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Review

In vitro skin irritation: facts and future. State of the art review of mechanisms and models

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Abstract

The skin is the main target tissue for exogenous noxes, protecting us from harmful environmental hazards, UV-irradiation and endogenous water loss. It is composed of three layers, whereas the outermost epidermis is a squamous epithelium that mainly consists of keratinocytes. These cells execute a terminal differentiation, which finally results in the assembly of the stratum corneum. This layer, consisting of cornified keratinocytes, is an effective barrier against a vast number of substances. Apart of this, keratinocytes play crucial roles in the immune surveillance and the initiation, modulation and regulation of inflammation in the epidermis. Regarding cutaneous inflammatory reactions, skin irritation is one of the most common adverse effect in humans. For reasons of human safety assessment new chemicals are still evaluated for irritant potentials by application to animals followed by visible changes such as erythema and oedema. Testing for skin irritation in animals potentially cause them pain and discomfort. Furthermore, the results are not always predictive for those found in humans. In order to replace animal testing and to improve the prediction of irritants, the cosmetic and toiletry industry, in Europe represented by Colipa, develops and uses several alternative in vitro test systems. In this respect, the use of in vitro reconstructed organotypic skin equivalents are mostly favored, because of their increasingly close resemblance to human skin. Due to ethical and scientific questions and on account of the 7th amendment of the European Council Directive 76/768/EEC, the authors see the requirement to drive the development of alternative tests for irritants. Therefore, this article centres on cosmetic ingredients and provides the readership an overview of the state of art of cellular mechanisms of skin irritation and summarizes the results of the commonly used skin equivalents to evaluate irritation in vitro.

Keywords: Apligraf; Calcium; Differential gene expression; Emulsifier; Epidermis; EpiDerm; Episkin; IL-1α; IL-1ra; IL-6; IL-8; IL-10; Prostaglandin; Proteases; Protease-inhibitors; Reactive oxygen species; Skin equivalent; Skin irritation; Skinethic; Solvents; Surfactants; TNF-α

Contents

Abbreviations: IL, interleukin; ROS, reactive oxigen species; PGE, prostaglandin E; SDS, sodium dodecyl sulfate; SLS, sodium lauryl sulfate; PLA, phospholipase A; LDH, lactate dehydrogenase; TNF, tumor necrosis factor.

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1. Introduction

The skin is the main target tissue for exogenous noxes, protecting us from harmful environmental hazards, UV-irradiation and endogenous water loss. It is composed of three layers: the epidermis, the dermis and the deeper localized subcutis. The outermost epidermis is a squamous epithelium that mainly consists of keratinocytes. These cells execute a terminal differentiation, which finally results in the assembly of the stratum corneum. This layer, consisting of cornified keratinocytes, is an effective barrier against a vast number of substances. Apart of this, keratinocytes play crucial roles in the immune surveillance of the epidermis. After stimulation they trigger inflammatory responses (Steinhoff et al., 2001). In correspondence with fibroblast, endothelial cells and immune cells, mostly resident in the dermis, keratinocytes play an important role in the initiation, modulation and regulation of inflammation (Coquette et al., 1999, 2000).

Regarding cutaneous inflammatory reactions, skin irritation is one of the most common adverse effect in humans. Clinically, skin irritation accounts for about 70% of all cases of contact dermatitis (Goh and Soh, 1984). The presence of erythema, oedema, dryness of the skin, fissures, desquamation, itching and pain characterizes both irritant contact dermatitis and allergic contact dermatitis. Some of these characteristics are signs of an inflammatory reaction and an altered homeostasis. All together, these symptoms are the ultimate physiological manifestation of a complex chain of biochemical, neural, vascular and cellular responses following the initial irritation signal (Weltfriend et al., 1996).

Visibly, the uniform pathology makes it difficult to distinguish between skin irritation and allergic contact dermatitis (Frosch, 1995). In contrast to allergic reactions, skin irritation is initiated through a direct inflammatory effect on the skin, excluding mechanisms of causation involving sensitization (Basketter, 1999). Therefore, skin irritation is defined as a locally arising, non-immunogenic inflammatory reaction, which appears shortly after stimulation and usually disappear during a few days (Harvell et al., 1995).

In general, chemically induced skin irritation can be divided into three basic subtypes, acute skin irritation, cumulative skin irritation and delayed acute skin irritation. Acute skin irritation occurs rapidly after a single exposure to a powerful irritant. The cumulative type is the most common skin irritation and arises after repetitive exposures to mild irritants. It often occurs in humans who do repetitive wet work and subsequently is often a cause of occupational skin disease (Goh and Soh, 1984).

In contrast to the predominant inflammatory reactions following the acute or cumulative form, chronic skin irritation is mainly characterized by hyperproliferation and hyperkeratosis which finally leads to hardening of the epidermis (Corsini and Galli, 1998).

For reasons of safety assessment new chemicals are still evaluated for irritant potentials by application to animals and the investigation of visible changes such as erythema and oedema (Draize, 1944). Testing for skin irritation in animals potentially cause them pain and discomfort and the results were not always predictive for those found in humans (Nixon et al., 1975; York et al., 1996). In order to replace animal testing and to improve the prediction of irritants, the cosmetic and toiletry industry, in Europe represented by COLIPA, develops and uses several alternative in vitro test systems. In this respect, the use of in vitro reconstructed organotypic skin equivalents are mostly favored, because of their increasingly close resemblance to human skin.

Nevertheless, to date there is no standardized protocol to assess irritation in vitro. Partly, this is caused by the use of various experimental protocols, which differ in the concentration of the applied irritants, the time of exposure and the time of incubation after the initial treatment. Moreover, even though considerable attention has been invested in attempting to understand the underlying mechanism(s) of skin irritation, to date, the molecular and cellular responses following contact with irritants are still poorly understood.

Due to ethical and scientific questions and on account of the 7th amendment of the European Council Directive 76/768/EEC, the authors see the requirement to

drive the development of alternative tests for irritants. Therefore, this article centres on cosmetic ingredients and provides the readership an overview of the state of art of cellular mechanisms of skin irritation and summarizes the results of the commonly used skin equivalents to evaluate irritation in vitro.

2. Mechanisms of skin irritation: presumption of the initiating steps

In this chapter we describe the known and presumed interactions of irritants and the skin. At the beginning we had the request to give a short overview about the classes of chemicals commonly provoking skin irritation: a challenging task since a vast number of chemicals belonging to different classes of substances could result in chemically induced skin irritation. Actually, every substance is an irritant to some degree and even water has possible irritant potential after long-term exposure (Tsen-Fang Tsai, 2000; Warner et al., 1999).

Problematic are those substances with double-sited effects. Due to their physico-chemical properties chemicals contribute to the efficacy of products, but some also react adversely to the skin of consumers. Regarding cosmetics and cleaning agents this holds true for molecules consisting of a hydrophobic tail and a hydrophilic head, like surfactants and emulsifier. Both are able to reduce the surface tension of and to form micelles in solution, which makes them essential ingredients in many chemical and cosmetically products. But they can also interact with lipids of the skin and that's how surfactants or emulsifier can lead to adverse effects. Not all surfactants or emulsifier have the same potential to induce irritation. The reason for this is obviously due to the chemical structure, but the underlying regularity is not well understood so far. It is discussed that this could be related to the critical micelle concentration, the structure of the alkyl tail, the functional groups or indeed a number of other factors, singly or in combination (Effendy and Maibach, 1995).

In general, solutions with lipid solving properties can lead to skin irritation. In this context, aromatic and aliphatic hydrocarbons and their derivatives cause problems with irritation (e.g. jet fuel). But it is also known that inorganic chemicals like acids, bases and salts can trigger irritation. Additionally, numerous pharmaceuticals are known to be potent irritants (e. g. dithranol).

According to the different classes and chemical structures of substances with irritant potential, it seems highly probable that different pathways must be involved in skin irritation. In general, it is accepted that chemicals can operate by at least two distinct pathways to initiate and modulate irritation (Berardesca and Distante, 1994). Firstly via damage to the barrier function of the stratum corneum and secondly by direct effects of irritants on cells of the skin. Both pathways lead to irritation either alone or in combination. As mentioned above, the stratum corneum is the outermost layer of the epidermis. The dead, keratin-rich corneocytes are tightly connected by desmosomes and the inter-cellular space is filled with the so-called "lipid cement", consisting of various lipids, mainly ceramides and neutral lipids such as free cholesterol, cholesterol esters and free fatty acids (Schürer and Elias, 1991). This composition represents an effective barrier against exogenous influences and endogenous water loss. Experiments have shown that the concentration of the irritant and the time of exposure dependence of the response are strongly related to the barrier capacity of the stratum corneum (Ponec and Kempenaar, 1995).

Irritants entering the stratum corneum can lead to delipidation and protein denaturation. Delipidation describes a process in which the balance of the different lipids is disturbed, subsequently, resulting in an altered "lipid cement" and loss of the barrier function (Ponec, 1992). In respect of delipidation, Hall-Manning et al. proposed that this process, triggered by surfactants, appears to be dependent on the critical micelle concentration of the raw surfactants (Hall-Manning et al., 1998).

These routes to barrier damage lead to enhanced transepidermal water loss and, more serious, to increased penetration of further irritants to deeper epidermal layers of living keratinocytes.

Whilst a great deal is already published on the effect of irritants on keratinocytes, it is still far from clear how substances lead to irritation.

The best-described mechanism by which chemicals lead to skin irritation is that triggered by surfactants. This is mainly rooted in the use of SDS in many in vitro experiments and as reference substance for assessing irritant potentials in vivo (Lee and Maybach, 1995; Tupker et al., 1997). Obviously, surfactants can disrupt cell membranes resulting in the release of cytoplasm (Osborne and Perkins, 1994; de Brugerolle de Fraisinette, 1999). The cytoplasm of keratinocytes of all epidermal layers contains the pro-inflammatory cytokine IL-1α, the so-called main switch in the induction of an inflammatory cascade (Luger, 1989; Nickoloff and Naidu, 1994; Corsini and Galli, 1998). Briefly, the passively released IL-1α induces the expression of further cytokines, IL-6 and IL-8. Furthermore, it activates phospholipase A_2 (PLA_2) which is a key enzyme in the arachidonic acid cascade (Terry et al., 1999). The mediators involved in this cascade will be discussed in detail later in this article.

In summary, Fig. 1 describes the proposed pathway by which surfactants initiate the release of IL-1α and subsequently leading to the induction of secondary mediators (molecular responses), followed by morphological alterations and finally the onset of typical symptoms of contact dermatitis.

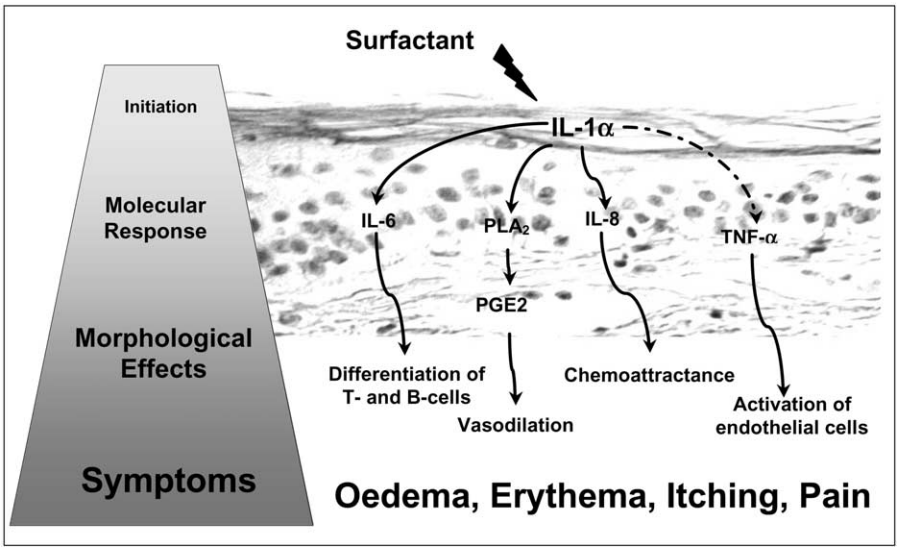

Fig. 1. Skin irritation induced by surfactants. Proposed mechanism of the pathway: surfactants initiate the release of IL-1α, subsequently leading to the induction of secondary mediators (molecular responses), followed by morphological alterations and, finally, the onset of typical symptoms of contact dermatitis.

In contrast to this mechanism, irritants without membrane-damaging characteristics probably do not initiate the inflammatory response solely by the release of IL-1α. At the cellular level, irritants induce profound alterations in the physiology of keratinocytes. One of the more recently identified effect is triggered by oxidative stress following irritation (Rogers et al., 2001; Willis et al., 1998; Camera, 1998). Keratinocytes constitutively produce reactive oxygen species (ROS), which function as second messenger and regulate the levels and activities of phosphorylated proteins and protein kinases (Coquette et al., 2000). At non-physiologic increased levels, ROS can cause cellular damage by oxidizing nucleic acids, proteins and membrane lipids, resulting in altered gene expression or cytotoxicity (Camhi et al., 1995; Allen and Tresini, 2000). Corsini et al. investigated the irritant response of tributyltin (TBT) applied to the ear of Balb/c mice. They detected dose-dependent production of IL-1α. Since TBT causes disturbances in the respiratory chain in mitochondria, the mechanism of its action may be the production of reactive oxygen species, which activate transcription factors and promote IL-1α synthesis (Corsini et al., 1996). Experiments with intra-dermally injected ROS or ROS-generating systems confirmed the involvement of ROS in skin irritation. Further evidence for the irritant-induced oxidative stress is given by experiments in which irritation induced by sodium lauryl sulfate (SLS) was diminished following application of ROS-scavenging vitamin E or superoxide dismutase (Shimizu et al., 1995).

Another conceivable mechanism by which irritants might provoke keratinocytes includes the modulation of the liposomal bilayer of cell membranes. At sub-cytotoxic concentrations, the interaction of irritants with cellular membranes or the "lipid cement" of the stratum corneum might result in substantial effects. The interference of irritants (e.g. fatty acids) with the cell membrane can modify the membrane fluidity (Fulbright et al., 1997; Zavodnik et al., 1997), which might impact receptor-mediated signal transduction (Rosette and Karin, 1996; Aragane et al., 1998) supporting or initiating irritant responses.

It seems also possible that interactions of chemicals with intracellular or membrane-bound proteins provoke keratinocytes either directly or indirectly by alteration of the epidermal environment (e. g. pH-values). Modification by or non-specific affinity of irritants to transmembranous receptors might also result in an altered signal transduction leading to irritant responses (Chou et al., 2003).

3. Test models to evaluate skin irritation in vitro

Independent of the affiliation of chemicals to the respective classes and the underlying mechanisms, the onset of skin irritation by chemicals follows general principles of toxicology. These biologic effects depend on many factors, including the concentration, the duration and frequency of exposure, the exposed skin site, the rate of penetration and the intrinsic toxic potential of the substance. In vitro, the first two parameters are simple to determine, whereas the rate of penetration and the irritant potential always has to be assessed experimentally.

This paragraph gives an overview of skin equivalents, common biomarkers and results of experiments conducted in the last years. As summary of this chapter we refer to Table 1. Here, the commonly used skin

Table 1
Overview of studies with various in vitro skin equivalents, test substances and used biomarkers

Models	Substances	Viability or Cytotox.	Morphology	Diff. gene expression	IL-1α	IL-1RA	IL-6	IL-8	PGE_2	References
					Released protein					
Skinethic RHE	SDS, TRA, Calcipotriol		+		+			+		De Brugerolle de Fraissinette et al., 1999
	Tween 80, TX, BC	+[a]			+[e,f]			+[e, f]		Coquette et al., 1999
	SDS	+[a,b]			+					Faller and Bracher, 2002
	SDS, BC, BA	+[a]			+[e, f]			+[e, f]		Coquette et al., 2003
EpiDerm	SDS	+		+[d]						Fletcher et al., 2001
	7 surfactants (anionic, nonionic and amphoteric betain)	+[a, b]			+					Perkins et al., 1998
	13 surfactants	+[c]			+[e]	+[e]	+[e]	+[e]		Bernhofer et al., 1999a
	Surfactants-based consumer products	+[c]			+	+				Bernhofer et al., 1999b
	10 Irritants, 10 Non-irritants	+[a]								Fentem et al., 2001
	SDS	+[a, b]			+					Faller and Bracher, 2002
	SDS, H_20				e					Portes et al, 2002
Episkin	10 Irritants, 10 Non-irritants	+[a]								Fentem et al., 2001
	10 Irritants, 10 Non-irritants	+[a]								Portes et al., 2002
	SDS	+[a, b]			+					Faller and Bracher, 2002
Prediskin	10 Irritants, 10 Non-irritants	+[a]								Fentem et al., 2001
LSE / HSE	7 irritants (e.g. BA, BC, SDS)	+[a]			+				+[g]	Gay et al., 1992
	SDS	+[b]	+		+[f]		+[f]	+[f]	+	Ponec and Kempenaar, 1995
	Fatty acids (7:0-12:0, 18:1, 18:2, 18:3, 20:4)				+[e]			+[e]		Boelsma et al., 1997
	SDS, TX, LA	+[a]								Sugibayashi et al., 2002
	CPC	+[b]								Watanabe et al., 2002
RE-DED	SDS	+[b]	+		+[f]		+[f]	+[f]	+	Ponec and Kempenaar, 1995
	Oleic acid		+		e			e		Boelsma et al., 1996
Apligraf	SDS, Vaseline, trans-retinoic acid, Calcipotriol	+	+		+[e]			+[e]		Medina et al., 2000
Skin[2]	16 surfactants	+[a]								Demetrulias et al., 1998
	13 surfactants	+[c]			+[e]	+[e]	+[e]	+[e]		Bernhofer et al., 1999a

(+) investigated biomarkers.
Abbreviations: BC, benzalkonium chloride, BA, benzoic acid, CPC, cetylpyridinium chloride, LA, actic acid, TX, Triton X-100, TRA, *trans*-retinoic acid.
[a] MTT.
[b] LDH.
[c] AlamarBlue.
[d] Differential Gene expression by cDNA array technology.
[e] Differential gene expression by RT-PCR.
[f] Detection of intracellular and released proteins.

equivalents, tested substances and biomarkers are listed together with the respective references.

3.1. Tissue models

The most prominent question guiding dermato-toxicologist is, whether it is feasible to use equivalents composed of keratinocytes and fibroblasts in order to evaluate irritant potentials of substances or cosmetic formulations. Since keratinocytes are discussed to play an important role in initiating, modulating and regulating skin irritation (Coquette et al., 2000), the use of epidermal skin equivalents for in vitro assessing is possible.

The 6th amendment of the European Union Cosmetics Directive (European Community directive 93/35/EEC) drew attention to alternative methods being developed to replace animal testing for assessing irritation, corrosivity and phototoxicity. These methodologies included the use of air-exposed reconstructed skin equivalents. The reasons to favor skin equivalents instead of monolayered keratinocyte cultures are obvious: compounds with a low water-solubility or finished formulations can be tested and the concentrations inducing irritant responses in monolayered keratinocytes cell cultures are several orders of magnitude lower. Therefore, results achieved from monolayered cell tests are often difficult to interpret or to correlate with the in vivo situation.

To date, two different kinds of reconstituted skin equivalents are available: (a) epidermal equivalents consisting of multilayering, differentiating human keratinocyte cultures grown on different synthetic matrices and (b) full skin equivalents with multilayering, differentiating human keratinocyte cultures grown on fibroblast containing collagen matrices. At the air-medium interface the keratinocytes of both systems develop an organized stratum corneum, which resembles a functional barrier. Currently various companies provide reconstituted human epidermal in vitro skin equivalents and the number of distributors is growing rapidly. Screening literature, the most prominent skin equivalents, which are still produced, are EpiDerm (MatTek, Ashland, MA, USA), Episkin (Episkin, Chaponost, France), Apligraf (Organogenesis Inc., MA, USA) and models engineered by Skinethic (Skinethic, Nice, France). All models consist of primary keratinocytes, seeded on matrices of either dermal components or non-biological origins. Using sophisticated protocols the keratinocytes fully differentiate and form a reconstituted epidermis. The source of the isolated keratinocytes is different. Some companies use keratinocytes from neo-natal foreskin, others keratinocytes from adult skin, widespread from cosmetic surgeries. For a more detailed characterization of the commonly used skin equivalents we refer to publications by Boelsma et al. (2000) and Ponec et al. (2002).

However, it has to be mentioned that skin equivalents differ from normal human skin in some characteristics. Compared to normal skin, equivalents vary in the penetration rate of substances through the stratum corneum with an approximately 10–30-fold higher permeability (Perkins et al., 1999; Ponec, 1992). Perkins et al. proposed that this could be an advantage in skin irritation testing of milder product formulations (Perkins et al., 1999). This probably results in an over-prediction of irritants due to the higher penetration rate of applied substances and the higher availability of the substances at the living keratinocytes.

Certainly, one of the most significant difference between in vitro equivalents and skin is the composition of the cells. For the evaluation of visible symptoms following irritation, in vivo the interaction of blood vessel endothelium, inflammatory cells, nerves etc. is essential. Using skin equivalents limited to keratinocytes and fibroblasts obliges the scientists to examine more sophisticated parameters.

3.2. Biomarkers

As a consequence of the absence of visible symptoms in vitro it is inevitable to investigate biomarkers to assess irritant responses. In this regard the term *biomarker* defines changes in the viability or cytotoxicity, morphological changes, differential expression of genes, differential distribution of proteins or other molecules following treatment with irritants.

In the following paragraphs the widespread investigated known biomarkers of skin irritation will be explained in detail.

3.2.1. Cell viability

The most commonly used parameters are measurement of cell viability (e.g. MTT conversion) (Gay et al., 1992; Triglia et al., 1991), and the membrane integrity (e. g. neutral red-uptake or LDH release) (Faller and Bracher, 2002; Osborne and Perkins, 1994).

Initial experiments showed that viability might be a suitable endpoint to evaluate the irritant potential of substances (Osborne and Perkins, 1994; Korting et al., 1994). The use of measuring viability for assessing irritant potentials was discussed at the workshop 38 of the European Center for the Validation of Alternative Methods (ECVAM). The members agreed that although there is a correlation between irritant potential and reduced cell viability, there are other important confounding factors (van de Sandt et al., 1999). However, at that time measuring viability has been proved to be a more robust endpoint than other mechanistic biomarkers. Therefore, ECVAM initiated a pre-validation study with five in vitro models and 20 substances, mainly using MTT conversion as endpoint (Fentem et al., 2001). As a result of this study, the ECVAM man-

agement team summarized that measuring cytotoxicity alone does not always reveal the right prediction between irritants and non-irritants (Fentem et al., 2001). For that reason, additional, more specific, biomarkers should be incorporated in further studies. In this respect, measuring the differential distribution (i.e. release and storage) of cytokines or other molecules might be a promising approach.

3.2.2. Interleukin 1 alpha (IL-1α)

As mentioned above IL-1α is a very important inflammatory mediator in the skin and is believed to be the main switch in the initiation of inflammation (Coquette et al., 2000). The 37 kDa IL-1α is constitutively expressed in keratinocytes and accumulates in the keratinocytes of all epidermal layers in the cytoplasm or as a membrane-bond form (Dinarello, 1998).

In an intact epidermis the IL-1α-reservoir is naturally eliminated by desquamation, due to the fact that IL-1α has no hydrophobic leader sequence for transmembrane secretion. Therefore, IL-1α is only released from leaky cells following cell injury or membrane perturbation (Dinarello, 1998). IL-1α induces the expression of itself, and other proinflammatory cytokines like IL-6 and IL-8 by binding to the IL-1 receptor I, which is expressed on the cell membrane surface of keratinocytes (Sims et al., 1993). The IL-1 receptor II, also detected on keratinocytes, has no intracellular protein domain, and binding of IL-1 to this receptor is not transmitting a signal. The proposed function of the IL-1 receptor type II is to protect cells from overwhelming IL-1 responses (Colotta et al., 1993).

At the molecular level IL-1 is a potent regulator of the NFκB pathway. The transcription factor NFκB controls the expression of various genes associated with the regulation of epidermal homeostasis and inflammatory responses. Following stimulation with IL-1, cells show a rapid phosphorylation of the NFκB regulating protein IκB, which results in a subsequent translocation of cytoplasmatic NFκB into the nucleus (Stylianou et al., 1992). Additionally, another transcription factor, the activating protein-1 (AP-1), also triggers the cellular response following IL-1 stimulation (O'Neill et al., 1998).

What are the findings on the regulation of IL-1 in different in vitro skin equivalents? Ponec and Kempenaar investigated the release and the cytoplasmatic concentration of IL-1 in two full thickness skin equivalents after SLS induced irritation. Intracellular, the IL-1 levels increased dose-dependently, but IL-1 was only released at cytotoxic SLS concentrations (Ponec and Kempenaar, 1995). Investigating the release of IL-1α after treatment of the full thickness skin equivalent SKIN^2 and an epidermal equivalent (EpiDerm) with surfactants revealed little difference in the amounts released (Bernhofer et al., 1999). This documents that following irritation IL-1α is expressed in and exclusively released by keratinocytes.

Boxman et al. investigated the regulation of further proinflammatory cytokines by IL-1α in vitro (Boxman et al., 1996). The release of IL-1α from activated keratinocytes may act as an inducer of IL-6 and IL-8 in dermal fibroblast, where two to three orders of magnitude more IL-6 and IL-8 was produced compared to keratinocytes (Boxman et al., 1996). They concluded that the modulation and the regulation of an acute inflammatory responses (e.g. IL-6 and IL-8 release) following irritation depends on the communication between keratinocytes and fibroblasts.

Additionally, the IL-1 receptor antagonist (IL-1ra), another member of the IL-1 family, is also of interest when looking at irritation. IL-1ra binds to the IL-1 receptors with nearly the same affinity as IL-1α, but without triggering a response (Dinarello, 1998). As a result IL-1ra effectively prevents tissues of overwhelming IL-1α responses. Comparison of the concentration of the constitutively expressed IL-1ra and IL-1α resulted in a ratio of 100–1000 times more receptor antagonist (Arend, 1993; Haskill et al., 1991; Hammerberg et al., 1992). The reason for this difference is the extensive amplification of the IL-1 signal following binding to the IL-1 receptor I. In this respect, the occupation of approximately 2–3% of IL-1R type 1 by IL-1 is sufficient to induce the biological response (Ye et al., 1992; Gallis et al., 1989). In contrast to IL-1α and IL-1β, the mRNA for IL-1ra is translated in the endoplasmatic reticulum and is transported to the Golgi. Although it possesses a leader sequence it is not glycosylated, and therefore keratinocytes do not appear to actively secrete IL-1ra (Arend, 1993).

IL-1ra has been detected in supernatants after irritation in vitro. Bernhofer et al. proposed that measuring the IL-1ra secretion in an epidermis model is the most sensitive predictor to differentiate between mild irritating surfactants (Bernhofer et al., 1999a,b). Recent clinically studies showed that the ratio of IL-1ra to IL-1α, detected in the stratum corneum can be used as a predictor of inflamed skin (Perkins et al., 2002).

3.2.3. Interleukin 6 (IL-6)

The 26 kDA IL-6 was initially discovered in fibroblasts following stimulation with proinflammatory mediators like IL-1 and TNF. It is a pleiotropic cytokine influencing inflammatory reactions, a potent B-cell differentiation-factor and stimulates keratinocyte proliferation (Grossmann et al., 1989). Although IL-1α induces the expression of IL-6 after activation of keratinocytes, not many studies have investigated the release of this secondary cytokine as a means of assessing irritation in skin equivalents. Bernhofer et al. detected IL-6 to be released at a relatively constant level from the full-thickness model SKIN^2, regardless of the treatment

(Bernhofer et al., 1999a,b). Ponec and Kempenaar described only limited modulation in IL-6 release after treatment of a re-epidermized de-epidermized dermis-model (RE-DED) with SLS. But treating the living skin equivalent (LSE^{TM}) with SLS they detected a more than 5 fold-increased release of IL-6 (Ponec and Kempenaar, 1995).

Obviously, these studies have been conducted with full thickness models. In our hands, experiments with epidermal equivalents (Skinethic and EpiDerm) showed no release of IL-6 (data not shown). These preliminary data are in concordance with results of Boxman et al. (1996), where the release of IL-6 was detectable only in co-cultures of fibroblasts and keratinocytes. In summary, IL-6 seems to be feasible as a marker for assessing skin irritation, but the test has to be carried out using full thickness models.

3.2.4. Interleukin 8 (IL-8)

IL-8 is a C-X-C class chemokine with strong chemotactic effects on polymorphonuclear neutrophils and lymphocytes. Additionally, it increases the intercellular Ca^{2+} concentration and induces granule exocytosis (Barker et al., 1991). Keratinocytes express IL-8 after stimulation with IL-1α, IL-1β, IFN-γ or TNF-α (Wilmer and Luster, 1995).

Enhanced expression of IL-8 has been shown to be a rather non-specific response to various noxes and therefore a more general parameter of tissue damage (Mohammadzadeh et al., 1994). Bernhofer et al. could not detect IL-8 secretion by an epidermal model in response to topical application of various facial creams. However, in the presence of fibroblast (full-thickness model) they detected elevated amounts of IL-8 in the culture (Bernhofer et al., 1999a,b). They speculated that following irritation, IL-8 is either secreted by fibroblasts or, alternatively, fibroblasts up-regulate production of IL-8 in keratinocytes.

Our initial experiments revealed that IL-8 is released following treatment of epidermal equivalents (Skinethic and EpiDerm) with recombinant human IL-1α (unpublished data). Very recently Coquette et al. (2003) showed that Skinethic models release IL-8 following irritation. Furthermore, they proposed that the ratio of released IL-8 to IL-1α is feasible to discriminate irritants from sensitizers (Coquette et al., 2003).

3.2.5. Tumor necrosis factor alpha (TNF-α)

TNF-α is a 17 kDa pleiotropic proinflammatory cytokine and influences the development of inflammation by inducing the expression of cutaneous and endothelial adhesion molecules (Groves et al., 1995). TNF-α is stored in the epidermal mast cells (Gordon and Galli, 1990). Additionally, it can be produced by keratinocytes following stimulation (Kock et al., 1990). The expression of TNF-α is transiently induced after treatment with various irritants and it is independent of the release of IL-1α (Corsini et al., 1996).

Using a full thickness skin model, Bernhofer et al. detected elevated mRNA quantities for TNF-α 16 h after the topical application of SDS. Some 24 h after the initial treatment the mRNA level had returned to baseline (Bernhofer et al., 1999a,b). However, TNF-α release was not detected following treatment of different skin equivalents with SDS and various other surfactants (Bernhofer et al., 1999a,b). Holliday et al. investigated the cutaneous expression of IL-6 and TNF-α in mice after provoking the skin with allergens oxazolone and 2,4-dinitrochlorobenzene and the irritant benzalkonium chloride (Holliday et al., 1997). In concordance to Bernhofer et al. (1999a,b), Holliday et al. also could not detect the release of TNF-α following treatment with a surfactant. They concluded that not all chemicals, which have the potential to cause skin irritation and cutaneous inflammation will elicit detectable TNF-alpha responses (Holliday et al., 1997).

3.2.6. Interleukin 10 (IL-10)

Initially IL-10 has been described as a cytokine produced by Th2 cells and which inhibits the release of Th1-derived cytokines. However, IL-10 is also expressed in keratinocytes (Enk and Katz, 1992a, b). Nickoloff and colleagues detected up-regulation of IL-10 mRNA in tape-stripped epidermis in vivo (Nickoloff et al., 1994). On the other hand, Luger et al. described no detectable up-regulated expression of IL-10 following contact with irritants but allergens (Luger et al., 1997).

Berg et al. examined the role of endogenously produced IL-10 in the regulation of inflammatory and immune reactions in the skin of genetically modified mice. In summary, they concluded that IL-10 is a natural suppressant of irritant responses, and it limits immunopathologic damage in the skin (Berg et al., 1995). Therefore, IL-10 might represent an important co-factor in the recovery phase and the cumulative irritation. This makes IL-10 particularly interesting for further investigation in skin irritation.

Apart low molecular weight proteins, derivatives of fatty acids and differential distribution of calcium are involved in skin irritation.

3.2.7. Arachidonic acid metabolites

Regarding inflammatory skin diseases, one of the commonly investigated groups of fatty acid derivatives are members of the arachidonic acid cascade. Metabolites of arachidonic acid (i.e. prostaglandins, thromboxanes, leukotrienes and hydroxyeicosatetraenoic acids) are actively synthesized in the skin (Ruzicka, 1989, 1990). Arachidonic acid, a long-chain polyunsaturated fatty acid, is released by enzymatic activity of the phospholipases A_2 and C (PLA_2, PLC) on membrane-sited

phospholipids (Greaves and Camp, 1988). The major enzyme responsible for the arachidonic acid release following various stimuli is believed to be the cytosolic PLA_2. As described elsewhere within this review, the activity of the cytosolic PLA_2 is strongly Ca^{2+} dependent and has to be activated by phosporylation triggered by kinases of the MAPK cascade (Leslie, 1997). Subsequently, the free arachidonic acid can undergo conversion into prostaglandins by the activity of cyclooxigenase 2 and PGE synthase.

Looking at skin irritation, prostaglandin E_2 (PGE_2) is the best-investigated prostaglandin. Following irritation with various irritants PGE_2 has been found to be released (Gay et al., 1992; Ponec and Kempenaar, 1995). Furthermore, Gay et al. investigated the release of an other prostaglandin PGI_2 in living skin equivalent (LSE) following irritation, whereas PGI_2 remained at background concentrations (Gay et al., 1992). Apart of prostaglandins, Dykes et al. demonstrated the release of leukotriene B4 and 15-hydroxyeicosatetraenoic acid from arachidonate prelabelled skin equivalents in response to benzoyl peroxide (Dykes et al., 1991).

Interestingly, not all in vitro skin equivalents release PGE_2 into the supernatant. Personal observation with the detection of PGE_2 following irritation in vitro revealed a discrepancy using EpiDerm and Skinethic epidermis models. EpiDerm releases PGE_2 into the medium after stimulation whereas no detectable amounts of PGE_2 were found using Skinethic models sized 0.63 cm^2.

3.2.8. Calcium

The epidermis harbors an well-organized calcium gradient with high levels at the upper stratum granulosum and lower stratum corneum, and low concentration in the stratum basale. damage to the stratum corneum is accompanied by radical alterations of the epidermal calcium-gradient. Within the first 30 min after barrier disruption the naturally abundant calcium gradient is completely decomposed (Denda and Kumazawa, 2002).

In the same context, Grängsjö et al. described changes in the intracellular concentration of various cations (e.g. Ca^{2+}, Mg^{2+}) in the stratum basale and stratum spinosum after topical treatment of human volunteers with SLS or nonanoic acid (Grängsjö et al., 1996). In this report topical exposure of nonanoic acid revealed a higher irritation score than SLS and these differences were also distinguished measuring the Ca^{2+}-levels.

Calcium is an important ion involved in the regulation of key mechanisms of all cells. In the skin, calcium is crucial for the maintenance of the epidermal homeostasis and is deeply involved in the restoration of the damaged stratum corneum (Mauro et al., 1998; Denda et al., 2000). For instance, it is an important co-factor in the activation of protein kinase C-triggered signaling (Bikle et al., 2001; Schmidt et al., 2000).

In respect to the restoration of the stratum corneum, Denda and Kumazawa recently showed that alterations in the magnesium and calcium gradient increases the secretion of lamellar bodies, which finally results in an accelerated barrier repair (Denda and Kumazawa, 2002). Additionally, Denda et al. described the acceleration of barrier recovery in tape-stripped skin of hairless mice following treatment of irritated skin with various mixtures of magnesium and calcium salts (Denda et al., 1999).

Calcium is also involved in the regulation of inflammatory processes, because intracellular Ca^{2+} triggers the translocation of the cytosolic phospholipase A_2 to the membrane and, subsequently, supports the initiating of the arachidonic acid metabolism cascade (Yoshihara and Watanabe, 1990; Leslie, 1997).

Some reports describe that irritants like SLS affect cell proliferation (Bloom et al., 1994) and differentiation (Le et al., 1996) and thus modify the epidermal homeostasis. This might partly be due to the altered Ca^{2+} gradient or the inflammatory response, but the underlying mechanism is not well understood.

4. Approaches to develop novel biomarkers

The currently used biomarkers described herein are potential options for the differentiation between irritants and non-irritants of some classes of substances.

It seems highly arguable whether one test system is sufficient to classify chemicals from different classes or we need separated tests for each class of substance. Therefore, investigations of further parameters are necessary in order to set up in vitro test systems, which resembles the in vivo situation as close as possible.

To date much effort is spend to find new markers involved in skin irritation. One possibility to unravel the mystery of skin irritation is comparing the expression pattern of irritated and non-irritated skin equivalents with sophisticated methods of genomics or proteomics. In this context, Corsini et al. (2002) started a project to discover new genes involved in the effects of allergens on keratinocytes using Differential Display PCR technique. Even though the draft human genome is sequenced, not all genes are functionally described or even known. In order to detect new differentially expressed genes in the context of skin irritation this seems to be an interesting approach.

A similar methodology to target differential gene expression was done by Flechter et al. (2001). They investigated the differential gene expression of a vast number of genes using cDNA array technology after treatment of EpiDerm with SLS for 15 min up to 24 h (Fletcher et al., 2001). They detected the up-regulation of transcription factors and genes for trafficking and transporters after 15–30 min of incubation. At later time points (1–3 h) DNA repair genes, tumor suppressors,

genes involved in protein translation and metabolism were up regulated.

Screening for differentially abundant proteins was conducted by Boxman and colleges in 2002 (Boxman et al., 2002). Comparing the expression profile of SLS-treated human skin by 2D-PAGE revealed 7 potential new epidermal markers for skin irritation. Among those, the heat shock protein 27 was the most prominently up-regulated one (Boxman et al., 2002).

Dealing with known genes to assess irritants, the list of chemokines, which is increasing continuously, presents a large pool of putative targets. Recently, the interaction of the new skin-specific chemokine CCL27 and the respective receptor CCR10 has been described to be responsible for the specific homing of CLA^+ memory T cells in inflammatory skin diseases like psoriasis, atopic or allergic contact dermatitis (Morales et al., 1999; Homey et al., 2002). At the 6th congress of the European Society of Contact Dermatitis in Rome (2002) Gibbs et al. presented a poster with data of the release of CCL27 from skin equivalents treated with irritants and allergens. They concluded that CCL27 is increasingly secreted early after treatment with non-cytotoxic concentration of both irritants and allergens. It looks promising to focus on the investigation of CCL27 whether it can be used as an early marker of skin irritation.

Considering that skin corrosivity, leading to excessive destruction, wounding and scarring, is the most aggressive form of skin irritation it can be proposed that skin irritation shows some similarities to skin wounding. In this respect, the skin-derived anti-leukoprotease SKALP/elafin is an interesting marker. SKALP/elafin has been described to be induced in inflammatory skin disorders, wound healing and irritation following treatment with SDS, oleic acid and tape-stripping in vitro (Schalkwijk et al., 1993; van Bergen et al., 1996; Boelsma et al., 1998). Recently, Tanaka et al. described a 4-fold increase in the release of SKALP/elafin following stimulation of cultured keratinocytes with TNF-α (Tanaka et al., 2000). The release of SKALP protects cells from polymorphonuclear leukocytes-derived elastase and proteinase-3 (Schalkwijk et al., 1990; Wiedow et al., 1991). Since release of SKALP following irritation can easily be detected in the cell culture media (Tanaka et al., 2000) it seems promising to evaluate SKALP for the use as a marker for skin irritation.

Additionally, other keratinocyte-specific proteinase-inhibitors might be involved in the regulation of inflammation and epidermal homeostasis. In this context the role of the latest described human serine protease inhibitor SERPIN B13/hurpin (Abts et al., 1999) might be of special interest, due to its skin-specific expression and its association with the inflammatory skin disease psoriasis. Additionally, SERPIN B13/hurpin seems to be involved in the regulation of keratinocytes homeostasis following UV irradiation (Welss et al., 2003).

The different markers and test-systems described above can just measure the effects following a treatment of biological material. In order to predict irritant potential of substances and to bypass "wet" experiments various scientists try to evaluate the irritant potential of substances in silico. Recently, Smith et al. developed a structure-activated relationship model, where 19 different physicochemical parameters were calculated for 42 investigated esters (Smith et al., 2000). In conclusion, their in silico model is applicable for prediction of human irritation of esters yet untested.

5. Perspectives of the future

In 1999/2000 a pre-validation study on in vitro tests in order to replace animal testing for risk assessment of chemicals has been conducted by ECVAM (Fentem et al., 2001). In the final phase III of this study a set of 20 coded chemicals, of 10 irritants and 10 non-irritants, were assessed for irritation in EPISKIN and EpiDerm by measuring cytotoxicity (MTT), and the skin integrity function test (SIFT) having TEWL and TER as endpoints. In conclusion, the predictive ability of EpiDerm and EPISKIN was not sufficient to meet the management team's prediction. The management team suggests to incorporate additional endpoints alongside the cytotoxicity into further testing (Fentem et al., 2001). Therefore, various follow-up activities to the prevalidation study are currently in progress (Zuang et al., 2002; Portes et al., 2003, Heylings et al., 2003), with the objective of having revised protocols suitable for inclusion in a formal validation study, which will start in late 2003 (Fentem and Botham, 2002).

In 2001 the Task Force Skin Tolerance of COLIPA started a research project on skin irritation. One focus within this project is to evaluate mechanisms by which different classes of substances induce skin irritation. These findings will hopefully lead to a new test system to assess irritants in vitro using new endpoints as well as so far known markers.

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