Desmoglein Versus Non-desmoglein Signaling in Pemphigus Acantholysis

CHARACTERIZATION OF NOVEL SIGNALING PATHWAYS DOWNSTREAM OF PEMPHIGUS VULGARIS ANTIGENS*

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Although it is accepted that pemphigus antibody binding to keratinocytes (KCs) evokes an array of intracellular biochemical events resulting in cell detachment and death, the triggering events remain obscure. It has been postulated that the binding of pemphigus vulgaris IgG (PVIgG) to KCs induces “desmosomal” signaling. Because in contrast to integrins and classical cadherins, desmoglein (Dsg) molecules are not known to elicit intracellular signaling, and because PV patients also produce non-Dsg autoantibodies, we investigated the roles of both Dsg and non-desmoglein PV antigens. The time course studies of KCs treated with PVIgG demonstrated that the activity of Src peaked at 30 min, EGF receptor kinase (EGFRK) at 60 min, and p38 MAPK at 240 min. The Src inhibitor PP2 decreased EGFRK and p38 activities by ~45 and 30%, respectively, indicating that in addition to Src, PVIgG evokes other triggering events. The shrinkage of KCs (cell volume reduction) became significant at 120 min, keratin aggregation at 240 min, and an increase of TUNEL positivity at 360 min. Pretreatment of KCs with PP2 blocked PVIgG-dependent cell shrinkage and keratin aggregation by ~50% and TUNEL positivity by ~25%. The p38 MAPK inhibitor PD169316 inhibited these effects by ~15, 20, and 70%, respectively. Transfection of KCs with small interfering RNAs that silenced expression of Dsg1 and/or Dsg3 proteins, blocked ~50% of p38 MAPK activity but did not significantly alter the PVIgG-dependent rise in Src and EGFRK activities. These results indicate that activation of p38 MAPK is a late signaling step associated with collapse of the cytoskeleton and disassembly of desmosomes caused by upstream events involving Src and EGFRK. Therefore, the early acantholytic events are triggered by non-Dsg antibodies.

Pemphigus vulgaris (PV)2 is an immunobullerizing disease associated with IgG autoantibodies that react with desmoglein (Dsg) 1 and/or 3 as well as non-Dsg target molecules on the cell membrane of keratinocytes (KCs) (reviewed in Ref. 1). It has been convincingly demonstrated that PVIgG binding to KCs evokes an array of intracellular biochemical events resulting in cell detachment and death. The binding of PVIgG to KCs activates phosphatidylcholine-specific phospholipase C, increases inositol 1,4,5-triphosphate and diacylglycerol production, elevates the intracellular free calcium level, and leads to activation of various kinases, including protein kinase C and p38 MAP kinase (MAPK) (reviewed in Ref. 2). Among the known substrates of PVIgG-dependent phosphorylation are the adhesion molecules E-cadherin, Dsg3, β-catenin, and γ-catenin (a.k.a. plakoglobin) (3), and also heat shock protein (Hsp)27 (4). PVIgG binding to KCs leads to depletion of Dsg3 from desmosomes, nuclear trafficking of plakoglobin, urokine-type plasminogen activator secretion, and c-Myc overexpression (reviewed in Ref. 5). DNA microarray assays demonstrated that PVIgG down-regulates expression of the genes encoding keratinocyte adhesion molecules, antigen-processing proteins, regulators of cell cycle and apoptosis, differentiation markers, Na+, K+-ATPase, protein kinases, phosphatases, and others (5). Overall, PVIgG decreased transcription of 198 genes and increased that of 31 genes. The pathobiologic outcome of the PVIgG-induced signaling is induction of the apoptotic and/or oncotic pathways, associated with collapse of the cytoskeleton, because of keratin filament retraction and actin reorganization, cell shrinkage, caused by cell volume decrease, and, finally, cell-cell dysheension (acantholysis) (reviewed in Ref. 6).

Elucidation of the signaling pathways mediating PVIgG-induced acantholysis has salient clinical implications. The in vitro and in vivo studies revealed not only the complexity of PVIgG signaling, but also clearly demonstrated the therapeutic potential of kinase inhibitors and pathway modifiers. PVIgG-dependent acantholysis in experimental animals could be ameliorated by inhibitors of phospholipase C, protein kinase C, p38 MAPK, other tyrosine kinases, calmodulin as well as cholinergic agonists (7–9). The triggering events, however, remain obscure.

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2The abbreviations used are: PV, pemphigus vulgaris; KCs, keratinocytes; MAPK, mitogen-activated protein kinase; RTK, receptor tyrosine kinase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; EGFRK, epidermal growth factor receptor kinase; Dsg, desmoglein.
The outside-in signaling of PVIgG may be elicited through both cholinergic receptors and desmosomal proteins, as well as other yet unknown PV antigens/receptors ligated on the cell membrane of KCs by an array of anti-keratinocyte autoantibodies produced in each PV patient, as explained by the “Multiple Hit” hypothesis (10). The major downstream signaling events resulting from activation/blockade of cholinergic receptors expressed in KCs have been characterized (11, 12), but only a little is known about the reputed “desmosome” signaling pathway (4). Recent studies have demonstrated activation of receptor tyrosine kinases (RTKs) at an early step of PVIgG-induced signaling (13), suggesting that such receptors are either novel specific targets of pemphigus autoimmunity or “innocent victims” of the “collateral damage” produced because of PVIgG binding to the keratinocyte plasma membrane, or both.

The purpose of this study was to identify the primary pathways mediating PVIgG-induced acantholysis that can provide a promising target for development of novel therapies. We performed a time course correlation study of biochemical and morphologic events induced by PVIgG binding to human KCs. The results indicate that activation of p38 MAPK is a late signaling step associated with collapse of the cytoskeleton and disassembly of desmosomes caused by upstream events involving c-Src kinase (i.e. the protein encoded by the cellular counterpart of the translation product of the src gene of Rous sarcoma virus) and epidermal growth factor receptor kinase (EGFRK). To dissect out the signaling pathways originating from of Dsg 1/3 and from those elicited due to ligation of non-Dsg antigens, we used KCs with the knocked-down expression of DSG1 and/or DSG3 genes by small interfering RNA (siRNA). The earliest acantholytic events appeared to be triggered by non-Dsg antibodies.

**MATERIALS AND METHODS**

**Chemicals and Tissue Culture Reagents**—A potent, cell-permeable, and selective p38 MAPK inhibitor PD169316, the inhibitor of Src family of protein tyrosine kinase PP2, and PhosphoDetect™ p38 MAPK ELISA kit were purchased from Calbiochem-Novabiochem Corp. (EMD Biosciences, Inc. La Jolla, CA). The FACE™ c-Src and EGFR kits were purchased from Active Motif (Carlsbad, CA). The predesigned and tested siRNA-targeting human DSG1 (NM_001942) mRNA (ON-TARGETplus SMARTpool reagent L-011644-00) (siRNA-Dsg1) and siRNA-targeting human DSG3 (NM_001944) mRNA (L-011646-00) (siRNA-Dsg3) were purchased from Dharmacon (Lafayette, CO). The negative control siRNA targeting luciferase gene with the target sequence 5’-CGTACGCGGAATCTTCCA-3’ that was employed in all RNA inhibition experiments was also from Dharmacon. The DeadEnd™ Fluorometric TUNEL System was from Promega (Madison, WI). Mouse monoclonal antibodies to Dsg1 and Dsg3 were purchased from R&D Systems (Minneapolis, MN) and secondary, FITC-labeled anti-mouse IgG antibody, from Sigma-Aldrich, Inc.

**Pemphigus and Normal Human IgG Fractions**—The results reported herein were obtained in experiments utilizing pooled IgG fractions isolated from sera of 6 PV patients with active lesions on both oral mucosa and the skin, and sera of healthy people purchased from Sigma-Aldrich, Inc. This study had been approved by the University of California Davis Human Subjects Review Committee. The diagnosis of PV was made based on the results of comprehensive clinical and histological examinations, and immunological studies, which included direct immunofluorescence of skin biopsies, indirect immunofluorescence of patient sera on various epithelial substrates, and immunoblotting following standard protocols. The titer of “intercellular” antibodies determined on monkey esophagus ranged from 1/640 to 1/2560. The presence of anti-Dsg1 and -Dsg3 antibodies in each serum was established using the MESACUP Dsg-1 & Dsg-3 ELISA test system (MBL, Nagoya, Japan). The index values for Dsg1 antibodies ranged from 64 to 136 and those for Dsg3 antibodies, from 82 to 176, i.e. were unequivocally positive. The IgG fractions were isolated by FPLC protein G affinity chromatography using the FPLC System purchased from Amersham Biosciences and following the manufacturer’s protocol, as detailed by us elsewhere (14).

**Keratinocyte Culture Experiments**—Human keratinocyte cultures were started from normal neonatal foreskins (15). The cells were grown in 75-cm² flasks (Corning Glass Works, Corning, NY) in serum-free keratinocyte growth medium containing 5 ng/ml epidermal growth factor and 50 µg/ml bovine pituitary extract (Invitrogen, Cambridge, MA) at 37 °C in a humid, 5% CO₂ incubator. The keratinocyte cultures used in experiments were between passages 2 and 4, ~80% confluent, grown from at least three different foreskin donors. The concentration of calcium in culture medium was adjusted to either 0.2 mM or 1.6 mM (see below). The IgG fractions were diluted in culture medium and added to the monolayers at the final concentration of 1 mg/ml. Prior to exposures, some monolayers were pretreated with kinase inhibitors (see “Results”). The control monolayers were left intact. All monolayers were incubated in culture media containing 1.6 mM calcium at 37 °C and 5% CO₂ for different periods of time (see “Results”), and then used in the Src, EGFRK, and p38 MAPK assays in accord with manufacturer’s protocols, quantitation of numbers of TUNEL (i.e. the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling)-positive cells, or for analyses of cell volume and keratin filament aggregation. The cytosolic volume was quantified by computing the cell diameter as detailed elsewhere (16), and the result expressed as % control values determined in intact culture. KCs exhibiting keratin filament aggregation were visualized by indirect immunofluorescence in at least three microscopic fields at magnification ×100, using anti-pan keratin antibody (BioLegend, San Diego, CA).

**siRNA Transfection Experiments**—For transfection with siRNAs, we followed the standard protocol described in detail elsewhere (17). Briefly, KCs were seeded at a density of 2.5 × 10⁵ cells per well of a 6-well plate, and incubated for 16–24 h to achieve ~70% confluence. To each well, increasing concentrations of siRNA in the transfection solution with the DharmaFECT™ 1 siRNA Transfection Reagent (Dharmacon) were added, and the transfection was continued for 16 h at 37 °C in a humid, 5% CO₂ incubator. The siRNA transfection efficiency was assayed using FITC-labeled luciferase GL2 duplex (Dharmacon). After transfection, the cells were grown for 24 h in culture with 0.09 mM calcium, and then switched to the medium containing higher concentrations of calcium.
concentration of calcium and incubated for additional time points (up to 72 h) to achieve maximum inhibition of the Dsg protein expression, as was experimentally determined by immunoblotting and immunofluorescence. For immunoblotting experiments, KCs were dissolved in a sample buffer, separated via SDS-PAGE, and electroblotted onto a 0.2-μm nitrocellulose membrane (Bio-Rad). The membranes were developed using the ECL Plus chemiluminescent detection system (Amersham Biosciences) and scanned with Storm™/FluorImager (Molecular Dynamics, Mountain View, CA). The PVIgG exposure experiments of transfected KCs were performed at 1.6 mM extracellular Ca²⁺. Some KCs transfected with siRNA-Dsg3 were cultured and exposed to PVIgG at 0.2 mM extracellular Ca²⁺. By immunoblotting and indirect immunofluorescence, at this concentration of Ca²⁺ KCs did not express Dsg1.

Electron Microscopic Experiments—Normal human foreskin KCs seeded on glass coverslips were transfected with normal control siRNA (siRNA-NC), siRNA-Dsg1, or siRNA-Dsg3, grown for 48 h in keratinocyte growth medium with 0.09 mM calcium, and then switched to the medium containing 2 mM calcium overnight. On the next day, the cells were fixed in 2% glutaraldehyde at 4 °C for 30 min, washed in cacodylate buffer (pH 7.4) and post fixed in 2% osmium tetroxide at 4 °C for 1 h, followed by embedding in epoxy resins. The ultrathin sections were stained with uranyl acetate and lead citrate, followed by observation under an electron microscope (JEM-100S, JEOL LTD, Tokyo, Japan) as detailed elsewhere (18). The traversing distance of the extracellular core domain (intercellular gap) of desmosome was calculated as follows: ~50 electron micrographs were taken from each sample at a magnification of ×30,000 and developed at ×2.5. From 30–60 desmosomes were analyzed. The distance was measured on the right-angled line with the line of clear plasma membrane, illustrating tri-lamelar structure.

Statistical Analysis—All experiments were performed in triplicate, and the results were expressed as mean ± S.D. Statistical significance was determined using the Student’s t test. Differences were deemed significant if the calculated p value was <0.05.

RESULTS

Time Course Study of Kinase Activities in KCs Exposed to PVIgG—To identify the effectors mediating intracellular signaling of PVIgG at the earliest stages of acantholysis, we studied the
activities of Src, EGFRK, and p38 MAPK. The time course studies demonstrated that the activity of Src peaked at 30 min, EGFRK at 60 min, and p38 MAPK at 240 min (Fig. 1, A–C).

To elucidate the hierarchy of signaling events, we used the Src inhibitor PP2, because the peak of Src activity was observed at the earliest point in time after addition of PVIgG, i.e. before that of EGFRK and p38 MAPK. The cells were pretreated with 10 μM PP2, incubated with PVIgG, and the activities of EGFRK and p38 MAPK were measured at 60 and 240 min of incubation, respectively, when the activities of these kinases peaked. Preincubation with PP2 decreased PVIgG-dependent EGFRK and p38 MAPK activities by ~45 and 30% (p < 0.05), respectively (Fig. 1, D and E), indicating that: 1) Src activation is upstream of both EGFRK and p38 MAPK; and 2) in addition to Src, binding of PVIgG to KCs engages other primary signaling pathways.

**Correlation between Biochemical and Phenotypic Changes in KCs Treated with PVIgG**—We have previously reported that PVIgG-treated KCs shrink, detach from neighboring cells, and die through a mixed apoptotic/ondotic pathway, featuring TUNEL positivity (14, 16). Keratinocyte shrinkage upon exposure to PVIgG (Fig. 2, A and B) is associated with collapse of the tonofilament scaffold supporting the polygonal shape of these epithelial cells (Fig. 2, C and D). To identify functional links between specific signaling events and alterations of keratinocyte morphology and viability resulting from PVIgG binding, we assayed changes in cell volume, keratin filament aggregation, and number of TUNEL+ cells in the monolayers at different time points after addition of PVIgG. The shrinkage of KCs became significant (p < 0.05) at 120 min, keratin filament aggregation at 240 min, and an increase of TUNEL+ cells at 360 min (Fig. 3, A–C).

To establish correlation between biochemical events and phenotypic changes developing in KCs upon PVIgG binding to their plasma membrane, we selected inhibitors of both “early” and “late” kinases, Src and p38 MAPK, respectively. Pretreatment of KCs with the Src inhibitor PP2, 10 μM, blocked PVIgG-dependent cell shrinkage and keratin aggregation by ~50% (p < 0.05), and TUNEL positivity by ~25% (p > 0.05). The p38 MAPK inhibitor PD169316, 10 μM, decreased these PVIgG effects by ~15% (p > 0.05), 22% (p > 0.05), and 70% (p < 0.05), respectively (Fig. 3, D–F). These results indicated that Src was mainly responsible for early events in PVIgG-induced anactinolysis, such as collapse of the cytoskeleton, whereas p38 MAPK, for late events, such as induction of apoptosis. Because neither kinase inhibitor could completely normalize studied parameters, as all remained significantly (p < 0.05) different from control values, PVIgG binding to KCs apparently engaged other signaling pathways contributing to the phenotypic changes.

**The Effects of Silencing of the DSG1 and DSG3 Genes on KCs and Their Response to PVIgG**—To elucidate the roles of desmosomal and non-desmosomal targets of pemphigus autoimmunity, we asked whether silencing of the DSG1 or DSG3 genes would affect the biochemical events elicited because of PV IgG binding to KCs. The efficacy of knocking down the expression of Dsg1 or Dsg3 proteins in KCs with the Dharmacon-predesigned siRNAs was controlled by immunoblotting and immunofluorescence. Already 24 h after transfection with either siRNA-Dsg1 or siRNA-Dsg3, the KCs contained no measurable amounts of Dsg1 or Dsg3, respectively (Fig. 4, A and B). siRNA-NC did not affect Dsg1 or Dsg3 expression. Transfection with either siRNA did not alter keratinocyte viability, evaluated by the trypan blue dye exclusion test and TUNEL staining (data not shown). The morphology of the monolayers comprised by KCs transfected with siRNA-Dsg1 or siRNA-Dsg3 or both was indistinguishable from that of intact cells or KCs transfected with siRNA-NC (not shown). The KCs transfected with either type of siRNA formed desmosomes visualized by electron microscopy.

To determine if gene silencing of Dsg1 and Dsg3 affected desmosome ultrastructure, we compared the ultrastructure of KCs transfected with siRNA-NC, siRNA-Dsg1 and siRNA-Dsg3 (Fig. 4C). KCs transfected with siRNA-NC exhibited normal ultrastructural features of desmosomes. Their desmosomes were comprised by the plasma membranes with the outer dense plaque and the inner dense plaque overlapped with keratin intermediate-sized filaments, and a ~22 nm-wide gap at the extracellular core domain (intercellular gap) without electron-dense midline. Similar desmosome structure was observed in desmosome produced by KCs transfected with siRNA-Dsg1. In Dsg3-deficient KCs, we observed fine filaments traversing the extracellular core domain of the desmosome with widened intercellular distance.

The traversing distance of the extracellular core domain of desmosomes in KCs transfected with siRNA-NC was 22.5 ± 4.4 nm. In the KCs with knocked-down expression of Dsg1, this

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**FIGURE 2. Effects of PVIgG on keratinocyte morphology and keratin filament framework.** Keratinocyte monolayers were treated with normal human IgG (A and C) or PVIgG (B and D) and then either examined by phase contrast microscopy (A and B) or fixed and stained with anti-pan keratin antibody described under “Materials and Methods” (C and D). Bar, 25 μm. A and B, shrinkage and intercellular dyshesion of KCs treated with PVIgG. Note, the polygonal shape of KCs in the monolayer changed to an irregular one, as the cells detached from each other, and then a spherical one, as cells rounded up, revealing large gaps in the monolayer. The cytosolic volume was measured as described under “Materials and Methods” (see Fig. 3, A and D). C and D, keratin intermediate filament aggregation. Note shrinkage of single KCs accompanied by keratin filament aggregation, which may spontaneously occur in intact monolayers (not shown), and also was seen in the monolayers treated with normal human IgG (C). Treatment with PVIgG, however, induced a massive collapse of intermediate filament framework and cell shrinkage (D). The keratin filament aggregation was measured as described under “Materials and Methods” (see Fig. 3, B and E).
distance was not altered, 19.9 ± 4.4 nm. However, silencing of the DSG3 gene significantly \( (p < 0.0001) \) increased the distance to 31.2 ± 11.8 nm \( (n = 37) \), illustrating the widening of desmosomes.

Transfection with siRNAs that inhibited expression of either Dsg1 and Dsg 3 alone or together in all cases blocked ~50% of p38 MAPK activity \( (p < 0.05) \), but only insignificantly \( (p > 0.05) \) altered PVlgG-dependent raise in Src and EGFRK activities (Fig. 5), indicating that anti-Dsg1 and -Dsg3 antibodies contributed predominantly to the pathobiologic events resulting from activation of p38 MAPK, such as development of keratinocyte apoptosis.

The experiments with the siRNA-Dsg3-transfected KCs that were performed at 0.2 mM of extracellular Ca\(^{2+}\) brought similar results (data not shown). Treatment of transfected cells with normal IgG did not elicit any measurable kinase activities at either concentration of extracellular Ca\(^{2+}\) tested, indicating that the signaling events observed in cells exposed to PVlgG occurred because of ligation of pemphigus autoantigen(s), rather than transfection with siRNAs.

**DISCUSSION**

The results of this study revealed the sequence of major intracellular biochemical events elicited due to PVlgG binding to the keratinocyte plasma membrane and their correlation with morphologic changes characteristic of acantholysis, and also defined relative contribution of both anti-Dsg and non-Dsg autoantibodies. The early signaling events were triggered predominantly by non-Dsg PVlgG, and involved activation of Src and EGFRK associated with collapse of the cytoskeleton. Anti-Dsg1 and Dsg3 antibodies contributed predominantly to the pathobiologic events associated with p38 MAPK activation, a late signaling step linked to keratinocyte apoptosis.

The exclusive roles in pemphigus immunopathogenesis of anti-Dsg 1/3 antibodies, which can cause steric hindrance at the adhesion points of KCs, has been largely reconsidered (19). Recent development of the “signaling” paradigm of the pathobiologic action of autoantibodies in PV has set the stage for well-controlled studies toward identification of the primary signaling pathway(s) responsible for keratinocyte detachment and
death during acantholysis. This study was designed to help answer the central question in elucidation of PV pathogenesis that has immediate clinical implications. To develop adequate anti-acantholytic therapies using kinase inhibitors and pathways modifies, it is essential to determine which pathway: 1) leads directly to a loss of cell-cell adhesion (primary pathway), 2) is being activated secondary to release of autocrine and paracrine cytokines and/or shrinkage and detachment of damaged KCs (secondary pathway), 3) contributes to utilization of altered proteins and organelles (scavenging pathway), and 4) represents a cell defense/survival effort (protective pathway) (20).

Src and RTKs are known to act either independently or in concert in regulation of cell adhesion (21). Activation of EGFR can occur by ligand-dependent and ligand-independent mechanisms (22). It has been recently demonstrated that the specific tyrosine kinase inhibitor AG1478 abrogated PVIgG-induced EGFR autophosphorylation, acantholysis, and keratinocyte death (13). In this study, we demonstrated that the peak of Src activity preceded that of EGFRK. The stimulation of Src is known to cause transactivation of EGFR, with Src binding to the EGFR (23). Because Src is not expressed to the cell surface, one of the self-antigens targeted by PVIgG on the keratinocyte plasma membrane apparently relayed the signal to Src. Thus, engagement of Src/EGFR may be a key step that relays the signal emanating from interaction of PVIgG with various self-antigens on the keratinocyte

**FIGURE 4. Silencing of the DSG1 and DSG3 genes with siRNAs.** Knocking down of the expression of Dsg1 (A) and Dsg 3 (B) was achieved through transfection of KCs with siRNA-Dsg1 and siRNA-Dsg3, respectively, followed by analysis of the monolayers by immunoblotting, indirect immunofluorescence, and electron microscopy (C), as detailed under “Materials and Methods.” A and B, immunoblotting assay of Dsg1 was performed using 10% SDS-PAGE gel and that of Dsg3 using 7.5% SDS-PAGE gel. As an internal control, both assays utilized anti-pan keratin antibody that visualized a double-band at ~46 kDa. Note, transfection with Dsg-specific siRNAs, but not with negative control siRNA (siRNA-NC), eliminated completely both Dsg1 and Dsg3 protein bands in Western blots of keratinocyte proteins and staining of the keratinocyte cell membranes with specific antibodies. Bar, 20 μm. C, electron microscopic analysis of keratinocyte desmosomes revealed that KCs transfected with control siRNA-NC or siRNA-Dsg1 exhibited normal ultrastructural features of desmosomes. In contrast, KCs transfected with siRNA-Dsg3 showed fine filaments traversing the extracellular core domain of the desmosome with widening of the intercellular distance. Bars, 0.2 μm.

**FIGURE 5. Silencing of the DSG1 and DSG3 genes modifies PVIgG effects on kinase activities in KCs.** The activities of Src (A), EGFRK (B), and p38 MAPK (C) were measured in control (untransfected) and experimental KCs transfected with negative control siRNA (siRNA-NC), siRNA-Dsg1, and/or siRNA-Dsg3 at the time points corresponding to the maximal activities of each kinase, i.e. at the 30th, 60th, and 240th min, respectively, after addition of PVIgG. Asterisks indicate significant (p < 0.05) differences compared with untransfected control cells.
plasma membrane structures to the pathway affecting the cytoskeleton and adhesion structures.

Likewise, PVIgG induces Fas-ligand production in KCs (14), and Fas-ligand acts synergistically with PVIgG and TNFα in acantholysis induction (16). Fas signaling leads to apoptosis through the p38 MAPK step (24), which may provide a mechanism for engagement of this stress kinase in the acantholytic pathway. Noteworthy, cell detachment/mechanical stress per se can trigger p38 MAPK, leading to overexpression of Fas ligand (25) and induction of Hsp27 and Hsp70 (26). Berkowitz et al. (4) reported that PVIgG from 2 PV patients induced p38 MAPK and Hsp27 phosphorylation in KCs. However, Frusic-Zlotkin et al. (13) could not confirm phosphorylation of p38 MAPK. This discrepancy has several possible explanations, including patient-to-patient variations in the repertoire of anti-keratinocyte antibodies. To avoid this potential caveat, we in this study used IgG fractions combined from 6 PV patients. Our results showed that the most likely explanation is variability in the timing of activation for different kinases when PVIgG binds to KCs.

Based on the timing of activation of Src, EGFRK and p38 MAPK and effects of inhibitors of specific kinases on the morphologic changes of KCs exposed to PVIgG, it appears that both Src and EGFR predominantly contributed to cell shrinkage and keratin filament aggregation. Because p38 MAPK peaked already after development of first signs of keratinocyte distortion, this kinase could be activated due to both mechanical stress and upstream biochemical stimuli. The alterations within the keratin filament network play a pivotal role in activation of stress kinases (27). Shrinkage of KCs treated with sorbitol has been shown to activate p38 MAPK (28). Mechanical stress of KCs can also activate ERK 1/2, c-Jun, and JNK (27, 29). In the epidermolysis bullosa cellular model, keratin aggregation was reminiscent of those seen in the epidermolysis bullosa simulant cells (31). In contrast to epidermolysis bullosa KCs, in which keratin filaments break, pemphigus KCs develop collapse of the tonofilaments, due to their dissociation from the cadherin/plakin complexes. The electron microscopic studies in pemphigus revealed that detachment of tonofilaments occurs before the desmosomes show any visible alterations (32–35). Although phosphorylation of the adhesion molecules seems to be a major mechanism by which PVIgG induces collapse of the cytoskeleton (36), the role for keratin hyperphosphorylation cannot be excluded (37).

In some recently published experimental works utilizing total PVIgG, the signaling elicited in KCs was interpreted as a result of action of anti-Dsg3 antibodies alone (4, 38, 39). Based on this assumption, Berkowitz et al. (4) coined the term “desmosomal” signaling. Although the idea that cell-cell adhesive structures are involved in signaling is not a new one (40, 41), the ability of desmosomal cadherins to directly elicit intracellular signaling has never been demonstrated directly. This, however, may result from internalization and processing of encycysed adhesion molecules triggered by the autoantibody assault. In addition to Dsg 1 and 3, PV patients develop antibodies to other adhesion molecules, i.e. Dsg 2 and 4, desmocollins 1, 2, and 3, desmoplakins I and II, plakoglobin, collagen XVII, and cell-membrane receptors, such as nicotinic acetylcholine receptor subunits α3 and α9, pemphxin (a.k.a. annexin 31), some other annexins, FceRlα, and thyroperoxidase (reviewed in Ref. 42). Ligation of the receptor antigens by PVIgG is more likely to trigger disassembly of the adhesion units.

To accurately detect signaling elicited solely by anti-Dsg antibodies from that evoked because of ligation of non-Dsg targets, we used the RNA interference technology. The siRNA-Dsg1 and siRNA-Dsg3 predesigned by Dharmaco proved to be very efficient silencers of gene expression of Dsg1 and Dsg3, respectively. We utilized the 20% concentration of extracellular calcium to match closely the experimental conditions employed to test PVIgG effects at high, 1.2 to 1.8 mM, Ca2+ in culture medium (e.g. Refs. 13, 39, 43). To match experimental conditions used by Berkowitz et al. (4), at which Dsg 1 was not expressed by KCs, and those reported by Calkins et al. (38), we also performed a series of experiments with siRNA-Dsg3 transfected KCs at low, 0.2 mM, extracellular calcium. At low calcium, the results were similar to those obtained at high calcium, when Dsg 1 was present, indicating that inhibition of signaling did not involve Dsg1, thus further illustrating the accuracy of our approach to silence selectively the DSG3 gene.

The elimination of Dsg targets of PVIgG only partially abolished signaling in KCs. This was not surprising, because previous studies convincingly demonstrated that PVIgG signaling could not result exclusively from anti-Dsg 1/3 antibodies. Berkowitz et al. (4) demonstrated that elimination of anti-Dsg3 antibody from the PVIgG fraction that did not contain anti-Dsg1 antibody, decreased only partially the effects of PVIgG on protein phosphorylation in cultured KCs. Had these proteins been phosphorylated as a result of exclusive signaling downstream of Dsg3, all activity should be eliminated. Or, treatment of DJM-1 cells with the anti-mouse Dsg3 acantholytic monoclonal antibody AK23 augmented by ~30% the phosphorylation level of Dsg3 (44). In marked contrast, stimulation of DJM-1 cells with the whole PVIgG fraction increased Dsg3 phosphorylation level by ~300% (3), which is ten times more compared with the effect of anti-Dsg3 antibody alone. Lack of appreciable effects of double transfection with siRNA-Dsg1 and siRNA-Dsg3 on kinase inhibition suggests that Dsg-dependent signaling is a secondary or tertiary pathway mediating processing and utilization of internalized Dsg molecules rather than a primary downstream signaling emanating from the cell membrane.

It is well documented that the mechanism of Dsg3 dissociation from the cell membrane adhesion structures with its subsequent intracellular processing involves serine phosphorylation (45, 46). This leads to formation of Dsg3-depleted desmosomes in cultured KCs (47). The desmosomes formed by KCs transfected with siRNA-Dsg3 were similarly distorted, as judged from significantly (p < 0.0001) increased intradesmosomal space. Silencing of the DSG1 gene, however, did not cause desmosomal widening, in keeping with the "desmoglein
compensation” hypothesis (48). Lack of obvious ultrastructural alterations in desmosomes formed by Dsg1-deficient KCs suggests that the role of this desmosomal cadherin in desmosome formation by cultured KCs is less important compared with Dsg3. In addition to widening the extracellular core domain, silencing of the DSG3 gene generated another interesting alteration in the desmosome structure. Dsg3-deficient desmosomes featured traversing filaments bridging neighboring cells. This may be caused by a reduced density of the extracellular core domain and/or weakening of the binding force.

In conclusion, while the Dsg 1/3 targets of pemphigus autoimmunity appeared to be important for initiation of apoptosis, the early events leading to acantholysis in PV patients are apparently triggered by non-Dsg antibodies. The synergy between anti-Dsg and non-Dsg antibodies required for acantholysis was predicted by the Multiple Hit hypothesis (10). The chronological scheme of signaling and pathobiologic events in KCs exposed to PVIgG is shown in Fig. 6. Future studies of the signaling pathways mediating acantholysis in individual PV patients should be directed to identification of the membrane proteins (receptors) triggering signaling along the primary pathways.

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FIGURE 6. Novel signaling pathways in PV. The chronological scheme of signaling steps and their correlation with the major pathobiologic events in KCs exposed to PVIgG. NDGs, non-desmoglein antigens.

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