Serial Cultivation of Strains of Human Epidermal Keratinocytes: the Formation of Keratinizing Colonies from Single Cells

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Summary

Human diploid epithelial cells have been successfully grown in serial culture. To initiate colony formation, they require the presence of fibroblasts, but proliferation of fibroblasts must be controlled so that the epidermal cell population is not overgrown. Both conditions can be achieved by the use of lethally irradiated 3T3 cells at the correct density. When trypsinized human skin cells are plated together with the 3T3 cells, the growth of the human fibroblasts is largely suppressed, but the epidermal cells grow from single cells into colonies. Each colony consists of keratinocytes ultimately forming a stratified squamous epithelium in which the dividing cells are confined to the lowest layer(s). Hydrocortisone is added to the medium, since in secondary and subsequent subcultures it makes the colony morphology more orderly and distinctive, and maintains proliferation at a slightly greater rate. Under these culture conditions, it is possible to isolate keratinocyte clones free of viable fibroblasts.

Like human diploid fibroblasts, human diploid keratinocytes appear to have a finite culture lifetime. For 7 strains studied, the culture lifetime ranged from 20–50 cell generations. The plating efficiency of the epidermal cells taken directly from skin was usually 0.1–1.0%. On subsequent transfer of the cultures initiated from newborns, the plating efficiency rose to 10% or higher, but was most often in the range of 1–5% and dropped sharply toward the end of their culture life. The plating efficiency and culture lifetime were lower for keratinocytes of older persons.

Introduction

Many mammalian cell types continue to resist attempts at serial cultivation. Fibroblasts taken from tissues can be routinely cultivated either through many cell generations as unchanged diploid cells (Hayflick and Moorhead, 1961) or indefinitely (in the case of rodents) as established lines (Rothfels, Kupelwieser, and Parker, 1963; Todaro and Green, 1965). For other cell types it is much more difficult to develop culture lines, and the majority have originated from tumors (Sato and Yasumura, 1966). For the most part, we are ignorant of the factors that permit any cell type to be serially propagated.

An accompanying paper (Rheinwald and Green, 1975) describes the establishment of a keratinocyte line (XB) derived from a mouse teratoma. Under the special conditions developed for its cultivation (the presence of 3T3 cells at the correct density), it could be propagated indefinitely, maintaining for at least a very long time its ability to express differentiated function. Since, as far as we knew, neither the necessary culture conditions nor the existence of a keratinizing cell line had been reported previously, we decided to study their relevance to the problem of the cultivation of normal human epidermal cells.

In addition to studies of epidermal growth in organ or explant culture (Feil, 1964; Prose, Friedman-Kien, and Neisten, 1967; Flaxman, Lutzner, and Van Scott, 1967), there have been numerous attempts to cultivate disaggregated epidermal keratinocytes in monolayers (Briggaman et al., 1967; Karasek and Charlton, 1971; Fusenig, 1971; Fusenig and Worst, 1974; Yuspa et al., 1970). In general, these studies have shown that disaggregated epidermal cells do grow in monolayers, but to a very limited extent, and have not been satisfactorily subcultured. It is well known that epidermal cells depend for their maintenance and growth upon the presence of fibroblasts or their products (McLoughlin, 1961; Dodson, 1963; Wessells, 1963, 1964; Moscona, 1964; Melbye and Karasek, 1973). The experiments described here show that the limitations observed previously in the cultivation of epidermal cells in surface cultures are not intrinsic, but are due to the complex relation of the epidermal cells to fibroblasts. When these relations are optimized, human epidermal keratinocytes can grow and differentiate very well.

Results

Skin biopsies were obtained from humans of different ages. Disaggregated cell suspensions (see Experimental Procedures) were inoculated together with approximately $3 \times 10^4$ lethally irradiated 3T3 cells (1/layer). The 3T3 cells quickly formed a monolayer on the surface of the dish, but the epidermal cells often required several days to attach. As in the case of the keratinocyte line derived from a teratoma (Rheinwald and Green, 1975), the human epidermal keratinocytes eventually make contact with the surface of the dish and grow as expanding colonies on the vessel surface, pushing away the 3T3 feeders at the periphery. Figure 1a shows a group of fixed and stained colonies resulting from single cells. Keratinocyte colonies are stained red by Rhodanil blue, while the background of the 3T3 monolayer is stained blue.
Among the disaggregated cells obtained from full thickness human skin are many fibroblasts. As noted earlier (Green and Todaro, 1967; Rheinwald and Green, 1975), the growth of fibroblasts is much suppressed by an $\frac{n}{3}$ 3T3 monolayer. Curiously, human fibroblasts are more effectively suppressed than mouse fibroblasts. A measure of the suppression was obtained by comparing cultures inoculated with fibroblasts together with $\frac{n}{3}$ and $\frac{n}{30}$ 3T3 cells. Figure 2 (right) shows dense colonies of human diploid fibroblasts in an 11 day culture containing $\frac{n}{30}$ 3T3. The same inoculum combined with $\frac{n}{3}$ gave no well defined colonies (Figure 2, left). Viable fibroblasts were still present in such a culture and could easily be detected by replating with $\frac{n}{30}$ 3T3, but their growth was obviously much suppressed by the $\frac{n}{3}$ layer. In this way, human epidermal cells could be serially cultivated in most cases without being overgrown, and could be obtained as pure clones uncontaminated with viable fibroblasts.

The growth of the epidermal cells and the appearance of the colonies resemble those of the established teratomal keratinocyte line XB, but there are a number of differences which we describe briefly below.

The Colonies

Round cells could be seen on top of the 3T3 layer a few days after inoculation of the mixture of 3T3 and human epidermal cells. These may have been the epidermal cells, but keratinocyte colonies could be definitely identified only later, after the cells made contact with the dish surface and adopted a typical epithelial pattern. This was easily seen as soon as 4 days after inoculation, when the colonies were quite small. In contrast to the teratomal keratinocyte line, the human epidermal keratinocytes, even at this early stage, were in close contact with each other and began to stratify. As the colonies grew laterally, the centers thickened and the cell boundaries became difficult to discern. The centers eventually acquired a cracked appearance. All human epidermal keratinocyte colonies stained red with Rhodamine B even when they were very small, whereas the teratomal keratinocyte colonies usually stratified and stained red only when the colonies were large.

Figures 3b–d show the appearance of vertical sections through the colonies after staining with hematoxylin and eosin. The stratified squamous epithelium is more regularly organized than that produced by the XB line (Rheinwald and Green, 1975). The surface of the colony is more uniform and there are no round cells. Electron micrographs of similar sections through the colonies confirm the stratified construction, the keratinization (most advanced in the upper cell layers), and the abundant desmosomes in all layers (Figures 4–6). The appearance resembles that of epidermis, and all the cells belong to the same type, keratinocytes. Though they are flattened, the cells closest to the petri dish surface correspond most closely to the germinative cells in normal epidermis, for cell division in stratified colonies takes place in the deeper layers. Colonies containing over 1000 cells were labeled for 1 day with tritiated thymidine and covered with photographic emulsion. Radioautography showed that the nuclei close to the perimeter of the colonies produced abundant grains, but those in the interior of the colonies showed very faint grain density or none at all. When the same cultures were labeled with C$^{14}$-thymidine, many nuclei in the internal regions of the colonies were labeled. These nuclei were presumably so deep within the colony that $\beta$ particles emitted from tritium could not reach the emulsion; but even after exposure to C$^{14}$-thymidine, unlabeled nuclei could be seen in large flattened cells with thickened cell membranes in the superficial layers of the thicker colonies. These nuclei appeared to belong to cells at a more advanced stage of differentiation. Radioautographs of cross sections of large colonies labeled with tritiated thymidine showed that in areas where stratification had occurred, no labeled nuclei were present in the upper layers.

The Effect of Hydrocortisone

Hydrocortisone has sometimes been used to improve growth in 3T3 cultures (Armelin, 1973; Gospodarowicz, 1974), although the reason for its effect remains obscure. The growth of the keratinocyte line XB was substantially enhanced by the ad-
dition of hydrocortisone at 0.1 µg/ml when the cells were growing in medium previously conditioned by 3T3 cells, but not when the 3T3 cells were present in the culture. In the presence of a hydrocortisone concentration greater than 0.1 µg/ml, XB colonies did not stratify and were not stained by Rhodamine B.

In primary culture, human epidermal keratinocytes formed colonies of the same appearance whether hydrocortisone was added or not. In subsequent cultures, they formed colonies in the absence of added hydrocortisone, but the cells did not have a regular epithelial appearance. The addition to the medium of hydrocortisone (0.4 µg/ml) restored the regular stratified epithelial appearance, and increased lateral expansion of the colonies (Figure 3a). The rate of cell proliferation was increased, since 2 week cultures usually showed a 2-3 fold increase in cell yield when hydrocortisone was added to the medium. The difference in appearance of the cultures as shown in Figure 3a is partly due to increased proliferation and partly to increased lateral expansion of the colonies.

Hydrocortisone has been reported to hasten keratinization in skin explants (Weissman and Fell, 1962). In intact animals it is thought to suppress epidermal growth (for references, see Jarrett, 1973); this effect is observed on cultured human keratinocytes at concentrations greater than 10 µg/ml. At 0.4 µg/ml, the improvement in growth and colony morphology of the human keratinocytes is sufficient to warrant its inclusion in the culture medium.

Plating Efficiency

When XB teratoma keratinocytes were allowed to grow into stratified colonies, a large fraction could reinitiate colony formation, for the plating efficiency of the cells after trypsin disaggregation was about 40%. The plating efficiency of human keratinocytes was variable but always considerably lower. Table 1 shows that the number of keratinocyte colonies produced by primary disaggregated skin cells was usually 0.1-1.0%. Since most epidermal cells in skin are probably not capable of division and the biopsies also contain dermal cells, this seems a reasonable value. Yet even on subculture, the plating efficiency was only occasionally as high as 10% and was usually 1-5%, even for newborn donors. Toward the end of their culture life, the epidermal keratinocytes of both newborn and older donors plated with an efficiency considerably below 1%.

It is clear that in the early passages the plating efficiency of human keratinocytes is much lower than can be explained by loss of ability to initiate DNA synthesis during the preceding culture. As noted above, examination of colonies by radioautography following exposure to C14-labeled thymidine showed that a large fraction of the cells synthesized DNA during a period of 1 day (>30%). Evidently many more cells divide in a growing colony than can initiate a new colony. Plating efficiency is affected by different batches of fetal calf serum and may be subject to improvement. Increasing the plating efficiency will have a great influence on the actual culture yields (expansion of keratinocyte population) (Table 1), but little influence on the number of cell generations through which the keratinocytes grow (see below).

Growth Rates

The average doubling time of the XB line of teratomal keratinocytes was about 19 hr. Human epidermal keratinocytes in primary or subsequent culture had a doubling time of about 32 hr over the period from the time of inoculation to the development of colonies containing an average of about 1000 cells. Since this includes their period of attachment, it is probable that the doubling time in the exponential phase is appreciably shorter. The growth rate of the keratinocytes did not seem to change much on serial subculture until close to the end of their culture life, when it declined sharply.

Growth Potential of the Epidermal Keratinocytes

Human diploid fibroblasts have a limited growth potential in serial culture (Hayflick and Moorhead, 1961). The number of generations may be as much as doubled by improving the culture conditions (Todaro and Green, 1964), but the cells always die out eventually. The number of generations is inversely related to the age of the donor (Hayflick, 1965). Finite growth potential in animal tissues has since been found to be a property of other cell types as well (Daniel et al., 1975). The keratinocyte strains initiated from humans at different ages from birth to 34 years also showed finite culture lifetimes; all grew for at least 2 transfers, but none grew through more than 6 (Table 1).

The number of cell generations could not be obtained from the dilution at each transfer since the plating efficiency was low. The number of colonies initiated at each transfer was estimated from plates inoculated with 10^3 to 10^5 cells (Table 1, column II). The final yield of cells from any plate (Table 1, column IV) was then related to the number of colony-forming cells. This gave the number of cell generations grown in each subculture.

These values and the cumulative totals are shown in Table 1. It can be seen that of 7 cultures initiated from biopsy, the number of cell generations grown was from 20 to 50. These values are probably on the low side. After stratification of the colonies, cells
<table>
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<th>Strain</th>
<th>Age of Donor</th>
<th>Date Plated</th>
<th>Passage Number</th>
<th>Inoculum (Cells)</th>
<th>Colonies per 100 Cells Inoculated</th>
<th>Keratinocyte Yield</th>
<th>Number of Generations</th>
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<tbody>
<tr>
<td>HFE</td>
<td>n</td>
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<td>1</td>
<td>$10^3$</td>
<td>0.7</td>
<td>10^3</td>
<td>700</td>
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<tr>
<td></td>
<td></td>
<td>9/24/74</td>
<td>2</td>
<td>$5 \times 10^4$</td>
<td>5</td>
<td>$3.8 \times 10^4$</td>
<td>2500</td>
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<td></td>
<td></td>
<td>10/04/74</td>
<td>3</td>
<td>$10^5$</td>
<td>2</td>
<td>$2.0 \times 10^5$</td>
<td>2000</td>
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<td></td>
<td></td>
<td>10/15/74</td>
<td>4</td>
<td>$10^6$</td>
<td>0.3</td>
<td>$2.0 \times 10^4$</td>
<td>300</td>
</tr>
</tbody>
</table>

*Expansion of keratinocyte population in culture: 1.5 x 10^4 fold.*

| HFE (f) | n            | 1/09/75     | 1              | $3 \times 10^4$ | 0.8                                | 0.04               | $2.6 \times 10^4$  | 240                | 10.3               |
|         |              | 1/24/75     | 2              | $10^6$          | 2.5                                | 0.7                | $1.4 \times 10^6$  | 2500               | 10.7               |
|         |              | 2/07/75     | 3              | $5 \times 10^6$ | 1.8                                | >10                | $2.6 \times 10^6$  | 8000               | 27.6               |

*Expansion of keratinocyte population in culture: 626 fold.*

| A      | n            | 2/11/75     | 1              | $5 \times 10^6$ | 0.9                                | 0.8                | $3.8 \times 10^6$  | 4500               | 10.3               |
|        |              | 2/24/75     | 2              | $3 \times 10^6$ | 2.3                                | 1.0                | $2.5 \times 10^6$  | 6900               | 10.7               |
|        |              | 3/06/75     | 3              | $10^6$          | 0.6                                | 1.6                | $7.0 \times 10^6$  | 500                | 10.7               |
|        |              | 3/21/75     | 4              | $10^6$          | 0.14                               | >10                | 287                | <9                 | <38

*Expansion of keratinocyte population in culture: 442 fold.*

| B      | n            | 2/11/75     | 1              | $5 \times 10^6$ | 2.8                                | 1.8                | $2.7 \times 10^6$  | 14000              | 8.3               |
|        |              | 2/24/75     | 2              | $2 \times 10^6$ | 1.7                                | 6.5                | $8.4 \times 10^6$  | 3000               | 10.7               |
|        |              | 3/06/75     | 3              | $10^6$          | 0.2                                | 4.7                | $10^6$            | 200                | 10.7               |
|        |              | 3/21/75     | 4              | $10^6$          | 0.02                               | >10                | 28                 | b                  | b

*Expansion of keratinocyte population in culture: 23 fold.*

| C      | n            | 4/04/75     | 1              | $10^6$          | 0.04                               | 0.2                | $2.7 \times 10^6$  | 40                 | 12.5               |
|        |              | 4/24/75     | 2              | $10^6$          | 3.6                                | 0.27               | $1.3 \times 10^6$  | 3600               | 8.5               |
|        |              | 5/08/75     | 3              | $2 \times 10^6$ | 7.5                                | 0.2                | $2.5 \times 10^6$  | 15000              | 7.5               |
|        |              | 5/21/75     | 4              | $3 \times 10^6$ | 0.7                                | 0.1                | $2.6 \times 10^6$  | 2100               | 7.5               |
|        |              | 6/05/75     | 5              | $1.1 \times 10^6$ | 0.5                             | 1.2                | $3.7 \times 10^6$  | 550                | 9.5               |
|        |              | 6/17/75     | 6              | $2 \times 10^6$ | 0.5                                | <0.1               | 1100               | 6                  | 51

*Expansion of keratinocyte population in culture: 1500 fold.*

| E (f)  | n            | 4/24/75     | 1              | $10^6$          | 0.15                               | 0.12               | $1.4 \times 10^6$  | 150                | 10.3               |
|        |              | 5/08/75     | 2              | $10^6$          | 8.2                                | 0.1                | $6.0 \times 10^6$  | 8200               | 6.5               |
|        |              | 5/17/75     | 3              | $10^6$          | 10.0                               | 0.0                | $5.8 \times 10^6$  | 10000              | 6.5               |
|        |              | 5/27/75     | 4              | $2 \times 10^6$ | 4.8                                | 0.5                | $4.6 \times 10^6$  | 9600               | 6.5               |
|        |              | 6/04/75     | 5              | $1.5 \times 10^6$ | 2.0                             | 0.2                | $7.5 \times 10^6$  | 3000               | 8.5               |
|        |              | 6/17/75     | 6              | $2 \times 10^6$ | 0.5                                | <0.1               | 1000               | 9                  | 45

*Expansion of keratinocyte population in culture: 560 fold.*

| E (f)  | n            | 4/24/75     | 1              | $10^6$          | 0.15                               | 0.12               | $1.5 \times 10^6$  | 150                | 10.3               |
|        |              | 5/10/75     | 2              | $5 \times 10^6$ | 15.7                               | <0.1               | $1.5 \times 10^6$  | 7850               | 8.5               |
|        |              | 5/21/75     | 3              | $3 \times 10^6$ | 3.3                                | <0.1               | $2.3 \times 10^6$  | 10000              | 8.5               |
|        |              | 6/04/75     | 4              | $10^6$          | 1.8                                | 0.2                | $5.1 \times 10^6$  | 1800               | 8.5               |
|        |              | 6/17/75     | 5              | $10^6$          | 1.2                                | 0.4                | $3.7 \times 10^6$  | 1200               | 8.5               |
|        |              | 6/30/75     | 6              | $3 \times 10^6$ | 0.6                                | 1.3                | 180                | 9                  | 51

*Expansion of keratinocyte population in culture: 2.2 x 10^4 fold.*

| GRE    | 3 years     | 2/10/75     | 1              | $10^6$          | 0.45                               | 5                  | $5.6 \times 10^6$  | 45                 | 10.3               |
|        |            | 2/25/75     | 2              | $2 \times 10^6$ | 0.3                                | 0.25               | $1.6 \times 10^6$  | 60                 | 11.5               |
|        |            | 3/21/75     | 3              | $1.4 \times 10^6$ | 0.007                          | 1.8                | b                  | 10                 | <5

*Expansion of keratinocyte population in culture: 45 fold.*

| HAE    | 12 years    | 10/25/74    | 1              | $3 \times 10^6$ | 0.7                                | 1.1                | $1.1 \times 10^6$  | 200                | 12.5               |
|        |            | 11/08/74    | 2              | $10^6$          | 0.15                               | 1.3                | $2.6 \times 10^6$  | 150                | 10.5               |

*Expansion of keratinocyte population in culture: 2.6 fold.*

| CS-1   | 34 years    | 4/16/75     | 1              | $3 \times 10^6$ | 0.1                                | <0.01              | $3.8 \times 10^6$  | 300                | 10.5               |
|        |            | 5/08/75     | 2              | $10^6$          | 0.3                                | 0.4                | $2.2 \times 10^6$  | 300                | 9.5                |

*Expansion of keratinocyte population in culture: 952 fold.*
in the upper layers do not divide and appear to differentiate toward squame formation; the remaining dividing population would therefore undergo more divisions than would be calculated from the total yield per cell colony.

It is probable that as in the case of human fibroblasts, the epidermal cells of older donors have reduced growth potential, since keratinocytes of ages 3–34 years grew through a total of 20–27 generations, whereas those from newborns grew through 25–51 generations. The plating efficiency of the keratinocytes of older donors was always less than 1%, whereas that of newborns was often in the range of 2–10% (Table 1, column II). It is also possible that the site of origin of the keratinocytes has some bearing on their behavior in culture, for the keratinocytes of the two oldest donors were derived from abdominal skin, while the others were derived from foreskin.

The minimum number of generations through which the keratinocytes grew in culture, 20 cell generations, corresponds to an increase in cell mass of approximately 10^6 fold if all progeny could initiate colony formation, but in view of the low plating efficiency on subculture, such increases in cell mass are not actually obtainable. It is therefore useful to calculate the actual expansion of the keratinocyte population in the course of serial cultivation without correcting for the losses due to low plating efficiency. Table 1 shows that the values varied from 2.6 to over 10^6 fold. The median value was 600 fold. It is quite possible that improvement of culture conditions will result in an increased plating efficiency and thereby permit greater expansion of the keratinocyte populations.

**Chromosome Complement of the Epidermal Cells**

The established keratinocyte line XB, derived from a mouse teratoma, was found, like many established mouse lines, to have a heteroploid chromosome complement (Rheinwald and Green, 1975). To examine the chromosomes of the human epidermal keratinocytes, it was necessary to obtain the cells completely free of human fibroblasts. This was accomplished by plating primary disaggregated foreskin cells in such number as to yield about 3 epidermal colonies per plate. A colony was isolated, trypsinized, and transferred to dishes containing n/3 and n/30 3T3. The purity of the isolated colony was confirmed by the absence of fibroblast colonies on the n/30 monolayer. Epidermal cells growing on the layers were treated with colchicine (2 × 10^-4 M) for 2 hr, and metaphase preparations were made by conventional methods and stained with orcein. In spite of their large dose of irradiation, abnormal 3T3 metaphases were often seen, but these were easily identified. Well spread human metaphases were counted and found to have the diploid number of chromosomes. The karyotype was not studied in detail.

**Dependence upon the 3T3 Cells**

Colony formation by human keratinocytes required the presence of the 3T3 cells. The dependence was different from that of the XB line in at least two ways: first, the XB line could grow (poorly) although not keratinize if inoculated at high density in the absence of 3T3, whereas the human keratinocytes could not even initiate colony formation; and second, medium conditioned by the growth of 3T3 cells could substitute for the 3T3 cells themselves in supporting the growth of the XB line, but human epidermal keratinocytes could not initiate colony formation in conditioned medium. We have reason to think that there may be separate factors required for colony initiation and for continued growth of an established colony, and that XB cells and human epidermal keratinocytes may differ in their dependence upon both factors.

Irradiated 3T3 cells were more effective than irradiated human diploid fibroblasts in supporting growth of the human epidermal keratinocytes. Colonies formed when human fibroblasts were used, but they grew more slowly and were less stratified.

**Discussion**

As a result of lessons learned from the study of a teratoma, it has become possible to cultivate human epidermal cells serially. As in the case of other diploid cell types, the human keratinocytes have a restricted lifetime of 20–50 cell generations. Whereas in the case of fibroblasts there is no way

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*Estimates from average colony size.

Indeterminate because of excessive fibroblast growth.

(n) Newborn.

(i) Cultures initiated from suspensions of trypsin-disaggregated cells stored viable in the frozen state.

(ii) Estimated from cultures inoculated with 10^9 cells together with n/3 3T3.

(iii) Estimated from cultures inoculated with 300–10^6 cells together with n/3 3T3.

(iv) Cell layers were treated with EDTA for 20 seconds, and the 3T3 cells and human fibroblasts were dislodged by vigorous pipetting and aspirated. The remaining cells were then removed with trypsin-EDTA and counted (any residual 3T3 cells were not included; see Experimental Procedures).

(v) Calculated from values in columns I and II. Expansion of keratinocyte populations in culture were obtained as the product of the increases at each passage listed in columns I and IV.
to relate their finite culture lifetime to their lifetime in tissues of the human, a comparison of this type can be made for the epidermal cells.

The average doubling time of cells in the basal layer of human epidermis has been estimated from thymidine labeling index to be about 12 days (Weinstein and Frost, 1969). This would permit 30 cell generations per year; over the lifetime of the human, the number of cell generations would be one or two orders of magnitude greater than the number of generations we obtained by measuring the number of progeny in culture. The difference may in part be due to an accelerated differentiation of the keratinocytes in culture and their removal from the dividing population; it is also possible that as in the case of fibroblasts (Todaro and Green, 1964), improvements in culture conditions may lead to extension of culture lifetime.

The use of serially cultivable strains of human epidermal cells should make possible the investigation of a variety of problems for which, up to now, only the fibroblast has been available. Such problems should include:

— the growth and differentiation of the keratinocyte;
— the effect of viruses, including oncogenic ones and especially those specifically affecting epidermal cells, such as the wart virus;
— the behavior of epidermal cells involved in human diseases;
— the testing of drugs affecting the human epidermal cell;
— the practical applications for skin grafting made possible by the production of human epidermal cells in quantity.

Experimental Procedures

Preparation of Cultures

Skin biopsies from foreskin or other sites were placed aseptically into growth medium containing 10% calf serum at room temperature. Within 3 hr, most of the subcutaneous tissue was removed with surgical scissors, and the remaining skin (1–3 cm² in area) was minced finely with scissors to pieces less than 1 mm in diameter. These were stirred in 10 ml of 0.25% trypsin at 37°C. After allowing 1 min for settling, the supernatant containing <95% single cells, was withdrawn at 30 min intervals and replaced with fresh trypsin solution. The cells were centrifuged, resuspended in medium containing 20% fetal calf serum and hydrocortisone (0.4 μg/ml), mixed with lethally irradiated 3T3 cells, and plated. The medium was changed 3 to 5 days later, when most epidermal cells had attached, and twice weekly thereafter until the cells were subcultured or fixed and stained.

Subcultures were made after removing nearly all 3T3 cells and viable fibroblasts by exposing the culture to 0.02% EDTA for 15 sec and pipetting vigorously. The keratinocyte colonies, which remained adherent, were then disaggregated to single cells in a solution containing equal parts of EDTA and 0.05% trypsin, and replated together with fresh irradiated 3T3 cells. The keratinocytes were usually subcultured when the average colony size reached about 1000 cells.

Efficiency of colony formation by keratinocytes was determined by plating 10⁴ to 10⁶ cells together with 1/3 3T3, fixing 2 to 4 weeks later, and staining with Rhodamine blue. The extent of contamination by human fibroblasts was determined by plating 300 to 10³ cells with 1/3 3T3. The cultures were fixed 1 to 2 weeks later and stained with hematoxylin for counts of fibroblast colonies. Culture conditions, preparation of lethally irradiated 3T3 cells, staining, and microscopy were carried out as described by Rheinwald and Green (1975).

Radioautography of Keratinocyte Colonies in Situ

Labeled thymidine was added to the medium of cultures for 24 hr (tritiated, 0.5 μCi/ml (50 Ci/m mole); C¹¹, 5 μCi/ml (54 mCi/m mole)). The medium was then removed and a solution of 0.5% NP40 was added. 2 min later the 3T3 cells were dislodged. The keratinocyte colonies remained attached, but the cytoplasmic compartments became substantially emptied (Tsai and Green, 1973), and the nuclei became easily visible. The cultures were then fixed, stained, dried, and covered with photographic emulsion. After 5–10 days the emulsion was developed.

Acknowledgments

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References

Figure 1. Human Epidermal Keratinocyte Colonies
All cultures were inoculated with a mixture of human keratinocytes and lethally irradiated 3T3 cells. After 2-3 weeks, cultures were fixed and stained with Rhodamine blue.
(a) Primary culture of strain HFE. (Inoculum $10^6$ cells.) Red staining colonies show typical blue cuff of displaced 3T3 cells.
(b) Secondary culture of strain HFE. (Inoculum $6 \times 10^6$ cells.) Colonies have grown to confluence.
(c) Fourth plating of strain HFE. (Inoculum $10^6$ cells.) The keratinocytes are close to the end of their culture life. Colonies are variable in size, but generally small and irregular in shape.
(d) Primary cultures of strain HAE. (Inoculum $3 \times 10^4$ cells.) Colonies formed from keratinocytes of older humans tend to pile up more strikingly in their centers and make keratinizing balls of cells.
Figure 3(a). Effect of Hydrocortisone on Growth of Keratinocyte Colonies of Strain HFE

A tertiary subculture was inoculated with $3 \times 10^4$ epidermal cells at their seventeenth cell culture generation, together with $\frac{2}{3} 3T3$ cells. Photograph shows the cultures fixed and stained with Rhodanil blue 15 days later. Left, no hydrocortisone; right, hydrocortisone (0.4 
$\mu$g/ml).

Figures 3(b-d). Vertical Sections through Keratinocyte Colonies of Strain HFE Showing Their Stratified Appearance.

Keratinization is more advanced in the layers closest to the medium. (b and d) primary culture 22 days after inoculation, during which the cells grew through about 12 generations; (c) tertiary culture 26 days after inoculation, total growth in culture about 29 generations.
Figures 4–6. Electron Micrographs of Sections through Superficial Layers of Keratinocyte Colonies

Tertiary cultures were made of strain HFE (17 generations in culture) together with 3T3 cells. 17 days later, the keratinocyte colonies were fixed and prepared for electron microscopy. Sections through the cells show numerous characteristic features of keratinocytes: abundant tonofilaments in longitudinal section (Figure 5, right) and cross section (Figure 6), numerous desmosomes (Figures 4–6), keratohyalin granules (Figures 4 and 5, left), and thickening of cell membranes (Figures 4–6). Keratinization is usually more advanced in the cell layer closest to the medium.

Figure 4, 18,000 X; Figure 5 (left), 30,000 X; Figure 5 (right), 75,000 X; Figure 6, 75,000 X.
Serial Cultivation of Human Epidermal Keratinocytes

Figure 6