ORIGINAL ARTICLE

Role of Human Skin Antimicrobial Peptides in Psoriasis

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Summary
Introduction. Psoriasis is a chronic inflammatory skin disease with various biochemical, immunologic and vascular changes, and a vague relationship to nervous system function. Skin defends the body by rapidly setting an innate immune response and providing a swift first–line defense against infection or injury. Hundreds of naturally occurring antimicrobial peptides have been discovered and it has been shown that psoriatic epidermis expresses high levels of host defense proteins.
Aim of the Study. To evaluate human antimicrobial peptides in correlation with inflammation level in skin biopsy material of psoriatic lesions.
Materials and methods. We evaluated 9 psoriasis patients and 1 healthy volunteer. Skin biopsies were obtained from using the routine punch biopsy method. All tissue specimens were stained with hematoxylin and eosin and by immunochemistry for human β defensin 2, PGP 9.5, MMP2. The intensity of immunostaining was graded semiquantitatively. For apoptosis evaluation, we used TUNEL method.
Results. We found a distinct inflammatory cell infiltration with diffusive character in subepithelial layer, epithelium and hair follicle outer epithelial sheath. Defensin–containing cell number, PGP 9.5–containing nerve fibers and number of MMP2 positive macrophages, fibroblasts and epitheliocytes varied from few to abundant in the visual field. Apoptosis affected epithelial cells, connective tissue cells and inflammatory cells focally.
Conclusions. Histological findings vary from marked inflammatory cell infiltration to granulation tissue. Psoriatic lesions of patients with no previous active psoriasis treatment feature marked activation of defensin, matrix metalloproteinase, apoptosis and neuropeptides–containing innervation. In skin of psoriasis patients with long–term ineffective treatment psoriatic lesions show abundance of positive structures of defensin, matrix metalloproteinase and neuropeptides–containing innervation. Close correlation between expression of defensin–containing cells, apoptotic cells, and inflammation in the skin was found suggesting about possible stimulation of AMPs and apoptosis by specific inflammation.
Key words: antimicrobial peptides, human keratinocytes, immunity, psoriasis.
Abbreviations: AMPs – Antimicrobial peptides; HBD–2 – Human beta defensin 2; HNP – Human neuthrophil peptides; IMH – Immunohistochemical method; MMP2 – Matrix metalloproteinase 2; PGP 9.5 – Protein gene product 9.5; PKC – Protein kinase C.

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease, with a strong genetic basis, characterized by complex alterations in epidermal growth and differentiation and multiple biochemical, immunologic and vascular abnormalities, and a poorly understood relationship to nervous system function (10).

The skin is situated at the interface between an organism's internal milieu and an external environment characterized by constant attack with potential microbial pathogens. Now it is evident that a major role of the skin is to defend the body by rapidly setting an innate immune response and providing a swift first–line defense against infection or injury. Both resident and infiltrating cells in the skin synthesize and secrete small peptides that demonstrate broad–spectrum antimicrobial activity against bacteria, fungi, and viruses. Antimicrobial peptides (AMPs) also act as multifunctional immune effectors by stimulating cytokine and chemokine production, angiogenesis, wound healing, cell proliferation, chemotaxis, immune induction, and protease–antiprotease balance (3, 5).

Hundreds of naturally occurring antimicrobial peptides have been discovered based on their potency to inhibit the growth of microbial pathogens. There are two major families of AMPs – defensins and cathelicidins. Current evidence shows that both above mentioned families of AMPs act as natural antibiotics and as well as signaling molecules that activate host cell processes involved in immune defense and tissue repair. Modifications in the expression pattern of AMPs have been associated with a variety of pathological processes (2).

Antimicrobial peptides are predominantly small cationic polypeptides that are classified together due to their capacity to inhibit microbes. Defensins and cathelicidins comprise the major families of antimicrobial peptides in the skin, although other cutaneous peptides, such as proteinase inhibitors, chemokines, and neuropeptides, also demonstrate antimicrobial activity. Together, these multifunctional antimicrobial peptides play an important role in skin immune defense and pathogenesis of disease (4).

Antimicrobial peptides are produced by leukocytes and epithelial cells not only in skin, but also in the gastrointestinal and genitourinary tracts, tracheobronchial tree. They include defensins, cathelicidin, histatins, cathepsin G, azurocidin, chymase, eosinophil derived neurotoxin, high mobility group 1 nuclear proteins, lactoferrin (17).

The defensins can be classified into two subfamilies (α– and β–defensins) based on their tertiary structure. The alfa defensins also are known as the human neuthrophil peptides (HNP 1–3) and are largely stored in the granules of neuthrophils and macrophages. There are also many β defensins (HBD). HBD1 is expressed constitutively by keratinocytes, HBD2–4 on the other hand is inducible and produced by keratinocytes and epithelial cells in response to proinflammatory stimuli. Evaluation of the human genome suggests that there may be an additional 25 β defensins that have not yet been identified (17).

Several studies compare psoriasis and atopic dermatitis as the two most common chronic skin diseases. Patients with atopic dermatitis, but not psoriasis, suffer from frequent skin infections. The expression of innate immune response genes and antimicrobial peptides has been found to be decreased in atopic dermatitis, as compared with psoriasis, despite that both skin diseases are characterized by defective skin barriers. Psoriatic epidermis expresses high levels of host defense proteins compared with atopic dermatitis epidermis (13, 14, 16). Psoriatic–scale extracts have been identified as a unique source of human–inducible antibiotic peptides and proteins, among them psoriasin, HBD–2, and RNase 7, as by far, the quantitatively dominating antimicrobial peptides. These peptides and proteins can also be found at much lower levels in healthy skin, where they are expressed focally (11).

Other researches consider protein kinase C (PKC) inhibitor AEB071 and type I interferon as potential therapeutic options or targets for psoriasis (20, 22).

Great involvement in the pathogenesis of psoriasis have demonstrated neuropeptides – increased findings of antibodies to vasoactive intestinal polypeptide, substance P, calcitonin gene–related peptide, neuropeptide Y, and the general neuronal marker protein gene product 9.5 have been found. These substances can not only be released by nerve endings, but may also be synthesized directly in the skin and released from various dermal cells (1, 18).

Despite to all above mentioned data, still there is a lack of knowledge about distribution and relative appearance in antimicrobial peptides in the skin of patients with psoriasis, especially in different stages of disease. Thus, the aim of this study was to research of appearance in skin antimicrobial peptides in correlation with inflammation level in ontogenetic aspects of patients with psoriasis.

AIM OF THE STUDY

The aim of the study was to evaluate human antimicrobial peptides in correlation with inflammation level in skin biopsy material of psoriatic lesions.

MATERIALS AND METHODS

Patients. All psoriasis patients were diagnosed by a dermatologist. Skin biopsies were obtained from the skin lesions of nine different psoriasis patients using the routine punch biopsy method and one healthy volunteer using excision. All patients were off topical and systemic medication for more than 4 weeks before their skin biopsy was taken.

Patient selection criteria were created to achieve as much as possible accurate results with less affecting side factors.

Inclusion criteria were developed in accordance to previously realized researches, as well as to knowledge of immunological changes in skin due to exposure to sun and treatment with topical vitamin D analogue calcipotriol (13, 16, 24): patient 18 and older; patient has psoriasis symptoms for at least 6 weeks, preferable diagnosed for the first time; visible characteristic psoriatic eruptions in typical localization sites; patient hasn't received any previous treatment for at least a month; skin is not fiercely tanned; patient hasn't received any antibiotic treatment during last month.

Exclusion criteria were following: patient has received psoriasis or antibiotic treatment during last month; other confirmed skin diseases – such as various origin urticaria, eczema, atopic dermatitis, folliculitis; much tanned skin; other systemic diseases – such as inflammatory bowel diseases, acute liver diseases, diabetes mellitus, plodding cardiovascular diseases, autoimmune diseases, and oncology.

As a result we selected nine psoriasis patients – seven male and two female in age group from 25 to 68, average age 44,7; volunteer – 33 years old female.

The study was approved by the Ethical Committee at Riga Stradins University, permit issued on September 10, 2009.

Methods

1) Skin biopsies were fixed in Stefanini's solution, dehydrated and embedded in paraffin. Four micrometer thick sections were prepared from each tissue specimen and stained routinely with hematoxylin and eosin (Lillie, 1969).

2) Immunohistochemical method (IMH). Human beta defensin 2 (cat No AF 2758, LOT VJU015051, obtained from goat, 1:100 dilution, R&D Systems, Germany), PGP 9.5 (code Z5116, obtained from rabbit, 1:600 dilution, DakoCytomation, Denmark) and MMP2 (cat No AF902, LOT DUB034081, obtained from goat, 1:100 dilution, R&D Systems, Germany) were used by biotin – streptavidin IMH (Hsu et al., 1981).

3) For TUNEL method we used In situ Cell Death Detection, POD cat No 1684817 (Roche Diagnostics, Negoescu et al., 1998).

4) For visual illustration of our findings we used Leica DC 300F digital camera and image processing and analysis software Image Pro Plus.

The intensity of immunostaining was graded semiquantitatively and few positive structures in the visual field were labelled with +, moderate number of positive structures in the visual field was labeled

with ++, numerous positive structures in the visual field were labeled with +++, and abundance of positive structures in the visual field was marked with ++++.
Results of TUNEL method were obtained by counting apoptosis positive cells in three unintentionally chosen fields of vision. The mean ± SD was calculated for each specimen.

RESULTS

Intraepithelial lymphocytes, a distinct inflammatory cell infiltration in subepithelial layer as well as the presence of epithelioid cells and macrophages were detected in all patients (Fig. 1 – 2). Similar inflammatory cell infiltration was detected also in hair follicle outer epithelial sheath and in epithelium. Arteriole sclerosis and sweat gland cell vacuolization were established (Fig. 3). Inflammatory infiltration showed diffusive character. Patient with long–drawn psoriasis demonstrated granulations in the tissue. Also vacuolization in epithelial layer and Munro's microabscess were observed focally.
Defensin–containing cell number varied from few to abundant (Fig. 4 – 6). Pronounced correlation was observed between defensin–containing cells and apoptosis findings. Explicit increase of defensin–containing cells and apoptosis was noticed in sites of active inflammation; meanwhile in areas of granulation both findings were negative.
PGP 9.5–containing nerve fibers were found in all specimens and their number varied from few to abundant (Fig. 7). Fine PGP 9.5 positive nerves occupied subepithelial area and reached the epithelium.
Number of MMP2 positive macrophages, fibroblasts and epitheliocytes varied from few to abundant mainly in the subepithelium (Fig. 8). The results are summarized in Table 1.
Marked apoptosis affected epithelial cells, connective tissue cells and inflammatory cells focally (Fig. 9 – 10). However, there was also an interesting observation found in patient with very long psoriasis anamnesis and previous unsuccessful treatment, when in the region of granulation tissue number of apoptotic cells notably decreased (Table 2).

DISCUSSION

Antimicrobial peptides represent efficient innate defense mechanism which protects interfaces from infection with pathogenic microorganisms. In human skin AMPs mainly are produced by keratinocytes, neutrophils, and sweat glands and are either expressed constantly or after an inflammatory trigger. In several human diseases there is an inverse correlation between severity of the disease and the level of AMPs production. Decreased levels of AMPs are associated with burns and chronic wounds. In contrast, overexpression of AMPs can lead to increased protection against skin infections as seen in patients with psoriasis and *rosacea*, inflammatory skin–diseases which rarely result in superinfection. Increased levels of AMPs are often found in inflamed or infected skin areas in patients with *acne vulgaris* indicating a role of these peptides in the protection of infection. These data indicate that AMPs have a therapeutical potential as topical agents in several skin diseases (6, 19).
Antimicrobial peptides have maintained broad–spectrum antimicrobial activity and resisted most microbial strategies, which suggests that antimicrobial peptides may be strong alternatives to current antibiotic regimens in select disease situations (15).
The number of human beta defensin 2 positive structures in our study varied very broadly. Abundance of positive structures was found in specimens of two patients, both with a very long anamnesis of the disease (15 and 44 years) and previous unsatisfactory outcome of the treatment. Meanwhile, the number of positive structures found in specimen of volunteer and other patients was moderate to numerous. Increased number of HBD–2 in psoriatic lesions as well as increased serum level compared to healthy controls earlier has been shown and is tightly related also with the macroscopical clinical activity (12).
In the present study, the number of PGP 9.5 positive nerve fibers was found at an average amount of numerous positive structures in the visual field. Likewise abundance of positive structures was found in specimen of the patient with 44 years of anamnesis of psoriasis and no treatment for past six months. On the contrary, in patient with 15 years of psoriasis diagnosis finding of PGP 9.5 was from few positive structures to moderate number. Various earlier studies have investigated the role of stress and changes in expression of neuropeptides in psoriasis. Recent study by El–Nour et al. (8) suggests a down–regulation of innervation during psoriasis exacerbation. An unexpected reduction in the number of nerve fibers labeled for PGP 9.5 was found in skin biopsies from involved skin from psoriasis patients who believed that their psoriasis was influenced by stress. No association with additional stress was observed in our study, thought acquired data propose more profound investigation in circumstances of the disease in patient with noticeably higher results of PGP 9.5.
Appearance of MMP2 positive cells in current study varied from few positive structures to abundance of positive structures in visual field in previously mentioned patient with long–drawn progress of the disease. In earlier studies higher levels of MMP2 in psoriatic lesions as compared to healthy individuals has been found in both involved, and uninvolved skin (9).
Apoptosis was detected by TUNEL method and acquired data diversified in very extensive limits. No positive staining was detected in two of our cases, material of control patient showed few positive structures in the visual field, meanwhile also maximal intensity of staining could be found in specimens from patients with recent onset of psoriasis. Apoptosis has been evaluated in accordance with topical vitamin D analogue calcipotriol therapy after which number of apoptotic cells is significantly higher than in non–treated psoriatic lesions (7, 21). Biopsies from all patients enrolled in our study were taken from places with reduced exposure to sun and no previous treatment for at least four weeks to exclude such outreach. For all that our broad data

suggests that apoptosis expression may also be regulated by other factors.

We discovered pronounced correlation between defensin–containing cells and apoptosis findings. Considerable increase of defensin–containing cells and apoptosis was noticed in sites of active inflammation; meanwhile in areas of granulation defensin–containing cells and apoptosis findings were negative. Our discovery shows firm correlation between expression of defensin–containing cells, apoptotic cells, and inflammatory infiltration in skin of psoriatic lesions. To our knowledge, there is no previous data of such revelation. While as a control we have examined one healthy skin sample which might not be sufficient for strong statistics, our material is unique, based on a generally accepted norm and gives the first insight in a tendency. Acquired data show the complicated nature of skin innate immune system.

As AMPs are of peptide nature, they could present such problems as high manufacturing costs, short half life, lost of activity in physiological conditions, application problems, unwanted systemic reactions, and interference with normal flora bacteria when trying to use as antimicrobial agents. And also there remain unresolved issues to consider: standardized techniques to assess the activity, molecular regulation mechanisms, the ability to target the site of disease, tolerance and toxicity issues, understanding of the role and expression of AMPs in health and disease remains a challenging area of research (23).

CONCLUSIONS

Histological findings vary from marked inflammatory cell infiltration to granulation tissue and are much inwrought with anamnesis of psoriasis: psoriatic lesions of patients with no previous active psoriasis treatment feature marked activation of defensin, matrix metalloproteinase, apoptosis and neuropeptides–containing innervation. In skin of psoriasis patients with long–term ineffective treatment psoriatic lesion showed abundance of positive structures of defensin, matrix metalloproteinase and neuropeptides–containing innervation. Exception from this should be validated as individual variations due to the other unknown systemic / local factors affecting tissue changes. Close correlation between expression of defensin–containing cells, apoptotic cells, and inflammation in the skin was found suggesting about possible stimulation of AMPs and apoptosis by specific inflammation.

Conflict of interest: None

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Table 1. Relative appearance of defensin, neuropeptides–containing innervation and MMP2 positive structures in patients with psoriasis

Patient	Human Beta Defensin 2	PGP 9.5	MMP2
Nr. 1	++/+++	++	+
Nr. 2	++	+/++	++
Nr. 3 first biopsy	+++	+++	+++
Nr. 3 recurrent biopsy	+++	+++	+++
Nr. 4	++++	+/++	++
Nr. 5	+++	+++	++
Nr. 6	+	+++	+
Nr. 7	++	++	++
Nr. 8	+++/++++	+++/++++	+++/++++
Nr. 9	+++	+/++	+++
Volunteer	++	++	++

+ few positive structures in the visual field,
++ moderate number of positive structures in the visual field,
+++ numerous positive structures in the visual field,
++++ abundance of positive structures in the visual field.

Table 2. Apoptosis detected with TUNEL method in patients with psoriasis

Patient	1	2	3,1	3,2	4	5	6	7	8	9	Volunteer
1.	39.29	46	23.1	52.05	75.32	62.5	0	0	13.46	25.93	20.6
2.	50.8	45.65	13.84	45.71	87.5	65.57	0	0	23.08	24.24	12
3.	41.66	52.5	27.14	56.81	27.27	60.15	0	0	26.54	24.58	15
Mean ± SD	43.92 ± 6.08	48.05 ± 3.86	21.36 ± 6.82	51.52 ± 5.57	63.36 ± 31.85	62.74 ± 2.72	0.00	0.00	21.03 ± 6.78	24.92 ± 0.89	15.87 ± 4.37

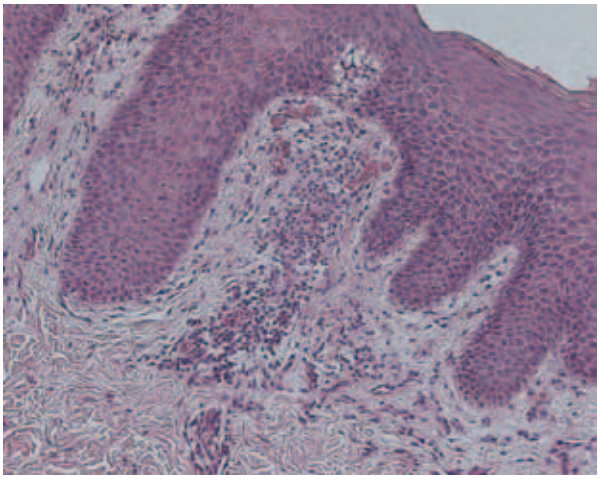

Fig. 1. Marked inflammatory cell infiltration in subepithelial layer. Hematoxylin and eosin, X 200

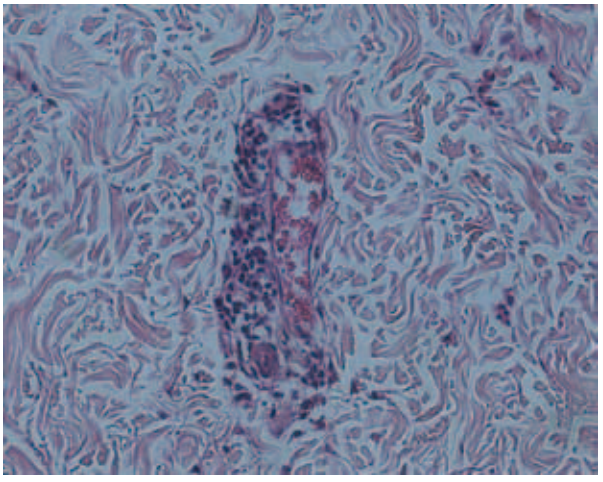

Fig. 2. Diapedesis in blood vessel due to pronounced inflammation. Hematoxylin and eosin, X 400

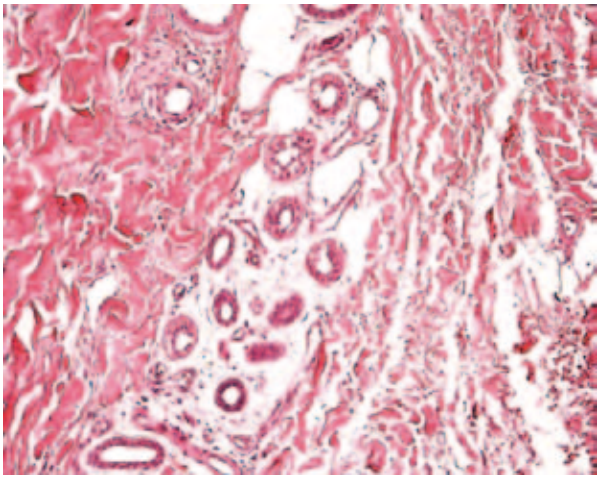

Fig. 3. Sweat gland cell vacuolizati on. Hematoxylin and eosin, X 250

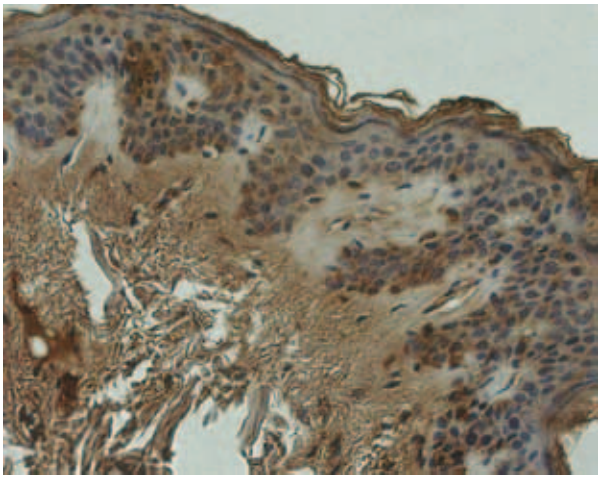

Fig. 4. Moderate number of defensin–containing cells in volunteer patient's biopsy material. Human beta defensin 2 IMH, X 400

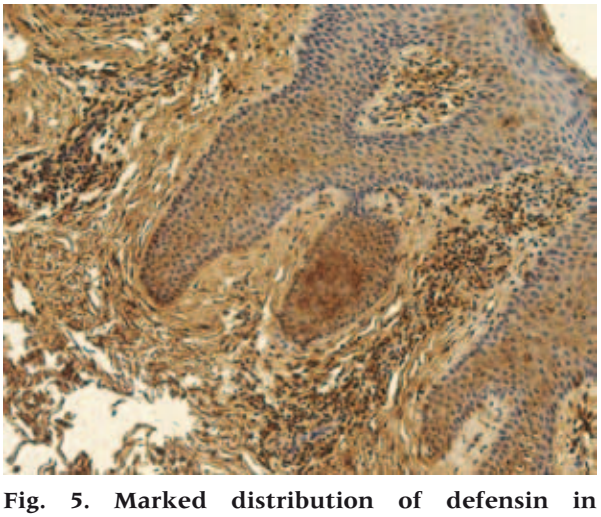

Fig. 5. Marked distribution of defensin in subepithelial tissue in psoriatic skin lesion. Human beta defensin 2 IMH, X 200

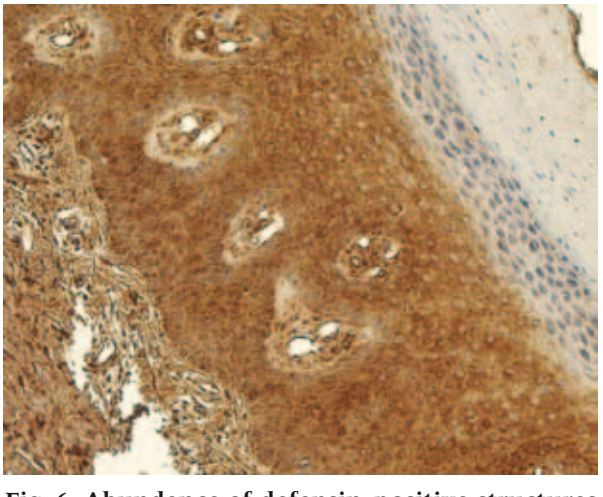

Fig. 6. Abundance of defensin–positive structures in the visual field, both epithelial and subepithelial tissue in psoriatic skin lesion. Human beta defensin 2 IMH, X 200

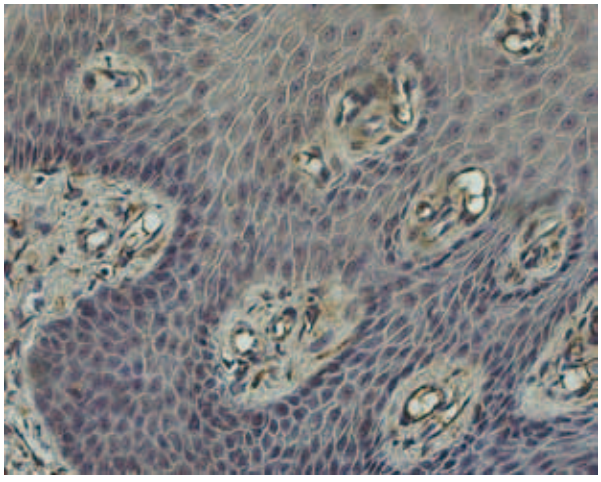

Fig. 7. Fine PGP 9.5–containing nerve fibres in subepithelium of patient's skin. PGP 9.5 IMH, X 400

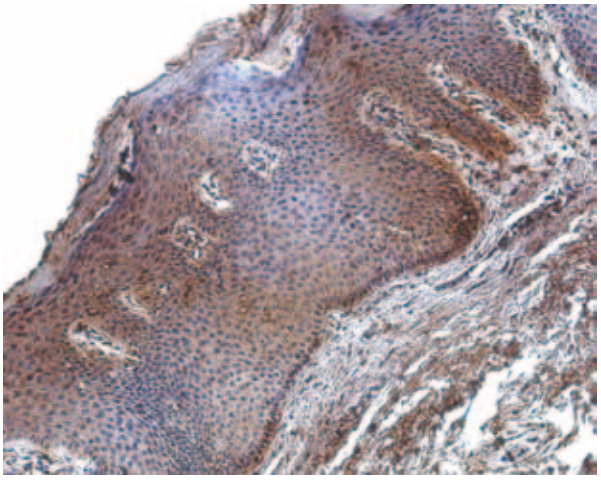

Fig. 8. Focal expression of matrix metalloproteinase 2 positive structures in epithelium and subepithelium of patient. MMP 2 IMH, X 200

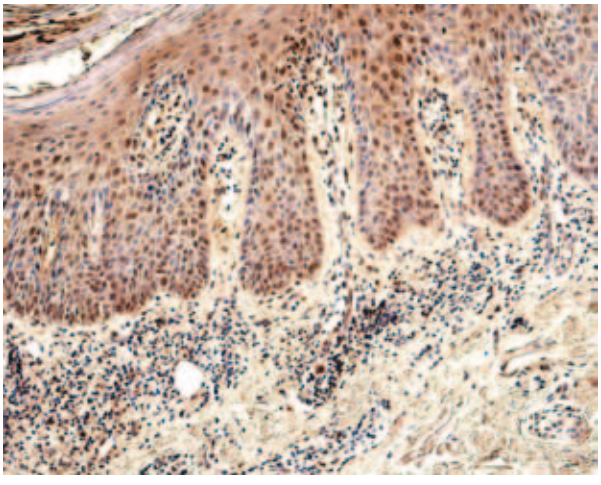

Fig. 9. Marked apoptosis of epitheliocytes and inflammatory cells in patient. TUNEL, X 200

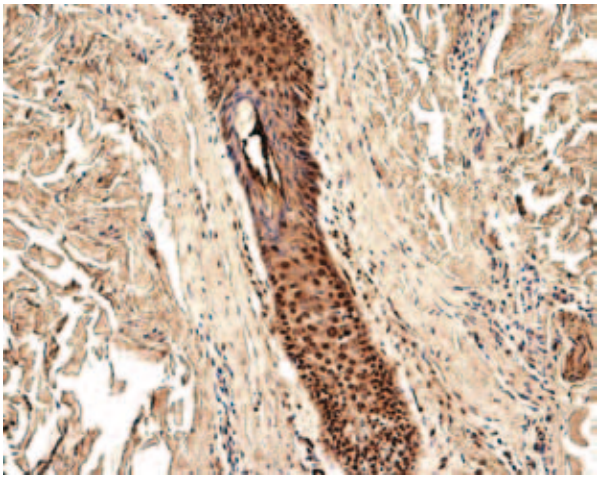

Fig. 10. Marked apoptosis in hair follicle cells of patient. TUNEL, X 250