Staphylococcal Exfoliative Toxin B Specifically Cleaves Desmoglein 1

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Staphylococcal scalded skin syndrome and its localized form, bullous impetigo, show superficial epidermal blister formation caused by exfoliative toxin A or B produced by *Staphylococcus aureus*. Recently we have demonstrated that exfoliative toxin A specifically cleaves desmoglein 1, a desmosomal adhesion molecule, that when inactivated results in blisters. In this study we determine the target molecule for exfoliative toxin B. Exfoliative toxin B injected in neonatal mice caused superficial epidermal blisters, abolished cell surface staining of desmoglein 1, and degraded desmoglein 1 without affecting desmoglein 3 or E-cadherin. When adenovirus-transduced cultured keratinocytes expressing exogenous mouse desmoglein 1 or desmoglein 3 were incubated with exfoliative toxin B, desmoglein 1, but not desmoglein 3, was cleaved. Furthermore, cell surface staining of desmoglein 1, but not that of desmoglein 3, was abolished when cryosections of normal human skin were incubated with exfoliative toxin B, suggesting that living cells were not necessary for exfoliative toxin B cleavage of desmoglein 1. Finally, *in vitro* incubation of the recombinant extracellular domains of desmoglein 1 and desmoglein 3 with exfoliative toxin B demonstrated that both mouse and human desmoglein 1, but not desmoglein 3, were directly cleaved by exfoliative toxin B in a dose-dependent fashion. These findings demonstrate that exfoliative toxin A and exfoliative toxin B cause blister formation in staphylococcal scalded skin syndrome and bullous impetigo by identical molecular pathophysiologic mechanisms. ***Key words: cadherin/impetigo/pemphigus foliaceus/skin infection/SSSS. J Invest Dermatol 118:845–850, 2002***

Staphylococcal scalded skin syndrome (SSSS) is a generalized blistering skin disease induced by the exfoliative (epidermolytic) toxin (ET) of *Staphylococcus aureus* (Melish and Glasgow, 1970; Melish *et al*, 1972). It was also known in the past as Ritter's disease, dermatitis exfoliativa neonatorum, or pemphigus neonatorum. It is a disease primarily affecting infants and young children, but adults can also be affected. Its clinical manifestations begin abruptly with fever, skin tenderness, and erythema, followed by large sheets of epidermal separation involving the entire skin surface within hours to days. The mortality is still 3% in children (Gemell, 1995), and over 50% in adults with underlying diseases despite antibiotic treatment (Cribier *et al*, 1984). SSSS caused by antibiotic-resistant strains of *S. aureus* has recently emerged as an even more serious problem (Yokota *et al*, 1996; Acland *et al*, 1998). The pathogenic role of ET in SSSS is well established. For example, ET injected into neonatal mice causes extensive blisters similar to the disease manifestations in human neonates (Melish and Glasgow, 1970). In SSSS, *S. aureus* is present at distant foci such as the pharynx, nose, ear, or conjunctiva, and ET produced by *S. aureus* gets into circulation and causes exfoliation at remote sites, whereas in bullous impetigo, a localized form of SSSS, *S. aureus* is present in the lesions.

ET has two major serotypes A and B (ETA and ETB). In the U.S.A. and Europe more than 80% of toxin-producing *S. aureus* produce ETA (Cribier *et al*, 1984), whereas in Japan there is a predominance of ETB-producing strains (Murono *et al*, 1988). The gene encoding ETA is located on the chromosome whereas the gene encoding ETB is found on a large plasmid. The genes for ETA and ETB have been cloned and their amino acid sequences have been deduced (O'Toole and Foster, 1986; Lee *et al*, 1987). The mature proteins of ETA and ETB are 242 and 246 residues, respectively, after their signal sequences are cleaved. The ETA and ETB amino acid sequences are about 40% identical to each other.

Although previous ultrastructural studies have shown intercellular cleavage with split desmosomes after injection of ET into neonatal mice, it was not clear whether disruption of desmosomes is an initial event or a secondary event that occurs only after edema caused by other intercellular pathology (Lillibridge *et al*, 1972; Melish *et al*, 1974; Elias *et al*, 1975; Dimond *et al*, 1977). ETs have also been reported to be superantigens (Choi *et al*, 1989; Kappler *et al*, 1989), which bind to major histocompatibility complex class II molecules on antigen-presenting cells and to the variable parts of the T cell receptor, resulting in polyclonal T cell activation. Other investigators have argued, however, that the previous demonstration of superantigenic activity with ETs was probably due to contamination with other staphylococcal enterotoxins (Fleischer and Bailey, 1992; Fleischer *et al*, 1995). Therefore, until recently, the molecular mechanism of the epidermal separation by ET had

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Abbreviations: Dsg1, desmoglein 1; ET, exfoliative toxin; ETA, exfoliative toxin A; ETB, exfoliative toxin B; SSSS, staphylococcal scalded skin syndrome.

been unclear, even 30 y after the pathogenic role of ET was demonstrated using neonatal mice in 1970 (Ladhani *et al*, 1999). The clue to how ETA might cause the blister was provided by studies of the pathophysiology of pemphigus foliaceus, an autoimmune blistering disease, in which inactivation of the desmosomal cadherin desmoglein 1 (Dsg1) by autoantibodies was shown to cause clinical and histologic blisters similar to those seen in bullous impetigo and SSSS (Stanley, 1993; Amagai, 1999; Mahoney *et al*, 1999). These observations led us to hypothesize and prove that ETA specifically cleaves Dsg1 (Amagai *et al*, 2000).

In this study, we determine that the molecular pathophysiology of blistering caused by ETB is identical to that of ETA, namely the direct cleavage of Dsg1.

MATERIALS AND METHODS

Construction of recombinant ETB ETB-producing *S. aureus* TY4 chromosomal DNA was prepared as described previously (Sugai *et al*, 1998; Yamaguchi *et al*, 2001) and used as a polymerase chain reaction (PCR) template. A primer set, 5′-AAGCTTCCACCTAATACCCT-AATAATC-3′ and 5′-GGATCCACAGAGGTTCAACTCATGGTT-3′, was designed to generate a 1148 bp DNA fragment containing the entire *etb* gene (M17348) (Jackson and Iandolo, 1986) with terminal *Hin*dIII and *Bam*HI sites. The PCR product was cloned into pGEM-T Easy vector to generate pTY231. The pTY231 was digested with *Hin*dIII and *Bam*HI, and the insert was cloned into *E. coli–S. aureus* shuttle vector pCL8 to generate pTY133. *S. aureus* RN4220 (NCTC8325-4r-), which is a standard strain with no production of ETA or ETB, was then transformed with pTY133, and one of the transformants was designated TY2134. The physical map and genetic determinants of NCTC8325 indicate that this strain does not possess *eta* or *etb* (Iandolo, 2000). The nucleotide sequence of the *etb* gene was confirmed by DNA sequencing.

Staphylococcus aureus TY2134 exponentially growing in Trypticase soy broth (Becton Dickinson Microbiology Systems, Cockeysville, MD) was inoculated in 1 liter of the same fresh medium and incubated with continuous agitation by a rotary shaker for 24 h at 37°C until the cells reached the stationary phase. The culture was centrifuged at 10,000 × *g* for 20 min at 4°C. Concentrated culture filtrate was prepared by 80% saturated ammonium sulfate precipitation of the culture supernatant. A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Coomassie Brilliant Blue staining of concentrated culture filtrate revealed a single major protein band of 27 kDa, which was not present in the concentrated culture filtrate of RN4220 pCL8. We therefore used Coomassie Brilliant Blue staining of SDS-PAGE gel for detection of the ETB-positive fraction with the 27 kDa band as the marker. Concentrated culture filtrate dialyzed against 10 mM phosphate buffer (pH 6.8) (buffer 1) was applied to a hydroxyapatite column (Wako, 15 mm × 90 mm), which was equilibrated with buffer 1. The column was washed with buffer 1 until most of the unbound proteins passed through. Bound proteins were eluted by stepwise elution with 100 mM, 250 mM, and 500 mM phosphate buffer (pH 6.8), respectively. Eluate with 100 mM phosphate buffer (pH 6.8) was dialyzed against 10 mM phosphate buffer (pH 6.8) (buffer 2) and concentrated to 500 μl. The sample was loaded onto TSKgel $SW3000_{XL}$ (Tosoh, 7.5 mm × 300 mm) and eluted with buffer 2 at a flow rate of 0.5 ml per min, and the ETB-positive fractions were collected. Those fractions were collected and further applied to Bioscale CHT2-I hydroxyapatite HPLC column (Bio-Rad, 7.5 mm × 52 mm), and eluted with a linear gradient from 10 mM to 500 mM phosphate buffer (pH 6.8) at a flow rate of 1 ml per min. The ETB-positive fractions were collected and extensively dialyzed against phosphate-buffered saline (PBS). This purified ETB was used for incubation with cryosections of normal human skin (see below) and recombinant Dsg1 and Dsg3 (see below).

We also cloned the *etb* gene by a similar approach using an ETB-producing *S. aureus* isolated from a patient as a PCR template. A primer set, 5′-ACCCTAATAATCCAAAAACAG-3′ and 5′-CACAGAGGTT-CAACTCATGGT-3′, was used to amplify the *etb* gene and promoter sequence, and the PCR products were ligated into the pCRII vector and then subcloned into the pCE104 vector (a kind gift from Dr. Patrick Schlievert) (Vath *et al*, 1997). The supernatant from *S. aureus* RN4220 transformed with this plasmid contained ETB as the major protein, which was greater than 90% pure as determined by SDS-PAGE. This partially purified ETB was used for incubation with transduced HaCaT cells (see below) and injected into neonatal mice prior to immunofluorescence and immunoblotting of epidermis (see below).

Neonatal mouse study with ETB To evaluate the exfoliative activity of recombinant ETB, neonatal ICR or BALB/C mice (< 24 h of age) were injected subcutaneously with a designated amount of ETB in 20–50 μl of PBS, and the skin was analyzed grossly and microscopically 1–24 h after injection.

Mouse back skin was homogenized on dry ice, and then extracted with Laemmli sample buffer. Samples with equal amounts of protein (protein assay kit; Bio-Rad Laboratories, Hercules, CA) were separated by 6% Tris–glycine PAGE (Novex gels; Invitrogen, Carlsbad, CA), and then transferred to nitrocellulose membranes (Trans-Blot; Bio-Rad Laboratories). The membranes were incubated with rabbit antiserum against mouse Dsg1 and Dsg3, and ECCD-2 rat monoclonal antibody against mouse E-cadherin (a kind gift from M. Takeichi) (Shirayoshi *et al*, 1986).

Cell culture study with ETB To transduce cells with constructs encoding mouse Dsg1 and mouse Dsg3 with FLAG tag, recombinant adenovirus was constructed. The cosmid cassette pAxCAw, control Ad Ax1w, and the parent virus Ad5-dLX were all kind gifts from Dr. Izumi Saito (Tokyo University, Japan) (Miyake *et al*, 1996). cDNA encoding mouse Dsg1-FLAG and mouse Dsg3-FLAG (Amagai *et al*, 2000) were subcloned into the Ad cosmid cassette pAxCAw. Adenovirus containing CA promoter and cDNA encoding mouse Dsg1-FLAG and mouse Dsg3-FLAG (AxmDsg1F and AxmDsg3F) were generated by the COS-TPC method (Miyake *et al*, 1996) as follows. The cosmid DNA was mixed with the *Eco*T22I-digested DNA-terminal protein complex of Ad5-dLX and used to cotransfect to 293 cells in which recombinant viruses were generated through homologous recombination. Virus stocks were prepared using standard procedures (Miyake *et al*, 1996), and were concentrated by the CsCl gradient method. The virus titer was checked with a plaque formation assay.

HaCaT cells were cultured in a 12-well plate and transduced with AxmDsg1F and AxmDsg3F. After 24 h the cells were incubated with 0, 0.05, 0.15, and 0.25 μg per ml of recombinant ETB in culture media and incubated for 10 min or 1 h. Then the cells were washed with PBS, and extracted with 200 μl 2 × SDS Laemmli sample buffer (Bio-Rad Laboratories). The transduced Dsg1 and Dsg3 were visualized by immunoblotting with anti-FLAG tag rabbit antibody (Zymed, San Francisco, CA).

Immunofluorescence Formalin-fixed, paraffin-embedded skin from neonatal mice injected with ETB or saline was used for indirect immunofluorescence to localize Dsg1, Dsg3, and E-cadherin, as previously described (Amagai *et al*, 2000). A rabbit antiserum against extracellular domain 5 of mouse Dsg3 was raised and affinity purified on the antigenic peptide. A rabbit antiserum, raised similarly against extracellular domain 5 of mouse Dsg1, and ECCD-2 were also used. Stained sections were photographed using confocal microscopy (Leica TCS 4D, Wetzlar, Germany).

Cryosections of nonfixed normal human skin were incubated with 0.1 μg per ml of ETA (Toxin Technology, Sarasota, FL) in PBS with 1 mM $CaCl_2$ (PBS-Ca), 0.1 μg per ml of recombinant ETB in PBS-Ca, or PBS-Ca alone for 1 h at room temperature. The sections were then stained with anti-Dsg1 sera obtained from patients with pemphigus foliaceus, anti-Dsg3 mouse monoclonal antibody, 5H10, which reacts with the extracellular domain (Proby *et al*, 2000), anti-Dsg1 + 2 mouse monoclonal antibody, DG3.10, which reacts with the cytoplasmic domain (Koch *et al*, 1990), antidesmocollin mouse monoclonal antibody, 52-3D, which reacts with the cytoplasmic domain of Dsc1–3 (a kind gift from Dr. D. R. Garrod) (Collins *et al*, 1991), and antidesmoplakin mouse monoclonal antibody, 11-5F (a kind gift from Dr. D. R. Garrod). Staining with DG3.10 in this study represents the expression of Dsg1 because there is no detectable expression of Dsg2 in normal human skin.

***In vitro* digestion of recombinant Dsg1 with ETB** The entire extracellular domain of mouse and human recombinant Dsg1 and Dsg3, with an E-tag on the carboxyl terminus, were produced as a secreted protein by baculovirus expression as previously described (Ishii *et al*, 1997; Amagai *et al*, 2000). These recombinant human Dsg1 and Dsg3 proteins have been shown to retain their native conformations enough to adsorb out all immunoreactivity of pemphigus foliaceus and vulgaris sera, respectively (Ishii *et al*, 1997). High Five cells (Invitrogen, San Diego, CA) cultured in serum-free EX Cell 405 medium (JRH Bioscience, Lenexa, KS) were infected with the recombinant viruses and incubated for 3 d. Culture supernatant containing each recombinant Dsg was incubated with the indicated amount of ETB for 1 h at 37°C, and subsequently subjected to immunoblot analysis with anti-E-tag mouse monoclonal antibody (Pharmacia Biotech, Uppsala, Sweden) for detection of the intact as well as digested recombinant proteins. Culture

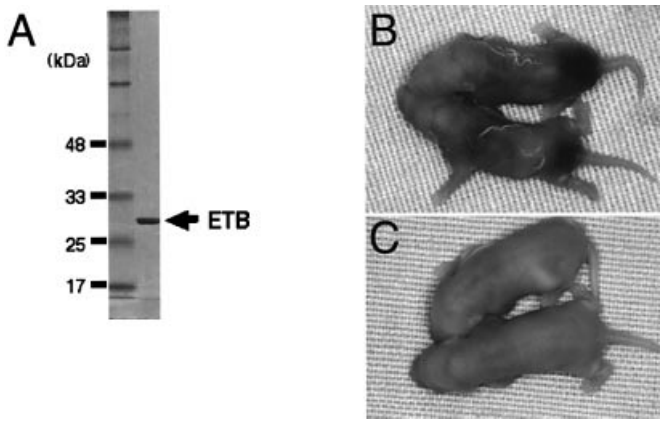

Figure 1. Production of recombinant ETB with exfoliative activity. Coomassie Blue stained SDS-PAGE shows recombinant ETB was produced as a 27 kDa peptide (*A*). Neonatal mice injected with ETB showed extensive blisters 2 h after injection (*B*), whereas neonatal mice injected with saline alone did not blister (*C*).

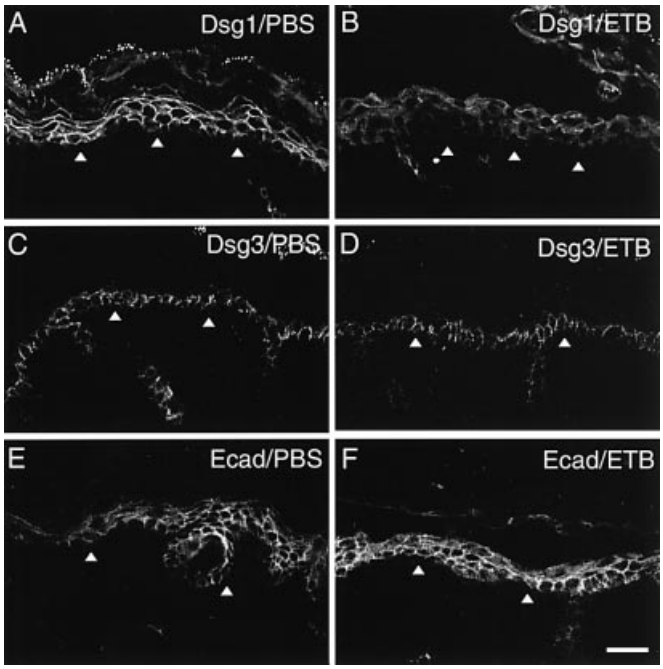

Figure 2. Immunofluorescence staining of cadherins in the epidermis of mice injected with ETB. Neonatal mice were injected with PBS (*A*, *C*, *E*) or ETB (*B*, *D*, *F*) and stained for Dsg1 (*A*, *B*), Dsg3 (*C*, *D*), and E-cadherin (*E*, *F*). Dsg1 staining showed that the cell surface staining of Dsg1 was removed by injection of ETB. The stainings of Dsg3 and E-cadherin were not affected by injection of ETB. *Arrowheads* indicate the location of the basement membrane zone. *Scale bar*: 50 μm

supernatant of RN4220 transfected with *etb*, but not that of RN4220 itself, digested recombinant Dsg1, indicating that RN4220 did not produce any ET (data not shown).

RESULTS

Production of recombinant ETB cDNA for ETB was PCR-amplified from ETB-producing *S. aureus* TY4, subcloned into an *E. coli–S. aureus* shuttle vector (pTY133), and used to transform *S. aureus* RN4220 (TY2134). TY2134 produced ETB in culture supernatant, which was recognized as a single predominant band of 27 kDa by Coomassie Blue. N-terminal sequencing of the protein band identified that it was the correctly processed form of recombinant ETB (data not shown). Finally 360 μg recombinant

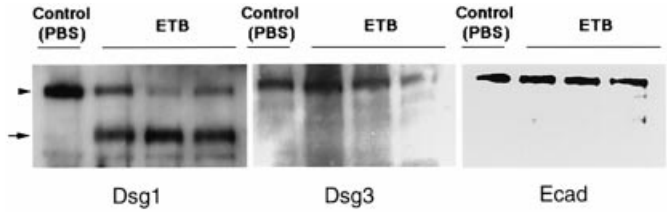

Figure 3. Immunoblot analysis of skin extracts from neonatal mice injected with PBS or ETB. Dsg1 was degraded by ETB injection, whereas Dsg3 and E-cadherin were not affected. Each lane represents an extract from a different mouse. *Arrowheads* and *arrows* indicate intact Dsg1 and cleaved Dsg1, respectively.

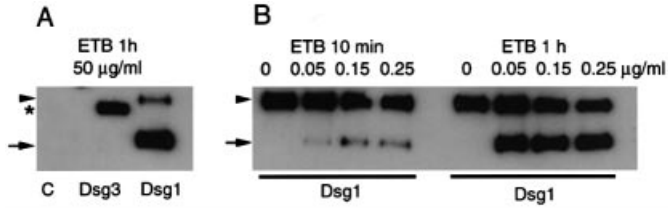

Figure 4. Immunoblot analysis of extracts from cultured keratinocytes incubated with ETB. (*A*) HaCaT cells were transduced with recombinant adenovirus with mouse Dsg1 (Dsg1) or mouse Dsg3 (Dsg3) or control adenovirus without any insert (C), and incubated with 50 μg per ml ETB for 1 h. Exogenous Dsg1 and Dsg3 was visualized anti-FLAG tag antibody. Dsg1, but not Dsg3, was degraded by ETB. *Arrowheads*, *asterisk*, and *arrows* indicate intact Dsg1, intact Dsg3, and cleaved Dgs1. (*B*) HaCaT cells transduced with recombinant adenovirus with Dsg1 was incubated with various amounts of ETB for 10 min or 1 h. Dsg1 was degraded by ETB in a dose- and time-dependent fashion.

ETB was purified to homogeneity from 1 liter of culture supernatant as described in *Materials and Methods* (**Fig 1*A***).

To confirm the exfoliative activity of the recombinant ETB, 20 μg of ETB in 100 μl of PBS was subcutaneously injected into neonatal mice. The mice started to show gross blisters as soon as 2–3 h after injection around the injected site (**Fig 1*B***; compare to control **Fig 1*C***).

Dsg1, but not Dsg3 or E-cadherin, was degraded *in vivo* in neonatal mouse skin injected with ETB To investigate the molecular mechanism of the blister formation by ETB and to determine whether ETB specifically affects Dsg1 as does ETA (Amagai *et al*, 2000), we examined the skin from neonatal mice injected with ETB by immunofluorescence with antibodies to Dsg1, Dsg3, and E-cadherin. When the skin was examined 1 h after ETB injection, the cell surface staining of Dsg1 was markedly diminished (**Fig 2*B***; compare to control **Fig 2*A***). In the same area, the cell surface staining of Dsg3 or E-cadherin was unaffected (**Fig 2*C–F***).

To determine whether this change in Dsg1 staining was caused by degradation of Dsg1 in the neonatal mouse skin, extracts of the skin from the mice injected with ETB or PBS were subjected to immunoblot analysis for Dsg1, Dsg3, and E-cadherin. A 160 kDa band for Dsg1 was degraded into a band of approximately 113 kDa in all three mice injected with ETB, whereas Dsg3 and E-cadherin were not degraded (**Fig 3**).

These results indicate that Dsg1, but not Dsg3 or E-cadherin, was cleaved *in vivo* in neonatal mouse skin after injection with ETB.

Exogenous Dsg1, but not Dsg3, was degraded by addition of ETB to transduced HaCaT cells To further demonstrate specific cleavage of Dsg1 by ETB, we transduced HaCaT cells, a human keratinocyte cell line, with recombinant adenovirus containing cDNA encoding mouse Dsg1 or Dsg3 with a FLAG epitope tag on their C-termini. When 50 μg per ml of ETB was

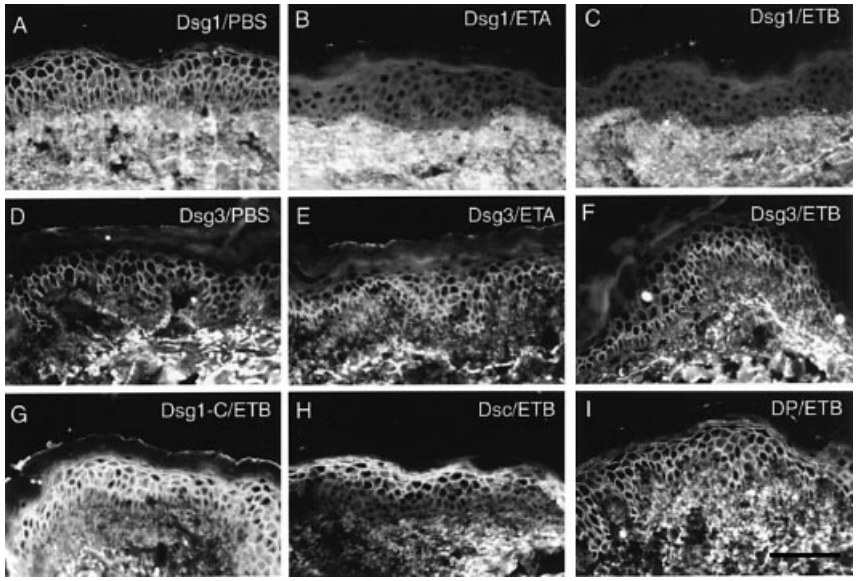

Figure 5. ***In vitro*** **ETB treatment of cryosectioned normal human skin.** Non-fixed cryosectioned normal human skin was incubated with ETA (*B*, *E), ETB (C, F, G, H, I)or PBS alone (A, D), and stained for extracellular domain of Dsg1 (A, B, C)*, extracellular domain of Dsg3 (*D*, *E*, *F*), cytoplasmic domain of Dsg1 (*G*), cytoplasmic domain of desmocollin (Dsc, *H*)or desmoplakin (DP, *I*). The staining for the extracellular domain of Dsg1 was removed by ETB (*C*)as seen with ETA (*B*), whereas the staining for Dsg3 was not affected. The staining for the cytoplasmic domain of Dsg1, desmocollins, or desmoplakin was not affected. *Scale bar*: 50 μm

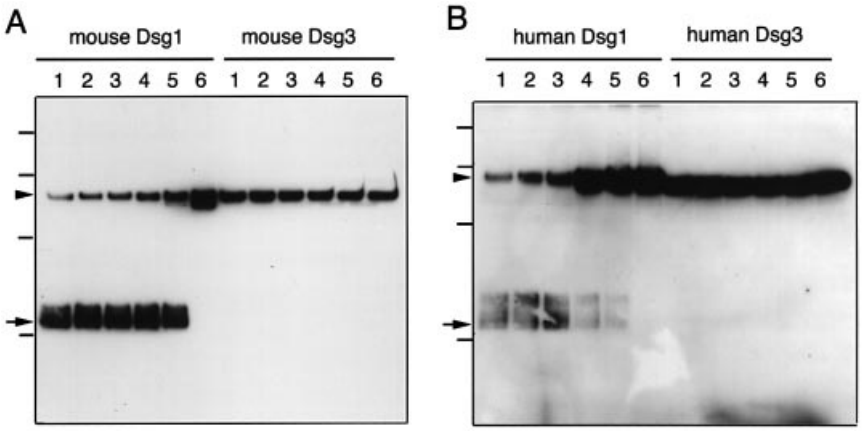

Figure 6. ***In vitro*** **ETB treatment of recombinant extracellular domain of Dsg1 and Dsg3.** Mouse (*A*) or human (*B*) Dsg1 and Dsg3 extracellular domains produced in baculovirus were incubated with various amounts of ETB (*A*: *lane 1*, 10 μg per ml; *lane 2*, 3 μg per ml; *lane 3*, 1 μg per ml; *lane 4*, 0.3 μg per ml; *lane 5*, 0.1 μg per ml; *lane 6*, 0 μg per ml; *B*: *lane 1*, 1 μg per ml; *lane 2*, 0.3 μg per ml; *lane 3*, 0.1 μg per ml; *lane 4*, 0.03 μg per ml; *lane 5*, 0.01 μg per ml; *lane 6*, 0 μg per ml), and subjected to immunoblots and visualized with anti-E-tag monoclonal antibody. The extracellular domain of Dsg1, but not that of Dsg3, was cleaved by ETB in a dose-dependent fashion. *Arrowheads* and *arrows* indicate the intact recombinant Dsg1 and cleaved product, respectively. *Bars* on the left side indicate molecular weight standards 250, 100, 50, and 25 kDa, from top to bottom.

added to the culture medium of these cells and incubated for 1 h, Dsg1 was degraded whereas Dsg3 was not (**Fig 4*A***). When these transduced HaCaT cells were incubated with various amounts of ETB (0, 0.05, 0.15, and 0.25 μg per ml) for 10 min or 1 h, Dsg1 was cleaved in a dose- and time-dependent fashion to a 113 kDa product (**Fig 4*B***).

Dsg1 cleavage by ETB is a direct effect The *in vivo* findings described above do not prove that ETB directly degrades Dsg1, as opposed to, for example, degradation through activation of other proteases in living cells. Therefore, to first demonstrate that living cells are not necessary for this inactivation of Dsg1, we incubated cryosections of normal human skin with ETB, ETA, or PBS, as a control, and stained them with antibodies against various desmosomal components (**Fig 5**). The cell surface staining of Dsg1 by pemphigus foliaceus sera, which react with the extracellular domain of Dsg1 (Amagai *et al*, 1995), was removed by ETB, whereas that of Dsg3 by 5H10, which reacts with the amino terminal extracellular domain of Dsg3 (Proby *et al*, 2000), was not altered at all (**Fig 5*A***, ***C***, ***D***, ***F***). These effects by ETB on the Dsg1 and Dsg3 staining were identical to those by ETA (**Fig 5*B***, ***E***). ETB treatment did not affect the staining pattern by monoclonal antibody DG3.10, which recognizes the cytoplasmic domain of Dsg1 (**Fig 5*G***). ETB treatment did not alter the staining of desmocollin by 52-3D nor that of desmoplakin by 11-5F (**Fig 5*H***, ***I***). ETA treatment did not change the staining by DG3.10, 52-3D, or 11-5F, either (data not shown). These findings indicate that ETB as well as ETA specifically affect the extracellular domain of Dsg1 in the absence of living cells, presumably by cleavage.

To demonstrate direct proteolysis of the extracellular domain of Dsg1 by ETB, we incubated a soluble recombinant form of the extracellular domain of Dsg1 and Dsg3 with ETB *in vitro* (**Fig 6**). ETB cleaved the 81 kDa recombinant mouse Dsg1 down to a 34 kDa peptide in a dose-dependent fashion, whereas ETB did not cleave mouse Dsg3 at all. In the same way, ETB cleaved the recombinant human Dsg1, but not human Dsg3. These findings indicate that ETB specifically recognizes and cleaves the extracellular domain of both mouse and human Dsg1.

DISCUSSION

The major physiologic function of skin is to form a protective barrier that hampers the penetration of microorganisms and inhibits the loss of water. This barrier has been shown to reside in the stratum corneum. In bullous impetigo, *S. aureus* is found in a blister cavity just beneath the stratum corneum, thus circumventing the barrier. This blister cavity is known to be caused by ET, released by the pathologic organisms. A similar blister occurs in patients with the autoimmune disease pemphigus foliaceus. In that disease IgG autoantibodies against Dsg1 block the cell adhesion function of Dsg1 with resultant superficial blisters in the epidermis where Dsg1 is expressed without coexpressed Dsg3 (Amagai, 1999; Mahoney *et al*, 1999). Although Dsg1 is also expressed in the deep epidermis and mucous membranes, blisters do not occur in these areas because Dsg3 is coexpressed and can compensate for the antibody-induced loss of function of Dsg1. Because pemphigus foliaceus shows identical tissue specificity and histology to SSSS and bullous impetigo, which are caused by ET, we previously suspected that the target molecule for a major type of ET, ETA, might be Dsg1 and proved that ETA specifically cleaves Dsg1 (Amagai *et al*, 2000).

In this study, we hypothesized that another type of ET that causes SSSS and bullous impetigo, ETB, also cleaves Dsg1. We have shown that injection of recombinant ETB into neonatal mice caused the specific removal of cell surface staining of Dsg1 and the degradation of Dsg1 *in vivo* with resultant blister formation, whereas Dsg3 and E-cadherin were not affected. Similarly, when ETB was added to cultured keratinocytes expressing tagged Dsg1 or Dsg3, ETB digested Dsg1 without affecting Dsg3. Specific loss of immunofluorescence of Dsg1, but not other cell surface molecules, after incubation of normal human skin with ETB, suggested that inactivation of Dsg1 by ETB did not require living cells and was probably a direct effect. Finally, we have demonstrated that ETB specifically cleaved the recombinant extracellular domain of Dsg1, but not that of Dsg3, *in vitro*. These findings indicate that ETB recognizes the extracellular domain of Dsg1 and cleaves it directly.

Histologic studies suggested that ET binds to a receptor in the granular layer of the epidermis, although to keratohyalin granules, not to the cell surface (Smith *et al*, 1989). Other studies have suggested binding to an epidermal GM4-like glycolipid (Sakurai and Kondo, 1979; Tanabe *et al*, 1995). Our studies demonstrate direct cleavage of recombinant Dsg1 in solution, however, suggesting that any epidermal receptor other than Dsg1 is unnecessary for activation of ET's proteolytic activity.

It is hypothesized that ETA and ETB are serine proteases. ETA and ETB are 25% identical to the staphylococcal serine protease V8. Structural studies show particularly striking homology in the region of the serine–aspartic acid–histidine catalytic triad that forms the active site of trypsin-like serine proteases (Dancer *et al*, 1990). In addition, the X-ray crystal structure of ETA and ETB showed significant structure similarity with known glutamate-specific trypsin-like serine proteases (Vath *et al*, 1997; 1999). It is interesting that ETA and ETB share identical binding specificity and cleavage specificity although overall identity is only 40%. We suspect that amino acid residues that are responsible for this specificity should be conserved. The final identification of ETA and ETB as glutamate-specific serine proteases has to await the determination of the cleavage site of Dsg1 by ETA and ETB.

Dsg1 is targeted by ETA as well as ETB in SSSS and bullous impetigo, resulting in a blister just below the stratum corneum, the major barrier in skin. These toxins, then, allow the bacteria to circumvent this barrier and spread just beneath it. These findings provide an important framework to understand the molecular mechanism for blister formation in these diseases as well as cell–cell adhesion of keratinocytes in the epidermis.

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