Fine structure of the apical rim–mesenchyme complex during limb morphogenesis in man

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SUMMARY

Ultrastructure of the apical rim–basal lamina–mesenchyme complex in man during Horizons 15–18 is correlated with the development of limbs.

1. Apical epithelium is a complicated tissue exhibiting surface microvilli, junctional complexes, free ribosomes, elaborate Golgi centers, glycogen and scanty granular endoplasmic reticulum.

2. The apical rim thickens during stages 15 and 16, remaining multilayered at digital tips and thinning adjacent to zones of necrosis by stages 17 and 18. Epithelial thinning may be a response to lack of ‘maintenance factor’.

3. A continuous basal lamina is present during these stages. Collagen-like fibrils are numerous in interdigital zones of necrosis and sparse at tips of digital blastemata.

4. The presence of oriented microfilaments in interdigital epithelium suggests active invagination during morphogenesis of fingers in man.

INTRODUCTION

Epithelial–mesenchymal interaction plays an integral role in the morphogenesis and regeneration of vertebrate limbs (reviews by Amprino, 1965; Hay, 1965; Milaire, 1965; Saunders & Gasseling, 1968; Faber, 1971) but remains a controversial and complex problem involving cell surfaces, extracellular matrices (e.g. basal lamina and collagen), and the physiology of inducing and reacting cells. Kölliker (1879) and Balfour (1885) were among the first to characterize a thickening of epiblast in the limb-buds of amniote embryos, but it remained for later investigators to demonstrate that an apical epidermal ridge (A.E.R.) was essential to normal limb development (Saunders, 1948). Not only does the A.E.R. ‘induce’ mesenchymal cytodifferentiation, but it may regulate proliferative (Camossi, Jacobelli & Pappalettera, 1960; Janners & Searls, 1970; Hornbruch & Wolpert, 1970) and necrotic (Saunders, Gasseling & Saunders, 1962) patterns in mesenchyme as well. In addition, Zwilling (1956) has demonstrated that a mesenchymal factor must exist for the maintenance of the A.E.R. (a ‘mesodermal maintenance factor’). Hence it is clear that vertebrate
limb development is dependent on mutual interaction between the apical epithelium and underlying mesenchyme.

Investigators are usually reserved in attempting to apply experimental results from one animal group to another, and since variation does exist (e.g. presence of an A.E.R. in amphibians is controversial; Dober & Tschumi, 1969; Tarin & Sturdee, 1971) this reservation seems merited. To study limb morphogenesis in man then, it is necessary to demonstrate structural features characteristic of other experimental animals (namely an apical rim–basal lamina–mesenchyme complex; Kelley, 1970).

Bardeen & Lewis (1901) described a thickened epithelium at the free edge of the human lower limb-bud, and Steiner (1929) illustrated an ‘ectodermal cap’ in the upper limb of a 9 mm embryo. More recently, O’Rahilly, Gardner & Gray (1956) reported the location, time of first appearance and temporal relationship of apical ectoderm to structural differentiation in limbs in a survey of collections at the Carnegie Institution of Washington. An ectodermal thickening was present on the ventro-lateral border of upper limbs by Horizon 12, forming an ectodermal ridge at Horizon 14 and disappearing during Horizon 17. Similar events in lower limbs were delayed to the next respective stage. Whether or not this A.E.R. is functionally similar to that studied thoroughly in the chick and mouse remains to be investigated; however, it is clear that an apical ridge exists in man.

Ultrastructural features of the epithelium–basal lamina–mesenchyme complex in mouse and chick limb-buds have been reported by Jurand (1965), and Hay (1965) reviewed the fine structure of such cell interaction during chick organogenesis. Surprisingly little attention has been directed to the fine structural differentiation of apical epithelium during limb morphogenesis concomitant with (a) developmental time and (b) association with adjacent digital and interdigital mesenchyme. Assuming that morphogenetic processes are reflected at the ultrastructural level, what submicroscopic changes occur in apical epithelium during stages 14–18 in man? Are epithelial cells structurally different over digital and interdigital blastemata? Is the basal lamina uniform but discontinuous (Jurand, 1965), or does it vary in composition, retaining continuity beneath the epithelial layer? Techniques of electron microscopy were chosen to investigate these questions in the human embryo.

MATERIALS AND METHODS

Limb-buds, hand and foot plates for this investigation were dissected from human embryos following therapeutic interruption of pregnancy. Developmental stage was determined by matching structures with corresponding developmental horizons of Streeter (1948, 1951) and descriptions of O’Rahilly et al. (1956). The terms ‘Horizon’ and ‘stage’ will be synonyms in this report. Sequential specimens from stages 15–18 were studied (approximately the late
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Fig. 1. Light micrograph of the epithelio-mesenchymal interface (Horizons 15–16). × 850.

Fig. 2. Light micrograph of thickened epithelium adjacent to digital tip during Horizons 17–18. × 850.

Fig. 3. Light micrograph of thinned epithelium adjacent to interdigital zone of necrosis during Horizons 17–18. × 850.

fifth to seventh postfertilization weeks), attempts to recover embryos in stage 14 being unsuccessful. Material was immersed in a number of fixatives (phosphate-buffered osmium tetroxide, veronal-acetate-buffered osmium tetroxide, phosphate-buffered formaldehyde, phosphate-buffered glutaraldehyde, cacodylate-buffered glutaraldehyde and veronal-acetate-buffered glutaraldehyde). Best preservation was observed in specimens immersed in 3% glutaraldehyde in 0.15 M cacodylate/HCl buffer (pH 7.3) at 4 °C for 4 h, postfixed in ferrocyanide-reduced 2% osmium tetroxide in cacodylate buffer (Karnovsky, 1971) at 4 °C for 2 h, rapidly dehydrated through an ethanol series to propylene oxide and embedded in Epon 812. Sections 1 μm thick were mounted on glass slides and stained with toluidine blue for light-microscopic examination. Thin sections (mounted on uncoated grids) were stained for 30 min in either saturated aqueous or ethanolic uranyl acetate at 35 °C, for 5 min in alkaline lead citrate at room temperature and examined in an Hitachi HU-11 C electron microscope.
OBSERVATIONS

Light microscopy

Horizons 15–16

Examinations of frontal sections through limb-buds with the light microscope reveal a uniform two-layered epithelium 30–40 μm in thickness (Fig. 1). Apical cells (periderm; Krause, 1902) are flattened and nuclei are fusiform. Basal cells (stratum germinativum; Krause, 1902) are cuboidal to low columnar in shape, nuclei are spherical to ovoid and extracellular spaces are apparent. Mitotic figures are numerous and germinative layer cytoplasm does not reveal specialization (e.g. glycogen bodies). Mesenchymal cells are uniformly distributed beneath a basement lamina.

Horizons 17–18

Digital zone. Frontal sections through hand and foot plates demonstrate epithelia of varying thicknesses. Epithelia adjacent to tips of digital blastemata (Fig. 2) possess squamous, superficial cells with features similar to earlier stages. However, the stratum germinativum exhibits a multilayered appearance, the entire epithelium being 45–55 μm in average thickness. Cells in the stratum germinativum are cuboidal to low columnar and those adjacent to the apical layer have no apparent attachment to the basement lamina. Nuclei are spherical to ovoid, extracellular spaces are evident, and numerous dense bodies are prominent near basal surfaces. Mitotic figures are present in both superficial and deep layers, and numerous mesenchymal cells abut the basal lamina.

Interdigital zone. Whereas digital epithelium is stratified and thickened, a bilaminar pattern is resumed adjacent to interdigital zones of necrosis (Fig. 3). Apical cells remain squamous and few are mitotic. Basal cells are cuboidal to squamous and extracellular spaces are diminished. Epithelial thickness is 15–20 μm. Macrophages are present in centers of mesenchymal necrosis beneath the basement lamina.

Figures 4–8

Fig. 4. Apical epithelium during Horizons 15–16. Note flattened apical cells and low columnar basal layer. Arrows indicate microvilli projecting into amniotic cavity from the periderm. bl, Basal lamina; g, Golgi center; m, mitochondrion; n, nucleus; nu, nucleolus; ger, granular endoplasmic reticulum. × 6000.

Fig. 5. Developing epithelial junctional complex (arrows) during Horizon 15. × 39000.

Fig. 6. Multilayered basal lamina during Horizons 15 and 16. × 43000.

Fig. 7. Apical epithelium–basal lamina–mesenchyme complex during Horizons 15–16. bl, Basal lamina; m, mitochondrion; n, nucleus; ger, granular endoplasmic reticulum; pm, plasma membrane. × 25000.

Fig. 8. High magnification of epithelio-mesenchymal interface. Brackets denote lamina densa. Note electron-dense, filamentous zone in epithelial cytoplasm (ec) adjacent to cell membrane. mc, Mesenchymal cytoplasm. × 80000.
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Electron microscopy

Horizons 15–16

Late in the fifth postfertilization week, superficial cells (Fig. 4) exhibit numerous apical microvilli which project into the amniotic space. These processes vary in length from 0.2 to 0.5 µm and possess an average diameter of 0.2 µm. Some microvilli appear to fold back to the cell surface, their distal extreme fusing with the plasma membrane (arrows, Fig. 4). Junctional complexes (Farquhar & Palade, 1963) develop between cells although differentiation is incomplete at this stage (Fig. 5). Nuclei are ovoid to fusiform, possess prominent nucleoli, and exhibit sparse condensed chromatin (heterochromatin). A particular cytoplasm contains free ribosomes, few mitochondria, scanty endoplasmic reticulum (ER) and few desmosomes at points of contact with basal cells.

Nuclei in the stratum germinativum possess a single nucleus and abundant dispersed chromatin (euchromatin). Discrete segments of granular ER and numerous free ribosomes are present in the cytoplasm. Golgi centers are located in the apical cytoplasm, mitochondria are sparse and small condensations of glycogen are visible in basal regions of the cell. Extracellular spaces are prominent, containing particulate and fibrillar material. Furthermore, a thin (0.1 µm) but continuous basement lamina separates epithelial from mesenchymal cells (Figs. 4, 6, 7). During Horizons 15 and 16 the lamina is multilayered in some areas (Fig. 6) but generally consists of a single dense inner zone (a lamina densa, approximately 0.05 µm in thickness) which grades into less dense regions exhibiting particles and fine fibrils. Plaques of filamentous material are frequently visible immediately adjacent to the basal lamina (Figs. 7, 8) but are absent in corresponding areas of subjacent mesenchyme.

Horizons 17 and 18

Digital zone. Major ultrastructural features of epithelial cells are unchanged by stages 17 and 18 although the germinative zone becomes stratified (Fig. 9). Junctional complexes (consisting of zonula occludens, zonula adherens and macula adherens) are differentiated between surface cells (Fig. 10) and, in addition, glycogen bodies become prevalent (Fig. 11). Apical Golgi centers exhibit

Figures 9–12

Fig. 9. Stratified squamous epithelium adjacent to digital blastemata during Horizons 17–18. bl, Basal lamina; m, mitochondrion; mv, microvilli; n, nucleus; ger, granular endoplasmic reticulum. × 5000.

Fig. 10. Differentiated junctional complex at apical epithelial surface during Horizons 17–18. × 14000.

Fig. 11. Profile of cytoplasmic glycogen body (gb) in basal cell. × 14000.

Fig. 12. Golgi centers (g) in apical cytoplasm during Horizons 17–18. Note numerous (secretory?) vesicles (v). n, Nucleus; ne, nuclear envelope; pm, plasma membrane. × 50000.
Fig. 13. Apical epithelium midway between digital tip and interdigital zone of necrosis. Note dense bodies (db), lipid droplets (l), nucleus exhibiting karyorrhexis (k), and numerous vesicles (v) in otherwise 'normal' cytoplasm (c). × 10000.
flattened cisternae and numerous, membrane-bound (secretory?) vesicles (Fig. 12). Isolated necrotic elements in the epithelial interzone between digital tip and centers of necrosis appear to be engulfed by adjacent cells in the stratum germinativum. The cell illustrated in Fig. 13 contains a normal, euchromatic nucleus with perinuclear features not unlike adjacent profiles. However, numerous dense bodies, vacuoles, lipid droplets and a nucleus exhibiting karyorrhexis are present in the cytoplasm towards the center of the illustration. Extracellular spaces remain prominent and the basal lamina is unchanged from previous description.

**Interdigital zone.** Epithelium adjacent to interdigital zones of necrosis resumes a double-layered appearance, individual cells exhibiting different cytoplasmic density following postfixation and staining (Fig. 14). Apical cell nuclei exhibit areas of diffuse and condensed chromatin whereas basal cell nuclei retain a predominance of euchromatin. Dense bodies are present in cells in both layers, and basal cells possess numerous lipid droplets. Extracellular spaces are diminished and cellular borders become interdigitated. In contrast to digital epithelium, cells in the developing interdigital layer exhibit bundles of oriented microfilaments along apical surfaces associated with chains of desmosomes (maculae adherens) (Fig. 15). Dense aggregates of microfilaments also form perpendicular abutments with the basal cell membrane (Fig. 16).

Fig. 17 illustrates the epithelial–mesenchymal border over an interdigital necrotic center. The basal lamina is thrown into a series of folds and collagen-like fibrils are prevalent. These fibrils lack the 64 nm periodicity of mature collagen (Fig. 18). In addition, glycogen abuts filamentous plaques on the cytoplasmic surface of basal cells adjacent to the basement lamina (Fig. 17, higher magnification Fig. 19).

**DISCUSSION**

Present observations reveal that apical epithelium in man is a complicated tissue with numerous subcellular systems: surface microvilli, junctional complexes, free ribosomes, elaborate Golgi centers, and a basal lamina which varies in composition between digital and interdigital zones. Numerous investigators (see review by Milaire, 1965) have demonstrated that the mammalian ectodermal ridge organizes only the distal limb segment. Hence, it seems necessary to correlate, if possible, epithelial ultrastructure with development of digital blastemata and interdigital spaces.

Diffuse chromatin, numerous free ribosomes and prominent Golgi centers are structural features associated with active protein synthesis and cytodifferentiation. These features are not appreciably altered in thickened epithelium during Horizons 15–18 in man. It is of interest, however, to note that epithelial cells which are closely associated with adjacent mesenchyme (either digital or interdigital) during stages 15–16 exhibit fibrillar zones (Figs. 7, 8). Since this structure is present in later stages at epithelio-mesenchymal
interfaces and zones of necrosis (Figs. 17, 19) one might anticipate a potential role either in formation of basal lamina or elaboration of material specific to mesenchymal morphogenesis in that region.

The presence of thinned interdigital epithelium exhibiting dense (lytic?) bodies and a thickened, multilayered digital zone corresponds, respectively, to subjacent mesenchymal necrosis (macrophagy) and proliferation in the digital tip (Kelley, 1970). Saunders & Gasseling (1968) reported a similar thinning of epithelium after grafting mesoderm of the posterior necrotic zone beneath the A.E.R. of a host chick wing-bud. They interpreted the thinned epithelium as a response to lack of ‘mesodermal maintenance factor’. Similar events in man suggest that maintenance activity is present at digital tips but absent in interdigital zones of necrosis. A principal ultrastructural correlate in these zones is the notable difference in collagen-like fibrils in the basal lamina.

Balinsky (1957), demonstrating induction of extra limbs by foreign tissue grafts to urodelan Wolffian crests, found that the basement ‘membrane’ in that area was very thin or absent. Reuss & Saunders (1965) suggested, utilizing similar experiments in the chick, that interruption of the ectodermal basal lamina was essential for supernumerary limb induction. This hypothesis gained support from the observations of Jurand (1965), who reported discontinuities in the basal lamina immediately below the median zone of the apical ridge during normal chick development. However, similar interruptions were absent in the mouse embryo. Current observations reveal that man, during normal limb development, exhibits an intact basement lamina (between stages 15 and 18 inclusive). It is difficult to believe, however, that such an extracellular layer could be an effective barrier to transfer of informational macromolecules. Since numerous experiments demonstrating the role of surface-associated, extracellular matrix components in cytodifferentiation have been reported, it is not unreasonable to suggest that visible or detectable differences in the basal lamina (either structural or chemical) might have morphogenetic importance. The presence of quantities of collagen-like fibrils in centers of necrosis (Fig. 17, interdigital zones) contrasts with the absence of such material subjacent to epithelium at developing digital tips. At present, it is uncertain which cell layer contributes to the fibrils visible at interdigital tips in man, although collagen

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**Figures 14–16**

Fig. 14. Thinned epithelium adjacent to interdigital zone of necrosis. Note dense bodies (db), lipid droplet (l), and light and dark cytoplasm in adjacent cells. × 11000.

Fig. 15. Profile of junction between adjacent peridermal cells. Note maculae adherens (arrows) and associated microfilaments (mf) in perpendicular orientation to amniotic surface. × 51000.

Fig. 16. Microfilaments in basal cell cytoplasm in perpendicular orientation to basal lamina (not illustrated). × 55000.
associated with epithelium during epithelio-mesenchymal interactions can be derived from both cellular participants (Hay & Revel, 1963; Kallman & Grobstein, 1965). Unfortunately, the role of the basement lamina during normal limb development remains unclear. It is interesting to speculate that an increase in collagen-like fibrils is associated with increased cell death, decreased maintenance activity and associated epithelial thinning.

As interdigital necrosis continues, the thinned overlying epithelium assumes an increasingly deep position (relative to digital tip outgrowth) so that by stage 19 and later, well-defined fingers are developed. Are these morphogenetic movements passive (i.e. the epithelium sagging into a cell-deficient space) or an active process of invagination? Gustafson & Wolpert (1963) have hypothesized that invagination of an epithelial layer might result from existence of greater adhesiveness between apical than basal portions of cells in that layer. Presence of numerous maculae adherens near apical junctions in interdigital epithelium may be morphological evidence of increased appositional adhesiveness (Fig. 15). Furthermore, cytoplasmic microfilaments have recently been implicated as contractile organelles responsible for changes in cell shape and intercellular organization during embryonic epithelial morphogenesis (Wessells et al. 1971). Fig. 15 demonstrates numerous cytoplasmic microfilaments oriented along the apical surface of interdigital epithelium. Basal surfaces exhibit dense bundles of microfilaments perpendicular to the basement lamina (Fig. 16). These structural features support the hypothesis that interdigital epithelium may actively invaginate into zones of necrosis. Experimental induction of syndactyly with agents which disrupt contractile filaments (e.g. cytochalasin B, the Vinca alkaloids) is in progress to provide further information on limb morphogenesis in man.

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**Figures 17-19**

Fig. 17. Portions of epithelium (above) and macrophage (below) in interdigital zone. Note collagen-like filaments (arrows) and convoluted nature of basal lamina (bl). $\times 9000$.

Fig. 18. High magnification of collagen-like fibrils. Note absence of 64 nm periodicity characteristic of native collagen. $\times 100000$.

Fig. 19. Filamentous plaque of cytoplasmic surface of interdigital basal epithelial cell. bl, Basement lamina; pm, plasma membrane. $\times 100000$. 

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