Ability of Normal Human Keratinocytes That Grow in Culture in Serum-Free Medium To Be Derived From Suprabasal Cells[1]

Mark S. Wilke, Maryanne Edens, Robert E. Scott[2,*]

The current studies were performed to determine from which regions of the skin keratinocytes that grow in vitro are derived. Normal human foreskin specimens were first separated by differential trypsinization into two suprabasal fractions and one basal fraction. Utilizing complete MCDB 153 basal nutrient culture medium containing epidermal growth factor and insulin, we then evaluated the clonogenic potential of cells in these three fractions. Suprabasal cell fractions demonstrated a colony-forming efficiency as great as or greater than that of the basal cell fraction, and 10%–15% of the keratinocytes that grew in primary and secondary cultures expressed involucrin, a suprabasal keratinocyte differentiation marker. Of such involucrin-containing keratinocytes, 80% also possessed the potential to undergo DNA synthesis, as determined by autoradiography following a 48-hour incubation with [^{3}H]thymidine. These observations support the conclusion that the human keratinocytes that grow in vitro in serum-free medium can be derived from suprabasal cells and, therefore, that a state of nonterminal keratinocyte differentiation exists. [J Natl Cancer Inst 1988;80:1299–1304]

The epidermis is a classic example of a tissue that is composed of a combination of proliferative and terminally differentiated cells that are maintained in a steady state. It has generally been assumed that proliferative cells are restricted to the basal layer of the epidermis. This concept evolved from studies 20–30 years ago in rodents in which [^{3}H]thymidine incorporation into DNA was noted to occur selectively in basal cell nuclei (*1–3*). Similar results were reported in studies on human epidermis (*4*).

However, Epstein and Maibach (*5*) subsequently presented evidence that some suprabasal cells could express proliferative potential. They found a small but significant number of proliferating cells in the suprabasal layer of the human epidermis, as determined by autoradiographic analysis of [^{3}H]thymidine incorporation in DNA. Penneys et al. (*6*) established quantitative evidence in support of this finding. They reported that in normal epidermis many proliferative cells in the skin were situated in the suprabasal layers. Lavker and Sun (*7*) later reported that many proliferative cells in monkey palm epidermis also were located in the suprabasal tissue.

The development of specialized culture medium by Ham and co-workers (*8,9*) has now made it possible to study the in vitro clonal growth characteristics of epithelial cells of normal human skin. In this regard, numerous studies have characterized the growth and differentiation of cultured keratinocytes (*8,10–12*). However, it has not been established from which epidermal layer keratinocytes that grow in culture can be derived. Therefore, in the current studies, the clonogenic potential of cells derived from various layers of the epidermis of normal human foreskin was evaluated. The results demonstrated that (*a*) keratinocytes derived from suprabasal regions of the skin can grow in tissue culture, (*b*) some of these proliferative cells contain involucrin, and (*c*) a state of nonterminal differentiation must, therefore, exist in epidermal keratinocytes.

Experimental Design

Growth Medium

For the culture of human epithelial cells of the skin, complete MCDB 153 medium devoid of serum was used. More specifically, basal nutrient medium formulation, designated MCDB 153, was prepared as previously described (*8,9*); it was then supplemented with 0.1 m*M* ethanolamine, 0.1 m*M* phosphoethanolamine, 5×10^{-7} *M* hydrocortisone, 0.1 m*M* Ca^{2+}, 5 μg of insulin/mL, 10 ng of epidermal growth factor/mL, and 140 μg of bovine pituitary extract/mL (*11,12*). Cells in this medium were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Isolation of Epithelial Cells for Clonal Growth Assays

The procedures for isolating epithelial cells from different layers of the epidermis were developed as described in this article. Neonatal foreskin specimens, obtained from the nurs-

[1]Received February 5, 1988; revised June 21, 1988; accepted July 21, 1988.

[2]Section of Experimental Pathology, Mayo Clinic/Foundation, 200 First St., S.W., Rochester, MN.

**Correspondence to:* Dr. Robert E. Scott, Section of Experimental Pathology, 511B Guggenheim Bldg., Mayo Clinic/Foundation, Rochester, MN 55905.

ery as surgical waste, were trimmed free of essentially all dermal connective tissue, cut into 4-mm^2 pieces, and floated on trypsin [0.17% in phosphate-buffered saline (pH 7.4); Sigma Chemical Co., St. Louis, MO] overnight at 4 °C. As a result of this treatment, a cleavage plane typically develops between the keratinocytes in the stratum spinosum. This effect is quite reproducible when the conditions described above are followed closely. Some variability does nonetheless exist in the precise location of the cleavage plane even in the same specimen.

With this procedure, it is generally possible to isolate three populations of cells from foreskin specimens. The keratinocyte fraction above the trypsin-induced cleavage plane can be isolated by peeling off the upper epidermal layer with a fine forceps. The cells in this fraction can then be dissociated in tissue culture medium by gentle agitation, i.e., holding the tissue fragment with the tip of a fine forceps and shaking it with short, rapid motions 10–15 times in tissue culture medium. The resulting cell suspension is passed through nylon mesh (Nytex; TETKO Inc., Elmsford, NY) and centrifuged at 250 *g* for 10 minutes to prepare a cell pellet that can be assayed for clonogenic potential. The cell fraction below the trypsin-induced cleavage plane remains attached to the dermis. This tissue typically consists of the three to four layers of epidermal cells above the basement membrane and the dermis.

By gentle agitation of this tissue as described above, suprabasal cells primarily of the lower stratum spinosum were dissociated and could be subsequently isolated by centrifugation. The residual basal cell fraction remained attached to the basement membrane–dermal tissue. The basal cells were finally isolated from the tissue fragment by incubation in trypsin for an additional 1 hour at 4 °C or by scraping the tissue surface with a fine metal rod. Thereafter, the basal cell fraction was recovered as a cell suspension. The clonogenic potential of all three cell specimens was then evaluated.

Intactness of cells was measured by the trypan blue dye exclusion test. Additional studies were performed in which both suprabasal cell fractions were trypsinized for an additional 1 hour in a manner comparable to that used for basal cells to assure that such treatment did not modify the clonogenicity of the suprabasal cell fractions; it did not in two separate experiments. For determination of cell size, preparations of spherical cells in suspension were examined in a Leitz photomicroscope, and randomly selected fields were photographed. The diameter of each cell was then measured on photographic enlargements. These results were substantiated by use of a flow microfluorometer that employed light-scatter techniques. All of these experimental procedures were reproduced on three or more foreskin specimens. Some tissue and cell specimens were evaluated for the expression of involucrin, a suprabasal keratinocyte differentiation marker, by use of immunofluorescent techniques as previously described (*12*). Some tissue and cell specimens were also incubated for various intervals in [^{3}H]thymidine in complete MCDB 153 culture medium. They were then processed by autoradiography to determine the extent of DNA synthesis in the various cell layers by the extent of nuclear labeling.

Clonal Growth Assays

Clonal growth assays were performed as previously described (*8,11,12*). Briefly, 500 viable cells were seeded onto 60-mm culture dishes containing complete MCDB 153 medium. This cell density was employed because it gave optimum clonogenicity. At the end of a 10-day incubation period at 37 °C in a humidified 5% CO_2 incubator, cells in dishes were fixed and stained, and colony-forming efficiency was determined (*8,11,12*).

Results

The ability to fractionate the epidermis into separate layers by differential trypsinization and the use of serum-free MCDB 153 growth medium to culture epithelial cells derived from human skin at clonal densities made possible the studies described in this article. More specifically, these studies were performed to determine the relative clonogenic potential and the biological characteristics of epithelial cells isolated from various layers of the human epidermis (fig. 1a).

Cell Preparations

Incubation of neonatal human foreskin preparations in trypsin for 16 hours at 4 °C typically results in the development of a cleavage plane within the bottom region of the stratum spinosum layer, as depicted in the routine histological section shown in figure 1b. The cleavage plane was evident as a clear zone. The portion of the tissue above the trypsin-induced cleavage plane could easily be peeled off by use of a fine forceps. Gentle agitation of this tissue in culture medium generated a cell fraction illustrated in figure 1c, designated fraction A. Fraction A represented the upper epidermal cell fraction and primarily included cells in the upper stratum spinosum and cells in the stratum granulosum.

After the fraction A cells superior to the trypsin-induced cleavage plane were removed, the remaining tissue (figs. 1d, 1e) could then be gently agitated in culture medium to dissociate the loosely attached suprabasal cells in the lower stratum spinosum, which are designated fraction B in figure 1e. Upon removal of the fraction B cells, only the basal cells remained attached to the dermis (figs. 1f, 1g).

Further trypsinization of the latter tissue fragment or mechanical dissociation yielded a suspension of enriched basal cells, designated fraction C in figure 1g. Once this cell fraction was isolated, the remaining tissues consisted solely of the dermal tissues (fig. 1h). This procedure was employed on numerous specimens and similar results were obtained, although some variability in the development of the cleavage plane was observed when different experimental conditions were employed.

Characterization of Cell Fractions

Table 1 presents the average relative yield of cells isolated in each fraction from a given foreskin sample. Fraction A cells and fraction B cells typically represented ≈44% of the total cell yield. In contrast, fraction C cells represented 12% of the total cell yield. Table 1 shows that cells in fractions A and B averaged 9.1 μm in diameter, whereas

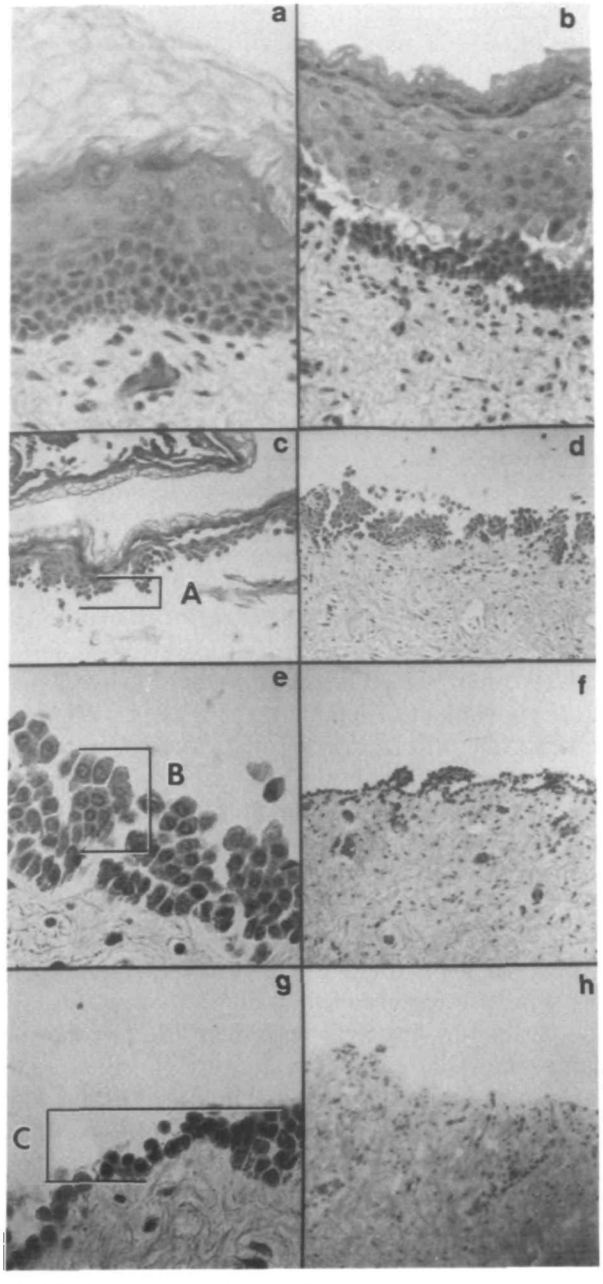

Figure 1. Sections of normal human foreskin before and after differential trypsinization and processing: (a) normal human foreskin before trypsinization; (b) foreskin after 16 hr in trypsin at 4 °C, demonstrating presence of clear zone representing a trypsin-induced cleavage plane; (c) fraction A containing cells above trypsin-induced cleavage plane; (d, e) tissue fragment remaining after fraction A cells are removed, illustrating presence of fraction B and C cells and specifically illustrating suprabasal cell fraction B that can be isolated by gently agitating this tissue fragment in tissue culture medium; (f, g) tissue fragment remaining after both fraction A and fraction B cells have been removed, specifically illustrating basal cell fraction C that can be subsequently isolated by additional brief trypsinization or mechanical dissociation; (h) residual dermal tissue fragment after all epidermal cells have been removed. Hematoxylin and eosin.

cells in fraction C averaged 6.4 μm in diameter. Statistical analysis established no significant difference in the sizes of the cells in the upper two fractions, but cells in both upper fractions were found to be significantly larger than cells in fraction C ($P < .001$) as determined by the paired t-test. Table 1 also documents that >90% of cells in all three

Table 1. Characteristics of cells isolated from different layers of human epidermis

Cell layer	Relative % isolation from foreskin*	Cell size (μm)†	% of cells showing trypan blue exclusion	% of involucrin-containing cells
Fraction A	44	9.2 ± 2.2	92	63
Fraction B	44	9.0 ± 1.7	94	≤10
Fraction C	12	6.4 ± 0.9	93	≤1

*Based on hemocytometer counts of cell suspensions in studies performed on more than three specimens.
†Values = means ± SD.

fractions excluded trypan blue dye. Somewhat similar results relating to keratinocyte size have been reported (*13*). In addition, table 1 shows that 63% of the cells in fraction A contained involucrin and that 10% of the cells in fraction B also contained involucrin. In contrast, essentially none of the basal cells in fraction C contained involucrin.

Clonogenic Potential of Cell Fractions

To determine the clonogenic potential of specimens isolated from each fraction, we seeded cells into culture dishes (500 viable cells/dish) in complete MCDB 153 growth medium (*8*). Ten days later, we scored those cells that formed colonies. Table 2 summarizes the data and shows that cells derived from fractions A and B demonstrated the highest colony-forming efficiency. In contrast, cells of the basal fraction C demonstrated relatively less clonogenic potential. Various other tissue culture media, with and without added serum, were also employed in selected studies to attempt to more efficiently grow cells of the basal fraction. However, no increase in cloning potential could be detected (data not shown).

These results raise several significant questions. First, can suprabasal cells within the three-dimensional confines of skin tissue fragments proliferate? In an attempt to answer this question, we cultured tissue fragments of human foreskin in complete MCDB 153 medium for ≥24 hours in [^{3}H]thymidine. We then determined by combined histochemical analysis and autoradiography the extent of nuclear labeling indicative of DNA synthesis in various layers of the skin. The results (table 3) demonstrated that, of the epithelial cells that incorporated [^{3}H]thymidine into DNA, ≥28% were localized to skin layers three to four cells above the basement membrane, which, therefore, certainly represented suprabasal cells.

Table 2. Clonogenic potential of cells isolated from different layers of human epidermis

Cell layer	Colony-forming efficiency*
Fraction A	0.36 ± 0.06
Fraction B	0.43 ± 0.22
Fraction C	0.09 ± 0.06

*% of cells that form colonies of four cells or greater after 10 days in culture. Values = means ± SD.

Table 3. Evidence that suprabasal keratinocytes possess proliferative potential: analysis of [^{3}H]thymidine incorporation in organ culture*

Keratinocyte position within epidermis above basement membrane	% of cells containing labeled nuclei
Basal cell layer 1	33
Cell layer 2	38
Cell layer 3	10
Cell layer 4	18
Cell layer ≥5	≤1

*Intact tissue fragments of skin were cultured in complete MCDB 153 medium containing 8 μCi of [^{3}H]thymidine/mL for ≥24 hr. Thereafter, frozen sections were prepared, followed by autoradiography. The extent of nuclear labeling was determined by light microscopy in hematoxylin and eosin-stained specimens.

Another question concerns whether any involucrin-containing cells isolated from the suprabasal zones of skin proliferate in culture. For the study of this question, rapidly growing cell populations within primary and secondary cultures in complete MCDB 153-0.1 m*M* Ca^{2+} were evaluated for involucrin expression by immunofluorescence. Table 4 demonstrates that in primary cultures 15% of the cells expressed involucrin. This percentage decreased slowly with extended time in culture. For example, after four population doublings and passage to secondary cultures, ≈10% of the rapidly growing cells continued to express involucrin; after eight population doublings, ≈1% of the cells continued to express involucrin. Table 4 also demonstrates that 80% of the involucrin-containing cells in primary cultures synthesized DNA and showed radiolabeled nuclei following incubation in [^{3}H]thymidine. Figure 2 illustrates five such involucrin-containing cells with labeled nuclei.

These results suggest that the keratinocytes that grow in vitro in the tissue culture environment of complete MCDB 153 medium in plastic dishes can be derived from suprabasal cells. The fact that suprabasal cells actually appear to be cloned best in this culture environment cannot easily be explained by suggesting that such specimens were contaminated with basal cells, because basal cells show a relatively lower clonogenic potential. Notwithstanding this fact, the question remains: Why do basal cells not proliferate better in this culture microenvironment? Perhaps when the basal cells are detached from the basement membrane by additional trypsinization or mechanical dissociation, they lose their proliferative potential for other unknown reasons; or perhaps basal cells require unique growth factors or a specific substrate to attach and/or grow.

Table 4. Evidence that cultured keratinocytes expressing involucrin can undergo DNA synthesis

Involucrin expression*	% of cell population	% of involucrin-containing cells demonstrating labeled nuclei†
−	85	99
+	15	80

*Only cells that demonstrate very bright immunofluorescence were designated to be involucrin-containing cells.

†DNA synthesis was determined by culturing adherent cells in [^{3}H]thymidine for 48 hr, followed by fixation, processing, and autoradiographic analysis with the use of combined light and fluorescence microscopies. Primary keratinocyte cultures were studied.

Discussion

Before the development of methods to grow epidermal cells in vitro, studies on the proliferative compartment of the epidermis depended either on the analysis of mitotic figures in fixed sections of the epidermis or on the evaluation of nuclear labeling indices of epidermal cells following in vivo injection of [^{3}H]thymidine. With the development of new tissue culture media, in vitro studies are now possible on the growth and differentiation characteristics of these cells (*8–10,12*). Nonetheless, very few studies have previously attempted to evaluate the precise anatomic origin of the epithelial cells of the skin that grow in tissue culture. In 1971, Vaughan and Bernstein (*14*) suggested that suprabasal cells showed no evidence of proliferation; rather they suggested that only basal cells proliferated in their cell culture system. In 1978, Liu and Karasek (*15*) also suggested that basal cells selectively produced proliferative primary cultures in vitro, whereas populations of suprabasal cells and malpighian cells were nonproliferative. However, those studies were severely limited by the lack of serum-free tissue culture media that support the extended proliferation of human epidermal cells and by the lack of other new tissue culture methodologies. More recently, Barrandon and Green (*13*) reported that human keratinocytes that are <11 μm in diameter can grow in a tissue culture microenvironment of 3T3 feeder layers, and some of these relatively large cells might be derived from suprabasal regions of the skin.

The current studies were performed to more specifically evaluate the proliferative characteristics of cells from different anatomical layers of human skin by use of the new culture methods described by Ham and co-workers (*8,9*). The results show that specimens enriched in cells isolated from the suprabasal stratum spinosum layer can be stimulated to proliferate when they are placed in serum-free medium in plastic dishes. In fact, when cells from each of the three layers of the skin were seeded at clonal densities in complete MCDB 153 medium, the fractions enriched in suprabasal cells (fractions A and B) had a colony-forming efficiency at least comparable with that of the fraction derived from basal cells (fraction C). Data are in fact presented showing that involucrin-containing keratinocytes can proliferate in vitro. This finding then suggests that a state of nonterminal differentiation exists in keratinocytes as well as in other cell types.

A variety of reasons could explain why the tissue fraction enriched in basal cells does not show the greatest proliferative capacity in our studies. Perhaps the most reasonable explanation is that basal cells normally have an intimate interaction with the basement membrane, and once this interaction is interrupted, basal cells undergo a relative decrease in their clonogenic potential. In addition, basal cells may require unique growth and/or survival factors that are not included in complete MCDB 153 culture medium. In this regard, it is important to stress that the colony-forming efficiency of human

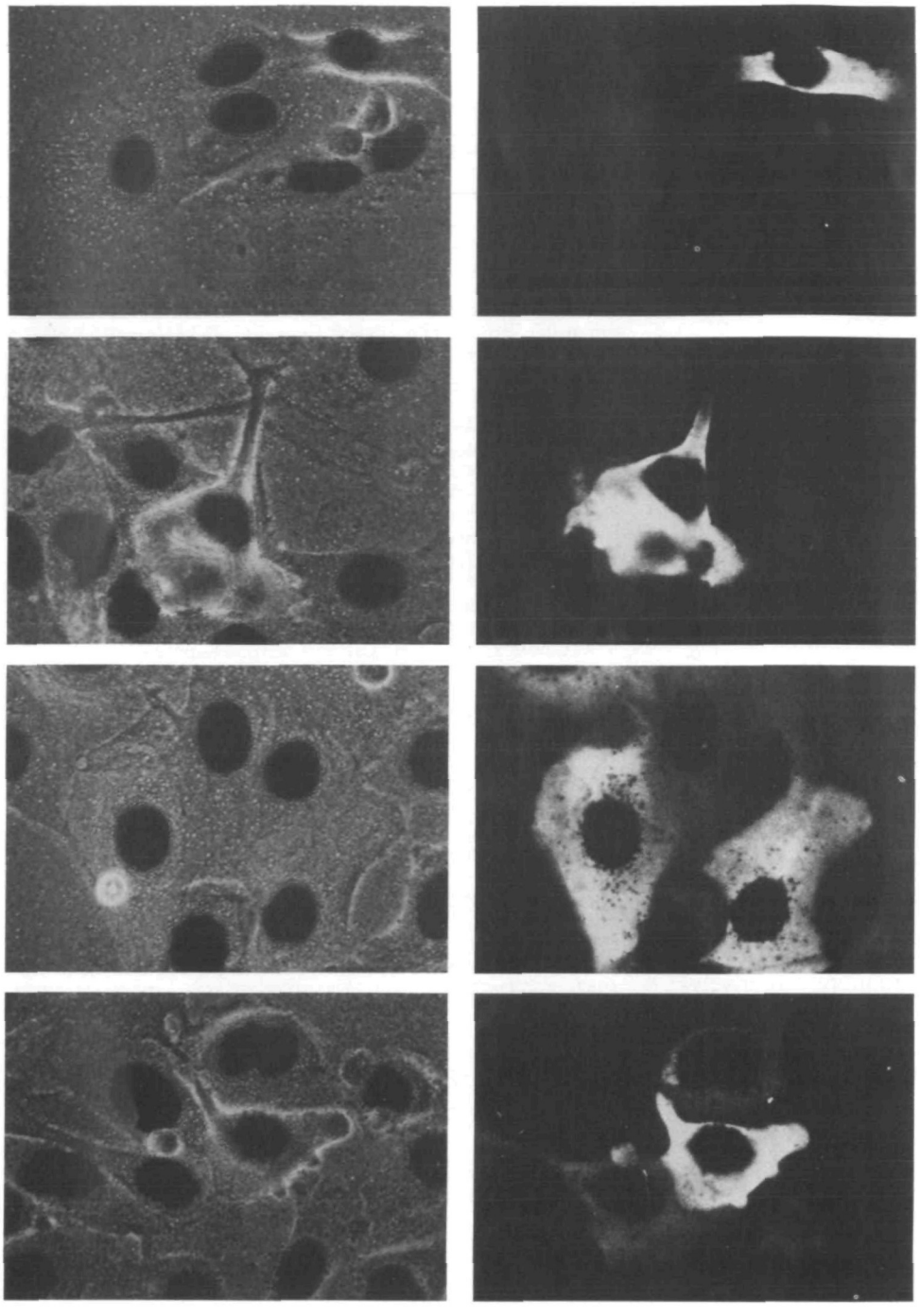

Figure 2. Demonstration that primary cultures derived from normal human skin contain involucrin-expressing cells that can undergo DNA synthesis. Five involucrin-containing cultured keratinocytes are illustrated by immunofluorescence as brightly stained cells in photomicrographs on the right. On the left are identical cells after autoradiographic detection of labeled nuclei following incubation in [^{3}H]thymidine, as visualized by phase microscopy. These data show that involucrin-containing keratinocytes in culture have the potential to synthesize DNA.

squamous epithelial cells is in general relatively low. In our best studies, a maximum colony-forming efficiency of ≈1% was obtained. In general, however, primary cultures of human and mouse keratinocytes usually show a colony-forming efficiency of ≤1% (*16,17*). In bronchial epithelial cells, the colony-forming efficiency of keratinocytes was even reported to be as low as 0.05% in serum-free medium on plastic dishes (*18*). Irrespective of the extent of clonogenicity and irrespective of whether suprabasal or basal cells of the skin primarily proliferate in vivo, suprabasal cells can unequivocally express clonogenic potential in vitro when they are cultured in complete MCDB 153 medium containing epidermal growth factor and insulin on a plastic substrate.

Another important question concerns the potential significance of a state of nonterminal squamous cell differentiation. In a physiological context this question cannot be answered, but in a biological context the existence of a nonterminal differentiation state emphasizes that the proliferative capacity of a cell need not be strictly linked to its differentiated phenotype. This situation is also true in other cell types. For example, many nonterminally differentiated cells, such as adipocytes and lymphocytes, have extensive proliferative potential. From this perspective, the expression of involucrin in keratinocytes need not be equated with loss of proliferative potential; i.e., some involucrin-containing keratinocytes are capable of proliferation in vitro.

The fact that at least some epidermal cells that grow in culture appear to be derived from previously nonterminally differentiated suprabasal keratinocytes raises another question: Are epidermal cells that grow in culture stem cells or are they more committed progenitor cells? Since epidermal stem cells in vivo are thought to be localized only to the specific sites of the basal cell layer of the skin (*19-21*), whereas suprabasal cells grow best in vitro in our system, it is highly probable that cultured keratinocytes in general represent progenitor cells, i.e., cells committed to a specific differentiation pathway. In support of this possibility is a recent study (*10*) showing that when cultured human keratinocytes are transplanted onto the dermis of a burned patient from whom the keratinocytes were initially derived, the transplanted epidermis remains viable and reproductive for years but is composed only of keratinocytes and not of other cell types thought to be derived from epidermal stem cells (i.e., hair follicle cells, sweat gland cells, and sebaceous gland cells). This phenomenon could, of course, also result at least in part from differential effects of dermal components at the burn site on cell differentiation.

The possibility that human keratinocytes that grow in vitro are progenitor cells rather than stem cells could help explain why they are so resistant to experimentally induced neoplastic transformation in vitro (*22*). This is so because it is probable that most cancers originate primarily by the transformation of certain classes of stem cells (*23*). If stem cells are indeed the primary target of carcinogenic agents and if differentiation toward more mature progenitor cells really does suppress a cell's transformation potential, as previously suggested (*24,25*), the reason that cultured epithelial cells of human skin are resistant to neoplastic transformation could be that they do not represent stem cells. To more definitively answer this important question, new approaches to culture true human epithelial stem cells need to be developed and well-controlled studies need to be performed to specifically determine if stem cells can indeed be more efficiently transformed than progenitor cells.

References

1. Fukuyama K, Bernstein IA. Autoradiographic studies of the incorporation of thymidine-H^3 into deoxyribonucleic acid in the skin of young rats. J Invest Dermatol 1961;36:321-326.
2. Leblond CP, Messier B, Kopriwa B. Thymidine-H^3 as a tool for the investigation of the renewal of cell populations. Lab Invest 1959;8:296-308.
3. Schultze B, Oehlert W. Autoradiographic investigation of incorporation of H^3-thymidine into cells of the rat and mouse. Science 1960;131:737-738.
4. Weinstein GD, Van Scott EJ. Autoradiographic analysis of turnover times of normal and psoriatic epidermis. J Invest Dermatol 1965;45:257-262.
5. Epstein WL, Maibach HI. Cell renewal in human epidermis. Arch Dermatol 1965;92:462-468.
6. Penneys NS, Fulton JE Jr, Weinstein GD, et al. Location of proliferating cells in human epidermis. Arch Dermatol 1970;101:323-327.
7. Lavker RM, Sun T-T. Epidermal stem cells. J Invest Dermatol 1983;81(suppl):121-127.
8. Boyce ST, Ham RG. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. J Invest Dermatol 1983;81(suppl):33-40.
9. Tsao MC, Walthall BJ, Ham RG. Clonal growth of normal human epidermal keratinocytes in a defined medium. J Cell Physiol 1982;110:219-229.
10. Pittelkow MR, Scott RE. New techniques for the in vitro culture of human skin keratinocytes and perspectives on their use for grafting of patients with extensive burns. Mayo Clin Proc 1986;62:771-777.
11. Pittelkow MR, Wille JJ Jr, Scott RE. Two functionally distinct classes of growth arrest states in human prokeratinocytes that regulate clonogenic potential. J Invest Dermatol 1986;86:410-417.
12. Wille JJ Jr, Pittelkow MR, Shipley GR, et al. Integrated control of growth and differentiation of normal human prokeratinocytes cultured in serum-free medium: clonal analyses, growth kinetics, and cell cycle studies. J Cell Physiol 1984;121:31-44.
13. Barrandon Y, Green H. Cell size as a determinant of the clone-forming ability of human keratinocytes. Proc Natl Acad Sci USA 1985;82:5390-5394.
14. Vaughan FL, Bernstein IA. Studies of proliferative capabilities in isolated epidermal basal and differentiated cells. J Invest Dermatol 1971;56:454-466.
15. Liu S-C, Karasek M. Isolation and growth of adult human epidermal keratinocytes in cell culture. J Invest Dermatol 1978;71:157-162.
16. Milo GE, Ackerman GA, Noyes I. Growth and ultrastructural characterization of proliferating human keratinocytes in vitro without added extrinsic factors. In Vitro 1980;16:20-30.
17. Yuspa SH, Harris CC. Altered differentiation of mouse epidermal cells treated with retinyl acetate in vitro. Exp Cell Res 1974;86:95-105.
18. Lechner JF, Haugen A, McClendon IA, et al. Clonal growth of normal adult human bronchial epithelial cells in a serum-free medium. In Vitro 1982;18:633-642.
19. Banks-Schlegel S, Green H. Involucrin synthesis and tissue assembly by keratinocytes in natural and cultured human epithelia. J Cell Biol 1981;90:732-737.
20. Al-Barwari SE, Potten CS. Regeneration and dose-response characteristics of irradiated mouse dorsal epidermal cells. Int J Radiat Biol 1976;30:201-216.
21. Potten CS. Cell replacement in epidermis (keratopoiesis) via discrete units of proliferation. Int Rev Cytol 1981;69:271-318.
22. DiPaolo JA. Relative difficulties in transforming human and animal cells in vitro. JNCI 1983;70:3-8.
23. Pierce GB, Shikes R, Fink LM. Cancer: a problem of developmental biology. Englewood Cliffs, NJ: Prentice-Hall, 1978:68-87.
24. Anders A, Anders F. Etiology of cancer as studied in the platyfish swordtail system. Biochim Biophys Acta 1978;516:61-95.
25. Scott RE, Edens M, Estervig DN, et al. Cellular differentiation and the prevention and treatment of cancer. In: Waxman S, Rossi GB, Takaku F, eds. The status of differentiation therapy of cancer. Serono Symposia, vol 45. New York: Raven Press, 1988:3-16.