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Research Article

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Degradation of Human Glomerular Basement Membrane by Stimulated Neutrophils

Activation of a Metalloproteinase(s) by Reactive Oxygen Metabolites

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Abstract

We examined the role of reactive oxygen metabolites in the degradation of human glomerular basement membrane (GBM) by stimulated human neutrophils. Neutrophils stimulated with phorbol myristate acetate (PMA) caused a significant degradation of GBM over 3 h resulting in 11.4±0.9% (SEM), $n = 11$ release of hydroxyproline compared with 0.3±0.09%, $n = 11$ release by unstimulated neutrophils. Superoxide dismutase, a scavenger of superoxide, did not inhibit the GBM degradation, whereas catalase, a scavenger of hydrogen peroxide, caused a marked inhibition (−60±7%, $n = 4$, $P < 0.001$) of hydroxyproline release. Neither alpha-1 proteinase inhibitor, an inhibitor of elastase, nor soya bean trypsin inhibitor, an inhibitor of cathepsin G, caused any significant inhibition of GBM degradation. GBM degradation by cell-free supernatants obtained from stimulated neutrophils was markedly impaired in the presence of metal chelators EDTA (−72±7, $n = 6$, $P < 0.001$) and 1,10,phenanthroline (−85±5%, $n = 3$, $P < 0.001$). Considering these results, we postulated that reactive oxygen metabolites generated by the stimulated neutrophils activate a latent GBM degrading metalloproteinase(s). GBM degradation by supernatants obtained from incubations with catalase, azide, an inhibitor of myeloperoxidase, and methionine and taurine, scavengers of hypochlorous acid, was markedly reduced. Our data thus indicate that degradation of the GBM by PMA-stimulated neutrophils is due to activation of a latent metalloproteinase by hypochlorous acid or a similar oxidant generated by the myeloperoxidase–hydrogen peroxide–halide system.

Introduction

Several human and experimental glomerulonephritides are characterized by the infiltration of glomeruli by neutrophils and/or monocytes (1, 2). The critical role played by leukocytes in mediating glomerular injury has been delineated by demonstrating that selective depletion of neutrophils (3) or monocytes (4) results in a marked reduction in proteinuria. It is generally accepted that leukocytes cause proteinuria by damaging the glomerular basement membrane (GBM),[1] which serves as the major ultrafiltration barrier to restrict the entry of proteins into the urinary space. Indeed, increased excretion of GBM fragments in urine has been observed in a variety of human and experimental glomerular diseases (1, 3, 5). Apparently even subtle damage to the GBM, composed primarily of type IV collagen, noncollagenous glycoproteins, and glycosaminoglycans, is sufficient to cause marked alteration in its function (3).

The manner in which leukocytes cause damage to the GBM in glomerulonephritis has been attributed to the release of lysosomal enzymes (1, 3, 5), in particular neutral proteinases (1, 5). For example, elastase and cathepsin G obtained from neutrophil homogenates were shown to degrade GBM (6). Similarly, a serine proteinase inhibitor inhibited degradation of GBM by intact stimulated neutrophils (7).

In response to plasma membrane perturbation by a variety of soluble and particulate stimuli, leukocytes not only release enzymes but also exhibit a burst of oxidative metabolism (8, 9) leading to the generation of several potentially toxic reactive oxygen metabolites (ROM). The primary species generated are the univalent and divalent reduction products of oxygen, superoxide, and hydrogen peroxide. These species may then participate in the formation of additional ROM, including the hydroxyl radical and hypochlorous acid by the hydrogen peroxide–myeloperoxidase–halide system. Whereas several studies have suggested an important role for proteolytic enzymes in GBM degradation, the role of ROM in GBM degradation has not been previously demonstrated.

Several lines of evidence suggest that the ROM generated by the stimulated leukocytes may either be required for or enhance the damage to GBM by neutral proteinases. Recent studies show that alpha-1 proteinase inhibitor (α-1-PI), the primary regulator of neutrophil elastase, can be inactivated by ROM generated by human neutrophils (10, 11), thus allowing the released elastase to degrade the extracellular matrix more readily (11). In addition, ROM may be involved in the activation of latent proteolytic enzymes (12, 13). For example, it was recently shown that triggered neutrophils release and simultaneously activate their latent collagenase (12), a metalloproteinase. The activation of the latent enzyme required the generation of hypochlorous acid, a highly reactive oxygen metabolite. Gelatinase, another collagen-degrading metalloproteinase also released by stimulated neutrophils (14), has several similarities to collagenase (15) and is thus likely to be activated by similar mechanisms. Taken to-

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1. *Abbreviations used in this paper:* α-1-PI, alpha-1 proteinase inhibitor; APMA, 4-aminophenylmercuric acid; GBM, glomerular basement membrane; PMA, phorbol myristate acetate; ROM, reactive oxygen metabolite; SBTI, soya bean trypsin inhibitor; SOD, superoxide dismutase.

gether, these studies suggest a potential role for ROM in GBM degradation.

The purpose of the present study was to examine the role of ROM in the degradation of human GBM by stimulated human neutrophils. Phorbol myristate acetate (PMA), a soluble stimulus, was chosen as the agent to trigger the neutrophils because it is a potent stimulus for both the generation of ROM (9) and for the release of collagenase (12) and gelatinase (14, 16). In addition, in a recent study the role of ROM in degradation of endothelial cell basement membrane by intact neutrophils was demonstrated with PMA as the stimulus (11).

Methods

Preparation of glomerular basement membrane. Human GBM was prepared from glomeruli isolated from kidneys obtained at autopsy and immediately stored at −20°C. The kidneys were slowly thawed at 4°C overnight, and the cortex carefully separated. All subsequent preparative steps were carried out at 4°C. Glomeruli were isolated by a combination of differential centrifugation and sieving as described in our previous study (17). The cortices were minced with a blade and gently squeezed through a stainless steel sieve (250-μm opening) using a metal spatula. The resulting suspension of cortical tissue was washed four times in Hanks' balanced salt solution (HBSS) by repeated centrifugation at 200 *g* for 2 min to eliminate small fragments in the supernatant. The resultant pellet was resuspended in the buffer and passed sequentially through Nitex nylon sieves (Tetko Inc., Elmsford, NY) of 390-, 250-, and 180-μm pore openings. To obtain glomeruli, the suspension resulting after filtering through the 180-μm sieve was passed through a screen cloth (Nitex No. 25, 60-μm pore opening). This step allowed debris and small fragments to pass through, with glomeruli retained on the cloth. The retained glomeruli were recovered by gently rinsing with buffer. Glomeruli prepared in this way were well preserved, and the purity of preparation, evaluated by counting glomeruli under light microscopy, was ~90%.

GBM was prepared as described by Blau and Michael (18). Briefly, glomeruli were resuspended in 1 M NaCl and disrupted by repeated sonication (15 s, setting 5, microtip, cell disrupter, model W-375, Heath Systems–Ultrasonics, Inc., Farmingdale, NY) at 0–4°C, with 1 min cooling interval between bursts. Disruption was monitored microscopically. GBM were collected by centrifugation (121 *g*, 15 min), washed three times with 1 M NaCl (centrifugation at 121 *g*, 15 min after each wash) and then four times with distilled water (centrifugation at 8,000 *g*, 5 min after each wash). The GBM was resuspended in water and lyophilized. Amino acid analysis, (kindly performed by Dr. Miriam Blitzer, Department of Genetics, Tulane Medical School, New Orleans, LA) with an amino acid analyzer (model 6300, Beckman Instruments, Inc., Berkeley, CA), after hydrolysis under nitrogen at 105°C for 25 h with 6 N HCl was similar to previously published data (19). The hydroxyproline content (percent of dry weight) of the GBM was also as previously reported. To determine the total solubilizable hydroxyproline content, the GBM was incubated with 50 μg of bacterial collagenase for 24 h. This resulted in ~90% release of the total hydroxyproline content.

We also examined the GBM preparations for the presence of interstitial and denatured collagen. Electron microscopy of the GBM preparations (performed by Dr. Patrick Walker, Department of Pathology, Tulane Medical School) showed the presence of membranes that, like the GBM in situ, demonstrated little organization. Importantly, the preparation was free of recognizable cellular elements or striated material characteristic of interstitial collagen. We also estimated the combined type I and type III collagen present in the GBM preparations as follows. Pepsin digests of the GBM were made 0.7 M with NaCl in order to precipitate type I and III collagen (20, 21). Each precipitate was then dissolved in sodium dodecyl sulfate (SDS) sample buffer and subjected to SDS–polyacrylamide slab gel electrophoresis under reducing conditions. A known amount of purified type I collagen (obtained from pig aorta, courtesy of Drs. J. Heath and G. Murphy, Strangeways Research Laboratory, Cambridge, England) was run simultaneously with the solubilized precipitates. In this system (uninterrupted reduction) type I and type III collagen co-migrate. The gel was stained with Coomassie Brilliant Blue, and the area under the curve corresponding to a1 (type I and type III) and a2 (type I) bands was quantitated by scanning densitometry. Comparison of these values with those obtained with purified type I collagen allowed us to estimate the combined amount of type I and type III collagen in the samples. The results of these studies revealed that in two separate GBM preparations the amount type I and type III collagen together accounted for 0.9 and 0.8% of the dry weight of GBM. The amount of denatured collagen in the GBM preparations was estimated by examining the amount of GBM that could be degraded by α-thrombin, which degrades denatured but not native type IV collagen at 25°C (22, 23). We incubated the GBM preparation with 1.5, 3, and 6 U of α-thrombin (3,080 U/mg protein) at 25° and 37°C for 20 h. At 25°C thrombin released <1% hydroxyproline, even at the highest concentration tested. At 37°C the maximal release with the highest dose (6 U) was 2±0.07% (n = 3). Under the incubation conditions used for the bulk of experiments in the study (3 h incubation at 37°C), α-thrombin (6 U) released only 0.7%±0.15 (n = 3) hydroxyproline. Finally we confirmed that the concentration of α-thrombin used in these studies was sufficient to show degradation of denatured GBM. GBM was heat denatured (30 min at 85°C) and incubated with 6 U of α-thrombin at 37°C for 20 h. This resulted in 31±1% release of hydroxyproline. Taken together these results indicate that insignificant amounts of either denatured or interstitial collagen were present in the GBM preparations.

Hydroxyproline assay. The release of hydroxyproline was used to quantitate the GBM degradation (5–7). Supernatants and pellets from the GBM incubations were made 6 N with HCl and hydrolyzed for 3 h at 105°C. After careful neutralization, the total volume was measured and the hydroxyproline content determined as described by Woessner (24). 200 μl of chloramine T solution was added to 400 μl of the hydrolyzed samples in duplicate. The tubes were vortexed and allowed to stand 20 min at room temperature, and 200 μl of perchloric acid (3.15 M) was added. After 5 min at room temperature, 200 μl of 20% *p*-dimethylaminobenzaldehyde was added, the tubes were heated at 60°C for 20 min and cooled in cold water for 5 min, and absorbance was determined at 557 nm. The hydroxyproline content was derived from the standard curve. The scavengers of ROM, the enzyme inhibitors, or other agents used in the study did not interfere with the hydroxyproline determinations.

Leukocyte isolation. Leukocytes were obtained with a combination of dextran sedimentation and hypotonic lysis, essentially as described in our previous publication (25). Cells were counted with a model ZF counter, Coulter Electronics, Inc., Hialeah, FL, and final suspensions were adjusted to give the desired concentration. Cell viability was determined with trypan blue exclusion and was ~99%. Differential cell counting with Wright's stain showed leukocyte preparations to be 85% neutrophils with lymphocytes composing the majority of contaminating cells. For a few experiments, neutrophils were obtained by first separating them from mononuclear cells on a Ficoll–Paque gradient, then by dextran sedimentation (26). Contaminating red blood cells were removed by hypotonic lysis. The preparations contained >95% neutrophils.

Incubations. (*a*) The incubation mixture consisted of human GBM incubated with neutrophils in HBSS, pH 7.4. Duplicate mixtures were incubated at 37°C for 3 h in the absence or presence of PMA (1 μg/ml). Other additions to this system included α-1-PI (250 μg/ml), soya bean trypsin inhibitor (SBTI, 250 μg/ml), superoxide dismutase (SOD, 100 μg/ml), catalase (25 μg/ml). At the end of the incubation, the samples were centrifuged for 15 min in a Microfuge B (Beckman Instruments, Inc.) (27). The supernatants and pellets were carefully separated, hydrolyzed with 6 N HCl as described above, and the hydroxyproline content was determined. The hydroxyproline released into the supernatant was expressed as the percentage released based on the total hydroxyproline content in each sample.

(*b*) Neutrophils were suspended in HBSS with or without PMA. The mixtures were incubated for 1 h at 37°C and then centrifuged. Portions of the supernatants, representing the released products from 5 million

neutrophils, were incubated with the GBM for 3 h at 37°C, and the amount of hydroxyproline released was determined as described above. Other additions to the incubation of supernatant with GBM included α-1-PI, SBTI, and the metal chelators EDTA (10 mM) (12, 16) and 1,10,phenanthroline (2 mM) (28).

(*c*) Human neutrophils were suspended in HBSS with or without PMA as described above. The mixtures were incubated for 1 h at 37°C. Other additions to this incubation included SOD, catalase, azide (1 mM), an inhibitor of myeloperoxidase (12), and scavengers of hypochlorous acid, methionine (5 mM) (11, 12), and taurine (5 mM) (11). After centrifugation, supernatants obtained from these incubations were incubated with GBM for 3 h at 37°C in the presence and absence of the activating agent for latent metalloenzymes, 4-aminophenylmercuric acid (APMA; 0.5 mM) (12).

Materials. PMA was obtained from Consolidated Midland Chemical Co., Brewster, NY. Bovine superoxide dismutase (3,000 U/mg protein) was from Diagnostic Data, Inc., Mountain View, CA, and HBSS from Grand Island Biological Company, Grand Island, NY. Human α-1-PI (lot 405895) was purchased from Calbiochem (Behring Diagnostics, American Hoechst Corp., San Diego, CA). The preparation showed a single line on an immunoelectrophoresis gel and migrated as a single band on SDS-reduced gel and was >95% by pure NH_2-terminal sequence analysis (Behring Diagnostics). Ficoll–Paque was obtained from Pharmacia Fine Chemicals, Piscataway, NJ. All other reagents, chemicals, and enzymes, including thymol-free catalase (17,000 U/mg protein), SBTI (type 1-S; chromatographically prepared and provided lyophilized), bacterial collagenase (type VI), α-thrombin (lyophilized powder from human plasma, containing 3,080 U/mg protein), sodium azide, L-methionine, APMA, taurine, *p*-dimethylaminobenzaldehyde, methyl cellosolve (ethylene glycol monomethyl ether), dimethylsulfoxide (DMSO), and chloramine-T were from Sigma Chemical Co., St. Louis, MO. Perchloric acid (70%) was purchased from Mallinckrodt Inc., St. Louis, MO).

Results

We first examined the ability of neutrophils triggered with a soluble stimulus, PMA, to degrade GBM. The complete incubation consisted of 5 million leukocytes and GBM containing ~20 μg of hydroxyproline. The release of hydroxyproline was used to quantitate GBM degradation (5–7). Although hydroxyproline represents only a small percentage of the total GBM, these measurements do not require correction for non–GBM proteins and are thus an accurate measure for GBM degradation (7). The hydroxyproline released in the supernatant was expressed as a percentage of the total amount of hydroxyproline. Only a negligible amount of hydroxyproline was present in the supernatant when GBM was incubated alone (Table I). When GBM was incubated with unstimulated neutrophils, 0.29±0.09% (n = 11) of the hydroxyproline was released. In contrast, leukocytes stimulated with PMA caused a significant degradation of GBM resulting in 11.36±0.89% (n = 11) release of hydroxyproline. In these experiments the total amount of hydroxyproline in the GBM was 19.6±1.7 μg, of which 2.02±0.2 μg (n = 11) was released by the stimulated neutrophils. This release was not due to some direct action of PMA on GBM, because incubation of GBM with PMA without neutrophils did not result in a significant amount of release. We considered the possibility that stimulated neutrophils may release some products that interfere with the hydroxyproline assay. This was not the case, because no significant amount of hydroxyproline was detected when neutrophils were incubated with PMA without GBM. We also confirmed, as have others (27), that centrifugation in the Microfuge centrifuge for 15 min results in complete sedimentation of non–solubilized GBM. Two sets of samples in triplicate containing GBM, neutrophils, and PMA were incubated as above, and the mixtures subjected to either routine centrifugation in the Microfuge or centrifugation at 78,000 *g* for 2 h in an ultracentrifuge (Beckman Instruments, Inc.). Similar amounts of hydroxyproline were present in supernatants of these samples.

Table I. Human Glomerular Basement Membrane Degradation by PMA-stimulated Neutrophils

	Percentage release of hydroxyproline*
GMB alone	0.02±0.01 (3)
GBM + neutrophils	0.29±0.09 (11)
GBM + PMA	0.25±0.08 (4)
Neutrophils + PMA	0.04±0.01 (4)
GBM + neutrophils + PMA‡	11.36±0.89 (11)§

* Percentage release expressed as mean percent±SEM of hydroxyproline released in the supernatant, based on total amount hydroxyproline present in each individual sample. Numbers in parentheses indicate number of experiments.

‡ Complete system consisted of GBM and 5×10^6 neutrophils. Stimulated with the addition of 0.4 μg of PMA in a final volume of 400 μl. Incubation was carried out for 3 h at 37°C.

§ $P < 0.001$ compared with GBM incubated with unstimulated neutrophils using paired Student's t test.

We next examined the relation between the GBM degradation and the number of neutrophils. The amount of degradation increased with increasing numbers of neutrophils with 3.5±0.5% (n = 6) release with as few as 100,000 stimulated neutrophils, 9.4±0.7% (n = 6) release with 1 million, and 12.3±0.4% (n = 6) release with 5 million cells.

We next examined the role of neutral proteinases, elastase, and cathepsin G in the GBM degradation. Neither α-1-PI, an inhibitor of elastase (11, 29), nor SBTI, an inhibitor of cathepsin G (29), caused any significant inhibition of the GBM degradation (Table II). The role of ROM in the GBM degradation was examined with SOD, a scavenger of superoxide anion, and catalase, a scavenger of hydrogen peroxide. Superoxide dismutase, in sufficient concentration to scavenge the generated superoxide, did not inhibit the GBM degradation (Table II). In contrast, catalase caused a marked inhibition (58.9±6.8%, n = 4, $P < 0.01$) of the hydroxyproline release (Table II). Heat inactivated catalase had no effect on the GBM degradation by the stimulated neutrophils. Thus, these results show that PMA-stimulated human neutrophils degrade human GBM and that hydrogen peroxide plays an important role in this degradation.

To further evaluate the role of released proteinases on the GBM degradation, the effect of cell-free supernatants obtained from stimulated neutrophils on hydroxyproline release was examined. The purpose of these experiments was: (*a*) to examine whether cell-free supernatants from stimulated neutrophils would degrade the GBM, because if the released proteinases were important in the GBM degradation then the cell-free supernatants should degrade the GBM to the same extent as the intact cells; (*b*) to examine the possibility that the complete lack of effect of α-1-PI may be due to its inactivation by oxidants released by the triggered neutrophils, because then the degradation by the cell-free supernatants should be inhibited by α-1-PI (11); (*c*) to examine the role of metalloenzymes (e.g., collagenase or gelatinase) in the GBM degradation.

*Table II. Effect of Proteinase Inhibitors and Scavengers of Reactive Oxygen Metabolites on GBM Degradation by PMA-stimulated Neutrophils**

	Percent hydroxyproline released‡		Percent change§
	−	+	
Complete system + α-1-PI (250 μg/ml)	11.3±0.8	11.9±1.3 (5)	6.5±4.5
Complete system + SBTI (250 μg/ml)	10.3±0.9	10.0±0.2 (3)	−1.0±7.1
Complete system + SOD (100 μg/ml)	10.8±0.7	9.5±0.7 (4)	−5.8±4.7
Complete system + inactive SOD	11.4±0.1	11.6±0.9 (3)	−1.9±0.9
Complete system + catalase (25 μg/ml)	10.4±0.5	4.3±0.9 (4)‖	−58.9±6.8¶
Complete system + inactive catalase	10.3±0.6	11.1±0.6 (3)	9.1±7.2

* Complete system consisted of GBM and 5×10^6 neutrophils stimulated with addition of 0.4 μg of PMA in a final volume of 400 μl. Incubation was carried out for 3 h.

‡ Percentage release expressed as mean percent±SEM of hydroxyproline released in supernatant based on total amount of hydroxyproline present in each sample. Number in parentheses indicates number of experiments. Data shown is percent release in the presence of inhibitor or scavenger (+) and the corresponding control values in the same experiments (−).

§ Data shown represent mean±SEM of percent change calculated for each experiment as follows: $(E - C)/C \times 100$, where E is the percent release in the presence of proteinase inhibitor or scavenger of ROM. C is the percent release in the absence of proteinase inhibitor or scavenger of ROM.

‖ $P < 0.001$ with paired Student's t test.

¶ $P < 0.01$ with paired Student's t test.

As shown in Fig. 1, cell-free supernatants obtained from 5×10^6 activated neutrophils caused significant release of hydroxyproline at all time points examined. At 3 h the hydroxyproline released (9.1±0.8%, $n = 6$) was similar to the release observed with intact cells (Table I). The amount of hydroxyproline released increased with time, with 14.8±0.5% ($n = 6$) release at 24 h by supernatants from activated cells compared with 4.1±0.3% ($n = 6$) release by supernatants from unstimulated neutrophils. To determine whether neutrophils stimulated with PMA could solubilize a greater percentage of the GBM, we incubated supernatants from 25 million neutrophils for 24 h at 37°C. This resulted in 32–36% release of hydroxyproline. In addition, we carried out experiments with smaller amounts (5 μg of hydroxyproline) of GBM. Under these conditions supernatants from 5 million stimulated neutrophils released 18.9±0.6% ($n = 2$) hydroxyproline over 3 h and 38.5±4.3% ($n = 3$) over 24 h compared with 3.9±0.4% release by supernatants from unstimulated neutrophils over 24 h.

When supernatants were incubated with GBM in the presence of α-1-PI, no inhibition was observed (Table III). Similarly, SBTI had no significant effect on hydroxyproline release. In contrast, the GBM degradation by cell-free supernatants from stimulated neutrophils was markedly inhibited in presence of EDTA (72.4±6.6%, $P < 0.001$) and 1,10,phenanthroline (85.3±5.3, $P < 0.001$) (Table III), suggesting that the degradation of GBM was mediated by a metalloproteinase(s). In addition, heat-inactivated supernatants from stimulated neutrophils did not cause significant release of hydroxyproline (0.9±0.4, $n = 3$), further suggesting an enzymatic mechanism for the GBM degradation. Recently, a novel mechanism whereby triggered neutrophil release and simultaneously activate their latent collagenase has been reported (12). According to this mechanism, hypochlorous acid generated by the myeloperoxidase–hydrogen peroxide–halide system activates the latent collagenase. The marked inhibition of the GBM degradation by catalase (Table II) and the effect of EDTA and 1,10,phenanthroline (Table III) led us to consider a similar mechanism for the GBM degradation by the stimulated neutrophils. This was examined by stimulating the neutrophils with PMA in the presence and absence of various scavengers and inhibitors (Table IV) and examining the effect of cell-free supernatants obtained from these incubations on the GBM degradation. In keeping with the results with intact cells, the GBM degradation by supernatants obtained from incubations with SOD was unaffected, whereas the degradation by supernatants obtained from incubations with catalase was markedly reduced (Table IV). The roles of myeloperoxidase and hypochlorous acid were examined by stimulating the neutrophils in the presence of azide, an inhibitor of myeloperoxidase, and methionine and taurine, scavengers of hypochlorous acid. The GBM-degrading activity of the cell-free supernatants obtained from these incubations was markedly impaired (Table IV). Neither azide nor the scavengers of hypochlorous acid interfere with the generation of hydrogen peroxide (reference 12 and data not shown). The effects of catalase, azide, methionine, and taurine

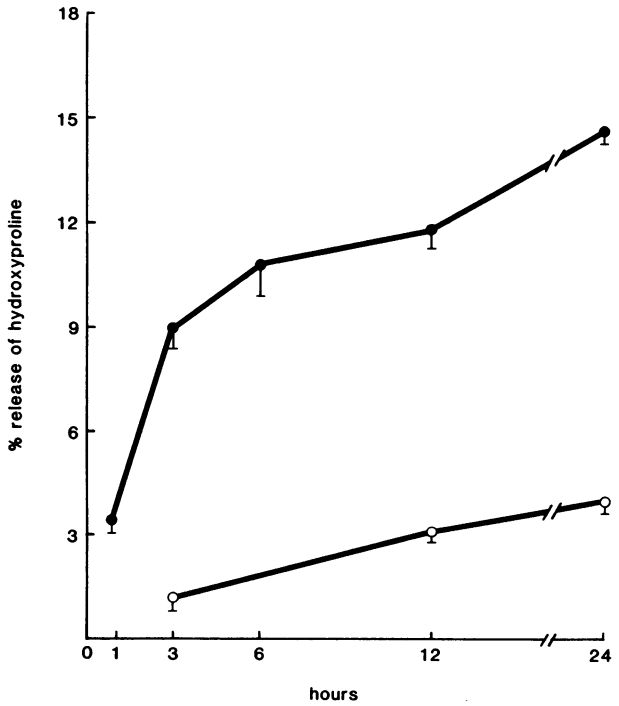

Figure 1. Time course of GBM degradation by cell-free supernatants obtained from 5×10^6 stimulated (*solid circles*) and unstimulated (*open circles*) neutrophils. Neutrophils were incubated at 37°C in HBSS with or without PMA for 60 min, then cell-free supernatants were obtained by centrifugation. These cell-free supernatants were then incubated with GBM for various time periods, as shown in the figure. Results shown represent mean±SEM of six samples.

*Table III. Effect of Proteinase Inhibitors and Metal Chelators on GBM Degradation by Cell-free Supernatants from PMA-stimulated Neutrophils**

	Percent hydroxyproline released‡		Percent change§
	−	+	
Supernatants from stimulated neutrophils + α-1-PI (250 μg/ml)	11.8±2.4	11.6±1.9 (3)	1.7±7.6
Supernatants from stimulated neutrophils + SBTI (250 μg/ml)	10.2±1.5	10.5±1.3 (3)	5.1±10.3
Supernatants from stimulated neutrophils + EDTA (10 mM)	11.0 + 0.7	3.1±0.6 (6)‖	−72.4±6.6‖
Supernatants from stimulated neutrophils + 1,10, phenanthroline (2 mM)	15.2±1.1	2.3±0.3 (3)¶	−85.3±5.3‖

* Neutrophils were incubated at 37°C in HBSS with or without PMA for 60 min, following which cell-free supernatants were obtained by centrifugation. These cell-free supernatants were then incubated with GBM for 3 h at 37°C with additions as noted above.

‡ Percent release expressed as mean percent±SEM of hydroxyproline released in supernatant based on total amount of hydroxyproline present in each sample. Number in parentheses indicates number of experiments. Values shown represent percent release in the presence of proteinase inhibitor and metal chelators (+) and corresponding control values obtained in the same experiments in the absence of these agents (−).

§ Data shown represent mean±SEM of percent change calculated for each experiment as follows: $(E - C)/C \times 100$, where E is the percent release in the presence of proteinase inhibitor or metal chelator. C is the percent release in the absence of proteinase inhibitor or metal chelator.

‖ $P < 0.001$ with paired Student's t test.

¶ $P < 0.01$ with paired Student's t test.

Table IV. Role of Reactive Oxygen Metabolites in GBM Degradation by PMA-stimulated Neutrophils

Supernatants obtained from PMA-stimulated neutrophils in presence or absence of:*	n	Percent hydroxyproline released‡	Percent change§	Percent release with APMA‖
SOD (100 μg/ml)	3	10.7±1.6	2.5±11.2	13.3±0.3
Catalase (25 μg/ml)	6	3.8±1.1¶	−49.7±4.4¶	10.7±1.3
Azide (1 mM)	4	3.2±0.8¶	−59.0±9.3¶	10.9±3.0
Methionine (5 mM)	4	2.5±0.5¶	−48.2±1.3**	9.4±0.4
Taurine (5 mM)	3	5.1±0.5¶	−50.8±3.0**	—

* Neutrophils were incubated in HBSS at 37°C and stimulated with PMA in the presence or absence of scavengers of reactive oxygen species as shown in the table. At the end of 60 min incubation, cell-free supernatants were obtained by centrifugation and incubated with GBM for 3 h at 37°C.

‡ Percent release expressed as mean percent±SEM of hydroxyproline released in supernatant based on total amount of hydroxyproline present in each sample.

§ Data shown represent mean±SEM of percent change calculated for each experiment as follows: $E - C/C \times 100$, where E is percent release in the presence of scavenger of ROM and C is the percent release either in the absence of the scavenger or in the presence of heat-inactivated scavenger (SOD, catalase).

‖ Supernatants obtained from PMA-stimulated neutrophils in presence of the scavengers were incubated with GBM and 0.5 mM APMA for 3 h at 37°C, and percent release of hydroxyproline was measured. Addition of APMA to supernatants from neutrophils incubated without scavenger resulted in 1.3±0.1% ($n = 4$) release from unstimulated neutrophils and 13.7±0.8% ($n = 6$) release of hydroxyproline from PMA-stimulated neutrophils.

¶ $P < 0.01$ with paired t test.

** $P < 0.001$ with paired Student's t test.

were not due to interference with the release of or direct inhibition of the GBM-degrading enzyme, because subsequent addition of the activating agent APMA completely restored these supernatants' ability to degrade GBM (Table IV). In addition, the ability of APMA to restore the GBM-degrading activity and the results with metal chelators suggest that GBM degradation is due to activation of latent metalloproteinase(s). No GBM-degrading activity was present in the supernatants obtained from unstimulated neutrophils, even after the addition of APMA to these supernatants (Table IV).

It has been recently reported that T lymphocytes secrete a metalloenzyme that can degrade articular cartilage proteoglycan (30). Therefore, we considered the possibility that the ROM generated by the stimulated neutrophils activates a metalloproteinase released by the contaminating lymphocytes rather than by neutrophils. PMA-stimulated neutrophils obtained by the Ficoll–Paque centrifugation and dextran sedimentation (95% purity) caused a significant (13±0.8%, $n = 6$) release of hydroxyproline, confirming the important role of neutrophils in the GBM degradation.

Discussion

Our results show that intact neutrophils stimulated by a soluble stimulus, PMA, degrade GBM and that the ROM generated by the stimulated neutrophils play a critical role in this GBM degradation. Our data also indicate that the GBM degradation is caused by a metalloproteinase that is released in a latent form and activated by hypochlorous acid or an oxidant with similar characteristics.

Because interstitial and denatured collagen are readily degraded by neutrophil metalloproteinases, we carried out several experiments to confirm that the observed effect was on native GBM (type IV collagen). SDS–polyacrylamide gel electrophoresis of salt-precipitated pepsin digests of the GBM demonstrated that type I and type III collagen together accounted for <1% of the GBM dry weight. In addition, electron microscopy of the GBM showed that the preparation was free of striated material characteristic of interstitial collagen. The amount of denatured collagen in the GBM preparations was estimated by examining the amount of GBM that could be degraded by α-thrombin, which degrades denatured but not native type IV collagen at 25°C (22, 23). Under the incubation conditions used for most of the experiments in the study (3 h at 37°C) α-thrombin released <1% of the total hydroxyproline. Taken together, these results indicate that stimulated neutrophils affect native GBM rather than denatured or interstitial collagen.

α-1-PI and SBTI, inhibitors of elastase and cathepsin G, did not inhibit GBM degradation by intact activated cells. We considered the possibility that the lack of effect of α-1-PI may be due to its inactivation by oxidants generated by stimulated neutrophils (10, 31). When stimulated neutrophils were incubated with GBM in the presence of catalase alone, α-1-PI alone, or both together, the inhibition observed with catalase alone was similar to the inhibition with α-1-PI and catalase together (data not shown). Weiss et al. showed that the stimulated neutrophils can inactivate α-1-PI not only by generating hypochlorous acid but also by generating long-lived oxidants (31). In a subsequent study (10) they showed that high concentrations of α-1-PI were required to inhibit the endothelial matrix degradation by PMA-stimulated neutrophils, whereas the degradation by cell-free supernatants from stimulated neutrophils was inhibited by as little

as 0.25 mg of α-1-PI. In our study, cell-free supernatants obtained from PMA-stimulated neutrophils degraded the GBM to the same extent as PMA-stimulated intact cells. However, no inhibition was observed even with high concentrations of α-1-PI. In addition it should be noted that SBTI inhibits not only cathepsin G but also leukocyte elastase (32). Taken together these data appear to indicate that, at least with PMA as a stimulus for the neutrophils, elastase and cathepsin G did not play an important role in GBM degradation. Nonetheless, because the oxidants generated by stimulated neutrophils have the capacity to inactivate substantial amounts of α-1-PI (10, 31), our studies do not entirely exclude the role of elastase in the GBM degradation. It must be emphasized that the present studies were designed to delineate a potential role for ROM in the GBM degradation. The use of stimuli that are more effective in causing release of the contents of azurophilic granules (33) may indeed demonstrate the role of elastase and cathepsin G in the GBM degradation (7).

The GBM degradation by cell-free supernatants was completely abolished by heat inactivation of the supernatants, suggesting a role of enzymes in GBM degradation. In addition, metal chelators EDTA and 1,10,phenanthroline cause a marked inhibition of the GBM degradation, indicating the enzyme involved in the GBM degradation is a metalloproteinase. The GBM-degrading activity of cell-free supernatants obtained from neutrophils stimulated in presence of catalase, azide (an inhibitor of myeloperoxidase), or methionine and taurine, scavengers of hypochlorous acid, was markedly impaired. Neither azide nor the scavengers of hypochlorous acid interfere with the generation of hydrogen peroxide (reference 12 and data not shown). The inhibitory effect of catalase, azide, methionine, and taurine were not due to the action of or their inhibition of the release of the GBM-degrading enzyme, because addition of activating agent APMA completely restored the ability of these supernatants to degrade GBM (Table IV). No GBM-degrading activity was present in the supernatants obtained from unstimulated neutrophils even after addition of APMA (Table IV).

Taken together, these data indicate that when stimulated with PMA, human neutrophils secrete a latent metalloproteinase activated by hypochlorous acid or a similar oxidant generated by the hydrogen peroxide–myeloperoxidase–halide system. The activated enzyme then causes degradation of the GBM. It must be noted, however, that none of the scavengers of ROM completely inhibited the GBM degradation. This suggests a possible contribution by other mechanisms (e.g., activation of a metalloproteinase by oxygen-independent mechanisms) in the GBM degradation.

The human neutrophil contains two metalloproteinases capable of degrading collagen: collagenase and gelatinase (12, 16, 28). The presence of GBM-degrading activity at neutral pH, inhibition by metal chelators, and activation by APMA and hypochlorous acid all suggest that the enzyme(s) responsible for the GBM degradation are collagenase and/or gelatinase (12, 15, 28, 34). Although GBM, consisting of type IV collagen, is resistant to the action of several specific mammalian collagenases (27), Uttio et al. (35) described a metalloproteinase other than collagenase from leukocyte extracts that degraded type IV collagen. Based on recent work (28), the metalloproteinase described by Uttio et al. is most likely gelatinase. Similarly, Hibbs et al. have recently shown that gelatinase from human neutrophils actively degrades type V collagen (16), which, like type IV collagen, is not readily degraded by several specific collagenases. In addition, Murphy et al. have also shown that degradation of insoluble type IV collagen by purified neutrophil collagenase was markedly enhanced by gelatinase (28). It appears likely that the collagenase and the gelatinase secreted by the neutrophils act in concert to degrade collagen.

Thus, in the present study we have described degradation of the GBM by a metalloproteinase activated by ROM and generated by PMA-stimulated neutrophils. Such a mechanism for GBM degradation has not been previously described. However, in a recent study, administration of catalase prevented proteinuria, in anti–GBM antibody disease in rats, a glomerular injury known to be neutrophil-dependent (36). Similarly, catalase also prevented neutrophil-dependent proteinuria after intraarterial injection of PMA (37). These observations suggest that the mechanism for GBM degradation described in this study may be quite relevant to human and experimental neutrophil-dependent glomerulonephritides.

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