Autoantibodies to DSC3 in Pemphigus Exclusively Recognize Calcium-Dependent Epitope in Extracellular Domain 2

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INTRODUCTION

Pemphigus is a group of autoimmune bullous diseases characterized by the presence of autoantibodies against adhesion molecules, desmogleins, and desmocollins (DSCs). The pathogenicity of anti-DSC3 antibodies in pemphigus has been demonstrated; however, its characteristics have not yet been elucidated. We aimed to analyze the characteristics of anti-DSC3 antibodies using DSC3 domain-swapped desmoglein 2 molecules in which the prosequence and five extracellular (EC) domains of desmoglein 2 were replaced with the corresponding domains of human DSC3. Using these proteins, we established an ELISA and analyzed sera from 56 patients with pemphigus. In 34 pemphigus sera positive for DSC3 full-EC domains, 15 sera (44.1%) were positive for EC2 domain, whereas other domains were rarely positive. We assessed the reactivity to a calcium-dependent epitope in DSC3 by ELISA with EDTA. The reactivity with the EC2 domain was mostly compromised in the presence of EDTA. In the in vitro assay, IgG from patients with paraneoplastic pemphigus pre-adsorbed with EC2 prevented both reduction of DSC3 and keratinocyte dissociation as compared with that with EDTA-treated EC2. This study revealed a predominant recognition of calcium-dependent epitopes in EC2 domain by anti-DSC3 antibodies and its pathogenicity on keratinocyte adhesion through DSC3 depletion.


Pemphigus is an autoimmune bullous disease characterized by impaired cell adhesion in the epidermis by autoimmune mechanisms (Bystryn and Rudolph, 2005). Desmosome is the most important machinery for keratinocyte (KC) cell–cell adhesion that confers mechanical integrity to tissues (Nekrasova and Green, 2013). Pemphigus develops circulating autoantibodies against desmosomal cadherins (Kasperkiewicz et al., 2017). Two types of desmosomal cadherins, desmogleins (DSGs) 1–4 and desmocollins (DSCs) 1–3, have been identified (Getsios et al., 2004). Among these desmosomal cadherins, DSG1 and DSG3 are well-known autoantigens in pemphigus foliaceus and pemphigus vulgaris (PV), respectively (Amagai et al., 1999).

The extracellular (EC) domain of desmosomal cadherins consists of four cadherin repeats of approximately 110 amino acids, EC1–4, and an EC anchor, EC5 (Figure 1) (Dusek et al., 2007). The adhesive capacity was conferred by EC1 and EC2 whose junctions were modified by calcium (Ca2+) at the Ca2+-binding sites (Cailliez and Lavery, 2005). Furthermore, our previous study suggested the involvement of a Ca2+-dependent epitope in EC3 (Ohyama et al., 2012). In DSG–DSC interaction, it has been reported that EC1–2 of DSC1 was required for its binding to DSG in a Ca2+-dependent manner (Chitaev and Troyanovsky, 1997). Typically, anti-DSG antibodies in pemphigus predominantly recognized the N-terminal domains, which consist of Ca2+-dependent conformational epitope (Chan et al., 2010; Ohyama et al., 2012).

Administration of AK19 and AK23 mouse anti-DSG3 mAbs, whose epitopes locate on N-terminal EC domain (targeting to EC1 and EC1–2, respectively), but not AK7, AK9, and AK20 mAbs, whose epitopes locate on C-terminal EC domain (targeting to EC4–5), into mice induced intraepidermal blistering at a microscopic level (Tsunoda et al., 2003). In addition, coinjection with exfoliative toxin A, which is known to enzymatically cleave DSG1, induced blister formation at the gross level. With this evidence, antibodies targeting EC1–2 but not targeting EC4–5 of DSG3 were considered to be pathogenic (Koga et al., 2013).

DSC1 was identified as an autoantigen in subcorneal pustular dermatosis—type intercellular IgA dermatosis (also known as IgA pemphigus) by cDNA transfection method (Hashimoto et al., 1997). Using the same detection system, anti-DSC antibodies were detected in some patients with atypical pemphigus (Preisz et al., 2004; Saruta et al., 2013; Ueda et al., 2013a, 2013b). Then, ELISAs using DSC1–3 recombinant proteins (RPs) produced in baculovirus system were developed; however, their sensitivities were not
sufficient (Hisamatsu et al., 2004). Therefore, we established more sensitive ELISAs using DSC1–3 RPs produced in mammalian expression system that detect IgG anti-DSC antibodies frequently in atypical pemphigus (paraneoplastic pemphigus [PNP], pemphigus herpetiformis [PH], and pemphigus vegetans) but rarely in typical pemphigus (pemphigus foliaceus and PV) (Ishii et al., 2015). DSC IgA ELISAs were also established that successfully detected IgA anti-DSC1 antibodies in subcorneal pustular dermatosis–type intercellular IgA dermatosis (Teye et al., 2016).

Among DSC1–3, the importance of DSC3 in cell adhesion was confirmed by skin-specific DSC3 knockout mice, which showed intraepidermal blistering, resembling that observed in patients with PV (Chen et al., 2008). The capability of anti-DSC3 antibodies to enforce the loss of KC adhesion was demonstrated by organ culture system with biopsy skin specimen and anti-DSC3 mAb (Spindler et al., 2009) and by KC dissociation assay, in which the number of fractions of cultured KC sheets were counted, after incubation with anti-DSC3 antibodies purified from sera of atypical pemphigus (Mao et al., 2010; Rafei et al., 2011).

Therefore, the pathogenicity of anti-DSC3 antibodies was elucidated, although that of anti-DSC1 and anti-DSC2 antibodies remains unknown. However, the characteristics of anti-DSC3 autoantibodies in patients with pemphigus have not yet been clarified. In this study, we aimed to analyze the epitope of anti-DSC3 antibodies in pemphigus using DSC3–DSG2 domain–swapped RPs produced in a mammalian expression system.

RESULTS

Establishment of DSC3 domain–swapped DSG2 recombinant proteins

Previously, to analyze the epitopes for anti-DSG1 or anti-DSG3 antibodies in patients with pemphigus, we generated domain-swapped RPs containing DSG3 or DSG1 with DSG2 as the backbone, taking advantage of the fact that PV sera show no reactivity with DSG2 (Ohyama et al., 2012). These swapped proteins allowed a precise mapping of the conformational epitopes embedded in the three-dimensional molecular structure because the swapped RPs had similar structures but distinct epitopes (Müller et al., 2008). With this concept, we aimed to generate domain-swapped molecules comprising DSC3 with DSG2 as the backbone.

We first generated RPs of full EC domains of DSG2 and DSC3 in HEK293T cells. DSG2 and DSC3 contain five EC domains, EC1–5, and a consequence (Pro). By replacing the five EC domains and Pro of DSG2 with the corresponding domains of DSC3, we obtained a series of six DSC3–DSG2 domain–swapped RPs: Pro, EC1, EC2, EC3, EC4, or EC5 of DSC3 swapped into the corresponding domain of DSG2. However, the RP of EC1 swapped into DSG2 was not expressed efficiently even though the cDNA in the plasmid was confirmed in the transfected cells. Eventually, the following swapped RPs were generated: Pro of DSC3–DSG2, Pro and EC1 of DSC3–DSG2, that is, Pro + EC1; EC2 of DSC3–DSG2; EC3 of DSC3–DSG2; EC4 of DSC3–DSG2; and EC5 of DSC3–DSG2 (Figure 1a). All the RPs and RPs with the full EC domains of DSC3 and DSG2 were found to be secreted into the culture medium, which were confirmed as bands detected at expected molecular weights by western blotting in TALON-purified fractions (Figure 1b). Two bands were detected in DSC3 and Pro + EC1 that suggested expressions of precursor and mature forms, as reported for DSG3 RPs (Yokouchi et al., 2009).

Predominant recognition of EC2 domain of DSC3 by anti-DSC3 autoantibodies in patients with pemphigus

With these RPs, an ELISA system was established. In total, 56 sera from patients with pemphigus (pemphigus foliaceus n = 3, PH n = 7, PNP n = 42, pemphigus vegetans n = 4), which were positive for anti-DSC3 antibodies in our previous study (Ishii et al., 2015), and 14 sera from healthy volunteers (normal) were analyzed by the ELISAs (Figure 2a). Interestingly, most sera showed relatively high optical density (OD) values for EC2 RP, similar to those for DSC3 RP. In contrast, a few sera demonstrated moderate to low OD values against the other swapped proteins. It was difficult to set a cutoff value using the ODs of healthy volunteer sera because each serum from the patients with pemphigus showed different reactivity (OD) to each protein; for example, some sera from patients with PNP showed relatively high OD values to all the swapped proteins, including the proteins purified from the culture medium of intact vector–transfected cells. Therefore, we set a cutoff value at triple of the OD for proteins purified from the culture medium of cells transfected with intact vector as a negative control for each serum. Among the 34 cases that were positive against DSC3 RP, 15 cases (44.1%) were positive for EC2 RP with the highest frequency, followed by 4 cases (11.8%) for EC3 RP, and minimally for the other domain RPs (Figure 2b).

Predominant recognition of Ca2+-dependent epitope of DSC3 by antibodies in pemphigus

A previous study indicated that anti-DSG3 antibodies recognizing Ca2+-dependent epitopes are pathogenic (Kamiya et al., 2012). In that study, an EDTA-pretreated ELISA plate was used to detect antibodies to non-Ca2+-dependent epitopes. Applying this method, the reactivities of pemphigus sera to DSC3 and EC2, which were treated or untreated with EDTA, were compared (Figure 3). The OD values for DSC3 RP in 27 samples significantly decreased in the presence of EDTA compared with that in the absence of EDTA (0.25 ± 0.42 and 0.55 ± 0.70, P = 0.032). This difference in OD values in the presence or absence of EDTA was not significant for EC2 RP (0.23 ± 0.37 and 0.35 ± 0.54, P = 0.44). In the analysis for each subtype of pemphigus, there were similar tendencies of decrease in the presence of EDTA, particularly noticeable in PH and PNP, with no significant differences, possibly owing to a small sample number.

Decrease of DSC3 expression by anti-EC2 antibodies recognizing Ca2+-dependent epitope in pemphigus

The above results indicate that anti-DSC3 antibodies in pemphigus predominantly recognize Ca2+-dependent epitopes in the EC2 domain. We further investigated whether anti-DSC3 antibodies recognizing Ca2+-dependent EC2 domain have a pathogenicity in pemphigus. For this purpose, we measured the decrease in DSC3 expression after the
bindings of anti-DSC3 IgG in cultured HaCaT cells because purified IgG from the sera of patients with PV was reported to cause a decrease of DSG3 on the cell surface through endocytosis, leading to loss of KC cell–cell adhesion (Calkins et al., 2006). Similar to DSG3, cell surface DSC3 was also shown to decrease by anti-DSC3 antibodies from patients with pemphigus in vitro (Mao et al., 2010). The serum from one patient with PNP, which was negative in commercial DSG1 and DSG3 ELISAs (MBL, Nagoya, Japan) and positive for DSC3 but not for DSC1 and DSC2 by DSC ELISAs (Supplementary Table S1), was used in this experiment. The cells incubated with normal human IgG (control) for 24 hours showed a high intensity of DSC3 staining at the cell surfaces (Figure 4a). Expectedly, the cells incubated with IgG from one patient with PNP (PNP-IgG) preadsorbed with the TALON-purified protein from the cultured medium with intact vector–transfected cells (vector) showed a significant reduction of DSC3 staining (Figure 4b). Notably, the cells incubated with PNP-IgG preadsorbed with DSC3 RP (Figure 4c) and EC2 RP (Figure 4d) showed a similar intensity of DSC3 staining as in Figure 4a. On the other hand, the cells incubated with PNP-IgG preadsorbed with EC2 RP pretreated
with EDTA (EDTA + EC2) significantly reduced the intensity of cell surface DSC3 staining (Figure 4e), similar to that in Figure 4b.

DSC3 expression levels were also measured in normal human KCs by western blotting (Figure 4ga and h). It was previously reported that IgG from the sera of patients with PV reduced Triton X-100 soluble (membrane) and insoluble (cytoskeleton) DSG3 on human KC cell lines (Aoyama and Kitajima, 1999). Owing to the limited amount of the IgG from the patient, the IgG from another patient with PNP was used in later assays (Supplementary Table S1). In Triton-soluble fraction, the cells incubated with vector and PNP-IgG preadsorbed with EDTA + EC2 significantly reduced DSC3 expression to “In Triton-soluble fraction, the cells incubated with PNP-IgG preadsorbed with vector or EDTA + EC2 significantly reduced DSC3 expression. The results indicated that PNP-IgG recognizing Ca^{2+}-dependent epitope in EC2 domain enforces the decrease of cell surface DSC3 expression as observed during DSG3 endocytosis (Calkins et al., 2006).

Attenuation of KC adhesion by anti-DSC3 EC2 antibodies recognizing Ca^{2+}-dependent epitope in pemphigus

Finally, a KC-based dissociation assay, which was previously established to assay adhesion effects (Ishii et al., 2005), was performed (Figure 5). The purified IgG from the serum of the patient with PH was used because KC dissociation induced by anti-DSC3 antibodies from patients with PH was reported previously (Rafei et al., 2011). The cells incubated with IgG from the serum of the patient with PH and PNP-IgG preadsorbed with vector resulted in a significantly larger number of fragments ($P = 0.036$ and $P = 0.047$), whereas the cells incubated with PNP-IgG preadsorbed with DSC3 RP and EC2 RP resulted in equivalent fragments compared with cells incubated with normal human IgG (control). The cells incubated with PNP-IgG preadsorbed with EDTA + EC2 also resulted in a relatively larger number of fragments than control, although it was statistically insignificant. These results supported that anti-DSC3 antibodies from PNP-IgG attenuated adhesion in KCs. These results also indicated that anti-DSC3 antibodies recognize Ca^{2+}-dependent epitopes in EC2, thereby evoking strikingly attenuated adhesion in KCs.

DISCUSSION

Using DSC3 domain--swapped DSG2 RPs, this study revealed that anti-DSC3 antibodies in pemphigus, particularly in PNP and PH, predominantly target Ca^{2+}-dependent conformational epitope(s) on the EC2 domain. Furthermore, the cell culture system using PNP-IgG demonstrated that such antibodies lead to a decrease of cell surface DSC3 expression by autoantibody binding--induced endocytosis, resulting in KC dissociation.

Our previous study using DSG3 domain--swapped DSG2 RPs revealed that the epitopes for anti-DSG3 antibodies in pemphigus located over several domains of DSG3 N-terminal domain; the reactivities to EC1, EC2, EC3, EC4, and EC5 were 91.0%, 71.2%, 50.4%, 18.9%, and 12.3%, respectively (Ohyama et al., 2012). Similarly, the reactivities of anti-DSG1...
antibodies in pemphigus foliaceus to EC1, EC2, EC3, EC4, and EC5 were 88%, 50%, 13%, 22%, and 0%, respectively (Chan et al., 2010).

Of note, unlike anti-DSG3 antibodies, our results indicated that anti-DSC3 antibodies predominantly recognize the EC2 domain. In comparison with the patients with reactivity to multiple domains in DSC3 RP ELISA, only one patient with PNP reacted with EC2 and EC3; two patients with PH reacted with EC2 and EC3 or with EC2 and EC5; and two patients with pemphigus vegetans reacted with EC2 and EC3 or all domains. Hence, anti-DSC3 antibodies in pemphigus seem to target the EC2 domain almost exclusively (Figure 6). Our results also indicated that anti-DSC3 antibodies in pemphigus predominantly recognized Ca\(^{2+}\)-dependent conformational epitopes, particularly in PNP. Although PH sera also showed a similar tendency, it was difficult to draw a conclusion owing to the small sample size.

This difference between anti-DSC3 antibodies and anti-DSG antibodies may indicate that a break of immunotolerance for DSC3 occurs differentially from other major
pemphigus autoantigens. Anti-DSC antibodies are frequently detected in patients with atypical pemphigus whose clinical and pathological features are distinct from those of classical pemphigus. Therefore, the immunoreactivity and pathogenic activity of anti-DSC3 antibodies in atypical pemphigus may be different from those in classical pemphigus.

The almost exclusive recognition of the EC2 domain by anti-DSC3 suggested that the epitope on EC2 of DSC3 is also highly specific and that there may be only a few unique epitopes. However, finer epitope mapping was restricted by the technical difficulties associated with the production of Ca\(^{2+}\)-dependent conformational epitopes.

However, the ELISA system using DSC3 domain–swapped DSG2 RPs established in this study showed a lower positive rate than the previously reported system using full DSC3 EC domain (Ishii et al., 2015); 22 of 56 sera from patients with pemphigus, which were positive in the previous study, were negative in the new ELISAs. This may be attributed to the difference in the cell culture system and to the RP purification method. The serum from a patient with PV, which was weakly positive for DSC3 (OD of 0.161) in the former study (Ishii et al., 2015), was negative in this ELISA; thus, we could not address anti-DSC3 reactivity to EC2 in PV in this study. Although most of the pemphigus sera that were positive with DSC3 RP reacted only with EC2 RP, the positive rate was not so high (44.1%) owing to negative cases for all swapped RPs. Further efforts are required to enhance the sensitivity of this system.

We showed that IgG antibodies to DSC3 EC2 domain in PNP sera reduced DSC3 expression on the surface of KCs and attenuated KC adhesion. It is still possible that antibodies to non-DSC3 antigens in the PNP serum participate in these phenomena. However, a previous study showed that IgG from the sera of patients with PV depletes only DSG3 but not DSC3 from the surface of KCs (Shu et al., 2005). Although the PNP-IgG used in Figures 4g and h and 5 contained anti-DSC2 antibodies, preadsorption with DSC3 and EC2 RPs attenuated DSC3 reduction and KC dissociation, indicating the pathogenicity of anti-DSC3 antibodies. These results indicate that antibodies to Ca\(^{2+}\)-dependent DSC3 EC2 domain in the patient serum indeed reduced DSC3 expression. The borders of HaCaT cells showed dotted staining patterns of DSC3 after 24 hours of incubation with PNP-IgG (the small columns in Figure 4b), although a time-course analysis for DSC3 endocytosis was not performed owing to the limited amount of the patient’s serum. These findings suggested that a reduction of DSC3 expression resulted from endocytosis of DSC3.

In this study, we found that anti-DSC3 in atypical pemphigus recognized EC2 domain almost exclusively. This uniqueness may lead to new approaches to investigate the pathogenicity of pemphigus, particularly with anti-DSC antibodies, which are different from those used mainly on DSGs.
to date. Further characterization of the immunoreactivities against DSC3 is required to fully understand their pathological relevance in DSC-related pemphigus.

**MATERIALS AND METHODS**

**Patients’ sera**

Sera from 56 patients with pemphigus, which were positive for anti-DSC3 antibody in our previous study (Ishii et al., 2015), were used. This study was approved by the ethics committee of the Kurume University, Japan, and was performed in adherence to the Declaration of Helsinki guidelines. Written informed consent was obtained from all patients and the controls.

**Plasmid constructs**

cDNA was obtained from normal human KC. pcDNA3.1 (Invitrogen, Carlsbad, CA) containing puromycin resistance and cMyc and histidine tag sequences at the C-terminus of the multiple cloning site was used. First, the complete EC domains of DSC3 and DSG2 were cloned into pcDNA3.1 using BamHI and XhoI; the plasmids for DSC3 full ECs and DSG2 full ECs are designated as DSC3 and DSG2, respectively. To produce the plasmid constructs of DSC3 domain-swapped DSG2 RPs, each PCR product for Pro, Pro⁺EC1, EC2, EC3, EC4, and EC5 of DSC3 was assembled with DSG2 vector using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA). The sequence for each domain of DSC3 and DSG2 was referenced from a previous report (Harrison et al., 2016). The construction and nomenclature of the plasmids are represented in Figure 1a. Primers were designed to overlap the swapped point of DSC3 and DSG2 (Supplementary Table S2). Plasmid sequences were confirmed using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

**Expression of recombinant proteins**

The plasmids were transfected into HEK293T cells using Lipofectamine LTX (Invitrogen, Carlsbad, CA). Transfected cells were cultured in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml puromycin, with medium exchange every 3 days. After culture media were collected, the RPs were purified by TALON metal affinity resin (Clontech Laboratories, Mountain View, CA) and eluted with tris-buffered saline containing 250 mM Imidazole. As a negative control, an extraction procedure was implemented on the culture medium of pcDNA3.1-transfected cells.

**ELISA**

ELISA was performed as described previously (Ishii et al., 2015). Concentrations for each purified RP-containing stock solution for ELISA were adjusted by direct ELISA. Purified protein from the culture media of intact vector-transfected cells was used as a negative control. Because its concentration could not be adjusted in ELISA, the mean concentration between different purified RP-containing stock solutions was used. In EDTA-treated ELISA, each RP was immobilized on the ELISA plate with a coating buffer: 10 mM Tris, 150 mM sodium chloride, pH 7.2 with 1 mM Ca²⁺ chloride (CaCl₂) or with 0.5 mM EDTA overnight at 4 °C. After coating, the followed steps were the same described earlier. ODs were measured at 450 nm by Infinite M200 pro Microplate Reader (Tecan, Männedorf, Switzerland).
KC-based DSC3 expression assay
For the evaluation by immunofluorescence, cells of an immortalized human KC cell line (HaCaT cells) were cultured with EpiLife medium supplemented with Human Keratinocyte Growth Supplement (Thermo Fisher Scientific, Waltham, MA) in a 12-well plate (BD Falcon, Franklin Lakes, NJ). At 70–80% confluence, the culture medium was changed to a 1.5 mM CaCl2-containing medium. PNP-IgG was purified by Spin column-based Antibody Purification Kit (Protein G) (Cosmo Bio, Tokyo, Japan). Intact vector-, DSC3 vector-, or EC2 vector–transfected cell culture media were incubated with anti-cMyc Agarose (Pierce Thermo Scientific, Waltham, MA) in the presence of 1 mM CaCl2 or 0.5 mM EDTA overnight at 4 °C. Each coupled anti-cMyc Agarose was incubated with PNP-IgG for 1 hour at 4 °C, and the supernatants were collected by spin column, followed by sterilization with a 0.22 μm filter. Twenty-four hours after HaCaT cells were cultured in 1.5 mM CaCl2-containing medium, the PNP-IgG containing supernatants diluted in EpiLife with 1.5 mM CaCl2 were added to the cells and was further cultured for 24 hours. For the evaluation by western blotting, normal human KCs were seeded on a 24-well plate and cultured in EpiLife with Human Keratinocyte Growth Supplement. At confluency, the medium was changed to that with 1.5 mM CaCl2. The next day, PNP-IgG, which were preadsorbed by anti-cMyc agarose preincubated with each cell culture media described earlier in the presence of CaCl2 or EDTA overnight at 4 °C, were added and incubated for 24 hours. The cells were rinsed with PBS and scraped in 1% Triton X-100 with protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). After centrifugation, the supernatants were collected as a Triton-soluble fraction.

Dispase-based KC dissociation assay
The adhesion of KC in the presence of the patient IgG was evaluated as described previously (Ishii et al., 2005). In brief, normal human KCs were seeded on a 12-well plate and cultured in EpiLife with Human Keratinocyte Growth Supplement. At confluency, the medium was changed to that with 1.5 mM CaCl2. The next day, PNP-IgG, which were preadsorbed by anti-cMyc agarose preincubated with each cell culture media described earlier in the presence of CaCl2 or EDTA overnight at 4 °C, was added and incubated for 24 hours. Two hours before the end of incubation, 0.5 μg/ml exfoliative toxin A (Toxin Technology, Sarasota, FL) was added to cleavage DSG1. After washing with PBS, the cells were incubated with 2.5 U/ml dispase (StemCell Technologies, Vancouver, Canada) to release the cells as monolayers. After rinsing with PBS, the monolayered cells were subjected to mechanical stress by pipetting with a 1-ml pipetman. Fragments were counted by Image J (NIH software, Bethesda, MD).

Statistical analysis
The data are presented as the mean ± SD. Statistical calculations were performed using GraphPad Prism (version 6.05; GraphPad Software, San Diego, CA). The tests used are indicated in the figure legends. P-value of 0.05 was considered to be statistically significant.

Data availability statement
No datasets were generated or analyzed during this study.


SUPPLEMENTARY MATERIALS AND METHODS

Antibodies

Peroxidase-conjugated monoclonal anti-6×Histidine antibody (9C11, FUJIFILM Wako, Richmond, VA) was used for ELISA. Monoclonal anti-desmocollin 3 antibody (desmocollin 3-U114, GenWay Biotech, San Diego, CA) and Alexa 488-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA) were used for immunofluorescence (Figure 4a–f) and western blotting (Figure 4g and h).

Immunofluorescence

The cells were fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 0.1% Triton X-100 for 5 minutes, followed by blocking with PBS containing 1% BSA and 10% goat serum for 20 minutes. Cells stained with anti-desmocollin 3 antibodies were observed under a fluorescence microscope (Olympus DP74, Olympus, Tokyo, Japan) and analyzed by Image J (National Institutes of Health software, Bethesda, MD). Fluorescence density at cell borders was calculated according to the methods used in a previous report (Huen et al., 2002). Briefly, instead of dividing the fluorescence signal (pixels²) by length (pixels) on the cell border, the percentage of the area containing fluorescence signals (pixels) over the threshold in 30-pixels width line at the cell border was calculated. In total, 10 representative regions of intercellular contact for each condition were analyzed.

Western blotting

The eluted fractions from culture media of respectively transfected HEK293T by TALON metal affinity resin (Clontech Laboratories, Mountain View, CA) were separated by SDS-PAGE with 5–20% gel, followed by transfer to polyvinylidene fluoride membrane. The bands were visualized using peroxidase-conjugated monoclonal anti-6×Histidine antibody (9C11) and tetramethylbenzidine substrate (EzWest; ATTO, Tokyo, Japan). For evaluation of desmocollin 3 expression in keratinocytes, images were observed under Typhoon 9500 (GE Healthcare, Piscataway, NJ). The intensities of each band were analyzed using Image J.

SUPPLEMENTARY REFERENCE


Supplementary Table S1. The Immunological Features of the Sera Used in this Study

<table>
<thead>
<tr>
<th>Sera</th>
<th>DSG IgG ELISA (MBL, Nagoya, Japan) (Cutoff Index &lt; 20)</th>
<th>DSC IgG ELISA (Cutoff OD: for DSC1 &lt; 0.2, DSC2 &lt; 0.07, DSC3 &lt; 0.12)</th>
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<tr>
<td>Serum from patient with PNP used in</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Figure 4a–f</td>
<td>DSG1: −1.95</td>
<td>DSC1: −0.075</td>
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<tr>
<td>Serum from patient with PNP used in</td>
<td>DSG3: −2.07</td>
<td>DSC2: −0.038</td>
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<td>Figures 4g and h and 5</td>
<td>&lt;5.0</td>
<td>DSC3: 2.439</td>
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<td>Serum from patient with PH used in</td>
<td>DSG1: 2.54</td>
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</tr>
<tr>
<td>Figure 5</td>
<td>DSG3: 0.62</td>
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</table>

Abbreviations: DSC, desmocollin; DSG, desmoglein; OD, optical density; PH, pemphigus herpetiformis; PNP, paraneoplastic pemphigus; PNP-IgG, IgG from one patient with paraneoplastic pemphigus; RP, recombinant protein.

In a preliminary experiment, adequate amounts of the purified PNP-IgG and DSC3 RP for preadsorption were determined (the OD in DSC3 RP ELISA decreased from 2.56 to 0.13 after adsorption by DSC3 RP).
**Supplementary Table S2. The Primers Used in this Study**

<table>
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<tr>
<th>Primer ID</th>
<th>Primer Sequence (5’–3’)</th>
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<tr>
<td>DSC3 forward</td>
<td>TTGGTACCGAGCTggatccACC1ATGGCCGCCGCTGGGCCCCG</td>
</tr>
<tr>
<td>DSC3 reverse</td>
<td>AAGGGCCCTCTAGActcgagTTTTCACATATTCTCCTGATCC</td>
</tr>
<tr>
<td>DSG2 forward</td>
<td>TTGGTACCGAGCTgatccAAACC1ATGGCCGCCGAGCCCCGGA</td>
</tr>
<tr>
<td>DSG2 reverse</td>
<td>AAGGGCCCTCTAGActcgagGGGAGCCGCTGACATGAGACACTC</td>
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</table>

The underlined sequences are the overlapped sequence for ligation of pcDNA3.1/myc-His A.

**The Primers Used in the Vector.**

| DSC3 EC1/DSG2: ligation of DSC3 sequence into DSG2 vector |
| DSC3 EC1 forward | GTGCGGAAAAGCGCAGATGGGCACCTATTCCTTG |
| DSC3 EC1 reverse | GAACACTGGTTCGATCTATTTTCCCATCTACCC |
| DSG2 for DSC3 EC1 forward | AACGAAACAGTTGTCAACA |
| DSG2 for DSC3 EC1 reverse | GCCGTTGCGGGCACACTAAAT |

**DSC3 EC2/DSG2: ligation of DSC3 sequence into DSG2 vector**

| DSC3 EC2 forward | CTTGGATCAATGACAGATGGGCACCTATTCCTTG |
| DSC3 EC2 reverse | TATACAGGATATTATGTTGACATGGCAGGT |
| DSG2 for DSC3 EC2 forward | AAATACCTGTGATAGAAAATAAGT |
| DSG2 for DSC3 EC2 reverse | GTGATGATTGAAACAGAAAATAC |

**DSC3 EC3/DSG2: ligation of DSC3 sequence into DSG2 vector**

| DSC3 EC3 forward | TTGGATGTGTAATGACAGATGGGCACCTATTCCTTG |
| DSC3 EC3 reverse | TTTAAAATGAATGCCCACATCCAGATCCCTCACAT |
| DSG2 for DSC3 EC3 forward | GGGGGGCAAGAGGCCCACAGTTTTCCAAG |
| DSG2 for DSC3 EC3 reverse | GTGATGATTGAAACAGAAAATAC |

**DSC3 EC4/DSG2: ligation of DSC3 sequence into DSG2 vector**

| DSC3 EC4 forward | AAAATGTTGAAGAGGCCGTGAATGCACTCTT |
| DSC3 EC4 reverse | CACGGACGCACGATTTATGCTT |
| DSG2 for DSC3 EC4 forward | AAGTGTACCTGTGATAGAAAATAAG |
| DSG2 for DSC3 EC4 reverse | GTGATGATTGAAACAGAAAATAC |

**DSC3 EC5/DSG2: ligation of DSC3 sequence into DSG2 vector**

| DSC3 EC5 forward | GAAGATCAACGAGACACCATCCACGACAAATATTTCTTAAGA |
| DSC3 EC5 reverse | CCCTCTAGAATGCTGATTTTCCCAAGTTG |
| DSG2 for DSC3 EC5 forward | CTGAGTCTAGAGGGCCCCT |
| DSG2 for DSC3 EC5 reverse | GTGATGATTGAAACAGAAAATAC |

**DSC3 Pro/DSG2: ligation of DSG2 sequence into DSC3 vector**

| DSC3 Pro 5’-5’ forward | AGGCCTGCGCAAGGCCGCCGGCTGATACCCGCCC |
| DSC3 Pro 5’-5’ reverse | CCCTCTAGAATGCTGATTTTCCCAAGTTG |
| DSG2 for DSC3 Pro 5’ forward | CTAGGATGAGG |
| DSG2 for DSC3 Pro 5’ reverse | GTGATGATTGAAACAGAAAATAC |

**Abbreviations:** DSC, desmocollin; DSG, desmoglein; EC, extracellular; His, histidine; ID, identification; Pro, prosequence. The lower cases are for restriction enzyme–recognized sequence.\(^{1}\)

\(^{1}\)The Kozak sequence.