Fas Ligand in Pemphigus Sera Induces Keratinocyte Apoptosis through the Activation of Caspase-8

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The Fas/Fas ligand system triggers the extrinsic apoptotic pathway and is involved in several inflammatory conditions, also at the skin level. The Fas/Fas ligand cell death pathway plays a major role in anoikis, a type of apoptosis characterized by cell detachment. As pemphigus is characterized by loss of cell to cell adhesion, we evaluated the role of anoikis and Fas ligand in this bullous disease. We report that, in suprabasal epidermis from perilesional pemphigus skin, most keratinocytes are apoptotic. Moreover, Fas ligand levels are markedly increased in sera from pemphigus patients, whereas they are undetectable in sera from patients undergoing steroid treatment. Sera from untreated patients but not from patients under steroids induce keratinocyte apoptosis. Pemphigus-sera-induced cell death is partially inhibited by pretreatment with anti-Fas ligand antibodies and by incubation with caspase-8 inhibitor Z-IETD-FMK. Finally, caspase-8 is activated in keratinocytes provided with sera from pemphigus patients, whereas cleavage is partially blocked by pretreatment of sera with anti-Fas ligand antibody. These results suggest that increased Fas ligand in pemphigus sera is responsible for keratinocyte apoptosis, which occurs through the activation of a caspase-8-driven extrinsic apoptotic pathway. ***Key words: acantholysis/anoikis/corticosteroids/Fas.***
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Apoptosis plays a fundamental role in the regulation of cellular homeostasis and is involved in many pathophysiologic processes, including viral infection and cancer. Apoptosis can follow both cell to cell detachment (Bergin *et al*, 2000; Rezgui *et al*, 2000) and loss of cell–matrix interaction (Giancotti and Ruoslahti, 1999). This particular type of cell death has been called "anoikis" and may operate in epithelial cells (Frisch and Francis, 1994; Grossman *et al*, 2001) as well as in normal epidermis (Tiberio *et al*, 2002). Although the signaling pathways involved in anoikis are still incompletely understood (Frisch and Screaton, 2001), evidence has been reported recently for a function of Fas-related proteins in this type of apoptosis (Frisch, 1999; Rytomaa *et al*, 1999).

Fas is a member of the tumor necrosis factor receptor superfamily that, upon binding with Fas ligand (FasL), triggers apoptosis in many cell systems (Sharma *et al*, 2000). Intracellular signaling of Fas–FasL-induced apoptosis operates via recruitment of a number of adaptor molecules such as FADD (Fas-associated death domain) and FLICE (FADD-like ICE, caspase-8), which in turn is inhibited by FLIP (FLICE inhibitory protein) (Juo *et al*, 1998). Fas–FasL interaction is involved in the pathomechanisms of several immune-inflammatory and infectious conditions, such as AIDS (Bahr *et al*, 1997) and systemic lupus erythematosus (Kovacs *et al*, 1997). Cutaneous diseases characterized by an implication of the Fas–FasL pathway include acute graft *versus* host disease, toxic epidermal necrolysis, and melanoma (Wehrli *et al*, 2000). It has been shown recently that Fas–FasL-induced cell death is inhibited by β1 integrin, a crucial adhesion molecule for preventing anoikis (Aoudjit and Vuori, 2001).

Pemphigus is characterized by loss of adhesion of suprabasal keratinocytes that round up in a process known as acantholysis. Pathogenesis of pemphigus is undergoing a major revision, mostly because, in addition to antidesmoglein antibodies, a new group of anticholinergic receptor antibodies can induce acantholysis (Kalish, 2000). Independently from the autoantibodies involved, the question whether apoptosis plays a role in the pathogenesis of pemphigus has never been addressed. Recently, a relationship between "anoikis" and acantholysis has been proposed (Gniadecki *et al*, 1998). We present evidence that increased levels of FasL in sera from pemphigus patients induce apoptosis in human keratinocytes, through the activation of caspase-8.

MATERIALS AND METHODS

Patients Sera were collected from 23 patients with pemphigus vulgaris and 13 patients with pemphigus foliaceous. Patients were divided into two groups: one group was on a regular steroid therapy (1–2 mg per kg per d) for at least 2 wk; a second group included sera from untreated patients. Sera were also collected from eight healthy volunteers.

Enzyme-linked immunosorbent assay (ELISA) Soluble FasL quantitation was performed by a two-site enzyme immunoassay (MBL, Nagoya, Japan) according to the manufacturer's instructions. Serum concentration was determined by absorbance at 450 nm against recombinant human FasL standard protein.

Keratinocyte cultures Normal human keratinocytes were obtained from foreskin and cultured as described previously (Pincelli *et al*, 1997). Briefly, keratinocytes were plated on mytomicin C-treated 3T3 cells (2.4×10^4 per cm^2, ATCC, Rockville, MD) and cultivated in Dulbecco's modified Eagle's medium and Ham's F12 medium. Subconfluent

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Abbreviation: FasL, Fas ligand.

secondary cultures were trypsinized with 0.05% trypsin/0.02% ethylenediamine tetraacetic acid and replated for the experiments in defined serum-free medium (KGM, Clonetics, San Diego, CA).

TUNEL staining Keratinocytes and skin sections were fixed in paraformaldehyde [4% in phosphate-buffered saline (PBS)], permeabilized with Triton X (0.1%) and sodium citrate (0.1%) at 4°C, and incubated with fluorescein-labeled nucleotides and terminal deoxynucleotidyl transferase (TdT) (TUNEL kit, Boehringer) for 1 h at 37°C. Approximately 100 cells were evaluated, in randomly selected high power fields, and the percentage of TUNEL-positive cells was counted. Each experiment was repeated three times. Negative control was obtained by replacing the primary incubation with a nucleotide mixture without TdT. Fluorescent specimens were analyzed by confocal scanning laser microscopy (Leica TCS4D) in conjunction with a conventional optical microscope (Leica DM IRBE).

Crystal violet staining Subconfluent keratinocyte cultures were seeded in a 96-well tissue culture plate. At 48 h after addition of sera, cells were fixed with formaldehyde solution and stained with 0.5% crystal violet in 20% methanol solution. After washing, the extractive solution was added and the samples were analyzed using a scanning multiwell spectrophotometer at 540 nm. Results are expressed as optical density units (OD) and as the mean ±SD of three different experiments. Student's *t* test was used for comparison of the means.

Western blot analysis Cells were washed with PBS and lyzed on ice in RIPA buffer pH 7.5, as described previously (Marconi *et al*, 1999). Thirty micrograms of total protein were analyzed on 12% polyacrylamide gels and blotted onto nitrocellulose membranes. To verify equal loading of total proteins in all lanes, the membranes were stained with Red Ponceau. The blots were blocked for 2 h in blocking buffer (PBS buffer, pH 7.4, with 0.2% Tween-20 and 5% nonfat milk) and incubated overnight at 4°C with 2 μg per ml antihuman caspase-8 monoclonal antibody (PharMingen, San Diego, CA) and with anti-β-actin monoclonal antibody (1:1000, Sigma, St. Louis, MO) as a control. Then membranes were washed in PBS/Tween-20, incubated with peroxidase-conjugated goat antimouse antibody (Biorad, 1:800, Hercules, CA) for 45 min at room temperature, washed, and developed using the enhanced chemiluminescence detection system (Amersham).

RESULTS AND DISCUSSION

We first evaluated the presence of apoptosis in epidermis from perilesional skin in sections from untreated pemphigus patients. In suprabasal layers from perilesional epidermis most keratinocytes are apoptotic, compared to normal skin (**Fig 1**). This seems to indicate that keratinocyte cell death (anoikis) occurs before the detachment of keratinocytes leading to acantholysis.

As the Fas/FasL system is implicated in many apoptotic processes also at the skin level (Wehrli *et al*, 2000), we measured FasL levels in sera from pemphigus patients. FasL levels were very high in sera from untreated patients and below the limit of detection in sera from patients treated with corticosteroids or in sera from healthy subjects (**Fig 2**).

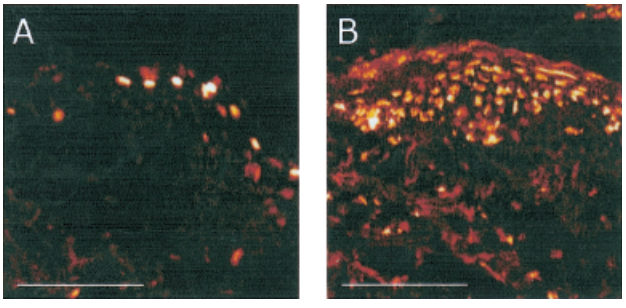

Figure 1. Apoptotic keratinocytes in pemphigus lesions. Skin sections from normal skin (*A*) and perilesional skin (*B*) were fixed in paraformaldehyde, permeabilized with Triton X (0.1%) at 4°C, as described in *Materials and Methods*. Sections were then incubated with fluorescein-labeled nucleotides and TdT (TUNEL) for 1 h at 37°C. *Scale bar*: 50 μm.

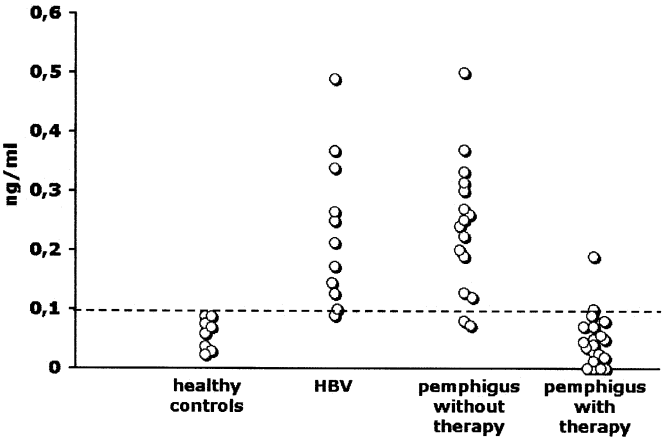

Figure 2. Detection of FasL in pemphigus sera. Pemphigus sera were collected before and after steroid treatment. Sera from healthy subjects and from patients with hepatitis B were collected as controls. FasL levels were then measured by ELISA.

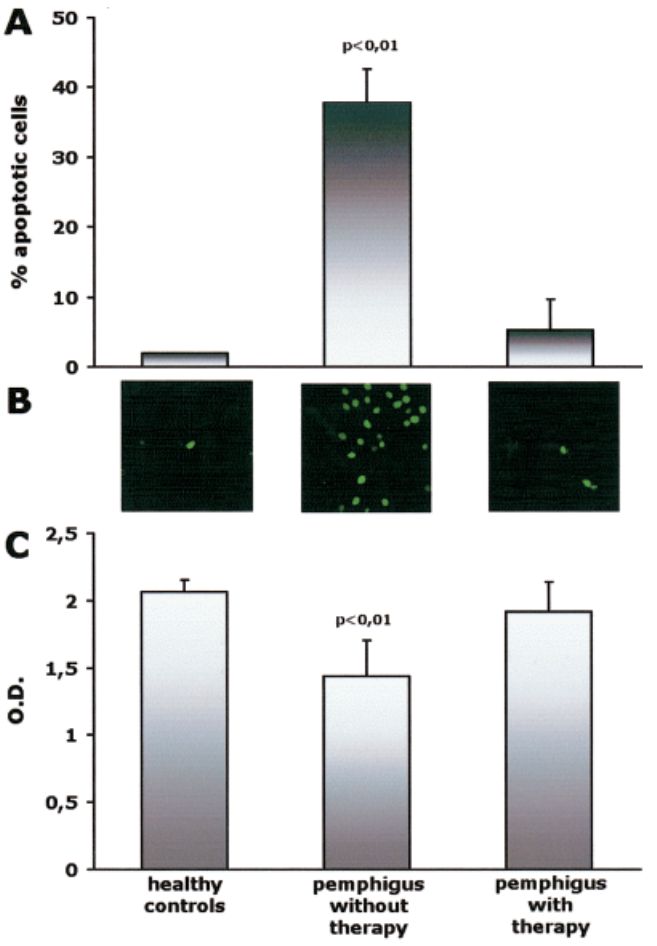

Figure 3. Pemphigus sera induce keratinocyte apoptosis. (*A*) Keratinocytes were plated in chamber slides and cultured in serum-free medium (KGM) up to preconfluence. Cells were then cultured in keratinocyte basal medium and treated for 48 h with the addition of 25% serum from either untreated patients ($N=4$) or patients treated with systemic corticosteroids ($N=5$). Sera from healthy subjects ($N=5$) were used as controls. Apoptosis was evaluated by TUNEL staining *in situ*. Approximately 100 cells were evaluated, in randomly selected high power fields, and the percentage of TUNEL-positive cells was counted. (*B*) Fluorescent specimens were analyzed by confocal scanning laser microscopy (Leica TCS4D). (*C*) Keratinocytes were seeded in 96-well plates and cultured as in (*A*). At 48 h, cells were fixed and stained with crystal violet. Cells were then eluted and analyzed by spectrophotometer. Each experiment was carried out in sextuplicate. Student's *t* test was used for comparison of the means.

As apoptotic keratinocytes are abundantly expressed in pemphigus, we wanted to explore whether pemphigus sera are capable of inducing apoptosis in normal human keratinocytes. To this purpose, keratinocytes were provided sera from patients with pemphigus, before or after corticosteroid treatment. Sera from pemphigus but not from healthy subjects or patients undergoing steroid treatment induced apoptosis in human keratinocytes (**Fig 3*A*, *B***). In addition, cell yield was significantly higher in keratinocytes treated with sera from healthy subjects or from patients on steroid therapy than in cells provided with pemphigus sera (**Fig 3*C***).

As pemphigus sera contain high levels of FasL and induce keratinocyte apoptosis, we asked whether this "death receptor" ligand could be responsible for cell death. To this purpose patient sera were pretreated with anti-FasL neutralizing antibody and added to keratinocyte cultures. Addition of anti-FasL neutralizing antibody partially inhibited pemphigus-sera-induced keratinocyte apoptosis (**Fig 4**). Consistent with our results, FasL plays a crucial role in the pathogenesis of toxic epidermal necrolysis, a disease that, similarly to pemphigus, is characterized by apoptotic keratinocytes (Viard *et al*, 1998). In addition, our observation is in good agreement with the recent report of anoikis being inhibited by anti-FasL antibodies (Aoudjit and Vuori, 2001).

The Fas/FasL system is the major trigger of the extrinsic apoptotic pathway, which implies the early activation of the initiator caspase-8 (Reed, 2000). In order to evaluate the involvement of this pathway in the FasL-induced keratinocyte apoptosis, we pretreated pemphigus sera with caspase-8 inhibitor Z-IETD-FMK before keratinocyte stimulation. Inhibition of caspase-8 activation significantly blocked pemphigus-sera-induced keratinocyte apoptosis (**Fig 4**). In addition, caspase-8 was markedly activated in keratinocytes treated with pemphigus sera compared to untreated cells, whereas caspase cleavage was partially inhibited by pretreatment with anti-FasL antibody (**Fig 5**). Taken together, these data suggest that pemphigus sera induce keratinocyte apoptosis through the extrinsic apoptotic pathway triggered by the Fas/FasL system. This evidence is consistent with the recent literature that confirms the caspase-8-driven apoptotic pathway as the predominating mechanism in anoikis (Frisch, 1999; Rytomaa *et al*, 1999). Moreover, it was reported that transfection of caspase-8 augments anoikis in gastric carcinoma cells (Nishimura *et al*, 2001). Finally, caspase-8 activation is an early event in anoikis determined by anti-β1-integrin treatment of human keratinocytes.[1]

In conclusion, our results suggest that high levels of FasL in pemphigus sera induce apoptosis in cultured human keratinocytes through the activation of the caspase-8 pathway. Moreover, FasL is most probably responsible for the high degree of apoptosis observed *in vivo* in keratinocytes from perilesional pemphigus skin. Finally, our results indicate that corticosteroids, the most effective therapy for pemphigus, strikingly reduce FasL, raising the idea that such a treatment also works through the modulation of the apoptotic process. Future studies will address whether anoikis and acantholysis are the same phenomenon or whether keratinocytes need to undergo DNA fragmentation and apoptosis before detaching from each other. In this respect, it is interesting to note that desmosomes are proteolytically targeted during apoptosis and caspase-3 has been shown to cleave desmoglein-3, the antigen of pemphigus vulgaris (Weiske *et al*, 2001).

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[1]Fila C, Marconi A, Krajewski S, Reed JC, Giannetti A, Pincelli C: Caspase-8-activated signal predominates in keratinocyte anoikis. 63rd Annual Meeting of the Society for Investigative Dermatology, Los Angeles, CA, May 15–18, 2002

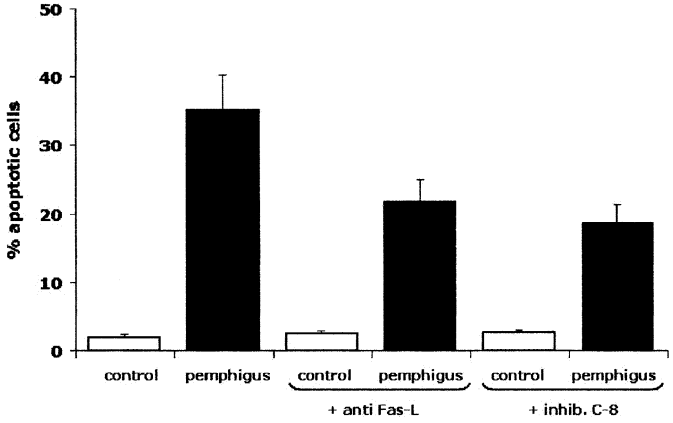

Figure 4. Inhibition of pemphigus-sera-induced keratinocyte apoptosis. Keratinocytes were cultured in KGM and treated with pemphigus sera ($N=3$) or with sera from untreated patients ($N=3$). Sera were pretreated with anti-FasL neutralizing antibody (2.5 μg per ml for 30 min, PharMingen) or caspase-8 inhibitor Z-IETD-FMK (100 μm for 30 min, Sigma). Apoptosis was evaluated by TUNEL staining, as described in **Fig 3**. Pemphigus sera *versus* pemphigus sera+anti-FasL, $p<0.01$; pemphigus sera *versus* pemphigus sera+caspase-8 inhibitor, $p<0.01$.

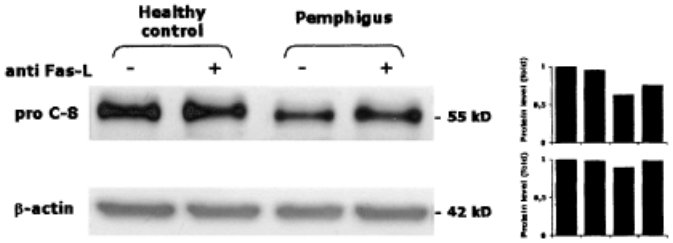

Figure 5. Inhibition of caspase-8 activation. Keratinocytes were treated as in **Fig 4** and provided with either anti-FasL antibody or irrelevant immunoglobulins. Cells were then lyzed in RIPA buffer, as described in *Materials and Methods*. Membranes were incubated with anti human caspase-8 or anti-β-actin antibodies. The relative intensity of bands on autoradiograms was quantified by scanning laser densitometry.

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