Defining the pathogenic involvement of desmoglein 4 in pemphigus and staphylococcal scalded skin syndrome

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Desmogleins (Dsgs), cadherin-type cell adhesion molecules, are targeted in skin-blistering diseases such as pemphigus and staphylococcal scalded skin syndrome (SSSS). The role of Dsg4, a new isoform, was investigated in these diseases. Dsg4 was recognized by 30 (77%) of 39 pemphigus sera containing anti-Dsg1 IgG but not by 16 pemphigus sera containing no anti-Dsg1 IgG or by 34 normal control sera. The Dsg4 immunoreactivity of these sera was abolished by removal of anti-Dsg1 IgG. Conversely, the removal of anti-Dsg4 IgG from pemphigus sera reduced the immunoreactivity against Dsg1 only 13.8% ± 8.8% (n = 23) and did not affect its ability to induce blisters in neonatal mice. IgG that was affinity-purified on Dsg4 recognized Dsg1 but failed to induce blisters, while IgG purified on Dsg1 from the same pemphigus foliaceus sera induced blisters. Thus, pemphigus sera show Dsg4 reactivity due to cross-reactivity of a subset of anti-Dsg1 IgG, and the Dsg4/Dsg1–cross-reacting IgG has no demonstrable pathogenic effect. In addition, Dsg4 was not cleaved by exfoliative toxins that induce blisters in SSSS. These findings suggest that Dsg4 may play a role other than adhesion and that the cross-reactivity of desmoglein autoantibodies should be factored into the framework of future studies of autoimmune mechanisms in pemphigus.

Introduction

Desmoglein (Dsg), a major transmembrane component in desmosomes, plays a critical role not only in the cell-cell adhesion of epithelial cells but also in the morphogenesis of epithelial tissues (1, 2). Since the identification of the desmoglein cDNAs in the early 1990s, 3 isoforms of desmoglein, i.e., Dsg1, Dsg2, and Dsg3, have been described (3–6). Dsg2 is expressed in all desmosome-containing tissues, which include simple epithelia and the myocardium, while the expression of Dsg1 and Dsg3 is restricted to stratified squamous epithelium (6). Recently, a fourth member of the desmoglein family, Dsg4, was identified based on an analysis of the genomic structure of the desmosomal cadherin gene cluster on human chromosome 18q12 (7, 8).

The desmogleins are targeted in inherited and acquired skin diseases as well as in skin infections. Mutations in the DSG1 gene cause striate palmoplantar keratoderma (MIM 148700), which is an autosomal dominant skin disease that is characterized by linear and focal hyperkeratosis of the palms and soles, probably owing to haploinsufficiency of Dsg1 (9). Dsg1 and Dsg3 are autoimmune targets in pemphigus, which is characterized by blisters and erosions of the skin and mucous membranes (5, 10). The classic forms of pemphigus are divided into 3 subtypes according to anti-desmoglein Ab profile (11, 12): patients with pemphigus foliaceus (PF) have only anti-Dsg1 IgG autoantibodies; patients with the mucocutaneous type of pemphigus vulgaris (PV) have both anti-Dsg3 and anti-Dsg1 IgG autoantibodies; and patients with the mucocutaneous type of PV have both anti-Dsg3 and anti-Dsg1 IgG autoantibodies. The disruption of desmoglein-dependent cell-cell adhesion by IgG autoantibodies represents the basic pathophysiology underlying blister formation in pemphigus, and the sites of blister formation in the skin and mucous membranes are explained by “the desmoglein compensation theory” (13, 14).

In addition to its involvement in these inherited and acquired blistering skin diseases, Dsg1 is also targeted by the exfoliative toxins (ETs) of Staphylococcus aureus, leading to bullous impetigo, which is a common bacterial infection of children, as well as staphylococcal scalded skin syndrome (SSSS), which is the generalized form found in newborns, young children, and adults with renal failure and/or immunocompromised status (15, 16). Among the 4 known isoforms of ET, ETA, ETB, and ETD bind and cleave the extracellular domain of Dsg1 once after glutamic acid residue 381 between the EC3 and EC4 domains in a specific manner (16–19). Thus, these ETs act as serine proteases with strict specificity for Dsg1, and they induce superficial blisters in the epidermis that are clinically and histologically similar to those seen in PF.

Mutations in the newly identified DSG4 gene cause impaired hair follicle development, which results in inherited hypotrichosis in humans (7, 20, 21) and abnormal hair with bulbous degenerative changes in the lanceolate hair mouse and rat (7, 22). It has been claimed also that Dsg4 acts as an autoantigen in PV, based on IB analysis showing that 2 PV sera recognized a bacterial recombinant protein that represented an extracellular domain of Dsg4 (7). It is puzzling, however, that the phenotypes of the inherited form and the acquired form are quite different; the phenotype caused by the mutated DSG4 is rather restricted to the hair and hair follicles, without apparent blister formation in the interfollicular skin or mucous membranes, as is found in patients with PV (23).

Nonstandard abbreviations used: Dsg, desmoglein; ET, exfoliative toxin; hDsg4, human Dsg4; mDsg4, mouse Dsg4; PF, pemphigus foliaceus; PV, pemphigus vulgaris; SSSS, staphylococcal scalded skin syndrome.

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In this study, we investigated the involvement of Dsg4, which is expressed in the superficial epidermis (7), in the skin-blistering diseases pemphigus and SSSS. We examined the reactivity against Dsg4 of sera derived from patients with different types of pemphigus and the specificity of anti-Dsg4 IgG autoantibodies. We compared anti-Dsg4 autoantibodies to other known pemphigus autoantibodies and investigated their role in the pathogenesis of blister formation. We also determined whether Dsg4 is a target of the ETs that cause superficial epidermal blisters in SSSS.

It is important to understand the involvement of Dsg4 in pemphigus and SSSS, not only to elucidate the functional role it may play in vivo but also to build a framework for future investigation into the autoimmune mechanisms of pemphigus.


Results

Pemphigus sera containing anti-Dsg1 IgG autoantibodies react with Dsg4. The secreted form of the entire extracellular domain of human Dsg4 (hDsg4) was fused with the E-tag and His-tag to produce hDsg4-His, which was produced by baculovirus expression as a 91.7-kDa protein in the culture supernatant (Figure 1, lane 1). The classic form of pemphigus is classified into 3 major forms according to the anti-Dsg IgG autoantibody profile: PF, mucosal dominant PV, and mucocutaneous PV (10–12, 24). To determine whether any of the pemphigus sera contained IgG autoantibodies that reacted with Dsg4, we performed IP-IB assays with hDsg4-His as the substrate on insect cell culture supernatants (25). Remarkably, 17 of 20 (85.0%) PF sera and 13 of 19 (68.4%) mucocutaneous PV sera contained anti-Dsg1 IgG autoantibodies, while the mucosal dominant PV sera contained no anti-Dsg1 IgG, but did contain anti-Dsg3 IgG autoantibodies. These disparate reactivity patterns raised the possibility that the reactivities against Dsg4 might be the result of cross-reactivities of anti-Dsg1 IgG autoantibodies.

Table 1

<table>
<thead>
<tr>
<th>Subtypes</th>
<th>n</th>
<th>Reactivity against Dsg1</th>
<th>Reactivity against Dsg3</th>
<th>Dsg4 reactivity</th>
<th>Elimination of Dsg4 reactivity by Dsg1</th>
<th>Reduction of Dsg1 reactivity by Dsg4 immunoadsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF</td>
<td>20</td>
<td>+</td>
<td>–</td>
<td>17 (85.0%)</td>
<td>17 (100%)</td>
<td>10.7% ± 5.8% (n = 11)</td>
</tr>
<tr>
<td>PV-MC</td>
<td>19</td>
<td>+</td>
<td>+</td>
<td>13 (68.4%)</td>
<td>13 (100%)</td>
<td>17.1% ± 10.5% (n = 12)</td>
</tr>
<tr>
<td>PV-M  #</td>
<td>16</td>
<td>–</td>
<td>+</td>
<td>0 (0%)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Normal</td>
<td>34</td>
<td>–</td>
<td>–</td>
<td>0 (0%)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

The reactivity against Dsg4 was examined by a combined IP-IB analysis using hDsg4-His as the substrate. The elimination of Dsg4 reactivity was examined by removing anti-Dsg1 IgG autoantibodies with hDsg1-IgHis. The reduction rate of Dsg1 reactivity was examined by competition ELISA against Dsg1 using hDsg4-IgHis as a competitor.  

When the anti-Dsg1 IgG autoantibodies were removed, Dsg4 reactivity was eliminated, while Dsg3 reactivity was unaltered. Similarly, the anti-Dsg4 IgG autoantibodies were preadsorbed from PF sera (B) or PV-MC sera (D) using various amounts of hDsg4-IgHis. Although the anti-Dsg4 IgG autoantibodies were removed, Dsg1 reactivity was not reduced significantly. The heights of the black triangles indicate the amounts of hDsg1-IgHis (A and C) or hDsg4-IgHis (B and D) that were used for preadsorption.

Figure 3

Elimination of Dsg4 reactivity by removal of anti-Dsg1 IgG autoantibodies. Anti-Dsg1 IgG autoantibodies were preadsorbed from PF sera (A) or PV-MC sera (C) using various amounts of hDsg1-IgHis. When the anti-Dsg1 IgG autoantibodies were removed, Dsg4 reactivity was eliminated, while Dsg3 reactivity was unaltered. Similarly, the anti-Dsg4 IgG autoantibodies were preadsorbed from PF sera (B) or PV-MC sera (D) using various amounts of hDsg4-IgHis. Although the anti-Dsg4 IgG autoantibodies were removed, Dsg1 reactivity was not reduced significantly. The heights of the black triangles indicate the amounts of hDsg1-IgHis (A and C) or hDsg4-IgHis (B and D) that were used for preadsorption.

supernatant via dialysis (Figure 2C). None of the 20 PF and 0 of 16 mucocutaneous PV sera reacted with the denatured hDsg4-His as examined by IB analysis (data not shown).

In addition, we expressed hDsg4-His in CHO cells as a 98.6-kDa protein (Figure 1, lane 2). The difference of the molecular weights between that expressed by baculovirus and that expressed in CHO cells was a result of glycosylation because the molecular weights for nonglycosylated forms produced in the presence of tunicamycin were identical (Figure 1, lanes 3 and 4). The PF sera (n = 6) and mucocutaneous PV sera (n = 5) that reacted with the baculovirus-expressed hDsg4-His also reacted with the hDsg4-His produced by the CHO cells (Figure 2B). Neither the mucosal dominant PV sera (n = 2) nor the normal sera (n = 2) showed any reactivity against the CHO cell–expressed hDsg4-His. This finding excludes the possibility that the reactivities against hDsg4-His are owing to the generation of neo-epitopes during baculovirus expression.

These data indicate that a subset of pemphigus sera react with calcium-dependent conformational epitopes on Dsg4. Interestingly, both the PF sera and mucocutaneous PV sera contained anti-Dsg1 IgG autoantibodies, while the mucosal dominant PV sera contained no anti-Dsg1 IgG, but did contain anti-Dsg3 IgG autoantibodies. These disparate reactivity patterns raised the possibility that the reactivities against Dsg4 might be the result of cross-reactivities of anti-Dsg1 IgG autoantibodies.

Reactivity of pemphigus sera against Dsg4 is eliminated by removal of the anti-Dsg1 IgG autoantibodies. To determine whether Dsg4 reactivity involved anti-Dsg1 IgG autoantibodies, we preincubated the sera that showed reactivity against Dsg4 with various amounts of hDsg1-IgHis (25) and performed IP-IB assays against hDsg1-His, hDsg3-His, and hDsg4-His. When the PF sera that reacted with Dsg4 were examined, the reactivity against hDsg4-His was eliminated in a dose-dependent fashion by preincubation with hDsg1-IgHis (Figure 3A). Dsg4 reactivity...
was abolished completely when anti-Dsg1 IgG was removed. The removal of Dsg4 reactivity by preincubation with hDsg1-IgHis was observed for 17 of 17 (100%) of the Dsg4-reacting PF sera (Table 1). When the mucocutaneous PV sera that reacted with Dsg4 were examined, the reactivity against hDsg4-IgHis was also eliminated in a dose-dependent fashion by preincubation with hDsg1-IgHis (Figure 3C). The removal of the anti-Dsg1 IgG autoantibodies by hDsg1-IgHis completely abolished the reactivity against hDsg4-IgHis, whereas it did not affect the reactivity against hDsg3-IgHis. The removal of Dsg4 reactivity with hDsg4-IgHis was observed for 13 of 13 (100%) of the Dsg4-reacting mucocutaneous PV sera (Figure 5). The competition rate by Dsg4 increased in a dose-dependent fashion, although its plateau level was no more than 25%.

We also removed the IgG autoantibodies that reacted with Dsg4 and then examined their reactivities against Dsg1 or Dsg3. In the case of the PF sera, the removal of Dsg4 reactivity by preincubation with hDsg4-IgHis (Figure 1, lane 5) did not significantly affect the reactivity against hDsg1-IgHis (Figure 3B). Similarly, in the case of the mucocutaneous PV sera, the removal of Dsg4 reactivity did not change the reactivity against hDsg1-IgHis or hDsg3-IgHis (n = 6) (Figure 3D). To further quantitate the IgG fraction cross-reacting with Dsg4 in anti-Dsg1 IgG autoantibodies, we performed competition ELISA against Dsg1 using various concentrations of hDsg4-IgHis as competitors. Competition increased in a dose-dependent fashion and reached a plateau at higher concentration (representative data are shown in Figure 4). The observed plateau level of competition for each serum should represent the IgG fraction cross-reacting with Dsg4 (n = 23).

Furthermore, we used the hDsg4-IgHis column to affinity-purify the IgG autoantibodies that reacted with Dsg4 from the PF sera and determined the reactivities of these sera against Dsg1 and Dsg3 (Figure 5). The affinity-purified anti-Dsg4 IgG autoantibodies reacted with hDsg1-IgHis but not with hDsg3-IgHis (n = 2).

Taken together, these findings indicate that there were essentially no IgG autoantibodies that reacted exclusively with Dsg4 in pemphigus and that the Dsg4 reactivity found in the PF sera and mucocutaneous PV sera is owing to the cross-reactivity of a subset of anti-Dsg1 IgG autoantibodies.

The Dsg4/Dsg1-cross-reacting IgG autoantibodies do not play a significant pathogenic role in blister formation in pemphigus. To evaluate the pathogenic relevance of Dsg4/Dsg1–cross-reacting IgG autoantibodies in the pemphigus sera, we performed passive transfer assays using neonatal mice (26). To ensure that the IgG autoantibodies that reacted with hDsg4 also reacted with mouse Dsg4 (mDsg4), we conducted IP-IB assays using mDsg4-IgHis (Figure 1, lane 6; Figure 2D). Similar to the findings with hDsg4, 11 of 12 PF sera and 3 of 6 mucocutaneous PV sera reacted with mDsg4-IgHis, while 0 of 6 mucosal dominant PV sera and 0 of 12 normal control sera recognized it. In addition, Dsg4 is indeed expressed in neonatal mouse epidermis. The expression of Dsg4 was demonstrated by RT-PCR in the various sites of the skin (ventral and dorsal skin, tail, and footpad) as well as in the tongue, but not in simple epithelia such as liver (Figure 6). In the same condition, the expression of Dsg1α, Dsg1β, Dsg1γ, and Dsg3 was demonstrated in the stratified squamous epithelia but not in liver, except for Dsg1γ, as previously described (5, 27, 28). Because essentially all IgG that reacts with Dsg4 cross-reacts with Dsg1 in pemphigus sera, the following experiments do not reflect sole effects by Dsg4 inhibition, but rather combined effects by Dsg1 and Dsg4 inhibition.

We removed the IgG autoantibodies that reacted with Dsg4 by immunoadsorption with hDsg4-IgHis and prepared IgG from the treated PF sera, which no longer reacted with Dsg4 (Figure 5). To compare the pathogenic strength of the PF sera with and without Dsg4 immunoadsorption in a quantitative fashion, the IgG fractions were serially diluted and injected into neonatal mice (Table 2). Mice injected with PF no. 1 serum IgG after the removal of anti-Dsg4 IgG developed extensive blisters 10 hours after injection (Figure 7B). The histology showed loss of cell adhesion in the upper part of the epidermis, a typical finding in PF (Figure 7F). Direct immunofluorescence of the mouse skin showed human IgG deposition on keratinocyte cell surfaces (Figure 7J). Mice injected with PF no. 1 serum IgG without hDsg4-IgHis immunoadsorption did not develop any blisters (Figure 7A). mDsg4-IgG fraction cross-reacting with Dsg4 (n = 23).

Figure 5
Immunoadsorption and purification of Dsg4-reactive IgG autoantibodies from PF sera. Two of the PF sera (A and B) were applied to a column that contained hDsg4-IgHis, and the Dsg4 reactivities of the sera were examined by IP-IB analysis both before and after column purification. We injected the IgG fractions, which were prepared from the sera with or without immunoadsorption by hDsg4-IgHis, into neonatal mice to examine their pathogenic activities (see Figure 7). IgG that was affinity-purified on the hDsg4-IgHis column was eluted (eluted fraction) and examined for its reactivities against Dsg1, Dsg3, and Dsg4. The IgG affinity-purified on Dsg4 cross-reacted with Dsg1 but not with Dsg3.
These findings indicate that the Dsg4/Dsg1–cross-reacting IgG fraction found in PF sera does not play a major pathogenic role in blister formation in PF.

Dsg4 is not digested by the exfoliative toxins of S. aureus. The ETs produced by S. aureus cause bullous impetigo, which is a common bacterial infection of children, as well as SSSS, which is its generalized form. Three isoforms of ET (ETA, ETB, and ETD) are known to digest Dsg1. To determine whether Dsg4 is cleaved directly by these ETs, we incubated hDsg4-His and mDsg4-His with ETA, ETB, and ETD in vitro at 37°C for 1 hour (Figure 8). None of the ETs cleaved hDsg4-His, while all of the ETs cleaved hDsg1-His. Similarly, none of the ETs cleaved mDsg4-His, while they all digested mDsg1α-His. These findings indicate that Dsg4 is not targeted by ETs. Therefore, Dsg4 does not appear to be involved in the pathogenesis of blister formation in impetigo or SSSS.

Discussion

Desmosomes are essential adhesion structures in most types of epithelium. Their transmembrane components are desmosomal cadherins, desmogleins, and desmocollins (1). Until recently, 3 human and mouse desmogleins had been characterized, namely, Dsg1, Dsg2, and Dsg3, which are expressed in tissue- and differentiation-specific manners. Recent detailed genomic analyses identified a fourth member of the desmoglein family, Dsg4, in humans and mice (7, 8). It has been elegantly demonstrated that mutations in the DSG4 gene cause inherited localized hypotrichosis in humans and the lanceolate hair phenotype in mice (7). In this study, we investigated whether Dsg4 is targeted in the skin autoimmune disease of pemphigus, as well as in the skin infection disease of impetigo and SSSS.

Because the IgG autoantibodies in pemphigus recognize conformational epitopes (29–31), we produced the secreted form of the extracellular domain of Dsg4 by baculovirus expression and examined the reactivities of pemphigus sera by IP-IB analysis. We found that 17 of 20 (85%) PF sera and 13 of 19 (68%) mucocutaneous PV sera, both of which contained anti-Dsg1 IgG autoantibodies, reacted with Dsg4, but none of 16 mucosal dominant PV sera and none of 34 normal control sera did (Figure 2, Table 1). Interestingly, Dsg4 reactivity was eliminated in all of the pemphigus sera that showed Dsg4 reactivity by the removal of anti-Dsg1 IgG by preadsorption with hDsg1-His (Figure 3, Table 1). IgG that was affinity-purified on Dsg4 immunoprecipitated not only Dsg4,

Table 2

<table>
<thead>
<tr>
<th>Serum used</th>
<th>Amount of IgG injected</th>
<th>Mice with gross blisters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No removal</td>
<td>Removal of anti-Dsg4 IgG</td>
</tr>
<tr>
<td>PF no. 1</td>
<td>Original 2/2 (10 h)*</td>
<td>2/2 (10 h)</td>
</tr>
<tr>
<td></td>
<td>2 Diluted 2/2 (13 h)</td>
<td>2/2 (13 h)</td>
</tr>
<tr>
<td></td>
<td>4 Diluted 0/2</td>
<td>0/2</td>
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<tr>
<td></td>
<td>8 Diluted 0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>PF no. 2</td>
<td>Original 2/2 (11 h)</td>
<td>2/2 (11 h)</td>
</tr>
<tr>
<td></td>
<td>2 Diluted 2/2 (12 h)</td>
<td>2/2 (12 h)</td>
</tr>
<tr>
<td></td>
<td>4 Diluted 0/2</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>8 Diluted 0/2</td>
<td>0/2</td>
</tr>
</tbody>
</table>

*Time required for development of gross blisters.

Figure 6

Dsg4 expression in neonatal mouse epidermis. Total RNA was extracted from various sites of the skin (lane 1, ventral skin; lane 2, dorsal skin; lane 3, footpad; lane 4, tail) and tongue (lane 5) and liver (lane 6) in neonatal mice and then subjected to RT-PCR for detection of expression for mouse Dsg4, Dsg1α, Dsg1β, Dsg1γ, and Dsg3. Note that Dsg4 is expressed in various sites of mouse stratified squamous epithelia, including footpad and tongue, where no hair follicles are present, but not in the liver.
B A

Dsg1–cross-reacting IgG autoantibodies play only a minor pathogenic role in blister formation in pemphigus. Therefore, anti-Dsg1 IgG autoantibodies alone are necessary but also Dsg1 (Figure 5). Conversely, the removal of anti-Dsg4 IgG from the PF sera did not significantly reduce the reactivity against Dsg1, but decreased it only 13.8% ± 8.8% (Figures 3 and 4; Table 1). These findings indicate that although many of the PF and mucocutaneous PV sera react with Dsg4, the Dsg4 reactivity is a result of the cross-reactivity of a small subset of anti-Dsg1 autoantibodies (Figure 9). There were essentially no IgG autoantibodies that reacted exclusively with Dsg4 in pemphigus, and all fractions of anti-Dsg4 IgG found in pemphigus sera cross-reacted with both Dsg1 and Dsg4. The 2 PV sera used in the previous study were obtained from patients with the mucocutaneous PV phenotype and should contain both anti-Dsg1 and anti-Dsg3 IgG autoantibodies (7); their reactivities against Dsg4 may also have been owing to the cross-reactivity of anti-Dsg1 IgG autoantibodies. Furthermore, the immunoadsorption of anti-Dsg4 IgG from the PF sera did not block the induction of blisters, while the removal of anti-Dsg1 IgG blocked blister formation in the passive transfer model using neonatal mice (Figure 7, Table 2). Affinity-purified Dsg4/Dsg1–cross-reacting IgG failed to induce blisters, and anti-Dsg1 IgG affinity-purified in parallel from the same volume of PF sera induced extensive blisters (D, H, and L). Scale bars: 100 μm.

Table 3
Pathogenic activity of affinity-purified IgG autoantibodies on Dsg4 and Dsg1 in passive transfer with neonatal mice

<table>
<thead>
<tr>
<th>Serum used</th>
<th>Amount of IgG injected</th>
<th>Mice with gross blisters</th>
<th>Purified Dsg4/Dsg1 cross-reacting IgG</th>
<th>Purified anti-Dsg1 IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF no. 1</td>
<td>Original 0/5</td>
<td>2/2 (11 h)</td>
<td>1/1 (12 h)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 diluted 0/2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF no. 2</td>
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<td>2/2 (9 h, 10 h)</td>
<td>2/2 (10 h, 20 h)</td>
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<td>2 diluted 0/2</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>4 diluted 0/2</td>
<td></td>
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</tbody>
</table>

4Time required for development of gross blisters.
Dsg4 is expressed in the suprabasal epidermis, as determined by immunostaining with chicken Abs that were raised against a specific peptide of Dsg4 (7). Dsg2 is not digested by ETA (Nishifuji and Amagai, unpublished data) but is expressed exclusively in the basal cell layer at a low level (35). Therefore, in the epidermis of patients with bullous impetigo and SSSS, ETs digest Dsg1, while leaving intact Dsg4 behind in the upper layers of the human epidermis. This raises the question as to why Dsg4 cannot compensate for the impaired adhesive function of Dsg1 in the human epidermis. Patients with mutations in the DSG4 gene show hypotrichosis, but do not manifest blisters or erosions in the skin (7). In addition, mice that lack Dsg4 expression show abnormal hair development, but they do not have blisters or erosions in the interfollicular skin (23). These findings suggest that Dsg4 may not play a significant role in the cell-cell adhesion of keratinocytes in the interfollicular epidermis and that Dsg4 may fulfill a role other than one in cell-cell adhesion in the epidermis.

Hair follicles are complex organs of the skin and contain compositionally different desmosomes in their different compartments (36, 37). In mice, Dsg3 plays an important role in the anchorage of telogen hair, as the inhibition of Dsg3, either by genetic abrasion or IgG autoantibodies, leads to telogen-specific hair loss (38–40). Dsg4 seems to be the principal desmosomal cadherin in the anagen hair follicle, and genetic disruption of Dsg4 leads to a transient, intermittent defect in hair follicle development and keratinization, with resultant periodic nodules along the hair shaft and increased hair fragility (7). It will be intriguing to determine whether Dsg4 is targeted in the development of acquired alopecia such as alopecia areata, which is postulated to be an organ-specific autoimmune disease, although the target antigen has not been identified. Future studies that explore the involvement of Dsg4 in various skin diseases represent an exciting field in medicine.

Methods

Human sera. Sera were obtained from 20 patients with PF, 16 patients with the mucosal dominant type of PV, and 19 patients with the mucocutaneous type of PV, each of whom had his or her diagnosis confirmed by clinical, histological, and immunopathological findings. All of the PF sera were positive for anti-Dsg1 IgG and negative for anti-Dsg3 IgG, as determined by ELISA (33, 41). Two of the PF sera that were obtained by plasmapheresis were used for the passive transfer study using neonatal mice (26). All of the mucosal dominant-type PV sera used in this study were positive for anti-Dsg3 IgG and negative for anti-Dsg1 IgG. All of the mucocutaneous-type PV sera used in this study were positive for both anti-Dsg3 and anti-Dsg1 IgG. The sera from 34 normal individuals were used as controls.

Production of Dsg4 recombinant protein by baculovirus and mammalian expression. The cDNA that encodes the entire extracellular domain of hDsg4, including the signal peptide and prosequence, was obtained by RT-PCR using total RNA extracted from normal human scalp skin and the following specific primers for hDsg4 (GenBank accession number, AY177664): forward primer, 5′-AAGCCATGGATTGGCTCTTCTTCAGA-3′, and reverse primer, 5′-TGGCTCGAGACCAACATTTGAAACTCC-3′. The amplified

Figure 8
Lack of in vitro digestion of Dsg4 by ETs. Human (A) and mouse (B) Dsg1(α)-His and Dsg4-His were incubated with ETA, ETB, ETD, or PBS alone (−) in vitro at 37°C for 1 hour. In both the human and mouse cases, Dsg1 (α) was cleaved by all of the ETs, while Dsg4-His was not digested by any of the ETs. The arrows and arrowhead indicate intact recombinant Dsg proteins and the cleaved protein, respectively. The bars indicate the molecular weight standards (from top to bottom: 100, 75, 50, 37, and 25 kDa). (C) Amino acid sequence alignment of human Dsg1, mouse Dsg1α, human Dsg4, mouse Dsg4, and human Dsg3 around the cleavage site of ETs (arrowhead). Amino acid residues that are conserved in human or mouse Dsg4 are shaded.

Figure 9
Anti-Dsg IgG autoantibodies in pemphigus. Two kinds of IgG autoantibodies have been characterized in pemphigus, anti-Dsg1 and anti-Dsg3 IgG. Cross-reactivity between Dsg1 and Dsg3 is only occasionally found in pemphigus sera. The identification of the Dsg4 isoform and the characterization of its involvement in pemphigus have revealed that anti-Dsg4 IgG is a cross-reacting subset of anti-Dsg1 IgG.
cDNA was digested with NcoI and XhoI and ligated into the pQE-TriSys-
tem vector (QIAGEN Inc.) to generate pQE-hDsg4-His (42). The E-tag and
His-tag were inserted in tandem at the C-terminal end of this protein.
The cDNA that encodes the entire extracellular domain of mDsg4 was also
obtained by RT-PCR of total RNA from ICR mouse skin with the follow-
sing specific primers for mDsg4 (GenBank accession number, NM181564):
forward primer, 5′-CGAAACATGGACTGGCTTTCTCTTCAGAAA-3′, and
reverse primer, 5′-TGCCCTCAGTCGAAGTTGATCTGCTAATCC-3′. The cDNA was digested with NcoI and XhoI and ligated into the pQE-TriSys-
tem vector, thereby generating pQE-mDsg4-His. In addition, a fusion
between the entire extracellular domain of hDsg4 and the constant region
of human IgG1 and the His-tag (hDsg4-HisIgG) was constructed in the bac-
culovirus transfer vector pEVmod (25). Nucleotide sequencing of the
hDsg4 constructs revealed 2 nucleotides that are different from the pub-
lished sequence (G285A and C495T, nucleotide numbering from the ATG
translation initiation codon; GenBank, AF177664). These nucleotides do
not cause any amino acid changes. The mDsg4 construct did not have any
nucleotide changes (GenBank, NM181564).

To produce the recombinant human and mouse Dsg4 proteins (hDsg4-His,
mDsg4-His, and hDsg4-IgGHis), the pQE-hDsg4-His, pQE-mDsg4-His, and
pEVmod-hDsg4IgHis plasmids were cotransfected with Saphhire Baculovirus
DNA (Oribgen Inc.) into cultured Sf9 insect cells. The culture supernatants
that contained recombinant viruses were infected into High Five cells, which
were then cultured in serum-free EX-CELL 405 medium (JRH Biosciences
Inc.) for 3 days. To express hDsg4-His in mammalian cells, pQE-hDsg4-His
was transiently transfected into CHO cells using the Superfect Transfection
Reagent (QIAGEN Inc.). To produce nonglycosylated forms of hDsg4-His,
tunicamycin (Sigma-Aldrich) was added to culture media for High Five cells
at 4.5 μg/ml and CHO cells at 0.5 μg/ml and incubated for 3 days.

IP-IB analyses for detection of reactivity against desmogleins. To determine
whether pemphigus sera contain IgG against Dsg4, a combined IP and
IB analysis was performed using culture supernatants that contained
either hDsg4-His or mDsg4-His as substrates. The culture supernatants
were incubated with pemphigus sera or normal control sera and then
immunoprecipitated with protein G-Sepharose or protein A-Sepharose
(Amersham Biosciences) at 4°C overnight with gentle rotation. The immu-
noprecipitates were then resuspended in SDS sample buffer (62.5 μM
Tris-HCL, pH 7.5, 1% SDS, 0.0025% bromophenol blue, 10% glycerol, 2.5%
2-mercaptoethanol), separated by SDS-PAGE, and transferred to a PVDF
membrane (Millipore Corp.). The IP hDsg4-His and mDsg4-His proteins
were visualized with the mouse anti-E tag mAb (Pharmacia Biotech).

To determine whether the recognition of Dsg4 by pemphigus sera was
dependent on protein conformation, the culture supernatants that con-
tained hDsg4-His were dialyzed against Tris-buffered saline in the absence
of calcium and subjected to IP, as described above.

Immunoadsorption of pemphigus sera with Dsg1 and Dsg4. Sera were
preincubated at 4°C for 1 hour with culture supernatant containing hDsg1-IgGHis
(25) or hDsg4-IgGHis without the E-tag or with control culture supernatant
without recombinant protein. Subsequently, the samples were incubated at
4°C overnight with culture supernatants that contained hDsg1-His,
hDsg3-His (33), or hDsg4-His, and immunoprecipitated with protein G-Sepharose.
The IP hDsg1-His, hDsg3-His, and hDsg4-His proteins were detected specifically by
IB analysis using the anti-E tag mAb.

Competition ELISA against Dsg1 using hDsg4-His as a competitor. To measure
Dsg4-reactive IgG in anti-Dsg1 IgG autoantibodies in pemphigus, we per-
formed competition ELISA against Dsg1 using hDsg4-His as a competitor.
Pemphigus sera that reacted with Dsg4 by IP-IB analyses (11 PF sera
and 12 mucocutaneous PV sera) were preincubated at 4°C overnight with
various amounts of hDsg4-His or hDsg1-His as a positive control for com-
petition. The sera were then subjected to Dsg1 ELISA. When necessary,
sera were diluted to keep the absorbance at 450 nm (A450) below 1.2 in
order to obtain a proper competition rate. Competition by immunoad-
soption was calculated using the formula: competition (%). = 1 - (A Asoxp
- A450pos)/(A450neg - A450pos)) × 100. A450pos and A450neg are the measures
obtained for sera preincubated with a maximum dose of hDsg1-His and
culture supernatant of uninfected High Five cells, respectively; A Asoxp is
the measurement obtained for sera preincubated with various concentra-
tions of hDsg4-His or hDsg1-His (31).

RT-PCR for detection of mouse Dsg expression. Total RNA was extracted from
various sites of the skin (ventral skin, dorsal skin, footpad, tail), tongue,
and liver of neonatal ICR mice (<24 hours old) using RNeasy mini kit (QIA-
GEN Inc.). For footprint and tail samples the skin was carefully removed under
a dissecting microscope. These RNA samples were used as templates to
amplify mouse Dsg4, Dsg1, Dsg1β, Dsg1γ, and Dsg3 by RT-PCR with
Superscript One-Step RT-PCR (Invitrogen Corp.) and the following primers:
mouse Dsg4 forward primer, 5′-CTCTGGTGACTGATGACATGTA-3′; mouse
Dsg4 reverse primer, 5′-CTCTGGTATTCGCCAAGATCTCCA-3′; mouse
Dsg1 forward primer, 5′-GGAGAATACGAAAGCTGTTAGT-3′; mouse
Dsg1 reverse primer, 5′-AGGCTCGAGGTGGAACGTGTTTCGTGAG-3′; mouse
Dsg1 reverse primer, 5′-AGGCTCGAGGTGGAACGTGTTTCGTGAG-3′; mouse
Dsg1 forward primer, 5′-CAGGTCAAGCTACAAACAAGT-3′; mouse
Dsg2 forward primer, 5′-CCGATACGACTGATGACATGTA-3′; mouse
Dsg3 reverse primer, 5′-TGCCCTGAGGAAGCATCACGTCTCAGTTGGATG-3′; mouse
Dsg3 forward primer, 5′-CCGATACGACTGATGACATGTA-3′; mouse
Dsg3 reverse primer, 5′-GCCCTAGATCATAGTAATCATC
TCCATCCCGAGCA-3′.

Passive transfer experiments with neonatal mice. Large-scale immunoad-
soption of PF sera was performed using the AKTA explorer HPLC sys-
tem (Amersham Biosciences) with the TALON affinity resin column (BD
Biosciences — Clontech). Typically, 5–10 ml of PF sera was applied to the
affinity resin column along with approximately 500–2,000 μg hDsg4-His or
hDsg1-His, and IgG was precipitated from the flow-through fraction with
40% ammonium sulfate. The IgG fraction was then dialyzed against PBS and
concentrated approximately tenfold using the microconcentrator Cen-
triprep 30 (Amicon; Millipore Corp.). The complete removal of the desired
IgG was confirmed by IP-IB analyses for anti-Dsg4 IgG and ELISA for anti-
Dsg1 IgG. For affinity-purification, IgG bound to hDsg4-His or hDsg1-His
was eluted with 0.1 M glycine-NaOH (pH 11.5), immediately neutralized
with 1 M Tris-HCl (pH 7.4), dialyzed against PBS, and concentrated to a
final volume of 600 μl in Centriprep 30.

To evaluate the pathogenic activities of the immunoadsorbed sera, a
passive transfer model with neonatal mice was used, as described previ-
ously (26, 43). Neonatal ICR mice at 12–24 hours of age (body weight,
1.6–1.8 g) were injected subcutaneously with the IgG fractions prepared
as above. To compare the pathogenic strength of PF sera with and with-
out Dsg4 immunoadsorption in a more quantitative fashion, the concen-
trated IgG fractions prepared by ammonium sulfate precipitation were
serially diluted and injected into neonatal mice. Similarly, to compare the
pathogenic activity of IgG affinity-purified on hDsg4-His or hDsg1-His,
purified IgG was serially diluted and injected into neonatal mice. The mice
were examined and a biopsy was performed when they developed
blister, or they were observed for 24 hours when they did not develop
apparent blisters. All mouse studies were approved by the animal ethics
review board of Keio University.

In vitro digestion of recombinant Dsg4 with ETs. Three isoforms of ETs, ETA,
ETC, and ET, were prepared as described previously (17, 18). The culture
supernatants that contained hDsg1-His, hDsg4-His, mDsg1-Exe-His (40),
or mDsg4-His were incubated with ETA, ETB, and ETD for 1 hour at 37°C. The
treated culture supernatants were subjected to IB analysis with the anti-E tag
mAb to visualize intact as well as digested recombinant proteins.
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30. Takeshi Nagasaka and Koji Nishifuji contributed equally to this work.