No Evidence of Apoptotic Cells in Pemphigus Acantholysis

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TO THE EDITOR

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 signaling has received a lot of attention in the past years, including the death signaling (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 (de Boer *et al.*, 2000; Hengartner, 2000).

Apoptosis has been suggested as an upstream event in acantholysis, but alternatively, also as a downstream event after loss of cell–cell adhesion (Schmidt and Waschke, 2009, Schmidt *et al.*, 2009, Lee *et al.*, 2009). 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 (Grando *et al.*, 2009).

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 (Schmidt and Waschke, 2009). Despite this, the caspase pathway has already been suggested as a therapeutic target in pemphigus (Pacheco-Tovar *et al.*, 2011). 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 *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.

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). Immunofluorescent analysis was performed for cleaved caspase 3, cleaved caspase 8, cleaved PARP, fractin, and TUNEL. Protocols and other details can be found in Supplementary Text S1 online. The apoptotic markers stained positive in the positive-control tissues but were absent in the negative controls (Supplementary Figure S1 online). None of the pemphigus skin biopsies showed positive staining of cl caspase 3, cl 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%).

Second, we used eight biopsies from a previously described pemphigus *in vitro* model experiment (Oktarina *et al.*, 2011). 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) (for additional details see Supplementary Text S1 online). 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 cl caspase 3, cl 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 $t=72$ (Supplementary Figure S2 online).

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. All details including used controls are described in Supplementary Text S1 online. All perilesional and lesional biopsies showed widening of intercellular spaces. The lesional biopsies show 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,

Abbreviations: Ab, antibody; Cl, cleaved; Dsg, desmoglein; HE, hematoxylin and eosin; PARP, poly (ADP-ribose) polymerase; PV, pemphigus vulgaris; PF, pemphigus foliaceus

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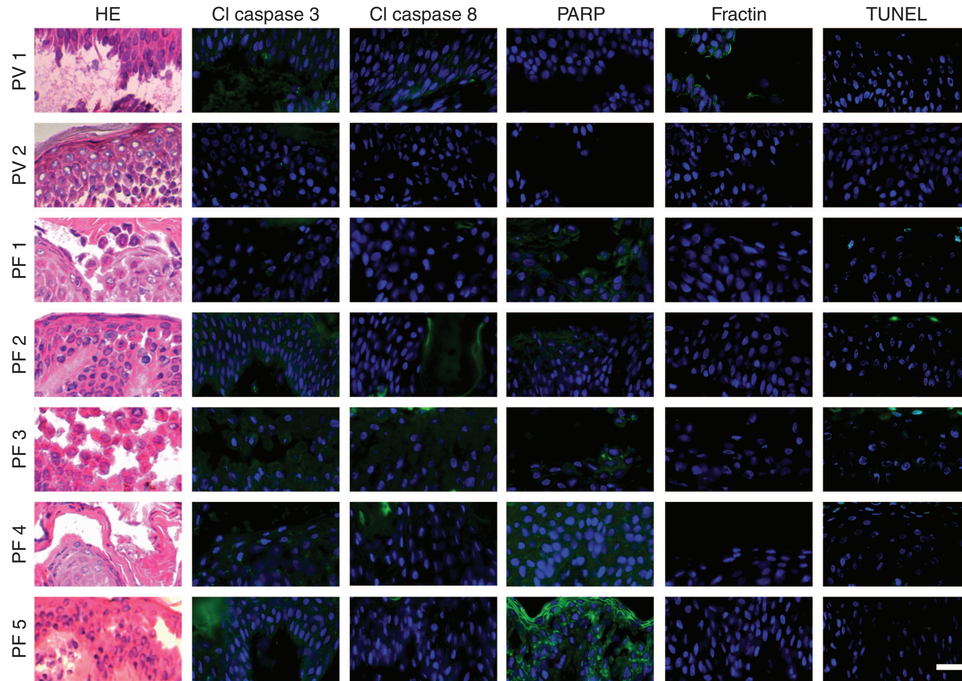

Figure 1. Immunofluorescence of lesional pemphigus patient skin. Hematoxylin and eosin (HE) staining shows pemphigus vulgaris (PV) skin with blistering in the suprabasal layer and pemphigus foliaceus (PF) skin with acantholysis within the granular layer. PV lesional (1–2) skin shows absence of staining 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.

plasma membrane blebbing, and engulfment by resident phagocytes (Figure 2).

In conclusion, we found no evidence of apoptotic cells in pemphigus acantholysis. First, apoptotic signaling 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 and one PV biopsies, 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 EM is considered to be the gold standard for the identification of apoptotic cells (Taatjes *et al.*, 2008).

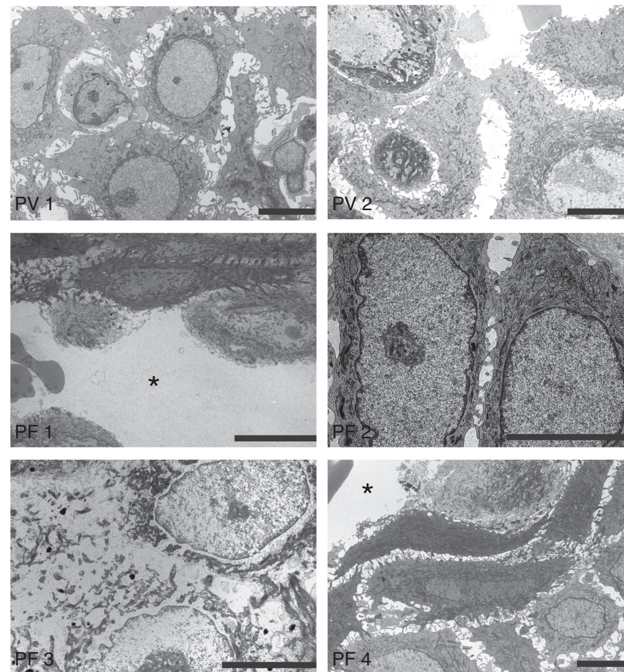

Figure 2. Electron microscopy of lesional pemphigus skin. Pemphigus vulgaris (PV) lesional skin (1, 2) and pemphigus foliaceus (PF) lesional skin (1–4) show acantholysis in absence of apoptotic features (rounding up of the cell, retraction of pseudopods, pyknosis, karyorrhexis, plasma membrane blebbing, 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.

Our results are in line with the findings of Schmidt *et al.* (2009) who conclude that apoptosis is not required for pemphigus acantholysis. Like us, they found no evidence of positive cl 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.* (2009) also disagreed that apoptosis is an upstream event in pemphigus acantholysis. In PF IgG–treated mice and PV IgG–treated keratinocyte cultures, they found activation of cl PARP, cl 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 (Schmidt and Waschke, 2009). 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 (van der Wier et al., 2010). As for mouse models, the repertoire of expressed genes involved in apoptosis in humans and mice is different (Reed *et al.*, 2003). Furthermore, previous data on pemphigus skin are scarce and based on a few lesional biopsies only (Wang *et al.*, 2004; Pacheco-Tovar *et al.*, 2009, Deyhimi and Tavakoli, 2012).

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 signaling pathways, which might be involved in dissociation of (inter)desmosomal adhesion complexes, cannot be excluded (Grando *et al.*, 2009).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Associations between Keloid Severity and Single-Nucleotide Polymorphisms: Importance of rs8032158 as a Biomarker of Keloid Severity

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TO THE EDITOR

A keloid is a fibroproliferative disorder of the skin (Tredget, 1994) that is caused by pathological wound-healing processes (Gurtner *et al.*, 2008). The development and progression of keloids relate closely to genetic factors (Marneros *et al.*, 2004; Nakashima *et al.*, 2010), systemic factors such as hypertension (Arima *et al.*, 2012) and pregnancy (Park and Chang 2012), and the local microenvironment (e.g., skin stretching force/tension, (Ogawa *et al.*, 2012a)). A genome-wide association

Abbreviations: CI, confidence interval; GWAS, genome-wide association study; NEDD4, neuronal precursor cell-expressed developmentally downregulated 4; SNP, single-nucleotide polymorphism

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