Degradation of Heparan Sulfate in the Subendothelial Extracellular Matrix by a Readily Released Heparanase from Human Neutrophils

Possible Role in Invasion through Basement Membranes

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Abstract

Freshly isolated human neutrophils were investigated for their ability to degrade heparan sulfate proteoglycans in the subendothelial extracellular matrix (ECM) produced by cultured corneal and vascular endothelial cells. The ECM was metabolically labeled with $Na_2(^{35}S)O_4$ and labeled degradation products were analyzed by gel filtration over Sepharose 6B. More than 90% of the released radioactivity consisted of heparan sulfate fragments 5–6 times smaller than intact heparan sulfate side chains released from the ECM by either papain or alkaline borohydride. These fragments were sensitive to deamination with nitrous acid and were not produced in the presence of either heparin or serine protease inhibitors. In contrast, degradation of soluble high molecular weight heparan sulfate proteoglycan, which was first released from the ECM, was inhibited by heparin but there was no effect of protease inhibitors. These results indicate that interaction of human neutrophils with the subendothelial ECM is associated with degradation of its heparan sulfate by means of a specific, newly identified, heparanase activity and that this degradation is facilitated to a large extent by serine proteases.

The neutrophil heparanase was readily and preferentially released (15–25% of the cellular content in 60 min) by simply incubating the cells at 4°C in the absence of added stimuli. Under these conditions, <5% of the cellular content of lactate dehydrogenase, lysozyme, and globin degrading proteases was released. Further purification of the neutrophil heparanase was achieved by its binding to heparin-Sepharose and elution at 1 M NaCl. It is suggested that heparanase activity is involved in the early events of extravasation and diapedesis of neutrophils in response to a threshold signal from an extravascular inflamed organ.

Introduction

Neutrophil mobilization constitutes an early and important event in the acute inflammatory process. On their way from the blood toward an affected tissue the cells must invade across the endothelium of postcapillary venules and the underlying basal lamina (1). This process involves adherence of neutrophils to the endothelial luminal surface, invasion and dissociation of endothelial cell junctions, and active participation of hydrolytic enzymes (2, 3). The same is true for virtually every epithelium in the body that is traversed by human neutrophils before they accumulate at the site of inflammation (4). Cell migration is thought to be driven by a chemotactic factor(s) gradient, which at the infected area, is high enough to induce discharge of granules and a subsequent O_2^- production (5). In contrast, while still in circulation the cells exhibit little or no enzyme release in response to the low concentration of the chemoattractant, and thus the vascular endothelial cell lining is protected. It was therefore postulated that neutrophils may contain an additional distinct secretory compartment whose enzymes are excreted in response to a threshold signal, and may participate in the early events of neutrophil mobilization and diapedesis without damaging the endothelial cells themselves. Such a role has been recently ascribed to a metallo-proteinase (gelatinase) that is preferentially released from human neutrophils even in the absence of added stimuli (6).

Recent research on degradation of extracellular macromolecules by invasive cells, whether normal or malignant, has emphasized the use of complex, naturally produced substrates because these resemble the in vivo situation better than isolated constituents of the extracellular matrix (ECM)[1] (7–9). Such a substrate is the subendothelial ECM deposited by cultured bovine endothelial cells (10, 11). This ECM has been shown to resemble the vascular basement membrane in its morphological appearance, platelet reactivity (12), and supramolecular arrangement and to contain characteristic constituents such as laminin, fibronectin, collagen types III and IV, and heparan sulfate (10–14). Proline-labeled matrices secreted by cultured venous endothelial cells were shown to be rapidly degraded by triggered neutrophils via a process dependent on elastase but not oxygen metabolites (15). Other studies on the interaction of cells with the subendothelial ECM revealed a correlation between the activity of a heparan sulfate degrading endoglycosidase (heparanase) and the metastatic potential of various mouse melanoma (16) and lymphoma (17) sublines. The enzyme has also been suggested as playing a role in the extravasation and homing of activated lymphocytes (18, 19) and macrophages (18) and to degrade heparan sulfate upon interaction of platelets with the endothelial cell surface (20) and subendothelial ECM (12).

The present study was undertaken to identify a heparan sulfate degrading endoglycosidase in human neutrophils and to characterize the requirements for its release and mediated degradation of heparan sulfate side chains in the subendothelial ECM.

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1. *Abbreviations used in this paper:* CPC, cetylpiridinium chloride; DME, Dulbecco's modified Eagle's medium; DFP, diisopropylfluorophosphate; ECM, extracellular matrix; FCS, fetal calf serum; FGF, fibroblast growth factor; Kav, variation in elution position; M_r, relative molecular weight; PMSF, phenylmethylsulfonyl fluoride; V_o, excluded volume; V_t, total volume.

Methods

Materials. Fibroblast growth factor (FGF) was purified from bovine brain as described (21). Dulbecco's modified Eagle's medium (DME), Roswell Park Memorial Institute medium (RPMI) 1640, calf serum, fetal calf serum (FCS), penicillin, streptomycin, and trypsin/EDTA solution were obtained from Gibco Laboratories (Grand Island, NY). Tissue culture dishes were obtained from Falcon Labware Division, Becton, Dickinson & Co. (Oxnard, CA). Zymosan, trypsin type III, soybean trypsin inhibitor, diisopropylfluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF), cetylpiridinium chloride (CPC), Triton X-100, and dextran T-40 were from Sigma Chemical Co. (St. Louis, MO). $Na_2(^{35}S)O_4$ and Biofluor scintillation fluid were from New England Nuclear (Boston, MA). Ficoll-Hypaque, dextran 70 (Macrodex), Sepharose 6B, and heparin-Sepharose were from Pharmacia Fine Chemicals (Piscataway, NJ). Sodium Heparin was from Diosynth (Oss, Holland). ^{14}C-globin prepared as described (22) was kindly provided by Dr. M. Mayer (Department of Biochemistry, Hadassah University Hospital, Jerusalem, Israel). All other chemicals were of reagent grade furnished from Sigma Chemical Co.

Cells. Neutrophils were prepared from fresh blood samples obtained from healthy human donors and were purified by dextran sedimentation followed by hypotonic lysis of contaminating erythrocytes and centrifugation over Ficoll-Hypaque as described by Boyum (23). The granulocyte pellet was washed in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (PBS) and suspended at 5×10^6 cells/ml in either PBS containing Ca^{2+} and Mg^{2+} or RPMI medium containing 10% FCS. Preparations obtained in this manner contained >95% neutrophils. The remaining 1–4% mononuclear cells could not account for any detectable heparanase activity as demonstrated in studies with purified preparations of human lymphocytes.

Clonal populations of adult bovine aortic endothelial cells were obtained as previously described (24). Cells were cultured in Dulbecco's modified Eagle's medium (DME, H-16) supplemented with 10% bovine calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml) at 37°C in 10% CO_2 humidified incubators. FGF (100 ng/ml) was added every other day during the phase of active cell growth. Cultures of bovine corneal endothelial cells were established from steer eyes as described (25) and stock cultures were maintained as in the case of vascular cells, except that 5% FCS was included in the growth medium. Both types of cells exhibited a massive secretion of an underlying ECM in a polar fashion and similar in organization and macromolecular composition to naturally occurring subendothelial basement membranes.

Preparation of sulfate-labeled ECM-coated dishes. Vascular or corneal endothelial cells were plated at an initial density of 4×10^4 cells per 35-mm dish and were maintained as described above except that 5% dextran T-40 was included in the growth medium. $Na_2(^{35}S)O_4$ (540–590 mCi/mmol) was added 3 and 7 d after seeding (40 μCi/ml) and the cultures were incubated with the label with no medium change. 5–7 d after reaching confluence (10–12 d after seeding) the cell layer was dissolved by exposure (10 min, 22°C) to 0.5% Triton X-100 in PBS (vol/vol), leaving the underlying ECM intact and firmly attached to the entire tissue culture dish (10, 11). Remaining nuclei, cytoskeletons, and cell debris were removed by a 2–3-min exposure to 0.025 N NH_4OH followed by four washed in PBS. Previous studies have shown that 70–75% of the total ECM bound radioactivity was incorporated into heparan sulfate side chains (12, 14, 17). Some of the ECM-coated dishes were purchased from International Biotechnology Ltd., Hadassah, Jerusalem, Israel.

Degradation of sulfated proteoglycans. $(^{35}S)O_4^=$-labeled ECM was incubated (37°C, 10% CO_2 incubator, 24 h, except when tested at shorter intervals) with 5×10^6 granulocytes suspended in 1 ml RPMI medium containing 10% FCS or with the supernate fraction of cells suspended in PBS and incubated for 1 h at 4°C. This supernate was first adjusted to pH 6.2 with 25 mM phosphate-citrate buffer. The medium was then collected and centrifuged at 10,000 *g* for 5 min. To evaluate the occurrence of proteoglycan degradation, a 0.5-ml aliquot of the centrifuged medium was applied for gel filtration on Sepharose 6B columns (0.7×35 cm in most experiments and 1.1×70 cm in some experiments) and 0.2 ml fractions were eluted with PBS at a flow rate of 5 ml/h. Fractions were counted for radioactivity using Bio-fluor scintillation fluid. The excluded volume (V_o) was marked by blue dextran, and the total included volume (V_t) by phenol red. The latter was shown to co-migrate with free $(^{35}S)O_4^=$ (17–19). Similar gel filtration profiles (Kav values) were obtained by using the ECM produced by either corneal or vascular endothelial cells. Recoveries of labeled material applied on the columns ranged from 85–95% in different experiments. Each experiment was performed at least four times and the variation in elution positions (Kav values) was <10%.

Biochemical assays. Lysozyme (26), lactate dehydrogenase (26), and proteolysis of ^{14}C-globin (22) were determined by previously published methods.

Results

Degradation of sulfated proteoglycans in the ECM by the neutrophil heparanase. Human neutrophils suspended in RPMI medium adhered to the subendothelial ECM within 30 min after seeding. ~30% of the cells attached firmly to the ECM, adopted a flattened and slightly elongated morphology, and showed filopodia and well developed ruffles in contact with the ECM. The other cells were either loosely attached or floating. Only 10–15% of the cells were firmly attached at 24 h after seeding but >95% of the entire cell population remained viable as determined by the trypan blue exclusion test. Degradation of sulfated macromolecules in the ECM was studied by allowing the cells to interact with sulfate-labeled ECM followed by gel filtration (Sepharose 6B) analysis of degradation products released into the incubation medium. In the presence of medium alone (RPMI + 10% FCS) there was a constant release of labeled material (30% of the total incorporated radioactivity over 24 h) that consisted almost entirely (>90%) of large M_r (peak I) components eluted with or next to V_o (14, 17). In contrast, as demonstrated in Fig. 1, incubation of the ECM with human neutrophils

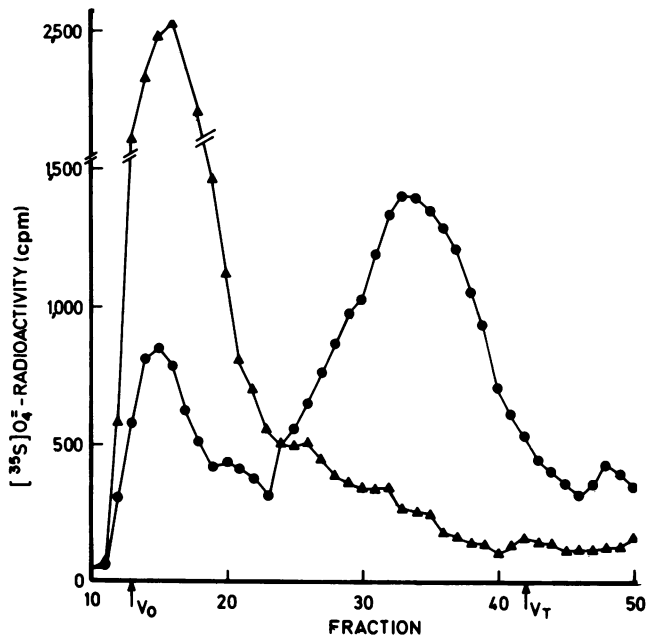

Figure 1. Degradation of sulfate-labeled ECM by human neutrophils. 5×10^6 neutrophils suspended in 1 ml RPMI medium containing 10% FCS were incubated (24 h, 37°C), pH 6.3, in contact with sulfate-labeled ECM in the absence (●) and presence (▲) of 10 μg/ml heparin. The medium was collected, centrifuged, and analyzed by gel filtration on Sepharose 6B as described in Methods.

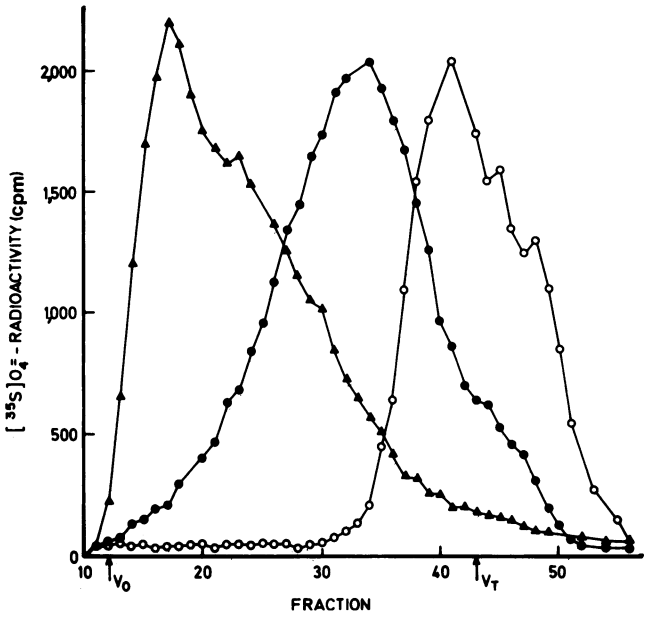

Figure 2. Degradation of sulfate-labeled ECM by the supernate fraction of neutrophils incubated at 4°C. Freshly isolated neutrophils suspended in PBS (5×10^6 cells/ml) were incubated at 4°C for 60 min. The cells were then centrifuged (300 *g*, 10 min) and the supernatant was adjusted to pH 6.2 (20 mM citrate-phosphate buffer) and incubated (24 h, 37°C) with sulfate-labeld ECM, in the absence (●) and presence (▲) of 10 μg/ml heparin. Radioactive material released into the incubation medium was analyzed by gel filtration over Sepharose 6B. Material released during incubation with labeled ECM at pH 6.2, as described above, was also subjected to deamination with nitrous acid (0.24 M $NaNO_3$ in 1.8 M acetic acid, 80 min, 24°C) before gel filtration on Sepharose 6B (○).

(5×10^6 cells/35-mm dish) suspended in RPMI medium and 10% FCS (pH 6.3) resulted in a release of low M_r labeled material (peak II, Kav ~0.68), which constituted >80% of the total released radioactivity. Low M_r material released from the ECM and eluted as peak II was detected already after 1 h incubation with the cells. Its amount increased progressively as time proceeded and reached a plateau at 24 h. ~70% of the total ECM incorporated sulfate was released during a 24-h incubation with 5×10^6 neutrophils. Release of low M_r (peak II) degradation products was inhibited in the presence of 10 μg/ml heparin (Fig. 1) or when the pH of the incubation medium was higher than 7.2 (not shown). Under these conditions 80–90% of the released radioactivity was eluted from Sepharose 6B with and next to V_o ($0.1 < Kav < 0.3$). Exposure of the ECM to papain (0.2 mg/ml, 24 h, 50°C) or alkaline borohydride (1 M $NaBH_4$ in 0.05 N NaOH, 24 h, 45°C) (27) resulted in a release of intact glycosaminoglycan side chains eluted with a Kav of 0.35 (17). This value corresponds to a M_r of about 45,000 daltons (28) which is five- to sixfold higher than that of fragments released during incubation of the ECM with the human granulocytes (Kav ~0.68; $M_r \sim 8.5 \times 10^3$ D). The latter were shown to be degradation products of heparan sulfate as indicated by their resistance to chondroitinase ABC and papain digestion, sensitivity to deamination with nitrous acid (27) (Fig. 2), and precipitation of 70–80% of the released radioactivity with 0.05% CPC in 0.6 M NaCl (29) (not shown). The extent of ECM heparan sulfate degradation was not affected by the presence of either zymosan activated serum (10% vol/vol) or phorbol myristate acetate (100 ng/ml). There was also no significant effect to first "charging" the labeled ECM by incubation with these chemoattractants followed by washing the unbound material before incubation with the neutrophils.

Release of heparanase activity. Heparanase activity is secreted by highly metastatic sublines of mouse lymphoma (16) and melanoma (17) cells and by activated lymphocytes (18, 19) and macrophages (18). As demonstrated in Fig. 2, incubation of freshly isolated human neutrophils (5×10^6 cells/ml PBS) for 1 h at 4°C resulted in a release of heparanase activity as detected by degradation of sulfate-labeled ECM upon incubation with a low-speed (300 *g*, 10 min) supernate fraction of the preincubated neutrophils. Similar results were obtained regardless of the presence or absence of calcium and magnesium in the PBS. Heparan sulfate degradation manifested by the appearance of peak II material was already observed after 3 h of incubation (37°C, pH 6.2) of the labeled ECM with 1 ml of the 4°C supernate fraction and reached a maximal value at 24 h (Fig. 3 *B*). Heparanase activity was detected upon incubation (24 h, 37°C, pH 6.2) of the ECM with as little as 0.1 ml of this neutrophil supernatant (Fig. 3 *A*) and was inhibited in the presence of heparin (Fig. 2). Incubation of ECM with PBS alone yielded small amounts (<10% of the total incorporated radioactivity) of labeled material eluted at V_o but no material eluted as peak II. ~90% of the

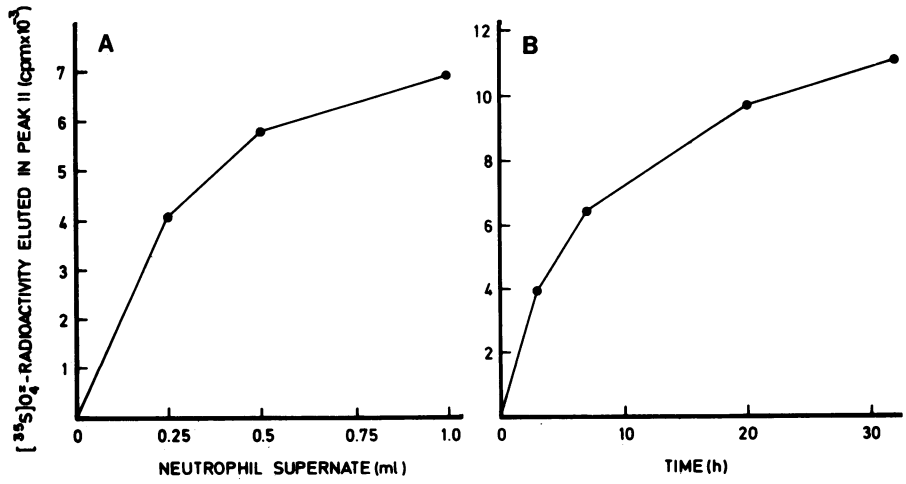

Figure 3. Degradation of sulfate-labeled ECM as a function of incubation time and amount of the neutrophil supernate fraction. Freshly isolated neutrophils were suspended in PBS (5×10^6 cells/ml) and incubated at 4°C for 60 min. Labeled ECM was then incubated with (*A*) various amounts of the neutrophil supernate fraction (24 h, 37°C, pH 6.2) or (*B*) 1 ml of the neutrophil supernate for various time periods. The total amount of released radioactivity eluted as peak II (sum of cpm eluted in fraction 25-45) is plotted against (*A*) volume of the 4°C neutrophil supernatant and (*B*) time of incubation with the labeled ECM.

enzyme activity was retained in the supernate fraction even after a high speed centrifugation (100,000 *g*, 40 min) of the 4°C supernatant, suggesting that the enzyme was released in a soluble form. Degradation of heparan sulfate by the soluble enzyme was optimal at pH 5.4 as reflected by both the amount of released peak II material and size of heparan sulfate cleavage products (Kav = 0.46 at pH 6.6 vs. Kav = 0.72 at pH 5.4) (Fig. 4). The heparan sulfate nature of these fragments was confirmed by exposing the labeled material eluted as peak II to nitrous acid (0.24 M $NaNO_3$ in 1.8 M acetic acid, 80 min, 24°C) (27). This resulted in further cleavage to low M_r (2.5×10^3 D) fragments that were not precipitated by CPC, were eluted with a Kav of 0.84 from Sepharose 6B (Fig. 2), and were retarded on Sephadex G-50.

By using our semiquantitative ECM degradation assay and testing various dilutions of both the 4°C supernate fraction and of lysed preparations of the resuspended cell pellet, it was estimated that 20–25% of the total cellular content of ECM-heparan sulfate degrading activity was released during the 1-h incubation of neutrophils at 4°C (Fig. 5, Table I). Under the conditions of these experiments <50% of the total incorporated radioactivity was released from the ECM and the amount of labeled material eluted as peak II was proportional to the amount of heparanase added. Table I shows that only 3–6% of the cellular content of lysozyme and globin degrading proteases (22) were released into the 4°C supernate fraction. Moreover, release was not due to cell damage since the cytoplasmic enzyme lactate dehydrogenase was fully retained (Table I). In the above estimation of percentage enzyme release it is assumed that the activity of the various enzymes is not affected by cell disruption or affected to a similar extent with all enzymes. 30-min incubation of neutrophils at

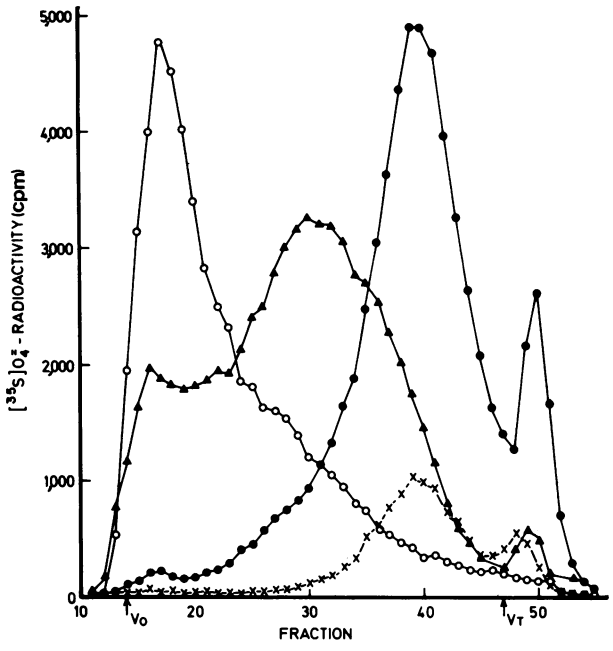

Figure 4. Effect of pH on degradation of ECM heparan sulfate by the neutrophil heparanase. 4°C supernatant of freshly isolated neutrophils, prepared as described in the legend to Fig. 2, was adjusted with phosphate citrate buffer (25 mM) to pH 7.2 (○); 6.6 (▲); 5.4 (●); and 4.6 (×) and incubated (37°C, 24 h) at the indicated pH with sulfate-labeled ECM. Radioactive material released into the incubation medium was subjected to gel filtration on Sepharose 6B.

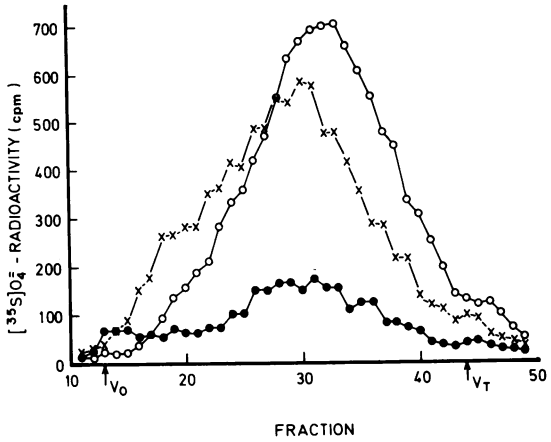

Figure 5. Degradation of heparan sulfate in the ECM by enzymes released from neutrophils at 4°C as compared with degradation by the remaining cell-associated enzymes. Freshly isolated neutrophils suspended in PBS (5×10^6 cells/ml) were incubated at 4°C for 60 min. The cells were centrifuged (300 *g*, 10 min) and the cell pellet was resuspended in the original volume of PBS and subjected to three cycles of freezing and thawing. A 1:8 dilution in PBS of the 4°C supernatant (●) and of the suspension of lysed cells (×) was incubated (24 h, 37°C, pH 6.2) with sulfate-labeled ECM and the released radioactivity was analyzed by gel filtration on Sepharose 6B. The total cellular content of ECM heparan sulfate degradation activity was determined after freezing and thawing of the original suspension of neutrophils and incubation (24 h, 37°C, pH 6.2) of a 1:8 dilution with the labeled ECM (○). Heparanase activity in the 4°C neutrophil supernatant was not affected by three cycles of freezing and thawing. Under the above described conditions the total amount of radioactivity eluted as peak II (fractions 25–42) was 2,250, 7,605 and 9,415 cpm for the 4°C supernate (●); remaining cell pellet (×); and the original cell suspension (○), respectively. Nondiluted preparations of each of these fractions yielded upon incubation with the ECM a total of ~20,000 cpm eluted as peak II. This value constituted ~90% of the total $SO_4^=$ -radioactivity that was incorporated into heparan sulfate.

37°C in the absence or presence of either opsonized zymosan or phorbol myristate acetate did not result in a significant increase of heparanase release despite a concomitant release of 25–30% of the cellular content of lysozyme (not shown).

Partial purification of heparanase activity. Because of the preferential release of heparanase activity, we attempted to further purify the enzyme from the 4°C neutrophil supernate fraction. For this purpose, human neutrophils were isolated from 400 ml normal human blood and suspended in 100 ml of PBS containing Ca^{2+} and Mg^{2+}. After 1-h incubation at 4°C, the supernate fraction was filtered through a heparin-Sepharose column (1.6 × 5 cm). As shown in Fig. 6, both the unadsorbed material and subsequent elution of the column with 0.45 M NaCl (100 ml) had no detectable heparanase activity. The column was then eluted with 1 M NaCl (20 ml in 2-ml fractions) and the material eluted in fractions 3–5 showed a six- to 10-fold higher heparanase activity per milliliter as compared with the original 4°C supernate fraction. The amount of protein in both the starting material and active fractions was too low for an accurate determination by absorbance at 280 nm or by the dye fixation (Bradford) assay. Preliminary analysis by $NaDodSO_4$ polyacrylamide gel electrophoresis and silver nitrate staining revealed, in some of the active fractions, a single faint band with an apparent molecular weight of ~80,000 (not shown).

Table I. Release of Heparanase, Lysozyme, Globin Degrading Proteases, and Lactate Dehydrogenase by Human Neutrophils During 60-min Incubation at 4°C

Enzyme	Release*
	% of total
Heparanase	24.0
Globin degrading proteases	3.3
Lysozyme	5.8
Lactate dehydrogenase	2.2

* Fresh donor neutrophils suspended in PBS (5×10^6 cells/ml) were incubated at 4°C for 60 min. Released enzyme activity was determined in the supernatant obtained by a 10-min centrifugation at 300 *g*. Total activity was determined in a sample of the original cell suspension that was subjected to three cycles of freezing and thawing (heparanase, proteases) or treated with 0.02% Triton X-100 (Lysozyme, lactate dehydrogenase). A similar estimated percentage of enzyme release was obtained when the neutrophil cell suspension was disrupted by sonication. Values for total enzyme activity were as follows: Lysozyme, 0.05 (A450/min/10^6 cells); lactate dehydrogenase, 53.2 (nmol/min/10^6 cells); proteases (82 μg ^{14}C-globin/min/10^6 cells). Percent heparanase release was calculated by comparing the amounts of sulfate-labeled material released from the ECM and eluted as peak II, as described in Fig. 5. The variation between different determinations of percent release (each experiment was repeated at least three times) did not exceed 15% of the mean.

Sequential degradation of ECM-bound heparan sulfate proteoglycan by a protease followed by heparanase. Exposure of sulfate-labeled ECM to the 4°C supernate fraction of human neutrophils at pH 8.0 resulted in a release of high M_r labeled material eluted as a broad peak (fractions 10–35) next to V_o (Fig. 7). A similar elution profile was obtained after exposure (12 h, 37°C, pH 8.0) of the ECM to proteolytic enzymes such as trypsin, or chymotrypsin (10 μg/ml) (17). The broadness of this peak I depended on the enzyme concentration and time of incubation with the ECM, but under no circumstances did such incubation yield fragments smaller than intact heparan sulfate side chains (Kav = 0.35). As demonstrated in Fig. 7 *A*, release of peak I material produced by the neutrophil supernate fraction was inhibited in the presence of a serine protease inhibitor such as PMSF, but there was no inhibition by heparin (not shown). Fig. 7 *B* shows that PMSF also inhibited the cleavage of ECM-bound heparan sulfate side chains by the neutrophil heparanase as shown by the greatly reduced amounts of peak II material released from the ECM. Similar results were obtained in the presence of DFP, indicating that a proteolytic activity is involved and most probably required for degradation of ECM-bound heparan sulfate by the neutrophil heparanase. The residual heparan sulfate degradation activity observed in the presence of protease inhibitors may be due to degradation of high M_r sulfate-labeled material that is constantly liberated, in small amounts, from the ECM (14, 17). In fact, as shown in Fig. 8, high M_r peak I material that was first released from the ECM was readily degraded into the low M_r labeled fragments characteristic of peak II, upon a subsequent incubation with the neutrophil 4°C supernate fraction at pH 6.2. This degradation was inhibited by heparin but, in contrast to that of ECM-bound heparan sulfate, was not affected in the presence of DFP or PMSF (Fig. 8).

The contributory role of a proteolytic activity was also demonstrated when degradation of ECM-bound heparan sulfate was attempted with small amounts (0.1–0.25 ml) of the 4°C neutrophil supernate. The addition of trypsin or chymotrypsin to the incubation mixture stimulated three- to fivefold the release of low M_r, heparanase-mediated heparan sulfate degradation fragments, and the released material had a M_r somewhat smaller than that observed in the absence of these proteases (not shown). Heparin inhibited both the basal and protease stimulated heparanase activity. In contrast, trypsin had no effect on degradation of soluble heparan sulfate proteoglycan that was first released from the ECM by incubation with the 4°C neutrophil supernatant at pH 8.0 (not shown).

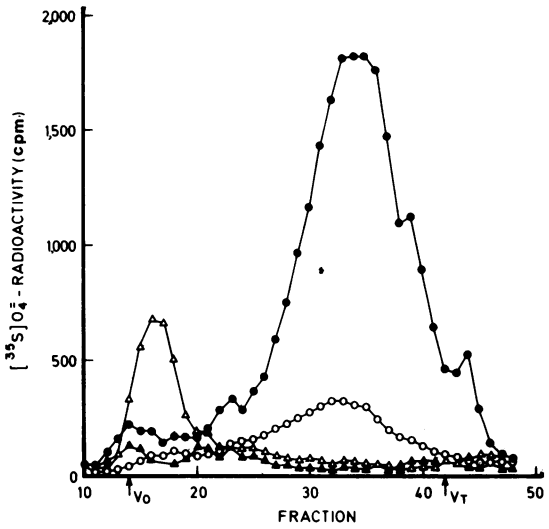

Figure 6. Partial purification by heparin-Sepharose chromatography of the neutrophil heparanase. Human neutrophils isolated from 400 ml blood were suspended in 100 ml PBS, incubated for 60 min at 4°C, and centrifuged (10 min, 300 *g*). The supernatant was filtered through a heparin-Sepharose column (1.6 × 5 cm) that had been equilibrated with 0.15 M NaCl in 0.01 M sodium acetate-acetic acid (pH 5.5). Fractions of 10 ml were collected during sample loading and column wash with 100 ml of 0.45 M NaCl in 0.01 M acetate buffer (pH 5.5). 1 M NaCl in 0.01 M acetate buffer (pH 5.5) was then added and 10 fractions of 2 ml each were collected. Chromatography was conducted at 4°C with flow rates maintained at 25 ml/h. 0.2-ml aliquots of each fraction were diluted with PBS and/or water to a final volume of 1 ml and to a salt concentration of 0.15–0.2 M. The pH was adjusted to 6.0 and the samples were incubated (24 h, 37°C) with sulfate-labeled ECM. Labeled degradation products were analyzed by gel filtration on Sepharose 6B. Unadsorbed material (▲) and material eluted with 0.45 M NaCl (△) showed no heparanase activity in all fractions, whether diluted or not, as compared with 0.2 ml of the original 4°C neutrophil supernatant (○) or to 0.2 ml of the 1 M NaCl eluant (●, fraction 3). A progressive decrease in heparanase activity was observed in fractions 4–10 of the 1 M NaCl eluant.

Discussion

We have previously reported that heparanase activity is correlated with the extravasation of lymphocytes (18, 19) and macrophages (18) toward an affected extravascular site. We now show that the ECM heparan sulfate is also degraded by human neutrophils and that the newly identified neutrophil heparanase is preferentially secreted by simply incubating the cells at 4°C without added stimuli. A somewhat similar situation was described for the neutrophil gelatinase where 30–40% of the enzyme cellular content was released under mild stimulatory conditions

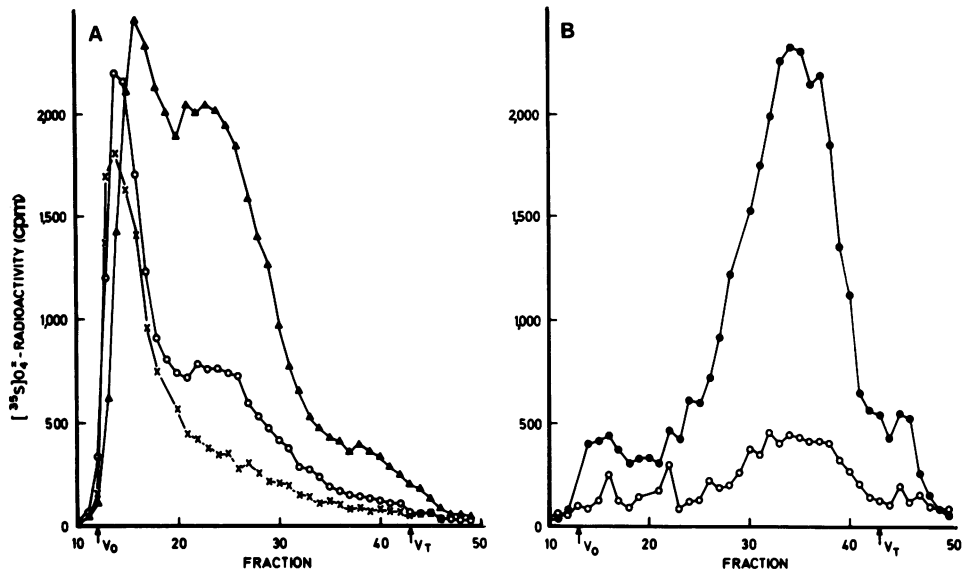

Figure 7. Effect of PMSF on degradation of sulfate-labeled ECM by enzymes secreted from neutrophils. Freshly isolated neutrophils suspended in PBS (5×10^6 cells/ml) were incubated at 4°C for 60 min. The cells were centrifuged and the supernatant was adjusted to pH 8.0 (*A*), and 6.2 (*B*). Supernatants were incubated (24 h, 37°C) at these pH values with sulfate-labeled ECM in the absence (▲, ●) or presence (○) of 5 mM PMSF. 1 ml of the pH 8.0 supernatant was also heated (95°C, 10 min) before incubation with the labeld ECM (×). Labeled material released into the incubation medium was applied for gel filtration onto Sepharose 6B columns.

(incubation at 37°C of cells that had been stored in the cold for 24 h) (6). It was proposed that the enzyme is localized within a novel secretory compartment of human neutrophils, and that together with other enzymes stored in this secretory organelle, is involved in the early events of neutrophil mobilization (6).

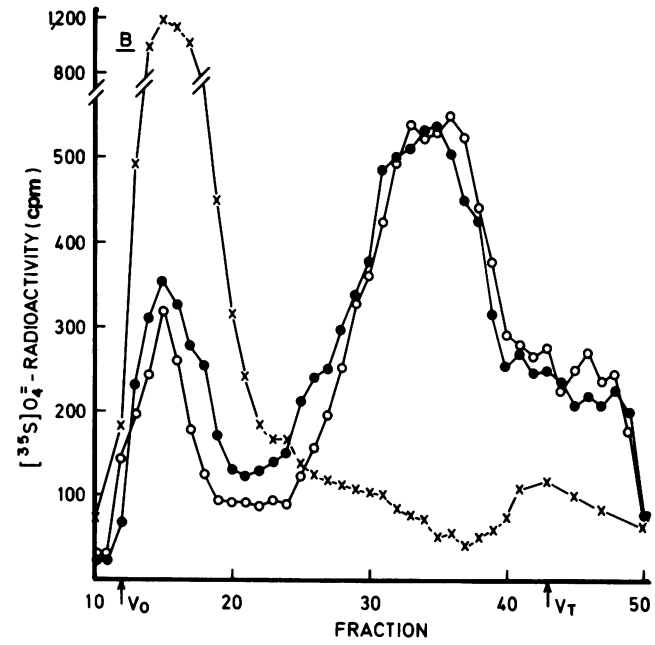

Figure 8. Degradation of soluble high M_r heparan sulfate proteoglycans first released from sulfate-labeled ECM. High M_r sulfate-labeled heparan sulfate proteoglycans (peak I material) was released from the ECM by incubation (48 h, 37°C, pH 7.5) with RPMI medium conditioned by Eb lymphoma cells (17). The high M_r material was then collected and 0.5-ml aliquots were taken for gel filtration (×) or for further incubation (24 h, 37°C) with 0.5-ml aliquots of the 4°C neutrophil supernatant at pH 6.2 in the absence (●) or presence (○) of 5 mM DFP. The reaction mixtures were then applied for gel filtration on Sepharose 6B. Similar results were obtained by using the high M_r material released from the ECM by the 4°C neutrophil supernatant at pH 8, except that the high M_r peak I material was eluted as a broader peak (fractions 10–30).

Due to their preferential release over various cellular enzymes, both the neutrophil gelatinase and heparanase activities may be involved in the initial impairment and dissociation of endothelial cell junctions and subsequent penetration of neutrophils through the subendothelial matrix. Thus, an extremely mild stimulation such as that produced by a gradient of a chemoattractant which is sufficient to induce cell migration but not degranulation, may facilitate the extravasation of neutrophils under conditions that would not cause a significant damage to the endothelial cell lining. Unlike the gelatinase that is secreted in latent form (30), heparanase-mediated degradation of heparan sulfate side chains does not require a prior activation of the enzyme in the microvessels or tissue invaded by the neutrophils.

A comparable ECM degrading heparanase was shown to be secreted by activated lymphocytes and highly metastatic sublines of mouse melanoma and lymphoma cells and postulated to play a role in the extravasation of blood-borne cells (16–19). In contrast, the platelet heparitinase was released only in response to a strong stimulus such as thrombin but was retained upon platelet aggregation induced by the ECM (12, 31). Although with all the cell types tested the low M_r heparan sulfate degradation products seem similar in size, it is not certain whether the same endoglycosidase activity is involved. In fact, different substrate specificities were shown for the platelet and B16 melanoma enzymes (32). We have also noted that at optimal reaction conditions (pH, enzyme concentration, time of incubation, etc.) the neutrophil heparanase yielded heparan sulfate cleavage fragments smaller than those obtained at less favorable conditions, as reflected by Kav values (Sepharose 6B) ranging from 0.46 to 0.72. Similar results were obtained upon incubation of neutrophils or their 4°C supernatant with the ECM produced by mouse PF HR9 teratocarcinoma cells (Vlodavsky et al., unpublished observation). This ECM is composed primarily of collagen type IV, heparan sulfate proteoglycans, laminin, and entactin and hence has a composition very close to that seen in vivo in epithelial and capillary endothelial cell basement membranes (33).

Heparin-Sepharose chromatography of the 4°C neutrophil supernate fraction showed that the neutrophil heparanase bound to the column and was eluted with 1 M NaCl. This procedure provided a mean for concentration and purification of the enzyme. Similar results were obtained when the ESb lymphoma,

serum-free conditioned medium (17) was subjected to heparin-Sepharose chromatography. Because the ESb-conditioned medium contained 50–100-fold more protein than the 4°C neutrophil supernatant, we could show that >95% of the total protein but almost no enzyme activity was eluted while loading the sample and in the 0.45 M NaCl eluant.

As shown with other cell systems (34), degradation of ECM-bound heparan sulfate, but not of a soluble heparan sulfate proteoglycan, was facilitated by a proteolytic activity. The presently observed almost complete inhibition of degradation of ECM-bound heparan sulfate by protease inhibitors, indicates that under certain conditions a proteolytic digestion of the ECM is a necessary prerequisite for subsequent degradation of heparan sulfate side chains by the neutrophil heparanase. Because the subendothelial ECM contains a number of elastase-sensitive targets including fibronectin, laminin, proteoglycans, and collagens (types I, III, and IV) (35), such a role can be fulfilled by the neutrophil elastase, which had already been shown to be primarily responsible for matrix degradation by triggered neutrophils (15). Our studies with the 4°C neutrophil supernatant showed that <4% of the total cellular content of globin degrading proteases was released under conditions that liberated at least 20% of the cellular heparanase content. Nevertheless, this low proteolytic activity was high enough to enable degradation of ECM-bound heparan sulfate by means of the neutrophil heparanase. Thus, degradation of heparan sulfate in the subendothelial ECM is likely to proceed in a sequential manner so that one enzyme (protease) provides a more accessible substrate for the next enzyme (heparanase). Such a combined enzymatic cleavage is characteristic of a supramolecular structure such as the ECM where degradation of a given constituent is affected by its covalent and noncovalent interactions with other components (7). These interactions may stabilize and/or mask the molecule so that it becomes less susceptible to cleavage by specific enzymes. Likewise, studies with the ECM produced by smooth muscle cells have shown that degradation of elastin and collagen by human sarcoma cells was accelerated by prior treatment of the matrix with plasmin or trypsin to remove connective tissue glycoproteins (36). It has also been proposed that collagen may serve to limit glycosaminoglycan degradation in the basal lamina (37).

Heparan sulfate proteoglycans interact with different attachment sites on plasma membranes and with macromolecules such as collagen and fibronectin in the matrix (38). This suggests a key role for this proteoglycan in the self-assembly and insolubility of the ECM as well as in cell adhesion and locomotion. Studies with various types of blood-borne cells have demonstrated that degradation of heparan sulfate side chains rather than release of the intact proteoglycan is correlated with the ability of cells to extravasate (16–19). A possible explanation is that in vivo the released high M_r proteoglycan is likely to be trapped in the tissue due to its size and noncovalent interactions with other extracellular constituents and hence can still create a barrier for cell invasion. In contrast, the low M_r glycosaminoglycan fragments are not restricted by the collagen fiber network and can more readily be cleared from the tissue. Moreover, the removal of glycosaminoglycans from the tissue exposes the collagen network and increases the susceptibility of collagen to cleavage by collagenolytic enzymes (39).

A fundamental question in the transendothelial migration of leukocytes is their homing to targets outside the vessels. Our studies with specifically activated T lymphocytes (19) suggested that ECM-bound antigens may dictate, via activation of ECM degrading enzymes, the site of extravasation and hence the homing of blood-borne cells. Although we could not demonstrate an effect of ECM-bound chemoattractants, the question of whether chemotactic substances sequestered by the ECM of an affected organ play a role in the recruitment of neutrophils to inflammatory sites requires further investigation. The present results with neutrophils, and our previous studies with lymphocytes (18, 19) and macrophages (18), suggest a mechanism for modulation of heparanase activity and release in response to various signals. On the other hand, blood-borne tumor cells either may exhibit a constitutive expression of the enzyme in correlation with their metastatic potential (16, 17) or can recruit normal cells (macrophages, neutrophils, lymphocytes, and platelets) and capitalize on their ability to infiltrate tissues (40, 41).

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