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Cell kinetic characterization of growth arrest in cultured human keratinocytes

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SUMMARY

In this study we have performed a cell kinetic characterization of growth and growth arrest of keratinocytes derived from normal human skin. Proliferative activity of the cell cultures was analysed with a flow cytometric technique, measuring relative DNA content and iododeoxyuridine (IdUrd) incorporation simultaneously. Normal human keratinocytes were grown in keratinocyte growth medium (KGM) and growth arrest was induced by using either keratinocyte basal medium (KBM) or KGM supplemented with TGF-β1. It was found that human keratinocytes grown in KGM plus TGF-β1 were growth-arrested within 52 hours. The rate of IdUrd incorporation into DNA decreased by more than 95% after 52 hours and parallelled the decrease of cells in S-phase. Within 52 hours after addition of TGF-β1, 79% of the growth-arrested cells were in the G_0/G_1-phase of the cell cycle, a situation that approaches that of the normal epidermis. Growth arrest of human keratinocytes in KBM showed a similar decrease in the rate of IdUrd incorporation. However, the decrease in IdUrd incorporation was not reflected in a decrease in cells in S-phase, suggesting that the cells were blocked in G_0/G_1, S or G_2/M-phase rather than selectively in the physiological growth arrest state of G_0/G_1. Secondly, we investigated the kinetics of the cells when they were restimulated after growth arrest. We found that after termination of the growth arrest in KGM supplemented with TGF-β1 the cells require 6 to 8 hours to initiate DNA synthesis, with a continued decrease in the G_0/G_1 population, suggesting that the cells are recruited as a cohort. After growth arrest induced by KBM, cells also require 6 to 8 hours in KGM to initiate DNA synthesis, but the cells are not recruited as a cohort. We conclude that growth arrest induced by TGF-β1 is the preferred system in which to study induction of keratinocyte proliferation, since it induces a state of quiescence that approaches that of normal human epidermis.

Key words: human keratinocyte, cell culture, flow cytometry, cell cycle, growth arrest, transforming growth factor β1

INTRODUCTION

Epidermis is a dynamic tissue in which highly coordinated mechanisms exist to control cell proliferation and differentiation. When this balance is disturbed (e.g. following trauma), human epidermis responds with an increased production of cells in order to restore its integrity as quickly as possible. We have previously advocated the concept of a G_0 population in normal human skin, and we have provided experimental evidence that recruitment from this pool is the mechanism used to restore homeostasis after epidermal injury (Boezeman et al., 1987). The importance of the G_0/G_1-phase of the cell cycle in the control of normal human keratinocyte proliferation is illustrated by the fact that 96% of the keratinocytes exist in G_0/G_1 in vivo, and are not cycling (Bauer et al., 1980). One of the growth abnormalities that occurs in psoriasis is that most, if not all, basal cells appear to be cycling, i.e. the total G_0 population has been recruited.

Experiments on cultured human keratinocytes are potentially useful in the study of growth arrest and recruitment from G_0 in vitro. Many culture systems have been developed to study the control of cell proliferation and differentiation of human keratinocytes in vitro. Early methods for culturing keratinocytes were not very convenient because of problems with mechanical dissociation of epidermal tissue, the need for very high inoculation densities, overgrowth by fibroblasts and the short lifespan of normal keratinocytes. The problem of fibroblast overgrowth has been overcome through the use of a feeder layer of lethally irradiated 3T3 cells (Rheinwald and Green, 1975). The multiplication of keratinocytes has been promoted using conventional Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.4 mg/l hydrocortisone and 20% foetal bovine serum (Rheinwald and Green, 1975). Methods have also been developed that eliminate the need for a feeder layer or the use of conditioned medium (Peehl and Ham, 1980a,b; Price et al., 1980; Maciag et al., 1981). The next step was the development and modification of serum-free culture media in order to obtain confluent and stratified cultures (Tsao et al., 1982; Boyce and Ham, 1983, 1985; Wille et al., 1984; Pillai et al., 1988). The culture systems that use serum-free media allow growth of keratinocytes to high density. The cells can be maintained in an undifferentiated state from which they can be induced to differentiate by several techniques.

Human keratinocytes can be growth-arrested in a reversible

and an irreversible manner. Keratinocyte basal medium (KBM) is currently the most widely used method to induce reversible growth arrest in keratinocytes (Coffey et al., 1987; O'Keefe and Chiu, 1988; Nickoloff et al., 1988; Grossman et al., 1989; Varani et al., 1989; Klein et al., 1992; Mitra and Nickoloff, 1992). In addition three types of treatment cause keratinocytes to arrest growth in a reversible manner: firstly, treatment of cells with medium lacking isoleucine (Pittelkow et al., 1986); secondly, exposure of the cells to lymphocyte conditioned medium (LCM; Pittelkow et al., 1986); and thirdly, treatment of cultures with purified transforming growth factor β1 (TGF-β1) isolated from human platelets (Shipley et al., 1986). All of these methods cause the majority of cells in the population to arrest growth in the G_0/G_1-phase of the cell cycle with a small but significant population of G_2/M-phase cells (Shipley and Pittelkow, 1987). Irreversible growth arrest can be induced in keratinocyte cultures by at least five different methods (Shipley and Pittelkow, 1987).

To get a better understanding of cell proliferation we studied the reversible G_0/G_1 growth arrest of normal human keratinocytes. In order to establish a model for studying growth arrest and G_0 recruitment of human keratinocytes, we evaluated two of these models. Using bivariate flow cytometric analysis we measured cell cycle kinetics of cultures that were growth-arrested either by the addition of a cytokine (TGF-β1), or by the deprivation of growth-promoting substances (KBM instead of KGM). The results show that there are considerable differences between the two systems.

MATERIALS AND METHODS

Biopsies

Biopsies (0.2 mm thickness) from healthy volunteers were taken with a keratotome as previously described (Schalkwijk et al., 1990) and used for primary keratinocyte cultures.

Keratinocyte primary culture

Human epidermal keratinocytes were initially cultured according to the Rheinwald-Green system (Rheinwald and Green, 1975). Primary cultures of keratinocytes were seeded on lethally irradiated (3000 rad, 3 minutes) Swiss mouse 3T3 fibroblasts in DMEM/F12 (3:1, v/v; Flow Laboratories, Irvine, Scotland) supplemented with 0.4 μg/ml hydrocortisone (Collaborative Research Inc. Lexington, MA, USA), isoproterenol (10^{-6} M; Sigma, St Louis, MO, USA), 100 i.u./ml penicillin plus 100 μg/ml streptomycin (Gibco, Breda, The Netherlands), 6% foetal calf serum (FCS; Seralab, Nistelrode, The Netherlands), and 10 ng/ml epidermal growth factor (EGF; Sigma, St Louis, MO, USA). Cells were grown at 37°C, 95% relative humidity and 8% CO_2 in air. EDTA-treated, trypsinized and liquid nitrogen-stored keratinocytes from the primary culture were used in further experiments.

Keratinocyte secondary culture

For the experiments the human keratinocytes were seeded at 10^5 cells in KGM in 60 mm culture dishes. KGM was composed of KBM (Clonetics, San Diego, CA, USA; 0.15 mM calcium) supplemented with ethanolamine (0.1 mM; Sigma, St Louis, MO, USA), phosphoethanolamine (0.1 mM; Sigma, St Louis, MO, USA), bovine pituitary extract (BPE; 0.4%, v/v; Clonetics, San Diego, CA, USA), epidermal growth factor (EGF; 10 ng/ml; Sigma, St Louis, MO, USA), insulin (5 μg/ml; Sigma, St Louis, MO, USA), hydrocortisone (0.5 μg/ml; Collaborative Research Inc. Lexington, MA, USA), penicillin (100 i.u./ml; Gibco, Breda, The Netherlands) and streptomycin (100 μg/ml; Gibco, Breda, The Netherlands).

Keratinocyte growth arrest

For experiments in which cells were required in the quiescent state, the medium was removed at 20-30% confluence (usually about 5 days), the culture was washed briefly with phosphate buffered saline (PBS) supplemented with calcium (120 μM; Merck, Darmstadt, Germany), and maintenance medium was added. During the experiments we used two types of maintenance medium (a) KBM and (b) KGM supplemented with TGF-β1 (10 ng/ml) (British Biotechnology Products Ltd, Oxon., UK). All cultures were grown at 37°C, 95% relative humidity and 5% CO_2 in air.

Keratinocyte growth stimulation

Growth-arrested keratinocyte cultures were washed briefly with PBS supplemented with calcium (120 μM), and new growth medium (KGM) was added.

IdUrd labelling

Cultured keratinocytes were either pulse-labelled (30 minutes) or continuously labelled with IdUrd (Sigma, St Louis, MO, USA), in either case at a final concentration of 10 μmol/l. After pulse-labelling the cultures were washed twice with PBS supplemented with calcium (120 μM) and refed with fresh medium. Experiments were terminated by washing the cultures twice with 3 ml PBS followed by one wash with 1.5 ml 0.25% trypsin (DIFCO Laboratories, Detroit, Michigan, USA), 0.02% EDTA and 0.1% glucose. After a 15 minute incubation with 0.5 ml of the same solution (to allow complete detachment of cells), the cells were collected in PBS containing 5% FCS. After centrifugation the keratinocytes were fixed by resuspending the pellet in 70% ethanol (–20°C), and the suspension was stored at –20°C until further use.

Inhibition of TGF-β1 with neutralizing antibody

To determine if TGF-β1 is effectively washed away from the culture dishes before restimulation we perfomed the growth restimulation experiment in the presence of anti-TGF-β1 polyclonal chicken antibody (1 mg/ml) (R&D Systems Europe Ltd, Abingdon, UK). To neutralize 1.3 ng/ml TGF-β1, 10 μl anti-TGF-β1 antibody was needed (W. van Rotterdam, Department of Cell Biology, University of Nijmegen, The Netherlands, personal communication). As a control, normal chicken IgG (NcIgG; 1 mg/ml) (Nordic, Tilburg, The Netherlands) was used. Human keratinocytes were growth-arrested and restimulated as previously described. Anti-TGF-β1 antibody and NcIgG were added during the restimulation. The cultures were continuously labelled with IdUrd. The effect of antibody addition on restimulation was expressed relative to the percentage of IdUrd-positive cells measured in an exponentially growing culture of normal human keratinocytes.

Measurement of cell viability

Cell viability was determined by staining cell suspensions or cells on coverslips with a 0.4% trypan blue solution (Freshney, 1988). After 1-2 minutes the cells were counted using either a haemocytometer for cell suspensions or a special grid for cells on coverslips.

Immunocytochemical staining of IdUrd and DNA labelling

Staining was basically as previously described (van Erp et al., 1988; Jensen et al., 1994). In brief, about 10^5 ethanol-fixed cells were washed once with PBS. The cells were hydrolysed for 30 minutes at room temperature with 0.2 mg/ml pepsin in 2 M HCl. Hydrolysis was terminated with excess 1 M Trizma base (Sigma, St Louis, MO, USA). The cells were pelleted and washed with PBS containing 0.1% Nonidet P40 (BDH Chemicals Ltd, Poole, England). After sedimentation the cells were incubated with a 1:50 dilution of the monoclonal antibody DAKO-BrdUrd (Dakopatts, Copenhagen, Denmark) for 30 minutes. This monoclonal antibody specifically detects BrdUrd and IdUrd. After washing the cells with PBS a second incubation step (15

minutes, 4°C) using a 1:50 dilution of fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulins (RAM-FITC; Dakopatts, Copenhagen, Denmark) containing 3% normal rabbit serum (NRS) was carried out to visualize the IdUrd incorporation. Following a final wash with PBS, the cells were resuspended in 300 μl PBS containing 40 μg/ml propidium iodide (PI; Calbiochem, San Diego, CA, USA) and incubated for 15 minutes with 50 μl of 1% (w/v) RNase A (Sigma, St Louis, MO, USA).

Immunocytochemical staining of involucrin and DNA labelling

Staining was basically as described earlier (van Duijnhoven et al., 1992). In brief, about 10^5 ethanol-fixed cells were washed once with PBS containing 1% newborn heat-inactivated calf serum (NHICS). After sedimentation the cells were incubated with a 1:20 dilution of MON-150, a monoclonal antibody against human involucrin (van Duijnhoven et al., 1992) for 30 minutes. After washing the cells with PBS/NHICS a second incubation step (15 minutes, 4°C) using a 1:50 dilution of RAM-FITC containing 3% NRS was carried out to visualize the anti-involucrin. Following a final wash with PBS/NHICS, the cells were resuspended in 300 μl PBS containing 40 μg/ml PI and incubated for 15 minutes with 50 μl of 1% (w/v) RNase A.

Flow cytometry

Cells stained with propidium iodide and fluorescein isothiocyanate (FITC) were analysed on the Epics® Elite flow cytometer (Coulter Corporation, Hialeah, Florida, USA) equipped with a 40 mW air-cooled argon-ion laser set at 15 mW and tuned at a wavelength of 488 nm. FITC and PI signals were separated by a 580 nm dichroic mirror. The FITC signals (green fluorescence) were detected through a 525 nm band-pass filter, and the PI signals (red fluorescence) were detected through a 630 nm long-pass filter.

Usually 10^4 cells were measured at a flow rate of approximately 50 cells per second. The data were recorded in listmode and analysed on the Epics® Elite workstation. The ratio area/peak of the red fluorescence is an excellent discriminator between artifacts due to doublets of diploid cells and real single tetraploid (or late S) cells when intact cells are used (Bauer and Boezeman, 1983).

The data were further analysed on the Epics® Elite workstation with the program Multicycle (Version 2.5; Phoenix Flow Systems, Sorrento Valley Rd., San Diego, CA, USA) to determine the percentages of G_0/G_1, S and G_2/M.

The results are presented in two-dimensional graphs in which error bars are drawn. Unless stated otherwise, these error bars indicate the standard error of the mean (s.e.m.). In case no error bars are visible the bar is smaller than the graphic symbol used.

Calculations of cell cycle time and duration of S-phase

Two-parameter flow cytometry of DNA versus IdUrd allows assessment of the number of cells in S-phase (N_S) and the duration of the S-phase (t_S) even from one single sample (Begg et al., 1985). The calculation of t_S is based on the assumption that there is a linear increase in mean relative DNA content of the IdUrd-labelled S-phase cells in time. At time zero after IdUrd pulse-labelling, the mean DNA content of the IdUrd-labelled S-phase cells is in the middle of the interval between the unlabelled diploid G_0/G_1 cell population and the unlabelled tetraploid G_2/M cell population. As the IdUrd-labelled cells move through S-phase, the mean DNA content of the population will approach the DNA content of the G_2/M population. The IdUrd-labelled cells that had divided and appeared in the (labelled) diploid G_1 region were excluded from the calculation of mean DNA content. Movement of IdUrd-labelled S-phase cells relative to the position of G_0/G_1 and G_2/M is expressed as relative movement (*RM*) and is calculated as follows:

$$RM = (F_{\mathrm{IdUrd}} - F_{\mathrm{G_0/G_1}})/(F_{\mathrm{G_2/M}} - F_{\mathrm{G_0/G_1}}),$$

where F_{IdUrd} is the mean DNA content of the IdUrd-labelled cell (IdUrd-labelled G_0/G_1 cells were excluded), $F_{\mathrm{G_0/G_1}}$ is the mean DNA content of the unlabelled diploid G_0/G_1 population (will also contain the differentiated cell population, if present) and $F_{\mathrm{G_2/M}}$ is the mean DNA content of the G_2/M cells.

RM will increase in time from *RM*=0.5 at time zero (IdUrd-labelled cells half-way between the G_0/G_1 cells and the G_2/M cells) to *RM*=1 when all IdUrd-labelled cells have reached tetraploid DNA content. The time needed for labelled cells to reach tetraploid DNA content is equal to t_S. t_S is calculated from one single sample using the formula:

$$t_S = 0.5/(RM - 0.5) \times \Delta t,$$

where Δt is the time between pulse-labelling and sampling.

Calculation of the absolute cell cycle time (t_C) depends on the assumption that cells are randomly distributed through the various phases of the cycle. Although this assumption has been questioned, it has been used by various investigators in this field (for example, by van Hooijdonk et al., 1993). It follows that:

$$\frac{N_S}{N_C} = \frac{t_S}{t_C},$$

where N_S is the number of cells in S-phase, N_C is the number of cycling cells (growth fraction, GF), t_S is the duration of the S-phase and t_C is the cell cycle time.

RESULTS

Although the KGM system is widely used, it is poorly documented with respect to cell cycle kinetics. Since we used KGM as the growth medium in this study we first characterized exponentially growing keratinocytes using bivariate flow cytometric analysis. Normal human keratinocytes from the first passage were seeded at 10^5 cells per 60 mm dish and were grown in KGM. At day 8 the cells were either pulse-labelled or continuously labelled with IdUrd and were harvested at different times. Flow cytometric analysis of the continuously labelled cells revealed a growth fraction (GF or N_C) of 92% after 48 hours as shown in Fig. 1. Fig. 2 represents the fraction of cells in the middle of S-phase during normal growth. In Fig. 2 it can be seen that the cells re-enter S-phase after approximately 20 hours, representing the length of the cell cycle. From the bivariate flow cytometric IdUrd/DNA analysis we calculated that the duration of the S-phase (t_S, with t=4 hours, *RM*=0.80) was 7 hours. With this result we calculated the cell cycle time (t_C, with t_S=6.6, N_C=91.9% and N_S=22.8%) that resulted in a t_C of 27 hours. These values are in agreement with those found for the Rheinwald and Green system and in vivo.

Kinetics of TGF-β1- and KBM-induced growth arrest

The morphology of exponentially growing human keratinocytes cultured in KGM is shown in Fig. 3a. The keratinocytes grow in colonies, forming a monolayer and continue to grow to full confluence. When normal human keratinocytes were growth-arrested in KGM supplemented with TGF-β1, a diffuse monolayer of keratinocytes was formed (Fig. 3b). Treatment with KBM also induced growth arrest; however, the morphology was distinctly different from keratinocytes treated with TGF-β1. Growth arrest induced by KBM resulted in colonies of tightly packed keratinocytes (Fig. 3c).

Normal first-passage human keratinocytes were grown in vitro in KGM until they reached about 25-30% confluence (day 5-6). From this point the cultures were switched to KGM sup-

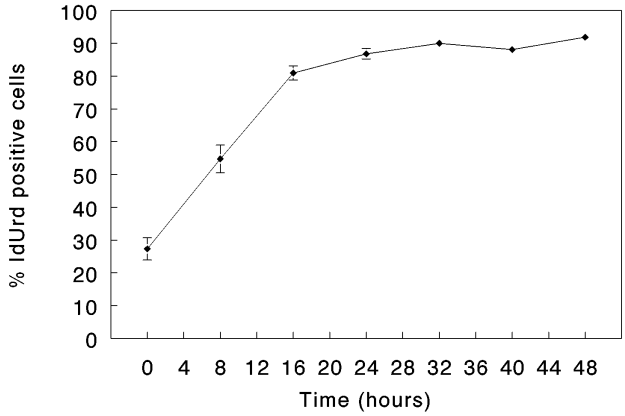

Fig. 1. Flow cytometric analysis of IdUrd-positive cells during normal growth. Keratinocytes cultured in KGM until 20-30% confluence were continuously labelled with IdUrd during a period of 48 hours, fixed and processed for IdUrd immunostaining, and counterstained with PI. Flow cytometric analysis revealed the population of IdUrd-postive cells after treatment and the different cell cycle stages. At least 10,000 cells were analysed. At the indicated times samples were taken and the IdUrd-positive cells were measured. (Bars in the figure indicate the data range of two dishes per time-point.)

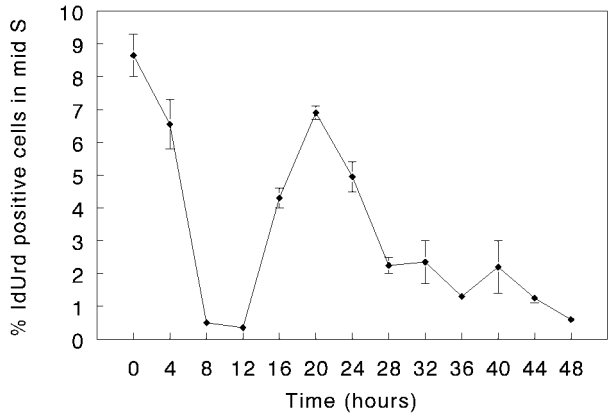

Fig. 2. Flow cytometric analysis of IdUrd-positive cells during normal growth. Keratinocytes cultured in KGM until 20-30% confluence were pulse-labelled for 30 minutes with IdUrd at several time points during a period of 48 hours. The cells were fixed and processed for IdUrd immunostaining, and counterstained with PI. Flow cytometric analysis revealed the population of IdUrd-positive cells after treatment and the different cell cycle stages. At least 10,000 cells were analysed. At the indicated times samples were taken and the IdUrd-positive cells in the middle of the S-phase was estimated. From this experiment we can follow the cells during the cell cycle. In this figure we can see that the population of cells starting in mid-S reach this point again after approximately 20 hours (the second peak). This indicates that the cells need about 20 hours to complete one cell cycle. (Bars in the figure indicate the data range of two dishes per time-point.)

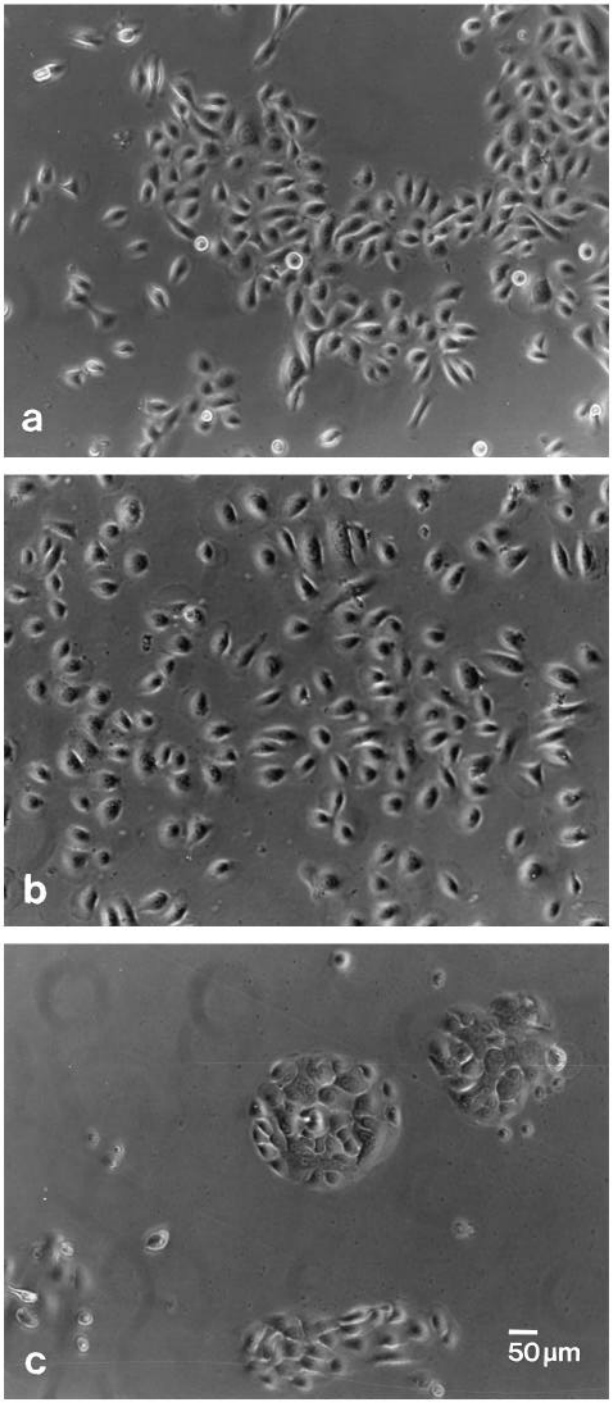

Fig. 3. Light microscopic visualization of normal human keratinocytes during growth under different culture conditions. (a) Growth of normal human keratinocytes in KGM; (b) growth arrest in KGM supplemented with TGF-β1 (56 hours); (c) growth arrest in KBM (56 hours).

plemented with 10 ng/ml TGF-β1 to induce growth arrest. TGF-β1 was shown to induce a dose-dependent decrease in IdUrd-positive cells. Fig. 4 shows that saturation is reached at 5 to 10 ng/ml TGF-β1. The cultures were analysed using bivariate flow cytometric analysis of DNA synthesis (IdUrd incorporation) and DNA content (PI). We measured the DNA synthesis and DNA content at different times during a period of 52 hours of growth arrest. As an example the flow cytometric data of 0 and 52 hours are depicted in Fig. 5. In Fig. 6 the cell cycle parameters of exponentially growing cells are shown, indicating that these remain constant over a 52 hour period. From the flow cytometric data we calculated the

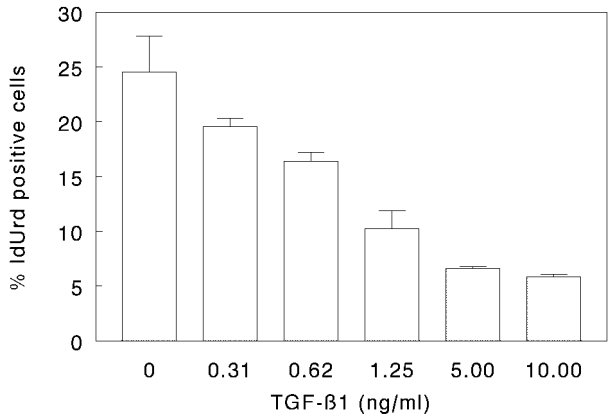

Fig. 4. The effect of different concentrations of TGF-β1 on the growth of normal human keratinocytes. Normal human keratinocytes cultured in KGM until 20-30% confluence were growth-arrested with the indicated concentrations of TGF-β1. After 48 hours of treatment the cells were pulse-labelled with IdUrd for 30 minutes, fixed and processed for IdUrd immunostaining, and counterstained with PI. Flow cytometric analysis revealed the population of IdUrd-positive cells after treatment and the different cell cycle stages. At least 10,000 cells were analysed. (Bars in the figure indicate the data range of two dishes per time-point.)

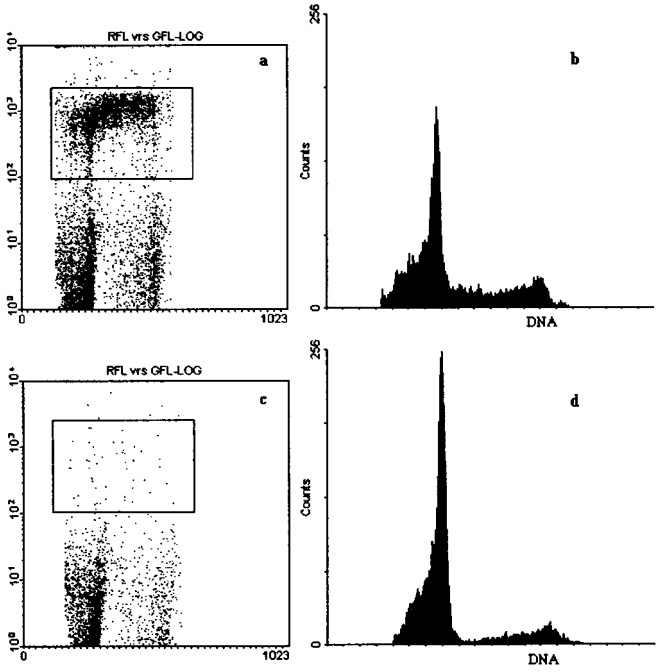

Fig. 5. Example of a flow cytometric analysis of IdUrd-positive cells and their DNA content. Comparison of: (a,b) exponentially growing human keratinocytes (*t*=0 hours); and (c,d) keratinocytes after 52 hours of TGF-β1 treatment. At the times indicated, cells were fixed and processed for IdUrd immunostaining with FITC and DNA content with PI. The IdUrd-positive cells can be calculated from the gate placed in the diagram, which represents the DNA content (red fluorescence (RFL)) versus the IdUrd-positive cells (Logarithm of the green fluorescence (GFL-LOG)). The gate, which represents all the IdUrd-positive cells, has been determined by using a sample without a specific antibody against IdUrd resulting in 0-1% IdUrd-positive cells. In (b) and (d) plots are shown of DNA distribution versus cell number.

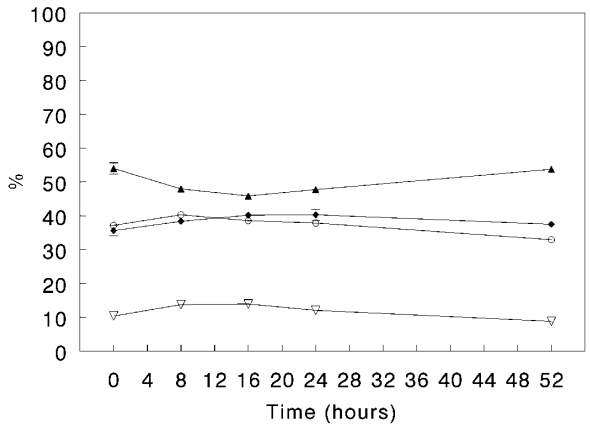

Fig. 6. A control group of normal human keratinocytes was cultured in KGM and starting at 20-30% confluence the cells were pulse-labelled with IdUrd for 30 minutes at several intervals during a period of 52 hours. Cells were fixed and processed for IdUrd and involucrin immunostaining, and counterstained with PI. The percentages of cells in the different cell cycle stages (G_0/G_1(▲), S (○) and G_2/M (▽)), and IdUrd-positive (◆) cells were determined by counting at least 10,000 cells. Note that the percentages remain constant in time during the 52 hour period. (Bars in the figure indicate the data range of two dishes per time-point.)

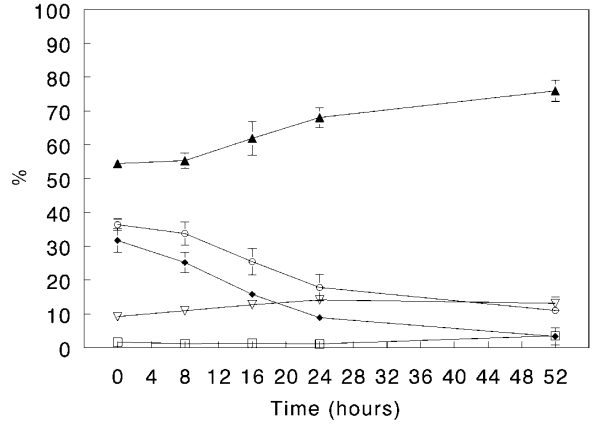

Fig. 7. Kinetics during growth arrest induced by TGF-β1. Exponentially growing human keratinocytes were growth-arrested at day 5 by adding 10 ng/ml of TGF-β1 to the culture medium. At different times, as indicated, cells were pulse-labelled with IdUrd for 30 minutes, fixed and processed for IdUrd and involucrin immunostaining, and counterstained with PI. The percentages of cells in the different cell cycle stages (G_0/G_1 (▲), S (○) and G_2/M (▽)), IdUrd (◆) and involucrin-positive (□) cells were determined by counting at least 10,000 cells. Note that the population of G_0/G_1 cells is increasing in time, and that the decrease in IdUrd-positive cells is parallelled by a decrease in cells in S-phase (4 dishes per time-point).

number of IdUrd-positive cells and the distribution throughout the cell cycle. Fig. 7 summarizes the kinetics of growth arrest of keratinocytes in KGM supplemented with 10 ng/ml TGF-β1 during the time course of 52 hours. This figure demonstrates that cells treated with TGF-β1 stop growing within 52 hours, as illustrated by a 96% decrease in IdUrd-positive cells. The figure furthermore characterizes the distribution of cells over

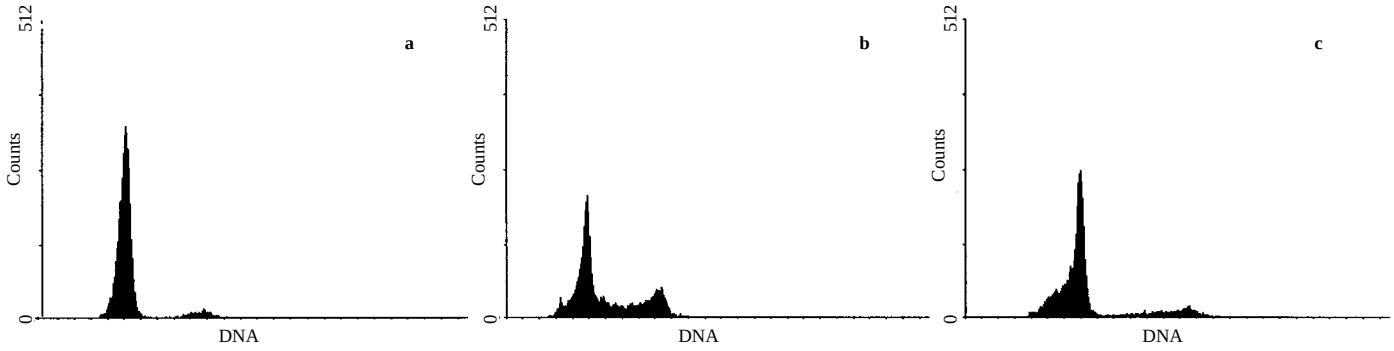

Fig. 8. Representation of the DNA distribution of: (a) normal human epidermis (in vivo); (b) cultured normal human keratinocytes; and (c) normal human keratinocytes growth-arrested with TGF-β1 for 52 hours. Normal epidermis was derived from a healthy volunteer. The keratinocytes were brought into suspension and stained with PI. The keratinocytes in culture were pulse-labelled with IdUrd for 30 minutes, fixed and stained with PI. The percentages of cells in the different cell cycle stages (G_0/G_1, S and G_2/M) were determined by counting at least 10,000 cells. Analysis of the DNA histograms revealed that: (1) normal human epidemis (in vivo) consists of 92% G_0/G_1, 2% S and 7% G_2/M; (2) normal cells in culture consists of 49% G_0/G_1, 37% S and 14% G_2/M; and (3) normal cells in culture growth-arrested with TGF-β1 consists of 79% G_0/G_1, 10% S and 11% G_2/M.

the cell cycle stages and shows that: (a) after 52 hours of exposure to TGF-β1, only 1% of the cells displayed evidence of DNA synthesis; and (b) 79% of the growth-arrested cells were in the G_0/G_1-phase of the cell cycle. The decrease in IdUrd incorporation is parallelled by the decrease of cells in S-phase of the cell cycle. This situation approaches the state in normal epidermis, in which we find 92% of the cells in G_0/G_1, 1% in S, 7% in G_2/M, and 3% IdUrd incorporation (as determined by in vivo IdUrd labelling; van Erp et al., unpublished data). In Fig. 8 we present DNA histograms of normal human epidermis (in vivo, (a)), normal human keratinocytes in culture (b), and normal human keratinocytes growth-arrested with TGF-β1 (c).

Growth arrest of normal human keratinocytes can also be induced by KBM. Fig. 9 shows that KBM inhibits cell proliferation and induces a 90% reduction in DNA synthesis within 52 hours as determined by the measurement of IdUrd incorporation into DNA. In contrast to growth arrest with KGM supplemented with TGF-β1, we see that the decrease in IdUrd incorporation into DNA is not parallelled by a decrease in cells in S-phase of the cell cycle. The population of cells in S remains on a permanent level of 35% during growth arrest. We also see that the population of G_2/M cells is increasing in time and that the G_0/G_1 population is stable. These results indicate that the cells are growth-arrested at random in either G_0/G_1, S or G_2/M.

During these experiments we analysed the number of differentiating cells using bivariate flow cytometric analysis of anti-involucrin and DNA content. The results, as shown in Figs 7 and 9, demonstrate that the cells are not induced to differentiate during growth arrest.

Treatment of cells during 52 hours with KBM causes a decrease in cell number. About 30% of the cells detach from the culture dishes and are floating in the medium. The cells remaining in the culture are all viable at the start of restimulation experiments. Treatment of the cultures with 10 ng/ml TGF-β1 does not result in a decline in cell number and has no effect on the viability.

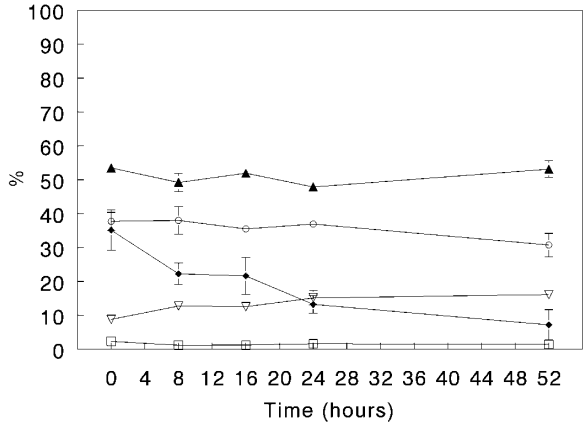

Fig. 9. Kinetics during growth arrest induced by KBM. Exponentially growing human keratinocytes cultured in KGM were growth-arrested by switching to KBM at day 5. At different times, as indicated, cells were pulse-labelled with IdUrd for 30 minutes, fixed and processed for IdUrd and involucrin immunostaining, and counterstained with PI. The percentages of cells in the different cell cycle stages (G_0/G_1 (▲), S (○) and G_2/M (▽)), IdUrd (◆) and involucrin-positive (□) cells were determined by counting at least 10,000 cells. Note that the population of G_0/G_1 cells is relatively constant in time, and that the decrease in IdUrd-positive cells is not parallelled by a decrease in cells in S-phase (4 dishes per time-point).

Kinetics of restimulation after growth arrest with TGF-β1 and KBM

Restimulation of KBM growth-arrested cultures results in a morphological change in the keratinocytes on the edge of the colony (Fig. 10a) and after 52 hours of restimulation the cultures grow to full confluence (data not shown). Restimulation of TGF-β1 growth-arrested cultures shows that after 52 hours the cultures have grown to full confluence (Fig. 10b), comparable to restimulation of KBM growth-arrested cultures.

On the basis of the reversibility of the TGF-β1 growth arrest, we investigated the kinetics of restimulation after growth arrest with TGF-β1 and KBM by switching these cultures to KGM. Before feeding new medium the cultures were washed to remove all of the previously added TGF-β1 and KBM. Fig. 11 gives the results of a typical experiment showing that in TGF-β1-arrested cells initiation of DNA synthesis occurs within 6 to 8 hours. This figure also demonstrates a decrease in the

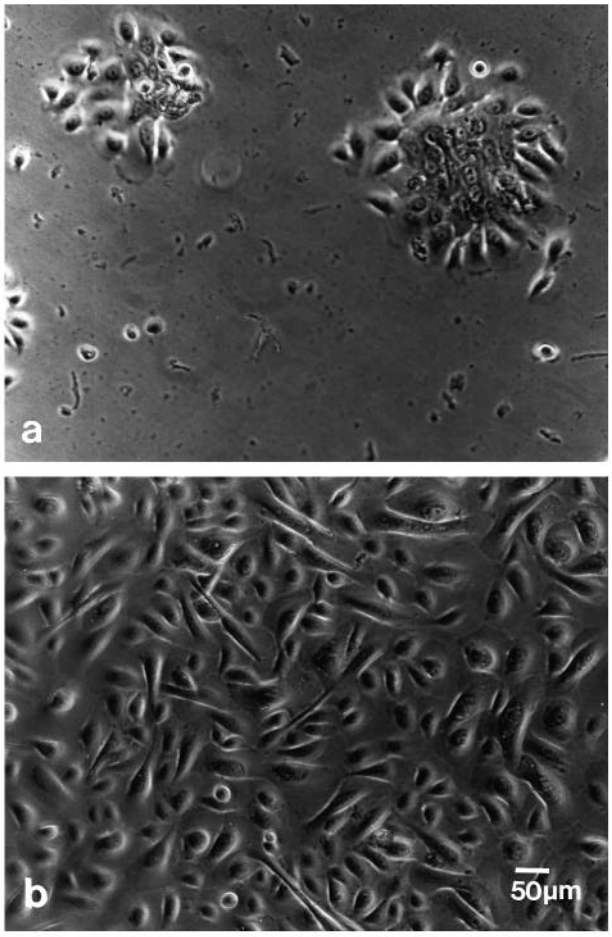

Fig. 10. Light microscopic visualization of normal human keratinocytes during growth under different culture conditions. (a) Restimulation after growth arrest induced by KBM (24 hours); and (b) restimulation after TGF-β1-induced growth arrest (52 hours) (4 dishes per time-point).

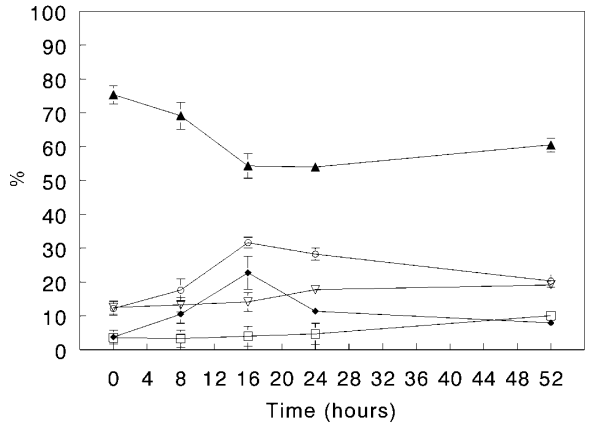

Fig. 11. Kinetics during restimulation of TGF-β1 growth-arrested keratinocytes. At day 5 exponentially growing human keratinocytes were growth-arrested by adding 10 ng/ml of TGF-β1 to the culture medium. After 48 hours of treatment with TGF-β1 the cultures were washed with PBS/calcium (120 μM) and refed with KGM (t=0 hours). At different times, as indicated, cells were pulse-labelled with IdUrd for 30 minutes, fixed and processed for IdUrd and involucrin immunostaining, and counterstained with PI. The percentages of cells in the different cell cycle stages (G_0/G_1 (▲), S (○) and G_2/M (▽)), IdUrd (◆) and involucrin-positive (□) cells were determined by counting at least 10,000 cells. Note that the population of G_0/G_1 cells is decreasing followed by an increase in IdUrd-positive cells and cells in S-phase. This indicates that the cells are recruited as a cohort (4 dishes per time-point).

G_0/G_1 population parallelled by an increase in the S-phase population and the incorporation of IdUrd into DNA. This means that the growth-arrested cells are recruited as a cohort from the G_0/G_1 population.

Restimulation of KBM growth-arrested cells results in initiation of DNA synthesis within 6 to 8 hours as shown in Fig. 12, which also makes it clear that the cells are not recruited in a cohort, but that they are recruited from within the G_0/G_1, S and G_2/M phases. Maximum DNA synthesis occurred 16 hours after growth restimulation for either KBM or TGF-β1 growth-arrested cultures as shown in Fig. 13.

During these experiments we also analysed the number of differentiating cells using bivariate flow cytometric analysis of anti-involucrin and DNA content. The results, as shown in Figs 11 and 12, demonstrate that the cells are not induced to differentiate during restimulation.

To get an estimate of the number of cells capable of restarting proliferation we continuously labelled KBM and TGF-β1 growth-arrested cultures during restimulation. Using this continuous labelling procedure, all cells that start to enter the cell cycle are capable of incorporating IdUrd. When keratinocytes are growth-arrested with TGF-β1, we see that after 32 hours of restimulation only 65% of the cells are cycling, which means that TGF-β1 reduces the number of cells that can restart proliferation (Fig. 14). On the other hand, when KBM growth-arrested cells are restimulated we see that after 32 hours about 85% of the cells are cycling (Fig. 14). This percentage is similar to the findings with keratinocytes maintained in KGM where we see that about 90% (Fig. 1) of the cells are cycling.

A possible explanation for the relatively small percentage of TGF-β1 growth-arrested cells that enter the cell cycle could be the persistence of TGF-β1 in the culture dishes (Rollins et al., 1989). To investigate if TGF-β1 was effectively washed away from the cultures before restimulation, we determined the effect of a neutralizing anti-TGF-β1 antibody on restimulation. The effect of the anti-TGF-β1 antibody was compared with the effect of NcIgG (normal chicken IgG). The growth fraction of normal growing keratinocytes was taken as a reference. From Fig. 15 we can see that addition of anti-TGF-β1 antibody during restimulation results in an increase in the growth fraction of approximately 30% compared to the addition of NcIgG during restimulation.

DISCUSSION

In this study we have determined cell cycle parameters of the KGM/KBM culture system during exponential growth, growth arrest and restimulation, using bivariate flow cytometric determination of DNA synthesis and DNA content. We compared TGF-β1- and KBM-induced growth arrest and we conclude that the former method is the preferred system, since it comes closest to the situation in vivo. This process is crucial in epidermal growth control in vivo.

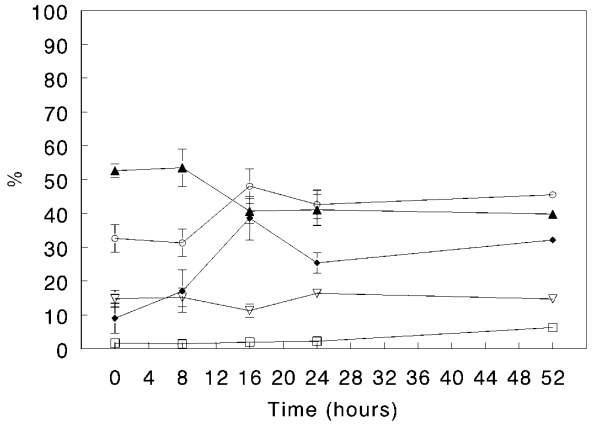

Fig. 12. Kinetics during restimulation of KBM growth-arrested keratinocytes. At day 5 normal human keratinocytes were growth-arrested by switching to KBM. After 48 hours of treatment with KBM the cultures were refed with KGM. At different times, as indicated, cells were pulse-labelled with IdUrd for 30 minutes, fixed and processed for IdUrd and involucrin immunostaining, and counterstained with PI. The percentages of cells in the different cell cycle stages (G_0/G_1 (▲), S (○) and G_2/M (▽)), IdUrd (◆) and involucrin-positive (□) cells were determined by counting at least 10,000 cells. The population of G_0/G_1 cells and the population of cells in S-phase increase during the first 8 hours. The increase in both populations means that the cells are recruited from both M-phase and G_0/G_1-phase, indicating that the cells are not recruited as a cohort (4 dishes per time-point).

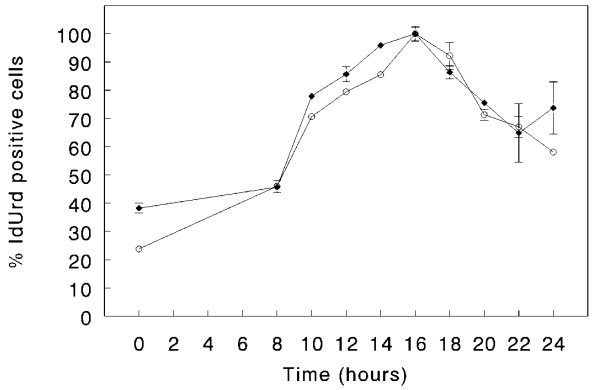

Fig. 13. Composite figure of the kinetics of appearance of IdUrd-positive cells during restimulation of KBM (◆) and TGF-β1 (○) growth-arrested cultures. At different times, as indicated, cells were pulse-labelled with IdUrd for 30 minutes, fixed and processed for IdUrd immunostaining, and counterstained with PI. Results from three different experiments were normalized relative to the maximum value, which is at 16 hours after restimulation. The percentage of IdUrd-positive cells was determined by counting at least 10,000 cells. (Bars in the figure indicate the data range of two dishes per time-point.)

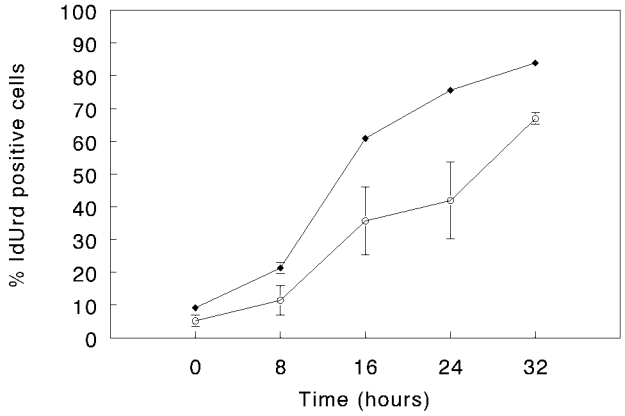

Fig. 14. Determination of the growth fraction of restimulated keratinocytes after growth arrest induced by either KBM (◆) or KGM supplemented with TGF-β1 (○). Growth-arrested keratinocytes were refed with KGM at the indicated times, and cells were continuously labelled with IdUrd, fixed, processed for IdUrd immunostaining, and counterstained with PI. The percentage of IdUrd-positive cells was determined by counting at least 10,000 cells. (Bars in the figure indicate the data range for KBM treatment (two dishes per time-point) and the standard deviation for the TGF-β1 treatment (4 dishes per time-point).)

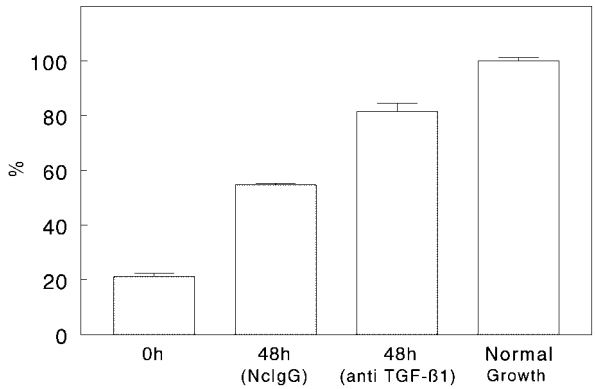

Fig. 15. Effect of anti-TGF-β1 antibody on restimulation of TGF-β1 growth-arrested cultures. Normal human keratinocytes cultured in KGM until 20-30% confluence were growth arrested with TGF-β1. Subsequently the cultures were restimulated with KGM in the presence of anti-TGF-β1 antibody and NcIgG. Normal untreated keratinocytes were used as a reference value (100%). Note that there is a 30% increase in IdUrd-positive cells in the presence of anti-TGF-β1 antibody compared to NcIgG. (Bars in the figure indicate the data range of two dishes per time-point.)

The cell cycle time of exponentially growing keratinocytes in KGM measured in this study is very similar to values obtained in previous studies for the cell cycle time in the Rheinwald-Green system and values obtained by in vivo labelling with IdUrd (van Erp et al., 1991, 1994). This finding supports the notion that cell cycle times of human keratinocytes are relatively constant regardless of the system used, and that cell production is controlled by the number of recruited (cycling) cells rather than by variation in cell cycle time, as suggested by others (Allegra and De Panfilis, 1974; Weinstein et al., 1985). Flow cytometric analysis of exponentially growing keratinocytes further revealed that the distribution over the different cell cycle compartments is constant in time. These results support the assumption of Begg et al. (1985) that cells are randomly distributed through the cell cycle.

Previously, other groups have studied TGF-β1-induced growth arrest using [^{3}H]thymidine incorporation and/or flow

cytometry (Moses et al., 1985; Shipley et al., 1986; Reiss and Sartorelli, 1987; Wilke et al., 1988; Matsumoto et al., 1990; Cook et al., 1991). We were able to confirm the findings of these studies with respect to the kinetics of growth arrest and restimulation. We have extended these findings by comparing TGF-β1-induced growth arrest with KBM-induced growth arrest. The latter method is currently often used to bring keratinocytes to quiescence before stimulating them with growth factors or cytokines (Coffey et al., 1987; O'Keefe and Chiu, 1988; Nickoloff et al., 1988; Grossman et al., 1989; Varani et al., 1989; Klein et al., 1992; Mitra and Nickoloff, 1992).

In our study we have used IdUrd incorporation followed by immunocytochemical staining and flow cytometric analysis, rather than [^{3}H]thymidine incorporation. IdUrd measurement is a more dynamic measurement and has advantages over [^{3}H]thymidine incorporation. Apart from the potential hazards of radioactive material, [^{3}H]thymidine is not detectable with flow cytometry, and the analysis is restricted to the total cell population. IdUrd measurement is fast compared to [^{3}H]thymidine autoradiography and single cells can be analysed using bivariate measurement of DNA content and IdUrd incorporation. Using IdUrd incorporation we found that DNA synthesis decreases during KBM-induced growth arrest, and is not parallelled by a decrease in cells in S-phase in contrast to TGF-β1-induced growth arrest.

Apart from the flow cytometric data, we also noted morphological differences between KBM- and TGF-β1-induced growth arrest. We see that TGF-β1 growth-arrested cultures form a diffuse monolayer of keratinocytes. The formation of a diffuse monolayer could be due to the increased motility of the keratinocytes that is caused by TGF-β1 (Nickoloff et al., 1988). In KBM the growth-arrested cultures seem to become more compact.

In conclusion, KBM-induced growth arrest results from a deprivation of essential growth factors, and blocks the cells in the various cell cycle stages. However, a high percentage of the cells (85%) can be stimulated to resume growth, starting from random positions in the cell cycle. TGF-β1-induced growth arrest blocks the cells in G_0/G_1 and appears to result in a state of quiescence that approaches the situation in normal epidermis. The cells can be stimulated to re-enter the cell cycle in a cohort-like fashion; however, only 65% of the cells are recruitable. It should be noted that the degree of confluence at the time of restimulation influences the percentage of IdUrd-positive cells at 32 hours; when restimulated TGF-β1-treated cultures become confluent before 32 hours, the percentage of IdUrd-positive cells drops below 65% (probably due to contact inhibition, data not shown). In the KBM-growth-arrested cultures we found that restimulation is strongly affected by the degree of confluence in the culture. When cell density in the dish is too low at the start of restimulation (less than 20% confluence) cells die, probably due to low levels of autocrine factors. During our experiments the densities of KBM-treated cultures were 20-30%, which resulted in a growth fraction of 85% after restimulation.

The experiment with the neutralizing antibody suggest that TGF-β1 cannot effectively be washed away from the cultures before restimulation. The presence of TGF-β1 explains the difference in growth fractions after 32 hours of restimulation between cells restimulated from KBM and cells restimulated from TGF-β1. To inhibit TGF-β1 activity from the cultures, large amounts of neutralizing antibodies or trypsinization of the cells should be used. Both methods have their restrictions, since large amounts of anti-TGF-β1 antibody are needed, and replating of the cells introduces additional variables.

In conclusion, both models for bringing keratinocytes to quiescence are useful in studying the effects of external stimuli on proliferation. However, one should bear in mind the limitations of these models when interpreting the results.

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