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ULTRASTRUCTURAL CHARACTERISTICS OF EPIDERMOCYTES CULTURED IN MEDIA WITH STANDARD AND REDUCED CALCIUM ION CONCENTRATIONS

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The problem of culture of autologous epithelium for subsequent use of a layer grown in vitro in clinical practice as an alternative method to skin grafting for various skin defects is of great importance [1, 2, 3, 5]. However, there have been few reports of the successful use of autologous skin in clinical practice [6, 7, 9, 10].

One of the basic conditions for culture of epidermocytes is the correct choice of nutrient medium. Reports have recently been published to show that proliferation of cells of the epidermis is accelerated in media with a low (not above 0.8 mM) calcium ion concentration [4, 8, 11]. We have studied ultrastructural changes in epidermocytes cultured in hypocalcium media and have compared them with epidermocytes cultured in media with the standard Ca^{++} concentration.

EXPERIMENTAL METHOD

Epidermocytes were obtained from slices of skin removed with the DRM-60 dermatome from patients during the operation of autodermoplasty (25 cases) and from cadaveric skin (15 cases). The skin graft, taken from different parts of the body, was placed in Eagle's nutrient medium with a high concentration of antibiotics and fungicides (sodium salt of penicillin $1 \cdot 10^4$ U/ml, streptomycin 0.1 g/ml, amphotericin B 500 μg/ml) for 3 to 24 h. The skin graft was then treated with a 0.02% solution of versene for 20 min. The epidermocytes were isolated in 0.25% trypsin solution of 4°C for 18-20 h. The action of trypsin was neutralized by the addition of an equal volume of calf serum. The epidermis was then separated from the dermis. The epidermocytes were washed off by frequent pipetting. The cells were counted in a Goryaev's chamber. The number of cells varied from $1 \cdot 10^5$ to $2.1 \cdot 10^6/cm^2$ of skin graft. The suspension thus obtained was centrifuged at 800 rpm for 10 min. The supernatant was poured off and the residue resuspended in nutrient medium (in hypocalcium medium and, in parallel experiments, in medium with the standard Ca^{++} concentration), and seeded into plastic culture dishes 60 or 100 mm in diameter (Nunc, Coster) with seeding density of $1 \cdot 10^5$-$2.5 \cdot 10^5$ cells/cm^2. The epidermocytes were cultured in a CO_2 incubator (Flow Laboratories) at 37°C, 80% humidity, and in an atmosphere of 95% air + 5% CO_2. The nutrient medium was

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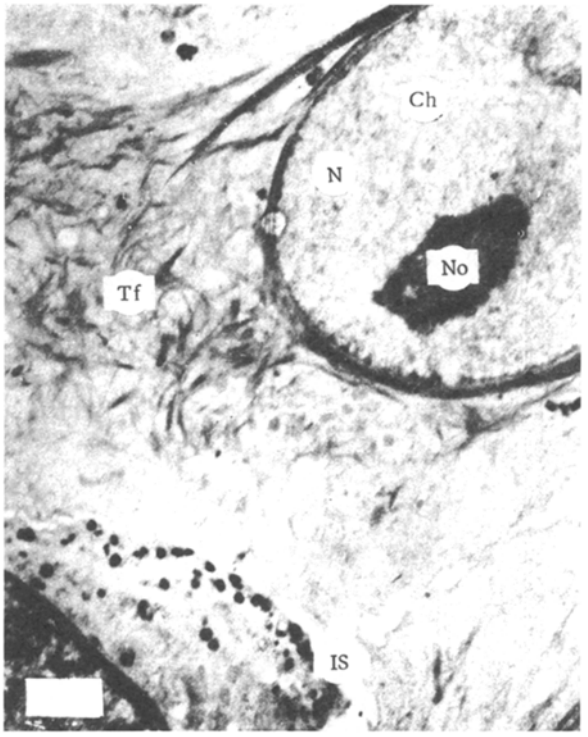

Fig. 1. Ultrastructure of epidermocytes in culture (medium with standard Ca^{++} concentration). Cytoplasm contains delicate bundles of tonofilaments (Tf), grouped together in some places; the nuclei are round (N), with a nucleolus (No) and finely granular chromatin (Ch); the intercellular spaces are widened in some places (IS). 4000 ×.

changed after 3-4 days. For the morphological study of the cells in culture, an inverted phase-contrast microscope (Opton) was used and the cells were photographed with a camera attachment (Winder). Cultures of epidermocytes in plastic culture dishes for electron-microscopic study were fixed in 1% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 12 h, and then treated with 1% OsO_4 solution at 4°C for 2 h. The material was dehydrated in ethanol solutions of increasing concentration and embedded in a Epon-Araldite mixture. Ultrathin sections were cut on an ultramicrotome (Reichert), stained with uranyl acetate and lead citrate, and studied in the JEM-100B electron microscope.

At the ultrastructural level the dispersed epidermocytes resembled cells in a state of acantholysis, which are formed in the epidermis in various diseases (pemphigus, Darier's dyskeratosis, familial benign pemphigus — Hailey–Hailey disease, senile keratosis, and also the vesicles in virus diseases). Unlike normal epidermocytes, these dispersed epidermocytes are more round in shape, the desmosomes are invaginated within the cytoplasm, and the tonofilaments are concentrated around the nucleus, but do not form bundles. It must be emphasized that otherwise the ultrastructural features of the dispersed and normal epidermocytes of the human skin were similar.

After monolayer formation in nutrient media with a standard Ca^{++} concentration (1-2 mM), which we observed on the 5th-10th day, the cultures became stratified by the 15th-20th days and contained from four to 12 cell layers depending on the period of culture. The bottom layer consisted of cells electron-microscopically similar to basal cells in vivo. The cells had large nuclei, one or two nucleoli, a peripheral arrangement of their chromatin, which was finely granular. Mitochondria, ribosomes, polysomes, and a rough, or rarely a smooth endoplasmic reticulum were observed in the cytoplasm. Unlike ordinary basal cells in vivo, however, the dispersed cells contained fewer tonofilaments, they were small in size, and had fewer desmosomes between the cells, and were more flattened and elongated in shape.

The next layer, consisting of more highly differentiated cells, was characterized by an even more flattened shape of cells, distinguishing them both from the underlying cells and from the spinous cells in vivo. The number of desmosomes between the cells was greater than in the subjacent layer. Junctions also were formed between the cells due to digitiform evaginations which insinuated themselves into corresponding depressions in neighboring cells (like spinous junctions in the epidermis). The diameter of the desmosomes was smaller, but expansions were observed between the cells more frequently and they were wider than in vivo. Although the tonofilaments were numerous they were not grouped into bundles and did not form tonofibrils, which distinguished these cells from those of the stratum spinosum of the epi-

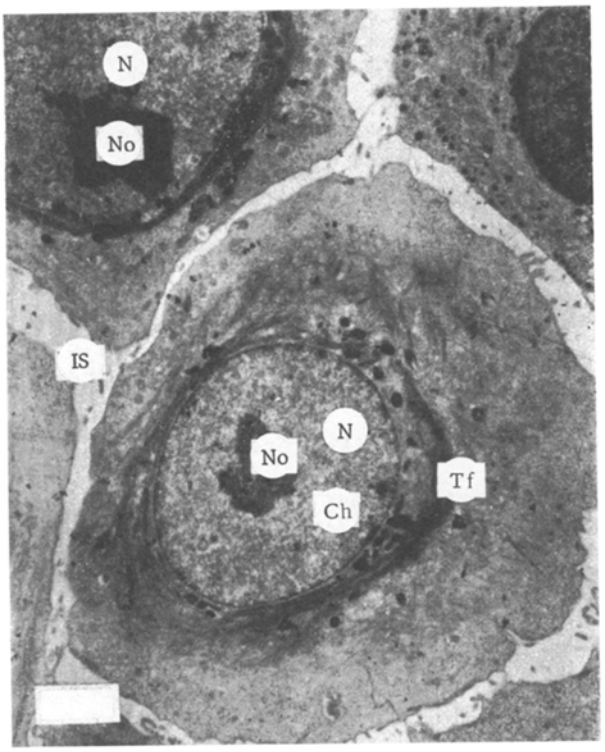

Fig. 2

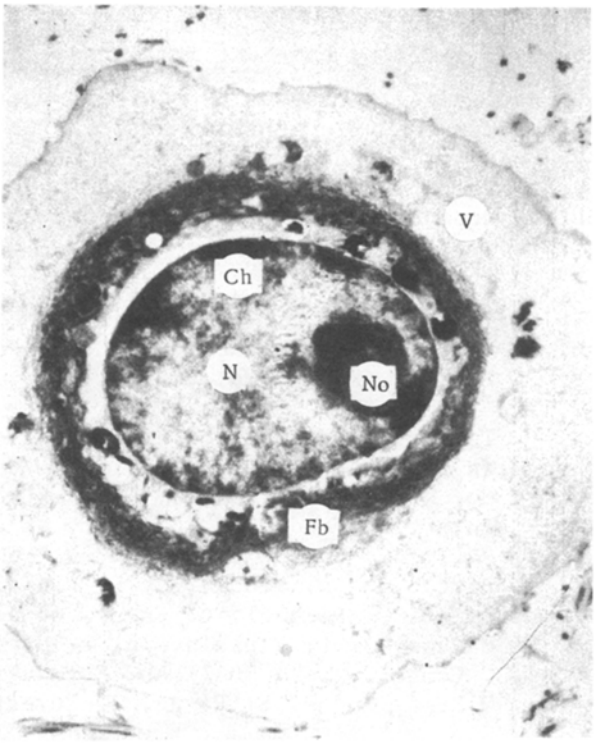

Fig. 3

Fig. 2. Ultrastructure of epidermocytes in culture (hypocalcium medium). Cytoplasm contains perinuclear tonofilaments, nuclei are round, with a nucleolus and finely granular chromatin; intercellular spaces widened so much that the cells are hardly in contact with each other. 2000 ×. Legend as to Fig. 1.

Fig. 3. Ultrastructure of epidermocytes in culture (hypocalcium medium). "Keratinizing" epidermocyte. Vacuoles (V) in cytoplasm; perinuclear concentration of electron dense fibrillary structures (Fb); nucleus reduced in size and chromatin in large clumps. 3000 ×. Legend as to Fig. 1.

dermis. This was evidently because of the absence of the mechanical factor, which stimulates the formation of bundles of tonofibrils in vivo. Keratohyaline granules, round in shape, appeared in the cytoplasm of the cells in the upper rows of this layer (in the stratum granules of the epidermis they are stellate in shape), and Odland's granules were rare.

The upper layers were composed of cells with a thickened karyolemma and with absence of granules in the cytoplasm. Many cells in the upper layers had a keratinizing cell membrane, manifested by a marked increase in its electron density. In the most superficial layers the cells lost their nuclei and formed a layer of cells resembling the stratum corneum in vivo, which were detached into the medium and contained keratin fibrils. A horizontal section through the monolayer of epidermocytes cultured in medium with the standard Ca^{++} concentration is shown in Fig. 1.

In hypocalcium medium (Ca^{++} concentration under 0.08 mM) the character of growth of the epidermocytes was greatly modified. Proliferation of the cells took place very quickly and the monolayer was formed in 3-5 days. Ultrastructural investigation of the epidermocytes revealed absence of desmosomal junctions between the cells. The intercellular spaces were widened even more than in media with the standard Ca^{++} concentration (Fig. 2). Stratification of the culture did not take place, but occasionally large cells in which bands of keratin fibrils were concentrated in the perinuclear region were given off into the nutrient medium (Fig. 3).

On the addition of Ca^{++} to the medium, increasing its concentration to 1.2 mM or more, the early ultrastructural changes took the form of the appearance of asymmetrical desmosomes between the contacting cells. Symmetrical desmosomes were observed after 24 h.

On electronmicroscopic investigation of the region of attachment of the epidermocytes to the culture dish or to the support, no structures resembling half-desmosomes were found. Structures resembling basement membranes likewise were not observed.

To conclude, the use of hypocalcium nutrient media accelerates the process of obtaining a continuous sheet of epithelium in culture. However, before separation of the formed sheet from the base of the culture vessel into the nutrient medium, Ca^{++} must be added in order to strengthen the intercellular connections. Thus by adding Ca^{++} to the medium, it is possible to control desmosome formation and, consequently, the formation also of a monolithic sheet of cultured epithelium.

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