

Eosinophil major basic protein: First identified natural heparanase-inhibiting protein

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Background: Heparanase and eosinophils are involved in several diseases, including inflammation, cancer, and angiogenesis.

Objective: We sought to determine whether eosinophils produce active heparanase.

Methods: Human peripheral blood eosinophils were isolated by immunoselection and tested for heparanase protein (immunocytochemistry, Western blot), mRNA (RT-PCR) and activity (Na_2^{35}S)-labeled extracellular matrix degradation) before and after activation. Heparanase intracellular localization (confocal laser microscopy) and ability to bind to eosinophil major basic protein (MBP) were also evaluated (immunoprecipitation). A model of allergic peritonitis resulting in eosinophilia was induced in TNF knockout and wild-type mice for *in vivo* studies.

Results: Eosinophils synthesized heparanase mRNA and contained heparanase in the active (50-kd) and latent (65-kd) forms. Heparanase partially co-localized with and was bound to MBP. No heparanase enzymatic activity was detected in eosinophils resting or activated with various agonists, including GM-CSF/C5a. Eosinophil lysates and MBP inhibited recombinant heparanase activity in a concentration-dependent manner (100%, 2×10^{-7} mol/L). Eosinophil peroxidase and eosinophil cationic protein, but not myelin basic protein or compound 48/80, partially inhibited heparanase activity. Poly-L-arginine at very high concentrations caused an almost complete inhibition. In allergic peritonitis, heparanase activity in the peritoneal fluid inversely correlated with eosinophil number.

Conclusions: MBP is the first identified natural heparanase-inhibiting protein. Its presence in the eosinophil granules might indicate a protective function of these cells in diseases associated with inflammation and cancer progression. (J Allergy Clin Immunol 2004;113:703-9.)

Key words: Heparanase, eosinophils, major basic protein, inflammation, angiogenesis, fibrosis, metastasis, allergic peritonitis

Eosinophils participate in allergic inflammation after infiltration from the peripheral blood into the tissue. In the inflamed tissue, eosinophils have been historically thought to cause damage mostly through the release of the granule-associated cationic proteins major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin.¹ MBP, localized in the core of the eosinophil secondary granules and comprising more than 50% of the granule protein, seems to play a role in host defense and in tissue damage.¹ Many of the biologic properties of MBP have been attributed to the strong positive charge of the molecule and its high arginine content,² even though its cationic nature does not fully explain its activity.³ More recently, however, a tissue-healing role has been ascribed to eosinophils and attributed to their ability to produce profibrogenic cytokines, metalloproteinases, and tissue inhibitors of metalloproteinases.⁴ In addition to allergy, eosinophils are associated with a number of chronic inflammatory and malignant diseases.^{1,5} This, and the increasing significance ascribed to heparanase in inflammation, angiogenesis, wound healing, and cancer progression,^{6,7} prompted us to investigate its presence in eosinophils.

Heparanase exerts its biologic effects primarily through specific intrachain cleavage of heparan sulfate (HS)⁸ and release of extracellular matrix (ECM)—resident heparin-binding growth and differentiation factors.^{7,9} Heparanase is synthesized as a latent protein of 65 kd that is processed at the N-terminus into an active 50-kd form.¹⁰⁻¹² Apart from tumor cells,⁷ heparanase activity is found primarily in platelets and activated cells of the immune system.^{13,14}

In this study, we report that MBP is a potent inhibitor of heparanase activity. Eosinophils that produce heparanase fail to express heparanase enzymatic activity in contrast to other cells. This is the first time that a natural protein inhibitor of heparanase has been described.

METHODS

Cells

Eosinophils were purified from the peripheral blood of mildly atopic volunteers according to the guidelines established by the Hadassah-Hebrew University Human Experimentation Helsinki Committee.⁴ For activation experiments, eosinophils (purity >99%, Kimura's, viability >99%, Trypan blue; Sigma, St Louis, Mo) were resuspended ($1 \times 10^6/\text{mL}$) in RPMI-1640 supplemented with

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Abbreviations used

ECM: Extracellular matrix
 ECP: Eosinophil cationic protein
 EPO: Eosinophil peroxidase
 HS: Heparan sulfate
 KO: Knockout
 MBP: Major basic protein
 WT: Wild type

200 µg/mL streptomycin, 200 U/mL penicillin, 2 mmol/L gentamicin, 2 mmol/L glutamine, 0.1 mmol/L nonessential amino acids, and 5% (v/v) heat-inactivated FCS (Biological Industries, Beit Haemek, Israel) and cultured in 96-well plates in medium alone or with either platelet-activating factor (1×10^{-7} mol/L) (Sigma), IL-2 (25 ng/mL) (R&D Systems, Inc, Minneapolis, Minn), human mast cell line HMC-1 sonicate (1×10^5 /mL) (a kind gift from Dr J. Butterfield, Mayo Clinic, Rochester, Minn), phorbol myristate acetate (2.5 ng/mL), or recombinant human skin β -tryptase (50 ng/mL) for 15 minutes or 18 hours as described.¹⁵⁻¹⁷ Eosinophils were also preincubated (20 minutes) in medium alone or in GM-CSF (50 ng/mL) (R&D Systems, Inc), followed by C5a stimulation (1×10^{-8} M) (R&D Systems, Inc) (25 minutes).¹⁵ EPO release was evaluated by an enzymatic colorimetric assay¹⁸ and IL-6 by a commercial ELISA assay in the supernatants. For lysate preparation, eosinophils and neutrophils (1×10^6 /mL) were resuspended in RPMI without FCS, adjusted to pH 6.0 by 20 mmol/L buffer citrate phosphate, and lysed by 3 cycles of freezing and thawing.¹⁴ For co-culture studies, confluent human foreskin fibroblast cell line HS68 (ATCC No CRL 1635, American Type Culture Collection) and primary bovine aortic endothelial and smooth muscle cells^{4,19} (3 to 5 passages) seeded in 50-mL flasks (Costar, Cambridge, Mass) were incubated with sonicated⁴ or viable eosinophils (1×10^6 /flask) in the presence of GM-CSF (10 ng/mL).

Heparanase activity assay

Cell-associated heparanase activity was determined as described.^{12,13,20} Metabolically $\text{Na}_2^{35}\text{S}\text{O}_4$ -labeled ECM, firmly bound to the tissue culture plastic and free of cellular debris,²¹ was incubated (4 hours, 37°C, pH 6.0) with cell lysates (eosinophils, neutrophils, fibroblasts) or with eosinophil supernatants, or MBP, EPO, ECP, or human recombinant heparanase in heparanase reaction mixture (50 mmol/L NaCl, 1 mmol/L CaCl_2 , 1 mmol/L dithiothreitol, and 20 mmol/L citrate-phosphate buffer, pH 6.0). To evaluate the presence of HS degradation products, the incubation medium was applied onto CL-Sepharose 6B columns (0.5×30 cm).^{12,13} We have previously demonstrated that nearly 80% of the ECM sulfate-labeled material is incorporated into HS proteoglycans and that heparanase releases from the ECM degradation fragments of HS that are eluted at $0.5 < k_{av} < 0.8$ (fractions 15 to 35, peak II). Nearly intact HS proteoglycans were next eluted to the V_0 ($k_{av} < 0.2$, peak I). Each experiment was performed at least 3 times, and the variation in elution positions (k_{av} values) did not exceed $\pm 15\%$.

Immunocytochemistry and confocal microscopy analysis

Cytospins (5 minutes, 1000g) of freshly isolated eosinophils (1×10^5) were fixed in methanol (3 minutes, -20°C) and stained with anti-human heparanase monoclonal antibodies (mAb-130, 10 µg/mL, 2 hours, 24°C) kindly provided by InSight Ltd (Rehovot, Israel), followed by secondary antibodies, by use of a ZIMED kit (ZIMED Laboratories Inc, San Francisco, Calif).²² For co-staining and confocal microscopy, either rabbit anti-human MBP IgG (0.5 µg/mL, 12 hours, 24°C) and Cy5 goat anti-rabbit fluorescent secondary antibodies or anti-heparanase mAb-130 followed by Cy2 goat anti-

mouse antibodies (Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa) were added.²³ Cells were examined and visualized as described.¹⁶

RNA isolation and RT-PCR

RNA was isolated from freshly isolated eosinophils (2×10^6) with Tri-Reagent (Sigma) and quantified.¹² After reverse transcription of 2 µg total RNA by oligo (dT) 17 mer priming (GIBCO, BRL, Life Technologies, Rockville, Md), the resulting single-stranded cDNA was amplified with Taq DNA Polymerase (Promega, Madison, Wis) and deoxyribonucleoside triphosphate mixture.¹² Human heparanase-specific oligonucleotide primers, 5'-GCAAACTCTATGGTCC TGATGT-3' and 5'-GCAAAGGTGTCGGATAGCAAG-3', yielding a 299-bp product, were used. The PCR conditions were denaturation (94°C, 2 minutes; 94°C, 15 seconds), annealing (45 seconds, 58°C), and extension (1 minute, 72°C) (33 cycles).¹² Aliquots (15 µL) of the amplified cDNA were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. A cDNA template of human heparanase was used as a positive control.^{12,24} Only RNA samples that gave completely negative results in PCR without transcriptase were analyzed.

Western blot and immunoprecipitation

For immunoblot analysis, lysates were mixed with heparin-Sepharose beads and incubated (1 hour, 24°C) with rotation. The beads were washed twice (PBS), boiled with Laemmli buffer, and centrifuged. The supernatants were separated by 10% SDS-PAGE. The proteins were transferred from the gel to Immobilon-P membrane (Millipore, Bradford, Mass) that was sequentially incubated with block solution, anti-human heparanase monoclonal antibodies, and horseradish peroxidase-conjugated rabbit anti-mouse antibodies (DAKO Corporation, Carpinteria, Calif).^{12,22,24} Enhanced chemiluminescence visualization was performed with SuperSignal West Pico Trial Kit (Pierce) and a subsequent exposure to Fuji film Super RX for 10 to 60 seconds. For immunoprecipitation, protein A-Sepharose beads were first saturated with anti-human MBP IgGs and incubated (1 hour, 24°C) with cell (eosinophils, fibroblasts) lysates. The protein A-Sepharose-MBP-heparanase complex was then boiled in Laemmli buffer and subjected to Western blot analysis as described previously.

Allergic peritonitis in TNF-knockout (KO) mice

Allergic peritonitis was induced in wild-type male C57BL/6 (WT) and C57BL/6 TNF-KO mice²⁵ (a gift from Prof H. P. Eugster, Department of Pathology, University of Bern, Bern, Switzerland), 8 weeks old and weighing 25 to 30 g, with ovalbumin, as described.²⁵ The experimental protocols were approved by the Animal Experimentation Committee of The Hebrew University of Jerusalem. Mice were put to death 3 days after challenge, and the peritoneal cavity was washed with 5 mL of Tyrode buffer (3 mmol/L potassium chloride, 10 mmol/L sodium hydrogen carbonate, 20 mmol/L sodium chloride, 0.36 mmol/L sodium phosphate, 0.1% glucose [all Sigma]) containing 0.1% gelatin (TG buffer).²⁵ Peritoneal lavage fluid was centrifuged (5 minutes, 150g), supernatants were saved for heparanase assessment, and cell pellets were resuspended in 2 mL of TG buffer for eosinophil quantification.²⁵

RESULTS**Evaluation of heparanase expression in eosinophils**

Expression of heparanase mRNA in freshly isolated human peripheral blood eosinophils was demonstrated by

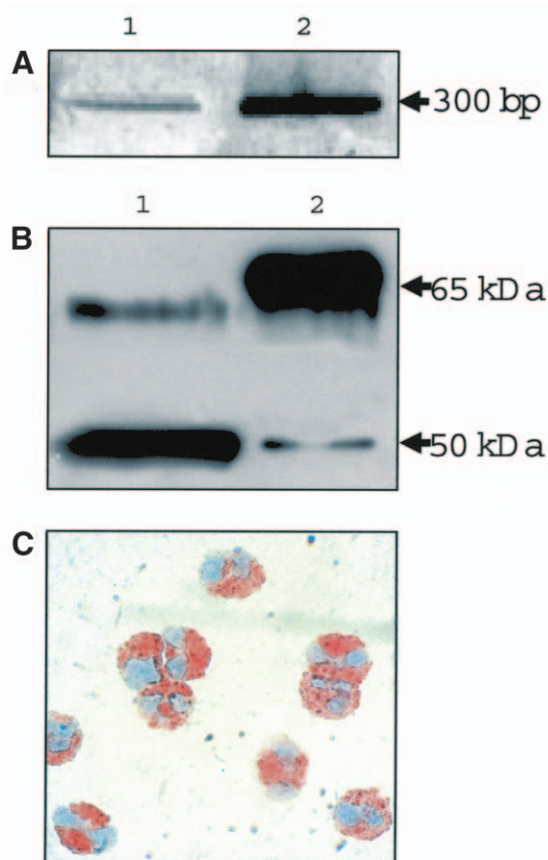


FIG 1. Heparanase expression in human eosinophils. **A**, RT-PCR. Lane 1, 300-bp band amplified from the mRNA of human eosinophils. Lane 2, Human heparanase cDNA. Control PCR (no RT) was negative (not shown). **B**, Western blot. Lane 1, Eosinophil lysates, incubated with HS-conjugated beads, electrophoresed, and blotted with anti-heparanase antibodies. Lane 2, Recombinant human heparanase, consisting mostly of the 65-kD latent enzyme. **C**, Immunostaining. Eosinophils were incubated with anti-heparanase antibodies and stained with peroxidase-conjugated secondary antibodies (red-brown).

RT-PCR with heparanase-specific primers (Fig 1, A). Both the processed (50-kD) and, to a lesser extent, the unprocessed (65-kD) forms of heparanase were detected by Western blot analysis of lysed freshly isolated eosinophils (Fig 1, B, lane 1). On the basis of this experiment, we estimated that 1×10^6 eosinophils contain 2 to 4 ng of heparanase similar to other cells of the immune system.^{13,14} By staining the eosinophils with anti-human heparanase monoclonal antibodies, it was found that all the examined cells contained preformed heparanase in their cytoplasm, appearing in a granular pattern (Fig 1, C).

Localization of heparanase in eosinophils

Confocal microscopy analysis of the eosinophils demonstrated that heparanase (Fig 2, B, green Cy2) partly co-localized with MBP (Fig 2, A, red CY2) in overlapping distinct yellow regions (Fig 2, C). Consequently, to evaluate the possible interaction between heparanase and

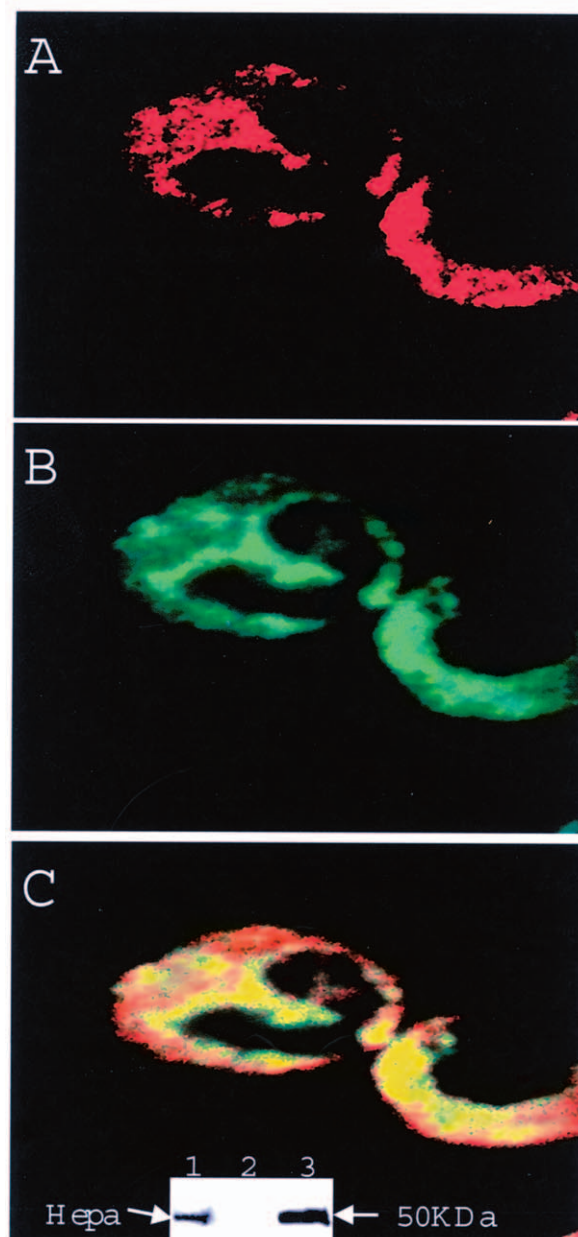


FIG 2. Heparanase and MBP co-localization in eosinophils: confocal microscopy. **A**, Staining of MBP (red). **B**, Staining of heparanase (green). **C**, Double immunostaining of heparanase and MBP. Partial co-localization of heparanase and MBP within the cells (yellow). **Inset**, Western blot. Lane 1, Eosinophil heparanase co-immunoprecipitated with anti-MBP and anti-heparanase antibodies. Lane 2, Human foreskin fibroblasts immunoprecipitated as in Lane 1. Lane 3, Recombinant human heparanase, consisting primarily of the 50-kD enzyme.

MBP, eosinophil lysates were incubated with anti-MBP antibodies, precipitated with protein A–Sepharose beads, and subjected to SDS/PAGE followed by immunoblot analysis with anti-heparanase antibodies. As shown (Fig 2, C, inset), the immunoprecipitate from eosinophils (lane 1) contained a 50-kD protein corresponding to

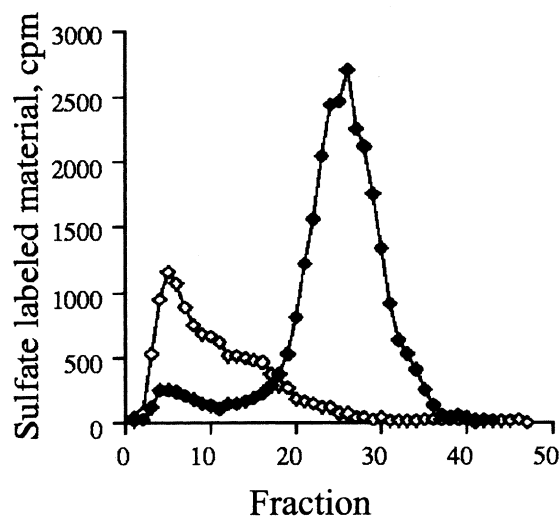


FIG 3. Lack of heparanase activity in resting human eosinophils. Lysates of human eosinophils ($\diamond$) and neutrophils ($\blacklozenge$) ($1 \times 10^6/\text{mL}$) were incubated with intact biosynthetically ^{35}S -labeled ECM. Labeled degradation fragments released into the incubation medium were analyzed by gel filtration on Sepharose 6B.

recombinant human heparanase (lane 3), whereas human foreskin fibroblasts, used as a negative control, did not (lane 2).

Evaluation of eosinophil-associated heparanase enzymatic activity

Next, we evaluated whether eosinophils display heparanase enzymatic activity. For this purpose, lysates of freshly isolated eosinophils were incubated with sulfate-labeled ECM. These samples failed to release sulfate-labeled HS degradation fragments (Fig 3), indicating a lack of heparanase enzymatic activity. In contrast, lysed neutrophils exhibited a high heparanase activity, releasing 60% to 70% of the total ECM incorporated radioactivity in the form of HS degradation fragments (fractions 20 to 35) (Fig 3). The biochemical nature of these cleavage fragments was characterized in previous studies.¹⁴ Subsequently, we tried to induce heparanase activity by activating the eosinophils for 15 minutes or 18 hours with either PAF, phorbol 12-myristate 13-acetate, recombinant human skin β -tryptase, IL-2, or sonicated HMC-1 cells. As observed with resting cells, none of these treatments induced heparanase activity, measured either in the eosinophil cell lysates or in their supernatants, even though they elicited eosinophil activation, as detected by EPO and/or IL-6 release (not shown).¹⁵⁻¹⁷ Even activation achieved by incubating the eosinophils with GM-CSF and C5a,¹⁵ which caused a 6.14-fold increase in EPO release over the control (Fig 4, A), did not result in secreted heparanase activity (Fig 4, B). Likewise, co-culture of eosinophils with either endothelial cells, smooth muscle cells or fibroblasts, or the addition of eosinophil sonicate to fibroblasts failed to yield an enzymatically active heparanase (not shown).

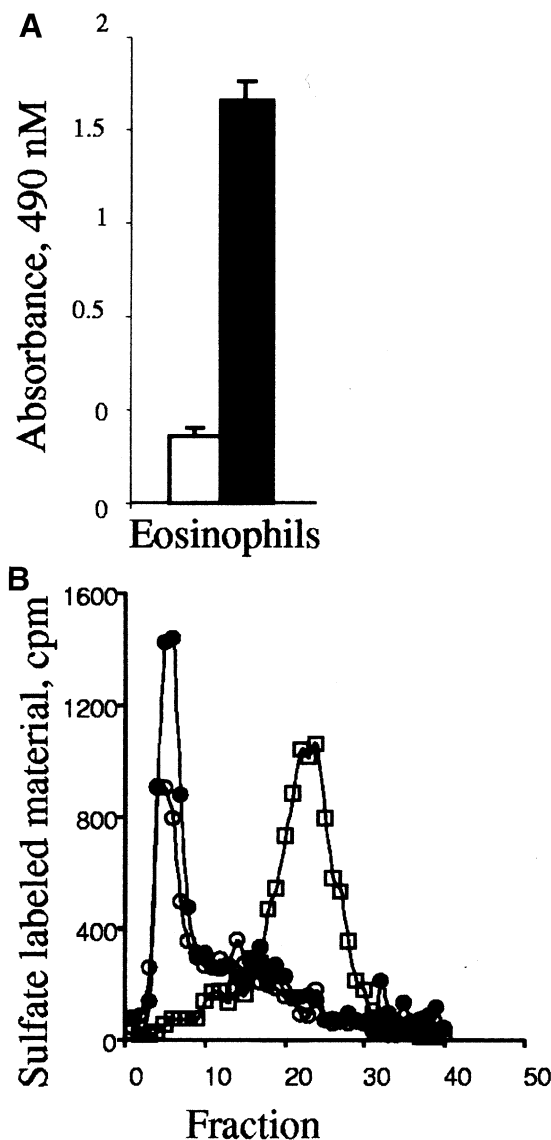


FIG 4. Lack of heparanase activity in activated human eosinophils. **A**, EPO release in the supernatants of eosinophils preincubated with medium in the presence ($\blacksquare$) or absence ($\square$) of GM-CSF followed by C5a stimulation (absorbance, 490 nmol/L).²⁶ **B**, Heparanase activity (see legend for Fig 3) of supernatants of GM-CSF/C5a unactivated ($\circ$) or activated ($\bullet$) eosinophils. The ECM was also incubated with of recombinant heparanase ($\square$).

Inhibition of heparanase activity by eosinophils and MBP

We next hypothesized that eosinophils might inhibit heparanase activity. Therefore, the ability of eosinophil lysates to inhibit heparanase-mediated degradation of HS in intact ECM was investigated. For this purpose, active 50-kd recombinant heparanase (10 ng/mL) was incubated with sulfate-labeled ECM in the absence or presence of either lysed eosinophils, or, as a control, lysed human foreskin fibroblasts. As shown in Fig 5, A, release of low-molecular-weight-labeled HS degradation fragments was specifically abolished by eosinophil lysates but

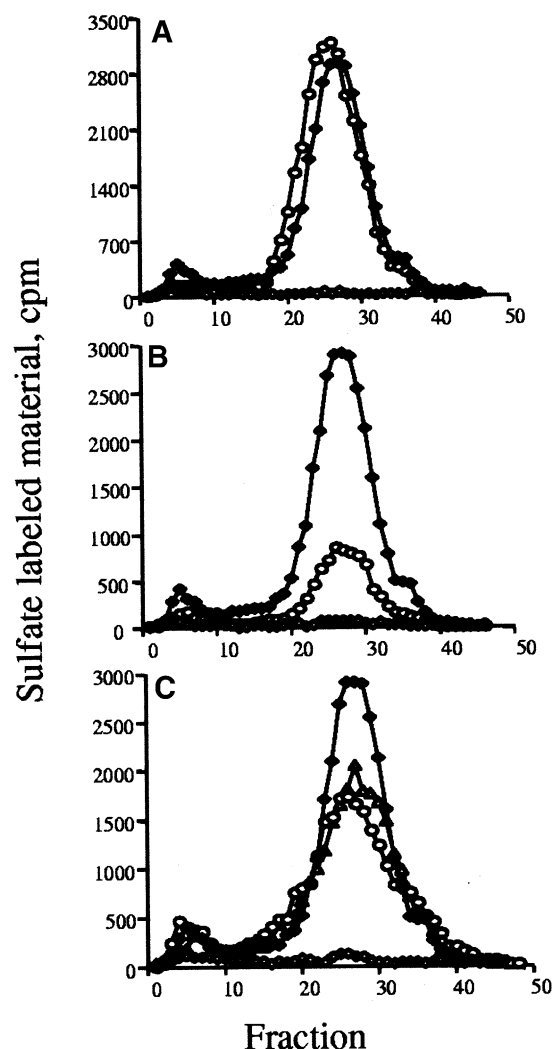


FIG 5. Inhibition of heparanase activity by eosinophils and MBP. Recombinant 50-kDa human heparanase (10 ng/mL) was incubated with ^{35}S -ECM in the absence ($\blacklozenge$) or presence of eosinophil lysates ($\diamond$), or human foreskin fibroblast lysates ($\circ$) (A); in the absence ($\blacklozenge$) or presence of 2×10^{-7} mol/L ($\diamond$), or 0.8×10^{-7} mol/L ($\circ$) purified MBP (B); in the absence ($\blacklozenge$) or presence of MBP ($\diamond$), EPO ($\circ$), or ECP ($\triangle$), each at a concentration of 4×10^{-7} mol/L (C) (see legend for Fig 3).

not by fibroblasts. In a subsequent experiment, the eosinophil lysates were incubated with the labeled ECM in the presence of increasing concentrations of recombinant heparanase. Complete inhibition of activity was obtained even at a heparanase concentration of 1 $\mu\text{g/mL}$, representing an estimated excess of MBP over heparanase of about 2.5 fold (not shown).

Because of the confocal microscopy showing that heparanase partially co-localizes with MBP, it was assumed that MBP and/or other granular constituents could function as specific inhibitors of heparanase. In fact, when purified MBP was added to recombinant heparanase, a concentration-dependent inhibition of its activity was observed with an almost complete inhibition at 0.8×10^{-7} mol/L and a complete inhibition at 2×10^{-7}

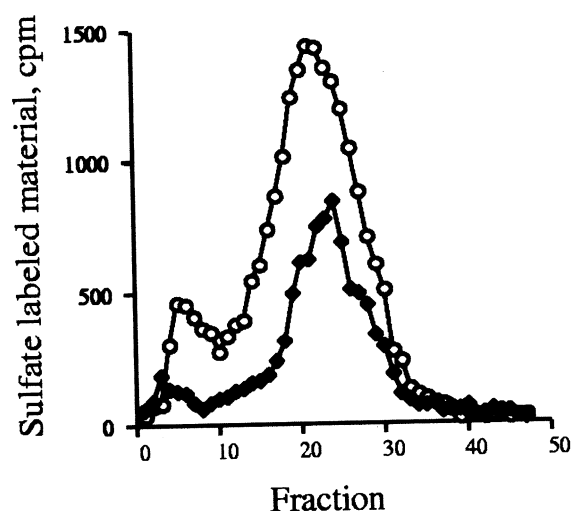


FIG 6. Heparanase activity in the peritoneal lavage of mice with allergic peritonitis. TNF-KO ($\circ$) and control WT mice ($\blacklozenge$) were sensitized and challenged with ovalbumin to induce allergic peritonitis. Three days after challenge, peritoneal lavage was performed, and the supernatants assessed for heparanase activity (see legend for Fig 3).

mol/L (Fig 5, B). As shown in Fig 5, C, ECP and EPO also exerted an inhibitory effect, although to a lesser degree than MBP at equimolar concentrations. Because MBP, ECP, and EPO share a high cationic charge, we tested other highly basic compounds (ie, compound 48/80; condensation product of N-methyl-P-methoxyphenethylamine with formaldehyde)²⁶ and myelin basic protein.²⁷ Even at 20 ng/mL, they did not show any inhibitory effect. A partial inhibition of heparanase activity was exerted by poly-L-arginine at 5 ng/mL and reached an almost complete inhibition at 13 ng/mL (not shown).

Correlation between heparanase activity and eosinophil numbers in murine allergic peritonitis

Heparanase activity was assessed in vivo and correlated with eosinophil numbers in TNF-KO and WT mice sensitized and challenged intraperitoneally with ovalbumin. As shown in Fig 6, in TNF-KO mice in which eosinophil numbers were significantly lower than in the WT mice ($1.9 \pm 0.3 \times 10^5$ cells/mL vs $5.9 \pm 0.7 \times 10^5$ cells/mL, respectively), heparanase activity determined in the peritoneal fluid was 2- to 3-fold higher.

DISCUSSION

This study demonstrates that eosinophil-derived MBP efficiently inhibits heparanase activity. This inhibitory effect might be relevant in vivo, because MBP is released from eosinophils in several diseases in which heparanase also participates. For example, eosinophils, besides being effector cells of allergic and parasitic reactions, have different roles in fibrosis, cancer, and autoimmune

diseases,^{1,4,5} whereas heparanase could degrade the ECM scaffold and hence facilitate the penetration of eosinophils into tissues.

In this study we found that eosinophils, as other inflammatory cells,^{13,14} produce heparanase. However, we could not detect any heparanase enzymatic activity in resting eosinophils or in eosinophils activated with various agonists,¹⁵⁻¹⁷ including GM-CSF/C5a, one of the most potent eosinophil degranulators.¹⁵ Likewise, co-culture of eosinophils with cells that did not express intrinsic heparanase but do interact with eosinophils²⁸⁻³⁰ or that process and activate exogenously added latent heparanase did not result in heparanase activity.

We therefore hypothesized that eosinophils might contain a substance that inhibits heparanase. Eosinophil lysates, as well as highly purified preparations of MBP, inhibited heparanase activity in a dose-dependent manner, reaching 100% at a concentration of 2×10^{-7} mol/L. Complete inhibition was observed even at an estimated lower excess (~2.5 fold) of cellular MBP over heparanase.

Although natural inhibitors of matrix-degrading proteases such as tissue inhibitors of matrix metalloproteinases and plasminogen activator inhibitors are well characterized,³² MBP is the first naturally occurring protein that efficiently inhibits heparanase enzymatic activity. Structurally, MBP is characterized by 14% arginine residues (17/117 amino acids) and only a single acidic residue, aspartic acid, yielding a calculated P value of 11.4.³³ Notably, both ECP and EPO, which were found to exert a partial inhibitory activity toward heparanase, also contain arginine,^{34,35} although to a much lower extent (3% to 5%) than MBP. MBP also contains 9 cysteines, 4 of which form 2 disulfide bonds that are homologous to the 2 disulfide bonds conserved in the carbohydrate recognition domain of C-type lectins.³ It is therefore conceivable that these polycationic proteins might interact with the polyanionic HS substrate and hence protect it from cleavage and/or diminish its accessibility to heparanase. In preliminary experiments, we found that heparanase preincubated with MBP co-precipitated when anti-MBP antibodies were added, indicating that a direct interaction between the 2 proteins is feasible. Other basic compounds such as compound 48/80 and myelin basic protein^{26,27} only partially inhibit heparanase activity. Poly-L-arginine at very high concentrations caused an almost complete inhibition. This, together with the previous results, strongly suggests a specific interaction of MBP with the heparanase enzyme, resulting in inhibition of its enzymatic activity.

In a murine model of allergic peritonitis,²⁵ we found that peritoneal cavity heparanase activity, probably originated from eosinophils and other inflammatory cells, was inversely related to the eosinophil number. This would indicate that heparanase can be inhibited by the "excess" of MBP released by activated eosinophils.

Given the potential tissue damage that could result from cleavage of HS, tight regulation of heparanase expression and activity is essential. An attractive regulatory target is

the apparently membrane bound, not yet identified protease that converts the heparanase from a latent 65-kd protein into an active 50-kd form.^{7,10} Regulation of heparanase promoter activity is being investigated, emphasizing the inhibitory effect of methylation and stimulation by estrogen.^{36,37} In vivo models of cancer metastasis and angiogenesis have shown that the potent pro-angiogenic and pro-metastatic properties of heparanase are tightly regulated by its cellular localization and secretion.²³ Thus, cell surface binding and routing of heparanase into late endosomes seem to control its activation, clearance, and storage within the cells.^{31,38} This study suggests that inhibition of heparanase by MBP might provide a protective feedback mechanism that can halt tissue damage in response to excess heparanase secreted at sites of massive inflammation.^{39,40}

The heparanase enzyme has been identified in platelets and neutrophils and is readily released in an active form on their degranulation. In contrast, this enzyme might be released from activated eosinophils as a complex with MBP and possible other granular mediators, but in an inactive form because of the inhibitory effect of MBP. The novel anti-heparanase activity of MBP contrasts sharply with the previously established involvement of eosinophils and MBP in tissue damage that seems to be important in allergy, parasitic diseases, and certain cancers. The mode of interaction, stoichiometry, and mechanism by which MBP inhibits the enzymatic activity of heparanase remains to be explained, as well as its role in allergy, inflammation, and other pathologic and normal conditions involving heparanase-mediated cell adhesion, migration, and invasion.

MPB is the first natural protein inhibitor of heparanase activity to be identified. In view of the increasing significance of heparanase as a target for anticancer drug development, MBP and related compounds are being evaluated in experimental models of cancer metastasis and angiogenesis as candidates for potential application in cancer treatment.

Note added in proofs

In a recent experiment, we have tested the effect of MBP on melanoma lung colonization. For this purpose, B16-BL6 melanoma cells were incubated (15 minutes) with or without purified MBP; this was followed by injection into the tail vein of C57BL6 mice (n = 7; 2×10^5 cells/mouse, 150 µg MBP/mouse). Mice were killed on day 20, and lungs were fixed and examined for the number of metastatic nodules on the lung surface (20). Preincubation with MBP resulted in an almost complete inhibition of melanoma lung colonization in this experimental metastasis model, correlated with the inhibition of heparanase activity.

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