Pemphigus Vulgaris IgG Cause Loss of Desmoglein-Mediated Adhesion and Keratinocyte Dissociation Independent of Epidermal Growth Factor Receptor

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Autoantibody-induced cellular signaling mechanisms contribute to the pathogenesis of autoimmune blistering skin disease pemphigus vulgaris (PV). Recently, it was proposed that epidermal growth factor receptor (EGFR) might be involved in PV signaling pathways. In this study, we investigated the role of EGFR by comparing the effects of epidermal growth factor (EGF) and PV-IgG on the immortalized human keratinocyte cell line HaCaT, and primary normal human keratinocytes. In contrast to EGF treatment, PV-IgG neither caused the canonical activation of EGFR via phosphorylation at tyrosine (Y)1173 followed by internalization of EGFR nor the phosphorylation of the EGFR at the c-Src-dependent site Y845. Nevertheless, both PV-IgG and EGF led to cell dissociation and cytokeratin retraction in keratinocyte monolayers. Moreover, the effects of EGF were blocked by inhibition of EGFR and c-Src whereas the effects of PV-IgG were independent of both signaling pathways. Similarly, laser tweezer experiments revealed that impaired bead binding of epidermal cadherins desmoglein (Dsg) 3 and Dsg 1 in response to PV-IgG was not affected by inhibition of either EGFR or c-Src. In contrast, EGF treatment did not interfere with Dsg bead binding. Taken together, our study indicates that the loss of Dsg-mediated adhesion and keratinocyte dissociation in pemphigus is independent of EGFR. Moreover, the mechanisms by which both EGF and PV-IgG lead to keratinocyte dissociation and cytokeratin retraction appear to be different. (*Am J Pathol* 2009, 174:475–485; DOI: 10.2353/ajpath.2009.080392)

Pemphigus is an autoimmune blistering skin disease caused by antibodies against keratinocyte surface antigens.^{1–3} Particularly, pathogenic autoantibodies are directed against epidermal cadherins desmoglein (Dsg) 3 and Dsg 1.^{4–6} In the mucosal-dominant form of pemphigus vulgaris (PV), antibodies against Dsg 3 are produced, whereas Dsg 1 is an additional target when epidermal involvement occurs. For pemphigus foliaceus (PF) and the Brazilian endemic variant fogo selvagem, Dsg 1 is the major autoantigen. However, non-Dsg targets have also been identified.⁷ Among those, pemphaxin, cholinergic receptors and E-cadherin are the best-studied so far.^{8–10} In addition, pathogenic non-autoantibody factors in pemphigus patients' sera such as Fas ligand are discussed.¹¹ Nevertheless, there is an ongoing debate whether acantholysis—the cellular hallmark of pemphigus pathogenicity—is induced by Dsg antibodies directly interfering with Dsg transinteraction or by cellular signaling mechanisms triggered by Dsg or non-Dsg autoantibodies.¹² At least for PF, cellular signaling seems to be important since no direct inhibition of Dsg 1-mediated binding by PF-IgG was observed by atomic force microscopy under conditions where autoantibodies caused keratinocyte dissociation.¹³ Nevertheless, recently we have demonstrated that PV-IgG directly interfere with Dsg 3 transinteraction.¹⁴

Over the past years, the involvement of several signaling pathways has been studied. However, the mechanisms involved in outside-in signaling as well as the interplay of the several pathways leading to acantholysis remain unclear.¹⁵ *In vitro* and *in vivo* studies have shown activation of p38 mitogen activated protein kinase (MAPK) by pemphigus IgG.^{16,17} Blocking p38 MAPK pre-

Supported by grants from IZKF Würzburg (TP A-51) and SFB 487 (TP B5).

Accepted for publication October 22, 2008.

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vented cell dissociation and cytokeratin retraction. In addition, activation of the small GTPase RhoA completely antagonized pemphigus IgG-mediated effects in cultured human epidermis and keratinocyte monolayers.¹⁸ Pemphigus IgG-induced Rho A inactivation was also p38 MAPK-dependent. Furthermore, plakoglobin depletion by pemphigus IgG is supposed to lead to diminished cell adhesion via c-Myc overexpression, which has been shown to result in keratinocyte hyperproliferation.^{19,20} Promotion of cell-cycle progression by PV-IgG-mediated upregulation of cyclin-dependent kinase 2 is another mechanism believed to cause acantholysis via continuing keratinocyte proliferation.²¹

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that activates a complex cellular signaling network involving the classical MAPK cascade (leading to activation of Erk and Akt), signal transducer and activator of transcription, phospholipase C γ , and RhoA.^{22,23} EGFR can be activated by extracellular ligands like EGF, by intracellular kinases such as c-Src or by G protein coupled receptors.^{24,25} Over a decade ago first work highlighted the interdependence of cell adhesion and EGFR function.²⁶ Stimulation of EGFR resulted in phosphorylation of catenins (cadherin family adapter proteins) and colocalization of EGFR with the cadherin-catenin complex. Moreover, epidermal growth factor (EGF)-mediated phosphorylation of plakoglobin caused depletion of desmoplakin from desmosomes as well as reduced cell adhesion.^{27,28} Activation of EGFR following PV-IgG treatment was speculated to up-regulate Fas receptor signaling resulting in apoptosis and finally in acantholysis.²⁹ Another study explored c-Src-dependent EGFR activation in pemphigus.³⁰ Blocking of c-Src diminished activation of EGFR as well as of p38 MAPK and also reduced pathogenic effects of PV-IgG.

Taken together, EGFR activation could explain various aspects of acantholysis in pemphigus. Therefore, in the present work we aimed to further evaluate the role of EGFR in pemphigus by the following approaches: (i) comparing the effects of EGF and PV-IgG on human keratinocytes, (ii) investigating PV-IgG-mediated EGFR phosphorylation and (iii) examining the requirement of EGFR for PV-IgG-mediated effects by inhibition of EGFR or of c-Src.

Materials and Methods

Cell Culture and Test Reagents

The immortalized human keratinocyte cell line HaCaT³¹ was grown in Dulbecco's modified Eagles medium (Life technologies) that was supplemented with 50 U/ml penicillin-G, 50 μ g streptomycin and 10% fetal calf serum (Biochrom) in a humidified atmosphere (95% air/5% CO₂) at 37°C. Normal human epidermal keratinocytes (NHEK) derived from juvenile skin were purchased from PromoCell GmbH (Heidelberg, Germany). Cells were grown in Keratinocyte Growth Medium 2 (PromoCell, Heidelberg, Germany) supplemented with 50 U/ml penicillin-G, 50 μ g streptomycin and supplement mix (PromoCell). NHEK

Table 1. Antibody Profile of Pemphigus Patients' IgG

ELISA	Dsg 1 (U/ml)	Dsg 3 (U/ml)
PV-IgG 1	—	1874
PV-IgG 2	60	1000
PV-IgG 3	535	1098
PV-IgG 4	54	1239
PV-IgG 5	—	1177

were grown in low Ca²⁺ (0.15 mmol/L), which was changed to high Ca²⁺ (1.2 mmol/L) one day before experiments were started. For experiments, EGF (Sigma-Aldrich, Taufkirchen, Germany) was used at 20 ng/ml. Pharmacological inhibitor of EGFR (GW2974) (Sigma-Aldrich) at 10 μ mol/L and neutralizing antibody against EGFR (LA-1) (Millipore, Schwalbach, Germany) at 1 μ g/ml. c-Src inhibitor PP2 (Calbiochem, Darmstadt, Germany) was applied at 10 μ mol/L. All inhibitors were pre-incubated for 2 hours. CNF-1 and CNF γ were used at 300 ng/ml and 900 ng/ml, respectively.

Purification and Preparation of Patients' IgG

Purification was performed as described previously.¹³ Sera from two patients with mucosal-dominant PV and three patients suffering from a mucocutaneous form of PV whose diagnoses were confirmed clinically, histologically, and serologically and from a volunteer without any skin disease (control IgG) were used for the present study. Patients' sera were tested by enzyme-linked immunosorbent assay (ELISA, Medical and Biological Laboratories, Nagoya, Japan) according to the manufacturer's protocols for reactivity against Dsg 1 and Dsg 3, respectively (see Table 1). The cut-off value was 14 U/ml for Dsg 1 and 7 U/ml for Dsg 3. All samples were run in duplicate. PV-IgG 1 and 5 contained Dsg 3 but not Dsg 1 autoantibodies. PV-IgG 2, 3, and 4 contained both Dsg 1 and Dsg 3 antibodies. IgG fractions were purified by affinity chromatography using protein A agarose. Final concentrations of IgG fractions were adjusted to 500 to 1000 μ g/ml for all experiments.

Cytochemistry

HaCaT cells were grown on coverslips to confluence and incubated with pemphigus IgG and indicated reagents for 24 hours or the indicated times at 37°C. After incubation, culture medium was removed, monolayers were fixed, permeabilized with ice-cold acetone for 2 minutes and washed with PBS. Afterward, HaCaT cells were incubated for 30 minutes with 10% normal goat serum and 1% bovine serum albumin in PBS at room temperature and incubated for 16 hours at 4°C with mouse monoclonal antibody directed against Dsg 3 (Zyotomed, Berlin, Germany; dilution 1:100 in PBS), mouse monoclonal antibody against EGFR (Millipore; 1:100), rabbit polyclonal antibody against Y845 phospho-EGFR (Abcam, Cambridge, USA; 1:50), or mouse monoclonal cytokeratin 5 antibody (Santa Cruz, Heidelberg, Germany; 1:100). After several rinses with PBS (3 $\times$ 5 minutes), monolayers

were incubated for 60 minutes at room temperature with Cy3-labeled goat anti-mouse or goat anti-rabbit IgG (Dianova). For visualization of filamentous actin (F-actin) or nuclear staining, ALEXA 488-phalloidin (Molecular Biology, Göttingen, Germany; diluted 1:60 in PBS, incubation for 1 hour at room temperature) and 4,6-diamidino-2-phenylindole (Roche, 1:3000 for 10 minutes at room temperature) were used, respectively. Cells were rinsed with PBS (3×5 minutes) and finally mounted on glass slides with 60% glycerol in PBS, containing 1.5% n-propyl gallate (Serva, Heidelberg, Germany). Monolayers were examined using a LSM 510 (Zeiss, Oberkochen, Germany). Images were processed using Adobe Photoshop 7.0 software (Adobe, München, Germany). For evaluation of PV-IgG binding to the surface of keratinocytes, images were quantitatively measured using ImageJ (National Institutes of Health, Bethesda, MD) software analysis. Therefore, after incubation of PV-IgG on HaCaT keratinocytes for the indicated times, cells were fixed and incubated with Cy3-labeled goat anti-human antibody to detect surface-bound PV-IgG. After washing, mean fluorescent intensities were measured in 3 independent areas of each condition with ImageJ. Each condition was repeated three times.

Electrophoresis and Western Blotting

After incubation with pemphigus patients' IgG or reagents for the indicated times, HaCaT cells were dissolved in sample buffer, heated at 95°C for 5 minutes and finally subjected to sodium dodecyl sulfate 7.5% or 10% polyacrylamide gel electrophoresis and immunoblotting to Hybond nitrocellulose membranes (Amersham, Buckinghamshire, UK). Membranes were blocked with 5% low fat milk for 1 hour at room temperature in PBS and incubated with the respective primary antibody overnight at 4°C. The rabbit antibodies against EGFR (Santa Cruz) and Y845 phospho-EGFR (Abcam) as well as the mouse Y1173 phospho-EGFR (Millipore) antibody were used at 1:400. As secondary antibodies HRP-labeled goat anti-mouse or goat anti-rabbit (both from Dianova, Hamburg, Germany) were used. Visualization was achieved using the enhanced chemiluminescence technique (Amersham).

EGFR ELISA

Phosphorylation of EGFR at Y845 was detected using chemiluminescence-based FACE EGFR (Y845) ELISA Kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. HaCaT cells were seeded on 96-well plates and grown to confluence before treatment with EGF or pemphigus patients' IgG in the absence or presence of various reagents for 1 hour.

Dispase-Based Keratinocyte Dissociation Assay

The assay was performed as described in the literature with the following modifications.^{19,32} HaCaT cells were seeded on 12-well plates and grown to confluence. After incubation for 24 hours under various conditions, cells

were washed with Hanks' buffered salt solution and treated for 30 minutes with 0.3 ml dispase II (2.4 U/ml, Sigma) at 37°C. Afterward, dispase solution was carefully removed and cells dissolved in 0.5 ml Hank's buffered salt solution (HBSS). Mechanical stress was then applied by pipetting 10 times with a 1 ml pipette. Finally, dissociation was quantified by counting and averaging cell fragments in three defined areas of each condition under a binocular microscope. Every condition was repeated at least four times.

Laser Tweezer

Expression and purification of recombinant Dsg 3 and Dsg 1, coating of polystyrene beads and the laser tweezer set-up were described previously in detail.¹³ Coated beads (10 μ l of stock solution) were suspended in 200 μ l of culture medium and allowed to interact with HaCaT monolayers for 30 minutes at 37°C before measuring the number of bound beads (= control values). Beads were considered tightly bound when resisting laser displacement at 42 mW setting. For every condition 100 beads were counted. Afterward, EGF or PV-IgG with or without test reagents (pre-incubated for 2 hours) were applied for 30 minutes or the indicated times. Percentage of beads resisting laser displacement under various experimental conditions was normalized to control values.

Statistics

Differences in ELISA values or bead adhesion between different protocols have been assessed using two-tailed Student's *t*-test. Mann-Whitney *U*-test was used for comparison of dispase-based dissociation assay experiments. Values throughout are expressed as mean $\pm$ SE. Statistical significance is assumed for $P < 0.05$.

Results

Effects of EGF and PV-IgG Treatment on Human Keratinocytes

In our study, we compared the effects of PV-IgG versus EGF treatment in various experimental setups to gain insight into the role of EGFR in pemphigus. First, we tested the effects of EGF and PV-IgG on desmosomes and the intermediate filament cytoskeleton in cultured human keratinocytes (HaCaT) using immunostaining. In addition, Alexa 488-phalloidin staining of filamentous actin (F-actin) was applied to sensitively detect keratinocyte dissociation, especially under conditions when Dsg 3 staining was altered and thus cell borders were hard to identify. Under control conditions, Dsg 3 was distributed along cell-cell borders together with F-actin (Figure 1, A and B). To examine pathogenic effects on the cytokeratin filament network, we stained the cells for cytokeratin 5 (CK5). In controls, CK5 was detected as a meshwork throughout the cytoplasm except of the cell periphery where staining was weak (Figure 1C). Incubation for 24

Figure 1. PV-IgG and EGF caused cell dissociation and cytokeratin retraction in keratinocyte monolayers. HaCaT cells were stained for Dsg 3 (**A**, **D**, **G**, **J**), F-actin (**B**, **E**, **H**, **K**), or cytokeratin 5 (CK5; **C**, **F**, **I**, **L**). In control monolayers (**A–C**) or cells treated with control IgG for 24 hours (**D–F**), Dsg 3 was distributed along cell borders (**A**, **D**) along with F-actin (**B**, **E**). Cytokeratin 5 (CK5) immunostaining was detected throughout the cytoplasm except the cell periphery where staining was weak (**C**, **F**). EGF treatment caused intracellular gap formation (arrows in **G**, **H**) and prominent CK5 retraction (**I**). Similarly, incubation with PV-IgG 1 (**J–L**) resulted in intercellular gap formation (arrows in **J**, **K**) and CK5 retraction (**L**) that additionally was accompanied by disruption of Dsg 3 staining (**J**). Scale bar = 20 μ m for all panels ($n = 4$).

hours with control IgG yielded a similar phenotype (Figure 1, D–F). In contrast, EGF caused formation of intercellular gaps and keratinocyte dissociation after 24 hours, paralleled by disorganization of the actin cytoskeleton including the generation of stress fibers and short F-actin aggregates (Figure 1, G–H). More significantly, CK5 staining revealed a strong retraction of keratin filaments from the cell periphery to the perinuclear area (Figure 1I). These effects already appeared after 2 hours (not shown), but were more prominent after 24 hours EGF treatment. Similarly, PV-IgG incubation caused keratinocyte dissociation, actin reorganization and cytokeratin retraction (Figure 1, J–L). However, in contrast to EGF, Dsg 3 staining shifted from the membrane to the cytoplasm and was fragmented along cell borders in response to PV-IgG indicating loss and aggregation of desmosomes. Experiments were reproduced with PV-IgG 2–4 (data not shown). Thus, prominent cytokeratin retraction and intercellular gap formation were induced by both EGF and PV-IgG. All experiments were repeated with NHEK cells, which yielded similar results

Figure 2. EGF but not PV-IgG caused phosphorylation of EGFR at Y1173 and internalization of EGFR. **A:** In contrast to treatment of HaCaT cells with EGF, PV-IgG 1–4 did not induce phosphorylation of EGFR at Y1173 as revealed by western blotting. Total EGFR amounts were similar under all conditions **G**. Comparable results were obtained in experiments with NHEK cells. **B–D:** As revealed by immunostaining, EGFR was continuously distributed along HaCaT cell borders (**B**). EGF treatment (1 hour) resulted in the appearance of intracellular punctuated EGFR staining likely reflecting EGFR internalization (arrows in **C**). In contrast, no EGFR endocytosis was observed following 1–4 hours PV-IgG treatment (**D–G**). Scale bar = 20 μ m for all panels. ($n = 4$).

(data not shown). These data suggested that EGFR signaling may account for at least some of the autoantibody-mediated effects, a conclusion that led us to further explore whether EGFR was activated by PV-IgG under these conditions.

EGF but Not PV-IgG Caused Canonical Activation of EGFR

Next we tested if PV-IgG were capable to activate EGFR via the canonical EGF pathway. It is known that EGFR is classically activated via ligand binding leading to dimerization and intracellular EGFR kinase activation.²² This finally results in Y1173 EGFR phosphorylation, subsequent initiation of the MAP kinase cascade as well as in EGFR endocytosis and termination of EGFR signaling.

We incubated EGF or PV-IgG on HaCaT cells for 1 hour and subsequently probed for Y1173 phospho-EGFR in Western blotting studies (Figure 2A) because previous studies from the literature reported significant phosphorylation of EGFR within 1 hour.^{29,30} As expected, EGF treatment resulted in strong phosphorylation of EGFR at Y1173. However, no enhanced signal was seen after PV-IgG 1–4 treatment. Comparable results were obtained when longer incubation periods for up to 24 hours were used. To exclude that EGFR activation is impaired in immortalized HaCaT keratinocytes, experiments were repeated with NHEK cells (Figure 2A). However, Western blotting studies using the Y1173 phospho-EGFR antibody

similarly showed that PV-IgG did not activate EGFR by phosphorylation at Y1173.

Immunostaining revealed EGFR to be localized continuously along cell borders in HaCaT cells (Figure 2B). EGF treatment for 1 hour resulted in punctuated EGFR staining within the cytoplasm of HaCaT cells likely representing endocytosed vesicles containing EGFR (Figure 2C). After PV-IgG treatment, localization of EGFR was not altered (Figure 2, D–G). This was also confirmed in EGFR-over-expressing A431 cells (data not shown). Therefore, canonical activation with phosphorylation of EGFR at Y1173 leading to EGFR endocytosis was not observed in response to PV-IgG, which is in contrast to previous findings from the literature.^{29,30}

EGF but Not PV-IgG Led to Phosphorylation of EGFR at Y845

As a next step, we looked for other modes of EGFR activation by PV-IgG. Y845 is not only an autophosphorylation site in the kinase domain of EGFR but also phosphorylated by c-Src.³³ Since it was recently reported that c-Src is activated after PV-IgG treatment,³⁰ it was important to test if EGFR was phosphorylated at Y845 in response to PV-IgG. We measured phosphorylation of EGFR at Y845 using a commercial ELISA kit (Figure 3A). Control IgG did not lead to an enhanced signal in ELISA experiments. EGF induced a strong phosphorylation (ELISA score $1585\% \pm 244\%$ of control). This signal could be efficiently blocked to $42\% \pm 5\%$, $45\% \pm 10\%$ and $61\% \pm 2\%$ of EGF values using neutralizing EGFR antibody (LA-1), pharmacological inhibitors of EGFR (GW2974) or c-Src kinase (PP2) (Figure 3B). Surprisingly, all PV-IgG tested also resulted in strong and significant EGFR phosphorylation (ELISA scores $590\% \pm 16\%$, $748\% \pm 55\%$, $680\% \pm 67\%$, Figure 3A). These signals, however, were also detected when the respective secondary antibody was applied without Y845 phospho-EGFR primary antibody (ELISA scores $686\% \pm 23\%$, $937\% \pm 38\%$, $476\% \pm 66\%$, respectively). This indicated that the ELISA signals were due to cross-reactivity of the ELISA secondary goat anti-rabbit (garb) antibody with pemphigus IgG bound to the keratinocyte surface. Consistent with this, PV-IgG-induced signals in ELISA experiments were not blocked by LA-1, GW2974, or PP2 (Figure 3B).

We further investigated phosphorylation of EGFR at Y845 by immunostaining. Under control conditions, HaCaT cells did not show any signal using the Y845 phospho-EGFR antibody (Figure 3C) but were positive for total EGFR (Figure 3D). EGF treatment for 1 hour resulted in Y845 phosphorylation of EGFR at the cell membrane as well as in endocytosis of phosphorylated EGFR (arrows in Figure 3E and F). Following treatment with PV-IgG 2, Y845 phospho-EGFR signals were detected at cell borders but no intracellular punctuated staining was observed (Figure 3 G and H). However, a similar signal at sites of cell borders was also present following PV-IgG 2-treatment when the secondary garb antibody was used in the absence of the Y845 phospho-EGFR antibody (Fig-

ure 3I). Again, this demonstrated that the secondary garb antibody cross-reacted with PV-IgG bound to the HaCaT surface. Consistent with this interpretation, deposition of PV-IgG on HaCaT cells after 1 hour incubation was confirmed using goat anti-human staining (Figure 3J).

To further substantiate this finding, we performed Western blotting of HaCaT cells treated with EGF or PV-IgG for 1 hour (Figure 3K). Total EGFR amounts were equal in the three experimental conditions. EGF treatment resulted in a strong Y845 phospho-EGFR immunosignal at the expected EGFR size (170 kDa). In controls as well as following incubation with PV-IgG 2, no band was detectable at 170 kDa. However, following incubation with PV-IgG a strong band was visible migrating at 55 kDa. This band was independent of the primary Y845 phospho-EGFR antibody as demonstrated by Western blotting using secondary garb antibody alone (Figure 3L). Similar results were obtained using a goat anti-human secondary antibody and confirmed for 3 additional PV-IgG (not shown). Therefore, this band most likely represented denatured heavy chain fragments of PV-IgG in the cell lysates. We further investigated EGFR phosphorylation at Y845 in NHEK cells (Figure 3M). However, consistent with results from HaCaT cells, only EGF treatment but not incubation with 4 different PV-IgG fractions resulted in Y845 phosphorylation as revealed by Western blotting (Figure 3M).

We then investigated if PV-IgG-induced phosphorylation of EGFR at Y845 occurred at other time points (Figure 4). However, when PV-IgG were incubated on HaCaT for 15, 30, 60 or 120 minutes, EGFR Y845 ELISA signals were indistinguishable to secondary antibody controls (Figure 4A). To investigate whether ELISA values of secondary antibody controls were caused by time-dependent binding characteristics of PV-IgG to the keratinocyte surface, we quantified PV-IgG binding to the surface of HaCaT cells following immunostaining during the same time course. Clearly, a time-dependent binding of PV-IgG to the keratinocyte surface was detected by fluorescence quantification on HaCaT cells, which had been treated with PV-IgG 5 for the indicated times and stained with Cy3-labeled goat anti-human antibody to detect surface-bound PV-IgG (Figure 4B). PV-IgG binding peaked after 1 hour incubation but was reduced after 2 hours. Taken together, ELISA, immunostaining and Western blotting studies showed that PV-IgG did not lead to phosphorylation of EGFR at Y845. Rather, secondary antibody cross-reactivity was observed in all three experimental setups.

Inhibition of EGFR and c-Src Blocked EGF-induced Keratinocyte Dissociation Whereas PV-IgG-induced Effects Were Not Prevented

Since we did not observe activation of EGFR following incubation with PV-IgG in our experiments, we further explored if blocking EGFR or c-Src function was able to prevent EGF- or PV-IgG-induced effects on keratinocytes (Figure 5, compare with Figure 1). EGF-induced intercellular gap formation and keratinocyte dissociation were blocked by pharmacological EGFR inhibition (GW2974,

Figure 3. PV-IgG did not induce phosphorylation of EGFR at Y845. **A:** ELISA studies revealed phosphorylation of EGFR at Y845 after EGF but not after control IgG treatment. All three PV-IgG tested also caused an apparent Y845 EGFR phosphorylation. These ELISA signals, however, were also detectable when experiments were performed in the absence of Y845 phospho-EGFR primary antibody indicating cross-reactivity of the secondary goat anti-rabbit (garb) antibody with pemphigus IgG bound to the keratinocyte surface ($n = 4$ to 6). **B:** EGF-induced EGFR phosphorylation at Y845 was reduced by EGFR neutralizing antibody LA-1, inhibition of intrinsic EGFR kinase activity by GW2974 and inhibition of c-Src via PP2. In contrast, these inhibitors did not reduce the PV-IgG 1-induced Y845 EGFR phosphorylation signal ($n = 3$). **C–J:** Moreover, immunostaining using a Y845 phospho-EGFR antibody did not reveal PV-IgG-induced Y845 EGFR phosphorylation. HaCaT cells were immunostained for Y845 phospho-EGFR (**C, E, G, I**) and total EGFR (**D, F, H, J**). Under control conditions, HaCaT cells showed no Y845 phospho-EGFR-specific signal (**C**) but were positive for total EGFR (**D**). EGF treatment (1 hour) resulted in Y845 phospho-EGFR immunostaining at cell borders as well as punctuated staining in the cytoplasm (arrows in **E** and **F**). Following incubation with PV-IgG, Y845 phospho-EGFR staining was detected at cell borders but was absent within the cytoplasm (**G** and **H**). Similar staining patterns were obtained after PV-IgG treatment when secondary garb or goat anti-human (gah) antibodies were applied in the absence of primary antibodies (**I** and **J**). Scale bar = $20 \mu\text{m}$ for all panels. ($n = 3$). **K:** Western blotting revealed that EGF but not PV-IgG caused Y845 EGFR phosphorylation. In controls and following incubation with PV-IgG, no band was detectable at 170 kDa indicating that EGFR was not phosphorylated at Y845 under these conditions. In contrast, EGF treatment resulted in a strong immunosignal at the expected EGFR size (170 kDa). After treatment with PV-IgG, a strong band was visible migrating at 55 kDa. This band likely represented PV-IgG heavy chains in cell lysates as demonstrated by Western blotting using secondary garb or gah antibodies in the absence of primary antibodies (**L**). ($n = 3$). **M:** Western blotting with NHEK lysates confirmed that treatment with EGF but not with PV-IgG resulted in phosphorylation of EGFR at Y845. Total EGFR bands revealed equal sample loading.

Figure 5A–B) and partially by inhibition of c-Src via PP2 (arrows in Figure 5D–E). EGF-induced cytokeratin retraction was largely abolished by inhibition of both EGFR and c-Src (Figure 5C and F). In contrast, effects of PV-IgG on HaCaT cells were neither altered by inhibition of EGFR nor of c-Src because intercellular gaps (arrows) and retraction of cytokeratin filaments were discernable under

these conditions (Figure 5G–L). Similar results were obtained using NHEK cells (data not shown).

Next, we quantified cell dissociation in response to EGF and PV-IgG treatment using a standard keratinocyte dissociation assay (Figure 6). Under control conditions, 8 ± 2 cell fragments were counted per cm^2 after applying mechanical stress to disperse-treated monolayers. EGF

Figure 4. No time-dependent phosphorylation of EGFR at Y845 but binding of PV-IgG to the keratinocyte cell surface. **A:** In time-dependent ELISA studies no specific phosphorylation of EGFR at Y845 after PV-IgG 5 treatment (+1. AB) was observed when directly compared with secondary antibody controls (−1. AB). No statistical differences were detected between different time points and experimental conditions (+1. AB vs. −1. AB). **B:** Binding of PV-IgG 5 to HaCaT cells was determined using quantitative immunostaining of HaCaT cells treated with PV-IgG 5 for the indicated times and stained with Cy3-labeled goat anti-human antibody to detect surface-bound PV-IgG. A clear time-dependent binding of PV-IgG to the keratinocyte surface was detected peaking at 1 hour but declining after 2 hours.

treatment led to a significant increase in fragments (67 ± 6) confirming the findings of the immunofluorescent studies (Figure 1G–I). All 3 PV-IgG tested in the assay (PV-IgG 1, 2, 4) also resulted in strong cell dissociation as marked by increased numbers of cell fragments (203 ± 33 , 177 ± 16 , 66 ± 7 , respectively). Simultaneous incubation of monolayers with EGF in the presence of GW2974 or PP2

partially blocked cell dissociation (15 ± 3 fragments for each condition). In contrast, acantholytic effects of PV-IgG 1, 2 and 4 were independent of EGFR kinase or c-Src because coincubation with GW2974 or PP2 did not significantly alter the number of cell fragments (156 ± 41 and 237 ± 44 for PV-IgG 1, 180 ± 23 and 159 ± 5 for PV-IgG 2 and 77 ± 6 and 78 ± 9 for PV-IgG 4, respectively). These data indicate that inhibition of EGFR signaling was not effective to block PV-IgG-induced cell dissociation. Therefore, to prove that acantholysis caused by PV-IgG can be inhibited by interference with cellular signaling, experiments were performed with Rho A-activating toxins cytotoxic necrotizing factor (CNF) 1 and CNFy. These toxins were shown to block PV-IgG-induced epidermal splitting in skin organ culture as well as autoantibody-mediated intercellular gap formation and loss of Dsg bead binding in laser tweezer assays in HaCaT cells.^{18,34} Consistent with these previous findings, selective activation of Rho A by CNFy was equally effective like activation of all three Rho family GTPases Rho A, Rac 1,

Figure 5. Inhibition of EGFR but not of c-Src blocked EGF-mediated keratinocyte dissociation whereas PV-IgG-induced effects were not affected. HaCaT cells were immunostained for Dsg 3 (**A, D, G, J**) and for CK5 (**C, F, I, L**) and F-actin was labeled using Alexa 488-phalloidin (**B, E, H, K**). **A–C:** EGF-induced effects on keratinocyte monolayers including intercellular gap formation and keratinocyte retraction (compare to Figure 1, G–I) were effectively blocked by pharmacological EGFR inhibition (GW2974). c-Src inhibition via PP2 did partially block EGF-induced intercellular gap formation (arrows in **D–E**) prevented keratin retraction (**F**). **G–I:** In contrast, neither inhibition of EGFR (**G–I**) nor of c-Src (**J–L**) was effective to reduce PV-IgG-induced keratinocyte dissociation (arrows in **G, H, J, K**) and cytokeratin retraction (**I, L**). Scale bar = $20 \mu\text{m}$ for all panels. ($n = 4$).

Figure 6. EGF but not PV-IgG caused keratinocyte dissociation dependent on EGFR and c-Src signaling. Keratinocyte dissociation was further quantified using a disperse-base assay. EGF treatment resulted in a strong increase of cell fragments compared with controls. This increase was partially blocked by simultaneous incubation with EGFR kinase inhibitor GW2974 or c-Src inhibitor PP2. In contrast, PV-IgG-mediated acantholysis was independent of EGFR kinase or c-Src because GW2974 and PP2 did not prevent increase in cell fragments. However, cotreatment with CNF1 and CNFy inhibited PV-IgG 4-induced cell dissociation. Similarly, in NHEK cells GW2974 and PP2 application were only sufficient to inhibit EGF- but not PV-IgG-mediated cell layer fragmentation ($n = 6$ for each condition).

and Cdc42 by CNF-1 to block pemphigus IgG-induced acantholysis (Figure 6).

We repeated these experiments using NHEK cells (Figure 6). EGF treatment led to cell dissociation ($473\% \pm 46\%$ compared with controls), which was significantly reduced by both GW2974 ($200\% \pm 85\%$) and PP2 treatment ($216\% \pm 55\%$). In contrast, PV-IgG 2 resulted in strong cell sheet fragmentation ($2082\% \pm 234\%$), which was blocked neither by GW2974 ($2000\% \pm 279\%$) nor by treatment with PP2 ($2188\% \pm 135\%$). Taken together, EGFR kinase seemed to be important for EGF-induced keratinocyte dissociation but not to be essential for acantholysis in pemphigus.

PV-IgG Interfered with Dsg-mediated Bead Binding Independent of EGFR or c-Src Whereas EGF Had No Effect

Next, we investigated whether EGFR and c-Src were required for PV-IgG-induced loss of Dsg binding. Previous studies using laser tweezers demonstrated that activation of Rho A by CNF-1 and CNF γ was effective to inhibit PV-IgG-induced loss of Dsg bead binding, consistent with experiments using the cell dissociation assay described above.^{18,34} Therefore, laser tweezers seem to provide an adequate and sensitive approach to evaluate the involvement of different signaling cascades in pemphigus-mediated loss of Dsg binding. In contrast to dissociation assays that quantify keratinocyte cohesion as a result of all of the different intercellular adhesion molecules involved, the laser tweezer assays allows to study the effect of PV-IgG on a single type of adhesion molecule on the microbead surface.

In this assay, binding of Dsg-coated microbeads was probed by laser beam-mediated displacement on the surface of HaCaT cells (Figure 7). Dsg 3- (black bars) and Dsg 1 coated beads (white bars) were allowed to settle on the surface of HaCaT cells for 30 minutes (control) and bound beads were counted. After application of PV-IgG or EGF, the number of bound beads was counted again. The numbers of bound Dsg 3- or Dsg 1-coated beads were not altered following incubation with control IgG but were reduced by incubation with EGTA (5 mmol/L, 30 minutes) to $41\% \pm 6\%$ and $22\% \pm 3\%$ of control levels, respectively. This proved that bead binding was mediated by Ca^{2+} -dependent Dsg transinteraction. Neither short term (30 minutes) nor long term (24 hours) treatment with EGF blocked Dsg 3- or Dsg 1-mediated binding. In contrast, incubation of monolayers and attached beads with PV-IgG 2 reduced adhesion of Dsg 3- and Dsg 1-coated beads ($75\% \pm 6\%$ and $54\% \pm 4\%$, respectively), similar to our previous studies.^{13,14,18,34} This reduction, however, was not altered by simultaneous inhibition of EGFR via GW2974 or of c-Src via PP2. In this context, our new data therefore indicate that EGFR and c-Src were not required for PV-IgG-induced loss of Dsg bead binding. Moreover, EGF obviously caused keratinocyte dissociation via mechanisms other than loss of Dsg 3 and Dsg 1 binding.

Figure 7. Inhibition of EGFR or c-Src did not prevent PV-IgG-induced loss of Dsg-mediated bead binding in laser tweezer experiments. Dsg 3- (black bars) and Dsg 1 coated beads (white bars) were allowed to settle on the surface of HaCaT cells for 30 minutes before the number of bound beads was counted (control). After application of PV-IgG or EGF, the number of adhering beads was counted again. The number of bound Dsg 3- or Dsg 1-coated beads was not altered after control IgG treatment but reduced by incubation with EGTA (5 mmol/L, 30 minutes) to $41\% \pm 6\%$ and $22\% \pm 3\%$, respectively. EGF did not block Dsg 3- or Dsg 1-mediated binding after incubation for 30 minutes or 24 hours. In contrast, incubation of monolayers and attached beads with PV-IgG 2 for 30 minutes reduced the numbers of Dsg 3 and Dsg 1 beads to $75\% \pm 6\%$ and $54\% \pm 4\%$, respectively. This reduction was unaffected by blocking of EGFR via preincubation with GW2974, or of c-Src via preincubation with PP2. ($n = 6$ for each condition).

Discussion

We investigated the role of EGFR and c-Src in pemphigus pathogenesis by directly comparing EGF- and PV-IgG-mediated effects on cultured human keratinocytes. Although in our experimental setup the effects of EGF and PV-IgG on keratinocyte morphology appeared to be very similar, we did not observe canonical or c-Src-dependent activation of EGFR in response to incubation with PV-IgG. Moreover, inhibition of EGFR or c-Src did not block PV-IgG-induced loss of Dsg binding, cytokeratin retraction and keratinocyte dissociation. Thus, EGFR and c-Src signaling may not be primarily required for PV-IgG-induced acantholysis *in vitro*. Moreover the mechanisms involved in EGF- and PV-IgG-induced keratinocyte dissociation and cytokeratin retraction seem to be different.

Mechanisms by Which EGF Reduces Keratinocyte Cohesion

EGFR activation leads to c-Src activation and plakoglobin phosphorylation, which in turn is known to weaken keratinocyte cohesion and to rearrange the cytokeratin filament network.^{27,28,35} In our experiments, EGF triggered intercellular gap formation and cytokeratin retraction. These effects were blocked by pharmacological inhibitors of EGFR kinase as well as of c-Src, suggesting that EGF-mediated keratinocyte dissociation is at least partly dependent on c-Src.

The mechanisms underlying EGF-induced loss of cell adhesion are only beginning to emerge. Gaudry and colleagues demonstrated that EGF-induced disruption of intercellular cohesion was at least in part mediated by loss of Dsg 2 binding.²⁷ It appeared that EGF caused uncoupling of the Dsg 2-plakoglobin complex from the cytokeratin cytoskeleton because desmoplakin, the primary molecule responsible for cytoskeletal anchorage, was not linked to plakoglobin following EGFR-mediated plakoglobin phosphorylation. However, the effects of EGF signaling on Dsg 3 and Dsg 1, the main targets in pemphigus pathogenesis, have not been characterized so far. Although resulting in intercellular gap formation, EGF failed to reduce Dsg 3- and Dsg 1-mediated bead binding in laser tweezer experiments. Therefore, one could speculate that EGF-mediated loss of cell adhesion is not primarily mediated by inhibition of Dsg 3 and Dsg 1 binding, but rather by other cell adhesion molecules such as Dsg 2 or E-cadherin. This is puzzling since Dsg 3 and Dsg 1 are also anchored to cytokeratin filaments via plakoglobin and desmoplakin and consequently should also be affected by EGFR-mediated plakoglobin phosphorylation. However, other mechanisms in addition to plakoglobin phosphorylation may contribute to EGF-induced cell dissociation because a plakoglobin mutant missing the phosphorylation sites required for EGFR-mediated phosphorylation only partially blocked cell dissociation in response to EGF treatment.²⁸ These mechanisms may involve proteases of the ADAM (a disintegrin and metalloprotease) family. It was demonstrated that EGF increased cellular ADAM10 and ADAM17 levels and thereby caused Dsg 2 and E-cadherin degradation.^{36,37} This is in line with the increased cell dissociation revealed by the keratinocyte dissociation assay in our study. Consistent with our observation that PV-IgG did not activate EGFR and the hypothesis that EGF caused cell dissociation via adhesion molecules others than Dsg 3 and Dsg 1, we did neither find PV-IgG-induced activation of ADAM17 nor shedding of Dsg 3 or Dsg 1 in HaCaT monolayers (unpublished observations). However, it has to be emphasized that in the laser tweezer setup we are only in the position to test binding of non-desmosomal Dsg molecules on the keratinocyte cell surface. Therefore, we cannot completely rule out that EGF-treatment reduces adhesion of Dsg 3 and Dsg 1 molecules within desmosomes.

Role of EGFR and c-Src in Pemphigus

The hypothesis that EGFR signaling plays a significant role in pemphigus pathogenesis is tempting because EGF-induced effects on keratinocytes such as a profound loss of cell cohesion as well cytokeratin retraction are hallmarks of acantholysis in pemphigus. Therefore, Dsg 3 molecules on the cell surface have been proposed to mediate autoantibody-triggered outside-in signaling at least in part via cross talk with receptor tyrosine kinases such as EGFR.¹⁵ This has already been demonstrated for other cadherins in keratinocytes: transinteracting E-cadherin molecules recruit EGFR resulting in phosphatidylinositol-3-kinase and Akt activation,^{38,39} which is thought

to finally result in cell contact-induced stop of cell proliferation. This mechanism is believed to be part of the so-called "contact inhibition". Because loss of E-cadherin binding should have the opposite effect and therefore lead to increased cell proliferation, it is interesting to note that autoantibodies against Dsg 1 in pemphigus have been shown to cross-react with E-cadherin.¹⁰ However, it remains to be elucidated whether these antibodies interfere with E-cadherin transinteraction. Nevertheless, in our experiments we did neither find canonical activation of EGFR at Y1173 followed by receptor internalization nor phosphorylation at Y845, although in parallel experiments EGF was effective to induce such effects. Accordingly, PV-IgG-induced keratinocyte dissociation, loss of Dsg 3 bead binding in laser tweezer experiments and cytokeratin retraction were not abolished by inhibition of EGFR and c-Src signaling. Thus, the pathogenic effects of PV-IgG under the conditions used in our study were independent of EGFR or c-Src.

It has to be emphasized that our findings contradict previous reports.^{29,30} However, to detect EGFR activation in these studies unspecific phospho-tyrosine antibodies instead of phospho-specific EGFR-antibodies were used.²⁹ In another study, the same ELISA-based measurements of EGFR phosphorylation were used like in our study,³⁰ which, as outlined below, may lead to unspecific signals by interaction of secondary antibodies with keratinocyte-bound PV-IgG. The authors used siRNA to reduce Dsg 3 and Dsg 1 expression and found that PV-IgG-mediated EGFR and c-Src activation was independent of autoantibodies against Dsg 3 and 1.³⁰ From these findings it was concluded that only non-Dsg autoantibodies could activate these signaling mechanisms. In contrast, our previous studies indicate that pathogenic effects of PV-IgG and PF-IgG were dependent on antibodies against Dsg 3 and Dsg 1 because immunoprecipitation using recombinant molecules completely abolished pathogenic effects of autoantibody fractions.^{13,14} Because direct inhibition of Dsg 1 binding by PV-IgG and PF-IgG was not detected in these studies, it is likely that autoantibodies against desmogleins are capable to trigger cellular signaling mechanisms. We characterized autoantibody fractions used in this study only in respect of anti-Dsg autoantibodies whereas identification of all other possible autoantigens was not possible. This is because a plethora of more than 20 different autoantibodies has been found in pemphigus patients' sera of which the pathological significance mostly has not been characterized yet.^{1,6-9} Therefore, it has to be emphasized that we cannot completely rule out that PV-IgG from other patients activate EGFR signaling and that this may be mediated by autoantibodies against autoantigens different from Dsg 3 and Dsg 1. Nevertheless this study demonstrates that PV-IgG-induced acantholysis in human keratinocytes is independent of EGFR signaling.

Cross-Reactivity of Secondary Antibodies with Patients' Autoantibodies in Pemphigus Research

Our data indicate that secondary antibody cross-reactivity with pemphigus autoantibodies bound to the keratinocyte cell surface may result in false-positive signals in several

antibody-based detection methods such as immunostaining, Western blotting or ELISA. Working with high concentrations of pemphigus autoantibodies leads to significant deposition of autoantibodies on the keratinocyte cell surface. Especially, when highly sensitive detection methods such as ELISA are used, the cross-reactivity of secondary antibodies with cell-bound autoantibodies may account for positive signals that cannot be reproduced by other methods.

Acknowledgments

We thank Lisa Bergauer and Tanja Reimer for excellent technical assistance and Volker Spindler for technical advice.

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