In vitro models for human skin disease

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Modern tissue culture technology has made it possible to generate human skin equivalents that represent either epidermis or epidermis plus dermis (full-thickness skin) *in vitro*. Commercially available skin equivalents and in-house models are used for safety analysis of cosmetics and toxicity screening of various pharmaceutical compounds. Recently, tissue culture technology has also been used to develop *in vitro* models of skin disease, in particular to promote cutaneous drug research while sparing experimental animals. The spectrum of model diseases available covers a range from inflammatory disease to cancer. It has, thus, been possible to gain more insight into the role of active pharmaceutical ingredients of various dermatologically relevant drug classes as well as conventional and innovative formulations.

In the past the preclinical development of drugs for the treatment of skin diseases was primarily based around animal experiments: as a paradigm the study of retinoid-induced influences on keratinization in the rhino mouse can be named [1,2]. Pertinent research is based on *in vitro* testing using human skin equivalents – either in addition to animal experiments [3] or, increasingly, alone. First approaches based on normal human keratinocytes (NHKs) proliferating and differentiating on de-epidermized dermis (so-called living skin equivalent; [4]) were soon followed by NHKs grown on supporting membranes (reconstructed human epidermis, RHE) that have become commercially available (e.g. SkinEthic®, Nice, France; EpiDerm™, MatTek, Ashland, MA, USA). Advances in tissue engineering allowed the generation of full-thickness skin models (FT models) based on fibroblast populated collagen matrices (dermis equivalent) and an epidermal overlay representing NHKs [e.g. EpiDermFT™, MatTek; Phenion® Full Thickness (FT) Skin Model, Henkel, Düsseldorf, Germany. Moreover, there are co-culture models such as melanocyte-populated RHE and RHE analogues for mucocutaneous tissues such as oral mucosa and vaginal mucosa (also commercially available from SkinEthic and MatTek).

Primarily developed to obtain new options in the treatment of burnt skin and chronic wounds [5,6], skin equivalents were also found to be useful for toxicity testing. Initially, this mainly applied to skin corrosion [7–9], but soon the spectrum of possibilities increased and today the OECD (Organization for Economic Cooperation and Development) has approved test guidelines that address the non-animal testing for phototoxicity (re-testing of poorly absorbed compounds; [10]), as well as skin irritation [11]; for a concise review see Ref. [12]. Test protocols are validated for the investigation of percutaneous absorption [13] and cutaneous biotransformation [14–16], and risk analyses of nanomaterials [17,18] seem to become other application domain.

Beyond this, advances in biotechnology enabled model generation for human skin disease. As an early example, models of localized candidosis can be named. In this context it is interesting to note that, with respect to this type of disease, not only skin diseases [19,20] in the stricter sense but also diseases of bordering mucosal tissues [20] were put into focus. In fact, similarities were demonstrated between candidosis models based on skin equivalents or experimental animals and the human infections [20,21], allowing researchers to compare the efficacy of antifungal treatments for cutaneous mycoses [22]. At the beginning of the 1990s medical devices became a major part of the dermatologist's therapeutic armamentarium and it was soon demonstrated that skin models could also be used for their characterization [23].

Today, a variety of skin disease models based on human skin equivalents are available in addition to animal and human models [24], and up to a point these models have been validated in drug

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research. To date, however, a comprehensive review on the subject has not been published. For this reason, in this article, a pertinent overview is given. At present, the available data do not generally enable characterization of advantages and disadvantages of the various options of human skin equivalents in relation to a given scientific question. Thus, it is a challenge in current research to use the best fitting model to obtain useful data [25].

Mucocutaneous candidoses

Several studies have addressed models of mucocutaneous and cutaneous candidoses based on reconstructed tissues. In particular, models have been described for oral and vaginal candidosis, and the value of pertinent models in therapeutically oriented pharmacological research has, at least in principle, been established.

Oral candidosis

As demonstrated in 1998, the inoculation of reconstructed human oral epithelium with *Candida albicans* induces tissue changes reflecting those seen *in vivo* – on morphological and molecular biological levels. Severe oedema and detachment of upper cell layers (acantholysis) were used as major morphological criteria, as was the expression of secreted aspartic proteinases (SAPs) judged by RT-PCR analyses [20]. Later the role of SAPs was characterized in more detail using the model drug pepstatin A. This well known inhibitor of aspartic proteinases markedly reduced the extent of lesions – which was also the case if SAP1-knockout mutants of *C. albicans* were used, with both a single mutant and a double mutant dubbed delta-SAP1/3 [26]. In fact, the linked, reduced expression of SAP1 and SAP3 attenuates virulence [27]. The *in vitro* model also characterizes the inflammatory reaction of the host: the most virulent species *C. albicans* correspondingly induced the most severe changes to be derived from the quantitative analysis of mRNA levels of various cytokines (Fig. 1) [28].

Later, the disease model was modified by adding polymorphonuclear leukocytes (PMNs) that induced a Th1-type epithelial response which was considered protective [29]. Moreover, reconstructed oral mucosa exposed to *C. albicans* clarified the role of toll-like receptors (TLRs) in antifungal host defense – attributing a major role to TLR-4-mediated signalling [30]. Pertinent investigations supported the clinical hypothesis that HIV-proteinase inhibitors might directly influence the structurally related SAPs of *C. albicans* and, thus, reduce the frequency of manifest fungal disease [31,32]. In fact, untreated AIDS patients are prone to develop oral candidosis as the most frequent opportunistic infection. Figure 2 illustrates the

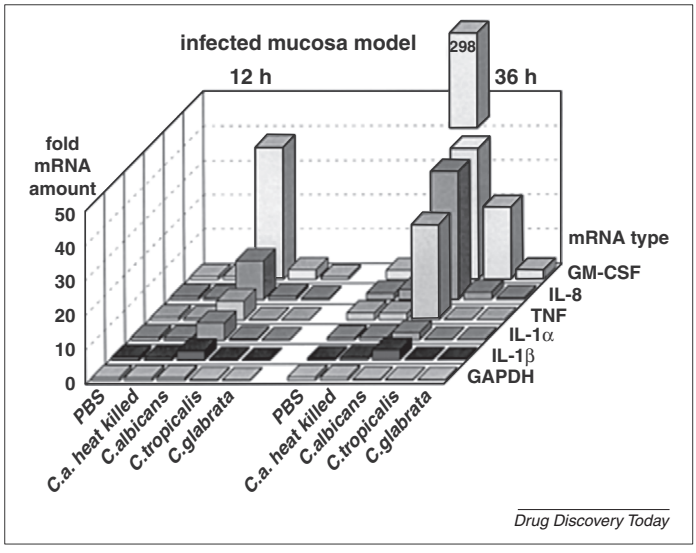

FIGURE 1

Expression of mRNA in uninfected and infected reconstructed human epitheliums addressing various Candida species. Figure reproduced, with permission, from Ref. [28].

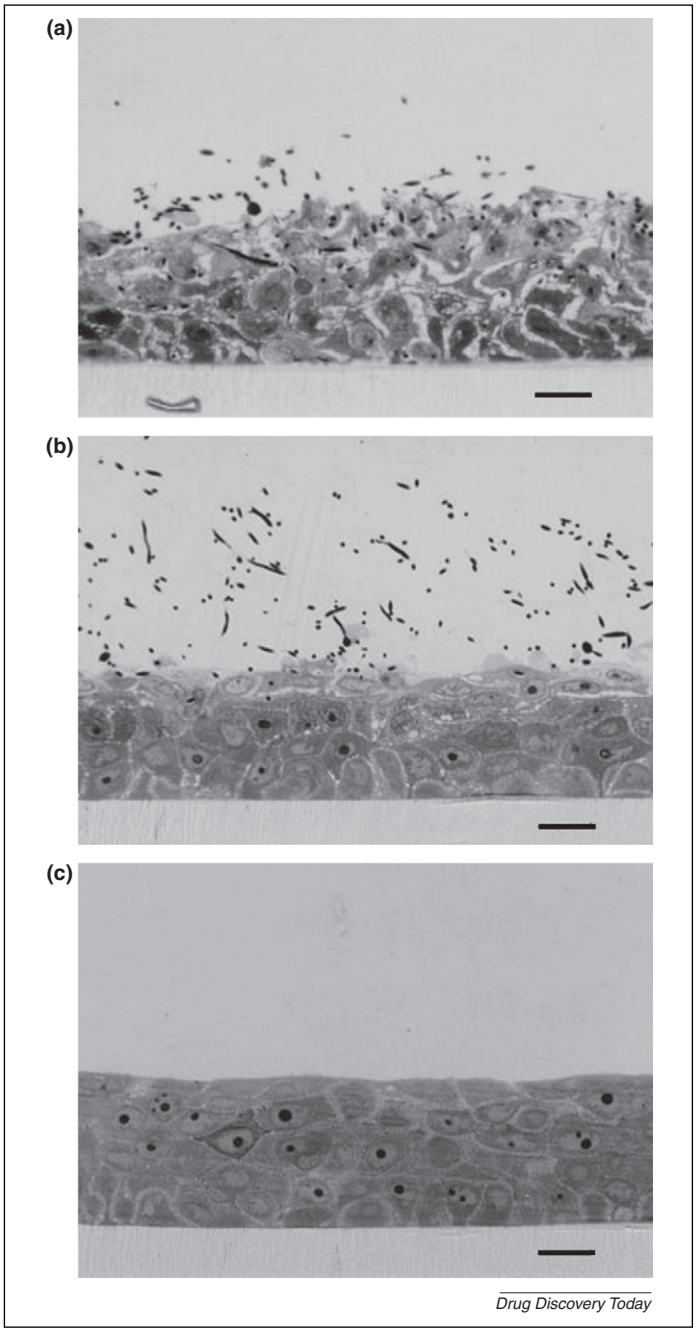

FIGURE 2

Morphological changes seen in reconstituted human epithelium. Changes seen after exposure for 12 h to *Candida albicans* alone (**a**) or *Candida albicans* together with saquinavir (0.3 μM; **b**), or saquinavir alone (0.3 μM; **c**). Inter- and intra-cellular oedema is a prominent finding representing tissue damage, being most prominent in the presence of fungus without antifungal (a). The bar represents 1 μm. Reproduced, with permission, from Ref. [32].

TABLE 1

Ultrastructural changes typical of cutaneous candidosis in the model based on reconstructed human epithelium (RHE) and in human tissue[a].

RHE	Patients
Enzymatic degradation of corneocytes	Electron-transparent areas, keratolysis
Scaling	Not observed
Hyperkeratosis	Not observed
Parakeratosis	Parakeratosis
Dyskeratosis	Not observed
Spongiosis	Spongiosis
Not observed	Intra- and sub-corneal micro abscesses

[a] Modified, with permission, from Ref. [9].

protective effect of the HIV proteinase inhibitor saquinavir – as judged on morphological grounds.

Vaginal candidosis

On the basis of reconstructed vaginal epithelium a similar approach was used to establish a model of vaginal candidosis. Again, an inhibitory effect was demonstrated with HIV-proteinase inhibitors [33].

Cutaneous candidosis

Although oral mucosa and vaginal mucosa lack stratum corneum this does not apply to human skin. This is reflected in RHE. With the exception of mucosal tissues, the protecting stratum corneum made it necessary to apply an irritant chemical (sodium dodecyl sulfate, SDS) before inoculation to ensure that the relevant damage occurred. Alternatively a mechanical insult can be obtained with an injection needle. The validation of the model was primarily based on ultrastructural criteria comparing the findings in the model with corresponding ones in human specimens [19,34] – details are presented in Table 1. Using RT-PCR the inflammatory host response was explored by analyzing various cytokines: interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α) were upregulated. Downregulation of transforming growth factor beta (TGF-β), a typical Th2 cytokine, supported the hypothesis of a protective Th1 response [35].

The RHE-based model of cutaneous candidosis enabled characterization of the relative therapeutical activity of econazole topical formulations; a liposomal formulation turned out to be superior to the conventional cream formulation [21]. In particular, hyperkeratosis was reduced – corresponding to only moderate detachment of stratum corneum and less-prominent vacuolization (Fig. 3). The higher *in vitro* activity of the liposomal formulation was reflected by higher clinical efficacy [22].

Dermatophytoses

The living skin equivalent was used to establish a model of dermatophytosis of glabrous skin. As dermatophyte of choice, a *Trichophyton mentagrophytes* strain was characterized by prolific production of arthroconidia. Terbinafin was the most active antifungal *in vitro* – dermatophytes prevented penetration of the pathogen into deeper strata of the tissue in a dose-dependent manner [36]. Efficacy of photodynamic treatment was also shown, using inoculated human stratum corneum as an *ex vivo* skin model of dermatophytosis [37].

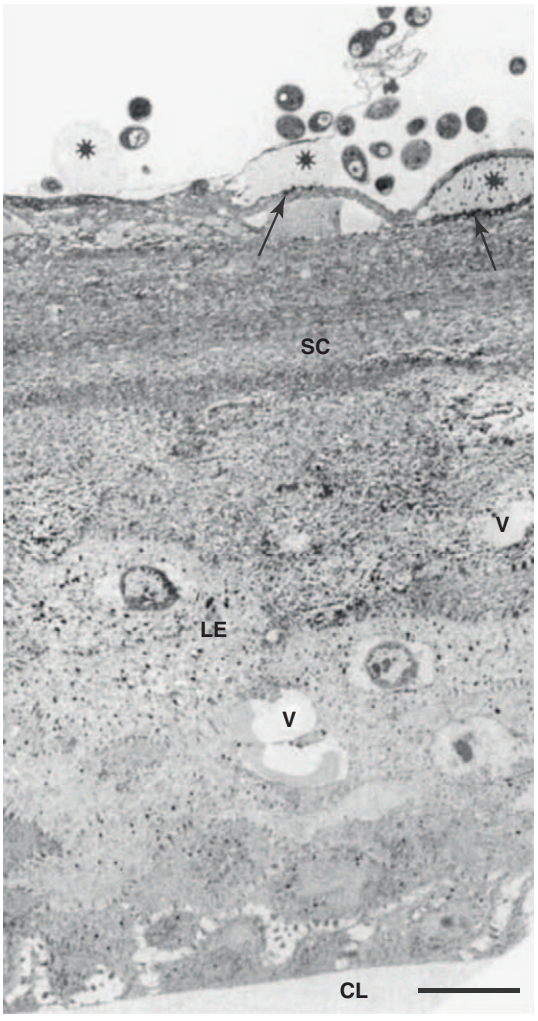

FIGURE 3

Reconstructed human epithelium together with *C. albicans* blastospores after 48 h treatment with econazole liposomal gel: econazole liposomal gel is seen attached to *C. albicans* blastospores as well as human reconstructed epithelium (stars) and within the stratum corneum (SC). V indicates vacuoles, LE represents lower epidermis and CL is collagen lattice. Reproduced, with permission, from Ref. [21].

Bacterial skin colonization

To date, reconstructed human skin has hardly ever been used to establish full-blown bacterial skin disease models. Yet, human skin equivalents have been used to characterize the interaction of cutaneous tissue with dermatologically relevant bacterial pathogens, in particular *Staphylococcus aureus*. *S. aureus* can increase its density by several orders of magnitude within a few days. In addition, there was also an increase in the colonization density over time with *Staphylococcus epidermidis*, *Propionibacterium acnes*

and the yeast *Malassezia furfur*; however, the density was remarkably lower with the yeast as compared with the densities of the bacteria. The less-marked growth of the lipophilic yeast *M. furfur* was explained by the relative lack of nutritious lipids within the skin model [38]. Moreover, the assay system compared the reaction of the host tissue to *S. aureus* and *S. epidermidis*. Unlike the results for *S. epidermidis*, large quantities of transcripts were markedly increased or decreased with *S. aureus* – as judged on the basis of gene expression change by a factor in excess of two. Differential upregulation of a variety of skin defence factors, such as TLR-2, beta-defensin4 and properdin, as well as pro-inflammatory TNF-α and IL-1, can help to explain the behaviour of the two Staphylococcus species with respect to skin colonization [39].

RHE also proved helpful for evaluating the therapeutic role of medical devices with *S. aureus* and *C. albicans* colonization. Hydrocolloid dressings were compared whether containing silver particles or not. Twenty-four hours after inoculation of a multiresistant *S. aureus* strain there was oedema within the entire epidermis leading to detachment and vacuolization of the keratinocytes. Staphylococci were found everywhere. In the presence of a silver-loaded hydrocolloid dressing significantly fewer bacteria were seen and deeper epidermal strata were essentially not invaded. Oedema and cell detachment were, by and large, restricted to upper strata. Using the corresponding silver-free hydrocolloid dressing tissue alterations were more marked than when the silver-particle-containing hydrocolloid dressing was used, but less striking when compared with the untreated control [29].

Cutaneous wounds

Repeatedly, FT models have been used to create models of cutaneous wounds or to characterize treatment procedures by their use. Following complete tissue bisection, re-epithelialization from the wound edges occurred within 8–12 h, whereas it took four days for complete epidermal restoration. Matrix metalloproteinases (MMPs) were overexpressed, as were several integrins [40]. Instead of cutting the FT model with a knife, standardized wounding can also be induced by laser irradiation. When compared with the Erbium-YAK and the high powered excimer laser, the low powered excimer laser excels because it does not only cut reproducible wounds but also leaves the surrounding surface virtually unaltered. Keratinocytes re-epithelialized the base of the wound within three days [41].

Wound healing can be characterized using optical coherence tomography and multiphoton microscopy. Laser-induced alterations in collagen microstructure organization and subsequent matrix reconstruction and fibroblast migration can be seen [42]. The former might be considered as particularly apt because it is also regularly used on clinical grounds for wound characterization.

Defined wounds induced by CO_2 laser served for a first characterization of topically applied active pharmaceutical ingredients (morphine) as well as drug carrier systems [43]. In experimental therapy opioids are used for severe local pain, and nanocarriers – in particular of the solid lipid nanoparticle type – are options for slow drug release and improved skin penetration [44–46]. In fact, experimental wounding enabled verification of enhanced keratinocyte migration as observed previously in keratinocyte monocultures. Morphine effects were caused by opioid receptor stimulation, whereas the glycerol esters used to produce solid lipid nanoparticles could suppress the antiproliferative and apoptotic protein p63 [18].

Autoimmune skin diseases

Disorders of the skin considered to be of autoimmune origin have, to date, only rarely been the subject of *in vitro* modelling. Although, it has been possible to shed some light on the pathogenesis of vitiligo using an elaborate living skin equivalent that comprises additional non-segmental non-lesional vitiligo (NSV) cells. The results support the hypothesis that NSV melanocytes show limited adhesion capability [47].

Psoriasis vulgaris

Living skin equivalents were constructed from skin biopsies from psoriatic skin and healthy individuals, respectively [48], or healthy skin only. Psoriasis-like morphology was obtained by cytokine stimulation [49]. Moreover, cells obtained from lesional and uninvolved skin of patients suffering from psoriasis vulgaris have been used to develop FT models of the human disease [50].

Among the four possible pairs of normal or diseased pertinent cells the combination of lesional keratinocytes and fibroblasts was found fairly representative for the morphology of human disease. Almost the same picture was seen if lesional keratinocytes were combined with normal fibroblasts. Disease models considered particularly apt were characterized by the thickening of the epidermal layer, in parallel involucrin was overexpressed and filaggrin and loricrin were underexpressed [48]. Reflecting the situation in the patient, the chemokine receptor CXCR2 was overexpressed in the disease FT model in the granular layer of keratinocytes. If the cells constituting the model were derived from lesional or non-lesional skin of psoriatic patients pro-inflammatory genes were highly expressed. In particular, this applies to TNF-α, interferon-γ and IL-8. Results were completely different with tissue based on normal cells [50]. The results support previous data obtained when keratinocytes from normal and lesional psoriatic skin were grown on de-epidermized dermis [51]. Using living skin equivalent, NHKs acted like typical psoriatic keratinocytes following stimulation with so-called psoriasis-associated cytokines (e.g. TNF-α and IL-1α) used alone or in combination. The related increased expression of hBD-2, SKALP/elafin, CK16, IL-8 and TNF-α was prevented by retinoic acid. The anti-psoriatic effect of ciclosporine could not be demonstrated, because the model is devoid of T lymphocytes [49].

Cutaneous effects of a retinoid in clinical use for psoriasis, namely acitretin, have only been analyzed in ''normal'' RHE – which allows for a study of side-effects only. Acitretin was considered to be of better tolerability owing to less-prominent release of pro-inflammatory cytokines, namely IL-1α and IL-8, as compared with other retinoids used for reference (i.e. retinoic acid and tazarotene) [3].

Atopic dermatitis

As in the disease itself, an atopic dermatitis model based on de-epidermized dermis and lesional keratinocytes showed decreased expression of cathepsin V and cystatin M/E, on the mRNA and the protein level. Regarding cathepsin L and transglutaminase 3 only the former was the case [52]. A second approach for tissue engineering resulted in advanced RHE and FT models made from

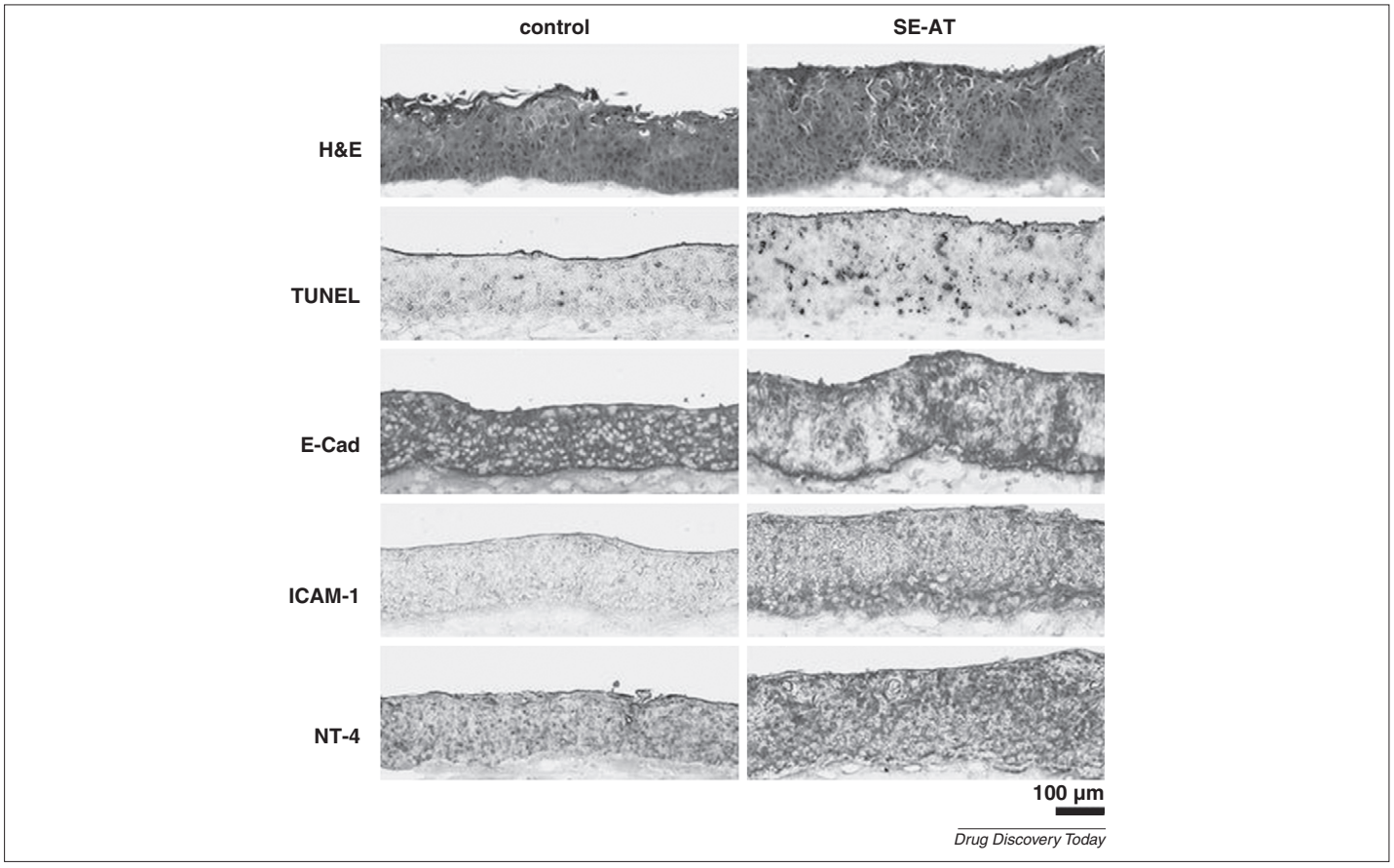

FIG. 4

Effect of activated T cells on various aspects of a dermatitis equivalent. Apart from the H&E as well as terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) aspect immunostaining is shown indicative for the presence of E-cadherin, ICAM-1 (inter/racellular adhesion molecule-1) and Neurotrophin-4. Reproduced, with permission, from Ref. [53].

immortalized keratinocytes (HaCaT cells) and human fibroblasts adding activated memory/effector T cells to the under side for the final three days of tissue culture. In the presence of the activated T cells, spongiosis and apoptosis turn up, the levels of pro-inflammatory cytokines increase and the barrier function is compromised (Fig. 4). Dexamethasone and tacrolimus induced a dose-dependent decline of cytokines and improvement of barrier function – relative electrical resistance served for readout [53].

Only recently, a homogenous nonsense mutation in the corneodesmosin-encoding gene (*CDSN*) was identified in a family suffering from general peeling disease. The skin phenotype could be reproduced by growing full-thickness skin models with fibroblasts and keratinocytes obtained from lesional skin. Permeability was increased for the standard test substances for hydrophilic and lipophilic compounds, caffeine and testosterone [54]. This is in accordance with the well-known poor skin barrier function and higher sensitivity for environmental noxae in atopic patients [55].

Irritant and allergic contact dermatitis

As a first approach to a model of irritant contact dermatitis RHE was exposed to irritants. The degree of irritation was rated using a scoring system discriminating between ''innocuous'', ''mild irritant'', ''moderate irritant'' and ''severe irritant''. Histopathology of the RHE showed slight oedema of the granular layer of the living epidermis as well as the cornified envelope with mild irritants such as SDS 0.2%, whereas severe irritants such as SDS 0.8% induced spongiosis, cellular necrosis and loss of cellular connections in the stratum basale. Morphological findings correlated with increased cytokine mRNA levels, in particular the IL-1. The *in vitro* findings corresponded to the findings in a double-blind, randomized, vehicle-controlled study in healthy human volunteers; for example, with respect to transepidermal water loss. The test compounds were applied for 22 days using the Finn chambers which were changed once a day [56]. The results are summarized in Table 2.

To the best of our knowledge, a full-fledged model of allergic contact dermatitis has not been described as yet, which is easy to understand considering the complexity required. Yet, there is an approach based on Langerhans cells integrated into the suprabasal layers of RHE. Established sensitizers, namely dinitrofluorobenzene and $NiSO_4$, induced a reduction in the number of Langerhans cells. Moreover, dendritic cells discriminate between haptens and irritants, which can be judged from the adaptation of their morphology and number [57].

Photodamaged skin

Photodermatitis

Current clinical terminology makes it somewhat difficult to define adequate *in vitro* correlates of photodamage, for a comprehensive review see Ref. [58]. This particularly applies to sunburn and phototoxic dermatitis. Obviously, stage 2 sunburn with its clinical

TABLE 2

Irritation potential of various compounds used in dermatology according to *in vitro* (day 3) and *in vivo* data[a].

Active principle	*In vitro* study (day 3)	Clinical study (day 3)	Clinical study (day 10)	Clinical study (day 22)
Vaselin	Mild to moderate	Innocuous	Innocuous	Innocuous
Calcipotriol	Moderate	Innocuous	Innocuous	Innocuous
Trans-retinoic acid	Moderate	Innocuous	Innocuous	Moderate
SLS 0.2%	Mild to moderate	Innocuous	Mild	Severe
SLS 0.4%	Severe	Mild	Severe	Severe
SLS 0.8%	Severe	Severe	Severe	Severe
SLS 1.0%	Not determined	Severe	Severe	Severe

[a] Healthy human volunteers have been exposed once a day for three weeks. Effects have been rated by visual scoring and measurement of transepidermal water loss. Modified, with permission, from Ref. [56].

characteristics of vesicles and blisters is a particular type of dermatitis that reflects the toxic effect of exposure to UV light. Thus, the model should qualify for the term phototoxic dermatitis (or toxic photodermatitis). Using current dermatological terminology this term, however, is reserved to a type of dermatitis seen upon UV light exposure in the context of the presence of a chemical agent increasing the damage caused by the physical strain (phototoxic compound). It might be helpful to consider photodermatitis as a possible designation for the entity. This might be particularly helpful if used together with the adjective ''acute''. In fact, today, acute photodermatitis can be reproduced eloquently in an *in vitro* model [10]. This model, however, can hardly be named sunburn because this term is virtually inseparable from the idea of redness linked to dilation of corial vessels – a feature, until today, not to be expected from a human skin equivalent.

Using an FT model ''sunburn cells'' were detected 24 h and 48 h after UVB exposure (50 mJ/cm^2), as observed in UV-exposed normal skin. Dermal fibroblasts disappeared after UVA exposure (25 mJ/cm^2). This reflects the major characteristics of dermatoheliosis *in vivo* (i.e. discoloration and wrinkles). Human skin equivalents also enabled the characterization of sunscreens, single agents and combinations; only a broad band filter inhibited UVA-related damage of the dermis equivalent [59–61]. The data obtained even enabled improved differentiation of UV filter capacity of various compounds as compared to conventional methods. Thus, in the future, FT models might be used for a pre-screen of sunscreens before entering into studies in healthy volunteers to establish the sun protection factor. Moreover, induction of reactive oxygen species (ROS) upon UVB exposure has been described. Pre-exposure of RHE to the antioxidants beta-carotene and superoxide dismutase prevented ROS formation [62].

Integrating dendritic cells into the epidermal layer of FT models, the inflammatory reaction of skin to the UV stimulus can be characterized in detail, TNF-α and IL-1β belonging to the parameters for readout [63]. Moreover, immunosuppression is seen in RHE populated by dendritic cells following UV irradiation [61].

DNA damage and DNA repair are clearly the focus of cutaneous photoaging research. IL-12 seems to be a major mediator in the repair of DNA damage caused by UV light exposure. Polyphenols from green tea increased IL-12 secretion and reduced apoptosis of keratinocytes following UVB exposure of an FT model. The effect is compromised by anti-IL-12 antibody [64]. Another important feature of extrinsic aging and photoaging is the formation of advanced glycation enzymes (AGEs) and related products. This can also be studied using FT models. Glycation inhibitors such as aminoguanidine reduce tissue damage caused by AGEs [65].

Xeroderma pigmentosum

An FT model for *Xeroderma pigmentosum* has been engineered using patient-derived keratinocytes and fibroblasts. The epidermal differentiation keratin 10 and loricrin are reduced, whereas proliferation of keratinocytes is more rapid. In fact, the epidermis tends to proliferate into the dermis reflecting early invasion [66].

Non-melanoma skin cancer

With respect to non-melanoma skin cancer the focus to date has been on squamous cell carcinoma. An early study elucidated the role of MMPs and the degradation of extracellular matrix proteins in the dissemination of tumour cells. The Ha-*ras*-transfected HaCaT cell line was used to reconstruct the epidermal compartment (RHE, FT model). For reference, the respective tissues were based on the spontaneously transformed (non-tumour) keratinocyte cell line HaCaT. Crosstalk of fibroblasts and the tumour cells induced MMP-9 at the tumour–stroma interface and is involved in tumour cell invasion [67]. Another important enzyme relevant for tumour growth is lysyl oxidase [68].

Later, squamous skin cancer biopsies were transferred to dermal constructs and allowed to grow. Many features are close to the human disease, in particular squamous cell nests formed mimicking the invasiveness of the hyperproliferating, transformed keratinocyte. This was not seen when building FT models with either NHK or the cancer cell lines SCC12 and SCC13, respectively [69]. Yet, an SCC12-based squamous cell carcinoma is also described. FT models comprising NHKs and fibroblasts having been seeded with the SCC12 cell line served for the evaluation of photodynamic therapy. Upon treatment, apoptosis and necrosis of the tumour cells led to a regression within five days – which was not the case when applying either the UV sensitizer alpha-laevulinic acid or UV irradiation alone [70]. Thus, this model appears suitable for preclinical testing of new anticancer drugs for squamous skin cancer and could have the potential to replace animal testing for this purpose in the future.

Melanoma

According to the current pathogenetic concept melanoma is less closely linked to UV exposure when compared with non-melanoma skin cancer. Also, this highly malignant skin tumour has been subjected to tissue engineering. Melanoma cells (HBL cell line) populating de-epidermized dermis invaded the dermis, whereas this was not the case with normal melanocytes [71]. The use of this model for pharmacological research, however, still has to be evaluated.

Concluding remarks

In conclusion, today several *in vitro* skin disease models offer new approaches to early drug development and formulation strategies aiming at new topical dermatologicals. In the future, this could replace corresponding animal models. Current *in vitro* skin disease models only reflect the situation in the patient up to a point. This makes further refinement look inevitable.

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