A Novel 105-kDa Lamina Lucida Autoantigen: Association with Bullous Pemphigoid

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Several cases have been reported of patients with immune-mediated subepidermal blistering disorders whose autoantibodies react to antigens present on both the dermal and epidermal side of 1 M NaCl-split skin. In this report, we identify, localize, and characterize the basement membrane zone antigen corresponding to the dermal staining in a patient whose serum stains both the dermal and epidermal side of 1 M NaCl-split skin. This patient's serum contains autoantibodies directed against a 10S-kilodalton (kDa) dermal antigen and the 230-kDa epidermal (bullous pemphigoid) antigen. This novel 10S-kDa protein was previously identified as the sole antigen in a patient with a unique bullous disease whose autoantibodies were directed against only the dermal side of 1 M NaCl-split skin. This 10S-kDa antigen was identical by one- and two-dimensional immunoblot analysis in these two patients. By immunoblot analysis, autoantibodies from our patient labeled a 105-kDa protein within various extracts of human skin basement membrane. Immunoblot analyses using epitope-selected autoantibodies directed against the 105-kDa protein demonstrated that this antigen is independent and distinct from other known basement membrane antigens. The 105-kDa antigen is an extracellular matrix component of the basement membrane, which is synthesized and secreted by both keratinocytes and fibroblasts. Identical electrophoretic migration of cellular and secreted forms of the protein suggested there is no major post-translational modification of the protein. Immunomapping of normal human skin fractured through the dermal-epidermal junction by incubation in 1 M NaCl or by suction blistering demonstrated that the location of the 105-kDa antigen within the basement membrane zone is between the bullous pemphigoid antigens and two other lamina lucida components, laminin and nic tin. These data demonstrate clearly that a subepidermal autoimmune bullous disease may have autoantibodies directed against two distinct components of the dermal-epidermal junction. Key words: bullous pemphigoid/dermal-epidermal junction/salt-split skin. J Invest Dermatol 103:78-83, 1994

Immune-mediated subepidermal bullous dermatoses are characterized by the patients' clinical presentation and the specific basement membrane antigens targeted by their autoantibodies. Currently identified basement membrane autoantigens associated with immune-mediated subepidermal bullous dermatoses include the bullous pemphigoid antigens (230-kilodalton [kDa] and 180-kDa antigens) [1-3], epidermolysis bullosa acquisita antigen (type VII collagen) [4-6], nicein (identified by circulating autoantibodies in some atypical cases of cicatricial pemphigoid) [7], the 97-kDa antigen targeted by autoantibodies in some cases of linear immunoglobulin (Ig)A bullous dermatosis [8], and a novel 105-kDa basement membrane autoantigen recently identified as the sole antigen in a patient with a unique bullous disease [9].

Several cases of patients with immune-mediated subepidermal blistering disorders whose autoantibodies bind to both dermal and epidermal sides of the dermal-epidermal junction (DEJ) have been reported by indirect immunofluorescence (IIF) on 1 M NaCl-split skin (SSS) [10-13]. In all cases, the epidermal staining of the DEJ corresponded to the bullous pemphigoid antigens. However, the antigen responsible for the dermal staining has not been identified. In this study, we investigated a patient with an atypical bullous disease who has autoantibodies that bind to both the dermal and epidermal sides of the DEJ by IIF on SSS. We demonstrated that the patient's circulating autoantibodies identify two independent and distinct basement membrane antigens corresponding to the dermal and epidermal staining.

MATERIALS AND METHODS

Case Report An 85-year-old white man with a history of bullous pemphigoid (BP) was referred to the dermatology clinic for a recent exacerbation of bullous pemphigoid in the gluteal cleft region that was refractory to topical steroids. Over the next 3 months, the inflammatory mucocutaneous bullous eruption became generalized. Bullous lesions were reminiscent of bullous pemphigoid. However, palmo-plantar blisters and extensive mucosal involvement (oral, pharyngeal, esophageal, conjunctival, and corneal) made the clinical presentation atypical for bullous pemphigoid. No scarring or milia was observed at the site of healing lesions. Treatment with a combination of azathioprine and prednisone (40-80 mg) ameliorated the patient's skin condition except for the persistent palmo-plantar and mucosal involvement. Histopathologic examination of lesional skin revealed a subepidermal blister with a mild mononuclear cell infiltrate and occasional eosinophils and neutrophils.
Preparation of Epidermal and Dermal Extracts from 1 M NaCl Buffer

Normal human skin specimens were floated in 1 M NaCl, 8.0), 0.1 M p-mercaptoethanol, and a vertical suction blister. The immunomapping serum-free culture medium was collected, concentrated, and resuspended as serum. Fibroblasts were trypsinized, harvested, extracted in detergent buffer, then sonicated, and the guanidine-extracted proteins were centrifuged at 10,000 X g for 15 min, then the supernatants were dialyzed against cold water for 24 h and lyophilized. The extracts were resuspended in sodium dodecyl sulfate (SDS) sample buffer.

Preparation of Epidermal and Dermal Extracts from 1 M NaCl-Split Skin

Normal human skin specimens were floated in 1 M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM ethylenediamine tetraacetic acid (EDTA) at 4°C for 72 h [5]. Separated epidermis and dermis were solubilized with 6 M guanidine hydrochloride, 400 mM Tris-HCl (pH 8.0), 0.1 M β-mercaptoethanol, 50 mM EDTA, and 10 mM cysteine. The guanidine-extracted proteins were centrifuged at 10,000 X g for 15 min, then the supernatants were dialyzed against cold water for 24 h and lyophilized. The extracts were resuspended in sodium dodecyl sulfate (SDS) sample buffer.

Preparation of Epidermal Extract from Suction Blister

Suction blisters were raised on the normal human skin with a Kiistala suction blistering device [18] as described previously [14]. The blisters were unroofed and the proteins solubilized with 2% SDS, 0.125 M Tris-HCl (pH 6.8), 0.1 M dithiothreitol (DTT), 0.1 mM EDTA, 1 mM PMSF, and 50 μM N-ethyl maleimide (NEM). The protein extracts were then used for immunoblot analysis.

Preparation of Keratinocyte and Fibroblast Cellular Extracts and Culture Media

Human neonatal foreskin keratinocytes were initiated into culture in a low-calcium, serum-free keratinocyte growth medium (KGM; Clonetics, San Diego, CA). Cultured cells were passaged three times to remove any contaminating fibroblasts [19]. At subconfluence, the keratinocytes were trypsinized, extracted in detergent buffer (1% Nonidet-P-40 [NP-40], 0.5 M NaCl, 5 mM EDTA, 2 mM DTT, 40 mM Tris [hydroxymethyl]-amino-ethane [Tris], pH 7.5), and resuspended in SDS sample buffer. Fibroblast cultures were initiated from newborn foreskins and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum. Fibroblasts were trypsinized, harvested, extracted in detergent buffer, and resuspended in SDS sample buffer. Fibroblasts were cultured for 24 h in serum-free DMEM supplemented with 2% lactalbumin hydrolysate. The serum-free culture medium was collected, concentrated, and resuspended as above. Protein concentrations of the cellular extracts and culture media were determined by Lowry methods and Bradford assays (Bio-Rad, Hercules, CA), respectively.

Immunoblot Analyses

Experimental serum and control sera were reacted by immunoblot analyses [21,22] with extracts of normal human cultured keratinocytes and fibroblasts, serum-free conditioned media, epidermal protein extracts of suction blister roosters and SSS, and dermal protein extracts from SSS. Our patient’s serum was compared with sera from two patients with well-characterized BP, one patient with cicatricial pemphigoid, five normal humans, and a monoclonal antibody against type VII collagen (L3D) [16]. Horseradish peroxidase-conjugated goat anti-human IgG (heavy chain specific) (Cappel) was used as the secondary antibody, followed by standard development with chloronaphthol (Bio-Rad).

To determine the cellular origin of the 105-kDa protein, equal amounts of total protein extracted from human keratinocyte and fibroblast cultures were reacted with sera from our patient and the previously reported patient with antibodies against only the 105-kDa antigen [9]. To determine whether the 105-kDa protein was a transmembrane, extracellular, or secreted protein, we reacted the serum from both patients with equal amounts of protein in keratinocyte and fibroblast culture media.

Immunoblot Analyses with Epitope-Selected Autoantibodies Against the 105-kDa Antigen

To demonstrate the absence of cross-reactivity between the 105-kDa antigen and the BP antigens or EBA antigen, and to exclude the possibility that the 105-kDa antigen was a breakdown product of either of these, patient autoantibodies against the 105-kDa antigen were epiphenomapped against normal human keratinocyte extracts. Briefly, extracts of keratinocytes were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to a nitrocellulose filter. A horizontal strip of the 105-kDa protein was cut out and incubated overnight at 4°C with the patient’s serum (1/50 dilution). After extensive washing of the membrane, the bound antibodies were eluted by vigorous agitation in 1 ml of elution buffer (5 mM glycine, 150 mM NaCl, 0.5% Triton X 100, pH 2.3) and then neutralized with a 1/10 volume of 50 mM Tris, pH 7.4 [23].

Figure 1. Direct and indirect immunofluorescence of the patient’s skin and serum. a) Direct immunofluorescence microscopy of a biopsy taken from the patient’s skin 1 inch from a lesion and then separated through the DEJ by incubation in 1 M NaCl. b) Indirect immunofluorescence using the patient’s serum diluted 1:100 to label normal human skin in which the DEJ has been fractured by incubation in 1 M NaCl. Both cryostat sections in a and b were reacted with fluorescein-conjugated goat anti-human IgG. Open arrows, epidermal side and solid arrows, dermal side of the fractured skin. Bar, 50 μm.
Figure 2. Immunoblot analysis of the patient’s serum against dermal and epidermal components. Immunoblotting of our patient’s serum (P1), epitope-selected antibodies of P1 against the 105-kDa antigen (eP1), bullous pemphigoid serum (BP), a monoclonal antibody against type VII collagen (L3D) and normal human sera were reacted against proteins extracted from keratinocyte culture (a) or dermal proteins isolated from SSS (b), followed by visualization with peroxidase-conjugated goat anti-human IgG (for human sera) or peroxidase-conjugated goat antimouse IgG (for the monoclonal antibody). Arrow, 230 kDa (a); 290 kDa (b).

RESULTS

Direct Immunofluorescence Microscopy DIF performed on the patient’s intact perilesional skin demonstrated linear deposits of IgG at the DEJ. When the patient’s perilesional SSS was used in the same procedure, a linear deposition of IgG on the epidermal and dermal side of the DEJ was observed (Fig 1a). Minor amounts of IgA and complement (C3) were deposited along the dermal and epidermal side of the fractured skin. No deposits of IgM were detected.

Indirect Immunofluorescence Microscopy IIF on monkey esophagus demonstrated that the patient’s serum contained IgG autoantibodies that bound to the BMZ. To locate the target of our patient’s autoantibodies, IIF was performed on normal human SSS. The staining pattern revealed that the patient’s serum antibodies bound both the epidermal and dermal sides of the DEJ (Fig 1b). Serial dilutions demonstrated an extinction titer of circulating IgG twofold higher on the dermal side (1:800) than on the epidermal side (1:400). No circulating IgA autoantibodies reactive to either the dermal side or the epidermal side of SSS were detected in our patient, whereas serum from three typical patients with linear IgA bullous dermatosis demonstrated IgA antibodies against the epidermal side of SSS (not shown).

Immunoblot Analyses To determine whether the epidermal staining seen with immunofluorescence microscopy corresponded to the bullous pemphigoid antigens, we reacted our patient’s serum and five well-characterized bullous pemphigoid sera against proteins extracted from cultured human keratinocytes. As shown in Fig 2a, our patient’s serum recognized the 230-kDa bullous pemphigoid antigen, as did the bullous pemphigoid sera. In addition, our

Figure 3. Comparison of the skin targets by immunoblot analysis of the autoantibodies of our patient serum (P1) and the previously described serum containing anti-105-kDa antibodies (P2) (please see reference [9]). The two sera were diluted 1:100 and reacted against proteins extracted from cultured keratinocyte monolayers or isolated from conditioned culture medium. The bound antibodies were visualized by a peroxidase-conjugated goat anti-human IgG. Arrow, 230 kDa.

Figure 4. The expression of the 105-kDa antigen by keratinocyte and fibroblast cultures. Immunoblot analysis was performed with our patient’s serum against equal amounts of protein extracted from cultured fibroblasts and keratinocytes and their corresponding conditioned media. A) Immunoreactivity of the 105-kDa antigen as detected by our patient’s serum. B) Dosimetry of the corresponding bands.
patient's serum clearly recognized another 105-kDa epidermal antigen (Fig 2a, lane PI) that was not recognized by any of the bullous pemphigoid sera (Fig 2a, lane BP).

To determine whether the dermal staining corresponded to the EBA antigen (type VII collagen), we compared our patient's serum with a monoclonal antibody to type VII collagen by immunoblot analysis against proteins extracted from dermis isolated from SSS. As shown in Fig 2b, the monoclonal antibody against type VII collagen identified the 290-kDa antigen (lane L3D), previously determined to be the alpha chain of type VII collagen (anchoring fibrils), whereas our patient's serum did not. Our patient's serum did, however, readily label a 105-kDa protein within this dermal extract (Fig 2b, lane Pt).

To demonstrate that the 105-kDa antigen was a distinct antigen and not a degradation product of the BP or EBA antigen, we performed immunoblot analysis against keratinocyte extracts with our patient's epitope-selected autoantibody against the 105-kDa antigen. As shown in Fig 2a (lane ePt) the affinity-purified antibody identified the 105-kDa antigen, but not the BP or EBA antigen.

We also compared our patient's serum with that of the previously identified patient whose autoantibodies react only against the 105-kDa antigen [9]. As shown in Fig 3, the 105-kDa band labeled by

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**Figure 5.** Immunomapping of the 105-kDa lamina lucida antigen and a comparison with the other known components of the DEJ. Indirect immunofluorescence was performed using normal human skin in which the DEJ was fractured either by incubation in 1 M NaCl (SSS) or by suction blistering. a, c, e) IIF performed on SSS; b, d, f) IIF performed on suction blister skin. a, b) IIF staining using a bullous pemphigoid patient's serum. c, d) IIF staining using the previously described serum [9], which only has antibodies to the 105-kDa antigen and not to the bullous pemphigoid antigen. e, f) IIF staining using anti-nicein antibody (GB3). Epidermis and dermis are labeled E and D, respectively. Solid arrows, side of the staining. Bar, 50 μm.
Table I. Comparative Immunolocalization of the 105-kDa Antigen to Other Basement Membrane Components on Salt-Split and Suction-Blistered Human Skin

<table>
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<tr>
<th>BP antigen</th>
<th>105 kDa</th>
<th>Laminin</th>
<th>NICE</th>
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* Location of basement membrane components was assessed by IIF on salt-split and suction-blistered human skin using sera from bullous pemphigoid patients, serum from the previously reported patient with autoantibodies to only the 105-kDa antigen, polyclonal antibodies to laminin, and monoclonal antibodies to ntecin/kalinin (gift from Dr. Patrick Verrando, University of Nice) [17], the L3D monoclonal antibody to type VII collagen [16], and a monoclonal antibody to type IV collagen [14].

our patient’s serum has electrophoretic migration identical to the protein labeled by the serum of the previously identified patient.

To examine the cellular origin of the 105-kDa antigen and to determine whether it is a transmembrane, intracellular, or secreted protein, we performed immunoblot analysis against proteins extracted from cultured monolayers of keratinocytes and fibroblasts and compared these with their corresponding conditioned culture media. As demonstrated in Fig 4A, our patient’s serum recognized a 105-kDa antigen in protein extracted from both monolayers and conditioned medium from both keratinocytes and fibroblasts. Because equal amounts of total protein were used in these experiments, dosimetry of the patient’s serum reactivity in these immunoblots reflects the relative production of the 105-kDa protein by fibroblasts and keratinocytes in vitro. As demonstrated in Fig 4B, the similar dosimetric values of the conditioned media and cellular extracts indicate that both keratinocytes and fibroblasts secrete a similar amount of the 105-kDa protein.

**Immunolocalization of the 105-kDa Antigen Within the BMZ**

To more precisely delineate the location of the 105-kDa protein within the DEJ, we performed immunomapping of SSS and suction blistered skin with the previously identified patient’s serum to the 105-kDa antigen [9], sera from two patients with BP, an EBA serum, a monoclonal antibody against type VII collagen [16], a monoclonal antibody against ntecin (GB3) [17], a monoclonal antibody against type IV collagen, and antibodies against laminin. As demonstrated in Fig 5 and Table I, the BP serum stains only the epidermal side of the DEJ on both suction-blistered and SSS (Fig 5a,b), whereas the antibodies against laminin, ntecin, type IV collagen, and type VII collagen/EBV antigen stain only the dermal side of both substrates (Fig 5e,f). In contrast, differential staining was observed only with the anti-105-kDa antigen serum. Whereas this serum stains only the dermal side of SSS, it stains both the epidermal and dermal side of suction blistered skin (Fig 5c,d). The labeling was identical on three suction blisters tested. These results were confirmed by immunoblot analysis of dermal and epidermal extracts obtained by suction blister and SSS. The anti-105-kDa antigen serum labeled a 105-kDa protein in both epidermal and dermal extracts from suction blisters, but only in the dermal extract from SSS (illustration not shown).

**DISCUSSION**

In this study, we analyzed the circulating autoantibodies of a patient with an atypical case of bullous pemphigoid whose serum reacts to both the dermal and epidermal side of the DEJ by IIF on SSS. We identified a 105-kDa antigen corresponding to the dermal staining, and the 230-kDa bullous pemphigoid antigen corresponding to the epidermal staining. Several reports have described patients with bullous disorders whose sera recognize separate epitopes on SSS in a pattern similar to our patient. In these cases, the epidermal antigens were identified as the bullous pemphigoid antigens, whereas the basement membrane antigen responsible for the dermal staining was not identified [10,11]. In this study, we have identified and characterized the 105-kDa dermal antigen as a candidate targeted by a subset of “bullous pemphigoid” serum that labels both the dermal and epidermal sides of the fractured DEJ [10–13].

Recently, a patient with a unique subepidermal blistering disorder was found to have circulating autoantibodies that bind only the dermal side of the DEJ. The target of these autoantibodies was identified as a new 105-kDa lamina lucida basement membrane antigen [9]. This previous report clearly demonstrated that a basement membrane antigen other than type VII collagen can be responsible for the dermal staining of the DEJ by immunofluorescence on SSS. Identical electrophoretic migration of the 105-kDa antigen identified by our patient and the previously reported patient suggest that this antigen is the same in both cases.

We further localized this novel 105-kDa basement membrane autoantigen in relation to other BMZ components by performing IIF on suction blister and SSS using anti-105-kDa antigen serum. The different staining patterns observed with SSS and suction blister skin demonstrate that the cleavage planes of the two substrates are separate and distinct. In SSS, the cleavage plane is between the BP antigen and the 105-kDa antigen. In suction-blistered skin, however, the cleavage plane is within the 105-kDa antigen area of the lamina lucida, thus lower than that of SSS. These results, along with the identification of the 105-kDa antigen by immunoblot analysis on suction-blistered epidermis, demonstrate that the 105-kDa antigen is a unique lamina lucida antigen that is distinct from other basement membrane components and is located between the BP antigen and two other lamina lucida proteins, laminin and ntecin.

It is known that keratinocytes and fibroblasts are the two major sources of production of the BMZ components. For example, human keratinocytes and fibroblasts synthesize fibronectin [24], type VII collagen [25,26], and laminin [27]. We have demonstrated that keratinocytes and fibroblasts, in vitro, secrete the 105-kDa antigen and produce similar amounts of the 105-kDa protein. As shown in this report (Fig 4A), the 105-kDa antigen has the same electrophoretic migration whether it is obtained from keratinocyte and fibroblast cellular extracts or from the corresponding conditioned media (i.e., the secreted form). This suggests that there is no significant post-translational modification of the protein.

Within the basement membrane beneath stratified squamous epithelium there is only one potential candidate macromolecule with a molecular weight of 105 kDa that could be the same as the 105-kDa protein described in this study. The candidate protein is a component of anchoring filaments, which are thin electron-dense structures below hemidesmosomes that traverse the lamina lucida. Anchoring filament proteins have been described as epiligrin [28], kalinin [29], and BM600/nicein [30]. Recent evidence strongly suggests that these molecules are identical [30]. This 105-kDa anchoring filament-associated protein is the product of processing an intracellular protein of 155 kDa. This anchoring filament component is synthesized only by keratinocytes and not by fibroblasts [28].

The 105-kDa basement membrane protein identified by the autoantibodies of our patient, and the previously identified patient [9], is clearly synthesized by both keratinocytes and fibroblasts, does not have an intracellular precursor of higher molecular weight, and does not co-localize with BM600/nicein on suction-blistered skin. For these reasons, we conclude that the 105-kDa antigen targeted by these two patients’ autoantibodies is distinct from epiligrin, kalinin, and BM600/nicein.

Another component of the BMZ within the lamina lucida that would have a molecular size close to the 105-kDa antigen is the antigen described by Zone et al [8]. This 97-kDa antigen is labeled specifically by IgA autoantibodies in the sera of patients with linear IgA bullous dermatosis. Our patient had no circulating IgA autoantibodies against the BMZ when tested by IIF. Furthermore, the 97-kDa antigen and the 105-kDa antigen are distinct based on their localization by immunomapping. The 97-kDa antigen always localizes to the epidermal roof of SSS [8], whereas the 105-kDa antigen always localizes to the dermal floor.
In conclusion, this study identifies the dermal antigen in a patient with an atypical bullous disease whose serum stains the epidermal and dermal side of 1 M NaCl-split skin. This antigen is a novel 105-kDa protein located in the lamina lucida above laminin and in some immunemediated bullous disease or may be a co-target of some immune-mediated bullous disease or may be a co-target associated with bullous pemphigoid antigen.

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