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Original Paper

Granulocyte-derived elastase and gelatinase B are required for dermal–epidermal separation induced by autoantibodies from patients with epidermolysis bullosa acquisita and bullous pemphigoid

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Abstract

Epidermolysis bullosa acquisita (EBA) and bullous pemphigoid (BP) are two clinically and immunologically distinct autoimmune subepidermal blistering skin diseases associated with IgG autoantibodies against the dermal–epidermal junction. BP antibodies are directed against the hemidesmosomal antigens BP180 and BP230, and those in patients with EBA target type VII collagen, a major component of anchoring fibrils. While the pathogenetic mechanisms of subepidermal blistering in BP have been previously studied using a passive transfer mouse model, the effector pathways of blister formation in EBA are largely unknown. Autoantibodies to type VII collagen and BP180 have recently been shown to induce leucocyte-mediated subepidermal cleavage in cryosections of human skin. The aim of the present study was to identify human leucocyte protease(s) instrumental in dermal–epidermal separation induced by autoantibodies to type VII collagen and BP180. When incubated with cryosections of human skin pretreated with IgG from patients with EBA or BP but not from patients with anti-laminin 5 mucous membrane pemphigoid or healthy controls, granulocytes were recruited to the dermal–epidermal junction and induced subepidermal splits. A combination of broad-range protease inhibitors as well as inhibitors of serine and matrix metalloproteases completely abolished dermal–epidermal separation induced by EBA or BP autoantibodies. When characterizing the proteases involved more specifically, selective inhibition of human leucocyte elastase or gelatinase B/MMP-9 was also found to result in suppression of blistering. These findings strongly suggest that elastase and gelatinase B are essential for granulocyte-mediated proteolysis resulting in dermal–epidermal separation in EBA and BP patients' skin.

Keywords: dermal–epidermal junction; *in vitro* model; leucocytes; matrix metalloprotease; protease; subepidermal blistering disease

Introduction

In various immune-mediated diseases, including vasculitis, systemic lupus erythematosus, rheumatoid arthritis, and Goodpasture's syndrome, tissue deposition of IgG contributes to enhanced effector cell activation which may result in fatal inflammatory responses [1]. Leucocytes, recruited and activated by IgG-containing immune complexes, may secrete proteases, reactive oxygen metabolites, and inflammatory mediators. Due to their capacity to degrade constituents of the extracellular matrix, leucocyte-derived proteases represent key effectors of tissue injury [2,3].

Autoimmune subepidermal blistering disorders are a group of organ-specific diseases characterized by subepithelial skin blisters and autoantibodies to various components of the dermal–epidermal junction (DEJ). In bullous pemphigoid (BP), the autoantibodies are directed against two hemidesmosomal proteins, BP230 and BP180 [4,5], while sera from patients with epidermolysis bullosa acquisita (EBA) recognize type VII collagen, a major constituent of anchoring fibrils [6,7]. The pathogenetic mechanisms of blister formation in BP have been extensively studied using an animal model based on the passive transfer of rabbit antibodies against the murine homologue

of BP180 into neonatal mice [8,9]. In this model, dermal–epidermal separation, induced by binding of antibodies to murine BP180, was dependent on complement activation and subsequent leucocyte infiltration into the skin of the mice [10,11]. Mice deficient for either elastase or gelatinase B/matrix metalloprotease (MMP)-9 were resistant to blister induction, demonstrating an essential role for these leucocyte-derived proteases in subepidermal cleavage in murine BP [12–14]. In contrast, little is known about mechanisms of blister induction in EBA, as all attempts to develop an animal model using EBA patients' autoantibodies transferred passively into neonatal mice, or adult severe combined immunodeficient (SCID) mice grafted with human skin, were unsuccessful [15–17]. Using a different approach, we recently demonstrated that IgG from patients with BP and EBA induces neutrophil-dependent dermal–epidermal separation in cryosections of human skin, suggesting the pathogenic relevance of these autoantibodies in human disease [18,19]. The aim of the present study was to examine the role of human leucocyte proteases in blister formation induced by autoantibodies to type VII collagen and BP180 in human skin.

Methods

For the experiments conducted, we obtained institutional approval issued by the ethics committee at the Medical Faculty of the University of Würzburg and by the ethics committee at the Medical Faculty of the University of Lübeck. In accordance with the Helsinki Principles, we obtained informed consent from all patients whose material was used in this study.

Antibodies

Serum samples were obtained from 5 EBA (3 with classic and 2 with inflammatory disease), 5 BP, and 3 mucous membrane pemphigoid patients prior to initiation of treatment and from 5 healthy volunteers. All patients were characterized by: (i) blisters on the skin, (ii) subepidermal blisters by histopathology, (iii) linear deposits of IgG and C3 along the DEJ by direct immunofluorescence microscopy, and (iv) reactivity to BP180 (BP patients), type VII collagen (EBA patients), or laminin 5 (mucous membrane pemphigoid patients) as detected by immunoblot analysis using cell-derived and/or recombinant forms of these proteins [18–20]. IgG fractions from serum of patients and controls were purified using Protein G Sepharose fast-flow affinity column chromatography (Pharmacia, Uppsala, Sweden) [19].

Isolation of peripheral blood leucocytes

Peripheral blood leucocytes from healthy volunteers were isolated by dextran sedimentation followed by three cycles of hypotonic red blood cell lysis in 0.2% NaCl. Granulocytes were obtained by density gradient centrifugation of leucocyte suspensions on Ficoll-Paque PLUS (Amersham Biosciences, Freiburg, Germany). Cells were washed twice in RPMI 1640 (Life Technologies, Karlsruhe, Germany) and resuspended in the same medium at a density of 3×10^7 cells/ml. Cell suspensions were kept on ice and cell viability was tested by flow cytometry and trypan blue exclusion as described below. Only preparations with viability $\geq$97% were used. Granulocyte preparations were demonstrated to contain >97% $CD16^{high}$ $CD14^{low}$ cells.

Flow cytometry analysis

The effects of protease inhibitors on leucocyte viability were analysed by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (both from Sigma, St Louis, MO, USA) to detect early apoptotic events and cell death, respectively. After incubation with protease inhibitors, 100 µl of cell suspensions, at a density of 5×10^6 cells/ml, were incubated for 10 min at room temperature with annexin V (5 µg/ml) and propidium iodide (20 µg/ml) in PBS containing 0.1% BSA and 0.02% NaN_3. The purity of granulocyte preparations was assessed by staining with monoclonal antibodies to CD14, CD16, and CD45. Cells (3×10^5) were incubated for 30 min at room temperature with a phycoerythrin (PE)-labelled monoclonal antibody to CD14 (clone UCHM-1) and FITC-labelled monoclonal antibodies to CD16 (clone 3G8) or CD45 (clone BRA-55) (all from Sigma) at concentrations recommended by the manufacturer, or with control isotype-matched irrelevant monoclonal antibodies at the same concentrations. After washing, two-colour flow cytometry analysis was performed on 10^4 events using a FACScan™ (Becton Dickinson, Mountain View, CA, USA) and CellQuest software (Becton Dickinson). Data were analysed using WinMDI version 2.8 software (Scripps Research Institute, http://facs.scripps.edu/software.html).

Protease inhibitors

Protease inhibitors used in this study included human α_2-macroglobulin (Calbiochem, Schwalbach, Germany), human α_1-proteinase inhibitor (α_1-PI) (Sigma), 4-(2-aminoethyl)-benzolsulfonylfluoride (AEBSF; Merck, Darmstadt, Germany), GM6001 (Calbiochem), E64 (Biomol, Hamburg, Germany), pepstatin A (Sigma), and methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone (MeOSuc-AAPV-CK; Bachem, Heidelberg, Germany). To block the activity of gelatinase B (GB), we used the recombinant single-chain variable fragment (3G12scFv) of the murine high-affinity inhibitory monoclonal antibody against human GB (REGA-3G12; Biophage Pharma, Montreal, Canada). REGA-3G12 does not cross-react with human gelatinase A, progelatinase A, or progelatinase B [21].

3G12scFv was demonstrated to possess the same specific activity as the intact monoclonal antibody [22]. Unless otherwise specified, reagents were used at the following working concentrations: α_2-macroglobulin 2 mg/ml, α_1-PI 5 mg/ml, AEBSF 1 mM, GM6001 100 μM, E64 100 μM, pepstatin A 100 μM, MeOSuc-AAPV-CK 200 μg/ml, and 3G12scFv 500 μg/ml.

Treatment of cryostat sections

Protein G affinity-purified IgG from patients' sera was diluted in PBS to an indirect immunofluorescence titre of 1 : 80 and the protein concentration was measured using absorbance at 280 nm. IgG from controls was used at the same protein concentration as patients' IgG. Six-micrometre cryosections of human skin were washed in PBS to remove embedding medium and incubated with 50 μl of diluted serum or antibody preparations for 90 min at 37 °C. Sections were washed twice with PBS and chambers were prepared as previously described [18,19]. Leucocyte suspensions were injected into the chambers and incubated for 3 h at 37 °C. Subsequently, cell suspensions were recovered from the chambers, centrifuged at 300 × *g* for 1 min, and the proteolytic activity of the supernatants was determined. Chambers were then disassembled and sections washed in PBS, air dried, and stained with haematoxylin and eosin. Each experiment was repeated twice to demonstrate the reproducibility of the results.

Elastase activity assay

Elastase activity in leucocyte supernatants was measured using methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (MeOSuc-AAPV-pNA) (Sigma), a substrate specific for human leucocyte elastase (HLE), as described previously [23] with some modification. Briefly, supernatants were diluted 10-fold with PBS and MeOSuc-AAPV-pNA was added to a final concentration of 0.2 mM. Baseline absorbance was read at 405 nm and the samples in the microtitre plates were then incubated for 2 h at 37 °C in the dark, followed by reading the absorbance at 405 nm again. Elastase activity was determined as the change of absorbance. OD readings were converted into moles using a standard curve obtained by serial dilution of purified HLE (Sigma). All samples were assayed in duplicate.

Gelatinase B activity assay

GB activity in granulocyte supernatants was determined as described [24] using the MMP gelatinase activity assay kit (Chemicon, Temecula, CA, USA). This highly sensitive assay utilizes biotinylated gelatin as a substrate and directly determines total gelatinolytic activity. As human neutrophils express GB but not gelatinase A [25], the gelatinolytic activity measured in our samples was considered to be mediated by GB. OD_{450} readings were converted to μg/ml from a standard curve obtained by serial dilution of purified human GB (Chemicon). All samples were assayed in duplicate.

Results

Protease inhibition abolishes leucocyte-mediated dermal–epidermal separation induced by EBA or BP autoantibodies

To investigate the role of leucocyte-derived proteases in subepidermal split formation, cryosections treated with autoantibodies from patients with BP, EBA, anti-laminin 5 mucous membrane pemphigoid, or normal human serum were incubated with peripheral blood leucocytes from healthy donors in the presence or absence of a cocktail of broad-spectrum protease inhibitors, including α_2-macroglobulin, α_1-PI, AEBSF, GM6001, E64, and pepstatin A. When incubated with cryosections pretreated with IgG from patients with BP (Figure 1a) or EBA (Figure 1c), peripheral blood leucocytes were recruited to the DEJ and induced subepidermal splits. Incubation of leucocytes in the presence of protease inhibitors completely abolished dermal–epidermal separation induced by BP (Figure 1b) or EBA autoantibodies (Figure 1d). No leucocyte recruitment or dermal–epidermal separation was seen in cryosections treated with serum from patients with anti-laminin 5 mucous membrane pemphigoid (Figure 1e) or healthy controls (Figure 1f).

Serine and matrix metalloproteases are essential for split induction in experimental EBA and BP

To determine the protease class responsible for induction of subepidermal splits in our experimental model, leucocytes were resuspended in RPMI without protease inhibitors or supplemented with (i) inhibitors of serine proteases, including α_1-PI and AEBSF; (ii) a broad-range hydroxamic acid-based inhibitor of MMPs (GM6001); (iii) a broad-range inhibitor of cysteine proteases (E64); and (iv) a broad-range inhibitor of aspartic proteases (pepstatin A). Treatment of cryosections with EBA or BP IgG, followed by incubation with leucocytes in the presence of serine protease inhibitors, abolished DEJ splitting and reproduced the findings observed when using the entire cocktail. In the presence of GM6001, EBA and BP autoantibodies recruited leucocytes to the DEJ, but dermal–epidermal separation did not occur. Cysteine protease inhibition by E64 had little influence on both recruitment of leucocytes and split induction. Interestingly, incubation of leucocytes with pepstatin A, an inhibitor of aspartic proteases, augmented dermal–epidermal separation. Control sections incubated with cells in the absence of protease inhibitors demonstrated leucocyte attachment at the DEJ and subepidermal split formation. Figure 2 shows representative results obtained using BP patients' sera. The viability of inhibitor-treated leucocytes was similar to that

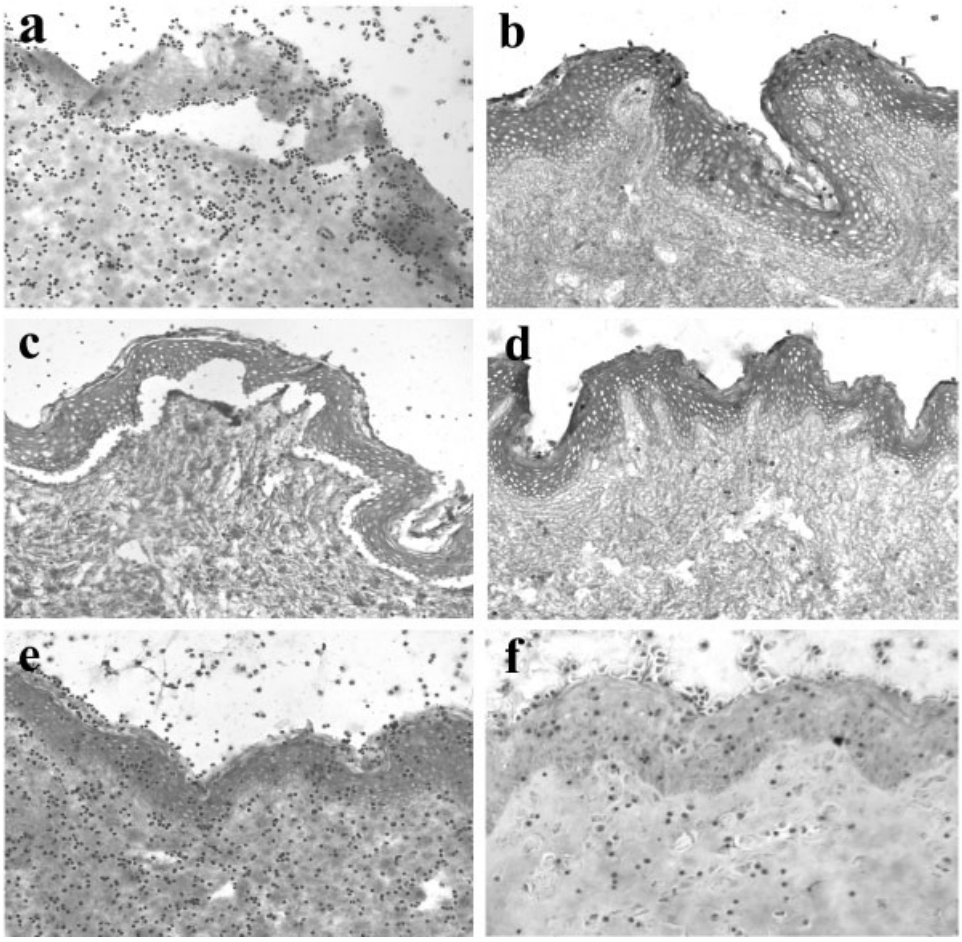

Figure 1. Dermal–epidermal separation in BP and EBA is mediated by proteolysis. Cryosections of human skin were treated with IgG from patients with BP (a, b), EBA (c, d), anti-laminin 5 mucous membrane pemphigoid (e), or normal human serum (f), and subsequently incubated with leucocytes from healthy volunteers. Antibodies from patients with BP (a) or EBA (c) induced dermal-epidermal separation in human skin, while those from patients with anti-laminin 5 mucous membrane pemphigoid (e) or healthy subjects (f) failed to do so. Incubation of leucocytes stimulated by IgG from patients with BP (b) or EBA (d) with a cocktail of broad-range protease inhibitors completely blocked subepidermal split induction. (H&E staining, original magnification $\times 200$)

of untreated cells ($\geq$97%) as shown by trypan blue exclusion and flow cytometry analysis using propidium iodide and annexin V staining as markers for cell death and early apoptosis, respectively (data not shown).

Leucocyte elastase plays a critical role in induction of subepidermal splits

HLE is the major serine protease responsible for extracellular proteolysis mediated by granulocytes [2]. To test the hypothesis that HLE is essential for dermal–epidermal separation in experimental EBA and BP, leucocytes were incubated with MeOSuc-AAPV-CK, a highly specific inhibitor of HLE [26]. Representative results of these experiments are presented in Figure 3 and Table 1. Leucocytes incubated without inhibitor induced dermal–epidermal separation (Figure 3a, c), while addition of 200 μg/ml MeOSuc-AAPV-CK completely inhibited the split formation induced by EBA or BP autoantibodies (Figure 3b, d). To demonstrate that these findings were due to specific and complete inhibition of HLE and not to a cytotoxic effect of the inhibitor, we determined levels of elastase activity and cell viability in MeOSuc-AAPV-CK-treated samples and controls. As shown in Table 1, treatment of leucocytes with MeOSuc-AAPV-CK resulted in a 10-fold reduction of HLE activity in the supernatants compared to untreated cells. In fact, HLE activity in supernatants of MeOSuc-AAPV-CK-treated cells was similar to the activity in supernatants of leucocytes incubated with α_1-PI plus AEBSF. The viability of inhibitor-treated leucocytes was demonstrated to be $\geq$97% by trypan blue exclusion and flow cytometry using propidium iodide and annexin V staining (data not shown).

Specific inhibition of gelatinase B/MMP-9 by 3G12scFv blocks dermal–epidermal separation in the cryosection model of subepidermal blistering disease

Based on the observation that the broad-range MMP inhibitor GM6001 completely inhibited dermal–epidermal separation, we subsequently investigated the role of GB in induction of subepidermal splits.

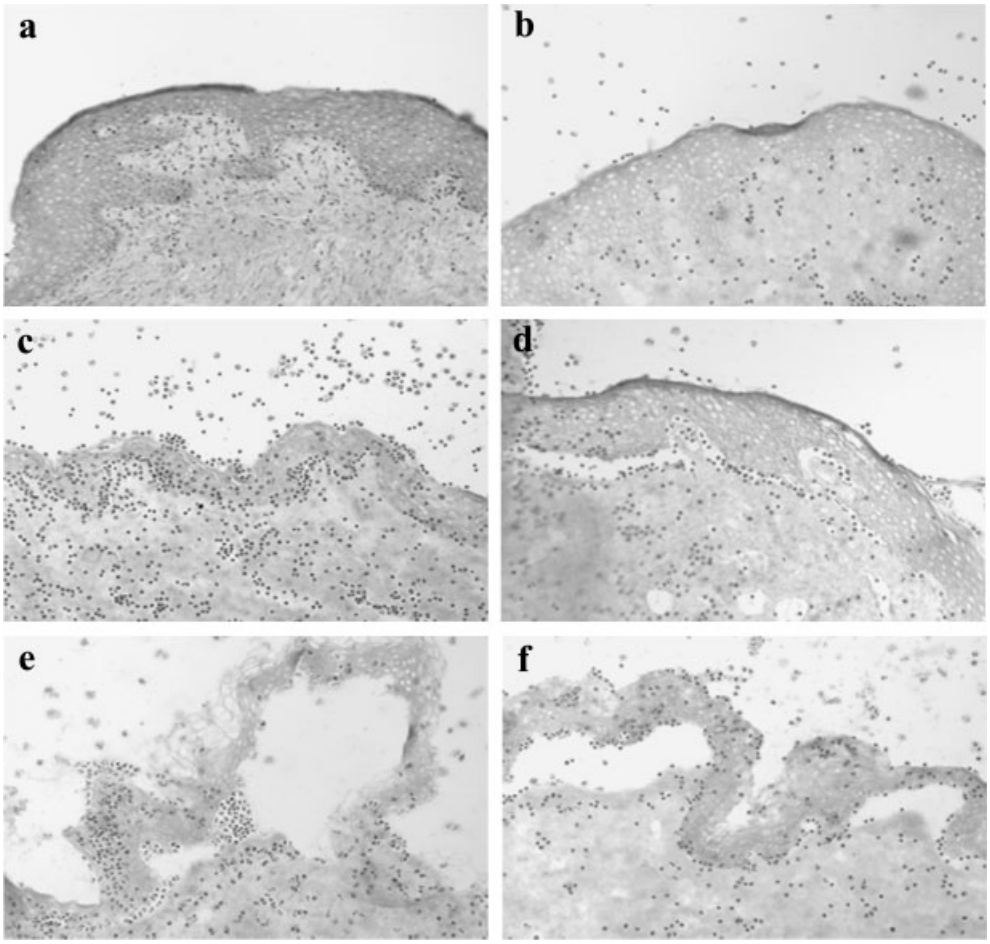

Figure 2. Serine and matrix metalloproteases are essential for split induction in experimental BP. Cryosections of normal human skin were treated with IgG from BP patients and incubated with peripheral blood leucocytes in the presence of (a) a cocktail of broad-range protease inhibitors (α_2-macroglobulin, α_1-PI, AEBSF, GM6001, E64, and pepstatin A), (b) α_1-PI + AEBSF, (c) GM6001, (d) E64, and (e) pepstatin A. Control sections were incubated with cells in the absence of protease inhibitors (f). (H&E staining, original magnification $\times$200)

Table 1. Elastase and gelatinase B activity in supernatants of human granulocytes triggered by autoantibodies from patients with BP or EBA

	Active elastase (nM)		Active gelatinase B (μg/ml)	
Protease inhibitor	**BP**	**EBA**	**BP**	**EBA**
None	16.68	18.75	0.139	0.149
α_1-PI + AEBSF	1.89	1.36	0.141	0.141
GM6001	16.81	17.71	0.032	0.024
MeOSuc-AAPV-CK	1.87	1.59	0.134	0.144
3G12scFv	16.33	18.60	0.029	0.031

Each enzyme inhibition experiment was performed twice and elastase or gelatinase B activity in granulocyte supernatants determined in duplicate as described in Methods. The table shows mean values of duplicate measurements from one representative experiment.

Figure 4 and Table 1 show representative results of this analysis. Granulocytes incubated without 3G12-scFv induced subepidermal splits in sections pretreated with BP or EBA sera (Figure 4a,c). In contrast, a recombinant single-chain variable fragment of inhibitory GB-specific monoclonal antibody REGA-3G12 completely blocked granulocyte-mediated dermal–epidermal separation induced by BP or EBA autoantibodies (Figure 4b,d). Incubation of granulocytes with 3G12scFv reduced GB activity to levels observed in the presence of GM6001 (Table 1). To ensure that we specifically measured GB activity in leucocyte supernatants, preparations of purified granulocytes expressing GB/MMP-9, but not MMP-2 [25], were used rather than whole leucocyte preparations.

Discussion

Activation of leucocytes through classically activated complement factors (C3a and C5a), and by engagement of their Fc receptors followed by the release of proteases, plays a crucial role in immune complex-induced inflammatory tissue destruction in various autoimmune diseases [1,3]. It has been suggested that dermal–epidermal separation in subepidermal blistering diseases also results from proteolytic digestion of the basement membrane mediated by inflammatory cells activated by autoantibodies bound to the DEJ [27]. The strongest support for this hypothesis comes from the work of Liu and coworkers,

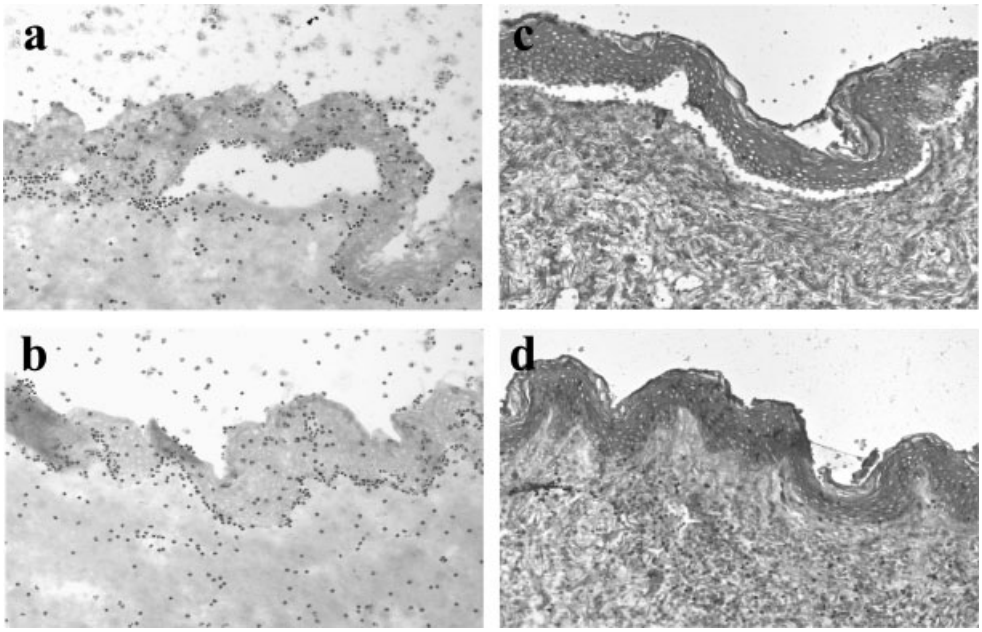

Figure 3. Leucocyte elastase plays a critical role in induction of subepidermal splits in the cryosection model of subepidermal blistering diseases. Cryosections of normal human skin were treated with IgG from patients with BP (a, b) or EBA (c, d) and then incubated with leucocytes in the presence (b, d) or absence (a, c) of MeOSuc-AAPV-CK, a highly specific inhibitor of leucocyte elastase. MeOSuc-AAPV-CK completely abolished subepidermal split induction (b, d), while leucocytes incubated without inhibitor continued to induce dermal–epidermal separation in sections treated with BP or EBA sera (a, c). (H&E staining, original magnification ×200)

who demonstrated that HLE- or GB-deficient mice are resistant to blister induction by passively transferred rabbit antibodies against murine BP antigen 180 [12–14]. However, it has remained uncertain if the same mechanisms apply to blister formation in the skin of patients with BP. EBA is another major autoimmune subepidermal blistering disease characterized by autoimmunity against type VII collagen. No information on the effector pathways of subepidermal blistering in EBA has been available to date. Recently, we have demonstrated that antibodies from patients with BP and EBA generate dermal–epidermal separation in human skin when co-incubated with leucocytes from healthy volunteers [18,19]. We now show that subepidermal splitting in this model system is completely abolished by a mixture of protease inhibitors. This finding demonstrates that subepidermal blistering in EBA, like that in BP, is a protease-mediated process. Interestingly, protease inhibition abolished dermal–epidermal separation induced by sera from patients with both the classic and inflammatory phenotype of EBA. Therefore, clinical differences observed between patients with EBA are probably related to *in vivo* factors that are not present in our model. In contrast to IgG from patients with EBA and BP, antibodies from patients with anti-laminin 5 mucous membrane pemphigoid bound to the cutaneous basement membrane but failed to recruit leucocytes to the DEJ and to induce dermal–epidermal separation in cryosections of human skin. Laminin 5-specific antibodies have been demonstrated to induce subepidermal blisters when passively transferred into neonatal mice or into SCID mice grafted with human skin [28,29]. Interestingly, these experimental blisters were completely devoid of leucocytic infiltrates and did not require complement activation for their induction [28–30]. The failure of laminin 5-specific antibodies to trigger subepidermal splitting in our *in vitro* model is probably due to a different mechanism of antibody-mediated blister induction in anti-laminin 5 mucous membrane pemphigoid as opposed to EBA and BP. It is likely that anti-laminin 5 antibodies directly interfere with the adhesive function of laminin 5 and do not initiate leucocyte-mediated proteolysis of the basement membrane. This mechanism of dermal–epidermal separation cannot be reproduced in our *in vitro* system utilizing non-vital cryosections of human skin.

Based on the biochemistry of the active site, proteases secreted by human leucocytes are classified into four major groups, including serine, metallo-, cysteine, and aspartic proteases [2]. Serine and metalloproteases, which are most active at a neutral pH, are thought to play a major role in the degradation of extracellular proteins. In contrast, cysteine and aspartic proteases preferentially function in an acidic environment and are predominantly involved in intracellular digestion of proteins [1,2]. Nevertheless, certain cysteine proteases, which have been shown to remain partially active at a neutral pH, are able to hydrolyse various extracellular matrix proteins [31,32] and may be involved in tissue destruction in chronic obstructive pulmonary disease [33] and arthritis [34]. Cathepsin D is the major aspartic protease of leucocyte lysosomes capable of degrading extracellular proteins such as fibronectin and proteoglycans [35]. To determine

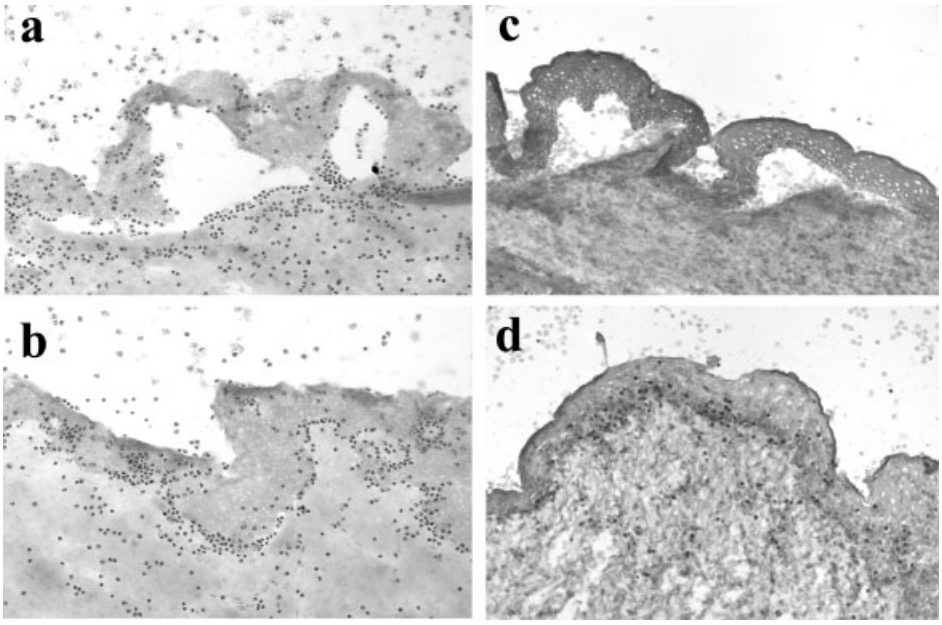

Figure 4. Specific inhibition of gelatinase B/MMP-9 by 3G12scFv blocks dermal–epidermal separation in the human skin *in vitro* model of BP and EBA. Cryosections of normal human skin were treated with IgG from patients with BP (a, b) or EBA (c, d) and then incubated with leucocytes in the presence (b, d) or absence (a, c) of a recombinant single chain variable fragment of monoclonal antibody REGA-3G12, which specifically blocks human gelatinase B. 3G12scFv completely abolished subepidermal split induction (b, d), while leucocytes incubated without inhibitor continued to induce dermal–epidermal separation in sections treated with BP or EBA sera (a, c). (H&E staining, original magnification $\times 200$)

the protease class responsible for subepidermal cleavage in our experimental model, we selectively inhibited each of the four major protease classes secreted by human leucocytes. While inhibition of either serine or matrix metalloproteases completely blocked dermal–epidermal separation induced by EBA or BP autoantibodies, split induction was only slightly diminished by a cysteine protease inhibitor. Interestingly, inhibition of aspartic proteases with pepstatin A enhanced blister formation. This unexpected finding may be related to the chemotactic and granulocyte-activating properties of pepstatin A [36,37].

The next set of experiments was aimed at characterizing the specific serine and metalloproteases responsible for split induction in our model of subepidermal blistering disease. For this purpose we focused on two major leucocyte proteases, namely HLE and GB. HLE is involved in the pathogenesis of tissue damage in a wide range of inflammatory conditions, including chronic obstructive pulmonary disease, rheumatoid arthritis, glomerulonephritis, and psoriasis [38], while GB plays an essential role in autoimmune encephalomyelitis [39] and antibody-induced arthritis [40,41]. Most importantly, the pivotal role of HLE and GB in autoantibody-induced basement membrane proteolysis is suggested by a large body of evidence originating from studies of BP: (i) high levels of HLE and GB may be detected in both blister fluid and lesional skin of BP patients [42–47]; (ii) knocking-out the gene encoding for HLE or GB renders neonatal mice resistant to the effects of rabbit antibodies against murine BP180 [12,13]; (iii) inhibition of HLE blocks subepidermal blister formation in the murine model of BP [13]; (iv) HLE and GB demonstrate broad substrate specificity for extracellular matrix proteins of the DEJ, including elastin, laminin, collagen types I, III, IV, VI, and XVII (BP180), proteoglycans, and fibronectin [13,14,46–49]. In contrast to BP, the role of HLE and GB in the pathogenesis of blister formation in EBA is unknown. In addition, direct experimental evidence of HLE and GB involvement in subepidermal blister induction by BP patient autoantibodies in human skin is missing due to the limits imposed by the rabbit/murine model system utilized for most studies on the pathogenesis of this disease. We now demonstrate that specific inhibition of HLE or GB completely blocks dermal–epidermal cleavage in cryosections of human skin treated with autoantibodies from patients with EBA or BP. Our findings strongly suggest that HLE- and GB-mediated proteolysis is critically important for blister formation in patients with EBA and BP and indicate that, despite divergent antigenic targets, dermal–epidermal separation in these two subepidermal blistering diseases occurs by a similar mechanism. The importance of HLE and GB as final effectors of subepidermal blister formation may open new approaches to topical treatment of these severe diseases.

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