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Degradation of glomerular basement membrane by purified mammalian metalloproteinases

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Neutral metalloproteinases degrade components of the extracellular matrix, including collagen types I–V, fibronectin, laminin and proteoglycan. However, their ability to degrade intact glomerular basement membrane (GBM) has not previously been investigated. Incubation of [^{3}H]GBM (50000 c.p.m.; pH 7.5; 24 h at 37 °C) with purified gelatinase or stromelysin (2 units) resulted in significant GBM degradation: gelatinase, 46 ± 2.2; stromelysin, 59 ± 5.8 (means $\pm$ S.E.M.; percentage release of non-sedimentable radioactivity; $n = 4$). In contrast, 2 units of collagenase released only $5.6 \pm 0.52\%$ ($n = 3$) of the [^{3}H]GBM radioactivity compared with $2.0 \pm 0.15\%$ ($n = 7$) released from [^{3}H]GBM incubated alone. Sephadex G-200 gel chromatography of supernatants obtained from incubations of [^{3}H]GBM with either gelatinase or stromelysin confirmed the ability of these enzymes to degrade GBM and revealed both high-(800000) and relatively low-(< 20000) M_r degradation products for both enzymes. GBM degradation by gelatinase and stromelysin was dose-dependent (range 0.02–2.0 units), near maximal between pH 6.0 and 8.6, and was completely inhibited (> 95%) by 2 mM-*o*-phenanthroline. Collagenase (2 units) did not enhance the degradation of GBM by either gelatinase (0.02 or 0.2 unit) or stromelysin (0.02 or 0.2 unit). Our results indicate that metalloproteinase-mediated GBM degradation by neutrophils and glomeruli may be attributable to gelatinase (neutrophils) and/or stromelysin (glomeruli) and suggest an important role for these proteinases in glomerular pathophysiology.

INTRODUCTION

The glomerular basement membrane (GBM), composed primarily of type IV collagen, laminin, heparan sulphate proteoglycan and fibronectin is the major ultrafiltration barrier of the glomerulus, restricting the passage of plasma proteins into the urinary space. Proteinuria, a major manifestation of glomerular disease, results from an increase in the permeability of the GBM to albumin and other plasma proteins. The specific mechanisms responsible for the increase in GBM permeability characteristic of glomerular disease are unknown. It has been suggested that proteinuria may result from GBM degradation by proteinases released from infiltrating neurophils (Hawkins & Cochrane, 1968; Sanders *et al.*, 1978; Holdsworth *et al.*, 1981; Shah *et al.*, 1987) or glomerular cells (Lovett *et al.*, 1983; Nguyen *et al.*, 1986). Collagenase, gelatinase and stromelysin are three well-characterized metalloproteinases which have been shown to degrade components of the extracellular matrix, including collagen, laminin, fibronectin and proteoglycan (Murphy *et al.*, 1982; Galloway *et al.*, 1983; Chin *et al.*, 1985; Okada *et al.*, 1986). Collagenase and gelatinase are well characterized metalloproteinases present in neutrophils (Murphy *et al.*, 1982; Hibbs *et al.*, 1985; Hasty *et al.*, 1987). Metalloproteinases have also been described in rat glomeruli (Nguyen *et al.*, 1986) and cultured glomerular mesangial cells (Lovett *et al.*, 1983). However, the ability of collagenase, gelatinase and stromelysin to degrade intact GBM and their potential role in causing proteinuria has not been previously investigated. In the present study, we have examined the ability of highly purified mammalian collagenase, gelatinase and stromelysin to degrade intact GBM *in vitro*.

A preliminary account of this work was presented at the Twentieth Annual Meeting of the American Society of Nephrology held in Washington, DC, U.S.A., 13–16 December, 1987.

EXPERIMENTAL

Materials

[^{3}H]Acetic anhydride (50 mCi/mmol) was obtained from New England Nuclear; bacterial collagenase (type VII), cytochrome *c* (horse heart, type VI), catalase (bovine liver) and bovine serum albumin were obtained from Sigma. Sephadex G-200 and Blue Dextran 2000 were obtained from Pharmacia. All other chemicals were reagent grade or higher.

Isolation of GBM

GBM was isolated from bovine kidneys by the method of Blau & Michael (1971) as described in detail in our previous paper (Shah *et al.*, 1987). We have shown that GBM prepared by this method is essentially free from interstitial and denatured collagen as determined by SDS/polyacrylamide-gel electrophoresis, lack of digestion by thrombin and electron microscopy (Shah *et al.*, 1987).

Abbreviations used: GBM, glomerular basement membrane; PMA, phorbol myristate acetate.

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Labelling of GBM

[^{3}H]GBM was prepared as described by Cawston & Barrett (1979), with minor modifications. The GBM (120 mg of protein) was suspended in 50 ml of 25 mM-$Na_2B_4O_7$ buffer, pH 9.5, containing 400 mM-$CaCl_2$, and 12.5 mCi of [^{3}H]acetic anhydride (in dry benzene) added in three portions over 1 h. At 30 min after the last addition, the GBM was collected by centrifugation (1450 *g*, 15 min, 4 °C) and washed (by repeated suspension and centrifugation) three times with 1M-NaCl, followed by several washes with distilled water. The final preparation had a specific radioactivity of 8.6×10^5 c.p.m./mg of protein. Radioactivity in the supernatant (1450 *g*, 15 min) after the last wash was less than 2% of that in the pellet. After incubation of the [^{3}H]GBM (approx. 50000 c.p.m.) with 10 μg of bacterial collagenase (0.1 M-Tris/HCl, pH 8.0, with 0.35 mM-$CaCl_2$) for 1 h, all of the radioactivity could be recovered in the 10000 *g* supernatant.

GBM degradation by purified metalloproteinases

GBM degradation was measured as the release of non-sedimentable radioactivity after incubation of purified enzyme with [^{3}H]GBM. GBM (approx. 50000 c.p.m.) was incubated with various amounts of each purified metalloproteinase in 400 μl (final volume) of buffer (see below). Incubations were carried out for 24 h at 37 °C in a Dubnoff shaking water path. Incubations were terminated by centrifuging each tube at 10000 *g* for 10 min. A 375 μl portion of each supernatant was carefully removed, mixed with 10 ml of Aquasol, and counted (Beckman liquid scintillation counter; model LS 3133P) for radioactivity. Results are expressed as percentage radioactivity release (mean ± S.E.M., three determinations). Unless specified otherwise, the following buffers were used: 50 mM-Tris/HCl, pH 7.6, containing 200 mM-NaCl and 5 mM-$CaCl_2$ for collagenase and gelatinase, and 100 mM-Tris/HCl, pH 7.6, containing 30 mM-$CaCl_2$, for stromelysin.

Gel chromatography of [^{3}H]GBM degradation products

Sephadex G-200 gel chromatography was carried out on supernatants (350 μl) obtained from [^{3}H]GBM (approx. 100000 c.p.m.) incubated with gelatinase or stromelysin or from [^{3}H]GBM incubated alone. The column (1.0 cm × 48 cm) was equilibrated with 0.2 M-Tris/HCl, pH 7.5, containing 0.02% azide, and calibrated with a mixture of Blue Dextran 2000 (M_r 2×10^6), catalase (M_r 250000), bovine serum albumin (M_r 66000) and cytochrome *c* (M_r 12400). The column was eluted with the same buffer (flow rate 8–9 ml/h; 0.9 ml/fraction). Each fraction was quantitatively transferred to a scintillation vial containing 10 ml of Aquasol and counted for radioactivity as described above.

Purification of metalloproteinases

Stromelysin was purified from rabbit calvariae culture medium as previously described (Galloway *et al.*, 1983). The purified stromelysin was homogeneous as determined by SDS/polyacrylamide-gel electrophoresis and autoradiography after labelling with ^{125}I and had a specific activity of 2400 μg of casein degraded/min per mg of enzyme protein. Gelatinase was purified from human polymorphonuclear leucocytes as previously described (Murphy *et al.*, 1982). The purified gelatinase was homogeneous as determined by Ultrogel AcA 34 chromatography after ^{125}I-labelling and SDS/polyacrylamide-gel electrophoresis and had a specific activity of 13000 units/mg of enzyme protein. Collagenase was purified from human gingivial fibroblasts as previously described (Cawston & Murphy, 1981). The purified collagenase (containing a minor impurity) had a specific activity of 2667 units/mg of enzyme protein.

Table 1. Degradation of GBM by purified metalloproteinases

[^{3}H]GBM was incubated in 50 mM-Tris/HCl, pH 7.5, containing 200 mM-NaCl and 5 mM-$CaCl_2$ (collagenase and gelatinase) or 30 mM-$CaCl_2$ (stromelysin) for 24 h at 37 °C. Results are expressed as means ± S.E.M. for *n* separate determinations carried out in triplicate.

Addition	Amount (units)	GBM degradation (% release) (*n*)
None		2.0 ± 0.15 (7)
Collagenase	2.00	5.6 ± 0.52 (3)
Gelatinase	0.02	7.3 ± 0.15 (3)
	0.20	35.8 ± 0.53 (3)
	2.00	45.7 ± 2.20 (4)
Stromelysin	0.02	2.9 ± 0.11 (3)
	0.20	16.2 ± 0.24 (3)
	2.00	59.2 ± 5.85 (4)

Other methods

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

RESULTS AND DISCUSSION

It is well established that metalloproteinases from different species and tissues have similar catalytic functions; that is, they degrade the same substrates, despite potential differences in immunological, kinetic and other biochemical properties. In the present study we have examined the ability of purified mammalian metalloproteinases to degrade intact GBM. As shown in Table 1, incubation of [^{3}H]GBM with purified gelatinase (2 units) or stromelysin (2 units) resulted in significant GBM degradation. In contrast, 2 units of collagenase released only 5.6 ± 52% ($n = 3$) of the [^{3}H]GBM radioactivity compared with 2.0 ± 0.15% ($n = 7$) released from [^{3}H]GBM incubated alone. GBM degradation by gelatinase and stromelysin was proportional to the amount of proteinase added (range 0.02–2.0 units; Table 1) and was completely inhibited (> 95%) by the presence of 2 mM-*o*-phenanthroline in the incubation mixture (results not shown). Sephadex G-200 gel chromatography (see the Experimental section) of supernatants obtained after incubation of [^{3}H]GBM with 2 units of either gelatinase or stromelysin revealed both high-(M_r > 800000) and relatively low-(M_r < 20000) M_r digestion products (Fig. 1).

These results are in keeping with previously published data concerning the ability of these metalloproteinases to degrade individual extracellular matrix components. For example, Murphy *et al.* (1982, 1985) and Hasty *et al.* (1987) have shown that purified mammalian collagenase degrades collagen types I, II and III, but has no activity against collagen types IV and V, which are present in

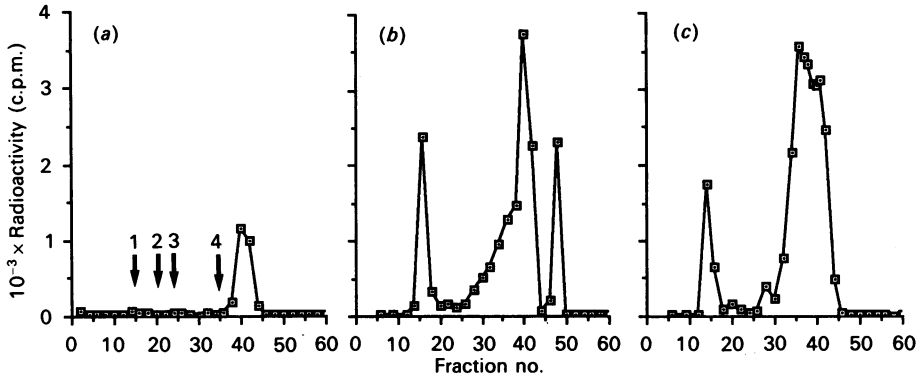

Fig. 1. Sephadex G-200 gel chromatography of supernatants obtained from [^{3}H]GBM incubated alone (*a*) or with 2 units of either stromelysin (*b*) or gelatinase (*c*)

Arrows in (*a*) indicate the elution positions of the molecular-mass markers Blue Dextran (1), catalase (2), bovine serum albumin (3) and cytochrome *c* (4). Further details are given in the Experimental section.

GBM. Gelatinase efficiently degrades gelatin (denatured interstitial collagen) and types IV and V collagens (Murphy *et al.*, 1982, 1985; Hibbs *et al.*, 1985), with significantly less activity towards elastin and proteoglycan (Murphy *et al.*, 1985). In contrast, stromelysin degrades a board range of extracellular-matrix components, including types IV and V collagens, as well as fibronectin, laminin, proteoglycan and elastin (Galloway *et al.*, 1983; Chin *et al.*, 1985; Okada *et al.*, 1986).

Murphy *et al.* (1982, 1985) reported that gelatinase and collagenase acted synergistically in the degradation of insoluble type IV collagen. Thus we examined the ability of collagenase to enhance the degradation of GBM by gelatinase and stromelysin. Collagenase (2 units) did not significantly enhance GBM degradation by either gelatinase (0.02 or 0.2 unit) or stromelysin (0.02 or 0.2 unit).

The extracellular location of the GBM suggests that at least the initial stages of GBM degradation must take place at the relatively neutral pH of the extracellular fluids. Thus it was of interest to examine the effect of pH on GBM degradation by gelatinase and stromelysin. As shown in Fig. 2, both gelatinase and stromelysin degraded GBM at near-maximal rates over a broad pH range from 6.0 to 8.6. These results clearly demonstrate that gelatinase and stromelysin can effectively degrade intact GBM in the pH range likely to be encountered in the glomerular capillaries *in vivo*.

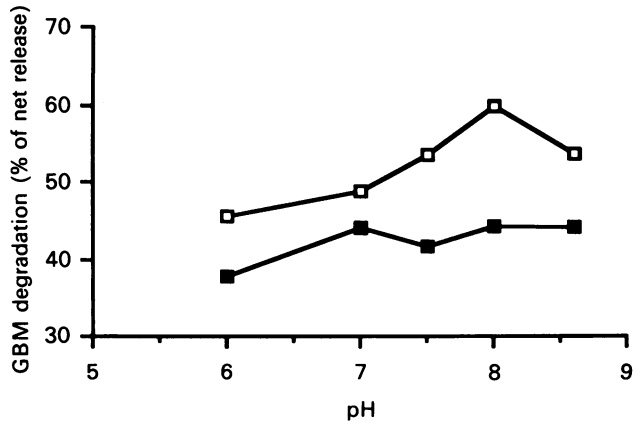

Fig. 2. Effect of pH on GBM degradation by purified gelatinase (■) and stromelysin (□)

[^{3}H]GBM was incubated in 50 mM-Tris/maleate buffer (containing 200 mM-Nacl and 5 mM-$CaCl_2$ for gelatinase, or 30 mM-$CaCl_2$ for stromelysin) at the pH indicated with 2 units of gelatinase or stromelysin. Results are expressed as the mean percentage release of radioactivity for triplicate incubations corrected for the appropriate blank (GBM alone incubated at each pH in the appropriate buffer).

The critical role of neutrophils in mediating glomerular injury has been established by demonstrating that selective depletion of neutrophils (Hawkins & Cochrane, 1968; Couser *et al.*, 1977) or monocytes (Holdsworth *et al.*, 1981) results in a marked reduction in proteinuria in experimental models of glomerular disease. This observation, coupled with our previous finding that PMA-stimulated neutrophils release a GBM-degrading metalloproteinase (Shah *et al.*, 1987) and the apparent lack of stromelysin in neutrophils, suggests that GBM degradation by the PMA-stimulated neutrophils was probably mediated by gelatinase.

GBM-degradation metalloproteinases have been described in glomeruli (Nguyen *et al.*, 1986) as well as in cultured glomerular mesangial cells (Lovett *et al.*, 1983), the latter having been attributed to gelatinase (Martin *et al.*, 1986). However, on the basis of the results presented here, the possibility that stromelysin may contribute to this activity must now be considered. In addition, the presence of stromelysin in fibroblasts (Chin *et al.*, 1985; Okada *et al.*, 1986) and the involvement of fibroblasts in crescentic types of glomerular disease (Glassock *et al.*, 1986) suggest a potential role for stromelysin in these types of glomerulonephritidies.

Taken together, these results suggest a potentially important role for gelatinase and/or stromelysin in glomerular pathophysiology. The recent report of Davin *et al.* (1987) concerning increased excretion of neutral metalloproteinase activity in the urine of rats with experimentally induced glomerular disease lends support to this suggestion.

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