Methods for Cultivation of Keratinocytes with an Air-Liquid Interface

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In ordinary cultures, cells are grown on artificial substrates and immersed in culture medium. In vivo, interfollicular epidermal cells grow on the basement membrane and are exposed to air. In a first effort to render the culture of these cells more physiological it seems legitimate to raise the cultured cells to the air-medium interface.

Epidermal cells can be raised by the use of collagen gels maintained on a rigid support. They can also be grown on nitrocellulose filters coated with collagen or coated with a basement-membrane equivalent (BME) previously deposited by bovine corneal endothelial cells. By raising the cultures to the air-medium interface there is some evidence of a more complete differentiation, as evaluated by morphologic criteria. However, biochemically, the raising of the cultures does not seem to induce the synthesis of those keratin polypeptides which are not expressed in immersed cultures.

Epidermal cells can also be raised by culturing them on dermal substrates or dermal equivalents. When they were cultured on inverted dead pig skin, epidermal cells synthesized membrane-coating granules (MCG). MCG were not found in immersed controls. By culturing epidermal-cell suspensions on deep epidermized dermis (DED), all morphologic markers of differentiation were seen except the keratin pattern. In addition, partial reexpression of high-molecular-weight keratin polypeptides occurred. However, the complete expression of keratins by cultured cells depends on the filtering action of the dermal substrate (the cultures are fed from underneath) more than on exposure to the air-liquid interface.

In summary, several methods are available to culture epidermal cells at the air-liquid interface that are of interest in an investigation of the response of these cells to epigenetic influences.

In ordinary cell culture systems, cells are attached on the bottom of culture vessels and covered with tissue-culture me-
addition to those seen in plastic cultures will be considered as indicative of an enhanced morphologic differentiation. Biochemically, the lack of expression of high-molecular-weight keratins is the rule in immersed cultures made on plastic [6,8]. The reexpression of these keratins in raised cultures will be taken as an indication of a more complete differentiation.

METHODS BASED ON RAISED COLLAGEN MEMBRANES

More complete differentiation of epithelial liver cells was obtained by raising collagen membranes to the air-medium interface by Michalopoulos and Pitot in 1975 [11]. In the method of these authors, collagen gels were prepared by pouring an acetic acid solution of rat tail collagen into plastic petri dishes. Six hours after having seeded the cells in suspension, the gels were loosened from around the periphery of the plate with the fused end of a Pasteur pipette. By shaking the plates, complete freeing of the gels ensued, which resulted in their floating together, with the loss of cells that had not attached to the gels. Twenty-four hours after seeding the cells, the floating gels, which were about 2 mm thick at the start, became thinner and appeared as thin membranes. Subsequently, the membranes shrank and their diameters decreased. Owing to this decrease in diameter, the cells on the membrane approached each other and formed aggregates. A schematic representation of the method of Michalopoulos and Pitot is given in Fig. 1.

By using a similar system, in 1977, Emermann and Pitelka [12] reported maintenance and induction of morphologic differentiation in dissociated mammary epithelium. The first interpretation was that better oxygenation favored differentiation. However, it was later shown by Shannon and Pitelka [13] that the crucial point in the floating-membrane method was the flexibility of the membrane and not the fact that the cells were maintained at the air-medium interface. If the membranes were made nonflexible by fixation, differentiation was the same in immersed as in air-exposed cultures. What was important was the change in cell shape resulting from the contraction of the collagen membranes.

More recently, Chambard et al. [14] and Hall et al. [15] have produced evidence that follicle formation from isolated thyroid cells or lumen formation by epithelial-cell lines resulted from embedding the cells into the collagen gel. When the cells were embedded into or cultured between two layers of collagen gel [14,15], their apical poles became oriented toward the lumen of the follicle, and differentiation occurred.

These experiments indicate that when collagen membranes are raised to the air-medium interface, two different factors have to be considered, namely, exposure to air and orientation.

![Fig 1. Schematic presentation (clockwise reading) of the culture method of Michalopoulos and Pitot](image-url)
of the cell axes. In order to evaluate the effect of exposure to air only, it is necessary to prevent changes in cell polarization by making sure that the collagen substrate will neither contract nor fold.

Lillie et al. [16] cultured cells of an established line of rat mucosal keratinocytes on collagen gels. After 4 days on the gel in immersed situation, the gels were lifted up and placed on grids in plastic organ-culture dishes. A schematic view of the Lillie et al. experiment is given in Fig. 2. The cells are placed on a substrate that is nonflexible and noncontractable because it is stuck to a stainless steel grid. The cells are fed from below and exposed to air. By light microscopy it was observed that the lifted cultures exhibited an enhanced degree of differentiation.

In particular, there was the development of as many as 10 suprabasal cell layers. By gross inspection of the cultures one could see that keratinization occurred across the entire surface of lifted cultures instead of the spotty keratinization that characterized the immersed cultures. Under the electron microscope at low magnification the cells in contact with the collagen gel had a cuboidal shape closely resembling basal cells of the parent tissue. However, this was not due to exposure to air, since in immersed cultures, basal-most cells were also cuboidal. At higher magnification, cytoplasmic vesicles with a lamellar content were seen that could be identified as membrane-coating granules (MCG). In some instances, this lamellar material has been seen between the cells in the intercellular space. MCG were also seen in immersed cultures, but less frequently.

A somehow similar observation though less detailed has been made by Fusenig et al. [17], who also cultured epidermal keratinocytes on collagen membranes lifted up above the tissue-culture-medium level so as to ensure feeding from underneath and exposure to air. The method of Fusenig et al. is schematized in Fig. 3. In this experiment, organoid organization of epidermal cells was observed, and this organization was lost when the cells were malignant.

More recently, adult human keratinocytes were cultured on nitrocellulose-coated filters and the synthesis of keratin polypeptides in immersed and lifted cultures was compared (unpublished data). Two different methods were used (Fig. 4A, B). In the first, 0.22-μm filters were coated on one side with soluble collagen I, and the cell suspension was placed on the collagencoated side. The coated filters with cells on top were laid down on stainless steel grids to allow both filter feeding and exposure to air.

In the second method, the filters were coated with collagen I on which bovine corneal endothelial cells were cultured in the presence of an eye-derived growth factor [18]. The endothelial cells were maintained on the collagen-coated filter until they achieved the deposition of a basement-membrane equivalent (BME). Endothelial cells were then removed and replaced by epidermal keratinocytes. Finally, the filters with collagen, BME, and keratinocytes were placed on grids and fed from below. The interest of this method lies in the fact that BME contains laminin and collagen IV [19]. Therefore, the cultures of keratinocytes on BME somehow mimic the in vivo situation in which epidermal keratinocytes are attached to the skin basement membrane.

Immersed control cultures were made by sticking the noncoated side of the filter onto the plastic bottom of the culture plate. As will be reported in another paper, the synthesis of keratins was not influenced by the raising of epidermal cells to the air-medium interface.

**METHODS BASED ON THE RECONSTRUCTION OF SKIN**

The idea of recombining epidermis and dermis has been widely used in developmental studies [20]. In culture, however,
be seen that the degree of terminal differentiation is higher in cultures on pig skin at the air-liquid interface. Heterogenous keratohyaline granules with dense homogeneous deposits were found. Desmosomes were consistently complete with their interdesmosomal dense line, which indicates full elaboration by the cells of desmosomal substructures. Intracellular membrane-coating granules (MCG) were observed. In cultures immersed in medium, MCG were still recognizable after 6 days in the explant itself, but no MCG could be seen in the outgrowing cells after 3, 8, 10, 17, and 30 days.

The fact that no MCG were found in immersed cultures does not mean that they were not produced at all, but it does mean that if they were produced, they were not numerous. On the contrary, in the outgrowths exposed to air, MCG were easy to see. Thus exposure to air may govern the synthesis of MCG.

This interesting system of Freeman et al. is nevertheless criticizable. Epidermal cells in vivo do not grow on the reticular aspect of the dermis. They grow on the basement membrane that covers the papillary dermis. In fact, when epidermal cells are dissociated and seeded as isolated elements on the reticular aspect of pig skin, they do not attach and they do not grow.

This prompted us to develop a novel culture system inspired from the pig skin model. The idea was to culture the epidermal cells at the air-liquid interface, as in the Freeman et al. method, and to provide the cells with a more "physiologic" substrate than the reticular aspect of dead pig dermis.

To do this, human split-thickness skin flaps were deepidermized and the remaining dermis was used as culture substrate. To deepidermize human skin flaps, they were maintained in PBS at 37°C for several days. After 2 days, the epidermis could be lifted up with fine forceps, but detachment of the epidermis was not always complete. After 5 to 10 days, epidermis and dermis could be totally and consistently separated. Histologic examination confirmed that all epidermal cells were removed [22].

After separation of the epidermis, it was also found that (1) the deepidermized dermis (DED) was dead (autoradiographs revealed no labeled uridine or amino acid uptake and cultures of dermal fragments yielded consistently negative results), and (2) the dead dermis was still covered by its lamina densa, as evidenced by electron microscopy [23]. By using specific antibodies it was found that the surface of the DED still reacted positively with antibodies against collagen IV (which confirms the presence of the lamina densa) but also with antibodies against laminin. However, the BP antigen was missing [24]. DED was placed on grids. Epidermal cells in suspension were seeded on top and cultured at the air-liquid interface in the same manner as in the Freeman et al. method. Figure 6 schematizes the novel culture system on DED. A vertical histologic section of a representative culture of human keratinocytes on human DED is shown in Fig. 7.

![Diagram](image-url)

**Fig 5.** Method of Freeman et al. [21]. See explanation in the text. Ex = skin explant with epidermis and dermis; E.O. = epidermal outgrowth; d = dermis; e = epidermis; ps = pig skin; G = stainless steel grid; TCM = tissue-culture medium; C = clot.

The recombination of epidermal cells with dermal elements has been proposed only recently. Such a recombination is interesting because it not only allows an evaluation of the effect that each component has on the biology of the other, but also permits raising of the cultured cells to the air-medium interface.

The first method of recombining epidermal and dermal elements was described by Freeman et al. in 1976 [21]. In this method, human skin explants were cultured on flaps of dead pig skin. Split-thickness flaps were removed by an electrokeratome. They were sterilized by exposure to gamma rays and kept for months or more. To initiate a culture, a square of dead pig skin is cut with scissors. The dead skin is laid down on a stainless steel grid with epidermis down, and 2 × 2 mm squares of living human skin are placed dermis down on the reticular aspect of the pig skin. The grids with the pig skin and the human explants on top are placed on petri dishes and enough culture medium is poured into the dishes to wet the grids from below.

Rapidly, epidermal cells start gliding down along the cut edges of the human skin explant, reach the surface of the pig skin dermis, and expand as circular outgrowths around the explants. After 2 to 3 weeks, individual outgrowths merge and the squares of dead pig skin are eventually covered by living cultures of epidermal cells.

Fibroblasts do not proliferate in cultures of this type, which means that subcultures of epidermal cells can be made, if so desired, by cutting explants of pig dermis with cultured cells on top and using them as in primary culture. Control cultures immersed in the medium are easily made by sticking the pig skin flaps on the bottom of the dishes with a fibrin clot (Fig. 5).

Table I compares the ultrastructural markers seen in cultures on pig skin at the air-liquid interface with those observed in cultures on plastic and immersed in tissue-culture fluid. It can
Table I shows that all but one marker (the keratin pattern) was found. In particular, not only intracellular membrane-coating granules (MCG), but also extracellular MCG material were seen. Complete hemidesmosomes were observed with intracellular pyramids and extracellular anchoring filaments. In addition, gap junctions were seen that had not been detected in plastic or in the Freeman et al. cultures.

When primary cultures of guinea pig keratinocytes (enriched in basal cells) were cultured on DED at the air-liquid interface, high-molecular-weight keratins, including a faint but definite 67 K band, were expressed. Autoradiography after labeling the cultures with $[^{35}S]$methionine confirmed that this 67 K band corresponded to actual protein synthesis by cultured cells. In addition, digestion with V8 protease yielded a peptide profile consistent with that of the 67 K keratin polypeptide (data not shown).

The reexpression of the 67 K protein was not observed in cultures on DED immersed in the medium. This result may be interpreted as evidence in favor of the action of air exposure on the synthesis of high-molecular-weight keratin.

However, in cultures made on DED, the medium has to pass (at least in part) through the dermal compartment of the system. This implies that the observed changes in cell differentiation may result not only from better oxygenation, but also from the filtering action of the dermis.

To address this point, Reichert and Jacques [25] have developed a culture system in which the dermal compartment is replaced by a cross-linked gelatin pad of known porosity. A view of this model is given in Fig. 8. The gelatin pad is stuck to the bottom of the dish by agarose blocks that are used as support. To allow epidermal cells to attach to the gelatin pad, a fibrin network is deposited on the gelatin.

The medium surrounds the agarose blocks and penetrates into the gelatin pad from underneath. It was found that under exposure to air, a reexpression of high-molecular-weight keratins could be obtained. However, this reexpression was also obtained (and even more pronounced) when the growth medium was serum-depleted independently of exposure to air. This suggests that exposure to air is not the crucial factor in the expression of keratin polypeptides in culture. On the contrary, it points to the probable impact of the filtering action of the substrate on which the keratinocytes are grown.

Another way to recombine epidermal with dermal elements has been discussed in this Symposium by E. Bell [26]. Cultures of epidermal cells on fabricated dermal equivalents, according to Bell, can be made at the air-medium interface. A schematic view of such cultures is given in Fig. 9.

**SUMMARY AND CONCLUSIONS**

The cultivation of keratinocytes at the air-liquid interface has been achieved by different methods. It is probable that exposure to air increases in some way epidermal morphogenesis and the synthesis of membrane-coating granules. However, exposure to air does not seem to influence the synthesis of keratins.

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**REFERENCES**

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Calcium-Regulated Differentiation of Normal Human Epidermal Keratinocytes in Chemically Defined Clonal Culture and Serum-Free Serial Culture

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An improved serum-free culture system has been developed for normal human epidermal keratinocytes (HK). Short-term clonal growth and differentiation studies are routinely performed in a defined medium consisting of optimized nutrient medium MCDB 153 supplemented with epidermal growth factor, insulin, hydrocortisone, ethanolamine, and phosphoethanolamine. A small amount of whole bovine pituitary extract (wBPE) is added for initiation of primary cultures, for frozen storage, and for serial culture. The need for feeder layers, conditioned medium, serum, and specialized culture surfaces has been eliminated entirely. With an optimal level of calcium ion (0.3 mM), colony-forming efficiency is about 90 percent and cellular multiplication rate is 0.96 doublings per day in the defined medium. A high-calcium concentration (1.0 mM) induces stratification and terminal differentiation, which can be quantified by counting cornified envelopes that are resistant to boiling in sodium dodecyl sulfate plus dithiothreitol. Under optimal conditions with wBPE present, cellular senescence occurs after about 40 population doublings. Scanning electron microscopy (SEM) has verified the occurrence of stratification during differentiation in the defined medium with high calcium. High-voltage electron microscopy (HVEM) after detergent extraction of human epidermal keratinocyte (HK) colonies grown in the defined medium with low and high calcium has revealed specific changes in the intermediate filament network and keratohyalin granules corresponding to changes in cellular differentiation. Indirect immunofluorescence studies have verified that the intermediate filament network observed with HVEM is composed of keratin proteins.

Reports from this laboratory have described development of an optimized basal nutrient medium for normal human epidermal keratinocytes (HK) [1] and clonal growth of second-culture HK cells in a hormone- and growth factor-supplemented defined medium [2], and improved methods for isolation, serum-free stock culture, and frozen storage of HK cells [3]. This report describes quantitative and qualitative characteristics of (1) growth and differentiation of third-culture HK cells in defined medium at clonal cell densities; (2) serial culture of HK cells in medium containing 0.1 mM Ca^++ chemically defined supplements, and whole bovine pituitary extract (wBPE); and (3) changes in surface morphology and intermediate filament organization corresponding to differentiation in vitro as mediated by calcium ion concentration in defined medium.