Major co-localization of the extracellular-matrix degradative enzymes heparanase and gelatinase in tertiary granules of human neutrophils

Faustino MOLLINEDO*[1], Motowo NAKAJIMA‡, Ana LLORENS§, Enrique BARBOSA†, Sagrario CALLEJO†, Consuelo GAJATE* and Angels FABRA§

*Laboratory of Signal Transduction and Leucocyte Biology, Instituto de Biología y Genética Molecular, Facultad de Medicina, Consejo Superior de Investigaciones Científicas-Universidad de Valladolid, C/ Ramón y Cajal 7, E-47005 Valladolid, Spain, †Department of Human Anatomy, Facultad de Medicina, Consejo Superior de Investigaciones Científicas-Universidad de Valladolid, C/ Ramón y Cajal 7, E-47005 Valladolid, Spain, ‡Bio-Organics Research Department, Ciba-Geigy Japan Limited, International Research Laboratories, Takarazuka, Japan and §Department of Cancer and Metastasis, Institut de Recerca Oncologica, Hospital Durani Reynals, Autovia de Castelldefels km 2.7, L'Hospitalet, E-08907 Barcelona, Spain

The expression of cell-surface adhesion proteins and the release of extracellular-matrix degradative enzymes constitute crucial processes for the attachment of neutrophils to the endothelium and for the subsequent extravasation of these cells through the endothelial layer. We have analysed in resting human neutrophils the subcellular localization of heparanase, a heparan-sulphate-degrading endoglycosidase that can degrade basement-membrane components, thereby facilitating neutrophil passage into the tissue during an inflammatory reaction. By subcellular fractionation of postnuclear supernatants from resting human neutrophils on continuous sucrose gradients, we have found that heparanase activity was mainly located in gelatinase-containing tertiary granules. Using a specific antibody, the 96-kDa heparanase protein was further located in the gelatinase-rich subcellular fractions. Following immunoblotting and immunoprecipitation analysis in the distinct subcellular fractions, we also found co-localization of heparanase and Mo1 (CD11b/CD18), a leucocyte integrin involved in the attachment of neutrophils to the endothelium, in the fractions enriched in gelatinase-containing tertiary granules. Treatment of human neutrophils with tumour necrosis factor or granulocyte/macrophage colony-stimulating factor induced an increase in the CD11b/CD18 cell-surface expression, as well as the release of both gelatinase (matrix metalloproteinase-9) and heparanase, but not of other granule markers, indicating a major co-localization of gelatinase, heparanase and CD11b/CD18 in the same organelle. Furthermore, confocal laser scanning microscopy using specific antibodies against gelatinase and heparanase revealed a major co-localization of both enzymes in intracellular cytoplasmic granules. The major localization of heparanase and CD11b/CD18 in the gelatinase-containing tertiary granule supports the notion that mobilization of this organelle can regulate extravasation of human neutrophils.

INTRODUCTION

Neutrophils play a major role in the surveillance system of the host organism against foreign invaders and in acute inflammation. An early event occurring in neutrophil activation is exocytosis of cytoplasmic granules. The neutrophil granule constituents, secreted or translocated into the cell surface on cell activation, seem to affect both the interaction of neutrophils with endothelial cells and the migration of neutrophils from the vascular lumen to the extravascular tissue. Four distinct granule populations have been reported in human neutrophils, namely azurophilic or primary granules; specific or secondary granules; gelatinase-containing granules, also named tertiary granules; and alkaline phosphatase-rich granules, also named phosphasomes or secretory vesicles [1–11]. Fusion of the different granule populations with the plasma membrane may occur independently. Thus mobilization of gelatinase-containing tertiary granules can be induced readily under conditions that mobilize slightly the specific granules and hardly mobilize the azurophilic granules [7,12–14]. As the gelatinase-rich tertiary granules contain proteins involved in cell-adhesion processes [6,15–20], as well as in diapedesis [3–7], we have previously postulated that the rapid mobilization of these granules can modulate early neutrophil responses upon cell activation [6].

Neutrophils have been shown to contain two matrix-degrading metalloproteinases: a 75 kDa interstitial collagenase [matrix metalloproteinase (MMP)-8; EC 3.4.24.34] located in specific granules [21,22], and a 92 kDa gelatinase (MMP-9; EC 3.4.24.35), also named gelatinase B, which is localized in tertiary granules [3,5–7,14,16,23–25]. Both metalloproteinases are produced in a latent form and can be physiologically activated by other proteinases present in the medium. Gelatinase degrades denatured collagens and some native types of collagen present in basement membranes [24]. Since basement membranes are a barrier for migrating cells, it has been suggested that gelatinase can facilitate tumour invasion and metastasis [26]. Neutrophils share with metastatic tumour cells the ability to invade solid tissue and to travel through, and extravasate from, the circulatory system. Thus some enzymes involved in cell invasion are present in both neutrophils and metastatic tumour cells.

Heparanase is an endo-β-D-glucuronidase that specifically cleaves carbohydrate chains of heparan sulphate proteoglycans present in the extracellular matrix and on the cell surface [27]. Thus heparanase can degrade basement-membrane components,

Abbreviations used: GM-CSF, granulocyte/macrophage colony-stimulating factor; MMP, matrix metalloproteinase; TNF, tumour necrosis factor.

[1] To whom correspondence should be addressed.

and it is thought to play an important role in tumour invasion [28]. The presence of heparanase activity in human neutrophils has been reported previously [29], as well as its subcellular localization in the specific granules [30]. However, the subcellular localization of gelatinase-containing tertiary granules was not examined in these subcellular fractionation studies [30]. Furthermore, the observation that heparanase release can occur under conditions that do not induce secretion of specific granule contents [29] suggests a putative intracellular localization for heparanase in another cytoplasmic organelle.

Here, we report evidence for a major co-localization of heparanase and gelatinase in the rapidly mobilizable tertiary granules in resting human neutrophils, supporting a key role for the mobilization of gelatinase-containing tertiary granules in the extravasation of human neutrophils. Also, we found a major co-localization and co-mobilization of these two extracellular-matrix degradative enzymes with Mo1 (CD11b/CD18), a glycoprotein composed of a non-covalently linked α (CD11b) and β (CD18) heterodimer, which functions as the receptor for C3bi opsonized particles and plays an important role in neutrophil adhesion processes [31], and whose intracellular localization in peroxidase-negative granules, including specific and gelatinase-containing tertiary granules, has been reported previously in resting human neutrophils [6,15–20,32].

MATERIALS AND METHODS

Materials

$Na^{125}I$ was purchased from Amersham (Little Chalfont, Bucks., U.K.). Acrylamide, bisacrylamide, ammonium persulphate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine were from Bio-Rad (Richmond, CA, U.S.A.). Low- and high-range pre-stained molecular-mass protein standards were from Bio-Rad. Protein A–Sepharose CL-4B was from Pharmacia LKB Biotechnology (Uppsala, Sweden). Recombinant human tumour necrosis factor-α (rhTNFα) was kindly provided by Dr. G. R. Adolf (Wien, Austria). Recombinant human granulocyte/macrophage colony-stimulating factor (rhGM-CSF) was from Amgen (Thousand Oaks, CA, U.S.A.). All other chemicals were from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, U.S.A.).

Antibodies

The anti-gelatinase rabbit polyclonal antibody [33,34] was raised against the synthetic peptide LGRFQTFEGDLKWHH [33,34], present in the amino acid sequence of the human MMP-9 gelatinase [35]. The anti-heparanase IgM monoclonal antibody 10E5 (ATCC-HB11403) was raised against murine melanoma heparanase and has been shown previously to recognize human heparanase [36]. The anti-CD11b monoclonal antibody Bear 1 [37] was kindly provided by Dr. J. E. De Vries (Unicet, Lyon, France) and was used as hybridoma culture supernatant. P3X63 myeloma culture supernatant was used as a negative control.

Cell isolation and subcellular fractionation

Human neutrophils were obtained from fresh peripheral blood by dextran sedimentation and centrifugation on Ficoll-Hypaque (Pharmacia LKB Biotechnology), followed by hypotonic lysis of residual erythrocytes as described previously [38]. The final cell preparation contained more than 98 % neutrophils, as assessed by Giemsa–Wright staining. Neutrophils were disrupted and subsequently fractionated in sucrose gradients as described previously [4,7]. Briefly, postnuclear supernatants were layered on to 15–40 % (w/w) continuous sucrose gradients, and centrifuged at 76000 ***g*** for 15 min at 4 °C in a SW-27 rotor (Beckman Instruments, Palo Alto, CA, U.S.A.). Subsequently, subcellular fractions were collected by pumping 60 % (w/w) sucrose into the bottom and stored at −20 °C in the presence of 2 mM PMSF. These subcellular fractions were used for enzymic activity determination, for PAGE or for membrane preparation. The marker enzymes for the distinct subcellular fractions were assayed as described previously [5,7,13]. Membranes from each subcellular fraction were obtained by diluting the fractions with 50 mM Tris/HCl (pH 8.0)/100 mM NaCl, and centrifugation in a 30-type rotor (Beckman Instruments) at 70000 ***g*** for 90 min at 4 °C as described previously [7]. For radiolabelling experiments, membrane proteins were solubilized in borate saline buffer [10 mM sodium borate/140 mM NaCl (pH 8.2)] containing 0.2 % (v/v) Triton X-100.

Heparanase assay

A 50 μl aliquot of ice-cold sample solution was transferred into an Eppendorf microfuge tube and 1 μg of N-[^{3}H]acetylated heparan sulphate, dissolved in 50 μl of 50 mM Tris/HCl/0.5 % (v/v) Triton X-100/0.05 % (w/v) sodium azide, was added. The sample was mixed with 20 μl of 1 M sodium acetate, pH 4.5 (final pH of the solution, 5.0), followed by incubation at 42 °C for 4 h. The reaction was terminated by placing the tubes in an ice bath and mixing with 13 μl of ice-cold 100 % (w/v) trichloroacetic acid. After a 30 min incubation at 4 °C, the samples were centrifuged at 18000 ***g*** for 5 min, and then the supernatant was analysed by high-speed gel-permeation chromatography using a Shimadzu LC7 system equipped with a TSK-Gel G3000PW XL column. Elution was performed at room temperature with 12.5 mM Tris/HCl/150 mM NaCl, pH 7.5, at a flow rate of 1.0 ml/min. Degradation of heparan sulphate was monitored by measuring the increase in radioactivity of heparan sulphate with a molecular mass lower than 10 kDa. The percentage of heparanase release was calculated by measuring the degradation of soluble [^{3}H]acetylated heparan sulphate (d.p.m.) in the supernatants of stimulated cells and in total cells.

Zymogram analysis

Zymographic assays for gelatinase were performed as described previously [39], using subcellular fractions and conditioned media of cytokine-activated neutrophils. Total protein (1 μg) from each subcellular fraction or equal volumes of conditioned medium from resting or activated neutrophils were used for zymographic assays. Gelatin (1 mg/ml) was dissolved in 0.3 % (w/v) SDS and then co-polymerized with 7.5 % (w/v) acrylamide. SDS/PAGE was carried out according to the method of Laemmli [40]. Removal of SDS was achieved by rinsing the gels twice (45 min each wash) in 2.5 % (v/v) Triton X-100/50 mM Tris/HCl, pH 7.5. The gels were then incubated overnight at 37 °C in 150 mM NaCl/10 mM $CaCl_2$/50 mM Tris/HCl, pH 7.5, containing 0.05 % (w/v) sodium azide. Gels were stained with 0.5 % (w/v) Coomassie Blue R-250 and the gelatinolytic activity was detected as a transparent band on slab gels.

Immunoblot analysis

Total proteins (30 μg) from the distinct subcellular fractions were separated by using SDS/PAGE under reducing conditions, transferred to nitrocellulose filters and subjected to immunological detection as described previously [41]. Immunodetection of gelatinase and heparanase were carried out by overnight incubation of the nitrocellulose filters with anti-gelatinase poly-

clonal antibody at a dilution of 1:400 in TBS buffer [50 mM Tris/HCl (pH 8.0)/150 mM NaCl] containing 0.05% (v/v) Tween 20, and with anti-heparanase monoclonal antibody at a dilution of 1:200 in TBS buffer containing 0.05% (v/v) Tween 20 respectively. Signal was developed using a biotin–streptavidin–horseradish peroxidase system (Amersham) or an ECL kit (Amersham) according to the manufacturer's instructions.

Immunofluorescence flow cytometry

Immunofluorescence flow cytometry analysis was performed in a FACStar-Plus flow cytometer (Becton-Dickinson, San Jose, CA, U.S.A.) as described previously [6]. Linear immunofluorescence values were obtained in each experiment, and the fluorescence observed using the myeloma P3X63 supernatant was considered as background. Specific linear fluorescence was obtained by subtracting the control fluorescence in which the monoclonal antibody was substituted by the myeloma P3X63.

Immunoprecipitation

Solubilized membrane proteins (50 μg) from the distinct subcellular fractions were radioiodinated in solution with chloroglycoluril (Iodo-gen, Pierce, Rockford, IL, U.S.A.), and processed for immunoprecipitation with anti-CD11b monoclonal antibody as described previously [16]. Samples were subjected to SDS/10%-PAGE, autoradiography and densitometry.

Neutrophil activation

Cells were resuspended at 3×10^6 neutrophils/ml in Hepes/glucose buffer [150 mM NaCl/5 mM KCl/10 mM Hepes/1.2 mM $MgCl_2$/1.3 mM $CaCl_2$/5.5 mM glucose (pH 7.5)] and incubated in the absence or presence of 250 units/ml TNFα or 50 ng/ml GM-CSF for 15 min at 37 °C. Subsequently, cells were pelleted by centrifugation at 300 ***g*** for 10 min. Supernatants were collected to determine the release of heparanase, gelatinase and other enzyme markers. The sedimented cells were saved to determine CD11b cell-surface expression by using immunofluorescence flow-cytometry analysis.

Confocal laser scanning microscopy

Resting cells were resuspended in Hepes/glucose buffer (10^7 cells/ml), and an aliquot (40 μl/ml) of the cell suspension was placed on slides and incubated at 37 °C for 5 min. Cells were washed with PHEM buffer [60 mM Pipes/25 mM Hepes/10 mM EGTA/3 mM $MgCl_2$ (pH 7)], and fixed in 4% (w/v) formaldehyde in PHEM buffer for 10 min. After extensive washing with PBS, cells were permeabilized for 5 min at −20 °C acetone, and then washed again with PBS. Incubation with primary antibodies was for 1 h at 37 °C in a humidified chamber. The antibodies used in this study were anti-gelatinase rabbit polyclonal antibody (diluted 1:400 in PBS) and anti-heparanase mouse IgM monoclonal antibody (diluted 1:200 in PBS). For co-localization studies, these antibodies were added consecutively. Then, cells were washed with PBS and incubated subsequently with phycoerythrin-conjugated anti-rabbit antibody (diluted 1:256 in PBS) and fluorescein isothiocyanate-conjugated anti-mouse antibody (diluted 1:200 in PBS) for 45 min in a humidified chamber at room temperature in the dark. Slides were then washed extensively with PBS and mounted in the aqueous-medium Crystal/Mount (Biomeda, Foster City, CA, U.S.A.). Negative controls were routinely prepared by omitting the primary antibodies or by using an irrelevant antibody, showing no fluorescence staining of the samples. Furthermore, no cross-reactivity was detected between the phycoerythrin-conjugated anti-rabbit antibody and the anti-heparanase antibody, and the fluorescein isothiocyanate-conjugated anti-mouse antibody and the anti-gelatinase antibody. Fluorescence was visualized with a Zeiss LSM 310 laser scan confocal microscope. Co-localization of both antigens was analysed by excitation of both fluorochromes in the same section.

RESULTS

In order to identify the subcellular location of heparanase in resting neutrophils we carried out subcellular fractionation studies on sucrose gradients as reported previously [7]. Post-nuclear fractions were separated by rate zonal sedimentation under conditions that resolved cytosol (lactate dehydrogenase), plasma membrane (5′-nucleotidase), gelatinase-containing tertiary granules (gelatinase), specific granules (lysozyme and lactoferrin) and azurophilic granules (peroxidase), as shown in Figure 1(A). Lysozyme, although present in both specific and azurophilic granules, was shown to be a reliable marker for specific granules [7]. Alkaline phosphatase has been widely used as a plasma membrane marker [19,42], but a latent alkaline phosphatase activity, i.e. the alkaline phosphatase activity that is measured only in the presence of detergent, has been used as a marker of a low-density cytoplasmic organelle in human neutrophils [9]. In our studies, alkaline phosphatase was measured in the presence of 1% (v/v) Triton X-100 to take into account the latent pool of this enzyme activity [9]. Nevertheless, we found that alkaline phosphatase co-fractionated with the plasma-membrane marker 5′-nucleotidase, indicating that under our experimental conditions we were unable to separate plasma membrane from the latent alkaline-phosphatase-rich organelle. We found that gelatinase activity was mainly enriched in fractions 4 and 5, with a lower content in fraction 6, when assayed enzymically in solution (Figure 1A). Gelatinase-containing tertiary granules (mainly fractions 4 and 5) were resolved from specific granules (mainly fractions 5 and 6), azurophilic granules (fraction 8) and from plasma membrane (fractions 2 and 3) (Figure 1A). The subcellular localization of gelatinase was further assessed by both immunoblotting and zymogram analysis (Figures 1B and 1C). Following these latter techniques on polyacrylamide gels, we found that gelatinase was mainly present in fractions 4–6, showing that fractions 4 and 5 had a higher enrichment of this enzyme, a similar result to that obtained assaying enzymic activity in solution (cf. Figures 1A–1C). The slight differences between the distribution data obtained for gelatinase subcellular location using an enzymic assay in solution or on polyacrylamide gels could be explained by the separation of a putative gelatinase inhibitor following gel electrophoresis. Thus we found practically the same subcellular fractionation pattern for gelatinase using both protein identification by immunoblotting and enzymic activity, thereby validating any of these methods to localize gelatinase-rich tertiary granules. This is consistent with a previous report by Morel et al. [25], who found identical subcellular distributions for both gelatinase activity and immunoblot analysis of gelatinase.

MMP-9 gelatinase activities with molecular masses of approx. 180, 110 and 92 kDa were observed by zymogram analysis (Figure 1C). Our results are in agreement to those reported previously with purified gelatinase from extracts of isolated neutrophil granules [43], or with secreted gelatinase from human neutrophils in response to PMA treatment [24,44]. The high-molecular-mass enzyme is suggested to be a homodimer of the 92 kDa MMP-9 gelatinase achieved in the absence of the tissue

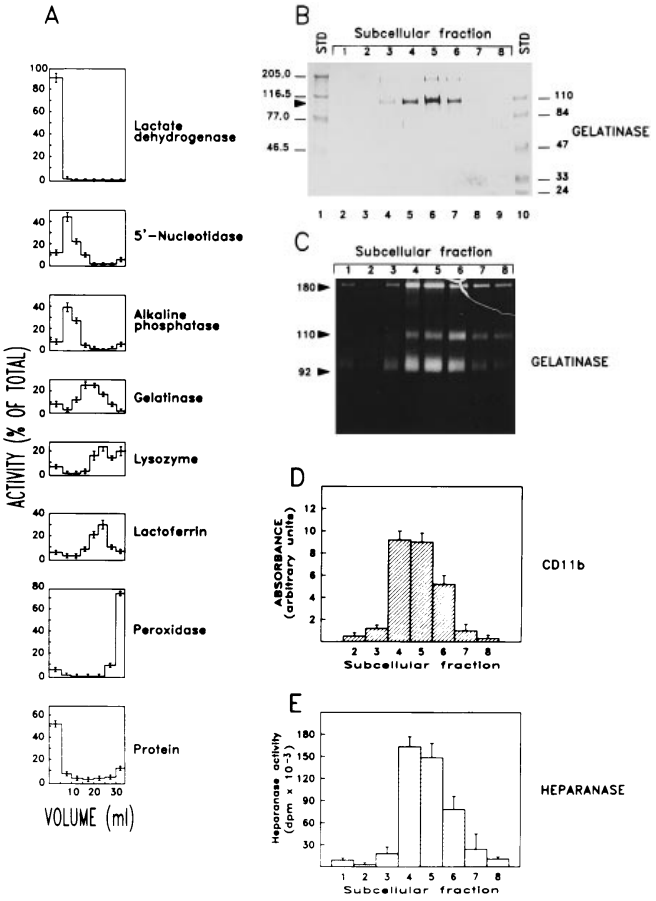

Figure 1 Subcellular localization of gelatinase, CD11b and heparanase in resting human neutrophils

(**A**) Subcellular distributions of marker enzymes after gradient centrifugation of postnuclear fractions obtained from resting human neutrophils, as described in the Materials and methods section. Plots of percentage of total activity in each fraction (versus volume) are shown. The total amounts of protein and lactoferrin, as well as the total enzyme activities for each marker enzyme in all the fractions, are taken as 100%. The actual activity values for the distinct enzyme markers in the postnuclear fraction of resting human neutrophils have been reported previously [4,13]. Values are shown as means ± S.E. of three independent determinations. The percentages of recovered activities were higher than 80% for all the assayed markers. (**B**) Equal amounts of total proteins (30 μg), prepared from the subcellular fractions of resting human neutrophils shown in (**A**), were run on SDS/10% polyacrylamide gels and analysed by immunoblotting using a specific anti-gelatinase antibody, as described in the Materials and methods section. Lanes 1 and 10, molecular mass protein standards (STD); lanes 2–9, subcellular fractions 1–8 shown in (**A**). The position of MMP-9 gelatinase is indicated with an arrowhead on the left. The molecular masses (kDa) of protein standards are indicated on the left and right. (**C**) Equal amounts of total proteins (1 μg), prepared from the subcellular fractions of resting human neutrophils shown in (**A**), were run on SDS/7.5% polyacrylamide gels to detect gelatinase activity by zymogram analysis through gelatin-embedded SDS gels, as described in the Materials and methods section. The positions and molecular masses (kDa) of the bands containing gelatinolytic activity are indicated with arrowheads. (**D**) Solubilized membranes proteins, isolated from the subcellular fractions shown in (**A**), were iodinated and immunoprecipitated with anti-CD11b monoclonal antibody. Immunoprecipitates were then subjected to PAGE, autoradiography and densitometric analysis as described in the Materials and methods section. Values are means ± S.E. of three independent determinations. (**E**) Degradation of soluble ^{3}H-labelled heparan sulphate (d.p.m.) by the distinct subcellular fractions shown in (**A**) was analysed as described in the Materials and methods section.

inhibitor of metalloproteases [45], which is consistent with the fact that neutrophils do not express this inhibitor [46]. The finding of a single band of approx. 92 kDa in Western blotting analysis (Figure 1B) and three bands (180 kDa, 110 kDa and 92 kDa) in zymogram analysis (Figure 1C) is due to the fact that

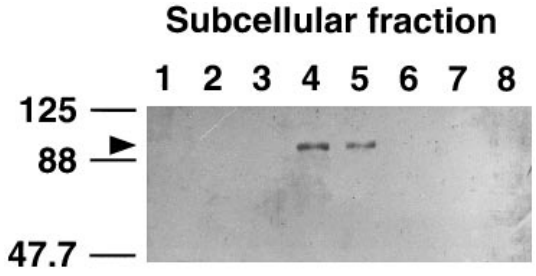

Figure 2 Subcellular localization of heparanase in resting human neutrophils by immunoblotting

Equal amounts of total proteins (30 μg), prepared from the subcellular fractions 1–8 shown in Figure 1(A) from resting human neutrophils, were run on SDS/8% polyacrylamide gels and analysed by immunoblotting using a specific anti-heparanase monoclonal antibody, as described in the Materials and methods section. The position of heparanase is indicated with an arrowhead. The molecular masses (kDa) of protein standards are indicated.

Western blotting was performed in the presence of 2-mercaptoethanol and zymogram analysis was carried out in the absence of this reducing agent. This result is in accordance with previous reports [14,24,44] showing that the SDS/PAGE of purified gelatinase rendered a single band of approx. 92 kDa under reducing conditions and three bands of molecular masses of approx. 225, 130 and 92 kDa under non-reducing conditions. The 130 kDa band of MMP-9 gelatinase has been demonstrated to be a complex of 92 kDa gelatinase and the 25 kDa protein named neutrophil gelatinase-associated lipocalin, which is a member of the lipocalin family [47].

On the other hand, we found that the leucocyte antigen CD11b was distributed in fractions 4–6, with a main location in fractions 4 and 5 (Figure 1D), a fractionation pattern practically identical with that of the gelatinase-containing tertiary granules. This result is consistent with previous reports showing a main localization of CD11b/CD18 in gelatinase-rich tertiary granules [15–20].

As shown in Figure 1(E), we found that heparanase activity was present in higher amounts in fractions 4 and 5, with a lower content in fraction 6. This subcellular distribution was practically identical with that of gelatinase-containing tertiary granules. The subcellular localization for heparanase was further assessed by immunoblotting analysis using a monoclonal antibody raised against heparanase which recognized a band of 96 kDa in human neutrophils (Figure 2), the same molecular mass as that reported previously for human and mouse melanoma heparanase [27]. As shown in Figure 2, we found a main location of heparanase in fractions 4 and 5. Thus we found practically identical patterns of subcellular distribution for heparanase using both enzymic activity (Figure 1E) and immunoblot analysis (Figure 2), with a major location in fractions 4 and 5 enriched in gelatinase-containing tertiary granules. No heparanase was found in cytosol, plasma membrane or azurophilic granules (Figures 1E and 2). Examination of the respective immunoblots for gelatinase and heparanase (cf. Figures 1B and 2) indicated that, although there is a major co-localization of both enzymes in fractions 4 and 5 of the subcellular fractionation, we found a somewhat broader subcellular distribution for gelatinase in resting neutrophils (Figures 1B and 1C). This could suggest the existence of subsets of tertiary granules containing distinct proportions of gelatinase and heparanase.

We also found that gelatinase activity was readily secreted on neutrophil activation with the cytokines TNFα or GM-CSF

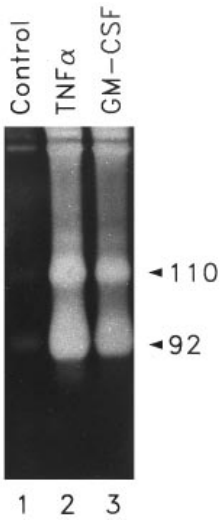

Figure 3 Release of gelatinase in human neutrophils treated with TNFα or GM-CSF

Detection of gelatinase activity by zymogram analysis in the extracellular medium from human neutrophils incubated for 15 min at 37 °C in the absence (control, lane 1) or in the presence of 250 units/ml TNFα (lane 2) or 50 ng/ml GM-CSF (lane 3), as described in the Materials and methods section. The positions and molecular masses (kDa) of the bands characteristic of MMP-9 gelatinase are indicated with arrowheads.

Table 1 Release of heparanase and granule enzyme markers, and increase of the CD11b cell-surface expression on incubation of human neutrophils with TNFα or GM-CSF

Neutrophils were incubated with TNFα or GM-CSF as described in the Materials and methods section. Then, the cellular supernatants were assayed for heparanase and for the granule enzyme markers: gelatinase (tertiary granules), lysozyme (specific granules) and β-glucuronidase (azurophilic granules). The sedimented cells were analysed for CD11b cell-surface expression by fluorescence flow-cytometry analysis. Values are means ± S.E. of three independent determinations.

Protein	Control	TNFα	GM-CSF
Heparanase*	14.1 ± 1.1	50.1 ± 7.8	52.8 ± 10.6
Gelatinase*	13.7 ± 2.8	47.6 ± 7.5	48.1 ± 8.7
Lysozyme*	6.0 ± 1.5	8.1 ± 2.2	8.2 ± 1.7
β-Glucuronidase*	1.3 ± 0.1	1.7 ± 0.1	1.6 ± 0.2
CD11b†	73.0 ± 4.5	121.6 ± 7.6	124.5 ± 8.5

* Percentage of the total cellular enzyme activity released to the extracellular medium. The total cellular enzyme content was measured after cellular disruption by addition of 0.2% (v/v) Triton X-100 and/or by thawing/freezing.

†Antigen cell-surface expression in arbitrary mean fluorescence units.

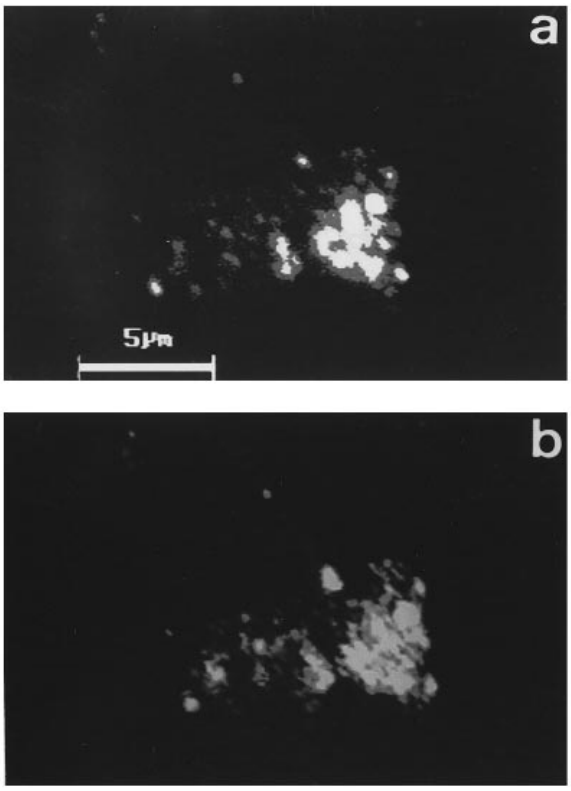

Figure 4 Localization of gelatinase and heparanase in resting human neutrophils by confocal laser scanning microscopy

Indirect immunofluorescence of gelatinase (polyclonal antibody) and heparanase (monoclonal antibody) in resting human neutrophils using confocal laser scanning microscopy, as described in the Materials and methods section. Cells were permeabilized and incubated with (**a**) anti-gelatinase (red fluorescence), and (**b**) anti-heparanase (green fluorescence) antibodies. Data shown correspond to a representative single cell. Bar = 5 μm.

(Figure 3 and Table 1). Accordingly, we found that treatment of human neutrophils with these cytokines induced release of heparanase, as well as an increase in the cell-surface expression of CD11b (Table 1). The percentages of both gelatinase and heparanase release following TNFα or GM-CSF treatment were very similar (Table 1), further supporting the localization of both enzymes in the same intracellular organelle which is readily exocytosed on cell activation. In contrast, TNFα or GM-CSF treatment did not induce release of specific or azurophilic granule contents (Table 1). These results show that heparanase, CD11b/CD18 and gelatinase share the same subcellular localization in an intracellular organelle, which is readily mobilized towards the cell surface on cell activation. Thus these release experiments indicated co-mobilization of heparanase, gelatinase and CD11b/CD18, and were correlated with a decrease in the content of these proteins in the tertiary granule fractions in subcellular fractionation assays of cytokine-stimulated neutrophils with no apparent changes in the content of other granule markers (results not shown). These data agree with those reported previously describing an increase in CD11b cell-surface expression on neutrophil treatment with TNFα [48] or GM-CSF [19,49], with no release of markers for specific and azurophilic granules.

We found that the anti-heparanase antibody used in the present study achieved a very poor signal in immunogold electron microscopy, but rendered appropriate immunofluorescent images. Thus in order to investigate further the putative co-localization of gelatinase and heparanase, as well as to visualize the location of both enzymes in intact resting cells, the intracellular gelatinase and heparanase were indirectly stained with specific antibodies conjugated with phycoerythrin and fluorescein respectively, and observed by confocal laser scanning microscopy. The antibodies used in this study were specific for gelatinase and heparanase respectively, as assessed by immunoblotting analysis (Figures 1B and 2). The antiserum against gelatinase recognized only a band of approx. 92 kDa, corresponding to the molecular mass of MMP-9 gelatinase (Figure 1B), and the monoclonal antibody against heparanase recognized only a band of approx. 96 kDa, corresponding to the molecular mass of heparanase

(Figure 2). As shown in Figure 4, in resting human neutrophils, gelatinase and heparanase were clearly located in intracellular cytoplasmic granules and showed similar fluorescence patterns. Side-by-side comparison of the fluorescence images from both gelatinase (red fluorescence in Figure 4a) and heparanase (green fluorescence in Figure 4b) showed an overlapping staining, indicating a major co-localization of gelatinase and heparanase in the same organelles. In this regard, superimposition of the above fluorescence images showed an extensive co-localization of both enzymes (results not shown). Negative controls, where the primary antibodies were omitted, or where an irrelevant antibody was used, showed no fluorescence staining of the samples. Confocal microscopy of TNFα-treated neutrophils showed a decrease in both gelatinase- and heparanase-stained granules when compared with untreated neutrophils in single labelling experiments (results not shown).

DISCUSSION

The results reported here indicate for the first time that two major extracellular-matrix degradative enzymes, heparanase and gelatinase, are mainly located in the same organelle in human resting neutrophils, namely the tertiary granule, which is prone to fuse with the plasma membrane on cell activation. In addition, we have found that the adhesive protein CD11b/CD18 is also mainly located in the tertiary granules. These conclusions are evidenced by: (a) a major co-localization of gelatinase and heparanase activities in subcellular fractionation studies of resting neutrophils; (b) a major co-localization of gelatinase, heparanase and CD11b/CD18 proteins assessed by immunoblotting and immunoprecipitation analysis in subcellular fractionation studies of resting neutrophils; (c) similar percentages of both gelatinase and heparanase release, as well as a parallel CD11b/CD18 up-regulation, following cell stimulation under experimental conditions that do not induce secretion of either specific or azurophilic granule contents; (d) a major co-localization between gelatinase and heparanase in resting human neutrophils assessed by confocal laser scanning microscopy.

Nevertheless, although we found a major co-localization of gelatinase and heparanase in resting human neutrophils by subcellular fractionation studies, release experiments and confocal laser scanning microscopy, we could also observe a somewhat broader distribution of gelatinase (cf. Figures 1B and 2). On these grounds, the existence of a major tertiary granule population could be envisaged, containing both gelatinase and heparanase, as well as the presence of subsets of granules containing these proteins in different proportions.

The localization of heparanase in the gelatinase-containing tertiary granules, which are readily mobilized on cell activation, gives an explanation for the previous finding that heparanase could be readily released from human neutrophils without apparent release of lysozyme [29]. On the other hand, Matzner et al. [30] have also reported that heparanase is mainly located in subcellular fractions enriched in specific granules in human neutrophils. However, gelatinase activity was not measured in that study [30], and the subcellular fractionation procedure used did not allow a proper separation of both specific and gelatinase-rich tertiary granules. In fact, specific and gelatinase-rich tertiary granules have almost identical densities, being difficult to resolve from each other, except under certain fractionation conditions using sucrose- or Percoll-gradient systems [3,5,7,14,18–20]. Previous reports have shown that most of the CD11b/CD18 protein is present in peroxidase-negative granules, including both specific and gelatinase-containing granules, and secretory vesicles in unstimulated human neutrophils [15–20,32].

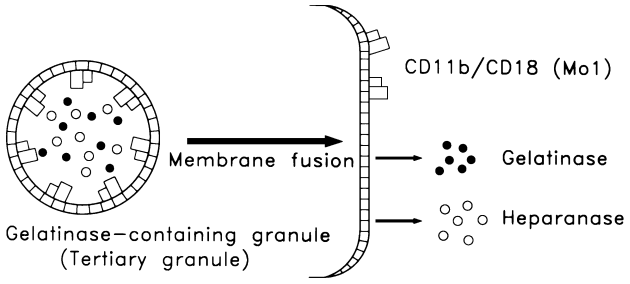

Figure 5 Release and translocation into the cell surface of proteins involved in human neutrophil extravasation on mobilization of gelatinase-containing tertiary granules

This is a schematic diagram designed to portray a currently plausible mechanism for the involvement of gelatinase-rich tertiary granule mobilization in neutrophil extravasation. Mo1 is composed of non-covalently linked CD11b and CD18 heterodimers. Evidence for the subcellular localization of CD11b and CD18 in gelatinase-rich tertiary granules has already been reported [15–20]. The gelatinase-rich tertiary granule components shown in the scheme are translocated to the cell surface (Mo1) or secreted to the extracellular medium (gelatinase and heparanase) following fusion of gelatinase-rich tertiary granules with the plasma membrane on neutrophil activation. The secreted gelatinase and heparanase, as well as the membrane-bound Mo1, may participate in the extravasation of human neutrophils, as discussed in the text.

Our present results corroborate previous findings showing a significant location of CD11b/CD18 in the gelatinase-containing granules [15–20].

Interestingly, the present findings indicate that CD11b, a protein involved in cell attachment to endothelium, and heparanase, an enzyme involved in cell invasion, share the same subcellular localization in the gelatinase-containing tertiary granules in resting human neutrophils. Neutrophil gelatinase (MMP-9) has been shown to degrade denatured collagen (gelatin) as well as native type IV, V and XI collagen [24,25,44,50], and potentiates the activity of interstitial collagenase (MMP-8), also produced by neutrophils [21]. This substrate specificity suggests that gelatinase might play an important role in the degradation of extracellular matrix and in the process of extravasation. Heparanase also degrades extracellular matrix by the cleavage of the carbohydrate chains of heparan sulphate proteoglycans, playing an important role in cell invasion [28]. On these grounds, it could be envisaged that the exocytosis of gelatinase-containing tertiary granule contents can play a key role in neutrophil diapedesis (Figure 5). The scheme depicted in Figure 5 summarizes the results obtained in the present work, which underline the putative involvement of tertiary granule mobilization in human neutrophil extravasation. Thus on appropriate cell stimulation, gelatinase-containing tertiary granules would fuse with the cell surface, exposing additional CD11b proteins at the cell surface and secreting gelatinase and heparanase into the environment (Figure 5). The CD11b up-regulation could enhance neutrophil attachment to the endothelium. The secreted gelatinase and heparanase may play a major role in neutrophil diapedesis by degrading partially the connective tissue and the basement membrane surrounding endothelium.

Although a great controversy has been raised regarding the existence of gelatinase-containing tertiary granules for a number of years [3-7,10,14,51], the existence of separate gelatinase-rich granules, also named tertiary granules [3–7], is confirmed by a different distribution pattern in subcellular fractionation studies [3–5,7,14,18,19,25,52], by a different granule mobilization [3,5–7,12–17,19,53], and by immunoelectron microscopy [10]. A number of functionally important proteins involved in signalling (CD45, Rap proteins, diacylglycerol lipase system) [16,41,54], in

superoxide anion generation (cytochrome b_{558}) [4,7,52], in vacuole acidification [5], in synthesis of platelet-activating factor [55], and in receptors (*N*-formylmethionyl-leucyl-phenylalanine receptor, C3bi receptor) [6,15–20], have been reported to be partially or mainly located in this gelatinase-containing tertiary granule. In summary, the results reported herein further support the notion that mobilization of gelatinase-rich tertiary granules can modulate early neutrophil responses during cell activation [6], such as neutrophil adhesion and diapedesis.

We thank the Blood Banks from the University Hospital and from Rio Hortega Hospital for blood supply. This research was supported in part by grants PM91-0034 (to A.F.), SAF95-539 (to A.F.) and PB95-0713 (to F.M.) from the 'Dirección General de Investigación Científica y Técnica' (DGICYT), and grant FIS96/1434 (to F.M.) from the 'Fondo de Investigación Sanitaria'. C.G. is the recipient of a fellowship from the 'Fondo de Investigacion Sanitaria'.

REFERENCES

1 Bainton, D. F., Ullyot, J. L. and Farquhar, M. G. (1971) J. Exp. Med. **134**, 907–934
2 Bretz, M. and Baggiolini, M. (1974) J. Cell Biol. **63**, 251–269
3 Dewald, B., Bretz, M. and Baggiolini, M. (1982) J. Clin. Invest. **70**, 518–525
4 Mollinedo, F. and Schneider, D. L. (1984) J. Biol. Chem. **259**, 7143–7150
5 Mollinedo, F., Manara, F. S. and Schneider, D. L. (1986) J. Biol. Chem. **261**, 1077–1082
6 Mollinedo, F., Pulido, R., Lacal, P. M. and Sánchez-Madrid, F. (1991) Scand. J. Immunol. **34**, 33–43
7 Mollinedo, F., Gajate, C. and Schneider, D. L. (1991) Mol. Cell. Biochem. **105**, 49–60
8 Smith, G. P., Sharp, G. and Peters, T. J. (1985) J. Cell Sci. **76**, 167–178
9 Borregaard, N., Miller, L. J. and Springer, T. (1987) Science **237**, 1204–1206
10 Kjeldsen, L., Bainton, D. F., Sengelov, H. and Borregaard, N. (1993) Blood **82**, 3183–3191
11 Borregaard, N., Lollike, K., Kjeldsen, L., Sengelov, H., Bastholm, L., Nielsen, M. H. and Bainton, D. F. (1993) Eur. J. Haematol. **51**, 187–198
12 Mollinedo, F. and Schneider, D. L. (1987) FEBS Lett. **217**, 158–162
13 Mollinedo, F. (1986) Biochim. Biophys. Acta **861**, 33–43
14 Kjeldsen, L., Bjerrum, O. W., Askaa, J. and Borregaard, N. (1992) Biochem. J. **287**, 603–610
15 Petrequin, P. R., Todd III, R. F., Devall, L. J., Boxer, L. A. and Curnutte III, J. T. (1987) Blood **69**, 605–610
16 Lacal, P., Pulido, R., Sánchez-Madrid, F. and Mollinedo, F. (1988) J. Biol. Chem. **263**, 9946–9951
17 Lacal, P., Pulido, R., Sánchez-Madrid, F., Cabañas, C. and Mollinedo, F. (1988) Biochem. Biophys. Res. Commun. **154**, 641–647
18 Jones, D. H., Schmalstieg, F. C., Dempsey, K., Krater, S. S., Nannen, D. D., Smith, C. W. and Anderson, D. C. (1990) Blood **75**, 488–498
19 Graves, V., Gabig, T., McCarthy, L., Strour, E. F., Leemhuis, T. and English, D. (1992) Blood **80**, 776–787
20 Sengelov, H., Kjeldsen, L., Diamond, M. S., Springer, T. A. and Borregaard, N. (1993) J. Clin. Invest. **92**, 1467–1476
21 Hasty, K. A., Pourmotabbed, T. F., Goldberg, G. I., Thompson, J. P., Spinella, D. G., Stevens, R. M. and Mainardi, C. L. (1990) J. Biol. Chem. **265**, 11421–11424
22 Murphy, G., Bretz, U., Baggiolini, M. and Reynolds, J. J. (1980) Biochem. J. **192**, 517–525
23 Devarajan, P., Johnston, J. J., Ginsberg, S. S., Van Wart, H. E. and Berliner, N. (1992) J. Biol. Chem. **267**, 25228–25232
24 Morel, F., Berthier, S., Guillot, M., Zaoni, P., Massoubre, C., Didier, F. and Vignais, P. V. (1993) Biochem. Biophys. Res. Commun. **191**, 269–274
25 Morel, F., Dewald, B., Berthier, S., Zaoui, P., Dianoux, A. C., Vignais, P. V. and Baggiolini, M. (1994) Biochim. Biophys. Acta **1201**, 373–380
26 Liotta, L. A., Steeg, P. S. and Stetler-Stevenson, W. G. (1991) Cell **64**, 327–336
27 Nakajima, M., Irimura, T. and Nicolson, G. (1988) J. Cell. Biochem. **36**, 157–167
28 Nakajima, M. and Chop, A. M. (1991) Semin. Cancer Biol. **2**, 115–127
29 Matzner, Y., Bar-Ner, M., Yahalom, J., Ishai-Michaeli, R., Fuks, Z. and Vlodavsky, I. (1985) J. Clin. Invest. **76**, 1306–1313
30 Matzner, Y., Vlodavsky, I., Bar-Ner, M., Ishia-Michaeli, R. and Tauber, A. I. (1992) J. Leukocyte Biol. **51**, 519–524
31 Carlos, T. M. and Harlan, J. M. (1994) Blood **84**, 2068–2101
32 Bainton, D. F., Miller, L. J., Kishimoto, T. K. and Springer, T. A. (1987) J. Exp. Med. **166**, 1641–1653
33 Juarez, J., Clayman, G., Nakajima, M., Tanabe, K. K., Saya, H., Nicolson, G. L. and Boyd, D. (1993) Int. J. Cancer **55**, 10–18
34 Nakajima, M., Morikawa, K., Fabra, A., Bucana, C. and Fidler, I. J. (1990) J. Natl. Cancer Inst. **82**, 1890–1898
35 Wilhelm, S. M., Collier, I. E., Marmer, B. L., Eisen, A. Z., Grant, G. A. and Goldberg, G. I. (1989) J. Biol. Chem. **264**, 17213–17221
36 Marchetti, D., McQuillan, D. J., Spohn, W. C., Carson, D. D. and Nicolson, G. L. (1996) Cancer. Res. **56**, 2856–2863
37 Keizer, G. D., Borst, J., Figdor, C. G., Spits, H., Miedema, F., Terhorst, C. and De Vries, J. E. (1985) Eur. J. Immunol. **15**, 1142–1148
38 Mollinedo, F., Nieto, J. M. and Andreu, J. M. (1989) Mol. Pharmacol. **36**, 547–555
39 Welch, D. R., Fabra, A. and Nakajima, M. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 7678–7682
40 Laemmli, U.K. (1970) Nature (London) **277**, 680–685
41 Mollinedo, F., Perez-Sala, D., Gajate, C., Jimenez, B., Rodriguez, P. and Lacal, J. C. (1993) FEBS Lett. **326**, 209–214
42 Parkos, C. A., Cochrane, C. G., Schmitt, M. and Jesaitis, A. J. (1985) J. Biol. Chem. **260**, 6541–6547
43 Murphy, G., Ward, R., Hembry, R. M., Reynols, J. J., Kühn, K. and Tryggvason, K. (1989) Biochem. J. **258**, 463–472
44 Hibbs, M. S., Hasty, K. A., Seyer, J. M., Kang, A. H. and Mainardi, C. L. (1985) J. Biol. Chem. **260**, 2493–2500
45 Goldberg, G. I., Strongin, A., Collier, I. E., Genrich, L. T. and Marmer, B. L. (1992) J. Biol. Chem. **267**, 4583–4591
46 Cooper, T. W., Eisen, A. Z., Stricklin, G. P. and Welgus, H. G. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 2779–2783
47 Kjeldsen, L., Johnsen, A. H., Sengelov, H. and Borregaard, N. (1993) J. Biol. Chem. **268**, 10425–10432
48 Díaz-González, F., González-Alvaro, I., Campanero, M. R., Mollinedo, F., del Pozo, M. A., Muñoz, C., Pivel, J. P. and Sánchez-Madrid, F. (1995) J. Clin. Invest. **95**, 1756–1765
49 Lopez, A. F., Williamson, D. J., Gamble, J. R., Begley, C. G., Harlan, J. M., Klebanoff, S. J., Waltersdorph, A., Wong, G., Clark, S. C. and Vadas, M. A. (1986) J. Clin. Invest. **78**, 1220–1228
50 Pourmotabbed, T., Solomon, T. L., Hasty, K. A. and Mainardi, C. L. (1994) Biochim. Biophys. Acta **1204**, 97–107
51 Hibbs, M. S. and Bainton, D. F. (1989) J. Clin. Invest. **84**, 1395–1402
52 Kjeldsen, L., Sengelov, H., Lollike, K., Nielsen, M. H. and Borregaard, N. (1994) Blood **83**, 1640–1649
53 Sengelov, H., Kjeldsen, L. and Borregaard, N. (1993) J. Immunol. **150**, 1535–1543
54 Balsinde, J., Diez, E. and Mollinedo, F. (1991) J. Biol. Chem. **266**, 15638–15643
55 Mollinedo, F., Gómez-Cambronero, J., Cano, E. and Sánchez-Crespo, M. (1988) Biochem. Biophys. Res. Commun. **154**, 1232–1239

Received 10 January 1997/8 July 1997; accepted 9 July 1997