

ULTRASTRUCTURE OF CULTURED HUMAN EMBRYONIC AND/OR FETAL KERATINOCYTES

D. HORKÝ, J. ADLER

Department of Histology and Embryology, Medical Faculty, Masaryk University, Brno, and Tissue Bank, University Hospital Brno-Bohunice, Brno

Received May 28, 1996

Accepted February 10, 1997

Abstract

Horký D., J. Adler: *Ultrastructure of Cultured Human Embryonic and/or Fetal Keratinocytes*. Acta vet. Brno, 1997, 66:27-32.

For treatment of large-surface skin defects such as burns the most suitable tissues derivatives are being looked for. Cultured embryonic and fetal keratinocytes have been used because of their vitality and ability to form a sufficiently thick cellular layer suitable for transplantation in a relatively short time and are well tolerated by the patients.

In the present study, the ultrastructure of human fetal keratinocytes was studied after 20-21 days of culture. After this period, keratinocytes acquired a form of stratified squamous epithelium. The appearance and structure of the cells revealed differentiated basal, middle, intermediary and superficial layers. The base was formed by 1-2 layers of low cylindrical or cubic cells with dense cytoplasm, striking light nucleus with a nucleolus of reticular type. The cytoplasm contained dark bodies of the type of secondary lysosomes, pigment granules or granules of keratohyaline or pseudomyeline figures. The middle layer was formed by about 3 layers of large cells with light voluminous cytoplasm. The nuclei of these cells were large, light, the nuclear membranes projected into numerous invaginations and formed false nuclear inclusions. Pronounced nucleoli were exclusively of reticular type. Numerous dark bodies and agranular endoplasmic reticulum (AER) were prominent within cytoplasm of some cells, respectively. The cytoplasm of flattened cells of the intermediary layer contained numerous autophagic vacuoles and glycogen deposits besides bundles of tonofilaments. Superficial 1-2 layers were formed by conspicuously flat cells usually without nuclei. Their cytoplasm involved thick bundles of tonofilaments with wavy course, and large dark bodies with homogenous contents. Among the basal cells of middle and intermediary layers there were only narrow intercellular spaces and numerous desmosomes, while among cells of the superficial layer the intercellular spaces became wider and desmosomes were less numerous. From the presented results it follows that the structure of cultured embryonic or fetal keratinocytes was similar to that of differentiated epidermis and was found suitable for treatment of skin defects.

Cultured keratinocytes, EM evaluation, burn treatment

Survival of patients with extensive burns (IIIrd degree) is particularly dependent on the rapid covering of the wound surface with suitable material. Various materials have been tested in the course of treatment of burns (for survey, see Compton et al. 1989). In recent 10 years, the method of covering the wound surfaces with human keratinocytes cultured *in vitro* (Green et al. 1979) has been used more and more often as these cells are able to create layers of sufficient area and thickness suitable for the grafting and covering of large defects (Arons et al. 1992). At first, either autografts of the epidermis or their combination with dermis allografts (see Cuono et al. 1986), both native or cryopreserved (Aoki et al. 1992), were used for such purposes. Numerous data on these methods, their advantages or disadvantages have been repeatedly presented in literature (see Compton 1993). Recently, attention of clinical hospital departments has been increasingly paid to utilizing keratinocytes produced *in vitro* in a form applicable for therapeutical purposes. Both culture without carriers, and culture on plates of different materials have been used (Bernstam et al. 1990; Rosdy and Claus 1990). However, all the above-mentioned authors have been

utilizing both the dermis alone, or combined with epidermis, or - in the case of keratinocyte culture - cells of adult individuals.

However, experience in clinical practice along with some earlier studies (Aoki et al. 1994; Brychta et al. 1994; Adler et al. 1996) has shown markedly better therapeutical results when embryonic or fetal keratinocytes were employed as far as the rapidity of reparation of the skin defects and tolerance by patients is concerned.

Therefore, the aim of our study was to use keratinocytes of human embryos or fetuses for culture to verify their growth potency and suitability for application in the treatment of skin defects.

Materials and Methods

Keratinocytes of human embryos or fetuses, aged 8-12 weeks after fertilization, obtained within 6-24 hours post mortem, were used for culture. Using two forceps, the periderm or epidermis (according to the age of the fetus) was easily removed from the dorsal surface of fetuses. The periderm or epidermis are formed by 2-5 layers of cells. The area of the fragments obtained by this method was about $1/2 \text{ cm}^2$. The fragments were separated mechanically without applying trypsin, and the cells were placed immediately into a culture medium DMEM-HAM (made by Sebak, Germany) and cultured in a usual way (Brychta et al. 1994) for 20 or 21 days.

After that period, a layer of keratinocytes with the area of several cm^2 was separated using dispase (made by Sigma, USA). The obtained tissue was divided into strips, $1 \times 2 \text{ mm}$, and fixed immediately for 2 hours in the solution of 300 mmol/l glutaraldehyde in cacodylate buffer, pH 7.4, and then for 1 hour in the solution of 20 mmol/l osmium tetroxide in cacodylate buffer, pH 7.4. Dehydration, immersion and embedding in Durcupan ACM were made by a routine method. Semithin sections for light microscopy were stained with methylene blue and Azur II. Ultrathin sections were made on ultramicrotome LKB Nova, and stained partly with lead citrate or uranyl acetate, and then by lead citrate. The sections were studied and photographed with electron microscope Tesla BS 500.

The experimental work with human embryonic and fetal keratinocytes was approved by the Ethical Committee of the Faculty Hospital Brno-Bohunice.

Results

After 20 days of culture, keratinocytes acquired the appearance of a stratified epithelium formed by 7 and more layers of cells. The cells were of various sizes, shapes, volumes of cytoplasm, and they differed in density. Basal, middle, intermediary, and superficial layers were distinguished. At the base, the cells were of cubic or low cylindrical or irregular shape, and in the middle layer they became polyhedral. Two superficial layers were made up of elongated or spindle-like elements with strikingly dense cytoplasm (Fig. 1, Plate VI., Fig. 2).

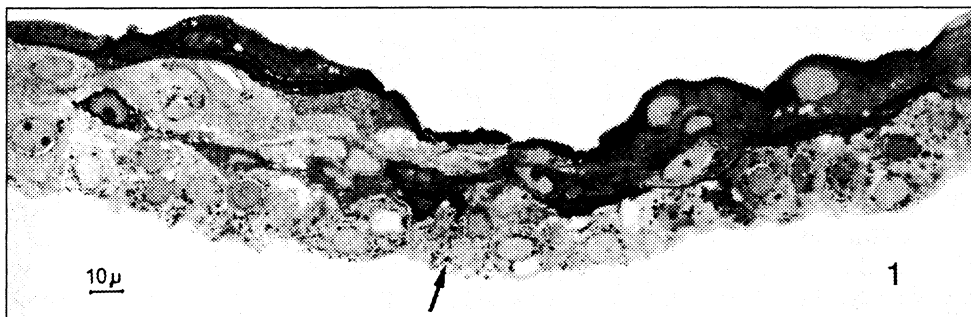


Fig. 1. Keratinocytes after 20 days of culture acquired an appearance of a layered epithelium consisting of 6-7 layers of cells. Near the base (→) they are of cubic or low cylindrical or polyhedral shape. Towards the surface, they change into spindle-like elements. One layer of flat cells with dark cytoplasm is situated on the surface. Semithin section, $\times 600$.

The basal layer was usually formed by 1-2 layers of cubic or polyhedral cells adhering to the plate (Figs. 1, 2). The cell sizes reached $15-25 \times 10-15 \mu\text{m}$.

The nucleus was oval, about $15 \times 10 \mu\text{m}$ in size. It was demarcated with a conspicuous nuclear envelope. Chromatin was finely dispersed on the section through the nucleus with a light appearance. Nucleolus of reticular type was found regularly (Plates VI., VII., Figs. 2, 3).

The cytoplasm was relatively dense. It contained sporadic mitochondria and dark bodies (Figs. 3, 4). Some of the dark bodies were probably secondary lysosomes, others had the appearance of pseudomyeline structures (Plate VIII., Fig. 4). Cytoplasm of some basal cells also contained pigment granules (Fig. 2). The cytoplasm projected towards the middle layer in numerous short processes connecting with the processes of the middle layer cells by means of many desmosomes (Fig. 3). The basal layer of cells was only apposed to the plate. Neither basal membrane, nor hemidesmosomes were formed. The granular endoplasmic reticulum occurred rarely, and it had the appearance of sporadic short cisterns. Cytoplasm contained a large number of tonofibrils forming larger or smaller bundles that were often encircling the nucleus. Among them, organelles were situated, and some cells contained also glycogen granules (Fig. 4).

The middle layer was the thickest. Here, the cells were situated in at least 3 layers on one another (Fig. 2). They were of polyhedral shape, reaching the size of $25-30 \times 15-17 \mu\text{m}$ (Fig. 2), and they became flattened towards the tissue surface.

The nucleus did not differ in its size, shape and appearance from the nuclei of the basal layer cells. The nuclear envelope, when compared to the basal layer, projected in relatively numerous narrow invaginations sometimes reaching quite deeply. Thus, irregular intranuclear inclusions can arise (on the nucleus section) (Plates IX., X., Figs. 5, 6). Nucleolus of reticular appearance occurred regularly, and it joined the inner membrane of the nuclear envelope (Figs. 5, 6). Karyosomes were of little density and small size (Figs. 5, 6). The cytoplasm of the middle layer cells adjacent to the basal layer contained mitochondria of oval and rod-like shapes, numerous short cisterns of the granular endoplasmic reticulum, bundles of tonofilaments. Among them numerous dark bodies were situated (Figs. 5, 6). Cytoplasm of some cells contained larger amounts of granular endoplasmic reticulum (Fig. 6).

The cytoplasm of the middle layer cells was dense compared to that of the basal layer cells (Fig. 2). Desmosomes were formed among numerous short processes of the cytoplasmic membrane of the middle layer cells (Figs. 5, 6, Plate XI., Fig. 7). Intercellular spaces were narrow.

Different appearance can be seen in the superficial cells of the middle (intermediary) layer that were adjacent to the superficial layer (Fig. 7). These elements were of elongated or spindle-like shape adopted even by the nucleus. The nucleus did not differ from the nuclei of the middle layer cells. The nuclear envelope did not project into deep invaginations, regularly occurring nucleoli were of reticular type.

Cytoplasm comprised organelles and bundles of tonofibrils like in the previous cell types. Moreover, more numerous pseudomyeline figures and large dark bodies, $2 \mu\text{m} \times 3-4 \mu\text{m} \times 1-1.5 \mu\text{m}$ situated near the nucleus were seen (Fig. 7). These bodies were probably autophagic vacuoles, although the demarcating membrane can be recognized with difficulties. The deposits of glycogen near the nucleus were observed only in the cytoplasm of these cells (Fig. 7).

The surface was formed by markedly flattened cells situated in 2 layers on each other (Plates XII., XIII., Figs. 8, 9). These cells contained no nucleus or contained only its residues. Cytoplasm contained sporadic damaged mitochondria (Figs. 8, 9) and quite short

flattened vesicles of granular endoplasmic reticulum. Bundles of tonofilaments running in a wavy form in the longitudinal axis of the cell and sometimes forming a thick bundle passing along the cell were very numerous (Fig. 8). In the cytoplasm of some cells, there were numerous, dense but short bundles of tonofilaments scattered in the cytoplasm and also oriented along the longitudinal cell axis. Among them, in the cytoplasm of some cells, numerous dark granules were located, about $0.5 \times 1 \mu\text{m}$ in size, whose appearance was very similar to keratine granules of epidermal cells. The cytoplasmic membrane ran into numerous projections that were longer than in the middle layer. Desmosomes were formed among them. Intercellular spaces were broad. Towards the tissue surface, cytoplasm projected into numerous short processes (Figs. 8, 9).

Discussion

The results of the present study on ultrastructure and arrangement of embryonic or fetal keratinocytes after 20 or 21 days of culture in a corresponding tissue medium can be compared, only to a certain extent, with literary data. To our knowledge, most published and available morphological studies have described the results of cultured keratinocytes obtained from adult individuals.

Therefore, our results can be compared only partially with reports published earlier, e.g. with research carried out by Compton (1989, 1993) and Bernstam et al. (1990). Their observations differ particularly in the use of carriers of various provenience for culture (e.g. dermis or collagenous tissue). Such base was covered mostly with allo-keratinocytes, only sporadically with auto-keratinocytes of adult individuals. The methodological procedure designed in that way was practically a guarantee of creating all the attributes of the intact skin during successful culture (e.g. an important phenomenon of forming the lamina basalis), and in some cases even of the basal membrane although this possibility occurred only after a longer period following the graft transplantation (Arons et al. 1992).

In the present study, a method differing from those in the above-mentioned experiments was used. The difference lies in the direct placing of embryonic keratinocytes into a culture dish without any carrier and continuous determination of the number of keratinocyte layers in dependence on the duration of cultivation. The approximate velocity of keratinocyte growth was followed, too. The optimal thickness of cultured layers and suitability of their application on an affected area were studied. Maintaining all the principles of culture (Adler 1996), our method of keratinocyte culture and use has been repeatedly confirmed to be the most suitable for this therapeutical purpose (Brychta et al. 1994).

After the interval of 20-21 days, the arrangement and shape of keratinocytes in culture is reminiscent of the stratified squamous epithelium present in the epidermis of intact skin. It included a keratinized layer. These findings are in agreement with those of Aoki et al. (1994), Compton (1993), Cuono et al. (1986).

The study of the keratinocyte ultrastructure revealed that the basal layer was formed mostly by cubic cells as in intact skin, however, the lamina basalis was not formed. Relatively numerous dark cells with strong cytoplasmic projections resembling dendritic cells can be seen among the cells of the basal layer (Compton 1989). The keratinocytes located above the basal layer in the direction to the surface form about 4-5 layers of polyhedral cells with light cytoplasm, large nucleus with diffusely arranged chromatin and reticular nucleoli. The adjacent cells are in contact by numerous short cytoplasmic projections, with many desmosomes created among them. The keratinocyte cytoplasm below the surface contains numerous and pronounced bundles of intermediary filaments and keratine granules. The surface is formed by about 2 layers of quite flattened cells with no nucleus in their cytoplasm,

while the bundles of tonofilaments are prominent. The cytoplasm of cells situated on the surface projects into numerous short processes. Our observations of the keratinocytes (their appearance and composition, special arrangement) correspond well with the findings of other workers (Compton 1989, 1993; Aoki et al. 1994). These cells have been generally considered vital and capable of further proliferation. The results of our study are in good agreement with the data published so far. The preliminary clinical results are promising.

Ultrastruktura kultivovaných lidských embryonálních a fetálních keratinocytů

Pro léčbu rozsáhlých defektů tělního povrchu (zejména při popáleninách) se stále hledají nejvhodnější tkáňové deriváty. Kultivované embryonální a fetální keratinocyty byly použity proto, že díky své vitalitě v relativně krátké době vytvářely dostatečně tlustou vrstvu vhodnou k transplantaci a byly příjemci dobře tolerovány.

Byla studována ultrastruktura fetálních keratinocytů po 20-21 dnech kultivace. Po této době mají keratinocyty podobu vrstevnatého epitelu. Podle vzhledu a struktury buněk lze odlišit vrstvu bazální, střední, podpovrchovou (intermediární) a povrchovou. Bazální vrstva je tvořena 1-2 vrstvami níže cylindrických až kubických buněk s denzní cytoplazmou, výrazným světlým jádrem a jadérkem retikulárního typu. V cytoplazmě se vyskytují tmavá tělíska typu sekundárních lysozomů, pigmentových granul nebo granul keratohyalinu či pseudomyelinových figur. Střední vrstva je tvořena asi 3 řadami velkých buněk se světlou objemnou cytoplazmou. Jádro je velké, světlé, jaderný obal vybíhá v četné invaginace a vytváří nepravé jaderné inkluze. Výrazná jadérka jsou výlučně retikulárního typu. V cytoplazmě promínuje množství tmavých tělísek a v některých buňkách AER. V cytoplazmě oploštělých buněk intermediární vrstvy kromě svazků tonofilament jsou přítomny četné autofagické vakuoly a depozita glykogenu. Povrchové 1-2 vrstvy jsou tvořeny nápadně plochými buňkami zpravidla bez jader. V jejich cytoplazmě se vyskytují vlnovité probíhající mohutné svazky tonofilament a velká tmavá tělíska s homogenním obsahem. Mezi buňkami bazální, střední a intermediární vrstvy jsou pouze úzké mezibuněčné prostory a četné desmosomy, zatímco mezi buňkami povrchové vrstvy se intercelulární prostory rozšiřují a desmosomy nejsou tak početné.

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Address for correspondence:

Prof. MUDr. D. Horký, DrSc.
Department of Histology and Embryology
Medical Faculty, Masaryk University
Joštova 10
662 43 Brno
Czech Republic

Phone: 420 5 42 12 62 13
E-mail: horky@med.muni.cz

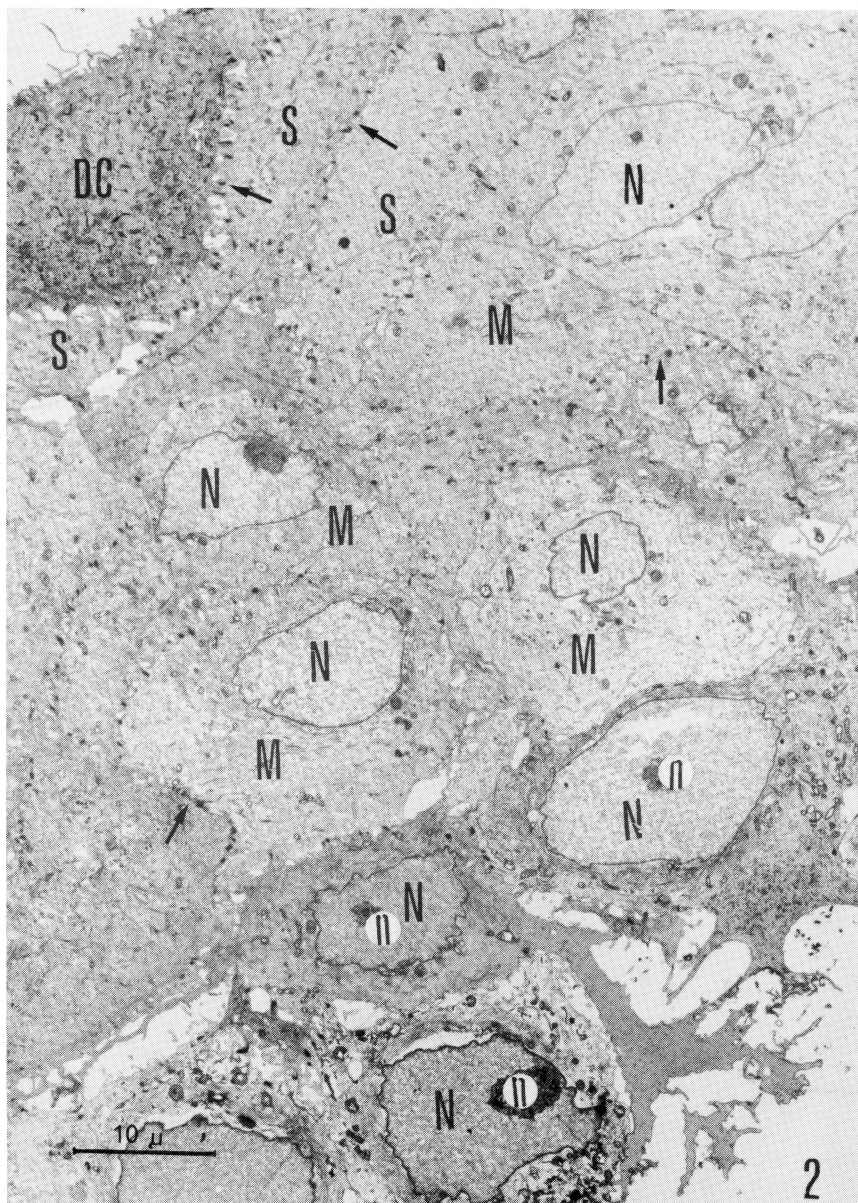


Fig. 2. Fetal keratinocytes after 20 days of culture have an appearance of a stratified squamous epithelium. The layer of dark cells with light nuclei (N) and marked nucleoli (n). Towards the surface of the middle layer of polyhedral cells (M) with light, voluminous cytoplasm. Nuclei (N) contain finely dispersed chromatin and conspicuous nuclear envelope with deep invaginations. Three superficial layers are made up of spindle-like or flattened cells (S) with numerous projections. Desmosomes are well formed among adjacent cells (→). On the surface, one layer of spindle-like or flat cells with dark cytoplasm (DC) is present, with bundles of tonofilaments running in different directions. x 2200.

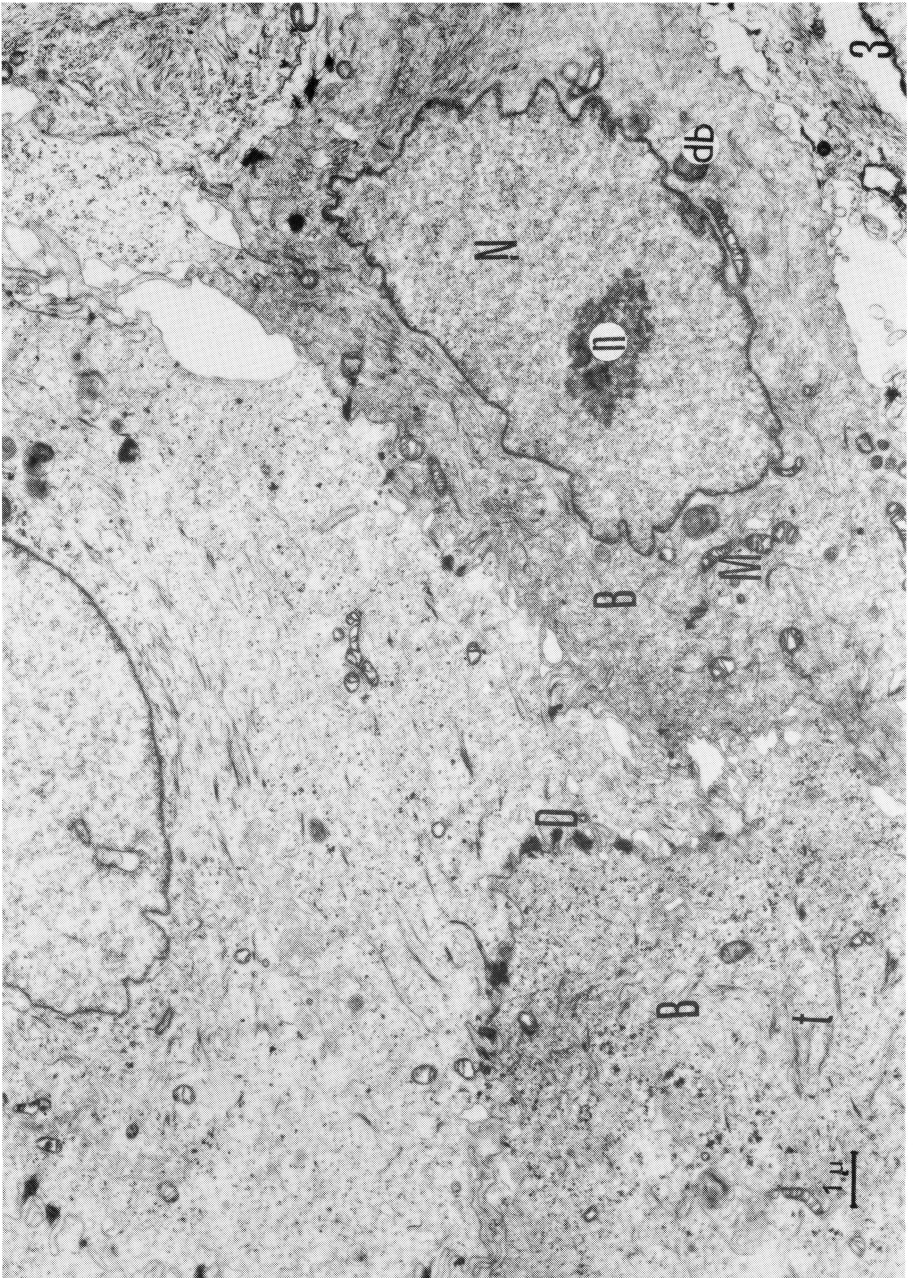


Fig. 3. Cells of the basal layer of keratinocytes after 20 days of culture (B), nucleus (N), nucleolus (n), nuclear envelope with numerous unevennesses. Mitochondria (M), dark bodies (db), bundles of tonofilaments (t). Desmosomes (D) are situated among numerous cytoplasmic projections. x 8000.

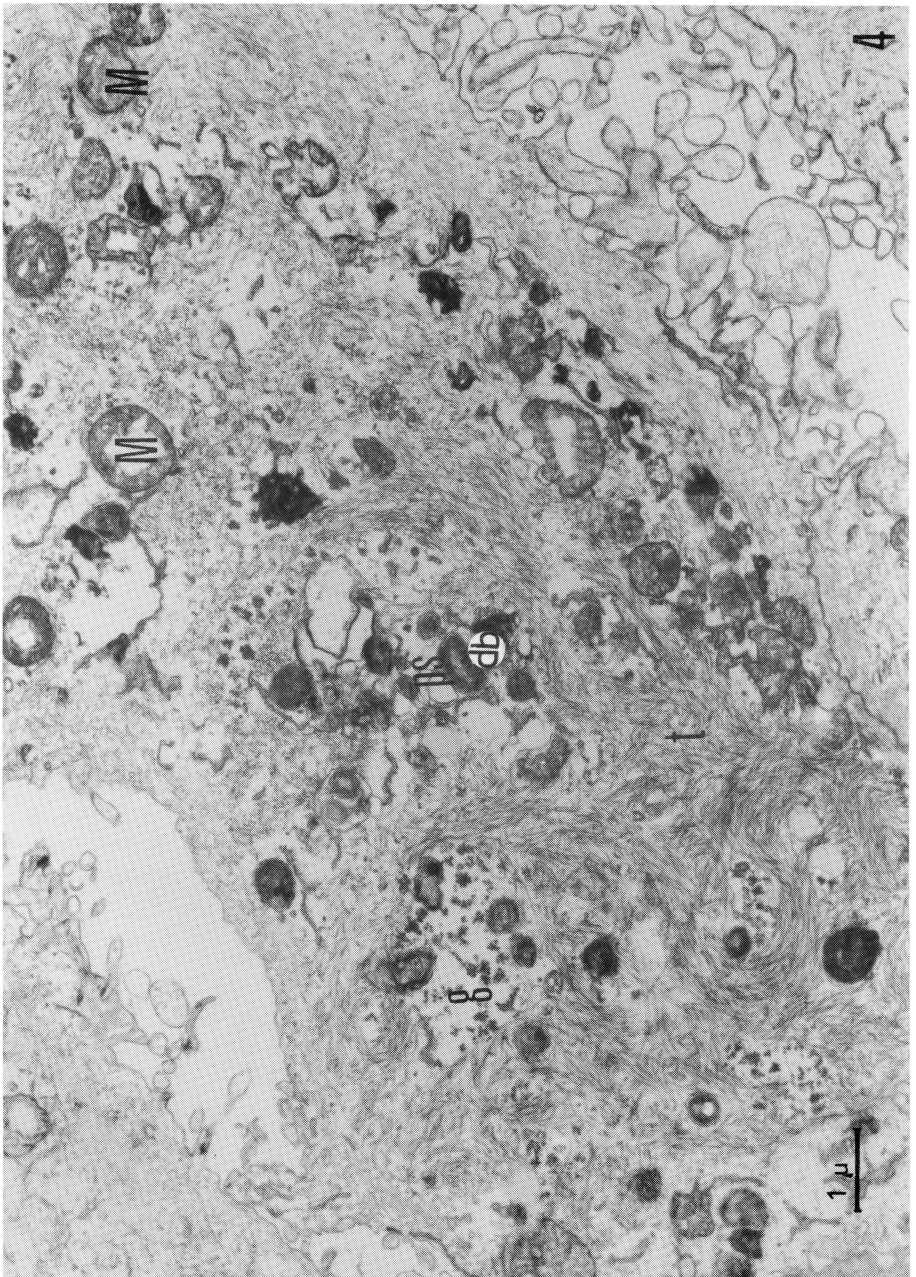


Fig. 4. Basal layer of keratinocytes after 20 days of culture. Numerous damaged mitochondria (M), dark bodies (db), pseudomyeline structures (ps), glycogen granules (g). Bundles of tonofilaments are encircling the organelles (t). x 12000.

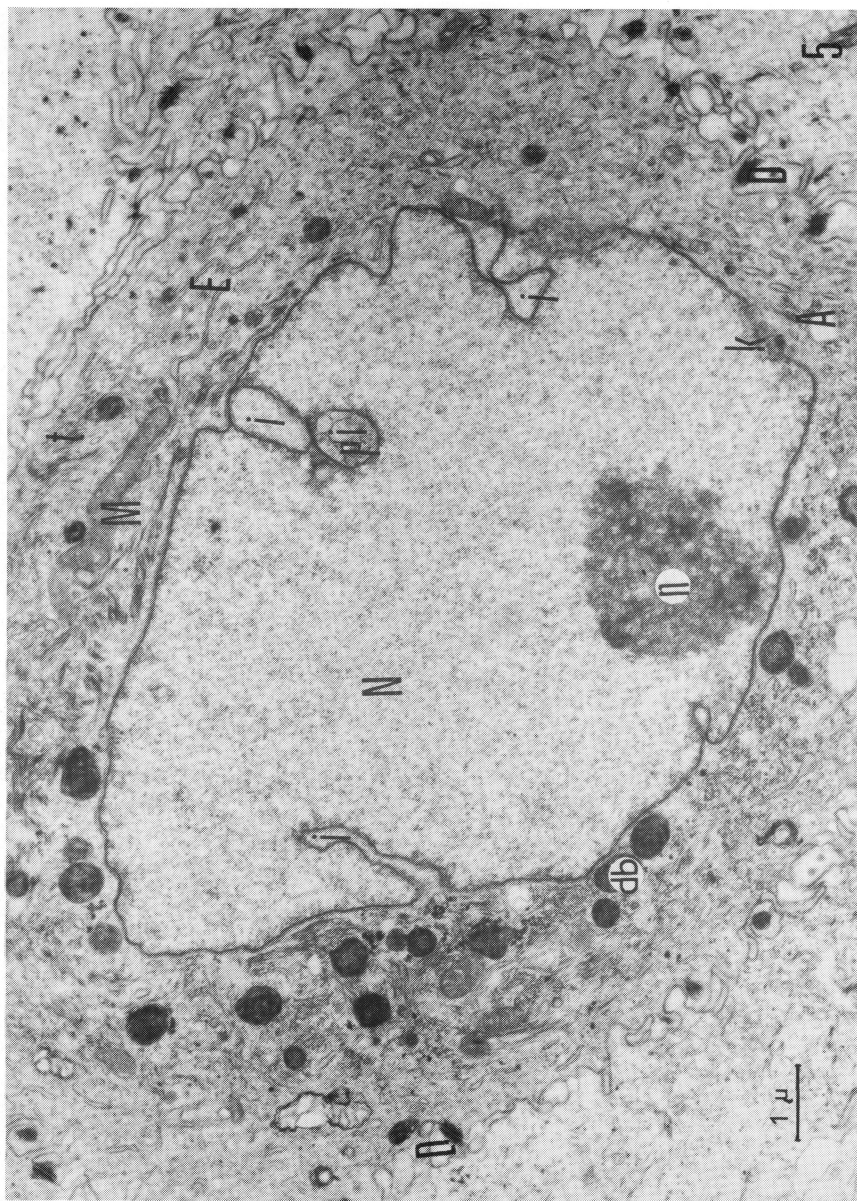


Fig. 5. The nucleus and part of cytoplasm of a keratinocyte of the middle layer after 20 days of culture. A nucleus (N) with finely dispersed chromatine, karyosomes (k) are small and joining the nuclear capsule. The nuclear envelope runs into deep invaginations (i), false nuclear inclusions (pi), a nucleolus of reticular type (n). Cytoplasm contains sporadic bundles of tonofilaments (t), long mitochondria (M), dark bodies (db), short cisterns GER (E), sporadic vesicles AER (A). Numerous desmosomes (D) are situated among adjacent cells. x 12000.

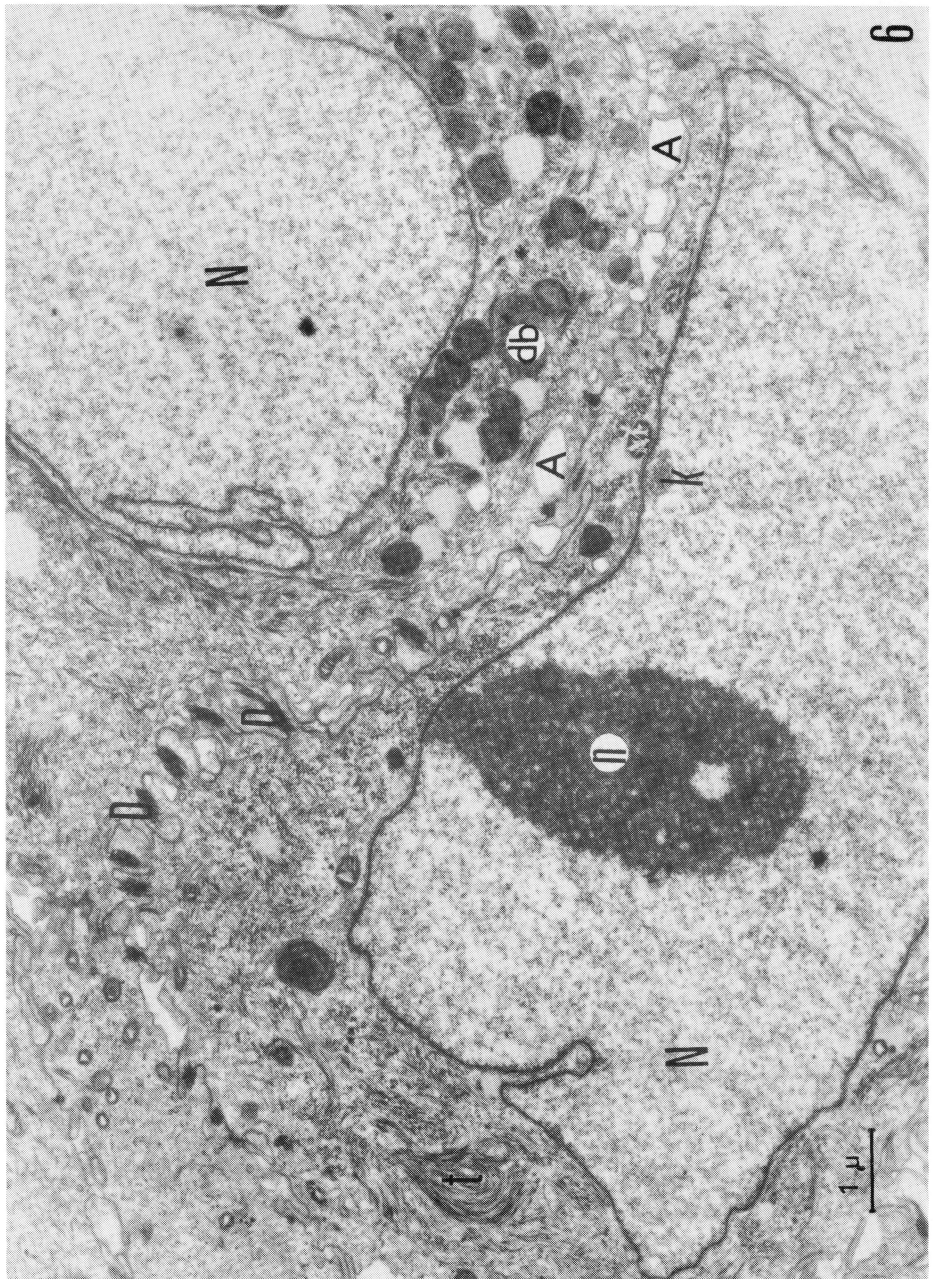


Fig. 6. Parts of nuclei and cytoplasm of keratinocytes of the middle layer after 20 days of culture. Nuclei (N), nucleolus of reticular type (n), sporadic karyosomes (k). Cytoplasm contains bundles of tonofilaments (t), dark bodies (db), numerous vesicles AER (A), small mitochondria (M). Desmosomes (D) are formed among numerous cytoplasmic projections. Intercellular spaces are narrow. x 12000.

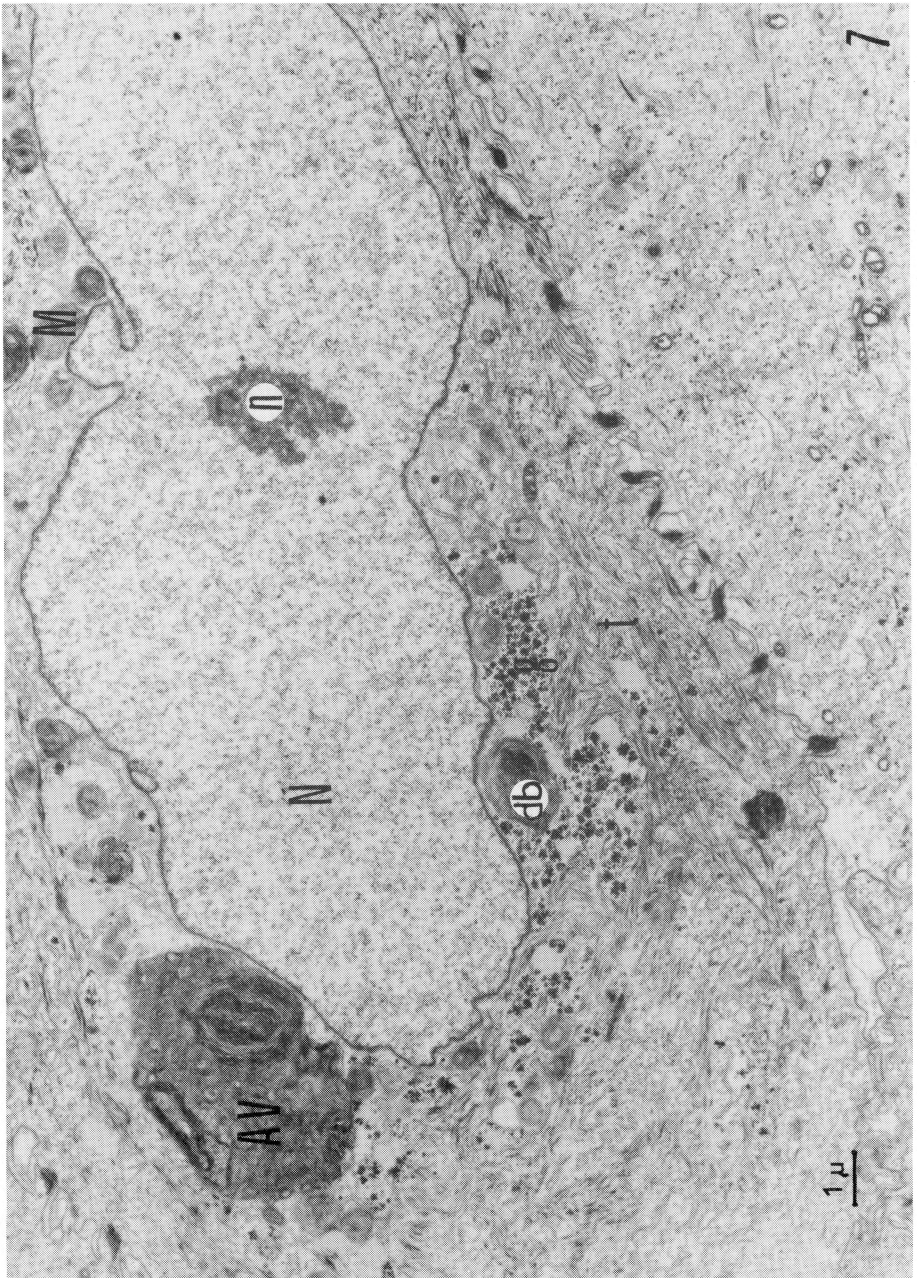


Fig. 7. Nucleus and part of cytoplasm of keratinocytes of the intermediary layer after 20 days of culture. The nucleus (N) is elongated in accordance with the shape of cells, chromatin is finely dispersed. Nucleoli are small, of reticular type (n). Nuclear invaginations are shallow. Cytoplasm contains numerous bundles of tonofilaments (t), mitochondria (M), numerous dark bodies (db), autophagic vacuoles (AV), often of large size. Glycogen deposits (g) in the vicinity of a nucleus. $\times 8000$.

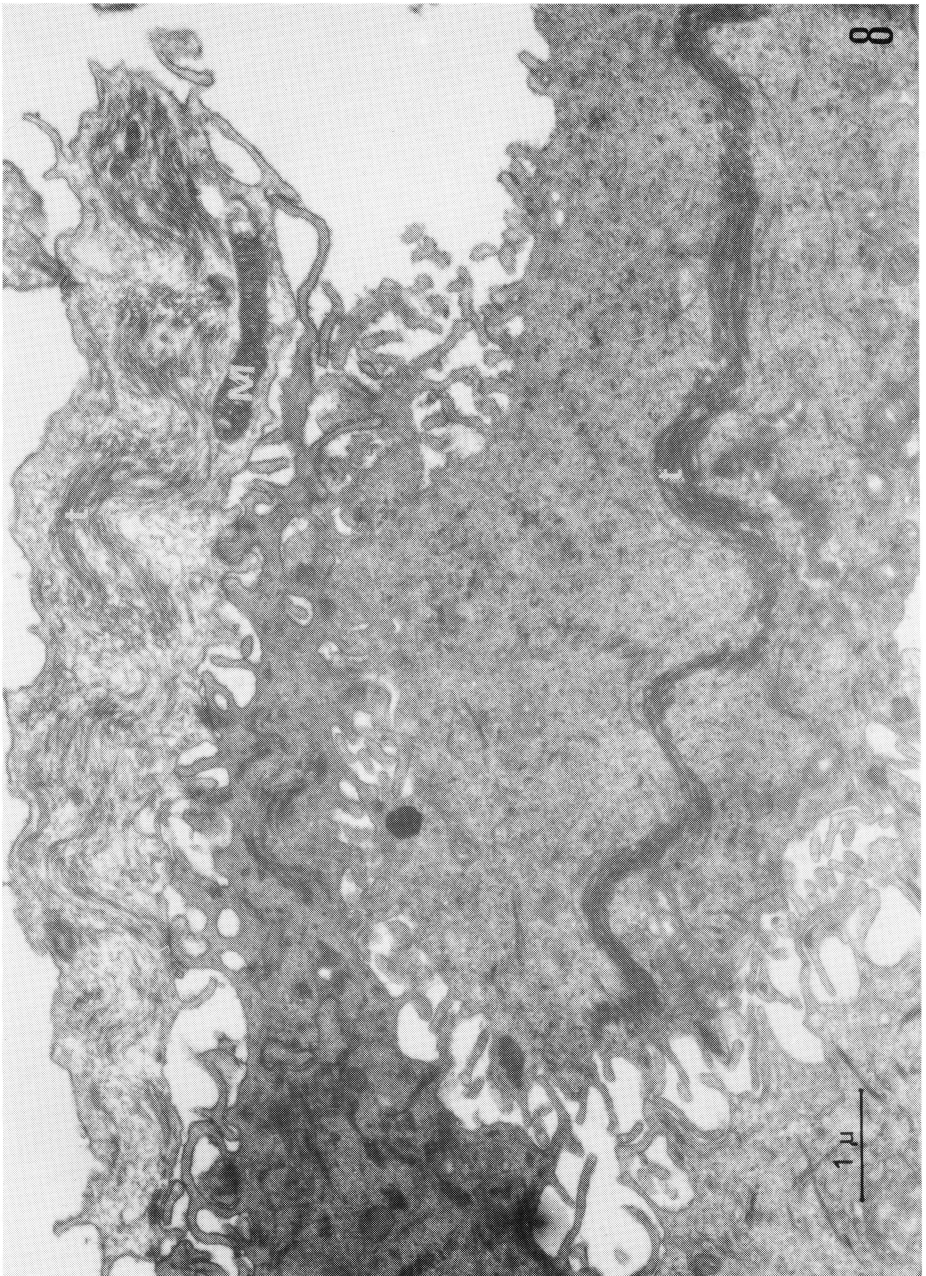


Fig. 8. A part of cytoplasm of flat keratinocytes of the superficial layer after 20 days of culture. Flattened cells are usually without nuclei. Dense cytoplasm contains residues of cellular organelles. Strong bundles of tonofilaments passing in a wavy form are prominent (t). On the surface, a separating cell with damaged mitochondrion (M) of rod-like type. Intercellular spaces are broad. x 16000.

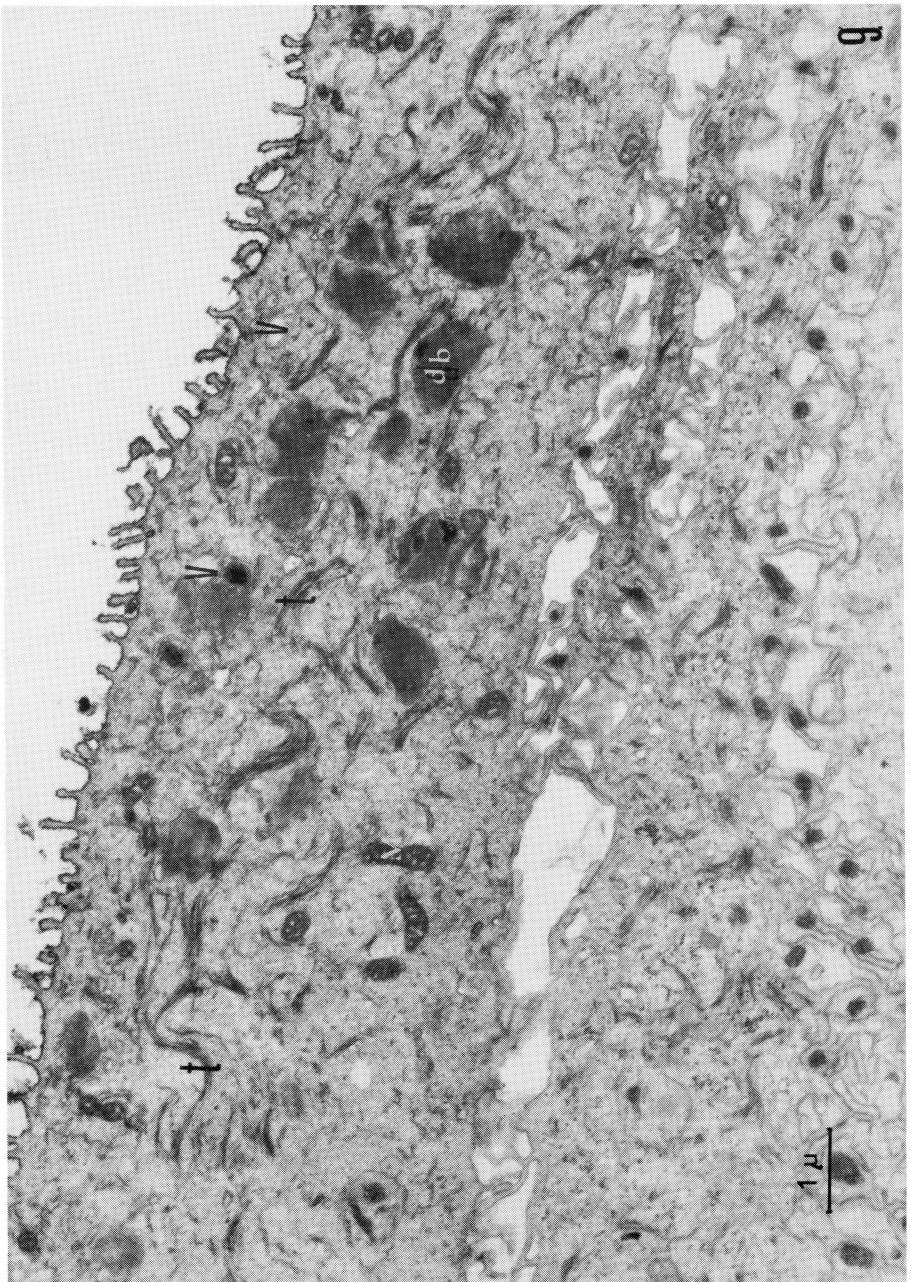


Fig. 9. A part of cytoplasm of the superficial layer of keratinocytes after 20 days of culture. Numerous bundles of tonofilaments are running in a wavy form among the residues of cellular organelles (t). Numerous dark bodies (db) with homogeneous contents, sporadic mitochondria (M), and smooth vesicles (V). Intercellular spaces are broad, the number of desmosomal connections is decreasing. x 12000.