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Human neutrophil gelatinase is a collagenase type IV

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Abstract : Secreted gelatinase from human neutrophils was purified as a 94 kDa polypeptide. Gelatinolytic and type IV collagenolytic activities of the purified protein were measured and compared. Immunoglobulins purified from antisera raised against gelatinase inhibited both the gelatinase and type IV collagenase activities. There was no cross-reaction in the inhibition with type I collagenase while the three metalloproteases were similarly inhibited by recombinant tissue inhibitor of metalloproteases. Purified gelatinase degraded denatured type I and native type IV collagens; there was no proteolysis of native type I collagen.

Human neutrophils secrete two matrix-degrading metalloproteases (MMP): a specific granule collagenase which, like interstitial collagenase from fibroblasts, cleaves fibrillar collagens, and a 92 kDa gelatinase similar to that synthesized by macrophages and tumor cells (1, 2). The 75 kDa neutrophil type I collagenase degrades preferentially native type I collagen (3, 4) while gelatinase which is localized in secretory vesicles distinct in human neutrophils from the granules (5) is thought of as having substrate specificity for denatured collagens (gelatins) and intact type IV basement membrane collagen (6). Some reports suggested however that native type IV collagen was a poor substrate for gelatinase (7,8). The two enzymes, type I collagenase and gelatinase, are secreted as inactive proenzymes, and they can be activated by oxygen metabolites or by serine proteases (4).

Abbreviations : **PBS,** Phosphate buffered saline; **HBS,** hank's balanced solution; **PMA,** Phorbol 12 - myristate 13 - acetate; **EGTA,** Ethylene glycol-bis (ß-amino ethyl ether) N,N,N,N', - Tetraacetic acid; **BCIP,** 5-bromo-4-chloro-3-indolyl phosphate, **NBT,** Nitroblue tetrazolium; **SDS PAGE,** Sodium dodecyl sulfate polyacrylamide gel electrophoresis; **MMP,** Matrix-degrading metalloproteinase; **APMA,** p-aminophenyl mercuric acetate; **TIMP,** Tissue Inhibitor of Metalloprotease.

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The metalloproteases are believed (6) to be mediators of the degradation of the extracellular matrix and the basement membrane which are a dense meshwork of collagen, glycoproteins and proteoglycans (9). Dissolution of the vascular basement membrane is associated with migration of endothelial cells, neutrophils and metastatic tumoral cells (10). Identity of neutrophil gelatinase with the well-characterized 92 kDa tumor gelatinase has been suggested by immunoblot analysis (11). A partial NH_2 - terminal aminoacid sequence of natural human neutrophil gelatinase was aligned to the cDNA-derived sequence of fibrosarcoma cell gelatinase (12). In order to answer the question about substrate specificity of neutrophil gelatinase (13,14), we have characterized the gelatinolytic and collagenolytic activities of the human neutrophil enzyme purified by a recently described procedure (15). The data allow us to conclude that the human neutrophil gelatinase has a type IV collagenase activity.

MATERIAL AND METHODS

Reagents. Reagents used in this work were obtained from the following sources : APMA, FMLP, Brij 35 (Sigma Chemical Co, USA) ; cyano ^{57}Co cobalamin, (Amersham, England) ; ^{14}C collagen I (Eliac, France) ; collagen (human type IV), N - [propionate-2,3-^{3}H], (NEN, USA); diisopropyl phosphoro- fluoridate (Fluka, Switzerland) ; Ficoll-paque, (Pharmacia, Sweden) ; affinity purified goat anti-rabbit IgG alkaline phosphatase conjugate, alkaline phosphatase color development reagent, BCIP and NBT (Biorad, USA). Recombinant tissue inhibitor of metalloprotease (TIMP) was a generous gift from Synergen, USA.

Cells. Human neutrophils were prepared from fresh blood or from donor blood stored overnight at 4° - 10° C (French Red Cross, Grenoble, France) (16). Single buffy coats diluted 4 fold with Ca^{2+} and Mg^{2+} free PBS containing 13 units/ml heparin were centrifuged through Ficoll/Na Metrizoate (Ficoll-paque) and the neutrophils were isolated from the cell pellets according to established methods (17). The cells (10^8/ml) were suspended in a solution of 0.154 M NaCl supplemented with 0.05 mM $CaCl_2$. Exocytosis was performed with suspended neutrophils as previously described (16,17).

Gelatinase purification and production of antiserum. Gelatinase was purified by the method of Hibbs (18) with some modifications (15). Briefly, released proteins from PMA stimulated neutrophils were fractionated by DEAE Sephacel chromatography and affinity gelatin agarose chromatography. The 94 kDa gelatinase was electroeluted from a preparative 5 to 15 % SDS polyacrylamide gel electrophoresis and injected into rabbits for polyclonal antibody production. In brief, the purified protein (50 µg - 100 µg) in PBS (1 vol) was mixed with Freund's complete adjuvant (1 vol) and injected into rabbits. This was followed by two booster injections, starting on day 15 using Freund's incomplete adjuvant. Immunoglobulins (Ig) were obtained from antiserum by 40 % ammonium sulphate precipitation at +4° C and further purified on DEAE cellulose for inhibition studies to remove all α_2-macroglo bulin (19). Finally the eluted antigelatinase Ig was affinity-purified by passage through a column of protein A sepharose; bound Ig were eluted by 1 M acetic acid.

SDS PAGE and immunoblotting. Purified fractions were subjected to SDS PAGE in a 5 to 15 % acrylamide gel with a 5 % stacking gel (20) . Separated proteins were stained with Coomassie brilliant blue R 250. The molecular mass standards were phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α lactalbumin (14.4 kDa).For immunoblotting, the polyacrylamide gel and a nitrocellulose membrane were rinsed in cold electrotransfer buffer containing 25 mM Tris-HCl, 129 mM glycine, 0.01 % (w/v) SDS, pH 8.3, supplemented with 10 % methanol. Electrotransfer was performed at 4° C for 5 h, at 0.8 mA/cm^2 (15 to 40 V) (21). After blotting, the membrane was incubated for 60 min on a shaker with 50 ml of a blocking buffer which contained 50 mM Tris HCl, 0.2 M NaCl, 0.02 % (w/v) NaN_3, 1 mg/ml polyethylene glycol pH 7.6 (Tris-buffered saline), and 3 % (w/v) serum albumin, at room temperature. The membrane was washed in Tris-buffered saline, and incubated for 2 h, with continuous shaking, in the presence of specific anti serum (1 : 200 dilution in PBS). After washing, the secondary goat antirabbit Ig alkaline phosphatase (1 : 1000 dilution in PBS) was added. After a 45 min incubation at room temperature, the membrane was washed, rinsed in assay buffer containing 0.1 M Tris-HCl, 0.1 M NaCl, 5 mM $MgCl_2$ pH 9.5, and stained with NBT-BCIP reagent.

Biochemical assays. Total protein was determined by the dye-binding method of Bradford (22). Gelatinolytic activity was estimated by measuring the degradation of denatured ^{14}C type I collagen (15) or it was detected with specific antiserum directed against gelatinase. Type I collagenase was quantitated by using ^{14}C acetylated type I collagen (15). Type IV collagenase was measured according to (23) with the following modifications : the enzyme present in the sample (50 µl of diluted sample) was activated by incubation with 50 µl of p-aminophenylmercuric acetate (APMA), 3 mM final concentration, for 1 hour at 37° C. N-[propionate-2, 3-^3H] human type IV collagen was diluted (1 : 50) with 1.4 mg/ml cold type IV collagen (50 µl) and dialysed against 0.15 M Tris.HCl, pH 7.7, 0.05 M NaCl and 0.05 % (w/v) Brij 35. Addition of 50 µl of the diluted propionylated human type IV collagen to the activated collagenase equilibrated in the previous Tris medium containing 0.02 M $CaCl_2$ (final volume 200 µl) started the reaction. After 1 hour of incubation at 37° C under stirring, non degraded collagen was precipitated by 50 µl of 90 % (w/v) trichloroacetic acid and the supernatant of centrifugation was recovered for radioactivity counting.

RESULTS AND DISCUSSION

Gelatinase from human neutrophils was purified as a 94 kDa protein (Fig 1 A and B) in a latent form which was efficiently activated at 37° C in the presence of 3 mM APMA. Upon activation, gelatinase degraded denatured type I collagen (gelatin) and type IV collagen, but was ineffective on native type I collagen (Fig 2). Antiserum raised against the purified gelatinase recognized the enzyme either in its purified form or in a crude extract (Fig 1B). As previously reported for tumor gelatinase (24), purified gelatinase from neutrophils self degraded to lower M_r forms. Data in Fig 1 B show that the two gelatinase subforms (67 kDa and 60 kDa) and the major 94 kDa form of gelatinase were immunologically identical. For immuno inhibition assays immunoglobulins (Ig) raised against purified

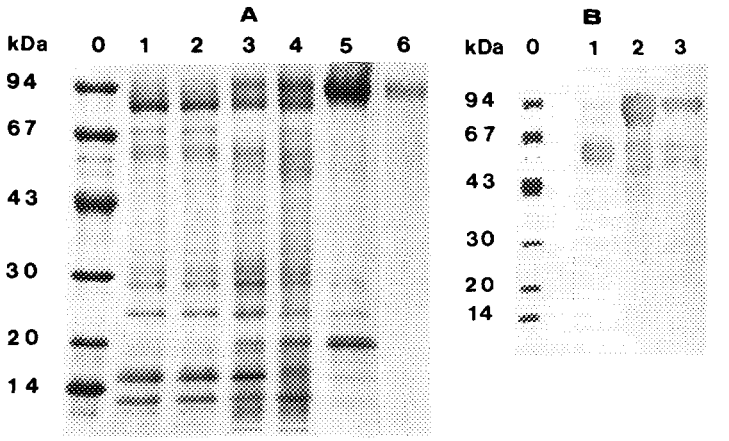

Figure 1. SDS-PAGE and immunoblotting analysis of protein fractions obtained during gelatinase purification from human neutrophils.

A. SDS PAGE : Fifty µg of the following fractions was loaded on the gel : lanes 1 and 2, exocytosis supernatant from neutrophils before and after 100 000 g ultracentrifugation; lane 3, recovered precipitate at 65 % ammonium sulfate saturation; lanes 4 and 5, fractions eluted from DEAE Sephacel and Gelatine agarose, respectively; lane 6, electroeluted 94 kDa protein. Lane 0, molecular mass markers. Proteins were stained with Coomassie brilliant blue.

B. Immunoblotting : lane 1, exocytosis supernatant; lane 2, gelatinase agarose fraction; lane 3, 94 kDa electroeluted protein. The proteins were immunodetected as described in Material and Methods. In a separate lane (0) are shown molecular mass markers stained with Coomassie blue.

gelatinase were purified from the antiserum in order to eliminate the α_2-macroglobulin that non specifically inhibits metalloprotease activities (cf Material and Methods). Gelatinolytic and type IV

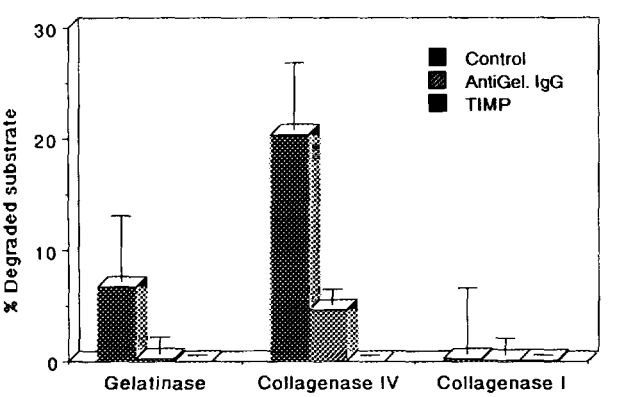

Figure 2. Inhibition of metalloprotease activities present in purified gelatinase by antigelatinase and rTIMP.

Gelatinase, collagenase type I and collagenase type IV activities were measured as described in Material and Methods and expressed as percent degraded substrates. For the inhibition tests, purified gelatinase (1.45 µg) was incubated with 280 µg antigelatinase (IgG) at 37° C for 30 min or with 3 µg rTIMP for 30 min at room temperature, before the activities were measured.

collagenolytic activities of purified gelatinase were similarly inhibited by increasing Ig concentration (Fig. 2 and 3 A). The antibodies had no effect on type I collagenase of a crude neutrophil extract (not shown). In a control assay, it was checked that there was no inhibition of enzyme activities with non immune Ig (Fig 3A). Recombinant inhibitor of metalloprotease (TIMP) strongly inhibited the gelatinase and type IV collagenase activities of purified gelatinase (Fig. 2), and the type I collagenase activity of a crude neutrophil extract (not shown). The purified gelatinase was found in a TIMP-free form, which is consistent with the fact that neutrophils do not produce TIMP (24), but type IV collagenase activity of the purified gelatinase was inhibited by TIMP in a non competitive manner (Fig 3 B).

In conclusion, the present work demonstrates that secreted gelatinase from human neutrophils has both gelatinolytic and type IV collagenolytic activities. Human neutrophils have been shown to secrete the necessary proteases for extravasation which implies the degradation of basement membrane proteins such as type IV collagen (2,25). The substrate specificity of the 94 kDa gelatinase including its ability to cleave type IV collagen suggests that gelatinase might play a role in the destruction of the extracellular matrix, perhaps

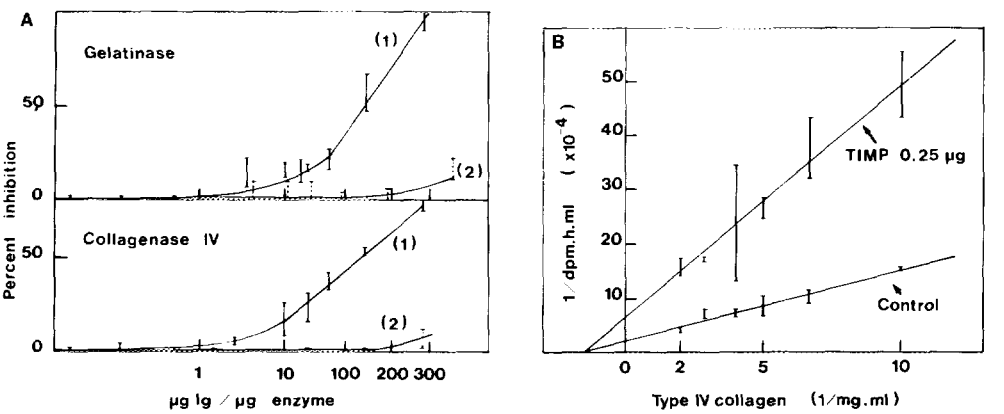

Figure 3.

A. Inhibition of gelatinase and type IV collagenase by antigelatinase IgG.

Gelatinase and type IV collagenase activities of the purified gelatinase were titrated with increasing concentrations of purified antigelatinase Ig (free from proteinase inhibitors). The purified 94 kDa polypeptide was preincubated with immune Ig **(1)** or non immune Ig **(2)** for 30 min at 37° C before initiating the APMA activation process of MMP which was carried out before starting the reaction.

B. Inhibition of type IV collagenase activity by rTIMP.

Various concentrations of type IV collagen were incubated with the purified gelatinase (0.23 µg in 50 µl) with or without 0.25 µg TIMP. Enzyme activity was expressed as degraded substrate (dpm/hour/ml enzyme).

acting in concert with the metalloproteases produced by activated endothelial cells (26).

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