Respective Contribution of Neutrophil Elastase and Matrix Metalloproteinase 9 in the Degradation of BP180 (Type XVII Collagen) in Human Bullous Pemphigoid

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Bullous pemphigoid is a blistering disorder associated with autoantibodies directed against two components of hemidesmosomes, BP180 and BP230. Autoantibodies to the extracellular collagenous domain of BP180 are thought to play a key role in the pathogenesis of the disease. In a murine model of bullous pemphigoid, neutrophil elastase and 92 kDa gelatinase (matrix metalloproteinase 9) have been implicated in subepidermal blister formation via proteolytic degradation of BP180. In this study we sought to elucidate the contribution of these two enzymes to subepidermal blister formation by assessing the expression, localization, and activity of the two proteases in lesional skin, serum samples, and blister fluids obtained from 17 patients with bullous pemphigoid. The results indicate that (i) neutrophil elastase is found in skin biopsy specimens from bullous pemphigoid lesions and is recovered as active enzyme in blister fluids, and (ii) although proform of matrix metalloproteinase 9 is present in lesional skin, it is present only as proenzyme in blister fluids, which also contain high levels of tissue inhibitor of metalloproteinase-1. Next, the capacity of matrix metalloproteinase 9 and neutrophil elastase to degrade a recombinant protein corresponding to the extracellular collagenous domain of the BP180 was studied. Our data illustrate that (i) recombinant matrix metalloproteinase 9, neutrophil elastase, and blister fluid from bullous pemphigoid patients are all able to hydrolyze recombinant BP180; (ii) the pattern of recombinant BP180 proteolysis with blister fluid was similar to that obtained with neutrophil elastase; and (iii) recombinant BP180 degradation by blister fluid could be inhibited by chloromethylketone, a specific elastase inhibitor, but not by batimastat, a wide spectrum matrix metalloproteinase inhibitor. Our results confirm the importance of neutrophil elastase but not matrix metalloproteinase 9 in the direct cleavage of BP180 autoantigen and subepidermal blister formation in human bullous pemphigoid. ***Key words: bullous pemphigoid/matrix metalloproteinase/neutrophil elastase. J Invest Dermatol 117:1091–1096, 2001***

Bullous pemphigoid (BP) is an autoimmune disease characterized by subepidermal blisters associated with autoantibodies directed against two components of adhesion complexes called hemidesmosomes, BP180 and BP230 (Stanley *et al,* 1984; Labib *et al,* 1986; Giudice *et al,* 1993; Zillikens *et al,* 1997). BP180, also known as type XVII collagen, is a transmembrane protein whereas BP230 is a cytoplasmic protein of the plakin protein family involved in the attachment of the keratin filaments to the plasma membrane (Borradori and Sonnenberg, 1999). BP180 is the main antigenic target of BP autoantibodies (Labib *et al,* 1986; Giudice *et al,* 1992; Zillikens *et al,* 1997). BP antibodies have been shown to predominantly react with antigenic sites clustered within a noncollagenous BP180 region adjacent to the membrane spanning domain, the NC16A domain (Giudice *et al,* 1993; Matsumura *et al,* 1996; Zillikens *et al,* 1997).

Recently, it has been shown that a protease-mediated shedding of the extracellular domain of BP180 from human keratinocytes occurs under physiologic conditions. It consists in the release by the cells of a 120 kDa proteolytic extracellular fragment (Hirako *et al,* 1998; Schäcke *et al,* 1998). The contribution of neutral proteinases in BP180 shedding in blistering diseases has been emphasized previously (Stahle-Bäckdahl *et al,* 1994).

In an experimental model of BP in mice, anti-BP180 autoantibodies trigger subepidermal blister formation via complement activation, recruitment of neutrophils, and liberation of proteases. Among these enzymes, 92 kDa gelatinase (matrix metalloproteinase 9, MMP-9) and neutrophil elastase (NE) probably play a prominent role. Indeed, both MMP-9- and NE-deficient mice were found to be resistant to experimental BP (Liu *et al,* 1995; 1998; 2000). In this mouse BP model, Liu *et al* (2000) recently showed that MMP-9 acts to regulate NE activity by inactivating α_1-proteinase inhibitor, whereas NE is the major tissue-damaging enzyme that produces BP180 degradation and dermal–epidermal separation.

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Abbreviations: BP, bullous pemphigoid; MMP, matrix metalloproteinase; NE, neutrophil elastase; TIMP-1, tissue inhibitor of metalloproteinase-1.

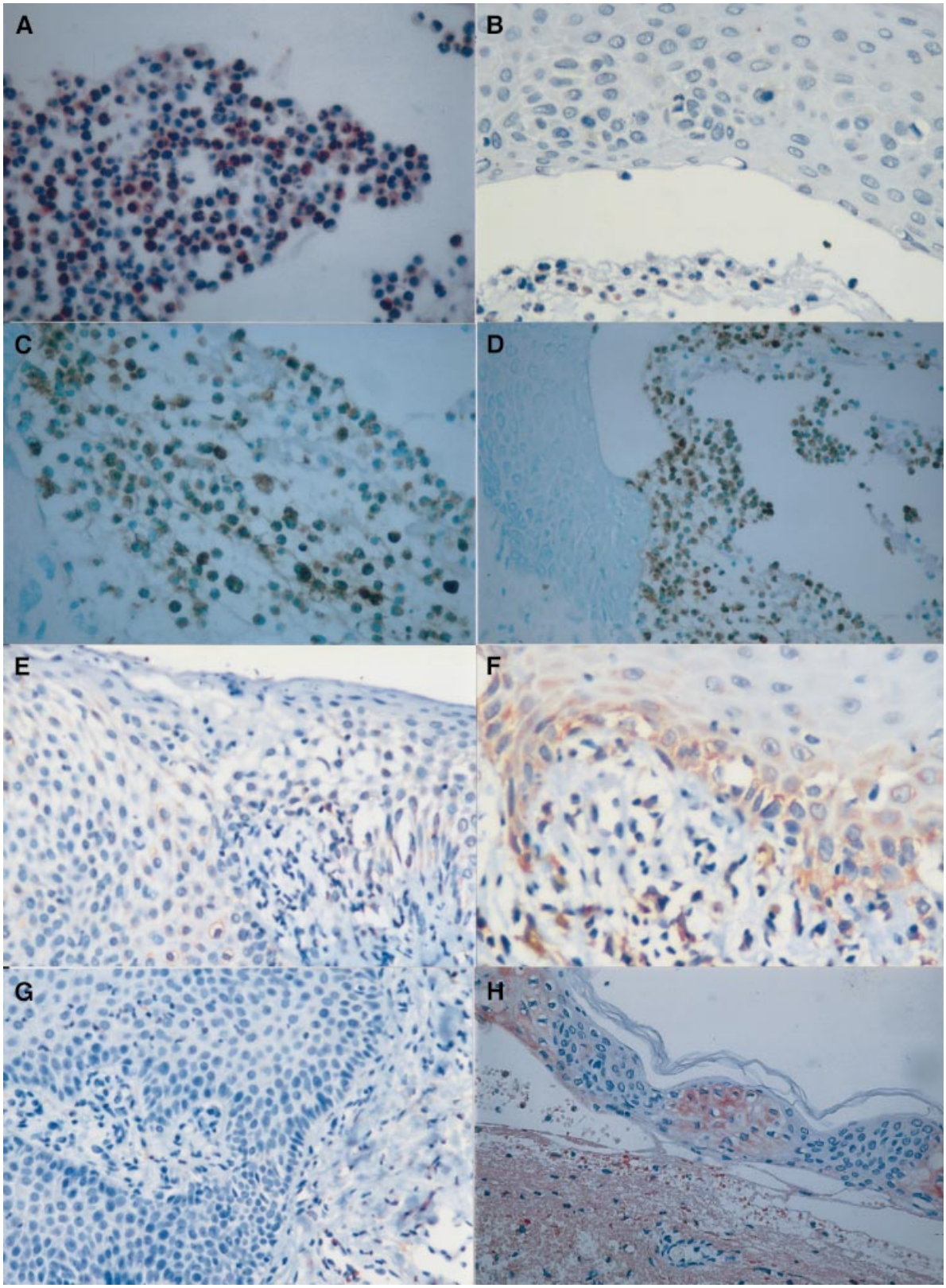

Figure 1. Immunolocalization of MMP-9 and human NE in skin lesions from BP and other inflammatory skin disorders. Labeling of gelatinase B (*B*, *E*, *F*, *G*, *H*) and human NE (*C*, *D*) on paraffin sections of skin biopsy specimens from a BP patient (lesional skin, i.e., recent blister), patients with other inflammatory skin disorders (eczema, lichen planus, psoriasis), and normal human skin using specific monoclonal antibodies as described in *Materials and Methods*. MMP-9 and NE labeling were found in inflammatory cells (eosinophils and neutrophils) (*A*, *C*) but not in adjacent keratinocytes (*B*, *D*). Positive immunostaining was found in altered keratinocytes of various inflammatory dermatoses: keratinocytes surrounded with spongiosis in eczema (*E*), necrotic basal keratinocytes in lichen planus (*F*), and keratinocytes in a several-days-old BP blister (*H*). Normal keratinocytes from psoriatic epidermis (*G*) or a fresh BP blister (*B*) did not show MMP-9 labeling.

In contrast to this elegant BP murine model, only few studies (Stahle-Bäckdahl *et al,* 1994) have been devoted to the respective contributions of these two proteases, i.e., MMP-9 and NE *in vivo* in human BP. *In vivo*, the activity of those proteases is controlled by specific inhibitors with molar concentrations that generally exceed enzyme levels (Basbaum and Werb, 1996).

In order to gain further insight into the pathophysiology of BP, levels of MMP-9, tissue inhibitor of metalloproteinase-1 (TIMP-1), and active NE were determined in blister fluids from patients. Furthermore, the ability of both purified enzymes and blister fluids from BP patients to degrade a recombinant form of the extracellular domain of BP180 was assessed. Finally the expression of MMP-9 and NE in diseased skin was assessed by immunohistochemistry. Our findings suggest that in human BP NE probably acts directly on BP180 autoantigen, whereas MMP-9, present at a much lower concentration and only as inactive zymogen, probably plays a minor role, if any, in the dermal–epidermal cleavage.

MATERIALS AND METHODS

Patients Seventeen consecutive patients with a mean age of 73 y (range 51–90 y) fulfilling the clinical and immunopathologic criteria of BP were studied. Serum samples were analyzed by indirect immunofluorescence microscopy studies (staining of the epidermal side of 1 M NaCl split skin) and by immunoblotting of epidermal extract as previously reported (Bernard *et al,* 1989). Circulating autoantibodies directed against BP180 and BP230 were detected in nine and five patients, respectively, whereas in three cases no serum reactivity was found by immunoblotting.

Blister fluid from six patients with a mean age of 75 y with widespread blistering was collected using a sterile syringe, centrifuged, and stored at –80°C until use. By immunoblotting, reactivity with BP180 and BP230 was detected in four and two, respectively, of the sera of these BP patients.

Immunohistochemistry Paraffin sections from lesional BP ($n = 17$) and other inflammatory skin diseases (eczema, lichen planus, psoriasis) as well as normal human skin biopsy specimens ($n = 8$) were studied. Sections were rehydrated and treated with 0.3% hydrogen peroxide for 5 min to quench endogenous peroxidase activity. Non-specific binding was blocked with phosphate-buffered saline (PBS) containing carrier protein and 15 mM sodium azide (Dako, Carpinteria, CA) for 20 min. Slides were incubated overnight at 4°C with anti-MMP-9 monoclonal antibody (2 μg per ml) (Oncologix, Gaithersburg, MD) or 30 min at room temperature with anti-NE monoclonal antibody (Dako). Negative controls were carried out by omitting the primary antibody. After three washes (10 min) in PBS, subsequent steps were performed using the peroxidase LSAB kit (labelled streptavidin–biotin method, Dako) according to the manufacturer's instructions. Peroxidase activity was revealed with 3-amino-9-ethylcarbazole chromogen, which gives a red-brown product. All slides were counterstained with Mayer's haematoxylin or green ethyl (Nawrocki *et al,* 1997).

Measurement of human NE activity in blister fluid Human NE activity was determined in blister fluid of BP patients ($n = 6$) using a specific chromogenic substrate: MeOSuc-Ala-Ala-Pro-Val-paranitro-anilide (Lestienne *et al,* 1980).

One nanogram or 5 ng of the active enzyme (Elastin Products Company) (standard curve) or a defined blister fluid volume was mixed in a 50 mM Tris-HCl buffer containing 150 mM NaCl, pH 8.0, and assay was initiated by addition of 0.6 mM chromogenic substrate. The rate of substrate hydrolysis by NE-containing blister fluid was linear from 0 to 180 min under our experimental conditions using a Titertek Multiskan Plus spectrophotometer at 405 nm. NE activity in blister fluid was expressed as ng HLE equivalent per mg protein.

Gelatin zymography Blister fluids were analysed in either the absence or the presence of 1 mmol per l acetylphenylmercuric acid (APMA) for 2 h at 37°C to activate gelatinases. Zymography was performed in 0.1% gelatin and 10% sodium dodecyl sulfate (SDS) polyacrylamide gels. After electrophoresis, gels were washed in 2% Triton-100 at room temperature and incubated at 37°C overnight in 50 mM Tris HCl, pH 7.6, 10 mmol per l $CaCl_2$, to allow renaturation of enzyme activity, without or with ethylenediamine tetraacetic acid in order to inactivate MMP. Gels were then stained with Coomasie blue, and gelatinase bands appeared white on a blue background (Emonard and Hornebeck, 1997).

Enzyme-linked immunosorbent assay (ELISA) Quantification of MMP-9 and TIMP-1 in blister fluids ($n = 6$ patients) was performed using ELISA kits from Calbiochem-Oncogene Research Products (France Biochem, Meudon, France) according to the manufacturer's instructions. Results were expressed as micrograms of enzyme or inhibitor per milligram of total protein. Total protein concentration in blister fluids was quantified using the Lowry method (Waterborg and Matthews, 1994).

CDNA construct and recombinant form of BP180 A eukaryotic expression plasmid (pcDNA3, Invitrogen, San Diego, CA) encoding for the entire extracellular domain of BP180 (residues 490–1497) including the last four COOH terminal amino acids of its transmembrane domain was used (Perriard *et al,* 1999). The recombinant form of BP180, which contained at its NH_2-terminus a c-myc epitope (MEQKLISQQDL) for immunodetection, was produced by an *in vitro* transcription and translation system (TNT T7 coupled Reticulocyte Lysate System, Promega, Madison, WI) as reported in detail elsewhere (Skaria *et al,* 2000). For radioactive control reactions, an amino acid mixture minus methionine with 40 μCi ^{35}S methionine was used.

Degradation studies The *in vitro* translation product was incubated with protease for 4 h at 37°C at a final concentration of 35.8 nM MMP-9 or 16.4 nM human NE, respectively, or with pure blister fluid from a patient with severe and active BP. To study inhibition of recombinant BP180 proteolysis by blister fluid, pure blister fluid was incubated for 30 min at 37°C with inhibitors of MMP-9 and NE, batimastat and a specific peptide chloromethylketone elastase inhibitor (MeOSuc-Ala-Ala-Pro-Val-CH_2Cl), respectively, at a final concentration of 1 μM. After incubation, samples were electrophoresed on 7.5% SDS polyacylamide gel and analyzed for BP180 proteolysis by autoradiography.

RESULTS AND DISCUSSION

Localization of proteases in BP lesions by immunohistochemistry In a previous study performed on human diseased skin of BP patients, *in situ* hybridization as well as immunohistochemistry showed that degranulated eosinophils produce large amounts of MMP-9, whereas the gelatinase mRNA signal was most pronounced at the floor of the forming blister where eosinophils accumulated (Stahle-Bäckdahl *et al,* 1994).

In our study, eosinophils as well as neutrophils of lesional BP skin showed positive immunostaining with anti-MMP-9 antibody whereas no signal was seen in sections processed without primary antibody (data not shown). Staining was negative within the epidermis of a recent blister (**Fig 1*A***). Interestingly, positive MMP-9 immunostaining was seen in necrotic or altered keratinocytes from several inflammatory skin diseases: keratinocytes surrounded with spongiosis (**Fig 1*E***), necrotic basal keratinocytes from lichen planus (**Fig 1*F***), and necrotic epidermal keratinocytes from an old BP blister (**Fig 1*H***). In contrast, no MMP-9

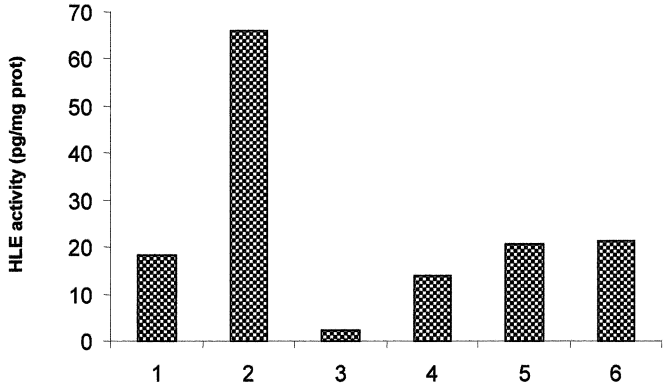

Figure 2. Human NE free enzyme activity in BP blister fluid of six patients. Human NE activity was assessed in blister fluids of six patients using a sensitive specific substrate (MeOSuc-$(Ala)_2$-Pro-Val-p-NA) as described in *Materials and Methods*. Variable NE activity was found in all blister fluids ranging from 1 pg per mg (patient 3) to 65 pg per mg (patient 2).

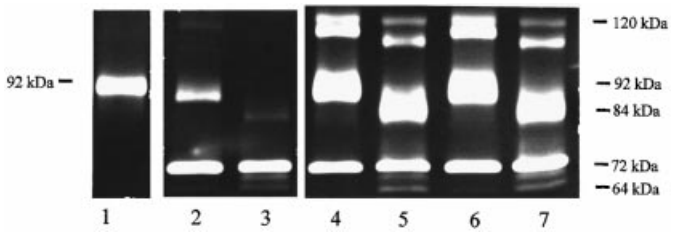

Figure 3. Presence of metalloproteinases in BP blister fluid by gelatin zymography. Blister fluid of three BP patients was analyzed using gelatin zymography (*lanes 2, 4, 6*) as described in *Materials and Methods*. Proenzymes present in blister fluids were activated by APMA (*lanes 3, 5, 7*). *Lane 1* is a control of recombinant MMP-9 (92 kDa). MMP-9 (92 kDa) and MMP-2 (72 kDa) were present in all blister fluids as proenzymes and could be activated by APMA. Higher gelatinolytic bands probably corresponded to dimer formation.

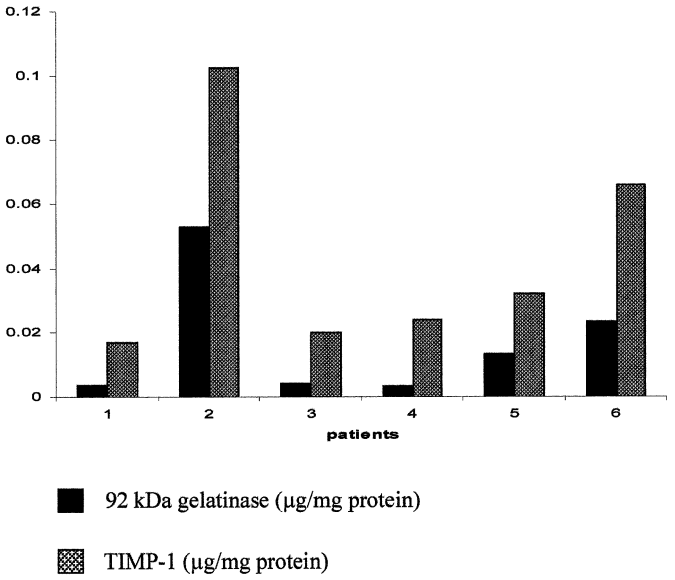

Figure 4. Level of MMP-9 and TIMP-1 in BP blister fluid of six patients. Levels of MMP-9 (*black column*) and TIMP-1 (*striped column*) were quantified in blister fluids from six patients using ELISA. On average, TIMP-1 concentration was 6.22-fold higher than MMP-9 level on a molar basis.

immunostaining was seen in acanthotic epidermis from psoriatic lesions (**Fig 1*G***), in a recent BP blister (**Fig 1*B***), or in normal human skin. Immunostaining of sections from lesional BP skin was also performed using NE antibody. Similarly, a strong labeling was observed predominantly in polymorphonuclear neutrophils close to the dermal–epidermal cleavage located within blisters (**Fig 1*C***), whereas no labeling was seen in keratinocytes (**Fig 1*D***).

Presence of elastase activity in BP blister fluids The serine protease NE is secreted as an active enzyme by azurophil granules of neutrophils, the concentration of which in cells is very high (5 mM). The proteolytic activity of this protease can be measured using MeOSuc-$(Ala)_2$-Pro-Val-p-NA, a specific chromogenic substrate for the enzyme (Lestienne *et al,* 1980). The presence of NE activity in various bullous diseases including BP has been documented in previous studies (Oikarinen *et al,* 1983; 1986; Welgus *et al,* 1986). In our study, blister fluids from all tested BP patients ($n = 6$) were found to contain active enzyme, the content of which varied from 1 to 65 pg NE per mg (mean 23 pg per mg ± 22) (**Fig 2**).

Quantification of MMP-9 and TIMP-1 in BP blister fluids MMP have been implicated in the pathophysiology of

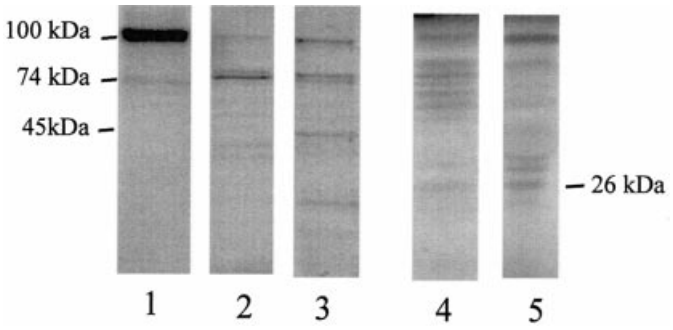

Figure 5. *In vitro* degradation of a recombinant form of the extracellular domain of BP180. ^{35}S-labeled recombinant BP180 (100 kDa) (*lane 1*, control) was incubated with 33 ng (35.8 nM) of MMP-9 (*lane 2*); 50 ng (62.4 nM) of MMP-9 (*lane 3*); 5 ng (16.9 nM) of NE (*lane 4*); or pure blister fluid from a BP patient (*lane 5*). Degradation of recombinant BP180 was observed with all tested proteases as with BP blister fluid. Main 74 kDa, 45 kDa, and 31 kDa proteolytic species were identified.

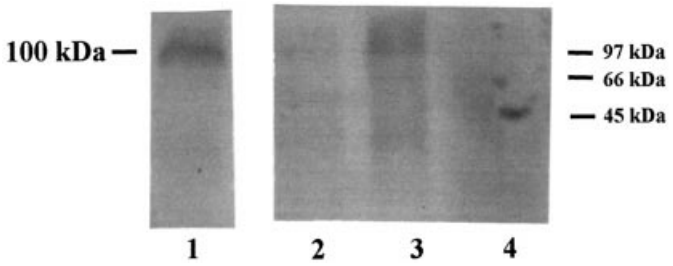

Figure 6. Inhibition of the degradation of the recombinant form of BP180 by protease inhibitors. The ^{35}S-labelled recombinant BP180 (100 kDa) (*lane 1*, control) was incubated with blister fluid from a BP patient, either alone (*lane 2*) or with peptide chloromethylketone elastase inhibitor (*lane 3*), or batimastat (*lane 4*). Only addition of elastase inhibitor prevented degradation of recombinant BP180 100 kDa protein, whereas batimastat did not.

several blistering diseases such as pemphigus, dermatitis herpetiformis, and BP (Oikarinen *et al,* 1983; Welgus *et al,* 1986). In BP, MMP-9 has been reported to be secreted in blister fluid as proenzyme (Oikarinen *et al,* 1993; Paquet *et al,* 1998).

In our study, the presence of gelatinases (MMP-9, MMP-2) within blister fluids was first examined by gelatin zymography. Several lysis bands at 120 kDa, 92 kDa, and 72 kDa could be seen in all samples (**Fig 3**). Proteolytic activity could be suppressed by adding to the incubation buffer either ethylenediamine tetraacetic acid (5 mM), which inhibits gelatinase activity by chelating Zn in the catalytic site of the enzyme, or batimastat (3 μM), a specific matrixin inhibitor (Yip *et al,* 1999) (not shown). Pretreatment with an organomercurial agent (APMA), which activates gelatinase through autocatalytic cleavage of the prodomain, led to the formation of lower gelatinolytic species at 84 kDa and 64 kDa. Consistent with previous observations (Paquet *et al,* 1998), BP blister fluids contained only proforms of the 92 kDa MMP-9 and the 72 kDa MMP-2. The gelatinolytic bands exhibiting lower electrophoretic migration most probably represented dimer complexes that could not be separated under the nondenaturing conditions used for the zymogram.

Previous studies have indicated that MMP-9 and TIMP-1 are coregulated (Buisson-Legendre *et al*, 2000) and are expressed in a tissue and temporal regulated manner during disease (Vaalamo *et al,* 1996). TIMP-1 level has never been previously studied in BP. In our study, ELISA was performed to assess the amounts of MMP-9

and TIMP-1 present in blister fluids from six BP patients. The results (**Fig 4**) indicate that MMP-9 levels in fluids vary from 0.03 to 0.05 μg per mg protein, a range close to values previously reported by Stahle-Bäckdahl *et al* (1994), whereas TIMP-1 concentrations vary from 0.01 to 0.1 μg per mg protein, with an approximately 5–10-fold molar excess of TIMP-1 over MMP-9. It is noteworthy that both MMP-9 and TIMP-1 appeared to be more strongly elevated in blister fluids obtained from the most inflammatory BP lesions. In addition, the relative levels of enzyme and inhibitors seemed to remain similar between patients, supporting the hypothesis that both proteinase and proteinase inhibitors were secreted by the same cell type such as polymorphonuclear cells in BP.

In some conditions, however, such as in cutaneous repair, MMP-9 and TIMP-1 can be produced by distinct cell types. In fact, transcripts for TIMP-1 are found confined exclusively to the dermis, whereas subpopulations of basal keratinocytes express MMP-9 after cutaneous injury (Parks, 1999). Our data confirm that MMP-9 is expressed in BP lesions as inactive zymogen but add new information about the presence in large excess at the same localization of its specific inhibitor TIMP-1. Taking these data together, we can conclude that, in human BP, MMP-9 probably could not act on its specific substrate within the basement membrane zone of the epidermis.

Degradation of recombinant BP180 by MMP and leucocyte elastase Murine BP180 was previously reported to be susceptible to proteolysis by MMP-9 and NE (Liu *et al*, 2000). Extensive degradation of this protein, however, was obtained at enzyme concentrations substantially greater than those found in blister fluid. A small portion of human recombinant BP180 ectodomain has also been reported to be efficiently degraded by 92 kDa MMP-9 (Stahle-Bäckdahl *et al,* 1994). We used the entire extracellular domain of BP180 to assess the capacity of the two proteinases present in BP lesions (MMP-9, NE) to degrade BP180. Under our experimental conditions, partial proteolysis was observed following incubation of the recombinant form of BP180 with 35.8 nM of MMP-9 or 16.9 nM NE for 4 h. For both enzymes the main proteolytic fragment exhibited a molecular weight of approximately 74 kDa, but degradation products of 45 kDa and 31 kDa could also be identified. At a final concentration of 35.8 nM, MMP-9 was able to degrade recombinant BP180 similarly to NE, whereas at a concentration of 3.5 nM MMP-9 did not demonstrate proteolytic activity on recombinant BP180. In parallel studies, we further analyzed the capacity of NE contained in blister fluid from BP patients to hydrolyze BP180 (**Fig 5**, *lane 4*). The pattern of proteolytic digestion of the recombinant form of BP180 obtained after incubation with blister fluid was similar to that obtained with NE. The different intensities of the signals in **Fig 5** (*lane 1*) and **Fig 6** (*lane 1*) are due to differences in the radiolabelling with ^{35}S of the recombinant BP180 fragment, as experiments were done at a different period of time with the radiolabeled protein although we used the same quantity of protein in both experiments. Importantly, protein degradation could be inhibited by a specific elastase inhibitor, peptide chloromethylketone (MeOSuc-Ala-Ala-Pro-Val-CH_2Cl), but not by batimastat, a wide spectrum MMP inhibitor (**Fig 6**, *lanes 3*, *4*).

In vivo, BP180, as one collagen type, exhibits triple helicoidal conformation with intervening sequences. *In vitro*, reconstitution of such structure proved to be rather difficult to achieve and only epidermal extracts were shown to contain trimeric complexes on Western blot run under nonreducing conditions (Pas *et al,* 1999). We first attempted to investigate BP180 proteolysis by human NE and MMP-9 using such epidermal extracts; data obtained were inconsistent due to the presence of protease inhibitors in the extracts. The capacity of NE and MMP-9 to degrade BP180 was therefore on a monomeric polypeptide fragment representing the entire extracellular part of the protein. Results obtained were similar to those reported with another baculovirus-encoded form of BP180 (Haase *et al,* 1998) (results not shown), indicating that both NE and MMP-9 could hydrolyse this polypeptide. In blister fluid from BP patients, however, human NE, but not MMP-9, was identified as an active enzyme able to degrade BP180. It suggested that, even at distant sites, control of that enzyme by its natural inhibitors, i.e., α_1-proteinase inhibitor and elafin, is impaired. As its concentration in the azurophilic granules (5 mM) is several orders of magnitude higher than any inhibitor concentration, local proteolysis of the dermal–epidermal junction proteins by human NE is probably uncontrolled. Furthermore, NE was shown to be able to bind to the cell surface of neutrophils, a mechanism that focuses and preserves catalytic activity of the enzyme.

Previous studies demonstrated that MMP-9 deficiency in mice did not modify neutrophil migration into the skin but inhibited subepidermal blistering of experimental BP (Liu *et al,* 1995). We here confirmed that, in humans, this enzyme could be identified only as a proenzyme at a molar concentration lower than active NE in BP. In addition, its natural occurring inhibitor TIMP-1 is present in molar excess, indicating that, contrary to human NE, this enzyme does not contribute to diffuse proteolysis. At the neutrophil pericellular environment, activated MMP-9 could degrade α_1-proteinase inhibitor, amplifying NE proteolysis by such mechanism. Binding of MMP-9 to neutrophil membrane, however, was shown to be very weak, and only cathepsin G, among skin elastases, was found to slowly activate membrane-associated pro-MMP-9. Together, our data further emphasize the crucial importance of NE in human BP; the contribution of other neutral endopeptidases, such as MMP-9, in disease progression is not as clear in humans as it is in mice (Liu *et al*, 2000). Finally, it must be emphasized that, contrary to dogs, whose BP180 autoantigen shares 87% identity with its human counterpart (Xu *et al*, 2000), mice do not develop spontaneous BP disease.

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