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Involvement of the Apoptotic Mechanism in Pemphigus Foliaceus Autoimmune Injury of the Skin

Ning Li, Minglang Zhao, Jinzhao Wang, Zhi Liu, and Luis A. Diaz

Pemphigus foliaceus (PF) and pemphigus vulgaris (PV) are the two classical forms of pemphigus characterized by detachment of epidermal cells known as acantholysis and anti-epidermal autoantibodies (1, 2). Whereas PF displays intra-epidermal blisters at the granular layer and autoantibodies to the desmosomal glycoproteins desmoglein (Dsg) 1, PV exhibits intra-epidermal blisters just above the basal cells and autoantibodies to Dsg3 (3, 4). The IgG fraction of the patients’ sera is pathogenic, as demonstrated by passive transfer experiments in neonatal mice (5, 6). Moreover, affinity-purified anti-Dsg1 and anti-Dsg3 IgG from patients’ sera are also pathogenic (7, 8). There are other autoantibodies detected in the sera of pemphigus patients, for example, autoantibodies against acetylcholine receptors (9) and E-cadherin (10). The pathogenic role of these autoantibodies remains to be determined.

The molecular mechanism responsible for the acantholysis induced by pemphigus autoantibodies has been a subject of intensive investigation in recent years. It has been generally believed that binding of pemphigus autoantibodies to the ectodo-

main of Dsg3 or Dsg1 impairs the adhesive function of these molecules, thus causing acantholysis (11, 12). However, recent single-molecule atomic force microscopy studies show that in a cell-free system, PF IgG does not inhibit homophilic trans interaction of Dsg1 molecules by steric hindrance (13), but PV IgG directly inhibits Dsg3-mediated trans interaction (14). Cell culture studies have shown that following the binding of PV autoantibodies to the cell surface, the Ag-Ab complex is internalized into cytoplasmic vesicles and subsequently fused with lysosomes (15, 16). The autoantibody-mediated Dsg3 or Dag1 internalization and depletion from the cell surface are accompanied by impaired desmosomal assembly/disassembly and decreased cell adhesiveness (16–21).

Besides the two possible mechanisms, the steric hindrance and desmoglein depletion from the cell surface, an increased body of evidence has demonstrated that pemphigus autoantibodies are able to trigger intracellular events that may indirectly lead to acantholysis by yet unclear mechanisms (22–30). Additionally, it has been hypothesized that keratinocyte apoptosis, in response to binding of pemphigus autoantibodies, results in pemphigus acantholysis (31–35). In vitro cell culture studies have shown that PV IgG or serum induces apoptosis (31, 32, 34–36). The proapoptotic changes are evident by various measurements, including annexin V binding, Hoechst 33342 staining, TUNEL labeling, DNA laddering, oligonucleosome formation, caspase activation, up-regulation of pro-apoptotic proteins (Fas, FasL, Bax, p53), and down-regulation of antiapoptotic proteins such as Bcl-2 and FLIP-L. Because most cell types are more susceptible to apoptosis in vitro than in vivo, demonstration of epidermal cell apoptotic response to pemphigus autoantibodies in an in vivo model is required to establish the pathogenic relevance of these mechanisms. In this study, using the passive transfer mouse model of PF, we provide the first evidence that a proapoptotic response in the epidermal cells occurs during the development of experimental PF. Moreover, we show that administration of caspase inhibitors abolishes PF IgG-induced intra-epidermal blisters and clinical disease in mice.

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Abbreviations used in this paper: PF, pemphigus foliaceus; Dsg, desmoglein; PV, pemphigus vulgaris.

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Materials and Methods

Pemphigus IgG preparation

Sera from two patients with classic clinical and histological features of PF were used for IgG preparation. The two sera contain IgG autoantibodies against the epidermal intercellular substance, showing the indirect immunofluorescence titer of 1:320 for PF1 and 1:640 for PF2. Both sera contained IgG autoantibodies to Dsg1 as determined by immunoprecipitation and ELISA. A normal donor serum with a negative anti-epidermal autoantibody titer was included as a control. The IgG fraction was prepared from the sera by ammonium sulfate precipitation, followed by extensive dialysis.

Animal

Breeding pairs of BALB/c mice were obtained from The Jackson Laboratory and maintained at the Division of Laboratory of Animal Medicine Facility, University of North Carolina. Neonatal BALB/c mice (1–2 days old with body weight between 1.4 and 1.6 g) were used for IgG passive transfer experiments. Animal care and animal experiments were approved by the Institutional Animal Care and Use Committee at University of North Carolina and were in accordance with National Institutes of Health guidelines.

Induction of experimental PF

Mouse models of PF were induced by IgG passive transfer experiments using a modified protocol originally described (5, 6). The IgG isolated from PF1 and PF2 sera were used individually for disease induction. Briefly, various doses of PF IgG in a total volume of 50 μl were administrated to neonatal mice by a single s.c. injection in the dorsal area. Twenty hours after IgG injection, the extent of skin disease in the dorsal area of the animals was evaluated and scored on a scale of 0 to 3, as described previously (37). IgG from PF1 and PF2 sera induced the same types of blisters clinically and histologically. The minimal dose of PF1 or PF2 IgG that produced skin lesions equal or larger than 2+ was used for the rest of the experiments. For time course studies, animals (two animals per time point) were sacrificed at various time points (up to 24 h) post-IgG injection. Skin samples from the dorsal area of each animal were harvested for H&E staining, TUNEL assay, and Western blotting.

Inhibitor administration

Peptide-based caspase inhibitors Ac-Asp-Glu-Val-Asp-Chl (Ac-DEVD-cmk) and Boc-Asp(OMe)-CH2F (Boc-D-fmk), purchased from Calbiochem, were dissolved in DMSO and diluted in TBS-Ca2+ buffer before use. The inhibitor (0.034–6.8 μg/g body weight) or vehicle in 50 μl total volume was administrated to mice by two s.c. injections. The first was given 1.5 h before the IgG injection, and the second was delivered concomitantly with IgG injection. The same dose of pathogenic PF IgG was used for comparison of the effects of the inhibitor and the vehicle treatment.

TUNEL assay

The experimental mouse skin samples were fixed in 10% buffered formalin and embedded in paraffin. Paraffin sections were subjected to TUNEL assay using the PopDETEK Green kit (Enzo Life Sciences), according to the manufacturer’s instructions. Briefly, following deparaffinization and rehydration, sections were treated with proteinase K and incubated with reaction mixture containing TdT and fluorescence-conjugated dUTP for 1 h at 37°C. Positive TUNEL labeling was revealed in the epidermis from mice (n = 6) injected with IgG from PF1 or PF2, but not from control mice (n = 3) injected with the normal human IgG. Representative results are shown.

FIGURE 1. Induction of DNA fragmentation in the mouse epidermal cells following PF IgG passive transfer. A, TUNEL staining. Neonatal mice were injected (s.c.) with pathogenic PF or normal human IgG. Skin samples were obtained 20 h post-IgG injections and subjected to TUNEL staining. Positive TUNEL labeling was revealed in the epidermis from mice (n = 6) injected with IgG from PF1 or PF2, but not from control mice (n = 6) injected with IgG from PF1 or PF2, but not from control mice (n = 3) injected with the normal human IgG. Representative results are shown. V: vesicle. B, Oligonucleosome releasing assay. DNA oligonucleosomes presented in the cytoplasmic fractions of mouse epidermal sheets obtained from control mice (n = 2) and PF mice (n = 2) were analyzed with an ELISA-based assay for presence of the histone-DNA complex.

ELISA-based nucleosome assay

Epidermal sheets were obtained from mouse skin pieces and used for the nucleosome detection assay using an ELISA kit from Roche Molecular Biochemicals, according to the manufacturer’s protocol.

Western blotting

Mouse skin proteins were extracted by homogenization. Radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, and 0.1% SDS) containing 2 mM PMSF and proteinase inhibitor mixture (Sigma-Aldrich) was used for Bax, Bel-1, and caspase expression. SDS buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 10% glycerol) was used for detection of cleaved actin. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were blocked with 5% nonfat dry milk in TBS containing 0.1% Tween 20 for 1 h. Blots were then incubated with primary Abs overnight at 4°C, followed by incubation with appropriate secondary Abs conjugated with HRP. Chemiluminescence was performed with Supersignal reagents (Pierce Biotechnology). The primary Abs used include Abs to Bax, Bel-1, cleaved caspase-3, and caspase-6 (Cell Signaling Technology), and to cleaved actin (Santa Cruz Biotechnology).

Statistics

The data are expressed as mean ± SD and were analyzed using Student’s t test. A value of p < 0.05 was considered statistically significant.

Results

PF IgG administered in vivo induced DNA fragmentation in the epidermal cells

To evaluate whether PF autoantibodies are able to induce an apoptotic response in vivo, we first conducted the IgG passive transfer experiments and then examined the epidermal cell DNA fragmentation by TUNEL staining. Twenty hours after IgG injection, TUNEL-positive epidermal cells were detected along the floor of the intraepidermal vesicle in the skin sections from mice (n = 6) injected with PF IgG isolated from PF1 or PF2 sera, but not from control mice injected with normal human IgG (n = 3). Representative results are shown in Fig. 1A. To verify the induction of DNA fragmentation by PF IgG, we further examined the presence of oligonucleosomes in the cytoplasmic fractions of
FIGURE 2. Temporal relationship between TUNEL-positive cells and histological blisters. Neonatal mice \((n = 2, \text{ each time point})\) were injected \((\text{s.c.})\) with pathogenic PF1 IgG and terminated at various time points post-IgG injection. Skin specimens were obtained and subjected to TUNEL labeling and H&E staining. As shown in these representative results, TUNEL-positive cells were first detected at 8 h, whereas initial histological cleft was revealed at 12 h post-IgG injection. Similar temporal relationship between TUNEL-positive cells and histological blisters was also observed using IgG isolated from PF2 serum.

Up- and down-regulation of apoptotic regulators

We next performed Western blot analysis to examine the expression of the proapoptotic factor Bax and the antiapoptotic factor Bcl-xL expression was reduced markedly at 6, 8, and 20 h. The intensity level was dramatically reduced at 6 and 8 h and moderately reduced at 20 h (Fig. 3B, the second panel from the top). The fully processed caspase-6 (p15) was detected at 6 and 8 h (Fig. 3B, the third panel from the top). Additional bands (p18 and p20) were observed at 6, 8, and 20 h, which are probably the products of partially processed caspase-6. In addition, we also detected the lysates from epidermis of mice injected with IgG using an ELISA-based assay. We found that PF IgG significantly increased the oligonucleosomes in these fractions, compared with the lysates of epidermis of mice injected with normal human IgG (Fig. 1A).

We then performed time course studies to determine the temporal relationship between the appearance of TUNEL-positive cells and the onset of histological blisters induced by PF IgG. PF1 IgG was injected into mice s.c. At various time points post-IgG injection, mice \((n = 2, \text{ each time point})\) were terminated. Skin specimens from the injected area of each animal were taken for TUNEL and histological examination. Fig. 2 shows the representative results of TUNEL and H&E staining on the same set of mouse skin specimens obtained at various time points following IgG injections. TUNEL-positive epidermal cells were first detected at 8 h post-PF IgG injection, whereas initial histological blisters appeared at 12 h. The number of TUNEL-positive cells increased at 12 h and remained steadily at 24 h, when clinical disease was fully developed. Consistent results were observed on the other set of samples from the duplicated mice. In addition, the experiment \((n = 2, \text{ each time point})\) was repeated using the second patient IgG. Similarly, IgG from PF2 also induced positive TUNEL epidermal cells from 8 h afterward and limited histological blisters at 12 h and extensive blisters at 24 h (data not shown). These results demonstrate that the appearance of TUNEL-positive epidermal cells precedes the onset of histological blisters.

Expression of the active form of caspase-3 and -6 and cleavage of caspase-3 substrate acinus

We further evaluated the activation of caspase-3 and -6 during the development of epidermal blisters in neonatal mice injected with PF1 IgG. As shown in the top panel of Fig. 3B, a mAb specific for the cleaved caspase-3 detected the processed active form of caspase-3 (p17) in the mouse skin extracts at time points 6, 8, and 20 h post-PF IgG injection. At 20 h, two bands (p17 and p19) with reduced intensity were detected, indicating that caspase-3 was not processed efficiently at this late time point. When the blot was reprobed with a polyclonal Ab that recognizes both the unprocessed (inactive) and processed (active) caspase-6, the unprocessed caspase-6 (35 kDa) was detected throughout the time points, but the intensity level was dramatically reduced at 6 and 8 h and moderately reduced at 20 h (Fig. 3B, the second panel from the top). The fully processed caspase-6 (p15) was detected at 6 and 8 h (Fig. 3B, the third panel from the top). Additional bands (p18 and p20) were observed at 6, 8, and 20 h, which are probably the products of partially processed caspase-6. In addition, we also detected the
cleaved acinus (23 kDa), a caspase-3 substrate, at 8 h post-PF1 IgG injection (Fig. 3C).

**Administration of caspase inhibitors protects mice from developing PF**

To test the role of caspase-3 in PF IgG pathogenicity, we further evaluated whether caspase inhibitors would have beneficial effect on PF model mice. We first assessed the effect of various doses of Ac-DEVD-cmk, a cell-permeable and irreversible inhibitor for caspase-3/7, on PF1 IgG-induced disease. Control mice that received the vehicle developed skin lesions 20 h post-PF1 IgG injection (Fig. 4, A and Ba). In sharp contrast, mice that received the inhibitor were protected from disease in a dose-dependent manner, in a range of 0.034 to 3.4 μg/g body weight (Fig. 4A). When the dose was equal or larger than 3.4 μg/g body weight, Ac-DEVD-cmk blocked disease completely (Fig. 4A; also see Table I). Fig. 4B shows the representative results derived from mice treated with the vehicle alone (left panel) or Ac-DEVD-cmk (6.8 μg/g body weight) (right panel). Twenty hours post-PF1 IgG injection, mice treated with the vehicle revealed skin lesions and intraepidermal cleft at the upper layer (Fig. 4B, a and b), mimicking the human disease. In contrast, the same dose of PF1 IgG failed to induce clinical or histological blisters in mice that received Ac-DEVD-cmk (Fig. 4B, d and e). TUNEL assay revealed a positive staining on the epidermis from mice treated with the vehicle (Fig. 4Bc), but a negative staining from mice treated with Ac-DEVD-cmk (Fig. 4Bf).

The effect of Ac-DEVD-cmk on skin blistering was also tested on mice induced by IgG from PF2 serum. The same beneficial effect was observed (Table I). In addition to Ac-DEVD-cmk, we also tested the effect of Boc-D-fmk, a broad-spectrum caspase inhibitor on experimental PF. Boc-D-fmk (6.8 μg/g body weight) exhibited a similar protective effect against PF IgG-induced histological blister and clinical disease (Table I).

**Discussion**

The aim of this study was to investigate the relevance of the apoptotic mechanism in cell/tissue injury in PF, an organ-specific autoantibody-mediated autoimmune blistering disease. Our study provides five lines of evidence that pathogenic PF IgG is able to provoke the biochemical response of apoptosis in epidermis of the mouse model. As a consequence, caspases are activated, which contribute to the development of acantholytic blisters and disease pathogenesis.

First, we demonstrated that administration of PF IgG to neonatal mice triggered DNA fragmentation of lesional epidermal cells as demonstrated by two techniques, the TUNEL assay and the nucleosome release assay. The former labels the free 3'-OH ends of the DNA strands resulting from DNA breaks, and the latter measures the amount of oligonucleosomes released in the cytosolic fraction of the epidermal cells. TUNEL-positive epidermal cells were detected on the roof and floor of the intraepidermal blisters of the PF mice (Fig. 1A). Consistent with the TUNEL result, the nucleosome release assay also reveals increased epidermal DNA fragmentation in lysates of the lesional skin of animals injected with PF IgG (Fig. 1B). These results are not only in line with previous findings in lesional epidermis of PF and PV patients (33–35, 38), but also demonstrate a cause-effect relationship between...
the injected PF autoantibodies and DNA fragmentation. Together, the data generated in the mouse model and the previous observations in patients strongly suggest that apoptotic DNA fragmentation is associated with the ongoing acantholysis induced by PF IgG.

Second, we observed that the appearance of TUNEL-positive epidermal cells precedes the onset of intraepidermal blisters in neonatal mice injected with PF IgG (Fig. 2). The time course study of the model mice post-PF IgG injections revealed that the TUNEL-positive epidermal cells first appeared at 8 h, whereas the initial histological blisters occurred at 12 h. This observation argues against the possibility that the apoptotic response to PF autoantibodies is the result of the intraepidermal blistering. It has been previously reported that TUNEL-positive epidermal cells are present not only in lesional epidermis, but also in perilesional or apparently normal epidermis of patients with PF and PV, suggesting that apoptosis may precede the onset of acantholysis (33–35, 38). The current time course study provides direct evidence that the occurrence of apoptotic DNA fragmentation of epidermal cells precedes intraepidermal blister formation and clinical disease.

Third, we detected up and down expression of Bax and Bcl-xL, two Bcl-2 family members exhibiting opposite effects in regulating apoptotic cell death via the mitochondrial pathway (39). Western blot analysis of skin lysates of PF IgG-injected animals revealed an increased amount of the proapoptotic factor Bax at earlier time points (2 and 4 h) and a decreased level of the antiapoptotic factor Bcl-xL at later time periods (6, 8, and 20 h) (Fig. 3A). These results indicate that the mitochondrial pathway of apoptosis might be evoked in the epidermal cells by PF IgG.

Fourth, we detected the activated form of two effector caspases, caspase-3 and caspase-6, by Western blotting of skin lysates from mice injected with PF IgG. Both caspase-3 and -6 were activated at later time points (6, 8, and 20 h) post-PF IgG injection (Fig. 3B). The experiment was repeated using a second IgG from PF2, and similar time-dependent expression of the cleaved caspase-3 was also observed (data not shown). Furthermore, cleaved caspase-3 was not detected in the skin lysate of the mice that were injected with normal human IgG (data not shown). To further verify the activation of caspase-3, we examined the cleavage of the protein actin, which is a substrate for caspase-3 and functions in inducing chromatin condensation (40). We found that the activated form of actin (23 kDa) was transiently expressed in mouse skin following the injection of PF IgG (Fig. 3C). However, we did not detect the 85-kDa cleaved fragment of poly(ADP-ribose) polymerase, another well-known substrate for caspase-3 (data not shown). Failure to detect the cleaved poly(ADP-ribose) polymerase fragment may indicate that the level of caspase-3 activity in the epidermis of mice injected with PF IgG is low or limited. Caspase-3 activity has been detected in cultured keratinocytes exposed to PV IgG (31, 35, 41). Interestingly, Frusic-Zlotkin et al. (41) found that the induced caspase-3 activity level roughly correlated with the immunofluorescence titers of the PV sera used. The result of the present study demonstrates for the first time that caspase-3, as well as caspase-6, is activated in vivo before the onset of intraepidermal blistering.

Finally, another novel observation reported in this study demonstrates that two caspase inhibitors were able to block the PF blistering in mice. Administration of the caspase-3/7 selective inhibitor Ac-DEVD-cmk abrogated DNA fragmentation and protected mice from developing intraepidermal blisters and clinical phenotype (Fig. 4B). The inhibitor was effective in a dose-dependent manner (Fig. 4A). Moreover, the pan-caspase inhibitor Boc-D-fmk was also effective in preventing skin disease in neonatal mice induced by PF IgG (Table I). The protective effect of these inhibitors against PF IgG-induced skin blistering is more likely a local effect because the inhibitors were delivered into the animals by local (s.c.) injections, and the skin blistering was examined only in the dorsal area where IgG was injected. However, it is also possible that a small fraction of the injected inhibitors may diffuse into the intravascular compartment, enter into the general circulation, and, thus, have some systemic effect.

It has been reported that many peptide-based caspase inhibitors, such as z-VAD-fmk, z-DEVD-fmk, and z-YVAD-fmk, also inhibit papain-like cathepsins at a higher concentration (100 μM) commonly used for cell-based studies (42, 43). However, the nonspecific effects are only observed in the fluoromethylketone (fmk)-modified inhibitors, but not in the chloromethylketone (cmk)- or aldehyde (cho)-modified inhibitors (42, 43). Indeed, z-DEVD-cmk and z-DEVD-cho do not exhibit any inhibitory effect on cathepsin activity at the high concentration of 100 μM when tested in cells (43). The finding in this study that local injection of Ac-DEVD-cmk blocked experimental PF at the low-micromolar concentration range (5 μg/50 μl, <0.2 μM) indicates that the possible nonspecific effect of this inhibitor on papain-like cathepsins is negligible, if it exists. Taken together, the protective effect of the caspase inhibitors on PF strongly suggests that caspase activation is critically involved in the formation of acantholytic blisters and subsequent clinical disease of PF.

Collectively, the results of this study suggest that PF IgG induces a proapoptotic response in epidermal cells of the neonatal mice. As a consequence, executioner caspases are activated and contribute to intraepidermal blistering. The observation that the cleaved caspase-3 and -6 are detected at late time points (6 h and afterward) indicates that activation of these executioner caspases is a downstream event proximal to the onset of histological blistering of PF. Possible upstream apoptotic pathways may include the mitochondrial pathway as well as the death receptor pathway. The former possibility is supported by the observation that Bax is upregulated at 2 h post-PF IgG injection. Activation of the death receptor pathway is also possible because increased expression of Fas, Fas ligand, and activated initiator caspase-8 in this pathway has been detected in lesional skin biopsy of PV patients and in cultured keratinocytes exposed to PV IgG (34, 35, 41). In addition, it has been found that two signaling events, the p38 MAPK activation and the RhoA inactivation, are involved in acantholysis induced by PF IgG as well as PV IgG (26, 28). The pathogenic role of these two signaling pathways in PF and PV is demonstrated by blocking skin blistering using pharmacologic inhibitors of p38 MAPK (22) or activator of RhoA (26). Although the relationship of the RhoA inactivation to the apoptotic pathway is not clear, p38 MAPK activation is known to be an upstream event of keratinocyte apoptosis (44). Indeed, s.c. injection of a p38 MAPK inhibitor prevents PF IgG-induced keratinocyte apoptosis in mice (D. Rubenstein, unpublished observations), indicating that apoptosis is one of the downstream events following p38 MAPK activation in PF mouse model.

An intriguing observation in this study is that although we detected the key biochemical hallmarks of apoptosis (caspase-3 activation and DNA fragmentation), the morphological characteristics of apoptosis are not obvious by H&E examination of the epidermis of mice injected with pathogenic PF IgG. This observation may suggest that activation of caspases leads to epidermal cell injury and dysfunction, which in turn are manifested as epidermal cell detachment and blister formation. Acantholytic blisters may occur before or without the end point of epidermal cell death. Caspase-3 activation without cell death has been reported in several physiological processes, such as terminal differentiation of various cell types, proliferation of resting peripheral T lymphocytes, and inhibition of the cell cycle in peripheral B cells (45). It
is unclear how cells are able to survive with an activated killer caspase. It has been suggested that compartmentalized or low magnitude activation of caspase-3 or -6 is prior and close to the onset of visible intraepidermal cleavage, it does not exclude the possibility that the acantholytic process has not started before executioner caspases are activated. It is probable that weakened intercellular adhesion and some extent of acantholysis already occurred at early course. Activation of executioner caspases may be one of the later events that aggravate this process. Therefore, although our time course study clearly shows that the activation of caspase-3 and -6 is a result of this process. Therefore, although our time course study clearly shows that the activation of caspase-3 and -6 may account for this enigma. Regardless of the possible explanations, it has been suggested that compartmentalized or low magnitude activation of caspase-3 or -6 is prior and close to the onset of visible intraepidermal cleavage, it does not exclude the possibility that the acantholytic process has not started before executioner caspases are activated. It is probable that weakened intercellular adhesion and some extent of acantholysis already occurred at early course. Activation of executioner caspases may be one of the later events that aggravate this process.

Disclosures

The authors have no financial conflict of interest.

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