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Pemphigus vulgaris autoantibodies induce apoptosis in HaCaT keratinocytes

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Abstract Pemphigus vulgaris (PV) is an autoimmune disease characterized by binding of IgG autoantibodies to epidermal keratinocyte desmosomes. IgG autoantibodies obtained from a patient with mucocutaneous PV reacted with plakoglobin (Plkg) in addition to desmoglein-3 (Dsg3) and Dsg1. Immunofluorescence analysis confirmed that IgG autoantibodies, unlike antibodies from a healthy volunteer, caused disruption of cell–cell contacts in HaCaT keratinocytes. Moreover, apoptosis was enhanced in cells treated with autoantibodies compared to those treated with normal antibodies. The apoptotic process induced by IgG autoantibodies was characterized by caspase-3 activation, Bcl-2 depletion and Bax expression. The present report demonstrates that PV IgG autoantibodies promote apoptosis in HaCaT keratinocytes.

Keywords: Pemphigus vulgaris; Desmoglein; Acantholysis; Apoptosis; Human keratinocyte; IgG autoantibody

1. Introduction

Pemphigus vulgaris (PV) is a bullous autoimmune disease of the mucocutaneous surfaces, characterized histologically by suprabasal acantholytic vesicles caused by the separation of epidermal cells. Immunofluorescence (IF) studies have demonstrated the presence of autoantibodies in epidermal lesions and in the serum of PV patients [1]. Impaired intercellular adhesion in PV seems to be due to binding of autoantibodies directed to the desmosomal transmembrane protein desmoglein-3 (Dsg3), a glycoprotein of the cadherin family of adhesion molecules [2]. It has also been described that PV patients can develop IgG antibodies against Dsg1 [3] and other antigens [4]. These autoantibodies have been shown to be pathogenic, since they are able to induce acantholysis in neonatal mice in passive transfer experiments [5,6].

Apoptosis is an important homeostatic mechanism that maintains correct cell numbers in tissues by balancing cell proliferation with cell death [7]. Epidermis is continuously renewed throughout adult life and adhesion seems to be one of the most important factors controlling cell growth and apoptosis in keratinocytes. It is now clear that loss of anchorage may cause apoptosis in epithelial cells [8,9], but little is known about regulation of apoptosis by cadherin-mediated cell–cell adhesion [10]. However, there is evidence indicating that disruption of cell–cell contacts in acantholytic skin diseases may in some cases provoke apoptosis of keratinocytes [11]. Moreover, previous studies have demonstrated that apoptosis can be induced in the human keratinocyte cell line HaCaT by several stimuli [12–14]. The apoptotic response is an active and physiological form of cell death which occurs by organized degradation of subcellular components. Although there are other types of cell death such as necrosis, apoptosis is distinguished by structural and morphological features including cell shrinkage, plasma membrane blebbing, mitochondrial swelling, and chromatin condensation [15]. The apoptotic process is usually accompanied by several changes in cellular biochemistry such as oligonucleosomal DNA fragmentation, caspases activation and modifications of the levels of Bcl-2 family members [16].

The aim of the present study was to investigate whether apoptosis is triggered by PV-IgG binding to HaCaT keratinocytes, on the basis of the specific characteristics of this process described above.

2. Materials and methods

2.1. Human sera

Serum samples were obtained from a PV patient and a normal human donor. The PV patient exhibited classical active mucocutaneous disease, confirmed by typical histological features and a titer of 1:320 of circulating anti-ICS antibodies. Sera from patient and control were tested by indirect IF using monkey esophagus as a tissue substrate. The presence of anti-Dsg1 and anti-Dsg3 autoantibodies was tested by enzyme-linked immunoadsorbent assay (ELISA) in the serum of the donors. Increased levels, expressed as index value (IV), of Dsg1 and Dsg3 immunoreactivity were found in the mucocutaneous PV patient, with values of 75.3 ± 5.2 and 82.5 ± 8.1, respectively, as compared to a healthy volunteer (1.4 ± 0.5 for Dsg1 and 0.8 ± 0.3 for Dsg3).

2.2. Cell culture

HaCaT cells, a human immortalized keratinocyte cell line, were a gift from Professor N. Fusenig (Heidelberg, Germany). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a 5% CO_2 humidified atmosphere. Subconfluent cultures maintained in serum-free medium were used in all the experiments.

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Abbreviations: PV, pemphigus vulgaris; Dsg, desmoglein; IF, immunofluorescence; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; ELISA, enzyme-linked immunoadsorbent assay

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2.3. Purification of IgG

IgG fractions from PV patients (PV-IgG) or normal donors (N-IgG) were obtained by precipitation with 50% ammonium sulfate followed by affinity chromatography on Staphylococcus protein A conjugated to agarose beads. Bound IgG was eluted with 0.2 M glycine/HCl, pH 3, dialyzed extensively against phosphate buffered saline (PBS), concentrated by ultrafiltration (Amicon), filter-sterilized, and stored at −70 °C until use. The IgG concentration in each fraction was estimated by nephelometry using goat anti-human IgG (Beckman Array 360 System). PV-IgG and N-IgG fractions were labeled with FITC and tested in preliminary binding studies to determine the optimal IgG doses for the experiments.

2.4. ELISA

The reactivity of test sera with the extracellular portions of the Dsg1 or Dsg3 peptide sequences, that contain pathogenic epitopes of the PV antigens, was determined using Dsg1 and Dsg3-coated ELISA plates purchased from MBL (Nagoya, Japan), and following the protocol provided by the manufacturer. All serum samples were tested in triplicate and the results were expressed as IVs, calculated as follows: $IV = (OD_{sample} - OD_{negative\ control})/(OD_{positive\ control} - OD_{negative\ control}) \times 100$. As recommended by the manufacturer, a serum sample was considered to be positive for Dsg1 or Dsg3 antibody if the IV exceeded 20.

2.5. Western blot analysis

HaCaT cells were incubated in the presence or absence of PV-IgG or N-IgG during 8 h. Western blot analysis of cell lysates was carried out after SDS–PAGE gels were transferred onto nitrocellulose membranes (Amersham). Membranes were incubated with a polyclonal rabbit antibody, anti-human Bcl-2 (Santa Cruz), or monoclonal antibodies, anti-human Bax, (Santa Cruz), or anti-human procaspase-3 (Transduction Laboratories), for 1 h at room temperature at a 1:2000 dilution. Membranes were washed and incubated for 1 h at room temperature with 1:10 000 donkey anti-rabbit (Promega) or sheep anti-mouse immunoglobulin horseradish peroxidase conjugated (Amersham), for polyclonal or monoclonal antibodies, respectively. Bound antibodies were detected by autoradiography with enhanced chemiluminescence with ECL-Plus (Amersham Pharmacia Biotech). Equivalent loading was confirmed by Coomassie staining of an identical gel.

2.6. Immunoprecipitation of HaCaT keratinocytes extracts

Sub-confluent HaCaT keratinocytes were washed and harvested. After sonication (five 5 s periods), cell homogenates were centrifugated and the pellet was solubilized in a buffer containing 10% (wt/vol) glycerol and 1% (wt/vol) Triton X-100, and stored at −70 °C. Protein extracts were immunoprecipitated with anti-plakoglobin antibody (Santa Cruz) and protein A–Sepharose (Sigma) overnight at 4 °C. Proteins were released from the beads by heating at 95 °C in Laemmli sample buffer for 5 min and separated on discontinued SDS–polyacrylamide gels. Blots of immunoprecipitated samples were incubated with 50 μg/ml of purified PV-IgG diluted 1:2000 for 2 h at room temperature and then with a goat anti-human antibody (Sigma) diluted 1:10 000. After stripping, the membrane was incubated for 2 h at room temperature with an anti-plakoglobin antibody (Santa Cruz) diluted 1:2000 and with a donkey anti-goat antibody (Santa Cruz) diluted 1:10 000.

2.7. IF microscopy

Cells were grown directly on glass coverslips and incubated in the presence or absence of 2.0 mg/ml of PV-IgG or N-IgG. After 24 h, HaCaT cells were rinsed with PBS at room temperature and dipped for 7 min in −20 °C methanol. Fixed cells were incubated with a monoclonal anti-human keratin antibody (LP34 clon, DAKO) at a 1:20 PBS dilution for 45 min and then incubated with a rabbit anti-mouse IgG-FITC (DAKO) at a 1:20 PBS dilution for 45 min. Coverslips were washed in PBS and mounted over glass slides in 50% glycerol in PBS.

2.8. Microscopic analysis

Morphologic examination of nuclei was performed using fluorescence microscopy. Cells were grown directly on glass coverslips and incubated in the presence or absence of 1.0 mg/ml of purified PV-IgG or N-IgG, during 8 h. Thereafter, cells were fixed with −20 °C cold methanol for 8 min, washed in PBS for 10 min, incubated with 5% BSA in PBS for 10 min, washed three times and stained by Hoechst 33342 (50 μg/ml) (Sigma) in PBS for 30 min. Coverslips were washed three times in PBS and mounted over glass slides in 50% glycerol in PBS.

2.9. Determination of oligonucleosomal (histone-associated) DNA fragments

The presence of soluble histone–DNA complexes was measured by the *Cell Death Detection Assay* (Boehringer Mannheim). Cells were seeded on 24-well plates and 8 h after addition of N-IgG or PV-IgG, or 10 μg/ml camptothecin (Sigma), supernatant was discarded and cell death ELISA assays were performed according to the manufacturer's instructions. Specific enrichment of mono- and oligonucleosomes released into the cytoplasm [Enrichment Factor, (EF)] was calculated as the ratio between the absorbance of the sample and the absorbance of the corresponding control sample (untreated cells).

2.10. Statistical analysis

The data were analyzed using the Kruskal–Wallis test to determine differences between all independent groups. When significant

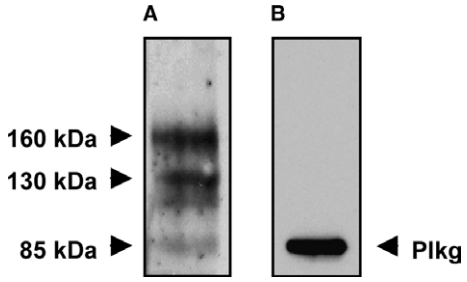

Fig. 1. Western blot analysis of the immunoprecipitated extracts from HaCaT cells obtained with an anti-plakoglobin antibody. (A) Immunoreactivity of IgG purified from a patient with mucocutaneous PV (PV-IgG). (B) Identification of Plkg after stripping of the same blot.

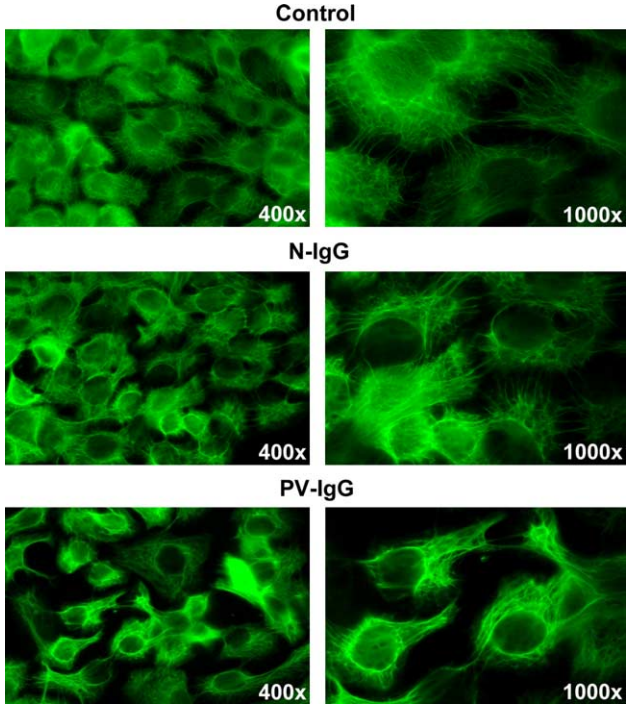

Fig. 2. IF staining of cytoskeletal proteins in HaCaT keratinocytes. Cells were treated for 24 h with 2.0 mg/ml of IgG purified from a healthy donor (N-IgG) or purified from a patient with mucocutaneous PV (PV-IgG). After treatment, cells, including untreated controls, were incubated with an antibody anti-cytokeratin as described in Section 2.

differences were obtained ($P < 0.05$), differences between two groups were tested using the Mann–Whitney U test.

3. Results and discussion

3.1. Characterization of IgG antibodies from a mucocutaneous PV patient

Immunoprecipitation of HaCaT keratinocytes extracts with an antibody against plakoglobin (Plkg) was used to identify the immunoreactivity of the autoantibodies obtained from a mucocutaneous PV patient (PV-IgG). Western blot analysis of immunocomplexes-containing samples with PV-IgG revealed the presence of three main immunoreactive bands, corresponding to 160, 130 and 85 kDa molecular weights (Fig. 1A). The two higher bands could contain Dsg1 and Dsg3, respectively, since their molecular weights were coincident and the presence of anti-Dsg1 and anti-Dsg3 antibodies had been already determined by ELISA (see Section 2). Although Dsg1 is the autoantigen characteristic of pemphigus foliaceus, it is now accepted that 25–66% of the patients with PV present antibodies anti-Dsg1, together with the specific PV autoantibodies anti-Dsg3 [17,18]. In fact, the detection of both antibodies in the serum of the PV patient agreed with his diagnosis of mucocutaneous PV, which is characterized by the presence of anti-Dsg1 and anti-Dsg3 [19].

Binding of PV-IgG autoantibodies to Plkg was assessed by Western blot analysis using an anti-plakoglobin antibody after stripping of the blot (Fig. 1B). Previous immunoprecipitation studies had detected the presence of anti-plakoglobin antibodies in the serum of PV patients [4]. Plkg plays an important role in the assembling of desmosomes and their interaction with intermediate filaments of keratin [20]. Interestingly, keratinocytes derived from Plkg knock-out mice are resistant to the pathogenic effect of PV antibodies, suggesting that the linkage of desmosomal Dsg3 to the cytoskeleton via Plkg might have a central role in the molecular events leading to acantholysis [21].

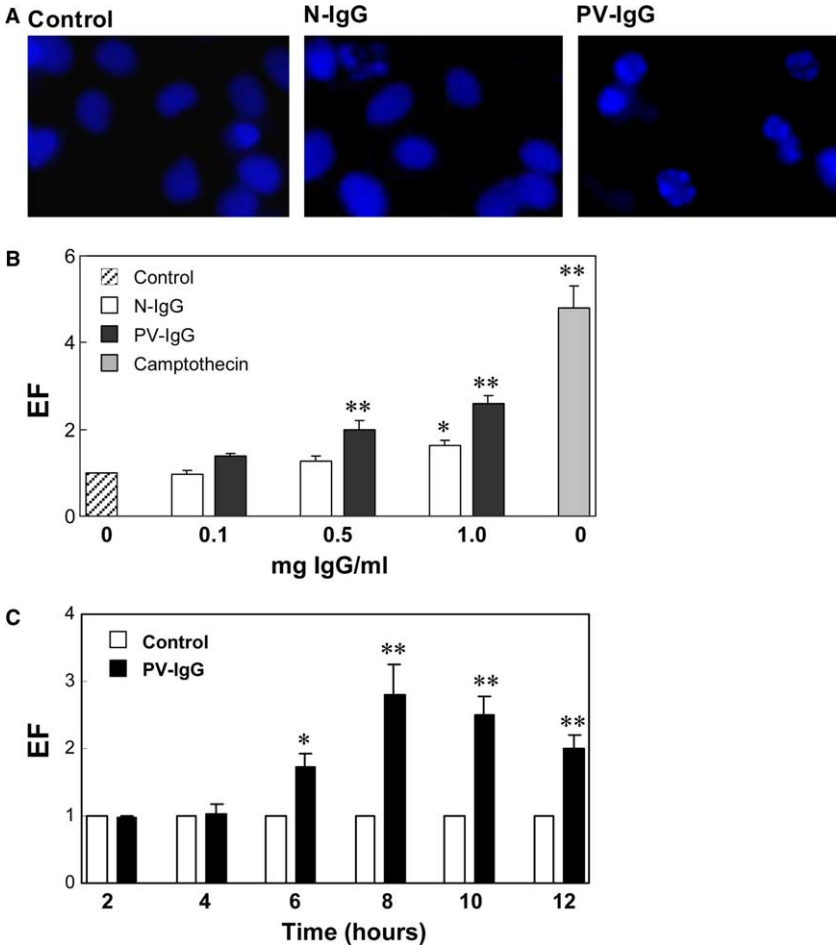

Fig. 3. Apoptosis of HaCaT keratinocytes induced by IgG from a mucocutaneous PV patient. (A) Nuclei staining of HaCaT keratinocytes using Hoechst 33342. Cells were incubated for 8 h with 1.0 mg/ml IgG purified from a healthy donor (N-IgG) or from a patient with mucocutaneous PV (PV-IgG). Original magnification 1000×. (B) Dose–response apoptotic effect of PV-IgG on HaCaT cells. HaCaT keratinocytes were treated for 8 h with the indicated concentrations of N-IgG or PV-IgG. Treatment with 10 μg/ml camptothecin was used as a positive control. (C) Time course study of the apoptotic effect of PV-IgG. HaCaT keratinocytes were treated with 1.0 mg/ml of PV-IgG fractions. Apoptosis was measured as accumulation of oligonucleosomal fragments in cytoplasmic extracts. Oligonucleosomal fragments content was expressed as EF, as described in Section 2. Each bar represents the mean ± S.D. of quadruplicate determinations from at least three independent experiments (*$P < 0.05$, **$P < 0.01$, vs control).

3.2. PV-IgG provokes disruption of cell–cell contacts in HaCaT keratinocytes

IF experiments performed in cultured HaCaT keratinocytes showed that incubation with PV-IgG induced specific changes in intercellular unions. When compared to untreated cells (Fig. 2A), or cells incubated with N-IgG (Fig. 2B), PV-IgG-treated cells presented loss of cell–cell interactions (Fig. 2C). The main histopathological characteristic of PV is acantholysis of epidermis provoked by the disruption of keratinocyte intercellular interactions. This disruption seems to be a consequence of signaling pathways triggered by the binding of PV-IgG antibodies to keratinocytes, as has been shown by different in vivo and in vitro studies [22–24]. Therefore, PV-IgG antibodies were able to produce in cultured HaCaT keratinocytes an effect similar to the features observed in animal models of PV and human patients affected by this disease. In the following experiments, we used this cell line to analyze other possible effects that PV-IgG could cause on human epidermal cells in PV disease.

3.3. Apoptotic effect of PV-IgG on HaCaT keratinocytes

We investigated whether PV-IgG induces the apoptotic response in HaCaT cells, which are susceptible to apoptosis caused by other inducers [12,25]. For this purpose, two different experimental approaches were used: detection of nuclear changes characteristic of apoptosis by staining of the cells with the fluorescent probe Hoechst 3342, and determination of oligonucleosomal fragments of DNA accumulated in cytoplasmic fractions as a consequence of the DNA fragmentation that occurs during the apoptotic process. As shown in Fig. 3A, PV-IgG-treated cells presented chromatin condensation that was not observed in untreated cells (control), or was observed only in few of the cells incubated with IgG from a healthy donor (N-IgG). On the other hand, PV-IgG induced internucleosomal DNA fragmentation in a dose-dependent manner in the concentration range from 0.1 to 1.0 mg/ml, with maximum values of 2.5-fold increase (Fig. 3B). N-IgG was also capable to provoke a slight increase in cytoplasmic oligonucleosomal content at a 1.0 mg/ml concentration, probably due to the binding of IgG to the FcR present in keratinocytes [26]. Indeed, binding of IgG to FcR in other cell types has been previously shown to induce apoptosis [27].

In additional experiments using IgG fractions from another patient affected by mucocutaneous PV, we also observed the capacity of these samples to cause apoptosis. However, the apoptotic effect was not observed with IgG fractions from mucous PV patients, indicating that autoantibodies against antigens other than Dsg3, not present in the mucous PV sera, were required to cause apoptosis (data not shown).

In time course studies, apoptosis induced by IgG from a mucocutaneous PV patient was only observed after 6 h of exposure of the cells with the autoantibodies. Increased levels of apoptosis were obtained at different time points of treatment between 6 and 12 h with the autoantibodies (Fig. 3C).

3.4. Mechanisms of apoptosis induced by PV-IgG in HaCaT keratinocytes

Caspases are key mediators in apoptosis of different cell types provoked by most apoptotic inducers [28]. Caspase-3 is an effector caspase that causes the cleavage of several proteins, such as PARP, DFF45, lamins, etc. [29,30]. The involvement of caspase-3 in the apoptotic process induced by IgG from a mucocutaneous PV patient in HaCaT cells was assessed by analysis of the activation of this enzyme by proteolysis of its inactive form, procaspase-3. Immunodetection by Western blot of the levels of procaspase-3 showed that a decrease in this form was produced by treatment of HaCaT cells with 0.5 or 1.0 mg/ml PV-IgG, indicating an increase of its proteolytic activity (Fig. 4A). These results were confirmed by pretreatment of HaCaT cells with the caspase-3 inhibitor Ac-DVED-CHO. The accumulation of oligonucleosomal cytosolic fragments induced by PV-IgG was totally prevented by this pretreatment (Fig. 4B).

The susceptibility of cells to apoptosis is controlled by the ratio of pro-apoptotic and anti-apoptotic members of Bcl-2 family proteins. It has been demonstrated that high levels of Bcl-2 protect HaCaT keratinocytes from apoptotic stimuli [31,32] and that Bax protein is enhanced by treatment of this same cell line with some apoptosis inducers [33]. In our experiments, levels of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 were determined by Western blot. We found that PV-IgG-treated HaCaT cells presented increased levels of Bax and decreased levels of Bcl-2 at the two concentrations tested, as compared to untreated cells (Fig. 5). As expected, N-IgG produced a slight effect on the apoptotic parameters analyzed only at higher concentration (Figs. 4A and 5B).

In the present work, apoptosis induction of human HaCaT keratinocytes by IgG autoantibodies obtained from a patient with mucocutaneous PV has been assessed. In epidermis apoptosis plays an important role in maintaining homeostasis in physiological situations, and also disturbed apoptosis is

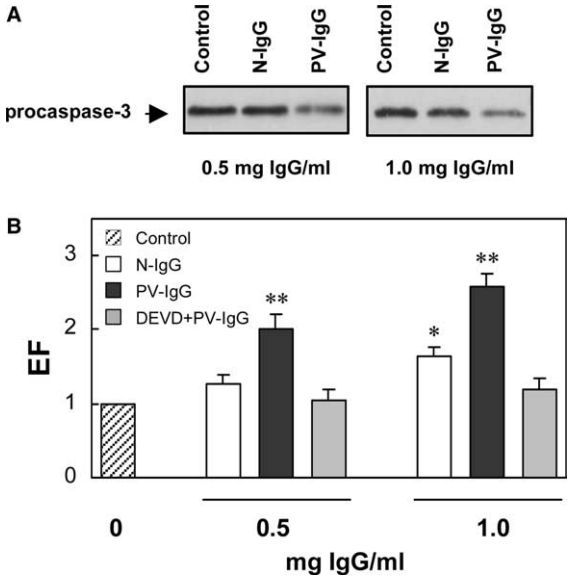

Fig. 4. Role of caspase-3 in the apoptosis of HaCaT cells induced by PV-IgG. (A) Western blot analysis of procaspase-3 levels in HaCaT keratinocytes untreated or treated for 8 h with N-IgG or PV-IgG (0.5 and 1.0 mg/ml). (B) Determination of oligonucleosomal fragments in cytoplasmic extracts from HaCaT keratinocytes incubated in control medium or in the presence of either N-IgG or mucocutaneous PV-IgG, after pretreatment with the caspase-3 inhibitor Ac-DEVD-CHO (30 μg/ml) during 30 min, when indicated. HaCaT keratinocytes were treated with 0.5 and 1.0 mg/ml N-IgG or PV-IgG for 8 h. Oligonucleosomal fragments content was expressed as EF, as described in Section 2. Each bar represents the mean ± S.D. of quadruplicate determinations from at least three independent experiments (*$P < 0.05$, **$P < 0.01$, vs control).

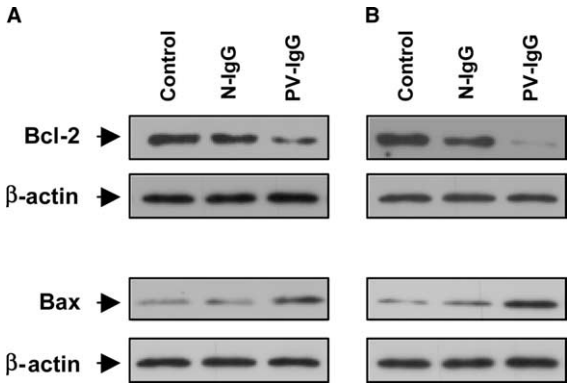

Fig. 5. Western blot analysis of Bax and Bcl-2 proteins in HaCaT keratinocytes incubated in the presence or absence of either N-IgG or PV-IgG. HaCaT keratinocytes were treated for 8 h with 0.5 mg/ml (A) or 1.0 mg/ml (B) of N-IgG and PV-IgG. β-Actin was used as a control for loading.

involved in a variety of pathological conditions. Moreover, the involvement of Fas-L-induced apoptosis in the development of acantholysis in PV has been previously shown [34]. The results obtained in this study suggest that PV-IgG autoantibodies could contribute to the pathogenesis of mucocutaneous PV by triggering apoptosis of epidermal keratinocytes. Dsg3 can in fact be cleaved by caspase-3 [35], also pointing to the involvement of apoptosis in PV development. In the same way, Nguyen et al. [36] have recently shown that the anti-acantholytic effect of methylprednisolone is well correlated with increased expression of several adhesion proteins, including Dsg3.

The contradictory results obtained by various investigators, related to the involvement of different pemphigus autoantibodies in acantholysis induction [37,38], might reflect that apoptosis triggered by several alternative inducers, including autoantibodies and other factors present in PV patients sera such as the Fas-L, could participate in the subsequent acantholytic process associated with these types of diseases.

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