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 effects 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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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 can be generated (7). 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
Biomedicals Inc., Costa Mesa, CA, USA), supplemented with sections (4

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**Tape-stripping**

EGF induces 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 and 1 ng/ml EGF to 0.5 μM and 0.1 ng/ml EGF 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 33°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 EGF-free conditions. Cultures grown in the absence of these growth factors (Table I). 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 33°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 (80–90% of the SKALP mRNA) than in monolayer 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
SKALP in reconstructed human epidermis

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×. (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 TGFα. 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

<table>
<thead>
<tr>
<th>Supplements</th>
<th>Concentration</th>
<th>Serum-containing medium</th>
<th>Serum-free medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth factors:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>1 ng/ml</td>
<td>†(33°C), ‡(37°C)</td>
<td>†(33°C), ‡(37°C)</td>
</tr>
<tr>
<td>EGF</td>
<td>10 ng/ml</td>
<td>†(33°C), ‡(37°C)</td>
<td>†(33°C), ‡(37°C)</td>
</tr>
<tr>
<td>TGFα</td>
<td>10 ng/ml</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>IGF</td>
<td>10 ng/ml</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>KGF</td>
<td>10 ng/ml</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Vitamins:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>50 µg/ml</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>10 µM</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>1,25(OH)2D3</td>
<td>10−8 M</td>
<td>n.d.</td>
<td>†(33°C)</td>
</tr>
<tr>
<td>All-trans-RA</td>
<td>10−9/10−7 M</td>
<td>n.d.</td>
<td>‡(33°C)</td>
</tr>
<tr>
<td>9-cis-RA</td>
<td>10−9/10−7 M</td>
<td>n.d.</td>
<td>†(10−6 M, 33°C)</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td></td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>+EGF</td>
<td>0.1 ng/ml</td>
<td>n.d.</td>
<td>=</td>
</tr>
<tr>
<td>1 ng/ml</td>
<td>n.d.</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>10 ng/ml</td>
<td>n.d.</td>
<td>=</td>
<td></td>
</tr>
</tbody>
</table>

† mild, †† moderate, ‡ strong induction; ‡ reduction; ‡ complete inhibition, n.d. not determined.

TGFα 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 and 37°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 intracellular 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 1).

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 1).

Retinoic acid and 1,25-dihydroxyvitamin D3 suppress SKALP expression. Supplementation of the medium with differentiation-modulating agents such as all-trans-RA, 9-cis-RA and 1,25(OH)2D3 significantly affected SKALP protein levels.

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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 $10^{-5}$ 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 I).

**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 200×. (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.
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 (TGFα, IGF, KGF) do not modulate SKALP and other differentiation markers. Therefore, a promising substitute for EGF may be TGFα, a growth factor that is structurally related to EGF and binds to the EGF receptor (22, 23). Although addition of TGFα 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 Ruisen, 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 TGFα, since – in contrast to EGF – no effects of TGFα 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 shown 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)2D3, 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 10−8 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 TGFα, 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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