Toxin in bullous impetigo and staphylococcal scalded-skin syndrome targets desmoglein 1

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Exfoliative toxin A, produced by *Staphylococcus aureus*, causes blisters in bullous impetigo and its more generalized form, staphylococcal scalded-skin syndrome[1–3]. The toxin shows exquisite specificity in causing loss of cell adhesion only in the superficial epidermis. Although exfoliative toxin A has the structure of a serine protease, a target protein has not been identified[4,5]. Desmoglein (Dsg) 1, a desmosomal cadherin that mediates cell–cell adhesion, may be the target of exfoliative toxin A, because it is the target of autoantibodies in pemphigus foliaceus, in which blisters form with identical tissue specificity and histology. We show here that exfoliative toxin A cleaved mouse and human Dsg1, but not closely related cadherins such as Dsg3. We demonstrate this specific cleavage in cell culture, in neonatal mouse skin and with recombinant Dsg1, and conclude that Dsg1 is the specific receptor for exfoliative toxin A cleavage. This unique proteolytic attack on the desmosome causes a blister just below the stratum corneum, which forms the epidermal barrier, presumably allowing the bacteria in bullous impetigo to proliferate and spread beneath this barrier.

In 1878, Baron Gottfried Ritter von Rittershain described a severe blistering disease of neonates that is now called staphylococcal scalded-skin syndrome (SSSS), or Ritter disease[6]. This disease was soon recognized to be a more severe form of what had been called pemphigus neonatorum, which occurred in epidemics in neonatal nurseries, and of sporadic localized bullous impetigo[2]. The blisters in these diseases are caused by toxins, most often exfoliative toxin A (ETA), produced by *Staphylococcus aureus*. In SSSS, a local infection releases the toxin into the circulation, whence it produces widespread skin blistering, whereas in bullous impetigo the toxin causes blistering locally at the site of infection. The amino-acid sequence of ETA indicates that it may belong to the serine protease superfamily, and mutational analysis indicates that ETA loses its ability to cause blisters in neonatal mice if residues homologous to those mediating the catalytic activity of serine proteases are mutated[7]. However, no substrate has been identified, protein cleavage has never been shown and the exquisite sensitivity of the superficial epidermis, but no other tissues including mucous membranes, to ETA has never been explained. ETA may cause specific cleavage of Dsg1, because in pemphigus foliaceus, loss of function of Dsg1 causes blisters only in the superficial epidermis, where Dsg1 is expressed without co-expressed Dsg3. In the lower epidermis and in mucous membranes, Dsg3 compensates for loss of function of Dsg1 (that is, there is 'desmoglein compensation')[8].

Like pemphigus foliaceus antibodies, ETA also causes blisters in the superficial epidermis when injected in neonatal mice[9]. Clinically and histologically, the blisters in mice injected with

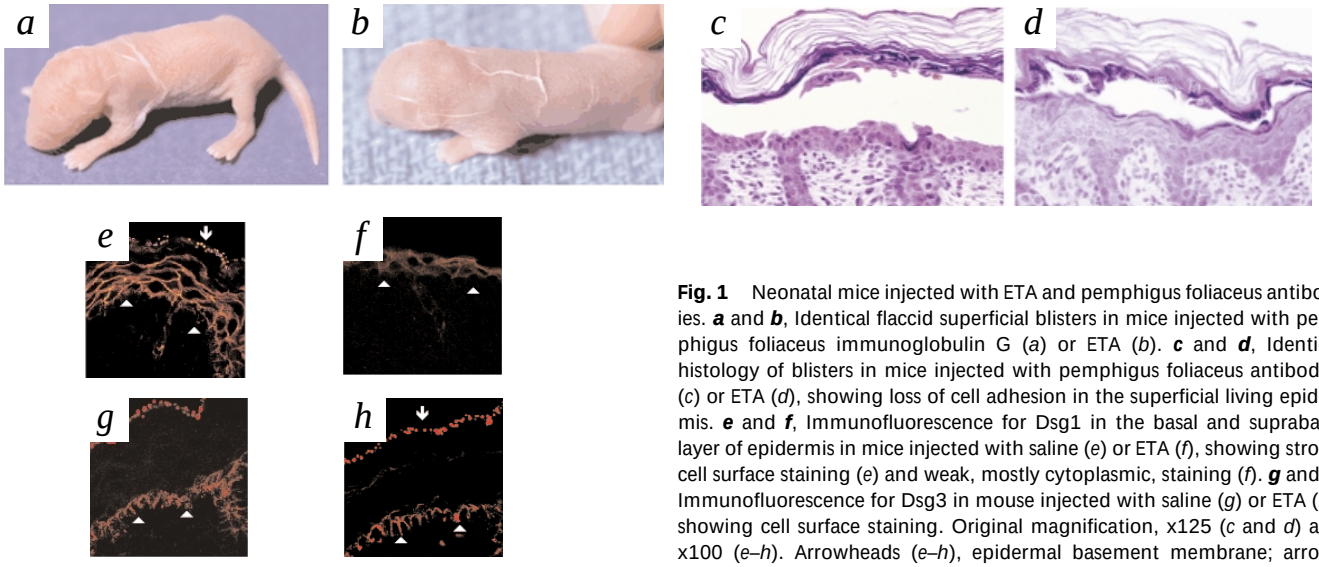

Fig. 1 Neonatal mice injected with ETA and pemphigus foliaceus antibodies. ***a*** and ***b***, Identical flaccid superficial blisters in mice injected with pemphigus foliaceus immunoglobulin G (*a*) or ETA (*b*). ***c*** and ***d***, Identical histology of blisters in mice injected with pemphigus foliaceus antibodies (*c*) or ETA (*d*), showing loss of cell adhesion in the superficial living epidermis. ***e*** and ***f***, Immunofluorescence for Dsg1 in the basal and suprabasal layer of epidermis in mice injected with saline (*e*) or ETA (*f*), showing strong cell surface staining (*e*) and weak, mostly cytoplasmic, staining (*f*). ***g*** and ***h***, Immunofluorescence for Dsg3 in mouse injected with saline (*g*) or ETA (*h*), showing cell surface staining. Original magnification, x125 (*c* and *d*) and x100 (*e–h*). Arrowheads (*e–h*), epidermal basement membrane; arrows (*e–h*), stratum corneum showing nonspecific fluorescence.

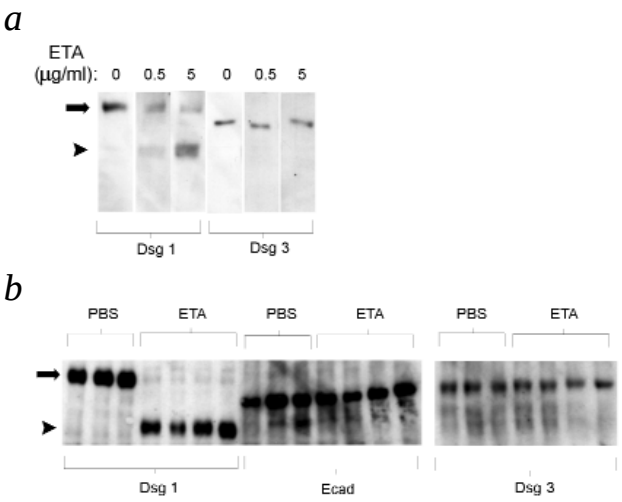

Fig.2 Western blot analyses showing degradation of mouse Dsg1, but not mouse Dsg3 or epithelial cadherin. ***a***, Antibody against FLAG on extracts of HaCat cells transfected with mouse Dsg1–FLAG or Dsg3–FLAG and incubated with ETA (concentrations (μg/ml), above blots). ***b***, Blots of extracts of skin from neonatal mice injected with ETA or saline (PBS), stained with antibodies against Dsg1, mouse epithelial cadherin (Ecad) or mouse Dsg3. Each lane represents an extract from a different mouse. ETA degrades Dsg1 (arrow), of approximately 160 kDa, to a product of approximately 113 kDa (arrowhead).

ETA and pemphigus foliaceus antibodies were identical (Fig. 1*a*–*d*). Therefore, to determine if ETA specifically affects Dsg1, we examined skin from neonatal mice injected with ETA by immunofluorescence with antibodies against Dsg1 and Dsg3. To avoid secondary effects from blister formation in ETA-injected mice, we focused on the basal and immediate suprabasal areas of the epidermis, because these areas contain Dsg1, but do not blister. ETA caused substantial changes in Dsg1 staining, which showed much less intensity and had increased cytoplasmic rather than cell surface staining (Fig. 1*e* and *f*). In the same area, Dsg3 staining was unaffected (Fig. 1 *g* and *h*). These results are consistent with cleavage of Dsg1 by ETA, internalization of the cleaved product and maintenance of adhesion by compensatory Dsg3.

To show cleavage of Dsg1 by ETA, we transfected HaCaT cells, a human kertinocyte cell line, with cDNA encoding mouse Dsg1 or Dsg3 containing a FLAG octapeptide epitope on their carboxyl termini, then incubated the transfected cells with ETA. Western blot analysis with antibodies against FLAG showed degradation of Dsg1, but not the closely related Dsg3 (Fig. 2a). To demonstrate this specific cleavage *in vivo*, we injected neonatal mice with ETA and used western blot analysis for Dsg1, Dsg3 and epithelial cadherin on extracts of skin from the mice that developed blisters in the superficial epidermis. Only Dsg1 was degraded (Fig. 2*b*). These results show that ETA results in cleavage of the 160-kDa Dsg1 to a peptide of approximately 113 kDa. As predicted from its crystal structure, ETA is thought to cleave at a glutamic acid[5]; there are many glutamic acid residues in the Dsg1 sequence around position 170 (Genbank AF097935)[10], where cleavage would be predicted to yield a carboxy-terminal peptide of approximately 113 kDa.

The studies described above demonstrate specific cleavage of Dsg1 but do not prove direct proteolysis by ETA, rather than secondary effects through other cellular proteases. If the cleavage is direct, it presumably occurs in the extracellular domain, as it is unlikely that ETA has access to intracellular peptides. To demonstrate direct cleavage, we isolated the extracellular domain of mouse Dsg1 and, as a control, mouse Dsg3, produced in baculovirus, and incubated these with varying concentrations of ETA. Mouse Dsg1, but not Dsg3, was degraded in a dose-dependent way (Fig. 3*a*). Finally, we used similar experiments to show direct and specific cleavage of human Dsg1 (Fig. 3*b*).

Our results have demonstrated exquisite specificity of the ETA protease for Dsg1. This type of molecule-specific proteolysis was predicted from the crystal structure of ETA, which indicated that the amino terminus might bind to a receptor that would change the conformation of ETA, allowing access to its proteolytic site[5]. Dsg1 is also the target of the autoantibodies in pemphigus foliaceus[8]. These autoantibodies and ETA caused a similar pathology of a blister just below the stratum corneum by producing dysfunction of Dsg1, which is the only desmoglein family member expressed in this area. The pathology of both pemphigus foliaceus and ETA-mediated staphylococcal disease indicates the necessity of functional Dsg1 in maintaining keratinocyte cell–cell adhesion in the superficial living epidermis. These results also emphasize the acumen of the physicians who, before any understanding of molecular pathophysiology of these diseases, realized that SSSS was clinically similar to pemphigus (hence the name pemphigus neonatorum). Finally, our findings have elucidated the molecular pathology of SSSS and bullous impetigo. By using this toxin to target Dsg1 and create a blister just under the stratum corneum, which forms the epidermal barrier, staphylococci have developed a very specific mechanism to allow their proliferation in the skin and their spread just underneath this barrier in bullous impetigo.

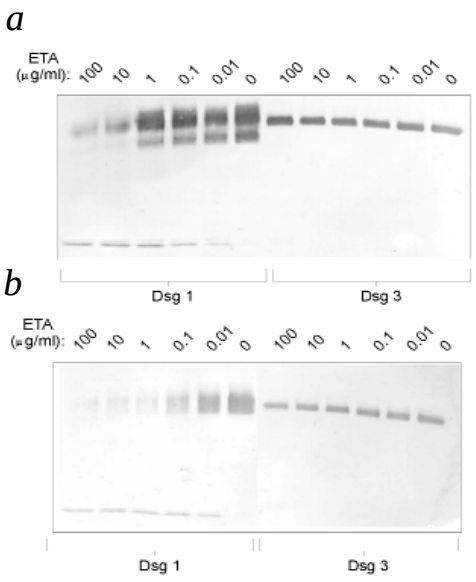

Fig. 3 Western blot analyses showing degradation of the recombinant extracellular domain of mouse and human Dsg1, but not Dsg3, with ETA. Mouse ***a***, or human ***b***, Dsg1 and Dsg3 extracellular domains produced in baculovirus incubated with ETA (concentrations (mg/ml), above blots).

Methods

Neonatal mouse model of SSSS and pemphigus foliaceus. Neonatal mice 1–2 days of age were injected with 20 μl of 1 μg/μl ETA (Toxin Technology, Sarasota, Florida) dissolved in PBS or with pemphigus foliaceus antibodies[8], and the skin was analyzed grossly and microscopically as described[8,9].

Immunofluorescence. Formalin-fixed, paraffin-embedded skin from neonatal mice injected with ETA or saline was used for indirect immunofluorescence to localize Dsg1 and Dsg3, as described[11]. A rabbit antiserum against extracellular domain 5 of mouse Dsg3 was raised as described[11], and was affinity-purified on the antigenic peptide. A rabbit antiserum raised similarly against extracellular domain 5 of mouse Dsg1 was also used. (M. Shapiro and P. Koch helped to produce and characterize antibodies against desmoglein) Stained sections were photographed using confocal microscopy (Leica TCS 4D; Leica, Wetzlar, Germany).

ETA incubation of cells transfected with cDNA encoding desmogleins. Mouse cDNA encoding Dsg3 with a FLAG octapeptide epitope[12] on the carboxyl terminus was cloned as described[13–15] (Genbank accession number, U86016) (F. Wang, Jefferson Medical College contributed to cloning). A lambda phage clone containing cDNA encoding mouse Dsg1 was provided by L. Pulkinen (Jefferson Medical College, Philadelphia, Pennsylvania). PCR was used, as with mouse Dsg3 cDNA, to add nucleotides encoding the FLAG peptide epitope to the 3′ end. The final Dsg3 FLAG and Dsg1 FLAG constructs were confirmed by nucleotide sequencing. Mouse Dsg3–FLAG was subcloned into the *Not*I site of the eukaryotic expression vector pcDNA3.1+ (Invitrogen, Carlsbad, California), and mouse Dsg1–FLAG was subcloned into the *Not*I and *Hin*dIII sites of pcDNA3.1– (Invitrogen).

FuGENE 6 (6 μl; Roche, Indianapolis, Indiana) was preincubated for 5 min at room temperature in 94 μl Dulbecco's modified eagle medium (DMEM) (FuGENE–DMEM mix). Then, 3 μg pcDNA in 10 μl water was incubated for 15 min at room temperature with 100 μl FuGENE–DMEM mix. This DNA in FuGENE–DMEM mix was then added to HaCaT cells that had been grown overnight in six-well plates in 3 ml 10% FBS in DMEM, then incubated for 48 h at 37 °C. The medium was changed to 2 ml 10% FBS in DMEM containing ETA (1 μg/ml diluted in PBS), and incubated for 1 h at 37 °C. Cells were then washed with PBS, and extracted with 200 μl 2× SDS in Laemmli sample (BioRad Laboratories, Hercules, California).

Western blot analysis. Mouse back skin was homogenized on dry ice, then extracted with Laemmli sample buffer. Samples with equal amounts of protein (Protein Assay Kit; BioRad Laboratories) were separated by 6% Tris–glycine PAGE (Novex gels from Invitrogen), then were transferred to nitrocellulose sheets (Trans-Blot; BioRad Laboratories). The sheets were incubated for 1 h in a blocking buffer of 5% fat-free milk powder in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS). The first antibody (rabbit antiserum against mouse Dsg3 and Dsg1, ECCD-2 rat antibody against mouse epithelial cadherin[16] (provided by M. Takeichi) or rabbit antibody against FLAG (Zymed, San Francisco, California), diluted in blocking buffer) was applied for 1 h at room temperature. After two washes with 0.1% Tween-20 in TBS, the sheets were incubated for 2 h with horseradish peroxidase-conjugated antibody against rabbit or rat immunoglobulin G (BioRad Laboratories), diluted 1:1,000 in blocking buffer. After the blots were washed, the signals were detected with chemiluminescence (ECL; Amersham).

ETA digestion of Dsg1 and Dsg3 produced by baculovirus. The entire extracellular domain of mouse or human recombinant Dsg1 and Dsg3, with an E-tag on the carboxyl terminus, was produced as a secreted protein by baculovirus as described[17,18]. In these conditions, recombinant human Dsg1 and Dsg3 retain their native conformations sufficiently to adsorb out all immunoreactivity of pemphigus foliaceus and vulgaris sera, respectively[17]. Approximately 1 μg purified mouse or human Dsg1 and Dsg3 was incubated for 1 h at 37° C with ETA in 50 μl PBS with 1 mM $CaCl_2$. The digested samples were assessed by immunoblot analysis with mouse monoclonal antibody against E-tag (Pharmacia) for detection of the recombinant proteins[17].

Acknowledgments

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