cosylation of proteins and lipids, the result of which may be loss of functionally important cell surface receptors and, in turn, this may be a new and interesting aspect of the problem of aging as a whole.

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ELECTRON-AUTORADIOGRAPHIC STUDY OF HUMAN EPIDERMOCYTES IN CULTURE

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KEY WORDS: tissue culture; autoradiography; cell multiplication and differentiation.

To treat patients with extensive burns, attempts have been made to graft layers of autologous epidermis, grown in the laboratory, to the affected region of skin. This advance in the treatment of burns has developed in recent years and is based on the most recent achievements of cell biology in the field of culture methods. At the present level of development of this science, it appears very promising. Its introduction into medical practice is entirely determined by the possibility of controlling proliferation and differentiation of cells in vitro. Several stimulators [6, 8-10, 12] and methods of cultures[4, 5, 7], capable of increasing the rate of multiplication of epidermocytes, have recently been suggested. Cytochemical and morphological criteria for assessing the level of differentiation of epidermocytes also have been described [3, 11]. An effetive method of studying cell proliferation and differentiation is electron-microscopic autoradiography, which combines an objective and precise

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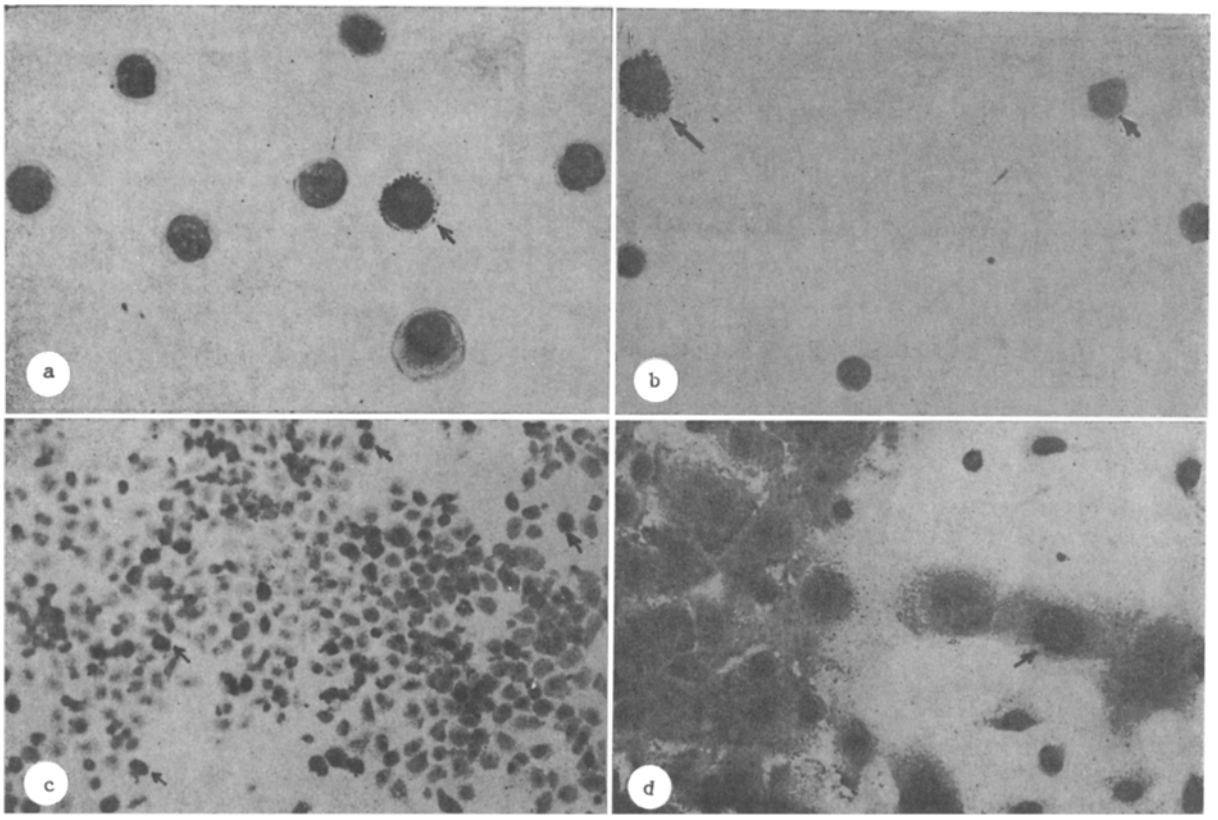

Fig. 1. Light-microscopic autoradiographs of cell suspensions and cultures. a) Basal cells after trypsinization became round in shape with a narrow border of cytoplasm; one basal cell is labeled with ^{3}H-thymidine (arrow; 630 ×); b) labeled (long arrow) and unlabeled (short arrow) fibroblasts (630 ×); c) culture of epidermocytes growing in medium with low calcium concentration; no colonies are formed, cells grow in the form of a monolayer, gradually filling the space on the bottom of the dish; labeled epidermocytes (arrows) are uniformly distributed in the cell layer (200 ×); d) some colonies of epidermocytes, growing in medium with normal calcium ion concentration. A labeled cell (arrow; 630 ×) can be seen at the periphery of the colony.

indicator of proliferative activity, namely DNA synthesis, with ultrastructural characterization of proliferating and nonproliferating cells. This paper gives the results of an electron-autoradiographic study of cultures of adult human epidermocytes.

EXPERIMENTAL METHOD

The test material consisted of small pieces of skin, taken from patients during autografting, and also split-thickness grafts from cadaveric skin. The skin flap 0.2-0.3 mm thick was immersed in trypsin solution for 18-20 h at 4°C. Depending on the thickness of the flap the trypsin concentration varied from 0.05 to 0.25%. After removal of the trypsin the flap was washed with phosphate buffer solution, pH 7.4, transferred to calf serum to neutralize the enzyme, after which the epidermis was separated from the dermis with forceps, and a suspension of epidermocytes was isolated by repeated pipetting. The cells were sedimented by centrifugation (at 500 rpm for 10 min), and resuspended in Eagle's medium with a low calcium ion concentration (0.14 mM) and with glutamine and 10-15% calf serum. The suspension of epidermocytes was adjusted to a concentration of $1 \cdot 10^5$-$2.5 \cdot 10^5$ cells/cm^2 with the same medium and transferred to plastic dishes. Incubation was carried out at 37°C in an atmosphere containing 5% CO_2 and with a humidity of 80%. After 24 h the nutrient medium was replaced by fresh, the calf serum being replaced by a 2% solution of Ultroser. Thereafter the medium was changed every 3-4 days. Some cultures were grown in Eagle's medium with a normal calcium ion concentration (1.5 mM) and with glutamine and calf serum as growth stimulator. The same medium was added 24 h before fixation to some of the cultures which had hitherto been grown in medium with low calcium concentration. Growth of the cultures was observed under the phase-contrast microscope.

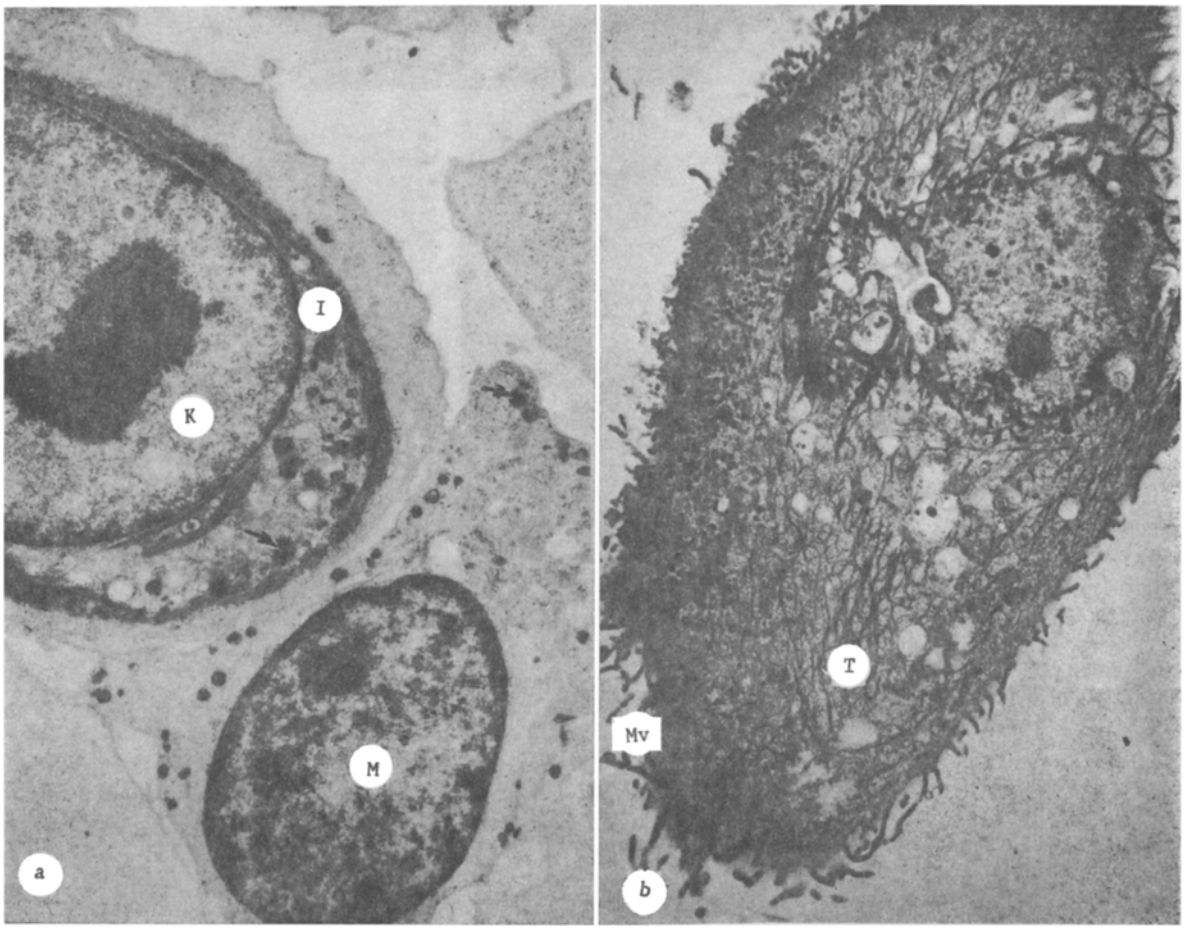

Fig. 2. Electron-microscopic morphology of epidermocytes in culture. a) Keratinocyte (K) and melanocyte (M), spreading on bottom of culture dish; melanocyte has a higher chromatin concentration in its nucleus; tonofibrils (T) form a ring around the keratinocyte nucleus; in both cells melanosomes are present (arrows; 20,000 ×); b) keratinocyte from second row of cells in culture, differs from cells of the bottom row by having numerous microvilli (Mv) on plasma membrane, with developed network of tonofibrils (T) and atrophy of nucleus (18,000 ×).

For the autoradiographic investigation a suspension of epidermocytes or a culture of them on the bottom of the dish was incubated for 2 h with ^{3}H-thymidine in a concentration of 10 μCi/ml. Either films (from the suspension) or electron-autoradiographic preparations were obtained from these materials by the method described previously [1]. In light-microscopic autoradiographs the number of labeled cells was counted and the distribution of labeled cells in the culture was determined. The ultrastructural features of labeled and unlabeled cells were investigated in electron-microscopic autoradiographs.

EXPERIMENTAL RESULTS

Suspensions of epidermocytes obtained after trypsinization were heterogeneous for cell composition, morphological characteristics, and proliferative ability. Significant variations also were noted in the number of epidermocytes obtained from unit surface area of skin (from $1 \cdot 10^5$ to $2.2 \cdot 10^6$ cells/cm^2.

Cells of the basal layer could be identified precisely by autoradiography by their ability to incorporate ^{3}H-thymidine (Fig. 1a). In suspensions they had the appearance of small, round cells with a narrow border of cytoplasm, and staining weakly with toluidine blue. These cells are possibly the only type of epidermocytes that are suitable for culture. They accounted for between 10 and 70% of the population in suspensions. The remaining cells belonged mainly to more differentiated types of keratinocytes: spinous, granular, and even keratin scales. Nearly always a few cells, larger than the basal cells and stained pink-lilac with toluidine blue, could be seen in the suspensions. The cytoplasm of these cells as a rule was almost

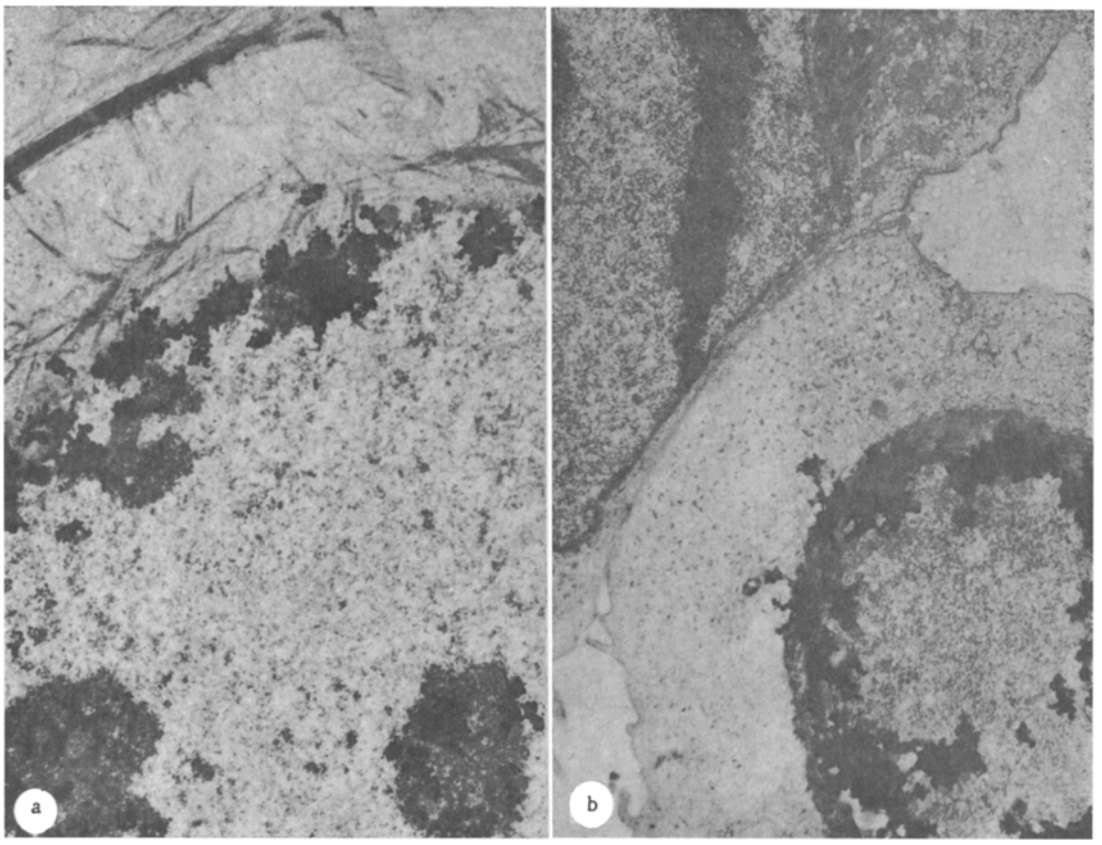

Fig. 3. Electron-microscopic autoradiographs of keratinocytes in culture. Cluster of grains of silver in heterochromatin regions observed above the nuclei of DNA-synthesizing cells. a) Medium with low calcium concentration. Plasma membranes of labeled and unlabeled cells lie side by side (in region indicated by arrows), but no desmosomes are formed and tonofibrils do not reach these areas of the membrane (23,000 ×); b) fixation of culture after increase in calcium ion concentration in medium to 1.5 mM. Desmosomes and bundles of tonofibrils reaching to them can be seen in region of junction (arrows) between labeled and unlabeled cells (47,000 ×).

completely destroyed by trypsin, but despite this damage, some of them were still able to incorporate ^{3}H-thymidine. The difference from epidermocytes in their staining properties, the size of the nucleus, lower resistance to the action of trypsin, and ability to synthesize DNA all suggest that the cells just described are fibroblasts (Fig. 1b). Of the basal cells from 0 to 5% were labeled. As a rule, suspensions in which no labeled basal cells were found, were unable to grow in culture.

The reasons why cell suspensions obtained after trypsinization differed greatly in the number of epidermocytes isolated from 1 cm^2 of skin, and in the number of labeled basal cells which they contained, were the unequal thickness of the split-thickness grafts and differences in activity of the trypsin used. The effect of trypsin on proliferative activity of the cell suspensions was demonstrated by treating the same material with 0.05 and 0.25% solutions of trypsin. In comparatively thin flaps, labeled basal cells were found only in samples treated with the dilute solution. During work with thicker skin flaps, only after treatment with 0.25% solution were suspensions containing labeled basal cells obtained; the number of epidermocytes obtained from 1 cm^2, moreover, was greater than after treatment with the 0.05% solution.

On the 1st day of culture in medium with a low calcium concentration many epidermocytes adhered to the surface of the bottom of the dish. The adherent cells were enlarged, and during observation from above they were polygonal in shape and had an extensive zone of cytoplasm, much larger than the zone of cytoplasm of the basal cells of the original suspension. On horizontal electron-microscopic sections, large nucleoli were observed in these cultures, and a few tonofibrils were arranged in the form of a ring around the nucleus (Fig. 2a); their bundles

did not reach the plasma membrane and there were no desmosomes on its surface. The membranes of two neighboring cells were either in close contact (Fig. 3a) or a short distance apart. Many of these keratinocytes contained melanosomes, sometimes single, more often multiple (Fig. 2a). From 17 to 37% of cells with the morphological characteristics just described incorporated ^{3}H-thymidine (Fig. 3a).

In vertical sections through the culture these cells had a considerably flattened shape and spread out on the surface of the plastic substrate. Later the number of cells spreading out on the plastic increased rapidly. However, colony formation did not take place, as is observed in media with normal calcium ion concentration, there were no clearly demarcated zones of epidermocytes, and labeled cells were distributed comparatively uniformly over the whole bottom of the dish (Fig. 1c), so that after 4-8 days the whole surface of the bottom of the dish was covered by a more or less uniform monolayer of epidermocytes, a little more widely separated from one another than in media with a normal calcium ion concentration. Stratification was not observed in these cultures. A second layer of cells was not formed, and only in a few areas were individual cells, strongly reflecting light and therefore, shining, observed in the second layer. Most of these cells were separated from the monolayer and floated freely in the culture fluid. In vertical sections these shining cells appeared to be more differentiated than cells of the epidermocyte monolayer (Fig. 2b). In such cells the network of tonofibrils was more highly developed than in cells of the bottom row; microvilli were located on the side of the nutrient medium. The network of tonofibrils contained keratohyalin granules. Besides keratinocytes, the monolayer contained quite a large number of melanocytes (Fig. 2a), but none of them, were found to be labeled. After the monolayer had spread over the whole surface of the bottom of the dish, the number of labeled keratinocytes fell sharply.

In medium with a normal calcium ion concentration (1.5 mM) growth of cells in the form of colonies was observed. The central parts of the colonies were covered with a layer of keratinizing cells. Labeled cells were found only at the periphery of the colonies (Fig. 1d). The number of labeled keratinocytes did not exceed 10%. As a rule, by the 4th-5th day proliferation of the cells had ceased, although among the colonies spaces remained on the bottom of the dish on which single adherent cells, not incorporating ^{3}H-thymidine, were established.

In cases when the calcium concentration in cultures grown in a medium with low calcium, was increased up to the normal level, changes were observed in the structure of the cells, their mutual arrangement, and their rate of proliferation. In cells of the bottom row bundles of tonofibrils lost their perinuclear orientation and reached the plasma membrane, terminating on the numerous desmosomes which appeared (Fig. 3b). A second or even a third row of cells was formed, firmly held by cells of the bottom row. Cells of the upper rows underwent keratinization. In the bottom row there was a sharp decrease number of cells incorporating ^{3}H-thymidine (Fig. 3b).

Data on the existence of a connection between migration and proliferation of keratinocytes have recently been obtained [2]. In particular, epidermocyte growth factor and growth transforming factor stimulate multiplication of cells and increase the rate of their migration. Our observations show that a decrease in the calcium ion concentration in the medium also acts in this same direction. Such conditions weaken bonds between cells of the epidermis, as is shown by disappearance of desmosomes, disturbance of stratification, and weakening of the tendency, characteristic of keratinocytes, to form groups and colonies. Weakening of the cell bonds enhances mobility and, as has been shown, is accompanied by an increase in the number of proliferating cells. Filling up of the free space on the bottom of the dishes prevents cell migration, and this is expressed autoradiographically as a sharp decrease in the number of epidermocytes labeled with ^{3}H-thymidine.

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EXPERIMENTAL MORPHOMETRIC STUDIES DURING THE ERYTHROCYTE FILTERABILITY TEST

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Several pathological states are linked with disturbances of the rheologic properties of the blood, which are largely dependent on erythrocyte deformability [6]. Consequently, assessment of erythrocyte deformability by the use of the filterability test on blood samples is becoming increasingly important. As a rule what is measured is the number of erythrocytes in suspension, passing without undergoing hemolysis through a microfiltration membrane with pores of known diameter in the course of a known time interval [1]. The microfiltration membranes used are made from various polymer materials and have pores of widely different shape – from straight cylinders in track membranes to twisted channels in membranes obtained from polymer solutions (Fig. 1). As was shown previously, the shape of the pores in the membrane has a significant effect on the degree and multiplicity of change in shape of the cells, which determines the pattern of movement of erythrocytes in the membrane [3].

A more precise and correct interpretation of results of the filterability test on blood samples could help with the development of a technique which would allow the shape of the cells to be investigated actually in the membrane. This would facilitate the transition from the phenomenologic level of examination of the filterability test to the analysis of the mechanisms of interaction of two discrete systems: the cell assembly and the porosity of the membrane-films, which constitute a periodic colloidal system [4].

In the investigation described below deformability of the blood cells was studied by the use of a combined method of morphometric analysis of filtration membranes and erythrocytes in them.

EXPERIMENTAL METHOD

Blood samples from healthy donors (men) and groups of rabbits kept on a high cholesterol diet served as the test objects. The hematocrit index varied from 0.003 to 0.2. Membranes used were obtained from the firms of Millipore and Nucleopore Pall, and were used under filtration conditions and also during statistical contact between the blood sample and membranes, soaked beforehand with buffer solution. Membranes with cellular material immobilized on them were fixed in 0.25% glutaraldehyde, after which the membrane was examined in a scanning electron microscope (Hitachi, Japan). (The photomicrographs were prepared by head of laboratory Dr. S. A. Gusev, to whom the authors are grateful.) Morphometric analysis of the cells and free regions of the membranes was carried out by means of our own program on the IBAS-2 system (Opton, West Germany). During morphometry of the membranes, image inversion was used. The program of morphometry of the cells, like that developed previously for evaluating the state of erythrocytes in patients with familial hyperlipoproteinemia [5], contained assessment of

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