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Human epidermal cell cultures: Growth and differentiation in the absence of dermal components or medium supplements

(skin *in vitro*/growth kinetics)

M. EISINGER, JI SOO LEE, J. M. HEFTON, Z. DARZYNKIEWICZ, J. W. CHIAO, AND E. DE HARVEN

Memorial Sloan-Kettering Cancer Center, New York, New York 10021

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ABSTRACT **Human epidermal cells grew and differentiated *in vitro*, provided that the pH of the culture medium was at 5.6–5.8, the seeding density was optimal (≈2.5 × 10^5 cells per cm^2), and the incubation temperature was maintained at 35–37°C. Under these conditions, epidermal cells from many different skin locations grew to confluency within 15–20 days and formed multi-layered sheets whose differentiated structure resembled that of the full depth of skin epidermis. Cell proliferation and differentiation did not require a feeder layer, a collagen substrate, a high concentration of fetal bovine serum, or added hormones. The sheets of differentiated epidermal cells could be dissociated from the plastic surfaces of the tissue culture flasks. The use of such cultured cells for wound dressing is proposed.**

The apparent dependence of human epidermal cell growth and differentiation *in vitro* on the presence of a mouse 3T3 cell feeder layer (1) or collagenized substrates (2, 3) has led to the supposition that epidermal cell growth and differentiation *in vivo* may depend on dermal cell products. Epidermal cell growth has also been observed in the absence of such supports, but then the medium had to be supplemented by hormone preparations such as pituitary extracts and a high concentration (20%) of fetal bovine serum (4). Thus, it has been difficult to study the role of cell-to-cell interactions, and the influences of substances such as hormones, chalones, etc. on the process of epidermal cell differentiation *in vitro* because of the obligatory presence of nonepidermal components in the system. We now present evidence tht epidermal growth *in vitro* demands neither dermal elements nor special nutrients, provided conditions of pH, seeding density, and incubation temperature have been optimized.

MATERIALS AND METHODS

Tissue Culture. More than 200 human skin specimens from different skin locations (scalp, face, neck, arms, breast, foreskin, legs, and trunk) as well as skin shavings from burn victims and cadavers (4–6 hr after death) have been successfully cultivated. Both full and split-thickness skin have been used. Skin samples were freed from fatty tissue and washed in minimal essential medium (GIBCO) with Earle's salts, containing, per ml, 1000 units of penicillin, 1 mg of streptomycin, and 2.5 μg of Fungizone for 30 min, followed by two washes, of 10 min each, in the same medium. Two additional washes 10 min each were in minimal essential medium with 1/10th the concentration of antibiotics. Discs of tissue were cut from the epidermal side of each specimen with sharp curved scissors, including as little dermis as possible. After washing in 0.02% EDTA (Sigma), the pieces were transferred to 0.25% trypsin (1:250, Difco) at 4°C for 12–15 hr. Subsequent to this incubation, the cut pieces were transferred to a fresh dish and the epidermis of each piece was detached from its dermis with fine forceps. Isolated epidermal samples were pooled in a trypsin/EDTA solution (5) [8 g of NaCl, 0.4 g of KCl, 1 g of dextrose, 0.58 g of $NaHCO_3$, 0.5 g of trypsin (Difco, 1:250), 0.2 g of Na_2EDTA, and distilled water to 1000 ml] and teased apart with forceps. The resultant suspension was transferred to a fresh vessel in which cell clumps were dissociated by gentle pipetting. Fresh trypsin/EDTA solution was added to the remaining piece of epidermis in the original dish, and the process was repeated until essentially only stratum corneum remained. The dermis adherent to each section of epidermis was either discarded or else used to initiate fibroblast cultures. The pooled epidermal cell suspensions, which were composed mainly of single cells, were filtered through three to four layers of sterile gauze to remove clumps and sheets of fully keratinized cells. These separated cells were then collected in fetal bovine serum, concentrated by centrifugation (10 min at 180 × *g*), and resuspended in medium [Eagle's minimal essential medium plus nonessential amino acids, 2 mM L-glutamine, hydrocortisone at 0.4 μg/ml (1), 10% fetal bovine serum, penicillin, streptomycin, and Fungizone]. The pH was adjusted to 5.6–5.8 with 1 M HCl and the cells were seeded at a density of 2.5 × 10^5 cells per cm^2. Repeated measurements of the pH of the cultures during cell growth showed fluctuation within the range of 5.6–6.0. Incubation was at 36°C in water-saturated air containing 5% CO_2.

Identification of Different Cell Types. Cells before and at different time intervals after initial plating were treated with trypsin/EDTA solution; cytocentrifuge preparations were fixed and stained with hematoxylin/eosin (6) for ATPase (7), with dopa stain (8), and with rhodamine B (9).

Single-cell suspensions were examined, immediately after trypsinization and after a 24-hr incubation period, for the presence of membrane receptors for IgG or complement. Complement receptors were detected by using guinea pig erythrocytes sensitized with rabbit IgM antibodies and mouse serum as a source of complement. IgG surface receptors were detected by using guinea pig erythrocytes coated with rabbit IgG (10). To test for the presence of lymphocytes, assays for membrane-bound immunoglobulins (11), rosetting with sheep

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erythrocytes (11), and reactivity with anti-T cell antiserum (12) were performed.

Enrichment for Langerhans Cells. Complement or IgG receptor-bearing cells were separated from nonrosetting cells by Ficoll/Hypaque gradients (13).

Electron Microscopy. For flat embedding, cells were grown on a surface of polymerized Epon. Postfixation was done with 2% osmium tetroxide in 0.1 M sodium cacodylate buffer. After ethanol dehydration, infiltration with propylene oxide, and Epon embedding, thin sections, cut with diamond knives, were double stained with uranyl acetate and lead citrate. An Elmiskop IA electron microscope, operated at 80 kV and at direct magnifications ranging from 1000 to 20,000, was used throughout this study.

Flow Cytometry. Cells were stained with the metachromatic fluorochrome acridine orange as described (14), under conditions in which DNA and RNA stain differentially (14, 15). The differential staining is due to the fact that the dye intercalates into double-stranded DNA and in this form fluoresces green (530 nm). Electrostatic binding of acridine orange to single-stranded RNA involves subsequent dye–dye interactions resulting in red metachromasia (640 nm). Denaturation of RNA prior to staining (15) is required to ensure that nearly all cellular RNA is stained metachromatically (16). Specificity of staining was evaluated by preincubation of cells with RNase or DNase, as described (17). Over 95% of green fluorescence was sensitive to DNase and over 85% of red fluorescence was sensitive to RNase. Cell staining with ethidium, or propidium, bromide (after RNase treatment) resulted in histograms of DNA distribution that were identical to those based on green fluorescence after staining with acridine orange. Fluorescence of individual cells was measured in an FC 200 cytofluorograph (Ortho Diagnostic Instruments, Westwood, MA) interfaced to a Data General microcomputer. All data were based on 10^4 cells measured per sample; cell doublets and higher aggregates were excluded (14–17).

RESULTS AND DISCUSSION

Microscopic examination of 0.5-μm sections of epidermal samples after the trypsinization step (Fig. 1*A*) showed that this procedure efficiently separated epidermis from dermis, as originally described by Medawar (18), and that cells of the uppermost layer were separated from one another. The upper layers of fully keratinized cells remained as sheets whose cells were strongly adherent to each other. Transmission electron microscopy of thin sections through pellets of cells centrifuged from the suspensions used to initiate tissue cultures revealed that only a small fraction could be identified as basal layer cells. The rest of the well-preserved cells were keratinocytes at different

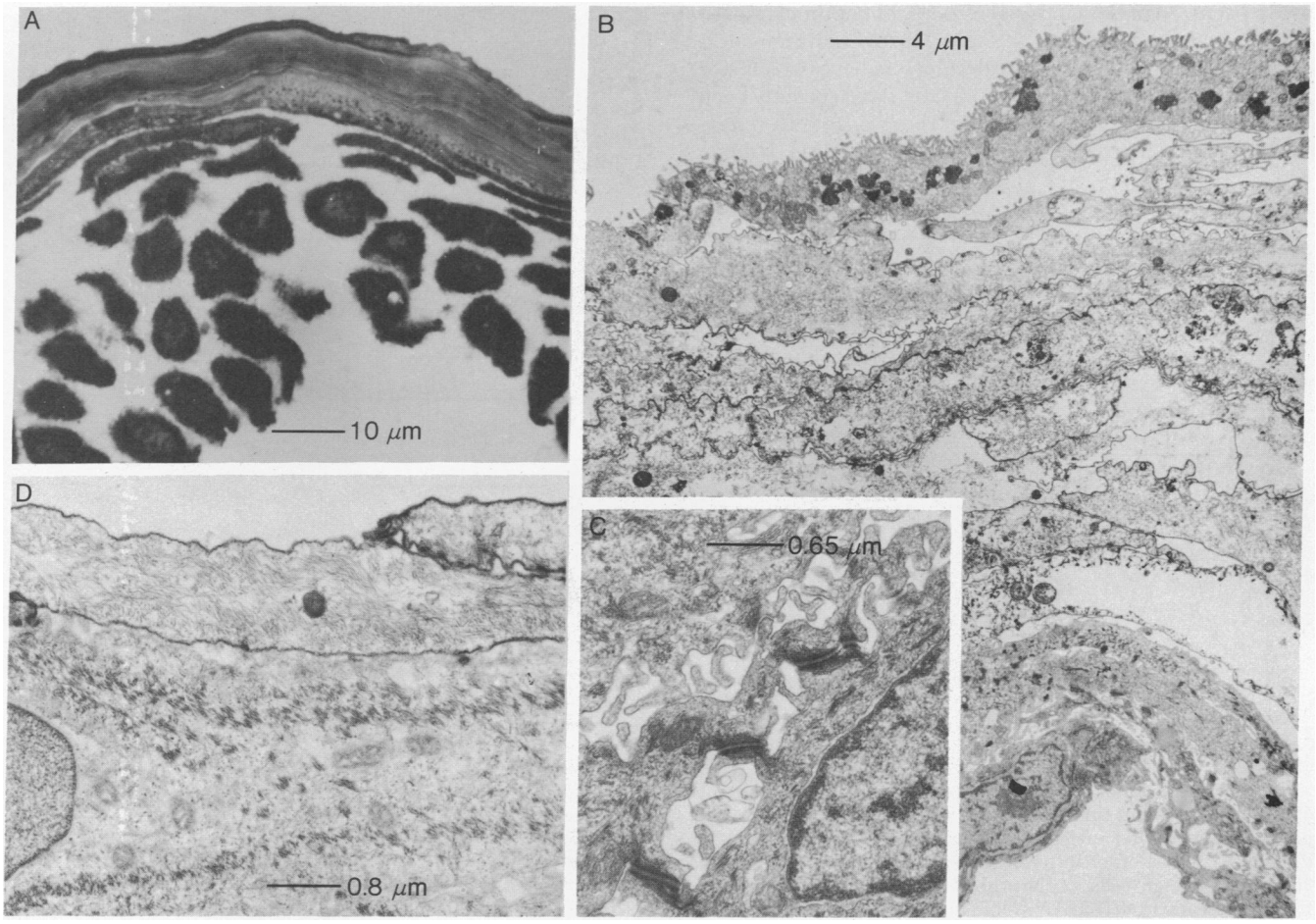

FIG. 1. (*A*) Light micrograph of section of epidermis split by the trypsin method described in the text. (*B*) Electron micrograph of thin section of cell layer peeled from a tissue culture initiated 18 days earlier from an epidermal cell suspension. Note villous surface. (*C*) Detail of basal cell and its attachment to a cell of the adjacent layer. Note desmosomes. (*D*) Electron micrograph of thin section of a second passage cell culture 8 days after seeding. Note surface thickening and many tonofilaments.

stages of differentiation.

When such cell suspensions were placed in tissue culture vessels, moderate clumping occurred; the clumps then settled and attached individually to the plastic substrate within 12–24 hr. At this stage, staining for ATPase (G. Potter, personal communication) revealed stained cells at the attachment site. A similar proportion of cells could be identified as possessing IgG for complement surface receptors (10, 11). Such characteristics render them identifiable as Langerhans cells (19). In many instances, long processes have been observed extending from these presumed Langerhans cells, sometimes forming connections with similar processes originating from other clumps. However, when 97% of the Langerhans cells were removed by rosetting with IgG antibody-coated erythrocyes and separation by a Ficoll/Hypaque gradient, cells remained that were still capable of attachment and of developing long processes. Whether these were residual Langerhans cells or whether some epidermal keratinocytes were capable of putting out long processes after attachment is uncertain.

When cultures were seeded with the optimum cell numbers ($2.5–4 \times 10^5$ cells per cm^2; minimum 1×10^5 per cm^2), growth took place toward the periphery of each clump, and the space between clumps eventually became filled with a contiguous sheet of epidermal cells. When fewer cells were seeded confluency was not achieved; cells either did not grow or grew into small colonies that did not coalesce.

Many attempts were made to cultivate cell suspensions at other pH values. Cells failed to grow at any pH above 7.0. Some growth occurred in the range pH 6.0–7.0, but optimal growth was achieved only at pH 5.6–5.8. The low pH requirement is in accordance with physiological values *in vivo* which, for human skin, are between 4.2 and 6.2 (20). It has recently been shown that a suitable temperature for the *in vitro* differentiation of neonatal mouse epidermal cells is 33°C (21). Human cells do not grow at this temperature; we have found the optimum to be somewhat higher (35–36°C).

Seventy to 80% of the cells originally plated became attached to the substrate in 24–48 hr. The majority of this population, consisting of more or less differentiated cells, detached during the next 4–5 days, and was removed when the medium was changed (see Fig. 2). Growth was initiated by only 20% of the cells originally plated, a fraction consisting predominantly of basal layer cells, some ATPase-positive cells, and a few melanocytes. After the initial decline there was a progressive increase in the number of cells from 4 to 13 days, when the numbers leveled off. After 18 days, the numbers began to decline once more, due to further detachment of differentiated cells. The period during which DNA synthesis took place was investigated by labeling with [^{3}H]thymidine (0.1 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) for successive 24-hr periods, followed by autoradiography. The percentage of labeled nuclei remained low ($\leq$8%) during the first 4 days but increased by day 8 to 30% and reached 68% by day 13, corresponding to the increase in cell numbers seen in Fig. 2.

Examination of cytocentrifuge preparations of epidermal cell cultures at different intervals after seeding showed that not only DNA synthesis but also cell multiplication had taken place. The proportion of cells in mitosis found (4 days, < 1/100; 8 days, 4/1000; 12 days, 11/1000; 16 days, 4/1000) again corresponded to the increased cell numbers shown in Fig. 2.

Further evidence of cell multiplication was obtained when cultures were examined by flow cytometry. Frequency distribution histograms of DNA content of individual cells from the representative 13-day old culture, as seen in Fig. 2*C*, indicated that 76% of the cells were in G_1 phase, 12% in S phase, and 12% in G_2 + M phase. Parallel cultures treated with Colcemid for 24 hr showed that 44% of the cells were in G_2 + M (Fig. 2*D*), thus indicating that 32% of the cells had completed their cell

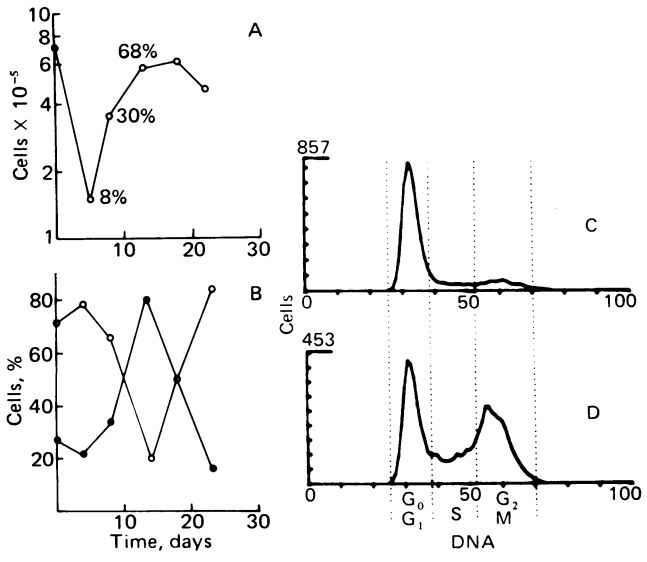

FIG. 2. Behavior of epidermal cells in culture. (*A*) Total number of cells after different times in culture. Figures beside graph indicate percentage of labeled nuclei after 24-hr incubation with [^{3}H]thymidine. (*B*) Proportion of different cell types after various times in culture. ●, "Basal" cells; ○, the other "differentiated" cells (for definitions, see text). (*C*) DNA frequency histogram indicating the proportion of cells in different phases of the cell cycle after 13 days in culture. (*D*) DNA frequency histogram of cells after 13 days in culture; 0.1 μg of Colcemid per ml was added 24 hr prior to harvest.

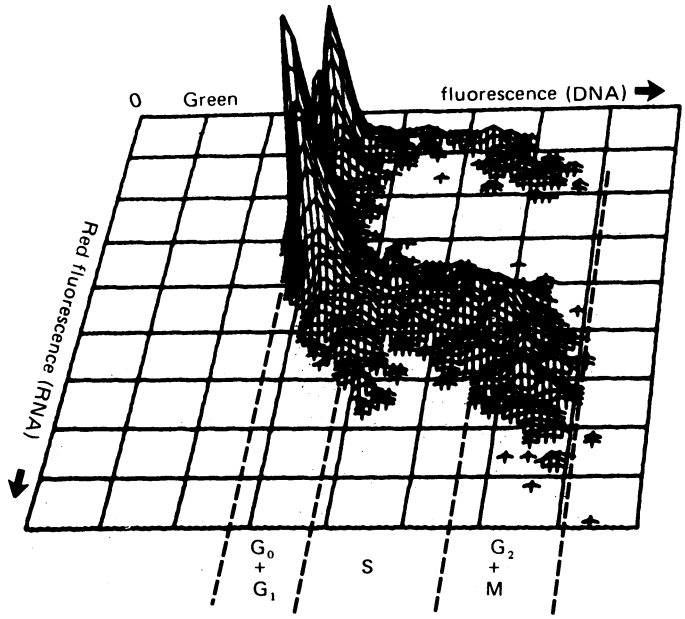

FIG. 3. Two-parameter frequency histogram of acridine orange-stained cells from a 14-day-old culture. The histogram illustrates the intracellular content of DNA and RNA as seen during the proliferative phase of cell growth. DNase-sensitive green fluorescence allows discrimination of cells in G_0/G_1, S, and G_2/M phases of the cell cycle. There are two cell subpopulations differing markedly in their RNase-sensitive red fluorescence. Peak height is 110 cells; 10,000 cells are represented altogether.

cycle and were arrested in mitosis during this 24-hr period. In repeated experiments, the number of cells in the S phase varied from 9 to 18% during the period of maximal cell proliferation in culture (10–16 days).

Fig. 3 illustrates two-parameter (DNA and RNA) frequency distribution histograms of cells during the proliferative phase. The characteristic feature of these cultures was the presence of two cell populations, markedly differing in RNA content. The majority of cells had the high RNA content and the DNA–RNA distribution generally characteristic of exponentially growing cells (15); however, a minority population with lower RNA values was present in all epidermal cultures. This minority population was composed primarily (over 90%) of cells with a G_0/G_1 DNA content but also contained cells whose DNA values were characteristic of S and G_2/M phases.

Care was taken during trypsinization to dissolve broken and dead cells and to measure only viable cells. Thus, the finding of a cell population with a low RNA content was not due to the loss of RNA from broken cells or isolated nuclei. These low-RNA cells may represent a noncycling quiescent subpopulation, because in a variety of other cell systems, noncycling quiescent cells are characterized by a low RNA content in comparison with their cycling counterparts (15–18).

Cytocentrifuge preparations of cells, both from human skin cell suspensions and from cultures of epidermal cells at different intervals after seeding, repeatedly showed the presence of small basophilic cells ($\leq$14-μm diameter) with large nuclei and a small rim of cytoplasm. Autoradiography showed this cell type to be the DNA-synthesizing cells previously mentioned. They have, therefore, been designated "basal" in contrast to "other" mostly larger cells ($\geq$30-μm diameter) that had smaller nuclei. In suspensions of epidermal cells from skin, 27% were "basal" cells. After 3–5 days in culture, 20% of the cells were still of the basal type, and 80% were differentiated (Fig. 2*B*). Most of the latter became detached and were removed when the medium was changed, so that after 10 days in culture basal and differentiated cells were present in equal numbers. On the 13th day, small basophilic cells predominated (80%), but there were also some differentiated cells (20%). At this time, cells could be subcultured after trypsinization, and in subsequent passages the cultures underwent the same cycle of multiplication followed by terminal differentiation as in the primary cultures. Epidermal cells from human foreskin have been successively transferred five times in this manner. In the case of breast skin, cells could not be subcultured more than three times, unless the medium contained cyclic AMP or cholera toxin (22) which stimulated the growth of cells and extended their life span in culture (unpublished data).

From the 13th to the 24th day of primary culture, the population of basal cells declined to 20% and large cells with pyknotic nuclei appeared, as well as cells that had lost their nuclei completely. It is, therefore, suggested that the first phase of the culture is one in which basal cell multiplication predominated. This ceased at about day 13 and was followed by a wave of differentiation.

Confluent monolayers stained with rhodamine B (9) showed strips of keratinizing cells after 15–20 days in culture, 2–3% ATPase-positive cells, but no cells possessing IgG or complement surface receptors. In addition, occasional melanocytes could be seen staining positively with dopa (8).

After having reached confluency, cultures regularly became multilayered, the lowermost layer consisting of small oval cells. The second layer usually had a regular pavement-like mosaic structure. Phase contrast microscopy of succeeding layers showed that their nuclei tended to be pyknotic. Finally, at the surface, long squames of keratinized cells could be seen, loosely attached to the rest of the culture.

The primary cultures from skin specimens grew into multilayered structures in 15–20 days after seeding. Electron microscopic examination of thin sections of the cell layer peeled from a tissue culture vessel after 18 days in culture showed approximately 11–13 layers of cells (Fig. 1*B*). To be sure that the specimen was properly oriented, multilayered structures were grown directly on Epon. The bottom layer consisted of cells morphologically resembling the basal layer cells seen in skin *in vivo*. Such cells had large nuclei, two nucleoli, and marginated chromatin. Mitochondria, free ribosomes, and rough and smooth endoplasmic reticulum were present, but very few microfilaments were detected (Fig. 1*C*). The cells of the bottom layer were attached to the next by many prominent desmosomes (Fig. 1*C*). This layer and more superficial ones consisted of more differentiated cells, characterized by flattened shape, prominent tonofilaments, desmosomes, keratohyalin granules, and membrane-bound granules. The uppermost layers consisted of cells showing an apparent thickening of the nuclear envelope, as well as the frequent absence of cytoplasmic granules. Many cells in the upper layers exhibited "cornified envelopes" (23); this cornification was reflected in an apparent increase in the electron density of the plasmolemmae (Fig. 1*D*). Finally, occasional cells, still with surface microvilli, were seen in the upper layers, presumably from areas in the culture where keratinization was not complete.

Confluent multilayered sheets of epidermal cells (18–21 days after initial seeding) could be removed from plastic surfaces by using adherence to a transfer medium (Biobrane, Woodroof Laboratories, Santa Ana, CA; pig skin, Genetic Laboratories, St. Paul, MN; or human skin), and applied to a wound (unpublished observations). It is obviously important to test the applicability of tissue cultured epidermal cell sheets for wound treatment.

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