In pemphigus vulgaris and pemphigus foliaceus (PF), autoantibodies against desmoglein-3 and desmoglein-1 induce epidermal cell detachment (acantholysis) and blistering. Activation of keratinocyte intracellular signaling pathways is emerging as an important component of pemphigus IgG-mediated acantholysis. We previously reported activation of p38 mitogen-activated protein kinase (MAPK) in response to pathogenic pemphigus vulgaris and PF IgG. Inhibition of p38MAPK blocked pemphigus IgG-induced cytoskeletal reorganization in tissue culture and blistering in pemphigus mouse models. We now extend these observations by demonstrating two peaks of p38MAPK activation in pemphigus tissue culture and mouse models. Administration of the p38MAPK inhibitor SB202190 before PF IgG injection blocked both peaks of p38MAPK phosphorylation and blister formation, consistent with our previous findings; however, administration of the inhibitor 4 h after PF IgG injection blocked only the later peak of p38MAPK activation but failed to block blistering. Examination of the temporal relationship of p38MAPK phosphorylation and apoptosis showed that apoptosis occurs at or after the second peak of p38MAPK activation. The time course of p38MAPK activation and apoptotic markers, as well as the ability of inhibitors of p38MAPK to block activation of the proapoptotic proteinase caspase–3, suggest that activation of apoptosis is downstream to, and a consequence of, p38MAPK activation in pemphigus acantholysis. Furthermore, these observations suggest that the earlier peak of p38MAPK activation is part of the mechanism leading to acantholysis, whereas the later peak of p38MAPK and apoptosis may not be essential for acantholysis.

Pemphigus is a group of related autoimmune diseases characterized by blistering in the skin. The histologic hallmark of these disorders is termed acantholysis, which describes the loss of adhesion between adjacent epithelial cells. The two major variants are pemphigus foliaceus (PF) and pemphigus vulgaris (PV). In PF, acantholysis is observed beneath the stratum corneum and within the granular layer of epidermal epithelia, whereas in PV, blister formation occurs above the basal layer of epidermal epithelia and mucosal epithelium. Passive transfer of IgG purified from both PV and PF patient sera reproduces the clinical, histological, and immunologic features of the human diseases, demonstrating that these autoantibodies are pathogenic (1, 2). In PF, autoantibodies target the desmosomal cadherin desmoglein (dsg) 1, whereas in PV, autoantibodies initially target dsg3 (3, 4) in mucosal PV and then subsequently target both dsg1 and dsg3 in mucocutaneous PV (5–7).

The mechanism by which pemphigus autoantibodies induce blistering has been under investigation. Work from a number of laboratories has suggested that activation of intracellular events is induced by binding of PF or PV IgG to dsg1 and dsg3, respectively (8–14). Previously, we have reported that PV IgG activate p38MAPK and heat shock protein (HSP) 27 in human keratinocyte tissue cultures (15). Significantly, p38MAPK inhibitors blocked PV IgG-induced keratin filament retraction and actin reorganization in human keratinocyte tissue cultures. Furthermore, we have demonstrated that both PV and PF IgG induce phosphorylation of p38MAPK and HSP25, the murine HSP27 homologue, in mouse models and that inhibitors of p38MAPK block blistering in both the PV (16) and the PF (17) passive transfer mouse models. Additionally, in human skin biopsies from both PV and PF patients, phosphorylation of p38MAPK and HSP27 has been observed (18). Collectively, these observations suggest that activation of p38MAPK within the target keratinocyte contributes directly to loss of cell-cell adhesion induced by pemphigus autoantibodies.

Both p38MAPK and HSP27 have been implicated in the regulation of the intermediate filament and actin cytoskeletons (19–25): the ability of p38MAPK inhibitors to block both pemphigus IgG-activated cytoskeletal reorganization and pemphigus IgG-activated blistering suggests that p38MAPK may be acting upstream of the cytoskeleton in the mechanism of acantholysis; however, p38MAPK signaling has been implicated in other cellular responses (reviewed in Ref. 26). For example, there is abundant evidence for p38MAPK involvement in apoptosis (27–29); however, the role of p38MAPK in apoptosis seems to be cell type- and stimulus-dependent. Although p38MAPK signaling promotes cell death in some cell lines, it also functions to enhance survival, growth, and differentiation.
in other cell lines (30). Several reports describe increased apoptosis of keratinocytes in pemphigus (31–35); however, the relationship between PV IgG-mediated p38MAPK signaling, the induction of apoptosis, and the relationship of apoptosis to blistering has not been defined. This study was undertaken to investigate the relationship between p38MAPK activation, apoptosis, and acantholysis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit polyclonal anti-p38MAPK antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), monoclonal anti-phospho-p38MAPK antibodies and anti-phospho-HSP 27 were from Cell Signaling Technology (Beverly, MA), and rabbit polyclonal anti-HSP25 antibodies were from StressGen (Victoria, BC, Canada). The p38MAPK inhibitor SB 202190 was from Calbiochem. A rabbit monoclonal antibody to cleaved caspase 3, a monoclonal antibody to human cleaved poly(ADP-ribose) polymerase (PARP), and a polyclonal antibody to murine cleaved PARP were from Cell Signaling Technology.

**IgG Preparation**—PV IgG and PF IgG were purified form PV and PF patient sera respectively by ammonium sulfate precipitation followed by affinity chromatography on protein G (HiTrap; Amersham Biosciences) as described previously (15). IgG fractions were dialedyed against PBS, sterile filtered, and purity-confirmed by SDS-PAGE; activity was assayed by indirect IF and enzyme-linked immunosorbent assay. Data from *in vivo* passive transfer mouse experiments utilized IgG purified from a single PF patient whose serum was available in sufficient quantities to carry out the described studies. The activity of this serum was determined by indirect IF on sectioned normal human skin with a titer of 1: 2560. Dsg3, not dsg1, is the predominant desmosomal cadherin in primary human keratinocyte monolayer tissue cultures; therefore, PV IgG was used for tissue culture experiments. The activity of this PV IgG was 1:640 by testing with indirect IF on sectioned monkey esophagus. Control IgG, which showed no activity by indirect IF, was prepared in parallel from normal human sera.

**Passive Transfer Mouse Model**—Breeding pairs of C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at the Division of Laboratory Animal Medicine Facility of the University of North Carolina in accordance with International Animal Care and Use Committee protocols. Neonatal mice, 24–36 h old and weighing 1.4–1.6 g, were used for passive transfer experiments. Neonates were injected intradermally with a sterile solution of either PF IgG or control IgG at a dose of 0.1 mg of IgG per gram of body weight in a total volume of 50 μl of PBS as described previously (16).

For time course studies of p38MAPK activation, samples were collected at 1, 2, 4, 6, 8, and 21 h after injection. For detection of apoptotic activity, additional time points at 24, 30, 48, and 50 h were collected. At the specified time point, mice were examined clinically and sacrificed. Skin biopsies were obtained at the IgG injection site and divided as follows. One-quarter was fixed and stained with hematoxylin/eosin for routine histological examination, and one-fourth was rapidly frozen and used for immunofluorescence staining; the remaining half was utilized for biochemical studies. Protein extracts were prepared from skin biopsies by glass pestle homogenization in Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 μM pepstatin, 100 μM leupeptin, 10 μM E-64, 1 mM phenylmethylsulfonyl fluoride, 500 μM Na3VO4, 50 mM NaF, 0.5 μM okadaic acid).

**Keratinocyte Cell Culture**—Normal primary human keratinocytes (from Cascade Biologics, Portland, OR) grown in Epilife medium containing 60 μM CaCl2 and human keratinocyte growth supplement were passaged and expanded as described (15, 36). Third passage keratinocytes were grown to 80–90% confluence when PV IgG, control IgG, or an equivalent volume of buffer was added and subsequently incubated for various times from 30 min to 10 h in the presence of 0.5 mM CaCl2. Cells were washed three times with ice-cold PBS and were harvested in isoelectric focusing lysis buffer (10 mM pepstatin, 100 μM leupeptin, 10 μM E-64, 1 mM phenylmethylsulfonyl fluoride, 500 μM Na3VO4, 50 mM NaF, 0.5 μM okadaic acid).

**Gel Electrophoresis**—Samples were equally loaded on and separated by 10% SDS-PAGE. Gels were transferred to polyvinylidene difluoride (Millipore, Billerica, MA) membranes and probed by immunoblot for proteins of interest. Western blots were developed by enhanced chemiluminescence (ECL) reaction (Amersham Biosciences). The signal intensity was quantified by scanning chemiluminescence on a GeneGnome HR scanner (Syngene, Frederick, MD) using GeneSnap software.

**Immunofluorescence and Confocal Microscopy of Murine Skin**—Frozen sections 7 μm in thickness were cut freshly on a Cryostat and transferred onto gelatin-coated glass slides. The sections were fixed and permeabilized in –20 °C methanol for 15 min and blocked in 5% normal goat serum for 1 h at room temperature. After rinsing, sections were incubated overnight at 4 °C in PBS with 0.1% Triton-100, 1% normal goat serum, and one of the following antibodies at the indicated dilutions: 1) 1:200 anti-p38MAPK antibody or 2) 1:100 anti-phospho-p38MAPK antibody. Primary antibodies were detected by exposure for 1 h to Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:400. Fluorescence patterns were examined using a Leica inverted DM IRB microscope and by confocal microscopy using a Leica SP2 AOBS confocal microscope with an excitation wavelength of 543 nm and capture at 580 nm using a ×63 objective with numerical aperture of 1.4.

**Immunofluorescence Microscopy of Human Keratinocytes**—Normal human keratinocytes were grown on 22-mm2 glass coverslips and then treated with PV IgG, control IgG, or an equivalent volume of serum-free medium, at 37 °C. Cells were fixed in 3.7% paraformaldehyde for 5 min at room temperature and permeabilized in 0.25% Triton X-100 for 10 min. Immunofluorescence analysis was performed using the mouse monoclonal anti-cleaved PARP antibodies at a 1:800 dilution followed by Cy3-conjugated secondary antibodies at a dilution of 1:200. Actin was stained with Alexa Fluor 488 phalloidin (Invitrogen) at a dilution of 1:60. After the final wash, the coverslips were mounted on slides with ProLong gold antifade reagent with 4’,6-diamidino-2-phenylindole (Invitrogen). For a positive control, cells were treated for 3 h with 0.3 μM staurosporine to induce apoptosis; the negative control consisted of omitting the primary antibodies. Images were obtained on a

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Leica DM IRB inverted microscope with a high sensitivity CCD camera and PCI imaging software (Version 6.5, Hamamatsu Corp., Sewickley, PA).

In a second set of experiments, primary human keratinocytes were grown on glass coverslips and either (i) pretreated with DMSO as a vehicle control or (ii) pretreated with SB202190 for 2 h prior to the addition of PV IgG. A third group of cells was treated with PV IgG and then SB202190 3 h after the addition of PV IgG. Six hours after the addition of PV IgG, cells were washed with ice-cold PBS three times and then fixed in 3.7% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked for 1 h at room temperature with 5% goat serum in PBS followed by an overnight incubation at 4 °C with mouse anti-human cytokeratin 5/8 antibodies (BD Biosciences). Samples were washed three times and probed with Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Images were captured with a Leica confocal microscope using the x63 objective (x2 zoom).

Terminal Deoxynucleotidyl Transferase-mediated Biotinylated UTP Nick End Labeling (TUNEL) Assay—A TUNEL assay kit was obtained from R&D Systems (Minneapolis, MN) and used on skin cryosections and freshly prepared human keratinocytes per the manufacturer’s instructions. Briefly, cell membranes were permeabilized with proteinase K for tissue sections or with cytonin for cultured cells. Endogenous peroxidase activity was quenched using 3% H2O2 for 5 min. The biotinylor conjugate was blocked with cytonin for cultured cells. Endogenous peroxidase activity was quenched using 3% H2O2 for 5 min. The biotinylated nucleotides incorporated into the DNA fragments by terminal deoxynucleotidyltransferase were detected using streptavidin-horseradish peroxidase conjugate followed by the substrate diaminobenzidine. Sections were counterstained with Methyl Green. Controls consisted of omitting the terminal deoxynucleotidyltransferase enzyme form the labeling solution (negative control) or pretreating the sections for 30 min with DNase to induce DNA strand breaks (positive control). The negative control gave no staining, whereas the positive control stained all nuclei in all layers of the epidermis. TUNEL staining at different time points was quantified by counting the TUNEL-positive cells in each sample. For each slide, three fields were randomly chosen using a defined rectangular area (x20 objective) for the average, and the assays were performed in triplicate. Statistical analyses were performed on the data with SPSS software (version 11.0, SPSS Inc., Chicago, IL). Values were expressed as mean ± S.E. Where indicated, data were compared using the Student’s t test. Statistical significance was defined as p < 0.05.

In Vivo Inhibitor Studies—For in vivo inhibitor studies, mice were assigned to each of the following three groups. In group A, mice were treated with only PV IgG as described above. In group B, we followed the established split dose protocol (16). Mice were preinjected with 6.25 μg of SB202190 in 50 μl intradermally and then reinjected intradermally with 6.25 μg of SB202190 plus PV IgG in 50 μl (total of 12.5 μg of SB202190). In group C, mice were injected intradermally with PV IgG first, and 4 h later, they received an injection of 12.5 μg of SB202190. All mice received the same dose of PV IgG (0.1 mg/g of body weight). For immunoblots, signal intensity from the ECL reaction for each band was quantified with a GeneGnome HR scanner (Syngene) using GeneSnap software (n = 3, S.D. shown by error bars in Fig. 6). The phospho-p38MAPK signal was normalized to total p38MAPK for each sample. Statistical analyses were performed on the data with SPSS software (version 11.0, SPSS Inc.). Results from three independent experiments were expressed as mean ± S.D. Where indicated, data were compared using the Student’s t test. Statistical significance was defined as p < 0.05.

RESULTS

Pemphigus IgG Induces Two Peaks of p38 MAPK Activation in Vivo—To examine the relationship of p38MAPK activation to apoptosis, time course studies were performed in the PF passive transfer mouse model. Neonatal C57BL/6j mice were injected intradermally with PV IgG as described previously (16, 17). After various time points, mice were examined clinically, and skin biopsies were obtained for histological, immunological, and biochemical analyses. Under the conditions used for these experiments, acantholysis was not observed within the first 8 h after injection of PV IgG (Fig. 1A). At later time points, blister formation was readily apparent both clinically and in histologic sections. Antibodies to phospho-p38MAPK were used to stain frozen sections from skin biopsies. Within 2–4 h after receiving PV IgG, increased phospho-p38MAPK activity could be detected in the epidermis of PF IgG-treated mice by immunofluorescent staining of frozen sections (Fig. 1B). Staining for phospho-p38MAPK was diminished at 6 h relative to the 4-h time point but increased again at 8 h and subsequent time points. To further analyze the in vivo time course of p38MAPK phosphorylation, extracts from skin biopsies of the above PF IgG-treated mice were prepared, separated by SDS-PAGE, and probed by immunoblot for phospho-p38MAPK and total-p38MAPK activity (Fig. 2). Confirming the results observed by IF staining, two peaks of phospho-p38MAPK activity were detected: (i) an early peak at 2–4 h after PV IgG injection and (ii) a later peak at 8 h and subsequent time points.
Pemphigus IgG Induces Two Peaks of p38MAPK Activation in Keratinocyte Monolayer Cultures—Pemphigus passive transfer mice more closely model in vivo biology when compared with tissue culture systems; however, it can be difficult to control for rates of absorption of PF IgG among the test animals. To ensure that the biphasic nature of p38MAPK activity was not due to variability in PF IgG absorption rates among the test animals, we next examined the time course of phospho-p38MAPK and total p38MAPK. Normal human keratinocytes were incubated with PV IgG for various times from 0 to 10 h; extracts were prepared, separated by SDS-PAGE, and probed with antibodies to phospho-p38MAPK and total p38MAPK. Similar to the in vivo experiments with PF IgG, biphasic activation of p38MAPK is observed in keratinocytes treated with PV IgG.

**FIGURE 2.** Time course of p38MAPK phosphorylation in cultured normal human keratinocytes treated with PV IgG. Normal human keratinocytes cultured to 80–90% confluence were treated with PV IgG (3.0 mg/ml) for the indicated times, and extracts (15 μg of protein/lane) were separated by 10% SDS-PAGE. Blots were probed with antibodies to phospho-p38MAPK (P-P38MAPK), stripped, and then reprobed with antibodies to p38MAPK. Similar to the in vivo experiments with PF IgG, biphasic activation of p38MAPK is observed in keratinocytes treated with PV IgG.

**FIGURE 3.** Time course of p38MAPK phosphorylation in mice treated with PF IgG. Two peaks of phospho-p38MAPK (P-P38MAPK) activity are detected by Western blot of skin extracts from PF IgG-treated mice. The first peak of p38MAPK activity is observed at 4 h, and the second peak is observed at 8–21 h. Neonatal C57BL/6J mice were injected intradermally with PF IgG (0.1 mg/g of body weight) or control (Con) IgG for the indicated times. Western blots of three independent time course experiments. Extracts (20 μg of protein/lane) from skin biopsies of mice were separated by SDS-PAGE, transferred to polyvinylidene difluoride, and immunoblotted with antibodies to phospho-p38MAPK and total p38MAPK. Signal intensity from the ECL reaction for each band was quantified with a GeneGnome HR scanner and GeneSnap software. Each time course (TC) was individually plotted. Biphasic activation of p38MAPK was induced by PF IgG in vivo.

**FIGURE 4.** Increased apoptotic activity is observed subsequent to activation of p38MAPK in PF passive transfer mice. Neonatal C57BL/6J mice were injected intradermally with PF IgG for various times from 1 to 48 h, and skin biopsies were obtained for biochemical analysis and for TUNEL A, immunoblotting of murine skin biopsy extracts (50 μg/lane) using antibodies to cleaved PARP and cleaved caspase 3, two markers of apoptosis. B, marked increases in TUNEL-positive keratinocytes were not observed until mice were treated with PF IgG for periods exceeding 21 h. Controls consisted of omitting the terminal deoxynucleotidyltransferase enzyme from the labeling solution (negative control (Negative)) or pretreating sections for 30 min with DNase to induce DNA strand breaks (positive control (Positive)).

**Pemphigus IgG Induces Two Peaks of p38MAPK Activation in Keratinocyte Monolayer Cultures**—Pemphigus passive transfer mice more closely model in vivo biology when compared with tissue culture systems; however, it can be difficult to control for rates of absorption of PF IgG among the test animals. To ensure that the biphasic nature of p38MAPK activity was not due to variability in PF IgG absorption rates among the test animals, we next examined the time course of phospho-p38MAPK activity in primary human keratinocyte cultures. Dsg3, not dsg1, is the predominant desmosomal desmoglein in primary human keratinocyte monolayer cultures; therefore, purified PV IgG was used for the tissue culture experiments. Normal human keratinocytes were incubated with PV IgG for various times from 0 to 10 h; extracts were prepared, separated by SDS-PAGE, and probed by immunoblot for phospho-p38MAPK and total p38MAPK. Similar to the above in vivo experiments, two peaks of phospho-p38MAPK activity were observed in normal human keratinocytes treated with PV IgG (Fig. 3): (i) an early peak observed within 30 min of the addition of PV IgG to cultures and (ii) a second later peak seen at 6–10 h after the addition of PV IgG.

**Apoptosis Is a Late Event, Occurring after the Second Peak of p38MAPK Activity**—Next, both PF IgG-treated mice and PV IgG-treated keratinocyte cultures were examined for markers of apoptotic activity. Extracts prepared from skin biopsies of PF IgG-treated mice were separated by SDS-PAGE and probed by immunoblot for two markers of apoptotic activity, cleaved PARP and cleaved caspase 3. Positive controls consisted of omitting the terminal deoxynucleotidyltransferase enzyme from the labeling solution (negative control (Negative)) or pretreating sections for 30 min with DNase to induce DNA strand breaks (positive control (Positive)).
PARP and cleaved caspase 3. Cleaved PARP and cleaved caspase 3 were not detected at 0–24 h after PF IgG injection but were readily detected at the late time points of 30 and 48 h (Fig. 4). As an additional probe for apoptosis, skin biopsies from PF IgG-treated mice were subjected to TUNEL staining (Fig. 4). TUNEL staining was not detected at early time points in vivo but could be detected at later time points in biopsies where acantholysis was already present.

Next, cleaved nuclear PARP and TUNEL staining were used to examine the temporal relationship of p38MAPK phosphorylation to apoptotic activity in PV IgG-treated normal human keratinocyte cultures (Fig. 5). Normal human keratinocyte cultures were treated with control IgG or pathogenic PV IgG or mock-incubated with buffer for various times from 0 to 10 h. Cells were fixed and stained with a monoclonal antibody that recognizes cleaved PARP (Fig. 5A). No cleaved PARP was detected in cells treated for 10 h with either buffer or control IgG. In contrast, time-dependent detection of cleaved PARP was observed in cells treated with PV IgG. No cleaved PARP was detected in cells treated with PV IgG for 1 h. A signal for cleaved PARP became detectable in cells treated with PV IgG for 6 h but was more readily apparent at 10 h. Similar results were observed when cultures were examined by TUNEL stain (Fig. 5B and C). Similar numbers of TUNEL-positive cells were observed in keratinocytes treated with control or PV IgG for 1 and 6 h. After 10 h, PV IgG-treated cells demonstrated more TUNEL-positive cells (five per high powered field) when compared with control IgG-treated cells (two per high powered field), although the number of TUNEL-positive cells observed after 10 h exposure to PV IgG was still relatively small.

Inhibition of the First, but Not Second, Peak of p38MAPK Activation Blocks Blistering in Vivo—Inhibitor studies were performed next to determine the association of each peak of p38MAPK activity with blister formation. Inhibition of the first peak of p38MAPK activation blocked blistering, whereas inhibition of the later second peak of p38MAPK activation failed to block blistering in vivo (Fig. 6 and Table 1). Neonatal mice were divided into three groups. The first group received PF IgG alone. The second group was pretreated with the p38MAPK inhibitor SB202190 before injection with PF IgG to block both the first and the second peaks of p38MAPK activation. The third group initially received PF IgG, and then 4 h later, was treated with SB202190 to block the second, but not the first, peak of p38MAPK activity. Mice injected with PF IgG alone developed blisters (Fig. 6A); using phospho-p38MAPK-specific antibodies, direct immunofluorescence of skin biopsies (Fig. 6B) and immunoblot of skin biopsy extracts (Fig. 6C) demonstrated phospho-p38MAPK activity in the epidermis of PV IgG-treated mice. Consistent with previous reports, PF IgG-induced p38MAPK phosphorylation and blister formation were blocked in mice pretreated with the p38MAPK inhibitor SB202190. In contrast, blister for-
In vivo: By treating neonatal mice with the p38MAPK inhibitor SB202190 2 h prior to the addition of PV IgG to block both the first and the second peaks of p38MAPK activity, blistering was observed. In contrast, treating keratinocytes with the p38MAPK inhibitor after the first peak of p38MAPK activity, but prior to the second peak, failed to block PV IgG-triggered cytokeratin retraction.

DISCUSSION

In previous studies, both in tissue culture and in vivo, we have demonstrated that both pathogenic PV and PF IgG induced blistering via p38MAPK-dependent signaling within the target keratinocytes (15–18). The activation of p38MAPK by pemphigus IgG has been confirmed by other investigators (10, 37). p38MAPK is a ubiquitous kinase that is involved in a variety of cellular processes, including regulation of the cytoskeleton, stress response signaling, and activation of apoptotic pathways. In our previous work (15), we had demonstrated that activation of p38MAPK was a rapid response, occurring within 30 min of the addition of PV IgG to normal human keratinocyte cultures. In these cultures, activation of p38MAPK preceded changes in the cytoskeleton and loss of cell-cell adhesion. Notably, pretreating keratinocyte cultures with p38MAPK inhibitors blocked PV IgG-induced keratin intermediate filament collapse and actin reorganization (15).
Biphasic p38 Activation in Pemphigus

TABLE 1
Inhibition of histological disease
A total of 50 mice were injected. The p38MAPK inhibitor SB202190 blocks blistering when injected 2 h before PF IgG but fails to block blistering when injected 4 h after PF IgG.

|                              | Numbers of mice with blisters | Numbers of mice without blisters |
|------------------------------|-------------------------------|----------------------------------|
| PF IgG (n = 17)              | 17                            | 0                                |
| p38 inhibitor (SB202190) 2 h | 1                             | 15                               |
| p38 inhibitor (SB202190) 4 h | 16                            | 1                                |

PV IgG

FIGURE 7. Inhibition of the first peak of p38MAPK activity blocked PV IgG-triggered cytokeratin retraction, whereas inhibition of the second peak did not. Primary human keratinocytes were treated with (A) vehicle (DMSO) control, (B) vehicle control 2 h prior to PV IgG, (C) SB202190 2 h prior to PV IgG treatment, or (D) SB202190 3 h after PV IgG treatment. Six hours after the addition of PV IgG, cells were fixed and stained for cytokeratin 5/8 and examined by confocal microscopy. Increased cytokeratin retraction is observed over time in keratinocytes treated with PV IgG (B). In cells pretreated with the p38MAPK inhibitor (prior to the first peak), cytokeratin retraction is blocked. In contrast, treating keratinocytes with the p38MAPK inhibitor after the first peak of p38 activity, but prior to the second peak, failed to block PV IgG-triggered cytokeratin retraction (D).

FIGURE 8. Model for the temporal relationship of pemphigus IgG-mediated activation of p38MAPK to blistering and apoptosis. Two peaks of p38MAPK phosphorylation occur subsequent to treatment with pemphigus IgG. Inhibiting the first, but not second, peak of p38MAPK activity blocks blistering. Markers of apoptosis, including caspase 3 cleavage, PARP cleavage, and TUNEL-positive staining, occur subsequent to the second peak of p38MAPK phosphorylation. Blocking the first peak of p38MAPK phosphorylation blocks blistering, indicating a role for the first peak of p38MAPK activation in loss of cell-cell adhesion. In contrast, blocking this second peak of p38MAPK phosphorylation fails to block blistering but blocks increases in apoptotic markers (e.g. caspase 3 cleavage). The second peak of p38MAPK phosphorylation is not part of the mechanism of acantholysis but may represent stress response signaling secondary to acantholysis.

Furthermore, in both PV and PF passive transfer mouse models, (i) p38MAPK and HSP 25 phosphorylation were observed in the skin and (ii) pretreating mice with p38MAPK inhibitors blocked blister induction by PV (16) and PF IgG (17).

In the present investigation, we have extended our studies on p38MAPK signaling using both the passive transfer PF mouse model and the in vitro PV keratinocyte culture system. In primary human keratinocytes, des3 is the predominant desmosomal cadherin; therefore, PV IgG was utilized to examine the time course of p38MAPK activation in primary human keratinocyte tissue culture. In vivo time course studies require large amounts of a single pathogenic IgG. To undertake the studies, we utilized a PF IgG, which was available to us in sufficiently large quantities to perform the necessary studies. We found that in mice, PF IgG triggers a biphasic activation of p38MAPK, producing an early peak at 2–4 h of the intradermal injection of the pathogenic autoantibody. The second peak appears at 8 h, and it is sustained until 30 h after IgG injection. Similarly, PV IgG in the cell culture system also induces a biphasic activation of p38MAPK, with the first activation at 30 min to 2.5 h and the second activation after 6 h of the introduction of the pathogenic autoantibody.

The temporal relationship of the first and second peaks of p38MAPK activation to blistering was tested in vivo and in vitro. By timing the administration of the inhibitor, either both peaks or the second peak of p38MAPK activation could be selectively blocked. It is likely that the first peak triggers a series of intracellular events that leads to acantholysis because administration of a p38MAPK inhibitor prior to the injection of IgG blocked acantholysis and disease in mice and PV IgG-induced cytokeratin retraction in keratinocyte cultures, whereas administration of the inhibitor to selectively block the second peak of p38MAPK phosphorylation failed to block acantholysis in vivo and cytokeratin retraction in vitro (Fig. 8).

In prior studies, we had demonstrated that PV IgG-induced acantholysis is mediated by activation of p38MAPK with subsequent phosphorylation of HSP27, actin reorganization, and cytokeratin retraction. Both p38MAPK and HSP27 regulate actin and intermediate filaments (19–25), suggesting a mechanistic role for p38MAPK and HSP27 in pemphigus-induced cytoskeletal alterations and acantholysis. In this current study, we have extended these initial observations to demonstrate two peaks of p38MAPK phosphorylation in response to pemphigus IgG. We interpret the data to indicate that the first peak of p38MAPK is part of the mechanism by which pemphigus IgG induces acantholysis, whereas the second peak of p38MAPK activation is not required for cytoskeletal reorganization nor blistering.
One of the potential mechanisms that could be set in motion following p38MAPK activation is the induction of proapoptotic pathways. A number of investigators have observed the induction of keratinocyte apoptosis by PV IgG in tissue culture (32–34) or in skin biopsy specimens from PV or PF patients (31), (i) raising the possibility that apoptosis is part of the mechanism by which pemphigus IgG induces blistering and (ii) implicating a role for p38MAPK in the induction of apoptosis. In the time course studies in the PV tissue culture model, markers of apoptosis including PARP cleavage and TUNEL stain were not observed at 1 h but became detectable at 6 h and more readily detectable at 10 h, correlating with the appearance of the second peak of p38MAPK activation. In the mouse model of PF, TUNEL staining is negative at 2 and 6 h after IgG injection but became apparent at 21 h when disease developed. However, cleaved PARP and caspase 3 were not detected until 30 h after IgG injection. Although we cannot completely rule out the possibility that the failure to detect the cleaved PARP or caspase 3 by Western blot at the earlier time points before 30 h was due to the sensitivity of the technique used, these data suggest that apoptosis occurs simultaneously or subsequently to the second peak of p38MAPK activity and acantholysis. Importantly, inhibition of the second peak of p38MAPK activation also blocked caspase 3 cleavage, suggesting that activation of this proapoptotic proteinase was dependent upon the second peak of p38MAPK activity.

Although our data suggest that the activation of proapoptotic pathways is a late event and may not be essential for blistering in pemphigus, the data do not exclude the possibility that activation of components of apoptotic signaling, including caspase family member proteinases, could augment the blistering response as downstream effects of p38MAPK activation. This hypothesis is supported by the observation that caspase inhibitors block pemphigus IgG-induced acantholysis in the passive transfer mouse model. The potential for caspase-dependent proteolysis to augment the pathogenic response in pemphigus is supported by the observation that components of the desmosome including dsg3, dsg1, plakoglobin, and desmoplakin (38, 39), as well as intermediate filaments (40, 41), have all been shown to undergo caspase-dependent cleavage. In A431 epithelial cells induced to undergo apoptosis by UV exposure, caspase 3-dependent and matrix metalloproteinase (MMP)-dependent cleavage of dsg1 has been observed (39). In this system, caspase 3 cleavage occurred within the cytoplasmic tail of dsg1, whereas MMP-dependent cleavage resulted in shedding of a 75-kDa fragment of the dsg1 ectodomain. Interestingly, UV-induced MMP cleavage of the dsg1 ectodomain was not only inhibited by the MMP inhibitor TAPI-0 but also by the caspase inhibitor ZVAD-fmk (39). In addition, the pathophysiology of staphylococcal scalded skin syndrome demonstrates that proteolysis of desmosome components can contribute to destabilization of desmosomes (42). Direct proteolysis of the dsg1 ectodomain induces loss of cell-cell adhesion in staphylococcal scalded skin syndrome, which, like PF, is characterized histologically by subcorneal blister formation. In staphylococcal scalded skin syndrome, exfoliative toxin A, a serine proteinase of Staphylococcus aureus, causes blistering (43). Thus, blocking caspase-dependent dsg degradation may augment cell-cell adhesion and decrease keratinocyte sensitivity to the acantholytic effects of pathogenic pemphigus IgG.

The presence or absence of adhesion represents a major biologic shift requiring coordination among various biological processes, including those regulating adhesion, migration, proliferation, differentiation, and cell death. For example, contact inhibition, in which proliferation is suppressed as cells contact and form adhesive interactions with one another, demonstrates the association between adhesion and proliferation. It is likely that changes in desmosome adhesion and signaling initiated by pemphigus IgG contribute to the regulation of these biological transitions. Whether a cell differentiates, migrates, proliferates, or dies when desmosome adhesion is disrupted is likely to be context-dependent; that is, additional non-desmosome-mediated signaling processes are likely to influence which of these fates ensues. Although we are beginning to identify molecular components of the mechanism by which pemphigus autoantibodies induce loss of cell-cell adhesion, the biology of acantholysis is likely to be complex. More work will need to be done to develop an integrated understanding of these transitions. Clearly, the ability of pemphigus IgG to induce changes in cell adhesion in vitro and in vivo provides not only a useful reagent to initiate adhesive transitions at the desmosome but also a physiologically relevant human disease-specific model system in which to further probe the molecular mechanism by which these processes proceed.

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