American Journal of Pathology, Vol. 143, No. 3, September 1993

Degradation of Endothelial Cell Matrix Heparan Sulfate Proteoglycan by Elastase and the Myeloperoxidase-H_2O_2-Chloride System

Seymour J. Klebanoff,* Michael G. Kinsella, and Thomas N. Wight†

From the Departments of Medicine and Pathology,† University of Washington, School of Medicine, Seattle, Washington*

The degradation of the heparan sulfate proteoglycans of subendothelial matrix by neutrophil elastase and the myeloperoxidase-H_2O_2-chloride system added separately, sequentially, or together at pH 4.5 to 7.5 was determined by the release of lower molecular weight ^{35}S-labeled material. Elastase alone and the myeloperoxidase system alone caused degradation, and when 4-hour exposure to elastase was followed by 15 minutes of exposure to the myeloperoxidase system, the effect was greater than additive. A greater than additive effect was not observed when elastase followed the myeloperoxidase system or the two were added together. Chloride (or sulfate) alone increased the release of ^{35}S-labeled material from elastase-treated matrix, although the effect of 0.1 M chloride was not as great as that observed when an equivalent concentration of chloride was combined with myeloperoxidase and H_2O_2. The release of these systems at sites of adherence of neutrophils to glomerular basement membrane may contribute to neutrophil-associated proteinuria. (Am J Pathol 1993, 143: 907–917)

Heparan sulfate proteoglycans (HSPGs) are widely distributed on the plasma membrane of cells and in the extracellular matrix where they are believed to be important to a broad spectrum of biological functions.[1,2] The heterogeneous family of proteoglycans known as HSPGs includes a number of macromolecules with distinct protein cores, encoded by different genes, and bearing one or more heparan sulfate glycosaminoglycan chains. Heparan sulfate chains are linear polysaccharides consisting of repeating disaccharide units composed of glucosamine and glucuronic acid, with the glucosamine residues either *N*-acetylated or *N*-sulfated, and the glycosaminoglycan chain variably modified by o-sulfation at various positions in both residues and epimerization of some glucuronate residues to iduronate. Thus, heparan sulfate chains are heterogeneous in structure, with specific sequences of modified residues implicated in interactions with other molecules, such as antithrombin III[3] and basic fibroblast growth factor.[4] One member of the family of HSPGs is the large matrix HSPG, named perlecan, which is consistently present as a component of basement membranes. Recently, the complete amino acid sequences of human and mouse perlecan have been deduced from overlapping cDNAs.[5–7] These studies indicate that the core protein of perlecan has a predicted molecular weight in excess of 450 kd and predict that perlecan bears three heparan sulfate chains near the NH_2-terminal of the core protein. Perlecan is present in large amounts in the glomerular basement membrane where its heparan sulfate chains appear to act as a negatively charged barrier to the passage of protein into the urinary space.[8,9] The nephrotic syndrome in rats is associated with a loss of glomerular basement membrane heparan sulfate,[10] and antibody to basement membrane HSPG is nephrotoxic.[11–13]

Myeloperoxidase (MPO) is a highly cationic protein present in the azurophil granules of neutrophils and monocytes that is released either into the phagosome or extracellularly when the phagocytes are stimulated.[14,15] Stimulated phagocytes also generate H_2O_2 during a respiratory burst and the MPO reacts with H_2O_2 and a halide such as chloride to form agents toxic to ingested micro-organisms or extracellular targets[14,15] MPO infused into the renal artery

Supported in part by USPHS grants AI07763 and HL18645 from NIH.

Accepted for publication April 30, 1993.

Address reprint requests to Dr. Seymour J. Klebanoff, Department of Medicine SJ-10, University of Washington, Seattle, Washington 98195.

binds to the negatively charged glomerular basement membrane, and when the MPO infusion is followed by infusion of H_2O_2, glomerular injury is induced as manifested by endothelial cell swelling, focal epithelial cell foot process fusion, a marked influx of platelets, and proteinuria.[16,17] When radioiodide is also infused, iodination of the glomerular basement membrane is observed,[16] indicating the interaction of MPO, H_2O_2, and a halide in the glomerular capillary wall. The infused neutrophil proteinases, elastase and cathepsin G, also bind to the glomerular basement membrane with the production of marked proteinuria without evidence of morphological damage.[18] These findings raise the possibility that the MPO-H_2O_2-halide system and the neutrophil proteinases elastase and cathepsin G act independently or together to contribute to the damage observed in neutrophil-dependent glomerulonephritis and that this damage is induced in part by an effect on the basement membrane heparan sulfate (perlecan).

In culture, endothelial cells assemble a basal extracellular matrix in which the predominant proteoglycan is perlecan.[19,20] This matrix can be labeled with ^{35}S-sulfate, and the release of the label as well as the demonstration of the presence of lower molecular weight degradation products in the supernatant fluid may serve as an *in vitro* correlate of our *in vivo* findings, thus allowing more detailed mechanistic analyses. In this study, ^{35}S-sulfate-labeled matrix, prepared from bovine aortic endothelial cells, was exposed to neutrophil elastase and to the MPO-H_2O_2-chloride system either independently, sequentially, or together and the release of lower molecular weight–sulfated fragments determined.

Materials and Methods

Special Reagents

MPO was purified from human leukocytes[21] and assayed by guaiacol oxidation.[22] Human neutrophil elastase was kindly provided by Margaret Vissers (Christchurch, New Zealand) and assayed using the specific synthetic peptide methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanalide (Sigma, St. Louis, MO).[18]

Preparation of Labeled Subendothelial Cell Matrix

Endothelial cells were isolated from calf thoracic aortas and maintained as previously described.[20] Cells between the 8th and 14th passage were collected by trypsinization and seeded onto gelatin-coated 24-well tissue culture trays (Costar, Cambridge, MA) at a 2:1 split ratio. The cells were kept in a CO_2 incubator for 9 to 12 days (generally 10) and labeled by incubation for 24 to 48 hours with 100 μCi/ml carrier-free $Na_2[^{35}S]O_4$ (ICN Radiochemicals, Costa Mesa, CA) in sulfate-free Dulbecco's modified eagle's medium containing 10% fetal bovine serum.

Subendothelial matrix was prepared from labeled cell cultures essentially as described by Kramer et al.[23] Briefly, the labeling medium was removed and the cultures incubated for 5 minutes with 0.5% Nonidet P-40 (Sigma) followed by three washes of 5 minutes each with sterile distilled water, which removed most nuclei and cytoskeletal elements. The labeled matrix adherent to the bottom of the wells was stored for no longer than 4 hours in distilled water at 4 C before use.

Harvest and Analysis of Samples

The distilled water was removed from the wells and the reagents added, which are indicated in the figure legends and tables. The plates were incubated at 37 C in a CO_2 incubator for the periods indicated, placed on ice, and processed immediately. The supernatants were removed from the culture wells and frozen. Matrix was extracted overnight at 4 C with 0.5 ml of 6 M urea, 0.25 M NaCl, 2% Triton X-100, 2.5 mM EDTA, 0.1 M 6-aminohexanoic acid, 5 mM benzamidine, 5 mM phenylmethylsulfonyl fluoride, and 10 mM *N*-ethylmaleimide in 50 mM Tris-HCl buffer (pH 7.5). The matrix was harvested with a rubber policeman. Duplicate-measured aliquots of the supernatant and solubilized matrix were spotted on filter paper that was dipped in cetyl pyridinium chloride to precipitate glycosaminoglycans.[24] The spots were washed to remove other labeled components and counted in a liquid scintillation counter. ^{35}S-labeled molecules were identified as heparan sulfate by sensitivity to nitrous acid deaminative cleavage at pH 1.5.[25]

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) separation of the components in the supernatant and matrix fractions was performed in some experiments on 5- to 12-% gradient slab gels essentially as described by Laemmli.[26] The positions of labeled bands were visualized by fluorography of fixed, dried gels previously treated with Enlightening (New England Nuclear, Boston, MA) enhancer and exposed at −70 C on Kodak XAR-2 film. In other experiments, samples were harvested as described above and

radiolabeled macromolecules separated by gel filtration on an analytical Sepharose CL-4B column (110 × 0.7 cm) in 4 M guanidinium hydrochloride plus 0.05% Triton X-100. Recoveries of radiolabeled macromolecules exceeded 90%. Radioactivity present in collected fractions was determined by liquid scintillation counting.

Immunochemical Analyses

Subendothelial cell matrices were prepared as described above, fixed for immunofluorescence studies in 3% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.4, for 30 minutes at 4 C and washed three times with cold phosphate-buffered saline. Fixed matrices were quenched by treatment for 10 minutes with 0.05 M glycine in phosphate-buffered saline and then incubated with 10% normal goat serum in 2% bovine serum albumin (BSA) saline for 30 minutes before incubation with the primary antibodies. Fixed matrices were incubated overnight at 4 C with either rabbit anti-bovine type IV collagen (Pel. Freez, Rogers, AR) diluted 1:100, or monoclonal antibodies specific for the core protein of basement membrane HSPG (HK102 rat hybridoma supernate, courtesy of Dr. K. Kimata, Nagoya, Japan) or the α-chain of laminin (2E8 mouse hybridoma supernate, Developmental Hybridoma Studies Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biology, University of Iowa, Iowa City, IA, under contract NO1-HD-6-2915 from the NICHD) at 1:10 in 2% BSA saline. Primary antibodies were localized by a 90-minute exposure to a 1:100 dilution of the appropriate fluorescein isothiocyanate-labeled goat secondary antiserum (Miles-Yeda, Naperville, IL) in 2% BSA saline, and the complex was visualized and microphotographs were made using a Zeiss photomicroscope. In addition, some matrices were directly stained with TRITC-phalloidin (Sigma) to visualize remnant portions of the polymerized actin component of the cytoskeleton.

Statistical Analysis

Triplicate wells were used in each experiment and the results averaged to obtain a single n for statistical analysis. The significance of differences were determined by Student's two-tailed *t*-test for independent means (NS, $P > 0.05$).

Results

Characterization of the Endothelial Cell Matrix

The fibrillar matrix deposited by endothelial cells in culture was detectable by phase contrast microscopy (Figure 1A), and was immunostained by antibodies for basement membrane HSPG (Figure 1B), type IV collagen (Figure 1C), and laminin (not shown), suggesting that the matrix has structural similarities to a basement membrane. The removal of the overlying endothelial cells by the Nonidet P-40 treatment and water washes was indicated by the absence of nuclei on visualization by phase

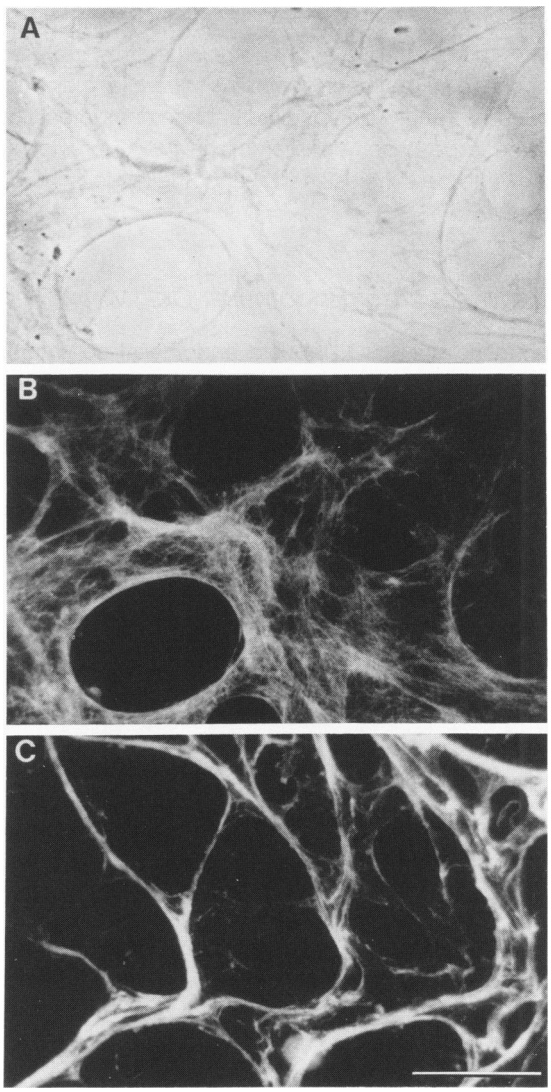

Figure 1. *Characterization of endothelial cell matrix. Endothelial cell matrix prepared as described in the Materials and Methods section was viewed under* (A) *phase contrast microscopy or after immunochemical staining for* (B) *basement membrane heparan sulfate proteoglycan in the same field or* (C) *type IV collagen. Bar = 20 μ.*

contrast microscopy and the absence of polymerized actin on staining with TRITC-phalloidin (not shown). The endothelial cells were initially plated on gelatin; the major Coomasie blue–stained sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) bands attributable to gelatin were no longer detectable in cultures 10 days after plating (not shown).

Radiosulfate-labeled proteoglycans extracted from prepared matrices ran on SDS-PAGE as a single band that just entered the resolving gel (Figure 2). This band was removed by treatment with nitrous acid at pH 1.5, which specifically degrades heparan sulfates. Chromatography of the extract of labeled matrix on Sepharose CL-4B gave a symmetric peak with a K_{av} of 0.25 (Figure 2). Taken together, these observations indicate that the basement membranelike subendothelial matrix prepared for these experiments contains a single major labeled proteoglycan species that has characteristics of the large basement membrane HSPG previously shown to be synthesized by endothelial cells in culture.[19,27]

Degradation of Endothelial Cell Matrix

Incubation of $^{35}SO_4$-labeled endothelial cell matrix for 4 hours at 37 C in buffer alone at pH 7.5 resulted in the release of 6.1% of the ^{35}S-labeled material into the supernatant fluid (Figure 3). This baseline release fell to very low levels as the pH was lowered, and thus may reflect the presence of endogenous proteinases active at neutral pH. The addition of neutrophil elastase significantly increased release above baseline at pH 7.5 ($P < 0.01$), 6.5 ($P < 0.01$), and, to a lesser degree, 5.5 ($P < 0.001$). Fifteen-minute exposure to the MPO-H_2O_2-chloride system also increased the release of ^{35}S-labeled material from the matrix at pH 6.5 ($P < 0.01$) and, to a lesser degree, at pH 5.5 ($P < 0.001$) and when the matrix was exposed sequentially to elastase for 4 hours and the MPO system for 15 minutes, the release was greater than additive at pH 6.5, 5.5, and 4.5. Chloride alone at the concentration used in the MPO system (0.1 M) significantly increased the release of ^{35}S-labeled material by elastase at pH 5.5 ($P < 0.01$) and 4.5 ($P < 0.05$) under the conditions used in Figure 3, although the potentiating effect of the complete MPO system was significantly greater than that produced by chloride alone at pH 6.5 and 5.5 ($P < 0.002$ and $P < 0.001$, respectively).

Samples of extracted matrix and supernatant collected after incubation with buffer alone, elastase at pH 7.5 for 4 hours, or elastase at pH 5.5 for 4 hours followed by the complete MPO system for 15 minutes were chromatographed on a gel filtration col-

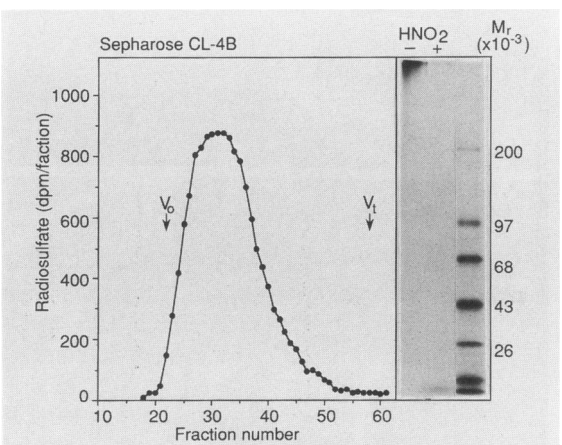

Figure 2. *Characterization of the radiosulfate-labeled proteoglycan. The radiolabeled matrix was extracted as described in the Materials and Methods section and separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis before and after treatment with nitrous acid at pH 1.5 for 90 minutes. A high molecular weight radioactive band was observed, which was lost on nitrous acid treatment. Chromatography of the extracted matrix on Sepharose CL-4B with elution by 4 M guanidinium hydrochloride with 0.5% Triton X-100 revealed a single peak with $K_{av} = 0.25$.*

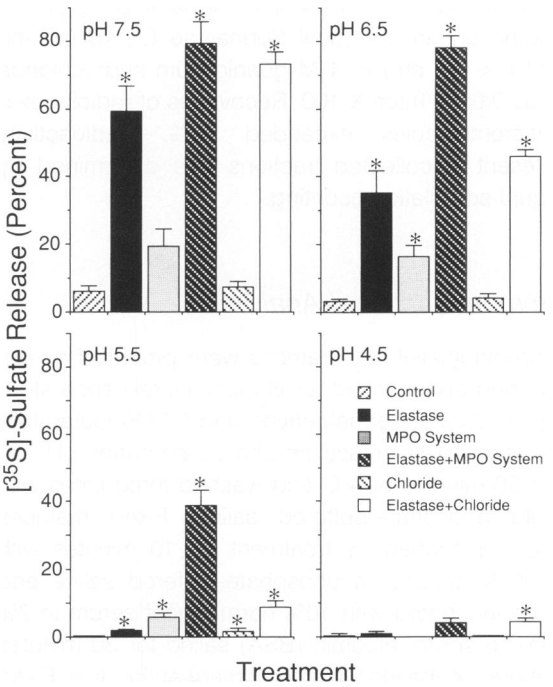

Figure 3. *Effect of elastase followed by the myeloperoxidase (MPO) system on ^{35}S release from labeled matrix. The labeled matrix was incubated either in buffer alone (10^{-2} M sodium phosphate buffer pH 7.5, sodium phosphate buffer pH 6.5, sodium acetate buffer pH 5.5, sodium acetate buffer pH 4.5) or in buffer containing 1 μg/ml elastase for 4 hours where indicated. 0.1 M Sodium chloride or 226 mU MPO/ml + 10^{-4} M H_2O_2 + 0.1 M sodium chloride were then added where indicated and the incubation continued for 15 minutes. The results are the mean + SE of three experiments. * Significantly different from control, $P < 0.05$.*

umn to obtain a more precise measure of hydrodynamic size (Figure 4). Although insufficient radioactivity is released by buffer incubation alone, radiolabeled matrix proteoglycan eluted from the column as a peak of K_{av} of ~0.21 (Figure 4A), comparable in size to the broader peak representing untreated matrix proteoglycan, eluted from a preparative column (Figure 2). Treatment of matrix proteoglycan with nitrous acid resulted in complete degradation, with radiolabel eluting near the column total volume, indicating that the matrix proteoglycan bears only heparan sulfate glycosaminoglycan chains. Similar column profiles were obtained for radiolabeled matrix incubated with acetate buffer at pH 5.5 (not shown). After digestion with either elastase alone (Figure 4B) or elastase plus the MPO system (Figure 4C), the bulk of the material shifts to smaller hydrodynamic sizes, with either treatment giving similar profiles for matrix or supernatant samples. In matrix extracts the major peak eluted with a K_{av} of 0.26 to 0.29, slightly smaller than matrix samples incubated with buffer alone. In addition, a poorly resolved shoulder (K^{av} ~0.45 to 0.47) in matrix samples eluted at the same position as the major peak of supernatant-associated radiolabel. The minor peak of supernatant radioactivity eluted with a K_{av} similar to that seen as the major peak from matrix samples.

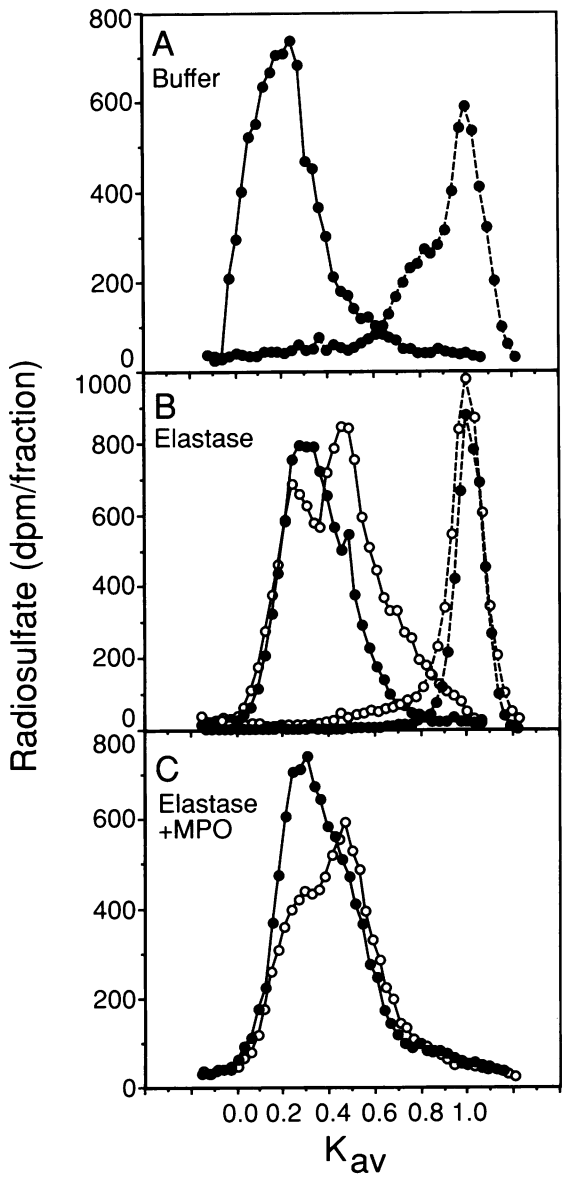

Figure 4. *Gel filtration analysis. Radiolabeled heparan sulfate proteoglycan was extracted from the matrix (●) and supernatant (○) after incubations either with* (A) *buffer alone for 4 hours,* (B) *elastase at pH 7.5 for 4 hours, or* (C) *elastase at pH 5.5 for 4 hours, followed by the myeloperoxidase system for 15 minutes (as described in the legend to Figure 3). Some extracts were treated after ethanol precipitation with nitrous acid to degrade heparan sulfate before chromatography (----).*

Figure 5 demonstrates the size distribution of the products of proteoglycan degradation at pH 5.5 (Figure 5A) and 7.5 (Figure 5B), as detected by SDS-PAGE. Both supernatant and matrix fractions were analyzed. On incubation with buffer alone, essentially all the radioactivity remained in the matrix at pH 5.5, whereas some release into the supernatant (<10%) was observed at pH 7.5. Incubation with buffer alone also did not change the SDS-PAGE profile of matrix radioactivity, which ran as a band that just entered the running gel. In contrast, after incubation with elastase at either pH, a large proportion of the radioactivity was present in fragments that ran with an apparent M_r of <250,000, with more material of smaller size (M_r < 100,000) present after elastase treatment at pH 7.5. Little radioactivity was released by elastase into the supernatant at pH 5.5, whereas most of the lower M_r radioactivity was recovered from the supernatant of matrices treated with elastase at pH 7.5. The MPO system (MPO + H_2O_2 + chloride) also increased the release of radioactive products into the supernatant at pH 7.5, and to a lesser degree at pH 5.5. When treatment with elastase for 4 hours was followed by treatment with the MPO system for 15 minutes, degradation at pH 5.5 was considerably greater than the sum of the effects of elastase and the MPO system alone. The increased release to the supernatant under the combined action of elastase and MPO is due largely to the release of lower M_r degradation products that remain matrix-associated after elastase or MPO treatment alone at pH 5.5. Chloride alone at 0.1 M had a small stimulatory effect on the release of labeled products in the presence or absence of elastase, particularly at

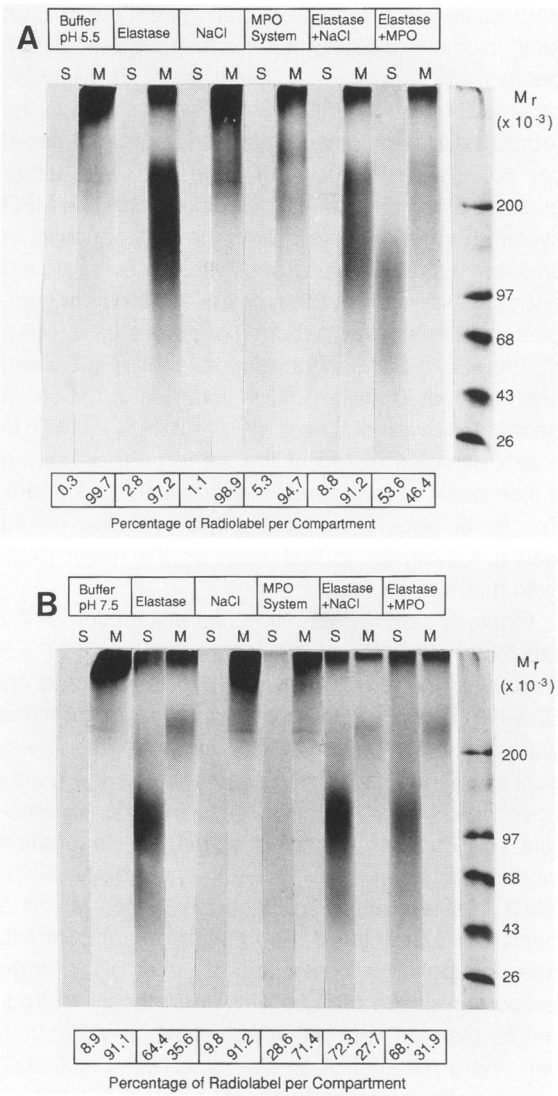

Figure 5. *Sodium dodecylsulfate–polyacrylamide gel electrophoresis analysis of elastase- and myeloperoxidase-treated matrix. The reaction mixture was as described in Figure 3 for pH 5.5* (A) *and 7.5* (B). *S, supernatant; M, matrix.*

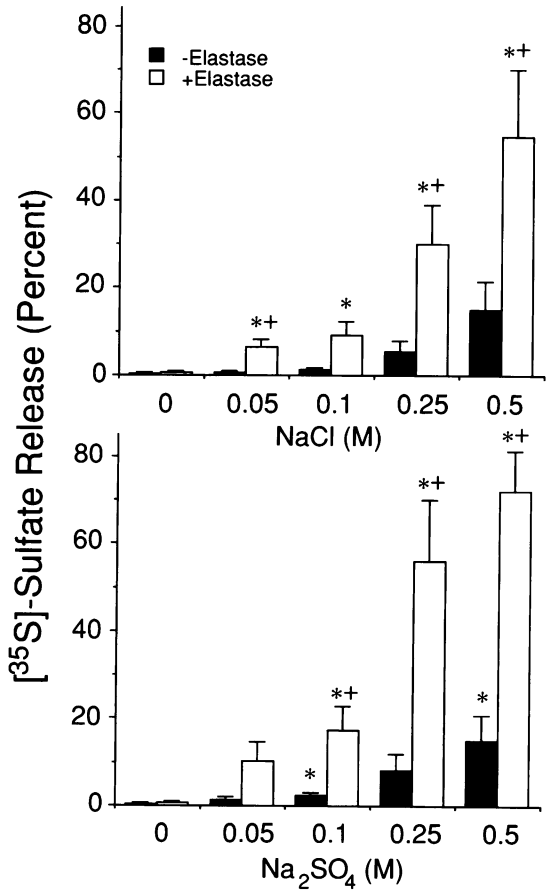

Figure 6. *Effect of chloride and sulfate concentrations. The labeled matrix was incubated either in 10^{-2} M sodium acetate buffer pH 5.5 alone (solid bars) or in buffer containing 1 μg/ml elastase (open bars) for 4 hours. Sodium chloride or sodium sulfate were added at the concentrations indicated and the incubation continued for 15 minutes. The results are the mean + SE of four experiments. * Significantly different from no chloride or sulfate, $P < 0.05$. † Significantly different from no elastase, $P < 0.05$.*

pH 5.5. All subsequent experiments were performed at pH 5.5.

The stimulatory effect of chloride alone on the release of ^{35}S-labeled material increased with an increase in the chloride concentration, and sodium chloride could be replaced in this regard by sodium sulfate (Figure 6). This stimulation of release by high salt concentrations was observed in the presence and, to a lesser degree, in the absence of elastase. When analyzed by SDS-PAGE, both chloride and sulfate at high concentration increased the release of matrix or its lower molecular weight fragments into the supernatant fluid, particularly in the presence of elastase (Figure 7).

Table 1 demonstrates the requirement for each component of the reaction mixture for the release of ^{35}S-labeled material by a 4-hour exposure to elastase followed by a 15-minute exposure to the MPO system. Release was lowest when chloride was omitted. The partial release observed when H_2O_2 or MPO was omitted is due to the effect of elastase plus chloride, and the partial release when elastase was omitted is due to the effect of the MPO system.

In Figure 3, labeled matrix was preincubated with elastase for 4 hours and then incubated with either chloride alone or the MPO-H_2O_2-chloride system for 15 minutes. In Figure 8, the order was reversed, with chloride or the MPO system (15 minutes) followed by elastase (4 hours). As in Figure 3, at pH 5.5, ^{35}S release was significantly increased by elastase, the MPO system and chloride either individually or in combination. ^{35}S release by a combi-

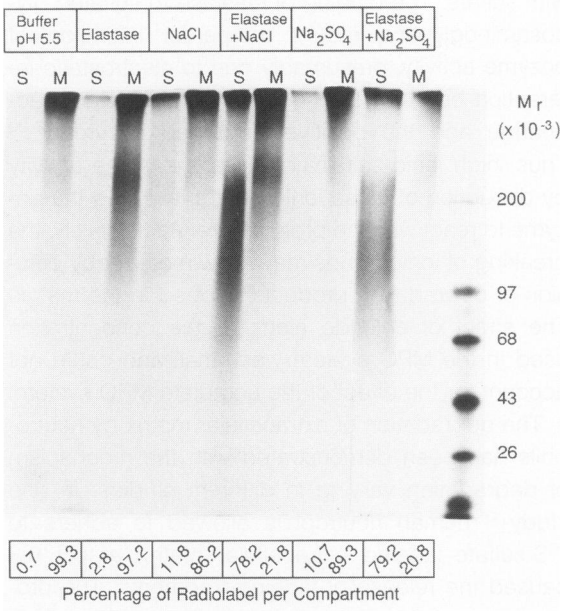

Figure 7. *Sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis of chloride- (or sulfate)-treated matrix. The reaction mixture was as described in Figure 5 with the use of 0.5 M sodium chloride or sodium sulfate.*

Table 1. *Effect of Deletion of Components*

Supplements	^{35}S release (%)	*P**	*P*†
None	0.4±0.3	<0.01	
Elastase + MPO + H_2O_2 + Cl	33.7±5.3		<0.01
Cl omitted	2.0±0.5	<0.01	NS
H_2O_2 omitted	7.6±3.1	<0.02	NS
MPO omitted	10.4±3.8	<0.05	NS
Elastase omitted	6.7±1.8	<0.01	<0.02

The reaction mixture was as described in Figure 3, except that 10^{-2} M sodium acetate buffer (pH 5.5) was used and components of the reaction mixture were omitted where indicated. Results are the mean ± SE of three experiments.

* *P* value for the difference from Elastase + MPO + H_2O_2 + Cl.

† *P* value for the difference from none.

nation of elastase and chloride was significantly greater than either elastase ($P < 0.001$) or chloride ($P < 0.001$) alone, or the sum of the two ($P < 0.002$). ^{35}S release by a combination of elastase and the MPO system was significantly greater than either elastase ($P < 0.001$) or the MPO system ($P < 0.05$) alone; however, it was not different than the sum of the two.

In Figure 9, elastase, the MPO system, and chloride, singly or in combination, were added to the labeled matrix at zero time and incubated together for periods of up to 4 hours. Under the conditions used, the combination of elastase and chloride produced a significantly greater release of ^{35}S-labeled compounds than did elastase and chloride alone (or the sum of the two) at all time periods. The com-

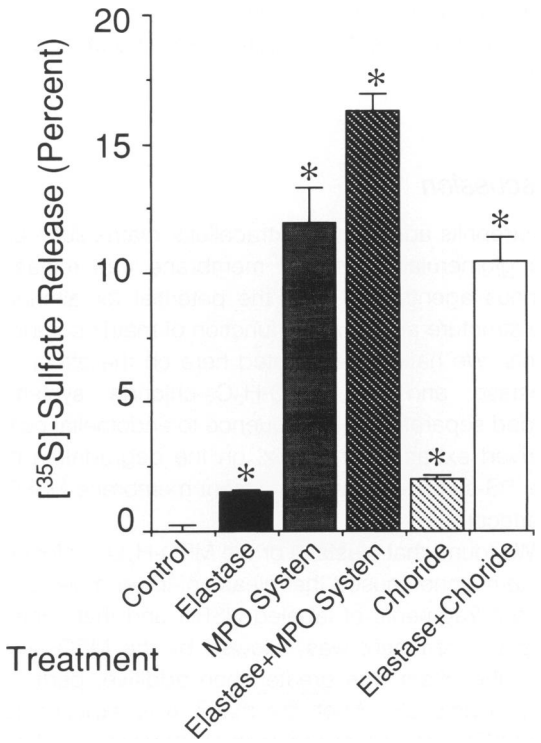

Figure 8. *Effect of the myeloperoxidase (MPO) system followed by elastase on ^{35}S release. The reaction mixture was as described in Figure 3 (pH 5.5) except that the labeled matrix was incubated with chloride or the MPO system for 15 minutes followed by a 4-hour incubation with elastase where indicated. The results are the mean ± SE of three experiments. * Significantly different from control, $P < 0.05$.*

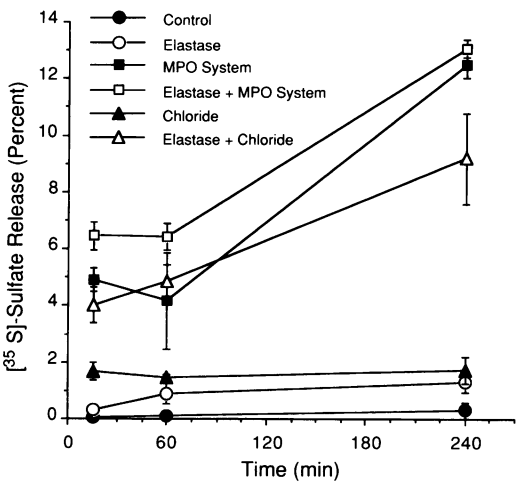

Figure 9. *Effect of elastase and the myeloperoxidase (MPO) system added together on ^{35}S release. The reaction mixture was as described in Figure 3 (pH 5.5) except that the components were all added at zero time and incubated for either 15, 60, or 240 minutes. The results are the mean of three experiments.*

bination of elastase and the MPO system also produced a significantly greater release than did elastase alone at all time periods; however, the effect of the combination was not significantly greater

than that of the MPO system alone (or the sum of the individual effects of elastase and the MPO system).

Discussion

Neutrophils adherent to extracellular matrix such as the glomerular basement membrane can release various agents that have the potential for altering the structure and thus the function of matrix components. We have concentrated here on the effect of elastase and the MPO-H_2O_2-chloride system, added separately or in sequence to endothelial cell-derived extracellular matrix, on the degradation of the ^{35}S-sulfate–labeled basement membrane HSPG (perlecan).

We found that elastase or the MPO-H_2O_2-chloride system alone caused the release of lower molecular weight fragments of labeled HSPG and that, when elastase treatment was followed by the MPO system, the effect was greater than additive, particularly at acid pH. When the matrix was exposed to the MPO system first and then to elastase, or when both the MPO system and elastase were added together, the effect of the combined treatment was not greater than the sum of the two systems added individually. Thus, both elastase and the MPO system degrade HSPG with the effect of the MPO system being enhanced by elastase pretreatment.

Elastase alone at pH 7.5, or together with the MPO-H_2O_2-chloride system at pH 5.5, appears to release HSPG degradation products of comparable hydrodynamic size. Both treatments generate several discrete HSPG fragments, with the smaller being released preferentially to the supernatant. The partial retention of larger HSPG fragments in the matrix suggests that elastase digestion alone is not sufficient to cause release of all HSPG. It is possible that larger fragments of HSPG may remain bound to other matrix components by electrostatic forces and that further proteolysis is required for release into the supernatant. In this context, it is important to note that the treatment did not cause complete morphological dissolution of the matrix. For example, despite the release of HSPG degradation products after treatment with elastase or the MPO-H_2O_2-chloride system, the remaining matrix appeared morphologically intact as indicated by phase contrast microscopy. In each instance, the matrix stained with an antibody to type IV collagen.

Chloride alone at high ionic strength increased the release of labeled products in the presence and absence of elastase, an effect that was also seen with sulfate. The binding of elastase to sulfated glycosaminoglycans results in partial inhibition of enzyme activity, presumably due to electrostatic interaction between the negatively charged polysaccharide and the positively charged enzyme.[28,29] Thus, high ionic salt may facilitate elastase activity by disruption of these ionic bonds, allowing the enzyme to react with the protein core. Alternatively, the breaking of ionic bonds may allow more ready diffusion of degradation products trapped in the matrix. The effect of chloride alone at the concentration used in the MPO system was small and could not account for the effect of the complete MPO system.

The degradation of extracellular matrix by neutrophils has been demonstrated with the mechanism of degradation varying in different studies. In one study,[30] human neutrophils allowed to adhere to ^{35}S-sulfate–labeled extracellular matrix at pH 6.3 caused the release of labeled fragments. The proteoglycan of the extracellular matrix was 70 to 75% heparan sulfate, and that the degradation products were derived from this proteoglycan was suggested by their resistance to chondroitinase ABC and papain digestion, sensitivity to nitrous acid deamination, and precipitation by cetyl pyridinium chloride.[30] Degradation was unaffected by stimulation of the neutrophil by zymosan-activated serum or phorbol myristate acetate, was lost when the pH was raised above 7.2 and was inhibited by heparin and proteinase inhibitors.[30,31] It was proposed that degradation of extracellular matrix heparan sulfate is due to the release of a heparanase from the specific granules of neutrophils,[32] whose action is facilitated by a proteinase. In another study[33,34] in which ^{35}S-sulfate–labeled extracellular matrix containing a smaller proportion (25%) of heparan sulfate was used, human neutrophils used predominantly intrinsic proteinases to degrade the proteoglycan. Degradation occurred at pH 7.4, was increased by the neutrophil stimulants, phorbol myrystate acetate or formyl-methionine-leucine-phenylalanine plus cytochalasin B, and was strongly inhibited by serum or a specific inhibitor of neutrophil elastase. Degradation was unaffected by catalase and superoxide dismutase. Degradation was induced by cathepsin G and the MPO-H_2O_2-chloride system; however, it was considerably less than that produced by neutrophil elastase. Both elastase and MPO-mediated degradation resulted from the cleavage of the core protein rather than the glycosaminoglycan of the peptidoglycan. Key et al[35] also have reported the cleavage of vascular endothelial cell–associated ^{35}S-labeled heparan

sulfate proteoglycan by stimulated neutrophils, cell-free neutrophil supernatants, and neutrophil elastase.

When subendothelial matrix was labeled with ^{3}H-proline, degradation was dependent on neutrophil elastase but not on oxygen metabolites.[35] However, when the elastase was inhibited by α_1 proteinase inhibitor, degradation could be facilitated by oxidants formed by the MPO-H_2O_2-chloride system because of their inhibitory effect on α_1 proteinase inhibitor.[35,36] Neutrophil-dependent degradation of glomerular basement membrane, as measured by the release of hydroxyproline, required the presence of aggregated immunoglobulin G and was inhibited by a serine proteinase inhibitor, implicating elastase and/or cathepsin G.[37] The MPO-H_2O_2-chloride system increased the susceptibility of the glomerular basement membrane to proteolysis; however, this potentiation was largely offset by oxidative inactivation of the proteinases.[38] Although H_2O_2, MPO, and oxidants released by stimulated polymorphonuclear leukocytes enhanced the degradation of matrix laid down by neonatal rat aortic smooth muscle cells, proteinases (elastase, collagenase, and/or gelatinase) appeared to be the major enzymes responsible for the degradation.[39]

In other studies, neutrophils or neutrophil extracts increased the degradation of cartilage proteoglycan, as measured by the release of ^{35}S-sulfate–labeled material.[40] Degradation was partially inhibited by a specific neutrophil elastase inhibitor. The product of the MPO system, HOCl, also degraded cartilage proteoglycan; however, the MPO-H_2O_2-chloride system had no consistent effect. It was concluded that neutrophil-mediated degradation of cartilage proteoglycan was due primarily to elastase with a minor role for the products of the MPO system. Neutrophil elastase and cathepsin G have been reported to be the primary proteinases responsible for neutrophil-mediated cartilage proteoglycan degradation, with the contribution by proteinase-3 being negligible.[41] The degradation of articular cartilage proteoglycan aggregates by hypochlorite[42] and the cleavage of hyaluronic acid by the MPO-H_2O_2-chloride system[43,44] has been reported.

Our studies support degradation of HSPG of the glomerular basement membrane as a contributing factor in the induction of proteinuria by renal artery infusion of MPO and H_2O_2[16] or elastase.[18] With MPO and H_2O_2 infusion, proteinuria is associated with marked morphological changes that are not observed after elastase infusion. Thus, glomerular damage, particularly produced by the MPO system, appears to also involve other mechanisms. In neutrophil-dependent glomerulonephritis, neutrophils adhere to glomerular endothelial cells and basement membrane and release oxidants and granule enzymes at the site of attachment.[45,46] Glomerular injury under these conditions may result in part from the disruption of basement membrane proteoglycans by proteinases and/or oxidants.

References

1. Gallagher JT, Lyon M, Steward WP: Structure and function of heparan sulphate proteoglycans. Biochem J 1986, 236:313–325
2. Yanagishita M, Hascall VC: Cell surface heparan sulfate proteoglycans. J Biol Chem 1992, 267:9451–9454
3. Lindahl U, Thunberg L, Bäckström G, Riesenfeld J, Nordling K, Björk I: Extension and structural variability of the antithrombin-binding sequence in heparin. J Biol Chem 1984, 259:12368–12376
4. Turnbull JE, Fernig DG, Ke Y, Wilkinson MC, Gallagher JT: Identification of the basic fibroblast growth factor binding sequence in fibroblast heparan sulfate. J Biol Chem 1992, 267:10337–10341
5. Noonan DM, Fulle A, Valente P, Cai S, Horigan E, Sasaki M, Yamada Y, Hassell JR: The complete sequence of perlecan: A basement membrane heparan sulfate proteoglycan, reveals extensive similarity with laminin A chain, low density lipoprotein-receptor, and the neural cell adhesion molecule. J Biol Chem 1991, 266:22939–22947
6. Kallunki P, Tryggvason K: Human basement membrane heparan sulfate proteoglycan core protein: A 467-kD protein containing multiple domains resembling elements of the low density lipoprotein receptor, laminin, neural cell adhesion molecules, and epidermal growth factor. J Cell Biol 1992, 116:559–571
7. Murdoch AD, Dodge GR, Cohen I, Tuan RS, Iozzo RV: Primary structure of the human heparan sulfate proteoglycan from basement membrane (HSPG2/Perlecan): A chimeric molecule with multiple domains homologous to the low density lipoprotein receptor, laminin, neural cell adhesion molecules, and epidermal growth factor. J Biol Chem 1992, 267:8544–8557
8. Kanwar YS, Linker A, Farquhar MG: Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion. J Cell Biol 1980, 86: 688–693
9. Rosenzweig LJ, Kanwar YS: Removal of sulfated (heparan sulfate) or nonsulfated (hyaluronic acid) glycosaminoglycans results in increased permeability of the glomerular basement membrane to ^{125}I-bovine serum albumin. Lab Invest 1982, 47:177–184
10. Mynderse LA, Hassell JR, Kleinman HK, Martin GR, Martinez-Hernandez A: Loss of heparan sulfate proteoglycan from glomerular basement membrane of

nephrotic rats. Lab Invest 1983, 48:292–302
11. Makino H, Gibbons JT, Reddy MK, Kanwar YS: Nephritogenicity of antibodies to proteoglycans of the glomerular basement membrane-1. J Clin Invest 1986, 77:142–156
12. Miettinen A, Stow JL, Mentone S, Farquhar MG: Antibodies to basement membrane heparan sulfate proteoglycans bind to the laminae rarae of the glomerular basement membrane (GBM) and induce subepithelial GBM thickening. J Exp Med 1986, 163:1064–1084
13. Abrass CK, Cohen AH: Characterization of renal injury initiated by immunization of rats with heparan sulfate. Am J Pathol 1988, 130:103–111
14. SJ Klebanoff: Oxygen metabolites from phagocytes. Inflammation: Basic Principles and Clinical Correlates. ed 2, Edited by Gallin JI, Goldstein IM, Snyderman R. New York, Raven Press, 1992, pp 541-588
15. SJ Klebanoff: Myeloperoxidase: occurrence and biological function. Peroxidases in Chemistry and Biology. volume 1. Edited by Everse J, Everse KE, Grisham MB. Boca Raton, FL, CRC Press, 1991, pp 1–35
16. Johnson RJ, Couser WG, Chi EY, Adler S, Klebanoff SJ: New mechanism for glomerular injury: Myeloperoxidase-hydrogen peroxide-halide system. J Clin Invest 1987, 79:1379–1387
17. Johnson RJ, Guggenheim SJ, Klebanoff SJ, Ochi RF, Wass A, Baker P, Schulze M, Couser WG: Morphologic correlates of glomerular oxidant injury induced by the myeloperoxidase-hydrogen peroxide-halide system of the neutrophil. Lab Invest 1988, 5:294–301
18. Johnson RJ, Couser WG, Alpers CE, Vissers M, Schulze M, Klebanoff SJ: The human neutrophil serine proteinases, elastase and cathepsin G, can mediate glomerular injury in vivo. J Exp Med 1988, 168:1169–1174
19. Saku T, Furthmayr H: Characterization of the major heparan sulfate proteoglycan secreted by bovine aortic endothelial cells in culture: Homology to the large molecular weight molecule of basement membranes. J Biol Chem 1989, 264:3514–3523
20. Kinsella MG, Wight TN: Modulation of sulfated proteoglycan synthesis by bovine aortic endothelial cells during migration. J Cell Biol 1986, 102:679–687
21. Rakita RM, Michel BR, Rosen H: Differential inactivation of *Escherichia coli* membrane dehydrogenases by a myeloperoxidase-mediated antimicrobial system. Biochemistry 1990, 29:1075–1080
22. Klebanoff SJ, Waltersdorph AM, Rosen H: Antimicrobial activity of myeloperoxidase. Methods Enzymol 1984, 105:399–403
23. Kramer RH, Vogel KG, Nicolson GL: Solubilization and degradation of subendothelial matrix glycoproteins and proteoglycans by metastatic tumor cells. J Biol Chem 1982, 257:2678–2686
24. Wasteson A, Uthne K, Westermark B: A novel array for the biosynthesis of sulfated polysaccharide and its application to studies on the effects of somatomedin on cultured cells. Biochem J 1973, 136:1069–1074
25. Lindahl U, Bäckström G, Jansson L, Hallén A: Biosynthesis of heparin. II. Formation of sulfamino groups. J Biol Chem 1973, 248:7234–7241
26. Laemmli UK: Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 1970, 227:680–685
27. Kinsella MG, Wight TN: Structural characterization of heparan sulfate proteoglycan subclasses isolated from bovine aortic endothelial cell cultures. Biochemistry 1988, 27:2136–2144
28. Baici A, Salgam P, Fehr K, Boni A: Inhibition of human elastase from polymorphonuclear leucocytes by a glycosaminoglycan polysulfate (arteparon R). Biochem Pharmacol 1980, 29:1723–1727
29. Baici A, Bradamante P: Interaction between human leukocyte elastase and chondroitin sulfate. Chem Biol Interactions 1984, 51:1–11
30. Matzner Y, Bar-Ner M, Yahalom J, Ishai-Michaeli R, Fuks Z, Vlodavsky I: Degradation of heparan sulfate in the subendothelial extracellular matrix by a readily released heparanase from human neutrophils. J Clin Invest 1985, 76:1306–1313
31. Bar-Ner M, Eldor A, Wasserman L, Matzner Y, Cohen IR, Fuks Z, Vlodavsky I: Inhibition of heparanase-mediated degradation of extracellular matrix heparan sulfate by non-anticoagulant heparin species. Blood 1987, 70:551–557
32. Matzner Y, Vlodavsky I, Bar-Ner M, Ishai-Michaeli R, Tauber AI: Subcellular localization of heparanase in human neutrophils. J Leukocyte Biol 1992, 51:519–524
33. McGowan SE, Thompson RJ: Extracellular matrix proteoglycan degradation by human alveolar macrophages and neutrophils. J Appl Physiol 1989, 66:400–409
34. McGowan SE: Mechanisms of extracellular matrix proteoglycan degradation by human neutrophils. Am J Respir Cell Mol Biol 1990, 2:271–279
35. Key NS, Platt JL, Vercellotti GM: Vascular endothelial cell proteoglycans are susceptible to cleavage by neutrophils. Arterioscler Thromb 1992, 12:836–842
36. Weiss SJ, Regiani S: Neutrophils degrade subendothelial matrices in the presence of alpha-1-proteinase inhibitor: Cooperative use of lysosomal proteinases and oxygen metabolites. J Clin Invest 1984, 73:1297–1303
37. Weiss SJ, Curnutte JT, Regiani S: Neutrophil-mediated solubilization of the subendothelial matrix: Oxidative and non-oxidative mechanisms of proteolysis used by normal and chronic granulomatous disease phagocytes. J Immunol 1986, 136:636–641
38. Vissers MCM, Winterbourn CC, Hunt JS: Degradation of glomerular basement membrane by human neutrophils in vitro. Biochim Biophys Acta 1984, 804:154–160
39. Vissers MCM, Winterbourn CC: The effect of oxidants on neutrophil-mediated degradation of glomerular

basement membrane collagen. Biochim Biophys Acta 1986, 889:277–286
40. McGowan SE, Murray JJ: Direct effects of neutrophil oxidants on elastase-induced extracellular matrix proteolysis. Am Rev Respir Dis 1987, 135:1286–1293
41. Kowanko IC, Bates EJ, Ferrante A: Mechanisms of human neutrophil-mediated cartilage damage *in vitro*: The role of lysosomal enzymes, hydrogen peroxide and hypochlorous acid. Immunol Cell Biol 1989, 67: 321–329
42. Janusz MJ, Doherty NS: Degradation of cartilage matrix proteoglycan by human neutrophils involves both elastase and cathepsin G. J Immunol 1991, 146: 3922–3928
43. Katrantzis M, Baker MS, Handley CJ, Lowther DA: The oxidant hypochlorite (OCI-), a product of the myeloperoxidase system, degrades articular cartilage proteoglycan aggregate. Free Radical Biol Med 1991, 10: 101–109
44. Baker MS, Green SP, Lowther DA: Changes in the viscosity of hyaluronic acid after exposure to a myeloperoxidase-derived oxidant. Arthritis Rheum 1989, 32:461–467
45. Green SP, Baker MS, Lowther DA: Depolymerization of synovial fluid hyaluronic acid (HA) by the complete myeloperoxidase (MPO) system may involve the formation of a HA-MPO ionic complex. J Rheumatol 1990, 17:1670–1675
46. Vissers MCM, Day WA, Winterbourn CC: Neutrophils adherent to a nonphagocytosable surface (glomerular basement membrane) produce oxidants only at the site of attachment. Blood 1985, 66:161–166