Original paper

Expression of selected pro-apoptotic proteins in pemphigoid

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Abstract

Introduction: Bullous pemphigoid (BP) is the most common blistering disease, developing especially among people over the age of 70. Apoptosis, also known as programmed cell death, seems to be involved in many inflammatory and malignant skin diseases.
Aim: Because of the scarcity of data concerning the role of apoptosis in BP, the study was aimed at analyzing pro-apoptotic factors (Bax, Fas, FasL, TRAIL and TRAIL-R) in lesional and perilesional skin using immunohistochemical methods.
Material and methods: The study was performed on 22 patients with bullous pemphigoid before treatment. Ten healthy volunteers, selected according to their sex and age, made up the control group.
Results: Bax protein expression was revealed in the cytoplasm of keratinocytes in samples of lesional skin, weaker in perilesional skin and the weakest in samples taken from healthy volunteers. Immunostaining of Fas in lesional skin was detected in the cytoplasm of keratinocytes, less intense in perilesional skin. Fas ligand expression was discovered in the basal layer of the epidermis and inflammatory infiltrates. None of the samples taken from healthy participants revealed Fas or Fas ligand expression. Immunostaining of TRAIL was detected in the cytoplasm of keratinocytes as well as in inflammatory cells and some fibroblasts in lesional skin. In perilesional skin and in healthy skin the expression was observed in keratinocytes and some fibroblasts, and it was less intense. Immunostaining of TRAIL receptor was revealed in inflammatory infiltration, in the cytoplasm of keratinocytes as well as in some fibroblasts in lesional skin, in perilesional skin and in healthy skin. The expression of TRAIL-R was more intense in perilesional than in lesional skin.
Conclusions: The more intense expression of TRAIL receptor DR4 on keratinocytes and cells of inflammatory infiltration in perilesional than in lesional skin may imply its role in maintaining the inflammatory process and damage to the dermo-epidermal junction. It is difficult to evaluate clearly the role of proteins in BP pathogenesis because they take part both in mechanisms of apoptosis and inflammatory process intensification.

Key words: pemphigoid, apoptosis, Bax, Fas, FasL, TRAIL, TRAIL-R.

Introduction

Bullous pemphigoid (BP) is the most common blistering disease, developing especially among people over the age of 70 [1]. In spite of many studies its pathogenesis is not yet fully known.

In the blood serum of most patients there are autoantibodies binding to autoantigens – glycoprotein 180-kD, BP180, BPAG2 – and in fact non-collagen NC16A domain localized in the upper part of the lamina lucida of hemidesmosomes [2]. In some patients there are autoantibodies against autoantigen BP230 (BPAG1), as well as autoreactive T cells against the same regions of BP180 and BP230.

Skin lesions in BP are actually the result of complement activation, inflammatory cell activation and release of proteases, which degrade various extracellular matrix proteins and extracellular BP180 domains [1].

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Recent studies have provided evidence indicating that anti-BP180 autoantibodies stimulate mast cell degranulation in skin and activate macrophage dependent and independent recruitment of neutrophils, directly by IgE or indirectly by IgG. The NC16A domain is probably a target one for IgE subclasses of autoantigens, and antigen-specific histamine release is observed only in those patients with detectable circulating IgE directed against this region [2].

Infiltrating neutrophils after activation of Fcγ receptor release proteases which cause dermo-epidermal junction separation [2]. Gelatinase B and elastase are the main proteases of neutrophils. Also free radicals released during the inflammatory process take part in tissue destruction.

The role of the process of apoptosis is investigated in the pathogenesis of many autoimmune diseases; however at present there is not much information about its role in subepidermal blistering diseases.

The death of a cell may occur in the process of apoptosis as well as necrosis. Necrosis is believed to be a kind of passive, accidental death of a cell, which results from strong and sudden damage involving physical, chemical and biological factors [3-5]. Apoptosis, also known as programmed death of a cell, is an outcome of intracellular cell "suicide", which is regulated by cellular pathways of passing the signal. Apoptosis seems to be a process observed in many inflammatory and neoplastic skin diseases such as contact dermatitis, toxic epidermal necrolysis, acantholytic dermatoses and systemic lupus erythematosus. It is an active process requiring the use of energy linked with the activation of many genes and it usually refers to single cells. Apoptotic death of a cell, depending on its type, can last from several hours (lymphocytes) up to a few days (48 to 72 hours in keratinocytes). The process takes an active part in not only the growth of the organism, but also in cell differentiation, eliminating the broken, infected and mutated cells. It also prevents many pathological processes, e.g. autoimmunization and neoplasm [3, 4, 6, 7].

To date, only two pathways of apoptosis have been described, i.e. intrinsic and extrinsic, and the basis for their distinction is the way of activation of the pro-caspases which initiate them [8, 9].

The intrinsic pathway, called mitochondrial, is connected with the activation of cytochrome c by the pro-apoptotic genes belonging to the Bcl-2 family (B-cell leukaemia/lymphoma-2) as a result of e.g. medicine administered or the destruction of DNA structure. The pro-apoptotic genes generate the process of translocation of cytochrome c and other pro-apoptotic proteins from the mitochondria into the cytosol, which, in turn, leads to the activation of caspases. In the intermembrane space of the mitochondria pro-caspases 2, 3 and 9 can be found; pro-caspase 3 easily undergoes the process of autoactivation by lowering the pH [10]. The features triggering the mitochondrial pathway of death can be: an increase in concentration of reactive forms of oxygen, nitrogen oxide, Ca^{2+} ions, thermal shock, active toxins, disturbance of electron transport or DNA damage. As a result of those features the mitochondrial pores (megachannels) open in the junction places of the external and internal mitochondrial membrane, through which the proteins promoting death of the cell (such as cytochrome c – also known as Apaf-2 – apoptosis protease activating factor 2, AIF protein – apoptosis inducing factor, pro-caspases-2, -3, -9, Smac/DIABLO protein) enter the cytosol. The key factor promoting the creation of the complex called apoptosome is cytochrome c, which consists of oligomers of cytochrome c, cytosol adaptor protein Apaf-1 and pro-caspase-9. Apoptosome is an equivalent of DISC (death-inducing signalling complex) in the receptor pathway [4, 9].

The extrinsic pathway is associated with attachment of the ligands to the receptor belonging to the superfamily of TNF receptors, which possess the so-called death domain (DD), by means of which activation of the pro-caspases inside the cell occurs [4].

The effector caspases, such as caspase 3, also called executive, are activated directly by caspase 8 or fission of caspase 8 and activation of the pro-apoptotic protein Bid. The death domain of the receptor connects with adaptor protein also containing DD, e.g. FADD (Fas-associated death domain) [6, 11-14]. The adaptor cell and the domain of the pro-caspase 8 or 10 create a DISC complex, as a result of which activation of the initiating caspase 8 or 10 occurs [3, 11].

The intrinsic and extrinsic pathways stimulating apoptosis are linked to each other by e.g. Bid protein, belonging to the Bcl-2 family [9].

The proteins composing the TNF receptor superfamily also take an active part in the process of apoptosis. These are: Fas, TRAIL-R1 (DR4), TRAIL-R2 (DR5, Apo2), TNF-R1, TNF-R2, TRAMP (TNF-related apoptosis-mediated protein) and DR-6 [15]. The pathway of operation of the Fas ligand on the Fas receptor has been most thoroughly described. At present it is believed that the constitutive co-expression of the receptor and the ligand Fas takes place in cells of rapid apoptotic turnover [16].

As TNF-R, Fas and TRAIL receptors appear on the keratinocytes, they can take part in the pathogenesis of some skin diseases, such as: toxic epidermal necrolysis, graft-versus-host disease, skin neoplasms and contact oversensitivity [15]. The expression of Fas and FasL on the keratinocytes is regulated by various factors such as UV radiation, cytokines, and antineoplastic medicines [17].

The executive phase is the final start of the programme of death of a cell, which is precisely controlled by the gene products, which can block, activate or delay apoptosis by their influence on the executive caspases.

The main inhibitor of apoptosis is Bcl-2 protein. Activated executive caspases carry out the process of proteolysis of many proteins critical for cell life, such the proteins of the cytoskeleton, the nuclear envelope, the enzymes

engaged in relaxation of DNA, as well as the division of chromosomes during mitosis. In the destruction phase the cells undergo the shrinking process as a result of reorganization of the nucleo- and cytoskeleton as well as by pumping out the ions K^+, Cl^- and organic osmolytes [4].

The fragments of the apoptotic cell are packed into the apoptotic bodies without any visible damage to the cell membrane, which limits the release of the potentially harmful intracellular substances into the extracellular space and the blood stream. Apoptotic bodies are then eliminated by the phagocytic cells and the inflammatory process around the apoptotic cell cannot be observed [3-5].

In tissue material *ex vivo* apoptosis is difficult to determine quantitatively because of the dynamics of the process; therefore the number of registered apoptotic cells often constitutes only a small percentage of the total number of cells which entered the state of apoptosis [5].

In general the majority of cells of the haematopoietic line atrophy in the process of apoptosis and manifest the typical features of apoptosis, while the death of epithelial cells is more complicated and very often hard to classify [18].

Anoikis is a special kind of apoptosis caused by the loss of intercellular connection, which seems to be a crucial factor triggering apoptosis in keratinocytes [19]. This process, however, is part of normal epidermal development, during which the keratinocytes from the layers located over the basal layer lose connections between each other and die out [20].

Material and methods

A study of selected pro-apoptotic proteins (Bax, TRAIL, TRAIL-R, Fas, Fas-L) was performed on 22 patients with bullous pemphigoid, aged 60-86 (average age was 70.5) who were treated in the Department of Dermatology and Venerology of the Medical University of Lodz. The patients were before treatment, at an active stage of the disease, i.e. with developed skin lesions (erythema, papules, blisters on erythematous surface). The lesions were accompanied by itching of various intensity. Pemphigoid was diagnosed based on the clinical picture, and histological and immunological findings. Patients who had malignancies, other immunological diseases or BP provoked by drugs were excluded from the study.

Ten healthy volunteers, selected according to their sex and age, made up the control group.

All the participants of the experiment gave explicit consent in writing before entering the study and the study protocol was approved by the Local Ethical Committee of the Medical University of Lodz (no. RNN/132/07/KE, 20.02.2007).

The biopsies from all patients were taken from lesional (blisters on erythematous surface) and uninvolved skin (trunk) before administration of any treatment (topical or systemic). In the control group biopsy specimens were taken from the buttock or abdominal skin of healthy volunteers.

Paraffin-embedded sections (3-4 μm) were used for routine H+E staining and for immunohistochemistry in the DAKO EnVision detection system using the immunoperoxidase method. The following primary monoclonal antibodies were used: Bax (Dako, Denmark), TRAIL (Abcam, USA), TRAIL-R (R&D, UK), Fas, Fas-L (Novocastra, UK).

For immunohistochemistry the paraffin-embedded sections were placed on adhesive plates and dried at 56°C for 24 hours. Later they were deparaffinized in a series of xylenes and alcohols with decreasing concentrations (96%, 80%, 70%, 60%). In order to retrieve the antigenicity of tissues and allow them to react with antibodies, sections were prepared in a bath (98°C – 1 h) or microwave oven. Then the sections were washed with TRIS buffer (pH 7.6) for 5 min. Activity of endogenous peroxidase was inhibited with 0.3% hydrogen peroxide solution in methanol for 30 min. Primary antibody solution directed against human antigens was applied to these sections. After incubation with diluted antibodies for 60 min at room temperature or for 12 hours at 4°C, they were washed with TRIS buffer twice. The DAKO EnVision double-step visualization system was then used in order to visualize the antigen-antibody reaction. In cases of positive immunohistochemical reaction cellular nuclei were stained with Meyer haematoxylin for 2 min. After dehydration and processing through a series of acetones and xylenes the sections were fixed in DPX. For every antibody a negative control was performed using TRIS buffer instead of antibody.

Expression of proteins was evaluated by two independent pathologists using an Olympus BX 40 (Japan). Magnification used was 100 × and 400 × respectively. A semiquantitative visual scale was employed to evaluate the intensity of TRAIL, TRAIL-R, Fas and FasL. The scale range consists of 4 grades: 0-3. Morphometric analysis was used for Bax (MultiScan 8.08 software, Computer Scanning System, Poland). All data are shown as mean ± SD. Student's *t*-test was applied where appropriate after evaluation of distribution. Mann-Whitney test was used where necessary. The difference was considered statistically significant when $p < 0.05$

Results

Bax protein expression was revealed in the cytoplasm of keratinocytes in samples of lesional skin (mean immunoexpression 32.886 ±9.190) (Fig. 1). The expression was weaker in uninvolved skin (20.703 ±6.174) (Fig. 2). The weakest Bax expression was revealed in samples taken from healthy volunteers (16.60 ±3.6) (Fig. 3).

Fas expression in lesional skin was detected in the cytoplasm of keratinocytes (0.393 ±0.177) (Fig. 4). In uninvolved skin the expression was less intense 0.137 ±0.183 (Fig. 5). None of the samples taken from healthy patients revealed Fas expression.
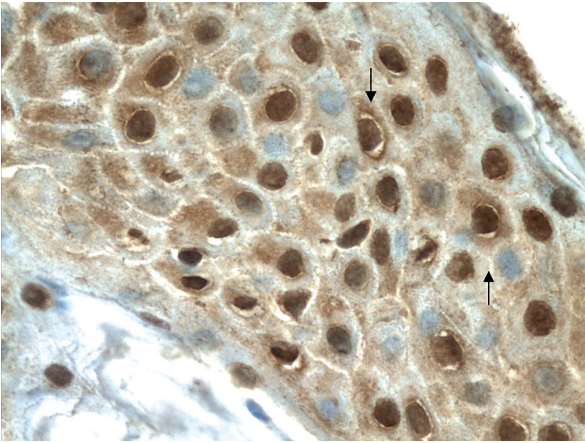
Fig. 1. Moderate expression of Bax in keratinocytes of all epidermal layers in lesional skin (blister). Mag. 400 ×
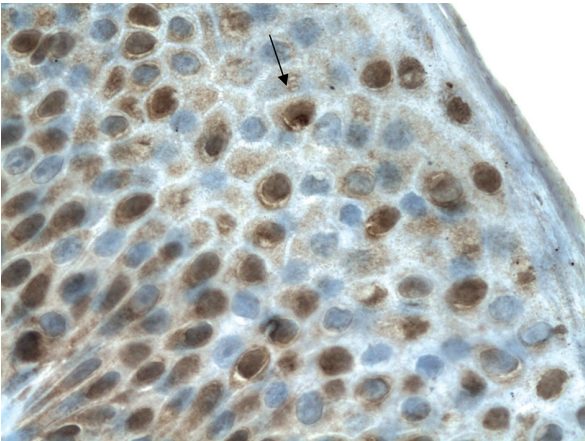

Fig. 2. Moderate expression of Bax in keratinocytes in perilesional skin. Mag. 400 ×
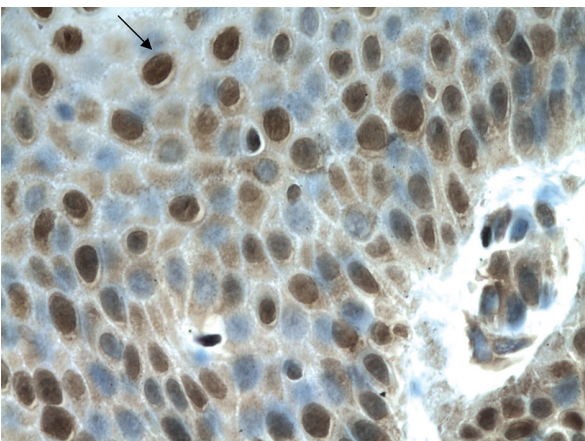

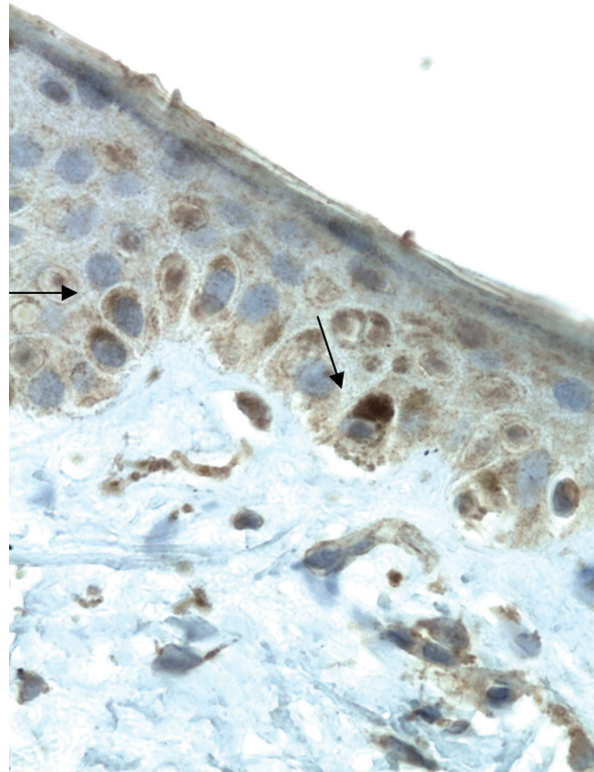**Fig. 3.** Weak expression of Bax in keratinocytes in skin from healthy control group. Mag. 400 ×

Fig. 4. Moderate expression of Fas in keratinocytes in lesional skin (blister). Mag. 400 ×
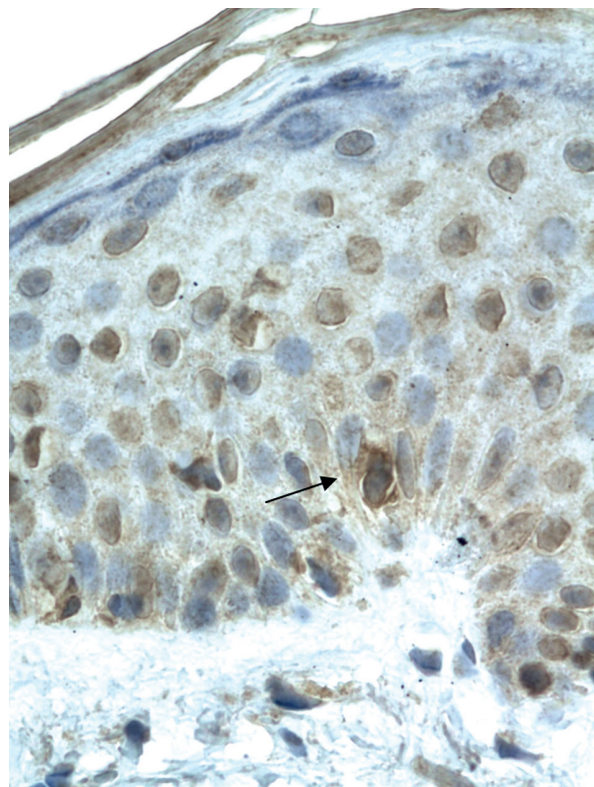

Fig. 5. Weak expression of Fas in keratinocytes of all epidermal layers in perilesional skin. Mag. 400 ×

Fas ligand expression was discovered in the basal layer of the epidermis and inflammatory infiltrates in lesional skin (0.70 ±0.404) (Fig. 6). In uninvolved skin the expression was weaker in the basal layer of the epidermis and in a few cells infiltrating the skin (0.61 ±0.364) (Fig. 7). Immunostaining for Fas ligand was negative in the control group.

Immunostaining of TRAIL was detected in the cytoplasm of keratinocytes as well as in inflammatory cells and some fibroblasts in lesional skin (0.896 ±0.782) (Fig. 8). In uninvolved skin the expression was seen in keratinocytes, mainly of the basal layer, and in some fibroblasts and was less intense (0.339 ±0.350) (Fig. 9). As for healthy skin, the expression was detected in keratinocytes and in some fibroblasts (0.25 ±0.191).

Immunostaining of TRAIL receptor was revealed in inflammatory infiltration, in the cytoplasm of keratinocytes as well as in some fibroblasts in lesional skin (0.657 ±0.697) (Fig. 10), in uninvolved skin (0.936 ±0.697) and in healthy skin (0.5 ±0.258) (Fig. 11). The expression of TRAIL-R was more intense in uninvolved than in lesional skin.

There were statistically significant differences between lesional, perilesional and healthy skin of the control group in Bax and Fas expression analysis. FasL expression was significantly higher in skin lesions and perilesional skin than in the control group. There was a significant difference between TRAIL expression in lesional and perilesional skin. In TRAIL-R analysis there was no statistically significant difference.

The statistical analysis is presented in Table 1.

Discussion

Caproni *et al.* [6], examining apoptosis in DH (dermatitis herpetiformis), also analysed 5 tissue biopsy specimens taken from perilesional skin in BP patients. Apoptosis was assessed by the TUNEL method (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling technique). It was seen in
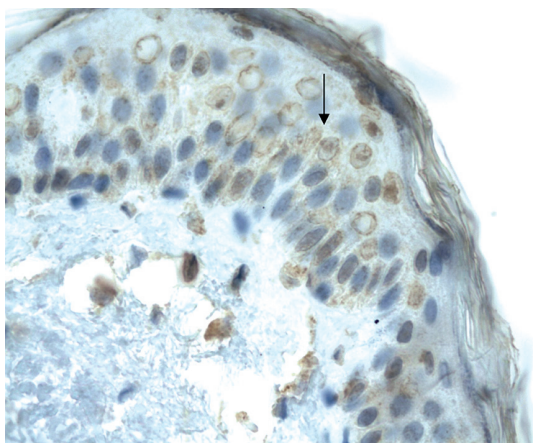

Fig. 6. Moderate expression of FasL in keratinocytes in lesional skin (blister). Mag. 400 ×
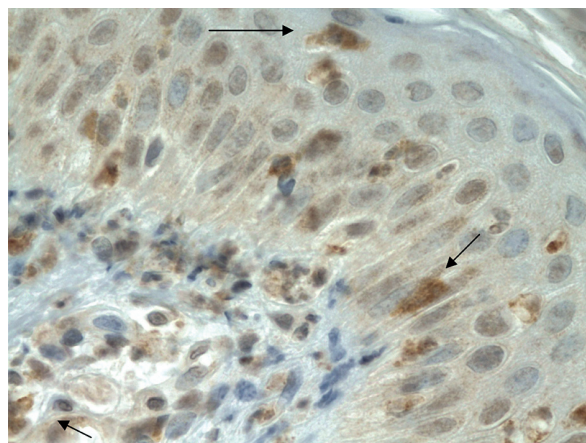

Fig. 8. Expression of TRAIL in keratinocytes and infiltrating cells in lesional skin (erythema). Mag. 400 ×
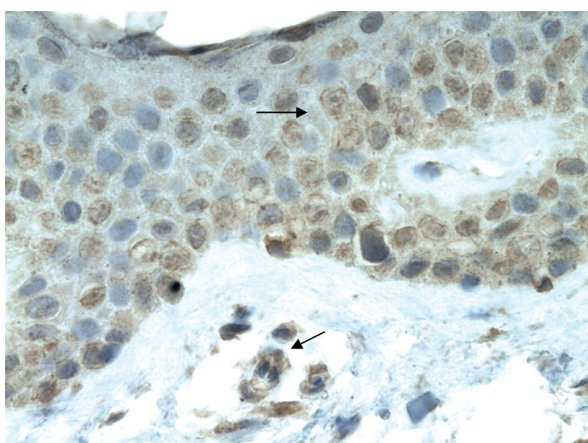

Fig. 7. Weak expression of FasL in keratinocytes and infiltrating cells in perilesional skin. Mag. 400 ×
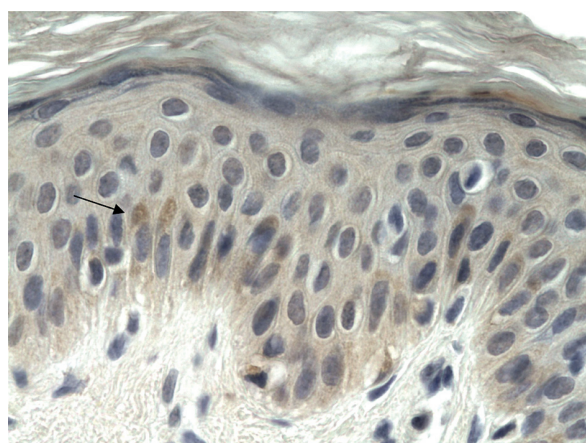

Fig. 9. Weak expression of TRAIL in basal keratinocytes in perilesional skin. Mag. 400 ×
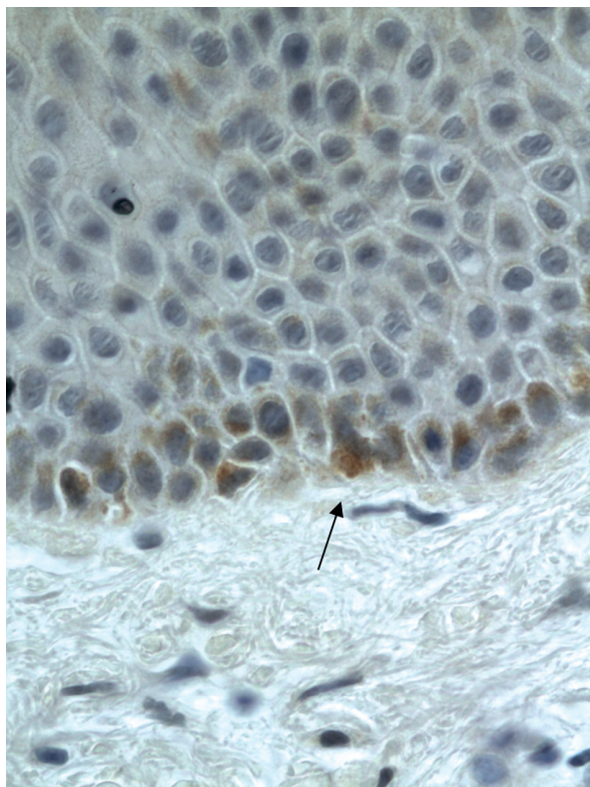
Fig. 10. Expression of TRAIL-R in keratinocytes of basal and suprabasal layers in lesional skin (erythema). Mag. 400 ×
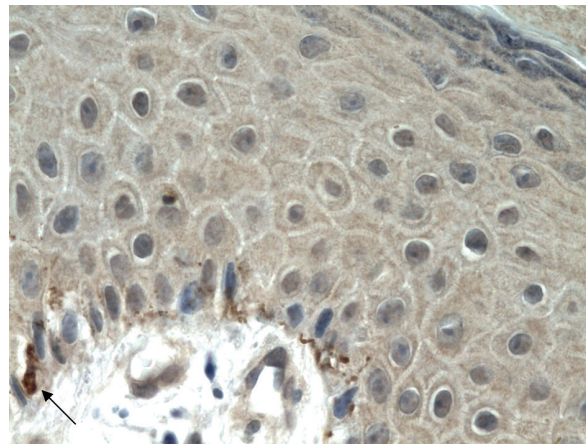

Fig. 11. Weak expression of TRAIL-R in basal keratinocytes in healthy skin. Mag. 400 ×

the basal and suprabasal layers of the epidermis. In the dermis, in the area of blood vessels, there were only a few apoptotic cells. In skin biopsies taken from participants from the healthy control group there were no apoptotic cells. The authors state that there were more apoptotic cells nearer dermo-epidermal detachment.

Bax protein belongs to the proapoptotic part of the Bcl-2 family and takes part in the intracellular pathway of apoptosis. In the cytosol Bax takes on a monomeric form. After receiving an apoptotic signal, this protein oligomerizes because of conformational changes started by Bid protein. Also Bax is incorporated into the external membrane of mitochondria and plays a role in ion channel formation [4]. In non-apoptotic cells Bax and Bcl-2 form heterodimers maintaining homeostasis. Protein p53 by Bax induction destroys this homeostasis [4]. In our study Bax expression was seen in keratinocytes of

suprabasal layers, which was confirmed in earlier studies [21, 22].

Our own study proves that there was expression of Bax protein in the cytoplasm of keratinocytes, and the expression was significantly higher in the lesional than in the perilesional or the control group skin.

Caproni *et al.* [6] also examined expression of pro-apoptotic protein Bax and anti-apoptotic Bcl-2 in lesional skin in BP. Bax expression was strong in the basal layer of the epidermis and in the papillary dermis. In BP the epidermal expression was significantly higher than in DH. Bcl-2 expression was present in the epidermis and in the dermis near superficial vessels. Both Bax and Bcl-2 expression was significantly higher in lesional skin than in the skin of the healthy control group. The ratio of Bax to Bcl-2 was similar in lesional and perilesional skin of DH and BP patients and in the healthy group skin. In the authors' opinion, the Bax/Bcl-2 way of apoptosis plays no important role in these disorders.

However, the findings of our study showed a significant difference in Bax expression between lesional, perilesional and healthy skin; the overexpression of Bax protein seems to take part in BP pathogenesis, but the explanation of the precise mechanism of starting the intrinsic pathway of apoptosis needs more studies.

The extrinsic pathway of apoptosis starts e.g. after activation of Fas receptor (also known as APO-1 or CD95) by its ligand FasL [7]. In physiological conditions Fas

Tab. 1. Analysis of statistical significance (*p*) – the comparison of immunoexpression of pro-apoptotic proteins between lesional skin (PHZ), perilesional skin (PHO) and control group (control)

	Bax keratinocytes (%)	Fas	FasL	TRAIL	TRAIL-R
control vs. PHO	0.2 (NS)	0.15 (NS)	< 0.004	0.62 (NS)	0.17 (NS)
control vs. PHZ	< 0.002	< 0.001	< 0.005	0.11 (NS)	0.66 (NS)
PHO vs. PHZ	< 0.001	< 0.001	0.51 (NS)	< 0.002	0.11 (NS)

expression is weak and is present in the cell membrane or in the intracellular compartment, which prevents spontaneous apoptosis [23]. Oishi *et al.* [24] and Lee *et al.* [25] showed Fas in keratinocytes of the spinous layer, basal cell layer, dendritic cells, and in the dermis in fibroblast and epithelial cells, but also skin glands, hair papilla, and the external hair shaft. FasL is present on keratinocytes in the granular layer, spinous layer and external hair shaft. Fas-FasL interaction takes part in pathogenesis of AIDS, GVHD, melanoma, and toxic epidermal epidermolysis [26-28]. Resistance of cells of Fas-induced apoptosis might result in cancer metastases [13].

The Fas way of apoptosis may lead to keratinocyte death. Activated cytotoxic T cells have FasL that binds to Fas on the surface of keratinocytes and leads to apoptosis. Some authors have reported expression of Fas on keratinocytes in disorders connected with damage to the basal layer of the epidermis (e.g. lichen planus, lupus erythematosus). Adding IFN-γ to keratinocyte cultures leads to cell apoptosis due to anti-Fas antibodies [27]. Apoptosis of keratinocytes can be induced by perforin and serine proteins [7]. FasL is also present as a soluble form after being cleaved by metalloproteinases, but has no pro-apoptotic nature in this form, and it also may inhibit the influence of FasL connected with the cell membrane [29].

In our own study there was expression of Fas and FasL in epidermal cells of BP patients, and FasL was also seen in inflammatory infiltration. Therefore we suggest that infiltrating cells with FasL on the surface act on keratinocytes with the Fas receptor. The connection of the ligand to the Fas receptor triggers an intracellular cascade leading to apoptosis; therefore the expression of Fas receptor on keratinocytes makes them susceptible to apoptosis. Expression of both Fas on keratinocytes and FasL cells in the same topographic place implies that after the ligand and receptor have joined, apoptosis is started.

Farley *et al.* [29] described the influence of FasL on Fas receptor which leads to its oligomerization without caspase activation. The effect of that process is not apoptosis, but expression of genes of pro-inflammatory factors, e.g. IL-6, IL-8, MCP-1. The chemotactic activity of FasL seems to be the effect of its influence on chemokine production rather than direct chemotactic activity [30].

The possibility of activation also by a pro-inflammatory way, not a proapoptotic one, by Fas could explain the overexpression of Fas in lesional skin in psoriasis, where no apoptosis is observed. It may be the effect of coexpression of anti-apoptotic protein Bcl-xL or Bcl-2 [31].

Also, studies of other authors confirm Fas and FasL expression in BP. Sayama *et al.* [27] by immunohistochemistry showed Fas on keratinocytes within BP lesions and minimal expression in skin of healthy people.

Caproni *et al.* [6] described Fas expression on basal keratinocytes and FasL in skin under the dermo-epidermal junction. The authors did not observe Fas expression in the dermis of BP patients or FasL in their epidermis.

The authors also did not observe Fas and FasL expression in skin biopsies taken from the healthy control group. Higher expression of epidermal Fas than FasL and inversely in the dermis of BP patients, in the authors' opinion, indicates the importance of the extrinsic pathway of apoptosis in BP skin lesion formation [6].

Aoudjit and Vuori [32] also showed that endothelial cells are protected before Fas-induced apoptosis by bonding with the extracellular matrix.

Expression of Fas in the epidermis is induced by many factors, including UV radiation, cytokines (e.g. IL-1β, TNF-α, IFN-γ, IL-15), and viral infections (hepatitis B or C, HIV infection) [7, 19]. More precise studies are needed to establish which of these factors can take part in BP.

TRAIL, also known as Apo-2L, takes part in activation of apoptosis. Pro-apoptotic activity is seen after it binds to receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2). Although both of them coexist on keratinocytes, DR4 seems to be more effective [33]. These receptors do not take part in terminal differentiation of keratinocytes [17]. There are also TRAIL receptors on keratinocytes which do not promote apoptosis, because recombinant TRAIL does not induce apoptosis on healthy keratinocytes [15]. By receptors DcR1, DcR2, which do not have DD, TRAIL activates NF-κB, which leads to transcription of genes that are antagonists of caspase 8 and promote the inflammatory process. In endothelial cell lines which avoid TRAIL-induced apoptosis, expression of E-selectin, ICAM-1, IL-8 and IL-1Ra is seen. That leads to lymphocyte adhesion. Induction of mRNA for MCP-1 by TRAIL was not observed [8, 12, 33, 34].

The role of TRAIL in physiological conditions is not completely known. TRAIL is a stronger pro-apoptotic factor than TNF *in vitro*. In spite of that it seems that most cells are resistant to TRAIL-induced apoptosis. TRAIL protein is constitutively present on many cells, but in T and NK cells and dendritic blood cells it is synthesized after their stimulation [4, 35]. Also on neutrophils after stimulation by interferon the expression of TRAIL is seen. mRNA for DR2 and DR4 – TRAIL receptors – is found in neutrophils [36].

Opinions on the role of TRAIL in autoimmune diseases vary. Some researchers suggest that TRAIL can exacerbate, others that it decreases the disease intensity. TRAIL can induce the process of apoptosis of dendritic cells and neutrophils and its high expression was seen in T cells infiltrating skin in atopic dermatitis [35].

In our study TRAIL and DR4 expression was present on keratinocytes and infiltrating cells. Higher expression of TRAIL receptor in perilesional rather than lesional skin seems interesting, but it was not statistically significant. The more intense expression of TRAIL receptor DR4 on keratinocytes and cells of inflammatory infiltration in uninvolved than in lesional skin may suggest its role in maintaining the inflammatory process and damage to the dermo-epidermal junction

The influence of TRAIL on human eosinophils did not induce a chemotactic effect, but led to apoptosis [36];

therefore the presence of TRAIL and its receptor in infiltrating cells seems to be related to apoptosis.

Also, patients with atopic dermatitis have overexpression of TRAIL on T cells and monocytes in peripheral blood and T cells in skin lesions. It seems to be related not to induction of apoptosis, but to activation of antagonist of interleukin-1 receptor expression. Antagonist of IL-1 receptor prevents massive tissue damage during the process of inflammation [35]. Schmidt *et al.* [37] reported that the level of antagonist of IL-1 receptor in blister fluid in BP patients was higher than in artificial blisters and higher than in the patients' serum. Having taken into consideration the role of TRAIL in atopic dermatitis, we suggest that its role in BP might be similar.

We examined proteins which take part in apoptosis started by the intrinsic (Bax) and extrinsic (Fas and TRAIL) pathway. However, some authors hold the opinion that the recognition of autonomy of these two apoptotic pathways is a simplification, as there are proteins which connect these two pathways, e.g. Bid protein [9]. Also, Suliman *et al.* [38] showed that TRAIL, besides activation of the extrinsic pathway, decreases the transmembrane potential in mitochondria and induces the intrinsic pathway of apoptosis.

Dahlman-Ghozlan *et al.* [39] in 2008 described apoptosis of eosinophils infiltrating tissue in BP during methotrexate treatment (small doses – 2.5-5 mg a week). The authors analysed tissue samples in 10 patients before and after a week of treatment. The study showed that the number of eosinophils decreased and the number of apoptotic eosinophils increased. There were also some apoptotic keratinocytes in the basal layer of the epidermis and other apoptotic cells. Earlier phases of apoptosis seem worth studying, e.g. expression of caspases or inhibitors of apoptosis, to decide whether accumulation of eosinophils is the result of apoptosis inhibition. Other researchers showed that small doses of methotrexate induce apoptosis of activated T cells [40].

Caproni *et al.* [6] state that apoptosis in DH seems to be induced by the extrinsic pathway, but other factors may also be influential: hypoxia due to mechanical tension of blister fluid or loss of link between the dermis and epidermis. The same mechanisms are seen in BP and they can also promote apoptosis in that disease.

Our results as well as studies conducted by other authors showed that the role of apoptosis in BP pathogenesis is not clear. Pro-apoptotic proteins also manifest pro-inflammatory properties [8, 29, 35]. Also there are other difficulties in evaluating the process of apoptosis in the skin. Some cells after apoptotic death are not removed from the organism, but stay there for some time. Keratinocytes after death are not phagocytosed, but go to the skin surface and are desquamated [5]. Next, *in vitro* research seems to be additionally needed to clarify the role of apoptosis in BP.

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