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Leiden reconstructed human epidermal model as a tool for the evaluation of the skin corrosion and irritation potential according to the ECVAM guidelines

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ABSTRACT

In the ECVAM validation studies two common skin protocols have been developed, the skin corrosion and skin irritation protocol. Both protocols include next to general and functional conditions that the skin model must meet, also the correct prediction of the activity of certain reference chemicals. For the skin corrosion protocol, the OECD TG 431 defined 12 reference chemicals that should be correctly predicted by the epidermal skin model. For skin irritation 20 test substances should meet the defined criteria. In this study we aimed to subject our Leiden human epidermal (LHE) model to both common protocols according to the ECVAM guidelines. The LHE model generated in this study has been fully characterized and shows very high similarities with the native skin. After minor technical changes in both protocols, corrosion classifications were obtained in concordance with those reported for the validated human skin models EpiSkin™ and EpiDerm™. The results obtained with the common skin irritation protocol were very similar to that of earlier studies with the SkinEthic, EpiSkin™ and EpiDerm™ models. This means that the protocols and prediction models developed during the validation studies with a specific skin model can be used with other similar skin models. This study demonstrates that reconstructed human skin equivalents have been proven to be efficient and reliable alternatives to animal testing.

1. Introduction

Various human skin recombinants have been reconstructed *in vitro*; many of these mimic the native tissue to a high degree. The tissues are made of keratinocytes that will differentiate during epidermal development and cultured for approximately two weeks at the air–liquid interface on cell-free dermal matrices such as de-epidermized dermis (DED), inert filters, fibroblast-populated collagen matrices or lyophilised collagen-GAG membranes (Bell et al., 1981; Boyce et al., 1988; Ponec et al., 1997; El Ghalbzouri et al., 2002a,b; Stark et al., 2006). These various types of skin models are used for research or commercial purposes. The full-thickness models (epidermis generated onto a dermal matrix) are e.g. used to study the interaction between the keratinocyte and fibroblasts or skin diseases (El Ghalbzouri et al., 2003). In addition, skin models generated with synthetic dermal matrices or DED are e.g. used for tissue engineering purposes (Gibbs et al., 2006). The reconstructed human epidermal (HE) skin models are much easier to generate and are therefore attractive for screening purposes. Nowadays, a large number of the skin models mentioned above are commercially available (e.g. SkinEthic™, EpiDerm™, EpiSkin™). Morphological studies have shown that these HE models form a multilayered epithelium, which display characteristic epidermal ultrastructures and express markers of epidermal differentiation (Boelsma et al., 2000; El Ghalbzouri et al., 2002a,b). In addition the LHE model shows features of a functional permeability barrier, which is one of the main functions of viable skin (Ponec et al., 1997).

The HE models have the advantage that they allow topical application of products used in daily life which may have the potential to (a) irritate the skin or the eyes (irritation), (b) elicit toxic responses in combination with UV-light (phototoxicity), (c) corrode the skin (irreversible damage) or (d) sensitise the skin (caused by immunological mechanisms). According to current international regulatory requirement, assessment of the skin corrosion is obligatory for all chemicals placed on the market (OECD, 2002).

In vivo skin corrosion testing causes severe pain to test animals. Therefore, reduction of the use of animals for testing of products is demanded by the general public, as well as by the relevant authorities. In the EU testing of cosmetic products is banned since 2004 and animal testing for cosmetics ingredients will be banned by 2009 (with some exceptions in 2013). This has triggered the development of *in vitro* alternatives.

Several alternative methods were evaluated during 1996 and 1998 in the ECVAM international validation study on *in vitro* tests for skin corrosion, and two assays were accepted as a full replacement of the *in vivo* procedure; the TER assay (based on measuring

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of the electrical resistance in the *ex vivo* rat skin) and the EpiSkin™ assay (using the reconstructed human epidermal model *in vitro*) (Fentem et al., 1998). In 2000 also EpiDerm™ was successfully validated in an ECVAM "catch-up" validation study (Liebsch et al., 2000). All three methods were finally implemented into the EU and OECD Guidelines for testing of dangerous substances as OECD TG 430 (TER assay) and OECD 431 (human skin models). From several studies it became clear that the skin corrosion assay performed on 3D reconstructed human epidermal models, which show a well developed epidermal architecture, will perform quite similar as the EpiDerm™ and EpiSkin™ skin models (Liebsch et al., 1997, 2000; Kandárová et al., 2006a).

In the present study we have optimized our reconstructed (LHE) model so that it can be used for screening purposes. This means that these epidermal models must meet certain criteria, such as a competent barrier function, appropriate number of viable cell layers and no side effects of the substrate (e.g. edge effects of the epidermis). For this purpose, different filter substrates were tested and LHE models were cultured at different time points to evaluate the ET50 (exposure time required to reduce cell viability by 50%) value. This ET50 value gives an indication about the robustness of the formed stratum corneum in the LHE model. The optimized LHE model was then subjected to the commonly used skin corrosion and irritation protocols. In the first part of this study we evaluated the performance and predictability using the EpiDerm™ skin corrosion protocol (Liebsch et al., 2000). After some minor modifications of the protocol, coded chemicals from the ECVAM validation study were tested using the EpiDerm™ Standard Operation Procedure (SOP) and prediction model (PM). Results obtained with this protocol were comparable to the published results of EpiDerm™, EpiSkin™ and SkinEthic models (Fentem et al., 1998; Liebsch et al., 2000; Kandárová et al., 2006b). From this study it became clear that a common test protocol and prediction model could be used with other similar skin models. Next to the corrosion protocol, efforts have also been made to replace the Draize skin irritation test by a common irritation protocol using reconstructed skin models. The ECVAM Skin Irritation Task Force (ESITF) proposed the development of such a protocol applicable to EpiSkin™ and EpiDerm™ skin models (Zuang et al., 2002). Late 2004 the common protocol was ready to proceed to a formal ECVAM validation study (Kandárová et al., 2005; Cotovio et al., 2005). The concept of a common irritation protocol for different reconstructed human skin models was later successfully applied also to SkinEthic RHE™ (Kandárová et al., 2006b).

In the second part of this study we used the EpiDerm™ and EpiSkin™ common irritation protocol. Also here coded test chemicals were included and some minor modifications were made for our LHE model.

The results obtained with both protocols were similar to the published results of EpiDerm™ and EpiSkin™ and SkinEthic models (Fentem et al., 1998; Liebsch et al., 2000; Cotovio et al., 2005; Kandárová et al., 2005; Kandárová et al., 2006a,b). This study shows that the LHE meet the acceptance criteria of a common test protocol and that the prediction model can be used in other skin models in which the epidermis contains a proper barrier function.

2. Material and methods

2.1. Cell culture

2.1.1. Keratinocytes

A culture of normal human keratinocytes was established from human mammary skin, as described earlier (Ponec et al., 1997). The keratinocyte medium used consisted of three parts Dulbecco-modified Eagle medium and one part Ham's F12 medium supplemented with 5% HyClone calf serum (Greiner, Nürtingen, Germany), 1 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin. In all experiments, secondary cultures were used.

2.2. Reconstructed epidermis

Reconstructed epidermis was obtained by seeding 0.4×10^6 keratinocytes onto filter insert (12 wells plate, polyester membrane, Costar) or 1.2×10^6 keratinocytes onto filter insert (6 well plate, polyethylene terephthalate membrane, Greiner Bio-One).

Some of these filters were coated with collagen type IV (Sigma) (50 mg/ml) to improve epidermal attachment. The cultures were incubated overnight in keratinocyte medium supplemented with 1% serum, 1×10^{-5} M L-carnitine (Sigma), 1×10^{-2} M L-serine (Sigma), 1 μM dL-α-tocopherol-acetate and a lipid supplement containing 25 μM palmitic acid, 15 μM linoleic acid, 7 μM arachidonic acid and 2.4×10^{-5} M bovine serum albumin (Sigma). The cultures were then lifted to the air–liquid interface and cultured for additional 17 days in the same medium except that serum was omitted, the concentration of linoleic acid was increased to 30 μM and 50 μg/ml ascorbic acid (Sigma) and 1 ng/ml EGF (Sigma) were added. Medium was refreshed every second day. After 17 days of air exposure, reconstructed epidermal models were used for screening experiments or processed for immunohistochemical analyses.

2.3. Morphology and immunohistochemistry

Harvested cultures were washed in PBS, fixed in 4% paraformaldehyde, cut into two fragments. One half was snap-frozen and the other half dehydrated and embedded in paraffin. Sections (5 μm) were cut, deparaffinized in xylene, rehydrated and used for morphological (haematoxylin staining) or immunohistochemical analysis of keratins listed in Table 1. Immunohistochemical analysis of basement membrane proteins and integrins listed in Table 1 was performed using 5 μm frozen sections, which after sectioning at –20 °C were air-dried overnight, and fixed in acetone for 10 min. The primary antibodies used in the present study are listed in Table 1. After incubation with primary antibodies, sections were washes and incubated with avidin–biotin–peroxidase complex system (streptABcomplex/HRP, DAKO), as described by the supplier. All sections were counterstained with haematoxylin. Evaluation of immunohistochemical analysis was performed by two independent observers.

2.4. ET50 assay with Triton X-100

To evaluate whether the stratum corneum in the skin models resist the rapid penetration of certain cytotoxic marker chemicals

Table 1
Primary antibodies used for immunohistochemical staining of tissue sections

Sections	Antibody designation	Source[a]
Paraffin-embedded	Keratin 6 (Ks6.KA12)	Sanbio B.V. Uden, The Netherlands.
	Keratin 10 (DE-K10)	ICN Biomedicals Inc., Aurora, Ohio, USA
	Keratin 16 (LL0025)	Dr. I.M. Leigh, London, England
	Keratin 17 (CK-E3)	Sigma, Saint Louis, Missouri, USA
Frozen	Integrin: a6 chain (JEB5)	Dr. A. Sonnenberg, Amsterdam, Netherlands
	Integrin: p4 chain (3E1)	Biomol, Hamburg, Germany
	Collagen VII (LH7.2)	Dr. I.M. Leigh, London, England
	Laminin 5 (P3E4)	Chemicon, Temecula, CA
	Nidogen (1025+)	Dr. R. Timple, Martinsried, Germany

[a] Antibodies not purchased from indicated sources were personal gifts from the investigator named.