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Skin constructs for replacement of skin tissues for in vitro testing

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Abstract

Reconstructed human skin equivalents as an alternative to animal experimentation offer not only a way to concede to demands of regulatory authorities, animal welfare organizations, consumers and scientists, but also provide a means to improve and extend our knowledge on biological processes in the skin. Presently, various skin reconstructs are available composed either of the epidermal compartment only or of both the epidermal and dermal compartments. Within each compartment various types of cells can be incorporated, including keratinocytes, melanocytes and Langerhans cells in the epidermal, and fibroblasts and endothelial cells in the dermal compartment. The quality of the human skin equivalents has now reached a point that their suitability for skin toxicity testing will make great progress. Next to the field of toxicity and safety standards, skin equivalents offer a well-characterized model for studies of the basic skin biology, wound repair, regulation of melanogenesis, pathogenesis of skin diseases and skin cancer.

Keywords: Human skin equivalents; Epidermis; Stratum corneum; Barrier function; Skin irritation; UV exposure; HSP 27; Melanin

Contents

1. Introduction

Skin represents the largest organ of the human body and therefore it suits as a potential anatomical site for application of drugs and cosmetic products. Depending on the site of action, these products should be delivered to their potential target sites, which may be the stratum corneum, epidermis, dermis, appendages or blood vessels, respectively. In

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most cases, it is necessary to predict the way in which materials penetrate the skin, since their effects will depend on the time they reach the target site and their concentration there. Skin penetration is a rather complex phenomenon, with the stratum corneum being the main barrier, in which a number of processes need to be taken into account, including (i) partitioning into the stratum corneum, diffusion within or through this layer; (ii) partitioning into the more hydrophilic viable epidermis and dermis, diffusion within and through these layers, metabolic processing, binding to the receptor; (iii) partitioning into the systemic circulation or fat depot.

Product safety testing and risk/benefit evaluations are conducted in order to assess the effectiveness of topical products and to identify substances which may induce adverse skin reactions, and to assess human environmental risks. In the past, animals have been often used for such testing and the outcome of the tests has been till now used by official authorities, in spite of the lower competence of barrier function, differences in skin architecture and metabolism. Recently, laws and regulations have become more stringent regarding the safety of products and reduction of laboratory tests on animals. Although during recent years numerous in vitro, non-animal systems have been developed, international agreements on in vitro methods as alternatives to animal studies are very limited. The need for rapid and reproducible methods for assessing the effectiveness of topical preparations, percutaneous absorption and cutaneous irritancy has encouraged many investigators to develop in vitro models. Since the time course and concentration of a skin irritancy reaction in vivo is strongly related to the barrier capacity of the stratum corneum (SC), a suitable in vitro model should adequately mimic skin barrier function.

2. Human skin recombinants

2.1. Generation and morphological characterization

Various human skin recombinants have been reconstructed in vitro, which mimic the native tissue to a high extent (reviewed in Ref. [1]). They are generated by growing differentiated keratinocyte cultures on acellular or fibroblast-populated dermal substrates, such as de-epidermized dermis (DED) [2–4], collagen matrices [5,6], inert filters [7,8], and lyophilized collagen-GAG membranes cross-linked by chemical agents [9]. Some of these models are commercially available, like SkinEthic™, EpiDerm™ and Episkin. Morphological studies have shown that these skin equivalents form a multilayered epithelium composed of an organized stratum basale, stratum spinosum, stratum granulosum and a stratum corneum (Fig. 1a–c). Furthermore, they display characteristic epidermal ultrastructure [4,10] and they express markers of epidermal differentiation [11–14]. Ultrastructurally, keratohyalin granules and lamellar bodies are present in the stratum granulosum (SG). The lamellar bodies are extruded at the SG/SC interface and the SC is filled with lipid lamellar structures (Fig. 1d and e).

2.2. Stratum corneum characterization

The permeability barrier resides in multiple lipid lamellae that fill the extracellular spaces between the keratinized cells (corneocytes) of the SC (reviewed in Refs. [15,16]). To assure that the penetration pathway is confined to the intercorneocyte space, the presence of an impermeable cornified envelope (CE) is required. The CE consists of a layer of protein that is about 15 nm thick and is formed beneath the plasma membrane during terminal differentiation. It is composed of several cross-linked precursors (reviewed in Ref. [17]). Detailed analysis of the composition of CE proteins of native and reconstructed epidermis revealed great similarities between the two (Table 1). The CE proteins are cross-linked together to form a rigid, chemically resistant, insoluble envelope by disulfide bonds and by the $N^{\varepsilon}(\gamma$-glutamyl)lysine isopeptide cross-link formed by the action of one or more known transglutaminases [17]. In superficial layers of the native SC the 'rigid' type (about 80%) of CE is the most abundant type [18] assuring that penetration occurs via the intercorneocyte pathway. Using confocal laser scanning microscopy, we could establish that also in the reconstructed epidermis the intercorneocyte penetration pathway is the predominating one [19]. This

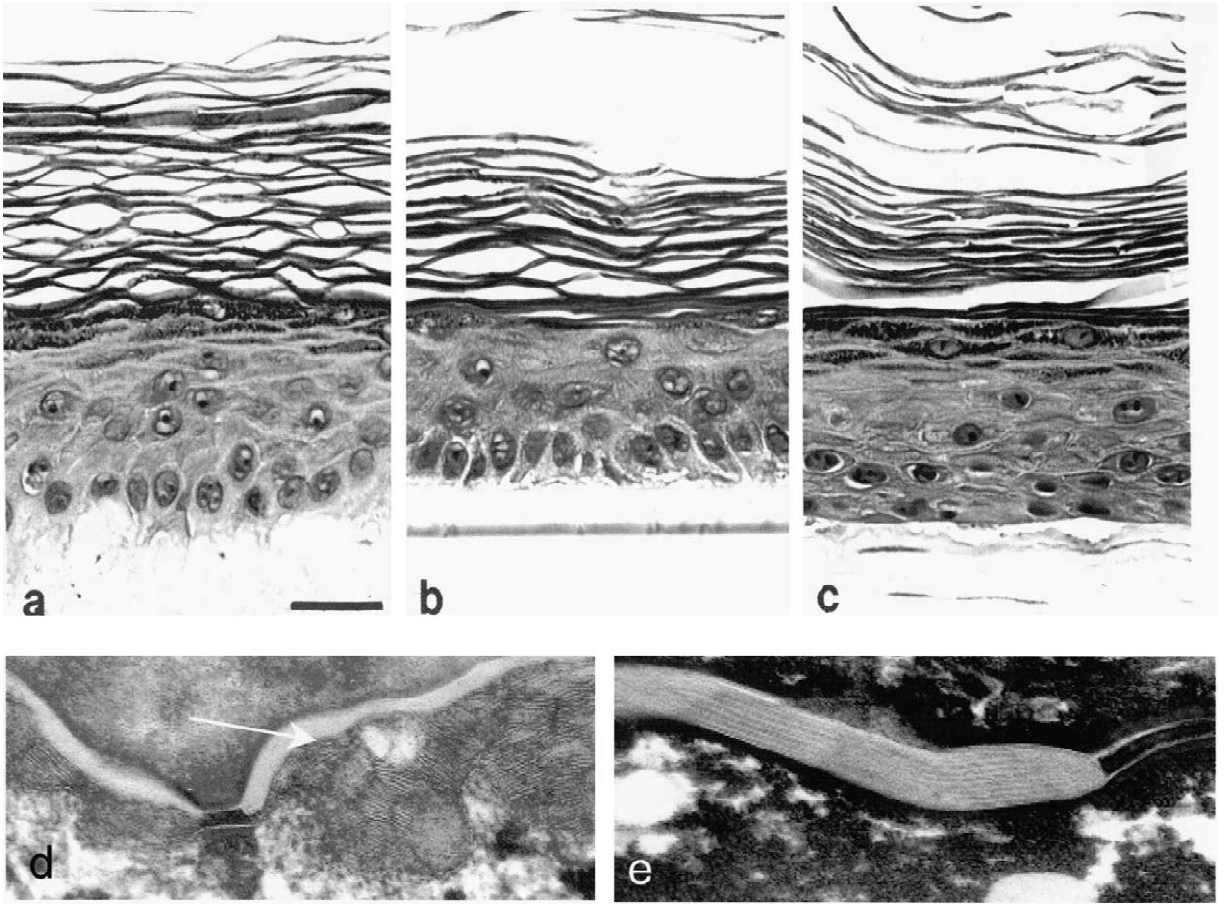


Fig. 1. Morphology of reconstructed epidermis using (a) de-epidermized dermis (DED), (b) inert filter, (c) fibroblast-populated collagen matrix as substrates. Light microscopic level (a–c) hematoxylin and eosin staining of paraffin-embedded cultures of keratinocytes grown for 14 days at the air–liquid interface in serum-free medium supplemented with vitamin C and E (scale bar 5 μm). Ultrastructural level (transmission electron micrographs; d, e); (d) the extrusion of lamellar bodies at the stratum granulosum interface. Notice the presence of a desmosomal plug (small arrow) and the cornified envelope surrounding the desmosomal plug (arrowhead) and the formation of lipid lamellar structures (large arrow), scale bar 0.1 μm. (e) Multilayered lamellar structures with the characteristic alternating electron-dense and electron lucent pattern with repeating units (arrowhead), scale bar 0.1 μm.

finding is indicative that also in vitro the rigid type of CE is formed.

The corneocyte lipid envelope (CLE) is a hydrophobic envelope that is situated externally to the CE and is composed of ω-hydroxyceramides, ω-hydroxyacids and free fatty acids [20]. The CLE is assumed to play an important role in the orientation of intercorneocyte lipid lamellae that are oriented approximately parallel to the corneocyte surface. It has been proposed that ω-hydroxyceramides, the major component of bound lipids, are attached to the glutamate side of involucrin in the CE. Analysis of covalently bound lipids revealed great similarities in their composition between reconstructed and native epidermis (Fig. 2) [21]. In both tissues, comparable amounts of ω-hydroxyceramides were detected. The ω-hydroxyceramide fractions were composed of three major components, which consist of a long chain ω-hydroxyacid amide linked to sphingosine (OS), phytosphingosine (OP) and 6-hydroxy-4-sph-

Table 1
Protein composition of cornified envelopes as estimated from amino acid analysis (%)

Cornified envelope proteins	Human epidermis	
	Reconstructed	Native
Loricrin	68	73
SPRRs	9	8
Involucrin	6	5
Keratins	5	4
Filaggrin	2	3
Elafin	3	3
Cystatin a	3	3
Plakins	2	–
Genbank	2	–

Cornified envelope isolation and purification was performed by S. Gibbs, Department of Dermatology, LUMC, NL; analysis of CE composition performed by P. Steinert, Bethesda, USA.

ingosine (OH), respectively [16,22]. These findings clearly indicate that human keratinocytes in vitro can generate a complete spectrum of covalently bound lipids that can act as a scaffold for the extracellular lipid lamellae.

Since the penetration route mainly resides in the intercorneocyte space filled with lipid lamellae, the proper composition and structural organization of SC lipids is of great importance for a proper barrier function. In native epidermis the extracellular lipid lamellae consist mainly of cholesterol, free fatty acids and ceramides (reviewed in Refs. [15,16]). Presently, at least nine different ceramides have been identified in the human native and reconstructed epidermis (Refs. [16,23–25] and Ponec et al., in press [43]). Individual ceramides differ in their head

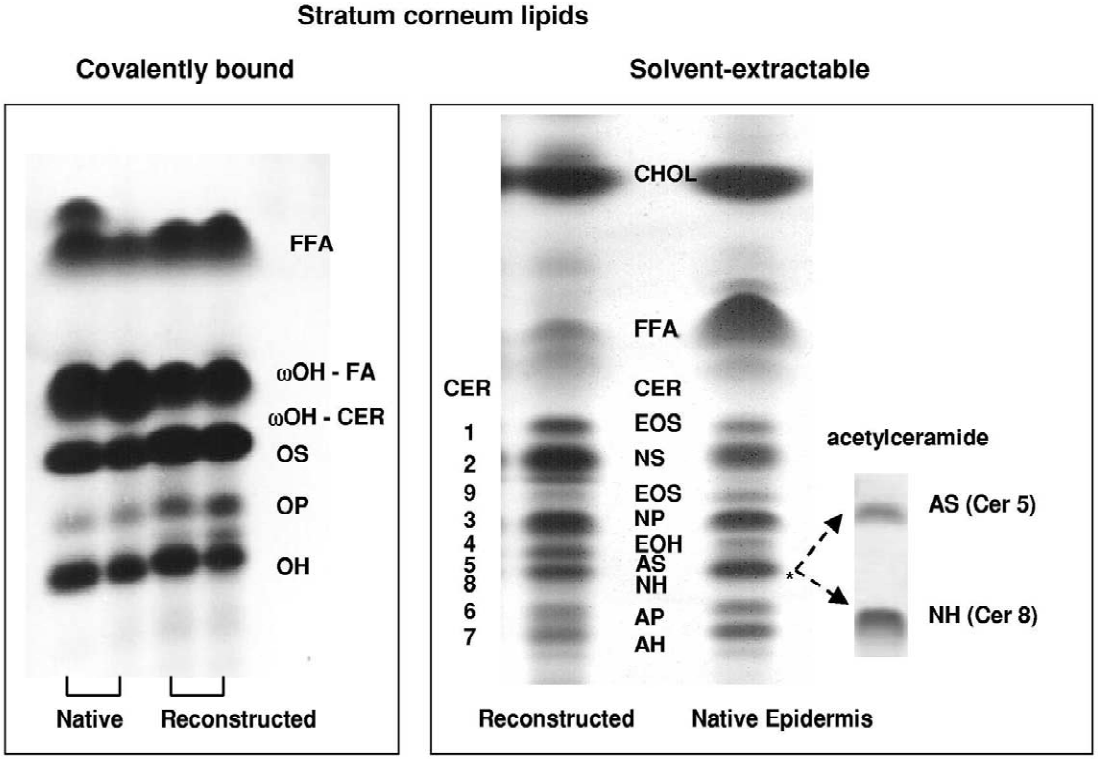


Fig. 2. Stratum corneum lipid profile in reconstructed and native epidermis. (A) Covalently bound lipids, (B) free lipids. Lipids from stratum corneum were extracted (free, extractable fraction) and the residues were subjected to mild saponification treatment (covalently bound, non-extractable fraction). Subsequently, the extracts were analyzed by HPTLC as described in detail by Ponec et al. [4,21].

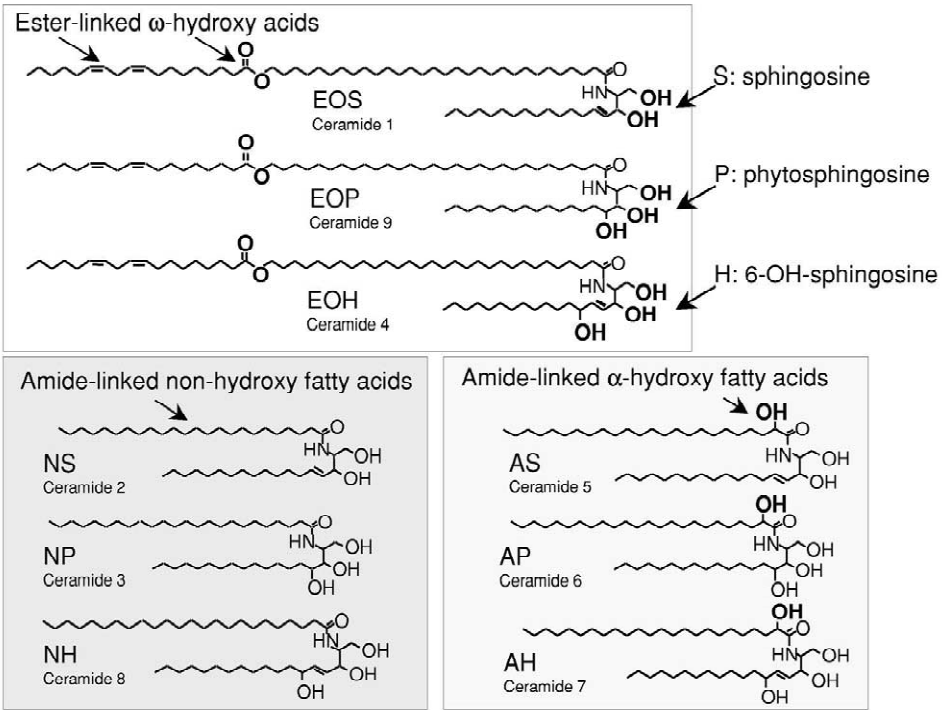


Fig. 3. Molecular structure of stratum corneum ceramides. The molecular structures are based on the data of Wertz et al. [16], Robson et al. [23], Stewart and Downing [24] and Ponec et al. (in preparation).

group architecture composed of sphingosine (S), phytosphingosine (P) or 6-hydroxy-4-sphingosine (H), to which amide-bond non-hydroxy (N) or α-hydroxy fatty acids (A) are linked. In addition, three acylceramides contain linoleic acid chemically bound to a long chain ω-hydroxyacid (EOS, EOP and EOH, respectively) (Fig. 3). Analysis of extractable lipids by thin layer and gas chromatography revealed great similarities in lipid profiles between native and reconstructed epidermis (Fig. 2) [4]. All major SC lipid classes, including all ceramide fractions, have been found to be synthesized under in vitro conditions. However, some differences have been noticed, such as a lower content of free fatty acids (FFA) [4] and of linoleic acid in acylceramides and more abundant presence of short fatty acids in some ceramide fractions (Ponec et al., in preparation). The observed deviations in the lipid composition may explain the differences in SC lipid organization between native and reconstructed epidermis [4]. Furthermore, using wide angle and electron diffraction techniques, it has been established that the lateral packing in native SC lipids is predominantly orthorhombic, while in reconstructed SC the hexagonal packing is prevailing [26,27]. These differences may account for the impaired barrier function of the reconstructed epidermis. The impaired barrier function is commonly seen in human epidermal equivalents, including commercially available SkinEthic, EpiDerm and Episkin models [28–30] and various in-house models [31]. In these models a 5- to 50-fold higher penetration rate was observed for most of the substances tested [32]. The deviations in SC lipid composition and organization demonstrated in these models [33] may account for the observed differences in SC barrier function.

3. Use of human skin equivalents for cutaneous irritancy testing

In spite of some still persisting differences be-

tween native and reconstructed tissue, the presence of the stratum corneum in the reconstructed epidermis makes it possible to apply topically a great variety of topical products used in daily life and examine their effectiveness, metabolic transformation and potential pathologic effects. For safety reasons with various topical products, a number of tests have been developed to detect the changes in stratum corneum integrity, epidermal morphology, in viability of epidermal cells, and in release of pro-inflammatory mediators (Fig. 4). To predict the value of the in vitro test system for the in vivo situation it is inevitable to use human skin in vivo for validation. For the in vivo tests, non-invasive methods, like assessment of changes in transepidermal water loss (TEWL) or cutaneous blood flow (CBF) are preferred. To illustrate the usefulness of such an approach, examples will be shown on the in vitro–in vivo correlation studies with sodium lauryl sulfate (SLS) and oleic acid. As reported by Agner et al. [34,35], SLS when applied at concentrations of about 0.5 to 2.5% induces increase in both TEWL and CBF. In this concentration range also, changes in tissue morphology and release of pro-inflammatory cytokines have been noticed with epidermis reconstructed on de-epidermized dermis [36] and excised human skin [37]. Due to the less competent barrier function in the commercially available Epiderm model, these SLS-induced changes in tissue morphology and IL-1α mRNA levels have been observed at lower SLS concentration [37]. In addition, increase of IL-1α mRNA level was observed upon topical application of water onto excised skin, whereas topical application of water to EpiDerm cultures did not alter the IL-1α mRNA levels (Fig. 5).

To extend the number of potential new markers for monitoring changes induced upon application of various test agents, next to changes in mRNA levels, also changes in protein levels can be assessed using proteomics. Proteomics, in which 2D-PAGE is combined with mass spectrometry, revealed that upon topical application of low SLS concentrations, seven proteins could be identified as potential new epider-

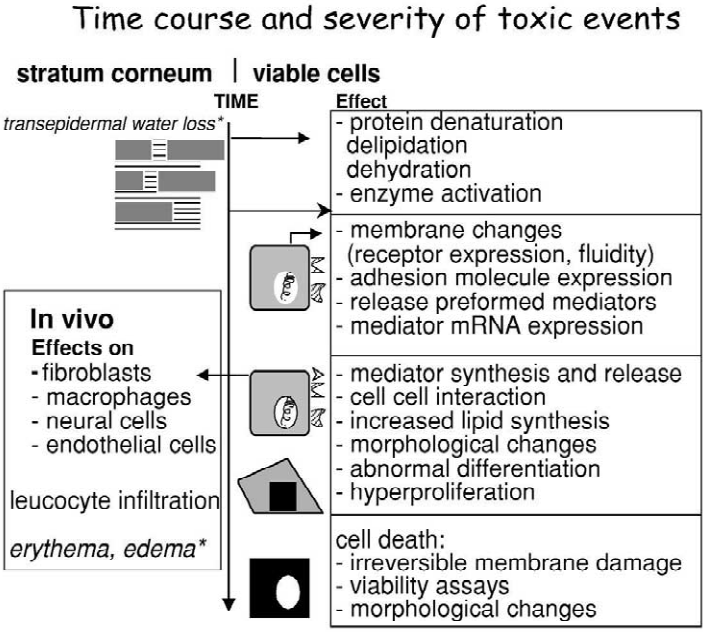


Fig. 4. Time-dependent effects evoked by topical agents. Topical agents may interact with the skin at three different levels: (1) they may impair the barrier function by interfering with either lipid or protein components. This may accompanied by increase in TEWL. (2) They may reach the viable epidermis and either stimulate cell metabolism and cause hyperplasia or inhibit cellular activity and finally destroy the epidermal cells. (3) They may reach the dermis and affect other cells involved in inflammatory responses.

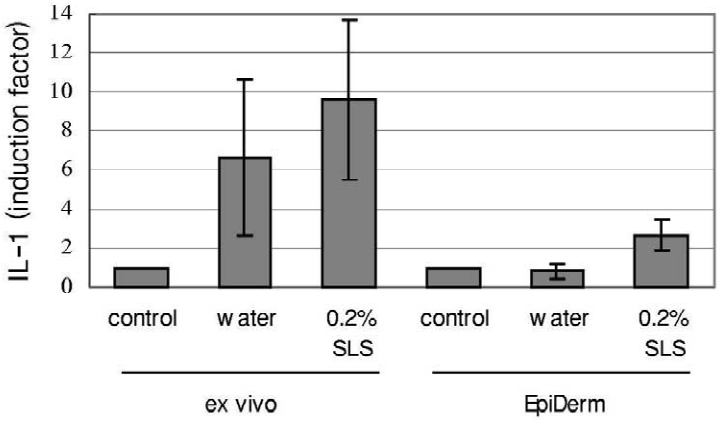


Fig. 5. SLS-induced IL-1α mRNA increase in native and reconstructed epidermis. SLS was applied topically onto native skin or Epiderm for 6 h and subsequently RNA isolated. Semi-quantitative RT-PCR of IL1-α mRNA expression in excised skin (ex-vivo) and EpiDerm cultures untreated (control) or after 8 h topical application of water (vehicle) or 0.2% SLS [37].

mal markers for skin irritation. Among these proteins, heat shock protein HSP27 was identified as the most prominent marker for skin irritation. Its level increased upon topical application of SLS onto excised human skin. Furthermore, nuclear translocation of HSP27 was observed upon topical application of SLS onto excised human skin, onto reconstructed epidermis and in vivo onto human volunteers [38,39] (Fig. 6).

In contrast to SLS, when oleic acid was applied on the surface of reconstructed epidermis or on excised human skin, no alterations in tissue architecture have been observed [40]. Moreover, no epidermal tissue damage was seen in vivo. In vivo skin irritation, a twofold increase in TEWL and CBF values has been observed after topical application of 5% oleic acid. At these concentrations also, increase in IL-1α mRNA levels was seen after application of oleic acid in vitro (Fig. 7). In contrast to the effects in these three-dimensional cultures, direct interaction of oleic acid and human keratinocytes in submerged cultures resulted in cell toxicity and increase in IL-1α mRNA levels at very low concentration of the fatty acid. The results with submerged and air-exposed cultures

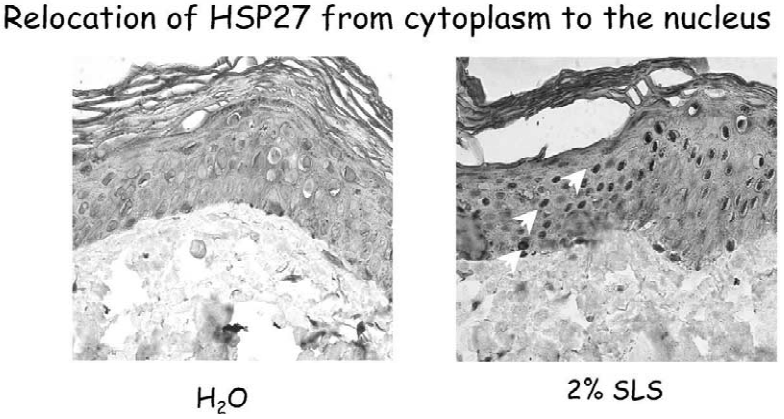


Fig. 6. SLS-induced nuclear translocation of heat shock protein (HSP) 27. To validate the value of HSP27 as a putative sensitive irritation marker, immunohistochemistry was performed on skin topically treated with water or 2% SLS. Note: water-treated specimens show cytoplasmic staining, SLS-treated specimens strong nuclear staining (arrow) [38].

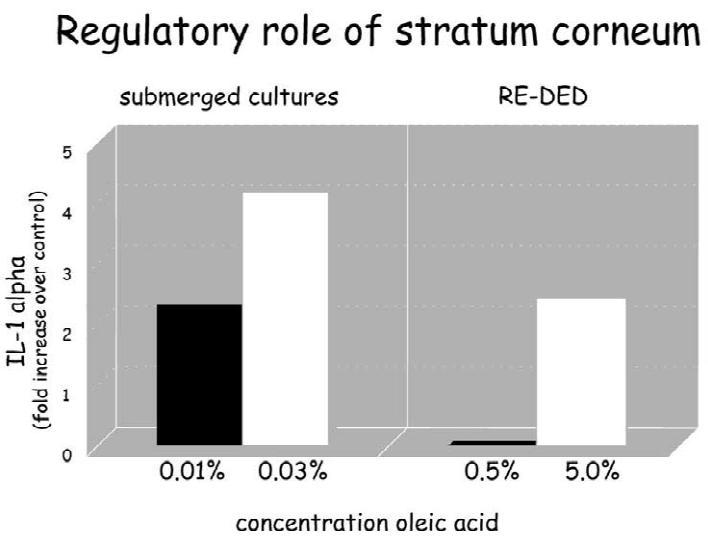


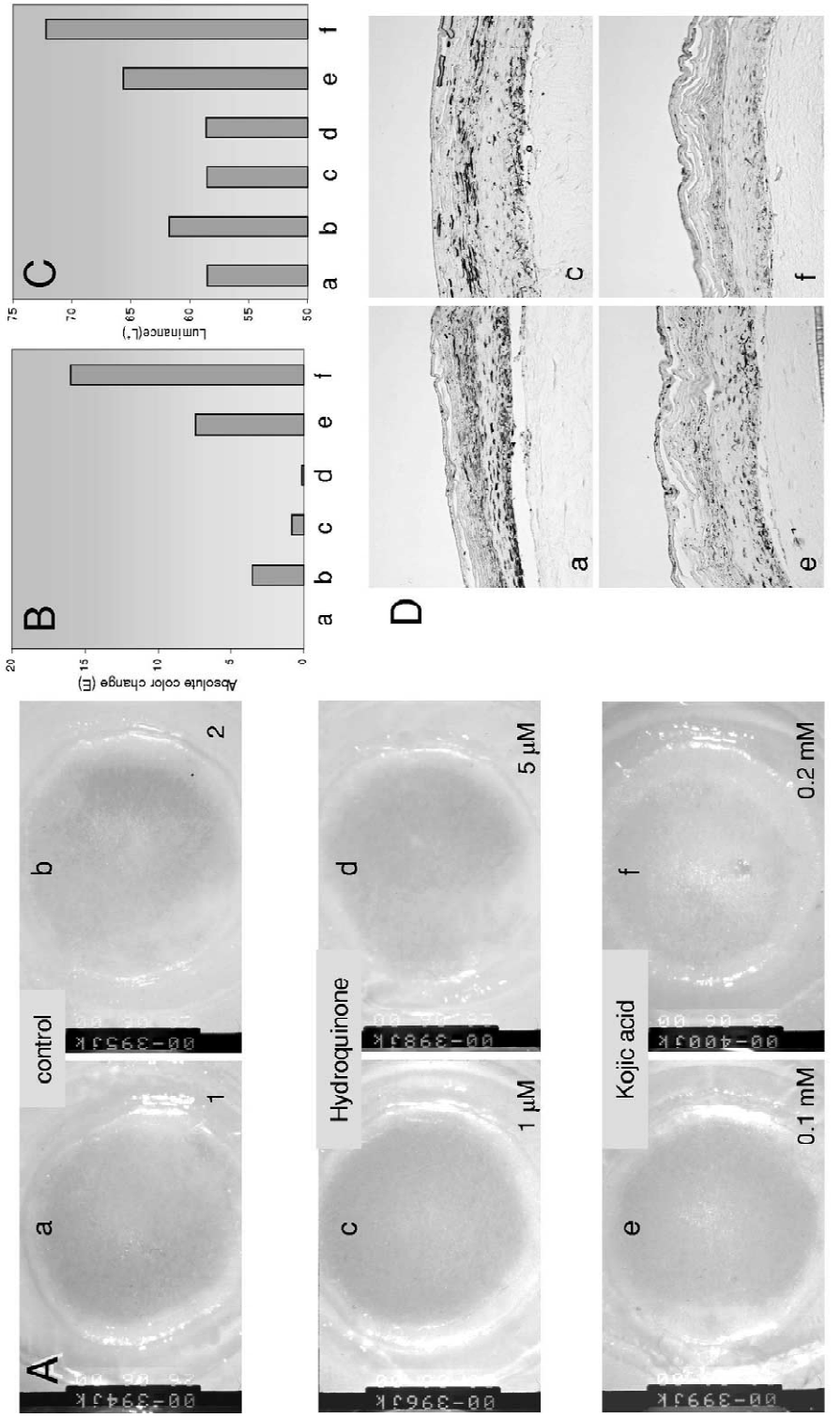
Fig. 7. Oleic acid-induced change in IL-1α mRNA levels in submerged (conventional) and air-exposed keratinocyte cultures. Oleic acid was either added to culture medium at 0.01% or 0.03% concentration (submerged cultures) or applied topically (0.5% or 5%) onto epidermis reconstructed on de-epidermis dermis (RE-DED). After 6 h exposure the cultures were harvested, RNA isolated and Northern blot analysis performed [40].

with respect to oleic acid-induced cell toxicity clearly indicate a key role of the stratum corneum in the control of the observed cell damage by oleic acid. Although the stratum corneum appeared to prevent induction of tissue damage by oleic acid, skin irritation by the compound was not inhibited. Therefore, an intact skin barrier should be considered of major importance for studies on cutaneous irritancy. Comparing the above mentioned results obtained with oleic acid with those obtained after topical application of sodium lauryl sulfate (SLS) onto reconstructed epidermis, it became evident that one should be careful in the choice of endpoints for studying the cutaneous irritancy. Namely, after topical application of SLS, next to increase in IL-1α protein and mRNA levels [36,37] also changes in tissue morphology have been noticed. These findings clearly indicate that the effects on tissue morphology occurring in irritant reactions may vary in accordance to the nature of the irritant, reflecting the different ways in which the chemical may interact with the skin. Therefore, it is likely that multiple mechanisms and thereby multiple endpoints are implicated in testing skin irritation.

4. Lack of desquamation affects the evaluation of screening tests

The progress made in recent years by improving

Fig. 8. Modulation of melanogenesis in vitro. Co-culture of keratinocytes and melanocytes on fibroblast-populated collagen matrices was established as described earlier [42]. After 1 week culture under submerged conditions, the cultures were lifted to the air–liquid interface and the medium was supplemented with the following additives: (a, b) none, (c) 1 μM hydroquinone, (d) 5 μM hydroquinone, (e) 0.1 mM kojic acid, (f) 0.2 mM kojic acid, and cultured for an additional 3 weeks. The medium was renewed three times a week. (A) Macroscopic appearance; (B) absolute color change (E); luminance (L*). (C) Melanin distribution in reconstructed epidermis generated on fibroblast-populated collagen matrix, as established by Masson–Fontana staining. After 1 week culture under submerged conditions, the cultures were lifted to the air–liquid interface and the medium was supplemented with the following additives: (a) none, (c) 5 μM hydroquinone, (e) 0.1 mM kojic acid, (f) 0.2 mM kojic acid, and cultured for an additional 18 days. The medium was renewed three times a week. Magnification 200×. Note the abundant presence of melanin in control cultures both in the viable epidermal layers as well in the stratum corneum. Upon administration of 5 μM hydroquinone, marked reduction of the melanin content was observed in viable epidermal but not in the stratum corneum layers. In the presence of kojic acid a dose-dependent decrease of melanin content was seen in the whole epidermis.


A
control
a
b
1
2
Hydroquinone
c
d
1 μM
5 μM
Kojic acid
e
f
0.1 mM
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B
Absolute color change (ΔE)
a b c d e f
C
Luminance(L*)
a b c d e f
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a
c
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the culture conditions led to a substantial prolongation of the epidermal life span. After 6 to 7 weeks in culture the viable epidermis showed all the signs of a normal differentiation program but the thickness of the stratum corneum was gradually increasing [13,32]. The stratum corneum is continuously being formed in the process of epidermal differentiation, in which a fraction of the basal keratinocytes leaves the basal cell layer and migrates, while differentiating, toward the skin surface. There the cells maturate and anabolically dead corneocytes are accumulating and form the stratum corneum. To maintain a constant stratum corneum thickness, the continuous generation of corneocytes is balanced by shedding of surface cells, the so-called desquamation [41]. Ultrastructural studies performed earlier revealed that in reconstructed epidermis the transformation of desmosomes into corneosomes at the stratum granulosum/stratum corneum interface and partial degradation within the stratum corneum seem to occur normally [4,19]. Also, the corneosome frequency within the stratum corneum in reconstructed and native stratum corneum are similar [19]. In spite of these findings, subsequent cell shedding at the surface does not occur. In a recent study all treatments (including mechanical stress, application of various agents that should decrease the surface pH and calcium level or application of α-hydroxyacids) failed to induce desquamation in vitro. The absence of desquamation, which is a tightly regulated process with the final sequence taking place abruptly in the uppermost stratum corneum layers, is probably due to the lack of appropriate microenvironmental conditions, where calcium, cholesterol sulfate, surface pH and water levels as well as the presence of various factors play an important role in activation of diverse enzymes.

The lack of desquamation under the in vitro conditions has a practical consequence. Namely, it may hamper the use of reconstructed epidermis for various screening studies aiming to examine the repetitive exposure to topical agents or UV irradiation. The gradual thickening of the stratum corneum will lead to its higher resistance to environmental stimuli and in this way affect the outcome of the tests. Furthermore, one should be careful in selecting endpoints when, for example, the effects of agents known to modulate the melanogenesis are examined. The visual scoring or colorimetric measurements, frequently used in vivo as endpoints, can often be inappropriate. This can be illustrated by experiments in which various compounds known to affect melanogenesis have been administered for a period of 2 weeks. Stereophotographs of individual cultures taken after administration of test substances for 18 days are shown in Fig. 8. There was a slight color difference observed only in kojic acid-treated cultures. Similar results have been obtained by colorimetric measurements. Only in kojic acid-treated cultures an increase in absolute color change (E) was noticed (Fig. 8B) due to an increase of luminance (lightness) (L*) (Fig. 8C). One should realize that topical agents should be applied onto the reconstructed epidermis at the time point at which a fully competent stratum corneum barrier is formed and that melanin is produced in the viable epidermis already during the culture period required to reach this situation. Even when the melanogenesis is suppressed, the already formed melanin can accumulate within the stratum corneum. In such studies, therefore, histological examination should replace the visual scoring and colorimetric examination (Fig. 8D).

5. Conclusion

A great deal of effort is being put into the development and validation of reconstructed human skin equivalents for their widespread applicability in testing of various topical agents or treatments. The models are nowadays valuable for short-term cytotoxicity assays to get the first impression of the toxic potential of a test compound. For the assessment of early stages of irritation, and long-term exposure to mild irritants, specific protocols have still to be developed. Presently, information gained on test-agent-induced changes in mRNA or protein expression may help to develop such protocols. Obviously, in vivo–in vitro correlation studies have to be performed to indicate the limitations of the data with regard to their interpretation in terms of assessing human risk. Since results obtained from human studies are necessary as reference values for in vitro assays, attention should also be paid to the generation of in vivo data and standardization of in vivo test protocols.

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