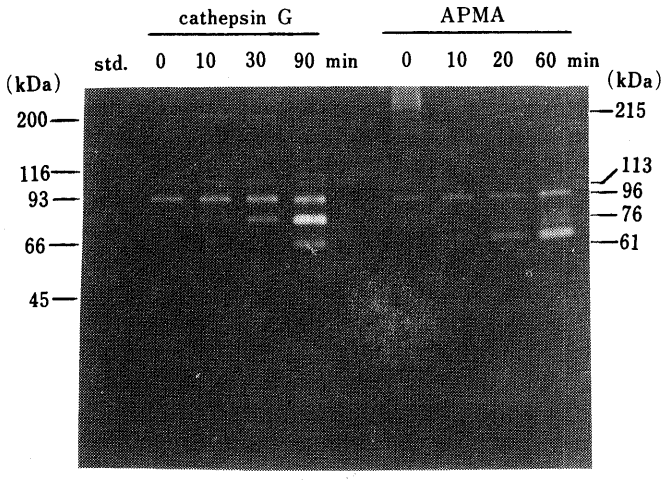

Fig. 3. Effect of Incubation Time on PMN Gelatinase Activation by Cathepsin G and APMA

Partially purified rat PMN latent gelatinase (0.02 units) was incubated with cathepsin G (0.62 μg) or APMA (0.64 mM) at 37 °C for 0, 10, 20, 30, 60 or 90 min. Activation with cathepsin G was stopped by the addition of 1 mM PMSF and the reaction mixture was subjected to SDS-substrate PAGE. std.: molecular-weight standards.

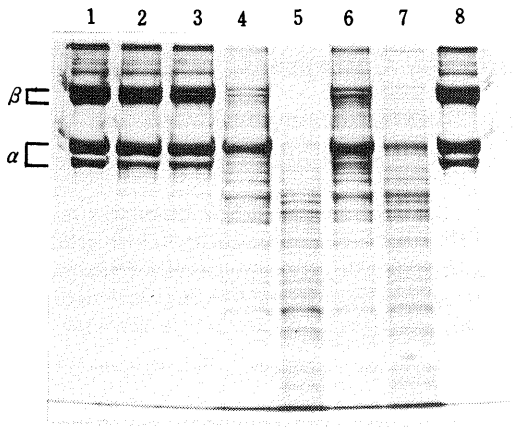

Fig. 4. SDS-PAGE of the Products of Gelatin Degradation by the Cathepsin G- or APMA-Activated PMN Gelatinase

Partially purified rat PMN latent gelatinase (0.01 units) was incubated at 37 °C with 0.8 mM APMA for 20 min and with cathepsin G (0.04 μg) or 1 mM PMSF-treated cathepsin G (0.04 μg) for 60 min. Gelatin (100 μg) from rat tail tendon type I collagen was incubated with the latent gelatinase for 3 h (lane 2) or 12 h (lane 3), with the APMA-activated gelatinase for 3 h (lane 4) or 12 h (lane 5), with the cathepsin G-activated gelatinase for 3 h (lane 6) or 12 h (lane 7), and with the gelatinase incubated with the PMSF-treated cathepsin G for 12 h (lane 8). The reaction was stopped by the addition of 10 mM 1,10-phenanthroline and the reaction mixtures were analyzed by SDS-PAGE (7.5% acrylamide). Lane 1, gelatin alone.

generated in the process of activation by cathepsin G of rat PMN latent 96-kDa gelatinase, but not intermediates in the degradation process of the gelatinase.

Both the cathepsin G- and APMA-treated latent gelatinases degraded the α and β chains of gelatin and gave similar degradation fragments as determined by SDS-PAGE analysis of the reaction mixture after incubation of gelatin with the activated gelatinases, while the latent gelatinase and the gelatinase treated with cathepsin-G which had been inactivated with 1 mM phenylmethylsulfonyl fluoride (PMSF) had no gelatinolytic activity (Fig. 4).

Discussion

SDS-substrate PAGE was performed to study the gelatinase species generated from rat PMN latent gelatinase by treatment with PMN elastase and cathepsin G. The cathepsin G treatment of the latent 96-kDa gelatinase resulted in the appearance of two lower molecular-weight species; firstly 76-kDa species and subsequently 61-kDa species appeared in accordance with the increase in active gelatinase (Figs. 1—3). These results suggest that the 61-kDa species may be generated from the 76-kDa one, and both species are active. A high-molecular-weight species (about 215-kDa species) appeared during storage of PMN latent gelatinase solution, and this species may be an aggregate of the 96-kDa species (Fig. 3). The clear band of latent 96-kDa species did not disappear but rather increased after activation with cathepsin G or APMA (Figs. 1 and 3). This increase in the 96-kDa band may be due to the conversion of the high-molecular-weight aggregate to lower-molecular-weight gelatinases including the 96-kDa species by the treatment with cathepsin G or APMA.

On the other hand, rat PMN elastase also converted the 96-kDa gelatinase to lower-molecular-weight species; 76-kDa species (a mojor species) together with 86-, 59- and 37-kDa species (minor species) were observed by SDS-substrate PAGE (data not shown). The PMN elastase-

treated latent gelatinase, however, had slight proteolytic activity toward gelatin (data not shown). It may be argued, therefore, that lower-molecular-weight species generated by the treatment with rat PMN elastase are intermediates in the process of degradation. Our results are consistent with the findings reported by Murphy *et al.*[8] that human PMN latent gelatinase was activated by cathepsin G but destroyed by human PMN elastase.

Recently, Vissers and Winterbourn[17] have reported that proteolytic activation of PMN gelatinase by PMN elastase is likely to be most efficient, and cathepsin G and oxidants are not involved in the activation. Their results were obtained in experiments using specific inhibitors of PMN elastase, cathepsin G and oxidative reactions under conditions where azurophil degranulation of human PMNs occurred. Although the reason for the discrepancy between our observations and the findings of Vissers and Winterbourn remains unclear, it sould be pointed out that our results were obtained by direct activation of partially purified rat PMN latent gelatinase by purified rat PMN elastase and cathepsin G, while Vissers and Winterbourn obtained indirect evidence by using *in vitro* incubation system of human PMNs with specific inhibitors of human PMN elastase and cathepsin G. In addition, the stability of the enzymes is different; rat PMN elastase is unstable compared with human PMN elastase.[18]

The myeloperoxidase-derived oxidant (HOCl) can activate latent gelatinase,[5-7] but antioxidants were not able to inhibit gelatinase activation completely.[6] Furthermore, PMNs isolated from patients with chronic granulomatous disease are incapable of generating any oxygen metabolites but are able to activate latent gelatinase.[6] These findings indicate the presence of an HOCl-independent pathway of gelatinase activation. A limited proteolysis of latent gelatinase is probably involved in gelatinase activation. In the present study, we have obtained evidence that cathepsin G has an ability to activate rat PMN latent 96-kDa gelatinase by converting it to 76- and 61-kDa species.

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