Arch Dermatol Res (2007) 298:361–369
DOI 10.1007/s00403-006-0709-6

REVIEW

Modelling the human epidermis in vitro: tools for basic and applied research

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Received: 3 August 2006 / Accepted: 29 September 2006 / Published online: 28 October 2006

Abstract Culture models of tissues and organs are valuable tools developed by basic research that help investigation of the body functions. Modelling is aimed at simplifying experimental procedures in order to better understand biological phenomena, and consequently, when sufficiently characterized, culture models can also be utilized with high potential in applied research. In skin biology and pathology, the development of cultures of keratinocytes as monolayers has allowed the elucidation of most functional and structural characteristics of the cell type. Beside the multiple great successes that have been obtained with this type of culture, this review draws attention on several neglected characteristics of monolayer cultures. The more sophisticated models created in order to reconstruct the fully differentiated epidermis have followed the monolayers. The epidermal reconstruction produces all typical layers found in vivo and thus makes the model much less simple, but only this kind of model allows the study of full differentiation in keratinocyte and production of the cornified barrier. In addition to its interest in basic research, the reconstructed epidermis is currently gaining a lot of interest for applied research, particularly as an alternative to laboratory animals in the chemical and cosmetic industry. Today several commercial providers propose reconstructed skin or epidermis, but in vitro assays on these materials are still under development. In order to be beneficial at long term, the validation of assays must be performed on a material whose availability will not be interrupted. We warn here providers and customers that the longevity of in vitro assays will be guaranteed only if these assays are done with well-described models, prepared according to published procedures, and must consider having a minimum of two independent simultaneous producers of similar material.

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Introduction

The human epidermis is the crucial tissue that produces a sturdy, flexible and self-repairing barrier between our internal body organs and our environment. Its major function is to protect the body from dehydration, loss of nutrients and unwanted effects of all the substances coming into contact with the skin in several forms depending on their physicochemical properties such as vapour deposition, liquid contact or solid contact such as contaminated soils or metals. Furthermore, it contributes to the protection against the living organisms we encounter around us, i.e. other animals, plants and fungi, as well as micro-organisms invisible for the human eye: essentially bacteria and viruses. In order to be and remain effective, the epidermal barrier must protect the body from most frequent potentially harmful physical, chemical or biological harassment. Therefore, most studies of the epidermis address questions that have a more or less close

relationship with production, maintenance, and repair of the epidermal barrier.

In this review, we will present how culture models of the epidermis have been developed in order to answer basic questions on this tissue:

- How do epidermal keratinocytes follow the finely tuned program of differentiation that is finally responsible for the formation of the epidermal barrier?
- How can we use in vitro models of the epidermis in order to evaluate safely and ethically whether a part of our environment is potentially harmful to our skin?

In other words, we will focus on models aimed at studying in vitro the role of keratinocytes, the main cell type of the epidermal tissue, and illustrate how these models can be valuable tools for basic and applied research on the epidermis. As a conclusion, we will discuss how much critical is guaranteed availability of a model before it can be utilized in routine evaluations.

Several practical limitations in the in vivo studies of the human epidermis have led to an ever increasing number of in vitro studies when the attention of researchers focused on the human epidermis or the human skin [41]. For this purpose, *in vitro* protocols using excised human or animal skin samples have been developed [18] and adopted in the OECD guideline 428 [25]. However, for legal and ethical reasons, the use of such kind of substrates is often limited and closely regulated especially for the evaluation of cosmetics. Moreover, the relevance of conclusions drawn from animal data for human skin has always been questionable. Therefore, OECD have stated, that i.e. for percutaneous absorption studies artificial human skin models could be used also, given equivalency is proven [24].

Such kind of in vitro models were developed in several research laboratories during the last 30–40 years, and skin models became progressively available from commercial companies [15]. Among their advantages, in vitro models avoid for instance the need to recruit volunteers for a specific study. They also reduce the number of laboratory animals required, even though certain models still require the sacrifice of donors (e.g. newborn mice) in order to set up the culture of epidermal cells. Most interestingly however, models in culture allow a clear separation between the cutaneous cellular components which thus can be analysed individually. More recently, some of these have become commercially available [23] and have undergone various validation programs in order to evaluate their suitability in cutaneous toxicology and pharmacology [8, 23, 45, 49]. They exhibit a similar differentiation pattern compared to the normal human epidermis in vivo, and represent reproducible models in a controlled environment [32, 43]. Consequently, factors such as variability of skin source, i.e. different donors or different anatomical body sites, which could influence the final results, can be ruled out.

Although they represent a major improvement over what was available just a few years ago, it is evident that no model is ideal. In general, each model exhibits advantages for a particular kind of studies, but is also restricted by limitations that impede its use in other kind of studies. The precise understanding of a model is based on the knowledge of multiple research data published in several, sometimes relatively old, research papers in which the relevant information is often not easy to retrieve from a large bunch of data that may have become obsolete. In this review, we focus on both simple and more sophisticated models available to date for in vitro investigation of the human epidermis, emphasizing largely on serum-free models that become increasingly popular. Indeed, serum-free cultures of human keratinocytes are made easier thanks to the availability through several companies of culture media and isolated cells (e.g. [47]). However, those serum-free cultures remain too often poorly understood because critical information on them is scattered in the literature and thus remains in the shade of the information available on older models using serum as a component of the growth medium. Particularly, we will try here to present and discuss uncommon advantages and limitations that appear ignored by too many skin researchers.

The epidermis

In order to produce and maintain the vital epidermal barrier, the keratinocyte, the main cell type in this tissue undergoes proliferation and differentiation. During the progressive terminal maturation of the keratinocyte, its cellular morphology changes from typically cuboidal in the undifferentiated proliferative cells anchored on the epidermo-dermal junction in the basal layer, into a squamous morphology in the dead cells of the cornified layer. Between these layers, morphological changes mean taking shape of a prickle cell within the spinous layer and intracytoplasmic accumulation of dark structures, named keratohyalin granules, inside the granular layer, underlying the cornified barrier.

The typical epidermal organization into four layers reveals that inside the keratinocytes, the differentiation program is intended to produce the epidermal barrier.

The appearance of different layers simply results from progressive maturation of this cell type inside the epidermis. Because desquamation, i.e. detachment of cornified keratinocytes, occurs regularly from the surface of the epidermis, a constant proliferation of cells in the lowest basal layer must be regulated in order to guarantee homeostasis of the epidermal tissue, i.e. an equilibrium between the number of cells lost from the surface of the body and the number of new keratinocytes produced deeply within the basal layer. Of course, this homeostasis is crucial for the tissue's well-being.

Keratinocytes represent the first epidermal normal cell type that has been grown successfully in culture [40]. Keratinocytes can be cultured in immersed conditions as a monolayer or as stratifying layers, but we will see below that it can further be grown in conditions where the cultured cells reconstruct the basis of an epidermis with three dimensional organization and production of a cornified barrier when the surface of the culture is exposed to the air [37].

Other minor cell types occupy the epidermis. Merkel cells are linked to neural sensitive endings and are not yet a cell type which can be grown in tissue culture, however the cell type can be isolated and cultured with keratinocytes. In the presence of nerve cells, Merkel cells establish synapses and their survival is promoted by the nerve cell [46]. A current hypothesis proposes that Merkel cell type differentiates by an unknown mechanism from keratinocytes themselves [48]. Thus, the identification of this hypothetical mechanism could one day render possible the differentiation of cultured keratinocytes into Merkel cells. Langerhans cells are antigen-presenting dendritic cells that enter the epidermis from blood circulation and become sentinels inside the spinous layer. Once stimulated by a foreign intruder, a Langerhans cell leaves the epidermis in direction of the lumen of lymphatic vessels in order to meet other cells of the immune system within lymph nodes. Langerhans cells can be produced in vitro from CD34-positive cells [4] and are eventually available for incorporation into more specialized epidermal in vitro models based on keratinocytes [14, 38]. Their differentiation into dendritic cells can occur in vitro, opening the possibility of preparation of immunocompetent reconstructed skin [12]. The incorporation inside a model made up of keratinocytes is also possible with melanocytes, the pigment-producing cell type in the epidermis. This cell type can indeed be successfully isolated and cultured from the human epidermis [29] and so it can be diluted into a suspension of cultured keratinocytes when those cells are plated in order to reconstruct in vitro a human epidermis [38].

The first model

The problem of growing large number of keratinocytes has been solved more than 30 years ago now, during an elegant study of teratogenic cell line by Rheinwald and Green [39]. Basically, they found that certain culture conditions induced in this teratogenic cell line a maturation that mimics epidermal keratinisation [39]. The clever deduction was that if those conditions were favouring an epidermal phenotype for the teratoma cell line, same conditions might be beneficial for normal epidermal keratinocytes themselves. This proved to be the case [40] and from then on normal keratinocytes were grown easily and confluent cultures were even used for grafting in patients with extensive burns for instance or for treatment of other conditions like giant naevi [17]. In this first model of cultured epidermal cells [40], serum is included in the culture medium, a feeder-layer of irradiated fibroblasts helps the growth of colonies of keratinocytes, the epidermal growth factor (EGF) and insulin are parts of the medium and cholera toxin is further used for its beneficial effect on cyclic-AMP production. When keratinocytes grow and expand their colonies in this model, they compete with fibroblasts of the feeder-layer for the plastic substrate, so the mesenchymal cells are shed into the culture medium, whilst keratinocytes form a basal layer of anchored cells able to proliferate, as demonstrated by incorporation of bromodeoxyuridine [33]. Some keratinocytes leave simultaneously the basal anchorage and migrate upwards in order to produce stratification and induce differentiation. Differentiating keratinocytes in this model exhibit strong expression of the marker involucrin as early as in the first suprabasal cell layer [1], a situation that differs from in vivo differentiation where involucrin is mainly induced in upper spinous and granular layers. In consequence, this culture model is certainly suitable for easy large production of keratinocytes and can be utilized for particular studies of epidermal cell growth and differentiation. However, the presence of serum and EGF with insulin makes studies of epidermal cytokines and growth factors quite difficult in such conditions where interference with molecules already present in the culture medium impedes clear determination of the roles assigned to those molecules. So proliferative cells and differentiating cells are present simultaneously in those cultures, early after seeding of keratinocytes, while fibroblasts of the feeder layer are also present, which makes difficult the discrimination between events occurring in proliferative keratinocytes and their modification or the appearance of new events in differentiating keratinocytes, or simply normal phenomenon in fibroblasts. This limitation

has probably been responsible for the frequent use of a particular technique in order to analyze the onset of terminal differentiation, the suspension of single keratinocytes in culture medium made viscous by the dissolution of methylcellulose, a situation quite distant from the natural environment of keratinocytes in the epidermis.

In summary, although this model has been used the most frequently, notably as revealed by a number of citations over 2000 since its publication, the first model of epidermal keratinocyte exhibits limitations for several studies of human keratinocytes. This probably explains the emergence of other models.

Cultures of keratinocytes in serum-free conditions: the controversial role of calcium and autocrine growth

The calcium concentration in regular culture media is usually between 0.3 and 2 mM. Investigations of mouse keratinocytes in culture identified calcium as a regulator for growth and differentiation[19]. It was indeed demonstrated that, by modulation of the extracellular calcium concentration, it was possible to modify proliferative and differentiating properties of mouse keratinocytes [51]. This tight link between the calcium extracellular concentration and the expression of precise differentiation markers had a considerable impact on the keratinocyte community: calcium was from then on considered has the regulator of choice for epidermal differentiation. The beneficial effect of reduced calcium concentration, first observed on the cell growth of murine keratinocytes, was explored rapidly with human keratinocytes and resulted in publication of successful methods for their culture in a serum-free medium [3]. However, is the whole differentiation of the human keratinocyte as tightly regulated by calcium as is the case with mouse keratinocytes ? The answer is: "probably not", but the influence of calcium on cell-cell interactions and particularly on stratification of keratinocytes through the formation of desmosomes arose from those early studies [3]. The effect of the stratification phenomenon itself on the induction of differentiation was then questioned. As early as in 1984, a largely under-considered study demonstrated that colony forming efficiency of human keratinocytes was more dependent on cell density in the culture plate than on the calcium concentration in the culture medium of keratinocytes, strongly suggesting that in those conditions the modification of extracellular calcium concentration has less effect on human keratinocytes differentiation than on mouse keratinocytes [50]. This was confirmed later, for instance when the expression levels of early markers of differentiation, the suprabasal keratins 1 and 10, were shown independent of calcium but regulated by cell density at confluence of the culture [13, 34].

This new idea that epidermal differentiation was not so tightly linked to calcium concentration in human keratinocytes did not gain attention from all researchers in the field since the reverse idea had been long ago accepted and could not be challenged so easily. Furthermore, the sensitivity of certain gene expression and the discovery of calcium-sensing proteins made the hypothesis of a central role of calcium in epidermal differentiation even stronger. However, recent observations have interestingly demonstrated that the role of calcium in epidermal differentiation has been strongly exaggerated [21]. It was even shown, using high density microarray analysis, that two known inducers of epidermal differentiation, i.e. cell detachment from the culture substrate (the suspension culture in methylcellulose) and cell density reaching confluence of the culture, are much stronger regulators of epidermal differentiation than the extracellular calcium concentration [2]. Unfortunately, those confirmations are still largely ignored today and publications still appear in which an increased concentration of calcium is presented as a certified way to induce keratinocyte differentiation, with no need to control at least one differentiation marker (e.g. [26]).

In summary, although the serum-free culture conditions for the growth of human epidermal keratinocytes utilize low extracellular calcium concentration in order to favour cell proliferation, epidermal differentiation of this cell type does not necessarily require an increase in calcium concentration. Increasing the extracellular calcium concentration certainly helps in the establishment of the cellular stratification of keratinocytes [28] and this triggers expression of differentiation markers, however keeping it low does not impede the commitment of keratinocytes towards irreversible growth arrest and terminal differentiation [34].

Another remarkable observation already performed 15 years ago in serum-free cultures was that rapidly growing keratinocytes cultured in absence of any peptide growth factor are able to grow continuously [5], simply by autocrine stimulation of the EGF receptor [30]. Indeed, a medium conditioned by growing keratinocytes was shown to contain a growth factor regulated by heparin sulphate and secreted by keratinocytes. This autocrine factor was identified as amphiregulin [6], one member of the EGF family of growth factors, able to bind and activate the EGF receptor. This observation is firstly of interest for those researchers who want to specifically study epidermal cytokines and

growth factors. Indeed, because no peptide component is added to the medium, the autocrine culture model allows the precise study of any added protein. The second interest of autocrine cultures is demonstrated in the study of differentiation of keratinocytes in such conditions. Indeed, it was then proven that addition of EGF in the medium of cells that attain culture confluence alters strongly the expression of the early differentiation markers keratins 1 and 10 [34]. Thus, autocrine conditions only provide a correct estimation of expression and accumulation of suprabasal keratins 1 and 10 when stratification is initiated. Simultaneously, the expression of involucrin is shown to increase slightly at confluence in autocrine conditions, but is further induced in postconfluence, mimicking the sequence of events described during the stratification and epidermal differentiation of keratinocytes in vivo [35].

These data argue in favour of the autocrine model when researchers are interested in the events linked to the onset of terminal differentiation in keratinocytes, since EGF and vitamin A from serum lipoproteins may account together for an inhibition of the suprabasal keratins, but also for a precocious upregulation of involucrin [35].

Culture at the air–liquid interface

The culture of keratinocytes as monolayers represents a tool highly useful in basic research on the biology of this cell type. However, since no production of the cornified layer can occur in those conditions, in vitro studies of the differentiation program at the level of granular and cornified layers was impossible as were studies of the epidermis as a tissue.

As early as in 1983, Pruniéras et al. [37] have demonstrated that it is possible to get full differentiation in vitro simply by raising cells up to the air–liquid interface. This technique has been first developed by culturing keratinocytes over a de-epidermized dermis [37] or on a gel of collagen, or on a lattice including fibroblasts and collagen [20]. Apparently, the interface with air stimulates in keratinocytes the synthesis of profillagrin and thus the appearance of the granular phenotype when keratohyalin granules develop. These granules never appear in immersed culture conditions and seem to be the missing link that allows the final gain-of-function for keratinocytes during cell cornification. In such culture conditions, keratinocytes located at the top of the granular layer scarify and leave their fully differentiated cell skeleton (represented by the aggregated intermediate filaments) or cell shell (represented by the cornified envelope formed after activation of transglutaminase) to the human body in order to maintain the superficial barrier.

The efficiency of the barrier is variable in vivo, but its efficiency when produced in vitro using the air–liquid interface as the triggering mechanism has been questioned as permeability measurements revealed a too much permeable interface [44, 52]. So far in vitro, no production of sebum occurs that will help to create an hydrophobic interface. However, even with minimal production of sebum, the human skin demonstrated better barrier function than the barrier of reconstructed epidermis. This difference was identified to result from an altered composition in lipids present in the cornified layer [31]. The inclusion of vitamin C in culture medium was then shown to improve the barrier properties of the reconstructed epidermis [27] due to an improved lipid composition in the stratum corneum [31]. We now obtain epidermis in vitro with increasingly efficient barrier.

Very recently, a thorough study has been undertaken in order to identify and understand molecules controlling epidermal stratification, but also barrier formation. Comparing immersed epidermal cultures with cultures incubated at the air–liquid interface, that study interestingly described important roles of several molecules from different, sometimes unexpected, metabolic pathways in establishing the fully differentiated phenotypes, proving that the process of terminal differentiation is more complex than a process involving structural molecules only [22].

The reconstruction of epidermis on filter

Growth at the air–liquid interface in vitro means feeding the epidermal cells from the bottom of the reconstructed tissue, through the basal layer. This is close to the situation in vivo, but must be done without any blood circulation: impregnating a collagen gel or a dermis with liquid culture medium containing all the components required for keratinocyte proliferation, stratification and differentiation. However, since the techniques of cell culture want first to reproduce the in vivo organs and tissue, but also since those techniques also try to simplify complex histological structures in order to isolate phenomena and consequently allow easier studies scheduled to improve our understanding and permit refined analysis of otherwise too complex phenomena, the 3D-reconstruction of the epidermis has been adapted to basal inert substrates such as porous filters [42]. Filters provide a solid mechanical support on which keratinocytes can attach through integrins, organize hemidesmosomes and then stratify

thanks to the formation of adherens junctions and desmosomes in order to form the typical epidermal layers. These layers can then be easily exposed to the air–liquid interface when the culture medium is only present in the compartment under the filter. The diameter of the pores in the filter must be small enough in order to impede keratinocyte migration through the holes and an eventual colonization of the other side of the filter.

Several porous substrate can apparently be used, as long as cell adhesion occurs easily, and in the reconstructed tissue, the pattern of gene expression is very similar to the pattern analysed from skin samples [16, 42].

Although the reconstructed skin is interesting in order to study particular interactions between different cell types, or at the epidermo-dermal junction, the presence of a complex biological structure and its components is a real problem when a study's aim is to focus on the measurement of epidermal phenomena. Furthermore, this problem is crucial when a study intends to detect and identify growth factors or cytokines that are secreted or released from keratinocytes at different levels of the epidermis. Indeed, in this case, the presence of the superficial barrier leaves as the unique possibility for the molecules to escape the tissue by diffusion towards the basal surface. Whereas a biological gel or dermis will then trap, sometimes avidly, the molecules escaping the epidermis, a filter will provide a relatively inert interface with holes big enough for the passage of macromolecules towards the medium bathing the tissue from the other side of the filter.

This type of reconstructed epidermis has been refined progressively and has been rendered available through a commercial source, but its production using commercially available reactives and media does not present real difficulties in any laboratory where batches of human cultured keratinocytes are available [36].

Furthermore, since autocrine conditions have been identified during the growth of keratinocytes, the culture conditions have been oriented towards serum-free conditions, assuming that the more simple the culture environmental conditions, the easier the study of cell and tissue reactions.

Use of the reconstructed epidermis for the testing of chemicals

The availability of cultured skin models didn't mean automatically the availability of validated biological assays on such material. The current availability of cultured cutaneous organ and tissue, plus their reproducibility and the multiplicity of sources from where this kind of material is available, indicate at first that the era of in vitro assays has begun to replace the tests formerly performed on living animals. Those assays expand quickly in order to follow the rule of the 3 "R"s and its enforcement in developed countries.

The reconstructed epidermis can be used for the testing of chemicals. Briefly, with the reconstructed epidermis, either commercially available or produced in our lab, we have shown that following suggestions made by Corsini and Galli [10, 11], very typical behaviours in term of viability and release of interleukins 1alpha and 8 differentiated a surfactant with sensitising potential from other detergents with weak, mild and strong irritation potential [7, 36]. In view of our data, the pressure to test such an easy procedure with other substances was very strong. Even though releases of cytokines similar to our first study were found, enlarging the number of compounds rendered the analysis of the data much more difficult at first glance [9]. Retrieving and comparing the data of different compounds at different concentrations, we realized that a standardization of the procedure was required [9]. A solution based on starting by the determination of an MTT50 value (the compound's concentration that preserves 50% of survival based on an MTT assay) was suggested and finally tested on the larger number of substances. This procedure gives us a common reference as it determines at which concentration of the substance the release of interleukin 1alpha and the release of interleukin 8 have to be measured. Then, ratioing the measurements clearly partition compounds with irritant potential from compounds known to induce sensitisation in vivo [9]. Since then, the procedure has been applied to other compounds and again, data and conclusions proved highly convergent with in vivo analysis (unpublished data), bringing great hopes for the validation of this procedure.

The continuous availability of a model

Since the elaboration of in vitro skin models has arisen as an elegant response to the requests of activists wishing to ban the use of laboratory animals, the first attitude has been to create in different institutions or companies different models of epidermal cell cultures, each research group claiming that its own model was certainly the best for very specific reasons. Then, over the past few years, different commercially available cultured human skin models have been developed by private companies such as MatTek Corporation, L'Oréal, SkinEthic, Organogenesis, Advanced Tissue Sciences, etc. This situation has created initially a really

beneficial competition between producers of skin cultures and the competition has accelerated significant improvements. However, when one turns to the development a highly specific assays aiming at the replacement of animals, when determining whether a new chemical exhibits irritative and/or sensitising properties, no company can reasonably afford relying on one unique producer of skin or epidermal models. Indeed, dependence on one source of a model for such a crucial part of the activity means that any interruption in the source of the model can become an economical catastrophe, whichever the cause of the interruption: collapse or re-orientation of the company, technical problem during the production of the model, unavailability of the model due to restrictions imposed by the owner of the producing company (who can be a direct competitor of the customer), etc. For example, in 1996–1997, the model Skin2TM and EPISKIN became unavailable due to marketing decisions of the producers. Therefore, ECVAM and a lot of laboratories had to support a new validation study to determine whether another human skin model (EpiDerm; available since 1992) could replace the previous tested commercial models.

Thus, contrarily to the current tendency that places back-to-back all the producers of epidermal models, and despite some concordance can be found when comparing different models [15], it would be the interest of everybody if customers do favour models developed hand-in-hand by different laboratories in different corners of the world. Indeed, this type of collaboration will be the only way to provide customers with models whose production could never be totally interrupted for any reason.

With this idea in mind, we demonstrated in 2004 that the model available from Skinethic and based on the publication by Rosdy and Claus [42] can be reproduced nicely with materials easily available, showing that adequate culture conditions provide an environment that produces a normal looking epidermis [36]. Only the large scale production remains a limitation.

Conclusions

Modelling the epidermis for in vitro analysis of its individual components, but also in order to identify and understand their behaviour in an environment either normal or conditioned by chemicals or pathogens, has been a challenge for several reasons:

– The progress of cell and tissue biology render possible detailed analysis of skin components
– Isolated components clearly demonstrate their contribution to the tissue's function
– The replacement or reduction of laboratory animals for the testing of chemicals and cosmetics requested by activists has found an echo in legal enforcement in more and more areas around the world.

Simple models give simple answers to questions asked on the functioning epidermis or its components. However, since the main function of the epidermis is found in the cornified layer, more sophisticated models allowed the assay of properties linked to this function. A true race at getting the best model was started. However, contrarily to classical economical requirements for a product, which customers want cheapest and best, it is progressively recognized that the main requirement is now for models that won't be discontinued before other tests become available. Thus, the first requirement in order to guarantee the longevity of a model is the publication of all its characteristics, but second that the model is ideally available with closely similar (if not identical) characteristics from at least two different and totally independent sources. No monopoly seems desirable for the future of living epidermal models.

Acknowledgments This research received support from FRFC grant 2.4506.01.

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