Two Functionally Distinct Classes of Growth Arrest States in Human Prokeratinocytes That Regulate Clonogenic Potential

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Rapidly growing normal human neonatal prokeratinocytes (HPK) cultured in serum-free medium can be induced to undergo either reversible or irreversible growth arrest at distinct cell cycle states. Reversible G_1 arrest was induced by culture of low-density cells in human lymphocyte conditioned medium, by culture in high-density stationary phase conditioned medium, and by culture in isoleucine-deficient medium. Irreversible arrest of HPK growth predominantly in G_1 was induced by culture in growth factor-deficient medium. Irreversible arrest of HPK growth in G_1 and G_2 was also induced by culture in suspension in methylcellulose prepared in complete MCDB 153 medium or by culture in serum-containing medium. Finally, the drug razoxane was employed to induce irreversible arrest of HPK in G_2. These data establish that there are 2 distinct classes of growth arrest states for HPK and suggest that each arrest mechanism may serve a unique role in the control of keratinocyte differentiation in normal cells. It is also possible that the development of selective defects in either of these processes could be of etiologic significance in certain epidermal disease states. *J Invest Dermatol 86:410–417, 1986*

Normal human proliferative keratinocytes [prokeratinocytes (HPK)] can be cultured in vitro and their proliferation and differentiation can be regulated. Initially, Peehl and Ham [1,2] reported the development of selective media for the culture of epidermal cells derived from skin. They showed that in this medium HPK grow in the absence of a feeder layer and in the absence of contaminating fibroblasts. Supplementation of this medium with epidermal growth factor (EGF), insulin, and other factors was indeed shown to support clonal cell growth from low-density cell inocula. Further improvements in this culture system were implemented by Ham and coworkers [3,4] and it was suggested that this culture system for HPK was a good model to study the physiologically significant regulatory process that modulates proliferation and differentiation. In this regard, it was shown that changes in medium calcium concentrations modulate cellular differentiation and that changes in medium growth factor concentrations modulate cellular proliferation [4].

Our previous studies extended these observations and established that the control of HPK proliferation and differentiation is integrally regulated [5]. More specifically, we showed that the proliferation and differentiation of HPK is regulated by a delicate balance of influences which either promote proliferation and thereby limit differentiation or which limit proliferation and thereby promote differentiation.

No studies, however, have been published which establish whether the control of HPK proliferation can be mediated by cell cycle-specific events nor whether there is a significant correlation between HPK growth arrest at a distinct cell cycle state and expression of the differentiated phenotype as has been demonstrated in other cell systems [6–9]. In this regard, the studies of Pardee [10], Holley and Kiernan [11], and others [12,13] documented that most nontransformed mesenchymally derived animal cells and normal human fibroblasts can be reversibly growth arrested in the G_1 phase of the cell cycle by culture in growth factor-deficient media (GFDM) or by density-dependent mechanisms. It has also been documented that various cell types, including normal human fibroblasts [14,15], can be growth arrested primarily in G_1 by culture in nutrient-deficient medium. Pledger and coworkers [16] have even formulated the relationship between these different restriction points into their commitment-progression model. Distinct cell cycle-associated mechanisms to control cellular differentiation have also been demonstrated by our studies on murine mesenchymal stem cells [6,17,18] and by other investigators who have confirmed these results [19].

In this paper we have therefore extended our previous studies on HPK and report that HPK can be growth arrested by 2 distinct types of mechanisms. Reversible arrest of growth in G_1 can be induced by human lymphocyte conditioned medium (LCM), by high-density stationary phase conditioned medium (SPCM), and by isoleucine-deficient medium (IDM). The data further show

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Abbreviations:
BPE: bovine pituitary extract
CFE: colony-forming efficiency
dFBS: dialyzed fetal bovine serum
EGF: epidermal growth factor
GFDM: growth factor-deficient medium
HPK: human prokeratinocytes
IDM: isoleucine-deficient medium
LCM: lymphocyte conditioned medium
PBS: phosphate-buffered saline
PHA: phytohemagglutinin
SP: stationary phase
SPCM: stationary phase conditioned medium

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that irreversible growth arrest in G_1 and G_2 can be induced by culture of HPK in suspension or in a monolayer microenvironment in medium containing serum. Culture of HPK in GFDM is also shown to induce irreversible growth arrest of a significant proportion of the cells in G_1. Finally, the data demonstrate that the drug razoxane can induce >95% of HPK to irreversibly arrest in G_2.

These data provide a basis for future studies to determine whether arrest at either reversible or irreversible states is required for keratinocyte differentiation and whether the development of defects in such cell cycle-dependent regulatory processes is etiologically significant in the pathogenesis of specific disease states, such as psoriasis, epidermal atrophy, epidermal metaplasia/dysplasia, or epidermal cancer.

MATERIALS AND METHODS

Cell Culture and Culture Media The procedures for preparing primary and secondary cultures of normal HPK are the same as those previously described [5]. Briefly, neonatal foreskin specimens were trimmed of connective tissue, cut into 3- to 4-mm pieces, and floated on trypsin [0.17% in phosphate-buffered saline (PBS), Sigma Chemical Co., St. Louis, Missouri] overnight at 4°C. The epidermis was separated from the dermis and washed gently with solution A [20]. The suspension was passed through nylon mesh (Nytex, Tetco Inc., Elmsford, New York) and the cells were pelleted by centrifugation at 250 *g* for 10 min. Cells were resuspended in complete MCDB 153 and seeded at $5 \times 10^3/cm^2$ in 75-cm^2 culture flasks. Primary cultures were harvested 6–8 days later by enzymatic dissociation in trypsin 0.25%-EDTA 0.1% solution. Cells were collected in solution A, 10% dialyzed fetal bovine serum (dFBS), centrifuged, and resuspended in complete MCDB 153 medium. Secondary cultures were seeded routinely at $1 \times 10^3/cm^2$. Adult HPK were isolated and cultured as previously described [5]. Both neonatal and adult secondary cultures of HPK were used in these studies. Cultures were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C in various media as described below. These include basal or complete MCDB 153, isoleucine-deficient complete MCDB 153, lymphokine conditioned MCDB 153, stationary phase conditioned MCDB 153, and others.

The basal nutrient medium formulation, designated MCDB 153, was prepared as previously described [4]. Standard medium is defined as basal nutrient medium supplemented with ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), hydrocortisone (5×10^{-7} M), and 0.1 mM Ca^{++}; complete medium MCDB 153 is standard medium supplemented with 5 μg/ml insulin, 10 ng/ml EGF, and 140 μg/ml protein of bovine pituitary extract (BPE).

Medium MCDB 153 deficient in isoleucine was prepared according to Boyce and Ham [4] except that stock no. 1 contained no isoleucine and isoleucine-free leucine (Gibco, Grand Island, New York) was used. All other amino acids were obtained from Sigma Chemical Co.

LCM was prepared as follows: peripheral blood mononuclear cells were obtained by centrifugation of heparinized venous blood from normal human volunteers over lymphocyte separation medium (LSM, Bionetics Laboratory Products, Kensington, Maryland). They were washed twice with PBS and once with MCDB 153 and the cells were then suspended at 1×10^6 cells/ml in MCDB 153 containing 5 μg/ml phytohemagglutinin (Sigma). After 2 days' incubation at 37°C, the supernatant was decanted after centrifugation for 10 min at 400 *g* and dialyzed exhaustively against MCDB 153 using 1000 M_r exclusion dialysis tubing (Spectrapor, Los Angeles, California). The conditioned medium was frozen at −70°C. Prior to use the material was thawed and sterilized by passage through a 0.45 μM filter (Gelman, Ann Arbor, Michigan). The effect of complete MCDB 153 containing phytohemagglutinin (PHA) without lymphocyte conditioning was also assayed.

Stationary phase conditioned medium (SPCM) was prepared by harvesting the spent complete medium from cultures of HPK that had ceased exponential growth and had entered stationary phase [5].

Razoxane (ICRF 159; Imperial Chemical Industries, PLC, Cheshire, England) is a cytostatic agent that is cell cycle dependent and phase specific acting at the end of the G_2 phase. It was prepared in 0.2 M HCl [21] and filter sterilized before use.

dFBS was prepared as described earlier [5]. It contained less than 0.04 mM Ca^{++} as measured by atomic absorption spectroscopy.

Suspension Culture Stock solutions of methylcellulose (Sigma) were prepared according to Stoker [22] as adapted by Green [23] except that the medium was basal MCDB 153. For suspension culture, approximately 2.5×10^5 cells were suspended in 2 ml of 1.3% methylcellulose solution which was supplemented with insulin (5 μg/ml), EGF (10 ng/ml), and BPE (1%), and either 0.1 mM or 2 mM Ca^{++}. The suspension was mixed and incubated at 37°C in a humidified 5% CO_2 atmosphere. To collect cells after suspension culture, 10 ml of solution A were added to the culture and the specimen was centrifuged at 400 *g* for 10 min. The supernatant was removed and the wash procedure was repeated.

Clonal Growth Assays Clonal growth assays were performed as previously described [4,5]. Briefly, 500 cells were seeded into 60-mm culture dishes containing complete medium and were grown for 10 days before fixation and counting of clone formation. Colony forming efficiency (CFE) and average colony area were calculated as earlier described [5].

Cell Kinetics, DNA Synthetic Rates, and Cytofluorimetry Procedures for cell counting, autoradiography, and DNA synthetic rate analysis were those previously reported [5] except that to determine the rate of DNA synthesis in suspension cultures, 4 μCi/ml of [^{3}H]thymidine were added to the culture, thoroughly mixed, and incubated for 1 h. Autoradiography in most cases was performed by incubating the culture with 4 μCi/ml [^{3}H]thymidine (New England Nuclear, Boston, Massachusetts) for 24 h prior to the termination point, fixing the culture with Trump's fixative, and processing for light microscopy. Autoradiography for restimulation experiments was performed by incubating cultures with [^{3}H]thymidine for sequential time periods after culture restimulation, then fixing and processing. Cell cycle analyses were determined by flow microfluorimetry as earlier described [5].

RESULTS

The mechanisms by which normal human keratinocytes regulate their proliferation are largely unknown. In an attempt to establish the role of cell cycle-mediated processes in the regulation of cellular proliferation, we have identified 6 methods that growth arrest randomly proliferating HPK low-density cultures. The procedures we employed include growth arrest induced by LCM, SPCM, IDM, and GFDM. These 4 selective media induce proliferation arrest in the G_1 phase of the cell cycle. G_2 proliferation arrest was induced by addition of the drug ICRF (razoxane) 159 to complete medium, and a combined G_1 and G_2 arrest was induced by suspension of cells in semisolid complete medium or by the addition of dFBS to complete medium. The detailed results for each of these procedures is presented below.

LCM-Induced Growth Arrest The detailed preparation of LCM is given in *Materials and Methods*. Briefly, it has been established that PHA-stimulated human blood peripheral lymphocytes produce an inhibitory activity on the growth of secondary cultures of HPK (manuscript in preparation). In our experiments, cultures of HPK displaying rapid exponential growth kinetics (24-h generation time) were seeded at low densities ($1–3 \times 10^3$ cells/cm^2) in complete medium and were allowed to undergo 1 or 2 population doublings; the growth medium was then replaced with LCM. Fig 1 compares the morphology of rapidly growing HPK (Fig 1*a*) with tightly packed, occasionally elongated, LCM-treated

HPK (Fig 1*b*). Table I presents data that show that in LCM less than one population doubling occurred during the 48-h treatment period while control cultures completed at least 2 full population doublings. The cessation in cellular proliferation was accompanied by a dramatic decline in the DNA synthetic rate as measured on day 2 of LCM treatment. Autoradiographic studies also documented the loss in proliferative capacity on day 2; only 2% of the cells labeled with [^{3}H]thymidine when incubated for 24 h. Flow cytofluorimetric analysis of untreated and LCM-treated cell populations show that by day 2 of treatment, LCM induced a preferential proliferation arrest in the G_1 phase of the cell cycle (81% of cells in G_1 compared with 46% G_1 cells in asynchronously dividing exponential cultures). Since less than 2% of the cells were in S, the results show that LCM blocks cells at some state in G_1. Such G_1 growth arrest was not induced by complete MCDB 153 containing PHA alone.

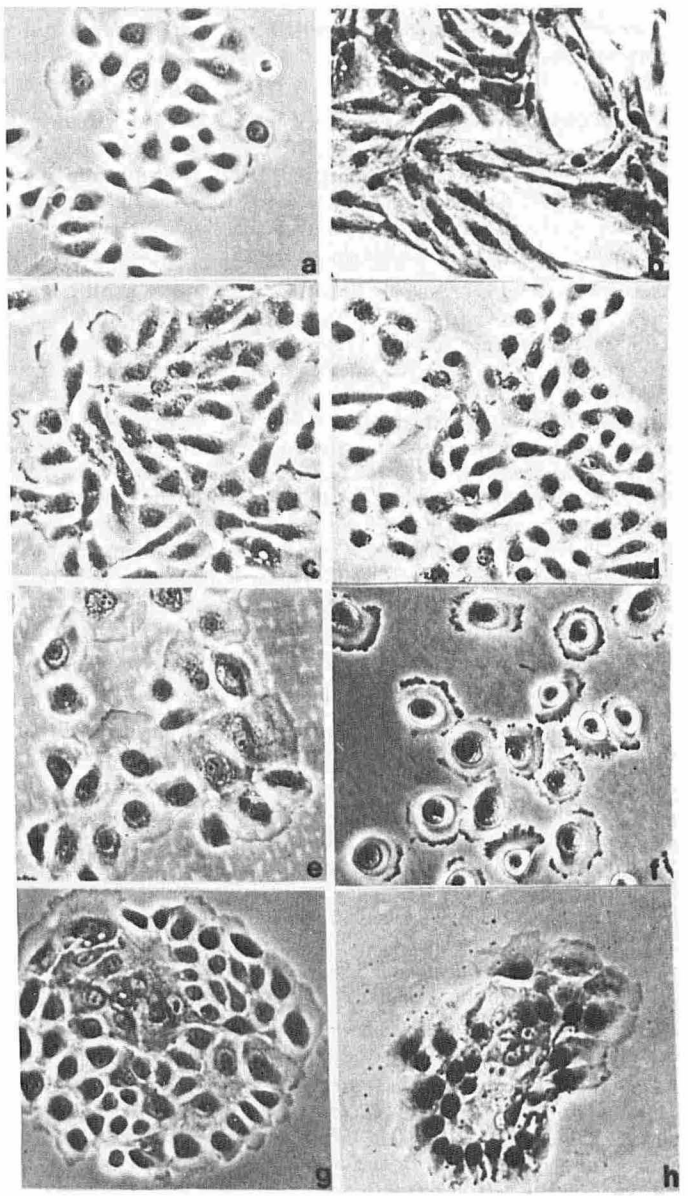

Figure 1. Typical morphology of cultures of HPK cells in rapid growth (*a*) and of cells cultured in: (*b*) LCM, (*c*) SPCM, (*d*) IDM, (*e*) serum, (*f*) razoxane, (*g*) GFDM. An autoradiograph of HPK colony that was fed GFDM is also illustrated. One day after growth factors were removed, [^{3}H]thymidine was added for 24 h (*h*). Note lack of nuclear radiolabeling of centrally localized prokeratinocytes. Phase micrographs, ×400.

The reversibility of the LCM-induced G_1 arrest was examined by studies on the kinetics of restimulation of DNA synthesis. This was accomplished by removal of the spent LCM, then incubating the arrested cells in fresh complete medium in the presence of [^{3}H]thymidine, fixing cultures after sequentially longer elapsed time intervals, and processing for autoradiography. Within 12 h after restimulation, 45% of the cells entered the S phase, and by 48 h 85% of the cells had completed S phase. At 48 h the total cell number had doubled. These data demonstrate that the majority of arrested cells can reenter the proliferative cycle from the LCM-induced G_1 arrest state. Clonal growth assays were also performed to ascertain whether cells arrested at the LCM G_1 arrest state for 2 days have a reduced proliferative potential. Growth arrest at the LCM-induced G_1 arrest state reduced only slightly the CFE (18%) (Table II) and no difference in average colony area resulted.

SPCM-Induced Growth Arrest We reported previously that exponentially growing cultures of HPK enter a spontaneous subconfluent stationary phase (SP) growth arrest [5]. These observations suggested to us that SPCM might exert an inhibitory effect on the growth of proliferating cultures. To test this possibility, SPCM was harvested from high-density, SP-arrested cultures and refed to low-density, exponentially growing HPK cultures. Growth kinetic and [^{3}H]thymidine pulse-labeling studies on control and SPCM-treated cultures were performed. Table III presents some of these results. Within 1 day, growth arrest was observed by measurements of cell density (data not shown). Autoradiographic data show that SPCM-treated cells fail to enter the S phase. Preferential accumulation in the G_1 phase of the cell cycle was established by flow microfluorimetry. The cell cycle data indicate that G_1 arrest occurs by blocking the transit of G_1 cells into the S phase. Fig 1*c* illustrates the disappearance of mitotic cells from SPCM-arrested cultures.

These results suggest that SPCM either contains a growth inhibitory activity or exerts an inhibitory action on growth because of nutrient depletion. Table III presents data on the restoration of growth-promoting activity of SPCM that had been dialyzed extensively against fresh basal MCDB 153 medium. These data support the hypothesis that SPCM either lacks some low-molecular weight nutrient supplied by dialysis against fresh basal medium or that a low-molecular weight inhibitor of growth had been diluted out.

This question was resolved in favor of the nutrient depletion hypothesis as detailed below (see *IDM-Induced Growth Arrest* below). The above results do, however, rule out the possibility that the G_1 growth arrest was due to the production at these cell densities of a chalone-like growth inhibitory protein. This was further confirmed by the repeated failure to concentrate such putative chalone by 100-fold volume reduction using the methods of ultrafiltration, dialysis against various carbowaxes, and dialysis with different exclusion limits above 1000 daltons.

IDM-Induced Growth Arrest Strong evidence that G_1 growth arrest induced by SPCM is due to nutrient depletion was obtained by studies wherein each of the individual component stocks of the basal MCDB 153 medium was replenished. Only the amino acid stock (MCDB 153 stock no. 1) restored rapid growth kinetics, and within stock no. 1 isoleucine was the only amino acid whose replenishment permitted recovery of exponential growth kinetics to low-density, randomly proliferating cultures (data not shown). Furthermore, we found that subconfluent growth arrest in SP cultures could be prevented, and the saturation density of exponential cultures raised by a factor of 10 if complete medium was further supplemented to have 5 times more isoleucine. From these observations, we conclude that basal MCDB 153 medium is primarily optimized for clonal growth, but must be supplemented with 5 times more isoleucine in order to avoid nutrient depletion-induced arrest at a subconfluent saturation density. In fact, other studies showed that isoleucine is not the only amino acid that is limiting in high-density cultures, only the first to limit

Table I. Preferential Growth Arrest in the G_1 Phase of the Cell Cycle Induced by Exposure of Randomly Proliferating Human Prokeratinocyte Cultures to Lymphocyte Conditioned Medium (LCM)

Culture Condition	Growth Responses: Density[a] (cells/cm^2 × 10^{-3})	DNA Synthesis[b] (cpm × 10^{-3})	Labeled Nuclei (%)[c]	Cell Cycle Phase[d] (± SE): G_1	S	G_2/M
Complete MCDB 153						
Day 0[e]	6	7	94	—	—	—
Day 2	28	34	91	46 (0.9)	39 (0.1)	15 (0.8)
LCM						
Day 2	10	1	2	81 (2.5)	2 (0.5)	17 (2.5)

[a]Cell density was determined by direct measurements of total cell number of duplicate untreated and experimentally treated samples as described in *Materials and Methods*.
[b]DNA synthetic rate was determined by pulse-labeling duplicate untreated and LCM-treated samples for 1 h with 4 μCi/ml [^{3}H]thymidine. The cells were harvested for scintillation spectrometric estimation of their contained radioactivity, as described in *Materials and Methods*.
[c]Percent labeled nuclei was determined by light microscopic autoradiographic analysis of duplicate control untreated and LCM-treated cultures as described in *Materials and Methods*. [^{3}H]Thymidine (4 μCi/ml) was added to the culture. Cells were fixed 24 h later and prepared for autoradiographic analysis.
[d]Flow microfluorimetric analysis of populations of control untreated and LCM-treated cultures was used to assess cell DNA contents. Mithramycin was the DNA stain and percent cell cycle phases were computed by procedures previously described (see *Materials and Methods*).
[e]LCM was fed to rapidly growing cultures 3 days after passage when they were at a density of approximately 5–7 × 10^3 cells/cm^2.

growth. We now employ a 6-amino acid supplement including histidine, isoleucine, methionine, phenylalanine, tryptophan, and tyrosine to increase the final concentration of each by 3–5 times.

The above results suggest that G_1 growth arrest induced by SPCM was the result of isoleucine deficiency. Table IV presents direct experimental verification of this hypothesis. When rapidly growing, low-density HPK cultures were refed isoleucine-deficient complete MCDB 153 medium (IDM), they underwent growth arrest at less than one population doubling within 1 day. They remained growth arrested at essentially the original cell density during the entire 48-h treatment period. In constrast, during this same 48-h period, control cultures of HPK fed complete medium underwent 2 complete population doublings. [^{3}H]Thymidine pulse-labeling experiments to measure the rate of DNA synthesis on days 1 and 2 after IDM-induced arrest show that all DNA synthetic activity has ceased. Likewise, 24- and 48-h pulse-labeling experiments and autoradiographic analysis showed that IDM-induced arrest prevented all cells from entering the S phase. Finally, flow cytometric analysis confirmed that fact that IDM results in G_1 arrest. Fig 1*d* illustrates that IDM-induced G_1 arrest results in colony arrangements that closely resemble SPCM-induced G_1 growth arrest (Fig 1*c*).

Clonal growth experiments were performed and showed that nutrient-deficient G_1 growth arrest is reversible, i.e., greater than 80% of the cells nutrient-arrested for 2 days retained their proliferative potential as measured by CFE (Table II) and average colony area was decreased by only 34%. Fig 2 presents results of kinetics of restimulation of DNA synthesis. The kinetic data show that DNA synthesis resumes in 10–12 h after replenishment of isoleucine to the nutrient-deficient culture. Therefore, the slight decrease in colony area noted above may be due simply to a delay in resumption of proliferation following growth arrest.

Table II. Clonogenic Potential of Growth Arrested Cultures

Culture Condition	CFE (%)[a]
Control	100
Reversible[b]	
Lymphocyte conditioned medium	82
Isoleucine-deficient medium	81
Irreversible	
Suspension	<1
Serum	*
Razoxane	9
Growth factor deficient medium	5

*The CFE of human prokeratinocyte culture in serum containing medium was variable depending on the lot of serum used. In all experiments the CFE in these specimens was, however, less than 45%; under prolonged culture in this medium less than 5% of the cells show CFE (see [5]).
[a]CFE was determined as described in *Materials and Methods*. Forty-eight hours after initiation of growth arrest, cultures were trypsinized and seeded into clonal assay with complete medium.
[b]Reversible applies to clonogenic potential of growth arrested cultures exceeding 50% CFE.

Suspension Culture-Induced Growth Arrest It has been reported that suspension of cultured keratinocytes results in a drastic loss of their proliferative potential, and that such suspended cells eventually undergo terminal differentiation as shown by the formation of detergent-resistant cross-linked envelope structures [23]. However, these studies did not establish whether suspension itself induces cell cycle-dependent growth arrest; hence, no conclusion can be drawn as to whether there is any cell cycle mediation of suspension-induced differentiation.

Cells resuspended in methylcellulose prepared in complete medium were incubated in the presence of [^{3}H]thymidine to assess their ability to synthesize DNA as a function of time in suspension. Table V presents results that demonstrate the capacity of suspended cells to temporarily continue to synthesize DNA. The data show that suspended cells maintain a high rate of DNA synthesis for the first 10 h, but by 12 h the rate of DNA synthesis declines significantly and by 14 h DNA synthesis had ceased.

Table V also presents results of studies on the effect of suspension on the distribution of cell cycle phases. The results show that suspended HPK cells continue to traverse the S phase and enter the G_2 plus M phases where they accumulate, while G_1 phase cells are completely blocked from entering the S phase. This conclusion was substantiated by treating HPK with hydroxyurea before and during suspension culture. Similar G_1 arrest was obtained in the presence of the G_1/S inhibitor and thymidine incorporation was inhibited indicating that scheduled DNA synthesis was occurring in suspension culture (data not shown).

The effect of suspension on the colony forming ability was assessed by clonal growth assay. Clonal growth assays were performed at 3-, 8-, and 16-h suspension. Whereas at 3 h all cells were capable of initiating colonies, virtually no colonies were

Table III. Growth Arrest and Preferential Accumulation in the G_1 Phase Induced by Stationary Phase Conditioned Medium (SPCM)

	Labeled Nuclei (%)[a]	Cell Cycle State (%): G_1	S	G_2/M
Complete MCDB 153	92	50	33	17
SPCM	0	81	2	17
Dialyzed SPCM	93	47	35	18

[a]Autoradiographic analysis of cells grown in the presence of [^{3}H]thymidine for 24 h.

Table IV. Isoleucine-Deficient Medium (IDM) Induces G_1 Phase Growth Arrest of Human Prokeratinocyte Cultures

Culture Condition	Growth Response[a]			Cell Cycle State (%) (± SE)		
	Density (cells/cm^2 × 10^{-3})	DNA Synthesis (CPM × 10^{-3})	Labeled Nuclei (%)	G_1	S	G_2/M
Complete MCDB 153						
Day 0[b]	8.2	15	94	—	—	—
Day 1	16.5	35	—	—	—	—
Day 2	34.1	62	90	49 (1.0)	33 (1.5)	18 (0.5)
IDM						
Day 1	10.1	0.17	0	—	—	—
Day 2	10.2	0.15	0	80 (3.5)	3 (0.5)	17 (3.0)

[a]See legend, Table I.
[b]IDM was fed to log growth cultures 3 days after their prior passage. The time at which IDM was added to designated day 0.

detected at 8 or 16 h after suspension (Table II). We conclude that there is a marked decline in clonogenicity which appears to accompany cessation of DNA synthesis and cell cycle partitioning into G_1 and G_2 compartments.

Serum-Induced Growth Arrest In previous studies we presented evidence that the addition of small amounts (0.5%) of dFBS to complete medium could virtually abolish the colony-forming ability of normal early-passage HPK cultures [5]. Here, we report the results of experiments on the effect of 5% dFBS on the growth arrest of rapidly growing HPK cultures. Table VI presents data showing that within 1 day after addition of 5% dFBS in complete medium to randomly proliferating low-density cultures, cell proliferation ceased after less than 35% increase in cell density and failed to subsequently increase further during the 48-h period of observation. In addition, measurements on the rate of DNA synthesis indicated that serum-treated cultures had only 3% of the control rate 2 days after treatment. Flow microfluorometric analysis on day 2 of serum treatment revealed that serum induces a combined G_1 and G_2 arrest. Fig 1 illustrates the morphologic appearance of HPK cultures growth arrested by serum (see Fig 1*e*). Note that serum arrested cultures contain predominantly enlarged flattened cells which adhere tightly to form rectangular-shaped colonies.

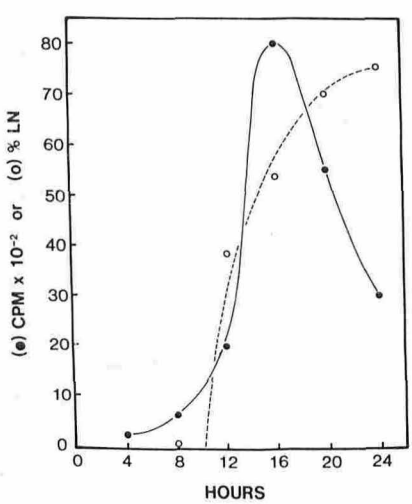

Figure 2. Kinetics of growth restimulation of HPK arrested in IDM. Following arrest by culture in IDM, the cells were refed complete MCDB 153 and the incorporation of [^{3}H]thymidine into DNA was assayed periodically thereafter. Percent of total nuclei that incorporated [^{3}H]thymidine [labeled nuclei (*LN*)] was determined by autoradiographic analysis (○– – – –○). Initiation of DNA synthesis occurred between 10–12 h. The maximum rate of DNA synthesis determined by 1-h pulse [^{3}H]thymidine incorporation was approximately 16 h after growth stimulation (●– – – –●).

As with other growth arrested cultures, the reversibility of serum-induced G_1 and G_2 combined arrest was assessed by clonal growth assay. Colony forming ability for serum-arrested cultures was markedly reduced (Table II) as was the average colony area. The efficiency with which serum induced irreversible growth arrest was somewhat variable and appeared to depend on the lot of serum that was used. In all studies, however, CFE was reduced by >50% and in certain experiments it was reduced by >80%, especially following prolonged culture (see [5]).

Razoxane-Induced Growth Arrest The drug razoxane has been employed to arrest the growth of PHA-stimulated lymphocytes in the G_2 phase of the cell cycle [21]. We therefore analyzed its effect on HPK. Preliminary studies showed that growth arrest could be achieved with little or no toxicity at concentrations below 20 μg/ml. Of considerable interest was the fact that growth arrest was not exerted at a mitotic stage but at some earlier part of the G_2 phase. Fig 1*f* shows HPK cells exposed to the drug (10 μg/ml) for 2 days are uniformly large and are not associated with other cells in aggregates. Table VII presents the results of a study showing the inhibitory effect of razoxane (10 μg/ml) on the kinetics of rapidly growing low-density HPK cultures and evidence for the highly selective accumulation of cells blocked in the G_2 plus M phase of the cell cycle. Growth arrest is not achieved unless cells undergo one full population doubling (5.8 vs 2.7). Thus, it requires 2 days of treatment to attain complete arrest.

The results of clonal growth assays on razoxane-treated cultures indicated that less than 10% of the cells retained colony-forming ability (Table II). Moreover, removal of the drug followed by extensive washing with fresh complete medium failed to initiate continued cell cycling as judged by autoradiographic analysis (data not shown).

Growth Factor Deficiency-Induced Growth Arrest We and others have reported that successful clonal growth of HPK requires the addition of protein growth factors including insulin

Table V. Induction of Growth Arrest in the G_1 and G_2 Phases of the Cell Cycle by Suspension of Rapidly Growing Human Prokeratinocyte Cells in Semisolid Medium[a]

Time	DNA Synthesis (cpm)	Cell Cycle		
		G_1	S	G_2/M
0	1500	47	28	25
6	1600	43	21	36
10	1800	—	—	—
12	750	43	3	54
14	240	—	—	—
21	230	44	3	53

[a]Trypsinized cells were resuspended in 1.3% methylcellulose in complete medium and the rate of DNA synthesis determined as described in *Materials and Methods*. Cell cycle analysis was performed by flow cytofluorimetric analysis of cells stained with a DNA stain, as described also in *Materials and Methods*.

Table VI. Fetal Calf Serum Induces Growth Arrest of Rapidly Growing Human Prokeratinocyte Cultures in Both the G_1 and G_2 Phases of the Cell Cycle

Culture Condition	Cell Density (cells/cm^2 × 10^{-3})			DNA Synthesis[b] (cpm)	Cell Cycle State (%)[b]		
	Day 0[a]	Day 1	Day 2		G_1	S	G_2/M
Complete MCDB 153	2.2	—	13.5	6000	56	25	19
5% dFBS	—	2.9	2.9	200	54	6	40

[a]Log growth cultures fed 5% dFBS 3 days after their prior passage. The day on which dFBS was added is designated day 0.
[b]Assays performed 2 days after addition of 5% dFBS.

and EGF to basal MCDB 153 [4,5]. In this regard, complete medium that contains both these growth factors in addition to 1% BPE is used routinely to subcultivate HPK cells.

We therefore examined the effect of removing all 3 of these protein growth factors on the cellular proliferation kinetics and distribution of cell cycle phases of low-density rapidly growing HPK cells.

Fig 1*g* illustrates the typical appearance of growth factor-deficient cultures. Within 1 day of culture in the absence of growth factors, a striking reorganization of colony morphology is observed, namely, all single cells disappear and are replaced by different sized aggregates of cohesive colonies. This radical colony reorganization occurs independently of calcium level (0.1–2 mM) but is similar to that observed when the calcium concentration was increased to 2 mM in complete medium [5].

Table VIII presents results on the effect of GFDM on the kinetics of growth and cell cycle distribution of HPK cultures. At 48 and 96 h after refeeding GFDM, cell density continued to increase but at a rate considerably less than exponentially growing cultures. For example, compared with rapidly growing control HPK cultures, the growth factor-deficient cultures expanded at only one population doubling for the first 2 days. Sequential autoradiographic analysis indicated that greater than 90% of the incorporation of [^{3}H]thymidine during this 48-h interval occurred during the first 24-h period (data not shown). Thus, the majority of cells continue to cycle for the first day and growth arrest begins to take effect on the second day. In agreement with this supposition, pulse-labeling of growth factor-deficient cultures begun 1 day after refeeding in standard medium revealed that only the peripheral or margin-located cells had incorporated, to any significant extent, radioactivity in their nuclei (see Fig 1*h*). This contrasts with exponentially growing HPK where nuclei uniformly label with [^{3}H]thymidine during a 24-h period of exposure (data not shown). By 4 days, growth factor-deficient cultures had growth arrested almost completely. However, if such cultures are refed complete medium and incubated with [^{3}H]thymidine after 3 days of growth in GFDM, autoradiographic studies showed that the marginal cells continue to incorporate label into nuclei and to proliferate, while the central region of the colony remains quiescent. These results suggest that growth factor deprivation results in the partitioning of the majority of the cell population into quiescent cells (90%) that are located in the interior of the colonies and very slowly cycling cells (10%) at the perimeter of the colonies.

Table VIII also reports results on cell cycle analysis 2 and 6 days after HPK cultures were induced to undergo proliferation arrest in GFDM. G_1 enrichment is observed at both times. Since, however, a small percentage of the cell population at the periphery of the cell aggregates continues to grow slowly, these cell cycle analyses are most likely slightly skewed. Nonetheless, significant enrichment of cells in G_1 is still apparent.

Finally, colony forming ability for growth factor-deprived cultures was dramatically decreased (Table II).

DISCUSSION

Cell cycle-dependent regulation of cellular proliferation has been documented in numerous cell types [6,13,17,24,25]. It has also been shown that defects in these regulatory processes are expressed by a variety of neoplastically transformed cell lines [25–29].

Cell cycle-dependent growth arrest in vitro can be induced by a large variety of pharmacologic and physiologic agents and by modification of a cell's culture microenvironment. For example, cells can be growth arrested by drugs such as hydroxyurea [30], razoxane [21], colchicine [31], and vinblastine sulfate [32] and by physiologic agents such as cyclic AMP [33]. Growth arrest can also be induced by placement of cells in nutrient [34] or growth factor [12] deficient medium, at high cell densities [35], and also in nonadherent culture conditions [36]. In essentially all such experimental systems the growth arrest that is induced is reversible.

Growth arrest at cell cycle specific states has also been demonstrated for certain epithelial cell types in in vivo microenvironments. For example, it has been shown that murine epidermal cells in vivo are physiologically capable of arrest in G_1 and G_2, and it has been reported that other quiescent cells can arrest their growth in G_2 [37–39]. In such experimental systems using epidermal cells it has not, however, been possible to establish whether or not growth arrest of a specific cell population can be reversed.

To accomplish this goal and to more clearly define the mechanism of growth arrest in epidermal cells, in vitro studies are required. However, in vitro studies on cultured epidermal cells have not been previously reported to characterize their cell cycle-dependent growth regulatory control mechanisms.

The results presented in this paper report the first such data generated to meet this deficiency. We show that cultured human epidermal cells can be growth arrested in vitro by at least 6 different methods. Two of these methods induced reversible arrest of HPK growth in G_1. That is, the culture of HPK in IDM or in LCM induced reversible growth arrest. By contrast, culture of HPK in suspension in complete medium or in a monolayer microenvironment in complete medium containing serum, the drug

Table VII. Selective Growth Arrest in the G_2 Phase of the Cell Cycle Induced by the Drug Razoxane

Culture Condition	Cell Density (cells/cm^2 × 10^{-3})			DNA Synthesis[b] (cpm)	Cell Cycle State (%)[b]		
	Day 0[a]	Day 1	Day 2		G_1	S	G_2/M
Complete MCDB 153	2.7	5.1	11.0	5800	46	36	18
Razoxane	—	—	5.8	100	2	1	97

[a]Razoxane (final concentration 10 μg/ml) was added to cultures 1 day after their prior passage and the time razoxane was added is designated day 0.
[b]Assays performed 2 days after addition of razoxane.

Table VIII. Induction of Growth Arrest in the G_1 Phase of the Cell Cycle by Growth Factor-Deficient Medium (GFDM)[a]

Culture Condition	Cell Density[b] (cells/cm^2 × 10^{-3})	DNA Synthesis[c] (cpm × 10^{-3})	Cell Cycle State (%)[d]		
			G_1	S	G_2/M
Complete MCDB 153					
Day 0	2.8	4.5	—	—	—
Day 2	10.0	22.7	47	37	16
Day 4	33.0	50.4	—	—	—
GFDM					
Day 2*	5.3	7.5	80	8	12
Day 4*	7.0	3.0	—	—	—
Day 6†	—	—	72	10	18

[a]GFDM is equivalent to standard medium, i.e., basal MCDB 153 supplemented with hydrocortisone, ethanolamine, and phosphoethanolamine, but it lacks any of the protein growth factors present in complete medium. The calcium concentration was either 0.1 mM* or 2.0 mM†.
[b–d]See legend, Table I, for further details.

razoxane, or in GFDM induced irreversible growth arrest in G_1/G_2. The fact that HPK can be growth arrested in an irreversible manner as well as in a reversible manner whereas other cells, especially other cell lines, can only undergo reversible growth arrest could be explained by the fact that HPK cells are of epithelial origin and are normally present in an organized structure associated with a basement membrane. Alternatively, this observation could be explained by the fact that HPK represent normal mortal cells that are preprogrammed to a specific proliferation and differentiation phenotype and that once their growth is inhibited, a commitment program is activated thereby limiting their subsequent proliferative potential.

It has previously been suggested that growth arrest at a specific cell cycle state precedes differentiation in some cell types [6,9,17]; it is therefore possible that reversible growth arrest of HPK might prevent their differentiation while irreversible growth arrest would promote differentiation. Preliminary evidence indeed supports this explanation (unpublished observations).

If the evidence that irreversible growth arrest at a distinct cell cycle arrest state(s) is requred for keratinocyte differentiation in HPK is substantiated by subsequent experiments, it should then be possible to further determine whether the development of defects in this biologic process is associated with specific human diseases, such as, epidermal dysplasia and cancer. In subsequent studies it should also now be possible to determine whether the development of defects in reversible growth arrest mechanisms in HPK is associated with other diseases, such as, inflammatory skin disease associated with epidermal atrophy.

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