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INVITED REVIEW

IN VITRO MODELS OF EPIDERMAL DIFFERENTIATION

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SUMMARY

The differentiation program followed by the epidermal keratinocyte in skin is intended to continuously produce and maintain a cornified layer made of fully keratinized cells. This outer layer of the body provides a certain protection against external pathogens and chemical or physical agents, together with a barrier that prevents loss of body fluids. Considerable knowledge of epidermal differentiation and understanding of its regulation has progressively emerged from the availability of keratinocyte cultures, and from the consecutive possibility of unlimited in vitro experimentation. This short review briefly presents the main current in vitro models of epidermal differentiation and emphasizes their advantages or pitfalls when studying particular steps of the differentiation program or analyzing their regulation.

Key words: keratinocyte, epidermis, cell culture, differentiation, *in vitro* models

Homeostasis of the epidermis is critical for the normal appearance and function of the human skin and depends essentially on the strict program of differentiation followed by the epidermal keratinocyte, the main cell type present in this tissue. To protect, maintain, or restore homeostasis of the epidermis, today's cosmetology and dermatology take their knowledge from the numerous *in vitro* studies of the keratinocyte and its cellular physiology. During the last two decades, particular culture conditions that allow isolated keratinocytes to proliferate and produce expanding colonies when anchored to a plastic substratum have been discovered. At the same time, researchers found accurate markers of epidermal differentiation and identified environmental conditions that favour, or conversely impede, the program of keratinocyte differentiation. In this review, we present the main currently available models of keratinocyte culture and discuss their respective potentials and pitfalls regarding their utilization in basic or pharmacological studies of epidermal differentiation.

The epidermis and the differentiation of keratinocytes

The human skin is covered by the epidermis, a stratified squamous keratinized epithelium composed mainly of proliferating and differentiating keratinocytes. Histologically, keratinocytes organize into four typical layers named, from the innermost to the one exposed on the surface of the epidermis, the basal, spinous, granular

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and cornified layers. Keratinocytes in normal skin proliferate exclusively in the basal layer. Cells in this layer are anchored to the underlying extracellular matrix through the epidermo-dermal junction. For this basal anchorage, keratinocytes express adhesion molecules of the integrin family and these integrins are responsible for the organization of hemi-desmosomes, the specialized anchoring junctions of the stratified epithelia[1]. The anchorage on the extracellular matrix through the formation and maintenance of hemi-desmosomes is absolutely critical for the polarized organization of the epidermal tissue[2,3]. Within the basal layer, keratinocytes are either slow cycling stem cells, assuring renewal of the tissue during the complete lifespan of a living organism, or transit amplifying cells that result from division of the slow cycling stem cells. The basal rapidly dividing keratinocytes are devoted to amplify the epidermal cell population and produce a sufficient number of daughter cells that migrate out of the basal layer towards the suprabasal layers at the onset of terminal differentiation[1].

Keratinocytes committed to terminally differentiate are growth-arrested first, then exhibit morphological changes that result from the progressive maturation of cell structures concomitant with their upwards migration. Indeed, differentiating keratinocytes enter the spinous layer probably because they lose their integrin adhesion molecules, express cadherin-related intercellular adhesion molecules to elaborate more numerous desmosomes than between basal keratinocytes, and synthesize suprabasal keratins 1 and 10, typical of the dense filament bundles that characterize cells of the spinous layer. Since they are expressed in all suprabasal layers (Fig. 1), keratins 1 and 10 serve as early markers of epidermal differentiation[1,4,5].

In upper spinous and granular layers, keratinocytes continue to prepare the keratinization process. They synthesize components of the cornified envelope (involucrin, loricrin) as well as transglutaminase-1, the enzyme that will elaborate crosslinks between those components at the making of the envelope[1]. The localization of involucrin in the normal epidermis (Fig. 1) makes involucrin a late marker when studying the process of epidermal differentiation. Lamellar bodies, containing glycosylated ceramides and hydrolases that will play respectively in intercellular sealing and in the final

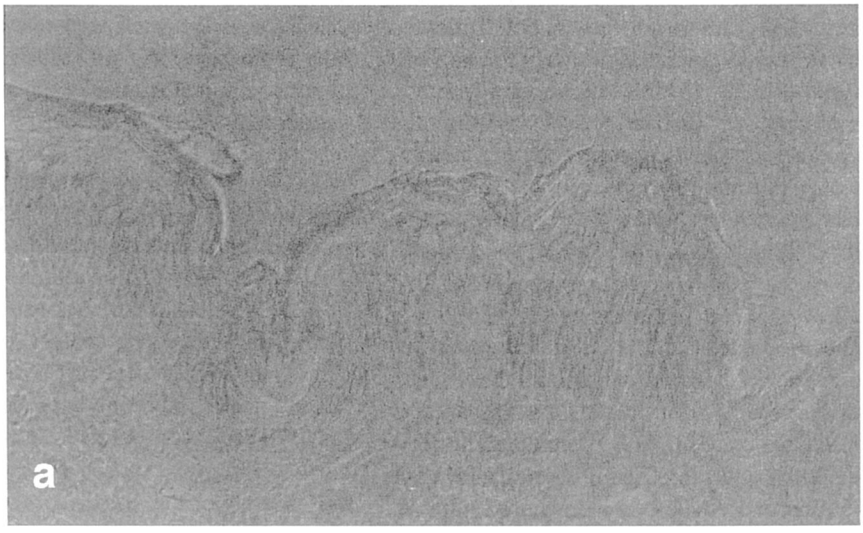

Fig. 1 (a)

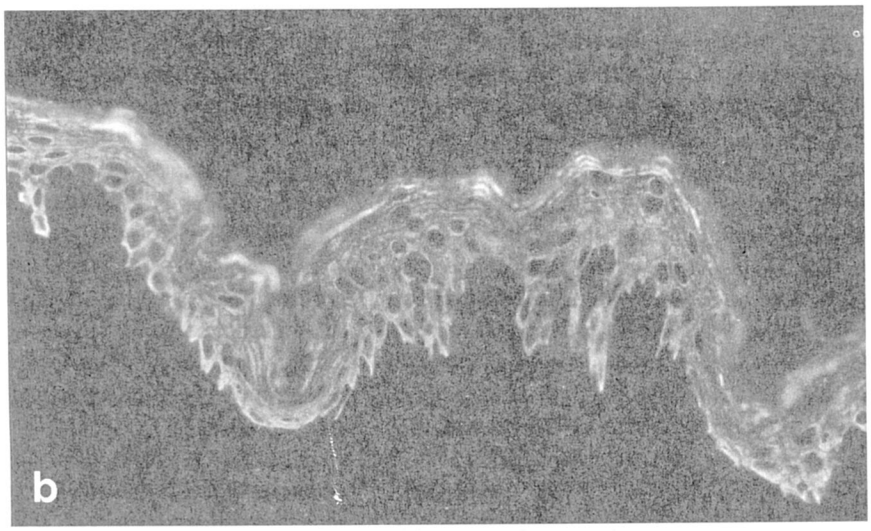

Fig. 1 (b)

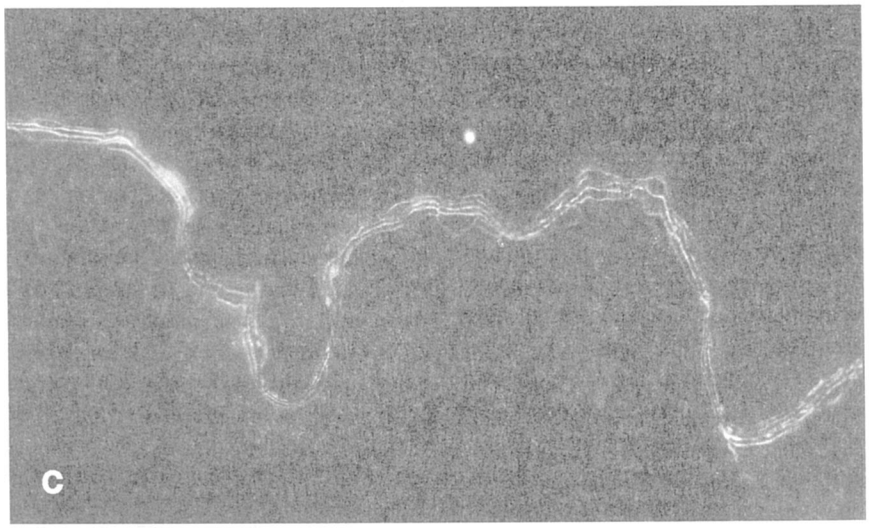

Fig. 1 (c)

Figure 1. Expression of early (keratin 10) and late (involucrin) differentiation markers in normal human epidermis.

A frozen section of adult normal human skin obtained at plastic surgery was labeled by double immunofluorescence with mouse monoclonal antibody DEK10 against keratin 10 (Cappel-ICN) followed by biotinylated anti-mouse antibody and Texas Red-conjugated streptavidin (Amersham) (b), and with rabbit polyclonal antibody against involucrin (BTI) followed by fluorescein isothiocyanate anti-rabbit antibody (Biosys) (c). Histology of the tissue was observed by differential interference contrast microscopy (a). Bar, 100 μm.

cell desquamation in surface of the tissue, appear in the same layers. Typical of the granular layer, keratinocytes synthesize profilaggrin, and store this precursor of filaggrin in the keratohyalin granules. Finally, with an apparently brief transition, keratinocytes enter the cornified layer, become enucleate dead cells sealed to each other, filled with keratin filaments aggregated by filaggrin inside a cornified envelope constructed just under the plasma membrane. These cornified cells stack onto each other in a regular way, then are lost in the surface of the skin by desquamation[1].

The feeder layer system for stratified cultures of human keratinocytes

In 1975, Rheinwald and Green first described culture conditions that allow large expansion of keratinocyte colonies in vitro and the formation of stratified epidermal sheets[6]. In this culture model, one uses growth-arrested (irradiated or mitomycin C-treated) 3T3 mouse cells in order to provide a feeder layer, and a culture medium based on DMEM and Ham-F12 solution (3:1) containing selected fetal bovine serum and several growth factors, including the epidermal growth factor (EGF). This type of culture exhibits an incomplete differentiation program as no typical granular or cornified layers is observed; however, keratinocytes cultured in this model produce cornified envelopes when they are detached by trypsin and then incubated in suspension in methylcellulose-containing culture medium[7].

It has been next demonstrated that a precursor of the cornified envelope, namely involucrin, is expressed by keratinocytes in such culture conditions and incorporated in the envelope upon activation of transglutaminase-1[8]. In vitro, the expression of involucrin has been shown to be directly suprabasal and its synthesis correlates with the size of the epidermal cultured keratinocytes[9,10]. In consequence, involucrin has been used extensively as a marker of choice in order to detect terminal epidermal differentiation in a large number of experiments conducted with this culture model. These include experiments designed to assess the role of cell shape and adhesion in controlling the process of epidermal differentiation[1,11,12]. The feeder layer system has afterwards allowed identification and understanding of the role of integrins as markers of the keratinocyte basal phenotype, as well as signal transducers from the extracellular matrix playing in the control of epidermal differentiation[11].

Historically, the culture model with a feeder layer has been the first system that allows pharmacological and toxicological studies of keratinocytes in vitro, in other words in absence of interference from the dermis or from body fluids. Nevertheless, probably the most spectacular application of this culture system has been the preparation of stratified cultured epidermal sheets, which can be detached by dispase from their substratum[13], and their utilization as autografts for the treatment of full thickness burns. These results are of very high clinical interest and prove that, although keratinocytes form an incompletely differentiated epithelium in submerged culture conditions, they pursue their normal differentiation program and form typical granular and cornified epidermal layers when grafted onto a wound bed[14].

Cultures in low calcium concentration

In 1980, studying mouse epidermal keratinocytes, Hennings and colleagues[15] identified the role of calcium concentration in the medium to control epidermal cell differentiation. They found that keeping calcium at 0.05-0.1 mM prevented cell-cell adhesion and consequently cell stratification[15], enabling investigators to keep keratinocytes in an undifferentiated phenotype,

or conversely to induce epidermal differentiation, as revealed by the accurately regulated expression of several markers[16]. This finding was then exploited by Boyce and Ham when defining serum-free culture conditions for human keratinocytes[17]. These conditions were used by Wille and coworkers who found that, although low calcium serum-free cultures allow serial cultivation of exponentially growing human keratinocytes, a drastic loss in their clonogenic potential (i.e. the capacity to initiate growing colonies) is suddenly observed when cell density attains confluence of the culture[18]. The critical role of cell density in serum-free conditions has been recently confirmed[4], but the role of calcium has become the matter of a controversy: low calcium conditions favour epidermal keratinocyte proliferation at subconfluence, but increasing the calcium concentration does not automatically induce epidermal differentiation in studies of mouse keratinocyte cultures[19], and in studies of human cells[4]. Moreover, because of species particularities, one cannot extrapolate observations made with mouse keratinocyte cultures to human cells and vice versa.

Regarding the potential clinical use of serum-free cultures, it must be noted that cell stratification, although not favoured in this model, can be used as a first step to expand cultured keratinocytes and cover culture area sufficient for grafting on burned patients[20]. Secondly, the expansion phase may be followed by the induction of cell stratification. For example, the medium calcium concentration of confluent cultures can be raised to 1.8 mM, and after addition of serum, the resulting stratified culture can be detached by dispase and then used for grafting[20].

Autonomous growth conditions

At the onset of human keratinocyte cultures, the epidermal cells are isolated by trypsinization. Then, keratinocytes plated on culture dishes need stimulation in order to initiate cell proliferation and colony formation. In serum-free cultures, this stimulation is usually provided by EGF and bovine pituitary extract[17,18]. In 1991, Cook and colleagues found that keratinocytes attaining a certain cell density do not require addition of any growth factor in the culture medium to continue their proliferation. When enough keratinocytes are present in the culture vessels, the stimulation to proliferate is provided by factors produced by keratinocytes themselves, in a situation named 'autonomous growth'[21]. This autocrine and/or paracrine stimulation results from the secretion of amphiregulin, a peptide with some properties similar to EGF[22]. Amphiregulin is able to activate the EGF receptor, a property demonstrated as responsible for the autonomous growth of human keratinocytes[23].

Using this type of culture only, it is possible to use the detection of suprabasal keratins 1 and 10 in order to assess the early onset of differentiation[4]. Indeed, we have shown that EGF, generally included in keratinocyte culture media, inhibits the expression of the suprabasal keratins, whereas in autonomous growth conditions, these keratins are suddenly upregulated at confluence of the culture. Interestingly, the particular properties of autonomous keratinocyte cultures have been nicely exploited to select culture conditions that mimic alterations observed in vivo in the epidermis affected by psoriasis, a disease characterized by hyperproliferation of keratinocytes and delayed incomplete differentiation[24].

Induction of differentiation by sodium butyrate

An easy way to induce terminal differentiation in keratinocyte cultures is certainly by means of incubation with sodium butyrate[25]. Sodium butyrate is largely known to be an

inducer of cell differentiation but is probably of poor specificity. Indeed, this compound probably acts on acetylation of histones and induces a general increase in transcription of several genes that results in cell differentiation at large, including in tumor cells[26]. Interestingly, one of the effects of sodium butyrate on keratinocytes is induction of the production of cornified envelopes[25]. In recent experiments, this in vitro model of epidermal differentiation and autonomous growth conditions allowed us to demonstrate some involvement of lysosomes during the process[27], an observation that will probably require further consideration in the future when establishing a general picture of keratinocyte differentiation.

Fully differentiated cultured epidermis

The culture of keratinocytes immersed in culture medium prevents most of the late steps of epidermal differentiation to occur. For example, no granular and cornified layers can be produced by cultured keratinocytes in a liquid environment. However, raising the culture to the air-medium interface was shown to induce a more complete differentiation that results in the production of keratohyalin granules and of a stratum corneum made of several layers of enucleated keratinized cells[28]. Although full differentiation is apparently induced by incubation at the air-liquid interface, the expression of keratins by those cells is not perfectly identical to the in vivo situation and further improvements are still required for the utilization of such models in pharmacological and toxicological tests. For example, studying retinoic acid applied topically on the cornified layer of an in vitro reconstructed epidermis, Rosdy and colleagues[29] demonstrated that discrepancies remained between the effects of retinoic acid in vivo and its effects in vitro. Those discrepancies may come from the use of EGF in the culture medium since we know that EGF alters the expression of the keratins 1 and 10[4].

Finally, the use of a lowered (33°C) temperature, closer to the effective skin surface temperature than the 37°C temperature usually chosen for the culture of keratinocytes, has been demonstrated recently to considerably improve the characteristics of differentiation in an *in vitro* reconstructed epidermis[5]. Furthermore, in such a fully differentiated model, the addition of EGF at this low temperature had deleterious effects on the tissue morphogenesis and differentiation[5]. Obviously, the importance of temperature on the quality of keratinocyte cultures will certainly need further investigation.

CONCLUSION

Epidermal differentiation is a crucial complex phenomenon involved in skin normal function as it maintains the essential cornified barrier between the body fluids and its environment. Studying the differentiation process and its regulation in keratinocytes is one of the main task of basic dermatological research. The availability of *in vitro* models for the study of epidermal keratinocytes has permitted significant breakthrough during the last two decades in the knowledge of many cellular events encountered during epidermal differentiation, but also in our understanding of their precise regulation.

The choice of one type of keratinocyte culture from among the models available at the moment depends on many criteria. These criteria include the price of culture media and reagents, as well as the duration of the culture. Also, one must consider the type of cellular mechanism that will be studied. For example, studies on cell stratification require a culture medium with high calcium concentration in order to allow the formation of intercellular junctions; studies on

growth factors are best conducted in autonomous growth conditions, and experiments on fully differentiated keratinocytes can only be performed on cultures raised to the air-liquid interface. Therefore the final choice of a particular model is often a compromise between the expectations of the researcher, and the financial and temporal limitations. Finally, but certainly important for most readers interested in dermatology, the knowledge of intrinsic characteristics of the different keratinocyte culture models is absolutely required in order to correctly appreciate published observations and interpretations made by others with those models.

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IN VITRO МОДЕЛИ ДЛЯ ЭПИДЕРМАЛЬНОЙ ДИФФЕРЕНЦИАЦИИ

Ив Пуме, М. Леклерк-Смекенс

РЕЗЮМЕ

Программа для дифференциации эпидермальных кожных кератиноцитов включает непрерывное производство и поддерживание опровергающего (корнифицирующего) слоя вполне кератинизированных клеток. Эта наружная оболочка тела обеспечивает некоторую защиту от внешних патогенных, от различных физических и химических агентов, и в то же время служит барьером, препятствующим потере телесных жидкостей.

Разработанные клеточные культуры кератиноцитов и возможность неограниченных in vitro экспериментов привели к накоплению значительных по обьему знаний об эпидермальной дифференциации и её регуляции. Этот обзор представляет собой краткое обобщение основных направлений в in vitro модели для эпидермальной дифференциации, рассматривает их преимущества и недостатки при изучении определённых этапов дифференционной программы или при анализировании их регуляции.