IN VITRO GROWTH AND MATURATION OF EPITHELIAL CELLS FROM POSTEMBRYONIC SKIN

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The in vitro growth and maturation of epithelial cells from normal human skin in organ culture, outgrowth culture, and isolated epithelial cell culture are described. In each model, the patterns of growth and maturation differ in growth rates, formation of keratohyaline granules, and keratinization. The differences and similarities in the behavior of epithelial cells in each system are described, some of the physical and chemical factors which alter these patterns are outlined, and the general advantages and limitations of each model as a research tool are discussed.

A central problem in skin biology is to identify the biochemical and physical forces which control the growth and maturation of epithelial cells. One experimental approach is to study the conditions in vitro that regulate the growth and maturation of these cells. In this study I shall review the growth patterns of epithelial cells in organ culture, outgrowth culture, and isolated cell culture, summarize the similarities and differences of their growth characteristics in each of these systems, and describe some of the factors and conditions which change these patterns.

ORGAN CULTURE

Organ culture of full-thickness skin was the first in vitro approach to the study of the growth and behavior of epidermis. In 1898 Ljunggren reported that adult human skin could be successfully grafted to an autologus host after incubation in ascitic fluid [1]. Current modifications of this technique consist of floating skin samples on the surface of a more defined nutrient medium [2], supporting the tissue in a medium by means of lens papers [3], stainless steel grids [4], and cellulose sponges [5], or submerging and holding the tissue under the nutrient medium in plasma clots [6,7]. These procedures produce only minor differences in the survival time and growth patterns of the epidermis.

General Growth Characteristics

Two patterns of epidermal growth can be distinguished in organ culture. In one, epithelial cells grow in the upward direction characteristic of the normal growth and maturation of epithelial cells in vivo. In the second, called epiboly, epithelial cells migrate from the edges of the tissue and encircle the underlying dermis with a layer of epithelial cells. Figure 1 shows the character of the epidermis after 6 days in organ culture and the process of epiboly.

In the first 48 hr in organ culture, the structure of the epidermis appears relatively normal at the light microscope level. At the ultrastructural level, however, pronounced changes in the upper epidermis include clumping of tonofilibrils, the appearance of a new granular material, and changes in the membrane-coating granules and in the granular layer [8]. At later intervals, marked changes appear in all epidermal cells [8-10]. Of particular importance is the character of the epidermis after long-term organ culture. After 3 weeks in culture, the epidermis converts to a single layer of cells attached to the dermis by a thickened basement membrane, retaining its full potential to re-form a normal epidermis after being regrafted to an autologus host [10]. These studies demonstrate that the marked changes in cell maturation induced by organ culture are modulations, not permanent somatic mutations, during a critical stage in the maturation process. These studies also demonstrate that current conditions for maintaining skin in organ culture only approximate the conditions for the normal growth and maturation of the epidermis in vivo.

Factors That Control Upward Growth and Maturation of Cells

Size of the stratum corneum. When the stratum corneum from adult human skin is removed by cellophane tape stripping in vivo, the number of mitotic figures in the basal layer increases in about 48 hr [11]. Similarly, when human skin sections are stripped with cellophane tape and maintained in organ culture, they increase their mitotic activity and the uptake of thymidine in 48 hr [12,13]. Hypotheses about this stimulation include the release of a tissue-specific mitotic inhibitor [14] and the synthesis of specific RNA and protein...
molecules controlled and mediated via the genome [15]. Since the epidermis can be separated at several time points before the burst in mitotic activity at 48 hr, this system offers an unusual opportunity to measure the biochemical changes in the epidermis and dermis that precede mitosis.

Dermis. When the epidermis is separated from the dermis by exposure to trypsin and maintained in culture, all epidermal cells degenerate and growth ceases. When it is separated and recombined with the dermis, growth and maturation resume. Some of the characteristics of connective tissue which support this growth and maturation have been reported by Briggaman and Wheeler [16,17]. These studies have shown that tendon sheath and fascia can be substituted for dermis, that the viability of dermal cells is not a growth requirement, and that the ability to induce these changes is destroyed when dermis is heated for 4 hr at 56°C.

Formation of Keratohyaline Granules

Two chemical factors may be involved in the formation of keratohyaline granules in organ culture. Hambrock, Lamberg, and Bloomberg [4] observed that the addition of glucosamine to the culture medium helped to increase the formation of a granular layer; other amino sugars such as glucosamine, n-acetylgalactosamine, or hyaluronic acid did not produce this effect. In a later study, Reaven and Cox [12] reported that an increase in pH from 7.2 to 7.6 resulted in an increased synthesis of a granular layer. How these changes in growth medium and pH promote the formation of keratohyaline granules is not known, but the synthesis may be related to nonspecific slowing in the turnover time of the epidermis in vitro. A recent observation supports this hypothesis, namely, that glucosamine added to the culture medium also inhibited the stimulation of DNA synthesis induced by stripping [13].

Control of Epiboly

When cells are maintained in a medium containing 10% serum, complete epiboly is often observed, but without serum, epiboly does not occur [18]. The serum components which appear to promote epiboly are present in the high-molecular-weight fraction remaining after dialysis; the low-molecular-weight fraction of serum is inactive.

In the epibolizing layer, no true keratinization, no keratohyaline granules, and no membrane-coating granules have been found. A new basal lamina, however, can be detected under the migrating epidermal cells by transmission electron microscopy [18].

Effects of Other Chemical and Physical Agents on Growth and Maturation

By applying the organ culture technique, Reaven and Cox studied the effects of oxygen tension, hormones, epithelia cell growth factor, and serum [12]. They observed that serum is not required for the growth and maturation of epithelial cells and that, at tensions greater than 40%, oxygen is toxic to epithelial cells.

OUTGROWTH CULTURES

When skin is attached to a suitable support (glass or plastic) by a fibrin clot, a third type of epithelial cell growth takes place. In addition to vertical migration and the epibolitic growth characteristic of organ cultures of skin described above, epithelial cells appear at the edge of the explant and grow onto the surface (Fig. 2). The morphologic description of the cells in the outgrowth was first described by Lewis, Pomerat, and Ezell [6]. Later studies defined the physical and chemical features of this growth [19] and the morphologic reorganization of the cells into a three-dimensional structure [20,21]. The behavior of the cells in the outgrowth has been used to evaluate drug toxicity [22], to study the specificity of chalone [23], and to determine the effects of allergens on sensitized and nonsensitized skin [24].

General growth characteristics. Figure 3 shows three phases in the outgrowth of cells from the explant. Phase I consists of the lag of 24 to 48 hr before the first epithelial cells appear, Phase II of a
rapid, almost linear rate of growth lasting up to 14 days, and Phase III of a plateau which sometimes continues up to 10 weeks. During Phase III, cells form an amorphous product and gradually detach from the surface. Phases I and II are strongly influenced by pH, serum concentration, oxygen tension, and the nutrient medium [19].

Factors that control Phase III. In the early stages of Phase III, if the peripheral edge of epithelial cells is wounded by removal of a part of the cells with a fine scalpel, cell repair is initiated anew at a level corresponding to Phase II. This potential for repair after Phase III is reached is illustrated in Figure 4. These findings indicate that the plateau of Phase III results from an equilibrium between the size of the cell population and the proliferative pool. The speed at which repair is instituted after wounding suggests that the cells are repressed and that wounding results in the loss of a repressor. That epithelial cells in outgrowth culture retain the ability to respond to an externally supplied epidermal chalone has been shown by Chopra and Plaxman [23].

Epithelial cell contact. In contrast to other skin cell types such as the fibroblast and the endothelial cell, epithelial cells do not appear in outgrowth cultures unless they are in contact with other epithelial cells through processes called “attachment bridges” [19]. When an epithelial cell appears to grow out of synchrony with the expanding sheath of peripheral epithelial cells, an attachment bridge is always present to maintain contact (Fig. 5). The nature of these interesting cellular projections or their function is not known, but they may be involved in maintaining electrical conductivity as well as in direct informational or nutritional exchange between epithelial cells. The putative importance of membrane junctions in epithelial cells from other tissues has been reviewed recently by Loewenstein [25].

Effect of oxygen. Of all the physical factors that affect Phases I and II of outgrowth cultures, changes in oxygen tension are particularly important. Whereas most cells in culture (e.g., skin fibroblasts) respond positively to an increase in oxygen tensions up to 100%, epithelial cells in outgrowth cultures are inhibited by tensions greater than 10% [19]. There is no experimental evidence to show how oxygen inhibits skin epithelial cells, but we do know that oxygen sometimes inhibits an early step in anaerobic glycolysis [26]. If this is so, then the integration of glycolysis and respiration differs in epidermis and in dermis.

Maturation of epithelial cells in outgrowth cultures. The ability of epithelial cells in outgrowth

![Fig. 3. Growth curve of epithelial cells in outgrowth culture. Three phases in appearance and rate of growth of cells are shown.](image-url)

![Fig. 4. Effect of wounding on stimulation of epithelial cell repair in outgrowth cultures.](image-url)

![Fig. 5. Top: Single epithelial cell showing contact with edge of outgrowth by an attachment bridge (Phase contrast micrograph, × 200). Bottom: Formation of a colony of epithelial cells in contact with edge of outgrowth culture by attachment bridge (Phase contrast micrograph, × 100).](image-url)
cultures to produce an ordered three-dimensional structure without connective tissue stroma has been reported by Flaxman, Lutzner, and Van Scott [21]. Their added ability to synthesize proteins with the staining characteristics of keratin has also been described [27]. These studies suggest that epithelial cells can mature even in the absence of direct contact with connective tissue, but this process is less ordered than when connective tissue is present.

How the dermis functions in initiating the maturation process remains unclear, but both outgrowth and isolated cell culture studies, described below, suggest that this function is permissive rather than essential. Since maturation occurs in isolated epithelial cells, it is unlikely that the dermis provides direct informational cues that initiate this process. More probably, the influence of the dermis is expressed at the basement membrane where the interaction of the surface membranes of basal cells and basement membrane plays a role in maintaining the normal proliferative cell population.

Effect of antimetabolites on outgrowth cultures. Since the outgrowth of cells can be measured directly with a calibrated microscope ocular, rate of growth can be used to determine the effectiveness of antimetabolites on growth and maturation. This technique was used to determine the antimetabolic effects of a series of known analogues of amino acids, proteins, carbohydrates, and vitamins [22]. Highly toxic compounds appeared to be analogues of guanine and uracil; those not so effective in inhibiting growth seemed to be analogues of amino acids and carbohydrates. The unusually high concentration (1000 \(\mu g/ml\)) of methotrexate required to produce an inhibition in vitro is important.

Modulation of epithelial cells in outgrowth cultures. Although tonofilaments, tonofibrils, and proteins with the staining characteristics of keratin can be demonstrated in outgrowth cultures [27,28], keratohyaline granule formation is rarely observed. The fact that the induction of Phase III in cultures and the failure to form keratohyaline granules are modulations of epithelial cell maturation has been shown by epithelial grafts of outgrowth cultures [29]. When transplanted to a suitably prepared site, outgrowth cultures complete the formation of a normal epidermis with a granular layer. The ability to graft epithelial cells after outgrowth culture and to observe the changes in the behavior of cells after grafting is a powerful research tool with which to determine what conditions induce somatic changes in epithelial cells in vitro.

ISOLATED EPITHELIAL CELL CULTURES

In 1941 Medawar reported that sheets of pure epidermal epithelium can be obtained from human skin by trypsinization of very thin split-thickness sections of human skin [30]. Further dissociation of epithelial sheets into viable composite cells was demonstrated conclusively by Billingham and Reynolds who grew isolated cells after transplantation [31]. In vitro techniques to isolate and maintain epithelial cells from postembryonic skin as isolated cell populations were later reported by Wheeler, Canby, and Cawley [32], Brigman et al [33], and by Karasek and Charlton [34].

General Growth Characteristics

After trypsin release and plating on a collagen gel, three stages in the reorganization and growth of the cell population can be defined [23]. In Stage 1, the cells settle to the surface and attach to it within 3 hr (Fig. 6). In Stage 2 (3-24 hr after plating), the cells migrate actively across the surface of the gel until a suitable receptor cell is found and a colony of several cells is gradually formed (Fig. 7). In Stage 3, the colony multiplies horizontally and vertically (Fig. 8).

Figure 9 shows the incorporation of tritiated thymidine into the epithelial cells from normal human skin at each stage of cell growth. In Stage 1 (0-3 hr), an immediate uptake of thymidine probably represents the completion of the S phase of a small percentage of cells in that stage of the growth cycle in isolation. In Stage 2 (3-24 hr), relatively little uptake of thymidine can be detected. In

Fig. 6. Pattern of attachment of epithelial cells in Stage 1. Minimal Essential Medium (Eagle) containing 10% calf serum (Phase contrast micrograph, \(\times 100\)).

Fig. 7. Pattern of epithelial cell growth in Stage 2. Numerous attachment bridges are seen (Phase contrast micrograph, \(\times 100\)).
Factors That Control the Growth of Isolated Epithelial Cells

Surface. Unlike epithelial cells in explant cultures, isolated epithelial cell populations are more sensitive to the plating surface. Whereas epithelial cells in outgrowth cultures grow equally well on either glass or plastic or a collagen gel, isolated epithelial cells attach and multiply on glass or plastic with a lower efficiency than on a collagen gel. Other cell types in skin (endothelial cells, fibroblasts) grow with equal efficiency on glass, plastic, or collagen. The possibility that trypsin alters the surface membranes of epithelial cells more destructively than those of fibroblasts and that the resynthesis of membranes occurs more rapidly on collagen may explain these differences. Alternatively, inherent differences in the chemical charge and surface qualities of glass and collagen may be accountable. Changes in the charges on the silicates in glass caused by extensive washing with strong acid or alkali also alter the plating efficiency of epithelial cells.

Since basal cells remain in direct contact with the basement membrane, changes in the net charge of acid mucopolysaccharides in this membrane would, on the basis of in vitro studies, be expected to alter the proliferative pool of epithelial cells because the acid mucopolysaccharides of connective tissue have a strong inhibitory effect on the growth of epithelial cells in vitro [35].

Connective tissue. The response of epithelial cells to the presence of connective tissue has been described by Briggaman and Wheeler [16,17] for postembryonic skin in organ culture. A growth-promoting effect of extracts of connective tissue and by medium conditioned by skin fibroblasts has also been demonstrated [36,37]. In connective tissue, one of the active components for epithelial cell growth is not soluble in organic solvents, is not adsorbed by charcoal, and is adsorbed by ion exchange resins capable of adsorbing substances with a net positive charge [37]. In addition to the small molecular factor, connective tissue also contains a higher-molecular-weight substance which inhibits epithelial cell growth. Preliminary studies suggest that this component is a protein.

Endothelial cells. Epithelial and endothelial cells respond in synchrony to both activation and inhibition of growth; these responses, which are characteristic of skin physiology, have been reviewed by Ryan [38]. A factor from epidermis that stimulates endothelial cells to divide in vivo has been described by Wolf and Harrison [39]. More recently a method to isolate endothelial cells from rabbit skin in cell culture has been described by Karasek and Charlton [40]. These studies suggest that in vitro models can be developed to study the communication between epithelial and endothelial cells and that the chemical cues responsible for these effects can be defined.

Maturation of Isolated Epithelial Cells

Epithelial cells grown on either glass or on collagen gels retain the capacity for partial maturation in vitro [34]. The agents which inhibit keratinization in vivo inhibit maturation of epithelial cells in vitro. The addition of retinal acetate in dimethyl sulfoxide to isolated epithelial cell cultures inhibits the formation of desmosomes and tonofilaments [41].

DISCUSSION

Over the past decades the data from many
Some degree of maturation takes place in outgrowth cultures and isolated cell cultures, but this capability is most clearly expressed in organ culture where keratohyaline granules and a stratum corneum are formed. In this system, basal cells remain in a partially repressed state and their growth rates approximate those in vivo more closely. Moreover, the tissue can be manipulated to produce an increase in growth rates.

In the early periods of organ culture, epithelial cells appear to be normal; at the ultrastructural level, changes in cell structure are more clearly evident. At later time intervals, more pronounced changes in maturation are observed. Since direct microscopic observation is not possible and both maturation and growth vary, larger numbers of specimens must be studied than in other methods; as a result, quantification of growth and maturation is more difficult.

In outgrowth culture, maturation is more disorganized and keratohyaline granule formation is infrequently observed. Since either full-thickness or split-thickness skin must be used as a source of epithelial cells, other cells from connective tissue frequently accompany the growth of epithelial cells and their contribution to both growth rates and maturation must be evaluated. Variations may also take place in the initial appearance and rate of outgrowth of cells. Since outgrowth can be directly observed and measured, these variations can be detected quickly. Cell morphology and rate of growth can also be documented by direct microscopic observation.

Of all the in vitro techniques, isolated cell culture produces the most disorganized maturation of cells. Formation of keratohyaline granules is not observed. Isolated epithelial cells are more demanding in their growth conditions and collagen gels must be used to achieve a high plating efficiency. Unlike organ culture and outgrowth culture, isolated populations of epithelial cells are studied independently of other components of the skin. Major contaminations with skin fibroblasts or endothelial cells, though infrequent, are present. Since large populations of cells can be isolated, biologic variations between individual samples are observed less frequently. As with outgrowth culture, however, direct microscopic observation is possible at all times.

A limited growth period is common to all in vitro procedures for epithelial cells from postembryonic human skin. Subculturing cells in outgrowth culture and isolated epithelial cell culture is not yet possible; hence reports of a method to establish permanent epithelial cell lines from human skin must be read with caution [42,43].

REFERENCES


