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Research Article

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92-kD Gelatinase Is Produced by Eosinophils at the Site of Blister Formation in Bullous Pemphigoid and Cleaves the Extracellular Domain of Recombinant 180-kD Bullous Pemphigoid Autoantigen

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Abstract

Eosinophils are prominent in bullous pemphigoid (BP), and proteases secreted from these and other inflammatory cells may induce disruption of the basement membrane. We used in situ hybridization and immunohistochemistry to localize the sites of 92-kD gelatinase expression in BP lesions. In all samples (20/20), a strong signal for gelatinase mRNA was detected only in eosinophils and was most pronounced where these cells accumulated at the floor of forming blisters. No other cells were positive for enzyme mRNA. Both eosinophils and neutrophils, however, contained immunoreactive 92-kD gelatinase indicating that active expression occurred only in eosinophils. Degranulated eosinophils were also seen near blisters, and as demonstrated by gelatin zymography, immunoblotting, and ELISA, 92-kD gelatinase protein was prominent in BP blister fluid. No other gelatinolytic activity was specifically detected in BP fluid, and only small amounts of 92-kD gelatinase were present in suction blister fluids. As demonstrated in vitro, 92-kD gelatinase cleaved the extracellular, collagenous domain of recombinant 180-kD BP autoantigen (BP180, BPAG2, HD4, type XVII collagen), a transmembrane molecule of the epidermal hemidesmosome. Our results suggest that production and release 92-kD gelatinase by eosinophils contributes significantly to tissue damage in BP. (*J. Clin. Invest.* 1994. 93:2022–2030.) Key words: bullous pemphigoid • 92-kD gelatinase • eosinophils • metalloproteinase • type XVII collagen

Introduction

Bullous pemphigoid (BP)[1] is a relatively common blistering disease affecting primarily the elderly. Although the precise etiology of this condition is not known, the pathogenic mechanism is considered to be an autoimmune-mediated process leading to inflammation and subsequent blister formation (1). Histologic and ultrastructural studies have shown that the epidermis detaches from the underlying dermis within the lamina lucida of the basement membrane (2, 3). Along with epidermal–dermal separation is a loss of various structural and cellular components, such as anchoring filaments and hemidesmosomes, and eventually fragmentation of the basement membrane. The extensive destruction of extracellular matrix and cell adhesive proteins suggests that proteases released by migratory cells play a key role in blister formation.

A predominant influx of eosinophils into the skin is a characteristic and early event in BP (2, 3), but other leukocytes, notably neutrophils, are found in the associated infiltrate, albeit in lower concentrations. Granulocytes are thought to release proteases that damage the basement membrane, and indeed, degranulated eosinophils are seen in close association with the basal epidermis in the early stages of blister formation in BP (2, 3). Furthermore, cytochemical studies suggest that eosinophils release their intracellular contents in the lamina lucida at sites of blister formation (4). Proteolytic activity is found in BP blister fluid (5–8), but the precise nature and origin of this activity and which enzyme or enzymes contribute to blister formation is not known. Although interstitial collagenase is present in BP blister fluid, the levels are much lower compared to other types of blisters and are not much different from the levels in suction blisters (6). Similarly, the level of serine protease activity in BP blisters is lower than that detected in pemphigus lesions (8).

Metalloproteinase activity has been detected in BP blister fluid (5), and because these enzymes have a broad substrate specificity for extracellular matrix proteins (9, 10), and because they are expressed by migratory cells in response to inflammation (11), members of this family of proteases are likely to be involved in BP blister formation. We have reported that dermal eosinophils express 92-kD gelatinase (also known as MMP-9) mRNA and protein (12, 13). This metallogelatinase degrades denatured collagen, or gelatin, as well as elastin and native type V collagen (14, 15). In addition to interstitial proteins, 92-kD gelatinase acts on various components of the basement membrane, such as type IV collagen, proteoglycans, entactin, and possibly laminin (10, 16).

Our in vivo findings, reported here, show that 92-kD gelatinase is produced by eosinophils and is an abundant component of BP blister fluid. Since the complete proteolytic profile of this enzyme is not known and because it is a prominent product of eosinophils, 92-kD gelatinase may cause the disruption leading to blister formation in BP by degrading important structural elements of skin. Interestingly, the two known BP autoantigens are components of epidermal hemidesmosomes,

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1. *Abbreviations used in this paper:* APMA, *p*-aminophenylmercuric acetate; BP, bullous pemphigoid; DAB, diaminobenzidine; GST, glutathione *S*-transferase.

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which attach the epidermis to the underlying stroma. The larger autoantigen, BP230 or BPAG1, is a 230-kD intracellular protein (17, 18) with high homology to desmoplakin I (19, 20) and plectin (21). Because of its intracellular location, BP230 is probably not degraded by extracellular proteases. The other autoantigen, BP180 (also called BPAG2, HD4, and type XVII collagen), is a 180-kD transmembrane hemidesmosome protein with a large collagenous extracellular domain that has recently been shown to extent into the basement membrane (22–26). Thus, degradation of the extracellular domain of BP180 may lead to dermal:epidermal separation, and, in fact, we demonstrate here that 92-kD gelatinase degrades the collagenous domain of a recombinant form this autoantigen. Our findings suggest that the release of 92-kD gelatinase by eosinophils is a critical event in blister formation and tissue damage seen in BP.

Methods

Tissues. Formalin-fixed, paraffin-embedded specimens of lesions from 20 patients with untreated BP were obtained from the Department of Dermatology, Karolinska Hospital (Stockholm, Sweden). Diagnosis of BP was confirmed by typical clinical and histopathologic features and by indirect immunofluorescence indicating that all patients had circulating autoantibodies against the basement membrane zone. Blood and fresh blister fluid were obtained within 24 h of blister formation sampled from seven inpatients with untreated BP. Suction blisters were induced on the ventral surface of the forearm of two healthy volunteers using a Dermovac device after continuous negative suction for 3–4 h at 200 mmHg (27). Fluid was collected at 1 and 24 h after cessation of suction. Two additional BP blister samples (Nos. 8 and 9, Fig. 4) and fluid from three patients with blisters secondary to wart treatment by either bleomycin (No. 1) or cryotherapy (Nos. 2 and 3) were provided by Dr. Luis Diaz at the Medical College of Wisconsin (Milwaukee, WI). These fluids were sampled ~ 24 h after blister formation. After centrifugation to remove cells, total protein concentration was measured by the Lowry method using bovine serum albumin as a standard, and blister fluid and serum samples were used directly for immunoblotting, zymography, and ELISA.

In situ hybridization. In situ hybridization was performed as described (28) with some modifications. All sections (5 μm) were treated with proteinase K (Sigma Chemical Co., St. Louis, MO) and were washed in 0.1 M triethanolamine buffer containing 0.25% acetic anhydride. Since eosinophils have been reported to non-specifically bind sulfated probes (29, 30), we minimized this potential artifact by pretreating specimens with 0.1 M iodoacetamide in 0.1 M triethanolamine, pH 8.2, for 30 min before hybridization. Sections were covered with 25–50 μl of hybridization buffer containing $2.5–4 \times 10^4$ cpm/μl of ^{35}S-labeled antisense or sense RNA probe or 10^5 cpm/μl of each ^{35}S-labeled 92-kD gelatinase-specific oligomers (see below). Specimens were incubated at 55°C (RNA probes) or 50°C (oligomers) for 18 h in a humidified chamber. After hybridization, slides were washed under stringent conditions, including treatment with RNase-A, except for sections hybridized with oligomeric probes, and were processed for autoradiography. After a 5–10-d exposure, the photographic emulsion was developed, and the slides were stained with Giemsa.

Probes for in situ hybridization. A 560-bp BamHI–XbaI fragment of 3′ end of the human 92-kD gelatinase cDNA subcloned in a Bluescript KS transcription vector (Stratagene, La Jolla, CA) was linearized to allow transcription of antisense or sense RNA. This part of the cDNA encodes for the carboxyl terminus of the 92-kD gelatinase which displays considerable sequence divergence from similar domains of other metalloproteinases (31). Specimens were also hybridized with two ^{35}S-labeled oligomers complementary to 92-kD gelatinase mRNA that were designed and prepared as described in detail (12). These oligomers are 23 and 25 nucleotides and are complementary to unique sequences within the region encoding the carboxy terminus of the 92-kD enzyme. A 15 nucleotide M-13 oligomeric universal primer was used as a negative control. We also hybridized sections for stromelysin-1 mRNA using a full-length human cDNA provided by Dr. Markku Kurkinen, Wayne State University. We subcloned a 217-bp EcoRI–XhoI fragment coding of a unique portion of the 3′ untranslated region of stromelysin-1 into a Bluescript KSII$^+$ plasmid to prevent crosshybridization to stromelysin-2 (32). Human collagenase (33) and matrilysin probes (34) were provided by Dr. Gregory I. Goldberg, Washington University (St. Louis, MO), and by Dr. Lynn Matrisian, Vanderbilt University (Nashville, TN). RNA probes were transcribed from linear cDNA templates and were labeled with α-[^{35}S]UTP (> 1,200 Ci/mmol) under conditions recommended by and with reagents from Promega (Madison, WI) except that the transcription reaction was extended to 4 h to allow for the relatively inefficient incorporation of sulfated ribonucleotides. Calculated probe-specific activities were between 10^8 and 10^9 dpm/μg. The specificity of the 92-kD gelatinase (12), collagenase (35), stromelysin-1 (36), and matrilysin (34) probes for the appropriate sized mRNAs has been demonstrated by Northern blot analysis under similar conditions as for in situ hybridization.

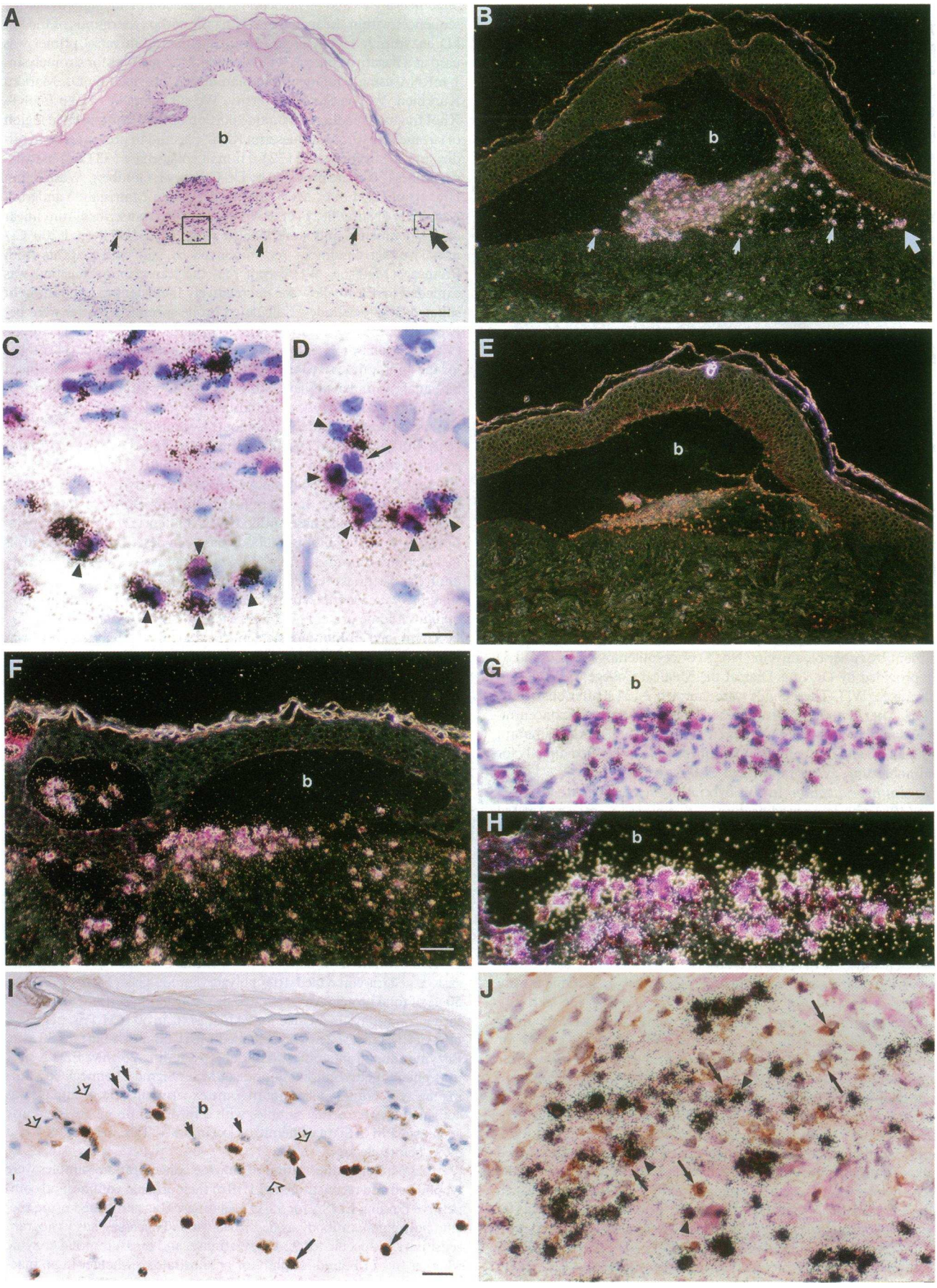
Immunohistochemistry. Immunohistochemistry was done by the peroxidase–antiperoxidase technique (Vectastain ABC Kit; Vector Labs, Burlingame, CA) using diaminobenzidine (DAB) as a chromogenic substrate. Sections were pretreated with 0.1% trypsin in PBS for 30 min at 37°C, and endogenous peroxidase activity was blocked by incubation 0.3% H_2O_2 for 30 min at room temperature. Affinity purified anti-92-kD gelatinase antibody was diluted 1:2,000. The preparation and specificity of this antibody has been described (13). Controls included pre-immune serum and sections processed without primary antibody. Sections were counter stained with Harris hematoxylin. Eosinophils were identified by immunostaining with an antibody to eosinophilic cationic protein (EG2; Pharmacia AB, Uppsala, Sweden).

Combined immunohistochemistry and in situ hybridization was done as described (37). First, tissue macrophages were identified using a monoclonal antibody (KP-1; DAKO Corp., Carpinteria, CA) which reacts with CD-68, a specific macrophages marker (38). The CD-68 antibody was diluted 1:300 and incubated on sections at 37°C for 1 h. Sections were processed without trypsin pretreatment, and all immunostaining solutions contained heparin to inhibit RNases (37). Next, the immunostained samples were hybridized for 92-kD gelatinase mRNA with ^{35}S-labeled RNA, processed for autoradiography, and stained with Harris hematoxylin.

Immunoblotting. Samples (25 μg per lane of total protein) were resolved by electrophoresis through precasted 10% polyacrylamide gels (Novex, San Diego, CA). Proteins were transferred onto Immobilon-P PVDF Transfer Membrane (Millipore Intertech, Bedford, MA) with a semi-dry blotting system. Affinity-purified 92-kD gelatinase antibody was diluted 1:500, and antigen was detected with a Vectastain ABC kit using DAB as a chromogen.

Zymography. Zymography was done as described (39) using precasted, gelatin-embedded 10% polyacrylamide gels (Novex). Briefly, 50 μg of total protein was separated by electrophoresis under nondenaturing conditions, incubated for 24 h at 37°C in 50 mM Tris, pH 8.0, 5 mM $CaCl_2$, 0.5 μM ZnCl, and stained with Coomassie blue for 30 min. Enzyme inhibition studies were done by adding 25 mM EDTA or 1 mM phenylmethylsulfonylfluoride (PMSF; Sigma Chemical Co.) during the zymogram incubation. In some experiments, progelatinase was activated by pretreating samples with 1 mM *p*-aminophenylmercuric acetate (APMA; Sigma Chemical Co.) in 0.1 M Tris, pH 8.0, for 1 h at 37°C before electrophoresis.

ELISA. For quantification of enzyme concentration, samples were lyophilized and reconstituted at 1:10 of their original volume, and competitive binding ELISA for 92-kD gelatinase was performed using specific polyclonal antibody as described (40). This assay has nanogram sensitivity, is specific for 92-kD gelatinase, and measures total enzyme whether free or bound to inhibitor or substrate or whether in an inac-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tive or active form. Other than dilution, samples were assayed without modification.

In vitro degradation assay. The pGEX-2T prokaryotic expression system (Pharmacia LKB Biotechnology, Piscataway, NJ) was used to generate a 42-kD fusion protein consisting of glutathione *S*-transferase (GST) at its NH_2 terminus and a portion of the BP180 ectodomain at its COOH terminus (26). The BP180 moiety of this fusion protein, GST-NΔ1, consists of 60 amino acids of the non-collagenous, NC16A, and 80 amino acids of the collagenous sequences from domains COL14 to COL15 (See Fig. 5 *A*). Recombinant GST-NΔ1 and GST encoded by pGEX-2T were expressed in *E. coli* and were purified by affinity chromatography as described (26, 41).

Purified human 92-kD progelatinase and activated human interstitial collagenase were provided by Dr. Howard Welgus, Washington University. Gelatin, used as a known substrate for 92-kD gelatinase, was denatured at 60°C for 15 min, then kept at 37°C until used. Purified progelatinase was activated with 1 mM APMA, 5 mM NaOH at 37°C for 1 h as described (16). Recombinant proteins (10 μg each) were incubated with or without 1.5 μg activated metalloenzyme or with 0.8 μg trypsin in 10 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$ in a total volume of 50 μl for 18 h at 25°C (16). The final concentration of metalloenzyme was 3.2×10^{-7} M for 92-kD gelatinase and 6.5×10^{-7} M for collagenase. Gelatin digestion was done for 1 h at 37°C to prevent reformation of triple helices. Reactions were terminated with an equal volume of reducing sample buffer and were heated at 100°C for 10 min. Reaction components (equivalent to 1 μg of test protein) were resolved by electrophoresis through 12% SDS-polyacrylamide gels under denaturing conditions and stained with Coomassie blue or with silver nitrate.

Results

In situ hybridization. All archival tissue samples of BP had morphologically identifiable eosinophils which accumulated in the upper part of the dermis and at the dermal–epidermal junction. Immunostaining for eosinophilic cationic protein confirmed the presence of numerous eosinophils in all samples (data not shown). In 15/20 samples, the epidermis was fully separated from the underlying dermis (for example, see Fig. 1 *A*). The remaining five samples represented earlier lesions with inflammation in the upper dermis and incomplete (see Fig. 1, *F* and *I*) or no blister formation.

As demonstrated by in situ hybridization, numerous cells were strongly positive for 92-kD gelatinase mRNA, and typically these cells clustered at sites of blister formation and along the dermal ridge of blister (Fig. 1, *A, B,* and *F*). Under high magnification, signal for 92-kD gelatinase mRNA was confined to eosinophils, as identified by Giemsa staining and by their characteristic bilobar nuclear morphology, and all eosinophils in all samples were positive for enzyme mRNA (Fig. 1, *C, D, G,* and *H*). No autoradiographic signal was seen in any other cell type, notably neutrophils (Fig. 1 *D*) and keratinocytes, both of which have been reported to secrete 92-kD gelatinase in culture (14, 42). Since macrophages also have the capacity to synthesize and release 92-kD gelatinase (11, 43), we combined immunohistochemistry for CD68, a macrophage-specific marker, with in situ hybridization to see if any of the cells positive for 92-kD gelatinase mRNA were macrophages (Fig. 1 *J*). No CD68-positive cells had autoradiographic signal for 92-kD gelatinase mRNA indicating that, at least in BP, macrophages do not activity produce this metalloproteinase. In addition, no signal for collagenase, stromelysin-1, or matrilysin mRNAs was seen (data not shown) indicating that 92-kD gelatinase is the principal metalloproteinase expressed in BP lesions.

No signal was seen on sections hybridized with sense RNA (Fig. 1 *E*). Because there are reports of nonspecific autoradiographic signal in eosinophils (29, 30), we also hybridized sections with two ^{35}S-labeled oligomers which are complementary to unique sequences in 92-kD gelatinase mRNA (12). Hybridization with these probes gave results that were identical to those obtained with the antisense RNA probe, namely strong signal for 92-kD gelatinase mRNA was seen only in eosinophils (results not shown). Sections hybridized with an ^{35}S-labeled M-13 primer, used as a nonspecific oligomeric sequence, had only background autoradiographic signal (results not shown).

Immunohistochemistry. We used an affinity purified antibody for human 92-kD gelatinase protein in an immunohistochemical assay to verify that eosinophils in the BP samples synthesized enzyme protein. In agreement with the in situ hybridization findings, eosinophils were immunoreactive for 92-kD gelatinase protein (Fig. 1 *I, arrowheads*). Consistent with our previous data (13), neutrophils also showed positive immunostaining (Fig. 1, *I, long arrows*). The lack of detectable 92-kD gelatinase mRNA in neutrophils indicates that a precursor cell produces the protein (13). No signal was seen in sections processed with preimmune serum or without primary antibody (data not shown). A number of morphologically identifiable

Figure 1. Expression of 92-kD gelatinase in bullous pemphigoid. (*A* and *B*) Paired bright-field and dark-field photomicrographs of a section of bullous pemphigoid hybridized with a ^{35}S-labeled antisense RNA for 92-kD gelatinase mRNA and stained with Giemsa. A blister (*b*) separates the overlying epidermis from the dermis. An acute inflammatory infiltrate is seen in the center of the specimen, which, as seen under dark-field illumination (*B*), is filled with cells having an intense signal for 92-kD gelatinase mRNA. Numerous positive cells are seen along the dermal ridge (*small arrows*) and are accumulated at the junction of intact and blistering epidermis (*large arrow*). Autoradiography was for 5 d. Bar, 100 μm. (*C* and *D*) The areas in the large and small boxes in *A* are presented under higher magnification in *C* and *D*, respectively. Autoradiographic signal for 92-kD gelatinase mRNA is seen only in eosinophils (*arrowheads*) along the dermal ridge, within the infiltrate, and at the blister edge. Other cells, such as neutrophils (*arrow*) and keratinocytes, had no signal for 92-kD gelatinase mRNA. Bar, 10 μm. (*E*) No autoradiographic signal was detected in sections hybridized with an ^{35}S-labeled sense RNA probe transcribed from 92-kD gelatinase cDNA. Autoradiography was for 10 d. Magnification is the same as *A*. (*F*) In another sample of bullous pemphigoid, many positive cells are seen on the dermal side of a blister (*b*). Autoradiography was for 5 d. Bar, 50 μm. (*G* and *H*) Under higher magnification, all cells with signal for 92-kD gelatinase were differentially stained with Giemsa and had a morphology of eosinophils. Bar, 20 μm. (*I*) Immunostaining for 92-kD gelatinase showed immunoreactive eosinophils (*arrowheads*) and neutrophils (*long arrows*) in the dermis near to a blister (*b*). Some eosinophils had weak to no immunoreactivity (*small arrows*) suggesting that the enzyme had been released. Indeed, punctate staining was evident in the extracellular space surrounding immunoreactive cells (*open arrows*). The section was counterstained with hematoxylin. Bar, 25 μm. (*J*) A section of bullous pemphigoid was immunostained for CD68, a macrophage-specific cell surface marker, using DAB as a chromogen, which forms a brown precipitate, then were assayed for 92-kD gelatinase mRNA by in situ hybridization. Macrophages (*arrows*) had no signal for 92-kD gelatinase mRNA (*arrowheads*). Magnification is the same as *I*.

eosinophils had little or no staining for 92-kD gelatinase protein (Fig. 1 *I, short arrows*). When we have assayed samples of other diseases, such as squamous cell carcinoma (13) or various lung disorders (our unpublished findings), eosinophils consistently stained much more intensely for 92-kD gelatinase protein than we saw in the BP samples, even though the specimens were fixed and processed by similar protocols. Thus, the relatively weak staining in the BP specimens suggests that eosinophils have degranulated and released the enzyme. Indeed, punctate staining was consistently seen in the extracellular space (Fig. 1 *I, open arrows*) around the weakly positive cells, which may indicate the presence of the secreted enzyme. No such extracellular staining was seen in sections processed with preimmune serum or without primary antibody (data not shown).

Gelatinase in blister fluid. To directly assess the presence of 92-kD gelatinase in BP blisters, fluid was collected from inpatients within 24 h of blister formation. Samples were gently centrifuged to remove any cells and were processed for gelatin zymography and immunoblotting. In all BP samples, a prominent gelatinolytic band was seen at 92 kD (Fig. 2 *A*), and immunoblotting with an affinity-purified antibody verified that this degradative activity was 92-kD gelatinase (Fig. 2 *B*). As a control, suction blisters were induced in healthy volunteers, and fluid was sampled at 1 h after blister formation and 24 h later, at which time the inflammatory response is maximal (44). Blisters induced by gentle suction and heat in healthy volunteers serve as an appropriate control since the mechanical separation occurs at the same level within the epidermal basement membrane as in BP (27). No gelatinolytic band near 92 kD was seen in these samples nor was any immunoreactive material detected (Fig. 2). In all samples, gelatinolytic activity was also detected at 72 kD (Fig. 2 *A*), which most likely represents 72-kD gelatinase, a closely related yet distinct gene product (31). The minor gelatinolytic bands at about 110 and 200 kD in BP samples 1 and 4 (Fig. 2 *A*) are probably complexes that were not separated under the nondenaturing condition used for the zymogram (45).

The identity of the 92-kD gelatinolytic activity as a metalloproteinase was determined by inhibition and activation studies. All gelatinolytic activity was fully inhibited by 25 mM EDTA (Fig. 3 *A*), but PMSF, a serine protease inhibitor, did not affect degradation (Fig. 3 *B*). These findings indicate further that the specific band in the BP samples was 92-kD gelatinase. As is expected for a metalloproteinase, organomercurial activation converted the 92-kD band to one at ~ 84 kD and the 72-kD band to one at ~ 63 kD (Fig. 3 *C*).

The amount of 92-kD gelatinase in BP blister was quantified by ELISA. Although the levels of enzyme varied among BP samples, the levels were markedly higher than those detected in 1- and 24-h suction blister fluid samples or in fluid from blisters resulting from wart treatment (Fig. 4). In fact, the values for the non-pemphigoid blister samples were not much above background levels of the ELISA. Consistent with 92-kD gelatinase being produced and secreted locally, only background levels of enzyme were detected in serum samples from BP patients and from volunteers for suction blisters (data not shown).

92-kD gelatinase degrades BP180. To assess if 92-kD gelatinase contributes to blister formation, we incubated purified, activated enzyme with a 42-kD recombinant fusion protein, GST-NΔ1, containing ~ 16 kD of the extracellular sequences of BP180, including a large portion of the first and largest collagenous domain (Fig. 5 *A*). Although the tertiary structure of native BP180 is not known, reactions were done at 25°C to provide conditions permissive for potential formation of triple helices among recombinant monomers. Based on a decrease of ~ 8 kD, 92-kD gelatinase cleaved recombinant BP180 at the beginning of the collagenous ectodomain, and as for gelatin, degraded the collagenous sequences into fragments too small to be seen on the gel (Fig. 5 *B*). The GST portion of the fusion protein was not cut by 92-kD gelatinase (Fig. 5 *B*). The degradation of recombinant BP180 by 92-kD gelatinase was specific since minor contaminating bacterial proteins were not digested by this enzyme (Fig. 5 *B*) and because the protein was not degraded by purified human interstitial collagenase (data not shown). Predictably, trypsin cleaved BP180 as well as the contaminating bacterial proteins (Fig. 5 *B*).

Discussion

A massive influx of eosinophils, especially at the dermal–epidermal junction, is a characteristic feature of BP. Although the ultimate cause of basement membrane injury is unknown, this granulocytic inflammation likely plays a key role in tissue destruction. Indeed, degranulated eosinophils accumulate along the epidermal side of the basement membrane, and this infiltration is temporally and spatially associated with blister for-

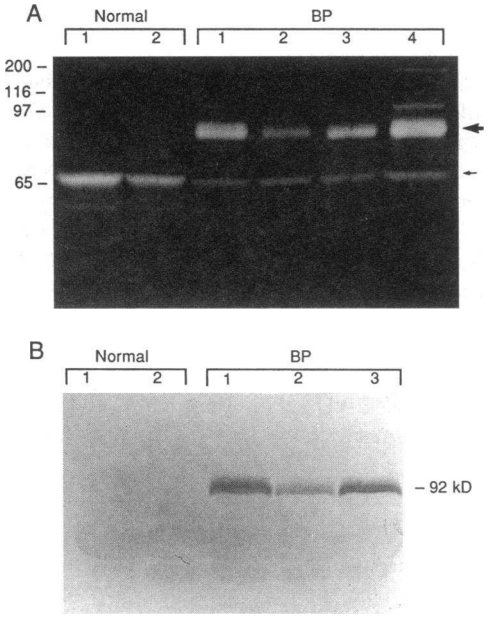

Figure 2. 92-kD gelatinase is present in bullous pemphigoid blister fluid. (*A*) Samples of blister fluid from 24-h suction blisters (*Normal*) from two volunteers and from four patients with bullous pemphigoid (*BP*) were analyzed by gelatin zymography as described in *Methods*. A prominent gelatinolytic band, which appears as a clear area on the Coomassie-stained gel, migrating at ~ 92 kD (*large arrow*) was seen only in the bullous pemphigoid samples. A smaller band, at ~ 72 kD, was present in all samples (*small arrow*). The higher molecular weight bands seen in BP samples 1 and 4 probably represent complexes of 92-kD gelatinase which are not separated in the non-denaturing gel. The white jagged line in the center is a crack in the gel. (*B*) Immunoblotting with an affinity-purified antibody to human 92-kD gelatinase demonstrated the presence of this enzyme only in bullous pemphigoid blister fluid.

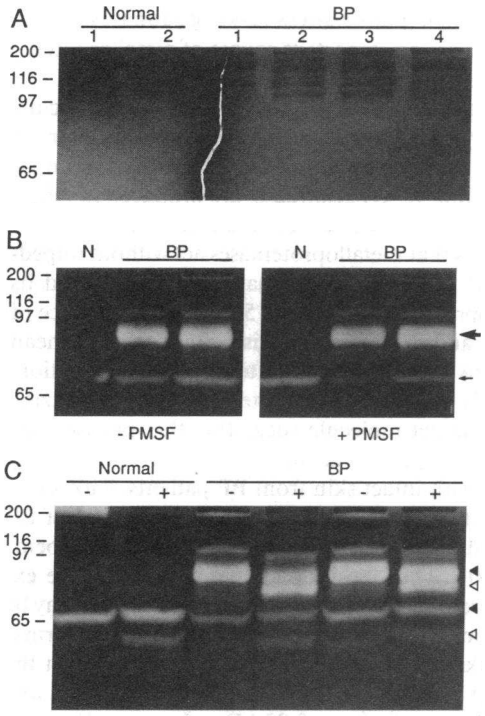

Figure 3. Characterization of gelatinolytic activity in bullous pemphigoid blister fluid as a metalloproteinase. (*A*) Samples of blister fluid from 24-h suction blisters (*Normal*) from two volunteers and from four patients with bullous pemphigoid (*BP*) were analyzed by gelatin zymography. Gels were incubated with 25 mM EDTA which blocked all gelatinolytic activity. (*B*) Incubating gels with 1 mM PMSF did not block gelatinolytic activity at either 92 (*large arrow*) or 72 kD (*small arrow*). Gelatinolytic activity at 92 kD was not detected in samples of suction blister fluid (*N*) but was prominent in samples from patients with bullous pemphigoid (*BP*). (*C*) Samples were treated with 1 mM APMA to activate metalloproteinases. The gelatinolytic bands at 92 and 72 kD (*solid arrowheads*) shifted to lower molecular weight species (*open arrowheads*) in samples pretreated with APMA (+).

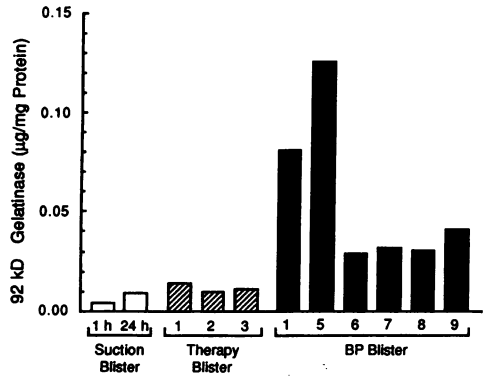

Figure 4. Quantification of 92-kD gelatinase in blister fluid. Fluid was sampled from a suction blister at 1 and 24 h after blister formation, from three patients with blisters secondary to wart treatment by bleomycin (*1*) or cryotherapy (*2* and *3*), and from six bullous pemphigoid (*BP*) patients (*1* and *5–9*). Samples were processed for ELISA as described. The data are the mean of triplicate determinations and are normalized to total protein.

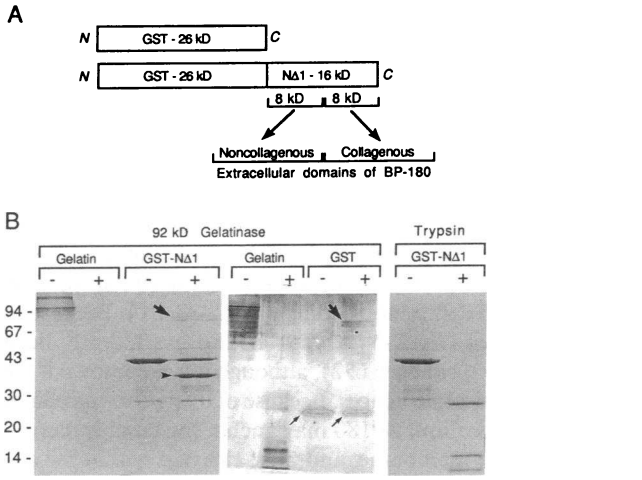

Figure 5. Degradation of recombinant BP180 by 92-kD gelatinase. (*A*) Illustration of the recombinant proteins used in these studies. *GST* is 26 kD. The fusion protein, GST-NΔ1, is 42 kD and contains GST and equal portions of two contiguous extracellular domains of BP180 as described in *Methods*. (*B*) Gelatin, the fusion protein GST-NΔ1, and GST were incubated with (+) or without (−) APMA-activiated 92-kD gelatinase, and the products were resolved by SDS-PAGE. Gelatin was completely degraded by 92-kD gelatinase. About 8 kD was cleaved from GST-NΔ1 resulting in a product of ~ 34 kD (*arrowhead*), but bacterial proteins, seen at about 45, 32 (triplet), and 28 kD, were not digested. As well, purified recombinant GST protein was not cut by 92-kD gelatinase. The GST products were silver stained, and the 26-kD GST bands are marked by small arrows. The large arrows mark activated 92-kD gelatinase, which migrates as a doublet (see Fig. 3 *C*). Trypsin effectively cleaved recombinant and bacterial proteins.

mation and destruction of the basement membrane (2, 3). In pemphigus vulgaris, another autoimmune blister disease, autoantibody binding to the epidermis induces endogenous protease production leading to detachment and acantholysis (46, 47). In contrast, BP autoantibodies do not induce protease production by keratinocytes (46), and models of BP blister formation using isolated cells or intact skin demonstrate the requirement of activated migratory cells (48) or of BP blister fluid (49). Thus, the proteases that cause epidermal–dermal separation in BP are most likely products of leukocytes, and our findings suggest that matrix-degrading enzymes, principally 92-kD gelatinase, produced and secreted by the abundant eosinophils, contribute to detachment of the epidermis in BP. Although 92-kD gelatinase is prominently expressed in BP, this metalloproteinase may contribute to tissue damage in various bullous diseases, as well as in other destructive lesions. We focused on BP because of the potential that large quantities of 92-kD gelatinase would be produced by eosinophils.

Blister formation in BP occurs primarily in the lamina lucida leaving the basal lamina at the floor of the blister. Still, in the fully developed blisters the lamina basalis is extensively damaged and eventually disappears (1, 2). Although 92-kD gelatinase degrades laminin and type IV collagen (10), immunohistologic findings with antibodies against basement membrane proteins indicate that blister formation in BP occurs adjacent to the basal surface of the epithelium and not within the basement membrane (50). Thus, if separation results from cleavage of an integral basement protein, immuno-

staining for type IV collagen or laminin should be evident on the upper face of BP blisters, which it is not.

Blister formation may occur by selective degradation of newly or not yet discovered components. Recently, two distinct autoantigens, BP230 and BP180, so named for their molecular mass, were cloned and characterized. The larger of these, BP230, is an intracellular protein (17, 19), and thus, it seems unlikely that it would be a target for degradation by extracellular proteases. BP180, however, is a transmembrane hemidesmosome protein with a large extracellular portion comprised of 15 collagenous domains (22, 23, 51). Because of its abundance of Gly-X-Y repeats, BP180 has recently been named type XVII collagen (52), although it is not known if the monomers form triple helices. Because of its rigid extracellular structure and location, BP180 may anchor the basal epidermis to the basement membrane, and thus, cleavage within the collagenous domain would be a likely site for dermal–epidermal separation. Our in vitro degradation results suggest that the first and largest collagenous domain of BP180 is efficiently degraded by 92-kD gelatinase. Although blister formation is probably more complex, involving the degradation of other structural molecules along with the disruption of cell–matrix contacts, our findings indicate that degradation of the extracellular domain of BP180 may be a critical event in the pathogenesis of BP. Our conclusion is tempered somewhat by the use of a truncated, recombinant substrate. As stated, the tertiary structure of native BP180 is not known, and confirmational alterations of the primary protein product, which would not be duplicated in the bacterial expression system, may affect the protease specificity.

In our studies, we found no evidence of specific production or release of other metalloproteinases. Other than the storage of 92-kD gelatinase by neutrophils, metalloproteinases are actively secreted by the cells that make them. Thus, our negative results for expression of interstitial collagenase, stromelysin-1, and matrilysin mRNAs would indicate that these enzymes are not produced in the area of blister formation in BP. Considering their substrate specificity, it is reasonable that these enzymes are not involved in BP. Collagenase cleaves only interstitial fibrillar collagens (types I, II, III, and X), which are not found in basement membrane, and in our studies reported here, human collagenase did not degrade recombinant BP180. Furthermore, studies by Welgus et al. showed that relatively little collagenase is present in BP blister fluid (6), and other studies by us showed that collagenase mRNA is not expressed by any cell near the site of blister formation in BP (50). Stromelysin-1 degrades various extracellular matrix proteins including components of the dermal basement membrane such as proteoglycans, type IV collagen, and laminin (10, 53). Since these components remain with the attached basement membrane, they are probably not the sites of tissue destruction in BP. Although matrilysin has a broad substrate specificity, its production seems to be limited to circulating monocytes (54) and glandular epithelium (34, 55).

In all blister fluid samples, we found detectable levels of 72-kD gelatinase. This enzyme is closely related to 92-kD gelatinase but typically is made by different cell types (11, 31). Because both metallogelatinases share the same substrate specificity (10), the 72-kD enzyme may contribute to degradation of the same molecules that the 92-kD enzyme recognizes. Preliminary observations (M. Ståhle-Bäckdahl, unpublished findings) suggest that eosinophils express 72-kD gelatinase indicating that these cells are an important source of metalloproteinases.

Metalloenzyme activity is regulated, in part, by specific inhibitors, and TIMP-1 is present in BP fluid (6). However, in other studies, we have usually found that TIMP-1 and metalloenzymes, including 92-kD gelatinase, are produced in different locations (12, 33, 50, 56). This spatially distinct pattern of expression suggests that metalloproteinases act without impedance from TIMP-1. Since TIMP-1 has functions beyond its role as a metalloproteinase inhibitor (57, 58), the presence of 92-kD gelatinase and TIMP-1 in BP blister fluid does not mean that these proteins had interacted at sites of tissue destruction.

Cells probably secrete metalloproteases upon recognizing the extracellular target molecule suggesting that precise cell–matrix interactions regulate enzyme release (50, 59). Interestingly, in studies with intact skin from BP patients, leukocytes bind preferentially to the basement membrane at sites of BP lesions compared to that of unaffected normal-appearing pemphigoid skin (60). Thus, eosinophil chemotaxis and the expression and secretion of 92-kD gelatinase by these cells may be influenced by some factor originating at the site of BP formation. It seems likely that local mechanisms originating at the site of BP blister formation direct eosinophil chemotaxis and the expression and secretion of 92-kD gelatinase. Whether these events are mediated by immune complexes or by some as-of-yet unknown stimulus remains to be elucidated.

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