Apoptosis is not required for acantholysis in pemphigus vulgaris

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Running head: apoptosis in pemphigus

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ABSTRACT

The autoimmune blistering skin disease pemphigus vulgaris (PV) is caused primarily by autoantibodies against desmosomal cadherins. It was reported that apoptosis can be detected in pemphigus skin lesions and that apoptosis can be induced by PV-IgG in cultured keratinocytes. However, the role of apoptosis in PV pathogenesis is unclear at present. In this study, we provide evidence that apoptosis is not required for acantholysis in PV. In skin lesions from two PV patients, TUNEL positivity but not cleaved caspase-3 was detected in single keratinocytes in some lesions but was completely absent in other lesions from the same patients. In cultures of human keratinocytes (HaCaT and normal human epidermal keratinocytes), PV-IgG from three different PV patients caused acantholysis, fragmented staining of Dsg 3 staining and cytokeratin retraction in the absence of nuclear fragmentation, TUNEL positivity and caspase-3 cleavage and hence in the absence of detectable apoptosis. To further rule out the contribution of apoptotic mechanisms, we used two different approaches which are effective to block apoptosis induced by various stimuli. Inhibition of caspases by z-VAD-fmk as well as overexpression of FLIP_L and FLIP_S to inhibit receptor-mediated apoptosis did not block PV-IgG-induced effects indicating that apoptosis was not required. Taken together, we conclude that apoptosis is not a prerequisite for skin blistering in PV but may occur secondary to acantholysis.

Key words: apoptosis, autoantibody, desmoglein 3, keratinocyte, pemphigus
INTRODUCTION

Pemphigus vulgaris (PV) is an intraepidermal blistering autoimmune disease that is morphologically characterized by loss of cell-cell contact between neighboring keratinocytes, referred to as acantholysis. PV is associated with autoantibodies against the transmembrane desmosome cadherins desmogleins (Dsg) 3 and Dsg 1 (2, 34). Autoantibodies with specificities for the non-desmosomal keratinocyte-surface molecules α9 acetylcholine receptor and pemphaxin have also been identified in PV sera (21).

While the pathogenic relevance of anti-desmosomal autoantibodies has been clearly established (2, 34), the molecular mechanism leading to acantholysis remains unsolved (42). Several hypotheses have been proposed (i) direct steric hindrance (32), (ii) activation of proteolytic activity (15), (iii) depletion of cell-surface Dsg 3 by its internalization (12), and (iv) transmembrane signaling first reported by Kitajima and coworkers (16). Since we have recently demonstrated that pemphigus IgG-induced direct inhibition of desmoglein-mediated transinteraction appears to contribute to acantholysis in PV but not in pemphigus foliaceus (PF) (22, 43) and PV Fab’ fragments are sufficient for blister formation in the passive-transfer mouse model of pemphigus even when complement-deficient mice are used (3, 26), cellular signaling events seem to be important to induce acantholysis in PV.

PV IgG-associated signaling has been shown to result in e.g. mobilization of intracellular calcium (16), activation of protein kinase C (14, 28) and Src (13, 14), phosphorylation of Dsg 3 (4), p38MAPK-mediated phosphorylation of heat shock protein 27 (8, 13), p38MAPK-triggered Rho A inactivation (44) and activation of EGF receptor kinase, p-ERK1/2, and c-Jun (13, 19). Among the potential downstream mechanisms that may result from binding of PV
autoantibodies to their cell-surface receptors, altered desmosome assembly (4, 11), alterations of the cytoskeleton (8, 44) and apoptosis are most widely discussed.

Increased apoptotic activity has been described in both lesional and perilesional skin of PV patients (20, 40). In addition, PV serum and/or PV-IgG have been reported to induce hallmarks of apoptosis such as DNA fragmentation (6, 13), increased expression of the pro-apoptotic molecules FasR, FasL, Bax, p53, depletion of anti-apoptotic Bcl-2 and FLIP_L, and activation of caspases 1, 3, and 8 in cultured keratinocytes (5, 18, 19, 29, 31, 40, 41). Hence, compelling evidence has been provided that programmed cell death found in some PV lesions and that apoptosis can be induced by PV-IgG/ PV serum in vitro. However, only few data support the concept that PV autoantibody-induced acantholysis is indeed mediated by apoptosis. The notion that in most experiments prolonged incubation times and high autoantibody concentrations have been used to provoke apoptotic events (6, 18, 19, 40, 41) led us to reinvestigate the role of apoptosis in PV.

Here, we show that acantholysis in PV patients’ skin and PV-IgG-induced cell-cell dissociation in cultured keratinocytes occurs in the absence of detectable apoptosis. In addition, neither the caspase-inhibitor z-VAD-fmk nor overexpression of FLIP_L and FLIP_S, which block death receptor-induced apoptosis, interfered with PV-IgG-induced acantholysis in cultured keratinocytes. These data do not support the assumption that apoptosis is a prerequisite for acantholysis in PV.
MATERIALS AND METHODS

**Pemphigus patients:** In this study sera from 5 PV patients (PV 1 to PV 5) and perilesional skin from 2 PV patients (PV 3 and PV 6) were used. In all patients diagnosis was based on (i) typical skin and/or mucous membrane lesions, (iii) histopathology that showed an intraepidermal split formation, and (iii) the detection of circulation autoantibodies that bound to monkey esophagus by indirect immunofluorescence microscopy and (iv) reactivity to Dsg 3 in patients’ sera. In some patients, serum autoantibodies to Dsg 1 were also detected. Sera were tested for reactivity against Dsg 1 and Dsg 3 by enzyme-linked immunosorbent assay (ELISA) performed according to the manufacturer’s instructions (Medical and Biological Laboratories, Nagoya, Japan) The cut-off value was 14 U/ml for Dsg 1 and 7 U/ml for Dsg 3; all samples were run in duplicate (Table 1). The study has been approved by the local ethics committee. In addition, sera from healthy volunteers were used.

**Skin biopsies from pemphigus patients:** Skin biopsies were taken from PV patients 3 and 6 before treatment was initiated. Following brief rinsing with phosphate-buffered saline (PBS, consisting of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄ and 1.5 mM KH₂PO₄, pH 7.4), skin specimen were mounted on copper plates using Reichert-Jung mounting medium (Cambridge Instruments, Nußloch, Germany) and frozen in liquid nitrogen. 5 µm thick cryosectiones were obtained using a Reichert-Jung 2800 Frigocut (Cambridge Instruments).

**Purification of IgG:** IgG fractions were purified by affinity chromatography using protein A agarose as described previously (43). For some experiments, a pool of four sera was used (PV-IgG pool: PV-IgG 1, 2, 4 and 5). The IgG fractions were diluted in culture medium to a final IgG concentration of 150 µg/ml. In some experiments IgG concentrations of 750 µl/ml were employed.
Cell culture and test reagents: The immortalized human keratinocyte cell line HaCaT and the corresponding FLIP<sub>L</sub> and FLIP<sub>S</sub> transfectants were grown in Dulbecco’s modified Eagles medium (DMEM, life technologies, Karlsruhe, Germany) supplemented with 50 U/ml penicillin-G, 50 µg streptomycin and 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany) in a humidified atmosphere (95% air/ 5% CO<sub>2</sub>) at 37°C. The cultures were grown to confluence and all experiments were performed using DMEM at 1.8 mM Ca<sup>2+</sup>. Normal human epidermal keratinocytes (NHEK) derived from adult skin were purchased from PromoCell GmbH (Heidelberg, Germany). Cells were grown in Keratinocyte Growth Medium 2 (PromoCell, Heidelberg, Germany) supplemented with 50 U/ml penicillin-G, 50 µg streptomycin and supplement mix (PromoCell). NHEK were grown in low Ca<sup>2+</sup> (0.15 mM) which was changed to high Ca<sup>2+</sup> (1.2 mM) one day before experiments were started. Staurosporine (Roche Diagnostics, Mannheim, Germany) was used at 1 µM. The caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (z-VAD-fmk; Bachem, Bubendorf, Switzerland) was used at 20-300 µM. Production and purification of Flag-TNC-TRAIL and Fc-Flag-FasL have been described elsewhere (7). Polyclonal HaCaT populations were stably transfected with empty pEGZ vector or the corresponding FLIP<sub>L</sub>- and FLIP<sub>S</sub>-encoding derivatives as previously reported (37).

Fixation: HaCaT and NHEK cells were grown on cover slips to confluence as described above and incubated with PV-IgG for 2 h, 6 h, 24 h or 48h at 37°C. After incubation with autoantibodies, culture medium was removed and monolayers fixed either for 2 min at room temperature (RT) with ice cold acetone for cytochemistry or for 25 min at 4°C with 4% formaldehyde in PBS (freshly prepared from paraformaldehyde) for TUNEL. These monolayers were treated with 0.1% Triton X-100 in PBS for 5 min at RT. Cryosections of
human skin were dried on a heat plate for 30 min, fixed with 4% formaldehyde at 4°C for 15 min and treated with proteinase K (20 µg/ml) for 10 min at RT.

Cytochemistry: After rinsing with PBS at RT, cryosections of human skin, NHEK or HaCaT cells were preincubated for 30 min with 10% normal goat serum (NGS) and 1% bovine serum albumin (BSA) at RT and incubated for 16 h at 4°C with mouse monoclonal antibodies directed to the ectodomain of human Dsg 3 (Zytomed, Berlin, Germany) to cytokeratin 5 (Santa Cruz, Heidelberg, Germany) or FLIP (Alexis, Grünberg, Germany,) (dilution 1:100 in PBS for all). After several rinses with PBS (3 x 5 min), monolayers were incubated for 60 min at RT with Cy3-labelled goat anti-mouse IgG (Dianova, Hamburg, Germany). For visualization of nuclei, DAPI stain (Roche Diagnostics, Mannheim, Germany; diluted 1:3000 in PBS) was used (incubation for 5 min at RT). Afterwards, cells were rinsed with PBS (3 x 5 min) and cover slips were mounted on glass slides with 60% glycerol in PBS, containing 1.5% n-propyl gallate (Serva) as antifading compound. Monolayers and cryosections were examined using a LSM 510 (Zeiss, Göttingen, Germany). Images were processed using Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA).

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling): Assay was performed in accordance to manufacturer’s protocols (Promega, USA). In brief, after permeabilization monolayers and cryosections were washed twice with PBS and cryosections were fixed again with 4% formaldehyde for 5 min and washed again. After incubation with equilibration buffer for 5-10 min at RT, cells and sections were transferred to incubation buffer (45 µl equilibration buffer, 5 µl nucleotide mix, 1 µl rTdT Enzyme) for 60 min at 37°C. Reaction was stopped by incubation with 2 x SSC buffer for 15 min at RT. After three washes with PBS, DAPI nuclear stain was added for visualization of all cells. Following another three PBS washes, cover slips were mounted on glass slides with 60% glycerol in
PBS, containing 1.5% n-propyl gallate (Serva) as antifading compound. For positive controls, fixed cells and sections were DNAse I-treated (10 U/ml, 15 min, 37°C) before equilibration.

**Quantification of apoptotic cells:** apoptotic cells were identified by nuclear condensation and degradation by DAPI stain or TUNEL-positivity. Cells of at least three microscopic fields at magnification x 40 were counted for each experiment and results were expressed as % of total cells from the respective microscopic field. Average cell numbers per microscopic field were 150 (HaCaT) and 50 (NHEK).

**Electrophoresis and Western blotting:** After incubation with pemphigus patients’ IgG or reagents for the indicated times, HaCaT cells were dissolved in sample buffer, heated at 95°C for 5 min and finally subjected to sodium dodecyl sulfate 7.5% or 10% polyacrylamide gel electrophoresis and immunoblotting to Hybond nitrocellulose membranes (Amersham, Buckinghamshire, UK). Membranes were blocked with 5% low fat milk for 1 h at room temperature (RT) in PBS and incubated with the respective primary antibody overnight at 4°C. The mouse monoclonal antibody against cleaved caspase-3 (Cell Signaling) was used at 1:1000. As secondary antibodies HRP-labelled goat anti-mouse (Dianova, Hamburg, Germany) were used. Visualization was achieved using the enhanced chemiluminescence technique (Amersham).

**Cell death assay:** HaCaT cells and transfectants were seeded at a density of 20x10³ cells/well in 96-well plates and were recovered overnight. Cells were then stimulated in triplicates with 300 ng/ml Fc-Flag-FasL or 300 ng/ml Flag-TNC-TRAIL oligomerized with 1 µg/ml anti-Flag mAb M2 (Sigma) for additional 16 hours. Cell viability was finally determined by crystal violet staining.
**Dispase-based keratinocyte dissociation assay:** The assay was performed as described in the literature with the following modifications (11, 23, 24). HaCaT cells were seeded on 12-well plates and grown to confluence. After incubation for 24 h under various conditions, cells were washed with Hank’s buffered salt solution (HBSS) and treated for 30 min with 0.3 ml dispase II (2.4 U/ml, Sigma) at 37°C. Afterwards, dispase solution was carefully removed and cells dissolved in 0.5 ml HBSS. Mechanical stress was then applied by pipetting 10 times with a 1 ml pipette. Finally, dissociation was quantified by counting and averaging cell fragments in 3 defined areas of each condition under a binocular microscope. For every condition, experiments were repeated at least 5 times.

**Statistics:** Differences in numbers of apoptotic cells were assessed using student’s T-test. In text and bar diagrams, values were expressed as mean ± standard error of the mean (SEM). Statistical significance was assumed for p < 0.05.
RESULTS

Acantholysis was observed in the absence of apoptosis in skin lesions from PV patients

We investigated biopsies from PV patients’ skin in order to determine whether apoptosis is absolutely required for acantholysis in PV. In this case, apoptosis would be expected to be present in all skin lesions as well as to involve a significant number of cells in the vicinity of the cleavage plane. Four biopsies from two PV patients were analyzed for apoptosis by staining with DAPI and TUNEL (Figure 1A). Untreated normal human skin was used as negative control (Figure 1A, a-c). From patients, biopsies were taken from macroscopically perilesional skin. Microscopically, intraepidermal split formation was present in some parts. In microscopically perilesional skin no nuclear changes and TUNEL reactivity was seen (not shown). In areas with intraepidermal splits, we found TUNEL reactivity that co-localized with initial nuclear condensation as detected by DAPI in the close vicinity of intraepidermal splits in one of the four PV biopsies (PV 1; Figure 1A, d-f). In another lesion (PV 2), TUNEL reactivity in the absence of nuclear condensation was found in cells from the blister roof only (Figure 1A, m-o). In the other two biopsies with microscopic PV lesions, no apoptosis was evident (Figure 1A, g-l). DNAse-treated normal skin served as positive control and showed TUNEL reactivity within the epidermis (Figure 1A, p-r). Moreover, cleaved caspase-3 was not detectable by immunostaining in control skin as well as in very early PV biopsies (shown for PV 1 and PV 2 in Figure 1B, e-g). HaCaT cells, in which apoptosis was induced with Fc-FasL (see below), served as positive control (Figure 1B, h). Collectively, these data demonstrate that acantholysis in PV patients’ skin can occur in the absence of apoptosis and hence apoptosis seems not to be required for lesion formation in the very early phase of the disease.

PV-IgG caused dissociation of cultured human keratinocytes without evidence of altered nuclear morphology and DNA fragmentation
Next, we investigated whether acantholysis in cultured keratinocytes was accompanied by apoptosis. To explore the apoptotic effects of PV autoantibodies, cultured HaCaT cells and normal human epidermal keratinocytes (NHEK) were incubated with affinity-purified IgG from 5 PV patients. We used NHEKs in parallel to HaCaT cells because the latter represent a spontaneously immortalized cell line (9). Nevertheless, HaCaT cells undergo apoptosis when treated with staurosporin, TRAIL of FASL (Figures 2, 3 and 5). Nuclear morphology was assessed by immunostaining with DAPI, DNA fragmentation by TUNEL assay, monolayer integrity and Dsg 3 expression by a Dsg 3-specific monoclonal antibody.

In the first set of experiments, the time-course of acantholysis in relation to nuclear morphology was addressed. HaCaT cells were incubated for 2, 6, 10, 24, and 48 h with IgG from 2 PV patients (PV-IgG 1 and 2), IgG pooled from 4 PV patients (PV-IgG pool), and normal IgG (control), respectively, at a final concentration of 150 µg/ml. In addition, staurosporin, a known inducer of apoptosis was applied. In controls, Dsg 3 was continuously located along cell junctions (shown for 48 h in Figure 2, a). After 2 h incubation with PV-IgG, no changes in Dsg 3 immunostaining (shown for PV-IgG 1 in Figure 2, b) were seen. After 6 h of PV-IgG incubation, fragmented Dsg 3 staining (shown for PV-IgG 1 in Figure 2, c) was visible. After 24h as well as after 48h, keratinocyte dissociation leading to formation of large intercellular gaps (arrows) and loss of Dsg 3-containing desmosomes occurred (Figure 2, d, e). Cell shrinkage, condensation of the nucleus, and karyorrhexis were noted after as early as 6h of staurosporin treatment (Figure 2, f). In contrast, no changes in nuclear morphology were seen in HaCaT cells treated with PV-IgG during the entire time course (Figure 2, b-e).

The nuclear changes induced in cultured HaCaT cells by incubation with PV-IgG (at final concentrations of 150 µg/ml) as well as staurosporin for 2, 6, 10, 24, and 48 h were quantified by evaluating 3 microscopic fields in 3 separate experiments after staining with DAPI (Figure
The number of apoptotic cells after treatment with control or PV-IgG was very low during the entire time course and slightly increased with longer incubation times to up to 4.6% (controls), 6.0% (PV-IgG 1), 3.4% (PV-IgG 2), and 1.8% (PV-IgG pool) after 48 h. At no time point, a significant difference (p<0.05) between control IgG- and PV-IgG-treated HaCaT cells was observed. In contrast, nuclear morphology was strikingly altered by incubation with staurosporin. After 2h, 2.7% of cells were apoptotic and after 6 h, 10 h, and 24 h of staurosporin, significantly more cells were apoptotic compared to incubation with control or PV IgG (p<0.05). The notion that after 24 h the number of apoptotic cells was reduced to 50.0% and to almost no cells after 48 h is explained by the increasing number of cells which have been completely degraded during prolonged apoptosis.

To exclude that apoptotic phenomena were missed in our system due to low IgG concentrations, the following experiments were performed using a 5-fold higher final concentration of 750 µg/ml IgG. No significant difference in the number of apoptotic cells was observed between controls and PV-IgG-treated HaCaT cells after 24 h (shown for staining with DAPI in Figure 2 h). As in all other experiments, the total cell number per visual field was controlled and did not differ significantly (p>0.05) between the various experimental groups.

In the next set of experiments, DNA fragmentation was assessed by TUNEL after incubation times of 2, 6, 10, 24, and 48h. Similar to controls (Figure 2, i, j), neither changes in nuclear morphology nor TUNEL reactivity were detected in cells treated with PV-IgG for 24 h, i.e. at the time-point when cell dissociation and reduced Dsg 3 staining were present, (shown for PV-IgG 1 and 2 in Figure 2, k-n). In contrast, using staurosporin, nuclear condensation, karyorrhexis and TUNEL reactivity were clearly detected already after 6 h (Figure 2, o, p). With time, a slight increase of apoptotic cells in PV-IgG-treated monolayers up to 6.0% and
3.4% was noted after 48h compared to 2.4% and 1.1% after 24h which was not significantly different to controls (Figure 2, q). When HaCaT cells were treated with staurosporin, TUNEL positivity was detected in 18.8% of cells after 6h and up to 70.9% after 24h which was significantly elevated compared to control and PV IgG-treated cells, respectively (p<0.05). After 48h of staurosporin, HaCaT cells were completely degraded and not evaluable for TUNEL reactivity.

To exclude that apoptosis in response to PV-IgG is impaired in immortal HaCaT keratinocytes, primary NHEK were employed in the following experiments. In controls, Dsg 3 was continuously distributed along cell junctions (Figure 3, a). Similar to experiments using HaCaT cells, after 24h incubation with PV-IgG acantholysis and fragmented Dsg 3 staining occurred but no change in the nuclear morphology was observed by DAPI staining and no significant DNA strand breaks were detected by TUNEL (shown for DAPI in Figure 3, b, c, e). Similar results were obtained after 48h (data not shown). In contrast, staurosporin mediated both nuclear condensation and DNA fragmentation after 6h and 24h, which was significantly increased compared to treatment with control and PV IgG, respectively (p<0.05), while after 48h, NHEK were completely degraded (Figure 3, d, e).

The caspase-inhibitor z-VAD-fmk and overexpression of FLIP\textsubscript{L} or FLIP\textsubscript{S} did not reduce PV-IgG-mediated acantholysis in cultured human keratinocytes

We found that PV-IgG-mediated cell dissociation and Dsg 3 degradation was not accompanied by apoptotic phenomena. However, it is not possible to completely rule out the contribution of apoptotic mechanisms only based on the finding that the assays used were not able to detect apoptosis under conditions where profound acantholysis was observed. To overcome this problem, we used the strategy to inhibit apoptosis by two different approaches to investigate whether PV-IgG-induced acantholysis is dependent on apoptotic mechanisms.
First, both HaCaT and NHEK were subjected to treatment with the caspase inhibitor z-VAD-fmk in addition to PV-IgG to investigate whether caspases were required for acantholysis. After 24h of treatment with PV-IgG 1 and PV-IgG 3, cell dissociation was initiated (arrows) and fragmented Dsg 3 immunostaining was obvious (Figure 4, a, c, e, g). These effects were not blocked by preincubation (20 µM, 1 h) of z-VAD-fmk (Figure 4, b, d, f, h). In control experiments, 2 known inducers of apoptosis, TNC-TRAIL, a fusion protein of the trimerization domain of tenascin-C (TNC) and TRAIL, and Fc-FasL, a fusion protein of 2 trimers of FasL and the Fc portion of immunoglobulin G1, were shown to induce apoptosis in cultured HaCaT cells which could then be greatly prevented by z-VAD-fmk (Figure 5, a). Moreover, even a longer preincubation time of 3 h or usage of an exceptional high dose of z-VAD-fmk (300 µM) failed to affect PV-IgG-induced reduced Dsg 3 staining (not shown).

In a second set of experiments, HaCaT cells stably transfected with FLIP<sub>L</sub> or FLIP<sub>S</sub> were analyzed. We used FLIP-overexpressing cells because it has been reported that PV-IgG lead to depletion of FLIP<sub>L</sub> whereas IVIG which blocked acantholysis increased expression of FLIP<sub>L</sub> indicating that FLIP<sub>L</sub> might be involved in blocking acantholysis (5). Under control conditions, FLIP expression was detectable in all cells of the FLIP-L and FLIP-S transfected HaCaT populations and was localized diffusely in the cytoplasm whereas endogenous FLIP in cells transfected with vector alone (mock-transfected) was not detectable (Figure 6, a-c). Because of homogenous expression of FLIP proteins in all cells potential effects of FLIP on PV-IgG treatment were expected to be observed throughout the monolayer. Following incubation with control IgG Dsg 3 was regularly distributed at cell borders similar to wild-type HaCaT cells (Figure 6 d-f). After 24h, incubation with PV-IgG 1 or 3 led to extensive fragmented Dsg 3 immunostaining in FLIP<sub>L</sub>- and FLIP<sub>S</sub>-expressing HaCaT cells as well as in mock-transfected cells (Figure 6, g-l). In control experiments, FLIP<sub>L</sub>- and FLIP<sub>S</sub>-transfected
HaCaT cells were shown to be almost completely protected by TNC-TRAIL- (Figure 5, b) and partly protected by Fc-FasL-induced apoptosis (Figure 5, c). Viability of keratinocytes was not significantly affected by treatment with PV-IgG 1 and 2 in FLIP<sub>L</sub>- and FLIP<sub>S</sub>-transfected cells compared to treatment with IgG from a healthy volunteer (control; p<0.05) as revealed by crystal violet staining (Figure 5, d). Taken together, these data indicate that caspase-mediated apoptosis is not required for PV-IgG-induced acantholysis.

**PV-IgG caused cytokeratin retraction and acantholysis in HaCaT monolayers in the absence of caspase-3 activation**

Finally, we analyzed whether cytokeratin retraction, which is a hallmark of PV pathogenesis both in vivo and in vitro (11, 45), and acantholysis occurred in the presence of the caspase inhibitor z-VAD-fmk or in FLIP<sub>S</sub>-protected cells. In controls, cytokeratin 5 formed dense meshworks in closely aligned wild-type, mock-transfected and FLIP<sub>S</sub>-expressing HaCaT cells (Figure 7, a-c). Treatment with PV-IgG 1 and 2 for 24 h caused strong cytokeratin retraction in all cell lines visible as detachment of filaments from cell junctions leading to accumulation of cytokeratin 5 staining around the nucleus (shown for PV-IgG 1 Figure 7, d-f), which was nor blocked by pre-treatment with caspase inhibitor z-VAD-fmk (20 µM, 1 h) in wild-type cells (Figure 7, g).

Because all experiments described so far showed that PV-IgG caused cytokeratin retraction, reduced Dsg 3 labelling and keratinocyte dissociation in the absence of apoptosis and caspase activation, it was important to investigate whether PV-IgG-induced effects were sufficient to cause significant acantholysis. Therefore, acantholysis was quantified using a standard dispase-based keratinocyte dissociation assay (Figure 8). PV-IgG 1 induced significant increase in the number of cell fragments and hence acantholysis in both wild-type and FLIP<sub>S</sub>-
expressing HaCaT cells. This effect was not significantly changed by z-VAD-fmk pre-treatment (20 µM, 1 h).

Finally, to rule out that early signs of apoptosis which may precede DNA fragmentation and nuclear condensation were present under conditions where PV-IgG caused acantholysis and morphologic alterations of keratinocyte monolayers, Western blotting was used to detect cleavage of caspase-3 which is required for activation of the enzyme (Figure 9). Under control conditions as well as in monolayers treated with PV-IgG 1 and 2 in the absence or presence of z-VAD-fmk (20 µM, 1 h pre-incubation), no p17 fragment indicative for caspase-3 activation was detectable. In contrast, Fc-FasL induced cleavage of caspase-3 in wild-type cells, but not in FLIP S-expressing HaCaT cells. Similarly, cleaved caspase-3 was detected by immunostaining following stimulation with Fc-FasL, but not when cell dissociation and cytokeratin retraction were induced by PV-IgG (data not shown).

Taken together, these results demonstrate that inhibition of death receptor-mediated apoptosis by FLIP overexpression as well as pharmacologic inhibition of the central effector caspase-3 did not prevent cytokeratin retraction and acantholysis in vitro. Moreover, activation of caspase-3 was not observed under these conditions.
DISCUSSION

Apoptosis is distinguished by structural and morphological features including cell shrinkage, plasma membrane blebbing, mitochondrial swelling, DNA fragmentation, nuclear condensation and karyorrhexis (25). The central event of the programmed cell death is the specific activation of caspases that cleave defined cellular target proteins finally leading to the morphologically hallmarks of apoptosis. Effector caspases, such as caspase-3, are activated by a complex process that involves the formation of the initiator caspase-8 activating death-inducing signalling complex (DISC) which is turn is triggered by activation of so-called death receptors (CD95/Fas, DR3, TNFR1, and TRAILR1/DR4 and TRAILR2/DR5) (reviewed in (30, 39)). Two isoforms of FLIP, FLIP⁰ and FLIP⁰, have been identified as potent inhibitors of DISC-mediated caspase-8 activation and, subsequently, of apoptosis (38).

In our study, acantholysis in lesions from PV patients as well as in cultured keratinocytes treated with PV-IgG, was found in the absence of detectable apoptosis. First, we investigated skin biopsies of PV patients. In order to minimize possible apoptosis occurring secondarily in response to cell dissociation we aimed at very early lesions. Therefore, perilesional skin biopsies were taken which macroscopically showed no signs of blister formation. When examined microscopically, however, both acantholysis and intraepidermal split formation were present in some parts of the biopsy. In none of them, apoptosis was detected in the intact parts of the biopsies and in only 2 out of 4 lesions, apoptosis was observed in close vicinity to areas where acantholysis was present. Moreover, hallmarks of apoptosis such as nuclear condensation and karyorrhexis were observed only in few cells in one lesion (PV 1) or were confined to the blister roof only (PV 2) and thus were unlikely to significantly contribute to acantholysis in these lesions. Activation of caspase-3 was completely absent in skin biopsies. In contrast, others did observe signs of programmed cell death in PV lesional skin (20, 40). In these reports, lesional skin was examined with inflammatory cells already present at the site
of acantholysis (40). It is therefore conceivable that apoptosis observed in these specimens appears more likely to have followed acantholysis rather than to be the cause of keratinocyte dissociation.

We then studied whether PV-IgG-induced cell dissociation in cultured keratinocytes was dependent on apoptosis. We were unable to demonstrate apoptosis in both HaCaT cells and NHEK. Especially, no TUNEL reactivity was observed and cleavage of caspase-3 was absent in Western blotting analyses while fragmented Dsg 3 immunostaining, cytokeratin retraction, cell dissociation and acantholysis were clearly detectable after 24 h of incubation with PV-IgG. In a different approach, we were also unable to prevent acantholysis in HaCaT Cells by addition of the caspase inhibitor z-VAD-fmk or by overexpression of FLIP<sub>S</sub> and FLIP<sub>L</sub>. Even after employing longer incubation times of up to 48h and higher PV-IgG concentrations of 750 µg/ml, no evidence of apoptosis was detected. In all experiments, no differences between the different PV IgG preparations were noted.

Remarkably, most other investigators who found apoptosis in PV have used longer incubation times of 48 h to 72 h (5, 6, 18, 19, 40, 41). Acantholysis, however, already occurs after 24h both in cultured keratinocytes and in neonatal balb/c mice treated with PV-IgG.(1, 2, 5, 33, 44) With regard to these results one may hypothesize that acantholysis is not caused by apoptosis but rather vice verse, acantholysis precedes apoptosis. In fact, loss of cell-matrix contact of epithelial cells including cultured keratinocytes has been described to induce apoptosis, a phenomenon termed anoikis (10, 17, 35). This notion would be compatible with the recent hypothesis that autoantibodies in PV inhibit the survival kinase Akt (27). The apoptotic phenomena observed in cultured keratinocytes after prolonged incubation with IgG may thus be explained, at least in part, by anoikis. Only Pelacho et al. showed soluble histone-DNA complexes, caspase 3 activation and Bcl-2 depletion after 8 h incubation of cultured
keratinocytes with PV-IgG at a concentration of 1 mg/ml (29). Chernyavsky and colleagues detected apoptosis by TUNEL reactivity starting after 6 h of PV-IgG treatment (13). However, according to their data, cell shrinkage and intercellular dyshesion was present as early as after 120 min, i.e. before apoptosis was detected by TUNEL reactivity.

A further reason why our data are not in line with results by other investigators may be due to different IgG concentrations used. While in our experimental system, a concentration of 150 µg/ml PV-IgG is sufficient to induce cell-cell dissociation of cultured keratinocytes and loss of Dsg 3-containing desmosomes (33, 43, 44), in the majority of reports that described apoptosis in cultured keratinocytes PV-IgG was applied in 15-50-fold higher concentrations of 2.5 and 7.5 mg/ml (18, 19, 40, 41). It may thus be speculated whether the apoptotic phenomena seen by these authors may be related to the high IgG concentrations used.

Interestingly, high levels of FasL have been observed in pemphigus sera (31). Contamination of concentrated PV-IgG with FasL, a potent activator of apoptosis, could have contributed to the observation of apoptosis in high-dose PV-IgG-treated keratinocyte cultures. The induction of p53 and HSP-70 as well as TUNEL reactivity and downregulation of Bcl-2 in cultured keratinocytes using 1:1 diluted PV serum (6) could be explained by the presence of FasL in this serum. Accordingly, the successful use of IVIG in another life-threatening blistering disease associated with high circulating FasL levels, toxic epidermal necrolysis, has been explained by the neutralization of FasL by IVIG (36). The authors demonstrated elimination of Fas-mediated keratinocyte apoptosis by preincubation with IVIG and the inactivation of lytically FasL in the patients’ serum (36).

Nevertheless, in some studies caspase inhibitors were effective to block PV-IgG-induced acantholysis in vitro indicating that if acantholysis was paralleled by apoptosis under
experimental conditions, apoptosis might be involved in the mechanisms contributing to keratinocyte dissociation (5, 40, 41).

Taken together, our study demonstrates that acantholysis in early pemphigus skin lesions as well as in keratinocyte cultures in vitro occurs in the absence of apoptosis and that inhibition of apoptosis does not ameliorate the effects of PV-IgG such as keratinocyte dissociation and loss of desmosomes. Therefore, apoptosis which is detectable in some PV skin lesions and also may occur under some experimental conditions in vitro, appears to arise secondary to acantholysis, a process which is referred to as anoikis.
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The authors declare that no conflict of interest exists for any part of the study.
References

16. **Esaki C, Seishima M, Yamada T, Osada K, and Kitajima Y.** Pharmacologic evidence for involvement of phospholipase C in pemphigus IgG-induced inositol 1,4,5-
**Figure legends**

**Figure 1**

Acantholysis was present in the absence of detectable apoptosis in PV patients’ skin. Four biopsies of 2 PV patients were analyzed. Biopsies were taken from macroscopically perilesional skin that microscopically, in some parts, revealed intraepidermal splits that thus represent very early lesions. To better identify pemphigus lesions and evaluate epidermal desmosomes, sections were stained with monoclonal antibody to desmoglein 3 (A c, f, i, l, o, r and B a-c).

A. Labelling with TUNEL was performed to visualize DNA fragmentation (a, d, g, j, m, p). Merged staining for TUNEL and DAPI (to detect nuclear changes) is shown in panels b, e, h, k, n, and q. Normal human skin served as negative control (a-c). In one of the 4 PV biopsies with microscopic lesions (PV 1), TUNEL reactivity that co-localized with initial nuclear condensation as detected by DAPI was found in the close vicinity of intraepidermal splits (d, e). In another biopsy (PV 2), TUNEL reactivity in the absence of nuclear condensation was restricted to cells of the blister roof (m, n). In the other 2 PV biopsies with microscopic lesions, no apoptosis was evident (g-l). DNAse-treated normal skin was used as positive control (p-r). Magnified views (x3) of selected areas are shown as inserts (e, n, q).

B. Immunostaining for cleaved caspase-3 was negative in control epidermis and in lesions from PV 1 and PV 2 (e-g). HaCaT cells treated with Fc-FasL served as positive control (d, h). Scale bar is 50 µm for all panels. Representative panels from at least 5 separate experiments are shown.

**Figure 2**

PV-IgG caused dissociation of cultured HaCaT cells without evidence of altered nuclear morphology and DNA fragmentation. Cultured HaCaT cells were incubated with IgG from 2 PV patients (PV-IgG 1, PV-IgG 2), 4 pooled PV sera (PV-IgG pool), and a healthy volunteer (control, as well as with staurosporin, respectively. HaCaT cells were then stained
with a monoclonal antibody to desmoglein 3 (Dsg 3) to label desmosomes, with DAPI to evaluate nuclear morphology, and with TUNEL to detect DNA fragmentation.

A time course study by incubation of HACaT Cells with 150 µg/ml IgG of PV-IgG 1 is shown in panels b-e, stainings for Dsg 3 and DAPI were overlaid (Dsg3 + DAPI). After 6 h of incubation with PV-IgG, initial fragmentation of Dsg 3 was present (c). After incubation periods of 24h and 48h, keratinocyte dissociation leading to formation of large intercellular gaps (arrows) and loss of Dsg 3-containing desmosomes occurred (d, e). After treatment with staurosporin for 6 h, cell shrinkage, condensation of the nucleus, and karyorrhexis were noted (f). In contrast, nuclear morphology remained unchanged after treatment with PV-IgG during the entire time course (b-e). Representative pictures of at least 5 separate experiments are shown. Changes in nuclear morphology were quantified after treatment with PV-IgG in a concentration of 150 µg/ml IgG for varying incubation times (g) and for 24 h with different IgG concentrations (h; 1:4, 750 µg/ml IgG; 1:20, 150 µg/ml IgG). HaCaT cells were labelled with DAPI and changes in nuclear morphology were recorded in at least three microscopic fields in 3 separate experiments and expressed as % of total cells. Average cell numbers per microscopic field were 150. In contrast to incubation with PV or control IgG, after incubation with staurosporin an altered nuclear morphology was seen as early as after 2h with in 3% of cells and increased over time to 58% of cells after 10 h. After 24 h the number of apoptotic cells was reduced to 50% and after 48 h almost no cells were visible which is explained by the increasing number of cells which were destroyed during prolonged apoptosis (h). No statistically significant difference was seen between control IgG- and PV-IgG-treated cells irrespective of the IgG concentration (h).

To detect DNA fragmentation TUNEL staining was applied (i-q) after treatment of HaCaT cells for 24 h with 150 µg/ml IgG from a healthy volunteer (control; i) and 2 PV patients (k-n) as well as with staurosporin (o, p), respectively. To evaluated nuclear morphology DAPI staining was also performed and pictures from DAPI and TUNEL stains were overlaid
(TUNEL + DAPI; j, l, n, p). No changes in nuclear morphology and TUNEL reactivity were detected in cells treated with PV-IgG (k-n) and control IgG (I, ja-e), respectively. In contrast, in cells subjected to staurosporin, nuclear condensation, karyorrhexis, and TUNEL reactivity were clearly visible (o, p). Representative pictures of at least 5 separate experiments are shown. Changes in DNA fragmentation as revealed by TUNEL reactivity were finally quantified after cultured HaCaT cells were incubated for 2 h, 6 h, 10 h, 24 h, and 48 h with 150 µg/ml IgG from a healthy volunteer (control) and 2 PV patients (PV-IgG 1, PV-IgG 2) as well as with staurosporin, respectively (q). Whereas no change was observed between cells incubated with control IgG compared to PV-IgG. Treatment with staurosporin resulted in TUNEL positivity in 19%, 42%; and 71% of cells after 6 h; 10 h, and 24 h incubation periods. The notion that after 48 h of staurosporin treatment no cells were seen is due to the complete degradation of cells. Mean and SEM from 3 different experiments are shown (q).

Figure 3

In normal human epidermal keratinocytes (NHEK) acantholysis induced by PV-IgG was neither accompanied by changes in the nuclear morphology nor by DNA fragmentation. Cultured NHEK derived from adult skin were incubated for 24 h with 150 µg/ml IgG from a healthy volunteer (control; a) and 2 PV patients (PV-IgG 1; b; PV-IgG 3, c) as well as with staurosporin (d), respectively. Labelling with DAPI to evaluate nuclear morphology and a monoclonal antibody to desmoglein 3 (Dsg 3) to visualize desmosomes were overlaid (a-d). In controls, signs of neither acantholysis nor apoptosis were present (a). In cells treated with PV-IgG, acantholysis developed as detected by keratinocyte dissociation leading to formation of large intercellular gaps (arrows) and loss of Dsg 3-containing desmosomes while nuclear morphology remained unchanged (b, c). Representative pictures of at least 5 separate experiments are shown.
Changes in DNA fragmentation as revealed by TUNEL reactivity were quantified. TUNEL reactivity was very low in cells incubated with PV-IgG and slightly increased in control IgG-treated cells. This increase was, however, not statistically significant compared to cells subjected to patients’ IgG. In contrast, strong TUNEL positivity resulted from incubation with staurosporin after 6 h and 24 h; after 48 h, absence of TUNEL reactivity was due to complete degradation of cells. Mean and SEM from 3 different experiments are shown.

**Figure 4**

**A caspase inhibitor did not block acantholysis mediated by PV-IgG in cultured human keratinocytes.** NHEKs (a, b, e, f) and HaCaT cells (c, d, g, h) were treated with (b, d, f, h) or without (a, c, e, g) the caspase inhibitor z-VAD-fmk at a concentration of 20 mM for 1 h followed by a 24 h incubation period with 150 µg/ml IgG from 2 PV patients (PV-IgG 1; a-d; PV-IgG 3, e-h) and a healthy volunteer (not shown). All cells were stained with a desmoglein 3-specific monoclonal antibody to visualize desmosomes. Acantholysis as characterized by both cell dissociation that resulted in the formation of large intercellular gaps (arrows) and loss of Dsg 3-containing desmosomes was seen in all PV-IgG-treated cells irrespective of the type of keratinocytes or preincubation with z-VAD-fmk. Scale bar is 20 µm. Representative pictures of at least 5 separate experiments are shown.

**Figure 5**

**Caspase inhibitor z-VAD-fmk and overexpression of FLIP-L- and FLIP-S efficiently blocked receptor-induced apoptosis whereas PV-IgG did not cause apoptosis in HaCaT.** A. HaCaT cells were stimulated in triplicates for 16 h with anti-Flag-oligomerized TNC-Flag-TRAIL and Fc-Flag-FasL in the presence (black columns) and absence (gray columns) of 20 µM z-VAD-fmk. Analysis of cell viability by crystal violet staining revealed that TNC-TRAIL-mediated apoptosis (central column pair) was substantially reduced and Fc-FasL-
induced apoptosis could be abrogated (right column pair) by the addition of z-VAD-fmk (z-VAD).

**B. and C.** HaCaT cells were stably transfected with the empty pEGZ vector (Vec.) and the corresponding FLIP_L- and FLIP_S-encoding derivatives, respectively. Black columns indicate subsequent stimulations for 16 h with anti-Flag-oligomerized TNC-Flag-TRAIL (B) and Fc-Flag-FasL (C). Unstimulated cells are represented by gray columns. Cell viability was evaluated by crystal violet staining and showed that FLIP_L- and FLIP_S-expressing cells were resistant to the TNC-TRAIL-mediated apoptosis (B) and less susceptible to Fc-FasL-triggered apoptosis (C) compared to cells transfected with the vector alone (VEC).

**D.** HaCaT cells stably transfected with empty vector (Vec.) or expressing FLIP_L and FLIP_S were treated with PV-IgG 1 and 2 (150 µg/ml) or Fc-FasL for 24 h. Cell viability was evaluated by crystal violet staining which showed that PV-IgG did not induce apoptosis in any cell line used. Fc-FasL caused apoptosis in empty vector-transfected cells only, whereas the corresponding FLIP_L- and FLIP_S-expressing transfectants were resistant. Means and SEM of 3 experiments are given.

**Figure 6**

HaCaT cells overexpressing FLIP_L and FLIP_S were still susceptible to the pathogenic effect of PV-IgG. FLIP_L (b, e, h, k) and FLIP_S (c, f, i, l) was stably overexpressed in cultured HaCaT cells. Cells transfected with the vector alone were used as controls (HaCaT-MOCK; a, d, g, j). FLIP was detected by immunostaining in FLIP_S- and FLIP_L- expressing cells but not in HaCaT cells transfected with empty vector (a-c). Cells were subjected to IgG from a healthy volunteer (control; d-f) and 2 PV patients (PV-IgG 1, g-i; PV-IgG 3, j-l) for 24 h at a concentration of 150 µg/ml. To visualize desmosomes cells were labelled with a monoclonal anti-desmoglein 3 (Dsg 3) antibody. In all cells, treatment with PV-IgG resulted in both cell dissociation presenting as large intercellular gaps (arrows) and loss of Dsg 3-containing
desmosomes reflected by the rarefaction of Dsg 3 expression at the cell borders (d-l). As expected, control IgG did not lead to any changes (a-c). Scale bar is 20 µm. Representative pictures of at least 5 separate experiments are shown.

**Figure 7**

*z*-VAD-fmk and overexpression of FLIP<sub>L</sub>- and FLIP<sub>S</sub> failed to block PV-IgG-induced cytokeratin retraction. Normal HaCaT cells and FLIP<sub>L</sub>- and FLIP<sub>S</sub>-expressing HaCaT transfectants were incubated with PV-IgG for 24 h and immunostained using a monoclonal antibody directed to cytokeratin 5. In untreated monolayers, cytokeratin 5 formed dense meshworks in the cytoplasm of all cell lines (a-c) whereas PV-IgG (150 µg/ml) caused profound cytokeratin retraction as revealed by perinuclear accumulation of cytokeratin filaments (d-f), an effect that was not reduced by pre-treatment with z-VAD-fmk in parental HaCaT cells (g). Scale bar is 20 µm, representative panels from at least 5 separate experiments are shown.

**Figure 8**

Apoptosis was not required for PV-IgG-induced acantholysis. Acantholysis was quantified using a dispase-based assay. Incubation with PV-IgG (150 µg/ml) resulted in a strong increase of cell fragments compared to controls. PV-IgG-mediated acantholysis was independent on apoptosis because both FLIP<sub>S</sub>-expression as well as pre-treatment with z-VAD-fmk did not prevent increase in cell fragments. Representative panels from at least 5 separate experiments are shown.

**Figure 9**
PV-IgG did not induce caspase-3 processing in HaCaT monolayers. Caspase-3 processing was evident by anti-caspase-3 western blotting in lysates of HaCaT cells undergoing apoptosis due to stimulation with Fc-FasL (200 ng/ml). No caspase-3 processing was detected by western blotting in samples derived from HaCaT cells or FLIP$_S$-expressing transfectants upon incubation with PV-IgG (150 µg/ml). Note that FLIP$_S$-expressing cells were resistant to treatment with Fc-FasL. Representative panels of 3 separate experiments are shown.
Tables

Table 1. Autoantibody profile of pemphigus vulgaris patients

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Dsg 1 (U/ml)</th>
<th>Dsg 3 (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV-IgG 1</td>
<td>-</td>
<td>1177</td>
</tr>
<tr>
<td>PV-IgG 2</td>
<td>55</td>
<td>1239</td>
</tr>
<tr>
<td>PV-IgG 3</td>
<td>-</td>
<td>1875</td>
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<tr>
<td>PV-IgG pool:</td>
<td></td>
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<tr>
<td>PV-IgG 1/2</td>
<td>see above</td>
<td>see above</td>
</tr>
<tr>
<td>PV-IgG 4</td>
<td>535</td>
<td>1098</td>
</tr>
<tr>
<td>PV-IgG 5</td>
<td>60</td>
<td>1000</td>
</tr>
</tbody>
</table>

ELISA, enzyme-linked immunosorbent assay

Dsg, desmoglein

1 performed according to the manufacturer’s instructions (Medical and Biological Laboratories, Nagoya, Japan) The cut-off value was 14 U/ml for Dsg 1 and 7 U/ml for Dsg 3; all samples were run in duplicate.
Figure 1A
Figure 1B
Figure 2
Figure 3
Figure 5
Figure 6
Figure 7
Figure 9