Reconstituted human epidermal, corneal and oral epithelial tissue models : alternatives to animal test models for the pre-clinical evaluation and risk assessment of topical products

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**Reconstituted human epidermal, corneal and oral epithelial
tissue models.**

**Alternatives to animal test models
for the pre-clinical evaluation and risk assessment
of topical products.**

Proefschrift

Ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. Mr. G.P.M.F. Mols,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op vrijdag 3 februari 2006 om 12.00h.

door

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Preface

The today's litigious social climate requires an extensive testing regimen for any new product that is being placed on the market by industry. A prudent corporation may spend several million Euros on in vivo procedures, utilizing animals such as dogs, cats and rodents. In pharmaceutical industry, the pre-clinical testing may run up to 7 years and costs billions. In fact, the expense is so great that the pharmaceutical industry now claims the testing required by regulatory authorities as one of the main reasons why their products cost so much. To offset some of the in vivo testing costs (both animal and human), there has been increasing emphasis on in vitro testing. More and more the entire idea of animal testing is called into question: for instance, the use of animals for the safety assessment of cosmetics has probably received the greatest criticism of all type of animal experimentation and several companies make their non-animal testing a sales point. Consequently, in 1993 the EC has set up ECVAM, the European Centre for the Validation of Alternative Methods to support the European policy in reducing the animal usage required for regulatory testing. In 2003, the European Parliament approved the 7th Amendment to the European Directive 76/768/EEC that phases out the use of animals for acute toxicity testing by 2009 and for long term toxicity effects by 2013 for all cosmetic products, including cosmetic ingredients, to be sold in the EC. Another 'regulatory' policy change that has been driving the development of alternatives to animal testing is the European Commission proposed REACH legislation (Registration, Evaluation and Authorization of Chemicals) requesting a toxicological re-evaluation of approximately 30.000 chemicals currently used in different industrial sectors across Europe. When the REACH program will come into effect, the testing required involve the use of several millions of animals and the total experimentation costs are estimated to be as high as 10 billion Euros. Hence ECVAM, in collaboration with industry, is currently developing test strategies for REACH, which involve the validation of alternative to animal testing methods so they can be used to meet the regulatory testing requirements.

In the last decade, a whole spectrum of in vitro methods have been developed including simple monolayer cell systems to more complex three dimensional tissue engineered models. Several testing approaches have been designed to replace animal methods, but their success has been quite limited as most of these methods have inherent limits, not least being that they had even less relevance than the animal test to the human situation, and the lack of physiologically relevant and highly predictive in vitro models has been a roadblock toward the adoption of in vitro test procedures in industrial R&D and safety testing strategies.

This thesis describes the characterization, validation and usage of physiologically relevant in vitro reconstructed tissue models entirely based on human tissue, including reconstructed human epidermis (Chapter 1, 2, 3 and 4), human cornea (Chapter 5, 6 and 7) and human oral and gingival epithelium (Chapter 8 and 9). All of these models can be produced in vitro in mass quantities, and with a high inter-tissue reproducibility, which is in sharp contrast with the relatively high variability amongst test subjects in in vivo studies. Additionally, these 3D tissue models allow direct topical application of test materials and therefore all types of products can be tested at 'in use' concentrations and at 'real-time' exposures. In other words, any

type of compound, whether formulated as cream, liquid or solid can now be screened on a batch of human tissues that are all identical and that will react in the same way to the same compound, under the same experimental conditions, week after week, month after month: the use of such 3D in vitro reconstructed human tissue models makes it possible for pharmaceutical, chemical and consumer product companies to:

- reproducibly differentiate compounds with irreparable toxicity without resorting to in vivo animal testing,
- provide accurate and measurable mechanistic information that can be utilized to determine whether a molecule or compound can be altered to reduce irritation or toxicity, without loss of efficacy,
- objectively compare the toxicity and efficacy of newly developed compounds with one already in use in the market place for the same purpose,
- reveal the precursor steps which lead up to toxic reactions, thus making reformulations possible, based on human response,
- evaluate the long term stability or shelf-life of finished products or raw materials in time,
- determine the health risks of employees, potentially exposed to chemicals (workers safety),

and it does this all at a fraction of the cost and time of in vivo animal or human testing. Additionally, the methodology is user- and implementation friendly, does not require large staff and facilities or supplies, and can potentially save the lives of then thousands of laboratory animals and eventually the lives of many humans.

In Chapter 1, the structural and biochemical characteristics of in vitro human reconstructed epidermis are described, compared to human skin in vivo. Additionally, the importance of an integrated in vitro testing strategy, called Multiple Endpoint Analysis or MEA, is being explained and illustrated. MEA includes the evaluation of toxic effects of compounds based on tissue morphology, tissue viability and the release of pro-inflammatory mediators by the epidermal keratinocytes present in the 3D human skin model. The combination of these 3 endpoints increases the effectiveness and predictability of pre-clinical testing methods for accurate in vitro assessment of skin irritation/sensitization, and phototoxicity. In Chapter 2, using large oligonucleotide micro-arrays, the gene expression pattern of in vitro reconstructed epidermis was compared with biopsies of normal human epidermis and with epidermal keratinocyte monolayer cultures. The epidermal model was found to closely reproduce the gene expression profile of normal human epidermis in vivo, opposite to the profile found with keratinocyte monolayer cultures, which do not stratify and differentiate. In Chapter 3, using a set of well defined sensitizers and irritants, the in vitro reconstructed epidermal model was assessed for its potential to differentiate irritants from sensitizers using an approach of differential release of pro-inflammatory mediators IL-1 α and IL-8, combined with dose-dependent cell viability measurements. This in vitro approach, once validated, could become a valuable, much less time and cost consumable, alternative test method to the classical in vivo sensitisation assays. In Chapter 4, the utility of the in vitro skin model for testing the corrosion potential of chemicals was successfully assessed through multi-centre validation according to the OECD guideline TG 431. Consequently, the assay is now being used as regulatory accepted alternative

method for skin corrosion testing. Chapters 5, 6 and 7 are dealing with 3D in vitro reconstructed human corneal epithelium, from biochemical and morphological characteristics (chapter 5 and 6) to successful pre-validation of the model as alternative to the Draize rabbit eye test for assessing the eye irritation potential of chemicals (Chapter 7). Finally the characteristics of in vitro reconstructed human oral and gingival mucosa were evaluated in Chapter 8, and a testing method for the evaluation of the toxic potential of toothpaste formulations is described in Chapter 9.

Chapter 1

An optimised in vitro approach to assess skin irritation and phototoxicity of topical vehicles.

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Dermatotoxicology, 6th Edition, Ed. Taylor & Francis, Chapter 43, 849-869, 2004.

Introduction.

The acceptance of new commercial products, cosmetics, household goods, industrial chemicals and pharmaceuticals is based both on ethical and legal requirements. The safety evaluation of new products is currently performed in part using non-animal methods. Over the last 20 years, there have been many attempts to utilize in vitro approaches to replace whole animal methods, but successful replacement has been limited since most of these in vitro tests were based on examining only a single toxicity parameter.

In this chapter we propose an integrated strategy for in vitro skin irritation and phototoxicity risk assessment based on the use of human epidermis, reconstituted in vitro in chemically defined medium, coupled to an optimized analytical evaluation of multiple in vitro toxicity parameters (MEA: Multiple Endpoint Analysis), including histology, epidermal tissue viability and the release of pro-inflammatory mediators by the epidermal keratinocytes. This approach increases the effectiveness and predictability of pre-clinical testing.

Human epidermis reconstituted in chemically defined medium: characteristics and advantages.

In the 1980's it was generally accepted by the scientific community that epidermal tissue regeneration could not be accomplished without the presence of bovine serum (undefined mixtures of growth factors and lipids), supplemented to culture medium, and dermal components or fibroblasts-collagen matrices (Asselineau et al, 1986; O'Keefe et al, 1987; Prunières et al, 1983; Régnier et al, 1988, Schürer et al, 1989, Boyce et al, 1990; Bell et al, 1991; Tinois et al, 1991; Gay et al, 1992; Fleischmeyer et al, 1993; De Wever et al, 1994; Gibbs et al, 1997). Three dimensional skin models produced in the presence of serum are however of limited success and major disadvantages result because of the undefined manufacturing conditions. These tissue products are of uneven reproducibility and relevancy, and the test results obtained with these epidermal models for skin toxicity, percutaneous absorption or skin efficacy, have been questioned (Lenoir et al, 1990). As a result, many companies have been reluctant to adopt such models in their testing programs.

Improvement of the cell culture conditions and the use of chemically defined medium has led to the optimization of epidermal tissue models (Rosdy et al, 1990). By cultivating second passage normal human keratinocytes in chemically defined modified MCDB-153 medium on inert filter substrates at the air-liquid interface for 17 days, a fully differentiated epithelium having features of normal epidermis has been obtained in vitro (Figure 1). In this model, the basal cells synthesize and secrete all major markers of hemidesmosomes, the lamina lucida, and a basement membrane-like structure is identifiable (Rosdy et al, 1993). This suggests that the presence of added serum and dermal components are not required for epidermal reconstruction. In fact, a structurally and biochemically normal permeability barrier is obtained when no serum or sphingolipids are included in the defined medium (Rosdy et al, 1996; Rosdy et al, 1997; Doucet et al, 1997a).

Figure 1: Human epidermis reconstituted in vitro in chemically defined medium Transversal section of human skin in vivo (a); human epidermis reconstituted in vitro in chemically defined medium on a polycarbonate substrate (b), and removed from the polycarbonate substrate after dispase treatment (c).

Fartasch and Poniec described the maturation of the barrier function in these epidermal cultures as comparable to the ontogeny of epidermal barrier formation in mammalian skin in vivo (Fartasch et al, 1996a; Fartasch et al, 1996b). In the early stages of epidermal differentiation of this tissue model (day 2-4), hyperproliferative markers such as keratin-13 and 16 are clearly expressed and increased thickening of the epidermal layers is observed. However at day 13-16 of culture, i.e. as soon as the formation of the barrier function is completed, epidermal thickness decreases, K13 and K16 are no longer expressed, and a normalization of the expression of differentiation and proliferation markers is seen. Additionally, biochemical analysis of epidermal lipids of the mature epidermal tissues shows a profile similar to in vivo human epidermis, with physiological amounts of free fatty acids and ceramides [Maria Poniec: personal communications]. The addition of Vitamin C analogs (Poniec et al, 1997) assists in the equilibration of the relative amounts of ceramides, the lipid structures which are responsible for the permeability barrier of the epidermis (Ramdin et al, 2001), which provide a primary function of human skin in vivo and which are a determining factor for any consequent skin reaction (Garcia et al, 2002) (Figure 2).

Recently, using a customized cDNA array system containing 475 skin-related genes, the gene expression pattern of reconstructed epidermis in chemically defined medium was compared with biopsies of normal human epidermis and with epidermal keratinocyte monolayers (Bernard et al, 2002). The epidermal model was found to closely reproduce the gene expression profile of normal human epidermis, which is not observed in keratinocyte monolayers. Additionally, topically applied retinoids induced gene expression modifications that confirmed previously reported retinoid effects (Rosdy et al, 1997b), but also provided new insights into their pharmacological activity on human skin.

Another very important advantage of using human epidermis reconstituted in serum-free chemically defined medium is the minimal presence of growth factors and protein in the culture medium, which makes these living epidermal models the most suitable for detecting, with very high precision, even minor toxicological and/or pharmacological effects of any product intended to be in contact with the epidermis.

Figure 2: Epidermal lipid analysis of the in vitro tissue construct (control and ascorbic acid treated) and human epidermis in vivo (Ramdin et al, 2001). Addition of ascorbic acid 2-phosphate to the culture medium during epidermal tissue reconstruction normalizes the ceramide subspecies to a profile reminiscent of that shown in ex-vivo samples. The relative percentages of fatty acid:cholesterol:ceramides were unaffected by the treatment.

For example, after topical application of test chemicals or finished products to the in vitro epidermal model at concentrations that would be used in vivo, a slightly irritating effect, typically occurring after prolonged 2-3 weeks repeat in vivo human clinical patch test application, can be measured objectively by quantifying pro-inflammatory mediators released by the epidermal keratinocytes in the chemically defined culture medium below the epidermal tissue.

Moreover, the quantification of these pro-inflammatory mediators, coupled to the analytical evaluation of other in vitro toxicity parameters including histology and tissue viability (we suggest the term MEA or Multiple Endpoint Analysis), provide an accurate prediction of skin irritation potential of the test product. Additionally, all parameters in the MEA can be quantified objectively and reproducibly, as opposed to the subjective measurements such as erythema and edema, often used in clinical evaluation. In this manuscript all studies cited were performed on commercially available SkinEthic® reconstituted human epidermis.

The bio-analytical analysis most frequently performed in toxicology and pharmacology screening tests using reconstituted human epidermal tissue models are based on biochemical parameters including tissue viability, either by mitochondrial enzymatic MTT conversion (Triglia et al, 1991; Roguet et al, 1994a; Edwards et al, 1994; Brozin et al, 1997; Spielmann et al, 2001), or cell membrane damage as measured by leakage of enzymes such as lactate dehydrogenase (Ponec et al, 1995), tissue damage (histological evaluation using hematoxylin-eosin staining (H&E)), and the release of pro-inflammatory mediators by epidermal keratinocytes including interleukin-1alpha (Slivka et al, 1993; Doucet et al, 1996; Corsini et al, 1997), interleukin-6 (Boxman et al, 1993), interleukin-8 (Ponec et al 1995), prostaglandins (Roguet et al 1992; Roguet et al, 1994b; Rheins et al, 1994; Lawrence et al, 1997), hydroxy-eicosatreonates and leukotrienes (LTB₄) (Dykes et al, 1991). Each of these parameters (biological endpoints) provides very specific information on the toxicological or pharmacological effect of the test product in contact with the epidermal model.

The MTT test is widely used for cell viability assessment of single-cell monolayer cell cultures in comparison to negative and positive controls (Mosmann et al, 1983). In three dimensional epidermal tissue cultures however, MTT is often used as a single parameter to assess the biocompatibility of test products and can induce false negative results. This incorrect outcome is the result of MTT being mainly converted by the basal cells of the epidermal tissue constructs and necrosis of the upper superficial layers of the tissue model is not detected (Figure. 3). To date, this can only be observed by histological analysis.

3a

3b

Figure 3: Histological analysis of in vitro reconstituted human epidermis: 3a untreated control and 4b treated with 2%SDS for 24 hours. Note the almost similar optical density readings (at 540 nm) after MTT assay: 0.944 ± 0.066 for the untreated control culture 3a, and 0.875 ± 0.040 or 92.2% of the untreated control tissue, whereas a clear necrosis of the upper epidermal cell layers can be observed in the 2%SDS treated tissue.

Furthermore, a very common need in the safety assessment of finished products, is the ability to discriminate between mild and very slightly irritating products, which cannot be detected by MTT assay or histology since slightly irritating products often induce the release of pro-inflammatory mediators (IL-1 α and IL-8) without affecting tissue integrity as measured by MTT and histology. Therefore, critical evaluation of each endpoint in the Multiple Endpoint Analysis (MEA) strategy (i.e.; tissue morphology, tissue viability and pro-inflammatory mediator release) is equally important for accurate in vitro cutaneous biocompatibility predictions (Figure 4). Different examples of the MEA approach are described below for assessing cutaneous irritation and phototoxicity.

Figure 4: In vitro skin biocompatibility determination using the MEA approach: epidermal tissues are being dosed with 2-10 $\mu\text{g}/\text{cm}^2$ of test formulation for 24 and 72 hours and consequently analyzed for tissue viability, tissue morphology and the release of pro-inflammatory mediators IL-1 α , IL-8 among others.

In vitro skin irritation and phototoxicity prediction based on the MEA approach.

The identification of test compounds that could potentially induce clinical skin irritation or phototoxicity are important steps in the product development phase of skin care products, and are very often performed in animal models such as the rabbit skin irritation test (Draize et al, 1944), the maximization test using guinea pigs (Magnusson et al, 1969), the mouse local lymph node assay (Kimber et al, 1989) and the mouse ear swelling test (Gad et al, 1986). Because of structural and physiological differences between animal and human skin, the relevance of these methods to the human situation is limited and can lead to misclassification (Nixon et al, 1975; Basketter et al, 1996; York et al, 1996). In the last decade, different in vitro skin models using de-epidermized dermis (Prunieras et al, 1983; Regnier et al, 1990; Ponc et al, 1991; Gibbs et al, 1997), and collagen matrixes (Bell et al, 1991; Slivka et al, 1993; Ponc et al 1995; Augustin et al, 1997) have been reported to be useful to predict skin irritation (Osborne et al, 1994; Boelsma et al 1996, Roguet et al 1999, Fentem et al 2001).

Using human epidermis, reconstituted in chemically defined medium however, a test method was validated against in vivo data from skin tolerability studies (De Fraissinette et al, 1997; De Brugerolles et al, 1999). Based on Multiple Endpoint Analysis (MEA), i.e., in vitro epidermal tissue viability modulation, morphological changes (Figure 5a) and the release of pro-inflammatory mediators interleukin-1alpha and interleukin-8 (Figure 5b), was compared to clinical skin reactions observed in vivo by visual scoring and biophysical measurement after repeat applications over 3 weeks could be predicted. All topical products that were non-irritating in the human clinical trial were non-cytotoxic in vitro and did not induce pro-inflammatory mediator release after 24 h of exposure. All strong irritating controls demonstrated specific in vitro tissue viability changes and cytokine release patterns. Based on the lack of cytotoxicity but the presence of specific pro-inflammatory mediator release patterns after 72 hours of exposure, ranking of mild to moderate skin irritation potential, clinically observed after 3 weeks, could be established (De Wever et al, 2000).

Figure 5a: Effect of SDS, vitamin D3 analogue Calcipotriol and a retinoid based formulation Retacnyl®. In vitro epidermal tissues were dosed topically for 24 and 72 hours with 10 mg/cm² of the test chemicals and consequently analyzed using the MEA approach. Histology and tissue viability analysis (% of untreated control in italic). Note the SDS induced tissue necrosis at 72 hours. Calcipotriol induces increased epidermal differentiation, whereas Retacnyl® treated tissue showed disappearance of the stratum granulosum layers.

Interleukin-1alpha release

Interleukin-8 release

Figure 5b: Pro-inflammatory release of interleukin-1alpha and interleukin-8 after topical treatment with SDS, Calcipotriol and Retacnyl®. The amount of mediator release correlates with the clinical trial data scored after 22 days: SDS 0.4% was irritating, Calcipotriol was innocuous and Retacnyl® moderately irritating (De Fraissinette et al, 1999).

Using a similar testing approach and confirming previously reported data (De Wever et al, 1999; Bernard et al, 2000b), a model system was designed to predict in vitro the phototoxicity potential of test chemicals and finished products (Medina et al, 2001). A set of phototoxic and non-phototoxic compounds was tested using reconstituted human epidermis grown in chemically defined medium. Test chemicals were topically applied for 24 hours, in the presence or absence of UVA light, and consequently analyzed for tissue viability (LDH release), tissue histology and the release and mRNA expression of the pro-inflammatory mediator interleukin-8.

Using this MEA strategy, the phototoxic potential of all chemicals tested were classified correctly: strong phototoxigants were detected by LDH release and morphological changes, whereas the weak phototoxic products (such as 6-methyl coumarin) did not induce changes in tissue viability or morphology, but increased interleukin-8 release and mRNA expression (Figure 6).

Figure 6: Tiered strategy for testing the phototoxic potential of test articles based on MEA (release of LDH, tissue morphology and release of interleukin-8 (with the courtesy of Novartis Pharma (Medina et al, 2001).

Differentiating skin irritants from skin sensitizers based on the MEA approach.

Both irritant and sensitizing compounds trigger clinical cutaneous responses that are very similar in terms of erythema, edema and epidermal scaling and thickening (acanthosis). The difference between a sensitizing and irritating compound lies in the ability of the sensitizer to induce a specific cutaneous response with immunological 'memory' (Coquette et al, 1999a). In either cases, epidermal keratinocytes releasing pro-inflammatory cytokines play a key role in the cascade of cellular and biochemical events that will eventually activate dermal microvascular endothelial cells and initiate accumulation of specific mononuclear cells (Anzel et la, 1990; Enk et al, 1992a; Enk et al, 1992b; Barker et al, 1991; Barker et al, 1992; Ponec et al, 1995; Knopp et al, 1995).

In other words, the critical signals that will lead to skin irritation and/or sensitization originate at the epidermal keratinocyte level and therefore the expression or release of such cytokines by reconstituted human epidermal models has been proposed as reliable signs to predict *in vivo* adverse reactions (McKenzie, 1990; Dickson et al, 1993, De Wever et al, 1994; Wilmer et al, 1994). However considering the complexity of the biochemical pathways underlying cutaneous cytokine production, the prediction of chemically induced contact dermatitis remains an area in which considerable effort is being directed towards the development of an *in vitro* test method that can replace the traditional *in vivo* assays.

Recent *in vitro* experimental data obtained using reconstituted human epidermis grown in chemically defined medium show that pro-inflammatory mediator interleukin-1 α and interleukin-8 expression induced by topical exposure to irritants is different from the expression pattern following exposure to sensitizing chemicals (Berna et al, 1998; Coquette et al, 1999b; Coquette et al, 2000). At the tested concentrations, the irritants (Triton x-100, Benzalkonium chloride and Tween-80) induced an elevated release of IL-1 α , but not IL-8, but whereas DNCB, a well-known sensitizer, did not induce an increase in IL-1 α but it provoked IL-8 release. Based on these preliminary results, additional experiments with 5 sensitizers (nickel sulphate, DNCB, DNFB, oxazolone, and TNSB) and 3 irritants (SLS, Benzalkonium chloride and benzoic acid) tested at 5 different concentrations were performed. MEA analysis (tissue viability, histology and IL-1 α and IL-8) demonstrated that indeed the sensitizers induced a dose dependent cytotoxicity, but with low IL-1 α release and high IL-8 release, and the irritants exhibited a high IL-1 α release with a low IL-8 release, when tested at doses that induced a cytotoxic response. Additional experiments are currently ongoing to provide further evidence to support these observations.

Conclusion.

The improvement of tissue culture techniques that allow the reconstitution of human epidermis *in vitro* in chemically defined medium results in highly reproducible epidermal constructs that demonstrate much closer physiological features of normal human skin in terms of structural, biochemical and barrier function properties as compare to 'conventional' epidermal models reconstructed in the presence of serum or dermal components. Consequently their use as alternative test models to predict cutaneous toxicological and pharmacological reactions of topically applied agents has proven to be highly reliable and reproducible (Doucet et al, 1996; Doucet et al, 1998; Gysler et al, 1999a; Gysler et al, 1999b; Doucet et al, 1999; Laugier et al, 2000; Bernard et al, 2000a, Garcia et al, 2000, Dagues et al 2001, Mavon et al, 2001) as opposed to animal test methods.

Using these tissue constructs in an optimized *in vitro* analytical evaluation of morphological epidermal assessment, cell viability measurement and consequent pro-inflammatory mediator release by the epidermal keratinocytes (MEA or Multiple Endpoint Analysis), a reliable pre-clinical test method is provided to accurately predict cutaneous irritation and phototoxicity. It should be noted that all *in vitro* parameters analyzed in MEA strategy are equally important for accurate data interpretation. To avoid false negatives and false positives, one should not perform tissue viability testing based on MTT without performing histological analysis: MTT is mainly converted by basal cells of the epidermal construct and tissue necrosis occurring only in the upper epidermal cell layers will not be detected.

Conversely, one should not assess IL-1 α and/or IL-8 release without performing tissue viability testing or histology, as some pro-inflammatory mediators (for instance IL-8) will not be synthesized and consequently released when the epidermal tissue is necrotic. Additionally, assessing tissue histology and tissue viability without quantifying the release of pro-inflammatory mediators can lead to false negative results as slightly irritating or weakly phototoxic agents will induce interleukin 1 α and/or IL-8 release without affecting tissue morphology and viability since their release are early signs of irritation or phototoxicity, undetected by either MTT or tissue morphology. Human epidermis reconstituted in vitro, in chemically defined medium, represents a very useful model for assessing skin biocompatibility, including irritancy, phototoxicity and hypersensitivity.

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Chapter 2

Transcriptional profiling of epidermal keratinocytes: comparison of genes in skin, cultured keratinocytes and reconstructed epidermis using large DNA microarrays.

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Transcriptional Profiling of Epidermal Keratinocytes: Comparison of Genes Expressed in Skin, Cultured Keratinocytes, and Reconstituted Epidermis, Using Large DNA Microarrays

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Epidermal keratinocytes are complex cells that create a unique three-dimensional (3-D) structure, differentiate through a multistage process, and respond to extracellular stimuli from nearby cells. Consequently, keratinocytes express many genes, i.e., have a relatively large "transcriptome." To determine which of the expressed genes are innate to keratinocytes, which are specific for the differentiation and 3-D architecture, and which are induced by other cell types, we compared the transcriptomes of skin from human subjects, differentiating 3-D reconstituted epidermis, cultured keratinocytes, and nonkeratinocyte cell types. Using large oligonucleotide microarrays, we analyzed five or more replicates of each, which yielded statistically consistent data and allowed identification of the differentially expressed genes. Epidermal keratinocytes, unlike other cells, express

many proteases and protease inhibitors and genes that protect from UV light. Skin specifically expresses a higher number of receptors, secreted proteins, and transcription factors, perhaps influenced by the presence of nonkeratinocyte cell types. Surprisingly, mitochondrial proteins were significantly suppressed in skin, suggesting a low metabolic rate. Three-dimensional samples, skin and reconstituted epidermis, are similar to each other, expressing epidermal differentiation markers. Cultured keratinocytes express many cell-cycle and DNA replication genes, as well as integrins and extracellular matrix proteins. These results define innate, architecture-specific, and cell-type-regulated genes in epidermis. **Key words:** epidermal differentiation/mitochondria, proteolysis/transcriptome/UV light. *J Invest Dermatol* 121:1459–1468, 2003

Skin is the most accessible target for testing new treatments of human diseases, from cosmetics and topical medications for local cutaneous symptoms to patches that treat systemic problems and even gene therapy. Keratinocytes, skin's main component, have been a target of extensive basic and applied research efforts. Animal models of skin are generally inadequate because they substantially differ from human skin, whereas experiments on people are ethically problematic. This led to the use of cultured epidermal keratinocytes as target for treatment. Keratinocytes are usually grown as monolayers, with or without fibroblast feeder cells (Rheinwald and Green, 1975). Nevertheless, the physiology of cultured keratinocytes differs from the *in vivo* physiology because cultured cells do not stratify and differentiate, and the growth conditions in culture resemble more the hyperproliferative conditions of wound healing than the normal, slow growth of skin. More recently, reconstituted epidermis comprising three-dimensional, differentiating keratinocytes grown on air-liquid interface, provided improved models, which more closely represent *in vivo* conditions (Rosdy and Claus, 1990; Bernard *et al.*, 2002). These systems are increasingly used both in basic and in applied research

as surrogates of human skin. Importantly, reconstituted epidermis not only represents a better and more economical model for human epidermis than previously available, but also can reduce the requirement for animal testing, a significant contemporary consideration. Until now, a systematic comparison of physiologic processes and genes expressed in the three systems, skin, cultured keratinocytes, and reconstituted epidermis, has not been done.

Comprehensive and systematic comparison of gene expression similarities and differences has not been possible until the advent of genomics. DNA microarrays are an ideal approach for systematic comparisons because they can simultaneously measure the expression of large number of genes (Iyer *et al.*, 1999; Wellford *et al.*, 1998; Li *et al.*, 2001; Rouillard *et al.*, 2002). Therefore, we decided to define the transcriptome of keratinocytes and compare it with those of nonkeratinocyte cell types, thus identifying keratinocyte-specific markers. Similar approaches in the literature probed much more limited sets of genes and did not use extensive replicate samples (Bernard *et al.*, 2002; Cole *et al.*, 2001). Furthermore, we compared the genes expressed in skin, reconstituted epidermis, and cultured keratinocytes, thus identifying a set of genes specific for the differentiating, three-dimensional architecture of the epidermis and a set presumably influenced by the additional, nonkeratinocyte cell types in the epidermis. For this, we used Affymetrix oligonucleotide microarray chips.

The microarray methodology is still very new, lacking codified approaches, controls, and even language. Its acceptance is slow because often a demonstration of reproducibility is lacking. Indeed, the massive amount of data may cause the inherent

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Abbreviation: ECM, extracellular matrix.

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experimental noise to match and obscure the real differences in expression, which mandates extensive replicate measurements (Lee *et al.* 2000). This becomes crucial in analyses of human samples, where inherent variation is difficult to eliminate. Therefore, we decided at the outset to use at least five replicates of each sample type in these studies, which allowed us to perform statistical analyses, determine confidence limits, and unambiguously identify the differentially expressed genes. Several computational approaches have been described to identify similarly regulated gene clusters. We have compared three of these, self-organizing maps, correlation coefficient clustering, and simple twofold difference in expression and realized that all three identify largely overlapping sets of genes. Because of the fivefold redundancy, the most reliable identification of differentially expressed genes derives from parametric and nonparametric statistical analyses, and therefore we adopted the *t* test and Mann-Whitney test for detection of such genes. An additional difficulty in performing and interpreting microarray analyses is an inadequate annotation, either too sparse or too extensive. Therefore, we compiled an extensive annotation table, which allowed us to identify quickly and efficiently the categories of genes and processes differentially expressed in the three samples.

The results of our studies categorize genes expressed in epidermal keratinocytes and identify those differentially expressed in skin, reconstituted epidermis, and cultured keratinocytes. Specifically, we find that skin and reconstituted epidermis are similar in most respects, except that skin expresses more transcription factors, secreted proteins, and cell surface receptors, reflecting its more complex cellular environment. As expected, the cultured cells do not express epidermal differentiation markers. They are further characterized by increased expression of cell cycle and DNA replication genes. At the same time, several apoptotic genes are suppressed in cultured cells, including four Bcl2-related ones. Unexpectedly, skin expresses much fewer mitochondrial proteins in comparison to both cultured cells and reconstituted epidermis, apparently reflecting its relatively slower metabolism. These results define the global patterns of gene expression in epidermal keratinocytes under a variety of experimental conditions and confirm the usefulness of reconstituted epidermis as differentiating keratinocyte monocultures. The human samples in our study were taken after obtaining informed consent from the donors, in accordance with the Helsinki principles as approved by the local IRB.

MATERIALS AND METHODS

Provenance and maintenance of the samples For each sample type, we attempted to widen the "normal" laboratory variations by preparing samples at different times, from different batches of cells, using different lots of reagents and by having different laboratory personnel perform the experiments.

Normal human skin samples were obtained from patients undergoing elective breast reduction surgery approximately 0 to 4 h after surgery. They were obtained in a protocol approved by the Institutional Review Board. For the experiments presented here, skin was obtained from three independent surgery patients, and two different experimenters prepared the microarray hybridization samples. The skin samples were obtained over a period of 6 mo. The fat layer and most of the dermis were removed using surgical scissors and by gentle scraping with a scalpel, leaving the epidermis as the predominant cellular structure (~0.2 mm deep). Samples were then cut into strips of approximately 0.5 × 3 cm and stored in RNAlater (Ambion, Austin, TX) overnight at 4°C.

The reconstituted human epidermis consists of a three-dimensional multilayered keratinocyte structure grown on air-liquid interface, without any other cell type (SkinEthic Laboratory, Nice, France). All media for cell culture were prepared without antibiotics and antimycotic agents (Roudy and Claus, 1990; Bernard *et al.* 2002). They originated from the same donor, but were expanded and delivered on several occasions, 6 mo apart.

Normal human foreskin epidermal keratinocytes were obtained from M. Simon (Living Skin Bank, Burn Unit SUNY, Stony Brook, NY). The cultures were initiated using 3T3 feeder layers as described (Rheinwald and Green, 1975; Simon and Green, 1984) and then frozen in

liquid nitrogen until used. Once thawed, the keratinocytes were grown without feeder cells in defined serum-free keratinocyte growth medium, supplemented with 5 ng per mL epidermal growth factor and 0.05 mg per mL bovine pituitary extract (Gibco, San Diego, CA) at 37°C, in 5% CO₂. The medium was replaced every 2 d and cells were expanded through two or three 14 passages for the experiments. They were trypsinized with 0.025% trypsin, which was neutralized with 0.5 mg per mL trypsin inhibitor. Cells were harvested by scraping 1 d after they re-ached full confluency. For this set of experiments, the cells were grown by two different experimenters, at three occasions, over a period of 12 mo.

Nonkeratinocyte cell types were grown, and hybridization samples were prepared by our colleagues, users of the NYU School of Medicine, Department of Dermatology Microarray Core Facility. They include six melanoma, four endothelial, two simple-epithelial, and four fibroblastic samples.

Preparation and hybridization of labeled probes Total skin RNA was isolated using 10 mL of Trizol (Invitrogen Life Technologies, Carlsbad, CA), which was followed by RNeasy kit (Qiagen, Chatsworth, CA) according to manufacturer's instruction. Trizol gives good yields and effectively disrupts the epidermis, but the purity of the RNA is inadequate for direct labeling; the RNA isolation kit gives adequate purity, but alone inefficiently disrupts the tissue, which is why we used the two in series. All solutions contain RNase inhibitors. Skin is particularly rich in RNases, necessitating their inhibition. If the sample is not immediately processed for RNA isolation, it is cut into 3-mm-wide strips and stored in RNAlater overnight at 4°C and then at -20°C. With this procedure, we routinely prepare RNA of high quality. From the reconstituted epidermis and cultured keratinocytes, total RNA was isolated using Qiaschredders to homogenize cell extracts and RNeasy kits procedure. DNA was removed with on-column DNase digestion using a Qiagen RNase-free DNase set. RNA samples were stored in water at -80°C until hybridization. To ensure good RNA quality, 28S and 18S ribosomal bands were visualized on a nondenaturing agarose gel and OD_{260/280} spectrophotometric ratio of at least 1.8 was obtained.

Approximately 5 to 8 µg of total RNA was reverse-transcribed, amplified, and labeled as described (Mahadevappa and Warrington, 1999; Li *et al.* 2001). Labeled cRNA was hybridized to HGU95A2 arrays (Affymetrix) with the capacity to display transcript levels of approximately 12,000 human genes. Arrays were washed, stained with anti-biotin streptavidin-phycoerythrin-labeled antibody using Affymetrix fluidics station and then scanned using the Agilent GeneArray scanner system (Hewlett-Packard).

Northern blotting For northern blot analysis, 5 µg of total RNA was electrophoresed on 1% formaldehyde-agarose gel and transferred to Hybond nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The GAPDH probe was synthesized using keratinocyte RNA and the RT-PCR kit (Promega) with the following primers: 5'-ACAGTCAGCCGCACTCTCTT-3' and 5'-GACAAGCTTCCCTCTCTCAG-3'. Gel-purified RT-PCR products were labeled with [³²P]dCTP (3000 Ci/mmol, Dupont NEN, Boston, MA) using a random-primed DNA labeling kit (Roche, Indianapolis, IN) and cleaned through a microspin column (Amersham Pharmacia Biotech). Hybridizations were performed using ExpressHyb solution (Clontech, Palo Alto, CA) at 68°C for 1 h. Membranes were washed with a 2 × SSC, 0.05% SDS solution, with continuous shaking, three times for 30 min at room temperature and with 0.1 × SSC, 0.1% SDS at 50°C for 30 min. The signals from the hybridized membrane were captured on Phosphor Screen (Kodak).

Array data analysis Generally, we used Microsuite 4.0 (Affymetrix) for data extraction, as before (Li *et al.* 2001). To compare data from multiple arrays, the signal of each probe array was scaled to same target intensity value. To eliminate genes exhibiting potential false-positive differential expression, we selected only those genes that possessed relative signal intensity greater than 1 SD above average for all genes scored as "absent" in the samples. GeneChip data mining software (Affymetrix-DM1, version 3.0) was used to derive scatterplots, self-organizing maps, and correlation coefficient clusters of the genes. For additional data interpretation, we used Cluster and Tree View software available at <http://rana.lbl.gov/scsmsoftware.htm> (Eisen *et al.* 1998). First, the data were imported into the Cluster and Tree View software in a tab-delimited format. A data set containing the expression patterns of the regulated genes was clustered in two ways, based on the similarity of gene expression over the time course of 24 h and based on the similarity between different time points. The clusters were observed using the TreeView program.

Differential expressions of transcripts were determined by calculating the statistical probability of differential expression using both parametric and nonparametric tests, specifically using the Student's *t* test and the Mann-

Whitney test, both included in the Affymetrix-JMT, version 3.0. Genes were considered differentially expressed if the both tests confirmed differential expression, and if expression levels differed twofold or more in at least one pairwise comparison, i.e., genes upregulated in skin are expressed at least twice as much in skin as in either reconstituted epidermis or cultured keratinocytes.

We developed an extensive gene annotation table describing the molecular function and biological category of the genes present on the chip (T. Baisio, D. Li, P. Ramphal, and M. Blumenberg, unpublished). The table is based primarily on the data by J.M. Rouillard (Rouillard *et al.*, 2002) and the Gene Ontology Consortium (<http://cgap.nci.nih.gov/Genes/GOBrowser>; <http://dot.pod.med.umich.edu:2000/ourimage/pub/shared/JMR.pubaffanno.html>). The regulated genes were functionally classified according to this table.

RESULTS

Keratinocyte-specific genes Because all three sets of samples, skin, reconstituted epidermis, and cultured keratinocytes, consist primarily, or exclusively, of the same cell type, they should express a common set of keratinocyte-specific genes. Therefore, we compared the 15 keratinocyte samples with a set of human nonkeratinocyte cells analyzed in our Core. The nonkeratinocyte set comprised six melanoma, four endothelial, two simple-epithelial, and four fibroblastic cells. We calculated the medians of the keratinocyte and nonkeratinocyte sets, as well as Student's *t* test and Mann-Whitney statistical calculations for difference in expression. Table 1 contains the most differentially expressed epidermal keratinocyte-specific genes. Among these, we find keratins, cornified envelope proteins, and desmosomal markers. Unexpectedly, we find many proteolytic enzymes as well as their inhibitors. This may suggest the presence of very active protein turnover and remodeling in keratinocytes, not common in other cell types.

Scatterplot analyses One of the important limitations of the use of microarrays today is the inherent variability due to sample differences and preparation, as well as intrinsic limits of instrument reproducibility (Zhao *et al.*, 2000). Indeed, a significant body of literature addresses this issue and the current standard is to perform the experiments in triplicate (Lee *et al.*, 2000). The reproducibility problem is not as serious when cell culture samples are used, but it can be quite significant when *in vivo* human samples from individuals are used. Therefore, we decided to use quintuplicates of each of our three sample types. We have calculated averages, means, and medians for each set of five sample types and found that medians are the least sensitive to the effects of outliers. The statistical analysis of redundancy and the effects of replicates will be published elsewhere (M. Blumenberg and P. Ramphal, manuscript in preparation). One of the main conclusions of that work is that triplicates are necessary and in general sufficient, but that quintuplicates do provide an additional margin of confidence. For example, when we compared scatter plots of individual pairs of microarray data with scatter plots of an array versus a median of all five arrays of the same type, we found that the points cluster much closer to the diagonal in comparison with the medians than in comparisons of individual arrays. This means that by using the medians of five experiments, we have eliminated much of the interexperiment variation (Fig 1). Therefore, we decided to use, in all subsequent comparisons, the medians of quintuplicates of each sample type.

When we compared the medians of skin, keratinocyte, and reconstituted epidermis microarrays using scatterplots, we found again that in all three comparisons most points fell close to the diagonal, which means that most genes are expressed at similar levels in the three sample types (Fig 1). Nevertheless, it appears that the skin samples and the reconstituted epidermis samples are more similar to each other than either is to cultured keratinocytes; comparisons with cultured keratinocytes have more

points threefold or more off the diagonals than the skin/skin equivalent comparison (Fig 1).

Nevertheless, focusing on particular functional categories of genes, we find characteristic similarities and differences between samples. For example, we identified the genes that contain in their functional description characteristic tags, such as "CDC" for cell division cycle, "ribosomal," "mitochondrial," or "growth factor." This last category includes growth factor receptors, their binding proteins or kinases, and even growth factor-like genes, all those that contain the phrase "growth factor" in their descriptions. Specifically, when we compare skin to cultured keratinocytes, we find that almost all CDC and ribosomal genes fall close to the diagonal, within the twofold difference lines, and all fall within the twofold difference lines (not shown). In contrast, although many mitochondrial genes fall close to the diagonal, many are expressed at more than threefold higher levels in cultured keratinocytes than in skin. Conversely, the growth factor category contains many genes expressed at more than threefold higher levels in skin than in cultured keratinocytes (Fig 2 and supplementary table available from <http://www.blackwellpublishing.com/products/journals/suppmat/jid12611/12611sm.htm>; <http://dot.pod.med.umich.edu:2000/ourimage/pub/Shared/JMR.pubaffanno.html>). Virtually identical results are obtained in the comparison of skin with reconstituted epidermis: the CDC and ribosomal genes are expressed similarly in the two sample types (not shown), mitochondrial genes are expressed at higher levels in reconstituted epidermis, and the growth factor category is expressed at higher levels in the skin (Fig 2). When we compared reconstituted epidermis to cultured keratinocytes, we found that these two sample types expressed similar levels of all four categories, CDC, ribosomal, mitochondrial, and growth factor genes (Fig 2).

Perhaps the most interesting differences were found in the category of "keratin," which contains, besides keratins, genes specific for keratinocytes (Fig 3). Here we find that skin and reconstituted epidermis express these genes at similar level, but that the expression in cultured keratinocytes differs (Fig 3). When we identified these genes individually, we found that the differentiation markers, keratins K1, K10, and K2e, are expressed at much higher levels in skin and reconstituted epidermis, than in cultured keratinocytes; this agrees well with the fact that keratinocytes in culture under our growth conditions do not differentiate. The activation-specific keratins K6 and K16 are also expressed at higher levels in the multilayered samples, perhaps reflecting the fact that *in vivo* these keratins are not expressed in the basal layer. We note that these two keratins are represented on the Affymetrix chips as two independent sets of spots and therefore are found in duplicates on the scatterplots. In all comparisons, these duplicates fall close to each other, substantiating the reproducibility of microarray analysis. The basal-layer-specific keratins K5 and K14 are among the most highly expressed genes in these samples and expressed equally in all three types of samples. K17 is also expressed at equal, but lower, levels. On the other hand, simple epithelia keratins, K8, K18, and K19, are expressed at higher levels in culture than *in vivo* and so is K15. Perhaps this demonstrates a certain level of "dedifferentiation" of keratinocytes when they are grown under artificial conditions as a monolayer in culture. Finally, the microarrays detect very low levels of "hair" keratins, at similar levels in the three sample types, as well as the gene encoding the keratinocyte growth factor, which was included in the selection because it contains the string keratin (Fig 3).

Selecting the differentially expressed genes While scatterplots compare gene expression in two dimensions, our 15 microarrays represent a 15-dimensional pattern of expression of over 7000 genes. To analyze such complex patterns, we used two of the approaches developed to reduce the dimensionality of the data: self-organizing maps and correlation coefficient clustering. The first uses a grid of nodes specified by the investigator,

Table I. Keratinocyte-specific genes^a

MEDIAN	FOLD DIFFERENCE	Gene	Symbol	Function
19.038	1904	Keratin 14	KRT14	Cytoskeletal
10.518	1052	Galectin 7	LGALS7	Secreted
9591	959	Keratin 5	KRT5	Cytoskeletal
9546	789	Keratin 6 A	KRT6A	Cytoskeletal
7399	740	Small proline-rich protein 1B, cornifin	SPRR1B	Epidermal differentiation
3678	368	S100 Ca-binding protein A8, calgranulin A	S100A8	Epidermal differentiation
3518	352	Desmoplakin, DPL, DPH	DSP	Adhesion, junction
3254	325	Serine protease inhibitor, clade B 5	SERPINF5	Protease inhibitor
3194	319	H10GF-binding proteing	H10P7	Secreted
3053	305	Desmocollin 1	DSC1	Adhesion, junction
5210	303	Keratin 16	KRT16	Cytoskeletal
2677	268	Collagen, type XVII- α 1	COL17A1	ECM
2651	263	Annexin A8	ANXA8	Regulator
4817	260	Aquaporin 3	AQP3	Transporter
2546	255	Filaggrin	FLG	Epidermal differentiation
2490	249	Cystatin E/M	CST6	Protease, peptidase
6245	245	S100 Ca-binding protein A2	S100A2	Epidermal differentiation
9621	225	Keratin 1	KRT1	Cytoskeletal
3027	212	Tumor-associated Ca signal transducer 2	TACSTD2	Regulator
2048	205	Lymphocyte antigen 6 complex, locus D	E48	Cell surface
1725	173	Gap junction connexin 43	CJAI	Adhesion, junction
6700	170	Keratin 2 A	KRT2A	Cytoskeletal
1652	165	S100 Ca-binding proteing A9, calgranulin B	S100A9	Epidermal differentiation
2674	158	Ataxia-telangiectasia group D-associated	ATDC	DNA replication, repair
1236	124	Cystatin A, stefin A	CSTA	Protease, peptidase
2701	122	Periplakin	PPI	Adhesion, junction
1200	120	Putative G0/G1 switch gene	G0S2	Cell cycle
1175	117	Kallikrein 11	KLK11	Protease, peptidase
1145	114	Interleukin 1 receptor antagonist	IL1RN	Receptor
1086	109	Bullous pemphigoid antigen 1	BPAG1	Adhesion, junction
1058	106	Trypsin 2	PRSS2	Protease, peptidase
1894	105	Interferon induced protein, 9-27	IFITM1	Interferon regulated
1589	103	Desmoglein 1	DSG1	Adhesion, junction
1000	100	Carbonic anhydrase XII	CA12	Small molecule enzyme
989	99	Trypsin 4	PRSS4	Protease, peptidase
947	95	Serine protease inhibitor, Kazal type, 5	SPINK5	Protease inhibitor
1274	94	Protease, serine, 11, IGF binding	PRSS11	Protease, peptidase
933	93	Interferon- α -inducible protein 27	IFI27	Secreted
928	93	CD24 antigen	CD24	Cell surface
1251	92	Serine protease inhibitor, clade B 2	SERPINF2	Protease inhibitor
904	90	Notch homolog 3	NOTCH3	Notch pathway
902	90	20- α ,3- α -hydroxysteroid dehydrogenase	AKR1C1	Metabolism, steroid
1054	84	Serine protease inhibitor, clade B 7	SERPINF7	Protease inhibitor
836	84	Kallikrein 7, chymotryptic, stratum corneum	KLK7	Epidermal differentiation
813	81	Keratin 15	KRT15	Cytoskeletal
12,578	80	Stratifyn	SFN	Epidermal differentiation
1161	69	Corneodesmosin	CDSN	Epidermal differentiation
6726	64	Loricrin	LOR	Epidermal differentiation
635	63	Epidermal growth factor receptor	EGFR	Receptor
2883	63	Tubulin- α 1	TUBA1	Cytoskeletal
619	62	Actin-binding LIM protein 1	ABLIM	Cytoskeletal
617	62	jun B proto-oncogene	JUNB	Transcription factor
596	60	Transcription factor AP-2 α	TFAP2A	Transcription factor
1196	58	Tumor protein 63-kDa homology to p53	TP63	p53 pathway
1246	58	Arachidonate 12-lipoxygenase, 12R type	ALOX12B	Prostaglandin pathway
2795	55	Involucrin	IVL	Epidermal differentiation
528	53	Cyclin D2	CCND2	Cell cycle
2075	52	Skin-specific protein	XP5	Epidermal differentiation
513	51	Serine protease inhibitor, clade B 3	SERPINF3	Protease inhibitor
532	51	Glutamate-ammonia ligase	GLUL	Metabolism, amino acid
490	49	Kallikrein 8, neuropsin/ovasin	KLK8	Protease, peptidase
483	48	Transglutaminase 1 epidermal type	TGM1	Epidermal differentiation
1063	47	G protein- α 15, Gq class	GNAI5	G-regulated protein
718	45	Peroxisinodoxin 2	PRDX2	Antioxidant
433	43	Laminin- α 3	LAMA3	ECM
413	41	Upregulated in carcinoma	DDP6	Cell surface

Table I. (Continued)

MEDIAN	FOLD DIFFERENCE	Gene	Symbol	Function
566	41	Aldehyde dehydrogenase 8	ALDH8	Metabolism, energy
3846	41	Junction plakoglobin	JUP	Adhesion, junction
393	39	Serine protease inhibitor, clade B 4	SERPINE4	Protease inhibitor
2000	37	CDK inhibitor 1 A, p21, Cip1	CDKN1A	Cell cycle
693	36	Integrin-β4	ITGB4	Adhesion, integrin
576	36	IGF-binding protein 7	IGFBP7	Secreted
372	36	Envoplakin	EVPL	Epidermal differentiation
448	35	Integrin-α3	ITGA3	Adhesion, integrin
842	35	Ncxin, plasminogen activator inhibitor 1	SERPINE1	Protease inhibitor
349	35	P-cadherin, placental	CDH3	Adhesion, junction

*The set of genes most differentially expressed in keratinocytes relative to other cell types examined. The genes are presented in the order of expression ratios, second column. The second column shows the ratios of medians of expression in keratinocytes and nonkeratinocyte cells. The symbols can be used to query public databases and obtain information about the listed genes, e.g., at <http://www.ncbi.nlm.nih.gov/90/entrez/query.fcgi?CMD=search&DB=unigene>, or <http://www.ncbi.nlm.nih.gov/80/entrez/query.fcgi?CMD=search&DB=omim>. NULL denotes genes without assigned symbols, and EST indicates that the transcript from the uncharacterized gene has been detected in at least some cell types. Some of the genes contain multiple entries (e.g., stratifin), because the same gene is probed multiple times on the DNA microarrays.

Figure 1. Scatterplots of individual versus individual microarrays, individual arrays versus medians, and the medians. Scatterplots compare the expression of all genes in two experiments; the identically expressed genes fall on the diagonal. The thin lines offset from the diagonals mark the 2-, 3-, 10-, and 30-fold differences in expression. The *top row* shows representative comparisons of two individual microarrays from each sample type. The *middle row* shows comparisons of one microarray with the median of all five. In the comparison with the medians, points fall much closer to the diagonal. The *bottom row* shows comparisons of the medians. The wider scattering of the points between different samples indicates an abundance of differentially expressed genes, including many differentially expressed 10-fold or more. K'cyt, cultured keratinocytes; REp, reconstituted epidermis.

Figure 2. Functional classes of genes identified in the scatterplots. Groups of functionally related genes can be selected from the gene annotations, identified and highlighted within the scatterplots. Here we show genes whose annotations contain the words "mitochondrial" (top) or "growth factor" (bottom).

assigns each gene to a node, and then, in an iterative process, moves the nodes to minimize the total distance between the genes and the nodes. In this way each gene is assigned to a node, the cluster of genes assigned to the same node having similar patterns of expression. A 4×6 grid of nodes is presented in Fig 4A. The leftmost five points of each node represent the expression levels in cultured keratinocytes, the middle five in skin, and the rightmost five in reconstituted epidermis (the sets of five are separated by thin lines in cluster 1, Fig 4A, top left). Whereas some of the clusters contain outlier genes aberrantly expressed in just one of the chips, e.g., clusters 4 and 6, others contain genes specifically expressed in cultured keratinocytes (cluster 3), skin (cluster 12), or reconstituted epidermis (cluster 21). Similarly, there are clusters of genes specifically not expressed in cultured keratinocytes, skin, or reconstituted epidermis (clusters 22, 13, and 10, respectively). The correlation coefficient clustering algorithm searches the 15-dimensional space for aggregations of genes that have similar expression patterns (Fig 4B). The two different approaches yield very similar clusters, however, and there is a significant overlap between the genes identified by the two methods (compare, for example, cluster 8 from correlation coefficient clustering with cluster 13 from self-organizing maps, Fig 4A vs. Fig 4B).

Arguably, the simplest way to discover the differentially expressed genes is to compare their level of expression and identify those that have differences of expression above a specified cutoff value. This method will overlook some of the highly expressed genes that are differentially expressed, but with less than the cutoff difference, as well as includes some of the poorly expressed but variable genes. Having the 5-fold replication of our data, we were able to use statistically superior

methods of selecting differentially expressed genes, methods that take into account the inherent variability of the measurements. We used both parametric and nonparametric tests, specifically, the Student's *t* test and the Mann-Whitney test; both are included in the Affymetrix data-mining programs. Only the genes found differentially expressed in both tests were analyzed. To reduce further the number of marginally relevant genes, we retained only those genes that have a 2-fold or higher differential expression of medians in at least one pairwise comparison. For example, if a gene is expressed 1.8-fold higher in cultured keratinocytes than in skin and in reconstituted epidermis, it was not retained for analysis, but if it is expressed 2.1-fold higher than in skin and 1.3-fold than in reconstituted epidermis, it was. A total of 3240 genes were selected this way.

In a two-way comparison, differentially expressed genes can be either "induced" in one sample or "suppressed" in the other one. But, in a three-way comparison, we can identify the genes that are induced and genes that are suppressed in a given sample, by comparing it with the other two. For example, to identify the genes induced in skin, we listed those genes that differentially expressed at higher levels in skin when compared to both cultured keratinocytes and reconstituted epidermis. Genes suppressed in skin are those expressed at higher levels in both cultured keratinocytes and reconstituted epidermis than in skin. Similarly, the cultured keratinocyte-specific expression lists includes genes that are twofold differently expressed versus both other sample types and *mutatis mutandis* for the reconstituted epidermis.

Functions of the differentially expressed genes We first focused on the expression of epidermal differentiation markers because the data in Fig 3, as well as a large body of literature,

Figure 3. Identification of keratin genes in scatterplots. Individual genes containing the word “keratin” are highlighted and identified in the scatterplot comparing medians of skin and cultured keratinocytes microarrays. Note that the keratin differentiation markers, 1, 2e, and 10, are expressed at higher levels in skin, whereas the simple-epithelia keratins, 8, 18, and 19, are expressed more in cultured cells. Several hair keratin genes appear expressed at low levels and are marked with “h”. KGFR is the gene for keratinocyte growth factor, and *a* represents a gene annotated as “cloned from a keratinocyte cDNA library”; both contain the string of letters “keratin” in their annotations. In the scatterplot of reconstituted epidermis versus cultured keratinocytes, we identified the same differentially expressed keratins (arrows), and in the scatterplot of skin versus reconstituted epidermis, most keratin genes fall at or close to the diagonal.

make it clear that keratinocytes in monolayer cultures do not differentiate. Indeed, the epidermal markers of differentiation, such as filaggrin, loricrin, and involucrin are suppressed in cultured keratinocytes (supplementary table, *a*). In addition, the differentiation-specific keratins, K1, K2e, and K10, were suppressed, but the simple-epithelial keratins, K7, K18, and K19, were induced in cultured keratinocytes. Interestingly, integrins are expressed at higher levels in cultured keratinocytes than in the other two samples. Similarly, several extracellular matrix (ECM) proteins are expressed at higher levels in cultured keratinocytes, particularly those that form microfibrils and the basal lamina. These include fibrillin 2, fibrillarlin, MFAP2, fibronectin, and laminin. Skin also expresses a set of ECM proteins; surprisingly, we find among these collagen type VI (supplementary table, *a*) (Olsen *et al.*, 1989; Watson *et al.*, 2001). This collagen may derive from the dermal fibroblast contaminating our preparations, but in that case, we would expect to see type I, type III, and other collagens as well. We consider the possibility that the immediately subepidermal, papillary fibroblasts specifically produce collagen type VI less likely that the possibility that, influenced by other cutaneous cell types, epidermal keratinocytes also contribute to the micro-fibrillar structures that anchor them.

Although the differentiation markers are suppressed, the cell cycle and DNA replication genes are greatly induced in cultured keratinocytes (supplementary table, *b*). These data correlate well with the high mitotic activity in the monolayers, compared to the other two samples. In parallel with the cell cycle and DNA

replication, the monolayer cultures enhance the expression of nucleoskeletal and RNA metabolism proteins. This would suggest that the keratinocytes in monolayers have significantly more active nuclear processes in general, compared to the multilayered structures.

Conversely, the mitochondrial proteins are strongly suppressed in skin (supplementary table, *c*). Over 50 mitochondrial genes are expressed less in skin than in the other two samples, which suggests that the skin is relatively quiescent and does not need to produce much energy. Apparently, the metabolism in skin is relatively slow. Nevertheless, when we directly examined the expression of enzymes participating in amino acid, carbohydrate, lipid, and steroid metabolism, we did not observe striking and meaningful differences (supplementary table, *d*).

In contrast to mitochondrial proteins, relatively higher levels of growth factors and cytokines, as well as their receptors, are found in skin (supplementary table, *e*). These include, on the one hand, several interleukin, growth factor, and chemokines receptors and their binding proteins and, on the other hand, the ligands, growth factors, and cytokines. In general, the induced ligands do not bind the induced receptors, which avoids establishment of autocrine feedback loops. In cultured keratinocytes, several receptors are suppressed, e.g., the ERTB family, interleukin-1, and interferon- γ receptors, as are several ligands. Perhaps the culture conditions attenuate some of these signaling processes. The behavior of reconstituted epidermis falls between the other two samples, with very few genes specifically regulated. We note again the absence of fibroblast-specific markers, such as

A **K S REp** SOM Clustering Algorithm; 24 clusters created.

B **Correlation Coefficient Clustering; 28 clusters created.**

Figure 4. Clustering of differentially expressed genes. (A) Self-organizing maps distributing the genes into 24 bins of a 6 × 4 grid. Of the 15 microarrays, the first 5 are cultured keratinocytes, the middle 5 skin, and the last 5 from reconstituted epidermis, as shown in the top left cluster. The lines represent the medians of relative expression of the genes in each cluster. Whereas many clusters contain genes specifically highly expressed in just one microarray, e.g., the top left, or bottom right clusters, and are presumably outlier artifacts, other clusters contain genes differentially induced or suppressed in cultured keratinocytes, skin, and reconstituted epidermis. (B) Correlation coefficient clustering. A set of 6 selected clusters of the 28 obtained is chosen to demonstrate similarities with the self-organizing maps.

keratinocyte growth factor or transforming growth factor- β , in skin, which indicates that the keratinocyte is the predominant cell in our preparations.

The differentially expressed signaling genes are presented in the supplementary table (f). For most, no clear indication of disparity is obvious, in many cases because too few genes are differentially expressed. Nevertheless, several pathways seem clearly different in the three sample types. The data in the supplementary table (f) are indicative of the differentially activated processes and can be used as a direction indicator for future studies, but are not in themselves indicators of activity of a given pathway. Signal transduction pathways result in activation of transcription factors. A relatively large number of transcription factors are differentially expressed in the three sample types (supplementary table, g). We have not tried to group them into categories, except to distinguish the homeobox proteins, which do not seem to be differentially expressed. Many transcription factors are induced in skin and suppressed in cultured keratinocytes, with the reconstituted epidermis relatively unaffected. We have no explanation for these differences at this time.

DISCUSSION

Using large-scale DNA oligonucleotide microarrays, we characterized the "transcriptome," the comprehensive global pattern of gene expression in epidermal keratinocytes. We compared the transcriptomes of keratinocytes with a collection of other cell types, as well as the three different experimental models of keratinocytes available, skin, reconstituted epidermis, and cultured keratinocytes. This allowed us to identify the keratinocyte-specific genes, the genes specific for the three-dimensional, multi-layered epidermal architecture, as well as the genes expressed in keratinocytes under the influence of neighboring cell types.

The keratinocyte-specific genes include, as expected, keratins, adhesion proteins and epidermal differentiation markers and, perhaps less expected, proteases and proteolysis inhibitors. We want to point out two additional genes highly expressed in keratinocytes, ATDC, the ataxia-telangiectasia D-associated protein and galectin-7. We note that the epidermis is the primary and perpetual target of UV light and therefore ATDC may be constitutively present in high amounts to stand sentinel against DNA damage (Gilestret, 1995; Li *et al.*, 2001). Galectin-7 is an epidermal differentiation marker also associated with UV damage and its responses (Madsen *et al.*, 1995; Maguadalo *et al.*, 1995; Bernerd *et al.*, 1999; Timmons *et al.*, 1999). These results suggest that human epidermal keratinocytes specifically recognize UV light as the major environmental insult and have evolved specific mechanisms to deal with it.

Among the most prominent differences between the sample types, as expected, is the lack of differentiation markers expressed in cultured keratinocytes. These include suprabasal keratins, as well as small proline-rich protein, loricin, and involucrin. Many actin-associated cytoskeletal proteins are expressed preferentially in the cultured cells, perhaps reflecting their motility, which stratified cells lack (not shown). If so, then these cytoskeletal proteins probably play important roles in wound healing and our data point to a potentially fruitful new area of research. Furthermore, cultured cells express high levels of integrins $\alpha 3$, $\alpha 6$, and $\beta 4$; this probably reflects the fact that in monolayer cultures every cell is attached to the substratum and therefore needs integrins to hold on, whereas in the stratified systems, only the basal cells do. It will be very interesting to examine the molecular regulatory mechanisms that cause such differences.

We expected to find more products of fibroblasts in skin, such as the ECM proteins, but we only detected collagen VI-a. This suggests that the contamination with fibroblasts in our epidermal preparations is minimal and that the keratinocytes are by far the predominant cell type in our samples. It also raises the question whether collagen VI-a is produced by keratinocytes; there are no

indications for this in the literature, although low-level expression in the epidermis may be obscured by the high levels in fibroblasts and disregarded as artifactual (Olsen *et al.*, 1989; Watson *et al.*, 2001).

Another prominent difference between the sample types is the high number of cell cycle and DNA replication proteins expressed in cultured keratinocytes. These cells are encouraged to proliferate by the culture conditions. In contrast, many such genes are suppressed in skin. In parallel, cultured cells express higher levels of nucleoskeletal proteins, which was perhaps expected, and RNA-processing enzymes, which was not. This parallelism may reflect similar requirements for these proteins in rapidly proliferating cells. The reconstituted epidermis is in between cultured keratinocytes and skin. We suspect that these genes are expressed in the basal layer of the reconstituted epidermis, which is stimulated to proliferate, while the suprabasal layers are postmitotic.

We find skin and reconstituted epidermis to be similar in most respects. Nevertheless, in skin we find elevated expression of cell-to-cell signaling molecules, such as secreted proteins and cell surface receptors, probably owing to the influence of additional cell types, besides keratinocytes. These results confirm the usefulness of reconstituted epidermis as a model for differentiating keratinocyte monocultures.

A characteristic set of genes is overexpressed in skin and reconstituted epidermis, genes specific for the stratified, differentiating samples. The set includes proteins that, presumably, play important roles in the suprabasal layers of the epidermis, which needs to be demonstrated directly. Importantly, the stratified samples specifically express cell surface receptors, as well as secreted signaling molecules. The receptors include interleukin-1 receptor, as expected (Groves *et al.*, 1994; Kupper and Groves, 1995; Groves *et al.*, 1996), but also ERBB2 and 3, which was controversial (De Potter *et al.*, 2001; Xie *et al.*, 1998), and ephrin receptors, which was unanticipated. Some of the secreted peptides were known to be associated with differentiation, but not others (Ali *et al.*, 2001; Tohyama *et al.*, 2001; Liu *et al.*, 2002). From these data, we conclude that cell-cell communications via diffusible peptides, and their receptors are extremely important in establishment and maintenance of the stratified epidermal structure.

The increased number of cell surface receptors and secreted signaling proteins in skin keratinocytes may reflect the induction by other cell types, absent from the two monoculture systems. Nevertheless, it is also possible that they are expressed by these other cell types and not by keratinocytes, and at present, we have not distinguished between the two possibilities. Additional experiments, either using immunohistology or using microarrays of epidermis completely separated from dermis, currently ongoing in our laboratory, will resolve this issue. Obviously, these results can lead to additional studies and we hope that they may inspire some of our colleagues or provide explanation for some of the unexpected results in other laboratories.

Several genes are expressed uniquely in skin, not even in the reconstituted epidermis, and although other cell types may produce some of them, others are induced in keratinocytes by the presence of additional cell types. These include matrix HLA markers, complement components, metalloproteases, etc. (not shown) (Suomela *et al.*, 2001; Dowezenski *et al.*, 1992; Haw, 1995; Pasch *et al.*, 2000). Quite unexpectedly, we find the mitochondrial proteins suppressed in skin. At present, we do not understand the causes for this difference. Nevertheless, the lower need for ATP and lower metabolic rate should be taken into account in studies of drug metabolism in epidermis, because in this aspect the differences between skin and reconstituted epidermis may be significant.

We believe that we have reliably identified the broad range of differentially expressed genes, because of the high number of repeats for each sample type. A fivefold redundancy allowed us greatly to reduce the variability often seen in comparison of microarrays. The sample-to-sample variations were greater between skin samples than other two types, as expected for in

in vivo human patient specimens. It is important to realize that we have not taken into consideration other variables. For example, all skin samples come from women, whereas the reconstituted epidermis and cultured keratinocytes derive from foreskins. The ages are also different: cultured cells come from neonates and reconstituted epidermis from children, whereas breast reductions come from adult women. Obviously, much larger samples will be needed to dissect the effects of age, sex, or ethnic background and multicenter, large-scale analyses are currently being developed for such purposes. From the data presented here, however, it appears that these variations are minor.

Finally, we would like to point out that different analysis software, developed using various approaches ideas and algorithms, produces very similar results. While direct and detailed comparisons of these will be published elsewhere, we were gratified to see the extensive overlaps of lists of differentially expressed genes obtained using self-organizing maps, correlation coefficient clustering, or Student's *t* test and Mann-Whitney test. In this, we were greatly aided by the extensive redundancy of our data.

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Chapter 3

Analysis of interleukin-1alpha (IL-1a) and interleukin-8 (IL-8) expression and release in in vitro reconstructed human epidermis for the prediction of in vivo skin irritation and/or sensitization.

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Analysis of interleukin-1 α (IL-1 α) and interleukin-8 (IL-8) expression and release in in vitro reconstructed human epidermis for the prediction of in vivo skin irritation and/or sensitization

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Abstract

In the present study, reconstructed human epidermis (RHE) was used as an in vitro model to discriminate 1-chloro-2,4-dinitrobenzene (DNCB), nickel sulfate (NiSO₄), oxazolone (OXA), 2,4-dinitrofluorobenzene (DNFB) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) as skin sensitizers from benzalkonium chloride (BC), benzoic acid (BA) and sodium lauryl sulfate (SLS) as skin irritants. Our criteria were (a) the differential IL-1 α and IL-8 synthesis and release (b) cytotoxicity assessment by MTT assay. When the RHE are topically treated with the sensitizers, very low levels of extra- and intracellular IL-1 α are observed although they induce significant cytotoxicity. In contrast, they exhibit a sharp maximum of IL-8 release. In the presence of the tested irritants, we observe the inverse cytokine release profile, although they induce dose-dependent cytotoxicity profiles similar to those observed with the sensitizers. Finally, IL-1 α mRNA upregulation is observed after topical application of both sensitizers and irritants, but only the latter significantly increase extracellular IL-1 α . In conclusion, our results suggest that the associated determination of IL-8, with IL-1 α , and MTT conversion are at least necessary to discriminate and classify, in a single assay, irritant and sensitizing agents and represent a potential in vitro alternative to two classical in vivo assays.

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1. Introduction

Skin exposure to chemicals, drugs or cosmetics can lead to a wide variety of skin reactions, such as altered

pigmentation, acne, cancer, irritant contact dermatitis and sensitization. Among those reactions, irritation and sensitization are the major causes of skin inflammation (Wahlberg, 1996). It is therefore important to identify chemicals or formulations which induce skin irritation and/or sensitization and this is the reason why in vivo data on animal skin irritation/sensitization are requested by regulatory authorities. Animal tests for the assessment of acute skin irritation are usually performed according to the method of Draize (Draize et al., 1944) while assays for skin sensitization in vivo are performed according to the guinea pig maximisation test (Magnusson and Kligman, 1970), according to the occluded patch test developed by Buehler (Buehler, 1965) or according to the local lymph node assay (Basketter et al., 2000). However, because of questionable significance of animal data and ethical opposition to animal experimentation, considerable efforts are being made towards the development and evaluation of in vitro methods to replace traditional in vivo assays (De Silva et al., 1996; Basketter et al., 1999).

Abbreviations: BA, benzoic acid; BC, benzalkonium chloride; DMSO, dimethyl sulfoxide; DNCB, 1-chloro-2,4-dinitrobenzene; DNFB, 2,4-dinitrofluorobenzene; GM-CSF, granulocyte/macrophage colony stimulating factor; IL-1 α , interleukin 1 α ; IL-6, interleukin 6; IL-7, interleukin 7; IL-8, interleukin 8; IL-10, interleukin 10; IL-12, interleukin 12; IL-15, interleukin 15; IP-10, interferon-induced Protein 10; LLNA, local lymph node assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NiSO₄, nickel sulfate; OXA, oxazolone; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PDC, 5-methyl-3-n-pentadecylcatechol; RHE, reconstructed human epidermis; RT-PCR, reverse transcription-polymerase chain reaction; SLS, sodium lauryl sulfate; TGF, transforming growth factor; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNF- α , tumor necrosis factor α .

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Culture of human skin explant (Rambukkana et al., 1996), epidermal Langerhans cells (Pineau et al., 1994), blood-derived dendritic cells (Hoppe et al., 1995) and conventional keratinocyte cultures (Ponec, 1992; Wilmer et al., 1994; Pastore et al., 1995; Corsini et al., 1996) has raised hope that the development of relevant *in vitro* test systems would be achievable. However, it is not yet clear whether one single *in vitro* assay could discriminate and classify sensitizing and/or irritant substances.

Keratinocyte represents the major cell type in the epidermis and plays a key role in skin inflammatory and immunological reactions (Barker et al., 1991; McKenzie and Snauders, 1990; Berardesca and Distant, 1995). Because of their ability to produce a large range of inflammatory mediators, immersed keratinocyte cultures were chosen for the development of *in vitro* methods to test irritants or sensitizers (Corsini et al., 1995, 1996). However, due to the absence of the stratum corneum, the concentrations inducing responses in these monolayer cell cultures are usually several orders of magnitude lower than those which induce inflammation *in vivo*. In addition, compounds with low water solubilities and/or final topical formulations cannot be tested.

On the other hand, cultures performed on de-epidermized dermis, collagen matrices or inert filters and raised at the air–liquid interface mimic the epidermis with a cornified barrier and metabolic activity (Rosdy and Clauss, 1990; Boyce et al., 1990; Régnier et al., 1990; Tinois et al., 1991; Bell et al., 1991; Ponec, 1995). So far, those promising reconstructed tissues have been used for metabolic studies of pharmaceutical products (Laugier et al., 2000; Bernard et al., 2000a), for the determination of absorption properties (Gysler et al., 1999), for the assessment of cutaneous irritancy (Ponec and Kempenaar, 1995; De Brugerolle de Fraissinette et al., 1999) and phototoxicity (Bernard et al., 2000b), and for studies of epidermal responses to irritants and sensitizers (Corsini and Galli, 1997; Coquette et al., 1999, 2000a).

In response to physical or chemical stresses, keratinocytes produce and release inflammatory cytokines [interleukins (IL) 1 α , tumor necrosis factor α (TNF- α)], chemotactic cytokines [IL-8, interferon induced protein 10 (IP-10)], growth-promoting factor [IL-6, IL-7, IL-15, granulocyte/macrophage colony-stimulating factor (GM-CSF)], transforming growth factor [TGF], cytokines regulating humoral versus cellular immunity [IL-10, IL-12] and other signalling factors which rapidly generate cutaneous inflammation (Williams and Kupper, 1996), suggesting that the measurement of such keratinocyte responses may allow the evaluation of toxicological properties of chemical in order to identify irritants and/or sensitizers. Unfortunately no specific marker has clearly been identified so far, probably because almost each chemical that causes contact

hypersensitivity is also a primary irritant, but also because of the existence of a complex network at molecular levels involved in both irritation and sensitization. Thus, instead of looking for specific markers, we proposed that differentiating sensitization and irritation effects might be possible by studying a group of cytokines and measuring the differential modulation of their respective levels (Coquette et al., 1999). Indeed, we chose to investigate keratinocyte IL-1 α and IL-8 synthesis and release, because among keratinocyte-synthesized cytokines, IL-1 α and IL-8 play critical roles in the epidermis. IL-1 α is constitutively produced and retained in keratinocytes, but in response to several stimuli, IL-1 α is released (Kupper and Groves, 1995), which is an essentially primary event of inflammation (Williams and Kupper, 1996). IL-1 α stimulates further release of secondary mediators, including IL-8. IL-8 promotes dendritic cells migration and recruitment of monocytes and neutrophils as key steps in the initiation phase of cutaneous inflammation (Barker et al., 1991). Indeed, preliminary *in vitro* results obtained in reconstructed human epidermis (RHE) triggered with DNCB, BC and Triton X100, showed that the amount of released IL-1 α and IL-8 did not correlate with cytotoxicity, but could be linked to the type of product applied, either irritant or sensitizer. Those results suggested that it could be possible in a single *in vitro* assay to classify and discriminate between irritant and sensitizing agents in function of induced IL-1 α and IL-8 production patterns (Coquette et al., 1999, 2000b).

Thus, since IL-8 and IL-1 α differential production and release seemed to be appropriate parameters to screen chemicals for their sensitizing/irritating potential, we decided to undertake an *in vitro* study using RHE topically exposed to DNCB, nickel sulfate (NiSO₄), oxazolone (OXA), 2,4-dinitrofluorobenzene (DNFB) and trinitrobenzene-sulfonic acid (TNBS) as sensitizing agents and sodium lauryl sulfate (SLS), benzalkonium chloride (BC) and benzoic acid (BA) as irritant reference chemicals. For each compound, the dose-dependent release, intracellular protein content and mRNA levels of IL-1 α and IL-8 were determined whilst their respective cytotoxic effect was assessed by MTT assay in order to identify a predictive model.

2. Materials and methods

2.1. Chemicals

BC was purchased from Merck Eurolab (Overijse, Belgium). SLS, BA, NiSO₄, OXA, DNFB, TNBS and DNCB were obtained from Sigma Aldrich (Bornem, Belgium) at the highest purity available. SLS, BC, NiSO₄ and TNBS were diluted in defined culture medium (SkinEthic Laboratories, Nice, France).

DNFB, OXA and DNCB were first diluted in Dimethyl Sulfoxide (DMSO) and a subsequent progressive series of dilutions with culture medium was prepared (Final DMSO concentration : 0.04% v/v). BA was dissolved in acetone:olive oil (1:15) and then diluted in olive oil (final acetone concentration 1.8% v/v).

2.2. Tissue culture and treatment

The RHE, featuring a normal cornified layer (Rosdy and Clauss, 1990; Rosdy et al., 1993, 1996), were obtained by culturing normal human keratinocytes in a biochemically defined medium on polycarbonate culture inserts lifted to the air–liquid interface for 16 days (SkinEthic Laboratories, Nice, France). The 0.63 cm² epidermal tissues were shipped in 24 well plates on medium containing agarose gels. At their arrival, the inserts were removed and replaced onto 0.5 ml of defined maintenance medium. The RHE were incubated for 24 h at 37 °C (5% CO₂) before testing.

A volume of 0.5 ml of fresh medium was placed under each insert and 20 µl of the diluted reference substances were applied directly on the stratum corneum of the RHE. Twenty microliters of medium was applied as control. RHE were also treated with 20 µl of DMSO (0.04% v/v) or olive oil/acetone in order to evaluate their eventual cytotoxic effect and their potency to induce IL-1α/IL-8 synthesis and release. After an incubation of 20 h at 37 °C (5% CO₂), the medium underneath the tissues was collected from each well and stored at –20 °C for extracellular IL-1α and IL-8 analysis. The RHE were washed twice with 1 ml PBS pH 7.4 (Life Technologies, Merelbeke, Belgium) and placed into appropriately labelled wells either for MTT cytotoxicity assay, or intracellular IL-1α and IL-8 analysis and/or mRNA extraction.

2.3. MTT assay procedure

A volume of 0.5 ml of MTT solution (Sigma Aldrich, Bornem, Belgium) at 0.5 mg/ml in defined culture medium was added to all test wells containing tissue or blank and incubated for 30 min at 37 °C (5% CO₂) on a shaking platform. After incubation, the MTT solution was discarded. The RHE were washed twice with 1 ml PBS pH 7.4 (Life Technologies, Merelbeke, Belgium) at room temperature for 2 min and 1 ml of isopropanol (Merck, Overijse, Belgium) was added to each well. The extraction process was performed during 1 h at room temperature on a shaking platform. Aliquots (200 µl) of blue MTT extraction solution were transferred from each well to corresponding wells of a 96-well plate. The Optical Density (OD) at 540 nm was determined spectrophotometrically using a MIOS microplate reader (Merck, Overijse, Belgium). The mean OD₅₄₀ of the untreated control tissues exposed to

culture medium was set to represent 100% of viability and the results were expressed as percentage of these controls.

2.4. IL-1α and IL-8 measurements

The levels of extracellular IL-1α and IL-8 were determined in the subnatants by a quantitative sandwich immunoassay technique (Quantikine, R&D Systems, Abingdon, UK) with a detection limit of 0.5 pg/ml for IL-1α and 10 pg/ml for IL-8. Briefly, 200 µl of test sample or standard were pipetted into the appropriate pre-coated wells and incubated for 2 h at room temperature. After washing, 200 µl of IL-1α or IL-8 conjugate were added and the plates incubated at room temperature for a further 1 h. The incubation mixtures were removed and the wells washed three times, then 200 µl of TMB/H₂O₂ solution were added to each well. Following incubation for 20 min at room temperature, 50 µl 2 N H₂SO₄ were added to stop the reaction. The microplates were read at 450 nm versus a substrate blank within 30 min. The concentrations of IL-1α or IL-8 in the samples were calculated on the basis of the calibration curve. For both kinds of molecule, standard curves were run for each assay. Values from specific control cultures were subtracted from the tests. Potential interference with each ELISA by the test chemicals was evaluated by assaying known amounts of each cytokine in the presence of appropriate concentrations of the products. The levels of IL-1α and IL-8 in intracellular proteins isolated together with total mRNA using TRIzol extracts (Life Technologies, Merelbeke, Belgium) were determined by the same method. In all cases, duplicate measurements were performed for each individual replicate sample.

2.5. Cytokine-specific mRNA extraction and detection by RT-PCR

Total RNA was isolated using TRIzol extraction. The RT-PCR was performed using GeneAmp RNA PCR kit (Perkin Elmer, Branchburg, NJ, USA) with 0.4 µM of each oligonucleotide primer in a total volume of 25 µl. The reaction was performed in a Perkin Elmer 2400 cyclor for 35 cycles. Each cycle consisted of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. This number of cycles was chosen because reactions had not yet reached a plateau. The oligonucleotide primer sequences used were the following: sense 5'-ATGGTCAACCCACCGTGT-3' and antisense 5'-GTGCTCTCCTGAGCTACAGA-3' for Housekeeping gene 1B15/cyclophilin (556 bp); sense 5'-GTCTCTGAATCAGAAATCCTTCTATC-3' and antisense 5'-CATGTCAAATTTCACTGCTTCATCC-3' for IL-1α (420 bp) (Wang et al., 1989); and sense 5'-CTAGGACAA-

GAGCCAGGAAG-3' and antisense 5'-AGTGTGGT-CCACTCTCAATC-3' for IL-8 (237 bp) (Paludan and Thestrup-Pedersen, 1992).

Aliquots of 15 µl of product from each PCR reaction were analysed by horizontal electrophoresis on a 2% agarose gel and detected by Southern blot. cDNA probes for the cytokine and IB15 transcripts were radiolabeled with [α -³²P]dCTP (Amersham, Gent, Belgium), using the Random Primers DNA Labeling System (Life Technologies, Merelbeke, Belgium). After X-ray film exposure, density measurements were performed with the NIH Image software 1.6 (National Institute of Health, Bethesda, MD, USA). The level of expression of the cytokines was normalised to the expression of the housekeeping gene IB15/cyclophilin. The results were expressed as the difference between the ratios of cytokine mRNA/IB15 mRNA determined in experimental tissues and the same ratio determined in control samples.

2.6. Data treatment and statistical analysis

The relative toxicity of the tested chemicals was estimated by determining of the MTT₅₀ concentrations (concentration in mg/ml which reduces the MTT-formazan formation by 50%).

The comparison of the effects of the individual agents on the cytokine release, intracellular level, and mRNA transcription, were based on the MTT₅₀ concentration. Dose-response curves were plotted and the cytokine concentration induced by the product dose equivalent to the MTT₅₀ value was calculated from each plot.

Analysis of variance, Student *t*-test and Dunnett comparison test were performed with multiple comparisons when appropriate.

3. Results

3.1. Cell viability after sensitizer and irritant exposure

The effects of the chemicals were first characterized by investigating the dose-response relationship of cytotoxicity. In order to encompass the different biological reactions of RHE, the tissues were topically treated with a large range of product concentrations. The aim was to determine for each compound five doubling concentrations ranging from low to high cytotoxicity (from $\pm 90\%$ to $\pm 20\%$ of surviving cells—data not shown). The following concentrations were selected: NiSO₄ and OXA: 1, 2, 4, 8 and 16 mg/ml; TNBS: 3.13, 6.25, 12.5, 25 and 50 mg/ml; DNFB: 0.125, 0.25, 0.5, 1 and 2 mg/ml; BC, SLS and DNCB: 0.25, 0.5, 1, 2 and 4 mg/ml and BA: 7.5, 15, 30, 60 and 120 mg/ml.

The dose-dependent toxicity of each compound was measured by a MTT test after 20 h of tissue exposure.

In the conditions of our experiment, the difference between the vehicles-induced cytotoxicity and the culture medium-induced cytotoxicity was <5% assessing that they do not interfere significantly with this induced by the tested chemicals (data not shown).

Our data indicate that cytotoxicity increases with increasing concentrations but also that the chosen concentrations vary from non-cytotoxic to cytotoxic (Fig. 1A–H). For all chemicals, the average percentage of surviving cells varies from 91.7% at the lowest concentrations, and exhibits an almost linear dose-dependent decrease with increasing concentrations. At the highest concentrations, the percentage of surviving cells is 18.2% on average. The concentrations (mean $\pm$ S.D.) of the individual compounds which reduce MTT-formazan formation by 50% (MTT₅₀) are respectively 0.414 $\pm$ 0.092 mg/ml for DNFB, 1.24 $\pm$ 0.123 mg/ml for DNCB, 9.66 $\pm$ 0.537 mg/ml for OXA, 10.0 $\pm$ 0.365 mg/ml for TNBS, 14.7 $\pm$ 0.343 mg/ml for NiSO₄, 0.792 $\pm$ 0.109 mg/ml for BC, 1.62 $\pm$ 0.046 mg/ml for SLS and 36.6 $\pm$ 0.893 mg/ml for BA. When the MTT₅₀ values are ranked according to increasing concentrations i.e. decreasing cytotoxicity, DNFB is the most cytotoxic followed by BC, DNCB, SLS, OXA, TNBS, NiSO₄ and BA. Comparison of the MTT₅₀ values shows that they are significantly different except for OXA and TNBS ($P > 0.05$). Consequently, the tested chemicals can be ranked as follow with regard to their relative cytotoxic potency: DNFB > BC > DNCB > SLS > OXA = TNBS > NiSO₄ > BA or DNFB > DNCB > OXA = TNBS > NiSO₄ for the sensitizers and BC > SLS > BA for the irritants (Table 1).

3.2. Extracellular release of IL-1 α and IL-8

Fig. 1 presents the results of IL-1 α and IL-8 release assays after 20 h of RHE treatment with the tested chemicals. For the quantitative evaluation of release, the concentrations, minus the contribution due to vehicles, are expressed in pg/ml. No significant differences were observed between culture medium and culture medium containing DMSO or olive oil. IL-1 α and IL-8 release induced by the vehicles were 22 $\pm$ 3 and 21 $\pm$ 7 pg/ml, respectively.

With increasing doses of test chemical, cell viability decreases and the amount of released interleukin increases. The most interesting finding is the discrepancy observed between the cytotoxic potency of the irritants or the sensitizers and the amount and type of concomitantly released interleukins (Fig. 1).

When RHE are treated with the sensitizers, the release of both interleukins in the culture medium is enhanced, however only low levels of released IL-1 α are observed despite significant cytotoxicity (Fig. 1A–E). The maximum release of IL-1 α was between 6 and 35 pg/mL range. On the opposite, treatments with TNBS, DNFB

Fig. 1. Influence of increasing doses of NiSO₄ (A), TNBS (B), DNFB (C), OXA (D), DNCB (E), SLS (F), BA (G) and IL-8 (H) on the extracellular release of IL-1α (○) and IL-8 (Δ). The tissues were exposed to the different test products for 20 h at 37 °C (5% CO₂) at which time IL-1α and IL-8 in the medium underneath the tissues were assayed. Each value represents the mean ± S.D. of three independent experiments performed on three different batches of tissues. The viability is expressed as percentage of surviving cells compared to untreated control and the release of IL-1α and IL-8 is expressed in pg/mL of culture medium. (*) $P < 0.01$; (**) $P < 0.05$.

Table 1
Chemicals concentrations reducing MTT- conversion by 50% (MTT₅₀) and corresponding IL-1 α and IL-8 extracellular, intracellular and mRNA levels in the RHE^a

Product	Type ^c	MTT	IL-1 α			IL-8			Ratio IL-8/IL-1 α ^b		
		MTT ₅₀ (mg/ml)	Extra (pg/ml)	Intra (pg/ μ g)	mRNA	Extra (pg/ml)	Intra (pg/ μ g)	mRNA	Extra	Intra	mRNA
DNFB	S	0.414 (0.092)	11.1 (0.3)	539 (107.0)	0.351 (0.233)	208 (56.2)	1278 (66.9)	2.36 (0.161)	18.8	1.3	6.7
DNCB	S	1.24 (0.123)	28.0 (0.2)	386 (50.7)	0.149 (0.005)	236 (29.7)	725 (151.5)	0.782 (0.213)	8.4	3.3	5.2
OXA	S	9.66 (0.537)	29.3 (0.7)	188 (7.8)	1.32 (0.043)	257 (40.1)	1010 (80.0)	2.19 (0.051)	8.8	5.4	1.7
TNBS	S	10.0 (0.365)	13.0 (1.5)	58.8 (1.1)	0.404 (0.036)	164 (5.6)	149 (68.3)	1.10 (0.039)	12.6	2.5	2.7
NiSO ₄	S	14.7 (0.343)	12.1 (0.4)	119 (1.5)	0.990 (0.002)	495 (6.2)	839 (36.8)	0.237 (0.009)	41.0	7.1	0.2
BC	I	0.792 (0.109)	134 (15.1)	711 (21.5)	0.774 (0.311)	32.9 (0.5)	180 (12.1)	0.430 (0.059)	0.2	0.3	0.6
SLS	I	1.62 (0.046)	236 (6.2)	715 (58.9)	1.14 (0.017)	168 (2.3)	99.7 (14.2)	0.418 (0.015)	0.7	0.1	0.4
BA	I	36.6 (0.893)	46.5 (0.4)	158 (0.9)	0.357 (0.007)	21.0 (0.4)	11.4 (1.2)	0.311 (0.010)	0.5	0.1	0.9

^a Each value represents the mean $\pm$ (S.D.) of three independent experiments performed on three different batches of tissues.

^b Each ratio was calculated on the basis of the overall average concentrations obtained in three independent experiments performed on three different batches of tissues.

^c S: sensitizer; I: irritant.

and OXA induces a sharp maximum of IL-8 release at low concentrations, followed at higher, more cytotoxic, concentrations by a drop to basal levels (Fig. 1B–D). For NiSO₄ and DNCB, the release of IL-8 exhibits an almost linear dose-dependent increase, and reaches a plateau at higher concentrations (Fig. 1A and E).

When the RHE are treated with irritants, the release of both interleukins is also enhanced. However, the relative profile of IL-1 α and IL-8 release is inverted compared to sensitizers, despite the fact that irritants produce dose-dependent cytotoxicity profiles similar to those produced by the sensitizers (Fig. 1F–H). At doses which significantly alter the cell viability, the extracellular IL-1 α exhibits an almost linear increase and reaches a plateau at the highest cytotoxic doses (Fig. 1F–H). The IL-1 α concentrations are 1.3–2.5-fold higher than IL-8 on average. On the opposite, the release of IL-8 is less increased by the treatment with irritants. BA, SLS and BC treatments induce a maximum of released IL-8 within a low dose range, then a drop to basal levels of IL-8 release is observed at higher cytotoxic concentrations (Fig. 1F–H).

At doses of sensitizers corresponding to the MTT₅₀ values, the release of IL-8 is 8–41-fold higher than the release of IL-1 α whereas the release of IL-8 induced by irritants is 2–4-fold lower than the release of IL-1 α (Table 1). Moreover, no apparent relationship is found between the MTT₅₀ values of sensitizers and the corresponding amounts of IL-8 or IL-1 α released (i.e. a low MTT₅₀ value does not imply a high release of IL-8 or IL-1 α). On the other hand, we observe a relationship between the MTT₅₀ values and the maximum amount of

IL-1 α released following treatment with the tested irritants (i.e. a low MTT₅₀ value implies a high release of IL-1 α). In summary, our data strongly suggest that the release of IL-8 could be linked to the sensitizing potency of NiSO₄, DNCB, DNFB, OXA and TNBS, whereas a good relationship between cytotoxicity and the release of IL-1 α (with IL-1 α > IL-8) is a characteristic of BA, SLS and BC (Table 1). Both observations suggest that the amount and type of released cytokine strongly depend on the nature of the compound tested, either irritant or sensitizer.

3.3. Intracellular levels IL-1 α and IL-8

Considering that both interleukins can be sequestered in keratinocytes before passive or active release, we have measured the intracellular IL-1 α and IL-8 levels. Fig. 2 presents the dose-dependent variations of the intracellular IL-1 α and IL-8 levels after 20 h of RHE treatment with the tested chemicals. The concentrations, minus the contribution due to the vehicles, are expressed in pg/ μ g of total cellular protein. As for the measure of the extracellular release, no significant differences were observed between the vehicles. The cytokine level induced by the vehicles alone was 45 ± 6 pg/ μ g for IL-1 α and 96 ± 20 pg/ μ g for IL-8.

As for the extracellular release, the amounts of intracellular interleukins are dependent on the type of chemicals tested and their concentration. At their lowest concentrations, none of the chemicals induced any increase in intracellular IL-1 α and IL-8 levels (Fig. 2A–H).

Fig. 2. Influence of increasing doses of NiSO₄ (A), TNBS (B), DNFB (C), OXA (D), DNCB (E), BC (F), SLS (G) and BA (H) on the intracellular levels (pg/μg of total cellular proteins) and mRNA expression (ratio of mRNA) of IL-1α and IL-8 in the RHE. The tissues were exposed to the different concentrations of the tested products for 20 h at 37 °C (5% CO₂) at which time total proteins or specific mRNAs were extracted and quantified by ELISA or by RT-PCR. Each value represents the mean ± S.D. of three independent experiments performed on three different batches of tissues. For cytokine intracellular levels, the results are expressed in pg/μg of total protein. For mRNA expression, the results are expressed as the difference between the ratios of cytokine mRNA/18S mRNA determined in treated samples and the same ratio determined in untreated samples (***) P < 0.001; (*) P < 0.05.

Upon sensitizers application, the intracellular IL-8 and IL-1 α levels are both enhanced. In good correlation with the extracellular release, the intracellular IL-1 α levels are lower than the IL-8 levels and amount a maximum of approximately 113–675 pg/ μ g of total protein. The dose-dependent profiles increase slowly and reaches rapidly a plateau (Fig. 2A–E). However, except for DNCB and DNFB, this increase must be considered as non significant when compared with the untreated tissues ($P > 0.05$). These results parallel the extracellular release profiles and suggest that the sensitizers do not induce IL-1 α neo-synthesis. Conversely, the cell-associated IL-8 is 1.3–7.1-fold higher than the intracellular IL-1 α at doses corresponding to the MTT₅₀ concentration (Table 1). For NiSO₄, OXA and DNCB, the intracellular level of IL-8 exhibits a dose-dependent increase similar to the respective extracellular release profiles (Fig. 2A, D and E).

The irritants BC and BA induce an increase of the intracellular level of both cytokines, but the IL-1 α level is more enhanced than the level of IL-8 (Fig. 2F–H). In the presence of BC and BA, the cell-associated IL-1 α is dramatically enhanced, according to the dose applied, with a maximum of 1850 pg/ μ g of total protein on average. However, IL-8 and IL-1 α levels are independently enhanced upon SLS application: IL-1 α increases at low doses to a maximum of 715 $\pm$ 59 pg/ μ g, and decreases when higher concentrations of SLS are applied, opposite to the intracellular IL-8 profile (Fig. 2G).

In correlation with our previous observations, no direct correlation is found between the MTT₅₀ values of the tested sensitizers and the corresponding amount of intracellular IL-8 or IL-1 α . On the opposite, for the tested irritants, we observe a correlation between the MTT₅₀ values and the intracellular IL-1 α level (Table 1).

3.4. Cytokine message profiles

In order to investigate the possible control exerted by the tested products on the IL-8 and IL-1 α gene transcription in the RHE, semi-quantitative RT-PCR was performed. Fig. 2 also shows the expression of IL-1 α and IL-8 transcripts.

Upon DNFB, DNCB, OXA and TNBS application, IL-1 α and IL-8 mRNA are both upregulated. At doses corresponding to the MTT₅₀ concentration, IL-8 mRNA transcript is 1.7–6.7-fold higher than the synthesis of cytokine-specific IL-1 α mRNA transcript (Table 1), suggesting a feature that paralleled the increase in extracellular protein levels (Fig. 2A–E). Upon SLS, BC and BA treatment, both IL-1 α and IL-8 mRNA are upregulated, but IL-1 α mRNA upregulation is more pronounced than that of IL-8 mRNA (Table 1).

Overall comparison of the results shows that protein expression does not necessarily correlate with mRNA

upregulation. Indeed, at concentrations equivalent to the MTT₅₀ values, OXA and NiSO₄, for example, induce a IL-1 α mRNA upregulation which is equal or higher than what observed with BC or SLS. This upregulation parallels an extracellular IL-1 α concentration of 20 pg/ml on average upon OXA or NiSO₄ application, whereas it is accompanied by 134 and 236 pg/ml extracellular IL-1 α after BC or SLS treatment (Table 1). Therefore, although IL-1 α mRNA and IL-8 mRNA upregulation is observed after topical application of both sensitizers and irritants, the latter significantly increased extracellular IL-1 α , whereas the sensitizers do not suggesting that high amounts of IL-1 α and IL-8 mRNAs do not correlate with protein transcription and extracellular release.

4. Discussion

NiSO₄, DNCB, DNFB, OXA and TNBS are strong sensitizers (Van Och et al., 2000; Coutant et al., 1999) but also known irritants (Müller-Decker et al., 1994; Lisby et al., 1995; Wilmer et al., 1994) while BC, SLS and BA are non-sensitizing strong and mild-moderate irritants (Müller-Decker et al., 1994; Wilmer et al., 1994). If we consider that RHE release high levels of IL-8 levels upon topical application of sensitizers while irritants induce the inverse profile, our results correlate well with the cutaneous toxicological characteristics of all the tested chemicals. But if we consider IL-1 α as an irritancy parameter, as suggested by others, the correlation is less pronounced (Gay et al., 1992; Corsini and Galli, 1997). Nevertheless, we show that in RHE a given pattern of cytokines production is chemical- and dose-dependent.

In the present experiments, no direct inverse correlation is found between the MTT₅₀ values of sensitizers and the amount of IL-8 or IL-1 α released (i.e. a low MTT₅₀ value does not imply a high release of IL-8 or IL-1 α). On the opposite, for the irritants, we observe an inverse correlation between the MTT₅₀ values and the maximum amount of IL-1 α released (i.e. a low MTT₅₀ value implies a high release of IL-1 α). Consequently, the suggestion that in vitro IL-1 α release and tissue viability are good parameters to assess irritancy must be qualified (Gay et al., 1992; Corsini and Galli, 1997). In fact, our results show that IL-1 α synthesis/release is a valuable in vitro parameter to identify and classify irritants but not to assess the irritant potency of sensitizers. In order to identify and classify sensitizers, our results seem to indicate that the associated determination of IL-8, with IL-1 α , and MTT conversion are at least necessary. For the first time, the data collected allow us to propose a prediction model which discriminates in vitro and in a single assay the two types of substances: we could consider as skin allergens those chemicals

where extracellular IL-8 > IL-1 α and as skin irritant those chemicals where extracellular IL-8 $\leq$ IL-1 α , and we could exclude as potential allergen or irritant those chemicals which fail to induce changes in IL-8 and IL-1 α production. Both parameters must be coupled to the corresponding dose-dependent viability data in order to determine the irritant potency of the tested sensitizers.

We have observed a lack of IL-1 α protein neo-synthesis and release when RHE are treated with sensitizers. IL-1 α plays a central role in initiating inflammatory response (Williams and Kupper, 1996) and is considered as a potent inducer of IL-8, important for controlling the recruitment of leukocytes in inflammatory skin disorders (Schröder, 1995). Surprisingly, the sensitizers induce essentially high IL-8 release while IL-1 α release is provoked when RHE are treated with irritants. Those observations are in accordance with results showing that in the presence of SLS, DNFB or 5-methyl-3-n-pentadecylcatechol (PDC), human keratinocytes synthesize 3–4-fold more IL-8 in the presence of PDC or DNFB than when treated with SLS (Mohamadzadeh et al., 1994). This could be considered as logical, since in allergic contact dermatitis, antigen presentation is pivotal. Thus by increasing IL-8 release, keratinocytes have a more generalized and functional role in promoting and facilitating leukocytes traffic into the epidermis. This is in accordance with *in vivo* observations showing that, on the one hand, BC induces epidermal necrosis without leukocyte migration into the epidermis, but on the other hand, leukocyte migration directly happens when cutaneous damages are induced by OXA (Wilmer et al., 1994).

Upon sensitizers application, the increase of intracellular IL-1 α levels can be considered as non significant, suggesting that sensitizers fail to induce IL-1 α neo-synthesis. In parallel, although IL-1 α and IL-8 mRNA are both upregulated, the synthesis of specific IL-8 mRNA is more increased than IL-1 α mRNA, indicating that a correlation exists between the level of IL-8 mRNA upregulation and the level of IL-8 protein production. Conversely, this correlation is not true for IL-1 α . Indeed, although IL-1 α mRNA upregulation is observed after topical application of both sensitizers and irritants, only the latter significantly induce an increase in extracellular IL-1 α . This is again in accordance with *in vivo* data showing that allergic contact dermatitis is characterized by a 2-fold increase in IL-8 mRNA when compared to contact dermatitis reactions provoked by an irritant (Morhenn et al., 1999).

A tendency to suppress IL-1 α gene expression was observed when normal human keratinocytes were treated with DNBS (Pastore et al., 1995). Recently, we demonstrated that the topical application of a sensitizer to an irritant-treated RHE decreased the release of IL-1 α (Coquette et al., 2000a). It is noteworthy that, in our system, the suppressive role exerted by the sensitizers on

IL-1 α gene expression could be due to cytotoxicity/corrosivity. However, such an effect is not observed with the irritants at similar cytotoxic concentrations. Finally, the expression level of the housekeeping gene 1B15 is not affected by the sensitizers in the tested range of concentrations (data not shown). In the case of the sensitizers, the correlation between IL-8 mRNA and protein levels suggests a regulation at a transcriptional level, while in the case of IL-1 α the regulation could take place at the posttranslational level. Intracellular signaling pathways of the keratinocyte are only beginning to be identified, assembled and integrated into an intricate stimulus-response network that mediates irritant and sensitizer reactions in skin. This network has the potential to either upregulate or downregulate a specific cutaneous response. IL-8 synthesis can result from the activation by a nuclear factor whose activity would be regulated by protein kinase C (PKC), of a responsive enhancer in the 5'-flanking region of the IL-8 gene (Chabot-Fletcher et al., 1994). In the case of the sensitizers, this nuclear factor would be the nuclear factor κ B (Goebeler et al., 2001). In contrast, NiSO₄ upregulates TNF α mRNA by a PKC-independent mechanism which does not increase TNF α promoter activity, but markedly increases the stability of the TNF α mRNA (Lisby et al., 1995). By analogy, this could be the same for IL-1 α . In fact we have demonstrated that the extracellular and intracellular levels of BC and BA-induced IL-1 α are decreased in the presence of sensitizers while mRNA expression of IL-1 α is not influenced by the presence of the sensitizers. On the opposite IL-8 protein and mRNA are upregulated (Coquette et al., 2000b).

In conclusion, our results show that divergent *in vitro* IL-1 α and IL-8 release profiles characterize the RHE response to irritants and sensitizers. For the first time, we demonstrate that the combination of dose-dependent cell viability measurements, with IL-1 α and IL-8 quantification, can provide enough information to allow, in a single assay, *in vitro* detection and discrimination of irritant and sensitizing agents. Moreover, our results indicate that in irritation/sensitization *in vitro* studies, the investigation of the balanced immune response and not the induction of a particular cytokine is a relevant concept. As a potential alternative to classical *in vivo* assays, when validated, this procedure will be much less time and cost consuming than usual *in vivo* procedures. The RHE used in our study recreates many of the structural and functional features of the epidermis, but is exclusively composed of keratinocytes. For example, Langerhans cells which play a critical role in cutaneous immune response and cytokine production are absent from the RHE (Srivasta et al., 1994; Cumberbatch et al., 1996). Moreover, the absence of blood-derived and resident leukocytes reduces the complexity of the observable cytokine network. The cytokine responses

documented here are only the potential contribution of the keratinocytes and thus represent a limited subset of the cutaneous immune response. Nevertheless, they are the predominant cell type in human skin and keratinocytes are the first cells with which test products are in contact during the initial stages of irritation and/or sensitization. Whether these initial and early molecular responses of the keratinocytes are sufficient to detect and discriminate all types of compounds which are sensitising and/or irritating to skin remains to be determined in further studies.

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Chapter 4

Assessment of the human epidermis model SkinEthic RHE for in vitro skin corrosion testing of chemicals according to new OECD TG 431.

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Abstract

Based on two successfully completed ECVAM validation studies for *in vitro* skin corrosion testing of chemicals (Fentem et al., 1998; Liebsch et al., 2000), the National Coordinators of OECD Test Guideline Programme endorsed in 2002 two new test guidelines: TG 430 'Transcutaneous Electrical Resistance assay' and TG 431 'Human Skin Model Test'.

To allow all suitable *in vitro* human reconstructed (dermal or epidermal) models to be used for skin corrosion testing, the OECD TG 431 defines general and functional conditions that the model must meet before it will be routinely used for skin corrosion testing. In addition, the guideline requires correct prediction of 12 reference chemicals and assessment of intra- and inter-laboratory variability.

To show that the OECD TG 431 concept works, in 2003 ZEBET tested several chemicals from the ECVAM validation trials on the SkinEthic™ reconstituted human epidermal (RHE) model. Based on knowledge that reconstructed human skin models perform similarly in toxicological studies, it was decided to adopt the validated EpiDerm™ skin corrosion test protocol and prediction model to the SkinEthic model. After minor technical changes, classifications were obtained in concordance with those reported for the validated human skin models EPISKIN™ and EpiDerm™. To allow adequate determination of inter-laboratory reproducibility, a blind trial was conducted in three laboratories - ZEBET (D), Safepharm (UK) and BASF (D), in which the 12 endorsed reference chemicals were tested.

Results obtained with the SkinEthic™ epidermal model were reproducible, both within and between laboratories, and over time. Concordance between the *in vitro* predictions of skin corrosivity potential obtained with the SkinEthic™ model and the predictions obtained with the accepted tests of OECD TG 430 and TG 431 was very good. The new test was able to distinguish between corrosive and non-corrosive reference chemicals with an accuracy of 93 %.

Keywords:

skin corrosion, OECD TG 431, SkinEthic™ RHE, alternative methods, validation, common protocol

Abbreviations:

C, corrosive; CI, confidence interval; BfR, Bundesinstitut für Risikobewertung (Federal Institute for Risk Assessment); ECETOC, European Centre for Ecotoxicology and Toxicology of Chemicals; ECVAM, European Centre for the Validation of Alternative Methods; EU, European Union; GHS, globally harmonised system for classification and labelling; MT, Management Team; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NC, non-corrosive; PM prediction model; R.G., reagent grade; SD, standard deviation; SDS, sodium dodecyl sulphate; ZEBET, Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch (National Centre for the Documentation and Evaluation of Alternative Methods to Animal Experiments)

1 Introduction

Skin corrosion is defined as the production of irreversible tissue damage in the skin following the application of a test material (OECD, 2002). According to current international regulatory requirement, assessment of skin corrosion is mandatory for all chemicals placed on the market.

Skin corrosivity testing *in vivo* may cause severe discomfort and pain to test animals. Therefore many attempts have been made to replace the *in vivo* test in laboratory animals. Several alternative methods were evaluated from 1996 - 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 of the electrical resistance in the *ex-vivo* rat skin) and the EPISKIN assay (using the reconstructed human epidermal model EPISKIN, EPISKIN SNC, France) (Fentem et al., 1998).

Later, another reconstructed human epidermal model - EpiDerm (produced by MatTek Corporation, MA, USA) was successfully validated in an ECVAM "catch-up" validation study performed during 1999 and 2000 (Liebsch et al. 2000). All three methods were finally implemented into the EU Methods for testing of dangerous substances and OECD Test Guidelines (TG) as TG 430 (TER assay) and TG 431 (human skin model test).

To allow other skin models to be used for the skin corrosion testing, the OECD TG 431 defines *general* and *functional* model conditions that need to be evaluated before the skin models will be routinely used. The most important *general conditions* are a multi-layered, functional stratum corneum with the necessary lipid profile, and absence of any contamination. The most important *functional conditions* specified in TG 431 are a stable and sufficiently high cell viability (expressed as metabolic conversion capacity), a sufficient resistance to a slowly penetrating cytotoxic marker chemical, reproducibility of data over time and between laboratories, and finally, capability to correctly classify twelve reference chemicals specified in TG 431 (OECD, 2004).

For the skin models SkinEthic, EPISKIN and EpiDerm the validity of the endpoint "metabolic conversion" (MTT reduction) has been demonstrated by Boelsma et al. (2000), Faller et al. (2002a, 2002b), and the tissue histology, ultrastructure, lipid composition and lipid organisation by Poncet et al. (Poncet et al 2000a, 2000b, 2001 and 2002). Therefore, in the current study, the SkinEthic model was regarded sufficiently well characterised and comparable to the models EpiDerm and EPISKIN validated for TG 431. Therefore, as a last step, it was necessary to evaluate if the SkinEthic RHE model will be able to correctly predict 12 OECD reference chemicals.

Based on knowledge that reconstructed human skin models perform similarly in toxicological studies (e.g. Perkins et al., 1996; Liebsch et al., 1997; Liebsch et al., 2000;

Kandarova et al., 2005; Cotovio et al., 2005) we have decided to adapt the EpiDerm skin corrosion protocol (and its prediction model) for the SkinEthic model.

The first phase of the study (screening of the model performance and its predictive ability with EpiDerm protocol) was performed at ZEBET in 2003. For this purpose chemicals were selected with specific properties included in previous ECVAM validation studies (Barrat et al. 1998; Fentem et al., 1998; Liebsch et al., 2000). After minimal optimisation of the protocol results obtained at ZEBET with SkinEthic RHE were comparable to published results of EpiDerm and EPISKIN models. Therefore, we concluded that SkinEthic model can be further evaluated to allow adequate determination of intra- and inter-laboratory variability.

Three laboratories - BASF AG, Germany; SafePharm Laboratories, UK and ZEBET at the BfR, Germany - decided to participate in an inter-laboratory study. Although not required by TG431, the study was conducted with coded test materials to increase credibility of the data. Predictivity and reliability of the new test method were evaluated by an independent biostatistician.

2 Material and methods

2.1 Skin model

The SkinEthic reconstituted human epidermis (RHE) model consists of normal, human epidermal keratinocytes cultured to form a multi-layer, well differentiated model of the human epidermis *in vitro*. SkinEthic RHE is histologically similar to *in vivo* human epidermis, and features a functional permeability barrier, which is one of the main functions of viable skin.

The epidermal model used in this study was fully differentiated three-dimensional reconstituted human epidermal cultures grown on the air-liquid interface for 17 days (surface 0.63 cm²; biological origins: foreskin; age of donors: usually 1-4 years or abdomen; age of donors: adult). The model is produced in polycarbonate filter inserts in a serum-free, chemically defined medium (Rosdy and Clauss, 1990; Rosdy et al., 1993).

The inserts (containing the SkinEthic human tissue models) are embedded in an agarose gel containing nutrient solution and shipped in multi-well plates at room temperature. Maintenance and growth medium are cooled with gel refrigerant during the shipment.

Each epidermal batch is controlled by the manufacturer. Information on histology (hematoxylin-eosin staining) and viability of tissues (MTT-test) is provided for each batch as a standard quality control. Optical density of formazan produced in the MTT assay must be higher than 0.8 and histology sections have to show a minimum of 4 healthy viable cell-layers under the stratum corneum. Tissues as well as culture media are tested for viral, bacterial, fungal and mycoplasma contamination. All information are archived to allow the retrospective controls.

In addition to quality controls performed by manufacturer, the *in vitro* skin corrosion assay defines an acceptance criteria and variability range, which have to be met for each run (see section 2.4.4 - Test protocol)

2.2 Test chemicals

The main objective of phase I was to screen performance of the SkinEthic model (correct prediction, inter-tissue variability) as well as the applicability of the assay to a diverse groups of chemicals before considering any inter-laboratory testing.

In this early phase only a limited number of tests was performed (one to four runs) with twenty non-coded chemicals selected from the ECVAM skin corrosion validation studies (Fentem et al., 1998, Liebsch et al. 2000) (see **Table 1**). Chemicals were selected to challenge the test protocol thus allowing to assess tissue quality with regard to barrier function.

The aim of Phase II was to evaluate the test performance of the SkinEthic skin corrosivity test with regard to its predictivity, reproducibility, intra- and interlaboratory reproducibility. The OECD TG 431 requires for this purpose to test 12 reference substances (see **Table 2**) which cover different chemical classes as well as a wide range of the corrosive potential. Although OECD TG 431 does not require testing under blind conditions, to increase the scientific reliability of the study we have decided to test the samples coded.

All test chemicals were purchased by ZEBET, coding and distribution was performed independently by the BfR biostatistics unit. From coding was excluded sulphuric acid 10%. It was decided, that this chemical will be tested in addition to 1h exposure also with 4-h exposure to evaluate, if the result from Phase I (false negative) can be improved by change of prediction model that would be than applied only for low concentrated solutions of inorganic acids.

2.3 Prediction model (PM) and Biostatistics

The *in vitro* skin corrosion test is based on the experience that skin corrosion (necrotic damage of viable skin cells) shows a high correlation with skin cell cytotoxicity induced by brief exposure of the skin barrier (stratum corneum) to a corrosive chemical (Perkins et al., 1996).

In this study, the EpiDerm test protocol and PM (Liebsch et al., 2000.) were directly applied to SkinEthic RHE. The corrosivity potential of the test material was predicted from the mean tissue viabilities obtained after 3 min and 1 hour treatments, and compared to the negative control tissues concurrently treated with H₂O. A chemical was classified as "corrosive" if the relative tissue viability was decreased below 50 % after 3 min of exposure to a test material. In addition, test materials classified "non corrosive" after 3 min (viability $\geq$ 50%) were classified "corrosive" if the relative tissue viability was decreased below 15 % after exposure for 1 hour.

Table 1: Chemicals tested in Phase I

No.	Chemical name	CAS No:	In vivo Class (C/NC)	Solid/ liquid	Supplier	Chemical group	Interaction with MTT (+ / -)
1	SLS (20 % aq)	151-21-3	NC	L	Aldrich	Surfactants/soaps	-
2	Boron trifluoride dihydrate	13319-75-0	C	L	Aldrich	Inorganic acids	+
3	Benzylacetone	2550-26-7	NC	L	Aldrich	Neutral organics	-
4	H ₂ SO ₄ (10 % wt.)	7664-93-9	C*	L	Aldrich	Inorganic acids	-
5	HCl (14,4 % wt.)	7647-01-0	C*	L	Aldrich	Inorganic acids	-
6	n-Heptylamine	111-68-2	C	L	Aldrich	Organic bases	+
7	2,4-Xylidine	95-68-1	NC	L	Aldrich	Organic bases	-
8	Tetrachloroethylene	127-18-4	NC	L	Aldrich	Neutral organics	-
9	Octanoic acid (caprylic acid)	124-07-02	C	L	Aldrich	Organic acids	-
10	55/45 Octanoic/decanoic acid	68937-75-7	C	L	Unichema	Organic acids	-
11	4-Amino-1,2,4-triazole	584-13-4	NC	S	Aldrich	Organic bases	-
12	2-tert-Butylphenol	88-18-6	C	L	Aldrich	Phenols	+
13	1,2- Diaminopropane	78-90-0	C	L	Aldrich	Organic bases	+
14	Phenethyl bromide	103-63-9	NC	L	Aldrich	Electrophiles	+
15	4-(Methylthio)-benzaldehyde	3446-89-7	NC	L	Aldrich	Electrophiles	+
16	Potassium hydroxide 10% aq.	1310-58-3	C	L	Aldrich	Inorganic bases	-
17	Isostearic acid	30399-84-9	NC	L	Unichema	Organic acids	-
18	Acrylic acid	79-10-7	C	L	Acros organics	Organic acid	-
19	1,6-dibromohexane	97-53-0	NC	L	Aldrich	Brominated	+
20	8N KOH	1310-58-3	C	L	Aldrich	Inorganic bases	+

C - corrosive; NC - non corrosive; S - solid; L - liquid

C* - classifications given in OECD Test Guide 431 and Fentem et al. don't agree with classification according to 67/548/EEC Annex I

Table 2: OECD reference chemicals - Phase II

No	Chemical name	CAS No:	In vivo Class (C/NC)	Remarks on data supporting classification / general comments
1.	1,2-Diaminopropane	78-90-0	C*	Interaction with MTT was observed.
2.	Acrylic Acid	79-10-7	C*	Published data with EpiDerm or EPISKIN model missing (corrosive on EpiDerm after 3 min at ZEBET - unpublished experiment).
3.	2-tert. Butylphenol	88-18-6	C	Borderline C/NC chemical, as judged from the proximity of the chemical to the classification boundary (SAR analysis) (Barratt et al., 1998) Interaction with MTT.
4.	Potassuim hydroxide (10% aq)	1310-58-3	C	C, but supporting data do not enable unequivocal classification as either R34 (II/III) or R 35 (I); more probable to be R 34 (II/III) (Barratt et al., 1998).
5.	Octanoic acid (caprylic acid)	124-07-02	C	Borderline C/NC chemical, as judged from the proximity of the chemical to the classification boundary (SAR analysis) (Barratt et al., 1998).
6.	Sulfuric acid (10% wt.)	7664-93-9	C	According to the classification mentioned in OECD Guideline, the chemical is classified as corrosive. According to Annex I of the Directive 67/548/EEC in range of concentration 5 -15 % the chemical is classified as irritant.
7.	4-Amino-1,2,4-triazole	584-13-4	NC	Non irritant.
8.	Eugenol	97-53-0	NC	Borderline NC/C chemical, as judged from the proximity of the chemical to the classification boundary (SAR analysis) (Barratt et al., 1998). Interaction with MTT.
9.	Phenethyl bromide	103-63-9	NC	Interaction with MTT.
10.	Tetrachloroethylene	127-18-4	NC	Classified as C in one of three EPISKIN laboratories in the validation study (Fentem et al., 1998). Very high scores for irritation <i>in vivo</i> in rabbits (ECETOC, 1995)
11.	Isostearic acid	30399-84-9	NC	Non irritant.
12.	4-(Methylthio)-benzaldehyde	3446-89-7	NC	Interaction with MTT.

C* - severely corrosive; C - corrosive; NC - Non corrosive

Standard statistical methods were used to assess the predictive value of the test in the phase I. In phase II, the concordance between laboratories and the predictive value of the assay were determined. Data obtained in phase II for 12 reference chemicals tested in three laboratories (each three times on separate occasions) were analysed by the following methods:

2.3.1 Assessment of inter- laboratory variability and systematic differences

The GLM (General Linear Model) multivariate procedure was used for analysis of intra- and inter- laboratory variances. The GLM Multivariate procedure is based on the general linear model, which provides a unifying framework for well-known approaches such as multiple regression and analysis of variance, and which also enriches the variety of models that may be routinely fitted to data. (Everitt et al., 2001, Landau S. et. al.).

The GLM multivariate procedure operates with the terms "factor" and "variable". All "factors" are usually determined by the assay design and are fixed. In our study the factors are "chemicals", "laboratories" and "runs". Inter-laboratory variability is represented by the factor "laboratories", and the factor "runs" is an indicator of intra-laboratory variability.

It is assumed that factors have linear relationships to the dependent variables (here, 3 minutes and 1 hour exposure times) and each factor can have a different effect on the value of the dependent variables. The contribution of all three factors to the assay variability was calculated and expressed by significance values. Effects with an error probability value smaller than 0.05 were assumed to be significant, and values below 0.001 were regarded as highly significant. The relative mean square values were calculated for both time-points as well, since this is a more straightforward way to assess the percentile contribution of each factor to the variability of the assay (Everitt et al., 2001, Landau S. et. al., 2003).

2.3.2 Evaluation of the prediction model (PM) adopted from the EpiDerm skin corrosion protocol

Evaluation of the PM was conducted by classifying each test chemical three times per laboratory by using the three single tests as predictor variables. The predicted classifications were presented with the known *in vivo* corrosivity classes in summarising tables for each laboratory. The degree of concordance between predicted and observed corrosivity classifications was evaluated using the 2 x 2 contingency tables, by calculating sensitivity, specificity, positive predictivity, negative predictivity, accuracy and significance.

2.4 Special technical steps and test protocol

2.4.1 Application technique for liquids

During adaptation of the EpiDerm protocol to the SkinEthic model, it was observed that spreading of hydrophilic liquids is difficult due to high surface tension. In the first experiments, this technical problem resulted in variability and under-predictions (unpublished data). Therefore, we decided to use the "mesh application technique" for the spreading of all liquids in a similar manner as described for the EpiDerm model (Kandarova et al., 2004). In this technique the chemical is directly applied on the tissue and a nylon mesh (8 mm diameter) is placed on the top. This technique helps to keep the chemical evenly distributed on the surface during the time of exposure (Kandarova et al., 2004).

2.4.2 Application technique for solids

Solids were ground with a mortar and pestle, and the powder was applied with a 25 mg calibrated spoon (Aesculap, #623) on the tissue surface, to give a constant bulk volume of about 25 ± 3 mg. 25 μ l sterile H₂O was then added to wet the test material. If necessary, the applied material was spread to match the size of the tissue.

The "spoon application technique" used in this study was already successfully used in the ECVAM skin corrosion study (Liebsch et al., 2000) with the EpiDerm model. In addition, in the routine (*in vivo*) toxicological praxis is the application of solids performed similarly.

2.4.3 Evaluation of the MTT conversion caused by chemicals bounded into the tissue

Initially, the ability of a test substance to directly reduce MTT (yellow stain which is used for determination of viability of cells/tissues (Mossmann, 1983)) was assessed. The test material was added into a 1.0 mg/ml MTT solution in Dulbecco's Modified Eagle Medium (DMEM). For liquids 50 μ l, and for solids 25 mg, of the test materials were added to the MTT solution (1 ml) and the mixtures were incubated in the dark at 37°C for 60 minutes. If the MTT solution turned to blue/purple, it was assumed the test chemical had reduced the MTT. However, only those test materials that remain bound to the tissue after rinsing present a problem, resulting in a false MTT reduction. To evaluate if residual test material was binding to the tissue, a functional check using freeze-killed control tissues was performed (for details see Liebsch et al., 2000).

Freeze-killed control tissues were prepared by a process of two times freezing (-20°) and thaw of viable tissues of the batch used in concurrent skin corrosion experiment. Two tissues per each time-point were exposed to 50 μ l (liquids) or 25 mg (solids) of MTT reducing test chemical. In case that the chemical remained bounded in the tissue, blue formazan product was formed. Consequently, the optical density of the extracted formazan was measured and the mean value was subtracted from the mean value obtained with viable tissues treated with the

same chemical. This procedure was used also in the EpiDerm skin corrosion assay (Liebsch et al., 2000) for n-Heptylamine, which is strongly reducing chemical. In our assay, we evaluated this procedure for Eugenol which seems to be able significantly reduce the MTT.

2.4.4 Test protocol

The test consists of topical exposure (3 min /1 hour) of the test material to the SkinEthic tissues followed by immediate determination of the cytotoxic effect. Cytotoxicity is assessed in the MTT assay (Mossmann, 1983).

SkinEthic RHE (0.63 cm²) tissues were shipped on Mondays and arrived at the laboratories usually on Tuesdays. The skin corrosion test was performed usually on Tuesdays or Wednesdays. Upon receipt, tissues were kept at room temperature (18-22°C) on the shipping agarose until use. On the day of testing, tissues were removed from transport agarose and conditioned by a 3 hrs pre-incubation in 1 ml assay medium (37°C, 5% CO₂, saturated humidity) in 6-well plates, to allow release of metabolites, cytokines and cellular debris accumulated during the shipment. After replacement of the medium, 50 µl (liquids and semi solids) or 25 mg (solids) of the neat test chemical were applied topically on 3 replicate tissues per chemical. Three tissues were dosed with 50 µl of H₂O (ultra-pure or distilled), serving as negative control, and 3 tissues were dosed with 50 µl 8N potassium hydroxide, serving as positive control.

Tissue dosing was performed consecutively at one minute intervals, which was the time needed to rinse off chemicals. After exposure tissues were carefully rinsed in a soft stream of PBS from a washing bottle, by filling and emptying the culture insert 20 times. Washed and blotted inserts were collected in a 24-well "holding plate" pre-filled with 0,3 ml of maintenance medium. Thereafter the inserts were transferred into a new 24-well plate containing 0.3 ml freshly prepared MTT medium (1 mg MTT/ml assay medium) and incubated for three hours at 37°C, 5% CO₂ and saturated humidity.

After three hours all tissues were rinsed with PBS, gently blotted and transferred to a new 24-well plate. 2 ml of formazan solvent (isopropanol, analytical grade) were added completely immersing the inserts. Extraction was either performed at room temperature for two hours on a plate shaker, or, alternatively, overnight without shaking, carefully sealed to prevent evaporation of the solvent. Following extraction, three 200 µl aliquots of each extract were transferred to a 96-well plate and the optical density determined in a plate spectrophotometer at 540 -570 nm.

The relative tissue viability of each tissue was calculated as a percentage of the viability of the mean of the negative controls. The acceptance criteria for the test to be valid were following (1). OD (optical density) of formazan extracted from negative controls had to be more than 0,8 (2). CV of identically treated tissues had to be less than 30%, with exception of cases

with OD below 0,3 (3) the positive control had to be classified as "corrosive" in the experimental run.

The mean of three identically treated inserts were used for the classification of the chemical according to the PM. All procedures were documented in "method documentation sheets", for GLP-compliant documentation.

3 Results

3.1 Phase I

The main objective of phase I was to screen the performance and predictive ability of the SkinEthic RHE model in the *in vitro* skin corrosion test, using the validated EpiDerm test protocol. In this early phase, we decided to test chemicals which are known for their special skin corrosion properties, and can thus be used for evaluation of the model's quality, and of the predictive ability of the test. Twenty non-coded chemicals (**Table 1**), covering diverse chemical group with known corrosive potential, were evaluated. The experimental outcome is summarised in **Table 3**.

The results obtained with SkinEthic RHE were in concordance with the *in vivo* classification, and with results obtained previously in the ECVAM skin corrosion validation studies with the EpiDerm and EPISKIN models (Fentem et al., 1998, Liebsch et al., 2000). A modification of the dosing procedure for liquids (for details see **2.4.1**) helped to reduce the under-prediction observed in the first experiments (data not shown). Only one chemical (10% wt. Sulphuric acid) was not classified in concordance with classification given in earlier studies (Fentem et al., 1998, Liebsch et al., 2000) and OECD TG 431. However the classification given Annex I of the Directive 67/548/EEC (EU COM, 1967; ECB, 2005) differs and supports the result obtained with SkinEthic RHE. The result which would be in concordance with OECD TG 431 was achievable only after 4h exposure (see **Table 3**).

In summary, the performance of the model in phase I was good, and the assay was ready for evaluation in an inter-laboratory study. To assure high formality of the study, phase II was performed as a blind trial. Before entering phase II, ZEBET distributed SOPs, spreadsheets, and trained the participating laboratories – BASF (Ludwigshafen, Germany) and SAFEPHARM (Derby, UK).

Table 3: Results obtained in Phase I

No.	Chemical name	In vivo Class (C/NC)	No of tests	Mean 3 min	Mean 1 hour	Mean 4 h	SkinEthic prediction	Remark
1	SLS (20 % aq)	NC	1	99,3	92,6	-	NC	
2	Boron trifluoride dihydrate	C	1	11,4	6,0	-	C	
3	Benzylacetone	NC	1	102,3	77,6	-	NC	
4	H ₂ SO ₄ (10 % wt.)	C	3	92,5	62,9	-	NC	in some regulations classified as NC up to 15 %
				103,3	86,7	5,7	C	
				94,7	61,4	3,7	C	
5	HCl (14,4 % wt.)	C	4	99	99	-	NC	in some regulations classified as NC up to 25 %
				86,4	4,4	-	C	
				88,3	5,4	-	C	
				92,5	5,3	5,4	C	
6	n-Heptylamine	C	1	66,1	102,3	-	NC	strong MTT reducer
	(results after correction with "killed controls")		1	36,0	1,0	-	C	
7	2,4-Xylidine	NC	2	78,3	14,4	-	C	borderline chemical
				82,1	10,2	-	C	
8	Tetrachloroethylene	NC	3	89,7	16,3	-	NC	
				92,7	14,7	-	C	
				101,4	25,6	-	NC	
9	Octanoic acid (caprylic acid)	C	1	24,8	6,5	-	C	
10	55/45 Octanoic/decanoic acid	C	1	38,4	10,1	-	C	
11	4-Amino-1,2,4-triazole	NC	2	104,1	106,8	-	NC	
				94,9	103,2	-	NC	
12	2-tert-Buthylphenol	C	1	26,4	21,3	-	C	
13	1,2- Diaminopropane	C	1	16,0	15,3	-	C	
14	Phenethyl bromide	NC	1	122,7	100,8	-	NC	
15	4-(Methylthio)-benzaldehyde	NC	1	108,2	112,4	-	NC	
16	Potassium hydroxide 10% aq.	C	1	79,8	8,9	-	C	
17	Isostearic acid	NC	1	100,0	94,4	-	NC	
18	Acrylic acid	C	1	3,7	3,6	-	C	
19	1,6 - Dibromhexane	NC	1	121,7	121,7	-	NC	
20	8 N KOH	C	4	78,3	3,3	-	NC	
				86,3	4,9	-		
				12,6	4,3	-		
				75,0	5,7	-		

C - corrosive; NC - non corrosive; S - solid; L - liquid

3.2 Phase II

3.2.1 Assay Prediction

In phase II, three independent test runs of 12 samples (using three tissues per time-point and chemical) were performed in each laboratory. From coding was excluded 10 % sulphuric acid and for reasons already mentioned in paragraphs 2.2 and 3.1 .

According to the PM (3 min/1hour) and classification given in OECD TG 431, in the group of 6 corrosive chemicals, misclassification for chemical #6 (10% wt. sulphuric acid) in all laboratories was obtained. In the group of 6 non-corrosive substances, an over-prediction was observed in 3 out of 9 tests for chemical # 10 (Tetrachloroethylene). For this chemical, viability values close to the classification cut-off were obtained. All results from phase II are summarised in **Table 4** and **Table 5**.

2x2 contingency table statistics were used to determine sensitivity, specificity, positive and negative predictive value and accuracy (**Table 6**). When compared to *in vivo* classification given in OECD TG 431, high sensitivity (85,2%) as well as high specificity (94,4%) was obtained. The overall accuracy of the method was 93,2%. It can be therefore concluded that the SkinEthic model provided excellent predictivity of the corrosive potential for 12 test chemicals in the skin corrosion assay.

3.2.2 Assay variability

The GLM multivariate analysis was performed simultaneously for the endpoint determinations at 3 min and 60 min. It took into account 3 independent factors: "chemicals", "laboratories" , and "run", which are contributing to the final variability (for details see **part 2.3**).

The results obtained in the GLM multivariate procedure are given in **Table 7**. Each multivariate statistic is transformed into a test statistic with a F-distribution, given as sign "F" in **Table 7**.

The variances can be divided and computed as the sum of squared deviations ("sums of squares") from the overall mean. The factors "chemicals" (for both time-points) and "laboratories" (for time-point 1 hour), shows significance values of less than 0.001. This means that both groups contribute to the variability of the assay. A more straightforward way to recognise the contribution to assay variability is to determine the relative mean square values (for details see **part. 2.3**). The relative mean square value for the factor "laboratories" for both endpoints is small (at 3 min - 3,1 % and at 1 hour - 6.5%). This means, that although the results are regarded as "significantly" variable, the contribution of laboratories to the final variability is not high. Intra-laboratory variability represented by the factor "run" is very small (for 3 min - 0,7 % and for 1 h - 0,6 %), showing good reproducibility of results within a laboratory.

Table 6: 2x2 contingency table statistic for Phase II

		In vivo classification		
		Corrosive	Non-corrosive	Total
In vitro prediction	Corrosive	46	3	49
	Non-corrosive	8	51	59
	Total	54	54	108
Statistics for shadowed area of 2X2 table				
Sensitivity:		85,2		
Specificity:		94,4		
Positive predictive value:		93,9		
Negative predictive value:		86,4		
Accuracy:		93,2		
Prevalence		1		

Table 7: Results of the analysis of variance for the two endpoint values “3 Minutes” and “1 hour”
by the factors chemicals, laboratories and run

Source	Dependent Variable	Sum of Squares	df ¹	Mean Square ²	Relative Mean Square (%)	F	Significance
Chemicals	3 Minutes	179575.5	13	13813.5	95.2	92.6	0.000000
	1 hour	211638.2	13	16279.9	92.3	155.4	0.000000
Laboratories (inter-laboratory variability)	3 Minutes	891.4	2	445.7	3.1	3.0	0.054515
	1 hour	2306.9	2	1153.4	6.5	11.0	0.000044
Run (intra-laboratory variability)	3 Minutes	202.3	2	101.2	0.7	0.68	0.509537
	1 hour	203.0	2	101.5	0.6	0.97	0.382687
Error (other variability)	3 Minutes	16104.4	108	149.1	1.0		
	1 hour	11312.4	108	104.7	0.6		
Total	3 Minutes	891673.6	126	14509.5			
	1 hour	485951.6	126	17639.5			

¹df= degrees of freedom, ² The mean square of each effect is calculated by dividing the sum of squares by its degrees of freedom, ³ F statistic is calculated by dividing the mean square by the mean square error (12 chemicals plus positive control)

The relative mean square value for the factor “chemicals” is very large (95.2% for time-point 3 Min, 92.3% for time-point 1 hour). This was expected, because the results obtained with a few test chemicals range from corrosives to non-corrosives. Also variance of the biological responses of different skin model batches may be expected.

In summary, no significant differences between runs in single laboratories were found indicating very good intra-laboratory performance of the assay. The differences between laboratories were low, and not significant for the endpoint value “3 minutes” and slightly significant for the endpoint value “1 hour”. Therefore, we conclude that the assay proved to be sufficiently reliable.

4 Discussion

4.1 Method performance in Phase I

In phase I of the study we proved that the EpiDerm protocol and PM can be reliably used with the SkinEthic RHE (see **Table 3**). Several disagreements in classification were observed at the beginning of the study (data not shown), these being due to the strong hydrophobic nature of the stratum corneum of SkinEthic RHE. This technical problem was overcome by using nylon mesh for spreading all liquid test chemicals.

Among the substances tested in phase I, some had been over-predicted or caused technical problems in previous validation studies (Fentem et al., 1998; Liebsch et al. 2000), e.g. 2,4-xylydine. This chemical has been consistently over-predicted by all reconstructed skin models in all skin corrosion and irritation studies (Fentem et al., 1998; Fentem et al., 2000; Liebsch et al., 2000; Portes et al., 2002; Heylings et al., 2003; Kandarova et al., 2005; Cotoví et al., 2005). The same result was obtained with xylydine in the SkinEthic RHE corrosion test. This seems to be due to the high toxicity of the substance. In addition, xylydine shows good solubility in lipids and may therefore easily penetrate the stratum corneum and damage the viable layers of reconstructed epidermis.

Tetrachlorethylene has also provided unbalanced results in previous studies. Like xylydine, it is highly soluble in lipids. In addition, tetrachloroethylene reacts with plastic inserts and can not easily be removed from the tissue surface during the washing procedure. An over-prediction has been observed with this test chemical in one of three tests in phase I. Similar results have been obtained with the EPISKIN model in the ECVAM skin corrosion validation study (Fentem et al., 1998). In one of three testing laboratories, this chemical has been even consistently over-predicted.

Minor problems were observed when testing solutions of inorganic acids at low concentrations. At the beginning of the study, we observed several under-predictions for

sulphuric acid 10 % wt and one under-prediction for Hydrochloric acid 14.4 %. As described above, we had problems with the spreading of this substance on the tissue surface, and in addition, due to interaction of these acids with nylon, the mesh couldn't be used as spreading tool. However, we assume that using a more resistant material than nylon would not improve prediction in case of 10 % sulphuric acid.

It is on the other hand important to notice that there exists disagreement in the classification of several inorganic acids and their solutions. For instance, hydrochloric acid 14.4.% and sulphuric acid 10 % wt. are classified as "in vivo corrosive" in the ECVAM validation study (Fentem et al., 1998), however, according to Annex I of the Directive 67/548/EEC (EU COM, 1967; ECB , 2005) solutions of hydrochloric acid should be classified as "irritating" in the range of 10 to 25 %. Similarly, 5 to 15 % solutions of sulphuric acid are classified in Annex I as "R38" EEC (EU COM, 1967; ECB , 2005).

4.2 Method Performance in Phase II

4.2.1 Predictions of classification

In phase II, clear and correct classifications were generally obtained. Only a few misclassifications or borderline results were observed (see **Table 4.**, **Figure 1 and 2**). To evaluate observation for 10 % sulphuric acid in phase I, we decided that the corrosive properties of this chemical should be evaluated applying the general PM (3 min/1 hour) and in addition exposure of 4 hours (as in case of *in vivo* skin corrosion test according to OECD TG 404). For that reason, the chemical was excluded from coding. All data obtained for this chemicals are summarised in **Table 5**.

According to the PM (3min/1hour), the chemical would have been classified "non-corrosive" in 8 of 9 cases. This classification is in agreement with Directive 67/548/EEC and its amendments (EU COM, 1967). However, according to the classification given in OECD TG 431, this chemical should be predicted "corrosive". This was, however, achievable only after 4 hours exposure.

According to OECD SIDS database "10 % sulphuric acid appears to be non-irritating nor corrosive to the skin of rabbit, guinea pig and human" (OECD SIDS, 2001). This statement is based mainly on two studies performed by Nixon G.A. (Nixon et al., 1975 Nixon et al. ,1990), where effects of 10 % sulphuric acid on intact and abraded skin of rabbits, guinea pigs and humans were evaluated. The exposure time was 4 hours and effects were scored after 4, 24 and 48 hours. Negligible or slight irritation effects were obtained.

Similar results were obtained with low concentrations of some other acids (10% hydrochloric and 10 % acetic acid) when tested in humans in the studies of Robinson et al. (2001) and Basketter et al. (1999; 2004).

Based on this knowledge, the OECD TG 431 should be amended with regard of this chemical and its classification.

4.2.2 Sources of variability

The statistical analysis shows that the intra-laboratory and inter-laboratory variability were acceptably low for most of cases.

Considerable variability was observed for 10 % sulphuric acid at the endpoint 1 hour which was caused mainly by spreading problems. For this chemicals nylon mesh could not be used as spreading support due to the chemical interactions. As the chemicals, due to the high hydrophobicity of SkinEthic surface, had tendency to collect at the edge of insert, multiple mechanical re-spreading of the chemical on the surface (using bulb headed glass stick) was necessary. This procedure could lead to a different degree of tissue damage consequently reflected in variable results in viability assay.

Next factor that may contribute to the inter-laboratory variability is the different order of testing the chemicals. The sample's coding procedure assured randomisation of the test order in the three testing laboratories. In addition, laboratories intentionally changed the testing order of the coded samples in the three independent runs .

It was known that there is a critical point in the test protocol which may contribute to variability. When tissues are washed with PBS after exposure and kept on the "holding plate" (for details see **paragraph 2.4.4 – Test protocol**), until washing of all inserts in the experimental set is completed. Thereafter, all inserts together are transferred into the MTT medium for 3 hours. Thus, tissues may stay on the holding plate for quite a long period of time (e.g. if in one testing set 6 substances, negative and positive controls were tested, each on 3 tissues, the first tissue must stay in holding plate for 24 min, the second for 23 min etc. until the last tissue has been washed). If a test chemical has not properly been removed from the tissue surface during the washing procedure, the remaining traces cause unwelcome post-exposure.

After decoding of samples and analysis of data provided by each laboratory, we conclude that these differences in contact time are responsible for overpredictions of tetrachloroethylene and for the variability observed after 3 min for potassium hydroxide 10% and octanoic acid. These three chemicals have induced slight, but significant differences between runs and also differences between testing laboratories (see **Figure 1, 2 and Table 4**)

Nevertheless, we conclude that the robustness of the assay and its predictive capacity were not affected.

Figure 1: In vivo corrosive chemicals

Six in vivo corrosive chemicals were tested three times in three laboratories. The results are displayed as means of each independent run $\pm$ standard deviation.

Figure 2: In vivo non-corrosive chemicals

Six in vivo non-corrosive chemicals were tested three times in three laboratories. The results are displayed as means of each independent run $\pm$ standard deviation.

4.2.3 Limitations of the assay

No special limitations of the SkinEthic skin corrosion assay were identified in this study, except for the well established interference of some test chemicals with the MTT assay. In the set of 12 OECD reference chemicals, 6 chemicals interacted directly with MTT. This fact did not affect the results for 1,2-diaminopropane, 2-tertbutylphenol and potassium hydroxide, since the chemicals were clearly predicted as corrosive. When testing phenethyl bromide and 4-(methylthio)-benzaldehyde, only slight MTT reduction was observed, which did not affect a correct prediction. However, problems were observed with Eugenol, which is a strong MTT reducer, and, in addition, has the capacity to spontaneously polymerise.

Therefore, in addition to the blind trial, we evaluated the chemical conversion of MTT of Eugenol. For this purpose, we used a procedure described by Liebsch et al, (Liebsch et al, 2000) applying Eugenol onto non-viable (freeze-killed) control tissues. The binding of the chemical to the tissue can be assessed and thus also amount of MTT converted in the corrosivity test by residual test material (for details see part 2.4.3 and Liebsch et al., 2000). It is important to notice, that this procedure is not yet properly evaluated and needs optimisation. Particularly, the preparation of freeze-killed controls seems to be a crucial step. We have observed that the binding of test material to frozen tissue can be higher than the binding to viable tissues. This result may be explained by formation of ice crystals during the freezing procedure. Crystals may damage structure of the skin and thus increase its permeability. Consequently, penetration of the test material into the damaged tissues will be higher and thus also the amount of residuals bound to the tissue.

From experiments performed in all of the laboratories, we conclude that Eugenol is able to significantly affect the viability of tissues (see **Table 4**). Because of the high conversion of MTT to formazan by Eugenol itself, a final estimation of the corrosive properties of this chemical based on above described technique may sometimes lead to borderline prediction.

5 Conclusion

The aim of the this study was (1) to verify if the SkinEthic RHE model is able to correctly distinguish between corrosive and non-corrosive chemicals in the sense of the new OECD TG 431, and (2) to support the idea of the use of a "common protocol" for different commercially available *in vitro* human skin models in sense of OECD TG 431.

We conclude from the presented data that the SkinEthic skin corrosion assay performed similarly to the validated EpiDerm and EPISKIN assays. The SkinEthic assay is also meeting criteria required by OECD TG 431, and therefore can be regarded as valid method for regulatory skin corrosion testing of chemicals.

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Chapter 5

Three dimensional construct of the human corneal epithelium for in vitro toxicology.

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Abstract

This project characterizes and explores the use of a 3-dimensional human corneal epithelial construct for *in vitro* toxicology testing. Immortalized human corneal epithelial cells were cultivated at the air-liquid interface in a chemically defined medium on a polycarbonate substrate for 7 days. Histologically, the 3-dimensional cultures appeared as a multi-layered, stratified epithelium resembling the normal human corneal epithelium. A layer of columnar basal cells, two or three layers of transitional wing cells, and two or three layers of superficial squamous cells could be distinguished. Ultrastructurally, numerous intermediate filaments, sparse mitochondria, and ribosomes, as well as intercellular desmosomal junctions, were seen. Laminin and a cornea-specific cytokeratin were identified on Western blots. Hemidesmosomes were present along the basal membranes of the basal cells, bridging the gap between the cell membranes and the growth surface. Incubation with benzalkonium chloride (0.001%, 0.01%, or 0.1%,) for 24 hours produced structural and biochemical changes correlated in severity with the concentration. These 3-dimensional human corneal epithelial constructs may reduce the need for short-lived corneal epithelial cell cultures and could provide a valuable tool for *in vitro* toxicology studies.

Introduction

The corneal epithelium, comprising 5 or 6 layers of differentiated ectodermal cells, protects the cornea and contributes to its optical properties [Lemp 1987; Beuerman and Pedroza 1996]. Injury to the cornea presents a substantial threat to vision [Gasset et al. 1974; Osgood et al. 1990; Clayton et al. 1985]. Thus, determining the risk of corneal injury from exposure to chemicals—both at home and in the workplace—is of critical importance.

The Draize test has been used as a standard method to evaluate the potential ocular toxicity of industrial and household chemicals, such as those used in cosmetics and soaps, for many years. However, problems with the Draize test, such as inaccuracy and nonspecificity, have prompted development of more reliable evaluation methods. Newer models include eye organ culture, primary cultures, and immortalized cultures of corneal epithelial cells from humans and other species [Kahn et al. 1993; Halenda et al. 1998; Nardone and Bradlaw 1983; Grant et al. 1992; Dason and Mustafa 1985]. Various parameters of epithelial physiology, such as electrical resistance, have also been correlated with the concentrations of some toxic materials [Kruszewski et al. 1997; Burstein and Klyce 1977]. These alternative methods also have several disadvantages, however, including quality control, low throughput, and relationship to normal structure and function of the human cornea.

The use of cultured cells for this purpose is attractive if the cells can be produced in quantity and in a standardized fashion. Immortalized cell lines can be used to replicate important properties of the ocular cells or tissues when primary cultures are not practical or not available [Araki-Sasaki et al. 1995; Nguyen et al. 1999]. Reconstituted human epithelial constructs have been reported to allow routine screening of cosmetic ingredients for assessment of eye irritation [Doucet et al. 1998; Doucet et al. 1999]. The purpose of the present study was to examine the properties

of the 3-dimensional corneal tissue construct in conjunction with an immortalized human corneal epithelial cell (IHCEC) line and to test the response of the cells in the construct to benzalkonium chloride, a cationic surfactant commonly used as a preservative in ocular medications and cosmetics. We found that this 3-dimensional human corneal epithelial cell construct provides a quality controlled, reproducible, and rapid *in vitro* test system that can be used for preliminary large-scale screening of potential corneal irritants and toxic compounds.

Materials and Methods

Three-dimensional corneal epithelial cell constructs. Immortalized human corneal epithelial cells (IHCEC) were placed in an inert, permeable polycarbonate insert (Nunc A/S, Roskilde, Denmark) and cultivated at the air-liquid interface in a chemically defined medium for 7 days. The 3-dimensional constructs were shipped overnight to the LSU Eye Center in New Orleans, Louisiana, from the SkinEthic Laboratories in Nice, France. Upon arrival, the constructs were aseptically removed and placed in 6-well culture plates containing 500 μ l of maintenance medium supplied by SkinEthic. The cultures were allowed to equilibrate in a humidified incubator at 37° for 24 hours, after which the medium was removed and replaced with fresh medium.

Histology. Constructs were transferred to 6-well plates filled with a solution containing 2% glutaraldehyde and 1% paraformaldehyde in a 0.1 M sodium cacodylate buffer (pH 7.4) containing 0.02% picric acid at 37° C [Beuerman and Pedroza-Schmidt 1996]. The fixative solution was also pipetted into the interior of the constructs. Specimens were washed in 0.1 M sodium cacodylate overnight, post-fixed in osmium tetroxide, dehydrated in a graded ethanol series, and embedded in plastic resin (Eponate 12, Ted Pella, Redding, CA). Ultrathin sections (70 nm) were stained for contrast with uranyl acetate and lead citrate (Ultrastainer, LKB, Bromma, Sweden) and viewed using a transmission electron microscope (EM10 C/A; Zeiss). Thick plastic sections (1 μ m) were stained with toluidine blue for light microscopy and photography.

In vitro toxicology assay. The effects of benzalkonium chloride were tested by pipetting 30 μ l of a 0.1%, 0.01%, or 0.001% stock solution of the preservative in phosphate-buffered saline (PBS) directly onto the constructs in 6-well culture plates. Benzalkonium chloride is a reference chemical for eye irritation testing as designated by the European Centre for Ecotoxicology and Toxicology of Chemicals. Control constructs received either vehicle alone (30 μ l of PBS, pH 7.4) or no additional material. Six separate constructs were used for each experimental condition. After 24 hours of incubation, constructs were frozen, fixed for light and transmission electron microscopy, or processed for Western blot analysis.

Western blot analysis. The 3-dimensional constructs were lysed with 150 μ l of ice-cold buffered PBS containing Triton X-100 (10%), sodium deoxycholate (5%), sodium dodecyl sulfate (1%), leupeptin (0.5 μ g/ml), EDTA (1 mM), pepstatin A (1 μ g/ml), aprotinin (1 μ g/ml), and phenylmethylsulfonyl fluoride (PMSF) (200 μ M) for 15 minutes. The lysate was transferred to a centrifuge tube and the polycarbonate insert was removed using a No. 11 scalpel blade and transferred to the same centrifuge

tube. Cultures were combined in groups of two. The combined specimens were mechanically processed using a disposable pestle (Kontes Glass Co., Vineland, NJ) and centrifuged at 4°C for 10 minutes at 5000 RPM. Total protein was determined by UV spectrophotometry and estimated using the A260/A280 correction ratio [Roby and White 1990]. Western blots were carried out as previously described [Nguyen et al. 1999]. Samples (25 µg) of the protein lysates were denatured by heating in equal volumes of 2x Laemmli buffer (4x buffer for the less concentrated protein samples) separated on 6%, 7.5%, or 10% polyacrylamide gels, and transferred to nitrocellulose membranes (Hybond-ECL; Amersham, Piscataway, NJ). Controls consisted of IHCEC cultured for 4 weeks, human donor corneal epithelial cells scraped from a pair of donor corneas obtained from the Tampa Eye Bank within 24 hours of death, and/or rabbit lacrimal gland. The membranes were subjected to 100 V for 75 minutes in an ice bath, then blocked in 5% powdered milk overnight, and incubated with various primary antibodies for 1 hour per antibody at room temperature: biochemical analysis—AE1 and AE3 (1:500) (Boehringer Mannheim Group Roche, Basel, Switzerland), cornea-specific 64 kDa AE5 (1:500) (Research Diagnostic, Inc., Flanders, NJ), and laminin (1:500) (Calbiochem, San Diego, CA); toxicology analysis—phospho-specific mitogen-activated protein kinase (MAPK) (1:250) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The membranes were then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000) (anti-mouse HRP, Santa Cruz Biotechnology). Determination of reactivity was carried out using electrochemical luminescence (ECL) detection reagents and visualized on X-ray film (Hyperfilm, Amersham).

Results

Histologic characteristics of the 3-dimensional corneal epithelial constructs. Histologically, the 3-dimensional constructs of IHCEC resembled the stratified cellular organization of human corneal epithelium (Figure 1). The nonkeratinized superficial cells were flattened in the plane of the construct. The outer membranes of these cells were thrown into microvilli measuring a few microns in length. Cells in all layers exhibited a well-developed cytoskeletal network and were attached to adjacent cells by desmosomes (Figures 2 and 3). Cells of the intermediate layer displayed more lateral cytoplasmic extensions than those in the basal layer, similar to wing cells in human corneal epithelium. Mean thickness of the constructs was $60\text{ }\mu\text{m} \pm 7\text{ }\mu\text{m}$ ($\pm$ s.e.; $n = 6$), similar to that of normal human corneal epithelium.

Figure 1. Light micrograph of a plastic section from a construct after 24 hours equilibration at 37°. The tissue organization reveals a stratified appearance with columnar basal cells, defined wing cells, and flattened superficial cells. Toluidine blue and basic fuchsin, original magnification 160x.

Figure 2. Transmission electron micrograph of the construct after 24 hours equilibration at 37°. The superficial cells give rise to numerous villus processes. The nucleus is oriented parallel to the surface. Some small, spot-like junction arrangements are seen between cells at the surface. Desmosomes are present between adjacent cells. Original magnification 12,600x.

Figure 3. Low power transmission electron micrograph of basal cells of the 3-dimensional construct. The nucleus of the basal cell is upright. Within the cell, there is an extensive cytoskeletal network. Mitochondria surround the nucleus and desmosomes join adjacent cells. Original magnification 10,000x.

Figure 4. High power transmission electron micrograph of the basal membrane of a basal cell revealing numerous mature hemidesmosomes. Also visible are the components of the hemidesmosomes: the intracellular membrane placode, filaments radiating through the membrane, and the typical band in what would be the lamina lucida. The amorphous band at the bottom is the polycarbonate support membrane of the construct. Original magnification 75,000x.

The basal cell layer consisted of a single layer of columnar cells whose basal membranes rested on the insert (Figures 1 and 3). At the ultrastructural level, the basal membranes revealed mature hemidesmosomes with internal placodes and filaments radiating through the cell membrane (Figure 4). The cytoplasm of the columnar cells was filled with mats of filaments and sparse mitochondria were located around the nucleus.

Biochemical characteristics of the 3-dimensional corneal epithelial constructs. The 3-dimensional constructs were assessed for immunoreactivity to the epithelial cell-specific keratins, a group of water-insoluble proteins that form the major components of the cytoskeletal complex. Keratins are divided into acidic and basic subfamilies and are found together in pairs [Sun et al. 1985]. In the cornea, acidic AE1 immunoreactivity is found in the hyperproliferative epithelium. As shown in the Western blots, the expression pattern of AE1 for the constructs was similar to that for IHCEC cultured for 4 weeks (Figure 5A). Basic AE3 expression for the 3-dimensional constructs was similar to the expression patterns seen in cultured IHCEC and freshly obtained human donor corneal epithelial cells (Figure 5B). Western blots using the AE5 antibody, a marker of corneal differentiation, showed positive immunoreactivity to a 64 kDa protein, indicating that the epithelial cells of the 3-dimensional construct expressed cornea-specific keratin 3 (Figure 6).

Figure 5. Western blots of the acidic (AE1) and the basic (AE3) keratin family in the 3-dimensional construct: A. The reactivity pattern for AE1 in the 3-dimensional construct (Lane 1) is similar to that of immortalized human corneal epithelial cells (IHCEC) cultured for 4 weeks (Lane 2). B. The reactivity pattern for AE3 (Lane 1) is similar to that of cultured IHCEC at 1 week (Lane 2) and 2 weeks (Lane 3) and human donor corneal epithelial cells obtained within 24 hours after death (Lane 4). The molecular weights of several cytokeratin isoforms recognized by the respective antibodies are indicated below under AE1 and AE3, respectively.

Figure 6. Western blot of the 64 kDa cornea-specific keratin AE5 in the 3-dimensional construct. Lanes show the construct on Day 0 (immediately upon arrival), Day 1 (24 hours equilibration at 37°C), and Day 2 (48 hours at 37°C), as well as the fresh human donor corneal epithelial cell positive control (+) and the rabbit lacrimal gland negative control (-).

In vivo, corneal epithelial cells are anchored via hemidesmosomes to the underlying structure called the basal lamina. These mechanical attachments are responsible for holding the epithelium tight to the stroma. Laminin and collagen IV are prominent extracellular components of the basal lamina and are synthesized by corneal epithelial cells. In this study, Western blot (Figure 7) showed two bands, one at 220 kDa and one at 440 kDa, indicating that the epithelial cells in the 3-dimensional

construct synthesized laminin. Connexin-43, a protein component of the gap junction was also present in these 3-dimensional constructs (not shown).

Response to benzalkonium chloride. Benzalkonium chloride, which is common used as a preservative in ophthalmic solutions in concentrations from 0.004% to 0.1%, may increase epithelial permeability.

Figure 7. Western blot of laminin in the 3-dimensional construct. One band is seen at 220 kDa and another at 440 kDa. Lane 1, 3-dimensional construct; Lane 2, fresh human corneal donor epithelial cells from cadaver eyes; Lane 3, immortalized human corneal epithelial cells cultured for 4 weeks.

The effects of benzalkonium chloride at several concentrations were determined after 24-hour exposure. Light microscopy showed no differences in the vehicle (PBS control) and the normal control constructs (not shown).

8B

8C

Figure 8. Light micrographs of the 3-dimensional construct showing cellular damage after treatment with benzalkonium chloride for 24 hours. A. 0.001%. B. 0.01%. C. 0.1%. Original magnification 126x.

With 0.001% benzalkonium chloride, cellular organization was similar to that of the controls, although some minor sloughing of the superficial cells was seen (Figure 8A). With 0.01% benzalkonium chloride, the organization of the constructs was similar to that of the constructs treated with 0.001% benzalkonium chloride, although the amount of cellular debris was greater (Figure 8B). Transmission electron microscopy showed remnants of superficial cells and increased vesiculation (not shown). With 0.1% benzalkonium chloride, the effect was severe (Figure 8C). Cellular damage was clearly evident and the constructs were completely separated from the basal membrane; there was much more vacuolation and the nuclei appeared pyknotic.

Figure 9. Top: Western blot analysis for phosphorylated (active) p42/p44 mitogen activated protein kinase (MAPK) after treatment with benzalkonium chloride. Positive control (Ctrl +), epidermal growth factor-stimulated A431 cells. Negative control (Ctrl -), unstimulated A431 cells. Bottom: The histogram shows the increase in intensity level (activity) of p42/p44 with increasing concentrations of benzalkonium chloride. The absence of activity with 0.1% benzalkonium chloride is likely due to cell death resulting from toxic insult.

The effects of benzalkonium chloride were further characterized by examining the induction of the MAPK pathway. Both p42 and p44 MAPK are activated by phosphorylation in response to extracellular and intracellular signals that are transduced to the nucleus, thus altering the types of genes expressed. Western blot using an antibody that recognizes phosphorylated (activated) p42 and p44 MAPK showed increased levels of activated p42 and p44 with both 0.001% and 0.01% benzalkonium chloride, compared with the vehicle control (Figure 9). With the highest concentration, however, (0.1%), the levels of p42 and p44 decreased below the control level, possibly due to the loss or death of cells as shown by transmission electron microscopy. These results suggest that the constructs may be able to demonstrate an incremental response to environmental stress, in this case benzalkonium chloride in concentrations up to 0.01%.

Discussion

In this study, we demonstrated similarities between the 3-dimensional IHCEC construct and normal human corneal epithelium in terms of the overall morphology and thickness (about 60 μm). The stratified appearance of the columnar basal cells and the wing cells overhanging the rounded surfaces of the basal cells was similar to the organization of epithelial tissue *in vivo*. Remarkably, the cultured construct cells produced mature hemidesmosomes along the basal membrane of the basal cells, and filaments were seen to radiate from the internal membrane placodes of the hemidesmosomes. These mechanical attachments were functional to some extent because 0.1% benzalkonium chloride affected their integrity, allowing the support membrane to fall away. This suggests an additional effect of this preservative that has not been previously noted.

Immunoreactivity to 64 kDa keratin 3 indicates that the cells of the construct retain this cornea-specific differentiation marker, i.e., IHCEC in the construct retain the ability to synthesize keratin and to differentiate as corneal epithelial cells [Kruse and Tseng, 1994]. The advantage of using this immortalized cell line may be found in the method used to immortalize the cells. The immortalizing vector was an amphotropic retroviral vector (LXSN16E6E7) that contained the E6 and E7 genes of human papillomavirus [Halbert et al. 1991]. This approach has also been used to produce an immortalized lacrimal gland epithelial cell line that expressed transferrin receptor and was able to express protein in response to pharmacological stimulation [Nguyen et al. 1999].

The surfactant benzalkonium chloride has been used as a preservative in cosmetics and ophthalmic medications for many years. The toxic effects of this and other surfactants, including corneal damage and delayed wound healing, are well known [Green et al. 1989; Scaife 1985]. The effects of benzalkonium chloride on epithelial

cell morphology and physiology have been verified in numerous studies [Burstein and Klyce, 1977; Clayton et al. 1985; Gasset et al. 1974]. However, the thrust of these studies has been to identify benzalkonium chloride-related problems in an effort to develop safer preservatives rather than a means of evaluating models suitable for testing the toxic potential of chemical additives.

It is not yet clear if the 3-dimensional constructs will support combined studies on morphology, physiology, and molecular expression. However, as the constructs can be produced in large numbers, cross-validation may be expected. Another potential advantage of the constructs is the ability to distinguish cellular and molecular markers of toxic effects. Commonly used toxicity markers, such as neutral red staining and the MTT assay, may work well *in vivo*, but may not be useful in predicting tissue recovery or in identifying the cellular mechanisms activated by the toxic stress. In this study, the intracellular signal transduction pathway MAPK was shown to be activated by benzalkonium chloride. This suggests that cellular stress responses were initiated; however, verification will require parallel studies using whole eyes and other stress-activated signal pathways at early time points with comparison to scores from the Draize test. Nevertheless, this cell-based strategy could provide a new approach to predicting the severity of injury as well as the ability to heal after chemical insult.

Engineered constructs such as those used in this study provide substrate reliability and the opportunity to standardize testing to facilitate broad comparisons between laboratories. This could result in the development of a data base of chemical responses. As with *in vitro* tests in general, finding the parameters that describe coarse measures of the response is not difficult. However, further work is required to develop and refine these measures and improve their ability to increase our understanding of the capacity for healing and the return to the normal state after exposure to a toxic event.

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Chapter 6

Differential expression of extracellular matrix metalloproteinase inducer (CD 147) in normal and ulcerated corneas.

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Matrix Pathobiology

Differential Expression of Extracellular Matrix Metalloproteinase Inducer (CD147) in Normal and Ulcerated Corneas

Role in Epithelio-Stromal Interactions and Matrix Metalloproteinase Induction

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Extracellular matrix metalloproteinase inducer (EMMPRIN) was originally identified on the tumor cell surface as an inducer of matrix metalloproteinase (MMP) production in neighboring fibroblasts. Here we demonstrate a role for EMMPRIN in MMP induction during corneal wound healing. MMP and EMMPRIN expression was analyzed in normal and ulcerated human corneas, as well as in corneal epithelial and stromal cells in culture using confocal microscopy, zymography, immunoblots, and real-time polymerase chain reaction. In normal cornea EMMPRIN was predominantly expressed in the epithelium but was markedly induced in the anterior stroma of ulcerated corneas. This coincided with MMP-2 induction that co-localized with EMMPRIN at the epithelio-stromal boundary. The role of epithelial-stromal interaction in MMP induction was investigated in an *in vitro* co-culture system and demonstrated an induction and co-localization of EMMPRIN and MMP-2 in the fibroblasts at the interface with epithelial cells. Direct contact of fibroblasts with

EMMPRIN-containing purified epithelial cell membranes also induced MMP-1, MMP-2, and EMMPRIN and this was inhibited by a blocking anti-EMMPRIN antibody, suggesting that EMMPRIN was primarily responsible for this induction. These findings, and the up-regulation of EMMPRIN by epidermal growth factor and transforming growth factor- β , demonstrate a role for EMMPRIN in wound healing and suggest that sustained local up-regulation of EMMPRIN and MMPs in chronic situations in which healing is delayed may lead to excessive matrix degradation and corneal melts. (*Am J Pathol* 2005, 166:209–219)

The cornea, first diopter of the eye, is a transparent anterior ocular tissue that has been extensively used as a model for studying wound healing because of its remarkably organized structure. It is composed of three distinct layers, an epithelium, a stroma, and an endothelium. The epithelium and the endothelium are separated from the stroma by three different structures, the epithelial basement membrane, Bowman layer, and the Descemet's membrane. The separation of these tissue compartments is necessary for the normal homeostasis of the cornea and the disruption of the basement membrane was shown to be associated with activation of the wound healing process that comprise fibroblast activation and stromal remodeling in which synthesis and degradation of the extracellular matrix are tightly regulated to restore tissue homeostasis.^{1,2}

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Matrix metalloproteinases (MMPs), a family of proteolytic enzymes able, collectively, to degrade all of the molecules of the extracellular matrix, are central to this process. Their induction during wound healing is thought to play a role in extracellular matrix remodeling, cell-matrix interactions, inflammatory cell recruitment, cytokine activation, and regulation of angiogenesis.³⁻⁵ This family currently includes more than 25 members that can be divided into collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3 and -10), matrilysins (MMP-7 and -26), and the membrane-type MMPs (MMP-14 to -17 and -24), according to their structure and substrate specificity.

Barely detected in unwounded cornea, MMPs are strongly induced during wound healing after excimer laser treatment, epithelial scrape, or alkali burn injury.^{3,6,7} They have a complex pattern of expression and can be induced in both the migrating epithelium and in the anterior stroma of the healing cornea. Some MMPs, such as MMP-1 or MMP-9, are induced in the basal layer of the regenerating epithelium. Although MMP-1 is detected in the epithelium and the anterior stroma only when the basement membrane is disrupted, MMP-9 induction in regenerating epithelial cells appears to be independent of the integrity of the basement membrane.⁸⁻¹² Other MMPs, such as MMP-2, MMP-3, and MT1-MMP were shown to be induced in the stroma of the healing cornea and are often detected in the subepithelial fibroblasts of the wounded area, even though MT1-MMP is normally expressed in the basal epithelial cells.¹³⁻¹⁵ The precise regulatory mechanisms involved in MMP induction in wound healing have not yet been elucidated despite extensive investigations, but are thought to involve cytokines, cell-matrix, and cell-cell interactions.^{2,4,16}

Although in physiological wound healing MMPs become rapidly undetectable after wound closure, their deregulation and prolonged accumulation in the anterior stroma may lead to ulcerations and perforations in chronic wounds.^{6,17,18} In such situations, the induction of MMPs in the subepithelial layer of the corneal stroma is reminiscent of their regulation in cancer whereby MMPs have often been seen localized in the stromal tissues directly in contact with the tumor.¹⁹ It was initially thought that tumor cells themselves were responsible for the production of MMPs, but it then became apparent that the surrounding stromal fibroblasts represent the major source of MMP activity, suggesting that the interaction between tumor cells and stromal fibroblasts is responsible for the elevated MMP levels detected in the tumors.²⁰ These observations led to the identification of EMMPRIN (extracellular matrix metalloproteinase inducer), or CD147, a 58-kd transmembrane glycoprotein enriched on the surface of most tumor cells that can stimulate stromal cells to produce elevated levels of several MMPs, namely MMP-1, MMP-2, and MMP-3.^{4,21-23} EMMPRIN has later been identified in several diseased human tissues such as in rheumatoid arthritis²⁴ and venous leg ulcers²⁵ as well as in normal keratinocytes and other epithelia.^{26,27} The fact that, during corneal wound healing, these same enzymes, MMP-1, MMP-2, and MMP-3 were shown to be up-regulated in the anterior stroma

underneath the injured epithelium incited us to look for EMMPRIN as the corneal inducer of MMP production through direct epithelial cell-fibroblast interaction.

In this study we used noninfectious ulcerated corneas as a model to study MMP regulation because they are associated with excessive extracellular matrix degradation that may lead to corneal perforation and where prolonged increase in MMP expression has been demonstrated.^{18,28} We report the differential expression and localization of EMMPRIN in normal and ulcerated corneas, its regulation by cytokines, and its biological function *in vitro*.

Materials and Methods

Tissue, Cells, and Materials

Corneal tissues ($n = 5$) were obtained from the French Eye Bank (Dr. P. Sabatier, Banque Française des Yeux, Paris, France) and Belgian Eye Banque (Dr. Lejeune, Lieges, Belgium). Chronic ulcerated or perforated corneal specimens ($n = 5$) were obtained from patients undergoing penetrative keratoplasty and use of specimen was performed in accordance with protocols approved by the institutional committee. Tissues were flash-frozen in optimal cutting temperature (OCT) compound and kept at -80°C for immunohistochemistry.

Human corneal reconstructed epithelia, stratified by air lifting *in vitro*, were obtained in collaboration with SkinEthic Inc. (Nice, France). The corneal epithelial cell line, 1384 HCE-T (Dr. Araki-Sasaki, Riken Cell Bank, Saitama, Japan) was maintained in supplementary hormonal epithelial medium (SHEM) [50% HAMS-F12 and 50% Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 ng/ml epidermal growth factor (EGF), 0.1 nmol/L cholera toxin, and 5 $\mu\text{g/ml}$ insulin] containing 10% fetal bovine serum. Corneal fibroblasts cell line were cultured in DMEM supplemented with 10% fetal bovine serum.²⁹ For experiments with cytokine treatment, cells were used at 60 to 80% confluence in 50% HAMS-F12 and 50% DMEM without supplements. Cells were incubated in serum-free medium 18 hours before cytokine addition.

The antibodies used were anti-EMMPRIN (anti-CD147) HIM6 monoclonal antibody (mAb), used for immunoblots and immunohistochemistry (Becton-Dickinson, San Diego, CA), and anti-EMMPRIN blocking antibody, UM-8D6, for inhibition studies (Research Diagnostics, Inc., Flanders, NJ). The neutralizing activity of this antibody has been confirmed in different assays and was shown to inhibit dose dependently and specifically both MMP-1 and MMP-2 production induced by recombinant EMMPRIN in fibroblasts, both of lung and dermal origin (L. Yan, unpublished data).

Anti-MMP-1 mAb was from Calbiochem (La Jolla, CA); anti-MMP-2 mAb CA 801 and polyclonal antibody (pAb)³⁰ were gifts from Dr. Raphael Fridman (Wayne State University, Detroit, MI); horseradish peroxidase-conjugated anti-mouse antibody was from Jackson ImmunoResearch Laboratories Immunotech (Marseille, France); and anti-

mouse IgG Alexa 488 and anti-rabbit IgG Alexa 594 were from Molecular Probes (Invitrogen, Cergy Pontoise, France). Other materials were gelatin from Sigma (St. Louis, MO), ECL+ reagent from Amersham Bioscience (Buckinghamshire, UK), and protease inhibitor cocktail Set V ethylenediaminetetraacetic acid-free from Calbiochem. Recombinant EMMPRIN used in the study was an affinity-purified Flag-tag protein consisting of the extracellular domain of human EMMPRIN expressed in mammalian cells.

Cell Treatment

Corneal epithelial cells 1384 HCE-T were maintained in SHEM medium supplemented with 10 ng/ml EGF, 0.1 nmol/L cholera toxin, 5 μ g/ml insulin, and 10% fetal bovine serum until they reached 70 to 80% confluence. The medium was then replaced with serum- and growth factor-free SHEM and cells were incubated for 18 hours before cytokine addition. Cells were then treated with either transforming growth factor (TGF)- β 1 (10 ng/ml), EGF (10 ng/ml), or tumor necrosis factor- α (10 ng/ml) in the serum-free medium and incubated for either 6 hours, after which total RNA was extracted with the RNeasy kit (Qiagen, Hilden, Germany) for real-time quantitative reverse transcriptase (RT)-polymerase chain reaction (PCR) measurements, or for 24 hours, after which the cells were lysed in the Tris-buffered saline-Nonidet P-40 solution containing protease inhibitors and subjected to immunoblot analysis of EMMPRIN, as described below. The incubation times were chosen on the basis of several time-course experiments.

To obtain epithelial cells at different densities, 5×10^5 cells suspended in complete SHEM media were seeded in either 25-, 75-, or 175-cm² dishes. Cells were allowed to adhere and grow for ~24 hours until a confluent monolayer was obtained in the 25-cm² dish and the cells in the larger flasks were at correspondingly lower densities. The different density cultures were then washed with serum-free SHEM and lysed for either quantitative RT-PCR or immunoblots analysis. In some experiments different density cultures were obtained by seeding 5×10^4 cells in 25-cm² dishes and allowing them to grow in complete media, collecting the cells each day up to 3 days when cells reached confluence.

Epithelial Fibroblast Co-Culture

Epithelial cell suspension containing 10^5 in 200 μ l (1:1 SHEM:DMEM) was applied on a sterile microscope slide that was placed in a culture dish. After cells within the drop became confluent (48 hours) forming an epithelial sheet of ~1 cm², fibroblasts (10^5 in 200 μ l) were added around the epithelial sheet and allowed to attach before the whole slide was completely submerged in medium. After 24 to 48 hours, a clear interface between the two cell types formed. The slides were then washed in phosphate-buffered saline (PBS); fixed in 4% paraformaldehyde; stained with 4,6-diamidino-2-phenylindole (DAPI),

anti-EMMPRIN, anti-MMP-2 mAb; and analyzed by confocal microscopy.

Epithelial Cell Membrane Preparation

Cell membranes were prepared according to Lindenmeyer and colleagues.³¹ Briefly, HCE-T cells were scrapped with rubber policemen in serum-free SHEM medium containing 1/100 protease inhibitor cocktail Set V (AEBSF, aprotinin, E-64, leupeptin, and 1 mmol/L ethylenediaminetetraacetic acid), disrupted on ice by controlled sonication: three cycles of 10 seconds at 40 W (Vibra cell Sonifier; Bioblock Scientific, France) and centrifuged at $1000 \times g$ for 10 minutes at 4°C to remove unbroken cells. The sonicate supernatant was centrifuged at $19,000 \times g$ for 30 minutes to sediment the granules and the resulting supernatant was further centrifuged at $100,000 \times g$ for 1 hour. After one wash in serum-free DMEM, the pellet containing the membrane fraction was resuspended in the same medium. Aliquots containing the membrane fraction diluted with serum-free DMEM (20 μ g/ml) were added to the fibroblasts cultures (10^6 cells per 25-cm² dish) and incubated for 24 hours.

Confocal Immunohistochemistry and Cytochemistry

Cryostat sections (8 μ m) were prepared from frozen cornea or stratified epithelium and were immunostained as previously described.³² Briefly, the sections were fixed in chilled acetone for 10 minutes, then rehydrated in PBS and incubated in blocking solution (3% bovine serum albumin) for 30 minutes. Sections were incubated for 1 hour with anti-EMMPRIN/CD-147 HIM6 mAb at 1/200 dilution, then for 30 minutes with conjugated affinity-purified donkey anti-mouse IgG (Alexa 488). For immunocytochemistry, cells were seeded on Lab-Tek glass slides, cultured for 2 days, fixed in 4% paraformaldehyde, and then processed as above using either CD-147 HIM6 or anti-MMP-2 pAb, and followed by conjugated affinity-purified donkey anti-mouse and/or anti-rabbit IgG (Alexa 488 and 594, respectively). The slides were mounted and examined with a laser-scanning confocal microscope (Leica Lasertechnik, Heidelberg, Germany). Negative controls were prepared using the same procedure, but phosphate-buffered saline was substituted for the primary antibody.

Immunoblot Analysis

Cells were lysed in Tris-buffered saline-Nonidet P-40 solution comprising 50 mmol/L Tris buffer, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% Triton X-100, 2 mmol/L ethylenediaminetetraacetic acid, and 1/100 protease inhibitor cocktail Set V. After incubation on ice for 20 minutes and scrapping, the lysates were centrifuged for 3 minutes at $15,000 \times g$ at 4°C. Protein concentration of the resulting supernatants was quantitatively determined using the Bradford Bio-Rad protein assay. Twenty

Table 1. List of the Primers and Probes Used

Gene and oligonucleotide	Location	Sequence	PCR product size (bp)
EMMPRIN			
Upper primer	766U	5'-GCAGCGGGCAGCACC-3'	
Lower primer	833L	5'-CCACCTGCCTCAGGAAGAGTT-3'	
Probe	788U	5'-CAAAGCAAGAACGTCGCCAGAG-3'	68
MMP2			
Upper primer	380U	5'-CCGTCGCCCATCATCAA-3'	
Lower primer	450L	5'-AGGTATTGCACTGCCAACTCTT-3'	
Probe	406U	5'-CGATGTCGCCCCCAAAACGGA-3'	71
MMP9			
Upper primer	610U	5'-CATTGAGGGAGACGCCCA-3'	
Lower primer	673L	5'-AACCACGACGCCCTTGC-3'	
Probe	629U	5'-TTCGACGATGACGAGTTGTGGTCCT-3'	64
β 2 Microglobulin			
Upper primer	20U	5'-CGCTCCGTGGCCCTTAGC-3'	
Lower primer	86L	5'-GAGTACGCTGGATAGCCTCCA-3'	
Probe	39U	5'-TGCTCGCGCTACTCTCTCTTCTG-3'	67

μ g of protein in Laemmli sample buffer were loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel for electrophoresis and then transferred to nitrocellulose filters. EMMPRIN or MMP-1 were revealed by incubation with 0.2 μ g/ml HIM6 mAb or anti-MMP1 mAb overnight at 4°C followed by a 1-hour incubation with horseradish peroxidase-conjugated anti-mouse antibody and visualized with ECL+ reagent. Protein loading was verified by comparing the intensity of actin bands. Band intensities were quantified using Image Quant 5.2 (Molecular Dynamics, Piscataway, NJ).

Gelatin Zymography

The presence of the gelatinases, MMP-2 and MMP-9, in the serum-free conditioned media (10 μ l) was determined by zymography in 10% polyacrylamide gels containing 1 mg/ml gelatin, as previously described.³³ Band intensities were quantified using Image Quant 5.2 (Molecular Dynamics).

Real-Time Quantitative PCR

To evaluate the expression levels of MMP-1, MMP-2, and EMMPRIN normalized to the housekeeping β 2-microglobulin gene in cultured corneal stromal cells, real-time quantitative PCR was performed using a LightCycler (Roche Diagnostics, France) according to described techniques.^{18,34} Selected sets of primers and labeled probes (Eurogentec, Biosense, Italy) are shown in Table 1. Standards for MMP-1, MMP-2, EMMPRIN, and β 2-microglobulin were prepared from total normal RNA, amplified by RT-PCR, and cloned using the TOPO II TA

Figure 1. Immunodetection of EMMPRIN in the human cornea. A-C: Confocal EMMPRIN immunostaining was observed in the peripheral (A) and mid-peripheral (B) limbal corneal epithelium and in the central human corneal epithelium (C). EMMPRIN staining was intense in the epithelial cells whereas the stroma was weakly stained. D: Corneal endothelial cells were also intensely stained. Note the gradient in EMMPRIN staining from superficial to basal cells in the central corneal epithelium, and the increased staining in the superficial peripheral corneal epithelium (b and s denote basal and superficial layers, respectively). Green, EMMPRIN staining; blue, DAPI nuclear staining. Scale bar, 25 μ m.

Figure 2. Cell surface distribution of EMMPRIN in corneal epithelial cells *in vitro*. A: Confocal imaging of EMMPRIN staining in monolayer cultures of epithelial cells. Staining in A and vertical section in inset A' show that EMMPRIN staining is distributed along the whole cell membrane and associated with small processes extending from the cells. B: EMMPRIN staining in stratified reconstituted epithelium *in vitro* shows a similar gradient to that seen in the central cornea. C: No signal was detected in control epithelium cultures when only the secondary antibody was used. Original magnifications, $\times 60$.

cloning kit (Invitrogen, France) following the manufacturer's recommendations.

Results

Distribution of EMMPRIN in the Normal Cornea

The presence and localization of EMMPRIN in the cornea was studied by confocal immunohistochemistry on 8- μ m-thick frozen human corneal sections (limbus, peripheral, and central cornea). Figure 1 shows the presence of EMMPRIN in the different regions of the cornea, with the strongest staining seen on the surface of epithelial cells and endothelial cells. Some staining can also be noted on the cell membrane of keratocytes in the corneal stroma. A gradient of EMMPRIN staining was observed in the central epithelium (Figure 1C), strongest in the basal cells, and decreased toward superficial layers. In the limbal epithelium, EMMPRIN staining (Figure 1A) appeared to follow columns of cells originating from the basal layer, presenting a staining pattern different from that in the central cornea. In addition, the most superficial layers of the limbal and mid-peripheral epithelium (Figure 1, A and B) were more intensely stained compared to the central epithelium (Figure 1C).

Distribution of EMMPRIN in Corneal Epithelial Cells in Culture

Corneal epithelial cell line in culture examined by confocal immunohistochemistry demonstrated intense immunoreactivity of EMMPRIN that was concentrated at the cell surface, forming small processes that projected from the cells (Figure 2). Confocal vertical section of the monolayer culture revealed that staining was homogeneously distributed around the cell surface (Figure 2, A and A'). Examination of reconstructed epithelium in culture, in which cells were allowed to stratify by air lifting, shows that stratification *in vitro* was associated with the formation of a gradient of EMMPRIN staining (Figure 2B) that contrasts with the homogenous staining of the monolayer cell culture, but resembles the one observed in the central epithelium of the cornea (Figure 1C), suggesting a regulation of EMMPRIN according to the differentiation state of the cells.

Regulation of EMMPRIN by Cytokines and Cell Density

The modulatory role played by cytokines on the regulation of EMMPRIN in corneal epithelial cells was assessed by immunoblot (Figure 3, A and B) and real-time PCR (Figure 3, C and D) after treating these cells with TGF- β 1, EGF, and tumor necrosis factor- α , cytokines implicated in wound healing. Immunoblot analysis of cell lysates revealed two bands at $\sim$ 40 and 60 kd corresponding to differently glycosylated forms of EMMPRIN.²⁰ An up-regulation of EMMPRIN by TGF- β 1 and EGF was obtained at both the mRNA and protein level (Figure 3, A and C).

Figure 3. Regulation of EMMPRIN in corneal epithelial cells by cytokines and cell density. Immunoblots (A, B) and real-time PCR (C, D) analysis of EMMPRIN regulation by cytokines (A, C) and cell density (B, D) in corneal epithelial cells. A and C: Cells were treated with TGF- β 1 (10 ng/ml), EGF (10 ng/ml), or tumor necrosis factor- α (10 ng/ml) in serum-free medium for 24 hours (A) or 6 hours (C). B and D: Different cell density cultures were obtained by seeding 10^6 of cells in 25-cm² (C1), 75-cm² (C2), and 175-cm² (C3) dishes. Cells were allowed to adhere for $\sim$ 24 hours to obtain confluent monolayer in the 25-cm² (C1). For immunoblots, 20 μ g of cell extracts were analyzed with either anti-EMMPRIN or anti-actin antibodies. C and D: Results are expressed as the mean of four separate experiments; bars, $\pm$ SD.

Tumor necrosis factor- α had a slight but not significant inhibition on EMMPRIN expression. To investigate the regulation of EMMPRIN as a function of the degree of confluence, cells were plated at different densities and cultured for 24 hours before immunoblot and PCR analysis. Figure 3, B and D, demonstrates an inverse regulation of EMMPRIN with cell density, with the highest level of EMMPRIN associated with sparse cultures. Similar results were obtained when cells were seeded at the same cell density and allowed to grow, EMMPRIN being analyzed each day up to confluence (not shown). Interestingly, the immunoblot analysis revealed in the sparse culture a greater increase in the higher M_r band at 60 kd (Figure 3B), suggesting a preferential up-regulation of the glycosylated form that was reported to be the most active.³⁵

Up-Regulation and Co-Localization of EMMPRIN and MMP-2 in Chronic Ulcerated Corneas

To assess the implication of EMMPRIN in wound healing we analyzed its expression and distribution, as well as that of MMP-2, in ulcerated corneas. To this end, tissue sections taken either from the center or at a distance of the ulcerated area were subjected to immunohistochemical and zymography analysis. Immunohistochemical analysis revealed a striking increase in EMMPRIN staining in the ulcerated area in all five corneas examined. This increase was predominantly observed in the wounded stroma, par-

Figure 4. Induction and co-localization of EMMPRIN and MMP-2 in the stroma at the epithelio-stromal boundary in chronic corneal ulcers. Tissue sections at a distance from the ulcerations (A–D) and in the ulcerated area (E–H) were subjected to double-labeled confocal immunohistochemistry using mouse anti-human CD147 (A, C–E, G, H; green) and rabbit anti-human MMP-2 pAb antibodies (B–D, F–H; red) and counterstained with DAPI (D and H, blue). Merged staining of EMMPRIN and MMP-2 (C, D, G, H) revealed induction of EMMPRIN and MMP-2 in the ulcerated area in the stroma at the epithelio-stromal boundary (G, H) whereas MMP-2 staining was weak without co-localization with EMMPRIN in the nonulcerated cornea (D). D and H: Higher magnification of the areas in squares in A and E. Note the absence of staining in the acellular layer of Bowman (D) and the intense staining when continuity exists between epithelium and stroma in the ulcerated area (H). Scale bar, 25 μ m.

licularly at the epithelio-stromal boundary (Figure 4). In addition, the gradient of EMMPRIN staining clearly seen in the epithelium at a distance from the wounded area that resembled closely that of normal cornea (Figure 1) was lost at the edge of the ulceration. Although only faint staining of MMP-2 could be observed in the unwounded stroma, it was markedly increased in the wounded stroma, in particular at the epithelio-stromal boundary, where MMP-2 and EMMPRIN were shown to be co-localized by double-labeled confocal immunohistochemistry. The same MMP-2 staining pattern was observed with either the monoclonal or the polyclonal MMP-2 antibody used. Some areas, which appear to correspond to the intercellular space, displayed only MMP-2 staining and may represent extracellularly located enzyme. The increased MMP-2 level in the ulcerated areas compared to nonulcerated areas was also observed by zymography (results not shown).

Corneal Epithelial Cells Up-Regulate MMP Expression in Corneal Fibroblasts through Direct Cell Contact

Because epithelial EMMPRIN is known to stimulate MMP expression in neighboring fibroblasts, we used a monolayer co-culture model of corneal epithelial cells and fibroblasts to evaluate MMP regulation in fibroblasts. For that, epithelial cell suspension was applied to one side of a sterile microscope slide and allowed to form a confluent epithelial sheet after which fibroblasts were added and

left to grow until a clear interface between the two types of cells was formed (Figure 5; A to C). Immuno-confocal analysis demonstrated a strong staining of MMP-2 on the surface of fibroblasts limited to the interface between the two cell types (Figure 5, F and J), suggesting a regulation of MMP-2 by direct interaction. This induction of MMP-2 was associated with a similar increase in EMMPRIN expression (Figure 5, E and I) and the two were co-localized in the fibroblasts at the interface with epithelial cells, as shown by the overlapping staining (Figure 5, G and K). Thus, the MMP-2 and EMMPRIN induction and co-localization at the interface between the two cell types resembles that observed at the subepithelial wounded stroma *in vivo* and advocate for an involvement of a direct cellular interaction as a mechanism of MMP-2 induction via EMMPRIN.

To further define the implication of direct cell contact in this induction, fibroblasts were incubated with purified epithelial cell membranes. Immunoblot analysis confirmed the presence of EMMPRIN in the epithelial membrane preparation (EpM) (Figure 6A) and these membranes stimulated protein secretion (Figure 6B) and mRNA expression (Figure 6C) of both MMP-1 and MMP-2 by fibroblasts. MMP-9 was not regulated by the epithelial cell membrane. No activity was detected in control experiments with isolated membranes incubated in the absence of fibroblasts (not shown). These experiments demonstrated that direct cellular contact mediated by the cell membrane can regulate MMP expression independently of secreted soluble factors. These results, together with the restricted localization of the induced MMP-2 to

Figure 5. Induction and co-localization of EMMPRIN and MMP-2 in corneal fibroblasts at the interface with corneal epithelial cells in an *in vitro* co-culture system. Epithelial cell (epi) suspension containing 10^6 in 200 μ l was applied, forming a large drop, on a sterile microscope slide that was placed in a culture dish. After cells within the drop became confluent (48 hours) forming an epithelial sheet, fibroblasts (f) were added and allowed to attach (A), proliferate, and migrate around the epithelial cells (B, C), forming after 24 to 48 hours a clear interface between the two types of cells (C). After further incubation for 24 hours, the slides were washed, fixed, and stained with DAPI (D and H, blue), anti-EMMPRIN/CD147, HIM6 mAb (E and I, green), and anti-MMP-2 pAb (F and J, red) and analyzed by confocal microscopy. G and K: Overlapping staining of EMMPRIN and MMP-2 in yellow. H–K: Higher magnifications of the epithelial/fibroblast interface shown in D–G, respectively. Original magnifications: $\times 10$ (D–G), $\times 60$ (H–K).

fibroblasts alongside epithelial cells, suggested that direct epithelio-stromal interaction (ESI) may represent an additional mechanism to that of cytokines in the regulation of MMP expression in the cornea.

MMP and EMMPRIN Induction in Corneal Fibroblasts by Epithelial Cell Membranes Is Mediated by EMMPRIN

To define the role of EMMPRIN in the induction of MMP-2 observed after fibroblasts were incubated with epithelial cell membranes, we used an EMMPRIN blocking antibody in this system. Zymography analysis of the conditioned medium demonstrated increased MMP-2 after treatment with corneal epithelial membranes (Figure 7A, lane 2) or recombinant EMMPRIN (Figure 7A, lane 4). Both inductions were markedly suppressed when anti-EMMPRIN antibody was present (Figure 7A, lanes 3 and 5). This inhibition by the antibody was confirmed using

real-time RT-PCR for both MMP-1 and MMP-2 (Figure 7B). Interestingly, we demonstrated that EMMPRIN mRNA was also induced by epithelial cell membrane and that this induction was inhibited by blocking the EMMPRIN antibody. Hence, EMMPRIN present at the surface of epithelial membrane is responsible for the induction of both MMP-2 and EMMPRIN in fibroblasts and may account for their induction at the interface with epithelial cells *in vitro* and represent a possible mechanism for MMP-2 and EMMPRIN induction *in vivo*.

Discussion

Although barely detected in normal cornea, MMPs are induced during wound healing processes. This induction may be mediated by different mechanisms because MMPs are known to be regulated by growth factors and cytokines, by integrin-mediated cell-matrix interactions, or by direct cell-cell interactions.^{5,16} EMMPRIN, a cell

Figure 6. EMMPRIN-containing corneal epithelial cell membranes stimulate MMP production in corneal fibroblasts in culture. **A:** Corneal epithelial cell membranes (EpM) were isolated by differential centrifugation and 10 μ g of membrane extract were analyzed for EMMPRIN content by immunoblotting. One major band at 60 kD and a lower M, faint band at ~40 kD, corresponding to the high and low glycosylated forms of EMMPRIN, can be observed. **B:** Fibroblast cultures (80% confluent) were incubated with 20 μ g/ml of EpM for 24 hours in serum-free medium after which the conditioned medium, 5X concentrated, was analyzed by immunoblot for MMP-1 and gelatin zymography for MMP-2. The amount of EpM added was derived from the same number of epithelial cells to that of fibroblasts. The graphs represent densitometry quantification of MMP-1 protein and MMP-2 gelatinolytic activity. **C:** Real-time PCR analysis of the same cultures as in **B** incubated with the membranes for 18 hours before RNA extraction. Results are expressed as the mean of triplicate experiments; bars, $\pm$ SD.

membrane glycoprotein enriched on epithelial cells and in particular on tumor cells, has been suggested to be responsible for MMP induction resulting from direct ESIs during carcinogenesis.^{20,22} In this investigation, we identified EMMPRIN in the cornea and demonstrated its im-

Figure 7. Anti-EMMPRIN-blocking antibody inhibits induction of fibroblasts MMPs and EMMPRIN by epithelial cell membrane. **A:** Corneal fibroblast cultures were incubated for 24 hours with 20 μ g/ml of corneal epithelial cell membranes (EpM) or with 5 μ g/ml of recombinant EMMPRIN (rec E), in the absence or presence of 20 μ g/ml of anti-EMMPRIN blocking antibody (anti E Ab) in serum-free medium, after which the conditioned medium was analyzed by gelatin zymography. The fibroblasts were preincubated with the antibody for 15 minutes before the addition of the membranes. The graph represents the densitometry quantification of MMP-2 gelatinolytic activity. **B:** Real-time PCR analysis of MMP-1, MMP-2, and EMMPRIN mRNA expression in fibroblasts incubated with EpM for 18 hours in the absence or presence of 20 μ g/ml of anti-EMMPRIN blocking antibody. Results are expressed as the mean of triplicate separate experiments; bars, $\pm$ SD.

plication in the localized MMP induction resulting from direct corneal ESI that may take place during corneal wound healing.

Direct ESI in the cornea is normally prevented by the presence of both the basement membrane and the Bowman layer.³⁶ During pathological wound healing processes, such as corneal ulcerations, breakdown of these barriers may facilitate direct ESI and favor MMP-mediated complications including corneal melts or perforations.^{6,18,37} EMMPRIN was shown, in studies using either tumor cells, EMMPRIN transfected cells, or directly the

recombinant molecule, to induce MMP-1, MMP-2, and MMP-3 in skin fibroblasts.¹⁹ These same proteinases have been detected in the subepithelial layer in corneal ulcerations/perforations of multiple origin,^{17,36} such as those associated with rheumatoid arthritis, Sjögren syndrome,²⁶ alkali burn injury,⁶ or iatrogenic corneal melts,¹⁶ suggesting that they may be induced by direct ESI in the cornea, mediated by epithelial cell EMMPRIN. Our immunohistochemical analysis identified EMMPRIN in the healthy cornea and demonstrated a marked increase in its expression in ulcerated corneas, in particular in the area of stromal thinning. MMP-2 was also induced in the wounded stroma, and the increase in both EMMPRIN and MMP-2 was concentrated at the epithelio-stromal boundary. The increase in EMMPRIN staining in the anterior stroma was concomitant with the loss of its normal polarized distribution seen in the epithelium at a distance from the wounded area, as well as in the normal corneas, with the highest expression in the basal cells, and corresponded to the disruption of the epithelial organization in this region. Furthermore, double-labeled confocal immunohistochemistry revealed a close co-localization of MMP-2 and EMMPRIN in the subepithelial area of the wounded stroma, except in areas that appear to represent the extracellular matrix, where MMP-2 predominated. This may indicate a localized induction of MMP-2 by the accumulated EMMPRIN at this region. MMP-9, although also induced in corneal ulceration, exhibited different localization and appeared to be expressed predominantly by regenerating epithelial cells and by the inflammatory cells recruited to the wounded area, as was previously shown.^{11,18,39} MMP-9, which is more implicated in the delay of epithelial wound closure,⁸ was not co-localized with EMMPRIN (results not shown), in accordance with the general consensus that EMMPRIN does not induce MMP-9 in fibroblasts.⁴⁰

The induction of both EMMPRIN and MMP-2 at the ESI interface observed *in vivo* was reproduced by our *in vitro* co-culture model, in which corneal epithelial cells and fibroblasts were allowed to come into direct contact, forming an epithelio-stromal border. The very strong staining of MMP-2 and EMMPRIN concentrated in the fibroblasts along the interface with the epithelial cells and their restricted localization is suggestive of direct cell-cell contact as a mechanism of induction. Although a paracrine regulation of MMPs involving secreted cytokines has been suggested^{3,41,42} and may also be involved to some extent in our co-culture system, it would be expected to result in a more generalized and homogenous staining. The experiments in which purified membrane preparations of epithelial cells induced both MMP-1 and MMP-2 in cultured fibroblasts confirms that EMMPRIN-containing epithelial cells are able to activate fibroblasts to produce more MMPs by direct cell-cell interaction. Furthermore, the induction of both MMP-1 and MMP-2 was greatly inhibited by a blocking antibody to EMMPRIN, indicating that the MMP induction observed in this system is mostly because of the effect of EMMPRIN. The increase in fibroblast EMMPRIN levels by the epithelial cell membrane is consistent with the increased EMMPRIN observed in the fibroblasts at the epithelio-

stromal interface, both in the ulcerated corneas and in the *in vitro* co-culture model. The inhibition of EMMPRIN induction by the blocking antibody to EMMPRIN suggests that EMMPRIN itself was primarily responsible for its own induction in the fibroblasts. This is in agreement with the previously reported demonstration of a positive feedback regulation of EMMPRIN.⁴³ However the importance of this new MMP-induction mechanism during corneal wound healing remains to be demonstrated in animal models *in vivo*.

In the normal cornea, EMMPRIN was detected mainly in the epithelium and endothelium. In the central epithelium, it was particularly concentrated in the basal cells with a gradual decrease toward the superficial cells. When isolated epithelial cells in culture were immunostained for EMMPRIN, a homogenous membrane staining was observed. However, the basoapical gradient observed in stratified epithelial cells *in vitro*, as in the central cornea *in vivo*, suggests that EMMPRIN expression may depend on the differentiation state of the epithelial cells, known to implicate cytokines and MMPs.^{9,44} Indeed, the growing list of MMP substrates, including cytokines, cell adhesion molecules, and signaling molecules, suggests that in addition to degrading the extracellular matrix, these proteases also contribute to communication between cells and their microenvironment and influence cell behavior such as cell growth and differentiation. However, despite the known role of EMMPRIN in regulating MMPs, an MMP-independent function in these cells cannot be excluded.

Although the cell surface localization of EMMPRIN both in cultured cells and tissue sections has been clearly demonstrated by our immunofluorescence staining as well as by numerous other studies, the release of a small fraction of soluble active EMMPRIN, either by proteolytic cleavage or by vesicular shedding has been previously described in tumor cell culture medium.^{43,45} The fact that soluble EMMPRIN retains its activity suggests that if EMMPRIN shedding can indeed occur *in vivo*, it may also be involved in mediating and modulating ESI in a diffusible paracrine manner, in a similar way to cytokines during corneal wound healing.

Although much attention was paid into the regulation of MMPs by EMMPRIN, little is known about how EMMPRIN itself is regulated and the nature of the factors that may induce its expression. During our investigations *in vitro*, we first observed an enhanced expression of EMMPRIN when corneal epithelial cells *in vitro* were incubated in the presence of serum (data not shown). We were able to demonstrate that at least two cytokines, TGF- β 1 and EGF, positively regulate EMMPRIN in these cells. The effect of EGF is in agreement with our previous results identifying EGFR signaling as critical for EMMPRIN regulation in mammary tumor cells.³⁴ Interestingly, the receptors for these two cytokines, T β R and EGFR, were also shown to concentrate in the basal layers of the corneal epithelium and were shown to be up-regulated during epithelial cell migration during wound closure.^{46,47} Taken together with the present results, it suggests that a complex regulation existing between EGFR signaling, cell-cell interaction, and EMMPRIN to regulate the prolif-

erative and migratory state of the basal epithelial cell. In addition, cell density, which has previously been shown to regulate the functional expression of TGF- β receptors in corneal epithelial cells,⁴⁶ also appears to be an important regulatory mechanism controlling EMMPRIN levels.

On the basis of these results the following scenario may be postulated on the possible events that follow deep wounding involving direct ESI. The decrease in epithelial cell density and the accumulation of cytokines known to be secreted during wound healing, such as TGF- β and EGF, would increase EMMPRIN levels in the epithelial cells in the wounded area. This can in turn induce fibroblasts, by direct interaction, to increase their own level of EMMPRIN. The resultant MMP induction would trigger either stromal remodeling to restore tissue homeostasis, or excessive MMP release and stromal destruction in chronic situations in which healing is delayed and direct ESI persists. The implication of EMMPRIN in stromal cell activation in response to injury, by increasing both MMPs and EMMPRIN, suggests that inhibition of EMMPRIN may represent a promising future therapeutic strategy in situations of excess extracellular matrix degradation associated with chronic wound healing.

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Chapter 7

Pre-validation of a new in vitro reconstituted human cornea model to assess the eye irritating potential of chemicals.

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Prevalidation of a new in vitro reconstituted human cornea model to assess the eye irritating potential of chemicals

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Abstract

This multicentre study aimed at evaluating the reliability (reproducibility) and relevance (predictivity) of a new commercially available human corneal epithelial (HCE) model (SkinEthic Laboratories, Nice, France) to assess acute ocular irritation. A prevalidation approach (protocol optimisation, transfer and performance) was followed and at each of the four participating laboratories, 20 coded reference chemicals, covering the whole range of irritancy, were tested. The compounds were applied topically to the HCE cultures and the level of cytotoxicity (tissue viability and histological analysis) was determined. Once a standardised protocol was established, a high level of reproducibility between the laboratories was observed. In order to assess the capability of the HCE model to discriminate between irritants (I) and non-irritants (NI), a classification prediction model (PM) was defined based on a viability cut-off value of 60%. The obtained in vitro classifications were compared with different in vivo classifications (e.g. Globally Harmonised System) which were calculated from individual rabbit data described in the ECETOC data bank. Although an overall concordance of 80% was obtained (sensitivity = 100% and specificity = 56%), the predictivity of the HCE model substantially increased when other sources of in vivo and in vitro data were taken into account.

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Keywords: Prevalidation; Alternative method; Eye irritation

1. Introduction

Adequate risk assessment in the context of ocular safety is mainly related to the generation of quality toxicological data on the eye irritation potential of chemicals, pharmaceuticals and cosmetics. These data are essential when establishing procedures for the safe handling, packing and transport of chemicals. Assessment

of the acute eye irritation potential is therefore included in international regulatory requirements for the testing of chemicals. Irritation testing using laboratory animals has largely remained unchanged for many years. The Draize eye irritation test (Draize et al., 1944) became a governmentally endorsed methodology and is described in OECD testing guideline 405 (OECD, 2002), which involves a standardised protocol for instilling materials onto the cornea and conjunctiva of rabbits. However, advances in ocular toxicology are challenging the validity, precision and relevance of the Draize eye irritation test (Wilhelmus, 2001). Significant levels of

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variability were observed since the test is based on a subjective (visual) scoring procedure (Weil and Scala, 1971). Besides the fact that the test causes considerable discomfort and pain to animals, it is also recognised that the response in the rabbit is not always predictive of that found in humans (Curren and Harbell, 2002). Taken together, both ethical and scientific reasons stimulated the development and validation of several alternative *in vitro* methods to assess eye irritation (Curren and Harbell, 1998; Balls et al., 1999; Worth and Balls, 2002).

Since the corneal epithelium provides an important barrier function and is the first point of contact when potentially hazardous material enters the eye, several *in vitro* models have been developed to assess the effects of chemicals on multilayered epithelial tissues. These models are generally reconstructed from human epidermal or corneal cells (primary or immortalised). Under appropriate conditions, the epithelial cells stratify and differentiate into three-dimensional non-keratinised structures after seeding on a specialised substrate. Test materials are applied directly on the epithelial tissue for a specified period of time and injury can be assessed by monitoring changes in cell viability (cytotoxicity), histology and/or the release of specific cytokines (e.g. interleukin-1 α and interleukin-6). Besides the possibility of analysing multiple endpoints (MEA: multiple endpoint analysis), these *in vitro* models have the advantage of assessing both water-soluble and water-insoluble materials. The selected test compounds can be tested either neat or at their final 'in use' concentration. One of the available reconstructed epithelial tissue alternatives is the human corneal epithelial (HCE) model of SkinEthic Laboratories (Nice, France). The reconstituted HCE model consists of immortalised human corneal epithelial cells cultured in chemically defined medium on an inert permeable polycarbonate insert at the air liquid interface. Histologically, the construct appears as a multilayered, stratified epithelium with an overall thickness of 60 micrometer, similar to that of the normal human corneal epithelium (Nguyen et al., 2003). Ultra-structurally, numerous intermediate filaments, desmosomal and hemidesmosomal junctions were found and Western blot analysis showed the presence of the cornea-specific differentiation marker cytokeratin-3 (Nguyen et al., 2003). Similar reconstituted human epithelial constructs have been reported to allow routine screening of cosmetic test formulations to assess eye irritation (Doucet et al., 1998, 1999; Courtellemont et al., 1999). These studies showed that the SkinEthic epithelial model was able to correctly identify 100% of the test products into two classes (irritant or non-irritant) by setting appropriate *in vitro* cut-off values.

The main objective of this study was to evaluate the relevance (predictivity) and reliability (reproducibility)

of the HCE model for ocular irritation testing of chemicals. This small-scale interlaboratory study, involving four participating laboratories, was based on the established ECVAM prevalidation scheme (Curren et al., 1995; Fentem and Balls, 1997; Worth and Balls, 2002) which addresses protocol optimisation (Phase I), protocol transferability (Phase II) and protocol performance (Phase III). In general, prevalidation is designed to prepare a test model for further progression to a formal validation study.

A detailed protocol was agreed on and made available to the participating laboratories. Test chemicals, covering the whole irritancy range, were selected from the ECETOC data bank (ECETOC, 1998) for eye irritation and were applied directly onto the surface of the epithelial cultures for different exposure periods (10, 20, 30 and 60 min). The induced cytotoxicity was monitored as a decreased MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction relative to the negative control cultures.

The percentage of viability was then calculated and both intra- and interlaboratory reproducibility was assessed. The model's predictivity was initially evaluated by investigating the relation of the *in vitro* HCE viabilities with the *in vivo* Modified Maximum Average Scores (MMAS), a scoring system which quantifies effects on the cornea, iris and conjunctiva. These scores are reported in the ECETOC data base and are used to rank the chemicals for eye irritation potential. However, the *in vivo* MMAS value is not considered to be the most suitable endpoint for the final evaluation of *in vitro* test models since recovery and/or irreversibility are not taken into account (Prinsen, 1999). Moreover, the use of the MMAS score has also been criticised since this scoring system shows considerable variability especially in the middle part of the irritation scale (Prinsen, 1999). For these reasons, *in vivo* classifications were assigned for each chemical based on individual rabbit data described in the ECETOC data bank. Individual tissue (cornea, conjunctiva and iris) scores were used to classify the chemicals according to the criteria of the European Union (EEC, 1993) and the recently implemented Globally Harmonised System (GHS) of classification (United Nations, 2003). In order to evaluate the predictivity of the HCE model to discriminate between irritant and non-irritant chemicals, a classification prediction model (PM) based on tissue viability was defined. To assess the validity of the assay, the HCE *in vitro* classification was compared with the GHS (United Nations, 2003). Besides the ECETOC report, additional (internet) sources of *in vivo* data (Toxnet; European Chemicals Bureau) and results obtained in other alternative test models (e.g. Bovine Corneal Opacity and Permeability test; Slug Mucosal Irritation test), were included in the final validity assessment of the HCE model.

2. Materials and methods

2.1. Media and reagents

The HCE cultures, maintenance medium and wash solution (saline phosphate buffered solution A) were all supplied by SkinEthic Laboratories (Nice, France). MTT was purchased from Sigma–Aldrich.

2.2. Test chemicals

The test chemicals were chosen primarily on the basis of eye irritation classifications derived from individual in vivo rabbit data described in the ECETOC database (ECETOC, 1998). Details on the 20 selected chemicals, together with their in vivo eye irritation classification, are shown in Table 1. The test materials covered the whole range of eye irritation potencies and represented different chemical classes. Chemicals with the same specification (e.g. purity) as those tested in vivo were obtained wherever feasible.

2.3. Control articles

Negative (Solution A vehicle) and positive control (0.5% and 1% w/v sodium dodecyl sulphate, SDS, Sigma–Aldrich) cultures were run in each assay to monitor intra- and interlaboratory variation and to provide the necessary data for defining possible acceptance criteria.

2.4. Participants and organisation

The four participating laboratories were Johnson and Johnson Pharmaceutical Research and Development (Belgium, coordinating laboratory), Novartis Pharma (Switzerland), Pfizer Global R&D (France) and SkinEthic Laboratories (France). During the whole project, team meetings were organised on a regular basis to discuss the obtained data and adjust the protocol where necessary. For Phase III, each laboratory received 20 blind coded chemicals, a detailed protocol and standard raw data forms on which all experimental variables (e.g. room temperature) and protocol deviations were recorded. The chemicals tested in Phase III were coded by the University of Ghent (Belgium) and distributed to the participating laboratories. The results of Phase III testing were submitted to the coordinating lab (Johnson and Johnson Pharmaceutical R&D) for decoding and quality control. Independent data analysis (statistics) was performed by the University of Ghent (Belgium) using SPSS software (v. 11.0). Data reporting forms (Microsoft Excel format) were agreed in advance, so that these could be submitted to the coordinator and statistician in consistent electronic formats.

2.5. The reconstituted human corneal epithelium (HCE)

To evaluate the irritating potential of different test chemicals, the reconstituted human corneal epithelial

Table 1
Selected test chemicals

Test chemical	CAS No.	Supplier	Purity (%)	In vivo Draize		
				MMAS ^a	EU ^b	GHS ^c
1 3,3-Dimethylpentane	562-49-2	Aldrich	99	0.0	NI	NI
2 3-Methoxy-1,2-propanediol	623-39-2	Acros	98	0.0	NI	NI
3 Polyethylene glycol 400	25322-68-3	Aldrich	–	0.0	NI	NI
4 Glycerol	56-81-5	Sigma	99.5	1.7	NI	NI
5 Methyl cyclopentane	96-37-7	Fluka	>95	3.7	NI	NI
6 Tween 20	9005-64-5	Sigma	–	4.0	NI	NI
7 Methyl isobutyl ketone	108-10-1	Fluka	>99	4.8	NI	NI
8 Toluene	108-88-3	Acros	≤99.5	9.0	NI	NI
9 Methyl amyl ketone	110-43-0	Aldrich	99	10.5/16.3	NI	NI
10 2-Methyl-1-pentanol	105-30-6	Acros	98.5	13.0	NI	2
11 Ethanol	64-17-5	Merck	≤99.8	24.0	NI	2
12 Sodium hydroxide (1%) ^d	1310-73-2	Merck	≤99	25.8	R36	2
13 Triton X-100 (5%) ^d	9002-93-1	Acros	SG	32.3	R36	2
14 1-Octanol	111-87-5	Aldrich	99	41.0	R36	2
15 2-Ethyl-1-hexanol	104-76-7	Aldrich	99	51.3	R36	2
16 n-Hexanol	111-27-3	Acros	98	64.8	R36	2
17 Acetone	67-64-1	Fluka	–	65.8	R36	2
18 Cyclohexanol	108-93-0	Aldrich	99	79.8	R41	1
19 Cetylpyridinium bromide (6%) ^d	140-72-7	Sigma	>99	85.8	R41	1
20 Benzalkonium chloride (10%) ^d	8001-54-5	Sigma	Ultra	108.0	R41	1

SG: scintillation grade.

^a Modified Maximum Average Score (ECETOC, 1998). For methyl amyl ketone, two tests were reported.

^b EU classification (EEC, 1993). NI: non-irritant; R36: irritating to the eye; R41: risk of serious damage to eyes.

^c GHS classification (United Nations, 2003). NI: non-irritant; category 2: irritating to the eye; category 1: irreversible effects on the eye.

^d Fixed amount of neat compound was distributed and solution was freshly prepared before treatment.

(HCE) model from SkinEthic Laboratories (Nice, France) was used. Immortalised human corneal epithelial cells (provided by Prof. Roger Beuerman, LSU Eye Center, New Orleans, USA) were cultured on an inert 0.5 cm^2 polycarbonate filter at the air liquid interface in chemically defined medium (Nguyen et al., 2003). All tissues were manufactured under high quality conditions (GMP-like) and each lot of tissues is Quality Assured according to specific QC standards including histology, tissue thickness, tissue viability, reproducibility and safety. A Quality Control Certificate was provided with every shipment of tissue. The inserts containing the human epithelial cultures were shipped in a multiwell plate filled with an agarose-nutrient solution in which they were embedded. Maintenance medium and saline phosphate buffered solution A, cooled with gel refrigerant, was shipped along with the human tissue models.

2.6. Culture preparation

After arrival, the human corneal epithelial tissue cultures were placed in $300\text{ }\mu\text{l}$ fresh maintenance medium (24-well plate) and, after the absence of air bubbles underneath the culture inserts was verified, incubated (overnight) at 37°C , $5\%\text{ CO}_2$ in a humidified incubator. Following this equilibration period, the cultures were transferred into a new 24-well plate (containing $300\text{ }\mu\text{l}$ SkinEthic maintenance medium in each well) for treatment. Since the nutrients in the medium are supplied through the basal cell layers, the surface of the corneal epithelium could be treated topically with the test material.

2.7. Culture treatment and viability determination

Quadruplicate *in vitro* cultures (0.5 cm^2) were placed in $300\text{ }\mu\text{l}$ fresh maintenance medium and dosed topically with $30\text{ }\mu\text{l}$ of each test chemical for different time points (10, 20, 30 and 60 min). The physical state of all tested chemicals was considered to be liquid (solution/suspension) in nature and each chemical was administered carefully onto the epithelial surface so that the complete area was covered. Non-hydrosoluble compounds or compounds that did not spread easily were spread carefully with a specially prepared (smooth curved) sterile glass Pasteur pipette. Negative control (Solution A) and positive control (0.5% and 1% w/v SDS) treated tissues were included in each experiment. At each time point, triplicate tissues were assessed for tissue viability (MTT assay), and one culture was fixed in a balanced 10% v/v formaldehyde solution and embedded in paraffin for histological analysis.

Following incubation, three tissues were carefully rinsed with Solution A (saline phosphate buffer) and transferred to a freshly prepared MTT solution

(0.5 mg/ml in maintenance medium; $300\text{ }\mu\text{l}$) in 24-well plates. MTT is a yellow tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) that is reduced to a purple formazan by mitochondrial succinate dehydrogenase in viable cells (Mossman, 1983). The plates were then incubated for 3 h at 37°C in a humidified atmosphere of $5\%\text{ CO}_2$ in air. The HCE tissues were removed from the MTT solution, blotted on absorbent material, and immediately transferred to a 24-well plate containing isopropanol ($750\text{ }\mu\text{l}$ per well). An additional $750\text{ }\mu\text{l}$ isopropanol was added onto each tissue where after the plate was sealed with parafilm to prevent isopropanol evaporation. The plates were gently shaken for at least 90 min at room temperature (in some cases overnight) to extract the reduced MTT (formazan crystals) out of the tissue. Three $200\text{ }\mu\text{l}$ aliquots of the extracted MTT solution were transferred to a flat-bottom 96-well plate and the optical density (OD) of each well was measured at 570 nm with a microtiter plate reader. The percentage viability of each of the treated cultured was calculated from the percentage MTT conversion in the test chemical treated cultures relative to the corresponding negative controls (100% viable). The following equation was used: percentage viability = $[\text{individual OD}_{\text{chemical}} / \text{mean OD}_{\text{negative control}}] \times 100$.

In general, all cultures were handled gentle, precise and short in order to avoid drying and damaging the tissues.

2.8. Histology

For each of the tested products or controls, at the end of each test period, one culture was rinsed and fixed in a 10% v/v formaldehyde solution. After overnight incubation at 4°C , the polycarbonate filter was carefully cut out of its plastic ring and the fixed tissue was divided in two parts with a sharp scalpel. Each half culture was stored in fixation solution until paraffin embedment. Finally, all tissues were further processed by the Histology Department of SkinEthic Laboratories and $4\text{ }\mu\text{m}$ vertical sections were prepared, stained with hematoxylin/eosin, and photographed under a microscope for further evaluation. To fulfil the experimental quality criteria, the negative control epithelial tissues must have a constant thickness devoid of terminally differentiated cells, and a regular and compact shape. In positive control cultures, most of the upper cell-layers of the epithelial tissues must be disintegrated. A (basic) histological evaluation of the treated epithelia obtained from the four laboratories was performed.

2.9. *In vivo* data and classification

Animal data for the 20 test chemicals were collected from the ECETOC Eye Irritation Reference Chemicals

Data Bank (ECETOC, 1998). This technical report includes comprehensive individual rabbit eye irritation data obtained on 132 chemicals of high purity covering different chemical classes and different degrees of irritancy. The chemicals have been tested in at least three rabbits and *in vivo* data have been generated since 1981. Dosing was by instillation of 0.1 ml into the conjunctival sac and observations were made at least at 24, 48 and 72 h after treatment. Effects on the cornea, iris and conjunctiva were quantified according to a weighted scoring system and the chemicals were ranked for eye irritation potential on the basis of a Modified Maximum Average Score (MMAS). This score represents the maximum average score calculated at 24 h or more following instillation.

In this prevalidation study, the individual rabbit data were used to assess the ocular hazard of the selected chemicals according to different classification schemes. Initially, the EU classification (EEC, 1993) was calculated and classified the chemicals as NI (non-irritating), R36 (irritating to eyes) or R41 (risk of serious damage to eyes). In addition, the recently implemented Globally Harmonised System (GHS) for classification (United Nations, 2003) was applied and classified the chemicals as NI (non-irritating), category 2 (irritating to eyes) or category 1 (irreversible effects on the eye). Additional information and background materials of the GHS can be found at <http://www.unece.org/trans/danger/publi/ghs/ghs.html>. This GHS classification was used to finally evaluate the predictive capacity of the *in vitro* HCE model to discriminate between irritants (GHS categories 1 and 2) and non-irritants.

2.10. Protocol refinement (Phase I)

The objective of Phase I was to refine and optimise the experimental protocol. The first preliminary experiments were performed based on the experimental protocol previously reported for testing cosmetic formulations (Doucet et al., 1999). However, the obtained data (not shown) demonstrated that modifications were needed since the 3 h and 24 h treatment times showed no discriminative power (all tissues were dead or no effect was observed for non-irritants). Consequently, the following exposure times were identified for testing chemicals: 10, 20, 30 and 60 min. In a face to face meeting, the participating laboratories discussed further protocol details and outlined the procedure for Phase II.

2.11. Protocol transfer (Phase IIa)

The main objective of Phase II was to assess transferability of the protocol defined in Phase I and to determine the interlaboratory reproducibility of the assay. For this purpose, five chemicals were selected (numbers

1, 2, 4, 7 and 14 in Table 1) and distributed to the four laboratories. Besides protocol transferability, also the sensitivity of the model was evaluated. Therefore, mainly non- to mild irritants were randomly selected. These chemicals, together with the negative control (Solution A) and positive controls (0.5% and 1% w/v SDS) were tested at four different time points (three cultures/time point, treatment at 37 °C).

After treatment, the relative viabilities were calculated and the interlaboratory reproducibility was evaluated by the coefficient of variation (CV) for the laboratory means. Additionally, one-way ANOVA was used to determine statistically significant differences between the mean viabilities obtained in the four laboratories. The normality of the residuals was tested with a Kolmogorov–Smirnov test. The homogeneity of variances was tested with Levene's test. If the variances were found not to be equal the data were transformed to their logarithm. To further compare the effects of the laboratories a multiple comparison among pairs of means was performed using a Scheffé test with $p < 0.05$ as significance level.

2.12. Further protocol optimisation (Phase IIb)

In order to simplify the experimental procedure (especially when short treatment times are used), it was investigated if cultures could be treated at room temperature. To evaluate the possible effect of temperature, 12 chemicals were selected and tissue cultures from the same batch were treated both at 37 °C and at room temperature by laboratory D. Altman Bland plots were used to assess the agreement between both temperature conditions and to identify eventual differences (Bland and Altman, 1987). For each chemical the difference between the viability measurements (room temperature – 37 °C) are plotted against their mean ((room temperature + 37 °C)/2).

To evaluate (preliminary) the predictivity of the HCE model, the relation between the obtained MTT-viabilities at both temperature conditions, with the *in vivo* MMAS values was investigated. In case a non-linear relationship was observed, the MTT-viabilities were transformed to acquire a linear relationship, then the Pearson correlation coefficient was determined to identify the best correlated time point. Consequently, it was investigated if one single exposure time could predict the irritating potential of the chemicals under investigation. This enabled us to establish a simple and straightforward test procedure, an approach which could lead to a reduced number of tissue cultures required in the final test protocol (cost and time saving effect).

At this stage, all participants again discussed the obtained results and identified possible improvements to be included in a final Phase III protocol.

2.13. Protocol performance (Phase III)

In Phase III, both the reproducibility and relevance (predictivity) of the assay were evaluated by using a standardised protocol based on the results obtained in the previous phases. Therefore, 20 compounds were independently coded and distributed by the University of Ghent to the four participating laboratories. In order to provide the necessary safety data, each laboratory received a sealed envelope containing information on chemical identities and classifications together with the corresponding codes for each laboratory. All chemicals were tested at room temperature at four different time points (four cultures/time point) together with the negative control (Solution A) and positive controls (0.5% and 1% w/v SDS). One culture was used for histological analysis. Four experimental runs were performed (5 test chemicals/run) and different tissue lots were used for each run. After treatment, the relative viabilities were calculated and the interlaboratory reproducibility was again evaluated by the coefficient of variation (CV) and using one-way ANOVA followed by a Scheffé post hoc test. For the positive controls intra- and interlaboratory reproducibility was assessed by a two-way ANOVA with replication, followed by a Scheffé post hoc test. If a significant interaction between the lab and the run was present, the simple effects of the run within each lab were investigated with a Bonferroni post hoc test.

To evaluate the predictive capacity of the HCE model to discriminate between irritants (I) and non-irritants (NI), two classification prediction models (PMs) were defined based on empirically determined viability cut-off values after 10 min treatment. When the relative viability (expressed by %MTT reduction of the control) is ≥ 50 or 60%, the test chemical is predicted NI. In order to identify the most relevant cut-off value, both reproducibility and relevance (predictivity) of the PMs were determined. The reproducibility of the classification PMs was analysed by calculating the κ -value, which is a measure of agreement between the classifications according to the different laboratories. A κ -value of 0.75 or higher suggest strong agreement above chance (Fleiss, 1981).

To assess the relevance of both PMs, the relation between the in vitro-derived irritancy classifications and those assigned based on in vivo data (GHS classification) was assessed by the use of contingency tables according to Holzhütter et al. (1996). Table statistics were calculated and the following parameters were determined: sensitivity (the percentage of I chemicals identified correctly); specificity (the percentage of NI chemicals identified correctly); positive and negative predictivity (the percentage of predictions for a particular classification which were correct) and concordance (the overall percentage of correct classifications) (Cooper et al., 1979).

2.14. Defining assay acceptance criteria for future studies

The scientific value of a toxicological test system (model and methodology) essentially depends on the quality and batch reproducibility (e.g. thickness) of the reconstituted tissue model. Criteria for evaluating the experimental outcome from these and future experiments, were based on the control data collected in this prevalidation study. Besides the epithelial tissue thickness, also the obtained MTT-derived OD-values after a 10-minute treatment with Solution A were analysed. To define acceptance criteria for this endpoint, the 95% prediction interval to contain future observations was determined (Hahn and Meeker, 1991). Further, the concentration-related effects induced by the positive controls (0.5% and 1% w/v SDS) were taken into account. In order to quantify these effects, the relative reduction in viability between both SDS formulation was evaluated.

3. Results

3.1. Protocol refinement (Phase I)

Preliminary experiments identified critical phases in the procedure and demonstrated (data not shown) that long treatment times (3 h and 24 h) had no discriminative power. Exposure times of 10, 20, 30 and 60 min were acknowledged and the experimental outline for Phase II was agreed on in a face to face meeting between all the participating laboratories.

3.2. Protocol transfer (Phase IIa)

Although HCE tissue cultures were treated with the test materials for periods of 10, 20, 30 and 60 min, only the results of the 10 min are reported since the final analysis in Phase III is based on the shortest exposure time. Besides the positive control solutions, five chemicals were tested and the relative viabilities were determined (Table 2). The positive controls (0.5% and 1% w/v SDS), induced a clear time-effect (data not shown) and concentration-effect in the four laboratories (Table 2). For both treatments, a Coefficient of Variance (CV) of 11% and 12% was obtained respectively (Table 2). This low level of interlaboratory variability was confirmed by one-way ANOVA testing, which revealed no statistically significant effect of the laboratory. In addition, both 3-methoxy-1,2-propanediol and glycerol induced no significant differences between the mean viabilities of the four laboratories, while two homogeneous subgroups (a and b) were identified ($p > 0.05$) after treating the cultures for 10 min with 3,3-dimethylpentane, methyl isobutyl ketone and 1-octanol. When the variation was assessed by the %CV, a high level (62%) was observed for methyl isobutyl ketone.

Table 2
Interlaboratory reproducibility of the viability (%) after 10 min treatment

Test chemical	J&JPRD ^a	Novartis ^a	Pfizer ^a	SkinEthic ^c	Mean ^d	%CV
0.5% SDS ^a	67.6 ± 8.6	83.7 ± 5.6	67.8 ± 12.4	80.5 ± 3.5	74.9 ± 8.4	11
1% SDS ^a	45.2 ± 6.7	46.6 ± 9.3	35.7 ± 10.2	47.0 ± 1.5	43.6 ± 5.3	12
1 3,3-Dimethylpentane	63.2 ± 5.9 ^{a,b}	53.8 ± 7.9 ^b	82.8 ± 9.1 ^a	74.8 ± 3.5 ^a	68.7 ± 12.8	19
2 3-Methoxy-1,2-propanediol ^a	80.2 ± 4.5	86.7 ± 6.6	73.6 ± 15.5	77.6 ± 0.6	79.5 ± 5.5	7
4 Glycerol ^a	95.4 ± 4.1	90.8 ± 10.9	101.9 ± 6.4	83.4 ± 3.7	92.8 ± 7.8	8
7 Methyl isobutyl ketone	8.8 ± 0.8 ^b	28.7 ± 2.8 ^a	10.6 ± 6.8 ^b	33.6 ± 6.9 ^a	20.4 ± 12.6	62
14 1-Octanol	5.7 ± 1.9 ^b	9.7 ± 2.2 ^{a,b}	9.7 ± 3.8 ^{a,b}	13.6 ± 1.7 ^a	9.7 ± 3.2	33

CV = coefficient of variation for the laboratory means.

^a One-way ANOVA testing revealed no statistically significant effect of the laboratory.

^{a,b} Mean viabilities with the same superscript are not significantly different from each other ($p > 0.05$, Scheffé test).

^c Mean ± SD, $n = 3$.

^d Mean ± SD, $n = 4$.

3.3. Further protocol optimisation (Phase IIb)

To assess the impact of treatment temperature (room temperature vs. 37 °C), tissue cultures were treated with 12 different chemicals for 10, 20, 30 and 60 min and the MTT viabilities were determined relative to the negative control cultures (Table 3). SDS was tested in three different concentrations (0.5%, 1% and 3% w/v). For both room temperature and 37 °C, a time-response effect was observed for most chemicals, although the effect was more pronounced for the experiments performed at room temperature and, as expected, less present for the non- and very severe irritants. In addition, SDS also induced a concentration-response effect at 37 °C and at room temperature. When both treatment conditions were compared, the viabilities obtained at room temperature were generally somewhat higher than those obtained at 37 °C as was demonstrated by the Altman Bland plots (Fig. 1). Especially the chemicals which induced an intermediate viability (between 20% and 70%) showed higher

viabilities at room temperature in comparison with 37 °C. The difference between both temperatures was most pronounced for the 10 min (Fig. 1A) and 20 min (data not plotted) treatment time and decreased in function of time, as is illustrated by the mean difference (continuous line) in Fig. 1B which is close to zero after 60 min treatment. Further, the discriminative power to rank the chemicals over the complete viability range, decreased also in function of time. At room temperature, the entire viability range was better covered (Table 3).

When the in vitro MTT-viabilities were compared with in vivo MMAS data, a non-linear relationship was observed. For this reason, the MTT-data were transformed to the inverse (1/MTT; Fig. 2) where after the Pearson correlation with the MMAS values was calculated for each treatment time (Table 4). When looking for the most predictive treatment time, the best correlation was obtained for those cultures which were exposed for 10 min. The correlation at room temperature was somewhat lower than at 37 °C (0.81 vs. 0.89), but this

Table 3
Tissue viabilities (%) determined at room temperature (RT) and at 37 °C after different treatment times (SkinEthic Laboratories)

Test chemical	MMAS ^a	RT				37 °C			
		10 min	20 min	30 min	60 min	10 min	20 min	30 min	60 min
1 3,3-Dimethylpentane	0.0	83.9	95.7	52.2	42.3	77.0	58.6	46.1	24.0
2 3-Methoxy-1,2-propanediol	0.0	87.1	75.7	72.5	63.5	82.6	88.0	70.4	69.8
3 Polyethylene glycol 400	0.0	82.0	99.6	87.1	85.5	91.2	93.2	85.3	77.5
4 Glycerol	1.7	100.9	96.5	101.6	101.9	101.5	102.8	108.5	101.3
5 Methyl cyclopentane	3.7	64.5	36.9	26.4	11.3	34.8	27.8	15.9	10.8
7 Methyl isobutylketone	4.8	47.4	12.6	1.5	4.7	20.4	5.4	3.7	5.0
10 2-Methyl-1-pentanol	13.0	6.3	5.9	5.2	4.8	6.2	4.9	5.4	3.9
14 1-Octanol	41.0	6.9	4.2	4.0	2.7	6.2	4.9	4.0	3.1
15 2-Ethyl-1-hexanol	51.3	28.4	15.9	7.5	3.7	9.4	4.7	3.8	3.2
16 1-Hexanol	64.8	4.7	3.8	4.3	2.9	4.3	4.2	3.7	2.8
18 Cyclohexanol	79.8	2.9	2.8	2.6	2.1	3.0	3.0	2.4	1.8
SDS 0.5%	–	78.4	70.7	38.6	24.3	67.6	47.0	44.2	34.1
SDS 1%	–	43.0	25.6	7.5	3.3	43.2	11.5	9.1	10.9
SDS 3%	16.0	6.6	1.5	2.1	1.1	6.3	1.9	1.7	1.2

^a Modified Maximum Average Score (ECETOC, 1998).

A

B

Fig. 1. Altman Bland plot of the difference between the viabilities obtained at room temperature (RT) and at 37 °C after a 10-minute (A) and 60-minute (B) treatment against the mean viability (continuous line = mean difference between both conditions; dashed lines = mean $\pm$ 2SD, representing the approximate 95% limits of agreement).

Fig. 2. Relation between the transformed in vitro (1/MTT) viabilities (10 min treatment) and in vivo MMAS scores, both at room temperature (RT) and at 37 °C. Regression lines are shown.

was mainly related to a single outlier (2-ethyl-1-hexanol). Without the outlier, a comparable (and higher)

correlation coefficient was observed for both temperature conditions (0.92 vs. 0.94).

3.4. Protocol performance (Phase III)

The aim of this final phase was to assess the performance (reproducibility and predictivity) of the HCE model. For this reason, each laboratory tested 20 blind coded chemicals at room temperature in four independent experimental runs. In each run, five chemicals were tested with the corresponding controls. Although it was shown in Phase II that the 10-minute treatment data were the most predictive, all chemicals were again tested at four different time points. This allowed us to enlarge the available data set and to confirm previous findings. Only the short treatment time data were reported.

In all laboratories, the positive controls induced a clear decrease in tissue viability in a time- (data not shown) and concentration-related manner. Since 0.5% and 1% w/v SDS solutions were tested in each run, the intra- and interlaboratory reproducibility was assessed. Two-way ANOVA revealed a significant interaction ($p < 0.001$) between the lab and the experimental runs for the 0.5% w/v SDS treatment, therefore the simple effects were investigated. Within each lab significant differences between the runs were observed (Table 5). Since there was no significant interaction for the 1% w/v SDS treatment (Table 6), the main effect can be investigated. There was no significant difference between the runs, however the viability at SkinEthic Laboratories was significantly higher when compared to the other labs (Scheffé, $p < 0.05$).

In Table 7, the relative viabilities for the 20 coded chemicals after 10 min treatment are shown and the (interlaboratory) variability was assessed. The viabilities obtained in the different laboratories were highly correlated as was demonstrated by the high correlation coefficients (Pearson correlation, $n = 20$) calculated between the four labs ($r \geq 0.82$) (Table 8). Moreover, the slopes of the regression lines were close to 1, which means the values obtained in the different labs were of the same magnitude. These findings were confirmed by ANOVA testing, which showed no significant differences between the viabilities obtained for eight chemicals in the different labs. The viability of the HCE cultures was significantly higher at SkinEthic Laboratories than at the other labs for nine compounds, and for seven of these compounds there were no significant differences between J&JPRD, Pfizer and Novartis. For three compounds there were at random significant differences between the laboratories. When only J&JPRD, Pfizer and Novartis were considered, the results were statistically comparable for 15 compounds. The coefficient of variation (CV) showed a mean value of 53%, while without SkinEthic, the mean CV was 35%. The interlaboratory relation of the viabilities obtained after a 10-minute

Table 4
Correlation between the in vitro viability (1/MTT) and the in vivo MMAS after different treatment times (SkinEthic Laboratories)

Pearson correlation	RT				37 °C			
	10 min	20 min	30 min	60 min	10 min	20 min	30 min	60 min
All chemicals ($n = 12$)	0.81	0.44	0.30	0.49	0.89	0.55	0.57	0.57
Without outlier 2-ethyl-1-hexanol	0.92	0.52	0.35	0.51	0.94	0.55	0.56	0.58

Table 5
Intra- and interlaboratory reproducibility of the viability (%) after 10 min treatment with 0.5% SDS

Laboratory	Run ^c				Main effect	
	1	2	3	4	Lab	%CV
J&JPRD	46.9 ± 7.7 ^{a,b}	36.3 ± 2.4 ^b	53.1 ± 5.9 ^{a,b}	58.6 ± 6.8 ^a	48.7 ± 9.5	20
Novartis	61.6 ± 1.9 ^{a,b}	52.5 ± 5.0 ^b	58.5 ± 1.5 ^{a,b}	66.8 ± 3.7 ^a	59.8 ± 6.0	10
Pfizer	75.3 ± 7.2 ^a	45.1 ± 6.9 ^b	31.6 ± 5.6 ^b	18.7 ± 16.5 ^b	42.7 ± 24.3	57
SkinEthic	83.2 ± 3.3 ^a	76.5 ± 5.3 ^{a,b}	82.8 ± 7.4 ^a	65.3 ± 3.6 ^b	76.9 ± 8.3	11
Main effect run	66.7 ± 15.2	52.6 ± 16.2	56.5 ± 19.6	52.4 ± 22.0	57.0 ± 15.0	26

Two-way ANOVA revealed a significant interaction ($p < 0.001$) between the lab and the run, therefore simple effects were investigated. Only the simple effects within the labs were displayed.

^{a,b} Within each lab, mean viabilities with the same superscript are not significantly different from each other ($p > 0.05$, Bonferroni).

^c Mean ± SD, $n = 3$.

Table 6
Intra- and interlaboratory reproducibility of the viability (%) after 10 min treatment with 1% SDS

Laboratory	Run ^d				Main effect	
	1	2	3	4	Lab	%CV
J&JPRD	27.2 ± 1.1	20.3 ± 0.4	21.5 ± 6.0	26.7 ± 6.4	23.9 ± 3.5 ^{b,c}	15
Novartis	28.3 ± 5.1	32.1 ± 1.0	32.3 ± 4.4	37.7 ± 3.5	32.6 ± 3.8 ^b	12
Pfizer	19.5 ± 14.3	10.5 ± 5.1	8.2 ± 1.3	11.9 ± 1.8	12.5 ± 4.8 ^a	39
SkinEthic	61.6 ± 13.3	56.8 ± 9.3	67.7 ± 1.8	51.6 ± 6.6	59.4 ± 6.8 ^a	11
Main effect run	34.2 ± 19.0 ^a	29.9 ± 18.6 ^a	32.4 ± 23.3 ^a	32.0 ± 15.8 ^a	32.1 ± 20.0	62

Two-way ANOVA revealed no significant interaction ($p > 0.05$), therefore the main effects were further investigated.

^{a,b,c} Mean viabilities with the same superscript are not significantly different from each other ($p > 0.05$, Scheffé test).

^d Mean ± SD, $n = 3$.

treatment are shown in Fig. 3. As an example, the comparison between J&JPRD and Novartis is given in Fig. 3A, while the increased viability levels in SkinEthic compared to the mean values obtained in the other three laboratories are shown in Fig. 3B.

The relevance (predictive capacity) of the HCE model was evaluated by defining different classification PMs based on a viability cut-off value of 50% or 60%. In order to identify the most relevant PM, κ -statistics were used to evaluate the reproducibility of the different PMs (Table 9). When 50% was used as cut-off value, J&JPRD classified three chemicals differently when compared with SkinEthic (methyl isobutyl ketone, toluene and methyl amyl ketone). Pfizer and Novartis classified four chemicals differently when compared with SkinEthic (methyl cyclopentane, methyl isobutyl ketone, toluene and methyl amyl ketone). There was a perfect agreement between Pfizer and Novartis. J&JPRD classified only 1 chemical differently when compared to Pfizer and Novartis. When 60% was selected as cut-off value there was a

much higher agreement between the laboratories resulting in a perfect agreement between J&JPRD, Pfizer and Novartis. SkinEthic classified only 1 chemical (methyl isobutyl ketone) different from the other labs.

In vivo classification based on the EU criteria identified eleven non-irritants and nine irritants; while based on the GHS classification, both 2-methyl-1-pentanol and ethanol were identified as irritants (Table 1). Since the GHS classification was found to be more realistic (see discussion), the relevance of both PMs was assessed by comparing the obtained in vitro classification with the in vivo GHS classification (Table 10). Table statistics were calculated and in both cases, a maximum level (100%) of sensitivity was obtained and thus, all irritating chemicals were classified correctly. When the 50% cut-off value was used, a better specificity was obtained for J&JPRD and SkinEthic. However, although the overall accuracy of this first PM is somewhat higher, the second PM (60% cut-off) was preferred since it showed higher interlaboratory reproducibility as was shown in Table 9.

Table 7
Interlaboratory reproducibility of the viability (%) after a 10-minute treatment

Test chemical	J&PRD ^d	Novartis ^d	Pfizer ^d	SkinEthic ^d	Mean ^c	%CV
1 3,3-Dimethylpentane	64.2 ± 3.5 ^b	70.0 ± 6.8 ^b	73.8 ± 5.3 ^b	117.6 ± 14.6 ^a	81.5 ± 24.4	30
2 3-Methoxy-1,2-propanediol [*]	75.3 ± 13.3	74.2 ± 6.3	97.0 ± 1.1	82.2 ± 1.3	82.2 ± 10.5	13
3 Polyethylene glycol 400 [*]	96.5 ± 4.4	100.6 ± 5.5	92.5 ± 5.0	87.6 ± 10.4	94.3 ± 5.6	6
4 Glycerol [*]	97.4 ± 5.1	94.8 ± 2.6	89.1 ± 2.2	91.2 ± 2.6	93.1 ± 3.7	4
5 Methyl cyclopentane	55.8 ± 3.8 ^a	46.7 ± 2.3 ^{a,b}	44.0 ± 2.5 ^b	52.5 ± 2.3 ^{a,b}	49.8 ± 5.4	11
6 Tween 20 [*]	90.1 ± 2.1	68.5 ± 15.3	86.3 ± 2.0	73.3 ± 0.9	79.5 ± 10.2	13
7 Methyl isobutyl ketone	1.7 ± 1.9 ^b	13.7 ± 11.2 ^b	22.1 ± 11.3 ^b	65.2 ± 3.2 ^a	25.6 ± 27.6	108
8 Toluene	19.9 ± 0.8 ^b	22.2 ± 2.4 ^b	15.0 ± 4.0 ^b	55.6 ± 0.3 ^a	28.1 ± 18.5	66
9 Methyl amyl ketone	7.5 ± 0.8 ^c	18.5 ± 3.3 ^b	15.3 ± 1.9 ^{b,c}	55.9 ± 3.6 ^a	24.3 ± 21.6	89
10 2-Methyl-1-pentanol [*]	3.3 ± 0.1	4.6 ± 0.7	3.0 ± 0.3	7.3 ± 3.7	4.6 ± 1.9	42
11 Ethanol [*]	3.7 ± 0.1	5.2 ± 0.1	3.7 ± 0.2	12.5 ± 5.8	6.3 ± 4.2	67
12 Sodium hydroxide (1%)	0.4 ± 0.0 ^b	7.7 ± 5.1 ^{a,b}	20.0 ± 4.6 ^a	1.1 ± 0.3 ^b	7.4 ± 9.1	124
13 Triton X-100 (5%) [*]	5.2 ± 4.6	2.3 ± 1.0	2.0 ± 0.6	5.0 ± 0.8	3.6 ± 1.7	46
14 1-Octanol	3.3 ± 0.4 ^b	7.1 ± 0.1 ^b	5.6 ± 2.1 ^b	24.0 ± 0.6 ^a	10 ± 9.5	95
15 2-Ethyl-1-hexanol	23.2 ± 2.4 ^b	25.2 ± 4.7 ^b	11.0 ± 0.4 ^c	45.1 ± 3.2 ^a	26.1 ± 14.1	54
16 n-Hexanol	3.2 ± 0.2 ^b	4.9 ± 0.2 ^b	4.2 ± 0.4 ^b	8.4 ± 1.5 ^a	5.2 ± 2.3	44
17 Acetone	3.9 ± 0.2 ^b	5.7 ± 1.5 ^b	3.4 ± 0.2 ^b	16.8 ± 3.9 ^a	7.4 ± 6.3	85
18 Cyclohexanol [*]	1.8 ± 0.2	1.8 ± 0.1	11.3 ± 7.9	6.6 ± 0.4	5.4 ± 4.5	83
19 Cetylpyridinium bromide (6%)	12.9 ± 2.5 ^{a,b}	11.0 ± 1.2 ^b	20.4 ± 2.6 ^a	21.4 ± 2.8 ^a	16.4 ± 5.2	32
20 Benzalkonium chloride (10%)	5.2 ± 0.3 ^b	6.7 ± 0.3 ^b	6.2 ± 0.4 ^b	14.9 ± 5.1 ^a	8.2 ± 4.5	55

^{*} One-way ANOVA testing revealed no statistically significant effect of the laboratory.

^{a,b,c} Groups with the same superscript are not significantly different from each other ($p > 0.05$, Scheffé test).

^d Mean ± SD, $n = 3$.

^e Mean ± SD, $n = 4$.

Table 8
Reproducibility of the viability after a 10-minute treatment

	J&PRD	Novartis	Pfizer	SkinEthic
J&PRD	–	0.98	0.96	0.82
Novartis	0.91 (3.5)	–	0.97	0.87
Pfizer	0.93 (4.4)	0.92 (0.9)	–	0.84
SkinEthic	0.84 (–6.7)	0.83 (–5.3)	0.84 (–4.1)	–

Note: Values above the dashes are the Pearson correlation coefficients ($n = 20$); values below the dashes are slopes of the linear regression lines; and values between the brackets are the intercepts. The correlation coefficients and slopes of the regression lines are significant ($p \leq 0.001$).

3.5. Assay acceptance criteria

In order to standardize data interpretation, acceptance criteria were defined based on results obtained according to the standardised protocol for the 10 min treatment time at room temperature. Since preliminary experiments demonstrated (data not shown) that low (<0.5) or high (>2.0) MTT-derived OD-values in negative control cultures can influence the relative viabilities in the positive controls, the 95% prediction interval was calculated (based on 23 experiments; $n = 66$). Consequently, future experiments will be considered valid when the mean OD-value of the negative control cultures fell between 0.700 and 1.150. In the current study, all negative control cultures used in Phase III fell within this range. For both positive controls (0.5% and 1% w/v SDS), a concentration-related decrease in the relative viability must be observed. The effects induced by both SDS formulations were quantified and based on 23 experiments (66 cultures from 9 different production

batches), performed between November 2001 and February 2003. The mean reduction in viability induced by 1% SDS (compared to 0.5% SDS) was $45\% \pm 19\%$.

Further, the thickness of the tissues of our valid experiments before transport was $65 \pm 10 \mu\text{m}$ (reported on the Quality Control Certificate included in the tissue delivery package).

3.6. Histological evaluation (Phase III)

When the histopathology was assessed for the vehicle control cultures, no histological alterations were observed. As expected, the positive controls (0.5% and 1% w/v SDS) induced reproducible effects in a time- and concentration-related manner (Fig. 4). In general, the effects ranged from alterations in superficial cell layers to complete detachment of the epithelium from the polycarbonate substratum. Chemicals classified as non-irritating by the PM60, induced no histological changes in the epithelial tissues and thus confirmed that no false

A

B

Fig. 3. Relation between the laboratories for the viability after a 10-minute treatment. The line of equality is shown. (A) J&JPRD vs. Novartis; (B) SkinEthic vs. mean of three other labs (the numbers correspond with the chemical's number as indicated in Table 7).

Table 9
Reproducibility of the classification prediction models (viability at 10 min)

	J&JPRD	Novartis	Pfizer	SkinEthic
J&JPRD	–	0.88 ± 0.12	0.88 ± 0.12	0.69 ± 0.16
Novartis	1.00 ± 0.00	–	1.00 ± 0.00	0.58 ± 0.17
Pfizer	1.00 ± 0.00	1.00 ± 0.00	–	0.58 ± 0.17
SkinEthic	0.88 ± 0.12	0.88 ± 0.12	0.88 ± 0.12	–

Note: Values above the dashes are the κ -statistics of PM1 (cut-off 50%) (significant at $p \leq 0.01$). Values below the dashes are the κ -statistics of PM2 (cut-off 60%) (significant at $p \leq 0.001$).

MTT-negatives were present. On the other hand, all irritating compounds induced various histological change (e.g. desquamation, vacuolisation, tissue disintegration, necrosis and/or detachment from polycarbonate sub-

Table 10
Relevance of the prediction models (viability at 10 min)

	J&JPRD	Novartis	Pfizer	SkinEthic
Sensitivity	100/100	100/100	100/100	100/100
Specificity	67/56	56/56	56/56	100/67
Positive predictivity	79/73	73/73	73/73	100/79
Negative predictivity	100/100	100/100	100/100	100/100
Accuracy	85/80	80/80	80/80	100/85

Note: Table statistics for PM1 (cut-off 50%)/PM2 (cut-off 60%).

stratum) hereby confirming the effects observed by quantitative viability (MTT) analysis. Consequently, this endpoint provided no added predictive value and it was decided not to include a detailed histology analysis in our proposed prediction model.

4. Discussion

The use of in vitro methods as screening tests to assess the eye irritating potential of test compounds is widespread in industry, since in-house evaluation studies have proven their usefulness. However, several multi-centre validation studies in which in vitro test results were compared with historical animal data, were not completely successful (Worth and Balls, 2002). One of the main reasons is related to the fact that the standard in vivo Draize test is based on a subjective scoring of tissue lesions in the eye, which provides variable estimates of ocular irritancy (Balls et al., 1999). Because of the subjective nature of the scoring and the inter-animal differences, a computer simulation revealed that, even if the alternative methods were perfectly reproducible ($CV = 0$), the variability in the maximum average scores (MAS) alone would restrict Pearson's correlation coefficients to the range 0.89–0.95 when these Draize scores are between 0 and 100, and to the range 0.65–0.80 when the Draize scores are between 0 and 40 (Bruner et al., 1997). Furthermore, the in vivo rabbit eye test may not be an appropriate model, because of the fundamental differences between the rabbit and human eye. Rabbits have a nictitating membrane (third eyelid); lack of significant tearing and a thinner cornea (Curren and Harbell, 2002). Due to these differences, Draize data correlate poorly with actual human experience. Indeed, the limited available human data have demonstrated the inadequacy of the Draize test. Freeberg et al. (1986a) reported 218 human ocular toxicity exposures to 14 household products, and they compared the findings to Draize test results. The human data differed from the Draize results significantly and the correlation coefficient between rabbit and human response was only 0.48 with $p > 0.1$. When animal data cannot be reliably extrapolated to man, substances “proven” safe in lab animals may in fact be dangerous to people. To simulate more closely accidental human exposures, the standard

Fig. 4. Histological appearance (hematoxylin–eosin staining, $\times 20$) of the human corneal epithelium after 10 min treatment with solvent control (normal epithelium, no histological changes); 0.5% and 1% w/v SDS (concentration-related changes in thickness and desquamation).

Draize test was refined (Griffith et al., 1980) and modifications were made in the amount of test material (0.01 ml vs. 0.1 ml) dosed and site of application (cornea vs. conjunctival sac). The low-volume eye test was reported to be less stressful to rabbits and more predictive for human ocular irritation (Freeberg et al., 1986a,b). However, *in vivo* animal models will always be compromised by the anatomical and physiological differences *between the experimental animal and man*. Since the purpose of any *in vitro* method is to predict human eye response and since (1) *in vitro* technologies constantly improve and (2) better test systems with objective endpoints can be developed, the *in vivo* Draize test can no longer hold his position as a “gold standard”. Although the limitations described above act currently as relevant hurdles in the evaluation of new *in vitro* test methods for eye irritation, it is our responsibility as toxicologists to continue to perform science driven research and dare to challenge the established ways of hazard identification. Indeed, qualitative risk assessment is directly depending on good hazard identification.

When taking these aspects into consideration, the use of human reconstituted *in vitro* models can help to better predict human eye irritation. The study described in this report must be regarded as an attempt to evaluate

the usefulness of a new 3-dimensional *in vitro* model for ocular safety testing and this in the context of the aspects described above. The four participating laboratories agreed to follow a prevalidation approach (protocol optimisation, transfer and performance) and assessed the reliability (reproducibility) and relevance (predictivity) of the HCE (Human Corneal Epithelial) tissue model. Twenty reference chemicals were selected (ECETOC, 1998) and although the limitations of the available animal data were known, individual tissue scores were used to classify the compounds into different eye irritation categories.

4.1. Protocol transfer

Protocol transferability was shown and good levels of interlaboratory reproducibility were obtained. Variability was initially assessed by the use of the Coefficient of Variation (CV) and a mean value of 22% was obtained. Although chemical no. 7 (methyl isobutyl ketone) showed a relatively high mean CV (62%), the same was observed in the animal test. When the inter-animal variability ($n = 4$) was determined based on the individual scores (MMAS) in the ECETOC data base, a CV of 63% was calculated. ANOVA testing revealed significant

differences between the viabilities of the different laboratories for three chemicals. However the differences observed for methyl isobutyl ketone and 1-octanol were considered to be biologically irrelevant since low viabilities were obtained in the 4 laboratories. Therefore, the test system was considered stable. At the end of Phase II, it was clear that the method was both reproducible and transferable.

4.2. Protocol optimisation

Based on the results obtained, a standardised protocol was put together and the optimal treatment temperature (room temperature) and treatment time (10 min) were identified. The HCE model required small amounts of test compound which allows a higher throughput when screening compounds for eye irritation. Treatment at room temperature creates a certain degree of flexibility especially when short exposure times are used for screening purposes. Moreover, at room temperature, the complete viability range was better covered which resulted in an improved discriminative power to rank the chemicals. Although 10 min was identified as the most relevant treatment time, cultures were treated for 10, 20, 30 and 60 min in all further phases in order to confirm these initial findings.

4.3. Protocol performance

The levels of reproducibility were confirmed in Phase III where ANOVA testing identified eight chemicals for which there were no significant differences between the viabilities obtained in the different laboratories. Although a mean CV of 53% ($n = 20$; 4 labs) was calculated, it was clear that compounds that induced lower viabilities showed high CVs (>40%). This indicates that the CV alone is not the most optimal endpoint to estimate variability.

The significantly higher viabilities observed at SkinEthic Laboratories for nine compounds, might be explained by the fact that the tissue cultures treated in this laboratory were not transported throughout Europe. Transport by aeroplane probably could have influenced the sensitivity of the epithelia (e.g. temperature and air pressure). When the SkinEthic data were excluded from the statistical analysis, 15 chemicals could be identified without significant differences between the viabilities obtained in the different laboratories.

A simple and straightforward PM was based on the obtained viability levels after a 10-minute treatment time. The reproducibility of the PM was illustrated by the use of κ -statistics, a measure of agreement between the classification according to the different laboratories. Since a perfect concordance between J&JPRD, Pfizer and Novartis was obtained when 60% viability was selected as cut-off value, this PM was used to further eval-

uate the predictive capacity of the HCE model when using the GHS classification system. The relatively low specificity values are probably related to the fact that the *in vivo* Draize test data reported in the ECETOC data base (1998) underestimate the irritation potential of three chemicals (no. 7, 8 and 9). This emphasizes the danger of using single Draize test data (originating from three or four animals) in (pre)validation studies. Methyl amyl ketone (chemical no. 9) for example, induced individual animal scores ranging from 4 to 24 (ECETOC databank), the latter score giving an irritant classification. For ethanol (MMAS = 24), a comparable situation exists since one animal was classified as non-irritant (score of 11), one as borderline (score of 22) and one as irritant (score of 39). Considering these inadequate data sets, additional *in vivo* and *in vitro* data were collected in order to better assess the eye irritating potential of the selected chemicals.

4.4. Additional *in vivo* data and other alternative tests for eye irritation

Irritation classifications obtained with the *in vitro* HCE model for chemicals 7, 8 and 9, were confirmed by additional *in vivo* data available in peer-reviewed literature and/or from freely available internet resources (e.g. <http://toxnet.nlm.nih.gov>). Additionally, the HCE outcome was also evaluated against *in house* (J&JPRD) classifications obtained in the Bovine Corneal Opacity-Permeability (BCOP) test (Vanparys et al., 1993; Gautheron et al., 1994) and the Slug Mucosal Irritation test (Adriaens et al., 2005). In Table 11, a classification overview of different available eye irritation data for the 20 chemicals is given. The HCE classification was based on the mean viability value from the four laboratories in combination with the 60% viability cut-off PM. The non-irritancy of chemicals 1, 2, 3, 4 and 6 and the irritancy of chemicals 12–20 was confirmed in all test models. For methyl cyclopentane (chemical no. 5), a classification of (borderline) irritancy was determined by the HCE model (mean viability ~50%) and mild effects were seen in the BCOP assay (*in vitro* Score = 7.5).

Surprisingly, chemical numbers 7, 8 and 9 were classified as non-irritants by the GHS. However, irritating effects were observed in all three alternative test models: HCE, BCOP and the Slug Mucosal Irritation test. Additionally, when other available data were taken into account, the irritating toxicological profile of these three chemicals could be confirmed.

Methyl isobutyl ketone (chemical no. 7) was labelled R36 (irritating to eyes) in the MSDS (Material Safety Data Sheet) of the supplier and the compound was described (peer-reviewed literature) as an eye irritant in the Hazard Substances Data bank (TOXNET®): undiluted methyl isobutyl ketone (0.1 ml) produced some irritation within 10 min when instilled in the rabbit

Table 11
Irritation potential of the test chemicals: in vivo/in vitro comparison.

Test chemical	GHS ^a	HCE ^b	BCOP ^c	SMT ^d
1 3,3-Dimethylpentane	NI	NI	NI (non)	NI
2 3-Methoxy-1,2-propanediol	NI	NI	NI (non)	NI
3 Polyethylene glycol 400	NI	NI	NI (non)	NI
4 Glycerol	NI	NI	NI (non)	NI
5 Methyl cyclopentane	NI	I	NI (mild)	NI
6 Tween 20	NI	NI	NI (non)	NI
7 Methyl isobutyl ketone ^e	NI	I	NI/I (mild/moderate)	R36
8 Toluene ^e	NI	I	I (moderate)	R36
9 Methyl amyl ketone ^e	NI	I	I (moderate)	R41
10 2-Methyl-1-pentanol	2	I	I (severe)	R36
11 Ethanol	2	I	I (moderate/severe)	NI
12 Sodium hydroxide (1%)	2	I	I (severe)	R41
13 Triton X-100 (5%)	2	I	I (severe)	R41
14 1-Octanol	2	I	I (severe)	R36
15 2-Ethyl-1-hexanol	2	I	I (moderate)	R36
16 <i>n</i> -Hexanol	2	I	I (severe)	R36
17 Acetone	2	I	I (severe)	R36
18 Cyclohexanol	1	I	I (severe)	R36
19 Cetylpyridinium bromide (6%)	1	I	I (moderate)	R41
20 Benzalkonium chloride (10%)	1	I	I (severe)	R41

^a GHS classification (United Nations, 2003). NI: non-irritant; category 2: irritating to the eye; category 1: irreversible effects on the eye (based on individual animal data from ECETOC database; 1998).

^b HCE viability classification prediction model: NI (viability $\geq 60\%$), I (viability $< 60\%$); $n = 4$.

^c BCOP classification based on the In Vitro Score (IVS): NI (IVS ≤ 25), I (25 < IVS ≤ 55) [non (IVS ≤ 3), mild (3 < IVS ≤ 25), moderate (25 < IVS ≤ 55) and severe (IVS > 55)].

^d Classification based on the Slug Mucosal Irritation Test (Adriaens et al., 2005).

^e Additional in vivo data from literature showed the eye irritating potential of the chemical (see text).

eye. Inflammation and swelling occurred in 8 h, and inflammation, swelling and exudate were present at 24 h (Clayton and Clayton, 1993–1994). In humans, methyl isobutyl ketone odor was objectionable and the vapor was irritating to the eyes at 200 ppm. The eye irritating potential of methyl isobutyl ketone was confirmed by other alternative test systems (Table 11). Duplicate BCOP experiments classified the chemical as mild to moderate, as an in vitro score of 22.7 and 28.3 was obtained respectively. It must be noted that in the BCOP test, an in vitro score of 25 is the cut-off value to discriminate between mild and moderate irritants. The Slug Mucosal Irritation test classified the compound as an eye irritant (R36). Gilleron et al. (1997) reported methyl isobutyl ketone as a severe eye irritant when using the HET-CAM (Henn's Egg Test-Chorioallantois Membrane) test.

The European Union Risk Assessment Report on Toluene (chemical no. 8) could be consulted via the European Chemicals Bureau web-site (<http://ecb.jrc.it>) and described ocular lesions (redness and chemosis) which were at the limit for R36 classification. In addition, Sugai et al. (1990) classified toluene as a moderate to severe rabbit eye irritant with corneal involvement (Draize test) and Guillot et al. (1982) found toluene to be irritating to the rabbit eye. Although there are no reports of human eye contact to liquid toluene, data of human eye irritation in response to vapour indicate that

the findings from animal studies are relevant for humans. The eye irritating potential of toluene was again confirmed by other alternative test systems (Table 11). The BCOP test classified the chemical as a moderate irritant (borderline of severe), since an in vitro score of 54.0 was obtained. The Slug Mucosal Irritation test classified the compound as an eye irritant (R36). Gilleron et al. (1997) reported toluene as an extreme eye irritant when using the HET-CAM test.

Methyl amyl ketone (chemical no. 9) was rated 2 on an irritation scale of 10 in rabbit eyes, and a slight to moderate irritation on the depilated skin of guinea pigs was described after a 24 h contact (Hazard Substances Data bank/TOXNET®). In the same data bank, the vapour was described as irritating to human eyes, nose and throat, while the liquid induced skin and eyes irritation. The BCOP test classified the chemical as moderate, as an in vitro score of 39.9 was obtained. The Slug Mucosal Irritation test classified the compound as a severe eye irritant (R41); (personal communication Adriaens).

In vivo classification based on the EU criteria identified both 2-methyl-1-pentanol and ethanol as non-irritants (Table 1) while based on the GHS criteria and the different alternative test methods, these chemicals were identified as eye irritants. 2-Methyl-1-pentanol (chemical no. 10) was rated 8 on an irritation scale of 10 in rabbit eyes (Clayton and Clayton, 1981–1982). In addition, the chemical was labelled R41 on the Material

Safety Data Sheet of the supplier. Based on the individual data in the ECETOC report, the mean time to clear all effects was 5.7 days. The BCOP test classified the chemical as severe, as an *in vitro* score of 76.6 was obtained. The Slug Mucosal Irritation test labelled the compound as R36. Ethanol (chemical no. 11) was rated 3 on an irritation scale of 10 and application of 70% alcohol to rabbit corneas injured and temporarily loosened the corneal epithelium (Grant, 1986). Weil and Scala (1971) described that ethanol was found to be an irritant in nineteen laboratories, a non-irritant in 3 and questionable in 2. A splash on human eye causes immediate burning and stinging discomfort with reflex closure of the lids (Grant, 1986). Based on the individual data in the ECETOC report, the mean time to clear all effects was 5.3 days. The BCOP test classified the chemical as moderate to severe, as *in vitro* scores ranging from 51.0 to 76.6 were observed. Surprisingly, no irritation effects were seen in the Slug Mucosal Irritation test, where the irritation potency of the chemicals is estimated during two contact periods of 60 min with a 1% and 3.5% w/v concentration of the test substance, respectively. However concentration response studies showed that a 10% (w/v) ethanol concentration induced severe membrane damage whereas concentration up to 10% w/v PEG 400, propylene glycol and glycerol did not induce irritation of the slug mucosa (Adriaens et al., 2005). Gilleron et al. (1997) reported ethanol as a severe eye irritant when testing it in the HET-CAM assay. Considering all the irritation data of 2-methyl-1-pentanol and ethanol, the GHS classification was found to be more realistic compared to the EU classification.

Taking into account the additional *in vivo* and *in vitro* irritation data, six real non-irritants were tested with the HCE model of which five were correctly identified and only 1 (methyl cyclopentane) was slightly over predicted. This approach resulted in a higher specificity (83%) without changing the sensitivity level (100%) of the *in vitro* HCE model. Consequently, an overall concordance of 95% was obtained. Therefore, the relevance of the HCE model markedly increased and resulted in an enhanced predictivity to discriminate between irritant and non-irritant chemicals. This approach made it clear that irritation scores from a single Draize test (ECETOC, 1998) are clearly insufficient on their own. Therefore, it is recommended that for future full validation exercises, the irritant/non-irritant classifications of the selected test chemicals should be independently evaluated using all available data prior to the start of the study.

In this study, histological analysis confirmed the classifications based on the MTT-viability and provided no added predictive value. Due to the subjective nature of the endpoint, histology was not included in our proposed prediction model. However, the evaluation of his-

tological changes when irritants fail to induce a significant reduction in the MTT-determined viability, can be a possible strategy in order to prevent false negatives in future studies.

4.5. Conclusion

This study illustrated that in the process of validating new *in vitro* test methods, the concept of prevalidation plays a critical role. The approach as reported by Curren et al. (1995) was used as a guidance during the different phases of the multicentre evaluation of the Human Epithelial Cornea model. This prevalidation study demonstrated the applicability of objective endpoints (e.g. MTT-viability) and provided a standardised detailed test protocol. The *in vitro* HCE model was found to be a quality controlled and easy to perform *in vitro* test system that was characterised by a high level of reproducibility (reliability). In accordance with recent EC-VAM and ICCVAM initiatives, it became clear that the selection of quality *in vivo* data on eye irritation is one of the major issues when (pre)validating new *in vitro* test methods. However, despite the known limitations of the available *in vivo* data, the proposed *in vitro* prediction model showed an adequate predictive capacity to discriminate between eye irritants and non-irritants (relevance). In conclusion, the authors are of the opinion that the *in vitro* HCE model is a good alternative test model for eye irritation and that a proof of principle was provided.

In order to increase the discriminative power of the assay, additional protocol modifications (e.g. treatment time, diluted test chemicals, cytokine analysis, etc.) may be considered.

Further, it is advised to check the suitability of the prediction model on a broader range of chemical classes and applications. For this purpose, additional testing can be performed hereby including for example acids, organic bases, phenols, electrophilic organic chemicals and inorganic chemicals. Subsequently, a full validation study should be initiated assessing further the usefulness of the MTT parameter as single and robust endpoint to discriminate between irritating and non-irritating test compounds. In this case, it is advised still to include histological analysis, especially when a broader range of chemical classes are tested.

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Chapter 8

Characterization of in vitro reconstituted human oral and gingival 3D epithelial cell cultures.

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Abstract

The present research describes the characterization of commercially available 3-dimensional (3D) oral mucosal and gingival epithelial constructs. The 3D oral mucosa construct was obtained by cultivating immortalized human oral epithelial cells (TR-146 cell line) at the air liquid interface in a chemically defined medium on a polycarbonate substrate for 7 days. The 3D gingival mucosa construct was obtained by cultivating primary human gingival epithelial cells (isolated from human gingival biopsy material) using similar culture conditions (SkinEthic Laboratories, Nice, France). A histological evaluation using light and electron microscopy was performed to compare the *in vitro* 3D constructs with human biopsy material. Both oral and gingival 3D cultures appeared as a multilayered stratified epithelium resembling respectively the normal oral lingual mucosa and gingival attached epithelium. Expression of tissue and site-specific differentiation markers involucrin, filaggrin and keratin-13 demonstrated a close resemblance of the *in vitro* 3D constructs with the human *in vivo* biopsies. Since these 3D human gingival and oral epithelium constructs are produced in a standardized and highly reproducible fashion, their use may reduce the need for animal experiments required to study the toxicology and inflammation potency of dental and orthodontic materials as well as oral care products. Structural differences however between both models suggest that different results may be obtained when used for *in vitro* toxicology studies.

Introduction

In humans, the gingival epithelia, which cover the underlying connective tissues, can be categorized into at least three different types based on their location and composition (Figure 1).

Figure 1: Anatomic relationship of the gingival tissues with the teeth. Clinical landmarks include the free gingiva at the cervical margin of the teeth (FGM); the interdental papilla (IP); and the mucogingival junction (MGJ), which separates the attached gingiva (AG) from the alveolar mucosa (AM).

The oral epithelium extends from the mucogingival junction to the tip of the gingival crest and is subdivided into the free marginal gingiva and the attached gingiva. The sulcular epithelium lines the gingival sulcus and extends from the tip of the gingival crest to coronal portion of the junctional epithelium. The junctional epithelium extends from the base of the gingival

sulcus to an arbitrary point approximately 2.0 mm coronal to the alveolar bone crest and is closely adapted to the tooth surface to form sealing and attachment structures. These three epithelia demonstrate ultrastructural differences (Schroeder and Listgarden, 1997). The oral mucosa consists of a stratified squamous keratinizing epithelium between 0.2 and 0.3 mm in thickness composed of the basal layer (stratum basale), the prickle cell layer (stratum spinosum), the granular layer (stratum granulosum) and the keratinized layer (stratum corneum) (Schroeder HE, 1991; Bartold *et al.*, 2000). Cells other than those of ectodermal origin are also be found in the oral epithelium of the gingiva and include Langerhans cells, melanocytes and Merckel cells (Newcomb GN and Powel RN, 1986, Mayer TC, 1973, Ramieri *et al.* 1992). The sulcular epithelium is the epithelial lining of the gingival sulcus, which is a small crevice of approximately 0.5 mm and often parakeratinized. Finally the junctional epithelium forms the tissue attachment from the gingival to tooth structures. This specialized epithelium ranges in thickness from a few cells at its most apical portion to 15-30 at its most coronal portion. Only two morphotypes of epithelial cell are evident in the junctional epithelium. The cells of the stratum basale proliferate rapidly, while those of the suprabasal layer have no mitotic capacity. Within the junctional epithelium the gaps between cells appear to be larger than in either the gingival or sulcular epithelium (Schroeder HE and Münzel-Pedrazzoli S, 1970). Throughout the junctional epithelium, polymorphonuclear leukocytes as well as lymphocytes are being found (Tonetti MS *et al.*, 1995; Tonetti MS *et al.*, 1998).

An orthodontic treatment procedure involves the use of different materials such as wires of different alloys, composite materials, plastics and many others. These materials are intended to stay in the mouth for different periods of time and they are subjected to several physical effects, including corrosion and abrasion caused by eating habits, temperature differences, exposure to chemicals and reaction with saliva (Eliades T. and Athanasiou AE, 2002). Additionally, metal components from almost all dental cast alloys can be detected in adjacent tissue (Garhammer *et al.*, 2003).

Assessing the toxic potential of these different materials is of critical importance prior to their usage in orthodontic treatment. Today no validated *in vitro* model exists to assess the potential toxicity of dental materials on oral mucosa. Some *in vitro* toxicology studies on dental materials were performed on monolayer cells (Schmalz *et al.*, 2000; Tomakidi *et al.*, 2000; Mockers O. *et al.*, 2002).

Monolayer cell cultures however have several disadvantages since they lack the differentiated architectural structure of the *in vivo* epithelial tissue (Nussler A.K. *et al.*, 2001). Schmalz *et al.* describe a commercially available model system based on a recombined co-culture of human fibroblasts and human epithelial cells. Tissue viability and prostaglandin E2 release from the cells were used as biochemical markers for determining the irritation potential of dental test materials (Schmalz *et al.*, 1997). In other studies, parameters of epithelial physiology such as electric resistance have been used to describe the toxic effect of dental materials (Kruszewski FH *et al.*, 1997; Burstein NL and Klyce SD, 1977) and the use of organotypic cultures of gingival cells has been suggested as a model for short-term tissue compatibility studies of

dental materials (Tomakidi *et al.*, 2003). Today industry and toxicology laboratories are actively seeking for alternative methods to replace and reduce the expensive animal as well as clinical testing. The use of cultured 3D human tissue models, produced under controlled laboratory conditions in a standardized fashion, provides an attractive tool for this purpose: in fact, the use of reconstituted human epithelial constructs has been reported for the assessment of possible toxic effects dental materials (Nahid M and Bottenberg P, 2003).

The purpose of the present study was to describe and characterize the *in vitro* 3-dimensional oral mucosa (RHOE) and gingival tissue constructs (RHGE) compared to *in vivo* biopsies of different regions in the mouth, both at light microscopical and transmission electromicroscopical level, as well as through the identification of tissue-specific differentiation markers using quantitative reverse transcriptase polymerase chain reaction (QRT-PCR).

Materials and Methods

Reconstituted human oral and gingival epithelial tissue models

The reconstituted human epithelial tissue models (SkinEthic Laboratories, Nice France) consist of airlifted, living, multi-layered epithelial tissue constructs of oral and gingival mucosa. The reconstituted human oral mucosal constructs (RHOE) were obtained by cultivating epithelial cells from the TR146 cell line (isolated from a squamous cell carcinoma) on polycarbonate inserts in serum-free and chemically defined medium for 7 days. The reconstituted human gingival epithelial tissues (RHGE) were obtained by cultivating primary human gingival epithelial cells using the same procedures. The inserts containing the RHOE and RHGE were shipped from SkinEthic Laboratories at room temperature in a multi-well plate filled with an agarose-nutrient solution in which they are embedded. The maintenance culture medium (SkinEthic Laboratories), cooled with gel refrigerant, was shipped along with the models. This medium is a chemically defined MCDB 153 based medium containing 5µg/ml insulin, 1.5 mM CaCl and 25µg/ml gentamycin. Both RHOE and RHGE tissue models and media are manufactured in the spirit of Good Manufacturing Practices (GMP) and their quality and sterility are guaranteed and verified systematically.

Human mucosal biopsies

2,5 mm² biopsies were taken from four healthy patients range between 22 and 31 years from 6 different regions in the mouth: the palatal mucosa, the lingual mucosa, the buccal mucosa, the attached gingival mucosa, the loose gingival mucosa below the free marginal gingiva and the interdental epithelium. All procedures involving human study subjects were approved by the Ethical Committee of the Free University of Brussels, and all study participants were provided with written informed consent prior to inclusion in the study.

Histological preparation

For histological preparation 10 cultures of RHOE and 5 cultures of RHGE were transported by air from SkinEthic Laboratories, Nice, France, to the Free University of Brussels, Belgium. Upon arrival the tissue constructs were transferred to culture medium and incubated at 37°C overnight, in accordance

with the directions for use provided by SkinEthic Laboratories. 5 tissues of RHOE and 2 of RHGE were used for fixation with formaldehyde and embedded in paraffin for light microscopy (LM). 4 μ m sections were made and stained following standard hematoxylin (0,7%) / eosine (1%) procedures. Semi-thin sections of 1 μ m were stained with 1% Toluidine Blue and ultra-thin sections of maximum 1000 Å or 0.1 μ m were coloured with uranyl acetate 3.5 % for 30 minutes. The sections were evaluated with the light microscope Leica® (DMR®, Wetzlar, Germany) at 10x, 20x, 40x and 63x magnification.

For transmission electron microscopy (TEM) (Tecnai 10 Philips®, Eindhoven, The Netherlands), 5 tissue constructs of RHOE and 3 tissue constructs of RHGE were put into a solution containing 1.5% glutaraldehyde in cacodylate buffer 0.1 M and sucrose 0.1 M, pH 7,4 for 30 minutes at 4°C. After 30 minutes, the solution was removed and 3 ml of osmiumtetroxide (OsO₄) 1% in 0.1 M cacodylate buffer was added again at 4°C (Wisse E, 1970). One hour later the cultures were rinsed three times with distilled water.

All remaining manipulations were carried out at room temperature. The cells were stained with 3.5 % of uranylacetate in H₂O for 10 minutes. After 3 short rinses with water, the dehydration was started. The dehydration consisted of 70° ethanol for 5 minutes, then ethanol 80° during 5 minutes followed by ethanol of 90°. Dehydration was stopped by transferring the cultures in pure ethanol for two times 10 minutes, followed by one time 20 minutes. The final step consisted of dipping the cell cultures into a mixture of ethanol 100° with epon 1/1 for 60 minutes. The cell cultures were consequently immersed overnight in epon at room temperature and the next day, the filter was carefully cut out, imbedded in fresh epon and placed in the oven at 60° for 2 days. Sections were made with an ultramicrotome Ultracut (Reichert®, Vienna, Austria).

For light microscopic histology preparation of the biopsies, the same standardised procedure was followed as described for the RHOE and RHGE tissue models. For Transmission Electron Microscopy, the oral mucosa biopsies were fixed overnight in 4% electron-microscopic grade glutaraldehyde in 0.1 mol/L of cacodylate buffer (Sigma, St. Louis, MO) at pH 7.4. After fixation with 2% osmium tetroxide (EM Sciences, Fort Washington, PA) buffered in 0.1 mol/L of cacodylate buffer, sections were dehydrated with graded alcohol to 2x 100% alcohol and 2x propylene oxide (EM Sciences). The samples were embedded in pure epon resin. One- μ m tissue sections were cut, stained with Toluidine Blue, and examined at the light microscopic level. Ultra-thin sections were cut from areas of interest, stained with lead citrate and uranyl acetate (all from EM Sciences), and observed in a Phillips 400 transmission electron microscope.

The different preparations were microphotographed (Leica DRMX equipped with a high resolution 4000x3000 Zeiss Axiocam digital camera) and LM and TEM histological evaluation and comparison of the tissue constructs with the biopsies was performed.

Quantitative reverse transcriptase polymerase chain reaction (QRT-PCR).

Total RNA was extracted from the 3D cell cultures using the TRIZOL reagent (Invitrogen, Merelbeke, Belgium). The extraction was performed according to the manufacturer's protocol. The concentration, purity, and amount of total RNA were determined by spectrophotometry. Total RNA was reverse-

transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The transcription procedure was performed according to the manufacturer's protocol. Reverse transcription was performed at 25°C for 10 min followed by 37°C for 120 min. QRT-PCR was performed using the ABI 7500 SDS (Applied Biosystems, Foster City, CA) reaction and detection system. Primers and probes for involucrin, filaggrin and keratin-13 were obtained from Taqman Gene expression assays (Applied Biosystems, Foster City, CA). Pre-Developed Taqman Assay reagent for 18S ribosomal RNA (18S rRNA) (Applied Biosystems, Foster City, CA) was used as an internal control. We used Taqman Universal PCR Master mix, cDNA template (50 ng per reaction) and Assays including two unlabeled PCR primers and an FAM dye labeled Taqman MGB probe. The conditions of QRT-PCR were as follows: 10 min at 25°C, 120 min at 37°C and then 40 cycles of amplification (denaturation for 15 seconds at 95°C and extension for 1 min at 60°C). Analysis was performed according to the Delta threshold (Ct) method and each Ct value was normalized to the respective 18S rRNA Ct value of the sample.

Results

1. Light microscopy:

The RHOE and the RHGE demonstrate a very different pattern of differentiation (Figure 2g and 2h). Histologically, the 3-dimensional constructs of the oral mucosa (RHOE) resembled most to the human oral mucosa biopsy taken at the lingual mucosa (2c and 2d); however opposite to the in vivo morphology, no keratinised layer is present in the in vitro model. Additionally, the RHOE tissues, cultivated from epithelial cells derived from the TR146 carcinoma cell line, and are poorly differentiated: all cells in the tissue construct, from the basal layer to the uppermost cell layers demonstrate equal cell morphology. This is in contrast with the morphology of the keratinocytes derived from hard-palate mucosa which are intrinsically different and appear to be more compressed. Additionally, the palatal mucosa is orthokeratinized and comprises a clearly visible stratum granulosum and stratum corneum, both of them lacking in the RHOE epithelial equivalent (Figure 2c), whereas buccal mucosa (figure 2f) is a typical non-keratinized epithelium.

The histology of human gingival epithelium (RHGE) demonstrates a differentiated tissue structure with stratum basale, stratum spinosum, stratum granulosum and stratum corneum (Figure 2h). The 3-dimensional constructs of the gingival mucosa resemble most the human attached gingival mucosa biopsy (Figure 2b).

Other cell types such as Langerhans cells and Merckel cells could be found in the human biopsies but were absent in the 3-dimensional constructs. In the semi-thin slices (Figure 3), the architecture of the cell cultures shows the inter-cellular hemidesmosomes, proof of the well organised tissue structure of the in vitro construct.

Figure 2 (a-h): Light microscopical histological comparison (H&E staining) of human oral biopsies with RHOE and RHGE cultures: 2a: lingual mucosa, demonstrating the different cell layers: stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG) and stratum corneum (SC). 2b: orthokeratinized attached gingival mucosa (compressed keratinocytes). The palatal mucosa comprises a clearly visible stratum granulosum and stratum corneum. cheek mucosa. 2f: buccal mucosa; 2g: reconstituted human oral epithelial construct. 2h: reconstituted human gingival epithelial construct.

Figure 3: High power histological appearance of inter-dental mucosa *in vitro* (a) compared to *in vitro* RHOE (b) 20x (semi thin slices, 2 μ m). The RHOE show clearly visible hemidesmosomes between the keratinocytes demonstrating normal tissue architecture.

A

B

2. Transmission electron microscopy

The ultrastructural appearance of *in vitro* RHOE and RHGE is presented in Figures 4-6. RHOE tissues show a uniform layer of keratinocytes only, presence of mitochondria, intermediate filaments, ribosomes and numerous desmosomes or intercellular desmosomal junctions (Figure 5). Mature hemidesmosomes are demonstrated in Figure 5a, c and d. Necrosis could not be observed in any of the tissues. The RHGE model (Figure 6) shows very close resemblance to the *in vivo* attached gingival biopsy. Infiltration of the cultures cells in the polycarbonate filter was noticed (Figure 6d). No evidence of the presence of other cells than keratinocytes was observed.

Figure 4: Ultrastructural appearance oral mucosal biopsies in vivo and RHOE in vitro. TEM, magnification 1650 x. 4a: lingual mucosa in vivo. Hemidesmosomes are clearly visible. 4b: ultrastructural appearance of interdental gingiva demonstrating flattening of the keratinocytes and more pronounced hemidesmosomes. 4c: ultrastructure of palatal gingiva with multiple desmosomes. 4d: ultrastructural appearance of attached gingiva. The attached gingiva shows a marked flattening of the keratinocytes and a thick keratinised layer (arrow). 4e: ultrastructural appearance of *in vitro* RHOE.

Figure 5: Details of the ultrastructural appearance of *in vitro* oral epithelium RHOE: Details of desmosomes: a:65000 x; c: 30000 x; d: 6500 x b. mitochondrial appearance 30000 x;

Figure 6: Ultrastructural appearance of *in vitro* gingival epithelium RHGE. (a (1650x), b (1650x), c (1650x), d (1200x)): RHGE structure (Fig 6a). comparable to *vivo* attached gingival (see Figure 4d). Presence of a thick keratin layer in the RHGE (Fig 6b). The basal cells of the RHGE construct tend to penetrate in the pores of the polycarbonate substrate (Fig 6C and d).

3.QRT-PCR results

The results of the quantification study are shown in Graphs 2 to 4. The average Ct values for endogenous control are shown in Table 5. All measurements were performed in triplicate. 18S r RNA showed a mean value for all samples of 7.114 (SD 0.797) and was accepted as reliable internal control, as less then 1 Ct value was obtained for all measurements. The measurements for keratin 13 are shown in Table 6 and Graph 2 a-d. There is a significant difference between RHOE and RHGE (Avg dCt 5 to 6 Ct) with higher values for RHOE. Buccal and lingual biopsies showed no Ct difference whereas gingival biopsies showed a difference of 6 Ct's. However values for RHGE are situated between both biopsies, while RHOE Ct values are located outside the biopsies (Graph c-d). Similar patterns can be observed for involucrin and filaggrin, however with small differences (Table 7 and 8, Graph 3a-d, Graph 4a-e). For involucrin the difference between RHOE and RHGE is 4 Ct, all biopsies show an avg dCt of less then 1 Ct compared to RHGE. The measurements of RHGE and RHOE samples nr

3 and 4 were performed 2 days later than the samples RHGE and RHOE 1 and 2. The observed difference in results may be due to the additional 2 day aging of the cell cultures.

Table 5: Endogenous control of measurements with 18S rRNA

Sample	Detector	Task	Avg Ct	Outliers removed
RHGE1	18s rRNA	ENDO	6.813	0
RHGE2	18s rRNA	ENDO	6.648	0
RHGE3	18s rRNA	ENDO	6.861	0
RHGE4	18s rRNA	ENDO	6.857	0
RHOE1	18s rRNA	ENDO	6.584	0
RHOE2	18s rRNA	ENDO	6.614	0
RHOE3	18s rRNA	ENDO	6.472	0
RHOE4	18s rRNA	ENDO	6.620	0
BB	18s rRNA	ENDO	7.697	0
LB	18s rRNA	ENDO	8.150	0
GB	18s rRNA	ENDO	8.937	0

Table 6: Keratin 13 values (threshold for 18S rRNA 0.203208, for keratin 13 0.203173)

Sample	Detector	Task	AvgCt	AvgdCt	dCt std err
RHGE1	Keratin 13	Target	17.635	10.895	0.058
RHGE2	Keratin 13	Target	16.946	10.299	0.083
RHGE3	Keratin 13	Target	22.539	15.695	0.037
RHGE4	Keratin 13	Target	23.164	16.324	0.041
RHOE1	Keratin 13	Target	25.494	18.759	0.060
RHOE2	Keratin 13	Target	27.196	20.462	0.058
RHOE3	Keratin 13	Target	29.158	22.631	0.065
RHOE4	Keratin 13	Target	30.116	23.482	0.059
BB	Keratin 13	Target	12.838	5.141	0.110
LB	Keratin 13	Target	12.998	4.850	0.051
GB	Keratin 13	Target	18.768	9.809	0.150

Table 7: Involucrin values (threshold for 18S rRNA 0.203208, for involucrin 0.065938)

Sample	Detector	Task	AvgCt	AvgdCt	dCt std err
RHGE1	involucrin	Target	19.166	12.427	0.065
RHGE2	involucrin	Target	18.771	12.124	0.067
RHGE3	involucrin	Target	19.009	12.165	0.033
RHGE4	involucrin	Target	19.167	12.328	0.034
RHOE1	involucrin	Target	23.131	16.397	0.063
RHOE2	involucrin	Target	23.494	16.761	0.070
RHOE3	involucrin	Target	24.868	18.341	0.096
RHOE4	involucrin	Target	28.080	18.446	0.063
BB	involucrin	Target	18.937	11.240	0.119
LB	involucrin	Target	19.079	10.931	0.071
GB	involucrin	Target	21.038	12.080	0.156

Table 8 Filaggrin values (threshold for 18S rRNA 0.203208, for filaggrin 0.256840)

Sample	Detector	Task	AvgCt	AvgdCt	dCt std err
RHGE1	Filaggrin	Target	23.870	16.982	0.065
RHGE2	Filaggrin	Target	22.941	16.291	0.048
RHGE3	Filaggrin	Target	20.910	14.032	0.092
RHGE4	Filaggrin	Target	21.722	14.848	0.048
RHOE1	Filaggrin	Target	29.563	23.130	0.030
RHOE2	Filaggrin	Target	30.252	23.757	0.098
RHOE3	Filaggrin	Target	29.838	23.422	0.47
RHOE4	Filaggrin	Target	31.786	25.180	0.126
BB	Filaggrin	Target	24.905		
LB	Filaggrin	Target	22.333	14.180	0.042
GB	Filaggrin	Target	25.178	16.262	0.120

Graph 2a-d: QRT-PCR measurements for keratin-13 (a) comparison between Ct values of RHGE (right) and RHOE (left). All RHOE (O1 and O2) values are situated right to those of RHGE (G1 and G2). (b) comparison between Ct values of biopsies: buccal and lingual biopsies (Lb/Bb) show no difference, the gingival biopsy (Gb) shows a different Ct value. (c) comparison between Ct values of RHGE and biopsies. Remark 2 biopsies (Bb, Lb)are situated left to RHGE, the gingival biopsy (Gb) is situated between the measurements of RHGE. (d) comparison between Ct values of RHOE and biopsies show a discrepancy of 6 Ct's compared to gingival biopsy and 12.5 Ct's compared to lingual and buccal biopsies.

Graph 3a-d: QRT-PCR measurements for involucrin (a) comparison between Ct values of RHGE (right) and RHOE (left). All RHOE (O1 and O2) values are situated right to those of RHGE (G1 and G2). (b) comparison between Ct values of biopsies: buccal and lingual biopsy's (Lb/Bb) show no difference, gingival biopsy (Gb) shows only a 2 Ct different Ct value. (c) comparison between RHGE and biopsies. 2 biopsies (Lb/Bb) coincide with RHGE G1/G2 and the gingival biopsy (Gb) shows a difference of 2 Ct's with RHGE. (d) comparison between RHOE and biopsies shows that all RHOE are situated on the right (2-4 Ct's).

a

b

c

Lb/Bb/G1/G2 Gb

d

Lb/Bb Gb O1 O2

Graph 4a-d: QRT-PCR measurements for fillagrin: (a) comparison between Ct values of RHGE (left) and RHOE (right). All RHOE (O1 and O2) values are situated right (6 Ct's) to those of RHGE (G1 and G2). RHGE and RHOE show at both time intervals a Ct difference of 2 Ct's. (b) comparison between Ct values of biopsies : buccal and gingival biopsies (Lb/Gb) show no difference, lingual biopsy (Lb) shows a 2 Ct different Ct value. (c) comparison between Ct values of RHGE and biopsies: 2 biopsies (Bb/Gb) appear 1 Ct to the right of RHGE (G1/G2) and 1 biopsy (Lb) appears in the middle between G1 and G2. (d) comparison between Ct values of RHOE and biopsies shows that all biopsies are situated left of O1 and O2 (4.5 to 7 Ct's).

Discussion

It is well described that stratified squamous epithelia differ regionally in morphology and differentiation (Gibbs S and Ponc M, 2000; Okazaki et al., 2003). We could also observe similar structural differences depending on the location of the oral biopsies in the oral cavity, for instance, histological observations demonstrated that keratinocytes at the hard-palate mucosa are morphologically quite different from those of non-keratinizing oral surfaces (Cho *et al.*, 2000). In contrast to classical monolayer cell cultures of oral epithelial cells, the 3D human oral and gingival epithelial tissues demonstrate an architectural structure closely resembling the human *in vivo* situation (Staab et al., 2004). The *in vitro* histology is comparable to the biopsy material tissue (Grafström, 2002) but regionally differences are shown. The expression profile of the differentiation markers in the *in vitro* 3D constructs demonstrated similarities in the expression of K13, involucrin and filaggrin compared to *in vivo* biopsies. Expression of filaggrin and keratin 13 in another 3D model of oral mucosa was suggested a premature keratinised state (Izumi K *et al.*, 2000). We identified the expression of K13 in all 3D *in vitro* samples, however differences were obtained for RHGE and RHOE. This could be explained by the fact that RHOE are cultivated using epithelial cells from the carcinogenic origin (TR-146 cell line). It is known from the literature that *in vitro* 3D tissues cultivated with carcinogenic cells show a lower differentiation level (Jacobson J *et al.*, 1999). We also found that 2 additional days of tissue culture of RHGE and RHOE induces a change the involucrin and filaggrin expression (graph 3a,3d and graph 4a,4b), indicating a differentiation shift due to continued tissue culture. Van Goethem et al reported that transport of 3D models by aeroplane may affect the sensitivity of 3D epithelial cultures as compared to untransported control tissues (Van Goethem et al, 2005), hence transport could be partially responsible for the observed changes in involucrin and filaggrin expression. Our present study demonstrated high reproducibility of the 3D constructs in terms of morphology as investigated using LM and TEM. This is a major advantage compared to several other models described in the literature (Sacks *et al.*, 1985; Kautsky *et al.*, 1995; Chung *et al.*, 1997; Grøn *et al.*, 1999; Hansson *et al.*, 2001; Salonen *et al.*, 1989, 1991; Gosselin *et al.*, 1989,1990; Schön and Rheinwald, 1996; Kandikonda *et al.*, 1996; Garlick *et al.*, 1996, Delcourt-Huard *et al.*, 1997; Matsuyama *et al.*, 1997, 2000; Tomakidi *et al.*, 1997, 2000a, 2000b; Papaioannou *et al.*, 1999; Yoo *et al.*, 2000; Cho *et al.*, 2000; Costea *et al.*, 2003). Therefore these 3D cell cultures present a promising choice for *in vitro* toxicology studies and biological research, including the study of periodontal tissue in the presence of different stimuli and for conducting immunotoxicologic experiments (Papaioannou *et al.*, 1999; Lamarque S. *et al.*, 2004). Additionally, oral and gingival organotypic cultures could also be recommended as a valuable model for studying the migration of Langerhans cells and their involvement in contact hypersensitivity and periodontal diseases.

Conclusion

In the present study we demonstrated similarities between the 3-dimensional constructs RHOE and RHGE and normal human oral mucosa in terms of the overall tissue morphology. Remarkably both cultured constructs produce mature hemidesmosomes. Ultrastructural differences however between both models suggest that different results may be obtained when used for *in vitro* studies. Engineered constructs such as those used in this study provide the opportunity to standardize testing and enable objectively quantifiable *in vitro* study of irritation. Additionally these constructs can be used to model important properties of the oral mucosa and the gingival epithelium when biopsies are not practical or not available. We conclude that these 3-dimensional human epithelial oral and gingival mucosa cell constructs provide a quality controlled, reproducible system for in "*in vitro*" testing to be used for preliminary large-scale screening of potential xenobiotics in dental and orthodontic treatment. Both cell cultures attain levels of differentiation resulting in a morphological resemblance with *in vivo* biopsies as demonstrated by LM and TEM and expression of differentiation markers. The rigid substrate design (polycarbonate base) allows easy handling and manipulation for repetitive testing purposes which is favourable when performing discriminating tests. Additionally these models are air-exposed and allow topical application of test materials at their 'in use' concentration. Hence, the 3D RHOE and RHGE tissue models can potentially reduce and replace animal toxicological studies and facilitate other studies of dental and orthodontic materials. Further research is needed to compare the immunoreactivity of the constructs with normal human oral and gingival mucosa.

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Chapter 9

The evaluation of sodium lauryl sulphate containing toothpaste toxicity on human gingival and oral mucosa: a 3D in vitro model.

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Abstract

Five different commercially available toothpaste formulations, labelled A, B, C, D and E, were evaluated for their toxic potential using *in vitro* reconstituted human oral and gingival mucosa cultures (SkinEthic Laboratories, Nice, France). Toothpastes A, D & E contained sodium lauryl sulphate (SLS) whereas toothpastes B and C did not. All toothpastes contained fluoride in different quantities. A 30 % solution was topically applied on triplicate oral and gingival mucosal tissues and incubated for 10 minutes, 1 and 3 hours. Distilled water served as negative control and SLS 0.5% and 1% as positive controls. Tissue viability (MTT), tissue morphology (LM and TEM) and release of pro-inflammatory mediator IL-1 α were evaluated. Topical exposure of the toothpastes on reconstituted human gingival mucosal tissues for 10 min, 1 h or 3 h did not affect MTT values. Exposure of reconstituted human oral mucosa however showed that the toothpastes B, E and D induced a significant loss of tissue viability after 1 h (respectively. 49.19%, 55.55% and 78.38%) ($p < 0.05$). After 3 hours toothpaste B demonstrated a loss of tissue viability of 51.41%, whereas tissue viability for toothpaste D and E dropped to 11.12% and 4.53% respectively. When assessing the release of pro-inflammatory mediator IL-1 α , only the gingival mucosal constructs exposed to toothpaste D and E showed a marked increase of IL-1 α after 1 hour, and toothpaste A, D and E after 3 hours exposure. Oral mucosa tissue exposed for 1 hour resulted in increased levels of IL-1 α for toothpastes A, B, D, & E, which significantly increased at 3 hours. Morphological analysis of the oral mucosal constructs demonstrated partial necrosis after exposure to toothpastes A, B and C, and severe necrosis to D and E. Although the MTT values were unaffected, the gingival mucosal cultures exposed to toothpastes D & E showed important morphological changes: this demonstrates that MTT cannot be used as single parameter to assess toxicity and should be confirmed by histological evaluation. We conclude that both *in vitro* oral and gingival mucosa constructs are suitable models to evaluate the toxicity of toothpaste formulations; however the 2 models show different sensitivity. The presence of SLS in the toothpaste formulations is presumably responsible for the toxicity observed *in vitro*: MTT, IL-1 α release and morphology were affected by the SLS containing toothpastes A, D & E. These observations confirm the clinical inflammatory effects of SLS in oral care products often reported in the scientific literature.

Introduction

Maintaining good oral hygiene is a key issue during orthodontic treatment. The terminology "oral hygiene products" was introduced relatively recent however there is evidence dating back at least 6000 years that formulations and recipes existed to benefit oral and dental health (Fischman 1997). Throughout the centuries, a variety of products including tooth powders, toothpastes and mouth rinses have been formulated, primarily for cosmetic reasons such as tooth cleaning and breath freshening, rather than to control dental and periodontal disease. Today however, toothpaste formulations focussing on effective oral disease prevention are available. Additionally, a set of specific safety standards for oral care products was introduced (BS: 5136, 1981, ISO: 11609, 1995, Lindhe *et al.*, 2003).

The ideal vehicle for the carriage of plaque control agents is toothpaste. A variety of ingredients including abrasives, detergents, thickeners, sweeteners, humectants, flavours and actives are being used to formulate toothpaste (Davis 1980; Forward *et al.*, 1997). The most commonly used detergent in toothpaste is the anionic compound sodium lauryl sulfate (SLS), which imparts the foaming and "feel" properties to the product. Additionally, SLS helps dissolve active ingredients and has both antimicrobial and plaque inhibitory properties. However SLS can also cause adverse effects: animal studies demonstrate that SLS induces prominent changes in the histological structure of animal cheek pouch epithelium, including hyperkeratosis, hypergranulosis, acanthosis and varying degrees of basal hyperplasia, resulting in a significant increase in epithelial thickness (Baert and Veys, 1997). The effects of SDS on mucosal permeability have been described extensively in literature (Siegel and Gordon, 1985). In humans it was reported that SLS can induce inflammation and desquamation of oral mucosa in the oral cavity (Babich and Babich, 1996; Veys *et al.*, 1992; Herlofson, Barkvoll, 1993). The aim of the present research project was to investigate whether commercially available toothpaste formulations containing SLS would alter the morphology and biochemical properties (viability and inflammatory cytokine release) of *in vitro* human 3D tissue constructs. Since tissue cell cultures are generally recognised in the literature as being a sensitive method of assaying the cytotoxic potential of test materials (Babich *et al.*, 1996; De Wever and Charbonnier, 2002; Nguyen *et al.*, 2003), the objective of this experiment was to measure the toxicity of 5 blinded marketed toothpastes on *in vitro* reconstituted human oral and gingival epithelial tissue constructs.

Materials and Methods

Test materials

Toothpastes were blinded and labelled A, B, C, D, E. Toothpastes A, D & E contained SLS whereas toothpastes B & C did not. All toothpastes contained fluoride. A detailed listing of the ingredients of each the tested toothpastes is provided in Table 1.

Tissue culture models and experimental treatments

0.5 cm² inserts containing reconstituted human oral epithelium (RHOE) and reconstituted human gingival epithelium (RHGE) were obtained by culturing either human oral epithelial cells (TR-146 cell line of oral squamous carcinoma) or human gingival cells (primary cells from a gingival epithelial biopsy) on inert polycarbonate culture inserts lifted to the air-liquid interface for 7 days. (SkinEthic Laboratories, Nice, France). Prior to testing, the RHOE and RHGE were placed onto 300 µl of maintenance medium (SkinEthic Laboratories, Nice, France). Thirty µl of a 30% dilution of each toothpaste product (A, B, C, D, and E) was applied onto triplicate cultures. Tissues were consequently incubated at 37°C, 5% CO₂ for 10 minutes, 1 hour and 3 hours. 30 µl of H₂O (negative control) and 30 µl of SDS 0.5% and 1% (positive control) was applied in parallel onto triplicate cultures. For each condition, at the end of the test period, duplicate cultures were placed into appropriated labelled wells for MTT cytotoxicity testing. Additionally, the medium underneath each culture was collected and stored at -20° C for the quantification of the extracellular release of pro-inflammatory mediator IL-1a.

Table 1: Ingredients of the tested toothpastes (as provided on the packaging by the product manufacturers)

Code	Toothpaste	Ingredients
A	Thera-med Original Batch number: 0520741 Schwartzkopf & Henkel, Dusseldorf, imp. B-1080 Brussels, Nieuwegein	Aqua, Hydrated Silica, Glycerin, Sorbitol, PEG-32, <u>Sodium Lauryl Sulfate</u> , Aroma, Cellulose gum, Trisodium phosphate, Disodium phosphate, Sodium saccharin, Zinc sulfate, Sodium benzoate, CI 14720, CI 42090, CI 69800, CI 42090 . Contains Sodium fluoride (1450 ppm F ⁻)
B	Elmex Batch number: 4385G2 Johnson & Johnson, GABA B.V., Postbus 1039, 1039 BA, Almere	Aqua, Hydrated Silica, Sorbitol, Hydroxy ethyl cellulose, Aroma, Titanium dioxide, Saccharin Contains Olafleur (1250 ppm F ⁻),
C	Zendium Classic Batch number: 125111; 152301; 7304742 Kortman intradal SA, Sara Lee H&BC, Belgium, 1702 Groot- Bijgaarden	Aqua, Hydrated silica, Glycerin, Sorbitol, Steareth-30, Carrageenan, Aroma, Titanium dioxide, Disodium phosphate, Citric acid, Sodium benzoate, Sodium saccharin, Potassium thiocyanate, Glucose oxidase, Amyloglucosidase, Lactoperoxidase Contains Sodium fluoride (1100 ppm F ⁻)
D	Signal Integral Batch number: 405671WB Unilever, Lever Fabergé Belgium, Unibel N.V., 1190 Vorst	Aqua, Hydrated Silica, Sorbitol, Calcium Carbonate, <u>Sodium Lauryl</u> <u>Sulfate</u> , Sodium Monofluorophosphate, Aroma, Sodium Fluoride, PEG-32, Cellulose Gum, Methylparaben, Propylparaben, CL 73360 Contains eucalyptus-extracts and Sodium fluoride (1450 ppm F ⁻)
E	Colgate Blue Minty Gel Batch number: 4146IT10305 Colgate-Palmolive, 1160 Brussel, 4041 Liege	Aqua, Hydrated Silica, Glycerin, Sorbitol, PEG-12, <u>Sodium Lauryl Sulfate</u> , Aroma, Cellulose gum, Sodium saccharin, Dicalcium phosphate dihydrate, CI 42090, CI 42090 Contains Sodium fluoride 0.1% (450 ppm F ⁻), Sodium monofluorophosphate 0.76% (1000 ppm F ⁻)

MTT assay procedure

The MTT assay was performed by placing the treated 0.5 cm² inserts containing reconstituted human oral epithelium (RHOE) and reconstituted human gingival epithelium (RHGE) in tissue culture wells containing 0.3 ml of 0.5 mg/ml MTT solution (Sigma-Aldrich St. Louis, MO, USA solution lot n° 02010AD0602). All tissues were consequently incubated at 37°C for 3 hours. For qualitative evaluation of cell viability, after 30 minutes incubation, the colour of each culture was noted: negative control cultures were dark blue colour, proof of cell's viability. Positive control cultures were

blue/white, evidence of cell death. For quantitative evaluation of cell viability, extraction was performed at room temperature in 1.5 ml of isopropanol for a minimum of 1.5 hours, by gentle shaking. 200 μ l of extracts were transferred in a 96 well plate and OD was measured at 570 nm by a spectrophotometer (Dynex Technology, Chantilly, Virginia USA). Reference filter: 690 nm. % of viability = $(DO_{570 \text{ nm sample}} / DO_{690 \text{ nm control}}) \times 100$.

IL-1 α quantification

The amount of pro-inflammatory mediator IL-1 α was quantified in the medium underneath the RHOE and RHGE tissues by quantitative sandwich immunoassay technique (Quantikine Human IL-1 α , R&D Systems, UK, Ref DLA50, lot 207423). After topical application, the cultures were incubated on 0.3 ml of defined medium for 1 hour and 3 hours. Conditioned media were collected and kept frozen for cytokine quantification. The amount of inflammatory mediators released in these conditioned media was then quantified using ELISA kits. For each sample and standard, 200 μ l was incubated in the pre-coated 96-well plate wells for 2 hours at room temperature. After washing, 200 μ l of IL-1 α conjugate was added and the plate was consequently incubated for 60 minutes. After incubation, all wells were washed 3 times and 200 μ l of TMB/H₂O₂ was added. Following incubation for 20 minutes, the reaction was stopped by adding 50 μ l of 2N H₂SO₄. All wells were consequently read in a spectrophotometer (Dynex Technology, Chantilly, Virginia, USA) at 450 nm (ref filter 570 nm). The concentrations of IL-1 α present in the samples were calculated on the basis of a calibration curve. In all cases, duplicate measurements were performed for each sample.

Morphology evaluation by light and electron microscopy

For each condition, at the end of each test period, one of the triplicate cultures was fixed in a 10% formalin solution and later embedded in paraffin. Four micron vertical sections were stained with hematoxylin/eosin and photographed under a light microscope. The sections were evaluated with the light microscope Leica® (DMR®, Wetzlar, Germany) at 10x, 20x, 40x and 63x magnification.

For Transmission Electron microscopy (TEM) (Tecnai 10 Philips®, Eindhoven, The Netherlands), RHOE and RHGE were put into a solution containing 1.5% glutaraldehyde in cacodylate buffer 0.1 M and sucrose 0.1 M (pH 7.4) at 4°C. After 30 minutes treatment, the solution was removed and 3 ml of osmiumtetroxide (OsO₄) 1% in 0.1 M cacodylate buffer was added again at 4°C (Wisse, 1970). One hour later, the cultures were rinsed three times with distilled water. All remaining manipulations were now performed at room temperature. The tissues were stained with 3.5 % of uranylacetate in H₂O for 10 minutes. After three short rinses with distilled water, the dehydration procedure was started. The dehydration existed of 70° ethanol for 5 minutes, then ethanol 80° during 5 minutes followed by ethanol of 90°. Dehydration was stopped by transferring the tissues in pure ethanol, two times for 10 minutes, followed by one time for 20 minutes. The final step consisted of dipping the biopsies and cell cultures into a mixture of ethanol 100° with epon 1/1 for 60 minutes. The biopsies and cell cultures were consequently immersed overnight in epon at room temperature and the next day, the filter was carefully cut out, imbedded in fresh epon and placed in the oven at 60° for 2 days. Sections were made with an ultra microtone Ultracut (Reichert®, Wien, Austria).

Statistical analysis

For each treatment, two-way ANOVA statistical analysis was performed on the viability and the IL-1 α release for both the RHOE and RHGE. The normality of the residuals was analyzed with a Kolmogorov-Smirnov test and the homogeneity of the variances was tested using the Levene's test. When the variances were found to be not equal, the data were log-transformed. To further compare the differences between the treatments for each time period, and to evaluate the time effect for each treatment, a Bonferoni post hoc test with a significance level of $p < 0.05$ was used. All statistical analysis was performed using SPSS version 12.0.

Results

Evaluation of cell viability

The effect of topical application of the different toothpastes formulations on the viability of the RHOE and RHGE in function of time is shown in Table 2. Using RHGE, none of the toothpaste tested had an effect on tissue viability and MTT values were statistically comparable with the negative controls up to the 3-hour treatment. However using the RHOE, toothpastes B, D and E induced a significant decrease of the viability after 1 hour. After 3 hours treatment with toothpastes D and E, a further statistically significant decrease of the viability as compared to the negative control could be observed. Toothpastes A and C did not affect the viability of the RHOE: after 3 hours the tissue viability was still comparable with the negative control. For both the RHOE and RHGE the positive controls (SLS 0.5% and 1%) resulted in a time and concentration dependent decrease of the viability.

Table 2: Effect of the different toothpaste formulations on the viability of RHOE and RHGE in function of time

Treatment	Viability MTT (% Untreated Control) ¹		
	10 min	60 min	180 min
RHOE			
Control	100.0 $\pm$ 1.4 ^a	100.0 $\pm$ 0.6 ^{a, b}	100.0 $\pm$ 2.7 ^{a, b}
SLS 0.5%*	93.2 $\pm$ 2.3 ^a	44.9 $\pm$ 6.4 ^{c, d}	29.4 $\pm$ 3.7 ^c
SLS 1%*	81.4 $\pm$ 3.4 ^a	30.3 $\pm$ 0.9 ^d	3.5 $\pm$ 1.7 ^e
A	109.5 $\pm$ 3.7 ^a	106.4 $\pm$ 5.4 ^a	103.1 $\pm$ 14.4 ^a
B*	104.7 $\pm$ 2.6 ^a	49.2 $\pm$ 3.1 ^{b, c, d}	51.4 $\pm$ 21.1 ^{b, c}
C	109.3 $\pm$ 2.1 ^a	92.2 $\pm$ 1.3 ^{a, b}	95.9 $\pm$ 1.5 ^{a, b}
D*	121.8 $\pm$ 9.8 ^a	78.4 $\pm$ 35.4 ^{a, b, c}	11.1 $\pm$ 5.1 ^d
E*	110.8 $\pm$ 6.9 ^a	55.6 $\pm$ 10.3 ^{a, b, c, d}	4.5 $\pm$ 0.4 ^e
RHGE			
Control	100.0 $\pm$ 1.8 ^{a, b, c}	100.0 $\pm$ 6.9 ^a	100.0 $\pm$ 3.2 ^a
SLS 0.5%*	87.4 $\pm$ 5.7 ^{b, c}	40.8 $\pm$ 0.8 ^b	33.1 $\pm$ 1.5 ^b
SLS 1%*	82.8 $\pm$ 1.4 ^c	28.0 $\pm$ 4.1 ^c	4.6 $\pm$ 0.2 ^c
A	100.4 $\pm$ 3.2 ^{a, b, c}	98.9 $\pm$ 6.0 ^a	102.4 $\pm$ 2.8 ^a
B	103.2 $\pm$ 3.0 ^{a, b}	103.3 $\pm$ 1.7 ^a	95.9 $\pm$ 6.1 ^a
C	97.8 $\pm$ 4.7 ^{a, b, c}	96.5 $\pm$ 2.7 ^a	106.2 $\pm$ 14.6 ^a
D	101.9 $\pm$ 5.2 ^{a, b, c}	98.1 $\pm$ 0.7 ^a	110.3 $\pm$ 2.8 ^a
E	108.5 $\pm$ 0.3 ^a	102.1 $\pm$ 7.4 ^a	100.4 $\pm$ 10.4 ^a

¹Values represent the mean $\pm$ SD, n=2

^{a, b, c, d, e} Within each time period, the same superscripts are not significantly different from each other.

* Treatment resulted in a significant decrease in viability in function of time.

Using qualitative tissue viability assessment, for both RHOE and RHGE models all negative control treated tissues were coloured blue and all positive control treated tissues coloured white after 1 hour.

Release of pro- inflammatory mediator IL-1α

Table 3 shows the release of the pro-inflammatory mediator IL-1α for the different treatments in function of time. Using the RHOE, topical application of toothpastes A, B, D and E induced a significantly increase of IL-1α after 1 hour when compared to the negative control. Toothpaste C did not induced any significant increase in IL-1α after 1 hour. After 3 hours, the IL-1α release continued to increased significantly for toothpastes A, D and E. The IL-1α release induced by toothpaste B remained unchanged at 3 hours, whereas with toothpaste C, the release after 3 hours was statistically lower when compared to the other toothpastes as well as to the positive control. Using RHGE constructs, toothpastes B and C did not have any affect on the IL-1α release: after 3 hours the IL-1α levels were still comparable with the negative control. Toothpaste A however induced a significant increase of IL-1α after 3 hours, whereas toothpaste D and E resulted in a significant increase of the IL-1α release already after 1 hour, and increasing after 3 hours, when compared to the negative controls. The positive controls induced a significantly increase of IL-1α after 1 hour in comparison with the negative control for both the RHOE and RHGE.

Table 3: Effect of the different toothpastes formulations on the IL-1α release in RHOE and RGHE in function of time

Treatment	IL-1α (pg/ml) ¹	
	60 min	180 min
RHOE		
Control	1.0 ± 0.0 ^a	1.0 ± 0.0 ^a
SLS 0.5%*	36.0 ± 3.9 ^c	66.6 ± 7.2 ^d
SLS 1%*	27.6 ± 10.8 ^c	63.3 ± 7.1 ^d
A*	8.0 ± 0.4 ^b	23.9 ± 2.6 ^c
B	27.7 ± 12.9 ^c	25.1 ± 7.7 ^c
C*	1.1 ± 0.3 ^a	9.0 ± 3.3 ^b
D*	11.7 ± 2.2 ^b	39.4 ± 2.0 ^{c,d}
E*	8.4 ± 3.5 ^b	35.2 ± 7.9 ^{c,d}
RHGE		
Control	1.1 ± 0.2 ^a	1.3 ± 0.1 ^a
SLS 0.5%	50.5 ± 21.5 ^b	68.9 ± 14.1 ^c
SLS 1%	34.6 ± 19.1 ^b	65.5 ± 9.7 ^c
A*	3.2 ± 1.7 ^a	15.0 ± 4.0 ^b
B	3.0 ± 2.7 ^a	2.1 ± 0.7 ^a
C	2.6 ± 1.2 ^a	1.5 ± 0.1 ^a
D	18.7 ± 12.3 ^b	28.5 ± 13.8 ^{b,c}
E	28.7 ± 18.3 ^b	57.4 ± 13.7 ^{b,c}

¹Values (pg/ml) represent the mean ± SD, n=3

^{a, b, c, d} Within each time period, the same superscripts are not significantly different from each other.

* Treatment resulted in a significant increase of IL-1α in function of time.

Histology

For each test material, at the end of each test period, half of each RHOE and RGHE tissue was fixed in a 10% balanced formalin solution and embedded in paraffin for LM, the other half was embedded in epon for TEM. The histopathological interpretation was performed by 2 patho-anatomologist who came to the same conclusions ($p < 0.05$). The histopathological examination of light microscopy pictures of RHOE and RGHE was performed according to the following classification scheme (Vande Vannet *et al.*, 2006a, 2006b) : non irritant (NI): constant thickness of the epithelium, regular and compact shape, cells attached to the others; mild irritant (Mil): minimal changes with slight oedema; moderate irritant (Mol): onset of spongeosis in upper layers and architectural atrophy, cellular irregularity; severe irritant (SI): disintegration of the upper cell-layers, spongeosis, cellular necrosis, loss of cellular junction in basic layer.

The results for both RHOE and RGHE are presented in Figure 1. Negative control tissues were scored NI (Figure 1a and 1c), positive control RHOE tissues and RHOE tissues treated with toothpastes D and E were scored SI (Figure 1b and 1d). RHOE tissues treated with toothpaste A, B and C for 1 hour are scored Mil. After 1 hour, partial necrosis was observed in all toothpaste treated RHOE tissues. The results of TEM showed similar effects including severe disturbances at the ultra-structural level, obtained after 1 hour and 3 hours treatment of both on RHOE and RHGE with toothpaste A, D and E (Figure 2). Additionally, apoptosis and necrosis could be observed after 1 hour and 3 hours.

Figure 1: Light microscopic evaluation of the effects of 3 hour topical treatment of RHOE (a-c) and RHGE (b-d) with toothpaste E. Untreated control tissues (a: RHOE and c: RHGE) demonstrate normal tissue morphology, compared to treated RHOE tissues (b) and RGHE tissues (d). Light microscopy (x40).

Figure 2: Transmission electron microscopic evaluation of the effects of 3 hour topical treatment of RHOE (a-c) and RHGE (b-d) with toothpaste E. Effects of toothpaste E on RHOE (b) and RHGE (d) compared to non treated cultures RHOE (a) and RHGE (c) both at 180 min. Notice the dramatic effects of toothpaste E at the ultra-structural level marked with necrotic and apoptotic cells (arrows). TEM (x6200)

Discussion

Only few papers report on the use of in vitro methods for evaluating the toxic potential of oral care formulations is not widely reported in scientific literature. Several publications however describe the use of reconstituted human epithelial models for the routine screening of cosmetic test formulations to assess the eye irritation potential (Doucet et al, 1998, Courtellement et al, 1999). We tested a similar in vitro concept by evaluating in blind 5 marketed toothpaste formulations for their toxicity irritation potential after topical application on RHOE and RHGE. A 30 % toothpaste solution was applied on the surface of the tissue constructs in order to simulate the normal 'in use' concentrations of toothpaste in the mouth during tooth brushing (Principe et al., 1995). Another important issue to take in consideration is that all tested toothpaste formulations contained fluoride (ranging from 1450 ppm to 1000 ppm, see Table 1). Although previous in vitro studies indicated that sodium fluoride can be toxic to oral mucosal fibroblasts in vitro through inhibition of protein synthesis, mitochondrial function and depletion of cellular ATP (Jeng et al., 1998; Chang and Chou, 2001), the potential toxic effect of fluoride was not taken into consideration in our study. One of the test formulations, toothpaste B, induced low viability of the

cultures at 60 minutes (Table 3). Although toothpaste B did not contain any SLS, the decreased viability and concomitant IL-1 α release could be explained by the presence of olafluor (bis(hydroxyethyl) amino-propyl-N-hydroxy-ethyl-octadecylamindihydrofluoride). Aminfluoride has been reported to induce contact dermatitis and stomatitis (Ganter et al; 1997).

Both RHOE and RHGE demonstrated very different toxicity patterns after topical exposure to toothpaste formulations. This is not surprising since the RHOE model demonstrates poor differentiation as compared to the RHGE where a stratum corneum is present, hence providing a physiological barrier to the exposed toothpaste formulations. In fact, using the reconstructed gingival epithelial tissues, only an effect was noticed at the level of IL-1 α release. None of the toothpastes induced tissue toxicity when evaluated with the MTT assay. However when morphological assessment was performed, some of the SLS containing toothpastes (toothpaste E) did alter the structure of the RHGE. This observation clearly demonstrated the importance of a Multiple Endpoint Analysis (MEA) approach for the assessment of the irritation potential of this type of test formulations and that MTT should not be used as a single endpoint to measure acute toxicity (De Wever et al, 2004). Based on the MEA (tissue viability, tissue morphology and the release of pro-inflammatory mediator IL-1 α) of the gingival RHGE tissues, the toothpastes could be ranked in order of increasing irritation potency as follows: (C) Zendium, (B) Elmex < (A) Thera-Med < (D) Signal < (E) Colgate. Using the oral RHOE tissues, lacking a differentiated ultra structure and stratum corneum, topical toothpaste application induced a decrease in tissue viability opposite to the effects observed with the RHGE constructs. Consequently, the SLS containing toothpastes more dramatically affected the morphology of the RHOE tissues and an increased the IL-1 α release (less discriminative) was also observed. Despite the different toxicity data obtained with the RHGE, multiple endpoint analysis of the oral RHOE tissues resulted in an almost similar toothpaste rank order of increasing irritation potency: (C) Zendium < (A) Thera-Med < (B) Elmex < (D) Signal ~ (E) Colgate. Only toothpaste A and B were classified differently: a higher viability was observed with toothpaste A after 180 min, however tissue morphological analysis demonstrated tissue necrosis.

Conclusion

In this study, we evaluated the usefulness of RHOE and RHGE as in vitro models for the toxicity assessment of topically applied toothpaste formulations. Based on the histological observations and the statistical interpretation of the quantitative evaluation of the biochemical endpoints, the following conclusions could be drawn:

1. The RHOE model seems more sensitive compared to RHGE. This is due to the structural differences between both models, the RHGE demonstrating a much more differentiated morphology featuring the presence of a thin stratum corneum, little parakeratosis and few or no granular cell layers, opposite to the few differentiated RHOE model.
2. False negative results are obtained when tissue viability (MTT assay) data are being used as a single endpoint to evaluate the toxicity potential of toothpaste formulations: Multiple Endpoint Analysis MEA, including simultaneous histological evaluation as well as the release of pro-inflammatory mediators (IL1 α) is therefore mandatory for accurate toxicity assessment.
3. Toothpastes containing SLS

demonstrate toxic effects on both RHOE and RHGE. This was clearly demonstrated for all endpoints. The use of tissue viability measurement, cytokine release and tissue morphological analysis can provide useful indications regarding the relative toxicity of toothpaste formulations *in vivo*. The clinical relevance of these findings can be resumed as follows : large amount of toothpaste, specially in children, should be avoided. Toothpastes containing SLS are not intended to stay in the mouth and proper rinsing after brushing is advised. Additionally, based on the literature and confirmed with this *in vitro* study, patients sensible to aphtuous lesions should be informed not to use toothpastes containing SLS. In fact, toothpaste formulations containing SLS should be reconsidered in general.

Clearly, further research is still needed to provide a better understanding of the potential toxic effects of toothpastes with or without SLS on oral and gingival mucosa. Both the RHOE and RHGE models provide very valuable tools for such research purposes. Additionally, these *in vitro* human epithelial tissue models could be very valuable as alternative to animal models for oral care product safety assessment. In order to validate the predictive value of both RHOE and RHGE tissue models and experimental protocols, more substances have to be evaluated in accordance to the ECVAM guidelines for validation (Curren et al, 1995, Fentem and Balls, 1997, Worth and Balls, 2002)

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General discussion

Chapter 1 reviews the morphological (ultra structural) and biochemical (differentiation marker expression, lipid analysis and gene expression) characteristics of in vitro reconstructed human epidermis reconstituted, obtained by cultivating primary human keratinocytes on inert polycarbonate filters for 17 days at the air-liquid interface in chemically defined medium. An optimised testing strategy called Multiple Endpoint Analysis or MEA is proposed for the evaluation of the biocompatibility of test compounds in general. The MEA strategy is based on the assessment of tissue viability, tissue morphology and the release of pro-inflammatory mediators after topical application of any given test compound for a specific time period. Too often only tissue viability (assessed by means of the MTT assay) is being used as a single toxicological endpoint to assess toxic effects: this can lead to false negative results since MTT is primarily converted by the basal and supra-basal cells of in vitro reconstructed tissue constructs: any superficial toxicity in the upper layers of these tissues is not detected by MTT testing, hence the MEA strategy proves to be crucial for the testing of milder products such as pharmaceutical skin care formulations. In Chapter 2 the gene expression profiles of human skin, cultured keratinocytes and reconstructed human epidermis were compared using large DNA micro-arrays. The results of this study show skin in vivo and reconstructed human epidermis are very similar in most respects, except that skin expresses more secreted proteins and cell surface receptors reflecting its more complex cellular environment. Also, the metabolism rate of human skin in vivo seems less than in in vitro reconstructed human epidermis. Nevertheless, a high overall correlation is found in most respects, opposite to the gene expression profile of cultured monolayer keratinocytes that lack differentiation and hence do not express epidermal differentiation markers, indicating the usefulness of reconstructed epidermis as a scientifically relevant tool for skin toxicology testing. Chapter 3 describes the utility of reconstructed epidermis for the assessment of skin irritation and sensitization. Using MEA, in this case the dose related tissue viability, in combination with the release of pro-inflammatory mediators IL-1 α and IL-8, it was possible to discriminate and classify, in a single assay, irritating and sensitizing test compounds. Although reconstructed epidermis features many of the characteristics of human skin in vivo, it is composed only of keratinocytes. Other cell types such as Langerhans cells as well as blood-derived leukocytes, which play a crucial role in cutaneous immune response in vivo, are absent. Nevertheless, keratinocytes are the predominant cell type of human epidermis and are the first cell type with which test compounds are in contact during the initial stages of irritation and/or sensitization. Since only a very limited set of well defined skin irritants and sensitizers were evaluated, additional testing needs to be performed to demonstrate that this approach can be used to classify other types of skin irritating/sensitizing compounds. Chapter 4 deals with the assessment of the SkinEthic in vitro reconstructed human epidermis model for in vitro skin corrosion testing, in accordance to the new OECD Guideline TG431. This OECD Guideline already endorsed the use of in vitro skin model systems for in vitro corrosion testing; hence the principle of 'catch-up' validation was applied to assess the SkinEthic reconstructed epidermis model. In a first phase, the test method was optimised and consequently, a set of 12 reference chemicals, specified in the OECD Test Guideline were tested in 3 independent runs in 4 laboratories. Results obtained with the SkinEthic skin model were reproducible,

both within laboratories and over time. Concordance between laboratories and the predictions obtained with other accepted OECD tests was also very good, since the SkinEthic skin model was able to distinguish corrosive from non-corrosive chemicals for all 12 tested OECD reference chemicals. Consequently, the assay can now be used as regulatory accepted alternative method for skin corrosion testing. Chapter 5 describes an *in vitro* reconstructed model of corneal epithelium by using immortalized corneal cells that are cultivated for 7 days at the air liquid interface which will eventually form a 3D differentiated epithelium that mimics the ultra structural and biochemical properties of human cornea *in vivo*. Hemidesmosomes are abundantly present along the basal membrane of the basal cells in the *in vitro* construct. Also the expression of Keratin-3, a cornea-specific differentiation marker was found. When the corneal construct was exposed to benzalkonium chloride, a dose dependent toxicity was observed, both by increased loss in epithelial viability and structure but also by induction of MAPK, a cellular stress response enzyme, indicating the potential applicability of this *in vitro* model as replacement for the animal Draize rabbit eye irritation test. Indeed, in Chapter 7, the successful pre-validation of this corneal model for eye irritation testing is reported (see below). In Chapter 6, an *in vitro* model for corneal wound healing was assessed based on the expression of EMMPRIN (extracellular matrix metalloproteinase inducer) to assess its role in epithelial-stromal interactions that occur during impaired corneal healing. The expression of EMMPRIN in the *in vitro* corneal construct demonstrated a similar gradient to the one observed in central cornea *in vivo*. This observation further substantiates the physiological features of *in vitro* 3D model. In Chapter 7 the pre-validation of the *in vitro* 3D corneal epithelial model to assess the eye irritation potential of chemicals is described. In a multi-centre study approach, 20 coded reference chemicals, covering the whole range of eye irritancy, were evaluated by topical exposure to the cultures for 10 minutes. Once a standardized protocol was established, a high level of reproducibility between the 4 participating laboratories was observed. Based on a tissue viability cut-off of 60%, a Prediction Model allowing differentiation between irritants and non-irritants was established. The *in vitro* 3D corneal model demonstrated an 80% overall concordance with published *in vivo* Draize eye irritation data. Although the sensitivity of the assay was 100%, the specificity was only 56%. However the predictive power of the *in vitro* corneal model increased substantially when other sources of *in vivo* and *in vitro* data were taken into account, indicating that the quality of the available Draize rabbit eye irritation data for some chemicals is questionable. A report of this pre-validation study is currently being evaluated by ECVAM (European Validation of Alternative Methods) for eventual full validation. In Chapter 8, the characterization of 2 recently developed 3D human constructs, human oral and gingival epithelium, is described. Using immortalized oral and primary gingival epithelial cells, airlifted cultivation in chemically defined medium for 7 days results in 3D constructs of oral and gingival epithelium respectively. A histological comparison, both using light and electron microscopy, was made using *in vivo* biopsy material. Both *in vitro* 3D tissue models demonstrated a high resemblance with normal *in vivo* oral lingual and gingival attached epithelium. In chapter 9, the oral and gingival tissue models were evaluated to assess the potential toxicity of toothpaste formulations after topical application. 5 different commercially available toothpastes (some of them containing SLS) were tested at a 30% dilution and exposed to both oral and gingival epithelial cultures for 10 minutes, 1 hour and 3 hours. Multiple Endpoint Analysis MEA (tissue viability, tissue morphology and the release of pro-inflammatory mediator IL-1a) was

assessed for each toothpaste. Both tissue models, although oral mucosa being more sensitive due to a less differentiated ultra-structure as compared to gingival epithelium) clearly demonstrated the toxic effect of SLS containing toothpastes. These in vitro observations confirm previous reports on SLS oral toxicity in clinical literature. Further studies are needed to validate the predictive value of both models and experimental protocols as alternative to animal methods for the risk assessment of oral care formulations.

Recent advances in tissue engineering allow today the reconstruction of a variety of human tissue models in large quantities and with high reproducibility. Both skin models (simple epidermal and more complex dermal-epidermal models), corneal constructs, oral and gingival mucosa models, vaginal, oesophageal, lung and others provide toxicologists reliable tools to develop relevant alternative methods to assess the toxicity of chemicals in stead of using animals. The present thesis work demonstrates that these in vitro models provide very valuable tools, especially to address the topical toxicity tests including skin irritation, eye and mucosal irritation, phototoxicity and corrosion. However in vitro assays to replace animal testing for sensitisation, chronic toxicity, and tissue recovery after eye injury remain challenges to be addressed: further R&D using these in vitro 3D human models is currently ongoing.

Samenvatting

Hoofdstuk 1 beschrijft de morfologische (ultrastructuur) en biochemische (differentiatie merkers, lipidenanalyse en genexpressie) karakterisatie van in vitro gereconstrueerd humaan epidermis, dat wordt verkregen door cultivatie van humane primaire keratinocyten op inerte polycarbonaat filters aan de lucht-water interface in chemisch gedefinieerd cultuur medium. Een geoptimaliseerde in vitro teststrategie, Multiple Endpoint Analyse of MEA genoemd, wordt voorgesteld om de biocompatibiliteit van testmaterialen te evalueren. De MEA strategie is gebaseerd op het bepalen van de weefselleefbaarheid (gemeten met behulp van de MTT assay), het analyseren van de weefselstructuur en de kwantificatie van de vrijstelling van pro-inflammatoire mediators na topische aanbrengen van de te testen stof voor een vastgestelde periode. Helaas wordt maar al te dikwijls enkel de MTT test gebruikt als enige eindpunt om de toxiciteit te bepalen: dit kan leiden tot valse negatieve resultaten aangezien MTT hoofdzakelijk wordt gemetaboliseerd door de basale en suprabasale cellen in de in vitro gereconstrueerde weefsels: elke superficiële toxiciteit (ttz ter hoogte van de bovenste cellagen van het 3D construct wordt niet gedetecteerd door de MTT assay. Om deze reden bewijst de MEA strategie haar doeltreffendheid voor de toxiciteitsmeting van mildere stoffen zoals farmaceutische skin care producten. In hoofdstuk 2 werd het genexpressie profiel van humane huid vergeleken met dat van in vitro gereconstrueerd humaan epidermis en van primaire humane keratinocyten, gekweekt als monolayer culturen. De resultaten van deze studie tonen aan dat humane huid in vivo en gereconstrueerd humaan epidermis in het algemeen goed vergelijkbaar zijn, hoewel in de huid in vivo meer proteïnen en cellulaire receptoren worden tot expressie gebracht, in overeenstemming met de grotere cellulaire complexiteit van de huid in vivo. Niettegenstaande bestaat er een grote correlatie tussen in vivo huid en in vitro epidermis, in tegenstelling tot het gen expressie profiel van niet gedifferentieerde monolayer keratinocyten culturen waar een volledige afwezigheid van differentiatie merkers werd vast gesteld. Hoofdstuk 3 beschrijft het gebruik van in vitro gereconstrueerd epidermis voor het bepalen van het irritatie en sensitizatie potentiaal van chemisch stoffen. Gebruikmakend van de MEA analyse, in dit geval door het meten van de dosisafhankelijke weefsel viabiliteit, in combinatie met de vrijstelling van de pro-inflammatoire mediators IL-1a en IL-8, was het mogelijk om de irriterende stoffen van de sensitizerende te onderscheiden in één en dezelfde test. Hoewel in vitro gereconstrueerde epidermis op verschillende vlakken overeenkomsten vertoont met humane epidermis in vivo, bestaat in vitro gereconstrueerde epidermis uitsluitend uit keratinocyten: andere celtypes zoals Langerhans cellen en uit het bloed afkomstige leukocyten, welke een cruciale rol spelen in de immuunrespons van de huid, zijn in dit in vitro model niet aanwezig. Niettegenstaande zijn de keratinocyten het dominante celtype in humane epidermis in vivo: zodra een chemische stof met de huid in contact komt zijn de keratinocyten de allereerste cellen die worden geactiveerd en die dus een determinerende rol spelen in het irritatie en/of sensitizatie reactieproces. Aangezien enkel maar een zeer beperkt aantal irriterende en sensitizerende stoffen werd getest, moet worden nagegaan of deze in vitro benadering kan worden uitgebreid tot andere types van huidirriterende en sensitizerende chemicaliën. Hoofdstuk 4 behandelt de evaluatie van het humaan in vitro epidermis model voor in vitro corrosiviteitstesting, in overeenkomst met de OECD testrichtlijn TG431. Deze testrichtlijn beveelt het gebruik van in vitro huidmodellen voor de bepaling van het

corrosief karakter van chemische stoffen aan. Bij gevolg werd het principe van 'Catch-up' validatie toegepast om het in vitro epidermis model te valideren. Eerst werd het test protocol geoptimaliseerd en vervolgens werden 12 referentiestoffen, gespecificeerd in de OECD richtlijn, getest in 3 afzonderlijke experimenten door 4 laboratoria. De resultaten verkregen met het in vitro huidmodel bleken reproduceerbaar, zowel tussen de test laboratoria als in de tijd (ie over de 3 afzonderlijke experimenten). Ook de overeenstemming tussen de labo's als tevens met eerder gepubliceerde OECD test methodes was zeer goed, aangezien door middel van dit in vitro huidmodel alle 12 referentiestoffen juist werden geclassificeerd als corrosief of niet-corrosief. Hoofdstuk 5 beschrijft een in vitro gereconstrueerd model van humaan cornea epitheel. Dit in vitro 3D weefsel wordt verkregen door geïmmortalizeerde cornea epitheelcellen te kweken op polycarbonaat filters gedurende 7 dagen (air-liquide interface) welke aldus een 3D epitheel vormen dat grote gelijkenis vertoont, zowel ultrastructureel als biochemisch, met humaan corneaweefsel in vivo. Zowel de aanwezigheid van hemidesmosomen ter hoogte van het basale membraan als de expressie van de differentiatie marker cytokeratin 3, een cornea specifiek eiwit, tonen dit aan. Wanneer dit 3D cornea model werd blootgesteld aan benzalkonium chloride, kon een dosisafhankelijke toxiciteit worden aangetoond door middel van de geïnduceerde verlaagde epitheliale viabiliteit en veranderde weefsel morfologie, maar ook door de inductie van MAPK (een cellulair stress response enzym), wat laat besluiten dat dit 3D in vitro model als potentieel alternatief zou kunnen worden aangewend ter vervanging van de konijnen Draize oogirritatie test. Inderdaad, in hoofdstuk 7 wordt de succesvolle prevalidatie van dit cornea model voor het bepalen van het oogirritatie potentiaal van chemische stoffen beschreven (zie verder). In Hoofdstuk 6 wordt het gebruik van het in vitro 3D cornea model voor corneaal wondhelingonderzoek beschreven, gebaseerd op de expressie van EMMPRIN (extracellular matrix metalloproteinase inducer), dat de vrijstelling van MMP's (metalloproteinases) induceert. De expressie van EMMPRIN in het in vitro 3D cornea model vertoont een gelijkaardige gradiënt als die wordt terug gevonden in het centrale deel van de cornea in vivo. Deze observaties ondersteunen verder het feit dat dit in vitro 3D cornea model fysiologische in vivo eigenschappen bezit. In hoofdstuk 7 wordt de pre-validatie van het 3D in vitro cornea model voor het testen van de oogirriterende eigenschappen van chemische stoffen beschreven. In een multi-center studie werden 20 gecodeerde referentiestoffen, die het ganse spectrum van oogirritatie omvatten, geëvalueerd door topische applicatie op de cornea weefsels gedurende 10 minuten. Vanzodra een gestandaardiseerd testprotocol werd gedefinieerd, werd een hoge reproduceerbaarheid van de test resultaten tussen de 4 participerende laboratoria bekomen. Gebaseerd op een weefselleefbaarheid cut-off waarde van 60%, werd een predictiemodel bepaald dat een classificatie van de stoffen als irriterend of als niet-irriterend toeliet. Het 3D in vitro cornea model vertoont een gemiddelde in vivo-in vitro overeenkomst van 80% met de gepubliceerde Draize konijnen oogirritatie gegevens. Hoewel de sensitiviteit van de assay 100% is, bleek de specificiteit maar 56% te zijn. Wanneer nochtans andere bronnen van in vivo en in vitro toxiciteit data mede in beschouwing werden genomen, verhoogde de predictive 'power' van het model aanzienlijk, wat er op wijst dat de beschikbare (gepubliceerde) Draize konijnen oogirritatie data voor sommige test stoffen in twijfel kunnen worden getrokken. Een rapport van deze pre-validatiestudie ligt ter discussie bij ECVAM (European Center for the Validation of Alternative Methods), om de volwaardige validatie van dit model verder te organiseren.

In Hoofdstuk 8 wordt de karakterisatie van 2 recenter ontwikkelde 3D humane constructen, tztz oraal en gingiva epitheel, beschreven. Door gebruik te maken van geïmmortalizeerde orale epitheelcellen enerzijds en primaire gingivale epitheelcellen anderzijds, kunnen na 7 dagen van air-liquid interface cultivatie op polycarbonaat filters, driedimensionale culturen van respectievelijk oraal en gingivaal epitheel bekomen worden. Een histologische vergelijking, gebruik makende van licht –en elektronenmicroscopie werd uitgevoerd en vergeleken met humaan biopsie materiaal. Beide in vitro 3D culturen vertonen grote gelijkenis met normaal in vivo oral-linguaal en gingivaal weefsel. In Hoofdstuk 9 tenslotte worden deze orale in vitro culturen geëvalueerd om het toxiciteitspotentiaal van tandpasta's te testen. 5 verschillende commercieel verkrijgbare tandpasta's (waarvan enkele SLS bevatten) werden verdund tot 30% en vervolgens topicaal aangebracht op in vitro oraal en gingivaal epitheel gedurende 10 minuten, 1 uur en 3 uur. Multiple End Point MEA analyse (weefselleefbaarheid, morfologie en de vrijstelling van interleukine-1alpha) werd vervolgens uitgevoerd voor elke tandpasta. Beide in vitro modellen (oraal mucosa in sterkere mate, daar dit model minder gedifferentieerd is dan gingiva epitheel) tonen de toxische effecten van SLS bevattende tandpasta's aan. Deze in vitro observaties bevestigen eerder gepubliceerde klinische toxiciteitsgegevens. Bijkomstige studies zijn uiteraard noodzakelijk om de predictiviteit van deze modellen en experimentele protocols te valideren zodat deze test approach kan worden aangewend als alternatieve testmethode voor de risico-toxiciteitsmetingen van oral care producten.

De recente wetenschappelijke vooruitgang in de weefselcultuurmethodiek laat heden ten dage toe om verschillende humane weefselmodellen te reconstrueren in grote hoeveelheden, en met intrinsieke hoge reproduceerbaarheid. Zowel huidmodellen (eenvoudige epidermale als ook complexe dermale-epidermale modellen), cornea constructen, orale en gingivale epitheelculturen, vaginale, oesofagale, long en andere 3D culturen kunnen door toxicologen worden aangewend om relevante alternatieve methoden voor toxiciteitstesten te ontwikkelen in plaats van gebruik te maken van proefdieren. Dit proefschrift toont duidelijk aan dat deze modellen als volwaardige testsystemen kunnen worden gebruikt, in het bijzonder om de irritatiegraad, fototoxiciteit en corrosiviteit te meten. Toch zijn een aantal testen waaronder bv. allergie, chronische toxiciteit, weefsel 'recovery' na oogirritatie, nog niet volledig door alternatieve methoden te vervangen. Verder onderzoek mede gebruikmakend van deze 3D in vitro culturen is aan de gang om ook voor deze complexere toxiciteitstesten volwaardige alternatieve methoden op punt te stellen.

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Bart De Wever
February 3, 2006.

Curriculum Vitae

Bart De Wever was born in Turnhout, Belgium, on October 11, 1962. He obtained a Bachelor degree in Pharmaceutical and Biological Techniques at the COOVI Institute in Brussels in 1984. From 1985 till 1992 he worked as a scientist at the Life Sciences Department of Johnson & Johnson Pharmaceutical R&D, in Beerse, Belgium, first as research assistant in the Laboratory of Cellular Biology and later on as Head of the In Vitro Cell Culture Facility where he was actively involved in the characterization and development of human skin models for dermato-pharmacology and wound healing. During this time, he continued to study at the Hoger School West-Brabant, Ettenleur, The Netherlands, where he obtained the degree of Engineer in Biochemistry in 1991. From 1993 till 1997 he worked as a consultant for Advanced Tissue Sciences, La Jolla, CA, USA, where he was responsible for the industrial implementation of their Skin 2TM in vitro reconstituted human skin and mucosal models as alternatives to animal testing, primarily in European cosmetic industry. From 1997 till December 2004, he was Director of Business Development of SkinEthic Laboratories, based in Nice, France, a human tissue production company that became a world leader in the development, production and commercialization of human tissue models for in vitro testing in pharmaceutical, cosmetic and chemical industry. Under his responsibility, 3D in vitro human models for skin, but also mucosa, including corneal, oral, gingival, esophageal, alveolar and vaginal mucosa were developed for testing purposes. Since January 2005, he works as a business consultant, in the field of industrialization of alternatives to animal test methods, for several companies and academic research centers including Henkel-Phenion, Frankfurt, Germany, (coordination of external collaborations for the development of in vitro methods for teratogenesis, mucosal irritation), The Flemish Institute for Technology Research VITO, Mol, Belgium (strategic plan development for alternative methods), Eggcentris NV, Brussels, Belgium (business development of in vitro model for fertility testing), and the University of Ghent, Belgium (business development and validation of an in vitro method for mucosal irritation testing). Since 2004, he is a member of the Society of Toxicology, USA. He is also sitting at the COLIPA Eye Irritation Task Force, and is an active member of the 'Europe goes Alternative' Task Force, recently endorsed by the European Commission. Besides his activities in the in vitro alternatives field, he is currently also Chief Executive Officer of MST Laboratories, based in Liechtenstein, a company that develops OTC products for fungal disinfection of oral care appliances.

Bart De Wever is married to Luta Wouters. They have 2 children: Louis and Jerom.

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This image shows a full page of white paper with horizontal dashed lines, typical of primary school writing paper. The lines are evenly spaced and run across the width of the page. There are no margins, text, or other markings on the paper.

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