ULTRASTRUCTURAL FEATURES OF DEFECTIVE IN VITRO KERATINIZATION OF CHICK EMBRYONIC SKIN

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SUMMARY

Thirty-four explants of epithelium and subjacent mesenchyme from the hind limb buds of 5-day chick embryos were cultured for 1 to 14 days. Controls consisted of a series of in ovo limb bud specimens from 5 day's incubation to hatching. Both experimental and control specimens were examined by similar electron-microscopic methods.

Differentiation of epidermis was precocious and that of dermis extremely retarded in vitro, contrasting with the events in ovo where epidermal differentiation occurred later and differentiation of dermis was early and vigorous. The two types of epidermal cells developed to a different degree in vitro. Percocytes and their derivatives at times developed precociously and reached full maturity, although their pattern of differentiation was asynchronous compared with that in ovo. Basal cells and their derivatives produced filaments precociously but mature keratinized cells were not found. Absence of keratohyaline granules in the epithelial cells appeared to be the initial defect in the keratinization process. Hyperplasia of mitochondria in basal and parabasal cells was also noted. The underlying mesenchyme, which in ovo begins to produce large amounts of collagen at an exponentially accelerating rate at 5 days, synthesized only scant collagen under the in vitro conditions employed. The hypothesis is presented that the mesenchyme alters epithelial differentiation by affecting keratohyaline synthesis which in turn controls keratinization.

INTRODUCTION

Using the light microscope epidermal 'keratinization' was found to be precocious (Fell & Mellanby, 1953; Litvac, 1939; McLoughlin, 1961a, b; Miszurski, 1937) and accentuated (Fell & Mellanby, 1953) in organ cultures of 5- to 8-day embryonic skin. The limit of resolution of the light microscope restricts the study of the process of keratin formation to some of its rather gross features. At the electron-microscopic level, in vitro differentiation of 12- to 13-day chick embryonic skin has been studied in organ culture (Fitton Jackson & Fell, 1963), but that of younger embryos has not been reported upon. Our research on this process revealed a peculiar epidermal cytoplasmic ultrastructure in chick embryonic skin explanted after 5 days' incubation. This finding gave impetus to report in detail the normal in ovo differentiation (Mottet & Jensen, 1968). This ultrastructure had apparently been observed before in the peridermal layer of mandibular epidermis (Fell, 1964). The ultrastructure referred to by us as corpuscula cribiformia (cc) was found to be a transient embryonic structure, disappearing as suddenly as it arose. It has subsequently been observed by others...
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(Parakkal & Matolsty, 1968). It occurs precociously in vitro and serves as an additional marker for the process of differentiation.

The present line of investigation was undertaken to establish the specific ultrastructural features of embryonic skin differentiation in vitro and to compare them with normal keratinizing and non-keratinizing epithelium. As is evident from the light-microscopic studies referred to above, the process varies depending on the methods employed. By selecting the in vitro environment one can alter the keratinizing process, which is as yet poorly understood. Under our in vitro conditions most aspects of normal cytodifferentiation occurred but some aspects of keratinization were markedly altered, an observation made at the ultrastructural level only. The appearance of flattened, deeply pink- or orange-stained cells on the surface of stratified epithelia as observed by light microscopy gives no significant information about the features of the keratinizing process or possible alterations.

Growing a piece of limb bud as an organ culture in many respects approximates natural conditions, since the epidermis and underlying mesenchyme are left undisturbed in close contact and large populations of cells maintain their intercellular relationships. This was a necessary prerequisite for evaluation of more complex in vitro changes such as replacing the mesenchyme with connective tissue from another source of the same animal after removal of epidermis by trypsinization. Studies of this nature have been performed at the light-microscopic level (McLoughlin, 1961a, b; Wessels, 1962) but one can expect to obtain further knowledge of the regulatory effects of mesenchyme on epithelial differentiation at the ultrastructural level. In conventional hanging drop cultures of chick embryonic skin, epidermal cysts commonly form (Fell & Mellanby, 1953; Litvac, 1939; McLoughlin, 1961a, b; Miszurski, 1937) and their mode of formation has been described (Fell & Mellanby, 1953; McLoughlin, 1961a, b; Miszurski, 1937; Strangeways & Fell, 1926). The pattern of growth will be illustrated in this report by photographs of the living cultures.

MATERIALS AND METHODS

Fertilized eggs from an inbred strain of white leghorn chickens (Heisdorf & Nelson) were incubated at 37.5 °C and the embryos harvested at 5 days, stage 26 or 27 (Hamburger & Hamilton, 1951). After removing and discarding the apical epidermal ridge the left hind limb bud of the embryos was cut into 5 pieces of approximately equal size. Each piece was grown in a medium consisting of 4 drops of chicken plasma and 2 drops of chick embryo extract. Freshly prepared chicken plasma and embryo (Parker, 1961) and a desiccated commercial product (Difco) were used without appreciably different results. The pH of the culture medium was maintained at 7.4 with a 95 % O₂, 5 % CO₂ gas mixture. On a 4-cm square glass slide the 2 ingredients were mixed as one drop and an explant was placed in its centre. After coagulation the clot was inverted and sealed with paraffin on to a depression slide. The explants were excised from their clots with large parts of their fibroblastic outgrowths and placed in fresh medium on the second day of growth in vitro; from then on transfer to a new medium was done every 24 h. This was necessary to avoid severe pH changes in the medium after the third day of growth in vitro.

After 1-14 days of growth in vitro the explants were fixed for electron microscopy. Pieces of 5-day hind limb buds were fixed concomitantly. The fixative used was 1 % OsO₄ in double strength Tyrode solution (Fitton Jackson, 1956) at pH 7.2 and a milliosmolarity of 600 to 620. After fixation for 1 h at 0-4 °C, the tissues were dehydrated in an ethanol series at 0-4 °C, taken
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through propylene oxide at 25 °C and embedded in Epon epoxy resin at 25 °C (Luft, 1961). Sections 1–2 μm thick were cut with glass knives at 20-μm intervals, stained with toluidine blue (Trump, Smuckler & Benditt, 1961) and examined in the light microscope to select a representative area for thin sectioning. Thin sections were cut with a diamond knife on an LKB ultramicrotome and mounted unsupported on copper grids. The sections were stained for 2 h with 2 % uranyl acetate (Watson, 1958) and subsequently for 2 min with lead tartrate (Millonig, 1961). The double-stained sections were examined in RCA EMU-3G electron microscopes.

Five explants of 1 day of growth in vitro, 6 of 2 days growth, 4 of 3 days, 6 of 4 days, 3 of 7 days, 2 of 8 days, 2 of 9 days and 6 of 14 days of growth in vitro were examined. Thirty specimens of 5-day skin from the hind limb bud were also studied. A control series of embryos consisted of 66 specimens of skin and subjacent tissue removed from the lateral thigh region from 5 days of incubation to hatching. Details of these observations have been reported separately (Mottet & Jensen, 1968).

RESULTS

Slight fibroblastic outgrowth emerged from the cut surfaces at the end of the first 24-h period in vitro. The plasma clot adjacent to the epithelial surface was devoid of fibroblasts (Fig. 1). Between day 2 and day 4 the epithelial outgrowth overlayed the lateral fibroblastic outgrowth. The epithelium growing along the surface continued to evert and subsequently a cyst of epithelium was created with the basal cells located peripherally (Fig. 2). Therefore, the centre of the epidermal cyst was free of fibroblastic cell outgrowth. When the epithelium was situated at or near the surface of the clot it was not directed upward by the fibroblastic outgrowth and maintained its original configuration. Occasionally a ‘snout’ covered with epithelium formed in the enlarging explant (Fig. 3). After 4–5 days in vitro the original explant comprised one-fourth to one-sixth of the surface area of the explant (Fig. 4), but the entire explant had become flattened during this time. After day 10 the epithelium was identifiable by its pearly-grey opaque appearance. Explants derived from the distal portion of the limb bud were indistinguishable from the proximal.

Five-day skin on day of explantation

The 2-cell layered epidermis was composed of cuboidal basal cells and a flattened superficial cell layer called the periderm (Krause, 1902). We refer to the cells of the latter as pericytes (Mottet & Jensen, 1968). The oval to slightly convoluted nuclei in the basal cells were located predominantly in the basal part of the cytoplasm with their long axes perpendicular to the skin surface. Their chromatin was finely granular and one nucleolus was present. The cytoplasm of the basal cells contained many free ribosomes. Granular reticulum in tubular profiles was quite sparse. A moderate number of elongated mitochondria with few mitochondrial granules was present throughout the cytoplasm. A few invaginations of the cytoplasmic membrane adjacent to the basal lamina were present and coated with electron-dense, fuzzy material. An occasional vesicle of a similar appearance and size was seen in the most basal part of the cytoplasm. No cytoplasmic filaments, junctional complexes on opposing or interdigitating cytoplasmic membranes adjacent to the basal lamina, or hemidesmosomes, were seen. The pericytes had a similar nuclear chromatin pattern and their elongated nuclei had their long axes horizontal to the epithelial surface. A moderate number of
microvilli was present on the free surface. The cytoplasm contained similar types and numbers of cytoplasmic organelles as did the basal cells, but no invaginations or vesicles coated with electron-dense, fuzzy material were present. The Golgi complex was not prominent. The cytoplasmic membranes of opposing pericytes showed an intertwining relationship, with an occasional short desmosome adjacent to the skin surface.

The continuous, relatively straight, basal lamina was 40 nm thick and followed the contour of the basal cell cytoplasmic membrane at a distance of 50–70 nm. Its fluffy appearance was quite homogeneous. The underlying mesenchyme was composed of cells with many slender and plump cytoplasmic processes. Their nuclear chromatin pattern was similar to that of the epidermal cells, but not uncommonly 2 nucleoli were present. The cytoplasm contained a marked number of free ribosomes; granular reticulum in tubular profiles was quite sparse. The Golgi complex was not prominent. A moderate number of mitochondria was present and these were similar in structure to those in the epidermis. Occasionally a small junction site (Greenlee & Ross, 1967) was found between 2 cells. Adjacent to the basal lamina and between mesenchymal cells the relatively narrow intercellular spaces in some specimens contained few fine collagen fibres, with the characteristic periodicity (Rhodin & Dalhamn, 1955; Wasserman, Roth & Minick, 1957). Mitotic figures were rarely seen in the epidermis or the underlying mesenchyme.

Five-day skin one day in vitro

The epidermis was 2 cell layers thick. Desmosomes, with no electron densities in the space between their attachment plaques and few fine filaments coursing into them, were present to a moderate degree between pericytes and between pericytes and intermediate cells, but were also occasionally seen between the basal cells. In the basal cells granular reticulum in tubular profiles had increased moderately, was devoid of ribosomes in many foci and contained finely granular, electron-dense material. Free ribosomes were less conspicuous. Intramitochondrial granules were increased in number. The Golgi complex was more prominent. Membrane-bound bodies 40 nm to 2 μm in diameter, containing amorphous material of varying density, were seen occasionally (Fig. 5). The invaginations and vesicles present in the distal part of the cells and coated with fuzzy, electron-dense material were more numerous. Fine filaments were present to a moderate degree in the cytoplasm adjacent to the basal lamina and coursed predominantly parallel to it. On the cytoplasmic membrane adjacent to the basal lamina electron-dense plaques 100–200 nm long and 25–40 nm tall were occasionally seen. The intermediate cells had prominent Golgi complexes and the same amounts of granular reticulum and free ribosomes as did basal cells. Their mitochondria, exhibiting more granules, were more numerous, but not to the same degree as in basal cells. The microvilli of pericytes were flattened in some cultures.

Five-day skin two days in vitro

The epidermis was 3 to 4 cell layers thick. Cytoplasmic processes and intercellular junction sites with desmosomal formations were increased in number. In all cell layers
fine lines of electron-dense material were present in the space between the attachment plaques of desmosomes and many fine filaments coursed into the latter (Odland, 1958). The quantity of fine filaments in the basal cell cytoplasm had increased (Fig. 6). In 2 of the 6 cultures several membrane-bound vacuoles containing myelin figures and indistinct irregular masses were seen in basal cells. In the same cultures pericytes were condensed and contained non-membrane-bound vacuoles up to 3.5 μm in diameter and of uniform, moderate electron density.

No vacuoles were evident in the mesenchymal cells in the two cultures that exhibited vacuoles in the epidermis.

**Five-day skin three days in vitro**

The epidermis was 4 cell layers thick and delicate, interdigitating cytoplasmic processes were numerous in all cell layers. In basal cells, granular reticulum in tubular profiles was increased and contained electron-dense, finely granular material. All epidermal cells, except pericytes, had prominent Golgi complexes.

Mesenchymal cells of the dermis had prominent Golgi complexes and an increased amount of granular reticulum in tubular profiles containing some electron-dense material. Invaginations coated with fuzzy, electron-dense material were conspicuous on the cytoplasmic membrane, as were intracytoplasmic vesicles of similar appearance and size. A slight amount of fine collagen fibres was present in the narrow intercellular spaces.

**Five-day skin four days in vitro**

Two of the 6 cultures showed the 4- to 5-cell-layered epidermis in cyst formation. In basal cells granular reticulum in tubular profiles predominated and in one of the epidermal cysts cisternal profiles were prominent. Both types of granular reticulum were distended with finely granular, electron-dense material (Fig. 7). Fine filaments were increased in the basal parts of most of the cells and coursed into the more prominent hemidesmosomes. The 2 cell layers above the basal cell layers had dilated profiles of granular reticulum containing granular, electron-dense material. Pericytes were seen only in the 2 cultures that had formed an epidermal cyst. Between the cells the cytoplasmic membranes showed a delicate intertwining pattern and contained up to 5 desmosomes in a row. Tight junctions (Farquhar & Palade, 1963) were not observed. These 2 cultures, from 2 different series of cultures, also exhibited the characteristic cells underlying the pericytes and referred to by us as subpericytes. In one culture a few small, well developed corpuscula cribiformia (cc) were observed in subpericytes containing nuclei. These cells did not contain prominent Golgi complex or granular reticulum and contained many free ribosomes and a moderate number of mitochondria. In the other culture the 3 cell layers located centrally contained cc (Fig. 8). In other areas of this culture pericytes contained no cc and the 3 or 4 cell layers basal to them contained cc in abundance. cc varied markedly in size even within the same cell and small, as well as larger, well developed ones up to 1.3 μm in diameter, were present. The anucleate cells containing these bodies had an electron-
transparent appearance with a decrease in free ribosomes. Intact mitochondria were quite prominent and strands of fine filaments coursed into desmosomes.

The basal lamina was moderately undulating, following the contour of the basal cells. It had increased in thickness, measuring 50–80 nm. The mesenchymal cells were closely approximated and junction sites were still seen (Fig. 9).

**Five-day skin, seven, eight, and nine days in culture**

Cultures grown for 7, 8, and 9 days showed a similar ultrastructure and each had formed an epidermal cyst. The epidermis was 5 cell layers thick and showed increased numbers of well developed desmosomes between the numerous cytoplasmic processes in all cell layers. Profiles of granular reticulum in basal cells were quite inconspicuous. Mitochondria with many granules had increased further in number and tended to occur in clusters (Fig. 10). Many smaller and larger bundles of filaments were present and often coursed adjacent to mitochondria (Fig. 11). The 2 cell layers overlying basal cells contained many bundles of filaments, of which they had more than the basal cells. Pericytes were present in all cultures. They contained similar amounts of cytoplasmic organelles as on the day of explantation. Their opposing cytoplasmic membranes were intricately intertwining, with many long desmosomes containing electron-dense material between their attachment plaques. No cc were observed.

The basal lamina was of varying density, tending to be denser opposite hemidesmosomes (Fig. 11). It undulated markedly. Fine collagen fibres were more abundant adjacent to the basal lamina than in the extracellular space between mesenchymal cells in general. Mesenchymal cells maintained a markedly prominent Golgi complex and the tubular profiles of granular reticulum contained a marked amount of electron-dense material. The narrow intercellular spaces contained a slight increase in fine collagen fibres.

**Five-day skin, fourteen days in culture**

Four of the 6 cultures had formed epidermal cysts and their walls were 15 to 20 cell layers thick. One of these (Fig. 12) had a particularly regular array of cell layers. In basal cells the invaginations and vesicles, lined by fuzzy, electron-dense material, were present to a moderate degree. The 2 cell layers superficial to the basal cells exhibited an increased number of larger and denser bundles of filaments distributed around nuclei and coursing into the increased number of desmosomes. The 4 cell layers superficial to them were anucleate, showed a gradual decrease of cytoplasmic organelles and became increasingly flattened toward the lumen of the cyst. Bundles of filaments were of increased density, at times becoming of homogeneous appearance, and tended to be located adjacent to the cytoplasmic membranes. The subsequent 5 to 6 anucleate cell layers were relatively electron-transparent, except for coarse, homogeneous strands of osmiophilic material, located adjacent to the cytoplasmic membranes (Fig. 13). In a rare situation, dense, irregular masses 160 nm to 1.3 μm in size were seen in the cell layer immediately below subpericytes (Fig. 13). Anucleate subpericytes contained cc. The cytoplasm of the pericytes was composed of lacy, finely homogeneous material throughout (Fig. 12), except adjacent to their plasma membranes.
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where the cytoplasm appeared granular, homogeneous and more electron-dense (Fig. 14). No intact desmosomes were seen between these cells. In the 3 other cultures with cyst formation some mitochondria in basal cells contained irregular, amorphous, electron-dense masses occasionally completely replacing the mitochondrial matrix. Other mitochondria showed a honeycomb pattern of cristae (Fig. 15). These 3 cultures did not show the above described gradual changes of cell-layer morphology; rather the cell layers were somewhat intermingled, so that a cell containing many cytoplasmic organelles and fine bundles of filaments could lie adjacent to an anucleate cell containing bundles and strands of osmiophilic material only. The subpericytes and pericytes could not be distinguished from each other. They formed smaller and larger conglomerations within the central part of the cyst wall. Cells with smaller cc, many free ribosomes and intact mitochondria would lie adjacent to cells containing larger cc in electron-transparent cytoplasm with few intact mitochondria and lacy strands of fibrils or adjacent to cells containing finely fibrillar, homogeneous material throughout. Occasionally bundles of electron-dense, elongated, fibre-like structures were located in the space between the opposing cytoplasmic membranes of these cells (Fig. 16). The central lumina of the epidermal cysts contained a variable amount of cells resembling pericytes and subpericytes (Fig. 12).

The two cultures that had not formed epidermal cysts but exhibited a ‘snout’ covered with epidermis (Fig. 3) had 4 basally located cell layers of epidermis that exhibited multiple membrane-bound vacuoles replacing a greater part of the cytoplasm. These cells had few, fine bundles of filaments, fewer mitochondria and no prominent Golgi complexes. No invaginations or vesicles coated with fuzzy, electron-dense material were seen in basal cells. Ten to 20 anuclear, flattened cell layers were located superficially and composed of intermingled, osmiophilic, dense strands as well as amorphous, irregular masses of dense material dispersed throughout the otherwise electron-transparent cells (Fig. 17). The cell membranes of these cells were markedly thickened and in an irregular manner; electron-dense material was present in small globular masses between opposing cytoplasmic membranes (Fig. 17). In these two cultures no pericytes or subpericytes were seen.

DISCUSSION

After any given period of growth, explants derived from the proximal part of the 5-day hind limb bud had a similar ultrastructure to those derived from the distal part. A comparison of the ultrastructural pattern of differentiation in vitro with that occurring in ovo from skin derived from the lateral mid-thigh (Mottet & Jensen, 1968) was therefore possible.

After several days' growth in hanging drop culture, epidermal cysts usually formed, an observation previously made by others (Fell & Mellanby, 1953; Litvac, 1939; McLoughlin, 1961 a,b; Miszurski, 1937). Better survival and more complete epidermal differentiation as judged by the light-microscopic appearance have been noted in such organ cultures (Fell & Mellanby, 1953; Miszurski, 1937; Weiss & James, 1955). In all cultures that had formed epidermal cysts, pericytes were present. In such an environ-
ment pericytes as well as basal cells and their derivatives seemed less exposed to
injury. Each of the two 14-day cultures that had not formed a cyst but exhibited a
'snout' covered with epidermis (Fig. 3) had severe vacuolization of the epidermis,
indicative of cellular degeneration. A few filaments had formed in the 3 cell layers
superficial to the basal cells, suggesting retarded differentiation. These 2 cultures were
recognized as having formed a 'snout' from their gross appearance and were selected
to determine the state of survival of epidermis when a cyst had not formed. Pericytes
were absent in the cultures that had not formed cysts after 4 to 14 days of growth in
vitro, an observation in accord with the study of in vitro differentiation of 12- to 13-
day-old chick embryonic skin, where pericytes were assumed to have been sloughed
after 2 days in vitro (Fitzon Jackson & Fell, 1963).

Five-day-old embryo tissue was used for explantation. At this stage the basal cells
and pericytes of the epidermis were immature and appeared very much alike in ultra-
structure. Equally important is the fact that the underlying dermal mesenchymal cells
were about to begin collagen synthesis (Mottet, 1967). Evidence has been presented
that collagen synthesis by the dermal mesenchyme influences epithelial differentiation
(Wessels, 1962; Dodson, 1967), though the exact mechanism remains to be defined.

After one day's growth in organ culture fine filaments were present in basal cell
cytoplasm adjacent to the basal lamina and small dense plaques on the cytoplasmic
membrane of the basal cells probably represented early hemidesmosomal formation;
the Golgi complex and granular reticulum had become more prominent, resembling
epithelium at 12 days in ovo (Mottet & Jensen, 1968) and presenting an appearance
consistent with light microscopy (McLoughlin, 1961a, b). The Golgi complex and the
granular reticulum became prominent in cell layers that subsequently contained
bundles of filaments. The initial sudden appearance of filaments in the cells coincided
with the disappearance of most of the material inside the cisternal and tubular profiles
of granular reticulum and occurred after 7 days in vitro. Similar ultrastructural
sequences were found in epidermis of skin in ovo on day 15 and 16. The epidermal
cells under our in vitro conditions start to form keratin precursors precociously
between the day of explantation and 7 days in vitro based on age in days after the
beginning of embryonic development (herein referred to as chronological age).

After 2 days in vitro the desmosomes between all cell layers appeared quite mature,
with linear densities in the space between their attachment plaques (Odland, 1958;
Farquhar & Palade, 1963). In ovo desmosomes increased in number first in relation
to pericytes and were well developed at these sites on day 15, whereas they were not
well developed in basal cells until day 17 (Mottet & Jensen, 1968). Therefore desmo-
somes matured at a much earlier age in vitro than they did in ovo.

Differentiation of cells derived from basal cells was less synchronized in vitro com-
pared with in ovo since keratinizing cells could lie next to cells that were much less
differentiated. The mature keratinized cells appearing in ovo at day 18 basal to sub-
pericytes in the form of several layers of anucleate, flattened, dense, homogeneous,
osmiophilic cells with thickened cytoplasmic membranes, were not observed after 14
days in vitro (a chronological age of 19 days). The cell layers immediately below the
subpericytes (Fig. 13) contained homogeneous, fibrillar material and thickened, cyto-
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plasmic membranes but had a loose general texture resembling the cells located below the fully keratinized cells of day 18 in ovo. A cell layer, referred to as 'moth-eaten', has been observed just basal to the fully keratinized cell layers during normal epidermal differentiation (Zelickson & Hartman, 1961). Ultrastructurally these 'moth-eaten' cells resemble the keratinizing cells of our study. Cells of a similar nature were seen in numerous layers in the two cultures with no cysts after 14 days in vitro (Fig. 17). Therefore, the completely keratinized cells did not develop in vitro, a conclusion also made from the electron-microscopic study of 13-day skin differentiating in vitro (Fitton Jackson & Fell, 1963). Keratohyaline bodies (Farbman, 1966) seen in ovo at day 16 and 17 when filaments were increasing in number were not observed at a comparable stage of differentiation on day 7, 8 or 9 in vitro. From our study in ovo the disappearance of keratohyaline bodies and the loss of distinctness of the bundles of filaments occurring concomitant with the appearance of dense, structureless, osmio-philic material suggested that the keratohyaline granules were incorporated into a product of keratinization (Brody, 1960; Farbman, 1966; Zelickson, 1961). We believe the faulty keratinization that occurs in vitro to result from the failure to form keratohyaline bodies. Foetal and adult skin from several species of mammals contained keratohyaline bodies in vivo but had none when grown in vitro (Pullar, 1964). The descriptions and illustrations of 12- to 13-day-old chick skin grown in vitro (Fitton Jackson & Fell, 1963) would indicate that the ultrastructural appearances of the keratinizing cells in their study are very similar to ours. Precocious loss of pericytes and imbibition of fluid were felt to have played a role (Fitton Jackson & Fell, 1963) but in our study the 'moth-eaten' appearance of the keratinizing cells was also evident when pericytes were present and an epidermal cyst had formed.

The precursor of subpericytes, the cell type destined to form cc, seen in 13-day-old skin in ovo, was not identifiable in these cultures. cc were present in pericytes and/or subpericytes in the 4-day cultures and the 14-day cultures exhibiting cysts, but for unknown reasons they were not seen in pericytes or subpericytes of the 7 cultures of 7, 8 and 9 days' growth that all had formation of cysts. In most cultures, after 14 days' growth, pericytes and subpericytes could not be distinguished from each other. Some contained cc only, whereas others contained cc as well as fine, fibrillar material (Fig. 16). Thus, even though these cells reached a level of maturation similar to those in ovo, their pattern of differentiation was asynchronous. In the 4 cultures that exhibited cysts after 14 days in vitro, many pericytes and subpericytes were swollen and resembled those of day 19 in ovo, their chronological age. Others had sloughed and were found in the central lumina of the cysts (Fig. 12).

In all cultures with epidermal cysts at day 14 in vitro small invaginations and vesicles coated with fuzzy, electron-dense material in the basal cells adjacent to the basement membrane were present. Other investigators (Bennett, 1963; Bennett & Luft, 1959; Nix, Nordquist & Everett, 1965; Odland, 1958) have interpreted similar ultrastructures as possible indicators of micropinocytosis. Hyperplasia of mitochondria in basal cells developed gradually and became conspicuous after 7 days of growth in vitro in cell layers superficial to basal cells. The cells therefore adapted by increasing the number of mitochondria (Fawcett, 1966a; Novikoff, 1961). The number
of mitochondria of voluntary striated muscle has been found to increase when the metabolic rate was increased by giving euthyroid. Occasionally, the pattern of mitochondrial cristae had changed in our explants and sharp angulations of adjacent cristae produced a honeycombed appearance. The latter morphology has been observed in tissues with markedly active metabolism (Fawcett, 1966), another indication that the \textit{in vitro} conditions may have increased metabolic needs over those \textit{in ovo}. Mitochondrial granules increased in number concomitantly with the increase in numbers of mitochondria. Evidence suggests these granules to be associated with the regulation and transport of ions (Peachey, 1964; Weiss, 1955).

The basal lamina increased in thickness \textit{in vitro}. At day 7 it was undulating to a marked degree, following the contour of basal cells, showing areas of increased thickness opposite hemidesmosomes. Since such an appearance was seen in the basal lamina of day 21 \textit{in ovo}, it may be stated that it matured precociously \textit{in vitro}. The presence of a well developed basal lamina in association with mature epidermis and immature mesenchyme is interesting, since other investigators indicate that epithelium plays a major role in the formation of the basal lamina (Hay & Revel, 1963; Pierce, Midgley & Sri Ram, 1963).

The very cellular and usually collagen-free mesenchyme underlying the epidermis on the day of explantation formed scant collagen fibres during the 14 days of growth \textit{in vitro}. The fibroblasts had a prominent Golgi complex and a moderate amount of material in the increasing number of tubular and cisternal profiles of granular reticulum after 3 days' growth \textit{in vitro}. Such an ultrastructural appearance suggests a secretory activity in a precursor stage of collagen synthesis (Ross & Benditt, 1963). However, a few collagen fibres were seen in intercellular spaces. Most collagen fibres were found adjacent to the basal lamina, as has been noted in other studies (Wessels, 1962). After 3 days \textit{in vitro} the fibroblasts also showed many invaginations and vesicles coated with fuzzy material, ultrastructures interpreted as indicating micro-pinocytosis and seen in other differentiating fibroblasts (Han, Avery & Hale, 1965). During the interval between 5 and 12 days \textit{in ovo}, the dermis becomes less cellular, with markedly increased amounts of collagen fibres in enlarged intercellular spaces and between day 12 to 21, collagen continues to increase in the vascularized connective tissue. The appearance of the collagen fibres and their increase during development as revealed in electron micrographs, correlates well with the biochemical measurements of hydroxyproline synthesis (Mottet, 1967). In the whole embryo, as well as in limb buds alone (N. K. Mottet, unpublished observations), the onset of synthesis occurs at 5 days of incubation and increases exponentially thereafter until the thirteenth day of incubation. The slopes of the limb-bud growth (weight) curve and hydroxyproline curve are similar.

In view of the purported role of collagen in the differentiation of keratinizing epithelium, the observed paucity of collagen fibre formation and the defective keratohyaline formation in our cultures suggests that they may be causally related. If subsequent experiments prove the hypothesis to be true, then an ultrastructural approach to the elucidation of the mechanism of mesenchymal-epithelial interactions in the selection of pathways of epithelial differentiation would be possible.
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REFERENCES


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Fig. 1. Living explant, 2 days *in vitro*. The very thin epithelium (e) covers a larger cell mass or mesenchyme (me). Fibroblastic outgrowth (fo) emerges from the cut surfaces of mesenchyme and is therefore not present adjacent to the surface of the epithelium. x 80.

Fig. 2. Living explant, 5 days *in vitro*. The epithelium (e) has formed a cyst and is surrounded by mesenchyme (me) on all sides. Into the cavity (ca) of the cyst, a small mass of mesenchyme is protruding. x 100.

Fig. 3. Living explant, 8 days *in vitro*. The mesenchyme (me) that is covered by epithelium (e) has the configuration of a 'snout'. x 125.

Fig. 4. Living explant, 7 days *in vitro*. The broken lines show the approximate surface area of the explant on the day of explantation. This part of the explant is partly covered by epithelium (e). The greater part of the explant is composed of mesenchyme (me) formed *in vitro*. Fibroblastic outgrowth (fo) is extensive and has formed a solid 'bridge' of mesenchyme (marked by an arrow) surrounding the epithelial covering. The space between the epithelium and the bridge of mesenchyme was eliminated by fibroblastic outgrowth after another day *in vitro*. x 13.
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Fig. 5. Five-day skin, 1 day in vitro. Membrane-bound bodies (ly) are present in a basal cell of the epithelium. A cluster of tubular profiles of granular reticulum (tp) is partially lined by ribosomes. The arrow points to some delicate filaments. The basal lamina (bl) is quite indistinct in this electron micrograph. Cytoplasmic processes of the underlying mesenchyme exhibit breakage of cytoplasmic membranes, evidence of suboptimal fixation. × 21,000.

Fig. 6. Five-day skin, 2 days in vitro. Part of the nucleus (n) of a basal cell of the epithelium is located adjacent to tubular profiles (tp) of granular reticulum, lined by many ribosomes. The arrow points to numerous delicate filaments in the cytoplasm, located in the vicinity of dense plaques (h) on the cytoplasmic membrane. The latter are interpreted as developing hemidesmosomes. The undulating basal lamina (bl) is distinct. × 31,000.
Skin differentiation in vitro
Fig. 7. Five-day skin, 4 days in vitro. The arrow points to the intercellular space between 2 basal cells of the epithelium. Mitochondria (m) are present in a cluster. Cisternal profiles (cp) of granular reticulum are prominent and are distended by finely granular, electron-dense material. The basal lamina (bl) is now of increased thickness. × 8000.

Fig. 8. Five-day skin, 4 days in vitro. The cavity (ca) of the epidermal cyst is surrounded by pericytes with many microvilli (mv). Corpuscula orbiforma (cc) of varying size are present in pericytes, subpericytes and underlying cell layers. Desmosomes (d) are well developed. Intact mitochondria (m) are seen in the cells and an arrow points to a strand of fine filaments. × 9500.
Skin differentiation in vitro
Fig. 9. Five-day skin, 4 days in vitro. The arrow points to a junction site between 2 of the mesenchymal cells, underlying the epithelium. Tubular profiles (tp) and cisternal profiles (cp) of granular reticulum are prominent and contain finely granular, electron-dense material. Collagen fibres (cf) are delicate and scanty in the narrow intracellular spaces between the cells. × 10 500.

Fig. 10. Five-day skin, 8 days in vitro. The nucleus (n) of a basal cell of the epithelium has been displaced centrally by a large cluster of mitochondria (m). Intramitochondrial granules are prominent. A bundle of filaments (f) is located adjacent to the nucleus. Desmosomes (d) between basal cells show delicate lines of electron density between their attachment plaques. The basal lamina (b) is of varying electron density. × 18 000.

Fig. 11. Five-day skin, 7 days in vitro. A basal cell of the epithelium contains a cluster of mitochondria with many intramitochondrial granules. Bundles of filaments (f) are coursing between and around the mitochondria. Many hemidesmosomes (h) are present on the cytoplasmic membrane. The basal lamina tends to be more electron-dense when located opposite a hemidesmosome. The arrow points to a cytoplasmic process of a mesenchymal cell. × 23 500.
Fig. 12. Five-day skin, 14 days in vitro. The dermo-epidermal junction is denoted by de. Clusters of mitochondria (m) are prominent in basal cells of the epithelium. Nuclei (n) of epithelial cells are surrounded by bundles of filaments. Bundles of filaments (f) are bigger in the more centrally located, anucleate cells. The arrow points to a 'moth-eaten' cell layer. Corpuscula cribiformia are composed of lacy, homogeneous material. The cavity of the cyst (ca) contains a sloughed pericyte-subpericyte-type cell (p) x 4000.
Skin differentiation in vitro
Fig. 13. Five-day skin, 14 days in vitro. Bundles of filaments (f) are located adjacent to the thickened cytoplasmic membranes. The arrow points to dense, homogeneous material in a 'moth-eaten' cell. Irregular dense bodies (db) are present in the cell layer below subpericytes. Subpericytes contain corpuscula cribriforma (cc). × 17000.

Fig. 14. Five-day skin, 14 days in vitro. The arrow points to the intertwining intercellular space between pericyte-subpericyte-type cells (p). No intact desmosomes are seen. Adjacent to the cytoplasmic membranes, the cytoplasm is granular and more dense, in contrast to the central lacy, homogeneous portion of the cells. × 27000.

Fig. 15. Five-day skin, 14 days in vitro. The hemidesmosomes (h) on a basal cell of the epithelium are prominent. The mitochondrion designated 1 contains several amorphous masses of moderate electron density. The mitochondrion designated 2 shows a honeycomb pattern of cristae. The cell peripheral to the basal cell contains an amorphous electron-dense mass (3), interpreted as part of a degenerated mitochondrion. The undulating basal lamina (bl) is of varying electron density. × 21000.
Fig. 16. Five-day skin, 14 days in vitro. All cells shown are of the pericyte-subpericyte variety. Some cells contain granular material only, whereas others also contain corpuscula cribriformia (cc). The arrows point to peculiar fibre-like material of marked electron density seen in the intercellular spaces. $\times 19000$.

Fig. 17. Five-day skin, 14 days in vitro. All cell layers are composed of 'moth-eaten' cells. Dense material (dm) has accumulated adjacent to the thickened cytoplasmic membranes. Intact desmosomes (d) are seen between some of the cells. The arrow points to a globular homogeneous mass located between opposing cytoplasmic membranes. $\times 18500$. 
Skin differentiation in vitro