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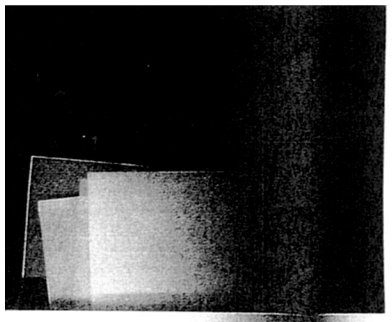

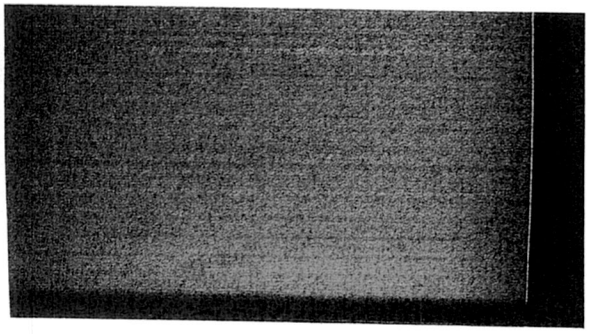

9 The Keratinocyte in Cutaneous Irritation and Sensitization

Alain Coquette, Nancy Berna, Yves Poumay, and Mark R. Pittelkow

CONTENTS

I. INTRODUCTION

Epithelial tissues, including epidermis, tracheobronchial epithelium of lung, gastrointestinal epithelium, and uterine cervical epithelium, play a critical role in protecting man and other mammals from external environmental threats. Epithelial cells such as epidermal keratinocytes have long been known to provide a relatively impermeable barrier to outside factors that challenge the structural integrity and resilience of epidermis and other epithelia. However, only more recently have we discovered the active role played by the keratinocyte in initiating, modulating, and regulating responses of the skin as well as organism to the multitude of irritant or allergic (sensitizing) reactions that are part of daily existence. Keratinocytes express and, in some cases, secrete a plethora of biologically active molecules that mediate these responses. As the identification and biological function(s) of factors produced by keratinocytes continue to expand, the complexity and functional sophistication of epidermis become more apparent.

This chapter provides an overview and update on the role of the keratinocyte in cutaneous irritant and sensitization reactions. These findings significantly impact how skin reactions in dermal and transdermal delivery can be biochemically modulated. We also summarize various models that have been developed to better assess and predict epidermal irritation and sensitization. The cellular and molecular mechanisms mediating these responses in man will also be delineated.

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The epidermis is a multilayered squamous epithelium that forms the interface between the organism and its environment. It is composed of several types of specialized resident or transient epithelial, neuroectodermal, and bone marrow-derived cells. These include epidermal keratinocytes to generate the protective barrier and provide for repair and regeneration of the epidermis, Langerhans cells, and T lymphocytes (T cells) for immunologic defense, melanocytes for pigment production and protection from ultraviolet radiation, and Merkel cells for neurocutaneous sensibility. Keratinocytes constitute the major cell type (>90%) and thus have the primary biologic role in providing both physical and biochemical attributes that maintain epidermal integrity and homeostasis. Epidermal keratinocytes also create a sentry function and compose the first level of communication with neighboring skin cells as well as other distant organs.[1]

The keratinocyte elaborates its protective function by undergoing a complex and finely coordinated program of cellular differentiation.[2] The basal layer consists of a single layer of proliferative and noncommitted keratinocytes, a fraction of which are functionally stem cells. The basal cell layer is anchored to the basal lamina via hemidesmosomes. These basal cells produce daughter cells that can either continue to populate the germinative layer or exit the basal layer to undergo terminal differentiation as they migrate to the epidermal surface. The spinous layer, constituting several or more cell layers, is located immediately above the basal layer and is characterized by the presence of extensive desmosomal connections between cells. The next morphologic layer, the granular layer, is distinguished by the presence of keratohyalin granules within the cytoplasm of the keratinocyte. Keratohyalin granules contain products of keratinocyte differentiation, such as loricrin, filaggrin, cystatin-α, and lipids that are used in the assembly of the corneocyte membrane and intercellular compartment. Another subcellular organelle, the keratinosome or lamellar body, is a specialized secretory vesicle present in the upper spinous and granular layers. Enzymes such as glucosylceramide synthase, lipid substrates/products such as glucocylceramides and sphyingolipids, as well as specialized proteins such as corneodesmosin that make up the corneodesmosomes of the cornified layers, are also present in keratinosomes.[3,4] The transition zone delineates the region between nucleated and anucleate cells in upper epidermal layers. Within this region, selected cellular organelles and nucleic acids are targeted for elimination by the action of specific proteases, nucleases, and other enzymes. The final stage in keratinocyte terminal differentiation results in the formation of the cornified layer. This outermost layer is made up of corneocytes or "bricks" that form a packaged, stabilized array of keratin filaments, proteins, peptides, and other breakdown products contained within a cross-linked protein envelope and united by a lipid-rich intercellular "mortar."

Each stage of epidermal differentiation is characterized by specific biomarkers of gene expression. During normal epidermal differentiation, keratins 5 (K5) and 14 (K14) are expressed in the basal keratinocyte layer, while keratin 1 (K1) and 10 (K10) are expressed in the suprabasal layers. Involucrin is expressed in the late spinous layers and granular layers, and loricrin and filaggrin are specific markers of granular layers.

In the last decade, it has become clear that keratinocytes are not simply a mechanical barrier to the external environment, but are also able to produce a number of cytokines and other mediators with immunologic, inflammatory, and cell-adaptive (e.g., proliferative) properties. Cytokines are relatively small, soluble (glyco)proteins which are synthesized and secreted by various cells, bind to specific receptors, and regulate activation, proliferation, and differentiation of immune as well as nonimmune cells. They include several subclasses, designated: (1) interleukins (IL), (2) colony-stimulating factors (CSF), (3) interferons (IFN), (4) tumor necrosis factor (TNF) family members, (5) growth factors, and (6) suppressor factors.[5,6] Selected cytokines produced by keratinocytes in sensitization or irritation reactions will be reviewed here as well as in Chapter 12. We also will briefly review other keratinocyte-produced factors that mediate these responses. These include arachidonic acid and metabolites, biogenic amines, small molecular weight factors, and second-messenger molecules, as well as nitric oxide (NO) and reactive oxygen species (ROS). Together, these constitutive or inducible gene products and cellular metabolites of the keratinocyte directly or indirectly regulate the epidermal response to irritant or allergic agents contacting skin.

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Figure 1 provides a schematic framework depicting the sequence of cellular and biochemical events that induce irritant or sensitization reactions in epidermis. The keratinocyte plays a central role in controlling and coordinating cutaneous responses by other immune and inflammatory cells within and between the epidermis, dermis, and microvasculature.

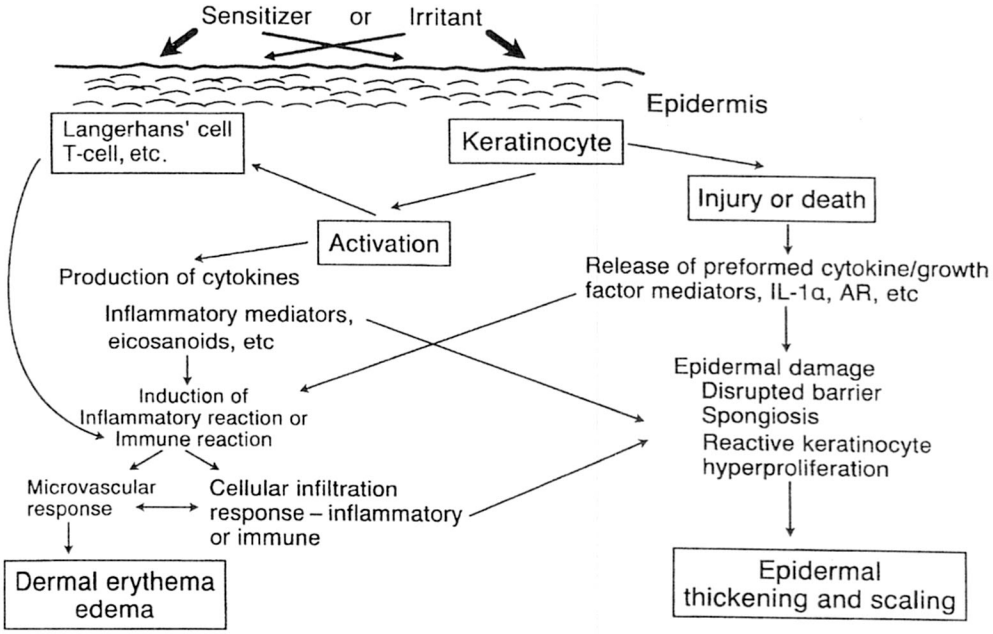

Figure 1 Sequence of events following irritant or sensitizer exposure to epidermis. (Modified from Corsini and Galli, *Toxicol. Lett.*, 102–103, 277–82, 1998.

Numerous protein and nonprotein factors are synthesized and secreted or released by keratinocytes that become "activated" by an irritant or allergen. A current, but inevitably incomplete list of these biologically active factors is presented in Table 1. The function(s) of some of these factors are well characterized while others are less well defined.

An important concept still to be comprehensively addressed for keratinocyte function in irritation and sensitization reactions is the hierarchy and ordering of events that take place within a single cell and the tissue to produce a given response. This concept is also critical for many other epidermal reactions to disease (e.g., psoriasis,[7] dermatitis, viral infections [verrucae-human papillomavirus, etc.]) In this context, some cytokines, such as IL-1 and TNF-α, have been considered to be "primary" cytokines, whereas others, such as IL-6, IL-8, and GM-CSF, are "secondary" since they are insufficient to induce an inflammatory response in the absence of other stimuli or primary cytokines.[8] However, the biological circuitry is no doubt much more complex and will likely require sophisticated mathematical modeling and application of neural network theory to fully describe the biological "output" of the keratinocyte that has been stimulated by an irritant or allergic "input."[9]

II. KERATINOCYTE IRRITATION OR SENSITIZATION: THE INTEGRATED CELL RESPONSE

As depicted in Figure 1, irritants and allergens (haptens) have the ability to initiate similar responses in epidermis. In fact, irritants and sensitizers have the potential to overlap in their activity profiles; that is, some sensitizers also have irritant properties. The difference lies in the ability of a sensitizer

TABLE 1
Keratinocyte Mediators of Irritation and Sensitization

Cytokines

Primary
- IL-1α
- IL-1β

TNF-α

Humoral/cellular immune regulation
- IL-10
- IL-12
- IL-18
- IFN-α
- IFN-β

T cell growth
- IL-7
- IL-15

Colony-stimulating activity
- IL-6
- G-CSF
- M-CSF
- GM-CSF

C-X-C chemokines
- IL-8
- Gro-α, -β, -γ
- IP-10

C-C chemokines
- MCP-1
- MIP-1α
- RANTES

Growth factors
- TGF-α
- AR
- HB-EGF
- NDF
- VEGF
- PDGF
- NGF
- FGFs
- Neurotrophin

Suppressive/antagonist
- IL-IRA
- TGF-β
- IL-10

Neuroendocrine

α-MSH

Eicosenoids

Arachidonate

PGE-2

12-HETE

LTB_4

Oxygen-derived

Nitric oxide (NO)

Hydrogen peroxide (H_2O_2)

Superoxide ($O_2^{\bullet-}$)

to induce a specific immune response with immunological "memory." By contrast, cutaneous irritation is a nonimmunologic, reversible, local inflammatory reaction that induces edema and erythema following a single or repeated epicutaneous exposure to the chemical at a defined skin site.

Upon exposure of the keratinocyte to an irritant or sensitizer, cell injury or cell death (due to sufficiently severe damage induced by agents such as nitrogen or sulfa mustard agents[10]) occurs and triggers a set of responses in the keratinocyte and epidermis. Key to this response is IL-1α release. Loss of barrier function by irritants, such as acetone, a strong delipidizing solvent, also can trigger rapid increase in expression of specific growth factors, such as amphiregulin (AR) and nerve growth factor (NGF).[11]

The keratinocyte becomes "activated" in response to irritant or sensitizer exposure. Specific sets of cytokines, as well as arachidonic acid metabolites and other inflammatory mediators, are expressed and secreted to trigger and modulate the inflammatory reaction. The ability of the keratinocyte to participate in generating effective signals for recruitment of Langerhans cells and T cells and propagating the afferent immune response places it within the central hub of the skin immune system (SIS).

Whether induced by an irritant or a sensitizer, similar morphologic and histologic features of erythema, edema, and epidermal scaling and thickening (acanthosis) are observed. Chapters 6, 7, and 10 delineate the unique roles of the Langerhans cell and the T cell in the epidermal immune response and allergic contact dermatitis.

In addition to expressing and releasing potent cytokines and other inflammatory mediators, the keratinocyte also modulates expression of various immune and nonimmune related cell surface receptors, cell adhesion molecules, and extracellular matrix (ECM) factors. These cell-associated molecules likely play important roles in orchestrating the keratinocyte response during irritant and sensitization reactions. These gene products include ICAM-1, HLA-DR, receptors of growth factor and other cytokine families, integrins, cadherins, fibronectin, heparin sulfate and related proteoglycans, and numerous other cell–cell and ligand–receptor factors.

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. The keratinocyte has the potential to either upregulate or downregulate a specific cutaneous response. For example, ultraviolet (UV) radiation induces cytokine cascades that have the ability to induce systemic immune suppression.[12] We have recently shown that H_2O_2 and other ROS induced in human keratinocytes by UVB rapidly, but transiently, enhance epidermal growth factor (EGF) receptor phosphorylation and differentially activate downstream protein kinase signaling pathways, including extracellular regulated kinase (ERK), p38, and c-jun N-terminal kinase (JNK), critical kinases of mitogen- and stress-related cascades in keratinocytes.[13,14] These pathways, in part, terminate in the nucleus where specific transcription factors such as activator protein (AP)-1, AP-2, γ-interferon activation site (GAS), NF-κβ, EGR, etc. regulate gene expression within the keratinocyte and many other cell types. In this regard, glucocorticoids are also known to be potent inhibitors of the inflammatory response. Recent studies have demonstrated that these steroid hormones strongly inhibit AP1, GAS, and NF-κβ DNA-binding activities and induction of the arachidonic acid metabolizing enzyme, cyclooxygenase-2 (COX-2), in IL-1β-stimulated keratinocytes.[15]

These findings link cytokines and other inflammatory mediators to signaling pathways that "activate" the keratinocyte, but also demonstrate that keratinocyte responses can be downregulated by UV or glucocorticoids, well known and potent modulators of cutaneous irritation and sensitization (see also Chapters 15 and 20).

In the following sections, we provide a concise review of selected cytokines and other inflammatory mediators produced by keratinocytes that regulate cutaneous sensitization and irritancy. This chapter also examines the progress and comparative evaluation of in vitro models to test irritants and sensitizers using keratinocytes or more complex multicellular systems.

III. KERATINOCYTE ELABORATED MEDIATORS

A. Interleukin 1 (IL-1)

IL-1 was originally described as a lymphocyte-activating factor produced only by monocytes. However, it is now well established that many cells, including epithelial cells, endothelial cells, fibroblasts, and various tumor cells, produce IL-1.[16] Two different forms of IL-1, IL-1α and IL-1β, encoded by distinct genes, have been identified. These two forms bind to the same two IL-1 receptor types, suggesting they have similar biological activities. IL-1α and IL-1β are synthesized as larger "pro-interleukins," which in the case of IL-1β must be cleaved by a specific converting enzyme to the shorter biologically active form. IL-1α is also cleaved, but this does not seem to be necessary for its activity. Keratinocytes are able to synthesize and secrete both forms of IL-1, but the predominant biologically active form released by keratinocytes is IL-1α,[17] since keratinocytes lack constitutive IL-1β converting enzyme (ICE) activity. However, ICE activity is induced in keratinocytes by both irritant chemicals and sensitizers, such as urushiol.[18] IL-1β activation may be induced in epidermis in vivo by a non-ICE mechanism.[19] This contrasts with observations in

vitro suggesting lack of IL-1β processing.[20] IL-1α appears to be retained intracellularly or in a membrane-bound form. As long as the epidermis is intact, IL-1 is eliminated by normal desquamation. Because IL-1 lacks a hydrophobic leader sequence necessary for transmembrane secretion, it has been proposed that it only can be released after some type of cell injury or membrane perturbation.[16] In human skin, the levels of IL-1 are 100 to 1000 times higher than in most other tissues. Keratinocytes are able to produce it constitutively without stimulation. Upregulation of IL-1 synthesis has been observed upon stimulation with lipopolysaccharides (LPS), phorbol myristic acetate (PMA), physical, chemical, or thermal injury, ultraviolet irradiation, and a variety of cytokines (i.e., GM-CSF, TNF-α, IL-6, TGF-α, and IL-1α and IL-1β itself).[21] Interestingly, IL-β appears to be specifically induced by hapten within 1 to 3 h of exposure, whereas IL-1α mRNA is not induced by either hapten or primary irritants, as measured by reverse transcriptase-polymerase chain reaction (RT-PCR).[22] Furthermore, IL-1β induces Langerhans cell migration out of epidermis and neutralizing antibody to IL-β, but not IL-1α, TNF-α, or GM-CSF, prevented allergen-induced migration of Langerhans cells, suggesting that IL-1β plays a role in irritation of contact hypersensitivity.[23] The effects of IL-1 are highly pleiotropic and space limits delineation of all of its biological effects. For further detailed information on IL-1, see Chapter 12.

IL-1 is a proinflammatory cytokine. It is chemotactic for monocytes, lymphocytes, and neutrophils. It stimulates the proliferation, differentiation, and activation of various cells and the production of other cytokines such as GM-CSF, IL-6, and IL-8. Keratinocytes, in addition to producing IL-1, express large amounts of specific IL-1 receptors and IL-1 receptor antagonists (IL-1ra).[24] This antagonist binds to the same receptor as IL-1, but it does not produce cell activation and so acts as a competitive inhibitor to prevent IL-1 effects unless IL-1 exceeds certain threshold levels. The reader is referred to Chapter 14 for further information on IL-1 and IL-1ra effects.

B. Interleukin 6 (IL-6)

IL-6 is a multifunctional cytokine released by many different cells, including monocytes, fibroblasts, endothelial cells, keratinocytes, and different tumor cells.[25] Unstimulated keratinocytes usually produce low levels of IL-6, but expression can be upregulated by the addition of stimulants such as IL-1, LPS, PMA, or UV-B irradiation, TNF-α, GM-CSF, IL-4, TGF-β, and injury.[6] Like IL-1, IL-6 has a variety of biological activities on different target cells. Many biological effects of IL-1 and IL-6 overlap. IL-6 may augment proliferation of keratinocytes. Moreover, some evidence suggests that IL-6 plays a role as mediator in inflammatory skin diseases such as psoriasis.[21] Compared to other cytokines and growth factors, the potency of IL-6 in these responses is less pronounced and likely secondary.

C. Interleukin 8 (IL-8)

In addition to monocytes, a variety of cells including endothelial cells, keratinocytes, fibroblasts, and T lymphocytes produce IL-8.[26] Keratinocytes do not produce IL-8 constitutively, but the production is stimulated by other cytokines (IL-1α, IL-1β, TNF-α, and IFN-γ), LPS, and phorbol esters.[27] IL-8 is strongly chemotactic for polymorphonuclear neutrophils and lymphocytes, increases cytosolic free calcium, and induces granule exocytosis.[28] IL-8 is also chemotactic for human basophils and stimulates them to release histamine.[27] Therefore, IL-8 is also classified as a potent chemokine of the C-X-C class.[26]

D. Interleukin 10 (IL-10)

Originally described as a product of bone marrow-derived cells, IL-10 is also produced by activated murine keratinocytes.[29] IL-10 is known to be an anti-inflammatory cytokine and may act as a suppressor factor of immune reactions. IL-10 expression is enhanced in UV-treated keratinocytes, and hapten-specific tolerance induced by UVB is mediated by IL-10.[30] It may inhibit the production

of cytokines such as IFN-γ, IL-1, and TNF-α. By inhibiting IFN-γ production by Th1 cells, it promotes induction of a Th2 response. One role of IL-10 may be to prevent severe damage to the skin by reducing the risk of necrosis by an ongoing inflammatory process.

E. Interleukin 12 (IL-12)

IL-12 is a heterodimeric protein and a potent costimulator of Th1 cells that are involved in cutaneous sensitization responses. Keratinocytes constitutively express the lower Mr (35 kDa) chain of IL-12 and are induced to express the 40-kDa chain following exposure to contact allergen, but not irritants.[31] IL-12 strongly stimulates T cell proliferation and mediates the primary immune response in skin.

F. Interleukin 15 (IL-15)

IL-15 has recently been shown to be induced in epidermal keratinocytes by culture and selected cytokines. IL-15 is a potent immunomodulator of T cell-mediated immune responses, similar in function to IL-2, and attracts and activates antigen-specific Th1 cells. IL-15 also stimulates the proinflammatory and antimicrobial properties of neutrophils. Both UVB exposure and corticosteroids downregulate IL-15 expression in keratinocytes.[32]

G. Tumor Necrosis Alpha (TNF-α)

TNF-α is a pleiotropic proinflammatory cytokine that mediates a range of biological responses, including proliferation, apoptosis, and inducing gene responses in TNF receptor-bearing cells. TNF-α also induces inflammation in skin following local synthesis and release or by injection as well as inducing ICAM-1 expression in keratinocytes.[33] Irritants such as SDS and PMA also have been shown to rapidly induce TNF-α expression as well as subsequent inflammation and edema in skin.[34] Selected allergens such as nickel and DNFB also induce TNF-α gene expression and protein in epidermis of sensitized animals.[35]

H. Chemokines — IP-10 etc.

Chemokines such as interferon-induced protein (IP)-10 and macrophage chemotactic protein (MCP)-1 have been shown to be upregulated in cutaneous delayed-type hypersensitivity reactions and other epidermal responses. Chemokines play an important role in inflammation via T cell chemotactic and adhesion-promoting activities . Interferon-γ strongly stimulates expression of IP-10 in keratinocytes.[36] IP-10 and other selected chemokines expressed by keratinocytes function in the epidermal signaling network to localize and induce specific responses that mediate cutaneous allergic and irritant reactions.

I. Miscellaneous Mediators

Products of the arachidonic acid metabolic pathway (termed "eicosenoids"), as well as arachidonate itself, are potent regulators of inflammation and allergic or irritant epidermal responses. The polyunsaturated fatty acid precursor, arachidonic acid, is produced by the enzymatic action of phospholipases (A_2 or C) on lipids of the cell membrane. In addition to the well-known actions of the cyclooxygenase, lipoxygenase, and monooxygenase metabolites of arachidonate in skin, arachidonic acid itself has been shown to trigger keratinocyte stress-activated responses, such as JNK activation.[37,38] A variety of the early events in skin inflammation are mediated by arachidonic acid and its metabolites. Tumor promoters and other irritants induce arachidonic acid metabolism in skin which may be used as relevant markers for cutaneous irritation.[39,40]

The keratinocyte also generates various free radicals following stimulation by chemical agents, UV radiation, etc. We have recently shown that superoxide and H_2O_2 are rapidly produced and eliminated in keratinocytes following exposure to UVB[13,14] and other agents. These ROS potently regulate levels

and activity of phosphorylated proteins and protein kinases within keratinocytes. These mediators may therefore be considered as second messengers mediating irritant or toxic responses in the epidermis.

IV. MODELS OF KERATINOCYTE IRRITANCY AND SENSITIZER TESTING

Human skin irritation and allergic contact dermatitis are common occupational and environmental health problems, resulting from skin exposure to man-made chemicals, waste products, and/or commercially marketed products such as solvents, soaps, organic dyes, cosmetics, pharmaceuticals, and skin protectants. Consequently, it is vital that the potential of a chemical compound to cause dermal irritancy and/or sensitization must be assessed accurately. For this purpose, various animal testing methods have been developed over the decades and have served industry very well. The most widely applied bioassays have been the rabbit skin irritation test,[41] the guinea pig maximization test,[42] the occluded patch test of Buehler,[43] the local lymph node assay,[44] and the mouse ear swelling test.[45] However, major problems of in vivo assays have been identified, including the (1) structural and physiological differences between the skin of rabbit, guinea pig or mouse, and human skin, (2) extrapolation from testing at fixed dose and time of application to the variable conditions of human exposure, (3) subjective nature of multi-end point assessment which can lead to interlaboratory differences, and (4) false-positive and false-negative responses.[46] Finally, when systemic effects of a product are estimated following topical exposure in vivo, metabolism in skin must also be considered. The general lack of data in this category is due to the difficulty in measuring skin metabolism in vivo that requires sampling from skin. Consequently, because of the multitude of problems associated with in vivo protocols as well as other restrictions emerging from ethical issues of animal use, the validity and propriety of in vivo testing methods have been increasingly challenged. As a consequence, in vitro methods offer alternatives to evaluate the interactions between chemical substances and a biological system such as skin and epidermis. Different types of excised skin have been used for in vitro screening studies to test a variety of biological properties, such as percutaneous absorption.[47,48] The major drawback of using this particular model for routine screening purposes is the time necessary to acquire both the specimens and data and the equipment needed to prepare the skin. Therefore, the development of new in vitro models and methods has become a focus of many academic and commercial laboratories. In this review, the usefulness of in vitro skin equivalent models will be illustrated and our experience with a system of human epidermis reconstructed on an inert filter substrate will be summarized.

Keratinocytes grown submerged in culture medium have often been used as in vitro alternatives for testing cutaneous toxicity, and a good correlation between skin irritation, cytotoxicity, and proinflammatory mediators release has been demonstrated.[49,50] However, under these conditions, keratinocytes organize to flattened, loosely associated layers, synthesize a different pattern of polypeptides, only sporadically form keratohyalin granules, and rarely contain lamellar bodies.[51] They lack a normal stratum corneum that acts as a barrier to chemical toxicity and, consequently, fall far short of simulating the in vivo situation. Moreover, these culture models are typically limited to water-soluble compounds.

The development of keratinocyte culture systems using de-epidermized dermis,[52,53] collagen matrix (with or without fibroblasts),[54–56] or inert filters (with or without fibroblasts),[57,58] coupled with living keratinocytes that undergo maturation to form a stratified epidermal tissue at the air–liquid interface, has led to the production of functional human skin equivalent models. They exhibit a considerable greater degree of tissue organization that closely resembles the in vivo state.[51,57,59–61] Over the past few years, different commercially available cultured human skin models have been developed and studied, including: (1) EpiDerm (MatTek Corporation, Ashland, MA), (2) Episkin (SADUC, Chaponost, France), the human reconstructed epidermis from SkinEthic (SkinEthic, Nice, France) with no fibroblasts, (4) Living Skin Equivalent (Organogenesis, Cambridge, MA), and (5) Skin2 (Advanced Tissue Sciences, La Jolla, CA), which are composed of both epithelial cells and fibroblasts.[58,62–65] These cultures exhibit a well-stratified epithelium and

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laminin, heparan sulfate,
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cornified epidermis with significantly improved barrier function and metabolic activity.[57,66–68] The presence of a stratum corneum makes it possible to apply topically a wide variety of products and/or complex formulations. Differentiation markers such as suprabasal keratins, integrin β4, integrin α6, fibronectin, involucrin, filaggrin, trichohyalin, type I, III, IV, V, and VII collagen, laminin, heparan sulfate, and membrane-bound transglutaminase have been found to be expressed similar to those of the epidermis.[58,69–71] Moreover, keratin synthesis and the production of cornified envelopes parallels that found in vivo. Spinous cells display abundant glycogen deposit, and keratohyalin granules are more abundant in the granular layer. Both the size and number of hemidesmosomes increase during maturation in vitro and anchoring fibrils are observed.[58,66,69,72–74]

Percutaneous penetration studies performed with human skin recombinant models have revealed that the stratum corneum forms a substantial barrier to ^{3}H-water,[53,63] pindolol, calcitonin,[75] toluene, carbazole, benzopyrene,[63] testosterone,[55,76,77] estradiol,[55] hydrocortisone,[55,76,78] benzoic acid,[15,77] cyclosporine,[79] salicylic acid, provitamin B5, theophylline, and scopolamine.[77] The results obtained are more consistent and reproducible than cadaver skin[79] and correlate well with those recorded for hairless guinea pig skin.[75] Nevertheless, the relative permeability of normal human skin compared to reconstructed skin is different and is likely to vary considerably from one compound to another. A good correlation for one class of chemicals is not necessarily indicative of a similar relationship for other chemicals.[53,55,63,77] This points to an impaired barrier function of reconstructed epidermis in vitro. In fact, despite the similarity in tissue architecture, reconstructed epidermis exhibits some deviations from normal epidermis, depending on the tissue culture method and the source of keratinocytes. Reduction of ceramides 4 to 7 and 6 to 7, integrin overexpression, premature expression of specific differentiation markers, and abnormal lipid composition have been observed under normal in vitro culture conditions.[71,80,81] By using freeze-fracture electron microscopy,[82] X-ray diffraction,[83,84] and confocal laser scanning microscopy,[85] it has been shown that, in some cases, reconstructed epidermis displays abnormalities in lamellar body delivery and extrusion, which manifests itself by a disturbance of the transformation of lamellar bodies into lamellar lipid bilayers by impaired structural organization and distribution of epidermal lipids into the intercellular space.[86,87] Furthermore, by using small-angle X-ray diffraction techniques, it has been shown that the stratum corneum lipids appear to be organized in multilamellar structures with a periodicity of 12 nm[87] in contrast to native epidermis, in which two lamellar phases with periodicities of 6.4 and 13.4 nm are typically detected.[83] Consequently, whereas for native epidermis the penetration pathway is confined only to the extracellular space, diffusion within the stratum corneum in the reconstructed epidermis likely occurs via both extracellular and intracellular pathways.[85] These findings may partially explain the divergent results obtained from various percutaneous penetration studies.

Improvements in the culture conditions, such as maintaining the cultures in delipidized serum, reduction of the relative humidity,[53] and use of chemically defined medium,[57,88] has led to further optimization of these models. Epidermal tissues generated at 33°C in absence of epidermal growth factor,[52,71] and in the presence of vitamin C[71] but absence of retinoic acid,[57,88] improves the stratum corneum architecture and lipid profile. In vitamin C-supplemental medium, the content of glucosylceramides and of ceramides 6 and 7 is markedly increased.[71] In absence of serum, the relative amounts of ceramides, free fatty acids, and cholesterol are comparable to native epidermis.[52] Epidermis reconstructed on fibroblast-populated collagen at 37°C in the presence of EGF has a similar morphology to that of native epidermis. However, irrespective of the culture conditions, involucrin is aberrantly expressed. EGF supplementation has a deleterious effect on epidermal morphogenesis and differentiation. The synthesis of K1 and K10 is suppressed on both protein and mRNA levels.[71]

Since 1990, a fully differentiated epithelium having the features of in vivio epidermis has been obtained in vitro by culturing second-passage normal human keratinocytes in a retinoic acid-free, chemically defined medium MCDB 153 on inert filter substrates exposed to the air–liquid interface for 14 days.[57] In this model, the basal cells synthesize and secrete all major markers of hemidesmosomes as well as components of the lamina lucida. Hemidesmosomes with major dense plaques and anchoring filaments and a basement membrane-like structure were identified, suggesting that

the presence of serum and dermal factors is not required.[58] Because of the restricted presence of exogenous growth factors and protein in the medium, this in vitro human living epidermis is approaching the most suitable system for detecting and testing the effects of any product that has the potential to be in contact with epidermis.[58,89]

An advantage of in vitro-reconstructed skin equivalents is the possibility of incorporating various additional cell types alone or in combination with keratinocytes. Recently, the introduction of melanocytes into epidermal reconstructs has expanded potential applications of these models.[90] As in the in vivo state, melanocytes appear as dendritic cells and are located in the basal keratinocyte layer. Melanin has been detected in both the melanocytes and the neighboring keratinocytes. Following UV radiation, increase in the number of dopa-positive melanocytes in the basal layer has been shown that results in increased pigmentation of the irradiated skin equivalent. More recent advances in culture techniques have made it possible to develop reconstructed epidermis containing not only keratinocytes but melanocytes and Langerhans cells as well. Cord blood-derived CD34+ hematopoietic progenitor cells induced to differentiate by GM-CSF and TNF-α were seeded onto a reconstructed epidermis composed of keratinocytes and melanocytes. This culture system gives rise to a reconstructed in vitro model displaying a pigmented epidermis with melanocytes in the basal layer and resident epidermal Langerhans cells located suprabasally and expressing major histocompatibility complex class II, CD1 antigen, and Birbeck granules.[91] It provides an attractive in vitro system to study the regulation of melanogenesis and melanocyte–keratinocyte interactions, and to investigate in a more defined model how these processes are affected by UV irradiation. In addition, this epidermal model can be used to test the phototoxic or photoprotective potential of various compounds as well as sunscreens, which is a distinct advantage over other animal models.

In vitro reconstructed epidermis allows testing of products at concentrations and in formulations that would be used in vivo. In addition, the dose–response relationship can be examined over a wide range of concentrations. Furthermore, the lower part of the tissue is bathed in the culture medium that can be withdrawn for analysis of released mediators. They provide quantifiable and objective end point measurements compared to those in vivo studies where more subjective parameters, such as erythema and edema, are often used. For these reasons, reconstructed human epidermis can be widely exploited for various research purposes, including studies of cutaneous biogenesis and skin wound healing, investigation of the regulation of keratinocyte differentiation, pharmaceutical agent metabolism studies and absorption properties,[63,79,92–95] assessment of cutaneous immunotoxicological response,[61] and responses to irritants[56,58,62,65,69,96–98] and to sensitizers.[99,100]

The end points most frequently used include histological analysis of tissue damage, cell membrane damage estimated by measuring leakage of enzymes such as lactate dehydrogenase (LDH);[56] cell viability determination by MTT conversion[62,65,89,96,101,102] or Neutral Red assay;[101] the modulation of the stratum barrier function and the release of proinflammatory mediators, such as IL-1α,[56,61,62,89,96,103,104] IL-1β, and IL-6,[56,103] IL-8;[56] TNF-α[61] prostaglandins;[56,62,96,105,106] hydroxyeicosanotretaeno (HETEs) and leukotriene B_4 (LTB_4);[107] plasminogen activator;[96] cytokine mRNA expression;[108–111] antileukoproteinase synthesis;[112] ICAM-1 expression;[81] integrin receptor modulation;[81] measure of intracellular ATP[113] and corneosurfametry.[114]

Upon reaching the living layers of the epidermis, irritant and sensitizing agents modulate cell membrane integrity. Irritation in vivo modulates integrin expression.[81] Keratinocytes in the basal layer of healthy epidermis express four different integrins, namely, α2β1, α3β1, α6β4, and $\alpha_v\beta5$; they participate in keratinocyte adhesion to the basement membrane that separates the epidermis from the dermis. Integrins have been shown to be involved in keratinocyte differentiation and activation, cell–cell adhesion between keratinocytes, and keratinocyte migration on extracellular matrix proteins.[115] Under inflammatory conditions, upregulation and suprabasal expression of these integrins coupled with the induction of α5β1 and intercellular adhesion molecule-1 (ICAM-1), a specific ligand for β2 integrins, have been demonstrated.[116] Finally, in skin reconstructed in vitro, UVB exposure leads to major epidermal developmental changes characterized by a downregulation of major markers of keratinocyte differentiation such as keratin 10, loricrin, filaggrin, and keratinocyte transglutaminase (Type I).[117]

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Irritants and sensitizing agents also trigger cutaneous responses by inducing epidermal keratinocytes to elaborate and/or to release proinflammatory cytokines at both the protein and mRNA levels. These cytokines activate dermal microvascular endothelial cells and induce accumulation of specific mononuclear cells in vivo, and they, therefore, are considered as critical signaling molecules in the cascade of events leading to in vivo skin irritation and/or sensitization.[28,29,56,118] Consequently, the expression and/or release of cytokines by human skin equivalent models have been proposed as reliable markers to predict in vivo toxicological effects.[56,65,119,120] Although the complexity of the skin response to injury has created significant challenges in the discrimination of irritant from sensitizing agents by in vitro methods exclusively, it is now evident that analysis of the cytokine mRNA expression and protein release by epidermal keratinocyte cells may provide one possible approach to detect which agents are irritants or sensitizers.

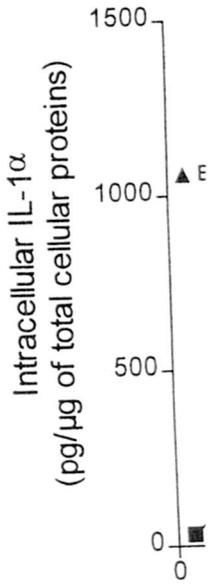
Cultured keratinocytes synthesize constitutively, or can be induced to produce a variety of cytokines, including IL-1α, -6, -8, and -10, GM-CSF, TGF-α, and TGF-β, TNF-α, monocyte chemotaxis and activating factor (MCAF), IP-10, and macrophage inflammatory protein 2 (MIP-2).[29,121] Cultured keratinocytes exposed to contact allergens exhibit a rapid increase in mRNAs for IL-1α, -8, -6, and -1β, GM-CSF, TNF-α, IP-10, and MIP-2,[28,29,122] with subsequent release of keratinocyte-derived IL-10, -8, -6, and TNF-α.[28,118,123]

Studies have shown that skin equivalent models constitutively express mRNAs for inflammatory and immunomodulatory cytokines. The MatTek model expresses mRNAs for IL-1α, -1β, -8, -6, and -15, GM-CSF, and TNF-α.[124] In vitro epicutaneous contact with irritants leads to mRNAs expression of IL-1α, -1β, -6, -8, and -10, GM-CSF, TNF-α, TGF-β, and IL-12.[109] In this case, the mRNA levels for IL-6 and IL-8 are higher. This may well be due to the presence of fibroblasts.[125,126] Finally, experiments performed on reconstituted human epidermis have shown that both the skin irritant sodium lauryl sulfate (SLS) and the sensitizing agent 1-chloro-2,4-dinitrobenzene (DNCB) induce an increase in IL-1α and IL-8 release. However, DNCB only upregulates TNF-α release. Constitutive message was expressed for IL-1α, IL-8, and IL-10 but not for IL-1β. Both DNCB and SLS increased message for IL-1α. The in vitro-reconstituted human epidermis, EPISKIN, was used to assess the molecular mechanisms of skin irritation and sensitization.[127] Studies were performed to assess the ability of irritants and contact allergens to modulate cytokine message in SKIN2™ and EpiDerm cultures and determine if a cytokine or panel of cytokines would identifiy and contact allergen and differentiate it from an irritant. For the EpiDerm model, two different irritants were evaluated, benzalkonium chloride and nonanoic acid, along with two moderate allergens, TNCB and Oxazolone. Both irritants and allergens increased steady-state message levels for IL-8 and decreased message levels for IL-1β in the epithelial cells. Only irritants increased message levels for TFN-α, whereas the allergens produced either no change or a decrease in TNF-α message. Effects on the message levels of IL-6 and IL-1α differed for each chemical in magnitude, timing, and concentration.[99]

For the SKIN2™, the irritants BC, SLS, and nonanoic acid (NA) were evaluated along with three contact allergens TNCB, DNCB, and oxazolone. All three irritants increased steady-state message levels for IL-6, IL-8, and TNF-α. The allergens DNCB and oxazolone increased message levels for IL-6, IL-8, and GM-CSF, whereas TNCB only increased message for IL-8. The steady-state level of IL-15 was increased by NA only.[100] These results suggest that different patterns of epidermal cytokines are stimulated during in vitro irritation and/or sensitization processes. Consequently, it should be possible to distinguish between skin sensitizing agents and irritants by investigating the differential upregulation and modulation of epidermal cytokines by keratinocytes. In this respect, the roles of IL-1α and IL-8 may be particularly relevant. IL-1α is produced by keratinocytes and sequestered in the epidermis. During irritation, the release of IL-1α causes autocrine regulation of epidermal cytokine synthesis which, in turn, induces accumulation of dendritic cells in lymph nodes, draining the site of irritation, and stimulates the maturation of Langerhans cells.[127,128] Moreover, previous experiments have demonstrated that allergens induce expression and release of IL-8 mRNA, which is a potent chemoattractant for polymorphonuclear neutrophils and T lymphocytes.[28,129]

Recently, a model of reconstructed human epidermis (RHE) was used as an in vitro skin model to discriminate the effects of Tween 80, Triton X100, and benzalkonium chloride (BC) as irritants and 1-chloro-2,4-dinitrobenzene (DNCB) as a sensitizing agent.[131] It is based on the model developed by Rosdy and Cross[59] and consists of a mitotically and metabolically active culture of human-derived epidermal keratinocytes that are differentiated into basal, spinous, granular, and cornified layers analogous to those found in vivo.[58] Specific markers of epidermal differentiation such as keratins 1/10, involucrin, filagrin, loricrin, and transglutaminase have been localized. The lipid profile analysis shows that this model contains free fatty acids and all classes of ceramides. These cultures exhibit barrier function and metabolic activity which allow direct application of the product to be tested, thus simulating in vivo human topical exposure and an in vivo skin irritation/sensitization test.[58,89] In the experiment, the extent of epidermal irritation and sensitization was evaluated morphologically and amounts of intracellular and extracellular of IL-1α and IL-8 were assayed. The corresponding constitutive mRNA levels of these interleukins were quantified and the cytotoxic response was assessed by a MTT assay. The RHE resembled normal human epidermis with all typical epidermal layers. Keratin 10 was typically confined to the suprabasal layers of the tissue, suggesting normal epidermal terminal differentiation. Topical application of each of the three surfactants resulted in significant changes of tissue morphology and was coupled with different dose-dependent decreases in cell viability corresponding to their in vivo irritant potency.[119,130,131] IL-1α release was shown to increase inversely with decrease in cell viability, but interestingly, the surfactants did not stimulate increase in IL-8 levels. In contrast, DNCB did not induce elevated IL-1α release, although it induced a rapid dose-dependent decrease in cell viability. By contrast, DNCB increased IL-8 release. RT-PCR demonstrated the presence of mRNA for IL-1α and for IL-8 as previously described in vivo.[132,133] IL-1α was the most abundant cytokine transcript. BC, Triton X100, and DNCB upregulated IL-8 mRNA expression, while only BC induced a significant increase in IL-1α mRNA expression. The results demonstrate that the production of IL-1α and its release into the extracellular medium were due not only to specific cytotoxicity, but also to the extent of direct epidermal tissue stimulation. Conversely, the production of IL-8 did not directly correlate with cytotoxicity but may be linked to the type of product applied and classified as either irritant or sensitizer. These findings emphasize the requirement to use substances of the same class as standard controls in order to test unknown compounds that will be coupled with the investigation of multiple end points. Our data demonstrate that divergence of the IL-1α and IL-8 releases profiles and corresponding mRNA upregulation differentiates between specific responses to irritants or allergens. These findings suggest that it may be possible in a single integrated assay to classify and discriminate between irritant and sensitizing agents as a function of patterns of induced cytokine production and cell viability measurements. It has not been determined which mechanism is responsible for the change in cytokine mRNA expression, but we have observed that mRNA levels do not necessarily correlate with protein expression, and we also find that the type of product appears to determine the pattern of cell mediator expression and release. This could explain the disparate results obtained with the EpiDerm or the Skin2 models where only mRNA expression was investigated.[100] Our results suggest that skin allergens and skin irritants could stimulate variable patterns of epidermal cytokine production in RHE. The stimulation seems to be nonspecific in terms of mRNA signal strength, but specific in terms of protein production and release. In fact, if the cytokine levels (intracellular vs. extracellular) are plotted, a strong correlation for IL-1α ($R = 0.999$) is observed, suggesting a direct relationship among synthesis, storage, and release. By contrast, we observe for IL-8 that BC and Triton X100 induces synthesis and storage without significant release, while DNCB induces a rapid synthesis and release of IL-8 without storage (Figure 2). These observations highlight the complexity of biochemical pathways underlying cytokine production, and suggest interactions with different specific cellular target sites.

Functional mitochondria seem to be required in keratinocytes for *de novo* IL-1α synthesis.[134] In fact, tributyltin, a well-known skin irritant in rodent and human, causes disturbance in the



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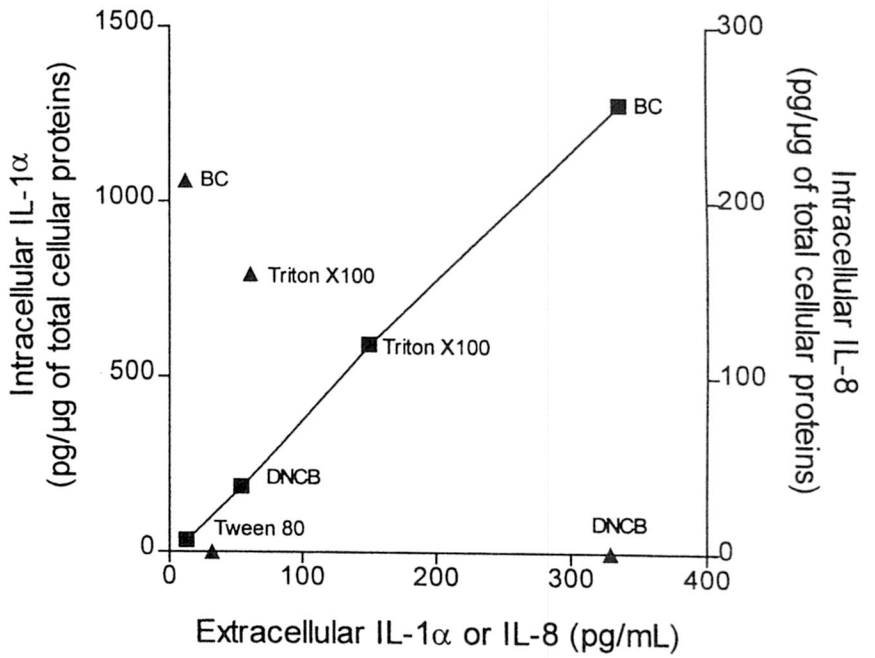

Figure 2 Correlation between the intra- and extracellular levels of IL-1α (■) and IL-8 (▲) in the RHE after topical application of Tween 80, Triton X100, BC, and DNCB (20 h, 37°C, 5%CO_2).

respiratory chain of mitochondria, probably by production of reactive oxygen intermediates at the ubiquinone site which activates transcription factor and promotes IL-1α synthesis.[134,135] In our experiments, the RHE treatments with Triton X100, BC, and DNCB reduce mitochondrial function as demonstrated by decreased MTT conversion and could partially explain the results. However, the release observed with DNCB suggests that mechanisms other than mitochondrial activity may be involved in the RHE cytokine production. In fact, DNCB increases NADPH oxidase enzymatic activity, producing reactive oxygen intermediates that mediate effects of this hapten on cells in vivo.[136] In addition, human keratinocyte IL-8 synthesis may be either positively or negatively regulated by protein kinase C depending on the stimulus.[137]

In conclusion, the reconstructed human epidermal equivalents more closely resemble native tissue in terms of their biosynthetic, morphological, and barrier properties than conventional submerged ones and than animal skins do. Due to the presence of the stratum corneum, water-insoluble as well as solid materials can be applied topically and are better suited than conventional cultures for predicting the irritation and sensitization potentials of topically applied agents. Divergent cytokine secretion profiles characterize the RHE response to irritants and sensitizers, suggesting that it is a complex array of signals that determines the type of protein released, not only in terms of mRNA upregulation, but above all in terms of interaction with the signal transduction. The combination of cell viability measurement with the quantification of IL-1α and IL-8 allows the classification and discrimination between irritant and sensitizing agents. The low interexperimental variations, irrespective of whether the experiments are performed on RHE derived from cells of the same or different donors, indicate that the RHE grown in defined medium represent a very useful in vitro model for toxicological studies which correlates with in vivo results. However, the number of products is not actually sufficient to extend the correlation across different classes of chemicals. The possibility that other irritant or sensitizing agents from different classes may exhibit specific patterns of inflammatory mediators would provide for the validation of in vitro models as alternatives to animal testing.

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