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Original Article

Plakoglobin-dependent disruption of the desmosomal plaque in pemphigus vulgaris

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Abstract: We recently reported that the pathogenesis of pemphigus vulgaris (PV), an autoimmune blistering skin disorder, is driven by the accumulation of c-Myc secondary to abrogation of plakoglobin (PG)-mediated transcriptional c-Myc suppression. PG knock-out mouse keratinocytes express high levels of c-Myc and resemble PVIgG-treated wild-type keratinocytes in most respects. However, they fail to accumulate nuclear c-Myc and loose intercellular adhesion in response to PVIgG-treatment like wild-type keratinocytes. This suggested that PG is also required for propagation of the PVIgG-induced events between augmented c-Myc expression and acantholysis. Here, we addressed this possibility by comparing PVIgG-induced changes in the desmosomal organization between wild-type and PG knock-out keratinocytes. We found that either bivalent PVIgG or monovalent PV-Fab (known to trigger blister formation in vivo) disrupt the linear organization of all major desmosomal components along cell borders in wild-type keratinocytes, simultaneously with a reduction in intercellular adhesive strength. In contrast, PV-Fab failed to affect PG knock-out keratinocytes while PVIgG cross-linked their desmosomal cadherins without significantly affecting desmoplakin. These results identify PG as a principle effector of the PVIgG-induced signals downstream of c-Myc that disrupt the desmosomal plaque at the plasma membrane.

Key words: adherens junction – catenins – desmosome – mouse keratinocytes – pemphigus vulgaris – plakoglobin

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Introduction

The autoimmune disease pemphigus vulgaris (PV) is characterized by blister formation following acantholysis (loss of cell adhesion) in the epidermis and/or mucous membranes of man and domestic animals (1–6). We recently showed that autoantibody binding to desmoglein (Dsg) 3 transiently enhances the turn-over of non-junctional Dsg 3 and associated plakoglobin (PG) at the plasma membrane (7). This subsequently abrogates signalling via glycogen-synthase kinase 3β (GSK3β), impairs nuclear translocation of PG and thus reduces c-Myc suppression. The resultant c-Myc accumulation was found to be pathogenic, as inhibition of either c-Myc or GSK3 was sufficient to prevent blister formation in a neonatal mouse model of PV. Consistent with this observation, disease progression and regression in the patient directly correlated with fluctuations in the levels of c-Myc (8).

Plakoglobin is an armadillo protein present in the plaque of both adherens junctions and desmosomes, which also associates with the cytoplasmic tail of Dsg3 (9,10). Although lack of PG does not abrogate junction formation in cultured keratinocytes from PG knock-out mice (9,11,12), their intercellular adhesive strength is reduced (9,13), these cells further are more migratory (13), fail to integrate growth factor-induced signals (11) and, consistent with their constitutively high c-Myc expression, are hyperproliferative (7). Moreover, they also are resistant to PV antibody-induced loss of intercellular adhesion even over prolonged periods of time (7,9). Together, these observations suggested that PG acts as an integrator of environmental cues and may in this function be responsible for the propagation of PV antibody-induced cellular alterations triggered by c-Myc upregulation.

We and others have observed that binding of PV antibodies in patients' epidermis disrupts the linear organization

Abbreviations: Dsg3, desmoglein 3; plakoglobin, PG; PVIgG, pemphigus vulgaris antibodies; nhIgG, normal human IgG; IF, intermediate filaments; GSK3β, glycogen-synthase kinase 3β.

of Dsg3 and associated PG in the deep epidermis, that is at the site of blister formation, but also in suprabasal layers (7,14–17). In cultured human keratinocytes, the direct PVIgG target Dsg3, associated PG and desmoplakin were reported to be disrupted (18) and PG in cultured mouse keratinocytes (9). However, the fate of the remaining desmosomal components so far has not been addressed and even less is known about the mechanisms involved. Interestingly, in human keratinocytes disruption of the linear organization of Dsg3 was dependent on intracellular signalling events, as it did not occur when the keratinocytes were held at 4°C in presence of the PV antibodies.

In light of the various cellular activities recently attributed to PG in keratinocytes (9,11–13) including its potential to mediate a cross-talk between the plasma membrane and the nucleus suggested by our previous study (7), it was of interest to address (i) whether PG was mediating the disturbance of Dsg3 and associated plaque proteins at the plasma membrane in PV, (ii) to which extent it affected the entire desmosomal organization and (iii) how this relates to loss of intercellular adhesion.

Materials and methods

Cell cultures

The isolation of wild-type and PG knock-out ($PG^{-/-}$) keratinocytes was described earlier (9,19). Cell culture and experimental conditions were as described (7). Briefly, at confluency, the calcium concentration in the Cnt-O2 medium (CELLnTEC, Advanced Cell Systems, Bern, Switzerland) was raised to physiological levels (1.29 mM $CaCl_2$, stated as 'high calcium') for 6 h. Cells were then treated with an excess of antibodies, namely 1 mg/ml control IgG [normal human IgG (nhIgG)], 1 mg/ml PVIgG or 0.66 mg/ml PV-Fab in high calcium medium for 24 h.

Sera and antibodies

The human PV serum and normal human sera pooled from healthy volunteers were purified as described (9). The Protein A affinity purified PVIgG fraction contained Dsg3-specific antibodies, but no Dsg1 or other major detectable antibodies, as evaluated by Western blot analysis, immunoprecipitation, ELISA and in metabolic labelling experiments (7,9). We will therefore refer to this antibody as anti-Dsg3 antibody. Other primary antibodies were against desmoplakin I and II (multi-epitope cocktail 2.15, 2.17, 2.20; Progen, Heidelberg, Germany), plakophilin 3 (PP3-270.8.2; Progen) and plakophilin 1 (11C6; 20), PG (Pg5.1; 21), the latter two from M. Wheelock, University of Nebraska, Omaha, NE, USA, β-catenin (BD Transduction Laboratories, San Jose, CA, USA), E-cadherin [DECMA; R. Kemler, Max-Planck Institute, Freiburg i.Br., Germany (22)], Dsg1/2 (DG 3.10; Progen), pan-Dsc 1/2/3 [Amber; D.R. Garrod, University of Manchester, Manchester, UK (23)], pan-keratin (LP34, DAKO, Glostrup, Denmark). Secondary anti-human antibodies were anti-IgG4 [HP6025; Southern Biotechnology Associates Inc., Birmingham, AL, USA; specific for the IgG_4 Fc portion representing the prevalent isotype of pathogenic PVIgG (24)]. Note that the secondary antibodies other than anti-human IgG used in this study do not cross-react with human IgG.

Generation of Fab fragments

Papain digestion of PVIgG was performed with immobilized papain (#20341; Pierce, Rockford, IL, USA) following the manufacturer's protocol. Fab fragments were separated from undigested IgG and Fc fragments via a Protein A-Sepharose column (Pharmacia, Uppsala, Sweden). The purified Fab fragments were dialysed against PBS, concentrated to 10 mg/ml using a Centricon YM-30 centrifugal filter unit (Millipore, Billercia, MA, USA) and tested for purity (Fig. 2a).

Immunofluorescence analyses

To assess surface-exposed Dsg3 in keratinocyte cultures, PVIgG- or nhIgG-treated wild-type and $PG^{-/-}$ cells were incubated prior to fixation with additional PVIgG (1 mg/ml) or equimolar PV-Fab (Figs 2b and 3) for 1 h on ice and washed with PBS containing 2 mM $MgCl_2$/2 mM $CaCl_2$ (PBS^+). Fixation was performed with precooled 100% methanol for 7 min at −20°C followed by treatment with 0.5% Triton X-100/2 mM PMSF/2 mM *N*-tosyl-L-phenylalanin chloromethyl ketone (Sigma, St Louis, MO, USA) in PBS^+ for 5 min to visualize intermediate filaments (IF), and otherwise with 1% paraformaldehyde in PBS^+ for 10 min followed by permeabilization with 0.5% Triton X-100 in PBS^+ for 10 min. In general, antibody incubation was performed for 1 h at room temperature in 1% BSA/PBS^+.

Electron microscopy

After 24-h PVIgG or nhIgG treatment, cells were fixed with 4% paraformaldehyde in PBS (pH 7.2) for 1 h at room temperature, postfixed with 1% osmium tetroxide in PBS for 30 min, washed with H_2O, treated with 1% aqueous uranyl acetate for 30 min, and then dehydrated through a graded series of ethanol. Subsequently cells were infiltrated for 1 h with EPON/EtOH (1:1) and embedded in EPON 812 resin. Ultrathin vertical sections were stained with uranyl acetate and lead citrate and viewed using a Philips TEM 300 at 60 kV.

Semi-quantitative adhesion assay

To evaluate intercellular adhesive strength in response to mechanical stress, keratinocyte monolayers seeded in dupli-

cates were treated with dispase for 30 min as described for the quantitative adhesion assay (9). Following this initial treatment, the assay was modified according to Huen et al. (25). In brief, the detached cellular sheets were carefully transferred onto a rocker and subjected to 100 inversion cycles. Generated fragments were then photographed in six-well plates and counted.

Results

PVIgG simultaneously disrupt the organization of PG and Dsg3 in wild-type keratinocytes

PVIgG disrupt the linear arrangement of PG along cell borders in wild-type mouse keratinocytes (Fig. 1a) (9). One of the binding partners of PG at the plasma membrane is Dsg3. Double-labelling immunofluorescence microscopy of wild-type mouse keratinocytes treated with PVIgG for 24 h revealed that disturbance of the linear staining pattern of PG simultaneously involved surface exposed Dsg3. Dsg3 and PG co-localized in most areas of the cell culture dish, but appeared in clusters. In areas of low cell density, reduced staining of PG and Dsg3 was seen which is consistent with the previously reported internalization (18). Disturbance in the desmosomal organization went along with reduced intercellular adhesive strength as shown by an adhesion assay that applies mechanical stress onto the monolayer culture (Fig. 1b) (25).

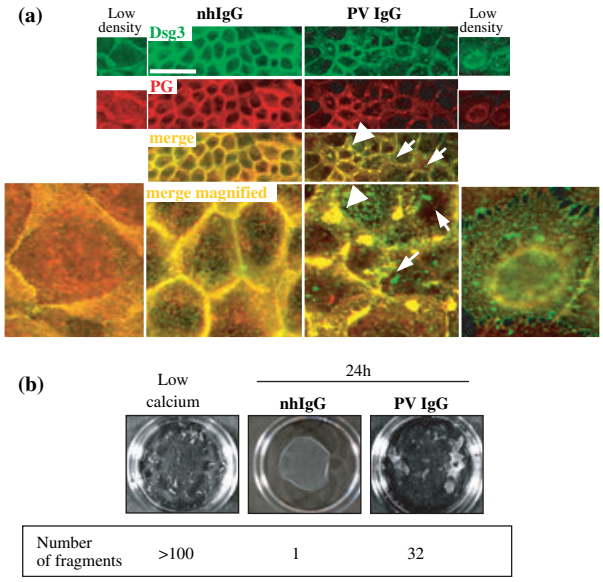

Figure 1. PVIgG disrupt the linear organization of antibody-targeted Dsg3/PG complexes in wild-type mouse keratinocytes. (a) Keratinocytes treated for 24 h with PVIgG or nhIgG were analysed by double-labelling immunofluorescence microscopy (middle panels). Photomicrographs from areas with lower cell density are also shown (external panels). Merged areas were magnified four times and are shown at the bottom. Experimental procedures and photographic exposures were identical for each antibody. The merge of both staining patterns demonstrates that Dsg3 and PG mostly co-localize (arrow head), while some Dsg3 lacks associated PG in the confluent areas (arrow). Magnifications are identical except for pictures at the bottom; scale bar, 25 μm. (b) The adhesion assay was performed and generated fragments counted to assess the adhesive strength in monolayer cultures.

PVIgG and PV-Fab disrupt the organization of all major desmosomal components in wild-type keratinocytes

Monovalent Fab fragments (PV-Fab) are sufficient to induce acantholysis in the neonatal mouse model of pemphigus (26) and are therefore expected to reproduce all major pathogenic events. We simultaneously treated wild-type keratinocytes with PVIgG or PV-Fab thereof to address whether the disturbance of Dsg3 and associated PG involves other desmosomal proteins.

Monovalent PV-Fab were prepared by papain digestion of bivalent PVIgG and their purity and ability to bind to the surface of keratinocytes were confirmed by Western blot and immunofluorescence analyses (Fig. 2a). Equimolar amounts to PVIgG of the PV-Fab further bound to the keratinocyte surface (Fig. 2a, lower right panel) and effectively induced IF reorganization as demonstrated by immunofluorescence analysis (Fig. 2b, keratin).

PVIgG and PV-Fab similarly disturbed the localization of all major desmosomal adhesion and plaque proteins as seen by immunofluorescence microscopy (Fig. 2b shows PV-Fab-treated keratinocytes). Of special interest is that desmoplakin, which is required to anchor the desmosomal complex to the IF network (27,28), was also visibly affected. In areas of lower cell density the PV-Fab induced prominent intercellular spaces indicative of retracted IF after exposure of the keratinocytes for 24 h (Fig. 2b, keratin, top panel). In contrast, in more confluent areas, the IF were still extending towards the plasma membrane. This is reminiscent of reports describing that the desmosomal connections are affected last during acantholysis (5,29) which in turn is consistent with the initial depletion of non-keratin anchored (soluble) Dsg3 from PVIgG-treated keratinocytes (7,30,31). To confirm that desmosome-like structures were still present despite weakened adhesion (Fig. 1b), PVIgG-exposed keratinocytes were double-labelled with keratin and anti-human IgG4 antibodies (Fig. 3a). Furthermore, electron microscopy was performed on the same cells to investigate whether desmosomes were still present in these areas (Fig. 3b). The immunofluorescence analysis showed prominent staining for PVIgG in interdesmosomal areas, the location of non-keratin anchored Dsg3, as well as along the extended IF, where desmosomes are expected to reside. Electron microscopy confirmed the presence of desmosomes Fabs in these areas. These results are consistent with immunoelectron microscopical and ''time labs''

studies performed on PVIgG treated JIM-1 cells that demonstrate simultaneous binding of PVIgG to fully assembled desmosomes as well as to the interdesmosomal space (32). More sophisticated experiments using shear forces (33) will be required to assess the capacity of PVIgG-targeted individual desmosomes to withstand mechanical stress, a scope which is beyond the present study.

In contrast to desmosomal proteins, adherens junction components forming the second type of junctions mediating intercellular adhesion between keratinocytes (34), still aligned at cell borders in an unperturbed fashion after 24 h (Fig. 2b). Collectively, these data demonstrate that PVIgG as well as PV-Fab binding, which to the best of our knowledge has not been reported so far, specifically disrupt the organization of desmosomal components at the plasma membrane.

PV-Fab-induced disruption of the desmosomal organization is compromised in $PG^{-/-}$ keratinocytes while PVIgG retain cross-linking activity

Desmosome-like structures assemble in $PG^{-/-}$ keratinocytes to which PVIgG bind to the same extent than to wild-type cells consistent with the preserved steady-state level of Dsg3 (7,9,11). However in these cells intercellular adhesion is weaker than in wild-type keratinocytes (9,13). Despite this, $PG^{-/-}$ keratinocytes do not respond to binding of PVIgG with loss of intercellular adhesion and retraction of keratin filaments from cell borders (9). In line with these previous studies, immunofluorescence microscopy revealed an unaffected IF network upon exposure to PV-Fab for 24 h (Fig. 4, keratin). Of particular interest, none of the investigated desmosomal proteins exhibited a disturbed localization (Fig. 4, left two panels). Consistently, in line with our previous findings (9) intercellular adhesive strength as defined by the adhesion assay was not affected (data not shown).

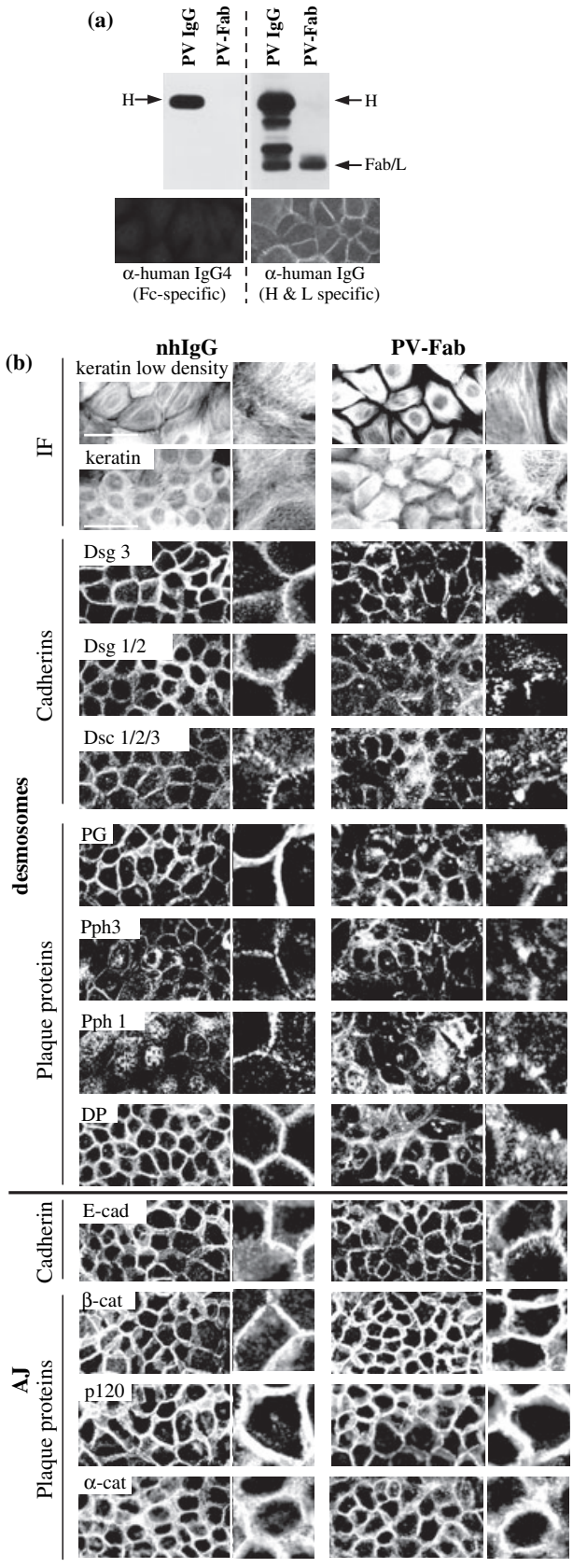

Figure 2. PV-Fab disrupt the linear organization of all major desmosomal components in wild-type keratinocyte cultures similar to PV antibodies in patients skin. (a) Upper panel: analysis of PV-Fab was carried out by Western blot and revealed with antibodies recognizing only the Fc portion of human IgG4 (Fc specific; left panel) or heavy and light chain of human IgG (H & L; right panel). Note that the PV-Fab preparation is devoid of IgG heavy chain (indicated with H). Lower panel: keratinocyte cultures were incubated with PV-Fab for 1 h at 4°C, and binding was assessed by immunofluorescence analysis using the same secondary antibodies as for Western blot analysis. Note that no heavy chain binding is detected (left panel). (b) The major desmosomal and adherens junction components were investigated by immunofluorescence microscopy in wild-type keratinocytes treated for 24 h with PV-Fab. The keratin IF network is shown in low density (top panel) and high density cultures. Three times magnifications of selected areas are shown on the right of each overview. Dsc, desmocollins; PPh 1,3, plakophilin 1,3; DP, desmoplakin; E-cad, E-cadherin; β-cat, β-catenin. Scale bar, 25 μm.

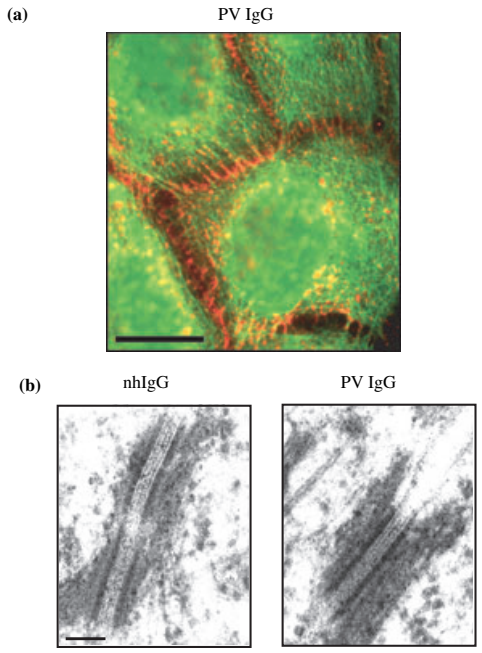

Figure 3. Disruption of desmosomes is a late event. (a) Double labelling immunofluorescence analysis using pan-keratin (green) and anti-human IgG4 (red) antibodies shows IF stretching out from PVIgG-exposed keratinocytes. (b) Electron microscopical studies confirmed that desmosomes are still present at the surface of PVIgG-treated mouse keratinocytes despite substantial loss of intercellular adhesive strength (Fig. 1). Scale bars, 5 μm (a), 100 μm (b).

Bivalent PVIgG have the capacity to cross-link surface proteins, however, the cross-linking activity can only be examined independently of the pathogenic activity in cells like $PG^{-/-}$ keratinocytes, as these lack reorganization of desmosomal proteins in response to PV-Fab. To address these cross-linking effects, $PG^{-/-}$ cells were incubated with bivalent PVIgG for 24 h and processed for immunofluorescence microscopy. Clustering was mainly seen for Dsg3 [the main target of the PVIgG (7,9)] and Dsg1/2 and to a lesser extent of Dsc1/2/3 (Fig. 3, right panel). The plaque proteins, in particular desmoplakin were only marginally affected. In agreement with the findings in wild-type keratinocytes, adherens junctions were not involved in the clustering event (Fig. 4, shows E-cadherin and β-catenin). Together this indicates that the bivalent PVIgG antibodies have the capacity to cross-link surface proteins, exert this activity on the $PG^{-/-}$ cells but fail to disrupt the desmosomal plaque in the absence of PG.

Discussion

Collectively our results demonstrate that treatment with either PVIgG or PV-Fab disrupts the linear organization of all major desmosomal components along cell borders in wild-type keratinocytes, and that this phenomenon fails to occur in PV-Fab-treated $PG^{-/-}$ keratinocytes. This provides a strong evidence that disruption of the desmosomal organization is dependent on PG. The timely correlation between the disrupted organization and loss of intercellular adhesion in wild-type keratinocytes and the absence of both events in $PG^{-/-}$ cells, further link the PG-dependent disturbance of desmosomal components, and in particular the desmosomal plaque to impaired intercellular adhesion and acantholysis. The reliance of acantholysis on a signalling molecule such as PG is in line with the involvement of an intracellular signalling event in PV pathogenesis, as was suggested already many years ago (35) and corroborated more recently [e.g. (7,9,36,37)].

Using the PVIgG-unresponsive $PG^{-/-}$ keratinocytes, we further demonstrate that bivalent PVIgG have the capacity to cross-link surface proteins independently of PG, predominantly Dsg3 but also Dsg1/2. Involvement of both types of cadherins, despite the fact that only Dsg3 antibodies were detected in the PVIgG fraction [as demonstrated by ELISA and metabolic labelling (7,9)], might relate to the postulated heterodimerization of desmosomal cadherins (38). Furthermore, as primarily non-keratin anchored (soluble) Dsg3 was affected in its turn-over during the first 24 h of PVIgG treatment (7,30,31), it is probably that mainly non-keratin anchored Dsg3 and Dsg1/2 are subject to cross-linking. The minor involvement of desmoplakin in the cross-linking event in $PG^{-/-}$ keratinocytes supports this possibility. Desmoplakin is required for the maintenance of intercellular adhesive strength by anchoring the desmosomal cadherins to the IF network (25,28), and if disturbed likely would trigger loss of intercellular adhesion as seen in wild-type cells. Consistently, for $PG^{-/-}$ keratinocytes the cross-linking by PVIgG was not pathogenic as these cells were resistant to PVIgG-induced alterations (7,9). In agreement with this, PV-Fab (that cannot cross-link surface proteins) were sufficient on their own to induce acantholysis in the neonatal mouse model of PV (26). With respect to desmoplakin and its disturbances, similar observations have been made both in vitro and in vivo. While alterations in the distribution of desmoplakin are observed in PVIgG-responsive keratinocytes of the basal layer, desmosomal components but not desmoplakin are affected in their organization in non-responsive suprabasal cells (15). As now suggested by the results of this study, the latter presumably occurs through PVIgG-induced non-pathogenic cross-linking.

The involvement of PG in the propagation of PV antibody-induced desmosmal alterations raises the question about the mechanism involved. One of the crucial events that takes place at the transition between proliferation and onset of terminal differentiation in basal keratinocytes is the inhibition of GSK3 (39). Evidence was provided that GSK3 inhibition is abrogated by PV antibodies presumably

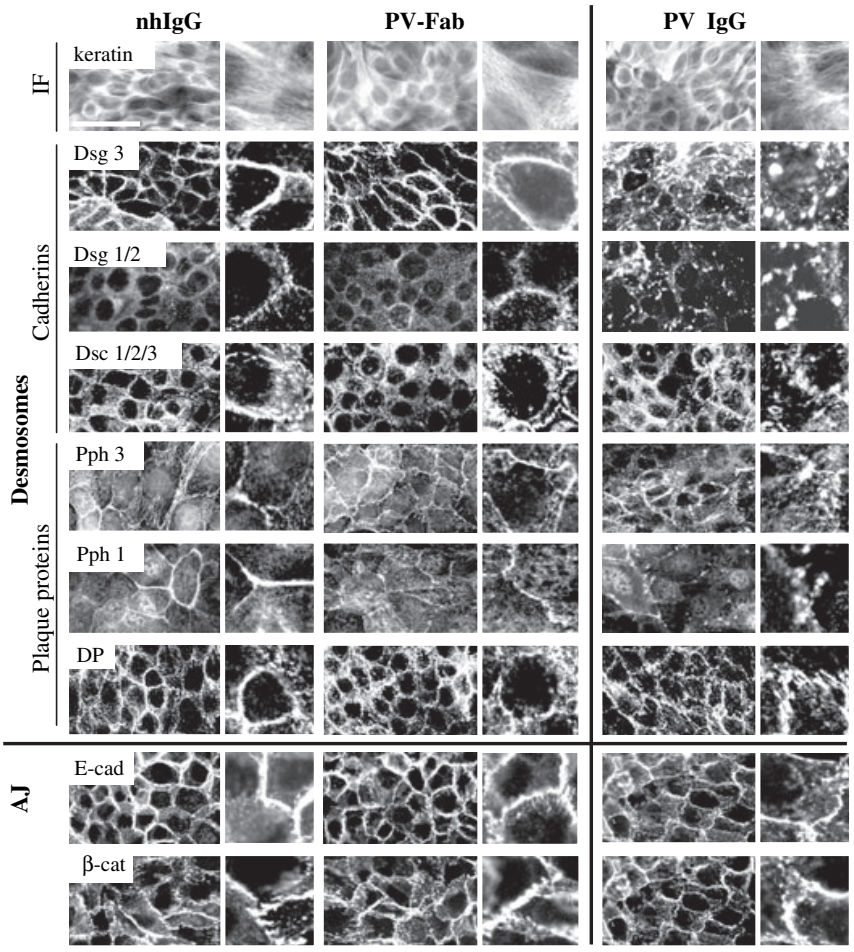

Figure 4. PVIgG but not PV-Fab disrupt the linear organization of desmosomal components in $PG^{-/-}$ keratinocyte cultures. Immunofluorescence analyses of nhIgG-, PV-Fab- and PVIgG-treated $PG^{-/-}$ keratinocytes for 24 h were carried out under the same experimental setting as in Fig. 2. Note that the most cross-linked desmosomal components in PVIgG-treated cells are Dsg3 and Dsg1/2 and that the staining pattern of β-catenin remained linear in these cells consistent with the observation that at that differentiation stage β-catenin does not associate with desmosomal cadherins (9). Weak staining for Dsg1/2 and Dsc 1/2/3 reflects low amounts of these proteins in $PG^{-/-}$ keratinocytes (9,11). Abbreviations as in Fig. 2. α-Catenin and p120 showed a similar linear staining (data not shown). Scale bar, 5 μm.

resulting in the sustained proliferation at the expense of terminal differentiation observed in both cultured PVIgG-treated wild-type keratinocytes as well as PV patients epidermis (7). Our current results now add disruption of the desmosomal organization to this previously described mitotic signal. A proliferative signal resulting in dissolution of desmosomal junctions is reminiscent of EGF-R-mediated events, which are intimately linked to cadherin signalling (39,40). Indeed, the disrupted desmosomal pattern reported in the PV patient's epidermis (7,14–17) as well as in wild-type keratinocytes in this study, closely resembles the pattern seen in cultured HaCat keratinocytes after EGF treatment (41). While inhibition of GSK3 prevents blister formation in neonatal mice injected with PVIgG (7), inhibition of GSK3 in EGF-stimulated HaCat keratinocytes prevents 'cell rounding' (41). The latter is an early event in EGF-stimulated keratinocytes and might compare with acantholysis in PV. More importantly cell rounding probably involves PG, as EGFR activation was shown to induce tyrosine phosphorylation of PG with subsequent loss of desmoplakin from desmosomes and a reduction in intercellular adhesive strength (11). In view of the similarity in intercellular alterations that are induced downstream of EGFR and PVIgG-targeted Dsg3 and the close association of cadherins and EGF-R (39,40), it is conceivable that some of the events downstream of EGFR activation are also triggered by Dsg3 subsequent to PVIgG binding. These could include PG phosphorylation and loss of desmoplakin from desmosomes (11), which seem a likely cause of the keratin filament retraction from the plasma membrane shown here and reported previously in wild-type mouse as well as human keratinocytes (9,18). The interesting finding is that if PG is deleted from keratinocytes, the EGFR-triggered signal cannot be integrated and is attenuated (11), which could also occur in $PG^{-/-}$ keratinocytes thereby explaining the unresponsiveness of these cells to PVIgG (7,9).

In conclusion, PV antibodies induce a mitotic signal via non-keratin-anchored Dsg3/PG (7). These events correlate, as is shown in this study, with the PG-dependent disruption of the desmosomal plaque, which likely lies downstream of GSK3 and increased c-Myc. While PG is involved in the switch between proliferation and terminal differentiation in the nucleus (7), the results of this study now suggest that PG also governs the distribution of desmosomal

components by integrating signals like those induced by growth factor stimulation or PV antibody binding to nascent Dsg3-meditated cell contacts. In the absence of PG, these signals cannot be generated. In general terms, this study provides yet another piece of evidence that PG serves as an integrator of both extracellular and intracellular cues (10), and therefore in addition to its role in adhesion also plays a multi-faceted and far-reaching role in epithelial signalling.

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