Elastase, but not proteinase 3 (PR3), induces proteinuria associated with loss of glomerular basement membrane heparan sulphate after *in vivo* renal perfusion in rats

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SUMMARY

Elastase, but not PR3, induces proteinuria associated with loss of glomerular basement membrane (GBM) heparan sulphate after *in vivo* renal perfusion in rats. PR3 and elastase are cationic neutral serine proteinases present in the azurophilic granules of polymorphonuclear leucocytes. Release of these proteolytic enzymes along the glomerular capillary wall may induce glomerular injury. Here, we investigated the effects of PR3 and elastase on the induction of proteinuria and glomerular injury after renal perfusion of these enzymes in Brown–Norway rats. Perfusion of active elastase induced a dose-dependent proteinuria 24 h after perfusion, while inactivated elastase did not. Perfusion of comparable amounts of active PR3 did not induce proteinuria. Light and electron microscopy showed no morphological abnormalities in any experimental group. However, immunohistology revealed that proteinuria occurring after perfusion of active elastase was associated with a strong reduction in intraglomerular expression of the heparan sulphate side chain and, to a lesser extent, of the protein core of heparan sulphate proteoglycans (HSPG). *In vitro*, both elastase and PR3 digested HSPG. However, PR3 bound to a lesser extent to HSPG than elastase. We conclude that elastase, but not PR3, induces proteinuria after *in vivo* renal perfusion. This differential effect probably relates to different binding to the GBM of those enzymes due to differences in their isoelectric points. Degradation of heparan sulfate proteoglycans, leading to the disappearance of their side chains that contribute to the polyanionic structure of the GBM, appears to be involved in the induction of proteinuria after perfusion of elastase.

Keywords serine proteases neutrophils glomerulonephritis extracellular matrix isoelectric point

INTRODUCTION

Polymorphonuclear leucocytes (PMN) contain various proteases in their granules which are released into the extracellular space upon activation. It has long been recognized that release of these proteases by PMN adherent to the glomerular capillary wall may play an important role in the induction of glomerular injury, as observed in PMN-dependent glomerulonephritis [1]. In this respect, most attention has been focused on the cationic neutral serine proteases elastase and cathepsin G, which are present in the azurophilic granules of PMN [2]. Evidence for the involvement of these neutral serine proteases in glomerular injury has been

provided by a number of studies. *In vitro*, it has been shown that elastase and cathepsin G degrade several constituents of the glomerular basement membrane (GBM), including fibronectin, laminin, and collagen type IV [3–5]. In addition, Johnson *et al.* showed that *in vivo* renal perfusion of μg quantities of active elastase and cathepsin G results in severe proteinuria, while the equally cationic but inactivated enzymes do not [6]. Moreover, Schrijver *et al.* found that Beige mice, whose PMN are deficient in elastase and cathepsin G, do not develop proteinuria after induction of anti-GBM antibody disease [7].

Recently, a novel proteolytic enzyme isolated from human PMN was described as PR3. PR3 has been characterized as a neutral serine protease with a mol. wt of 29 kD and an isoelectric point of 9·1 [8]. Like cathepsin G and elastase, it is present in the azurophilic granules of PMN and released after stimulation [9].

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Since the discovery that the majority of anti-neutrophil cytoplasmic antibodies (ANCA) in Wegener's granulomatosis (WG) are directed against PR3, the enzyme has attracted considerable interest and has been the focus of several studies [10,11]. Intratracheal insufflation of PR3 in hamsters has been shown to cause emphysema, and, *in vitro*, PR3 is capable of degrading several constituents of the GBM [8,12]. Furthermore, we recently showed that in renal biopsies from patients with WG, PR3 and elastase are localized extracellularly in renal tissue, suggesting that release of these proteolytic enzymes may contribute to glomerular damage as seen in WG [13]. However, *in vivo* evidence concerning the role of PR3 in glomerular injury is lacking. We therefore studied the effects of PR3 on the induction of proteinuria after renal perfusion of μg quantities of the active enzyme in Brown–Norway rats and compared the effects with those obtained after renal perfusion of elastase.

Recently, Klebanoff *et al.* showed that elastase is capable of degrading heparan sulphate proteoglycans (HSPG) of subendothelial matrix *in vitro*, suggesting that proteolytic cleavage of HSPG may be involved in neutrophil-associated proteinuria [14]. HSPG consist of a core protein bearing negatively charged heparan sulphate glycosaminoglycan chains and are considered to be main determinants for the charge-dependent permeability of the glomerular capillary wall [15]. In rats, the nephrotic syndrome is associated with a loss of GBM HSPG, and antibodies to basement membrane HSPG core protein and heparan sulphate are nephritogenic [16–18]. Therefore, we studied glomerular HSPG core protein and side chain expression after *in vivo* renal perfusion of elastase and PR3 in relation to the occurrence of proteinuria. We compared the results with the *in vitro* binding and degradation of HSPG by both enzymes.

MATERIALS AND METHODS

Animals
All experiments were performed in conventionally housed, 3-month-old Brown–Norway rats. Animals were fed *ad libitum* with standard chow (Hope Farms, Woerden, The Netherlands).

Enzyme activity assay
Enzymatic activity of PR3 and elastase was quantified by the cleavage of the synthetic ester, Boc-alanine-*p*-nitrophenyl ester (Sigma Chemical Co., St Louis, MO), according to the method of Rao *et al.* [12]. Assays were carried out in a 200-μl final volume of 0·05 M phosphate buffer pH 7·5 containing 0·5 mM substrate and were run and quantified in a 96-well microtitre plate (Greiner Inc., Alphen a/d Rijn, The Netherlands) using a Titertek multiscan (Titertek Flow Labs, Zwanenburg, The Netherlands) for analysis read at 405 nm. One unit of enzymatic activity was defined as the amount which cleaved 1 μmol of substrate in 1 min at 22°C.

Proteolytic assay
Cleavage of casein was assayed according to the methods of Twining [19]. For each sample, 0·04% (w/v) resorufin-labelled casein (Boerhinger Mannheim Biochemica, Mannheim, Germany), 0·2 M Tris–HCl pH 7·8, 0·02 M CaCl₂, and 1 μg PR3 or elastase were incubated in a total volume of 200 μl at 37°C for 16 h. The reaction was stopped by adding 480 μl 5% (w/v) trichloro acetic acid (TCA). A precipitate was allowed to form at 37°C for 10 min, after which the samples were centrifuged at 13 000 *g* for 5 min. Next, 400 μl of TCA-soluble supernatant were neutralized with

600 μl of 0·5 M Tris–HCl pH 8·8. Absorbance was read at 574 nm against sample blank. Total hydrolysis was determined by incubation with 1 mg pronase (Boehringer Mannheim Biochemica) for 16 h at 37°C.

ELISA for leucocyte enzymes
An antigen capture ELISA was used to detect PR3, myeloperoxidase (MPO) or elastase, as described [20]. Briefly, microtitre plates (Inotech, Breda, The Netherlands) were incubated with goat anti-mouse IgG (Jackson, West Grove, PA). After being washed, plates were incubated with mouse MoAbs against PR3, MPO (MoAb 12.8 and MoAb 4.15, respectively; Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands) or elastase (Dakopatts, Glostrup, Denmark). After another wash, 100 μl of different dilutions of the test samples in the incubation buffer were added for 1 h at 37°C. Bound leucocyte enzymes were then detected with rabbit anti-PR3, rabbit anti-MPO and rabbit anti-elastase (both from Dakopatts). Bound antibody was detected with alkaline phophatase-labelled goat anti-rabbit IgG and *p*-nitrophenyl phosphate disodium (both from Sigma Chemical Co.) as a substrate. Plates were read at 405 nm.

Proteinase 3
PR3 was purified using the methods of Ballieux *et al.* with minor modifications, as described previously [21]. Briefly, α-granules were isolated from normal human leucocytes by sequential nitrogen cavitation and centrifugation on 84% Percoll. Pooled α-granules were subjected to two rounds of 1% Triton X-100 (Kock Light Labs, Ltd, Colnbrook, UK) extraction. This α-granule extract was used as the starting material for a two-step chromatographic isolation procedure for the isolation of PR3. Initially, the α-granule extract was applied to a 28 × 1·5 Biorex 70 (BioRad Labs, Richmond, CA) column followed by fast performance liquid chromatography (FPLC) gel filtration over a 2·6 × 60 cm Superdex 75 column (Pharmacia LKB). Fractions were tested for esterolytic activity on Boc-alanine-*p*-nitrophenyl ester and for detection of PR3 antigen by ELISA. Purity was checked by SDS–PAGE, and contamination with elastase, cathepsin G or MPO was excluded by ELISA. The PR3-positive fractions were pooled, dialysed against distilled water, lyophilized and resuspended in PBS pH 7·4. Protein concentration was measured according to Bradford using bovine serum albumin (BSA) as standard. Aliquots of 500 μl containing 500 μg enzyme were stored at −80°C until use.

Elastase
Lyophilized purified human neutrophil elastase was purchased from Calbiochem-Behring Co. (La Jolla, CA). This elastase was dissolved in PBS pH 7·4 just before use. In some experiments, elastase was irreversibly inactivated by the specific elastase inhibitor MSAAPV-chloromethyl ketone (Sigma Chemical Co.).

Stimulation of normal human PMN
For comparative purposes, the amounts of elastase and PR3 released by activated human PMN were measured. Briefly, EDTA blood was drawn from six healthy donors and PMN were isolated by centrifugation on a lymphoprep density gradient (Nycomed Pharma AS, Oslo, Norway). Contaminating erythrocytes were lysed with ammonium chloride and cell concentrations were adjusted to 10⁷/ml in Hanks' balanced salt solution (HBSS; GIBCO, Breda, The Netherlands). PMN were activated by preincubation with cytochalasin B 5 μg/ml (18015; Serva, Heidelberg,

Germany) for 5 min at 37°C followed by a 30-min incubation with 10^{-7} M formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma Chemical Co.) or HBSS to estimate spontaneous release. Subsequently, cells were centrifuged at 1000 **g** for 5 min and supernatants were assayed for PR3 and elastase content by antigen capture ELISA, as described above, using the enzyme preparation used in the perfusion studies as standard. Values were corrected for spontaneous release.

Renal perfusion

Perfusion of the left kidney was performed according to the methods of Hoyer *et al.* with minor modifications, as described [22] Briefly, rats were anaesthetized with halothane, O_2, and NO_2. The aorta and vena cava were then exposed and the tributaries ligated. A temporary clamp was placed on the aorta above the left renal artery and on the renal vein. A needle was inserted in the aorta through a puncture hole and advanced up to the level of the left renal artery. A puncture in the left renal vein allowed blood and perfusion fluids to escape which were collected in cotton wool. Initially, PBS (37°C, pH 7·4) was perfused until no visible blood escaped from the punctured renal vein. Then, rats were perfused with different doses of active elastase (15 μg, $n = 3$; 25 μg, $n = 6$; and 50 μg, $n = 6$) or PR3 (50 μg, $n = 4$; 100 μg, $n = 3$; and 300 μg, $n = 3$) in 0·5 ml PBS to establish a dose–response relationship for both enzymes. In addition, four rats were perfused with 50 μg/0·5 ml of MSAAPV chloromethyl ketone-inactivated elastase. Control rats were perfused with PBS alone. After 3 min, kidneys were cleared with PBS and blood flow was restored. Total ischaemia time was always <11 min. After surgery, rats were allowed to recover from anaesthesia for 2 h under a heat lamp. Rats were then placed in metabolic cages for 24 h with free access to water, and urine was collected.

Urine analysis

Total protein content of the urine samples was measured with the quantitative biuret method. Proteinuria was further characterized by 12% SDS–PAGE under non-reducing conditions.

Light microscopy

Twenty-four hours after perfusion rats were killed. Blood was displaced from both kidneys by perfusion of PBS at 4°C. Tissue from both kidneys was processed for light microscopy and immunofluorescence. For light microscopy, tissue was fixed in 2% paraformaldehyde in PBS and embedded in Technovit 8100 glycolmethacrylate (Kulzer GmbH, Wehrheim, Germany) for morphological detail, as described [23]. Sections (2 μm) were cut on a Reichert Jung Supercut plastic microtome and stained with periodic acid-Schiff (PAS) or periodic acid silver methamine (MZ).

Electron microscopy

Renal tissue was fixed overnight in 2% glutaraldehyde in 0·1 M phosphate buffer pH 7·4 at 4°C. Tissue was post-fixed in 1% OsO_4 for 2 h at 4°C and serially dehydrated in ethanol, then embedded in Epon and thin sections (60 nm) were cut. Sections were stained with uranyl acetate and lead citrate and examined with a Philips EM 201 electron microscope.

Localization of PR3 and elastase

Separate experiments were performed to study the localization of elastase and PR3 after perfusion by indirect immunofluorescence. In these experiments, 50 μg of active elastase ($n = 2$) or PR3

($n = 2$) were perfused as described above. Ten minutes after reperfusion, rats were killed. For localization of PR3 and elastase, renal tissue was snap-frozen in isopenthane and 2-μm crysostat sections were cut. Sections were fixed in acetone-buffered formalin 9% according to Pryzwansky *et al.* [24] followed by incubation with 10% normal goat serum. PR3 and elastase were detected by incubating sections with rabbit anti-PR3 and rabbit anti-elastase (Dakopatts). Binding was visualized with FITC-labelled goat F(ab)$_2$ anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL).

Immunofluorescence

For indirect immunofluorescence (IIF) renal tissue was snap-frozen in isopenthane and 2-μm sections were cut. Sections were fixed in acetone and subsequently incubated with 10% normal rabbit or goat serum, depending on the conjugate used. Detection of heparan sulphate (HS) side chain was done using hybridoma supernatant of mouse anti-rat HS side chain (MoAb JM-403) [21]. Binding was visualized by FITC-labelled goat anti-mouse IgM (Nordic, Tilburg, The Netherlands). To detect rat HSPG, we used goat antiserum against human HS core protein, which has been shown to cross-react with rat HSPG [21]. FITC-labelled rabbit anti-goat IgG was used as conjugate (Dakopatts). The intraglomerular expression of S-laminin, fibronectin, and collagen type IV was investigated using mouse anti-rat S-laminin, rabbit anti-human fibronectin (Sigma Chemical Co.), and goat anti-human collagen type IV (Southern Biotechnology), which have been shown to cross-react with rat fibronectin and collagen type IV. The following conjugates were used: goat anti-mouse IgG FITC (Southern Biotechnology), goat F(ab)$_2$ anti-rabbit, and rabbit F(ab)$_2$ anti-goat FITC (both from Dakopatts). For all stainings, staining intensity was scored on a semiquantitative scale (3 +): −, absent; ±, weak; +, mild; ++, intermediate; +++, strong, using the right non-perfused kidney as reference. In addition, kidney sections were double-stained with anti-HS MoAb JM-403 and goat anti-HSPG core protein. For this purpose, sections were sequentially incubated with: (i) MoAb JM-403; (ii) TRITC-labelled goat anti-mouse IgM (Southern Biotechnology); (iii) goat anti-HSPG core protein; (iv) FITC-labelled rabbit F(ab)$_2$ anti-goat (Dakopatts). The double-staining experiments included the following controls: (i) omitting MoAb JM-403 or goat anti-HSPG core or both; (ii) incubation of MoAb JM-403 or goat anti-HSPG core with the conjugate used for the detection of the other antibody in the double staining.

In vitro *studies*

First, the effects of PR3 and elastase on HSPG degradation were studied *in vitro*. For this purpose, microtitre plates (Greiner) were coated with HSPG from the mouse EHS tumour (Sigma; 125 ng/well) overnight at room temperature. Plates were washed with PBS and incubated with elastase, MSAAVP chloromethyl ketone-inactivated elastase, PR3 or PMSF-inactivated PR3 in concentrations ranging from 40 μg/ml to 2·4 ng/ml for 1 h at 37°C. After washing with PBS–Tween (0·05%), plates were incubated with the MoAb directed against HS (MoAb JM-403) for 1 h at 37°C, followed by peroxidase-labelled rabbit anti-mouse (1 h, 37°C). Binding was detected using tetramethyl benzidine (TMB; Sigma) as substrate. Plates were read at 450 nm. To assess the binding of PR3 and elastase to EHS–HSPG, microtitre plates were coated with EHS–HSPG and incubated with inactivated elastase and PR3 as described above. Then bound elastase and PR3 were detected by

incubation with rabbit anti-elastase or rabbit anti-PR3 for 1 h at 37°C. Bound antibody was detected by incubation with perox-idase-labelled goat anti-rabbit (Sigma Chemical Co.) for 1 h at 37°C. Plates were developed and read at 450 nm as described above.

Statistical analysis
Results were analysed for statistical significance by Student's *t*-test. All results are reported as mean ± s.e.m. $P < 0.05$ was regarded as significant.

RESULTS

Characterization of PR3 and elastase
On SDS–PAGE the purified PR3 preparation migrated as a triplet around 30 kD (Fig. 1). Furthermore, by ELISA, no contamination with MPO, cathepsin G or elastase was detected. As shown in Table 1, enzymatic activity of purified PR3 was 2·5 or 5 times less than that of elastase as tested on resorufin-labelled casein and the synthetic substrate Boc-alanine-*p*-nitrophenyl ester, respectively. To estimate the amounts of PR3 and elastase which could be released by activated PMN, 10^7 isolated PMN from six normal donors were pretreated with cytochalasin B and stimulated with 10^{-7} M fMLP. From Table 2 it can be seen that under these conditions ≈ 3 times more PR3 than elastase was released extra-cellularly, as quantified by ELISA.

Localization of PR3 and elastase
Infused PR3 and elastase were localized for only a short period in the glomeruli. Ten minutes after perfusion no glomeruli stained
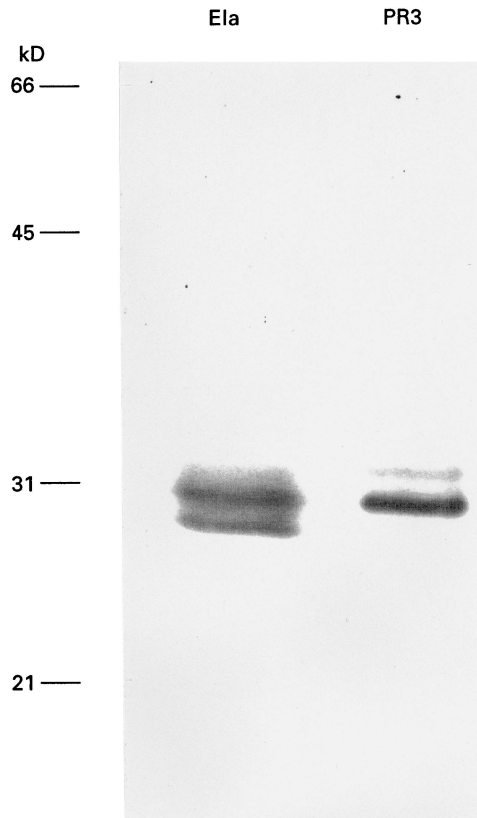
Table 1. Cleavage of Boc-alanine-*p*-nitrophenyl ester and casein by PR3 and elastate

Enzyme	Boc-alanine-*p*-nitrophenyl (mU/mg protein)*	Casein resorufin-labelled (%)†
PR3	1086	21 ± 3
Elastase	5140	52 ± 6

*Cleavage of Boc-alanine-*p*-nitrophenyl ester is expressed in milli-units, where 1 unit is defined as the amount of protein which cleaves 1 μmol of substrate in 1 min at 22°C.

† Proteolytic activity on resorufin-labelled casein is expressed as a mean percentage of total hydrolysis by pronase. Values represent means ± s.e.m. of three separate experiments done in triplicate

positive for elastase and PR3, as detected by IIF. However, strong staining was observed in tubular epithelial cells (Fig. 2). Twenty-four hours after perfusion elastase or PR3 were detected no more in glomeruli or in tubuli.

Proteinuria after renal perfusion of elastase and PR3
Renal perfusion of 50 μg active elastase induced severe proteinuria 24 h after perfusion (Table 3). The degree of elastase-induced proteinuria was dose-dependent. Proteinuria was not observed in rats perfused with 50 μg of inactivated elastase or PBS. Further-more, perfusion of active PR3 did not result in proteinuria at all doses studied. Further characterization of elastase-induced protei-nuria by SDS–PAGE revealed that most urinary proteins were low molecular weight proteins (Fig. 3).

Morphology
Twenty-four hours after perfusion, samples from left and right kidneys were taken and studied morphologically by light and electron microscopy. By light microscopy, no morphological abnormalities such as disruption of the GBM or infiltration of PMN and monocytes were seen in any group. In some elastase-perfused rats protein absorption droplets were observed in tubular epithelial cells. In addition, electron microscopy did not reveal glomerular damage in any group. No detachment or lysis of glomerular endothelial cells or fusion of epithelial foot processes were noted.

Fig. 1. Migration of PR3 on a 12% SDS–PAGE gel. For comparison, migration of elastase is also shown (Ela).

Table 2. Release of PR3 and elasstase from normal donor granulocytes afer stimulation with 10^{-7} M fMLP as measured by antigen capture ELISA

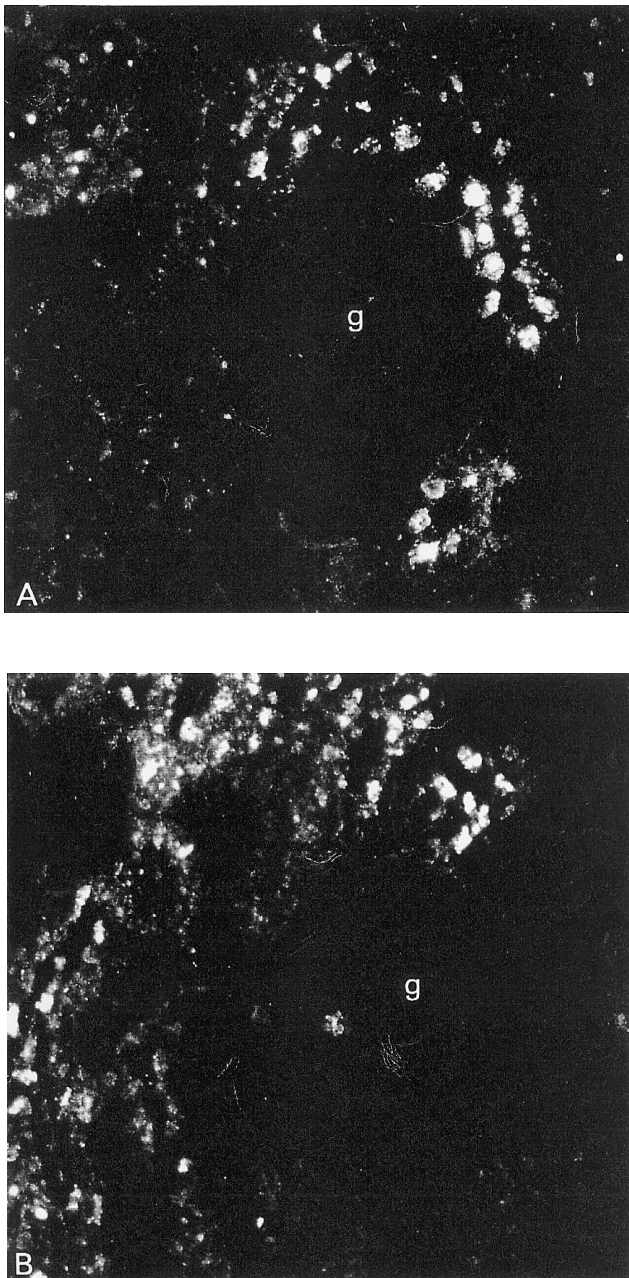| Donor	PR3	Elastase
1	12	4·5
2	10	4·7
3	7·7	3·2
4	14·5	3·3
5	10·3	3·1
6	8·8	2·4
Mean	10·6 ± 2·2	3·5 ± 0·8

Values represent μg ± s.e.m. per 10^7 PMN.

Fig. 2. Renal localization of elastase and PR3 10 min after unilateral perfusion of the left kidney as detected by indirect immunofluorescence. Localization of elastase (A) and PR3 (B) in tubular epithelial cells. g, Glomerulus. (Original mag. × 400.)

Glomerular staining of GBM components
GBM staining of HSPG and HS was studied in kidney sections from all groups by IIF using a MoAb directed against HS (MoAb JM-403) and goat antiserum directed against the core protein of HSPG (Table 3). In kidney sections of PBS-perfused rats strong staining of the GBM and Bowman's capsule in a fine granular to linear pattern was observed with MoAb JM-403 (Fig. 4A). Essentially the same staining pattern was obtained with polyclonal antiserum against the HSPG core protein (Fig. 4B). Furthermore, the pattern and intensity of staining was not different from that in right, non-perfused, kidney sections for both antibodies. However, after perfusion of 50 μg active elastase, staining for HS with MoAb JM-403 was almost completely absent in the glomeruli (Fig. 4E). In some cases staining of Bowman's capsule was still positive. In these sections expression of HSPG core protein was also reduced compared with sections from the right non-perfused kidney and PBS-infused rats, although to a lesser extent than for HS (Fig. 4F). Infusion of lower doses of active elastase also resulted in a reduction in staining for HS along the GBM, but no apparent decrease in expression of HSPG core protein was noted (not shown). In contrast to the elastase-perfused kidneys, no decrease in glomerular HS or HSPG core protein expression was observed after infusion of 50 μg inactivated elastase nor of active PR3 at any dose studied (Fig. 4C,D).

In addition to HS and HSPG core protein, glomerular expression of S-laminin, fibronectin, and collagen type IV was studied by IIF. In kidney sections of PBS-perfused rats a linear staining along the GBM together with a prominent mesangial staining was obtained with the mouse antibody against S-laminin. No differences in staining intensity were observed in kidney sections from rats perfused with active elastase and all other groups. Furthermore, for fibronectin and collagen type IV also no changes in expression were observed in all groups (not shown).

Effects of PR3 and elastase on HSPG in vitro
The *in vivo* results led us to investigate HSPG degradation by elastase and PR3 *in vitro*. As shown in Fig. 5a, incubation of EHS–HSPG coated to microtitre plates with active PR3 or elastase resulted in a dose-dependent loss of HS as detected with MoAb JM-403 for both enzymes. The loss of anti-HS reactivity could be prevented when the enzymes were inactivated. However, at lower concentrations elastase seemed to be more active than PR3 in degrading HSPG. In order to assess whether this difference was due to a lower degree of binding of PR3 to HSPG, we incubated microtitre plates coated with EHS–HSPG with inactivated PR3 and elastase and detected the amount of enzyme binding to HSPG. As shown in Fig. 5b, PR3 seems to bind less strongly to HSPG than elastase.

DISCUSSION

Activation of PMN adherent to the glomerular capillary wall leads to the production of reactive oxygen species and the release of their granule constituents, including cationic neutral serine proteinases. At the site of degranulation high concentrations of these proteolytic enzymes may be reached which may cause glomerular injury by proteolytic cleavage of GBM constituents and lysis or detachment of glomerular endothelial cells. In the present study we evaluated the effects of the human PMN-derived neutral serine proteinases elastase and PR3 on the induction of proteinuria and the degradation of GBM components after *in vivo* renal perfusion. We showed that infusion of μg quantities of active elastase induced a dose-dependent proteinuria 24 h after perfusion, while inactivated equally cationic elastase did not increase the urinary excretion of proteins, demonstrating that the proteolytic activity of elastase was responsible for this effect. These results confirm the data of Johnson *et al.*, who were the first to show the ability of PMN-derived neutral serine proteinases to induce proteinuria after renal perfusion [6].

In contrast, we further showed that rats perfused with highly purified active PR3 in concentrations up to 300 μg did not develop

Table 3. Urinary protein excretion 24 h and glomerular staining of heparan sulphate (HS) side chain and heparan sulphate core protein after renal perfusion of elastase and PR3

Perfusate	Concentration (μg/0·5 ml)	Activity (mU)	Proteinuria (mg/24 h)	HS	HSPG
Elastase	50 (6)	257	147·3 ± 35·6*	±	++
	25 (3)	129	38·4 ± 4·2*	+	+++
	15 (3)	77	12·6 ± 2·2*	++	+++
Elastase inactivated	50 (4)	0	1·5 ± 0·5	+++	+++
PR3	300 (4)	326	1·3 ± 0·2	+++	+++
	100 (3)	109	1·8 ± 0·3	+++	+++
	50 (3)	54	1·7 ± 0·3	+++	+++
PBS	(4)		3·4 ± 1·9	+++	+++

Values represent mean ± s.e.m.

* $P < 0.01$ compared with PBS perfusion.

Rats were killed 24 h after perfusion. Intraglomerular expression of HS side chain and the core protein of HS proteoglycan (PG) was estimated by indirect immunofluorescence using MoAb JM-403 (HS side chain) and a polyclonal goat antibody directed against the core protein of HSPG. Labelling intensity was estimated on a semiquantitative scale (3+): −, absent; ±, weak; +, mild; ++, intermediate; +++, strong.

proteinuria. The inability of PR3 to induce proteinuria might have been due to perfusion of too low amounts of active enzyme in comparison with elastase. As shown, *in vitro* activation of PMN results in the release of ≈ 3 times more PR3 than elastase. In our perfusion experiments, however, perfusion of 300 μg PR3 (i.e. 20 times more than the lowest dose of elastase inducing proteinuria) did not result in proteinuria. More importantly, the total proteolytic activity of the perfused PR3 preparation was comparable to that of elastase at the different dosages studied. So, it seems unlikely that the absence of proteinuria was due to unsufficient amounts of

active PR3 in comparison with elastase. Recently, Johnson *et al.* also mentioned in their review that infusion of 50 μg active PR3 did not result in proteinuria [25]. However, in their hands an additional rat perfused with 385 μg PR3 developed severe proteinuria without any histological evidence of glomerular injury.

Besides their ability to degrade extracellular matrix proteins, several studies have shown that neutral serine proteases can also injure endothelial cells [26]. Recently, a difference was noted between PR3 and elastase in their effect on cultured endothelial cells, showing that both elastase and PR3 caused detachment of endothelial cells, while PR3 also induced cytolysis [27]. Despite the severe proteinuria induced after perfusion of active elastase, we found no evidence for glomerular endothelial cell injury by light and electron microscopy, which observation also corroborates the findings of Johnson *et al.* [6].

The absence of cellular injury suggests that degradation of glomerular basement components is involved in elastase-induced proteinuria. As mentioned before, elastase is highly cationic at neutral pH, and binding to the GBM probably occurs by charge interaction with anionic sites present in the GBM. Heparan sulphate, the anionic side chains of HSPG, is recognized as one of the major determinants of this negative charge and is a main determinant of the charge-dependent selectivity of the GBM. In this study we showed that proteinuria occurring after renal perfusion of 50 μg elastase was associated with a strong decrease of HS expression as estimated by IIF, while staining for HSPG core protein was also decreased, but to a lesser extent. Infusion of lower amounts of elastase also reduced staining for HS, while no
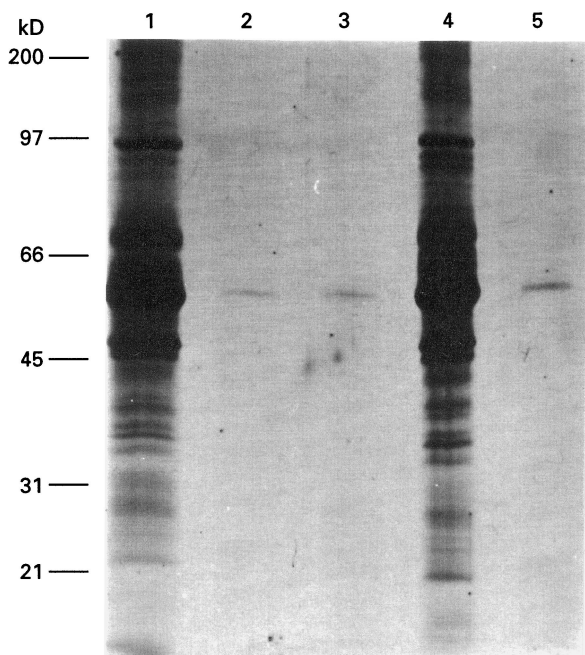

Fig. 3. Characterization of 24 h proteinuria by 12% SDS–PAGE after perfusion of 50 μg elastase (lanes 1 and 4), 50 μg inactivated elastase (lane 2), 300 μg PR3 (lane 3) and PBS (lane 5).

Fig. 4. Glomerular expression of heparan sulphate (HS) (A,C,E) and HS proteoglycan (PG) core protein (B,D,F) by indirect immunofluorescence double-staining 24 h after perfusion. Strong staining of the glomerular basement membrane (GBM) is shown by both antibodies in kidney sections of PBS- (A,B, PBS) and PR3- (C,D, proteinase 300 μg) perfused rats. After perfusion of 50 μg active elastase staining for HS (E) was almost completely absent, while staining for HSPG core protein was somewhat reduced (F). (Original mag.: A,B, × 250; C–F, × 400.)
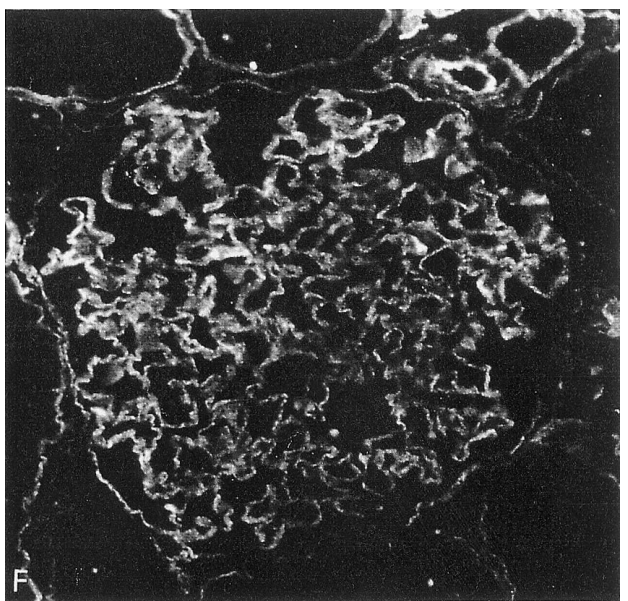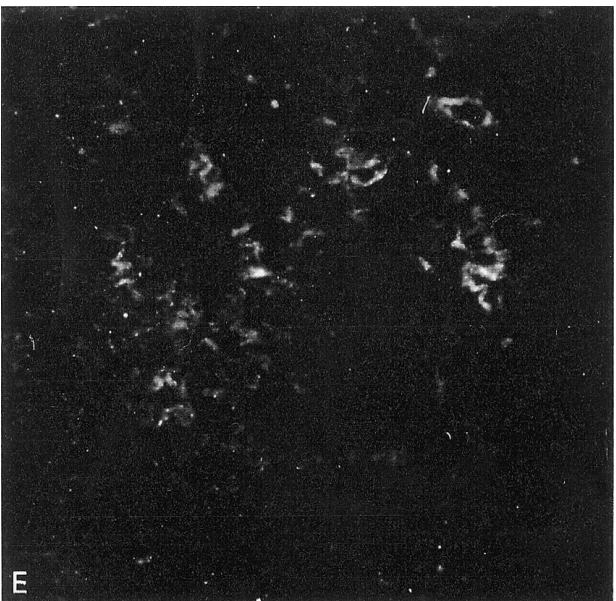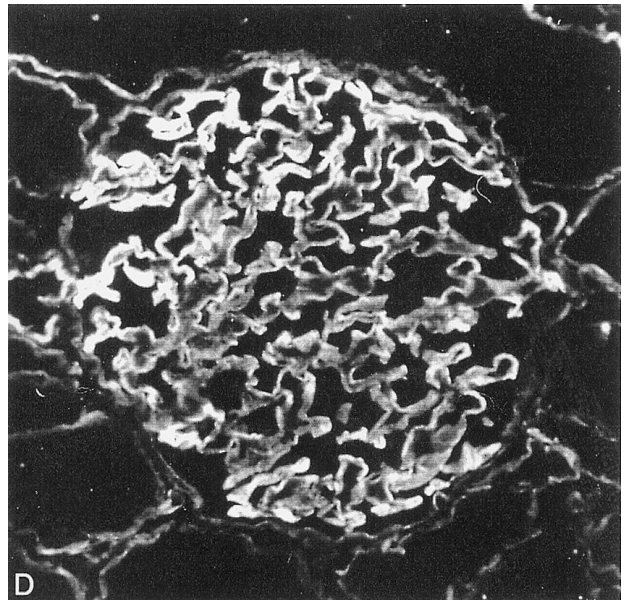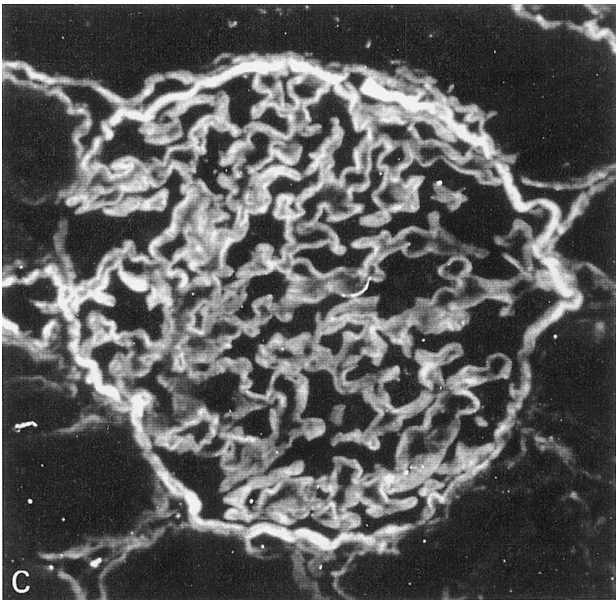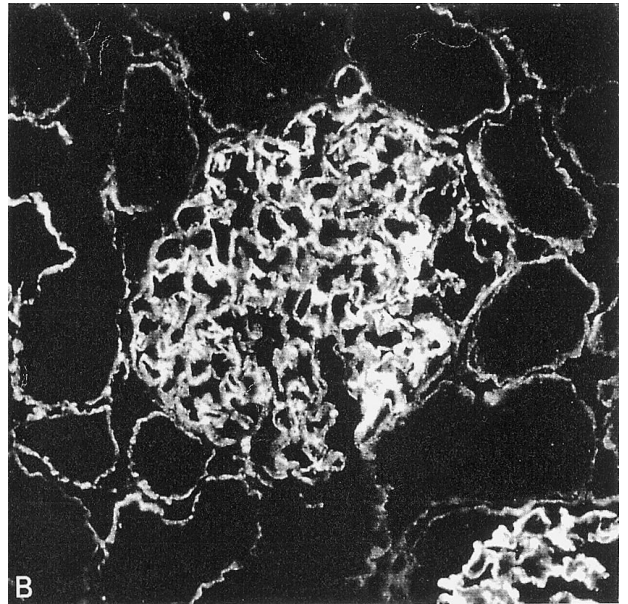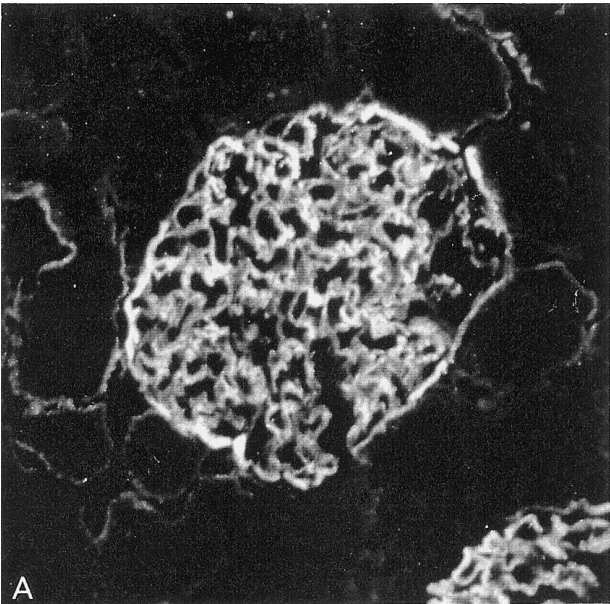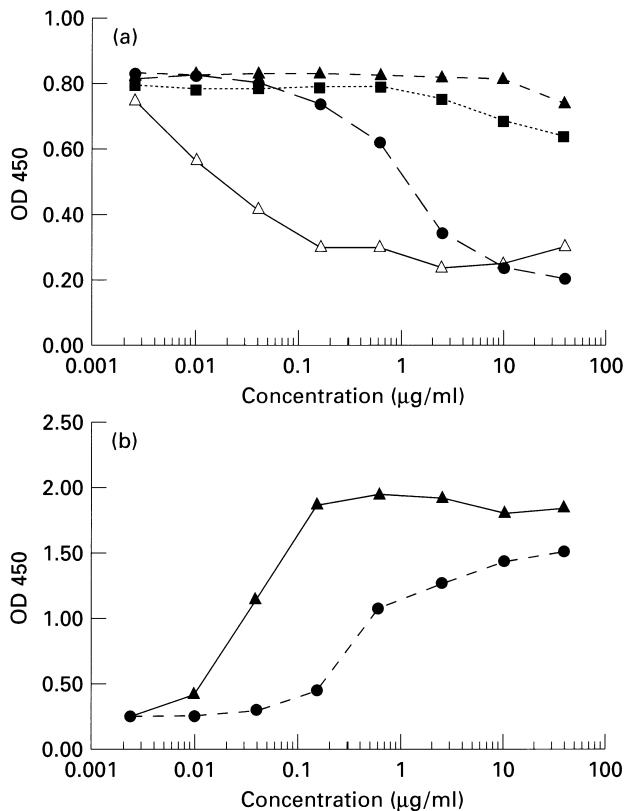
Fig. 5. (a) *In vitro* degradation of EHS–heparan sulphate proteoglycan (HSPG) by elastase and PR3 as demonstrated by ELISA. Anti-HS reactivity as detected with MoAb JM-403 is lost after incubation with active elastase (△) and PR3 (●). Elastase seems to be more active than PR3. No decrease in anti-HS reactivity is seen after inactivation of PR3 (■) and elastase (▲). (b) Binding of inactivated elastase (▲) and PR3 (●) to EHS–HSPG as measured by ELISA. Elastase seems to bind more strongly than PR3.

apparent decrease in staining for HSPG core protein was seen at those concentrations. One explanation might be that elastase cleaves the HSPG molecule close to the NH_2 terminus. Recently, it has been shown that the HS side chains are attached to the core protein very close to the NH_2 terminus [28]. Therefore, enzymatic removal of (a part of) the NH_2 terminus leads to a complete loss of HS side chains, whereas the major portion of the core protein remains in the GBM. Moreover, Klebanoff *et al.* showed that elastase-induced degradation of subendothelial cell matrix HSPG *in vitro* generates several HSPG fragments of different size [14]. They further found that the smaller fragments are preferentially released in the supernatant, while larger fragments remain bound to the matrix. This might, additionally, explain why in our study HSPG core protein still stained strongly positive after elastase perfusion. Several other studies have indicated a prominent role for neutrophil serine proteinases, especially elastase, in the degradation of HSPG [29–31]. In this respect, an interesting study was done by Kowanko *et al.*, who showed that neutrophil-mediated degradation of cartilage proteoglycan was primarily induced by elastase and cathepsin G, with only a minor role for PR3 [31].

The inability of PR3 to induce proteinuria was somewhat surprising, since *in vitro* studies have shown that PR3 is similar to the other PMN-derived neutral serine proteinases elastase and cathepsin G in exhibiting a broad substrate specificity towards

GBM components [8,12]. Our *in vitro* data also show that PR3, like elastase, is capable of digesting HSPG, although to a lesser extent. The differences between elastase and PR3 in their ability to cleave HSPG and inducing proteinuria *in vivo* may be explained by differences in charge between these enzymes. Studies by Vogt and colleagues have shown that glomerular binding of cationic molecules is dependent on charge and size of the molecules [32]. In their studies intravenously injected cationized ovalbumin, a small molecule of 40 kD, only bound to the GBM when its isoelectric point was > 10. PR3 and elastase are both small molecules, but differ significantly in their isoelectric point (9·1 for PR3 *versus* > 11 for elastase). Therefore, binding of elastase to negatively charged molecules within the GBM will be much stronger than for PR3. This was also shown by our *in vitro* studies comparing the binding of elastase and PR3 with EHS–HSPG, although the binding characteristics of the detecting antibodies may influence the results to some extent. *In vivo*, the binding of PR3 to negatively charged molecules is probably too weak to cause any damage. Because of its low molecular weight, PR3 will then be rapidly filtrated.

GBM components other than HSPG are also susceptible to proteolytic attack by elastase *in vitro* [1]. We therefore wondered whether the digestion of HSPG by elastase was selective for this protein. Earlier studies have shown that the enzymatic removal of HS from the GBM increases the permeability of the GBM for positively charged, low molecular weight proteins like albumin and native ferritin [33,34]. Furthermore, i.v. injection of the anti-HS MoAb used in this study was shown to induce an acute selective proteinuria [18]. Analysis of the elastase-induced proteinuria in this study by SDS–PAGE showed the predominance of lower molecular weight proteins. In addition, by IIF we found no decrease or alteration in expression of S-laminin, fibronectin or collagen type IV compared with control perfused rats. These observations may be explained by the preferential binding of elastase to the negatively charged HSPG. Once bound, elastase will cleave HSPG immediately, resulting in loss of the HS side chains. Cleavage products of HSPG, but also elastase itself, will then readily be filtrated without digesting other GBM components. In addition, loss of negatively charged HSPG leads to proteinuria.

In conclusion, *in vivo* perfusion of μg quantities of active elastase induced a dose-dependent proteinuria, while infusion of active PR3 did not. The elastase-induced proteinuria was associated with a strong reduction of glomerular HS expression and, to a lesser extent, of HSPG core protein. These studies indicate that proteolytic cleavage of HSPG by elastase may be a contributing factor to glomerular injury in PMN-dependent glomerulonephritis, and suggest a minor role for PR3.

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