Fine structure of the regressing interdigital membranes during the formation of the digits of the chick embryo leg bud

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SUMMARY

There is recent evidence showing that in addition to the well-known mesenchymal necrotic mechanism involved in the disappearance of the interdigital membranes, the ectodermal tissue may also play an active role in the formation of the free digits of most vertebrates. Ultrastructural study of the regressing interdigital membrane of the chick leg revealed significant changes at the epitheliomesenchymal interface. Disruptions of the ectodermal basal lamina and an intense deposition of collagenous material were the most conspicuous changes observed in the extracellular matrix. In addition the basal ectodermal cells showed prominent cell processes projected into the mesenchymal core of the membrane, and mesenchymal macrophages appeared to migrate through the epithelial tissue to be detached into the amniotic sac. It is concluded from our results that the elimination of the interdigital membranes is a complex process requiring the interaction of all the tissue components of the membrane.

INTRODUCTION

The development of the digits of most vertebrates takes place by their detachment from an initial hand or foot plate. On the basis of many descriptive and experimental studies this process is usually considered as a classic case of controlled cell death shaping morphogenesis (Saunders & Fallon, 1967; Ballard & Holt, 1968; Pautou, 1974, 1975, 1976; Fallon & Cameron, 1977; Hinchliffe, 1981). There are however studies showing that the interdigital necrosis (interdigital necrotic zones, INZ) is accompanied by conspicuous changes in the shape and structure of the ectodermal cells. On the basis of the ultrastructural features of the interdigital ectoderm of human embryos, Kelley (1973) suggested that the ectoderm may actively invaginate into the necrotic zones contributing to the detachment of the free digits. In a recent SEM study we have observed that the interdigital ectoderm of the chick differs significantly from that of the duck (Hurle & Colvec, 1982), supporting the hypothesis of such an ectodermal involvement in the detachment of the digits. Furthermore a significant rupture of

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the interdigital membrane, the ectodermal tissue being detached into the amniotic sac, was also observed in that study. These facts suggest that in the same way that distal outgrowth of the limb is carried out by a permissive interaction between apical ectodermal ridge (AER) and the marginal mesenchyme, the reabsorption of the interdigital tissue may be due to a change or a cessation in the interaction between the interdigital mesenchyme and the ectoderm, resulting in the cessation of growth and disruption of the interdigital membranes.

Epitheliomesenchymal interaction is a basic embryological mechanism accounting for the morphogenesis of many organs (Grobstein, 1967). The two tissues are separated by an interface of extracellular material which is a critical element in the interaction process (Hay, 1978, 1981). To summarize briefly, the characteristics of these extracellular matrix components can modify the differentiation of the epithelium and the behaviour of the underlying mesenchymal tissue.

The importance of the epitheliomesenchymal interface in relation to the growth of the limb buds has motivated several studies of the ectoderm–basal lamina–mesenchyme complex in different vertebrates (Jurand, 1965; Kelley, 1973, 1975; Kelley & Bluemink, 1974; Ede, Bellairs & Bancroft, 1974; Smith, Searls & Hilfer, 1975; Kaprio, 1977). However, most of these studies have focused on the early growing stages of limb organogenesis, while the structure of the epitheliomesenchymal interface of the regressing interdigital spaces has received little attention (Kelley, 1973).

In view of the reported information and on the basis of recent studies showing that modifications of the basal lamina of regressing epithelial structures might play a role in the disappearance of the tissue (Trelstad, Hayashi, Hayasi & Donahoe, 1982), we have undertaken a detailed ultrastructural study of the regressing interdigital membranes of the chick leg bud. Our results reveal significant changes in the epitheliomesenchymal interface during degeneration of the interdigital membranes. Evidence of an active involvement of the ectoderm in the detachment of the digits and the existence of a transepithelial migration process of mesenchymal macrophages were also observed.

**MATERIALS AND METHODS**

The third interdigital membrane (IM) of the leg bud of white Leghorn chick embryos was chosen for this study due to the high intensity and long duration of the degenerative process present during the regression of this membrane.

Thirty-six embryos ranging from 7 to 11 days of incubation were sacrificed at 12 h intervals. The leg buds were fixed in 2.5% glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.2) for 4 h, washed in buffer solution in which the third IM was carefully microdissected and postfixed in 1% osmium tetroxide. In some specimens 0.1% ruthenium red was added to the fixatives to stain the extracellular material according to the Luft (1971) procedure. The selected fragments
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were then dehydrated in a graded series of acetones and propylene oxide and embedded in Araldite. To ensure that the IM would be sectioned longitudinally, the fragments were embedded in flat capsules and carefully oriented under the binocular dissecting microscope. Serial semithin section were cut with a LKB ultratome III and stained with 1% toluidine blue. Ultrathin sections of selected areas were then made, mounted on uncoated copper grids, stained with uranyl acetate and lead citrate and examined with a Zeiss EM 109 electron microscope.

RESULTS

During the whole period studied, the interdigital membranes (IM) are relatively simple structures consisting of a core of mesenchymal cells covered by the ectoderm.

**Day 7-7-5**

The mesenchymal core of the IM shows a central loose zone rich in blood vessels and a more condensed region located peripherally under the ectodermal jacket. Abundant dying cells and macrophages are observed in the proximal region of the IM (Fig. 1). No dying cells are present in the distal zone of the IM. The extracellular space appears very poor in electron-dense components. Only small patches of amorphous material and occasional unbanded fibrils which are positive for ruthenium red are observed (Fig. 2). The epithelial layer of the ventral and dorsal surfaces shows a basal layer of cuboidal cells covered by flattened peridermal cells rich in microfilaments. At the margin of the IM the AER is observed (Fig. 1). The presence of gap junctions is a characteristic feature of the AER at this stage.

In the epitheliomesenchymal interspace a basal lamina, amorphous material and collagen fibrils are observed (Figs 3, 4). Ruthenium red revealed a conspicuous periodic staining of the externa and interna lamina rara of the basal lamina (Fig. 4). Under the basal lamina, amorphous material and fibrillar ruthenium red-positive material are abundant (Fig. 4). Crossbanded collagen fibrils arranged along the major axis of the IM are also observed. Cytoplasmic cell processes of the mesenchymal cells often traverse the sublaminar space establishing contacts with the basal lamina.

**Day 8**

The mesenchymal core of the IM shows a very prominent necrotic focus located in the marginal zone of the membrane (Fig. 5). Abundant isolated dying cells and some macrophages are present within this focus. In contrast with this marginal necrotic zone the proximal zone of the IM also shows a necrotic area but large macrophages are the most prominent features while isolated dead cells are scarce. As in previous stages, electron-dense extracellular materials are
scarce within the mesenchymal core of the IM, although they can be enhanced by ruthenium red staining (Fig. 6).

The epithelial layer of the IM shows a structural pattern similar to that observed at day 7–7·5. The only changes worth mentioning are present in the AER. At this zone the ectodermal cells lose the elongated shape of the preceding stages, taking on a rather rounded appearance. Gap junctions are now unusual and numerous dead epithelial cells are observed.

The epitheliomesenchymal interface is also similar to that observed at day 7–7·5 but discontinuities of the basal lamina are occasionally observed in the AER.

**Days 8·5–10**

In this stage the IM shows a very different structure from that observed in previous stages. The changes are present both in the mesenchymal and in the epithelial tissues and especially in the epithelial–mesenchymal interface. The mesenchymal tissue appears very condensed and consists mainly of healthy mesenchymal cells and large macrophages (Fig. 7). However, isolated dying cells and cell debris are also present. The morphology of the healthy mesenchymal cells is similar to that of the earlier stages but they now can show cytoplasmic invaginations filled with collagen fibrils (Fig. 8). All the degenerating cells, including macrophages, tend to be located in the proximity of the ectoderm, establishing in many instances close contacts with the ectodermal cells. We shall describe these features later. The extracellular matrix is now extraordinarily rich in crossbanded or faint-banded collagen fibrils which are preferentially located

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**Fig. 1.** Longitudinal semithin section of the IM at day 7·5. The ectodermal tissue still displays the AER (arrows). The necrotic area (n) is only present in the proximal zone of the membrane (n). Scale bar = 0·1 mm.

**Fig. 2.** 7·5-day IM stained with ruthenium red showing a dense staining of the basal lamina and few ruthenium red-positive particles in the extracellular space. Note the stellate shape of the mesenchymal cells with cell processes establishing contact with the ectodermal basal lamina. Scale bar = 5 μm.

**Fig. 3.** Electron micrograph of the epitheliomesenchymal interface of the dorsal face of a 7·5-day IM illustrating the continuous basement membrane along the basal surface of the ectoderm. Scale bar = 1 μm.

**Fig. 4.** Detailed view of the basal membrane of a 7-day IM after ruthenium red staining. Ruthenium red granules are attached to the externa and interna lamina rara of the basal lamina. Note ruthenium red granules attached to the sublaminar fibrillar material (arrows). Scale bar = 0·5 μm.

**Fig. 5.** Longitudinal semithin section of a 8-day IM. A prominent necrotic focus is present in the mesenchyme of the marginal zone of the membrane. The AER appears flattened displaying degenerating cells. Scale bar = 75 μm.

**Fig. 6.** Mesenchymal tissue of a 8-day IM after ruthenium red staining. All the cellular components, including dead cells, display a prominent ruthenium red-positive (coat). The extracellular matrix only shows occasional scattered ruthenium red-positive components. Scale bar = 2 μm.
in the most marginal zone of the IM (Fig. 9). They are grouped in large clumps located between the mesenchymal cells and in the epitheliomesenchymal interface.

The epithelial tissue of the IM in this stage shows clear regional differences in structure. In the ventral and dorsal faces of the IM the peridermal cells take on a rounded appearance and display abundant microvilli. They are joined to each other and to the underlying basal cells by desmosomes. Bundles of microfilaments are abundant within the cytoplasm. The ectodermal basal cells are low cuboidal or rather flattened and show large bundles of microfilaments parallel to the basal surface of the cells. In some instances dark condensations located at various intervals are present along a bundle of microfilaments. Solitary cilia projecting into the intercellular space are often observed. Cell junctions between these basal cells are only present at the basal zone of the lateral cell profiles and consist of maculae adherentes. Large macrophages are often observed within the epithelial cells. In some cases they are located between the basal ectodermal cells. In these cases the macrophages are separated from the underlying mesenchymal tissue by zones of junction between basal cell processes of the ectodermal cells (Fig. 10). Clumps of extracellular material including collagen fibrils are often located in the space between the macrophages and the ectodermal cells (Fig. 10). In other cases the macrophages are located between the peridermal cells and the basal ectodermal cells. In these cases the peridermal cells display cell processes which surround the surface of the macrophage (Fig. 11). We have never observed cell junctions between macrophages and ectodermal or peridermal cells.

In the marginal zone of the IM the AER is no longer observed. As can be seen in Fig. 7, the epithelium of this zone is a multilayered structure. It has a layer of basal ectodermal cells surrounded by a prominent ridge of peridermal cells. The basal cells differ from those of the dorsal and ventral faces of the IM in that they are more irregularly shaped. Flattened, rounded and cylindrical cells poor in cell junctions are present. Within the cytoplasm, the most prominent feature is the
presence of cell invaginations or vesicles filled with collagen fibrils. These vesicles are more often observed at the basal pole of the cells (Fig. 12), but in some instances cisternae showing a collagen fibril are observed at the apical pole of the cells. The presence of ruthenium red staining in some of these vesicles suggests that, at least in part, they are invaginations of the cell membrane. The peridermal cells form multilayered cords protruding towards the amniotic sac. They are joined by desmosomes. In some instances the presence of constrictions within the cords suggests that clumps of peridermal cells are detached into the amniotic sac. Large macrophages are often observed in the core of the peridermal cords (Fig. 7). The peridermal cells form a continuous sheet around these macrophages but cell junctions between the macrophages and the peridermal cells were never observed. Most of the peridermal cells show a healthy appearance but in some cases they show prominent vacuolization and degenerated organelles, although the cell nucleus retains a normal morphology. Gap junctions which are prominent features of the AER were never observed in the marginal epithelium at this stage.

The most characteristic feature of the epitheliomesenchymal interface is the presence of discontinuities in the basal lamina. This feature is especially prominent in the marginal zone of the epithelium. In this zone the ectodermal cells are underlain by a wide space rich in collagen fibrils, clumps of amorphous basal lamina-like material and cell detritus (Fig. 7). Small extracellular-matrix vesicles 0.01–0.03 μm in diameter are also abundant in this space (Fig. 12). Both in the marginal and dorsoventral zones of the IM, the ectodermal cells show abundant cell processes projecting into the mesenchymal tissue (Figs 13, 14). These processes originated most often at the edges of the basal surface of the ectodermal cells but they can be present in any zone of the basal surface. Contacts between these processes and the underlying mesenchymal cells and necrotic cell fragments (Fig. 13) are often observed. The pattern of ruthenium red staining showed conspicuous changes at these zones. The basal lamina appears irregularly stained. Patches of ruthenium red-positive material alternate with zones lacking stain (Fig. 15). Macrophages are frequently found in association with the basal surface of the ectodermal cells. Very often small cell processes of the ectodermal cells establish contacts with macrophages in zones in which the basal lamina shows discontinuities. In some instances a large segment of a macrophage appears in clefts of the ectodermal layer (Figs 16, 17). In these cases the ectodermal cells usually show cell processes rich in microfilaments apposed to the segment of the macrophage located under the epithelial tissue (Fig. 17).

**Day 10.5–11**

In this stage a progressive transformation of the regressing IM into the definitive interdigital tissue is observed. Fig. 18 shows the appearance of the IM at day 10.5. The necrotic zone of the mesenchymal tissue is restricted to the tip of the remaining IM. Some very large macrophages are observed while isolated
dead cells are exceptional. A prominent clump of collagen fibrils can still be seen under the marginal epithelium. The number of macrophages located within the epithelial tissue is significantly reduced from that of previous stages. Detaching peridermal cell cords are still prominent at the margin of the membrane. However, macrophages located within the cords are scarce or absent. The basal lamina tends to be continuous. At day 11 the IM reaches a rather stable appearance. In
the mesenchymal tissue degenerating cells are no longer present. Between the cells, scattered crossbanded collagen fibrils are observed. A prominent clump of collagenous material is still present near the free margin of the membrane. However, the size of the collagenous clump is significantly reduced with respect to that of previous stages. The epithelial tissue shows a basal layer of columnar cells covered by flattened peridermal cells. Occasional gap junctions are again observed between the basal cells of the free margin of the IM. As can be seen in Fig. 19, peridermal cell cords which now show a striking ballooning appearance are still observed at the marginal zone of the membrane. No macrophages are present within the peridermal cords at this stage. The epithelial–mesenchymal interface consists of continuous basal lamina underlain by occasional crossbanded collagen fibrils.

DISCUSSION

Our results show that the regression of the chick IM involves a wide range of structural changes affecting both the ectodermal and the mesenchymal tissue. As reported in many previous studies, the most remarkable feature of the regressing mesenchymal tissue is the establishment of a prominent necrotic focus. It has also been pointed out that two distinct components, proximal and distal (marginal) can be distinguished within the chick INZ (Pautou, 1975). Our observations show that the marginal part of the INZ appears simultaneously with AER degeneration, suggesting a causal relationship between the two processes.

The extracellular matrix of the mesenchymal tissue of the IM also shows dramatic changes during the degeneration process. Until day 8 of incubation the morphological characteristics and the pattern of ruthenium red-staining of the extracellular matrix are similar to those reported in other stages of limb development (Kelley, 1975; Sawyer, 1982). At day 8-5, however, an enormous accumulation of collagen fibrils is observed. A similar process has been reported in the

Fig. 16. Large mesenchymal macrophage showing a segment located between the basal ectodermal cells (e). Arrows show the limits of the ectodermal cleft. Note the abundance of collagenous material in the intercellular space. 8-5-day IM. Scale bar = 2 μm.

Fig. 17. Electron micrograph of the ectodermal layer of a 9-day IM showing a large macrophage in course of being incorporated into the epithelium. Arrow shows the presence of a ectodermal cell process contacting the subepithelial segment of the macrophages. Scale bar = 2 μm.

Fig. 18. Semithin section of a 10-5-day IM. Note the prominent peridermal marginal ridge and the abundance of extracellular matrix component in the subepithelial zone. Arrows show a large macrophage located within the epithelial tissue. Scale bar = 20 μm.

Fig. 19. Semithin section of the marginal zone of a 11-day IM showing the presence of prominent peridermal cell cords protruding towards the amniotic sac. Scale bar = 30 μm.
regressing IM of human embryos (Kelley, 1973). Both the origin and the significance of these collagen deposits are intriguing questions unsolved in our study. Neither the structure of the mesenchymal cells which are poor in rough endoplasmic reticulum, nor that of the ectodermal cells, support the possibility of an intense secretion process accounting for such a quick appearance of large clumps of collagen. In consequence, the only possibility which could be suggested is that the origin of collagen may be due to the polymerization of procollagen molecules.

The possible involvement of the ectodermal tissue in the regression of the IM has received little attention (Kelley, 1973; Hurle & Colvee, 1982). The first morphological change in the ectodermal tissue observed in this study was the degeneration of the AER. The basal ectodermal cells of the ridge degenerate and gap junctions which are a distinctive feature of the AER (Fallon & Kelley, 1977) disappear. By day 8-5 the basal cells of the marginal epithelium flatten and the peridermal cells of the IM become rounded. At the same time a prominent ridge consisting mainly of peridermal cell cords appears at the marginal zone of the regressing membrane. The change of shape of the peridermal cells and the abundance of oriented microfilament bundles within these cells have been interpreted as suggestive of an active invagination of the ectodermal epithelium during the morphogenesis of the digits (Kelley, 1973; Hurle & Colvee, 1982). Our observations do not lead us to discard such a possibility, however alternative explanations can be proposed. It has been observed that the growth patterns of the epithelial and mesenchymal components of the developing limb bud differ significantly. While the mesenchymal tissue grows by proliferation or directed outwards migration of the marginal tissue due to the inductive effect of the AER (Reiter & Solursch, 1982), the epithelial tissue, especially the peridermal component, undergoes a proximodistal sliding process (Roncali, 1973; Milaire, 1973). Based on these facts it can be suggested that in the regressing IM the disappearance of the mesenchymal tissue is followed by a process of sloughing off of the ectodermal cover at the margin of the membrane. In this respect it could be suggested that the AER, in addition to the inductive effect on the underlying mesenchymal tissue, might also play a stabilizing role, synchronizing the proximodistal sliding process of the limb ectoderm with the distal outgrowth of the mesenchyme.

There are several reports suggesting that the cessation of limb-bud outgrowth involves changes in the epithelial–mesenchymal interface (Jurand, 1965; Kelley, 1973, 1977). Alterations in the epitheliomesenchymal interspace were also reported in chick mutants with defective limb-bud development (Sawyer, 1982). In our study we have observed that the epithelial and mesenchymal modifications present in the regressing IM are correlated with conspicuous changes in the epitheliomesenchymal interface. As reported in the IM of the human embryo (Kelley, 1973, 1977), we found that the amount of collagen increases under the marginal ectoderm; but in contrast with the human embryo,
this was accompanied by prominent interruptions of the basal lamina. It is difficult from our study to ascertain the cause of the disruption of the basal lamina. However, the presence of matrix vesicles in zones of basal lamina interruption is a significant feature. During the development of the tooth organ, the basal lamina of the inner enamel epithelium appears to be degraded by the liberation of proteolytic enzymes from morphologically similar matrix vesicles formed by budding from the plasma membrane of the preodontoblast cell processes (see Slavkin, Trump, Brownell & Sorgente, 1977). If we compare our observations with those obtained during tooth organ development, it can be suggested that the disruption of the basal lamina of the IM could be related to the presence of the matrix vesicles. These vesicles presumably originate from the disintegration of the dying mesenchymal cells.

An interesting question in the present study is the significance of the ectodermal cell processes which pass across basal lamina discontinuities to establish contacts with the underlying mesenchymal components. Several cases of epithelial–mesenchymal interaction involve epitheliomesenchymal cell contact, which is assumed to play a role in inductive process (see Saxen, Ekblom & Thesleff, 1980). However, since the IM is in fact a regressing structure, the changes at the epitheliomesenchymal interface may well be a consequence of the controlled regression rather than reflecting active tissue inductive interaction. This consideration does not discount the possibility that such modifications might result in a change in the shape of the IM. The rupture of the basal lamina plus possible compositional changes in the subepithelial matrix might well explain the presence of ectodermal cell processes projecting into the mesenchymal space (see Hay, 1982). On the other hand, it appears that most embryonic cells in the presence of necrotic cells develop a phagocytic activity, surrounding such degenerating cells by means of cytoplasmic processes and eventually internalising them (see Hurle, Lafarga & Ojeda, 1978). The presence of contacts between ectodermal cell processes and underlying cell fragments might well indicate a phagocytic stimulus. Epithelial cell processes passing through disruptions of the basal lamina and participating in the phagocytosis of extracellular fragments have been reported during the formation of the chick lens primordium (Garcia-Porrero, Collado & Ojeda, 1979). However, we found no evidence that the contacts between the epithelial cell processes and the dead cells result in the engulfment of these cells.

The association of large macrophages with the ectodermal tissue is a striking feature which merits detailed discussion. Macrophages were observed in the following positions: i) under the ectodermal layer establishing close contacts with the ectodermal cell processes; ii) located partly between the basal ectodermal cells and partly in the mesenchymal space; iii) between the basal ectodermal cells; iv) between the basal ectodermal cells and the peridermal cells; v) within the peridermal cell cords of the marginal epithelium of the IM. In many cases evidences of a process of detachment of these cords into the amniotic sac were
observed. Cell junctions between the epithelial cells and the macrophages were never observed, nor was there any evidence of an intense phagocytic activity of the epithelial cells supporting the possibility of a transformation of the epithelial cells into macrophages. All these facts suggest the existence of a migration process of the mesenchymal macrophages through the epithelial tissue being finally detached into the amniotic sac along with the epithelial cells.

In conclusion, the present observations show that the disappearance of the chick IM involves major alteration of the ectodermal–basal lamina–mesenchyme complex. Due to the descriptive nature of our observations, the possible cause–effect relationships between the changes observed in different components of the IM cannot be established, nor can their precise role in the elimination of the IM be determined. However, it seems clear from the present study that cell death is only one of the factors involved in the detachment of the chick digits.

Our thanks are due to Mrs Pilar Elena-Sinobas for her excellent assistance in making the electron microscopic sections. One of us (M. A. F.-T.) wishes to acknowledge receipt of a grant ‘Para la formación de personal investigador’ of the Spanish Ministry of Education and Science.

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(Accepted 18 July 1983)