Arch Dermatol Res (2004) 296: 203–211
DOI 10.1007/s00403-004-0507-y

ORIGINAL PAPER

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A simple reconstructed human epidermis: preparation of the culture model and utilization in in vitro studies

Received: 16 February 2004 / Revised: 23 July 2004 / Accepted: 9 August 2004 / Published online: 2 September 2004
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Abstract The preparation of a reconstructed human epidermis is described with examples of its utilization in in vitro studies. The model was obtained by culturing normal human keratinocytes at high cell density for 14 days in serum-free and high calcium (1.5 m *M*) medium on an inert polycarbonate filter at the air-liquid interface. These stratified cultures showed histological features similar to those observed in vivo in the epidermis: a proliferating basal layer and differentiating spinous, granular, and cornified layers. Electron microscopy illustrated lamellar bodies, junctions and keratohyalin granules. Immunofluorescent localization of epidermal markers (keratins 14 and 10, involucrin and filaggrin) revealed typical differentiation. This in vitro reconstructed tissue was used in studies of toxic effects of chemicals. The modelled tissue showed progressive cytotoxicity of a skin irritant (benzalkonium chloride) and a sensitizer (dinitrochlorobenzene) as assessed by MTT assay. Moreover, differential release of interleukin-1α and interleukin-8 were measured after 20 h of incubation allowing the irritant to be distinguished from the sensitizer. Permeation studies indicated efficient barrier function of the reconstructed epidermis, as well as metabolizing properties towards hormones. This model can be custom-made and is potentially useful for studies involving keratinocytes in the epidermis, in basic science, dermatology or toxicology.

Keywords Reconstructed human epidermis · Keratinocyte · Barrier function · Irritant · Sensitizer

Introduction

The advent of improved cultures of keratinocytes together with maturation of the cultured keratinocytes into cornified cells when exposed to the air-liquid interface (Pruniéras et al. 1983) has led to the reconstruction of realistic cultured epidermis. So far, models of reconstructed human epidermis (RHE) have been used for metabolic studies of pharmaceutical products (Bernard et al. 2000a), for the determination of absorption properties (Gysler et al. 1999), for the assessment of cutaneous irritancy (De Brugerolle de Fraissinette et al. 1999) or phototoxicity (Bernard et al. 2000b), and for studies of epidermal responses to irritants and sensitizers (Coquette et al. 1999, 2003).

During the last decade, different cultured models of human skin have been made commercially available from several companies. Nonetheless, although this availability is of real benefit for researchers especially in the industry, academic laboratories and small or medium-sized enterprises can rarely afford to buy such tissues for their investigations. Furthermore, for any user of commercially available models, such a dependence on the availability of a model is not devoid of problems and limitations. For instance, as has already occurred with the Skin2 model, discontinuation of production can put in jeopardy the work of laboratories relying on such a model for their routine analysis and investigations (Southee et al. 1999). Moreover, some researchers may complain about difficulties in controlling certain culture parameters (culture conditions, origin of cells, etc). Thus, the best way to avoid those problems and limitations is to make a preparation procedure open to everyone.

Partial information on methods allowing the preparation of cultured skin models can be found in the lit-

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erature (reviewed in Coquette et al. 2000), but this information is never complete enough to allow extensive and reproducible production of tissues. Hence, we decided to combine our knowledge of serum-free cultures of human keratinocytes (Poumay and Pittelkow 1995; Poumay et al. 1999) with some information available in the literature (Rosdy and Claus 1990; Ponec et al. 1997) in order to develop a procedure using easily available materials and reagents, which demonstrates satisfactory characteristics of a tissue useful for any kind of investigation in epidermal biology. Several methods and culture media have been developed since 1980 to considerably expand keratinocyte populations in chemically defined conditions. All the described media (MCDB153, EpiLife, KSFM or KGM-2) contain low calcium concentrations since such culture conditions have been found to favour keratinocyte proliferation (Hennings et al. 1980; Boyce and Ham 1983), but the calcium concentration can be enhanced afterwards as values above 1 m *M* are apparently required in order to allow the establishment of intercellular junctions, when the epidermis is reconstructed as a stratified culture of keratinocytes (Pittelkow and Scott 1986). Furthermore, culture at the air-liquid interface stimulates the formation of the cornified barrier (Pruniéras et al. 1983) and supplementation of the medium with vitamin C significantly improves the barrier characteristics of the reconstructed tissue (Ponec et al. 1997; Pasonen-Seppänen et al. 2001).

Here we describe in detail the preparation of a fully differentiated cultured epidermis anchored on polycarbonate filter. This technique can be easily reproduced and the model exhibits a typical morphology with basal, spinous, granular and cornified epidermal layers, together with a very efficient barrier exhibiting properties close to the in vivo barrier. As previously described with a similar model (Coquette et al. 1999, 2003), the fine measurement of molecules released by treatment with irritant chemicals can be efficiently performed since no extracellular matrix can trap released molecules. Finally, besides application in cell biology and in vitro toxicology, this kind of model can also be used in transdermal studies. Indeed, since transdermal application of drugs has been shown to have several advantages over oral intake (Potts et al. 1992), studies of in vitro percutaneous absorption have been carried out using excised human skin from surgery or autopsy. However, the increasing difficulties in obtaining reliable human skin samples has urged researchers to develop new models, and stratified cultures of keratinocytes seem to represent an interesting alternative (Doucet et al. 1998). Since the metabolic activity of the cutaneous tissues used for those studies has to be taken into account (Altenburger and Kissel 1998), another aim of our study was to investigate the transepidermal absorption and metabolization of estradiol in order to determine whether the present model could be useful for predicting in vitro human percutaneous absorption and metabolization.

Materials and methods

Production of stratified cultures of keratinocytes

Samples of superficial normal adult skin, obtained with a dermatome, were collected from plastic surgery (abdominoplasty) after obtaining informed consent (Dr. B. Bienfait, Clinique Saint-Luc, Namur-Bouge). The samples were cut into pieces of area approximately 1–2 cm^2, then incubated overnight at 4°C floating on 10 ml of a 0.17% trypsin (from bovine pancreas, Sigma) solution in sterile 100-mm diameter Petri dishes (Becton-Dickinson) according to the method described by Wille et al. (1984). The trypsin was dissolved in solution containing glucose (10 m*M*), KCl (3 m*M*) and NaCl (130 m*M*), and buffered at pH 7.4 with sodium phosphate (1 m*M*) and Hepes (30 m*M*). During the following steps, all the solutions and vessels were kept on ice. At the end of the incubation, the epidermis of each piece of skin was rapidly separated from the dermis with fine forceps, then the epidermal tissue was triturated and the cell suspension filtered over a cell strainer with 70-μm pores (Becton-Dickinson). The cells were centrifuged (5 min at 1000 rpm at 4°C) and the pellet resuspended in culture medium containing 0.1% calcium-free fetal bovine serum.

Primary cultures were initiated at a density of approximately 5×10^4 cells/cm^2 in 175-cm^2 cell culture flasks in keratinocyte growth medium (KGM-2, Biowhittaker, Walkersville, Md.) containing 0.15 m *M* $CaCl_2$, 10 ng/ml human recombinant epidermal growth factor, 5 μg/ml human recombinant insulin, 5 μg/ml transferrin, 0.5 μg/ml epinephrine, 5×10^{-7} *M* hydrocortisone and 50 μg/ml bovine pituitary extract. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Before reaching confluence of the culture, proliferating keratinocytes were harvested by trypsinization in 0.025% trypsin/0.01% EDTA (Merck) in PBS, and then replated at 5×10^3 cells/cm^2 for subculture in EpiLife medium (Cascade Biologics, Portland Ore.) containing 0.06 m *M* $CaCl_2$ and HKGS (Cascade Biologics; 0.2% bovine pituitary extract, 5 μg/ml bovine insulin, 0.18 μg/ml hydrocortisone, 5 μg/ml bovine transferrin, 0.2 ng/ml human recombinant epidermal growth factor). The EpiLife medium was chosen for its higher efficiency regarding the proliferation of keratinocytes, while KGM-2 was retained for the primary culture because in our hands this medium was more efficiently allowed the growth of colonies from primary keratinocytes just isolated from the epidermis.

For the reconstruction of the tissues, second- to third-passage proliferating keratinocytes were used. In order to prevent variability between different batches of the RHE, keratinocytes from five different donors were pooled before inoculation at a density of 5×10^5 cells/cm^2. These cultures were performed on polycarbonate culture inserts (0.63 cm diameter) with 0.4 μm diameter pore size (Millipore) in EpiLife medium (150 μl) con-

taining HKGS and to which $CaCl_2$ had been added to reach a calcium concentration of 1.5 m *M*. The inserts then received 500 µl of medium and were placed in six-well multiplates (Becton-Dickinson) containing 2.5 ml of the same medium. After 24 h of incubation at 37°C in a humidified atmosphere containing 5% CO_2, the cells were exposed to the air-liquid interface by removing the culture medium in the upper compartment of the insert. In the multiwell, 1.5 ml of the EpiLife medium containing HKGS, 1.5 m *M* calcium, and 50 µg/ml vitamin C was then added to feed the keratinocytes from the bottom of the polycarbonate filter. The medium was then changed every 2 days and keratinocytes were cultured for another 13 days in order to obtain stratified cultures with characteristics of the epidermis which were named "reconstructed human epidermis" (RHE).

Histological and immunofluorescent analysis of the RHE

The RHE were fixed in 4% formaldehyde for 30 min, dehydrated by three incubations in methanol for 10 min, and then incubated in toluene (three incubations of 10 min) before embedding in paraffin. The embedding procedure allowed separation of the resistant polycarbonate filter bearing the cells from the rest of the insert which dissolved in the paraffin solvent. Tissue sections (6 µm thick) perpendicular to the filter were prepared for histological evaluation of stratified cultures and their comparison with human epidermis.

For immunofluorescent labelling of epidermal differentiation markers, tissue sections were rehydrated, then washed in PBS, and finally incubated in 0.1% bovine serum albumin and 0.02% Triton X-100 for 15 min. Tissue sections were then incubated for 2 h with appropriate antibodies. The antibodies used were: a rabbit polyclonal antibody to involucrin (Harbor Bioproducts, Norwood, Mass.; dilution 1:20), a mouse monoclonal antibody to keratin 14 (Novocastra, Newcastle upon Tyne, UK; dilution 1:40), a mouse monoclonal antibody to keratin 10 (Dako, Glostrup, Denmark; dilution 1:50), and a mouse monoclonal antibody to filaggrin (BioTrend Köln, Germany; dilution 1:100). After washing with PBS, the sections were incubated with secondary FITC-conjugated goat anti-mouse IgG, or FITC-conjugated goat anti-rabbit IgG (P.A.R.I.S., France; dilution 1:200).

Electron microscopy

RHE incubated for 13 days at the air-liquid interface were fixed in PBS-buffered 2.5% glutaraldehyde (pH 7.2) for 1 h at room temperature and then postfixed in the same buffer containing 1% osmium tetroxide for 1 h. After washing with PBS, tissues were dehydrated in an ascending ethanol series, substituting sequentially with epoxy resin. Ultrathin sections (50 nm) were stained with uranyl acetate and then lead citrate. Transmission electron microscopy was performed using a Tecnai 100 microscope and pictures were taken using the AnalySis camera and software (Germany).

Measurement of the toxicity and release of interleukin-1α and interleukin-8

All chemicals used for toxicology in vitro were purchased from Sigma-Aldrich (Bornem, Belgium). Benzalkonium chloride (BC) was diluted in EpiLife medium to obtain concentrations ranging from 0.25 to 2 mg/ml, and 1-chloro-2,4-dinitrobenzene (DNCB) was first dissolved in dimethylsulphoxide (DMSO), then diluted in the culture medium to obtain concentrations between 0.25 and 2 mg/ml, the final DMSO concentration being 0.1%. For application of compounds onto the RHE, 20 µl of the solution containing either BC or DNCB was topically applied to the stratum corneum of the tissue which was then incubated for 20 h at 37°C under culture conditions (atmosphere containing 5% CO_2). At the end of the incubation time, the inserts were removed, placed in wells containing MTT solution, and the medium was collected for interleukin-1α (IL-1α) and IL-8 ELISAs.

A 0.5 mg/ml fresh MTT solution was prepared by dissolving MTT (Sigma) in high-calcium culture medium. Each insert was incubated in 500 µl of this solution for 1 h at 37°C (in an atmosphere containing 5% CO_2). After this incubation, each insert was removed and then replaced in a well containing 500 µl of isopropanol for 30 min on a shaking platform in order to dissolve the formazan derivate. Blue MTT extraction solution in aliquots of 100 µl was pipetted and dispensed in the corresponding wells of a 96-well microplate. Isopropanol (100 µl) was added to dilute this solution and the optical density was spectrophotometrically measured in a microplate reader. The mean OD_{550} value of the untreated RHE was considered as representing a cell viability of 100% and results were expressed as percentage of these control tissues. For each chemical, four doubling concentrations were used as follows: 0.25, 0.5, 1 and 2 mg/ml.

Release of IL-1α and IL-8 was quantified by ELISA. Kits were purchased from R&D Systems (Abingdon, UK). Each triplicate sample was quantified in duplicate following the manufacturer's instructions. Potential interference of each chemical was checked by spiking a dilution of each cytokine with the four tested dilutions of the chemical. The mean value of untreated tissues was subtracted from that of each chemically treated epidermis. The optical density was determined at 540 nm using a MIOS microplate reader (Merck, Overijse, Belgium).

Permeation of estrone and metabolism assay

17-β-Estradiol (E2) at the highest purity available was provided by Sigma Aldrich (Bornem, Belgium) and was first diluted in 100% ethanol (13 mg/ml). Subsequent

progressive dilution was performed in EpiLife culture medium in order to obtain a final E2 concentration of 13 μg/ml. The diffusion assay was performed as described by Doucet et al. (1998). Briefly, a volume of 0.5 ml fresh medium was placed under each insert and 100 μl E2 solution was applied directly onto the stratum corneum of the RHE. A volume of 100 μl medium was applied as control. At different incubation times (0, 0.5, 1, 2, 3, 6, 8, 24 and 48 h), the culture medium was removed from the bottom of the tissue, stored at −80°C before E2 and estrone (E1) pending analysis by a radioimmunoassay (RIA), and replaced by fresh culture medium. The pH of the medium was kept constant during the experiments. Control incubations were conducted under the same conditions in the absence of tissue.

The levels of extracellular E2 and E1 were determined in the culture medium by RIA methods developed by Orion Diagnostica (Espoo, Finland) with detection limits of 1.5 pg/ml (E2 analytical range 2.72 to 545 pg/ml; E1 analytical range 15.0 to 900 pg/ml). The kits were obtained from Zentech (Liège, Belgium). All the assays were performed in duplicate. The methods are based on a competition between the radioactive and nonradioactive antigen for a fixed number of antibody binding sites. The amount of ^{125}I-labelled E2 or E1 bound to the antibody is inversely proportional to the concentration of unlabelled product. The separation of free and bound antigen is achieved by using a double antibody system. The radioactivity was counted using a Cobra gamma counter (Perkin Elmer, Zaventem, Belgium). Both methods were revalidated before the experiment following recommendations of the FDA analytical validation conference (Washington, 2001) and EU guidelines. With regard to the assay linearity, the target concentrations plotted against the back-calculated concentrations indicated an excellent linear proportionality with a coefficient of correlation above 0.999. Steady-state fluxes were determined from graphs in which the cumulative amount of penetrated E2 was plotted against time. Fluxes were calculated from the slope of the steady-state portion of the curves.

Results

Morphology of the RHE

After 13 days of culture in high-calcium serum-free culture medium at the air-liquid interface, the RHE was morphologically fully differentiated. Paraffin sections (Fig. 1) were prepared, and showed that the histological features of the RHE (Fig. 1B–D) were similar to those observed in vivo (Fig. 1A). The basal layer with mostly flattened to columnar cells was anchored on the polycarbonate filter, the spinous layer contained cells attached through desmosomes to the basal cells and to other spinous cells, the granular layer contained typical keratohyalin granules, and the superficial cornified layer was made up of acidophilic cells showing no nucleus. Thus, the keratinocytes cultured at the air-liquid interface behaved as they would in vivo, forming a stratified tissue which exhibited the general pattern of cell layers observed in normal epidermis (Fig. 1).

Localization of epidermal differentiation markers

In order to demonstrate that the epidermal differentiation observed in the RHE was closely similar to differentiation in vivo, immunofluorescent staining of differentiation markers was performed. The differentiation markers were correctly expressed: keratin 14 expression was localized mainly in the basal cell layer (Fig. 2A), keratin 10 was expressed in the living suprabasal cell layers (Fig. 2B), involucrin (Fig. 2C) was mainly expressed in the upper spinous and granular cell layers, and filaggrin (Fig. 2D) was essentially found in the granular cell layer.

Ultrastructural features of the RHE

Electron microscopy of the RHE revealed ultrastructural features similar to features observed in vivo in

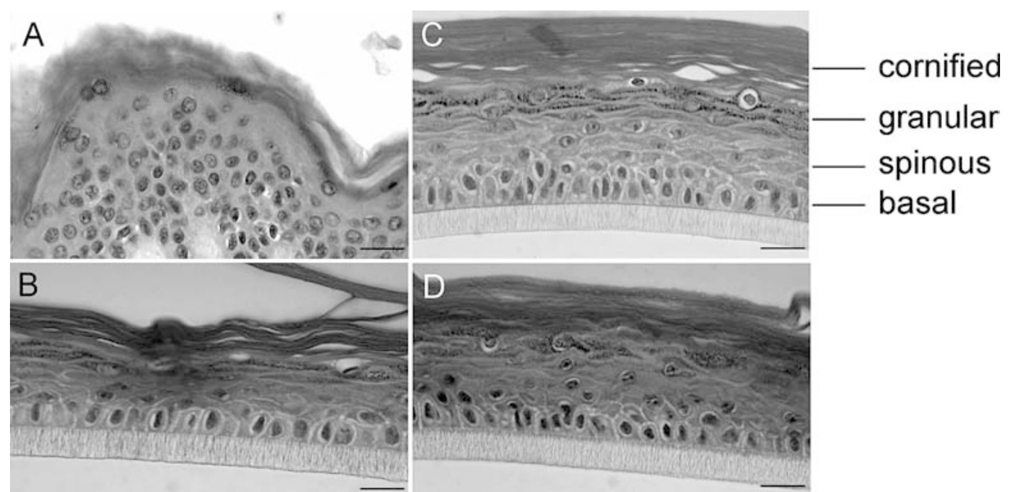

Fig. 1A–D Histology of the normal epidermis in vivo compared with histology of the RHE. Sections perpendicular to the surface of a sample of normal skin (**A**) or of RHE grown on polycarbonate filter (**B–D**) were obtained after fixation of the tissues with formaldehyde, embedding in paraffin and staining with hematoxylin and eosin. The centre of the basal, spinous, granular and cornified cell layers are indicated on the right of **C** (*bars* 50 μm)

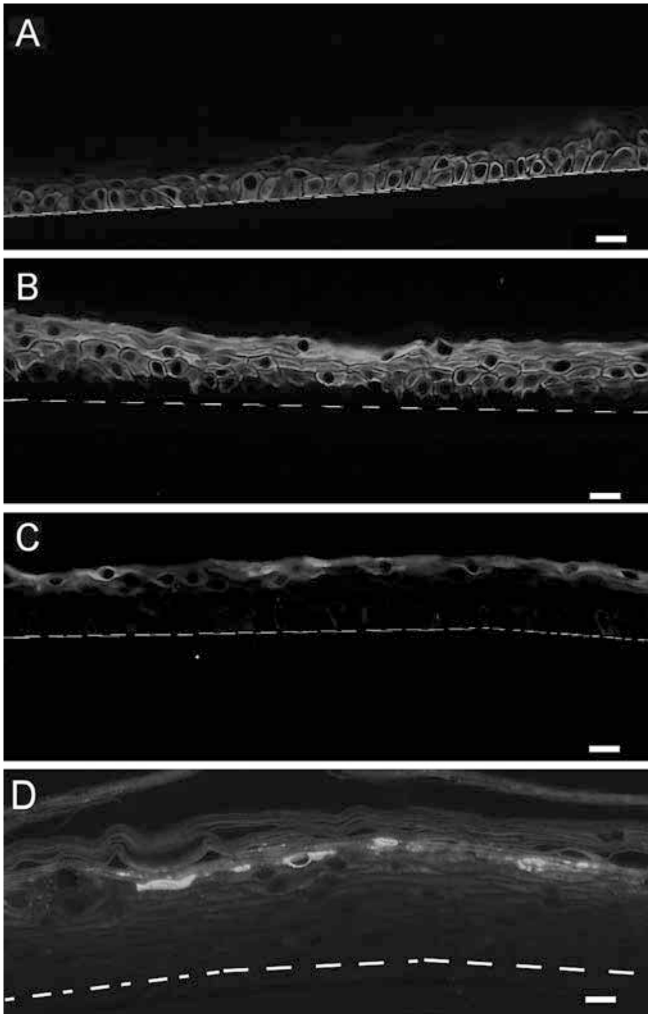

Fig. 2A–D Immunofluorescent staining of histological sections perpendicular to the surface of the RHE. Primary antibodies labelling keratin 14 (**A**), keratin 10 (**B**), involucrin (**C**), and filaggrin (**D**) were used, followed by FITC-conjugated secondary antibodies. The *dotted lines* delineate the interface between basal keratinocytes and the polycarbonate filter (*bars* 50 μm)

normal epidermis. Numerous keratohyalin granules were synthesized in the keratinocytes of the granular layers (Fig. 3A), junctions were localized at anchoring sites of basal keratinocytes on the polycarbonate filter (Fig. 3B), and desmosomes were present between keratinocytes at intercellular junctions (Fig. 3C). Moreover, lamellar bodies were synthesized and secreted at the interface between the granular layer and the first electron-dense cornified cell layer (Fig. 3D).

Cell viability after treatment with an irritant (BC) and a sensitizer (DNCB)

In order to estimate its relevance for in vitro toxicology testing, our model of RHE was used to study the effects of BC as a skin irritant, and the effects of DNCB as a sensitizer. This study was performed in order to check whether these substances could be discriminated as we have previously shown in a commercially available model of RHE (Coquette et al. 1999). The chemicals were topically applied to the stratum corneum. The RHE was incubated for 20 h at 37°C under an atmosphere containing 5% CO_2, then the cytotoxicity of these chemicals was assessed by the MTT assay. A dose-dependent decrease in the viability of the RHE was found upon treatment with the two chemicals, as shown in Fig. 4. The percentage surviving cells in the treated RHE varied from $89.4 \pm 11.8\%$ at the lowest concentration of BC, to $14.1 \pm 1.8\%$ at its highest concentration. BC-treated RHE exhibited a rapid decrease in cell viability (Fig. 4A). Indeed, the mean viability was $37.7 \pm 14.9\%$ at 1 mg/ml. The loss in cell viability following treatment with DNCB was slower and more progressive and dose-dependent (Fig. 4B).

Cellular release of IL-1α and IL-8

The release of these two cytokines was quantified by ELISA in the medium present under the polycarbonate filter during the 20-h incubation of the RHE with both the irritant (BC) and the sensitizer (DNCB). As also shown in Fig. 4, and in good accordance with data previously obtained with another model (Coquette et al. 1999, 2003), the release of cytokines first increased when the percentage of surviving cells was decreasing in relation to the increasing concentrations of the tested chemicals. However, at the highest concentration of DNCB (2 mg/ml), the release of IL-8 was decreased, an observation which differed from the findings of our previous study of DNCB in a commercial model (Coquette et al. 1999). Interestingly, in a subsequent study, decreased IL-8 release was found with several sensitizing molecules, including DNCB, studied at concentrations able to induce an important loss in cell viability (Coquette et al. 2003).

The RHE treated with BC released large amounts of IL-1α and IL-8 (Fig. 4A). On the other hand, the RHE treated with DNCB released only small amounts of IL-1α but larger amounts of IL-8, except at the highest cytotoxicity (Fig. 4B). BC induces a dose-dependant release of IL-1α and IL-8. The IL-8 release (Fig. 4A) was progressive between the different tested concentrations, whereas the IL-1α release seemed to reach progressively a plateau above 1 mg/ml (Fig. 4A). Even though DNCB exhibited a potentially high cytotoxicity, it did not necessarily induce a high release of IL-1α (Fig. 4B). However, the release of IL-8 was more important, reaching 392 ± 181 pg/ml at a concentration of 1 mg/ml. Nevertheless, this release of IL-8 was clearly decreased at 2 mg/ml of DNCB at which it reached 53 ± 61 pg/ml only. Thus, these findings suggest that our model of RHE responded to irritant and sensitizer in a similar manner to commercial tissues from SkinEthic studied previously (Coquette et al. 1999, 2003), making our RHE model potentially useful for the identification of irritating and sensitizing compounds.

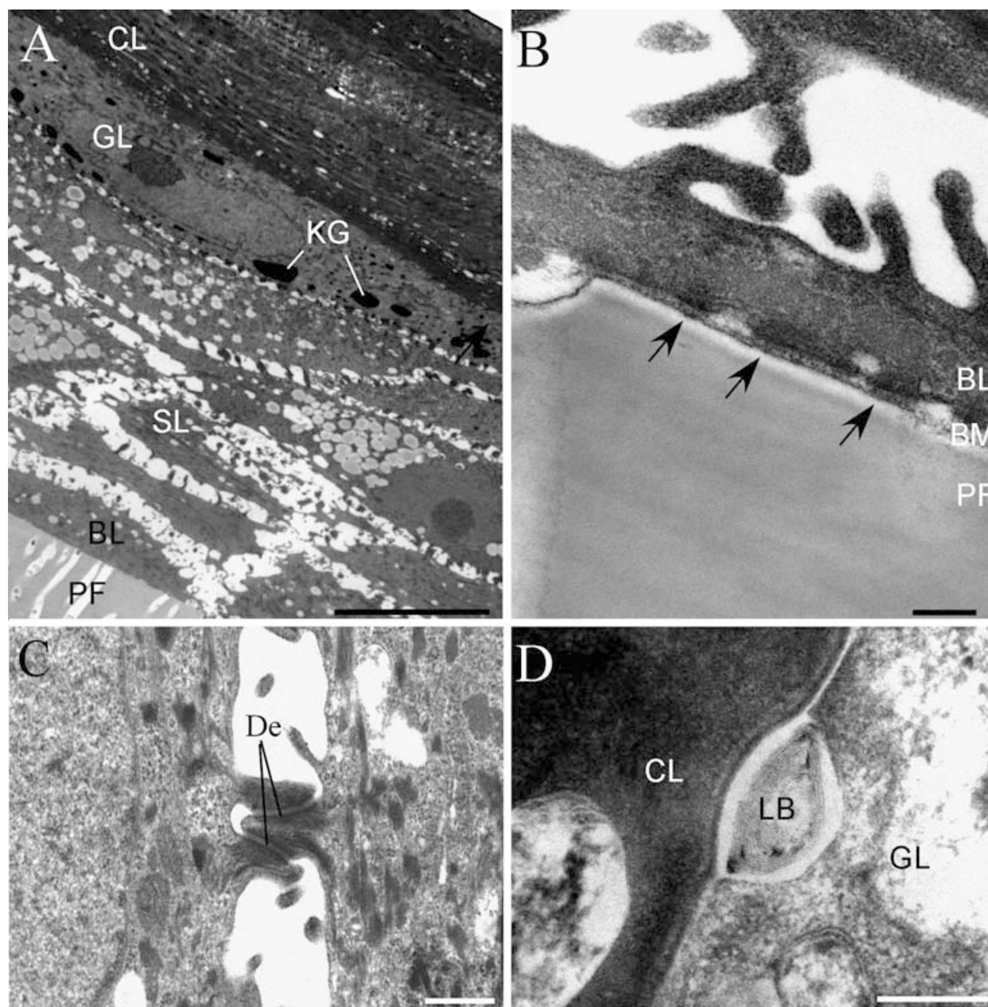

Fig. 3A–D Ultrastructure of the RHE. Ultrathin sections were obtained from RHE fixed and resin-embedded for electron microscopy. **A** At low magnification, stratification of keratinocytes into the basal layer (*BL*) can be seen localized above the polycarbonate filter (*PF*), the spinous layer (*SL*), the granular layer (*GL*) containing typical keratohyalin granules (*KG*), and the superficial cornified layer (*CL*) (*bar* 10 μm). **B** At higher magnification, the contact between keratinocytes of the basal layer (*BL*) and the polycarbonate filter (*PF*) at the level of the basement membrane (*BM*) zone reveals numerous junctions (*arrows*) (*bar* 200 nm). **C** Desmosomes (*De*) can be seen between adjacent keratinocytes in the spinous layer (*bar* 500 nm). **D** Secretion of typical lamellar bodies (*LB*) can be seen in the upper part of the granular layer (*GL*), just below the cornified layer (*CL*) (*bar* 200 nm)

Permeation and metabolism of estradiol

Transdermal estradiol flux rates during each respective time interval are shown in Fig. 5A and demonstrate that estradiol passed readily through the RHE. These data were calculated from the cumulative amounts penetrating during the in vitro incubation. The flux rate increased from 0 to 1 h and reached a value of 232 ± 76 ng/cm^2 per hour. Then it remained elevated between 1 and 3 h of incubation, but decreased below 18 ± 7 ng/cm^2 per hour after 24 h (Fig. 5A). According to previous studies, estradiol is actively metabolized by keratinocytes into estrone (Altenburger and Kissel 1998). In control incubations in the absence of keratinocytes, no estrone was found, indicating that there was no spontaneous degradation of estradiol (data not shown). In the presence of the RHE, the estrone formation rate was slow and increased between 0 and 8 h of incubation to reach a maximum value of 2.92 ± 0.67 ng/cm^2 per h. Then, its formation was drastically decreased down to 1.22 ± 0.29 ng/cm^2 per hour after 24 h of incubation. Despite a real barrier function, our RHE was apparently more permeable than normal human epidermis (Regnier et al. 1992). However, our results confirm the presence of 17β-hydroxysteroid dehydrogenase inside the RHE. Furthermore, since the rate of estrone formation did not seem to be limited by the diffusion of estradiol across the RHE, our findings suggest that the model exhibits a compartmentalization of enzyme systems as observed in vivo (Altenburger and Kissel 1998).

Discussion

In order to make available for our own studies a custom-made epidermal tissue in culture, we adapted and characterized a reproducible and easy model of RHE, free of dermal components and cultured under serum-free conditions. In this model, keratinocytes were indeed cultured at high cell density on a polycarbonate filter membrane with 0.4-μm pore diameter. Under such culture conditions, the keratinocytes formed a fully differentiated epidermis which showed histological features similar to those observed in vivo: a basal layer, a spinous layer, a granular layer and a stratum corneum. This RHE was comprised only keratinocytes, without Langerhans and Merkel cells, and without melanocytes, the

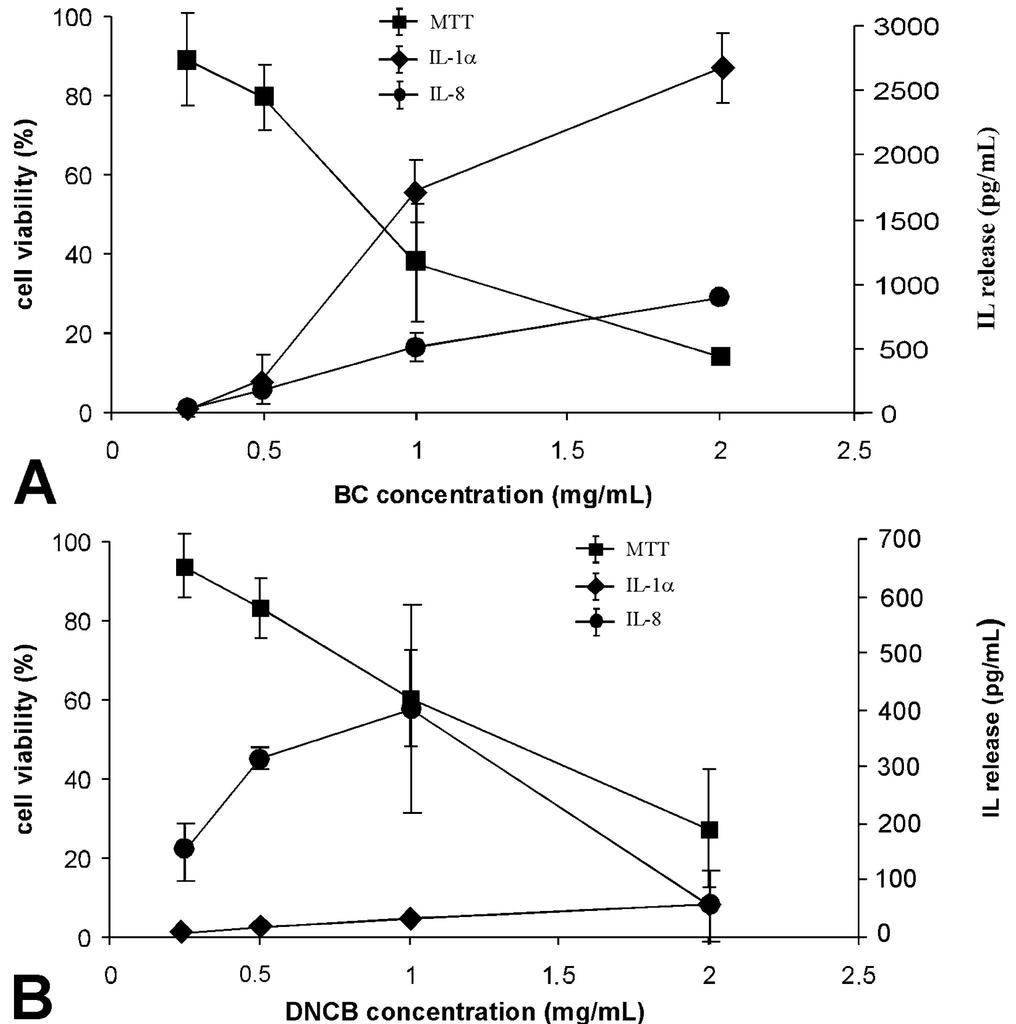

Fig. 4A, B Viability and cytokine release of RHE treated with the irritant BC (**A**), or the sensitizer DNCB (**B**). RHE were treated with the indicated concentrations of BC or DNCB for 20 h, then the cell viability of the RHE was determined using the MTT assay, and the medium was collected for measurement of IL-1α and IL-8 by ELISA in order to calculate the amounts released during the incubation periods. The data are presented as means ± SEM of three experiments, each performed in triplicate cultures. Viability is expressed as a percentage of the viability measured in control RHE fixed to 100%

three other epidermal cell types normally observed in vivo. Keratinocytes in the RHE underwent epidermal differentiation as evaluated by the formation of the typical epidermal layers, but in addition to the histology, the expression of several differentiation markers, i.e. K10, K14, filaggrin and involucrin, showed an identical cell layer localization to that found in vivo (Poumay and Leclercq-Smekens 1998). Moreover, electron microscopy demonstrated ultrastructural features typical of normal epidermis. Together, our data show that under culture conditions based on serum-free medium, stratified cultures of keratinocytes naturally underwent terminal epidermal differentiation (Rosdy and Claus 1990) and produced at the air-liquid interface a stratum corneum which exhibited typical in vivo morphology (Franz and Lehman 2000).

Next, the barrier function of the stratum corneum covering the RHE was evaluated. The response of the RHE to treatments with a known irritant or sensitizer revealed that the barrier function of our RHE was similar that of the SkinEthic model studied previously (Coquette et al. 1999), as the cytotoxicity at the same concentrations of the substances had the same order of magnitude, and the release of cytokines revealed that our RHE behaved similarly (Coquette et al. 1999). Indeed, the skin irritant induced the release of large amounts of IL-1α and much smaller release of IL-8, whereas the sensitizer induced a strong release of IL-8 only. These findings indicate that DNCB is cytotoxic, but induces a weak release of IL-1α whilst its induction of IL-8 release is much stronger. The release of inflammatory cytokines was nearly dose-dependent but the identity of the released cytokines further depended on the properties of the chemical, either irritant or sensitizer.

Studies including measurements of the RNA encoding both interleukins have been performed in other RHE (Coquette et al. 1999, 2003) allowing us to hypothesize about the effect of irritants and sensitizers. Chemicals such as BC, with high irritant properties, are thought to disrupt the barrier function, penetrate towards the living cell layers in the RHE and affect directly the integrity of the keratinocytes. Since these epidermal cells constitutively produce and retain IL-1α in their cytoplasm, irritants induce a massive release of this cytokine which reflects damage to keratinocytes of the living cell layers.

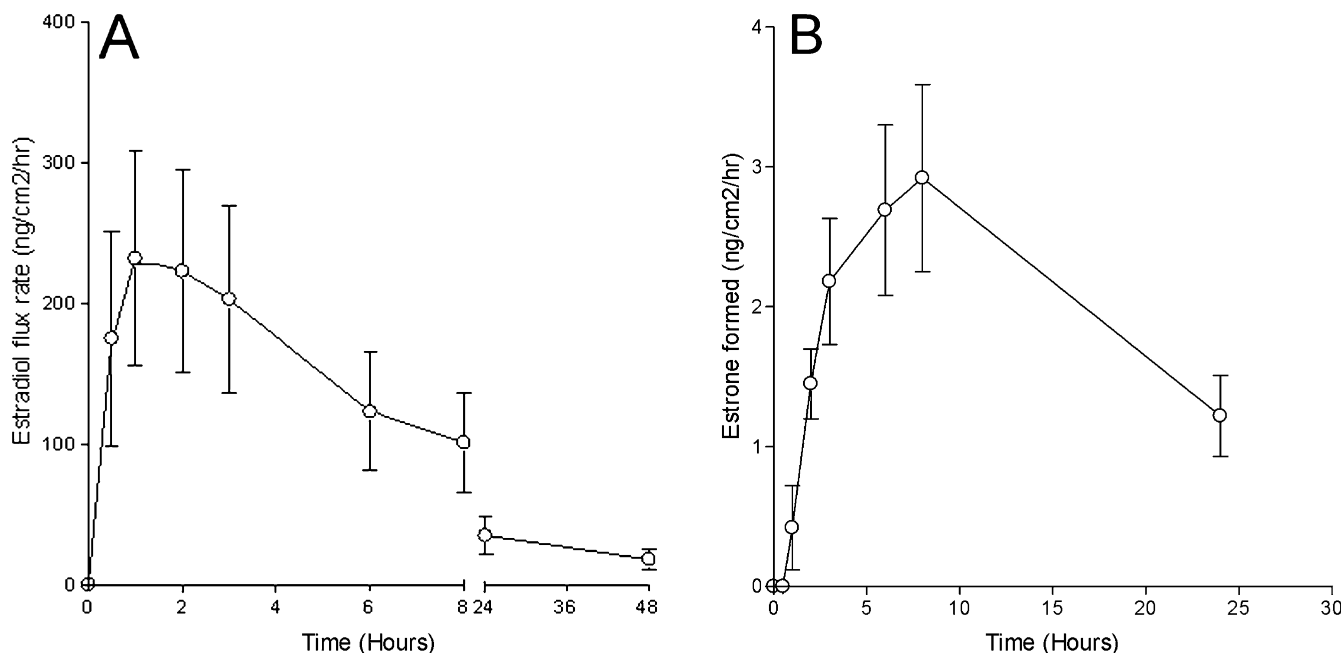

Fig. 5A, B Measurement of the permeation of estradiol through the RHE and measurement of the formation of estrone. The flux rate for estradiol (**A**) was calculated at different times after applying estradiol onto the cornified layer of the RHE. The formation of estrone (**B**) was determined under the same conditions by measurement of the compound in the culture medium bathing the basal side of the RHE. The data are presented as means ± SEM of the results from three independent tissues

DNCB also induces de novo synthesis and release of IL-8 but no storage, while BC probably induces synthesis and storage (Coquette et al. 1999, 2003). These observations further suggest that keratinocytes in the RHE respond to foreign substances and barrier disruption by the activation of signalling pathways which result in the synthesis and release of certain cytokines (Hobbs and Watt 2003; Kobayashi et al. 2003). Activation of such signalling pathways is currently under investigation in RHE treated with an irritant.

Finally, our permeation and metabolism studies further provide data which demonstrate that our model of RHE could be of interest also for comparing drug delivery processes and is a model at least suitable for studies of epidermal metabolism (Stinchcomb 2003).

In conclusion, this new model of RHE showed histological features similar to those of skin, and good chemical and physical properties which indicate its usefulness in future in vitro assessment of the behaviour of the epidermal tissue in many particular situations.

Acknowledgements The authors wish to thank Dr. B. Bienfait (Clinique Saint-Luc, Bouge) for providing samples of normal skin. The occasional technical assistance of R. Déom, D. Van Vlaender, F. Herphelin and C. Devignon is gratefully acknowledged. S. Marcoux was in receipt of a fellowship from the *Fonds pour la Formation à la Recherche dans l'Industrie et l'Agriculture* (FRIA). This work was partly supported by grant 2.4.506.01F from F.R.F.C. to Y. Poumay.

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