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Growth of cultured human epidermal cells into multiple epithelia suitable for grafting

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ABSTRACT **Owing to several recent developments, the cultivability of epidermal keratinocytes, particularly those of the human, has been greatly improved. Under the conditions used, single cultured cells generate stratified colonies that ultimately fuse and form an epithelium that is a reasonable approximation of the epidermis. It will be shown here that large amounts of cultured epithelium can be generated from a small piece of epidermis in a short time. We wish to bring to the attention of surgeons and cell biologists the possibility of using culture-grown epithelium derived from the same individual to restore defects in the epidermis.**

General description of behavior of cultured human epidermal cells

There have been many studies of the epidermis in culture (for review of early work, see ref. 1). Most studies were carried out on explanted fragments, a form of cultivation in which multiplication is usually limited. Although there were difficulties in expanding an epidermal cell population in this way and there was no effective means of controlling fibroblast proliferation, such cultures were shown to be transplantable onto animals by Medawar (2) and Karasek (3), and hopes were expressed very early for application to human transplantation (4, 5). Methods of expanding the size of an epidermal cell population by growth from tissue fragments have since been improved by Freeman *et al.* (6, 7); the proliferated epithelium remained transplantable (8).

When the superiority of disaggregated cell culture for obtaining increased proliferation of other cell types was recognized, attempts were made to grow epidermal cells in this way by Pruniéras *et al.* (9), Briggaman *et al.* (10), Yuspa *et al.* (11), Karasek and Charlton (12), Fusenig and Worst (13), and Marcelo *et al.* (14). Some multiplication took place in such cultures, but the cells generally were not serially cultivable, either because of overgrowth of fibroblasts or possibly because the epidermal cells when pure were deprived of fibroblast support. In such systems the inoculation density was high, and separated single cells were probably not able to give rise to colonies efficiently.

In studies of a keratinocyte line of teratomal origin by Rheinwald and Green (15), it was discovered that the cells could be cultivated serially from small inocula by supporting them with cocultivated lethally irradiated 3T3 cells or other fibroblasts. It was then found (16) that human epidermal cells could be serially cultivated under the same conditions. The following points were established.

(*i*) By use of supporting 3T3 cells, epidermal cells of newborn infants can be serially transferred through numerous subcultures.

(*ii*) Each colony initiated by a single cell forms a stratified squamous epithelium. The basal cells (those attached to the surface of the culture vessel) resemble basal cells of the epidermis in that they account for all cell multiplication, whereas the cells that leave the basal layer become terminally differentiated.

(*iii*) Living fibroblasts, which were present in the original tissue and whose multiplication in culture was formerly uncontrollable, can be controlled by the inhibitory influence of the 3T3 cells.

(*iv*) As revealed by electron microscopy, the cultured epidermal cells have the principal cytologic features of keratinocytes.

(*v*) The cells retain the diploid chromosome number, and no established lines develop.

Subsequent studies by Sun and Green (17) and Rice and Green (18) showed that the differentiated behavior of the cultured human epidermal cells imitates to a remarkable degree that of the epidermis. Ultimately, the epidermal colonies formed from single cells grow together to make a confluent multilayered epithelium in which colonial borders disappear. The cells in the superficial layers enlarge, and when they reach the most superficial layer of the epithelium they acquire crosslinked protein envelopes. They later detach as squames and destroy their nuclei (19) as do the cells of stratum corneum, although in the reverse order.

Although cultured epidermal cells contain abundant keratins (20), not all aspects of keratin synthesis are carried out exactly as in the epidermis; the molecular sizes of the keratins made by the cultured cells are similar but not identical to those made in the intact epidermis (21–23). These differences need not be irreversible; they are more likely the result of modulating effects of the local environment.

No evidence was obtained for the development of an abnormal proliferative behavior suggesting loss of growth control. Disaggregated cells obtained from cultures have been injected subcutaneously into athymic mice. Inocula of 10^7 cells grew to nodules about 0.5 cm in diameter within about 10 days and then regressed. The nodules had the histological appearance of benign keratinizing cysts, quite similar to epidermal inclusion cysts.

Recent improvements in cultivation

The first improvement in the culture method after 1975 was the addition to the medium of the epidermal growth factor (EGF) of Cohen *et al.* (24, 25). This polypeptide increases the rate of colony expansion, an effect at least partly related to cell mobility (26). As the colonies grow larger, the cells retain a higher growth rate in the presence of EGF than in its absence. Even after the cells become confluent, the cultured epithelium supports a higher rate of squame detachment from its surface (19). Cells growing in the presence of EGF retain a higher colony forming efficiency on retransfer and have a much longer culture life-time (26).

The second improvement in the cultivation was the addition to the medium of agents known to increase cellular cyclic AMP (cAMP) (27, 28). From most earlier studies on the relation

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Abbreviations: EGF, epidermal growth factor; *cAMP*, *cyclic* AMP.

Table 1. Primary cultures of human epidermal cells

Strain	Age of donor	Time elapsed before cultivation, hr	Plating efficiency, %	Time to confluence, days: For 10^4 cells	Time to confluence, days: For 10^5 cells
AA	Newborn	2	—	—	15
AB	Newborn	4	3.4	14	11
AC	Newborn	3.5	4.4	19	15
AK	Newborn	1.5	2.1	—	14
AE	45 yr	18	1.5	—	15
AI	49 yr	2	0.9	21	17
AD	70 yr	48	—	—	19
AJ	84 yr	5	0.6	—	20

Half the cultures were derived from foreskin of newborns. Cells of older donors were derived from skin over the knee or thigh, removed in the course of scar revision for orthopedic surgery on patients with chronic arthritis, and provided through the courtesy of Robert Poss, to whom we are greatly indebted. All cultures were grown in the presence of cholera toxin (10^{-10} M), beginning at the time of inoculation, and EGF (10 ng/ml) beginning 3–5 days after inoculation.

of cAMP to growth control (29), these agents might not be expected to increase multiplication; but, as in the case of some other cell types (30–34), these agents do greatly improve the growth of cultured human epidermal cells. The most effective agents were cholera toxin—which is not toxic even at very high concentrations (10^{-8} M) and is maximally effective in promoting multiplication at 10^{-11}–10^{-10} M nM—and the β agonist isoproterenol—which is effective at 10^{-6} M (27). These two agents are known to increase the activity of adenyl cyclase by different means (35, 36). Cholera toxin is also effective on disaggregated rodent epidermal cells and increases their cAMP level (28). In the presence of the toxin, the ratio of small proliferating cells to large terminally differentiating cells is increased (27).

Formation of primary culture epithelium under optimized conditions

Using 3T3 support and including in the medium cholera toxin at 10^{-10} M (beginning at the time of inoculation) and EGF at 10 ng/ml (beginning several days later), we reexamined the behavior of primary cultures derived from skin of donors of various ages. Trypsin-disaggregated cells (10^3, 10^4, and 10^5) were inoculated into 50-mm tissue culture petri dishes together with or after 4×10^5 lethally irradiated 3T3 cells, and cultivated as described (15, 16, 26, 27). Keratinocyte colonies appeared within a few days and, as they expanded, they displaced the 3T3 cells from the surface in typical fashion (16). The time required for the colonies to become confluent in cultures inoculated with 10^4 or 10^5 cells is shown in Table 1. The colony-forming efficiency was derived from the cultures inoculated with 10^3 cells; these cultures were fixed and stained with rhodanile blue (15) when the colonies were still discrete.

Most cultures inoculated with 10^5 cells reached confluence in 14–17 days. Cultures inoculated with 10^4 cells required an additional 4 days to become confluent. The colony-forming efficiency ranged from slightly less than 1% for older donors to as high as 4% for newborns. Particularly for older donors, the values obtained for primary cultures are much higher than those obtained in earlier experiments without the use of the toxin (16).

The colony-forming efficiency is an average for all cells obtained from the trypsinized skin. These values do not tell us the colony-forming efficiency of the multiplying subpopulation of epidermis, the basal cells. Although excess subcutaneous tissue was trimmed from the skin before enzymatic disaggregation, half the cells obtained could easily be connective tissue cells. Of the epidermal cells, probably more than half are terminally differentiating and cannot start colonies. The colony-forming efficiency of the basal cells in the skin might easily be >4-fold higher than the overall values listed in Table 1.

This line of reasoning is consistent with the much higher colony-forming efficiency of the cells of subconfluent primary and secondary cultures when they are trypsinized and transferred (Table 2). Whether derived from old or young donors, these cells formed new colonies with an efficiency of 6 to nearly 30%. If there was any effect of donor age, it is not clearly evident.

Table 2. Plating efficiency of human epidermal cells of expanding (subconfluent) cultures

Age of donor	Strain	Plating efficiency (%) of cells from: Primary culture	Plating efficiency (%) of cells from: Secondary culture
Newborn	AB	28.5	—
Newborn	AK	11.7	—
34 yr	CS-1	—	16.9 (clone)
49 yr	AI	6.7	—
70 yr	AD	28	23
84 yr	AJ	9.6	—

Most strains are those described in Table 1. CS-1 originated from abdominal skin taken for revision of a scar during Caesarian section. In early studies of its growth in the absence of EGF and cholera toxin, a secondary culture of CS-1 plated with an efficiency of 0.3% (16). Part of the original specimen preserved frozen for several years was thawed and grown under present conditions. A clone was isolated from the primary culture and expanded in secondary culture, and its plating efficiency was measured in tertiary culture. Note the high value obtained.

Logistics of large-scale cultivation

Some estimates of the amounts of culture epithelium that can be grown in primary cultures and the time required are shown in Table 3. By starting with 1 cm^2 of newborn skin and using

Table 3. Logistics of large-scale primary epidermal cultivation*

Size of biopsy: 1 cm^2
Number of cells obtained: 3×10^6
Number of primary cultures inoculated with 10^4 cells: 300
Time to reach confluence: 14–19 days
Area per culture: 20 cm^2
Total epithelium harvested†: $20 \times 300 = 6000$ cm^2 (increase, 6000-fold)
Total number of cells harvested: $5 \times 10^6 \times 300 = 1.5 \times 10^9$ (increase, 500-fold)

* Based on epidermal cells of newborns. Because primary plating efficiency of cells of elderly donors may be lower by as much as 5-fold, their primary cultures should be inoculated with 5×10^4 cells, and the total yield would be correspondingly decreased.

† Uncorrected for shrinkage after detachment of the epithelium.

Table 4. Retention of colony-forming ability by cells of confluent cultured epithelia detached with Dispase

Cell strain	Inoculum size, no.	Serial transfer of cells forming epithelium tested	Time at confluence, days	Subsequent colony-forming efficiency, %	
				Detached with Dispase, then disaggregated	Disaggregated directly
AN*	10^5	Primary	1	15	23
AN*	10^5	Primary	4	23	18
AO*	10^5	Primary	1	30	33
AQ*	10^5	Primary	2	27	25
AB*	10^4	Tertiary	5	11	13
AD	10^4	Secondary	1	4.0	5.1
AD	10^4	Secondary	4	4.3	1.9
AJ	10^4	Secondary	1	0.8	0.9
AJ	10^4	Secondary	3	0.8	0.4

* Derived from skin of newborns.

an inoculation density of 10^4 cells, the area of culture epithelium can be expanded to 0.6 m^2 in 14–21 days. The increase in area is about 6000-fold. Because the epithelium shrinks after its detachment (see below) to about one-fourth its area while attached, the overall expansion of epithelium might be reckoned as 1500-fold. The growth may also be evaluated from the number of cells produced; because each confluent 50-mm primary culture contains about 5×10^6 cells, the overall increase of cell number would be about 500-fold.

The possible yield of epithelium from a secondary subculture may be estimated from the following example. If 30 primary cultures were initiated with 10^5 cells each and allowed to grow to 10^6 cells each, the yield would be 3×10^7; this requires about 12 days. The cultures would be subconfluent, still growing fairly rapidly, and able to form colonies on transfer with high efficiency (Table 2); 2×10^4 cells could then be transferred into each of 1500 cultures. These cultures should be confluent in about 10–12 days, yielding 3 m^2 of epithelium. Of course, the inoculation density, the time allowed, and the yield can be varied according to requirements. In subconfluent secondary cultures, there seems to be little difference in performance between the cells of newborns and of older donors.

Transfer of intact sheets of culture-grown epithelium

It is known from earlier studies by Billingham and Reynolds (37) and Yuspa *et al.* (11) that disaggregated epidermal cells obtained directly from the epidermis or from briefly cultured cells can be applied to a graft bed and can reconstitute an epidermis. This is probably not the most efficient method of grafting cultured cells. Because the stratification in the cultures is such that the multiplying cells lie on the surface of the dish, it would be desirable to retain this polarity in the graft by applying the intact culture epithelium, rather than disaggregated cells, of which a large fraction would be incapable of multiplication. A method of doing this by growing cultures on transplantable collagen surfaces has been described by Worst *et al.* (38). We have examined several possible methods of detaching confluent epidermal sheets from the petri-dish surface without dissociating the cells. After numerous failures, an effective method was discovered.

The neutral protease Dispase, first described by Irie,* was used to disperse monolayer cultures of various cell types by Matsumura *et al.* (39, 40) and is available commercially (Boehringer Mannheim). It was noted in the earlier studies that epithelial cells were poorly dissociated from each other by the enzyme.

*Y. Irie, U.S. Patent 3,930,954.

Confluent epithelia were treated with Dispase II (the less-purified form) at 1.2 units/ml in serum-free medium, the volume being sufficient to permit access of the enzyme to the free edge of the epithelium which, by 2 weeks after inoculation, extended partway up the side wall of the petri dish. After about 30 min at 37°C, the process of detachment began at the free edge and moved downward and then centrally; it usually was complete in about 1 hr. The epithelium separated from the plastic surface as a disc with a slightly curled edge derived from the cells that had grown on the wall of the dish. The epithelium was very elastic and contracted to as little as 2 cm in diameter, becoming thicker in the process (Fig. 1) and acquiring a puckered appearance under low-power magnification. Very few epidermal cells detached from the epithelium, and none were left on the dish surface; but if any human fibroblasts were present, some could be detected after detachment of the epithelium because they remained attached to the surface of the dish. The detached epithelium could be picked up with a forceps and transferred to another vessel, and its polarity could be determined at any time by the orientation of the curled edge.

In order to determine the viability of the epidermal cells after this treatment, detached epithelia were washed free of Dispase

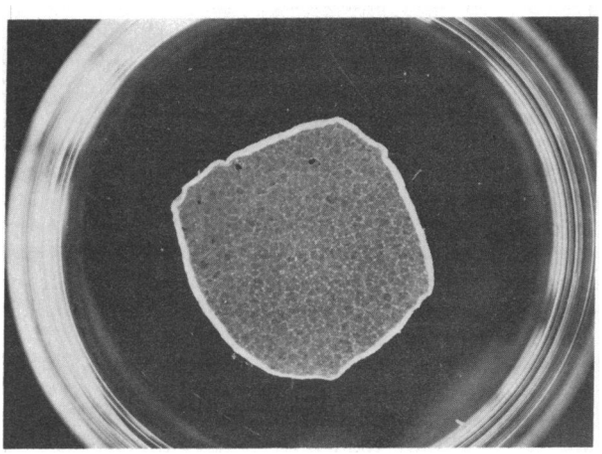

FIG. 1. Cultured epithelium after detachment with Dispase. A confluent culture of epidermal cells in a 50-mm dish was treated with Dispase in the usual way. The epithelium detached completely from the surface of the dish and contracted typically, retaining the curled edge of cells formerly on the wall of the dish. This epithelium was derived from the progeny of a single epidermal cell and is therefore a clone, at this stage in its fifth serial transfer. The epithelia formed from primary and secondary cultures were similar although usually a little thicker.

and disaggregated with trypsin and EDTA. The colony-forming efficiency of the cells was then compared with that of the cells of a duplicate epithelium disaggregated directly with trypsin and EDTA. Table 4 shows that the Dispase treatment had virtually no effect on the viability of the epidermal cells. This result is consistent with the absence of deleterious effects of Dispase on other cell types (39). The epidermal cells of newborns preserved high colony-forming efficiency even after several days in the confluent state, whereas that of cells from elderly donors (AD and AJ) decreased sharply in comparison with their behavior in expanding cultures (Table 2).

Some additional considerations

The experiments described here were carried out on cells derived from very few epidermal sites, and the possibility of site-dependent differences in cellular behavior must be borne in mind. In these experiments, no effort was made to eliminate human fibroblasts other than by the inhibitory effect of the irradiated 3T3 cells. Because EGF stimulates growth of fibroblasts, some are seen in the cultures; as the epidermal colonies reach confluence, the fibroblasts are mainly squeezed into cuffs between the colonies, but they may occupy space between the epidermal cells and the surface of the dish. They did not seem to interfere with the ability of the confluent epidermal cells to make a tissue-like structure that could be transferred as a unit.

Surprisingly, the fibroblasts were less numerous in cultures made from older donors; in the case of the oldest, either no fibroblasts (AD) or very few fibroblasts (AJ) were observed. It is known that fibroblasts of older persons grow more poorly in culture and have a shorter culture life-time (41). Under the culture conditions used here, the dermal fibroblasts of older donors evidently are less efficient at forming colonies than are those of young donors.

The use of cholera toxin increases the growth rate of the cultures and improves colony-forming efficiency, an effect that is marked in all cultures (27) but especially in cultures of cells derived from older donors. Use of the toxin would have another advantage: because the activation of adenyl cyclase by the toxin is irreversible (35), multiplication of the epidermal cells is increased for a period after the toxin is removed (27). Even if a graft is washed to remove all free toxin, cell multiplication should continue to be stimulated in the epithelium for some time after it is grafted. If, for some reason, this should prove disadvantageous, isoproterenol could be used in place of the toxin nearly as effectively and its action should quickly terminate when the epithelium is removed from the culture (27).

In preliminary experiments, the application of human epithelium cultured as described above to graft beds prepared in athymic mice was followed by an initial take of the graft in nearly all cases (S. Banks-Schlegel, personal communication); but it is not yet clear how well such grafts will carry out the functions of normal epidermis. The cultures are not likely to contain the minority cell types of epidermis. For example, there are probably no Langerhans cells, although these cells seem unnecessary for keratinocyte growth and function (42). The cultured epithelium is not likely to contain pigment cells or Merkel cells or to re-form hair follicles or sweat glands. Pure epidermal grafts are thought to be less durable than those containing dermis (37). In spite of these shortcomings, the large amount of autologous epithelium that could be made available suggests applications to the therapy of burns, for which large amounts of epidermis may be required. These considerations might be applied to other stratified squamous epithelia such as corneal, conjunctival, oral, and pharyngeal because the cells of these epithelia behave similarly to epidermal cells in cultivation (21, 27).

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