

Expression of Skin-derived Antileukoproteinase (SKALP) in Reconstructed Human Epidermis and Its Value as a Marker for Skin Irritation

ESTHER BOELSMA, SUSAN GIBBS and MARIA PONEC

Department of Dermatology, Leiden University Medical Center, Leiden, The Netherlands

For the investigation of the skin irritancy potential of chemicals in an in vitro model, it is necessary to have sensitive end-points that predict the effects on native human skin. Our aim was to investigate whether the induction of the proteinase inhibitor SKALP in reconstructed epidermis can be used as a marker.

The influence of culture conditions and the effect of topical application of sodium lauryl sulfate and oleic acid on SKALP expression were evaluated using immunohistochemistry and Northern blotting.

SKALP expression was induced by serum, epidermal growth factor and fibroblasts. In the presence of retinoic acid and 1,25-dihydroxyvitamin D₃ SKALP expression was inhibited, whereas supplementation with ascorbic acid and α -tocopherol had no effect. Tape-stripping of excised skin and topical treatment with sodium lauryl sulfate induced SKALP protein expression. Application of sodium lauryl sulfate and oleic acid on reconstructed epidermis also induced SKALP at the protein level but no significant effects could be demonstrated at mRNA levels.

In conclusion, SKALP expression, which was increased upon application of sodium lauryl sulfate and oleic acid, can be used as an in vitro end-point for skin irritancy, irrespective of the modifying effects of culture conditions. Key words: culture conditions; sodium lauryl sulfate; oleic acid; immunohistochemistry.

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M. Ponec, Department of Dermatology (P4-Q), Leiden University Medical Center, P.O. Box 9600, NL-2300 RC Leiden, The Netherlands.

The main function of the epidermis is to generate a coherent stratum corneum, which forms a solid, protecting barrier between the internal milieu and the environment. To maintain epidermal integrity, keratinocytes follow a highly coordinated differentiation program. However, when the skin is assaulted mechanically or chemically, homeostatic mechanisms may be deregulated, leading to non-specific cutaneous inflammation, increased epidermal proliferation and modulation of cell differentiation. These processes are guided by the release of inflammatory mediators, which for the greater part originate from keratinocytes (1).

The early phase of inflammation is generally characterized by the infiltration of polymorphonuclear leukocytes (PMNs), which is accompanied by the release of a number of proteolytic enzymes. Although the most important function of these proteinases is to act as a defense mechanism against the attack from the environment, they can also be destructive for the tissue itself. Therefore, the enhanced synthesis of proteinase inhibitors under these circumstances may provide a negative feedback mechanism to protect the tissue against excessive proteolysis by proteinases derived from invading PMNs and monocytes (2).

Skin-derived antileukoproteinase (SKALP) has been shown to be a specific inhibitor of PMN-derived elastase and proteinase-3 (3, 4) and the substrate specificity of SKALP for these elastolytic proteinases suggests that this enzyme may be involved in the regulation of cutaneous inflammation. SKALP activity has not been demonstrated in normal epidermis but it can be found in several inflammatory skin diseases, including psoriasis and in the epidermis after tape-stripping in vivo (3, 5). Besides its potential involvement in inflammatory skin conditions, SKALP may also play a role in situations of an abnormal differentiation program. Linkage of this proteinase inhibitor with epidermal homeostasis has been demonstrated in tumour cells which display a disturbed differentiation program (6).

In many inflammatory skin disorders epidermal hyperproliferation accompanies cutaneous inflammation. The induction of SKALP activity in situations of a localized imbalance of epidermal homeostasis implicates that this inhibitor may be important in decreasing the inflammatory reaction and/or may be involved in the restoration of the damaged tissue.

For the screening of potentially skin-irritating agents in vitro, skin models are needed that reliably predict the effects of compounds on human skin in vivo. Human skin equivalents can be generated by growing human keratinocytes on various dermal substrates, e.g. on de-epidermized dermis (RE-DED). After exposure to the air-liquid interface, a stratified epidermis with a coherent stratum corneum is formed, which closely resembles native skin. However, the expression of various protein-specific differentiation markers reveals some important differences compared with native skin, suggesting incomplete homeostasis (7). Nevertheless, by variation of the culture conditions the balance between cell proliferation and differentiation has been greatly improved (8, 9).

The aim of the present study was to investigate the effects of culture conditions on SKALP expression in RE-DED, including the use of serum-containing versus serum-free medium, culturing at 33°C versus 37°C and variation in the concentrations of growth factors and vitamins. In addition, the effect of human fibroblasts on epidermal SKALP expression was examined.

To determine whether SKALP can be used as a marker for skin irritation, excised skin specimens were tape-stripped in vitro to induce a standardized epidermal trauma or treated with increasing concentrations of sodium lauryl sulfate (SLS). In addition, the response of RE-DED following topical application of SLS and oleic acid (OA) was examined. The expression of SKALP was evaluated at the protein and mRNA level, using immunohistochemistry and Northern blotting.

MATERIAL AND METHODS

Reconstructed human epidermis

Secondary cultures of adult human keratinocytes, cultured as described previously (10), were seeded on RE-DED obtained from human

cadaver skin (11). The cultures were grown under submerged conditions for 3 days in medium consisting of a mixture of 3:1 Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (ICN Biomedicals Inc., Costa Mesa, CA, USA), supplemented with 5% Hyclone serum (Hyclone Laboratories Inc., Utah, USA), 1 μM hydrocortisone (Sigma Chemical Co., St. Louis, USA), 1 μM L-isoproterenol (Sigma), 1 μM insulin (Sigma), 100 IU/ml penicillin (ICN) and 100 $\mu\text{g}/\text{ml}$ streptomycin (ICN). After the cultures had been lifted to the air-liquid interface, they were incubated either at 33°C or 37°C. A mixture of DMEM: Ham's F12 (3:1) medium was used, supplemented with *i*) as described above but with addition of 10 ng/ml epidermal growth factor (EGF; Sigma), or with *ii*) 5% Hyclone serum, 0.5 μM hydrocortisone, 1 μM L-isoproterenol, 0.1 μM insulin and 10 ng/ml EGF. For serum-free conditions, cultures were first grown under submerged conditions in a mixture of DMEM: Ham's F12 (3:1) medium, supplemented with 1% Hyclone serum, 0.5 μM hydrocortisone, 1 μM L-isoproterenol, 0.1 μM insulin, 1 ng/ml EGF, 10 μM L-carnitine (Sigma), 10 mM L-serine (Sigma) and enriched with a lipid supplement as described by Boyce & Williams (12), with final concentrations of 25 μM palmitic acid, 25 μM OA, 15 μM linoleic acid, 7 μM arachidonic acid, 24 μM essential fatty acid free BSA (Sigma) and 1 μM α -tocopherol (Sigma). The fatty acids were all obtained from Sigma. After air-exposure the cultures were grown in the same medium except that serum was omitted. Variable supplementation: 10 ng/ml transforming growth factor- α (TGF α ; Sigma), 10 ng/ml insulin-like growth factor I (IGF-I; Sigma), 10 ng/ml keratinocyte growth factor (KGF; Sigma), 50 $\mu\text{g}/\text{ml}$ ascorbic acid (Sigma), 10^{-8} M 1,25-dihydroxyvitamin D₃ (1, 25(OH)₂D₃) dissolved in isopropanol (final concentration of isopropanol in the culture medium was 0.025 $\mu\text{l}/\text{ml}$) (kindly provided by Dr. L. Binderup, Leo Pharmaceutical Products, Ballerup, Denmark), 10^{-6} and 10^{-7} M all-trans-retinoic acid (all-trans-RA; Sigma), 10^{-6} and 10^{-7} M 9-cis-retinoic acid (9-cis-RA; Sigma) all dissolved in DMSO (final concentration of DMSO in the culture medium was 0.1 $\mu\text{l}/\text{ml}$). Each experimental condition was examined at least twice in independent experiments both under serum-containing and serum-free conditions. Culture medium was renewed three times a week, and cultures exposed to the air for 2 weeks were used for the experiments. In some experiments human fibroblasts were seeded on the basolateral site of DED and cultured for 3 weeks prior to seeding of keratinocytes.

Excised skin

Human breast skin obtained from plastic surgery was processed immediately upon arrival within 24 h after surgery. Subcutaneous fat was removed with a scalpel and the skin was cut into small pieces, which were placed epidermal side up onto a sterile underlay prewetted with PBS. The specimens were incubated at 37°C and 5% CO₂ maximally for 24 h.

Test agents

OA (Sigma) was used as a 0.5% or 5% solution in propylene glycol (PG; J.T. Baker B.V., Deventer, The Netherlands). SLS (Merck, Darmstadt, Germany) was dissolved in water and used at concentrations of 0.1% to 5%. The solutions were applied in stainless steel rings (80 $\mu\text{l}/0.78 \text{ cm}^2$) for 4 or 24 h. This application system excluded direct contact between the solution and the lower levels of the three-dimensional models. Each experimental condition was examined in duplicate in three independent experiments.

Tape-stripping

Freshly excised skin obtained from surgical operations was cleaned and subsequently stretched on polystyrene foam. The stratum corneum was gradually removed by repeated applications of cellophane tape (Scotch Tape, 3M). The tape-stripped skin specimens were prepared for light-microscopy. Three individual experiments were performed using skin from different donors.

Tissue morphology

Samples of RE-DED or excised skin were fixed in buffered 4% paraformaldehyde and processed for embedding in paraffin. Vertical sections (4 μm) were cut and stained with haematoxylin and eosin for light-microscopic examination.

Immunohistochemical staining

For SKALP analysis, the avidin-biotin-peroxidase complex method was used essentially as described by the suppliers (strep-ABCcomplex/HRP, DAKO). In brief, paraffin sections (4 μm) were deparaffinized, rehydrated and pre-incubated with normal human serum in PBS/0.1% BSA, followed by incubation with anti-SKALP serum (kindly provided by Dr. J. Schalkwijk, University of Nijmegen, The Netherlands). After incubation with biotinylated-swine-anti-rabbit Ig and strepABCcomplex/HRP, the sections were developed with amino-ethylcarbazole as the chromogenic substrate.

Total RNA isolation and Northern blotting

Epidermis was isolated by placement on a drop of water and heat-splitting at 60°C for 1 min and processed for mRNA extraction according to the method of Chomczynski & Sacchi (13). Briefly, isolated epidermis was placed in 1 ml GITC-buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) with 3.3 ml/l 30% antifoam (Sigma), and immediately stored at -70°C. Samples were homogenized using a Kinematic AG polytron PT 3000 with a PT-DA 3007/2 dispersing tool. After extraction, 10 μg of total RNA per sample was electrophoresed through a formaldehyde/1% agarose gel, transferred to nylon membranes and hybridized overnight at 60°C with a ³²P-labelled SKALP probe. Each experimental condition was examined in three independent experiments. The probe used was a 420 bp *PvuII/EcoRI* fragment of the SKALP cDNA clone pGESKA (kindly provided by Dr. J. Schalkwijk, University of Nijmegen, The Netherlands). Control hybridizations for equal loading were performed using a human 28S ribosomal RNA probe.

RESULTS

Effect of culture conditions on SKALP expression

In the experiments DMEM/Ham's F12 (3:1) medium was used, in which hydrocortisone, L-isoproterenol and insulin were standardly present. Reduction of the hydrocortisone and insulin concentration from 1 μM to 0.5 μM and 0.1 μM respectively, in serum-free medium did not affect SKALP expression as compared to control cultures.

EGF induces SKALP expression. Addition of 1 ng/ml EGF to cultures grown at 37°C in serum-containing medium did not affect SKALP expression compared to EGF-free conditions. Supplementation with 10 ng/ml EGF caused a small increase in SKALP expression compared to the control cultures, concentrated intracellularly below the upper cell membrane. Supplementation with 10 ng/ml EGF of cultures grown at 33°C in serum-containing medium resulted in a much more pronounced induction of SKALP protein levels in suprabasal layers and an expression which was located intracellularly throughout the cytoplasm (Fig. 1A). At 33°C, SKALP expression was already slightly affected when 1 ng/ml EGF was added to the medium. The presence of other growth factors in the medium, like TGF α , IGF or KGF, did not affect SKALP expression either at 33°C or 37°C when compared to cultures grown in the absence of these growth factors (Table I). In accordance with immunohistochemical data, supplementation of serum-containing medium with 10 ng/ml EGF resulted in an increased number of SKALP mRNA transcripts both at 33°C and 37°C, whereas the addition of 10 ng/ml

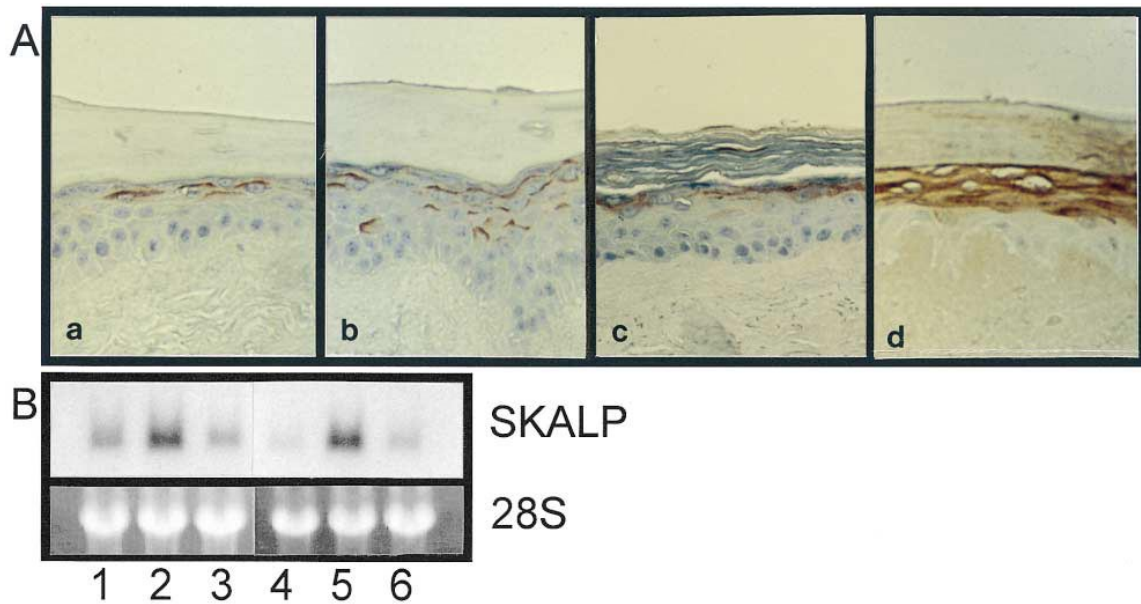


Fig. 1. (A) Immunohistochemical staining of SKALP in RE-DED cultured in serum-containing medium at 37°C (a, b) or at 33°C (c, d). (a, c) No growth factors were added to the medium. (b, d) The medium was supplemented with 10 ng/ml EGF. Original magnification 200 \times . B. Northern blot of SKALP mRNA expression in RE-DED cultured in serum-containing medium at 37°C (lanes 1–3) or at 33°C (lanes 4–6). (lanes 1 and 4) No growth factors were added to the medium. (lanes 2 and 5) The medium was supplemented with 10 ng/ml EGF or with (lanes 3 and 6) 10 ng/ml TGF α . The lower panel shows a photograph of the ethidium bromide-stained agarose gel to demonstrate equal RNA loading.

Table I. Effect of culture conditions on SKALP expression in RE-DED

Supplements	Concentration	Serum-containing medium	Serum-free medium
Growth factors			
EGF	1 ng/ml	\uparrow (33°C), =(37°C)	\uparrow (33°C), \uparrow (37°C)
	10 ng/ml	\uparrow (33°C), \uparrow (37°C)	\uparrow (33°C), \uparrow (37°C)
TGF α	10 ng/ml	=	=
IGF	10 ng/ml	=	=
KGF	10 ng/ml	=	=
Vitamins			
Ascorbic acid	50 μ g/ml	=	=
α -tocopherol	10 μ M	=	=
1,25(OH) $_2$ D $_3$	10 $^{-8}$ M	n.d.	\downarrow (33°C)
All-trans-RA	10 $^{-6}$ /10 $^{-7}$ M	n.d.	\downarrow (33°C)
9-cis-RA	10 $^{-6}$ /10 $^{-7}$ M	n.d.	\downarrow 10 $^{-6}$ M, = 10 $^{-7}$ M (33°C)
Fibroblasts			
+ EGF	0.1 ng/ml	n.d.	\uparrow
	1 ng/ml	n.d.	\uparrow
	10 ng/ml	n.d.	\uparrow

\uparrow mild, $\uparrow\uparrow$ moderate, \uparrow strong induction; \downarrow reduction; $\downarrow\downarrow$ complete inhibition, n.d. not determined.

= no change in SKALP expression compared to controls. No temperature indication means that the data were similar both at 33 and 37°C.

TGF α had no effect on SKALP mRNA expression compared to control levels (Fig. 1B).

In serum-free medium SKALP was less expressed than in serum-containing medium. In the absence of serum and growth factors, weak SKALP staining was observed in the upper granular layers. This applied to cultures grown at both 33°C (Fig. 2) and 37°C (data not shown). Supplementation of serum-free medium at 33°C with 0.1 ng/ml EGF affected neither the thickness of the epidermis nor the expression of SKALP. However, addition of 1 ng/ml EGF resulted in an increased number of living cell layers in which SKALP was expressed intracellularly below the upper cell membrane in suprabasal layers (Fig. 2c). This effect was even more pronounced when 10 ng/ml EGF was added to the medium; the epidermis was abnormally thick and SKALP was clearly present in all suprabasal layers (Table I).

Fibroblasts induce SKALP expression in the absence of growth factors. To investigate whether dermal factors influence the expression of SKALP in epidermal cell layers, DED was populated with human fibroblasts. After 3 weeks of culture, keratinocytes were seeded on top of this dermal equivalent. This resulted in the formation of a thicker epidermis and concomitantly in a clear SKALP induction in the upper suprabasal layers compared to cultures grown on DED without fibroblasts (Fig. 2b). Supplementation of these cultures with 0.1, 1 or 10 ng/ml EGF did not result in an additional induction of SKALP (Table I).

Retinoic acid and 1,25-dihydroxyvitamin D $_3$ suppress SKALP expression. Supplementation of the medium with differentiation-modulating agents such as all-trans-RA, 9-cis-RA and 1,25(OH) $_2$ D $_3$ significantly affected SKALP protein levels.

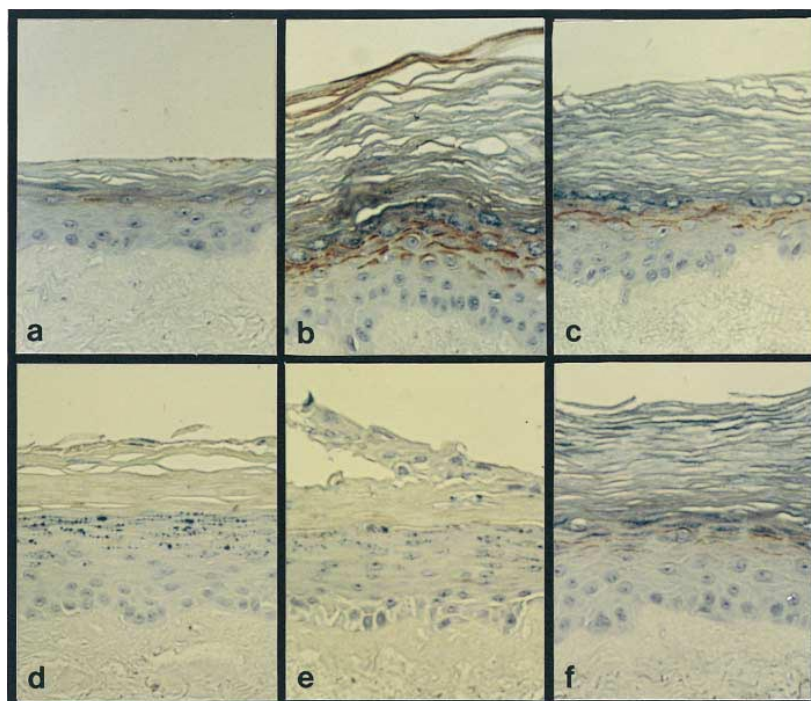


Fig. 2. Immunohistochemical staining of SKALP in reconstructed epidermis cultured at 33°C in serum-free medium (a) on DED in the absence of growth factors, (b) on DED populated with fibroblasts, (c–f) on DED supplemented with 1 ng/ml EGF, (c) in the absence or in the presence of (d) 9-*cis*-RA (10^{-6} M), (e) all-*trans*-RA (10^{-7} M), or (f) 1,25(OH) $_2$ D $_3$ (10^{-8} M). Original magnification 200 \times .

SKALP was completely absent when the concentration of 9-*cis*-RA was increased to 10^{-6} M. Low concentrations of 9-*cis*-RA ($<10^{-7}$ M) had no effect. Addition of all-*trans*-RA resulted in a complete absence of SKALP in the epidermal layers at both 10^{-6} and 10^{-7} M. The presence of 10^{-8} M 1,25(OH) $_2$ D $_3$ in the medium caused a reduction in SKALP expression (Fig. 2). As serum-enriched culture medium contains small amounts of retinoic acid the influence of this supplement was only investigated in serum-free medium.

Ascorbic acid and α -tocopherol do not affect SKALP expression. Supplementation of culture media with α -tocopherol and ascorbic acid did not affect the expression of SKALP (Table 1).

SKALP as a marker for skin irritation

After having determined the culture conditions which affect SKALP expression, we evaluated its value as a marker for skin irritancy after mechanical and chemical trauma.

Tape-stripping in vitro induces SKALP expression. Removal of the stratum corneum from excised skin specimens by tape-stripping in vitro induced SKALP expression in suprabasal cell layers within 24 h and increased in parallel with the number of stratum corneum layers that were removed. In accordance with previous findings by others, no SKALP was present in normal skin (Fig. 3).

SKALP is induced after SLS and OA application in vitro. Treatment of excised skin specimens for 24 h with increasing concentrations SLS resulted in an induction of SKALP at

0.5%, 1% and 2% SLS. At 1% staining showed a focal distribution in the granular and upper spinous layers, which was slightly increased after the application of 2% SLS (data not shown). Topical application of 0.5% and 1% SLS on RE-DED induced SKALP which was distributed evenly in the upper layers of the suprabasal compartment. Treatment with 2% SLS was toxic to the epidermal cells, so no conclusion could be drawn concerning SKALP expression. Exposure of RE-DED to 0.5% and 5% OA for 24 h resulted in an induction of SKALP protein expression, mainly intracellularly on the apical site of the granular cells (Fig. 4A, a–c). Air-exposed cultures which had been exposed to both the test agents for 4 h showed a very clear induction of SKALP at 48 h after treatment, which was more pronounced than the induction seen after 24 h of exposure. At 0.5% SLS, 0.5% and 5% OA, SKALP was present in all suprabasal layers, except the basal cells. After exposure to 0.5% SLS and 0.5% OA the intracellular staining was distributed throughout the cytoplasm (Fig. 4A, d, e). The effect of treatment with 5% OA was comparable with the expression induced by 0.5% OA. At the mRNA level, no significant effect on SKALP expression was detected after topical application of 0.5% SLS or 0.5% OA for 6, 12 and 24 h (Fig. 4B).

DISCUSSION

The suitability of in vitro models for the purpose of investigating whether compounds have a potential to irritate human skin is an important subject in a great number of current

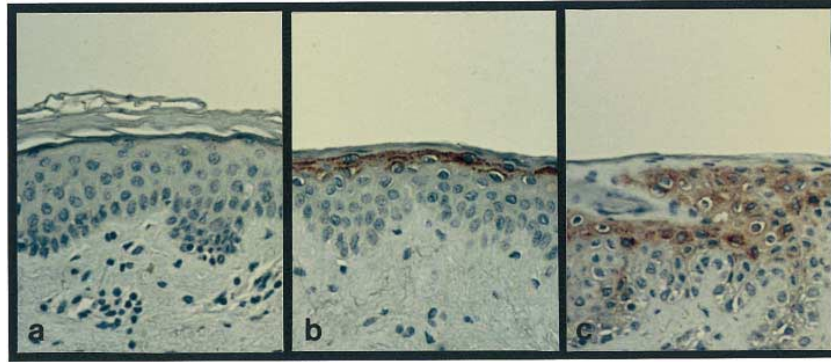


Fig. 3. Immunohistochemical staining of SKALP in excised skin after 24 h incubation of (a) untreated skin or of tape-stripped skin (b) 18 × and (c) 25 ×.

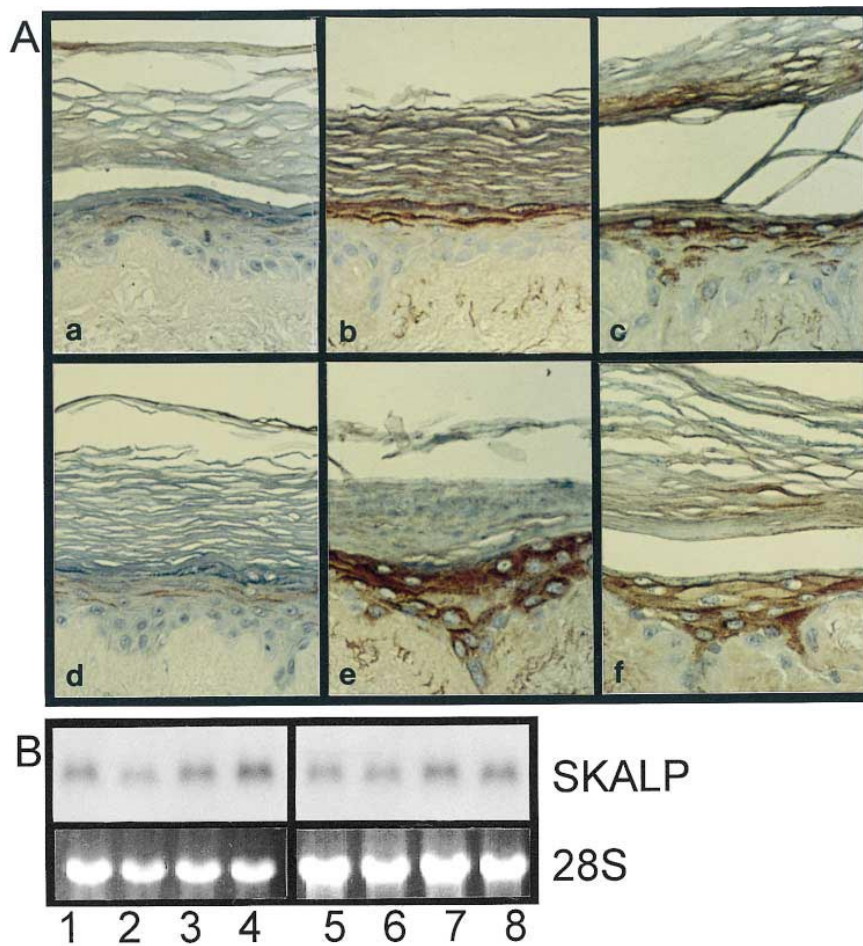


Fig. 4. (A) Immunohistochemical staining of SKALP in RE-DED cultured at 33°C in serum-free medium in the presence of 1 ng/ml EGF after topical application of SLS or OA. (a-c) 24 h treatment, (d-f) 4 h treatment after which the test agents were removed and the cultures were further incubated for 48 h, (a, d) untreated cultures, cultures treated with (b, e) 0.5% SLS, or (c, f) 0.5% OA. Original magnification 200x. (B) Northern blot of SKALP mRNA expression in RE-DED cultured at 33°C in serum-free medium with 1 ng/ml EGF after topical application of 0.5% SLS (left panel) or 0.5% OA (right panel) for (lanes 2 and 7) 6 h, (lanes 3 and 7) 12 h, and (lanes 4 and 8) 24 h. Lanes 1 and 5 = untreated cultures. The lower panel shows a photograph of the ethidium bromide-stained agarose gel to demonstrate equal RNA loading.

studies. In the present study, the effects of potential skin irritants were evaluated using an in vitro generated reconstructed epidermis, consisting of a well-differentiated epidermis closely approaching native tissue (14). A major problem encountered in studies dealing with irritancy screening is to find appropriate markers that cover such a wide range of biological parameters that they can predict whether a compound is likely to cause irritation in vivo (14–16).

The aim of the present study was to investigate whether SKALP can be used as a sensitive end-point. Since it has been shown that the infiltration of PMNs is a non-specific common feature in skin inflammation following various types of skin injury (17, 18), the release of serine proteinase inhibitors such as SKALP, is likely to be a key mechanism during inflammatory processes (19). Moreover, its marked presence in psoriatic epidermis in which hyperproliferation is accompanied by cutaneous inflammation and an abnormal barrier function may also indicate that induction of SKALP activity is related to barrier disruption. This concept is supported by the finding that tape-stripping in vivo has been shown to result in a gradual increase in SKALP expression in the epidermis (3). In accordance with the in vivo situation, we demonstrated a similar induction pattern after tape-stripping of excised skin specimens. In addition, topical application of SLS on excised skin also resulted in SKALP expression. Parallel treatment of air-exposed cultures with SLS and OA resulted in a dose-dependent increase in SKALP protein expression. However, at the mRNA levels no significant changes were observed, suggesting that the irritants may affect SKALP expression primarily at translational levels. On the other hand, the induction of SKALP mRNA levels in the presence of EGF suggests that this growth factor acts at transcriptional levels.

Investigation of SKALP expression in RE-DED cultured in serum-containing medium, resulted in a positive staining which varied slightly in extent but which was independent of the presence or absence of other supplements. The differences observed in SKALP expression in the various experiments may have been caused by the use of different batches of serum to prepare the culture media. The influence of serum has previously been demonstrated in keratinocyte monolayers, in which the addition of fetal calf serum to the medium resulted in the induction of SKALP (20, 21). The finding that serum positively affected SKALP expression in RE-DED may be linked to previous studies, indicating that RE-DED cultured in the presence of serum shares some common features with hyperproliferative epidermis (7, 9).

By using serum-free media and provided that EGF was absent or that only low concentrations were used (0.1 ng/ml), very little staining for SKALP was detected. Both at 33°C and 37°C the protein was absent or appeared as a faint band in the upper granular layers. This could be explained by the finding that under serum-free conditions complete epidermal homeostasis has not yet been achieved (8, 9). In this respect it should be noted that in the absence of serum and growth factors the epidermis is thin and only one layer of granular cells is present. The addition of EGF resulted in an increase of epidermal thickness with concomitant thickening of the stratum granulosum and an induction of SKALP expression. This effect of EGF was dose-dependent. Most remarkable was the effect of 10 ng/ml EGF in cultures grown at 33°C; SKALP was abundantly present and located intracellularly throughout the cytoplasm. The negative effects of EGF on terminal

differentiation at 33°C are supported by recent studies showing the overall aberrant expression of a number of differentiation markers at 33°C after EGF supplementation (8). Strikingly, other growth factors (TGF α , IGF, KGF) do not modulate SKALP and other differentiation markers. Therefore, a promising substitute for EGF may be TGF α , a growth factor that is structurally related to EGF and binds to the EGF receptor (22, 23). Although addition of TGF α to submerged keratinocyte cultures has been shown to induce SKALP up to a level comparable to the expression found after supplementation with fetal calf serum (van Ruissen, personal communication), this growth factor did not affect SKALP expression in air-exposed cultures. This may be explained by different mechanisms of action between EGF and TGF α , since – in contrast to EGF – no effects of TGF α were found on morphogenesis and the expression of K1, K10 and cornified envelope precursors (8, 24).

Careful control of optimal conditions not only include supplementation with the right combination of growth factors but also the provision of a balanced environment. It has been found that the addition of ascorbate, an aqueous antioxidant, results in a normalization of epidermal ultrastructure (25). In contrast to the marked improvement of skin barrier formation in ascorbic acid-supplemented medium, SKALP was still present under these conditions, which indicates that epidermal homeostasis is still not reached.

With respect to other vitamins, their role in proliferation and differentiation processes in vitro has been questioned, although lipid-soluble vitamins are essential in vivo. However, it has been shown that 1,25(OH) $_2$ D $_3$, all-*trans*-RA and 9-*cis*-RA have strong, modulating effects on cellular proliferation and differentiation (26–28). Our results show that in the presence of these vitamins SKALP was markedly suppressed. In air-exposed cultures, a concentration of 10 $^{-6}$ M all-*trans*-RA inhibited terminal differentiation, as demonstrated by the absence of granular layers. A lower concentration of 10 $^{-7}$ M showed a less dramatic effect on the differentiation pattern, but in spite of the formation of a stratum granulosum SKALP was still absent. The 9-*cis* analogue did not counteract formation of the stratum granulosum but prevented SKALP expression at both concentrations tested. So, the addition of these compounds may be beneficial for regulating aberrant epidermal proliferation.

When fibroblasts were present in the dermal compartment a strong induction of SKALP was seen. This occurred both in the presence and in the absence of EGF. The influence of dermal factors on epidermal growth and differentiation has been the subject of extensive studies. Fibroblasts have been shown to be of prime importance for the regulation of epidermalization (29). Although it is not known what growth factors may be responsible, we observed that neither TGF α , IGF nor KGF could mimic the fibroblast-induced SKALP expression.

Despite the sensitivity of SKALP expression to variations in the culture conditions, a slight basal presence of SKALP does not necessarily interfere with its induction by skin irritants. From our study it can be concluded that SKALP may be used as a potential marker for non-specific skin injury, although the mechanisms involved in SKALP induction, in particular with respect to its use in irritancy screening, need further examination.

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REFERENCES

- Nickoloff BJ, Turka LA. Keratinocytes: key immunocytes of the integument. *Am J Pathol* 1993; 143: 325–331.
- Baumann H, Gauldie J. The acute phase response. *Immunol Today* 1994; 15: 74–80.
- Schalkwijk J, Chang A, Janssen P, de Jongh GJ, Mier PD. Skin-derived antileukoproteinas (SKALPs): characterization of two new elastase inhibitors from psoriatic epidermis. *Br J Dermatol* 1990; 122: 631–641.
- Wiedow O, Lüdemann J, Utrecht B. Elafin is a potent inhibitor of proteinase-3. *Biochem Biophys Res Comm* 1991; 174: 6–10.
- Schalkwijk J, van Vlijmen IMJJ, Alkemade JAC, de Jongh GJ. Immunohistochemical localization of SKALP/Elafin in psoriatic epidermis. *J Invest Dermatol* 1993; 100: 390–393.
- Alkemade JAC, Molhuizen HOF, van Vlijmen-Willems IMJJ, van Haelst UJGM, Schalkwijk J. Differential expression of SKALP/Elafin in human epidermal tumors. *Am J Path* 1993; 143: 1679–1687.
- Ponec M. Reconstruction of human epidermis on de-epidermized dermis: expression of differentiation-specific protein markers and lipid composition. *Toxic in Vitro* 1991; 5: 597–606.
- Ponec M, Gibbs S, Weerheim A, Kempenaar J, Mulder A, Mommaas AM. Epidermal growth factor and temperature regulate keratinocyte differentiation. *Arch Dermatol Res*, 1997; 289: 317–326.
- Gibbs S, Vicanova J, Bouwstra J, Valstar I, Kempenaar J, Ponec M. Culture of reconstructed epidermis in a defined medium at 33°C shows a delayed epidermal maturation, prolonged lifespan and improved stratum corneum. *Arch Dermatol Res* 1997; 289: 585–595.
- Ponec M, Havekes L, Kempenaar J, Lavrijsen S, Wijsman M, Boonstra J, et al. Calcium-mediated regulation of the low density lipoprotein receptor and intracellular cholesterol synthesis in human epidermal keratinocytes. *J Cell Physiol* 1985; 125: 98–106.
- Régnier M, Pruniéras M, Woodley D. Growth and differentiation of adult human epidermal cells on dermal substrates. *Frontiers Matrix Biol* 1981; 9: 4–35.
- Boyce ST, Williams ML. Lipid supplemented medium induces lamellar bodies and precursors of barrier lipids in cultured analogues of human skin. *J Invest Dermatol* 1993; 101: 180–184.
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid-guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162: 156–159.
- Ponec M, Kempenaar J. Use of human skin recombinants as an in vitro model for testing the irritation potential of cutaneous irritants. *Skin Pharmacol* 1995; 8: 49–59.
- Boelsma E, Tanojo H, Boddé HE, Ponec M. Assessment of the potential irritancy of oleic acid on human skin: evaluation in vitro and in vivo. *Toxic in Vitro* 1996; 10: 729–742.
- Boelsma E, Tanojo H, Boddé HE, Ponec M. An in vivo-in vitro study to the use of a human skin equivalent for irritancy screening of fatty acids. *Toxic in Vitro* 1997; 11: 365–376.
- Chang A, de Jongh GJ, Mier PD, van de Kerkhof PCM. Enzymatic quantification of polymorphonuclear leukocytes in normal and psoriatic skin following standard injury. *Clin Exp Dermatol* 1988; 13: 62–66.
- Lammers AM, van de Kerkhof PCM. Response of polymorphonuclear leukocytes to topical leukotriene B₄ in healthy and psoriatic skin. *Br J Dermatol* 1987; 116: 521–524.
- Briggaman RA, Schechter NM, Fräki J, Lazarus GS. Degradation of the epidermal-dermal junction by proteolytic enzymes from human skin and human polymorphonuclear leukocytes. *J Exp Med* 1984; 160: 1027–1042.
- Alkemade JAC, Molhuizen HOF, Ponec M, Kempenaar JA, van Zeeuwen PLJ, de Jongh GJ, et al. SKALP/elafin is an inducible proteinase inhibitor in human epidermal keratinocytes. *J Cell Science* 1994; 107: 2335–2342.
- van Ruissen F, de Jongh GJ, Zeeuwen PLJM, van Erp PEJ, Madsen P, Schalkwijk J. Induction of normal and psoriatic phenotypes in submerged keratinocyte cultures. *J Cell Physiol* 1996; 168: 442–452.
- Hudson LC, Gill GN. Regulation of gene expression by epidermal growth factor. In: *Genetic engineering, principles and methods*. New York: Plenum Press; Vol 1991; 13: 137–152.
- Cha D, O'Brien P, O'Toole EA, Woodley DT, Hudson LG. Enhanced modulation of keratinocyte motility by transforming growth factor- α (TGF α) relative to epidermal growth factor. *Cell* 1996; 50: 1131–1137.
- Gibbs S, Boelsma E, Kempenaar J, Ponec M. EGF and TGF α differentially regulate epidermal morphogenesis and the expression of cornified envelope precursors. *Cell Tissue Res*, in press.
- Ponec M, Weerheim A, Kempenaar J, Mulder A, Gooris GS, Bouwstra J, et al. The formation of competent barrier lipids in reconstructed human epidermis requires the presence of vitamin C. *J Invest Dermatol* 1997; 109: 348–355.
- Régnier M, Darmon M. Human epidermis reconstructed in vitro: a model to study keratinocyte differentiation and its modulation by retinoic acid. *In Vitro Cell & Dev Biol* 1989; 25: 1000–1008.
- Smith EL, Walworth NC, Holick MF. Effect of 1 α ,25-dihydroxyvitamin D₃ on the morphologic and biochemical differentiation of cultured human epidermal keratinocytes grown in serum-free conditions. *J Invest Dermatol* 1986; 86: 709–714.
- Gibbs S, Backendorf C, Ponec M. Regulation of keratinocyte proliferation and differentiation by all-*trans*-retinoic acid, 9-*cis*-retinoic acid and 1,25-dihydroxy vitamin D₃. *Arch Dermatol Res* 1996; 288: 729–738.
- Coulomb B, Lebreton, C, Dubertret L. Influence of human dermal fibroblasts on epidermalization. *J Invest Dermatol* 1989; 92: 122–125.