Actin reorganization contributes to loss of cell adhesion in pemphigus vulgaris

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PEMPHIGUS VULGARIS (PV) is an autoimmune skin disease characterized by blisters in the deep epidermis and antibodies against keratinocyte surface antigens (36, 39). The main antigens are cadherins desmoglein (Dsg) 1 and Dsg3, but other targets may also exist (28, 29, 38). In mucosal-dominant PV, autoantibodies are directed against Dsg3, whereas in mucocutaneous PV involving the skin, antibodies also target Dsg1. Although antibody profiles of different types of pemphigus are well defined, the mechanisms leading to blister formation are still unclear (1). At the moment, several mechanistic theories are proposed. On one hand, the direct inhibition hypothesis states that antibodies directly interfere with Dsg transinteraction. This view is supported by atomic force microscopy studies with anti-Dsg3 antibodies that were sufficient to block Dsg3 transinteraction in a cell-free environment (20). Moreover, peptides stabilizing Dsg transinteraction have been shown to be protective against PV-IgG-induced cell dissociation (19). On the other hand, a huge number of intracellular signaling pathways have been found to contribute to pemphigus pathogenesis (11, 12, 16, 22, 24) involving p38 mitogen-activated protein kinase (MAPK) (3, 4), RhoA (40), and plakoglobin (7, 41).

In addition, recent studies reported that loss of keratinocyte adhesion in response to PV-IgG was accompanied by profound changes of the actin cytoskeleton such as displacement of the peripheral junction-associated actin belt or increased stress fiber formation, which were generally summarized as actin reorganization (3, 34, 40). Actin filaments have important functions for mechanical stability of cell-cell and cell-matrix contacts based on their anchorage to adherens junctions and focal contacts (31). It is noteworthy that actin filament functions are regulated by cellular signaling pathways such as Rho GTPases (17, 26, 40) and p38MAPK (30), which are also considered to be important signaling molecules in pemphigus pathogenesis.

In this study, we addressed the question whether actin reorganization in pemphigus is a secondary phenomenon that results from loss of Dsg function and cell dissociation or rather plays a primary role in loss of cell adhesion as proposed in the “basal cell shrinkage theory” (6). Using different approaches such as time-course studies, fluorescence recovery after photobleaching (FRAP), and pharmacological modulation of actin polymerization, we found that actin reorganization contributes to pemphigus pathogenesis.

MATERIALS AND METHODS

Cell culture and test reagents. The immortalized human keratinocyte cell line HaCaT was grown to confluence (5–7 days) in DMEM in a humidified atmosphere at 37°C. Culture medium (DMEM, Sigma) was supplemented with 50 μg streptomycin, 50 U/ml penicillin, and 10% FCS for all experiments. Cytochalasin D (Sigma-Aldrich) was used at 100 nM (3 h preincubation), latrunculin B (Calbiochem) at 200 nM (1 h preincubation), and jasplakinolide (Calbiochem) at 0.5 μM, and cytoxic necrotizing factor-1 (CNF-1) was used at 300 ng/ml (30 min preincubation), respectively.

Constructs and transfection. Human keratin (HK) 14-enhanced yellow fluorescent proteins (14-EYFP) and actin-red fluorescent protein (RFP) expressing constructs were kindly provided by R. Leube (RWTH Aachen) (42). For transfection cells were seeded on 9-mm glass coverslips. After 1 day, cells were transfected using Turbofect (Fermentas) according to manufacturer's instructions and imaged 1–2 days later.

Purification and preparation of patients’ IgG. For all experiments IgG fractions from two PV patients suffering from a mucous and mucocutaneous PV form were used, which yielded similar results. In
all experiments presented, the mucous-dominant PV-IgG was used except in Fig. 5 in which the mucocutaneous PV-IgG is shown. The diagnosis was based on the typical clinical appearance, intercellular deposits of IgG, and C3 in the epidermis by direct immunofluorescence microscopy of a perilesional biopsy and detection of circulating Dsg antibodies by ELISA (MBL). The mucous PV serum was ELISA positive for Dsg3 only (6,586 U/ml), whereas the mucocutaneous PV serum was positive for both Dsg3 (1,239 U/ml) and Dsg1 (60 U/ml). Purification of patients’ sera was performed by affinity chromatography by using protein A agarose as part of previous studies (33, 35). Concentration of IgG fractions was adjusted to 500 µg/ml for all experiments.

Cytochemistry. HaCaT cells were grown to confluence on cover-slips (5–7 days) and subsequently incubated with pemphigus IgG and various test reagents for 1 to 24 h depending on each experiment. All cells, except transfected, were fixed/permeabilized with ice-cold acetone for 2 min and then washed three times with PBS. Fixation of transfected cells was achieved by a 10-min incubation with formaldehyde, followed by three washes with PBS, permeabilization with Triton-X-100 for 5 min, and again with three washes with PBS. To block unspecific bindings, cells were incubated with 10% normal goat serum and 1% BSA at room temperature for 30 min. As primary antibody (1:100), a polyclonal Dsg3 antibody (Santa Cruz Biotechnologies) or a monoclonal E-cadherin antibody (BD Biosciences) was added overnight at 4°C. After being washed three times with PBS (each 5 min), cells were incubated with Cy3-labeled goat anti-rabbit or goat anti-mouse secondary antibodies (1:600, from Dianova) for 2 h at room temperature. To detect actin filaments, Alexa488-labeled phalloidin (Invitrogen) was added for 2 h at room temperature. Afterwards cells were washed again three times with PBS (each 10 min) and covered with 60% glycerol in PBS containing 1.5% n-propyl gallate.

Triton protein extraction assay. HaCaT cells were grown to confluence in 12-well plates for 6 days. After incubation under the

Fig. 1. Time course of pemphigus vulgaris autoantibodies (PV-IgG)-induced loss of keratinocyte cell adhesion. The time-dependent increase of PV-IgG-induced cell dissociation was measured using keratinocyte dissociation assays. Fragmentation of monolayers started after 0.25 h of incubation with PV-IgG (A, C) with a continuous increase up to 24 h (A, C–E) (n = 6).

Fig. 2. Time-dependent effects of PV-IgG on desmoglein 3 (Dsg3) localization and actin organization. Distinct and time-dependent effects of PV-IgG incubation on desmosomes were identified by Dsg3 immunostaining and correlated with effects on actin filaments visualized by staining with Alexa488-labeled phalloidin. Insets: a 3× magnification of representative areas. In controls, Dsg 3 was located along cell junctions, and the peripheral junction-associated actin belt was clearly visible (A–C). Starting at 15 min after addition of PV-IgG, Dsg3 and actin reorganization were observed (D–F). Both effects proceeded up to 24 h where strong fragmentation of Dsg3 staining and a complete loss of the peripheral actin band were discernible (G–U). Scale bar: 20 µm for all panels (n = 4).
imaging chambers. In PV-IgG conditions, cells were treated for 2 h with PV-IgG and subsequently analyzed in FRAP studies. On a Leica SP5 microscopic setup, cellular regions of interests comprising the respective cytoskeletal component were defined, and photobleaching was achieved by illumination with full laser power. Recovery of fluorescence was automatically captured by Leica advanced fluorescence suite software package, and obtained images were analyzed with ImageJ.

Statistics. Assessment of differences in displace-based dissociation assays was done using two-tailed Student’s t-test after data distribution was confirmed to be Gaussian. All values are expressed as means ± SE. Statistical significance was assumed for P < 0.05.

RESULTS

Time-dependent effects of PV-IgG on cytoskeleton organization and keratinocyte dissociation. First, we combined the displace-based keratinocyte dissociation assay (Fig. 1) with immunostaining studies to correlate the loss of cell adhesive strength with reorganization of Dsg3 and actin. In control keratinocytes the number of fragments was 18 ± 2. As soon as 0.25 h of PV-IgG incubation, cell coherence decreased with a significant (P < 0.05) elevation of fragments (82 ± 12). The number of fragments increased to more than 1,560 ± 78 fragments when PV-IgG treatment was continued for 24 h. Under control conditions (in the absence of autoantibodies, Fig. 2A) and after treatment with control IgG (data not shown) Dsg3 was found along cell borders. Under these conditions actin filaments were also continuously distributed along cell borders forming a junction-associated actin belt (Fig. 2B). After 0.25 h of PV-IgG incubation, fragmentation of Dsg3 immunostaining started to occur (Fig. 2D). In the following, PV-IgG effects on Dsg3 intensified with only few membranal Dsg3 clusters detectable after 24 h (Fig. 2S). Similarly, actin reorganization began 15 min after addition of PV-IgG with weakening of the cortical junction-associated actin belt (Fig. 2E) leading to an enhanced cytoplasmic distribution pattern and proceeded up to 24 h (Fig. 2T).

To further investigate changes of actin dynamics in response to PV-IgG, FRAP studies were performed in cells expressing green fluorescent protein (GFP)-variant fusion proteins actin-RFP and human CK 14 (HK14)-YFP (Fig. 3A). We observed actin-RFP and HK14-YFP to display a rather moderate recovery, with a small mobile fraction of molecules reaching a
plateau about 40 s after photobleaching. However, the actin-RFP mobile fraction was substantially greater than that of HK14-YFP, indicating a higher motility for actin filaments and confirming prior reports (9, 43). Interestingly, after addition of PV-IgG, fluorescence recovery of actin-RFP was significantly increased ($P < 0.05$), whereas recovery of HK14-YFP remained unchanged during the time of measurements. No changes in total protein levels of β-actin were observed in Western blotting experiments using up to 24 h of PV-IgG treatment, ruling out the possibility that PV-IgG interfere with actin synthesis and/or degradation (Fig. 3, B and C). Thus FRAP experiments supported the hypothesis that PV-IgG interfere with actin dynamics.

Pharmacological actin modulation affected PV-IgG-mediated Dsg3 fragmentation and cell dissociation. To further characterize the role of actin reorganization during pemphigus pathogenesis, we used cytochalasin D, which is known to inhibit actin polymerization (10), and jasplakinolide, which stabilizes existing filaments and induces polymerization of new filaments (5). Under control conditions, actin, Dsg3, and E-cadherin remained localized at cell borders (Fig. 4, A–C). After 2 h of PV-IgG incubation, Dsg3 staining became fragmented and first actin filament reorganization was observed, whereas no pronounced effects on E-cadherin distribution were detectable (Fig. 4, D–F). Coincubation of cytochalasin D (5 h) and PV-IgG (2 h) led to reorganization of actin filaments. Actin accumulated in clusters close to the cell membranes (Fig. 4H). Furthermore, Dsg3 fragmentation was significantly enhanced (Fig. 4G) compared with experiments using PV-IgG alone. Dsg3 was nearly completely removed from membranes and accumulated in the cytoplasm, whereas only modest changes were detected for E-cadherin (Fig. 4I). These results indicated that actin depolymerization enhanced PV-IgG-mediated effects on Dsg3. Next, we tested whether stabilization of actin filaments would ameliorate PV-IgG-induced effects. Compared with 6 h of PV-IgG incubation alone (Fig. 4J), Dsg3 fragmentation was significantly reduced by stabilization of actin filaments. After coincubation of cells with PV-IgG (6 h) and jasplakinolide (7 h), Dsg3 remained at cell borders and was only slightly fragmented (Fig. 4P). Again, distribution of E-cadherin staining was only mildly affected under these conditions (Fig. 4, O and R). Incubation with either cytochalasin D (3 h) or jasplakinolide (1 h) alone only slightly affected the immunoreactivity of Dsg3 as well as of E-cadherin (Fig. 4, J, L, S, U). In parallel, cytochalasin D treatment resulted in distinct alterations of actin filament organization (Fig. 4K). The optimal concentration of jasplakinolide was adjusted in preliminary experiments using Alexa488-coupled phalloidin, which competed with the same binding site of F-actin (5) and therefore did not stain actin filaments in jasplakinolide-treated cells (Fig. 4, Q and T).

To examine the effects of actin-modulation on desmoglein turnover, we performed Western blotting analyses of cytoskel-
to Dsg3 similar to endogenous actin (Fig. 7A). After incubation with PV-IgG for 2 h, Dsg3 fragmentation was visible in transfected and untransfected cells (Fig. 7B). Similarly, keratinocyte dissociation assays (Fig. 8) showed that overexpression of actin was not sufficient to affect pathogenic effects of PV-IgG. Under control conditions, the monolayer was largely intact (4 ± 1 fragments), whereas PV-IgG increased the number of fragments (254 ± 38). Actin-RFP transfection alone had no significant effect on cell cohesion in the absence (10 ± 1 fragments) and presence of PV-IgG (228 ± 16 fragments).

To substantiate the hypothesis that actin reorganization is important in pemphigus pathogenesis, we investigated whether Rho-GTPases, which are potent regulators of the actin cytoskeleton, modulate PV-IgG-induced keratinocyte dissociation via regulation of actin dynamics.

This is based on our previous findings that both, pemphigus IgG-induced keratinocyte dissociation and actin reorganization were prevented by activation of RhoA (18, 34, 40). Therefore, we used the Rho-GTPase activator CNF-1 together with PV-IgG in HaCaT keratinocytes. CNF-1 blocked PV-IgG-mediated Dsg3 fragmentation and actin remodelling (Fig. 7, D and E). Since CNF-1 additionally strengthened the peripheral junction-associated actin belt (Fig. 7E), we hypothesized that this

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**Fig. 5.** Protein levels of Dsg3 in Triton-X-100-soluble and -insoluble pools were largely unaffected by PV-IgG treatment and pharmacological actin modulation. In Western blot analyses of the Triton-X-100-insoluble fraction PV-IgG, cytochalasin D and jasplakinolide had no impact on cytoskeleton-bound Dsg3 amounts (A, B). Western blots of the Triton-X-100-soluble fraction revealed a slight but significant increase of noncytoskeletal-anchored Dsg3 after incubation with cytochalasin D, whereas under other conditions no significant effects were detectable (A, C). n = 6.

**Fig. 6.** Pharmacological modulation of actin depolymerization affected PV-IgG-mediated cell dissociation. In dispase-based keratinocyte dissociation assays, actin depolymerization by cytochalasin D (A) further enhanced PV-IgG-induced loss of cell coherence whereas stabilization of actin by jasplakinolide (B) was partly protective. For both experiments PV-IgG was added for 2 h. Cytochalasin D slightly increased fragment numbers, whereas jasplakinolide alone had no impact on keratinocyte dissociation (n = 6).

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Our previous experiments suggested a critical involvement of actin filaments in PV-IgG-induced cell dissociation. We transfected cells with actin-RFP before addition of PV-IgG to test whether overexpression of actin would affect PV-IgG-induced Dsg3 fragmentation and loss of cell adhesion. In controls, actin-RFP was concentrated at cell membranes in close vicinity of Dsg3 similar to endogenous actin (Fig. 7A). After incubation with PV-IgG for 2 h, Dsg3 fragmentation was visible in transfected and untransfected cells (Fig. 7B). Similarly, keratinocyte dissociation assays (Fig. 8) showed that overexpression of actin was not sufficient to affect pathogenic effects of PV-IgG. Under control conditions, the monolayer was largely intact (4 ± 1 fragments), whereas PV-IgG increased the number of fragments (254 ± 38). Actin-RFP transfection alone had no significant effect on cell cohesion in the absence (10 ± 1 fragments) and presence of PV-IgG (228 ± 16 fragments).

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mechanism may be important for RhoA-mediated protection against PV-IgG-induced loss of cell adhesion. Indeed, latrunculin B, which inhibits actin polymerization (13), abolished the capability of CNF-1 to block PV-IgG-mediated Dsg3 fragmentation (Fig. 7F). Incubation with latrunculin B alone did not influence the distribution of Dsg3 (Fig. 7G). Similarly, in dispase-based dissociation assays (Fig. 8) the Rho GTPase activator CNF-1 was effective to block PV-IgG-mediated cell dissociation (46 ± 7 fragments; \( P < 0.05 \) compared with PV-IgG alone). However, in the presence of latrunculin B, CNF-1 was not effective to reduce PV-IgG-induced cell dissociation (1,253 ± 62 fragments; \( P < 0.05 \) compared with PV-IgG + CNF-1 incubation). Latrunculin B alone led to a slight increment of fragments (17 ± 2 fragments). Taken together, these results indicate that the protective effect of Rho GTPase activation was at least in part dependent on strengthening of the junction-associated actin cytoskeleton.

**DISCUSSION**

The main outcome of this study is to demonstrate for the first time that reorganization of actin filaments plays an important role in PV-IgG-induced loss of keratinocyte adhesion. Profound reorganization of the actin cytoskeleton correlated with PV-IgG-induced Dsg3 fragmentation and cell dissociation. Moreover, FRAP studies supported the idea that PV-IgG interfere with actin dynamics. This is in line with previous observations that loss of keratinocyte adhesion was paralleled by actin reorganization (3, 34, 40). Strikingly, PV-IgG-mediated Dsg3 fragmentation as well as loss of adhesive strength were enhanced under conditions of actin depolymerization but were reduced by jasplakinolide-mediated actin stabilization, suggesting a crucial role of actin filament dynamics in pemphigus pathogenesis.

We therefore hypothesize that the junction-associated peripheral actin band is primarily affected in pemphigus patho-
A corolling function of the cortical actin cytoskeleton leading to clustering of cell adhesion molecules has previously been convincingly demonstrated (2, 15, 32). Thus, it is possible that the corolling function of the actin cytoskeleton is disturbed by PV-IgG, which is supported by our experiments, indicating increased actin mobility under PV-IgG treatment. Vice versa, stabilization of the actin network by jasplakinolide or strengthening the cortical actin by CNF-1, as presented in this study, may enhance clustering of Dsg3 molecules and promote cell adhesion. The mechanisms involved hereby are unknown at present; however, clustering may affect both cytoskeleton-anchor and non-cytoskeleton-bound Dsg3 molecules. It will remain a matter of future studies to investigate the contribution of non-cytoskeleton-linked desmogleins to intercellular adhesion. Moreover, it needs to be determined whether clustering is important for Dsg3 signaling and whether this process is altered upon PV-IgG binding. Nevertheless, at this stage our data are in line with the hypothesis that the actin cytoskeleton may contribute to PV pathogenesis at least in part due to autoantibody-induced alteration of its Dsg3 corolling function.

Alternatively, regulation of actin in desmosomal adhesion may be mediated by adherens junctions, which, besides desmosomes, represent the most important intercellular contacts providing cell cohesion in keratinocytes. Reduced function of adherens junctions may indirectly interfere with desmosome assembly, as adherens junctions have been identified to be the first contacts established between cell membranes of neighboring cells that are later stabilized by desmosomes (25, 37). However, under the conditions used in our study only mild changes in distribution of the major adherens junction protein E-cadherin following PV-IgG treatment were observed. Moreover, changes induced by modulation of the actin cytoskeleton alone were not different from experiments using simultaneous incubation with PV-IgG. This is in line with other studies suggesting that adherens junctions appear not to be a primary target of PV-IgG-induced mechanisms as they remain rather unaffected in anacantholytic cells (8, 14, 21, 40). In contrast, another recent publication presented considerable changes in E-cadherin distribution, indicating that the capacity of autoantibody fractions to cause alterations of adherens junctions may be different or may be dependent on the conditions used (12). Thus, in view of these data, the involvement of adherens junctions in pemphigus pathogenesis remains speculative.

Taken together, we provide evidence for an important role of actin filament reorganization in pemphigus pathogenesis. Therefore, further investigations on the role of actin reorganization in pemphigus seem to be worthwhile to identify new therapeutic approaches in pemphigus.

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