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Research Article

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Human Keratinocyte Culture

Identification and Staging of Epidermal Cell Subpopulations

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Abstract

Stratification of human epidermal cells into multilayered sheets composed of basal and suprabasal layers (resembling the stratum germinativum and stratum spinosum of the epidermis) was studied in a dermal component-free culture system. Although no stratum corneum developed in vitro, this culture system provided a method to study early events in human keratinocyte differentiation. Multiparameter flow cytometric analysis of acridine orange-stained epidermal cells from these cultures revealed three distinct subpopulations differing in cell size, RNA content, and cell cycle kinetics. The first subpopulation was composed of small basal keratinocytes with low RNA content and a long generation time. The second subpopulation consisted of larger keratinocytes, having higher RNA content and a significantly shorter generation time. Finally, the third subpopulation contained the largest cells, which did not divide, and represent the more terminally differentiated keratinocytes. This in vitro approach provides discriminating cytochemical parameters by which the maturity of the epidermal cell sheets can be assessed prior to grafting onto human burn patients.

Introduction

Cultures of human epidermal cells have been successfully initiated using both explants and enzymatic digests (single-cell suspensions) of adult skin (1, 2). Improvements of these early methods involved culture of epidermal cells on irradiated 3T3 fibroblast feeder layers, collagen matrices, and use of fibroblast-conditioned medium (3–5).

Recently, culture systems were developed that support growth of epidermal cells in the absence of added dermal components (6, 7). To gain a better understanding of keratinocyte proliferation and differentiation in the dermal-free culture system, multiparameter flow cytometric (FCM)[1] techniques were utilized to analyze population kinetics at various times after the initiation of culture (7, 8) using cellular RNA content as a discriminator of cell cycle traverse (9, 10). At least two distinct subpopulations could be cytochemically resolved based on total cellular RNA content. Both subpopulations defined as having either low or high cellular RNA content contained cells in the G_1, S, and G_2 + M phases of the cell cycle. As part of the overall effort to develop optimal conditions for culture of human epidermal cells suitable for grafting on burn patients, the kinetic state of the low- and high-RNA content subpopulations were examined as a function of culture growth status and stage of differentiation.

Methods

Culture of human epidermal cells

Specimens of split-thickness skin (obtained from cadavers 1–3 d after death) were collected with a Goulian hand dermatome and immersed in sterile Eagle's minimum essential medium ([MEM] Gibco, Grand Island, NY) with Earle's salts containing penicillin (100 U/ml), streptomycin (0.1 mg/ml), and fungizone (2.4 μg/ml) (Gibco). Samples were stored at 4°C, washed three times in MEM with antibiotics, then incubated in a solution of 0.5% trypsin (Difco Laboratories, Detroit, MI, 1:250) in Ca^{++} and Mg^{++} free phosphate-buffered saline ([PBS] Gibco) for 90 min at 37°C. Single-cell suspensions of epidermal cells were prepared by vigorous stirring in a solution of 0.25% deoxyribonuclease I (DNase I; Sigma Chemical Co., St. Louis, MO) and 1% fetal bovine serum in PBS and filtered through sterile gauze; FBS was added to the cell suspensions to neutralize trypsin activity. After centrifugation and resuspension in complete culture medium (MEM, 20% fetal bovine serum, 2 mM L-glutamine, hydrocortisone [0.5 μg/ml], penicillin [100 U/ml], streptomycin [0.1 mg/ml], and fungizone [0.25 μg/ml]), the viability of epidermal cells prepared in this manner was determined to be 90–95% by trypan blue dye exclusion. Plastic tissue culture flasks containing 2×10^5 epidermal cells/cm^2 were incubated at 37°C in a humid 95% air/5% CO_2 environment; the medium was changed every third day.

Preparation of cultured keratinocytes for morphologic analysis

During the initial phase of culture the keratinocytes grew as small colonies which were examined by phase-contrast microscopy. The cultures generally became confluent by the seventh day after plating. At this time, and at weekly intervals thereafter, cells could be lifted from the plastic substratum as intact sheets using the enzyme dispase (Boehringer Mannheim, Federal Republic of Germany). Briefly, the medium was decanted off and the cultures were washed three times with sterile PBS. A 0.5% solution of dispase was added to cover the entire cell sheet (50 ml of dispase/150-cm^2 flask) for incubation at 37°C. After 20 min the cultures detached from the plastic as intact sheets and the sheets (which were pliable) were placed into sterile petri dishes. Dispase was removed by careful washing three times with PBS and the cells fixed by dropwise addition of 10% buffered formalin. Fixed sheets were embedded in paraffin, sectioned and stained with hematoxylin-eosin for microscopic examination.

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1. *Abbreviations used in this paper:* AO, acridine orange; FALS, forward-angle light scatter; FCM, flow cytometry; MEM, minimum essential medium.

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Immunocytochemistry

Sections of paraffin-embedded, human full-thickness skin and confluent sheets of cultured epidermal cells were cleared in xylene, rehydrated in a graded series of alcohol rinses, and reacted with monoclonal antibodies I/2 (11, 12) and EL-2 (13). Indirect immunocytochemical staining methods have been previously described in detail (14). I/2 is reactive with basal and suprabasal keratinocytes but not (or minimally so) with keratinized epithelial cells (see below). EL-2 is specific for keratinocytes of the basal layer of epidermis (13).

Cell cycle

Stathmokinetic examination of the cell cycle kinetics of low- and high-RNA keratinocyte subpopulations (15, 16). Cell cycle kinetics of cultured keratinocytes were examined both during peak growth (days 7–11) and at plateau phase (days 12–21) of culture. The mitotic inhibitor, colcemid (Gibco) was chosen as the stathmokinetic agent. Dose-response experiments indicated 0.05 μg/ml to be optimal for mitotic arrest and further experiments were carried out using this concentration of colcemid. A total of six experiments were carried out during peak proliferation (days 7–11) and three experiments during the plateau phase of growth in culture (days 12–21). Experiments were carried out in two ways: (*a*) Colcemid was added to a series of keratinocyte cultures at time 0 h. At hourly intervals of up to 8 h, cultures were trypsinized and harvested. Because it is known that mitotic cells have a tendency to round up and lose their attachment to plastic, care was taken to harvest both the floating and nonfloating cells. Cell culture aliquots were stained with acridine orange (AO; Polysciences, Inc., Warrington, PA) and measured by FCM (see below). Control cultures which had not received colcemid were also measured in parallel; (*b*) colcemid was added to keratinocyte cultures at hourly intervals for 8 h (−8, −7, −6 h, etc.). After the eighth hour, all of the cultures were harvested and measured by FCM. The DNA distributions were determined by gated analysis of low-RNA ("A") and high-RNA ("B") subpopulations and the rate of entry into G_2 + M determined.

Simultaneous staining of intracellular RNA and DNA by acridine orange (AO) (15–18). Human keratinocyte suspensions (0.2-ml aliquot containing 1–4 × 10^5 cultured cells in complete culture medium) were mixed with 0.4 ml of 0.08 N HCl, 0.15 N NaCl, and 0.1% Triton X-100 (Sigma Chemical Co.) at 4°C. Cells were stained 30 s later by addition of 1.2 ml of a solution containing 0.2 M Na_2HPO_4, 0.1 M citric acid buffer, pH 6.0, 1 mM disodium EDTA, 0.15 N NaCl, 6 μg/ml AO. Under these conditions, interactions of the dye with DNA resulted in green fluorescence with a maximum emission of 530 nm (F530) whereas interactions with RNA gave red metachromasia at 640 nm (F640); the intensities of these reactions are proportional to the DNA and RNA content, respectively (18). Specificity of staining was evaluated by treatment of cells with RNase A (Worthington Biochemical Corp., Freehold, NJ) or with DNase I as previously described (15–18).

Fluorescence measurements and cell sorting

Fluorescence of individual cells was measured in the FC200 cytofluorograf interfaced to a Data General Minicomputer or in an Ortho System 50H cell sorter (Ortho Diagnostic Instruments, Westwood, MA). Fluorescence signals were generated by each cell as it passed in single file fashion through a 488-nm argon ion laser beam. The red and green fluorescence emissions from each cell were separated optically and the integrated values of the pulses were quantitated by separate photomultipliers. Background fluorescence was automatically substracted. 10^4 cells were counted per sample. Forward angle blue light scatter was used to measure cell size. Keratinocytes were sorted using a Coulter Epics V cell sorter (Coulter Electronics Inc., Hialeah, FL) based upon their RNA/DNA content.

Results

Within 18 h of plating, human epidermal cells exhibited attachment frequencies of 30–40% in this culture system. The majority of these adherent cells were small and rounded, consistent with an origin from the basal layer of epidermis (see below). Transfer of the nonadherent cellular fraction to new flasks did not result in further adherence, suggesting that the ability to attach to plastic was not a function of the initial seeding density.

A basal layer-specific monoclonal antibody, EL-2, was used to determine what proportion of the 18-h adherent cell fraction expressed this basal cell-specific marker. Approximately 95.9±0.05% of the adherent cell population stained positively with EL-2 (Fig. 1). Thus the initial keratinocyte cultures were composed predominantly (>95%) of cells with the morphologic and immunochemical characteristics of epidermal basal cells.

Histologic appearance of cultured epidermal cells

During the early days of growth in culture, the keratinocytes proliferated as small colonies, which coalesced into confluent sheets of cells within 7 d of seeding. At this time the cultures could be lifted from the plastic as intact sheets (Fig. 2). Histologic sectioning of a 7-d-old epidermal cell sheet revealed such cultures to be between one and two cell layers thick (0.04 mm, Fig. 2 *A*). These cultures subsequently became more highly stratified and typically attained thicknesses ranging between 10 and 15 cell layers (0.1–0.2 mm; Fig. 2 *C*), similar to stratification observed in vivo. During the latter stage of growth (days 14–21), the cultures were composed of small rounded basal cells attached to the plastic substrate and suprabasal layers consisting of enlarged, irregularly shaped vacuolated cells resembling the stratum

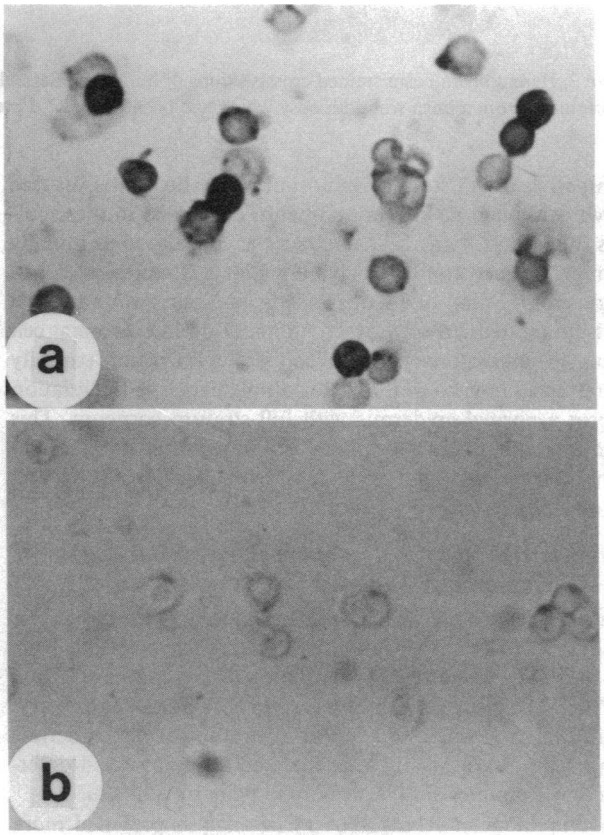

Figure 1. Immunoperoxidase staining of human epidermal cells using a basal cell-specific monoclonal antibody EL-2. Human epidermal cells were seeded onto coverslips; 18 h after plating, the coverslips containing the adherent cell fraction were treated as follows: (*a*) adherent cells were stained with EL-2, (*b*) negative control. ×110.

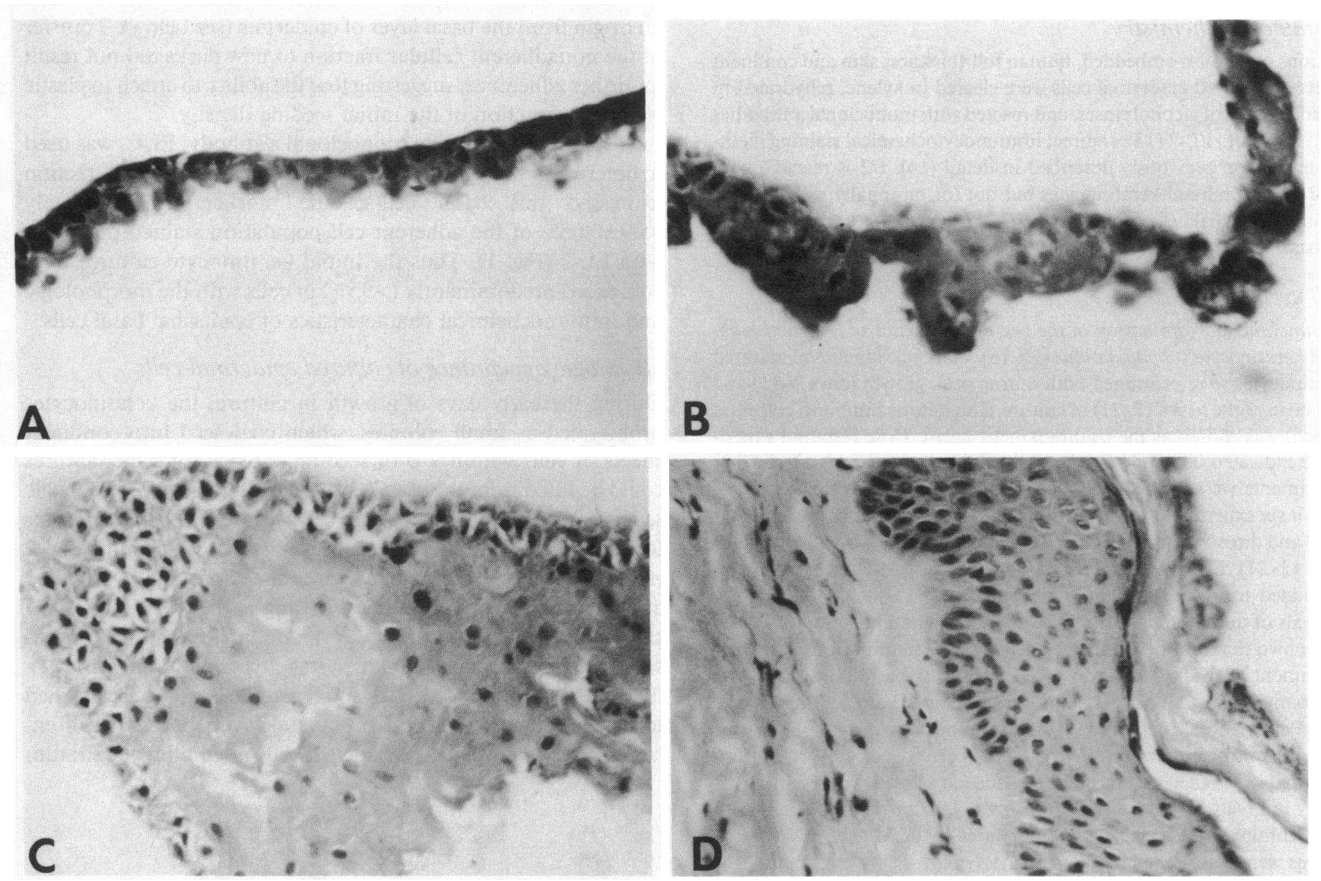

Figure 2. Hematoxylin-eosin stained cross-sections of human epidermal cell sheets after (*A*) 7 d, (*B*) 14 d, and (*C*) 21 d in culture. (*D*) Cross-section taken from a burn wound biopsy which had been grafted 7 d previously with a 21-d-old cultured epidermal cell sheet.

germinativum and stratum spinosum of in situ epidermis (see below). Although no stratum corneum developed in these cultures (Fig. 2 *A–C*), the cells retained the capacity to terminally differentiate once grafted in vivo (7). Fig. 2 *D* represents a histologic cross-section of a biopsy site from a burn wound which had been grafted 7 d previously with a 21-d-old epidermal cell culture. The keratinocytes that had proliferated and partially differentiated in vitro were able to complete their differentiation to form a normal epidermis with full stratum corneum. This laboratory and others have previously shown that cultured epidermal cell sheets can be used as grafts that provide healing and coverage of a variety of wounds (7, 19).

Identification of keratinocyte subpopulations during growth and differentiation in culture

Monoclonal antibody I/2 has been shown to stain the basal layer of the epidermis intensely and the suprabasal layer weakly (11). This antibody was unreactive with the stratum corneum (Fig. 3 *a*). The I/2 staining pattern observed in a 21-d-old keratinocyte culture was the same as that observed in the normal epidermis (Fig. 3 *b* an *c*), confirming that these cultures grow and differentiate in a manner similar to normal epidermis in situ.

Multiparameter RNA/DNA FCM analysis provided more detailed information regarding the individual subpopulations of keratinocytes during their growth and partial differentiation in vitro.

Initial phase of growth in culture. The adherent cell population consisted of small, rounded basal cells with G_1 DNA content and relatively uniform low RNA content (A compartment; Fig. 4 *A*). During initial growth in vitro, a number of the low-RNA A compartment cells increased in RNA content (Fig. 4 *B–D*). The proportion of cells recruited from the low-RNA A compartment into the high-RNA B compartment increased from ~5% after 24 h in culture to almost 50% by day 5 in culture (Table I). An examination of the DNA distribution of the A and B subpopulations indicated that with time more and more cells entered the S + G_2 + M phases of the cell cycle. Cultures, in which the keratinocytes did not undergo this initial transition from the low-RNA A compartment to the high-RNA B compartment, grew as isolated colonies that never coalesced or stratified and were thus unsuitable for use as grafts. Although the high-RNA B subpopulation, in the early phase of growth, was derived from and continuous with the low-RNA A subpopulation, these two subpopulations became independent of each other with time (see below).

Phase of rapid proliferation in culture. Generation of a maximum number of cycling (S + G_2 + M) cells occurred between days 7 and 11 of culture. Three subpopulations of cells could be distinguished based upon RNA content at this time (Fig. 5). The low-RNA A and high-RNA B compartments are the same as described above. During this growth stage, however, these subpopulations appeared to be independent of each other with few transitional cells in between. In addition, a third compartment of cells (6.7–9.3% of total), designated "C" was observed. This cluster extended from the G_1 phase of the B subpopulation and consisted of cells with no distinct S + G_2 + M phases.

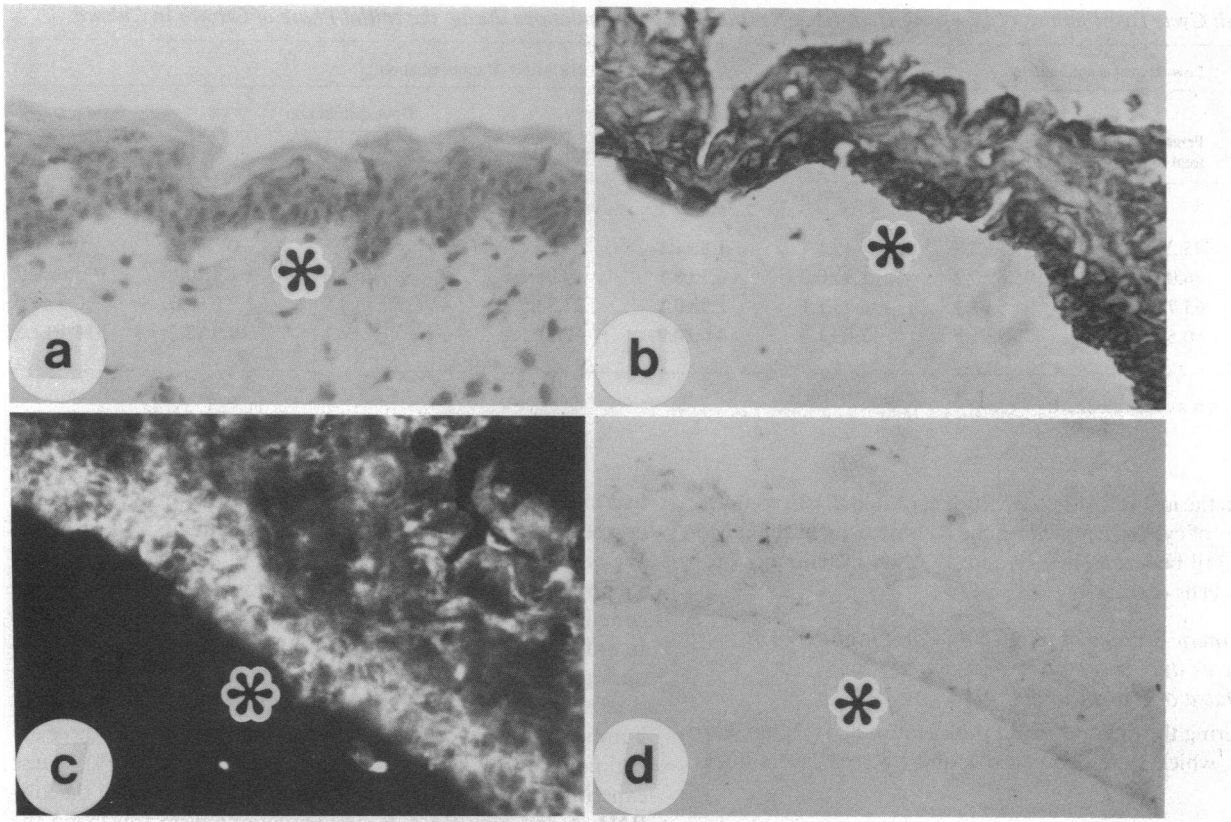

Figure 3. Immunoperoxidase and/or immunofluorescence micrographs of normal human epidermis and cultured epidermal cell sheets reactive with monoclonal antibody I/2; (*a*) cross-section of normal human epidermis, (*b*) cross-section of a 21-d-old cultured epidermal cell sheet, (*c*) immunofluorescence micrograph of a cross-section of 21-d-old cultured epidermal cell sheet, and (*d*) negative control. Asterisk indicates the basal cell layer of normal epidermis and the cell layer of the cultured sheet which was attached to the plastic. ×110.

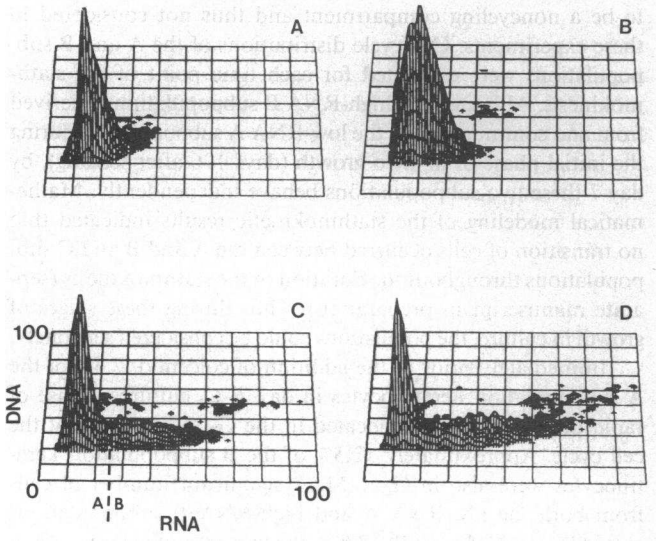

Figure 4. A representative two-parameter RNA/DNA isometric display of human epidermal cells from one donor after (*A*) 1 d, (*B*) 2 d, (*C*) 4 d, and (*D*) 5 d of growth in culture. Those keratinocytes with low-RNA content were designated as compartment A. Greater than 95% of keratinocytes were in the A compartment 24 h after plating. With time in culture, a proportion of the low-RNA A cells had increased in RNA content. We have designated this the high-RNA B compartment.

Gated analysis based upon RNA content and forward-angle light scatter as a sizing measurement (FALS) revealed that the smallest cells were in the low-RNA A compartment (FALS = 45.2±11.2), intermediate sized cells comprised the high-RNA B compartment (FALS = 70.5±11.3), and the largest cells localized to the C compartment (FALS = 86.0±8.0). Sorting of the cells based upon RNA content followed by light microscopic examination confirmed these results (data not shown). Immunocytochemical results with the I/2 antibody are consistent with the largest cells (C compartment) being equivalent to the more differentiated suprabasal cells of normal epidermis.

Analysis of the overall cell cycle profiles revealed that the number of cells in S + G_2 + M remained relatively constant during days 7–11 in both the A and B compartments (Table II). The high-RNA B subpopulation of keratinocytes predominated during this time (64.8–73.2%) and significantly greater numbers of S + G_2 + M cells were observed in the high-RNA B subpopulation of cells when compared to the low-RNA A subpopulation.

Plateau phase of growth in culture. By the 12th day after initial seeding the keratinocyte cultures reached a plateau phase where the total number of cells remained relatively constant. The three subpopulations of keratinocytes observed during days 7–11 could also be distinguished at this time. Compartment C continued to comprise just 5–6% of the total cell number up to 21 d in culture. A decrease in the number of cycling cells was

Table I. Cell Cycle Distributions of Low- and High-RNA Keratinocyte Subpopulations during the Initial Phase of Growth in Culture

Days in culture	Low-RNA A subpopulation				High-RNA B subpopulation			
	Percentage of total cells	DNA distribution			Percentage of total cells	DNA distribution		
		G_1	S	G_2/M		G_1	S	G_2/M
	%	%	%	%	%	%	%	%
1	95.2±1.8	97.1±2.9	1.1±1.1	1.8±1.1	4.8±2.4	100.0	0	0
2	86.0±1.0	95.8±0.2	1.9±0.2	2.3±0.1	14.0±1.1	94.7±1.3	1.7±0.6	3.6±0.7
4	63.7±1.7	91.5±1.1	5.7±1.1	2.9±0.1	37.3±5.2	80.7±4.9	9.0±3.3	10.3±1.6
5	50.5±1.4	78.5±1.2	17.0±1.3	4.6±0.9	49.5±1.7	71.1±2.1	14.9±2.2	14.1±1.5

All values given as mean±SEM.

observed in the low-RNA A compartment (Table III). In contrast, the number of cycling cells within the B compartment remained constant until between days 19 and 21 when the number of S + G_2 + M cells decreased.

Stathmokinetic studies of low-RNA A and high-RNA B keratinocytes during the proliferative and plateau phase of growth in culture

Upon entering the phase of rapid proliferation (day 7), the keratinocytes, which were between one and two cell layers thick, had to undergo a number of cell divisions before achieving the degree of stratification observed in early and late plateau phase. We have described above the existence of a low-RNA, basal, A subpopulation and a high-RNA B subpopulation, both of which contained cells within the S + G_2 + M phases of the cell cycle. In order to assess the kinetic status of these two subpopulations during their growth in culture, a series of six stathmokinetic studies were done during the phase in which the maximum number of cycling cells was observed (days 7–11) and three experiments during the early plateau phase (days 12–14). These experiments enabled us to determine the rates at which the low-RNA A and high-RNA B subpopulations were traversing the cell cycle during the progression from rapid proliferation through early plateau phase of growth in culture.

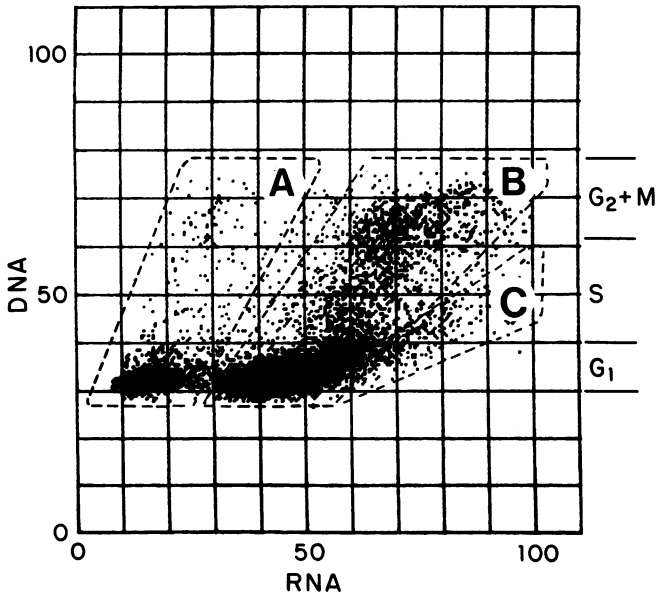

Figure 5. Representative RNA/DNA cytogram of cultured human epidermal cells during the phase of rapid proliferation (days 7–11 of culture). Each point of the cytogram represents the RNA and DNA values of an individual cell. Three subpopulations of keratinocytes can be identified and are marked by the broken lines. The low-RNA A and high-RNA B compartments are the same as described in Fig. 4. However, during this phase of growth, these subpopulations are well defined with few transitional cells in between. Distinct G_1, S, and G_2 + M phases of the cell cycle could be identified in these two subpopulations. In addition, a third C compartment of cells was identified which originated from the G_1 phase of the B subpopulation. No distinct S or G_2 + M could be distinguished in this compartment.

After incubation of the keratinocytes in colcemid for varying periods of time, cultures were harvested and cells stained with AO as described above. The low-RNA A and high-RNA B subpopulations were individually analyzed using gating analysis based upon RNA content. The C compartment was observed to be a noncycling compartment and thus not considered in these experiments. Cell cycle distributions of the A and B subpopulations were estimated for each time point of the stathmokinesis. Although the high-RNA B subpopulation is derived from and continuous with the low-RNA A subpopulation during the initial phase of in vitro growth (days 1–6 after seeding), by day 7 these two subpopulations behave independently. Mathematical modeling of the stathmokinetic results indicated that no transition of cells occurred between the A and B and C subpopulations throughout the duration of the stathmokinesis (separate manuscript in preparation). Thus during these stages of growth in culture, the populations could be considered separately.

Immediately prior to the addition of colcemid, 7.4% of the A subpopulation keratinocytes in day 7–11 cultures (phase of rapid proliferation) were located in the G_2 + M phases of the cell cycle. Approximately 12.5% of the B subpopulation keratinocytes were also in G_2 + M. A significant number of cells from both the low-RNA A and high-RNA B subpopulations entered G_2 + M during the 7 h in the presence of colcemid (Fig. 6). The accumulation of cells into G_2 + M is represented by the straight exponential slope. A lag time of ~2 h was observed. The rate of entry into G_2 + M of high-RNA B cells indicated a population-doubling time of between 30 and 40 h. In contrast, the cell-doubling time of the low-RNA A subpopulation ranged between 100 and 120 h. Thus the doubling time of the high-

Table II. Cell Cycle Distributions of Low- and High-RNA Keratinocyte Subpopulations during Peak Growth in Culture

Days in culture	Low-RNA A subpopulation				High-RNA B subpopulation			
	Percentage of total cells	DNA distribution			Percentage of total cells	DNA distribution		
		G_1	S	G_2/M		G_1	S	G_2/M
	%	%	%	%	%	%	%	%
7	26.8±1.7	68.0±2.4	24.0±1.5	8.0±0.9	65.1±3.8	58.3±6.6	22.3±2.1	19.4±4.7
8	17.5±3.5	74.6±3.1	18.0±1.6	7.4±1.7	73.2±4.1	64.2±4.3	22.1±2.7	13.7±3.1
9	28.5±2.3	69.1±2.7	23.2±1.4	7.7±2.1	64.8±3.3	59.3±2.1	23.0±1.7	18.7±1.3

Values given as mean±SEM.

RNA B subpopulation was approximately two and one-half to three times faster than that of the low-RNA A subpopulation.

The cell cycle kinetics of the A and B subpopulations were also determined during the early plateau phase of culture (days 12–14; Fig. 7). The cell doubling time of the high-RNA B subpopulation remained the same during this phase of culture (~40 hours). In contrast, the doubling time of the low-RNA A subpopulation was considerably longer during the plateau phase of culture (200–300 h). Thus during the early plateau phase of culture, the doubling time of the high-RNA B subpopulation was five to seven times faster than that of the low-RNA A subpopulation.

Relationship of kinetic status of epidermal cultures and graft survival after transplantation

As described above, RNA/DNA FCM can accurately assess the kinetic status of human keratinocytes grown in vitro. As part of the overall effort to determine optimal conditions for the use of cultured epidermal cell sheets as transplantable grafts, a series of 20 deep second-degree or third-degree burn wounds were grafted with either rapidly proliferating, early plateau phase, or late plateau phase cultures (Table IV). The minimal time required for complete closure of the wounds after grafting was documented. Table IV presents preliminary data on the relationship between the kinetic status of the epidermal cell sheet as determined by FCM, and the subsequent healing of the wound. The present data indicate that epidermal cell sheets derived from rapidly proliferating cultures (days 7–11 of growth) had the lowest incidence of survival after grafting (one of four total). In contrast, epidermal cell sheets derived from both early and late plateau phase of culture had significantly higher incidences of survival after grafting (3 of 5 and 8 of 11, respectively). Thus, human keratinocyte cultures that had progressed through the rapidly proliferative phase of growth and entered the more differentiated phases of culture were more suitable for use as grafts. Although the data also suggest that wounds grafted with late plateau phase cultures healed on average more rapidly (15.0±3.0 d) than wounds grafted with early plateau phase cultures (24.3±7.8 d), greater sample numbers must be accumulated to determine the statistical significance of this finding.

Discussion

Cultures of human epidermal cells grown according to the present method are composed of three distinct keratinocyte subpopulations distinguishable on the basis of cell size, RNA content, immunochemical properties, and cell cycle kinetics. Subpopulation A consisted of small basal keratinocytes with low cellular RNA content and long cell-doubling times. Subpopulation B cells were two to three times larger, had a higher RNA content, and significantly shorter cell-doubling times than A keratinocytes. Subpopulation C consisted of large nondividing cells with the highest RNA content.

Single-cell suspensions of human epidermal cells clearly possess different capabilities for attachment and growth. During the initial phase of culture, the majority of the larger, more differentiated keratinocytes were unable to adhere to the plastic substratum. Those cells capable of attachment during this phase were small basal cells. Such adherent basal cells gave rise to multilayered sheets of keratinocytes. Two distinct types of cells could be identified in these stratified sheets: a basal layer of small

Table III. Cell Cycle Profile of Low- and High-RNA Keratinocytes in the Plateau Phase of Culture Growth

Days in culture	Low-RNA A subpopulation				High-RNA B subpopulation			
	Percentage of total cell number	DNA distribution			Percentage of total cell number	DNA distribution		
		G_1	S	G_2/M		G_1	S	G_2/M
	%	%	%	%	%	%	%	%
12–14	51.4±0.1	76.8±1.3	13.1±0.1	10.2±1.3	42.2±0.9	52.8±2.1	32.1±0.8	15.1±3.0
19–21	56.8±2.0	78.8±0.2	12.7±1.2	8.5±0.6	38.1±1.8	66.8±2.4	17.7±2.2	15.5±0.2

Values given as mean±SEM.

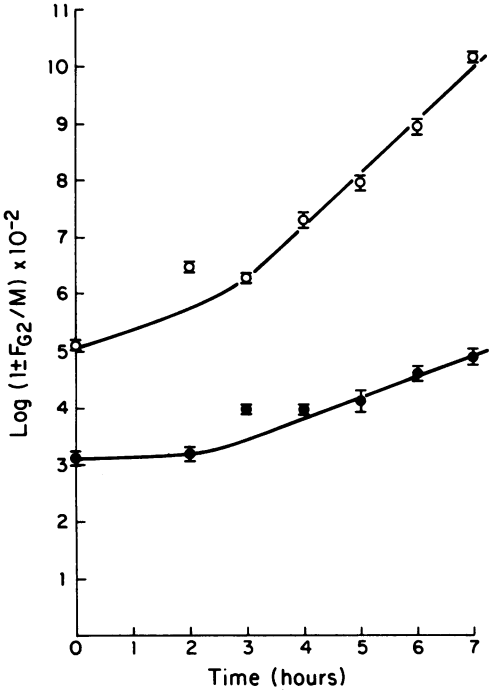

Figure 6. Stathmokinetic studies of the rate of entry into G_2 + M of the low-RNA A (•) and high-RNA B (○) compartments of rapidly proliferating keratinocytes (days 7–11 of culture; mean of six experiments ± standard error of the mean). A series of parallel cultures of human keratinocytes were incubated in the presence of colcemid (0.05 μg/ml) for various periods of time as described in Methods. Cultures were harvested at the designated time points stained with AO and measured by FCM. The DNA distributions of the low-RNA A and high-RNA B subpopulations were analyzed separately by setting electronic windows based upon RNA content. The rate of entry into G_2 + M was calculated as a function of time on the presence of colcemid. The accumulation of cells into G_2 + M after approximately a 2-h lag is represented by the straight exponential slope. The rate of entry into G_2 + M was two to three times faster in the B subpopulation as compared to the low-RNA A subpopulation.

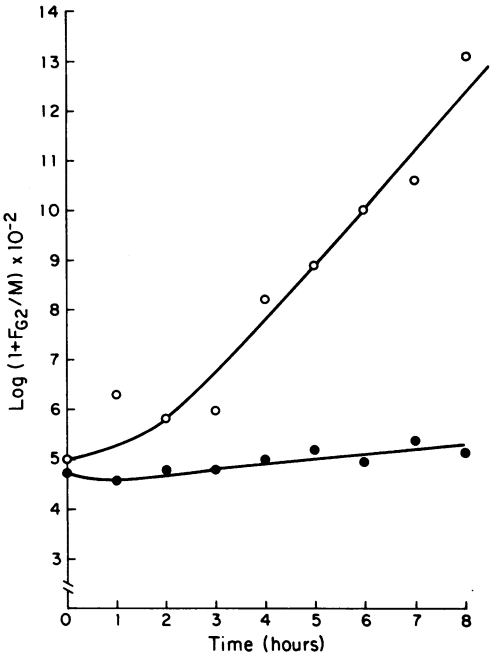

Figure 7. Stathmokinetic studies on the rate of entry into G_2 + M of the low-RNA A (•) and high-RNA B (○) compartments of keratinocyte cultures during early plateau phase of culture (days 12–14). Stathmokinetic studies were carried out as described in Fig. 6. The rate of entry into G_2 + M of the high-RNA B subpopulation of keratinocytes was six to eight times faster as compared to the low-RNA A subpopulation during the early plateau phase of culture.

rounded cells attached to the plastic and suprabasal layers of larger cells with greater amounts of vacuolated cytoplasm, reminiscent of stratum germinativum and stratum spinosum cells in vivo. This analogy was further confirmed by the immunohistochemical findings using monoclonal antibody I/2 in which staining patterns of the cultured epidermal cells approximated that of normal epidermis. No stratum corneum has been observed in this system; however, when these cultures are grafted onto human burn wounds, a mature stratum corneum will develop with 7–10 days (Fig. 2 *D;* 7).

Until recently, FCM measurements of keratinocytes have focused on single-parameter measurements of DNA content (with or without calculation of percent labeled mitoses) to assess cell cycle distributions and ploidy levels of diseased and normal epidermis (20–23). These observations, in conjunction with mathematical modeling data (24), have suggested that keratinocytes are highly heterogeneous regarding both cell type and rate of cell cycle traverse. Consistent with this mathematical analysis, Lavker and Sun described two morphologically distinct types of basal keratinocytes (25, 26). One such subtype was shown to have a relatively low rate of cell division subsequently giving rise to a rapidly dividing subpopulation of suprabasal keratinocytes.

The present data directly discriminate two distinct subpopulations of cycling keratinocytes based upon RNA content. The low-RNA A and high-RNA B subpopulations both contain cells within the G_1, S, G_2, and M phases of the cell cycle. The stathmokinetic experiments presented here show that both the A and B subpopulations are proliferating albeit at different rates. The A compartment keratinocytes have a generation time of ~100–120 h during days 7–11 of culture. In contrast, the B compartment cells have a generation time of ~30–40 h during this period of culture growth. During the latter stages of culture (days 12–21), the growth rate of the culture decreased while the total

Table IV. Relationship between Kinetic Status of Cultured Epidermal Cell Sheets and Graft Survival on Deep Second-degree and Third-degree Burn Wounds

Growth phase of culture	Ratio of high-RNA/low-RNA keratinocytes (B/A)	Total number of grafted wounds	Number healed	Mean healing time
		n	*n*	*d*
Rapid proliferation	2.8	4	1	—
Early plateau	0.8	5	3	24.3±7.8
Late plateau	0.7	11	8	15.0±3.0

number of cells remained relatively constant. This phase was characterized by the continued presence of both the A and B compartments. At this time the number of A compartment keratinocytes increased in proportion to the number of B compartment keratinocytes. Stathmokinetic experiments performed at this time indicated that the low-RNA A keratinocytes had slowed in their doubling time from 100 h during peak proliferation to 200–300 h during the plateau phase. By contrast, the higher RNA B keratinocytes maintained their doubling time of between 30 and 40 h throughout culture. One hypothesis for this shift in the proportion of the A and B subpopulations is a continued recruitment of B cells into the C compartment which subsequently desquamate. In our culture system, therefore, the stemlike A compartment keratinocytes of low-RNA content give rise to the more rapidly dividing B cells which have a higher RNA content. After several rounds of rapid division, B cells appear to terminally differentiate (C compartment). This model is in agreement with the findings of Lavker and Sun (25, 26) of a functional heterogeneity within the epidermal stem cell compartment.

These FCM data provide cytochemical parameters to rapidly assess the pregraft suitability of cultured epidermal cell populations prior to actual application to burn wounds. Keratinocyte cultures can be monitored at daily intervals during the initial phase of growth in vitro in order to quantitate the transition of low-RNA A basal keratinocytes to the high-RNA B compartment. If within the initial 6 d of growth, we do not observe this recruitment of keratinocytes from the A compartment into the B compartment, the cultures will not reach confluency or stratify. These conditions produce cultures that grow as isolated colonies and do not produce sheets of epidermal cells suitable for grafting. However, identification of a rapidly proliferating B subpopulation of keratinocytes within these early cultures is highly correlated with the subsequent ability of these cells to coalesce, stratify, and reach the level of maturity necessary for clinical use.

In order to define the relationship between culture growth phase (e.g., rapid proliferation, early plateau phase and late plateau phase) and graft suitability, a series of 20 deep second-degree or third-degree human burn wounds were grafted with cultured epidermal cell sheets harvested at various time during their growth and differentiation in culture. Attempts to use cultured grafts at an early stage of maturation (days 7–11 of growth) have proven difficult and resulted in a low rate of survival (25%). In contrast, attempts to use cultured grafts at later stages of maturation, i.e., from early plateau phase or late plateau phase of growth in vitro, resulted in a significantly higher rate of survival after grafting (60% and 72%, respectively). Furthermore, preliminary evidence suggests that wounds grafted with late plateau phase cultures may heal more rapidly (15.0±3.0 d) than wounds grafted with early plateau phase cultures (24.3±7.8 d). Thus there is a relationship between the kinetic status of the epidermal cell sheets measured by RNA/DNA FCM, and their subsequent ability to survive after grafting. Studies are continuing to further optimize the time during the plateau phase of culture that will provide the most rapid healing of burn wounds after transplantation.

In conclusion, RNA/DNA FCM can provide an objective way to evaluate: (*a*) whether human keratinocytes seeded into culture will reach confluency and stratify, and (*b*) the most appropriate stage for successful graft transplantation.

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