

INVESTIGATIVE REPORT

Transgenic Mice Over-expressing a Serine Protease in the Skin: Evidence of Interferon γ -independent MHC II Expression by Epidermal Keratinocytes

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Stratum corneum chymotryptic enzyme (SCCE; also known as kallikrein 7) is a serine protease that is preferentially expressed in cornifying epithelia and possibly involved in the desquamation process. We have recently described transgenic mice over-expressing human SCCE in the epidermis showing increased epidermal thickness, hyperkeratosis, and an apparent dermal inflammation with pruritus. This suggests that SCCE may be involved in the pathophysiology of inflammatory skin diseases. We therefore carried out a further characterization of the skin changes observed in *scce*-transgenic mice. An increase in number of dermal cells was verified by stereological measurements showing a more than twofold increase of the volume fraction of dermis occupied by cell nuclei. In some, but not all, animals the number of dermal mast cells was increased. The dermal cell infiltrate was shown to consist mainly of macrophages and granulocytes. The number of epidermal and dermal T-lymphocytes was not increased. Dermal changes were found in transgenic animals before the age they became pruritic. No increase in interferon- γ expression could be detected in the skin of transgenic animals. In spite of this, keratinocytes of adult transgenic mice were found to express MHC II antigen. We suggest that increased expression and/or activity of epidermal SCCE may lead to skin changes that contribute to development and maintenance of inflammatory skin diseases. **Key words:** epidermis; inflammation; kallikrein 7; serine protease-transgenic mice.

(Accepted April 11, 2003.)

Acta Derm Venereol 2003; 83: 322–327.

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Proteases constitute a large group of biologic catalysts with a wide spectrum of specificities and functions. Consequently they have a part to play in numerous reactions, physiological as well as pathological, in cells and tissues. The possibility of designing specific inhibitors makes proteases interesting targets for new drugs for treatment of diseases.

Evidence in support of a role of serine proteases in skin pathophysiology has come from the elucidation of the genetic aetiology of Netherton's syndrome. This disease, which is characterized by severe skin problems and a defect in skin barrier function, is caused by mutations in a gene (SPINK5) (1, 2) encoding a protein (LEKTI) consisting of 15 domains with structural similarities to serine protease inhibitors of the Kazal type (3). A protein derived from one of these domains has been purified and shown to have serine protease inhibitor activity *in vitro*. The proteolytic activity in the epidermis of patients with Netherton's syndrome is increased (4). In addition, an association between atopic dermatitis and polymorphic variations in the SPINK5 gene has been demonstrated (5).

Taken together, these findings suggest that serine proteases produced by keratinocytes and present in active form in the stratum corneum, and with functions in normal epidermal homeostasis and desquamation, become potential actors in disease processes in the skin. To this group of enzymes belongs stratum corneum chymotryptic enzyme (SCCE; EC 3.4.21.-; Swiss Prot P49862, also named kallikrein 7) (6, 7). SCCE is preferentially expressed in cornifying epithelia and several studies have suggested that it may take part in desquamation by means of degrading intercellular parts of desmosomes connecting the corneocytes (for review see (8)). In stratum corneum extracts, SCCE is responsible for a major part of the total proteolytic activity along with another serine protease, stratum corneum tryptic enzyme (kallikrein 5) (9–11).

In an attempt to further elucidate the possible involvement of SCCE in skin pathology we recently reported the production and initial characterization of transgenic mice over-expressing human *scce* mRNA under a viral promoter (12). The only phenotypic changes observed in the mice were found in the skin, which showed several histological changes similar to those seen in chronic inflammatory skin diseases in humans. These mice expressed human SCCE in suprabasal epidermal keratinocytes and were found to develop pathological skin changes with increased epidermal thickness, hyperkeratosis, an apparent dermal inflammation, and in the majority of the transgenic animals signs of severe itch.

In order to gain more insight into the possible mechanisms by which an over-production of SCCE could cause these changes we carried out further studies of the skin changes seen in *scce*-transgenic mice, including an immunohistochemical characterization of invading inflammatory cells.

MATERIALS AND METHODS

Transgenic mice

Production of the transgenic mice over-expressing the human *scce*-gene (founder line Tg1010^{SV40e-hscce}) was described recently (12). Transgenic males were bred with C57BL/6J female mice (M&B A/S, Ry, Denmark) for one to two generations to generate the mice used in this study. The Regional Ethics Committee for Animal Experiments approved the experimental protocols and procedures.

Genotyping

To identify transgenic animals, DNA was extracted from tail tips of mice and polymerase chain reaction (PCR) with primers (5' GCT CTC CCA TTA GTC CCC AGA GA 3'; forward, 5' CCA CTT GGT GAA CTT GCA CAC TTG 3'; reverse; Cybergene AB, Huddinge, Sweden) designed to recognize human but not murine genomic *scce* DNA was performed. PCR analysis was done according to standard procedures using AmpliTaq Gold taq polymerase (PE Biosystems, Stockholm, Sweden).

Tissue preparation

Mice were killed by cervical dislocation, and samples of organs for RNA preparation (spleen, thymus and skin) were removed immediately and snap-frozen in liquid nitrogen. For immunohistochemical analyses, tissues were embedded in O.C.T compound Tissue-Tek[®] (Sakura, Zoeterwoude, The Netherlands) and frozen in isopentane chilled with dry ice. Skin for haematoxylin/eosin and toluidine blue staining was fixed in formaldehyde and paraffin-embedded according to routine procedures. Samples were taken close to the midline dorsally in order to avoid scratched regions of transgenic mice. Immunohistochemical analyses were carried out on skin from three transgenic animals and three normal littermates from each of the age groups 3 days, 7–8 weeks and 13–14 weeks.

Stereology

Paraffin sections (4 µm thick) stained with haematoxylin and eosin were examined under a light microscope equipped with a square lattice with 121 points and one side covering 125 µm at magnification ×400 in the eyepiece. The number of grid

intersections falling on cell nuclei or mast cells (hits) in the dermis was counted and the volume density, i.e. the volume fraction of the dermis occupied by cell nuclei, calculated as hits_(nuclei or mast cells)/hits_(dermis) according to Weibel (13), as applied by Franck-Lissbrant et al. (14). The sections were oriented to allow one side of the lattice to touch the epidermis, and 3–6 arbitrarily chosen areas were counted. Groups were compared using the Mann-Whitney U-test.

Antibodies

Purified primary monoclonal antibodies were purchased from BD PharMingen (Stockholm, Sweden), (see Table I). As isotype controls we used purified monoclonal immunoglobulins from rat IgG_{2b} (cat. No. 553985, clone A95-1), rat IgG_{1κ} (cat. no. 11011D, clone R3-34) and from hamster IgG_{1κ} (cat. no. 11151D, clone A19-3) from BD PharMingen. The following biotinylated secondary antibodies were used: mouse anti-hamster IgG (cocktail) (cat. no. 12102D, clones G70-204, G94-56) (BD PharMingen), goat anti-rabbit immunoglobulins (cat. no. E0432) and rabbit anti-rat immunoglobulins (cat. no. E0468) (DAKO A/S, Glostrup, Denmark).

Immunohistochemistry

Cryosections (6 µm) were fixed in cold acetone for 10 min at room temperature and blocked with 5% normal rabbit or goat serum (DAKO) in phosphate-buffered saline (PBS) for 10 min at room temperature. Primary and secondary antibodies were diluted in PBS containing 0.1% bovine serum albumin. Incubation with primary antibodies diluted as indicated in Table I, or the appropriate isotype control, was performed for 1 h at 37°C. Incubation for 30 min at room temperature with biotinylated secondary antibodies was followed by incubation with streptavidine peroxidase from the StrAviGen MultiLink[®] kit (BioGenex, San Ramon, CA, USA), and development with 3,3'-diaminobenzidine chromogen solution (BioGenex). The sections were counterstained with haematoxylin. Sections of thymus and spleen were stained with the same antibodies as used for skin and served as positive controls.

RNA isolation and analysis

Total RNA was isolated from mouse skin and spleen using TRIzol (Life Technologies AB, Täby, Sweden) according to the manufacturer's instructions. cDNA was synthesized from 2.5 µg total RNA in a reverse transcription (RT) reaction performed according to standard protocols using oligo(dt) (PE Biosystems, Stockholm, Sweden) and AMV-RT reverse transcriptase (Promega, Madison, USA). PCR was performed using REDtaq DNA polymerase (Sigma, St. Louis, Mo., USA) with primers specific for mouse interferon gamma (IFN-γ): 5' TGC ATC TTG GCT TTG CAG CTC TTC CTC ATG GC 3' (forward); 5' TGG ACC TGT GGG TTG TTG

Table I. Primary antibodies: name, clone, specificity, type, and dilutions for immunohistochemistry

Antibody	Clone	Specificity	Type	Dilution
CD45	30-F11	All cells with haematopoietic origin, except erythrocytes	Rat IgG _{2b, κ}	1:200
CD3e	145-2C11	T-cell receptor-associated CD3 complex expressed on thymocytes and mature T cells	Armenian hamster IgG _{1, κ}	1:400
CD11b	M1/70	Granulocytes, macrophages, dendritic cells, NK cells, B-1 cells	Rat IgG _{2b, κ}	1:100
Ly-6G	RB6-8C5	Granulocytes	Rat IgG _{2b, κ}	1:200
I-A/I-E	M5/114.15.2	I-A ^b , I-A ^d , I-A ^q , I-E ^d and I-E ^k MHC class II alloantigens	Rat IgG _{2b, κ}	1:1000

ACC TCA AAC TTG GC 3' (reverse) (Cybergene) (15). As an internal control, specific primers to human cyclophilin A (5' TGC AGA CAA GGT CCC AAA GAC AGC 3'; forward, 5' CAA AGC GCT CCA TGG CCT CCA CA 3'; reverse) were used (16), as these were found to detect and amplify the mouse cyclophilin A gene as well. PCR was performed by 40 cycles of denaturation at 94°C for 1 min, annealing at 64°C for 1.5 min and elongation at 72°C for 2 min (15). The products, 323 bp for IFN- γ and 362 bp for cyclophilin, were analysed by 1% agarose gel electrophoresis. The 1 kb DNA ladder standard was from Life Technologies AB, Täby, Sweden.

RESULTS

Skin biopsies from *scece*-transgenic mice and wild-type control littermates of ages 3 days, 7–8 weeks and 13–14 weeks, a total of 46 mice, were analysed by microscopy of sections stained with haematoxylin/eosin or toluidine blue, and by immunohistochemistry. No morphological differences between transgenics and wild types could be detected in 3-day-old mice. In both groups (not shown) the epidermis was thicker than in older animals. Also the volume density of nuclei in the dermis, as measured by stereology, was similar and high compared to older animals (Table II). In the older age groups there was a marked thickening of the epidermis of transgenic animals, and an apparent increase in the number of cells in the dermis (Fig. 1A, B). The major part of the epidermis showed an orthokeratotic pattern with a well-defined granular layer. In some sections, however, areas with parakeratosis were found, often close to epidermal accumulations of granulocytes. In sections stained with haematoxylin and eosin, granulocytes, often eosinophilic, could be identified in the dermis of transgenic animals. The increased number of dermal cells in older transgenic animals could be verified by stereology (Table II), which showed that the volume density of nuclei in transgenics was at least two times that of the wild-type controls.

Toluidine blue staining disclosed areas of papillary dermis with a marked increase in number of mast cells (not shown) in some transgenic animals belonging to the two older age groups as compared to controls. An

increased number of dermal mast cells in transgenics was not a consistent finding, however. When groups of animals were compared with stereology, no statistically significant differences between transgenics and controls were found.

In mice 3 days of age no differences between wild-type controls and *scece*-transgenic animals could be detected with any of the antibodies used. Findings in older mice are presented below. No differences were found between animals 7–8 weeks and 13–14 weeks of age. With CD45 antibodies specific for the leucocyte common antigen the number of stained cells in the dermis was markedly increased in transgenic animals (only shown for transgene; Fig. 1K). This suggested that bone marrow derived cells contribute substantially to the dermal cellular infiltrate observed in 7–14 weeks old *scece*-transgenic mice.

With T-cell specific CD3e antibodies cells could be detected in the epidermis of transgenic as well as wild-type control animals (Fig. 1C, D). Only very few cells were stained in the dermis. No differences between transgenics and controls were seen. We conclude that T-lymphocytes are not responsible for the increase in number of dermal cells of *scece*-transgenic animals. With the CD11b antibodies (Fig. 1E, F) as well as with Ly-6G antibodies (Fig. 1G, H) there was markedly increased staining of the dermal compartment of 7–14 weeks old transgenic mice as compared to controls. An increased dermal staining in transgenics was seen also with I-A/I-E antibodies (Fig. 1I, J). Since the CD11b antibody is specific for the complement receptor 3, the Ly-6G antibody is specific for granulocytes, and the I-A/I-E antibody is reacting with cells expressing the MHC class II alloantigens, these results suggest that antigen-presenting macrophages and granulocytes are major constituents of the observed dermal infiltrate in *scece*-transgenic mice. An unexpected finding was obtained with the I-A/I-E antibody as regards staining of epidermal cells. In 3-day-old wild-type controls and transgenics as well as in 7–14 weeks old wild-type controls this antibody stained singular dendritic cells spread throughout the epidermis. However, in 7–14 weeks old *scece*-transgenics, in addition to dendritic cells, large number of I-A/I-E positive cells in the lower epidermis were stained in a pattern suggesting they were keratinocytes (Fig. 1J). The pattern of I-A/I-E staining was a general finding in the epidermis, including hair follicles, of transgenic mice. As compared to the I-A/I-E antibody the CD45 antibody, specific for bone-marrow-derived cells, labelled far fewer cells, the majority of which appeared dendritic (Fig. 1K). This supported the conclusion that a majority of the I-A/I-E positive epidermal cells in transgenics were keratinocytes. No evidence of increased ICAM expression paralleling the increased MHC II expression was found (results not shown).

Table II. Volume density of cell nuclei in dermis

Group	Volume density			Tg value in % of Wt value
	Mean	SEM	<i>n</i>	
<i>scece</i> -transgenics 3 d	0.34	0.038	3	110 (n.s)
Wild types 3 d	0.31	0.033	3	
<i>scece</i> -transgenics 7–8 weeks	0.16	0.017	10	232**
Wildtypes 7–8 weeks	0.069	0.0079	9	
<i>scece</i> -transgenics 13–14 weeks	0.17	0.024	10	246**
Wildtypes 13–14 weeks	0.069	0.0035	11	

The volume density is the volume fraction of the dermis occupied by cell nuclei. Tg = *scece*-transgenic mice; Wt = wild-type mice; n.s. = non-significant; ** $p < 0.01$ (Mann-Whitney U test).

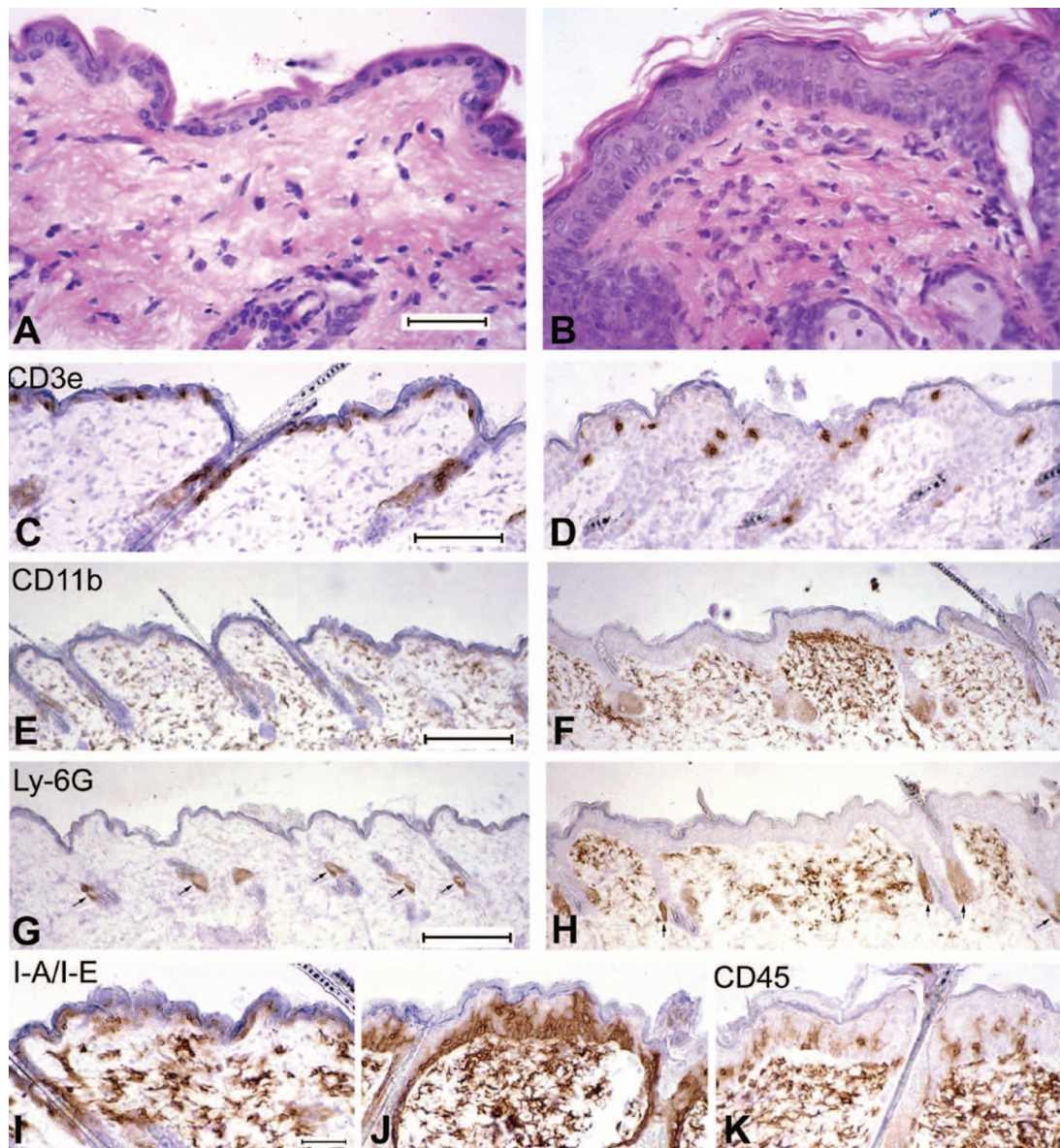


Fig. 1. Histology and immunohistochemistry of skin from *scee*-transgenic mice and normal wild-type littermates. Skin samples from 7–8 weeks old wild-type (A, C, E, G, I) and 7–8 weeks old *scee*-transgenic mice (B, D, F, H, J, K). A, B haematoxylin and eosin staining. Immunoperoxidase staining with CD3e-specific antibodies (C, D), CD11b-specific antibodies (E, F), Ly-6G-specific antibodies (G, H), I-A/I-E-specific antibodies (I, J), CD45-specific antibodies (K). Scale bars 50 μ m (A, B); 200 μ m (C–H) and 25 μ m (I–K). Arrows in G and H show background staining in sebaceous glands.

Since MHC II expression by keratinocytes is known to be induced by IFN- γ (17), we looked for increased production of this cytokine in *scee*-transgenic mice by RT-PCR. As shown in Fig. 2, we found higher expression of IFN- γ in 3-day-old mice than in older mice. No differences were found between wild-type control animals and transgenics in any of the age groups examined.

DISCUSSION

The aim of the present study was to characterize skin changes caused by an increased proteolytic activity in

the epidermis, caused by over-expression of the serine protease SCCE, an enzyme that is normally produced by keratinocytes. The postulated role of SCCE in desquamation implies that its activity is delicately regulated (8). We have asked whether dysregulation of SCCE activity can cause pathological changes and thus possibly contribute to the pathophysiology of common skin diseases.

The type of changes seen in *scee*-transgenic mice, i.e. epidermal hyperproliferation and invasion of the epidermis and dermis of inflammatory cells is, in a broad sense, a common feature of many acquired skin diseases. Hyperproliferation of keratinocytes can occur

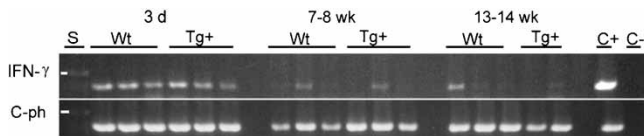


Fig. 2. Detection of mRNA for murine IFN- γ in skin from *scce*-transgenic and wild-type mice by means of reverse transcriptase-PCR. Three wild-type (Wt) and three *scce*-transgenic mice (Tg+) from each age group (3 days, 7–8 weeks and 13–14 weeks) were analysed for the presence of IFN- γ (expected product 323 bp). Cyclophilin A (C-ph) (expected product 362 bp) was used as an internal control. S=DNA standard; white bar denotes 506 bp. As a positive control (C+) spleen from a C57BL/6J mice was used and in the negative control (C-) water instead of RNA was added to the reverse transcriptase mixture.

as a response to stimuli by mediators produced by inflammatory cells, e.g. as postulated according to current views on the pathophysiology of psoriasis (18). It is also clear, however, that keratinocytes have the potential to produce cytokines and other factors, e.g. interleukin-1, which can induce keratinocyte proliferation as well as an inflammatory response including invasion of inflammatory and immune cells (19). It thus seems likely that there should be skin diseases in which the disease process is maintained by mutual interactions between invading and stationary immune and inflammatory cells on the one hand and keratinocytes on the other. The elucidation of this type of interaction may be expected to provide new potential targets for therapeutic interventions.

In several aspects our transgenic animals differ from other reported transgenic mice serving as models for human skin diseases (20 and references therein, 21, 22). Transgenic mice over-expressing a wide variety of genes in the epidermis have been reported and proposed to provide disease models. In most published cases epidermal over-expression has been achieved by means of constructs with the genes of interest or their cDNA under epidermis-specific promoters. In this way high production of gene products normally produced by for example immune cells has been induced in keratinocytes (20–22). Keratinocyte specific promoters have been used also to drastically increase the expression in the epidermis of genes, which are normally expressed by many cell types in most tissues, including keratinocytes (20, 23, 24). In this respect the *scce*-transgenic mice seem to be unique in over-expressing a gene, which is relatively tissue specific (9, 25). Moreover, the highest expression of SCCE in the epidermis of transgenics was seen in high suprabasal keratinocytes, i.e. a site where normal expression also occurs (12). The skin phenotype seen in *scce*-transgenic mice can therefore be considered as the result of an increased production of a skin-specific enzyme at a site where it is normally produced. The fact that similar changes may be present in common human skin diseases (12, 26) may be taken as an argument in favour of the relevance of our model.

Our results suggest that lymphocytes are not involved in the development of the skin changes seen in the *scce*-transgenic mice. The inflammation-like increase in dermal cell density could be ascribed to an increased number of granulocytes and macrophages. Based on analyses of keratin expression patterns (Ny & Egelrud, manuscript in preparation), the increased epidermal thickness in the transgenic mice appears to be the result of an increased keratinocyte proliferation. So far our results give no information as regards any possible causative relationship between the keratinocyte hyperproliferation and the dermal invasion of inflammatory cells, or vice versa. Similarly, we cannot tell whether the observed changes are caused by direct effects of SCCE or by mediators produced as a result of an increased proteolytic activity due to increased amounts of catalytically active SCCE in the epidermis.

We found that epidermal keratinocytes in transgenic animals stained positively for the I-A/I-E antigen, suggesting that they expressed MHC II. To our knowledge the only cytokine described so far, which can induce expression of MHC II by keratinocytes, is IFN- γ (17, 27). We could not, however, detect increased amounts of mRNA for IFN- γ in the skin of transgenic animals. Our findings thus indicate an alternative mechanism for MHC II induction in keratinocytes. Whether this is true or not remains to be elucidated. In this regard it may be of interest that whereas all *scce*-transgenic mice examined so far had developed the typical histological phenotype as described at the age of 4–5 weeks. At this age the animals show no signs of itch, in contrast to older animals (12), suggesting additional pathophysiological events in older animals not disclosed by routine histology or immunohistology. Could it be that an increased production of active SCCE predisposes the skin for the development of a pruritic skin disease, e.g. by induction of a dermal invasion of macrophages and granulocytes and an elevated MHC II expression by keratinocytes?

To conclude, we propose that increased expression and/or activity of epidermal proteases may be one of several contributing factors in the development and maintenance of inflammatory skin diseases. Based on our results and the results of others (1–5), there is substantial evidence that this type of proteolytic enzyme may contribute to skin pathophysiology, and should therefore be considered in forthcoming efforts to develop new treatment strategies.

ACKNOWLEDGEMENTS

We thank Astrid Lundgren for technical assistance. This work was supported by grants from the Swedish Research Council (grant no. K2002-74X-11206-08A), the Welander and Finsen Foundations, the Swedish Psoriasis Association, and Arexis AB. TE is a consultant of Arexis.

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