IN VITRO
Volume 15, No. 10, 1979

GROWTH CHARACTERISTICS OF HUMAN EPIDERMAL KERATINOCYTES FROM NEWBORN FORESKIN IN PRIMARY AND SERIAL CULTURES[1]

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(Received December 13, 1978; accepted March 20, 1979)

SUMMARY

Using gels of acid-soluble collagen as a culture surface, trypsin-released keratinocytes from 0.1-mm split-thickness sections of newborn foreskin may be plated with high efficiency and subcultured at a 1:5 split at 2- to 3-week intervals for three subpassages. When plated at a density of 3.2×10^4 cells per cm^2, keratinocytes attach to the gel with an efficiency of over 70%; after a lag phase of 3 days, the cells multiply exponentially with a doubling time of 60 hr. Cultures reach a growth-plateau phase at a density of 47.7×10^4 cells per cm^2. Both hydrocortisone and epidermal growth factor (EGF) stimulate slightly the growth of primary cultures; both factors are required for proliferation of the 2nd and further passage of keratinocytes. As the cultures reach confluence, multilayers of stratified cells are formed and cells of squamous morphology are spontaneously released from the surface. When the released cells and the attached cells are pulsed with [^{3}H]-histidine and [^{14}C]-leucine, a higher ratio of histidine to leucine is observed in the released cells indicating the biochemical onset of maturation. Orange G-Aniline Blue staining of the released cells show some of the cells to be completely keratinized. Fibrous proteins extracted from the cultured cells and analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis display the characteristic stratum corneum proteins of 60,000 and 66,000 daltons.

Key words: keratinocytes; primary and serial cultures; collagen; hydrocortisone; EGF.

INTRODUCTION

In 1967, Briggaman et al. described a method to release viable suspensions of adult human keratinocytes from split-thickness sections of skin (0.4 mm) by short-term exposure to trypsin (1). Cell viability, estimated by Nigrosin dye exclusion, was 80% and fibroblast contamination was infrequent. Using a modification of the split-thickness approach, Karasek and Charlton reported the superior plating efficiencies of the trypsin-released cells on collagen gels when compared to plastic or glass and described the growth characteristics and properties of the single cells on collagen gels (2).

Although primary cultures could be established and maintained from both human and other animal species (3–8), long-term growth and serial cultivation had been difficult to achieve until 1975 when Rheinwald and Green reported that serial cultivation of newborn keratinocytes was possible if cells were plated and maintained in the presence of irradiated 3T3 cells at the correct density (9). Addition of hydrocortisone and EGF increased cellular proliferation (10). Using split-thickness adult human skin and dexamethasone, Liu and Karasek described the isolation of proliferative and nonproliferative single cell populations and their growth characteristics on collagen gels following dispersion by trypsin and ethylenediaminetetraacetic acid (EDTA) (11).

In this study we describe a technique for the rapid isolation of keratinocytes from newborn foreskin, the growth profiles of primary and passaged cells and evidence for the onset of differentiation.

[1]Supported in part by Grants AM 14121 and AM 19595 of the U.S. Public Health Service.

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Materials and Methods

Materials Used Included

Dulbecco's modified Eagle's medium without sodium pyruvate in powder form (GIBCO, Grand Island, N.Y.); fetal bovine serum (Irvine Scientific Sales Co., Irvine, California); hydrocortisone and streptomycin (Pfizer Pharmaceuticals, Chicago, Illinois); epidermal growth factor (Collaborative Research Inc., Waltham, Massachusetts); plastic petri dishes, 35-mm diameter (Lux Scientific Corp., Thousand Oaks, California); trypsin 1-250 (hog pancreas) (ICN Pharmaceuticals, Cleveland, Ohio); 3,5-diaminobenzoic acid (Sigma Chemical Co., St. Louis, Missouri); [^{3}H]-histidine (37.5 Ci per mmol) and [^{14}C]-leucine (312 mCi per mmol) (Schwarz/Mann Laboratories, Orangeburg, New York).

Culture Surface

Cells are cultured on plastic coated dishes with a thin layer of acid-soluble collagen extracted from adult rabbit skin. The isolation of acid-soluble collagen and the preparation of the culture surface have been described in detail previously (11).

Preparation of Primary Cultures

Newborn foreskins are obtained at the time of circumcision and collected in 0.15 N NaCl containing 400 U per ml penicillin and 200 μg per ml streptomycin. The underlying elastic tissue is carefully removed using a No. 10 scalpel on a sterile glass surface. The trimmed foreskin is transferred to a dissecting board covered with a moistened sterile paper towel to increase adhesion of the foreskin to the towel and to prevent slippage during the removal of the epidermis. The width of the foreskin should not exceed the width of the cutting blade. A uniformity of cut is obtained by grasping the edge of the foreskin with fine forceps and cutting the tissue from the center out using a Castroviejo keratotome set to cut at a depth of 0.1 mm. The foreskin is reversed, and the second half is cut by the same procedure. The resulting thin split-thickness sections are transferred to Hanks' balanced salt solution containing 400 U penicillin per ml, 200 μg per ml streptomycin and

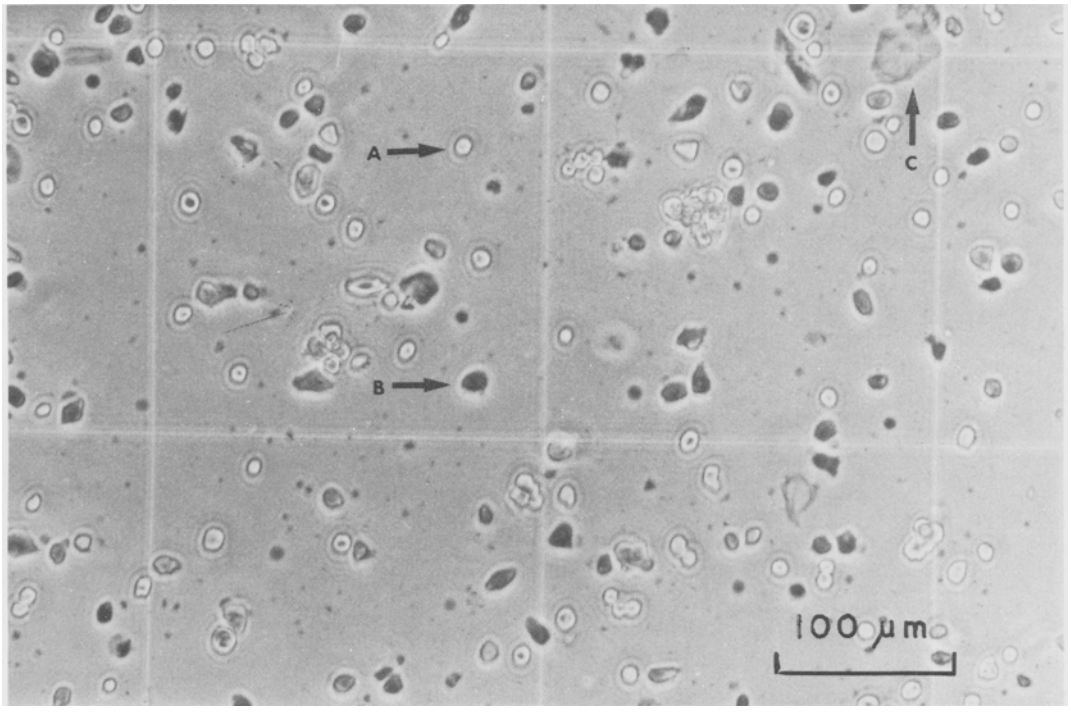

FIG. 1. Phase contrast light micrograph of trypsin-released human epidermal keratinocytes from foreskin. Three types of cells are observed; translucent (A), opaque (B) and keratinized (C).

75 U per ml mycostatin. The samples are soaked for 1 hr at 12° C.

To separate the epidermis, the cut sections are floated on 5 ml of 0.3% trypsin in GKN (0.15 N NaCl, 0.04% KCl and 0.1% glucose adjusted to pH 7.6 with $NaHCO_3$) in a 60-mm petri dish for 20 min at 37° C. The skin sections are rinsed twice with sterile 0.15 N NaCl, transferred to 5 ml of Dulbecco's modified Eagle's medium containing 20% fetal bovine serum, 200 U per ml penicillin, 100 μg per ml streptomycin and 50 μg per ml Gentamicin. The epidermis is separated from the dermis with two scalpels with No. 10 blades, and additional keratinocytes are released into the medium by agitation of the dermis and epidermis between blades. The released cells are counted in a hemocytometer, diluted, and 100 × 10^4 translucent cells (Fig. 1) plated on each 35-mm thin-gel plastic dish. The cells are allowed to settle and attach for 24 hr and are refed with medium containing 0.4 μg per ml hydrocortisone. Unless otherwise stated, cells are maintained at 37° C in an atmosphere of 94% air:6% CO_2 in medium containing 0.4 μg per ml hydrocortisone. EGF (10 ng per ml) is added to the medium 5 days after plating.

Serial Cultivation

Confluent cultures reached 10–16 days after plating are incubated with Ca^{2+}-, Mg^{2+}-free phosphate buffered saline (PBS) containing 0.3% trypsin and 1% EDTA, pH 7.3 for 15 min at 37° C. The attached cells are dispersed into single cells by gentle pipetting with a Pasteur pipette, and the cells are collected by centrifugation at 800 × *g* for 1 min. The packed cells are resuspended in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum and hydrocortisone and plated at not less than 30 × 10^4 translucent cells per dish. A 1-to-4 or 1-to-5 split is made when cells are subcultured into first and second passages. The cultures are fed with medium containing hydrocortisone after allowing the cells to attach overnight. EGF, 10 ng/ml, is added to the cultures on days 4–5 after plating.

Determination of Cell Growth

Cell growth is determined by direct cell count and estimation of DNA content per culture dish. After the medium is removed from the culture dish, the cells are incubated with 1.5 ml trypsin solution (0.3% trypsin + 1% EDTA in Ca^{2+}-, Mg^{2+}-free PBS, pH 7.3) for 15 min. A single cell suspension is obtained by gentle agitation of cell sheets in the trypsin solution. A small aliquot of cell suspension is placed in a hemocytometer, and the number of translucent and opaque cells (Fig. 1) is counted in triplicate in a Nikon microscope under phase contrast. An equivalence of cold 10% trichloroacetic acid (TCA) is added to a 0.5-ml aliquot of cell suspension, and the precipitate is collected by centrifugation at 800 × *g* after chilling at 4° C overnight. The DNA content is determined by the fluorometric procedure of Kissane and Robins (12).

Incorporation of [^{3}H]Histidine and [^{14}C]-Leucine Into Keratinocytes

Keratinized cells shed into the culture medium are collected by centrifugation at 800 × *g* for 1

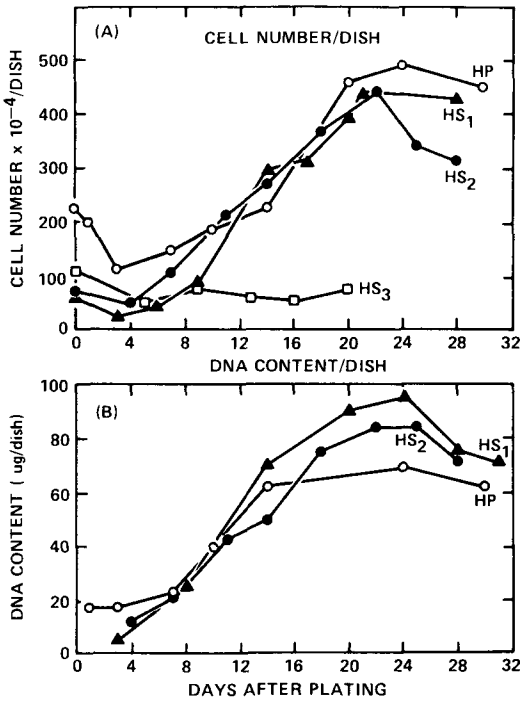

FIG. 2. Growth curves of human foreskin epidermal keratinocytes in primary (HP) and subsequent passages. Cells are plated on a thin-gel dish, maintained in Dulbecco's medium containing 20% fetal bovine serum, hydrocortisone (0.4 μg per ml) and EGF (10 ng per ml). First passage (HS_1) is subcultured on day 12 of primary culture at a 1-to-4 split. Second passage (HS_2) on day 16 of first passage at 1-to-5 split and third passage (HS_3) on day 16 of second passage at 1-to-2 split. Each point represents the mean values derived from three separate experiments.

min at 3- to 4-day intervals during the lag, proliferative and plateau phases (Fig. 2). These cells are resuspended in 1.5 ml of complete medium containing [^{3}H]-histidine (37.5 Ci per mmol), 5 μCi per ml and [^{14}C]-leucine (312 mCi per mmol), 0.5 μCi per ml; and incubated for 3 hr at 37° C. Keratinocytes remaining in the dishes are also pulsed with the same medium. After centrifugation at 800 × *g* for 1 min, released cells are suspended in 1 ml PBS, whereas attached cells are incubated with trypsin solution as described above. The cell suspensions are precipitated with an equal volume of 10% cold TCA after a small aliquot is removed for a cell count. The TCA precipitate is collected on filter paper and washed, and the radioactivity is determined as described previously (13).

Keratin Staining

Keratinized cells released into medium are collected by centrifugation at 800 × *g* for 1 min. The

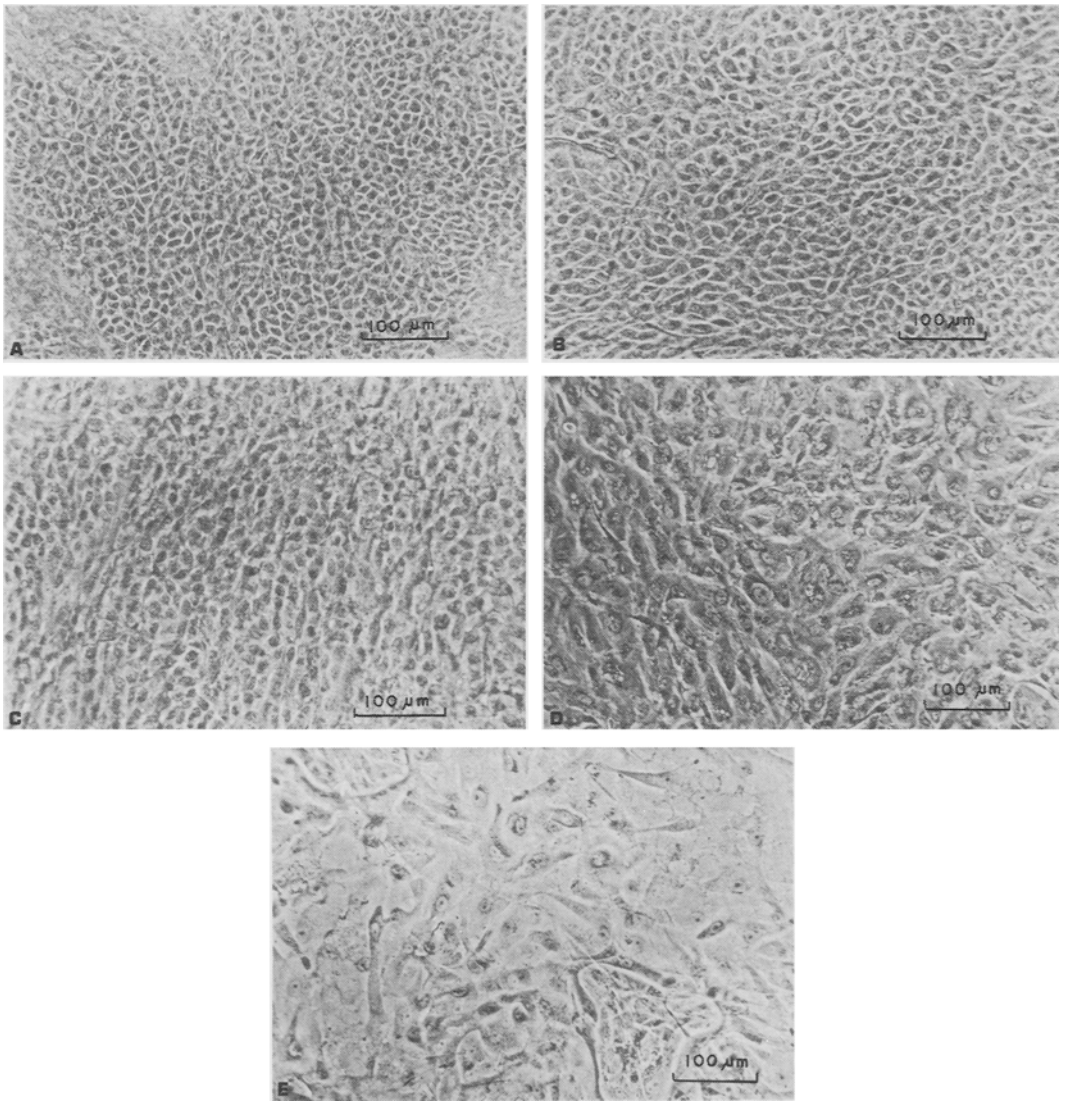

FIG. 3. Light micrograph of human epidermal keratinocytes. *A*, 12-day-old primary culture, proliferative stage; *B*, 13-day-old first passage, proliferative stage; *C*, 13-day-old second passage, proliferative stage; *D*, 10-day-old third passage, lag stage; *E*, 60-day-old primary culture, plateau phase.

TABLE 1

EFFECT OF CELL DENSITY AT PLATING ON GROWTH OF FORESKIN KERATINOCYTES IN PRIMARY, FIRST AND SECOND PASSAGE CULTURES

Passage	Plating Cell No. × 10^{-4}/Dish		Cell No. After 14d × 10^{-4}/Dish	
	Total	Translucent	Total	Translucent
Primary	203	157	358±36[a]	197±17
	101	78	187±31	94±13
	50	39	42±21	16±13
	20	16	7	1
First passage	124	104	369±63	265±66
	62	52	319±59	221±39
	43	36	282±43	202±36
	31	26	223±31	134±20
	15	13	24± 9	4± 2
Second passage	103	75	259±44	159±32
	69	50	232±17	154±15
	35	25	84± 9	37± 9
	20	15	35± 8	7± 5

[a] Mean ± SD of 3 dishes.

cells are washed once with PBS and recentrifuged, and the cell pellet is fixed in 0.1 ml 10% neutral buffered formalin (NBF) for 30 min. A drop of fixed cells is air dried on a slide. Stratum corneum from normal human skin is scraped off using the edge of a slide, fixed in NBF and air dried on a slide as described above. The cultured cells and stratum corneum are stained with Orange G-Aniline Blue according to the technique described by Ayoub and Shklar (14).

Analysis of Fibrous Proteins

Extraction of polypeptides. Stratum corneum from newborn foreskin, cultured human fibroblasts and keratinized cells released into medium are collected as described above. Soluble polypeptides in stratum corneum, keratinized cells and fibroblasts are extracted four times in 100 vol 20 mM Tris-buffer, pH 7.4, at room temperature. Insoluble proteins are collected after centrifugation at 10,000 × *g* for 10 min and extracted with 50 vol urea solution (8 M urea, 0.05 M Tris-HCl pH 9.0, 1% SDS and 0.1 M β-mercaptoethanol) for 4 hr at room temperature.

Polyacrylamide gel electrophoresis. SDS polyacrylamide electrophoresis is done using the gel system described by Steinert and Isler (15). Urea-soluble proteins are incubated for 2 hr at 37° C, and 50–100 μg is applied to the top of 9% cross-linked gels. A calibration curve of molecular weight against relative mobility is prepared using lysozyme (mol wt 14,300), β-lactoglobulin (mol wt 18,400), trypsinogen (mol wt 24,000), pepsin (mol wt 34,700), ovalbumin (mol wt 45,000) and bovine albumin (mol wt 66,000). The gels are stained in Coomassie Brilliant Blue R, for 30 min, the excess dye is washed out with 7% acetic acid

TABLE 2

COMPARISON OF THE TYPE OF CULTURE SUBSTRATE ON PLATING EFFICIENCY AND GROWTH OF FORESKIN KERATINOCYTES IN PRIMARY AND FIRST PASSAGE CULTURES[a]

Culture Substrate	Primary Culture			First Passage		
	Plating Efficiency	Cell No. × 10^{-4}/Dish		Plating Efficiency	Cell No. × 10^{-4}/Dish	
		Total	Translucent		Total	Translucent
	%			%		
Plastic	33	16	3	90	307±57[b]	221±48[b]
Collagen-coated plastic	64	286±43	179±44	89	369±54	277±49
Thin gel on plastic	73	178±20	78±11	96	346±28	246±42
2mm-Gel on plastic	65	238±20	130±12	94	250±57	163±38

[a] Cells are originally plated at 95 × 10^4 per 3.5-cm dish in primary culture and 52 × 10^4 per dish in first passage. Cell numbers after 7 days in culture (primary) and 14 days (first passage) are mean values ± SD of three dishes. First-passage cells are subcultured from cells in primary cultures maintained on thin-gel dishes.

[b] About 5% of the confluent cells are fibroblastlike.

5% methanol, and the molecular weights are determined from relative mobilities of the standard proteins.

RESULTS

Growth Characteristics

Growth curves. The growth curves of primary (HP), first passage (HS_1), second passage (HS_2) and third passage (HS_3) are shown in Fig. 2. A similarity in growth profiles is observed in HP, HS_1 and HS_2: a lag phase of 3 days during which a decrease in the number of cells attached to the culture surface occurs, a proliferative phase of approximately 19 days and a plateau phase. During the proliferative phase, total cell number increases by as much as 4-fold in HP, 17-fold in HS_1 and 9-fold in HS_2. As cells reach the plateau phase the total cell number and DNA content fall. For unknown reasons keratinocytes in HS_3 remain in the lag phase for 3 weeks and deteriorate.

Morphology. The appearance of keratinocytes under phase contrast in primary and subsequent passages during the proliferative stage is shown in Fig. 3. Cells in the growth stage are small, compact and polyhedral in primary (*A*), first (*B*) and second (*C*) passages. The cell diameter in the plane of the cell surface increases approximately fourfold in the third passage (*D*), and the cultures become less compact although the cells preserve the characteristic appearance of keratinocytes.

When keratinocytes in HP, HS_1 and HS_2 are kept in the plateau phase for longer periods of time without subculturing, the cell size continues to increase, and the cultures become less compact although they still remain polyhedral in shape (*E*).

TABLE 3

RATIO OF [^{3}H]-LEUCINE INCORPORATED INTO FIRST PASSAGE KERATINOCYTES DURING VARIOUS GROWTH STAGES

Growth Stage	[^{3}H]Histidine/[^{14}C]-Leucine	
	Attached Cells[a]	Detached Cells[b]
Lag	0.36±0.07[c]	1.36±0.28
Proliferative	0.23±0.03	1.50±0.04
Plateau	0.26±0.06	1.09±0.20

[a] Cells remaining on the culture surfaces.
[b] Cells sloughed into the culture medium.
[c] Means ± SD that were obtained from duplicate dishes on the following days: lag phase (days 3, 7); proliferative phase (days 1, 13, 17, 21, 24); plateau phase (days 28, 31, 35, 38, 45, 52).

Effect of cell density on growth. The influence of cell density at plating on primary and subsequent cultures on thin gels is shown in Table 1. A proliferative primary culture is obtained when the culture is seeded above 78×10^4 translucent cells per dish. The minimum plating density for proliferation at the first passage is 26×10^4 per dish and 50×10^4 per dish for the second passage.

Cell substrate. The influence of collagen on the growth of epidermal keratinocytes is shown in Table 2. When keratinocytes freshly isolated from split-thickness skin pieces are plated on a plain plastic surface, a plating efficiency of 33% is observed, and the cultures do not proliferate. When the cells are plated on collagen substrate (collagen-coated, thin-gel or 2-mm-gel dishes),

TABLE 4

EFFECT OF EGF AND HYDROCORTISONE ON GROWTH OF FORESKIN KERATINOCYTES IN PRIMARY-, FIRST- AND SECOND-PASSAGE CULTURE[a]

Medium	Primary[b]		First Passage[c]		Second Passage[d]	
	Cell No. × 10^{-4}/Dish	Control	Cell No. × 10^{-4}/Dish	Control	Cell No. × 10^{-4}/Dish	Control[e]
		%		%		%
Control	343±10	100	289±27	100	130±50[f]	100
EGF	403±75	117	198±28	68	128± 8	98
H-Cortisone	353±33	102	315±37	109	186±59[f]	143
EGF + H-Cortisone	414±37	120	337±35	117	393±53	302

[a] EGF (10 ng per ml) is added to cells on days 4–5 after plating and is included in the medium throughout the culture period. Hydrocortisone (0.4 μg per ml) is included in the medium at time of plating and throughout the entire culture period.
[b] Initial plating number: 100×10^4 translucent cells per dish.
[c] Initial plating number: 35×10^4 translucent cells per dish.
[d] Initial plating number: 50×10^4 translucent cells per dish.
[e] Expressed as a percentage of the control culture determined after 16–20 days in culture.
[f] About 50% of the confluent cells are fibroblastlike.

TABLE 5

OPTIMUM CONCENTRATION OF EGF FOR GROWTH OF FORESKIN KERATINOCYTES IN PRIMARY-, FIRST-PASSAGE AND SECOND-PASSAGE CULTURES

	Primary		First Passage		Second Passage	
Conc., ng/ml	Cell No. × 10^{-4}/Dish[a]	Control	Cell No. × 10^{-4}/Dish	Control	Cell No. × 10^{-4}/Dish	Control
		%		%		%
0	352±100	100	344±41	100	100±64[b]	100
1	337± 37	96	456±95	132	444±63	444
10	430± 26	122	400±55	116	406±30	406
30	403± 21	114	476±37	138	385±50	385

[a] Cell number per dish is determined on day 16–20 after plating. Hydrocortisone (0.4 μg per ml) is present in the plating medium and is added on days 4–5 after plating.
[b] About 50% of the confluent cells are fibroblastlike.

the plating efficiency increases twofold, and the cultures become proliferative. The preparation of each of these substrates has been described in detail previously (11).

In the first passage, keratinocytes attach to plain and collagen-treated plastic dishes equally well with plating efficiencies above 90%, and proliferative cultures are obtained. However, fibroblastlike contamination is frequently observed when the culture is on noncoated dishes.

[^3H]-histidine to [^{14}C]-leucine. The incorporation of [^{3}H]-leucine into first passage keratinocytes is summarized in Table 3. In cells remaining attached to collagen, the relative incorporation of histidine is low during all three stages of growth. However, when cells are released into the medium, a fourfold-to-sevenfold increase in the ratio of histidine to leucine is observed in the shed cells.

Effect of EGF and hydrocortisone. As shown in Table 4, keratinocytes in primary culture can be maintained as a proliferative culture without the addition of exogenous hydrocortisone or EGF. However, in subcultured cells, particularly in the second passage, a marked stimulation of growth (300%) is observed. When EGF is omitted, a frequent overgrowth of fibroblastlike cells is observed following the second passage.

Optimum concentration of EGF. The effect of EGF at concentrations from 1 to 30 ng per ml on cell growth is shown in Table 5. Only slight differences in the stimulation of growth at these concentrations are observed in the primary and subcultured cells.

Orange G-Aniline Blue staining. A comparison of the histology of keratinized cells scraped from the stratum corneum of skin with cells released from the culture surface is shown in Fig. 4*A,B*. When stained with Orange G-Aniline Blue, fully keratinized cells from the stratum corneum stain a characteristic orange-red (Fig. 4*A*). Cells in culture show many cells with the keratin reaction (Fig. 4*B*). Although morphologically many of the shed cells are flattened, squamous and nonnucleated, not all of the released cells exhibit the char-

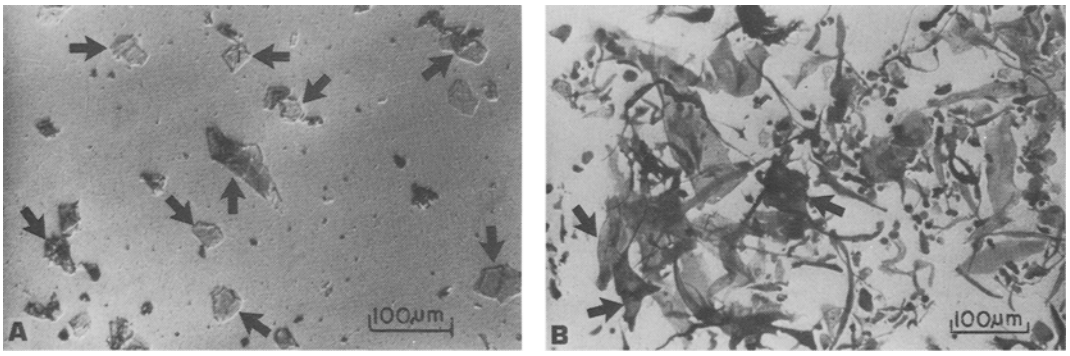

FIG. 4. Orange G-Aniline Blue staining of cells from the stratum corneum and from cell culture. *A*, Keratinocytes from the stratum corneum of normal skin; *B*, keratinocytes collected from cells released into the culture medium. *Arrows* indicate orange-red cells.

acteristic keratin stain, indicating incomplete keratinization.

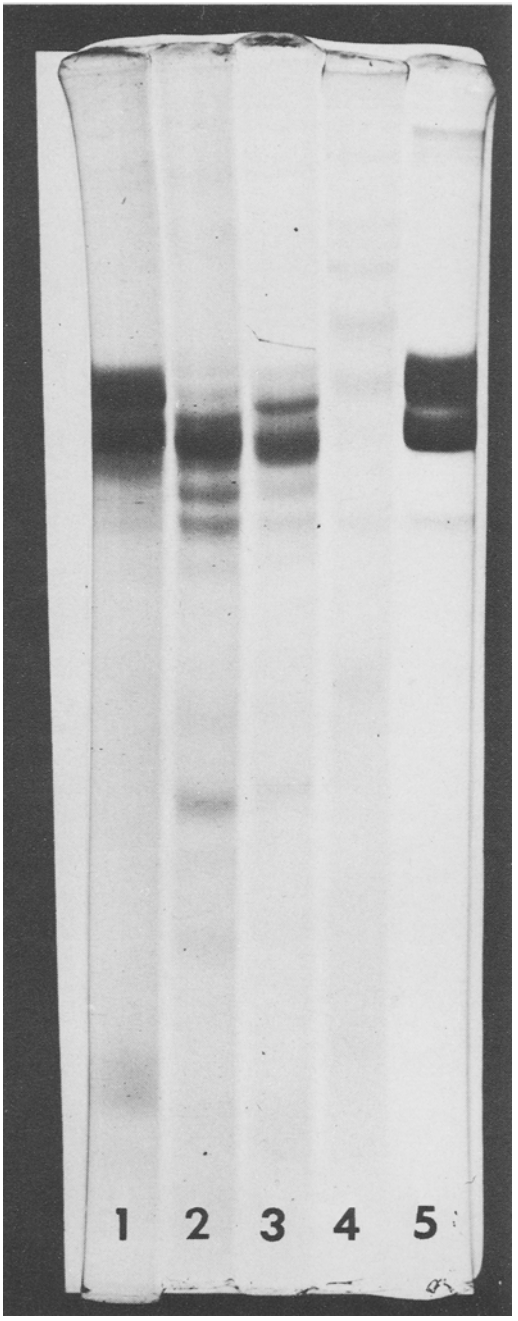

FIG. 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of fibrous proteins. 1, Stratum corneum from human newborn foreskin; 2, keratinized cells detached from culture surface; 3, cochromatography of 1 and 2; 4, cultured human fibroblasts; 5, bovine serum albumin (mol wt 66,000) and catalase (mol wt 60,000).

Gel electrophoresis of insoluble proteins. Gel electrophoresis profiles of the insoluble proteins extracted from stratum corneum, cultured human keratinocytes and fibroblasts are shown in Fig. 5. Two major protein bands extracted from stratum corneum migrate to the distances of bovine serum albumin (mol wt 66,000) and catalase (mol wt 60,000). A keratin band of 60,000, which co-electrophoreses with the isolated protein bands from stratum corneum, is evident; however, the 66,000 band is less obvious and a number of additional bands are also present. The fibrous proteins extracted from fibroblasts do not contain the 66,000 and 60,000 dalton bands.

DISCUSSION

In this report we describe a method to release large numbers of viable single keratinocytes from foreskin with a minimal contamination of other cell types present in skin. The isolated keratinocytes, when plated and grown on a collagen gel and in the presence of EGF and hydrocortisone, increase 80-fold after two serial passages (Table 6).

The ability to obtain significant numbers of viable cells from the epidermis without significant release of other cell types present in the skin depends on the ability to obtain split-thickness sections of foreskin of no more than 0.1 mm in thickness and a short exposure to trypsin. Foreskin, because of its elastic connective tissue and small size, is more difficult to cut than skin from other regions of the body. However, when an appropriate thin cut is made, an exposure time no longer than 20 min to 0.3% trypsin is required to completely separate the epidermis from the dermis and to release viable keratinocytes. Attachment and migration usually begin within 3 hr after plating.

Although keratinocytes isolated from explants of skin can be maintained as a dividing population for longer periods of time when grown in the presence of irradiated 3T3 cells as originally described by Rheinwald and Green (9), growth and serial cultivation of keratinocytes can be obtained in the absence of 3T3 or other feeder cells. Where high plating efficiencies of primary cultures and the absence of other cell types are important, the isolation of single cell suspensions and their attachment and growth on a collagen gel may be of advantage.

In these studies we have confirmed the observation of Rheinwald and Green that hydrocortisone

TABLE 6

CHANGES IN TOTAL CELL NUMBER DURING SERIAL CULTIVATION OF HUMAN EPIDERMAL KERATINOCYTES FROM FORESKIN[a]

Passage	No. of Dishes	Days in Culture	Cell No. × 10^{-4}/Dish		Total Cell No. × 10^{-4}
Primary	1	12	108[b]	260±40[c]	260
First passage	4	21	65	438±58	1750
Second passage	20	22	59	446±48	8920

[a] Four dishes of proliferative primary cells are obtained from one newborn foreskin of 1 × 2.5 cm. First passage occurred on day 12 using a 1-to-4 split, and second passage on day 16 of the first passage with a 1-to-5 split. Cells are cultured on thin-gel dishes and maintained in Dulbecco's medium containing 20% fetal bovine serum, hydrocortisone (0.4 μg per ml) and EGF (10 ng per ml).

[b] Original cell number plated.

[c] Cell number after 3 weeks in culture.

and EGF stimulate the growth of foreskin keratinocytes (10). It is however of interest that in primary cultures grown on a gel, EGF stimulates keratinocyte growth either in the presence or the absence of hydrocortisone, whereas in subsequent passages, hydrocortisone is required. The role that hydrocortisone may play in the interaction of growth factors such as EGF is unknown, but one possibility may be that this steroid facilitates the synthesis of membrane components required for the interaction of EGF with keratinocytes. In other systems dexamethasone facilitates the recovery of trypsin damage during the lag phase of growth (16,17), and Baker et al. (18) have observed that dexamethasone enhances the mitogenic effect of EGF in quiescent fibroblasts by increasing the binding ability of EGF.

The present studies demonstrate that newborn keratinocytes grown on a collagen gel in vitro retain their ability to keratinize in primary cultures and after serial cultivation. When cells spontaneously released from the surface of a confluent culture are fixed and stained with Orange G-Aniline Blue, the characteristic orange-red stain seen only in keratinized cells is obtained. Similarly, a higher ratio of [^{3}H]-histidine-to-[^{14}C]-leucine incorporation into the released cells when compared to the proliferative population is seen. Keratinocytes in vivo, during the process of maturation, increase their incorporation of histidine (19,20). The cultures also continue to synthesize two proteins resembling the major keratin proteins described by Steinert and Yuspa (21). Further study of the biochemistry of the retained and released populations of cells on a collagen gel may provide an additional model for the factors that regulate the differentiation of keratinocytes in situ.

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The authors acknowledge the technical assistance of Ms. Mary Ellen Charlton.