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THE COURSE OF CULTURE OF HUMAN KERATINOCYTES: INCREASE OF HEAT-LABILE FRACTION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

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Abstract. Human epidermal keratinocytes in cell culture are unique in reorganizing the formation of a stratified cellular sheet and performing terminal differentiation. The course of keratinocytes in culture was delineated over 50 days in the primary culture. The rapid increase in total DNA content commencing 2 weeks after plating corresponded to the appearance of small polygonal keratinocytes in groups. The DNA content reached its maximum after 25 days of cultivation, decreasing gradually thereafter. The protein content increased slowly until 16 to 18 days of cultivation, and then increased rapidly, though slightly behind the rapid increase in DNA. The maximum value of protein content was reached at 32 days, after which the value decreased when the keratinized cells of the uppermost layer began to detach. The heat-labile fraction of glucose-6-phosphate dehydrogenase was increased in 33- and 43-day cultures, when the DNA content was decreasing.

Key words: Tissue culture; Keratinocyte; Glucose-6-phosphate dehydrogenase; Ageing

Keratinocytes in cell culture are unique in forming a multilayered cellular sheet in which the keratinocytes are arranged to form a structure resembling the epidermis in vivo, and perform terminal differentiation to become keratinized cells (1–3). On the other hand keratinocytes in cell culture seem to have a limited lifespan. Subcultures could be made from the primary culture by dispersing a sheet of keratinocytes into single cells after incubation in a solution containing trypsin and EDTA and transferring to new culture dishes. After a few passages, however, the rate of proliferation decreased until further subcultivation was impossible (1, 2), although Rheinwald & Green reported the success of serial cultivation of human keratinocytes by the use of lethally irradiated 3T3 cells (4).

Since Hayflick proposed the concept that the cellular senescence observed in cultured cells has an analogy to the process of ageing in vivo (5, 6), almost all studies on in vitro ageing have been made by the use of fibroblasts. In aged cells certain enzymes become altered. These altered enzymes can be recognized by their reduced specific activity (7). The heat-labile fraction of glucose-6-phosphate dehydrogenase (G6PD) is increased during the senescence of human fibroblasts.

We demonstrate here that the heat-labile fraction of G6PD is increased in the primary culture of human keratinocytes when the culture decline becomes apparent.

MATERIALS AND METHODS

Isolation of keratinocytes and preparation of cultures

Human epidermal keratinocytes were isolated from skin of normal subjects as reported previously (8).

Human skin was obtained by plastic surgery, and soaked in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline. The full-thickness skin was trimmed on the dermal side as far down as possible with a fine curved scissors. The specimens were cut into pieces of about 3×10 mm, treated with 0.02% EDTA solution for 5 min at room temperature, and kept in 0.25% trypsin (Difco) in Ca^{2+}- and Mg^{2+}-free PBS for 12 to 24 hours in a refrigerator (4°C). An epidermal cell suspension was obtained by dissociating the epidermal sheet which had been peeled from the dermis with fine forceps. The viability of cells exceeded 90%, measured by the eosin dye exclusion test. The number of basal cells was determined by the criteria of Vaughan & Bernstein (9). The culture was initiated by seeding 8×10^5 basal cells suspended in 4 ml of culture medium into a 60×15 mm plastic tissue culture dish. This was incubated in an atmosphere of 95% air and 5% CO_2 at 37°C. The medium consisted of Eagle's minimal essential medium supplemented with 20% fetal bovine serum.

Methods of assay

To measure the total DNA and protein contents, cultures were washed three times with Ringer solution, and stored at −20°C until all cultures were ready for assay. The cells were scraped into 3 ml 0.5 N PCA, and hydrolysed at 70°C for 1 hour. After centrifugation, the supernatant was taken (sample 1). The pellet was resuspended into 3 ml 0.5 N PCA, and hydrolysed again at 70°C for 30 min. After

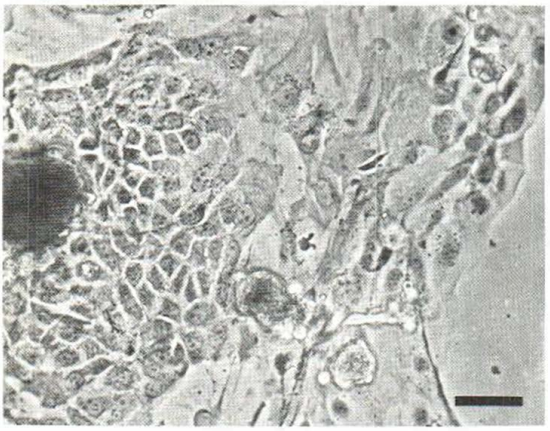

Fig. 1. Early, proliferating culture. Small polygonal keratinocytes are observed in groups. 9-day culture. Phase contrast. Bar represents 10 μm.

centrifugation the supernatant was taken (sample 2). Samples 1 and 2 were assayed for DNA *ad modum* Burton (10). Total DNA content was expressed as a sum of DNA in samples 1 and 2. The pellet after the second extraction in 0.5 N PCA was dissolved in 1 N NaOH, and assayed for total protein *ad modum* Lowry et al. (11).

The assay procedures of glucose-6-phosphate dehydrogenase (G6PD) followed those of Holliday & Tarrant (7). Briefly, keratinocytes (at 19, 33 and 43 days of cultivation) were harvested by scraping. Then the cells were rinsed with PBS, and resuspended in 0.05 M Tris/HCl buffer (pH 8.0) containing 10^{-3} M EDTA, 4×10^{-3} M ε-amino-*n*-caproic acid, 10^{-3} M mercaptoethanol and 10^{-3} M NADP. The cells were disrupted by sonication followed by centrifugation at 30 000 *g* for 30 min. The supernatant served s an enzyme sample.

To determine the percentage of heat-labile enzyme in the preparation, aliquots of supernatant were put into thin glass tubes, and the tubes heated in a water bath at 55°C for varying periods. The remaining activity in each tube was determined. Enzyme activity was measured by following the increase in absorption at 340 nm due to the production of NADPH in the presence of glucose-6-phosphate, as recorded by a Hitachi-200 spectrophotometer. The results are expressed as a percentage of the non-heated enzyme.

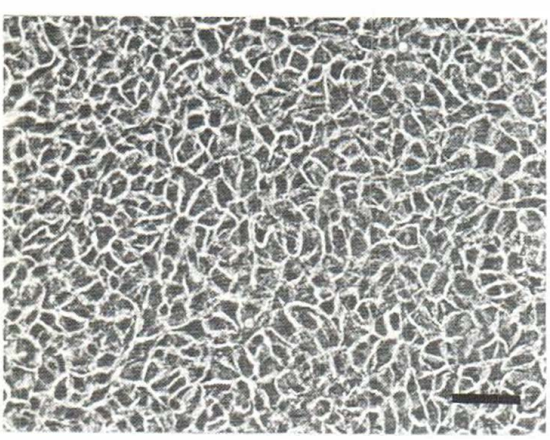

Fig. 2. Confluent sheet of keratinocytes. Intercellular bridges can be seen in the wide spaces between the cells. 34-day culture. Phase contrast. Bar represents 10 μm.

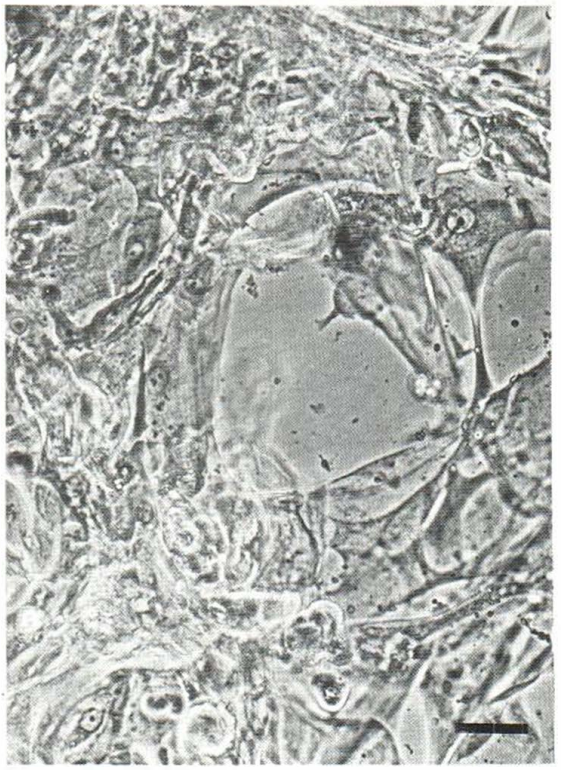

Fig. 3. Old culture. Keratinized cells are floating on the sheet of keratinocytes. There are holes in the sheet. 43-day culture. Phase contrast. Bar represents 10 μm.

RESULTS

Phase contrast microscopy

Keratinocytes attached to the bottom of a culture dish either singly or in small aggregates. At 24 hours after the onset of cultivation a few keratinocytes distended flatly, but most were spherical. Within a few days, the singly attached keratinocytes were distended, showed ruffling membrane activity along the margin of the cytoplasm, migrated around, and connected with one another upon collision. From the aggregate, keratinocytes grew out, and formed a circular sheet composed of tightly packed polygonal

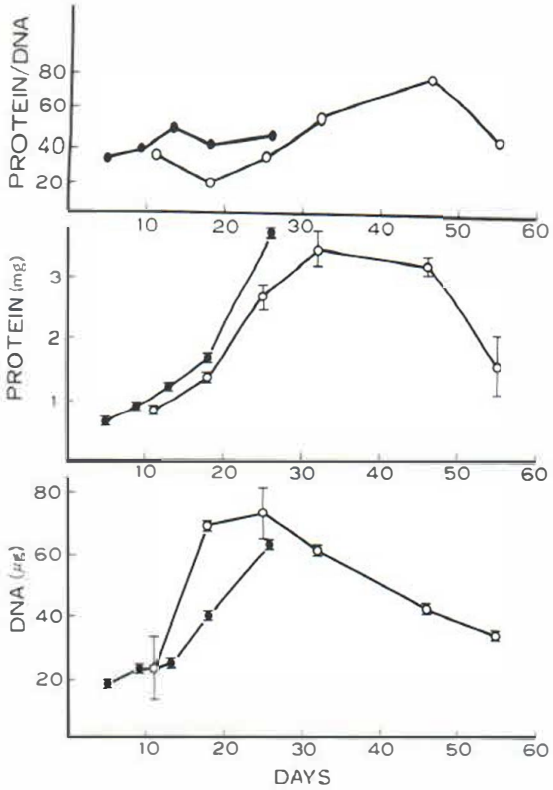

Fig. 4. DNA and protein content of cultures and ratio of amount of protein to amount of DNA according to days of cultivation. Each point represents the mean of three culture dishes ± S.E.M. ●: culture from 32-year-old male. ○: culture from 54-year-old female.

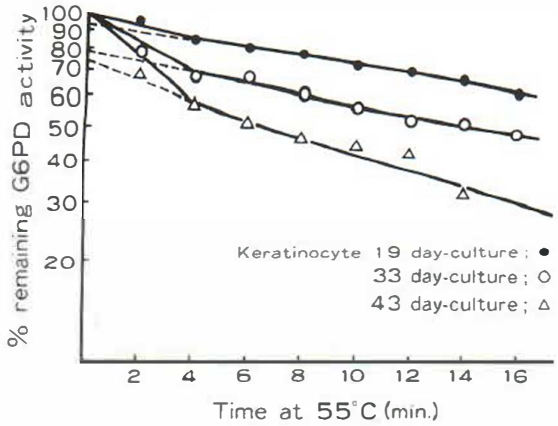

Fig. 5. Heat-lability of G6PD in 19-, 33- and 42-day cultures. A semilogarithmic plot of percentage activity remaining (*ordinate*) versus minutes at 55°C (*abscissa*) was prepared. Each point represents the residual activity after heat treatment at 55°C. Extrapolation of the linear portion of the curve to the ordinate gives an estimation of the proportion of the initial enzyme activity, and the ordinate-intercepted value gives the proportion of abnormally heat-labile enzyme.

cells. About 5 to 7 days of cultivation most keratinocytes became large, with a flatly distended cytoplasm and a relatively small nucleus in the center. This condition lasted for several days without apparent proliferation or movement of the cells. Small polygonal cells then appeared in groups in the sheet of flatly distended large cells (Fig. 1). There were numerous mitotic figures among these small cells.

The small polygonal keratinocytes increased rapidly in number, covered the entire culture surface, and then stratified (Fig. 2). Intricate fibrillar structures could be observed in the cytoplasm and intercellular bridges became prominent. In some areas large polygonal cells with a clear cellular margin and a small pyknotic nucleus were observed in the surface of the sheet. Desquamating cells were occasionally seen. After 30 days of cultivation the sheet of keratinocytes was covered with apparently keratinized cells. On the top of the sheet, these cells formed a loose network or aggregates, and occasionally floated in the medium. When the culture was 40–50 days old, the sheet of keratinocytes became thin here and there, and no small polygonal cells could be observed (Fig. 3).

DNA and protein content

During the early days of cultivation the amount of DNA in a dish was about 20 μg and increased slowly (Fig. 4). Then DNA content in a dish began to increase rapidly about 2 weeks after plating, when small polygonal cells appeared in groups, reaching its maximum value of 60 to 70 μg at about 25 days of cultivation. Thereafter the amount of DNA decreased gradually, and was found to be half of its maximum value at 55 days. The amount of protein increased slowly until 16 to 18 days of cultivation, and then increased rapidly, though not quite so fast as the increase in the amount of DNA. Contrast of the phase of slow and fast increase of protein was less conspicuous than that of DNA. Protein content in a dish reached the maximum value at 32 days, and the value decreased as shedding of the uppermost layer became prominent. There was a slight downward shift in the protein: DNA ratio at 18 days when DNA was increasing

rapidly, while the increase in protein was still in the initial slow phase. Then the ratio increased as the stratification and keratinization progressed.

Heat-lability of G6PD

The kinetics of heat-inactivation of G6PD obtained from cultured keratinocytes were studied. Fig. 5 shows the results of an assay for heat-labile G6PD obtained after varying periods of cultivation. In the 19-day culture, the activity of G6PD decayed almost exponentially during heat treatment. But, in the 33-day culture, when the keratinocytes were stratified, and desquamation began to occur, an initial rapid decrease in the activity of G6PD could be seen during heat treatment, after which the activity decreased much more slowly. About 7% of the total enzyme activity was detected as heat-labile in the 19-day culture, while in the preparations from the 33-day culture, 21% was detected as heat-labile. In the 43-day culture, when cellular proliferation could not be observed, and desquamation was prominent, the enzyme activity decreased more rapidly during the initial 2–4 min of heat treatment. About 26% of the total G6PD activity was detected as heat-labile in the same way. In comparison with the younger culture, the proportion of heat-labile enzyme is high in the older, 33- and 43-day cultures when the proliferative population decreased.

In order to exclude the possibility that long-term cultivation without subculturing may have caused an increase in the heat-labile fraction of G6PD, human skin fibroblasts were cultured in a regular subcultivation of every 10 days and without subcultivation for 43 days, and then assayed for G6PD. As a result, no difference in percentage of the heat-labile fraction was detected between them.

DISCUSSION

In cultures of human epidermal keratinocytes there occurred a roughly three-fold increase in the amount of total DNA, and a four-fold increase in the amount of total protein. When compared with the findings by phase contrast microscopy and electron microscopy, the rapid increase in DNA content after the initial slow increase corresponded to the appearance of small polygonal keratinocytes in groups, and the decline in DNA content after having reached its maximum value at 25 days correlated with the progress of differentiation and the decrease in proliferative population. Furthermore the decline in DNA content corresponded to the increase in the heat-labile fraction of G6PD.

Delescluse et al. (12) and Marcelo et al. (3) demonstrated that the DNA content in the culture of keratinocytes did not increase during the course of culturing. On the other hand Liu & Karasek observed a two- to three-fold increase in DNA content in their proliferative culture (13). This discrepancy might be due to a difference in the number of inoculated keratinocytes. Marcelo et al. inoculated 5×10^6 trypan blue-excluding cells into a T-25 flask, and observed a complete monolayer formation by days 3–4 and stratification at days 5–6 (3). We inoculated 8×10^5 basal cells into a 60×15 mm dish, and observed complete monolayer formation after 2 weeks.

Several of the cytoplasmic enzymes are known to alter during the ageing process. These altered enzymes can be recognized by their lowered specific activity. In addition, three cytoplasmic enzymes: G6PD, 6-phosphogluconate dehydrogenase and hypoxanthine-guanine-phosphoribosyl-transferase have been shown to have lowered heat stability during senescence (7, 14). In this study, the heat-labile portion of G6PD was increased in 33- and 43-day cultures. Human fibroblasts from different organs had different in vitro lifespans (15). Epithelial cells are believed to have much shorter in vitro life-span. Epithelial-like cells from human embryo kidney had a shorter in vitro life-span compared with fibroblast-like cells from human embryo lung (16). The early decline of keratinocytes in culture was attributed to the differentiation which proceeded faster than proliferation (1). We observed in cultures of more than 30 days' cultivation that the proliferative component of cultured keratinocytes decreased, some of the cells facing the bottom of the culture dish had degenerated, while the heat-labile fraction of G6PD had increased. Long-term cultivation without subculturing does not seem to be responsible for the increase in the heat-labile fraction of G6PD. In comparison with fibroblasts, keratinocytes are unique in performing terminal differentiation in terms of cell death rather than secretion of a special product. Then it would not be logical to draw the simple conclusion that the change in enzyme is due to cellular ageing. It is not certain as yet whether the increase in heat-labile G6PD in the cultured keratinocyte is related to cellular ageing or to terminal differentiation.

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