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INVESTIGATIVE REPORT

Epidermal Hyperproliferation and Decreased Skin Barrier Function in Mice Overexpressing Stratum Corneum Chymotryptic Enzyme

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Stratum corneum chymotryptic enzyme (SCCE; also known as kallikrein 7) is a serine protease that may have an important role in the skin desquamation process. We have recently described transgenic mice overexpressing human SCCE in suprabasal epidermal keratinocytes, leading to increased epidermal thickness, hyperkeratosis, dermal inflammation and signs of severe pruritus in older animals. In order to further evaluate the *scce*-transgenic mice as a potential disease model, we compared transgenic animals and wild-type littermates for patterns of epidermal keratin expression, *in situ* hybridization of *scce*-mRNA, scratching behaviour and measurements of transepidermal water loss (TEWL). In 3-day-old mice, despite readily detectable amounts of human *scce*-mRNA in the epidermis of transgenic animals, there were no histological differences in skin appearance, and no differences could be found in epidermal expression of the keratins 5, 6 and 10. In mice 7–8 weeks of age and older, there was strong suprabasal expression of keratins 5 and 6 in the epidermis of transgenic animals, suggesting that the thickened epidermis in these animals is the result of keratinocyte hyperproliferation. In transgenic animals 11 weeks of age and older there was an increased frequency of scratching, suggestive of pruritus, and also signs of a deteriorating skin barrier function, as reflected by an increased TEWL. There was no correlation between increased TEWL and increased frequency of scratching in individual animals, suggesting that the defect barrier function was not an effect of skin damage caused by scratching. *Key words: epidermis; kallikrein 7; pruritus; serine protease; transepidermal water loss; transgenic mice.*

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Protein degrading enzymes have a wide spectrum of specificities and functions. Consequently they take part in numerous reactions, physiological as well as pathological, in cells and tissues. The possibility to design specific inhibitors makes proteases interesting targets for new drugs for treatment of diseases.

The serine protease stratum corneum chymotryptic enzyme (SCCE; EC 3.4.21.-; Swiss Prot P49862, also named kallikrein 7; 1–2) is preferentially expressed in cornifying epithelia. Several studies have suggested that SCCE may take part in desquamation of cornified cells by means of degrading intercellular parts of desmosomes (3). In stratum corneum extracts, SCCE is responsible for a major part of the total proteolytic activity together with another serine protease, stratum corneum tryptic enzyme (kallikrein 5; 4–5). In our ongoing efforts to elucidate the wider function of epidermal serine proteases, we considered a potential role for SCCE and related relatively skin-specific enzymes in skin pathophysiology, e.g. by acting as activators of precursors of pro-inflammatory cytokines (6), or of protease activated cell surface receptors (7). Our observations that SCCE may be up-regulated in psoriasis lesions (8) and in chronic lesions of atopic dermatitis (9) have provided further incentives for these studies.

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 (9). The only phenotypic changes observed were found in the skin, which showed several histological changes similar to those seen in chronic inflammatory skin diseases in humans. The transgenic mice expressed human SCCE in suprabasal epidermal keratinocytes, and were found to develop pathological skin changes, including increased epidermal thickness, hyperkeratosis and a dermal infiltrate consisting of macrophages and granulocytes. There was also an induction of MHC II expression by keratinocytes. Furthermore, with increasing age the majority of the transgenic animals showed signs of severe itch (9, 10).

Since *scce*-transgenic mice may provide a useful model for further studies on the role of epidermal proteases in skin pathophysiology, and for the development of new treatment strategies, we have undertaken a further characterization of these animals. We analysed *scce*-expression in the skin by *in situ* hybridization, assessed the proliferative state of the epidermis by studies of keratin expression patterns, and

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determined skin barrier function by measuring trans-epidermal water loss.

MATERIALS AND METHODS

Transgenic mice over-expressing the human *scce*-gene were bred and genotyped as described recently (9, 10). The Regional Ethics Committee for Animal Experiments approved experimental protocols and procedures. Skin samples taken close to the midline dorsally were prepared as described (9). *In situ* hybridization and immunohistochemical analyses were carried out on skin from groups of mice, three animals in each group, aged 3 days, 7–8 weeks and 13–14 weeks, respectively.

RNA probes and in situ hybridization

Human *scce* cDNA (nucleotides 111–756) was PCR amplified and cloned into the pCR®-II TOPO plasmid in accordance with the manufacturer's instructions (Invitrogen Ltd, Paisley UK). Before transcription, plasmids were linearized so that antisense or sense RNA could be obtained. Transcription was performed using an *in vitro* transcription system from Roche Diagnostics (Bromma, Sweden) with digoxigenin-labelled UTP and the appropriate RNA polymerase. *In situ* hybridization was performed essentially as described by Sharen-Wiemers and Gerfin-Moser (11), but with the following modifications: 0.05% TWEEN 20 was included in the blocking solution, the colour reagent and the first washing step after the colour reaction, and slides were incubated upside down with the colour reagent.

Antibodies and immunohistochemistry

Purified rabbit polyclonal antibodies, raised against specific peptides to mouse keratin 5 (cat. no. PRB-160P; dilution 1:20 000), mouse keratin 6 (cat. no. PRB-169P), mouse keratin 10 (cat. no. PRB-159P; dilution 1:2000), mouse filaggrin (cat. no. PRB-417P; dilution 1:5000) and mouse loricrin (cat. no. PRB-145P; dilution 1:5000) were purchased from Nordic Biosite AB (Stockholm, Sweden). Biotinylated goat anti-rabbit immunoglobulins were from DAKO A/S, Glostrup, Denmark. Staining procedures of freeze-cut sections were performed as described (10).

Transepidermal water loss (TEWL) and scratching behaviour

TEWL was assessed using equipment from DermaLAB (Cortex Technology ApS, Hadsund, Denmark). The mice were lightly anaesthetized with a mixture of Dormicum (Midazolam, Roche) and Hypnorm (fentanyl citrate and fluanisone, Janssen Pharmaceuticals, Beerse, Belgium). The probe was applied on the back of the animals and two consecutive stabilized measurements of TEWL were recorded. At each session, measurements of wild-type and transgenic animals were carried out in random order. Scratching behaviour was studied as described (9) the day before each TEWL measurement. In brief, mice were transferred to individual cages, and episodes of scratching with hind or front paws were counted during three 5-min periods with 2.5 min lapsing from the transfer to the cage to the first counting, and between counting periods. The numbers of animals observed are given in Table I.

RESULTS

Expression of keratin and other differentiation markers

In the epidermis of animals 3 days of age, labelling patterns with keratin antibodies were identical in wild-type and transgenic mice. Keratin 5 could be detected in basal cells only; keratin 6 was sparsely found in hair follicles and a few interfollicular keratinocytes; and keratin 10 was present in suprabasal cells (Fig. 1A–F). No differences were seen between the two groups of mice with antibodies to filaggrin and loricrin (not shown). This was in accordance with results from a recent study, where we were unable to detect any differences in the skin by routine histology or immunohistochemistry of inflammatory cells between controls and transgenics in this age group (10). This was in contrast to older animals, where marked differences in keratin staining patterns were seen. As expected, keratin 5 was detected in basal cells, keratin 6 in very few interfollicular keratinocytes and keratin 10 in suprabasal cells of 7–8 weeks old wild-type mice (Fig. 1J–L). In *scce*-transgenic mice of this age, essentially all keratinocytes of the thickened interfollicular epidermis, as well as of hair follicles, were stained with antibodies towards keratin 5 and keratin 6 (Fig. 1G, H). Suprabasal cells of transgenic mice expressed keratin 10 (Fig. 1I). No differences in keratin staining patterns between animals aged 7–8 weeks and 12–13 weeks were found (results for the older age group not shown). Antibodies to filaggrin and loricrin (not shown) labelled high suprabasal keratinocytes in 7–8 and 12–13 weeks old transgenic as well as wild-type mice.

Table I. *Transepidermal water loss (TEWL) measurements and itch in wild-type and scce-transgenic mice*

Wild-type mice				*scce*-transgenic mice			Tg^{scce}/Wt	
Age (weeks)	*n*	TEWL (g /h/m^2)	Itch (scratch/min)	*n*	TEWL (g/h/m^2)	Itch (scratch/min)	TEWL	Itch
5	14	4.58±0.23	0	10	4.48±0.18	0	0.98	–
11	14	6.10±0.22	0.014±0.01	10	7.22±0.91	0.17±0.08	1.2	12.1*
17	14	3.41±0.43	0.043±0.02	10	5.82±0.91	0.29±0.13	1.7**	6.7**
25	29	3.34±0.16	0.028±0.01	21	7.12±1.12	1.74±0.79	2.1***	62.1***

Values for TEWL and itch are given as mean±SEM. *n*=number of animals, Tg^{scce}=*hscce*-transgenic mice, Wt=wild-type mice. *$P<0.05$, **$P<0.01$ and ***$P<0.0001$ with the Mann-Whitney U test.

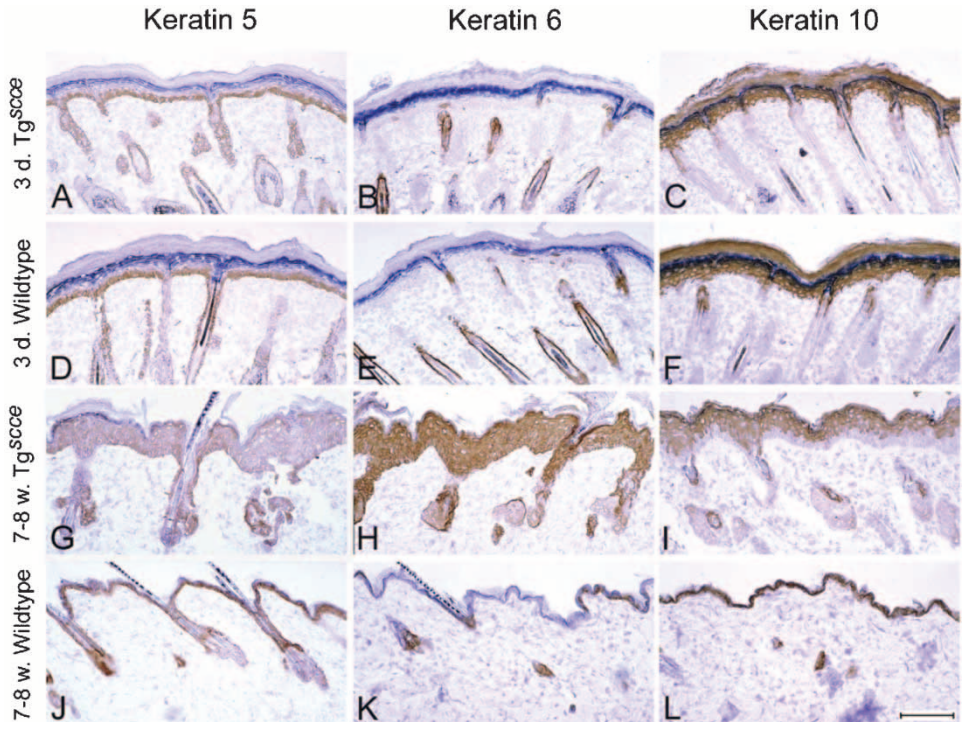

Fig. 1. Immunohistochemistry with keratin antibodies of skin from *scce*-transgenic mice (Tg^{scce}; *A–C; G–I*) and normal wild-type littermates (*D–F; J–L*) at age 3 days (*A–F*) and 7–8 weeks (*G–L*). Immunoperoxidase labelling was performed with antibodies specific for keratin 5 (*A, D, G, J*), keratin 6 (*B, E, H, K*) and keratin 10 (*C, F, I, L*). Bar = 50 μm.

In situ hybridization

In situ hybridization with anti-sense RNA probes for human *scce* gave significant signals only in skin samples from transgenic animals (Fig. 2). Hybridization with control sense probes showed no signals (results not shown). A clear hybridization signal was found in the epidermis of 3-day-old transgenics, although much weaker than in older animals (cf. Fig. 2A, C). Very little hybridization was seen in the dermis. In transgenic animals of all age groups the hybridization signal was generally stronger in suprabasal than in basal keratinocytes; the latter cell type often being apparently devoid of expression of human *scce*-mRNA. In

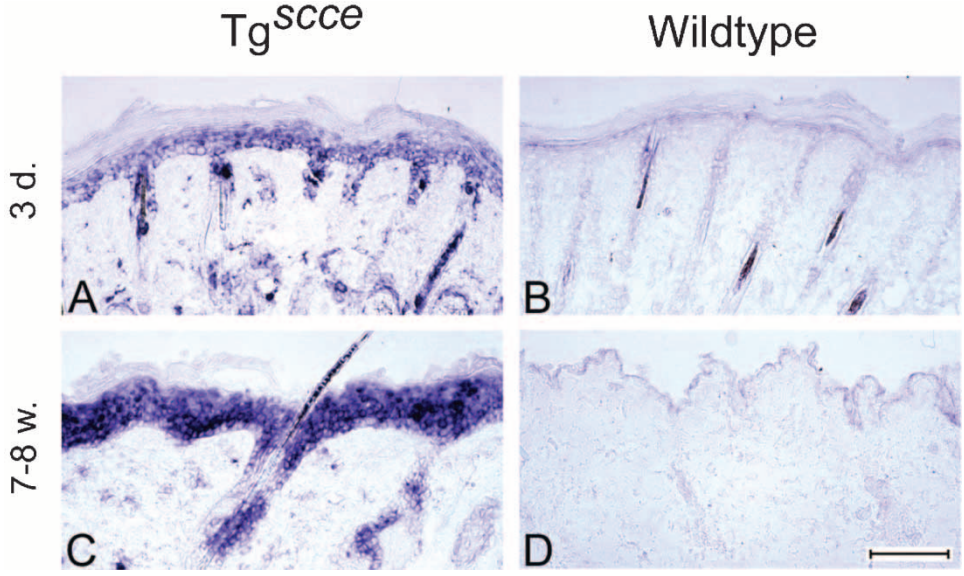

Fig. 2. In situ hybridization with an anti-sense RNA probe to human *scce* of skin from *scce*-transgenic mice (Tg^{scce}; *A,C*) and normal wild-type littermates (*B,D*). *A* and *B*: Age 3 days; *C* and *D*: age 7–8 weeks. Bar = 50 μm.

accordance with the *in situ* hybridization findings, an increased expression of SCCE could be detected in suprabasal epidermal cells of 3-day-old transgenic mice, as compared to wild-type littermates, also by immunohistochemistry with SCCE-specific antibodies. The SCCE-specific epidermal staining, however, was much weaker in 3-day-old than in 7–8 weeks old transgenic mice (9; results for 3-day-old mice not shown).

Transepidermal water loss and itch

The results of measurements of TEWL and apparent itch are given in Table I. A statistically significant increase in TEWL in transgenic animals as compared to controls was found in mice 17 weeks of age and older. Itch, measured as scratching behaviour, was more frequently observed in transgenic than in wild-type mice; a statistically significant difference was found from age 11 weeks and onwards. For individual animals there appeared to be no correlation between scratching behaviour and increase in TEWL in the oldest (25 weeks) group of transgenic mice (Fig. 3).

DISCUSSION

Mice transgenic for the human *scce*-gene under the viral promotor *SV40e* show no macroscopic or histological phenotypic changes at birth. The animals grow normally and appear healthy. From the age of 4–5 weeks it is possible to identify transgenic littermates by close inspection of the fur, especially around the eyes. At this age, all transgenics have aberrant skin histology with increased epidermal thickness, hyperkeratosis, which is usually orthokeratotic but in some areas parakeratotic, and an increased density of cells, mainly macrophages and granulocytes, in the dermis. In spite

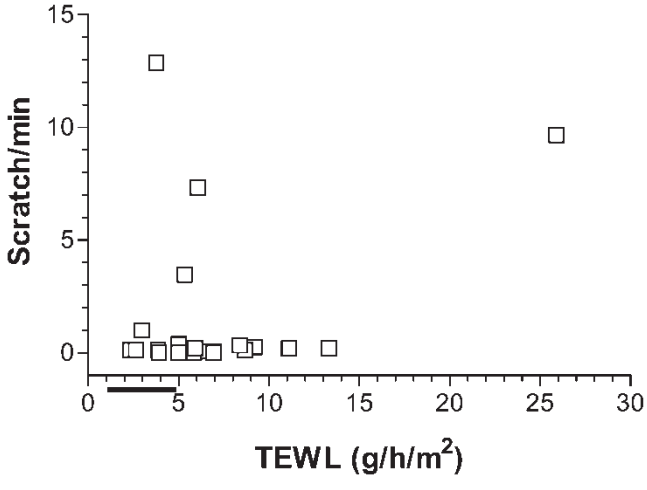

Fig. 3. Correlation between transepidermal water loss (TEWL) and scratching behaviour in 25-week-old *scce*-transgenic mice. Each open square represents one *scce*-transgenic mouse; bar = mean and range for TEWL of wild-type littermates ($n = 29$) of the same age.

of the absence of increased levels of interferon gamma in the skin, the transgenic keratinocytes show hitherto unexplained expression of MHC class II antigen (10). As the animals grow older, an increasing number show signs of pruritus, with frequent scratching with the front or hind paws. So far we have seen no correlation between histological or immunohistological changes in non-scratched skin and the incidence of scratching behaviour. Although expression of human SCCE can be detected in several tissues in addition to skin, the skin changes are the only pathological changes in the *scce*-transgenic mice detected so far (9, 10). We have therefore concluded that our transgenic animals may serve as a useful model for studying the potential role of SCCE and related enzymes in skin pathophysiology.

In this work we aimed to increase our understanding of the mechanisms by which an elevated production of SCCE causes the observed skin changes. An obvious possible explanation for the increased thickness of the transgenic epidermis is keratinocyte hyperproliferation. The pattern of keratin expression presented supports this hypothesis. We found expression of keratin 6, a marker of hyperproliferative keratinocytes, throughout the interfollicular epidermis of transgenics. The finding of a similar pattern for keratin 5, usually expressed by basal keratinocytes only, may suggest that the transgenic epidermis, in addition to being hyperproliferative, also has a disturbed differentiation. The fact that a major part of the epidermis had a well-developed granular layer, and the finding of an apparently normal expression pattern of keratin 10 and other differentiation markers, however, did not support major defects in keratinocyte differentiation.

In previous work we have found that the increase in expression of SCCE protein in transgenic mice, as revealed by immunohistochemistry with SCCE-specific antibodies, is most pronounced in suprabasal keratinocytes. Little or no staining was seen in basal keratinocytes and dermal cells (9). Corroborating results were obtained in the present work with *in situ* hybridization. Although not as distinctive as with immunohistochemistry, there was a clear tendency towards higher *hscce* RNA expression in suprabasal epidermal cells and in hair follicle cells closest to the inner root sheet than in basal cells. This expression pattern, which shows similarities to expression of SCCE in human skin, was unexpected. The *SV40e* promoter usually has its highest activity in proliferating cells. A possible explanation could be that the transgenic mice were constructed with the promoter linked to genomic *hscce* DNA with as yet unknown regulatory elements, which facilitate expression in differentiating keratinocytes. Although transgenic *hscce* expression in the skin appeared to be confined to keratinocytes, we know from previous studies that expression also occurs in other tissues, e.g. the intestines and lung (9).

In the studies on TEWL, a major concern was to rule out the possibility that the increased TEWL in *scce*-transgenic mice was a result of skin damage caused by scratching. To avoid this the measurements were made on the backs of the animals, as far away as possible from the neck region. This site, from which we also took samples for histological analyses, is difficult to reach for the animals, and does not become ulcerated even in animals with severe scratching. Our conclusion that the increase in TEWL was not primarily caused by scratch-induced skin damage was supported also by the results for individual animals shown in Fig. 3.

Although lower than in older transgenic animals, significant expression of *hscce* was seen also in the epidermis of 3-day-old mice. In spite of this, no macroscopic or microscopic phenotypic changes were found in this age group. A possible explanation for the histological skin changes observed in *scce*-transgenic mice might be that they reflect adaptation to skin barrier defects caused by increased SCCE activity, giving excessive degradation of cohesive structures in the stratum corneum. We found significantly increased TEWL, a measure of barrier function, in transgenic mice as compared to wild-type controls. This difference, however, was detectable only in older animals. At the age of 11 weeks, when the aberrant skin histology was fully developed, TEWL was similar in transgenics and controls. This might imply that the skin changes are not related to barrier function. Alternatively, the epidermal changes may indeed be an adaptive response, which can compensate for barrier deterioration up to a given point only. The fact that the difference in TEWL between transgenics and controls increased with increasing age supports this hypothesis.

A tentative explanation could be that skin changes caused by an increased production of SCCE evolve with time, possibly as the result of interactions between an increased SCCE activity in the superficial layers of skin, age dependent development of other parts of the skin, and external factors. This explanation would fit with the observations that histological skin changes in the transgenic animals precede the occurrence of decreased barrier function and pruritus.

There is now substantial evidence, presented by us and by others (12–15), that increased activity of epidermal serine proteases could contribute to the development and maintenance of pathological skin conditions. This provides a new principle for the development of treatment strategies, which may alone or in combination with other modalities give new therapeutic tools in dermatology.

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