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STUDIES ON GUINEA PIG SKIN CELL CULTURES

I. *Separate Cultures of Keratinocytes and Dermal Fibroblasts*[1]

M. Regnier, C. Delescluse and M. Prunieras

From the Laboratory for Research on Human Skin Tumours, Adolphe de Rothschild Foundation, Paris, France

Abstract. Both epidermis and dermis have been used as a source of cells. After splitting the skin with trypsin, epidermal cell suspensions have been made and cultures established. Primary cultures consist of cells which retain their specific fibre desmosome structures under the electron microscope. They are Leucinaminopeptidase (LNase) negative, as are epidermal keratinocytes in situ. They incorporate tritiated thymidine, with a peak at day nine after planting. Long-term cultures are composed of fibroblast-like cells with no distinct ultrastructural feature. These fibroblast-like cells remain LNase negative. Dermal cells have been isolated by dissociation of the dermis with collagenase, and cultured. Primary cultures are composed of fibroblast-like cells. They are consistently LNase-positive, like connective-tissue cells in situ. They incorporate tritiated thymidine, with a peak at day 5. Long-term cultures are composed of fibroblast-like cells resembling those derived from epidermal primary cultures. However, they are strongly and consistently LNase-positive. It is concluded that two different cell lines can be established from one single piece of skin. One consists of epidermal keratinocytes, partially dedifferentiated, the other of dermal connective tissue fibroblasts.

It has been shown in previous studies that after guinea pig ear skin has been split with trypsin, epidermal cells (keratinocytes and melanocytes) can be grown and recombined in primary cultures (12).

It has also been shown that subcultures can be obtained by transferring confluent primary cultures to new culture flasks (13) and that long-term cultures can be propagated by subsequent transfers (or passages) for as long as 15 months or sixty-one passages (15). These long-term cultures, however, are composed of cells with fibroblast-like morphology under the light microscope, and without the ultrastructural markers of keratinocytes (5).

The first interpretation is that these fibroblast-like cells are nothing but ordinary fibroblasts which have been detached from the dermis during the process of trypsination and have overgrown and replaced the keratinocytes. That this may not be the case was suggested by a study on leucine aminopeptidase (LNase) reaction on frozen sections of guinea pig skin and on subsequent epidermis-derived cultures (8).

On frozen sections of guinea pig skin, the LNase reaction is negative in the epidermis. It is strongly positive in dermal connective tissue cells. Epidermis-derived cultures have been shown to remain consistently negative as opposed to regular control fibroblasts in culture which were repeatedly found to react positively (8).

However, the evidence so given was only partially convincing because the so-called "regular" fibroblast cultures which have been used as control in the LNase test were derived from the bone marrow of the guinea pig and not from the dermis of the skin. Therefore, the possibility remained that dermal fibroblasts behave differently, and, in particular, loose their capacity to react to LNase after having spent some time in culture.

In the present paper, it will be shown that besides LNase-negative, LNase-positive cell cultures can be obtained from the same piece of guinea pig skin. In addition, just as negative cultures remain negative, positive cultures remain positive through serial passages in vitro.

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MATERIAL AND METHODS

1. *Material*

Both ordinary tricolor and albino (Hartley strain) adult guinea pig (250–300 g) were used. Hartley strain were germ-free certified animals (EVIC-CEBA, Blanquefort, France).

Methods of culture

(*a*) *Primary cultures of epidermal cells* were prepared according to the following technique: The hairs of the skin of the ears were plucked with wax. The skin was washed three times with saline solution and once with 70% ethanol. It was then rubbed with mycostatin. Immediately before its removal, the skin was washed three times with sterile saline solution followed by a rinse with 70% ethanol, and dried.

Strips of Steri-strip (3M-Minnesota) were stuck on to the back of the ear and the skin was cut with Castroviejo's electrokeratome, set at level one. The strips of skin with the Steri-strip attached were floated on preheated 0.15% Trypsin (Difco) in Phosphate Buffer Solution (PBS) at pH 7.2 for 90 min at 37°C. The pieces of skin were then drained and placed, keratin layers down, in a Petri dish. The dermis was lifted up with fine forceps.

Basal cells which had remained attached to the dermis were loosened by shaking the piece of dermal tissue in an hemolysis tube containing 2 ml of Tissue Culture Medium (TCM) for 30 sec, with a Vortex agitator at medium speed. After shaking, the TCM was filtered on sterile gauze. The yield was about 4 to 5×10^6 viable cells for one ear. Coverslip cultures in Leighton tubes were prepared by seeding 5×10^5 cells in 2 ml of TCM per tube.

(*b*) *Primary cultures of dermal fibroblasts* were made by treating the piece of dermal tissue which had just been shaken to loosen basal cells, with 0.1% collagenase (Worthington, crystallized) in glucose, potassium, sodium (GKN) solution according to Hinz & Syverton (7) for 60 min at 37°C.

After complete dissociation of the piece of dermal tissue, freed dermal cells were harvested. Viable cells were counted and seeded in Leighton tubes using 5×10^5 cells per 2 ml of TCM. The same TCM was used for both cultures. It consisted either of BME (Eagle, 1955) supplemented with 10% calf serum or MEM (Eagle, 1959) supplemented with 25% horse serum.

(*c*) *Subcultures.* They were made by using either the trypsin technique described for human diploid cells by Hayflick & Moorhead (6) or detachment with collagenase according to Lasfargues (9).

(*d*) *Freezing of cells and reconstitution of cultures.* Cells to be frozen were detached by trypsin treatment as indicated above and suspended at a concentration of 2×10^6 per ml in TCM supplemented with 10% dimethyl sulfoxide (DMSO). Ampoules containing 1 ml were then filled and sealed. They were frozen in liquid nitrogen according to the following technique: after having been kept for 60 min at 4°C they were transferred first to −20°C for 45 min, second into dry ice for 45 additional minutes, and then directly to liquid nitrogen.

Cultures were reconstituted by quick thawing of one or two ampoules under running lukewarm tap water and seeding of the cells into Falcon plastic F30 flasks in 5 ml of TCM.

Excess DMSO was eliminated by a TCM change 4 to 6 hours after seeding.

Methods of characterization

(*a*) *Electron microscopy.* Cultures of keratinocytes and cultures of fibroblasts were made in plastic Petri dishes (Falcon) and prepared for electron microscopy according to the technique already described (5).

(*b*) *Leucine aminopeptidase.* Coverslip cultures were processed according either to the technique of Nachlas et al. (10) with L-leucyl naphthylamide as substrate, or the improved method with L-leucyl-4-methoxy-naphthylamide (11). The reaction product was examined under oil immersion. Only those cells totally devoid of reaction product were scored as negative.

Assessment of growth capacity

The growth capacity of primary cultures has been estimated by tritiated thymidine incorporation. Coverslips were seeded with 5×10^5 viable cells both for epidermis- and dermis-derived cultures.

After a given time, TCM was removed and replaced with a new TCM containing tritiated thymidine (spec. act. 14.9 Ci/mM (C.E.A., Saclay, France)) at a final dilution of 1 μCi/ml. The cells were given a 30 min pulse and then processed for radioactivity measurement by direct counting of coverslip cultures according to the technique of Fallot et al. (2). Such pulse were made on day 1, 3, 5, 7, 9 and 11 for epidermal cultures, and on day 1, 3, 5 and 7 for dermis-derived cultures.

The growth of long-term cultures has been roughly calculated by considering the number of weeks necessary for a cell line to reach a given number of subcultures, each subculture being made by adjusting the split to 5×10^5 cells per flask.

RESULTS

A. *Primary cultures of cells derived from trypsinized epidermis*

The cultures were roughly similar to those obtained with previous techniques, as regards cell morphology. After 3 days in culture, numerous small sheets of cells were scattered throughout the coverslip. Many branched cells, identifiable as melanocytes, were visible and frequently formed bridges in between adjacent groups of growing cells.

With the electron microscope, typical fibre desmosome complexes were reconstituted as early as the second day. They remained unchanged until day 9. An example of such a fibre desmosome complex at day 7 is shown in Fig. 1.

Epidermis-derived cells were consistently non-

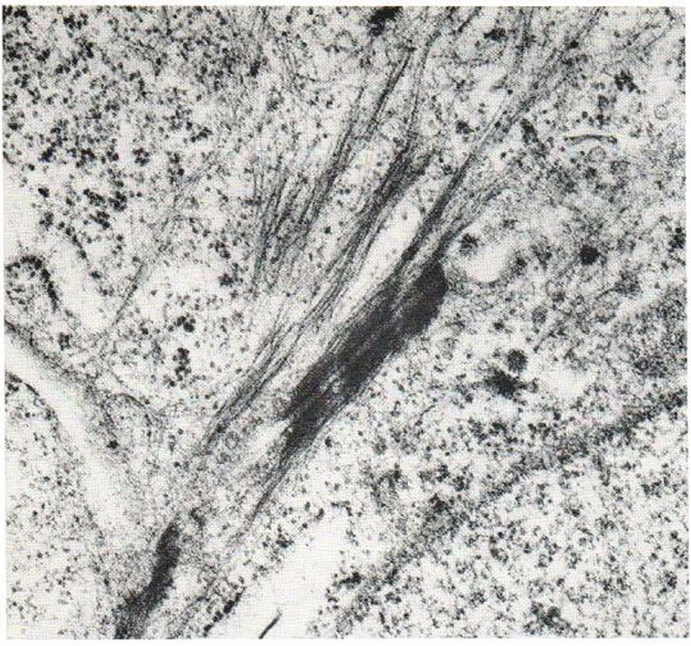

Fig. 1. Primary culture of epidermal cells at day 7. Reconstituted fibre desmosome complex. This is taken as evidence of the keratinocytic nature of the cultured cells. Electron microscopical preparation, × 35 000.

reactive to LNase. Melanocytes were also negative. Daily examination of LNase reaction in these cultures, revealed that occasional positive cells could be seen during the first 2 days. Such positive cells were not clearly identified. Some of them may be Langerhans cells. In rare instances,

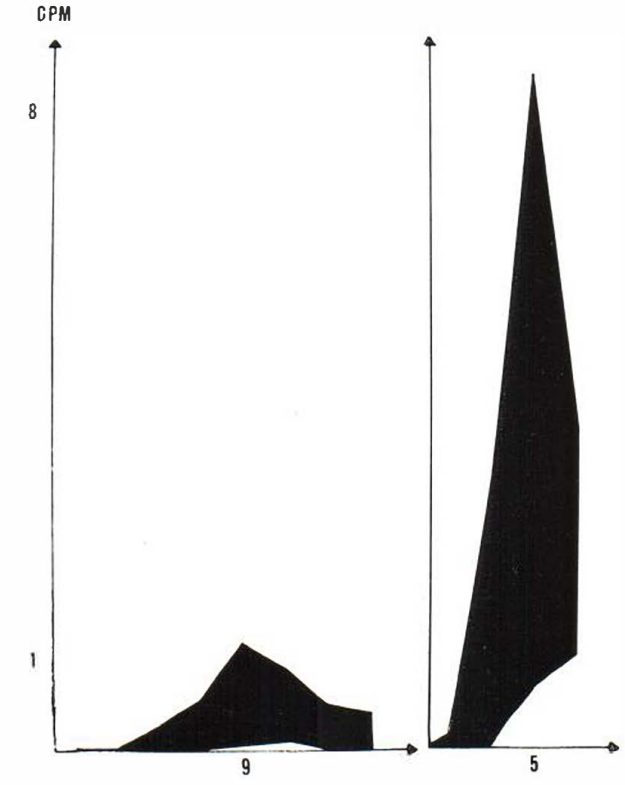

Fig. 2. Thymidine incorporation (CPM) according to number of days in ten series of primary cultures. (*Left*) epidermal cells. The peak of incorporation is reached at day 9. (*Right*) dermal cells. The peak (about eight times higher) is reached at day 5. The number of viable seeded cells is the same in both cases.

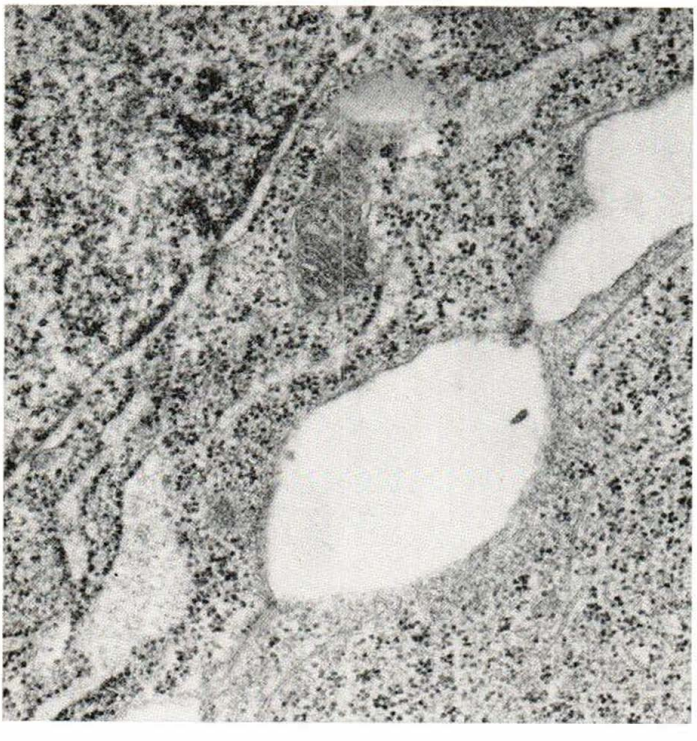

Fig. 3. Primary culture of guinea pig connective tissue cells at day 7. There is no fibre desmosome complex, as in epidermal cells. Instead, one can see well developed ergastoplasmic cisternae, which are in relation with collagen synthesis. Electron microscopical preparation, × 50 000.

one or two colonies of strongly positive fibroblast-like cells were seen after several days in culture, surrounded by a vast majority of non-reactive cells. As regards the shape and morphology of these non-reactive cells, they exhibited epithelial-like morphology during the first week of culture. They had a much more fibroblast-like appearance from the seventh day on.

The growth capacity of epidermis-derived primary cultures has been estimated from 10 series of 3HTdR-labelled coverslip cultures. Minimal and maximal values representing the two extremes of 3HTdR incorporation in at least 20 cultures have been plotted for a given day.

They are shown schematically in Fig. 2.

One can see on this diagram that the growth is exponential from day 5 to day 9. After the peak of day 9, three is a sharp decline in growth ability.

B. *Primary cultures of cells derived from collagenase-treated dermis*

As regards morphology, the cultures were composed of fibroblast-like cells since the first day after seeding. When the cultures were derived from a pigmented area, numerous coarse melanin granules were visible in the cytoplasm of some of the cells. Branched cells, identifiable as melanocytes, were rare or absent.

Confluence and cell crowding was reached at day 5.

Under the electron microscope the cells exhibited a well developed rough endoplasmic reticulum and no fibre-desmosome complexes (Fig. 3).

Dermis-derived primary cultures were strongly positive for LNase. An example of a positive cell is given in Fig. 4.

The growth capacity of dermis-derived cells is displayed in the right half of Fig. 2. Here, the diagram has been made like the left one for epidermis-derived cultures. Minimal and maximal values were extracted from at least 20 cultures for days 1, 3, 5 and 7.

One can see that the growth is exponential from day 3 to 5. After the peak of day 5, there is a sharp slope till day 7. Beyond this point the cultures become overcrowded and detach themselves from the coverslip.

C. *Subcultures*

1. Subcultures of primary cultures derived from trypsinized epidermis were composed of fibroblast-

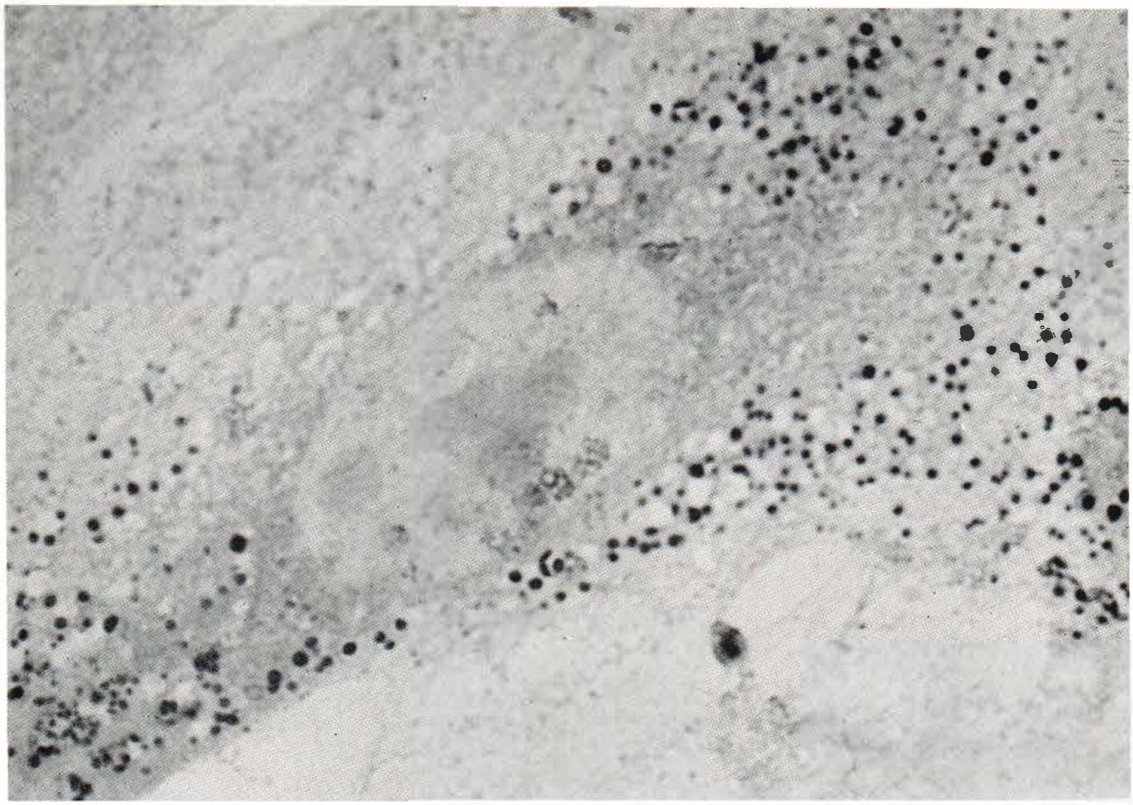

Fig. 4. Connective tissue cell in long-term culture, positive for Leucinaminopeptidase. The reaction product is seen as small, dense, black dots of variable size. Light microscope, under oil immersion 95 × objective.

like cells. These cells had no fibre desmosome complexes. These cultures were consistently negative for LNase reaction. No positive subcultures have been obtained so far. Repeated freezing and reconstitution of cultures did not alter the reaction, which remained negative. The growth capacity of LNase-negative cells has been estimated for two cell lines. In both cultures the eleventh passage was reached between 8 and 9 weeks after seeding.

2. Subcultures of primary cultures derived from collagenized dermis were composed of fibroblast-like cells. These cells had no fibre desmosome complexes.

Three lines of cells were tested for their reactivity to LNase. They were all found positive.

The cells were also strongly positive after two consecutive freezings in presence of DMSO and reconstitution by rapid thawing.

The growth capacity of three LNase-positive cell lines was evaluated as for LNase-negative cell lines. The eleventh passage was reached between 6 and 7 weeks after seeding the cells.

DISCUSSION

For the first time it has been shown that two distinct and separate primary cultures can be obtained from one single piece of adult guinea pig skin. Previous studies indicated that explant cultures could yield two different cell types in the same culture flask (8). One cell type is epithelial-like and LNase-negative. The other cell type, growing after a lag period of about 11 days, is fibroblast-like and LNase-positive.

In the present study, these two cell types were cultivated in two different tissue culture flasks, after dissociation of both epidermis and dermis.

The epithelial-like cells exhibited fibre desmosome complexes and remained LNase-negative, like keratinocytes in situ. They are therefore considered as being keratinocytes in culture.

These primary cultures of keratinocytes contain melanocytes. Such melanocytes do not multiply in culture (14) and they will therefore not overgrow the keratinocytes in subsequent subcultures.

In addition to keratinocytes and melanocytes, primary cultures derived from trypsinized epidermis contain from time to time rare but definite LNase-positive cells. These LNase-positive cells may be dermal fibroblasts, since dermal fibroblasts are strongly LNase-positive in situ (1). They may also be Langerhans cells, which are a normal component of the epidermis and are known to react positively to LNase, both in situ (16), and during the first days of culture (3).

An interesting fact is that these LNase-positive cells, whatever their origin may be, do not develop in the subsequent stages of cultivation, since all cultures derived from trypsinized epidermis have so far reacted negatively to the LNase test (8, 4). The hypothesis, according to which dermal fibroblasts, strongly LNase-positive in situ may become negative after a certain period of time in culture, is not supported by the experiments made in the present study. Indeed, all primary cultures prepared by collagenase dissociation of the dermis have yielded LNase-positive cells, and these cells have remained strongly LNase-positive throughout subsequent subcultures.

Furthermore, LNase-positive (and negative) reaction is not modified by repeated freezing and reconstitution procedures.

Thus, not only are two different primary cultures obtainable from guinea pig ear skin, but two different cell lines can also be propagated.

These two cell lines can hardly be distinguished on a morphological basis since both cultures are ultimately composed of fibre-desmosome complex free cells. However, they do remain different on the basis of their reactivity to LNase, as discussed above, and also their growth capacity.

In primary cultures, the growth curves are obviously dissimilar. Keratinocytes reach their peak at day 9 whereas dermal fibroblasts reach it in 5 days. In addition, the level of thymidine incorporation for a comparable number of seeded cells is about eight times higher in the latter cells.

In subcultures, there seems to exist a noticeable difference, since it requires 2 weeks more for keratinocytes than for fibroblasts to achieve the same number of passages.

However, such a difference is apparent only because it reflects the fact that LNase-positive cells grow faster in primary cultures.

In conclusion, two different cell lines can be initiated and propagated through subcultures from adult guinea pig ear skin. One is LNase-negative. It is thought to be composed of more or less dedifferentiated keratinocytes. The other is LNase-positive, and composed of dermal fibroblasts.

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M. Prunieras, M.D.
Fondation Ophtalmologique Adolphe de Rothschild
29, rue Manin
75019 Paris
France