No evidence of apoptotic cells in pemphigus acantholysis

I.C. Janse, G. van der Wier, M.F. Jonkman, H.H. Pas, G.F.H. Diercks

Centre for Blistering Diseases,
Department of Dermatology,
University of Groningen,
University Medical Centre Groningen,
Groningen, The Netherlands

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Abstract

Background The exact mechanism by which autoantibodies against desmogleins induce acantholysis in pemphigus remains unsolved.

Objectives In this study we explore the role of apoptosis in pemphigus acantholysis.

Patients/Methods Twenty-two skin biopsies from pemphigus vulgaris (PV) and pemphigus foliaceus (PF) patients and eight biopsies from a PV and PF in vitro skin model were included. In these biopsies immunofluorescence staining of cleaved caspase 3, cleaved caspase 8, poly (ADP-ribose) polymerase (PARP), fractin and TUNEL was performed. Fourteen skin biopsies from PV and PF patients were studied by electron microscopy for morphological features of apoptosis.

Results In pemphigus patient skin and the in vitro skin model there was no expression of apoptotic markers. A few TUNEL positive cells were seen in three lesional PF biopsies and in the biopsies from the in vitro skin model. However, these observations did not differ from control biopsies. None of the PV and PF skin biopsies showed electron microscopic morphological features of apoptosis.

Conclusions The absence of specific apoptotic signalling and morphological features of apoptosis in pemphigus patient skin does not support the hypothesis that apoptosis is involved in pemphigus acantholysis.

Introduction

Pemphigus is a group of rare mucocutaneous autoimmune bullous diseases that are characterized by intra-epidermal IgG deposition and loss of cohesion between keratinocytes, known as acantholysis. Although the pathogenic relevance of anti-desmosomal IgG has been clearly demonstrated, the exact mechanism by which IgG induces loss of adhesion remains unsolved. Cell signalling has received a lot of attention in the past years, including the death signalling (apoptotic) pathways. Apoptosis can be activated via an extrinsic or intrinsic pathway. In the extrinsic pathway, Fas L binds to the Fas receptor, which leads to the activation of caspase 8. In the intrinsic pathway, subsequently p53, bax, cytochrome c, and caspase 9 are activated. Both caspases 8 and 9 activate the common pathway caspase 3. Caspase 3 induces DNA fragmentation, which can be detected with poly (ADP-ribose) polymerase (PARP), fractin, and TUNEL.\(^1,2\) Apoptosis has been suggested as an upstream event in acantholysis, but alternatively, also as a downstream event after loss of cell–cell adhesion.\(^3\) Furthermore, it has been hypothesized that IgG might induce apoptotic enzymes but that these do not lead to cell death but instead to acantholysis, a mechanism referred to as apoptolysis.\(^6\)
A thorough examination of the literature on apoptosis in pemphigus revealed that the possible involvement of apoptosis in acantholysis was mainly studied in cultured cell and/or mouse models, but rarely in patient skin.\(^3\) Despite this, the caspase pathway has already been suggested as a therapeutic target in pemphigus.\(^7\) This, together with, as mentioned before, conflicting results from various studies, propelled us to reinvestigate this topic. We therefore searched for evidence of apoptosis in pemphigus patient skin and in an \textit{in vitro} skin model, wherein we induced acantholysis by patient IgG. We checked for activation of both the intrinsic and the extrinsic pathway by immunofluorescence and, furthermore, used electron microscopy to look for hallmarks of apoptosis.

\textbf{Materials and methods}

\textbf{Pemphigus patient skin for immunofluorescence}

For immunofluorescence, we included 11 biopsies from 9 mucocutaneous pemphigus vulgaris (PV) patients (5 from healthy skin, 3 from perilesional skin, and 3 from lesional skin), and 11 biopsies from 7 pemphigus foliaceus (PF) patients (4 from healthy skin, 2 from perilesional skin, and 5 from lesional skin). Pemphigus biopsies were selected from patients with increased anti–Dsg antibodies determined by enzyme–linked immunosorbent assay (ELISA) (increased anti–Dsg1 and anti–Dsg3 index for mucocutaneous PV, increased anti–Dsg1 and normal Dsg3 index for PF). Moreover, all pemphigus biopsies needed to show pemphigus specific epithelial cell surface (ECS) IgG depositions. All samples were immediately frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\). All used patient tissue was residual tissue, taken in the past for diagnostics. For this reason patient consent for experiments was not required. Immunofluorescent analysis was performed for cleaved caspase 3, cleaved caspase 8, cleaved PARP, fractin, and TUNEL. For all the stainings except TUNEL, one healthy skin sample was used as negative control\(^9\) and one basal cell carcinoma tissue sample, one colon carcinoma tissue sample and one toxic epidermal necrolysis (TEN) skin sample served as positive controls.\(^10\)\(^-\)\(^12\) For TUNEL four normal human skin samples served as negative controls and four samples of the same skin treated for 10 minutes with DNAse 1 and one TEN skin sample served as positive controls. The control samples were evaluated in the same experiment as the pemphigus specimens.

\textbf{Pemphigus in vitro model for immunofluorescence}

We used eight biopsies from a previously described pemphigus \textit{in vitro} model experiment.\(^8\) In these experiments, healthy breast reduction skin biopsies had been incubated with purified
pemphigus IgG (for 4, 16, and 24 hours with PV IgG and for 24, 48, and 72 hours with PF IgG). The PV IgG dose was 12.2 mg/ml, the PF IgG dose was 10.8 mg/ml. After harvesting, the biopsies were frozen in liquid nitrogen and stored at –80°C. Again, the aforementioned controls (pemphigus patient skin for immunofluorescence) were evaluated in the same experiment as the in vitro model samples. Additionally, control incubations included three healthy IgG and two omission of IgG.

**Staining procedure**
The following antibodies were used to detect apoptosis: cleaved caspase–3 rabbit monoclonal antibody (Cell Signalling, Danvers, U.S.A) in a dilution of 1/50, cleaved caspase–8 rabbit monoclonal antibody (Cell Signalling, Danvers, U.S.A) in a dilution of 1/200, cleaved PARP rabbit monoclonal antibody (Cell signalling, 1 Danvers, U.S.A.) in a dilution of 1/200 and fractin rabbit polyclonal antibody in a dilution of 1/200 (Mybiosource, San Diego, U.S.A). Cryosections of 4 μm were incubated overnight with one of the selected antibodies in phosphate buffered saline containing 1% ovalbumine (PBS–OVA) at 4°C. Next, the sections were incubated with donkey anti rabbit fluorescein isothiocyanate (FITC) (dilution 1/100 in PBS–OVA) labeled secondary antibody for 1 hour at room temperature. Finally, the sections were counterstained with bisbenzimide (BB) (1:10000 diluted in PBS) for 5 minutes at room temperature and coverslipped under SlowFade® Antifade reagent (Invitrogen, Paisley, U.K.). TUNEL (Roche, Mannheim, Germany) was performed as follows. After fixation with paraformaldehyde (4% in PBS, pH 7.4) for 30 minutes, the sections were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Then, the sections were incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein dUTP (diluted 1:10 in label solution) in a humidified chamber for 60 minutes at 37°C in dark (sections covered with cover slip during incubation). After that, the sections were counterstained with BB (1:10000 diluted in PBS) for 5 minutes at room temperature and coverslipped under SlowFade® Antifade reagent.

**Evaluation of the samples**
Per sample 5 high power fields were evaluated. If staining was present, between 45 and 259 keratinocytes were evaluated in the most representative high power fields, and the percentage of TUNEL was counted by two independent observers.
Pemphigus patient skin for electron microscopy

Finally, six skin biopsies (two lesional, one perilesional, and three non-lesional) from four mucocutaneous PV patients and nine skin biopsies (five lesional, one perilesional, and three non-lesional) from eight PF patients were investigated by electron microscopy for ultrastructural signs of apoptosis. Electron microscopy was performed as described before. For electron microscopy, pemphigus patient skin biopsies were selected that met the same inclusion criteria as described under ‘pemphigus patient skin for immunofluorescence’. The number of sections and cells examined differed per sample. PV lesional 1: 7 sections (1–10 cells), PV lesional 2: 5 sections (2–40 cells), PV perilesional 1: 5 sections (2–15 cells), PV non-lesional 1: 4 sections (1–8 cells), PV non-lesional 2: 4 sections (2–25 cells), PV non-lesional 3: 5 sections (3–15 cells), PF lesional 1: 4 sections (3–10 cells), PF lesional 2: 4 sections (2–22 cells), PF lesional 3: 4 sections (2–9 cells), PF lesional 4: 3 sections (2–22 cells), PF lesional 5: 3 sections (2–8 cells), PF perilesional 1: 1 section (8 cells), PF non-lesional 1: 4 sections (2–5 cells), PF non-lesional 2: 3 sections (2–10 cells), PF non-lesional 3: 5 sections (2–20 cells). Basal cell carcinoma served as a positive control (Figure S3).

![Figure S3](image_url) – Electron microscopy of basal cell carcinoma (BCC). Basal cell carcinoma tissue shows nuclear fragmentation. * = nuclear fragmentation. Scale bar = 5 μm.
Results
The apoptotic markers stained positive in the positive-control tissues but were absent in the negative controls (Figure S1). None of the pemphigus skin biopsies showed positive staining of cleaved caspase 3, cleaved caspase 8, fractin, or nuclear PARP. In PV, TUNEL was positive in 0.46% (range, 0–0.97%) of lesional epidermis and in 1.25% (range, 0–2.94%) of perilesional epidermis. In PF, these numbers were 4.42% (range, 0–17.78%) for lesional epidermis and 0.84% (range, 0–1.91%) for perilesional epidermis (Figure 1). In healthy pemphigus skin, positive cells were only sporadically present. In healthy control skin, 1.11% of the cells were TUNEL positive (range, 0–4.44%).

Acantholysis was present in PV IgG-incubated biopsies after 16 hours and in the PF IgG-incubated biopsies after 48 hours. None of the in vitro model biopsies showed positive staining for cleaved caspase 3, cleaved caspase 8, fractin, or nuclear PARP. An intercellular PARP staining was present in the in vitro model biopsies. This intercellular staining pattern was also present in healthy skin and in the additional controls of the in vitro model. Some TUNEL positive cells were found in the stratum granulosum of all biopsies but were absent in the layers beneath, including in the acantholytic biopsies. The percentages of TUNEL-positive cells found in the PV model were as follows: 7.46% at t=0 (non-incubated skin), 0% at t=4, 4.03% at t=16, and 3.17% at t=24. For the PF model, these percentages were 1.26% at t=0, 2.32% at t=24, 3.46% at t=48, and 1.62% at t=72 (Figure S2).

All perilesional and lesional biopsies showed widening of intercellular spaces by electron microscopy. The lesional biopsies showed a decreased number of desmosomes. None of the biopsies, however, showed any morphological features of apoptosis, i.e., rounding up of the cell, retraction of pseudopods, pyknosis, karyorrhexis, plasma membrane blebbing, and engulfment by resident phagocytes (Figure 2).
Figure S1 – (a) Immunofluorescence of controls (cl caspase 3, cl caspase 8, PARP, fractin) in the HE staining colon carcinoma tissue, basal cell carcinoma tissue and toxic epidermal necrolysis (TEN) tissue show apoptotic hallmarks while healthy skin shows no abnormalities. Colon carcinoma tissue, basal cell carcinoma tissue, and TEN tissue show cytoplasmic staining of cl caspase 3 and cl caspase 8. PARP shows nuclear staining in basal cell carcinoma tissue, colon carcinoma and TEN tissue and an intercellular staining pattern in the healthy skin. Besides cytoplasmic staining fractin shows staining of apoptotic bodies in the positive controls. Healthy skin shows negative staining for cl caspase 3, cl caspase 8 and fractin. Scale bar = 20 μm (only for Immunofluorescence) (b) Immunofluorescence of controls (TUNEL) TUNEL was extensively expressed in DNAse 1 treated skin and TEN tissue while it was only focally present in label treated healthy control skin. Scale bar = 20 μm
Figure 1 – Immunofluorescence of lesional pemphigus patient skin. Hematoxylin and eosin (H&E) staining shows pemphigus vulgaris (PV) skin with blistering in the suprabasal layer and pemphigus foliaceus (PF) skin with acantholysis within the granular layer. PV lesional (1–2) skin shows absence of staining of cl caspase 3, cl caspase 8, poly (ADP–ribose) polymerase (PARP), and fractin. PF lesional skin (1–5) shows absence of staining of cl caspase 3, cl caspase 8, and fractin. PARP shows staining with an intercellular pattern in PF lesions 4–5, whereas it is absent in PV lesions 1–3. PV lesion 2, PF lesions 2, 4–5 show absence of TUNEL, whereas PV lesion 1 and PF lesions 1,3 show a few TUNEL–positive cells. Bar = 50 μm.
Figure S2 – Immunofluorescence of pemphigus–IgG treated redundant breast reduction skin. HE staining shows no abnormalities after zero and 4 hours of pemphigus vulgaris (PV)–IgG incubation and after zero and 24 hours of pemphigus foliaceus (PF)–IgG incubation. After 16 and 24 hours of PV–IgG incubation acantholysis is seen within the suprabasal layer. After 48 hours of PF–IgG incubation acantholysis is seen in the subcorneal layer. In the PF model PARP is present with an intercellular pattern at all time points while all the other apoptotic markers are absent. A few TUNEL positive cells are seen in all biopsies except for PV T4. Scale bar = 20 μm
Figure 2 – Electron microscopy of lesional pemphigus skin. Pemphigus vulgaris (PV) lesional skin (1, 2) and pemphigus foliaceus (PF) lesional skin (1–4) show acantholysis in absence of apoptotic features (rounding up of the cell, retraction of pseudopods, pyknosis, karyorrhexis, plasma membrane blebbing, and engulfment by resident phagocytes). In all of the lesional biopsies, a widening of the intercellular spaces and a decreased number of desmosomes were seen. *Blister. Bar = 5 μm.
Discussion

In conclusion, we found no evidence of apoptotic cells in pemphigus acantholysis. First, apoptotic signalling of both the intrinsic and the extrinsic pathway was absent in 22 skin biopsies of pemphigus patients and in the in vitro model at any time point. Second, although some TUNEL staining was seen in two acantholytic PF biopsies and one PV biopsy, and in the pemphigus model, we also found TUNEL-positive cells in normal healthy skin, especially in the granular layers. The expression of TUNEL in the pemphigus skin and model was also seen in the stratum granulosum, above the blisters. If apoptosis would be a cause of acantholysis, then TUNEL positivity should have been present below the blister cavity. Third, ultrastructurally no morphological features of apoptosis were present. It should be noted that electron microscopy is considered to be the gold standard for the identification of apoptotic cells.13

Our results are in line with the findings of Schmidt et al. who conclude that apoptosis is not required for pemphigus acantholysis.4 Like us, they found no evidence of positive cleaved caspase 3 and TUNEL when analyzing lesional PV patient skin. Moreover, in cultures of human keratinocytes treated with PV IgG, they also found acantholysis in absence of positive TUNEL staining as well as caspase 3 cleavage. Lee et al. also disagreed that apoptosis is an upstream event in pemphigus acantholysis.5 In PF IgG–treated mice and PV IgG–treated keratinocyte cultures, they found activation of cleaved PARP, cleaved caspase 3, and TUNEL, but only after acantholysis. The belief that apoptosis is an upstream event in acantholysis is largely based on studies in model systems.3 We question whether these model systems are suitable for answering such questions on pemphigus pathogenesis, as they differ from the in vivo situation. For instance, in cultured keratinocytes the desmosomal makeup in terms of molecular composition does not accurately reflect the in vivo situation.14 As for mouse models, the repertoire of expressed genes involved in apoptosis in humans and mice is different.15 Furthermore, previous data on pemphigus skin are scarce and based on a few lesional biopsies only.16–18

In conclusion, this study does not support the hypothesis that apoptosis is involved in pemphigus acantholysis. However, although we studied morphological hallmarks of apoptosis, the involvement of certain apoptotic caspases or signalling pathways, which might be involved in dissociation of (inter)desmosomal adhesion complexes, cannot be excluded.6
References
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