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Fine structure of the interdigital membranes during the morphogenesis of the digits of the webbed foot of the duck embryo

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SUMMARY

Fine structural study of interdigital membranes during formation of the digits of the duck foot reveals that the interdigital necrosis is accompanied by a high deposition of collagenous material in the epithelio–mesenchymal interface, rupture of the basal lamina and detachment of ectodermal cells into the amniotic sac. These changes are similar to those observed in the regressing interdigital membrane of the chick although their intensity and temporal extension are less pronounced in the duck. It is suggested that these changes account for the disappearance of the marginal zone of the duck interdigital membranes. The possible causal relationship between the different structural changes are discussed.

INTRODUCTION

The digits of most vertebrates develop by detaching from an initial hand or foot plate. The mechanism of digit detachment has received considerable attention from embryologists and interdigital cell death (interdigital necrotic zones, INZ) has been widely accepted as the main factor involved in the process (Saunders & Fallon, 1967; Pautou, 1974, 1975; Hinchliffe, 1982). The production of soft tissue syndactyly in chicks with genetic or drug-induced inhibition of INZ has been considered as an experimental proof to that hypothesis (Menkes & Deleanu, 1964; Hinchliffe & Thorogood, 1974; Pautou, 1976). However, there is now an increasing amount of information suggesting that the interdigital ectodermal tissue might also play a significant role in digit detachment (Kelley, 1973; Hurle & Colvee, 1982). We have recently studied the structure of the regressing interdigital membranes of the chick foot and our results suggest that the mechanism of membrane regression might involve the interaction of the mesenchymal and epithelial components (Hurle & Fernandez-Teran, 1983). In that study we found three main morphological features in the regressing interdigital membranes which may be involved in the tissue disappearance: i) mesenchymal cell death; ii) deposition of collagenous material in the epithelial–mesenchymal interface accompanied by rupture of the ectodermal basal lamina;

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and iii) detachment of the ectodermal tissue into the amniotic sac. However, the possible correlation among these tissue changes and their contribution to the elimination of the membranes remain unclear.

Comparative analysis of the development of animals having free digits with those having webbed digits is one of the tools which has been employed for study the role of cell death in the development of the free digits (Saunders & Fallon, 1967; Pautou, 1974; Fallon & Cameron, 1977). From these studies it has been observed that the INZ in the duck have a reduced extension but there is little information about possible changes in the epithelial tissue (Hurle & Colvee, 1982) and the epithelio–mesenchymal interface.

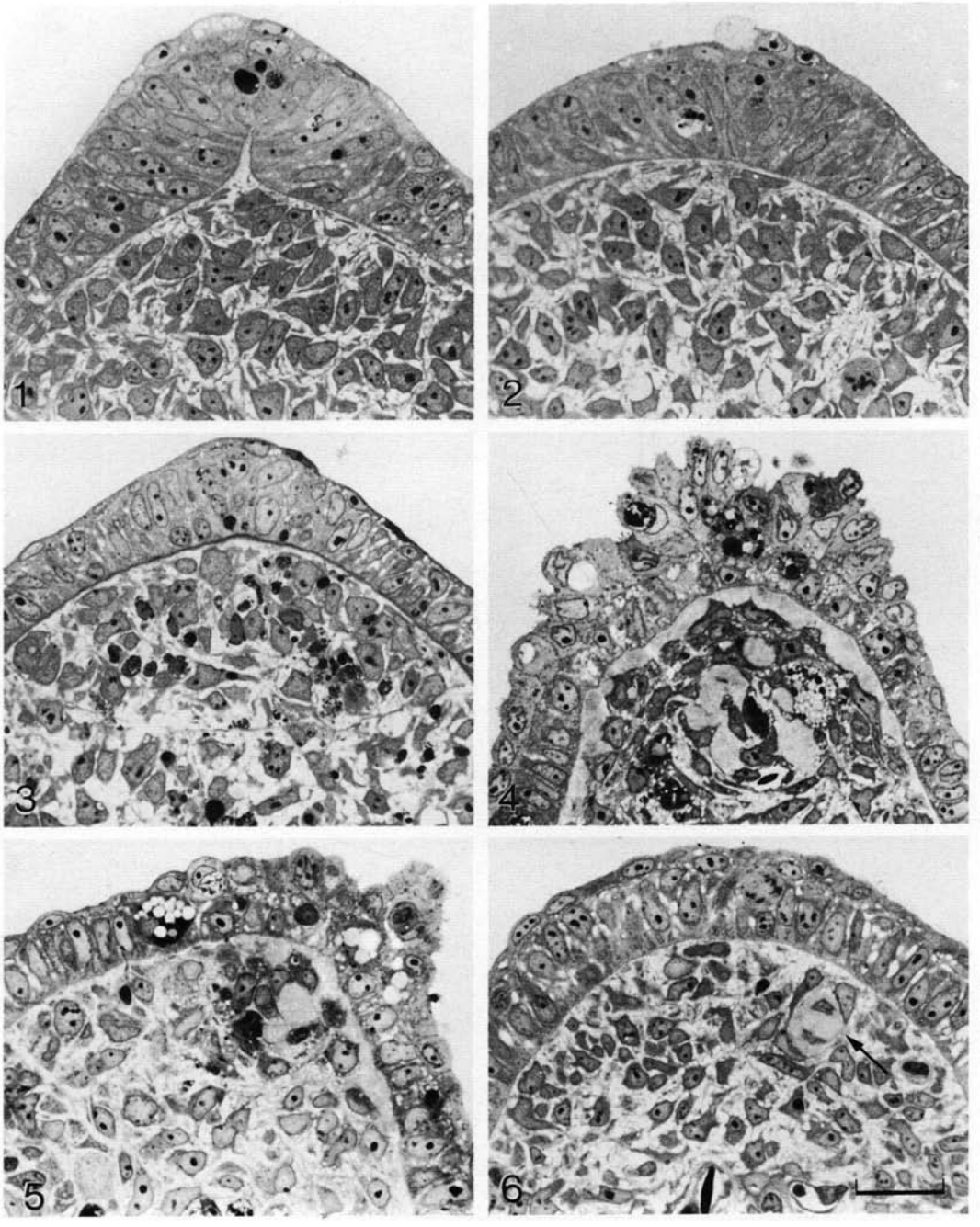
In the present study we have undertaken an ultrastructural examination of the interdigital membranes of the developing foot of the duck in order to analyse possible changes in the epithelial, mesenchymal and extracellular matrix components of the interdigital membranes during the development of the digits.

MATERIALS AND METHODS

Royal Pekin duck embryos ranging from 8·5 to 12 days of incubation were employed for this study. The embryos were sacrificed at 12 h intervals and the leg buds were fixed in 2·5 % glutaraldehyde in 0·1 M-cacodylate buffer (pH 7·2). After 4 h of fixation the leg buds were rinsed in buffer alone and the interdigital membranes were microdissected and postfixed in 1 % osmium tetroxide. The specimens 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 sections 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 10 electron microscope.

RESULTS

As observed in the chick embryo (Hurle & Fernandez-Teran, 1983) the interdigital membranes of the developing duck are simple structures. They consist of a core of mesenchymal tissue covered by the ectoderm. Significant structural changes were observed in the tissue components of the three interdigital membranes (IM). The changes were similar in the different membranes although some minor quantitative and temporal differences can be established among them. In the following description we shall describe only the development of the third IM but the results can be extended to the remaining membranes. Figs 1–6 show semithin sections of the IM at the different stages. As can be seen in these figures the changes were restricted to the marginal (distal) zone of the membrane. In all the stages studied the dorsal and ventral ectoderm consisted of a



Figs 1–6. Longitudinal semithin sections of the third interdigital membrane at days 8·5 (1), 9 (2), 9·5 (3), 10·5 (4), 11 (5) and 12 (6). The AER becomes progressively flattened from day 8·5 (1) to 9·5 (3). At day 10·5–11 (4–5) the ectoderm of the margin of the membrane forms a prominent ridge of detaching peridermal cells. At day 12 (6) the marginal ectoderm achieves a double layered appearance. Note the abundance of extracellular matrix in the epithelio–mesenchymal interface of the margin of the membrane at days 10·5 and 11 (4, 5). At day 12 (6) a prominent clump of extracellular matrix is still observed (arrow). Magnification bar = 25 μm.

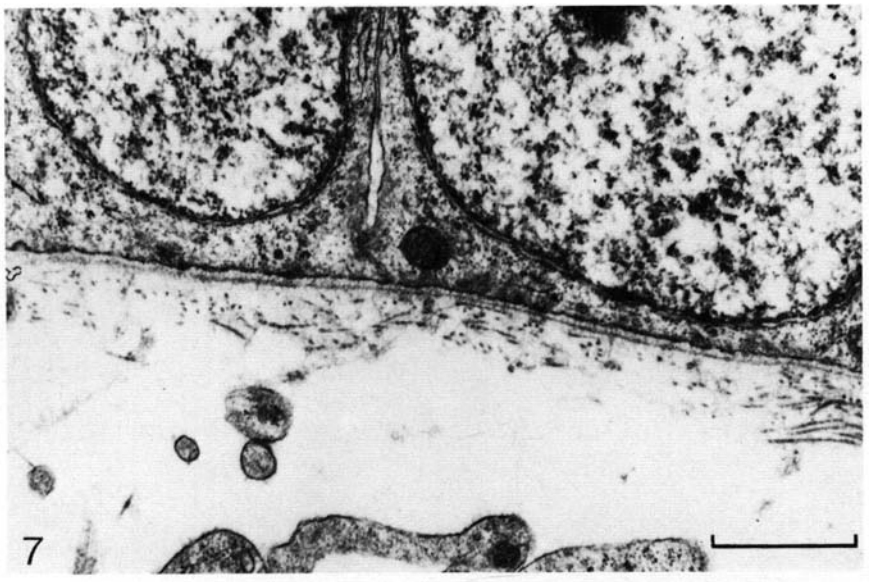


Fig. 7. Electron micrograph of the epithelio–mesenchymal interface of the dorsal surface of a 10-day IM. A continuous basal lamina and sublaminar matrix rich in collagen fibrils is present. Magnification bar = 1 μm.

double-layered epithelium underlain by a basal membrane. As shown in Fig. 7 the basal membrane contained a continuous basal lamina and sublaminar matrix rich in crossbanded collagen fibrils. The proximal zone of the mesenchymal core of the membrane also showed a stable morphology with stellate mesenchymal cells and abundant blood vessels. In contrast with the proximal zone of the

Fig. 8. Electron micrograph of the epithelio–mesenchymal interface at the AER of a 8·5-day IM. A basal lamina and abundant sublaminar matrix are present. Arrows show gap junctions between the basal cells of the ridge. Magnification bar = 1 μm.

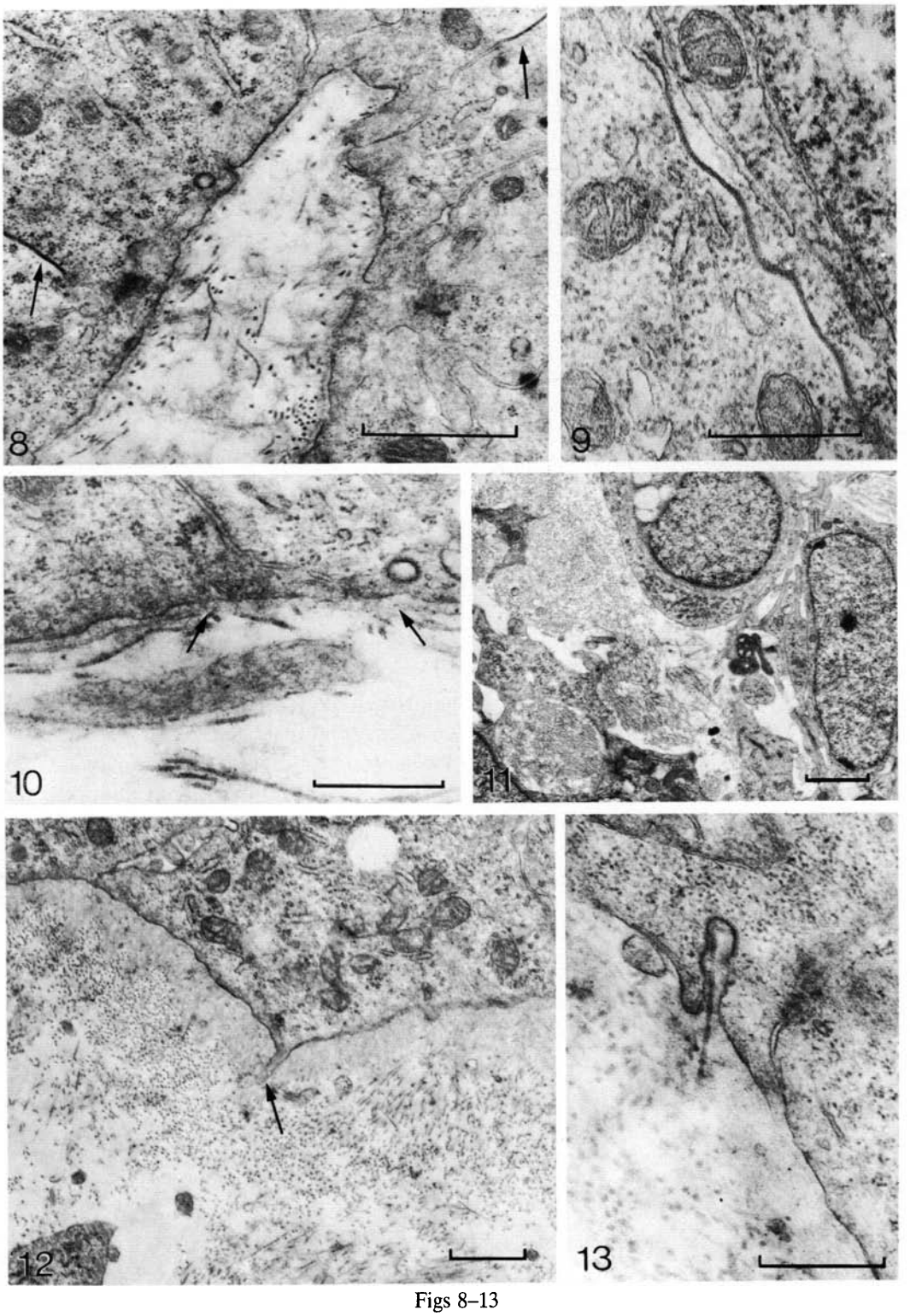
Fig. 9. Electron micrograph showing a prominent gap junction between two basal cells of the AER of a 9-day IM. Magnification bar = 0·5 μm.

Fig. 10. Electron micrograph of the epithelio–mesenchymal interface at the AER of a 9·5-day IM. Arrows delimit a zone of the basal lamina displaying a fuzzy appearance. Magnification bar = 0·5 μm.

Fig. 11. Electron micrograph of the marginal mesenchyme of a 11-day IM illustrating the abundance of collagen fibrils in the intercellular matrix at this stage. Magnification bar = 2·5 μm.

Fig. 12. Electron micrograph of the epithelio–mesenchymal interface of the marginal zone of an 11-day IM. The interface consists of an outer zone of amorphous basal-lamina-like material underlain by collagen fibrils. Note the presence of an ectodermal cell process passing across the subepithelial matrix (arrow). Magnification bar = 1 μm.

Fig. 13. Electron micrograph of the epithelio–mesenchymal interface of the marginal zone of a 10·5-day IM showing a collagen fibril located in a cytoplasmic invagination of a basal ectodermal cell. Note the absence of a basal lamina and the abundance of subepithelial extracellular matrix. Magnification bar = 0·5 μm.



Figs 8–13

membrane the marginal zone showed prominent structural changes. At day 8·5–9 the AER was prominent in the margin of the membrane although some degenerating cells were often present (Figs 1, 2). The most significant feature of the AER at the ultrastructural level was the presence of abundant gap junctions (Figs 8, 9). Up to five or six gap junctions were usually identified in each ultrathin section. These junctions were only present between the basal ectodermal cells and were located close to the basal surface (Fig. 8). The marginal mesenchymal tissue at this stage consisted of healthy mesenchymal cells, which were stellate in shape with a central rounded nucleus and a prominent nucleolus. Within the cytoplasm, free ribosomes were abundant while other organelles were scarce. Dividing mesenchymal cells were abundant. The extracellular matrix was very poor in electron-dense components except at the epithelial–mesenchymal interface where a prominent basal membrane was present (Fig. 8).

At day 9·5–10 the AER was still recognizable (Fig. 3) but degenerating cells were more numerous. Gap junctions between the basal cells of the AER were now scarce (usually no more than one or two per section). In the mesenchymal tissue a prominent necrotic focus was observed. As in the previous stage the extracellular matrix was very poor in electron-dense components. In the epithelio–mesenchymal interface the basal membrane was prominent (Fig. 3). The basal lamina consisted of a lamina densa and externa and interna lamina rara. However small segments of basal lamina showing a fuzzy appearance were occasionally observed (Fig. 10). In the sublaminar matrix crossbanded collagen fibrils and clumps of amorphous and unbanded fibrillar material were abundant.

At day 10·5–11·5 the AER was no longer present. As can be seen in Figs 4 and 5, the marginal ectoderm consisted of a basal layer of low-cuboidal cells covered by a thickened peridermal layer. In the basal layer, gap junctions were no longer observed. The peridermal cells formed cords of cells protruding into the amniotic sac. Some degenerating cells can be observed within the cords (Figs 4, 5). The marginal mesenchymal tissue also showed prominent structural changes. Degenerating cells and phagocytes were scarce. The healthy cells appeared to be in course of transformation into fibroblasts. As can be seen in Figs 5 and 11 the extracellular matrix showed large clumps of collagenous material. The collagen fibrils were randomly distributed without any appreciable organization. The epithelial–mesenchymal interface appeared very enlarged due to the deposition of extracellular materials (Figs 4, 5). Two distinct segments could be distinguished within this matrix interface. The outer segment located under the basal ectodermal surface consisted of an accumulation of amorphous basal-lamina-like material (Fig. 12). The normal appearance of the basal lamina with the laminae rara and densa was not observed. In some instances the basal ectodermal cells showed cell processes projecting into the mesenchymal zone (Fig. 12). Cross-banded collagen fibrils were located on the mesenchymal side of the interface (Fig. 12) although invaginations of the basal ectodermal cells filled with a collagen fibril were occasionally observed among the interface components. In

some instances large macrophages appeared in the proximity of the ectodermal basal surface but no evidence of a transepithelial migration of these cells was observed.

At day 12 the IM achieved a stable appearance (Fig. 6). In the ectodermal tissue the protruding peridermal cell cords typical of the previous stage were scarce or absent. Furthermore, the mesenchymal core of the membrane now has the appearance of a developing connective tissue. As shown in Fig. 6 a clump of collagenous material can be occasionally observed under the marginal ectoderm.

DISCUSSION

In a previous study we described three main structural changes in the regressing IM of the chick: mesenchymal cell death, deposition of collagenous material accompanied by rupture of the ectodermal basal lamina and desquamation of the ectodermal cells (Hurle & Fernandez-Teran, 1983). In this paper we have shown that similar changes occur in the duck IM. The results of these two studies raise two important questions for the understanding of digit development: i) What role do these changes play in the mechanism of IM regression?; ii) Are these changes interrelated? In answering the first question one should emphasize the fact that the duck foot is only partially webbed and that the distal zone of the IM is lost in the course of its development (Fallon & Cameron, 1977; Hurle & Colvee, 1982). Bearing this in mind, the structural changes observed in the developing IM of the duck might well be involved in the disappearance of the distal segment of the IM thus producing a limited regression of the interdigit. As would be expected both the intensity and temporal extension of the structural changes presumably related to IM regression appear less pronounced in the duck than in the chick. Furthermore, there are structural details of the regressing IM of the chick, such as the elimination of mesenchymal macrophages towards the amniotic sac, which we did not find in the duck. According to these facts it can be concluded that the structural differences between the regressing interdigital tissue of the chick and the partially webbed foot of the duck are quantitative rather than qualitative. However it cannot be discarded that the developmental differences between the webbed and non-webbed foot might also be due to changes in other components of the interdigit, such as the blood vessels, which were not analysed in the present study.

A definitive demonstration of a link between the different structural modifications of the IM would require further experimental analysis. However, there are some aspects which are worth mentioning. The proximodistal growth of the embryonic limb bud depends on the interaction between the marginal thickened ectoderm (AER) and the underlying mesenchyme (see Zwilling, 1968). The AER maintains the underlying mesenchyme in an undifferentiated growing state and in turn the mesenchymal cells provide an unknown factor which is required for the persistence of the AER. The AER cells die if they are grafted or cultured

in the absence of limb mesoderm (Searls & Zwilling, 1964; Zwilling, 1964). Furthermore, in the early stages of limb development AER-removal induces the degeneration of the underlying mesenchyme (Rowe, Cairns & Fallon, 1982; Janners & Searls, 1971) the limb mesenchymal cells die when they are cultured in the absence of AER (Cairns, 1975). Our observations show that both the disorganization of the AER and the establishment of a marginal necrotic focus take place at day 9·5–10. This suggests that the two processes may be closely related but does not allow us to establish which is the first. In the chick, the marginal necrotic zone of the interdigital space is more intense than in the duck and it is also simultaneous with the disappearance of the AER (Hurle & Fernandez-Teran, 1983). There is, however, a significant difference between the duck and the chick. The disappearance of the AER of the IM in the chick is very rapid; it takes place at day 7·5–8 (Hurle & Fernandez-Teran, 1983). In the duck, on the other hand, the AER appears progressively reduced in size and displays degenerating cells from day 8·5 to day 10 of incubation. Furthermore, rupture of the basal lamina at the level of the AER is a more precocious phenomenon in the chick than in the duck. Based on these findings it can be suggested that the intensity of the mesenchymal degeneration might depend on how rapid the interruption of the AER's influence takes place. Kelley & Fallon (1983) have suggested that the functional activity of the AER depends on the existence of a cell coupling mechanism mediated by gap junctions. They have even suggested that a rapid uncoupling of the AER cells may evoke cell death in the subridge mesoderm. Our results show that the regression of the AER is accompanied by a rapid reduction in the number and extension of the gap junctions. There are, however, possible additional roles in which the coupling of the AER cells might be involved. Our previous observations in the chick and the present observations in the duck show that once the AER disappears the ectodermal cells of the margin of the membrane become detached into the amniotic sac and gap junctions are lost. This fact suggests that gap junctions might also play some kind of signalling role which maintains the integrity of the limb by coordinating the growth of the mesenchymal and the ectodermal components of the limb. It is interesting to note that the alterations of the marginal ectoderm of the IM after the regression of the AER are quite similar to the changes observed in the ectoderm during wound healing induced experimentally in chick embryos (Thévenet, 1981).

The interaction between epithelial and mesenchymal tissues is often mediated by changes in the composition and arrangement of the intervening extracellular material (see Saxen, Ekblon & Thesleff, 1980). Considering this fact, it does not seem speculative to assume the existence of a relationship between the epithelial and mesenchymal structural changes and the deposition of collagen fibrils in the matrix of the IM. A similar increase of collagenous material has been reported in the chick (Hurle & Fernandez-Teran, 1983) and in the human embryo (Kelley, 1973) suggesting that the process of IM-regression displays similar mechanisms

in different vertebrates. Studies are now required to clarify how the collagen fibrils are elaborated and eliminated in the course of the regression of the membrane.

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REFERENCES

CAIRNS, J. M. (1975). The function of the ectodermal apical ridge and distinctive characteristics of adjacent distal mesoderm in the avian wing bud. *J. Embryol. exp. Morph.* **34**, 155–169.

FALLON, J. F. & CAMERON, J. (1977). Interdigital cell death during limb development of the turtle and lizard with an interpretation of evolutionary significance. *J. Embryol. exp. Morph.* **40**, 285–289.

HINCHLIFFE, J. R. (1982). Cell death in vertebrate limb morphogenesis. In *Progress in Anatomy* Vol. 2 (eds J. R. Harrison & V. Navaratnam), pp. 1–19. Cambridge: Cambridge University Press.

HINCHLIFFE, J. R. & THOROGOOD, P. V. (1974). Genetic inhibition of mesenchymal cell death and the development of form and skeletal pattern in the limbs of talpid^3 mutant chick embryos. *J. Embryol. exp. Morph.* **31**, 747–760.

HURLE, J. M. & COLVEE, E. (1982). Surface changes in the embryonic interdigital epithelium during the formation of the free digits: a comparative study in the chick and duck foot. *J. Embryol. exp. Morph.* **69**, 251–263.

HURLE, J. M. & FERNANDEZ-TERAN, M. A. (1983). Fine structure of the regressing interdigital membranes during the formation of the digits of the chick embryo leg bud. *J. Embryol. exp. Morph.* **78**, 195–209.

JANNERS, M. Y. & SEARLS, R. L. (1971). Effect of removal of the apical ectodermal ridge on the rate of cell division in the subridge mesenchyme of the embryonic chick wing. *Devl Biol.* **24**, 465–476.

KELLEY, R. O. (1973). Fine structure of the apical rim-mesenchyme complex during limb morphogenesis in man. *J. Embryol. exp. Morph.* **29**, 117–131.

KELLEY, R. O. & FALLON, J. F. (1983). A freeze-fracture and morphometric analysis of GAP junctions of limb bud cells: initial studies on a possible mechanism for morphogenetic signalling during development. In *Limb Development and Regeneration Part A* (eds J. F. Fallon & A. I. Caplan), pp. 119–130. New York: Alan R. Liss.

MENKES, B. & DELEANU, M. (1964). Leg differentiation and experimental syndactyly in chick embryo. *Rev Roum. Embryol. Cytol. ser. embryol.* **1**, 69–77.

PAUTOU, M.-P. (1974). Evolution comparée de la nécrose morphogène interdigitale dans le pied de l'embryon de poulet et de canard. *C. r. hebd. Seánc. Acad. Sci., Paris ser D.* **278**, 2209–2212.

PAUTOU, M.-P. (1975). Morphogenèse de l'autopode chez l'embryon de poulet. *J. Embryol. exp. Morph.* **34**, 511–529.

PAUTOU, M.-P. (1976). La morphogenèse du pied de l'embryon de poulet étudiée à l'aide de malformations provoquées par le vert janus. *J. Embryol. exp. Morph.* **35**, 649–665.

ROWE, D. A., CAIRNS, J. M. & FALLON, J. F. (1982). Spatial and temporal patterns of cell death in limb mesoderm after apical ectodermal ridge removal. *Devl Biol.* **93**, 83–91.

SAUNDERS, J. W. JR. & FALLON, J. F. (1967). Cell death in morphogenesis. In *Major Problems in Developmental Biology* (ed. M. Locke), pp. 289–314. New York: Academic Press.

SAXEN, L., EKBLON, P. & THESLEFF, I. (1980). Mechanisms of morphogenetic cell interaction. In *Development in Mammals vol. 4* (ed. M. H. Johnson), pp. 161–202. Amsterdam: Elsevier/North Holland Biomedical Press.

SEARLS, R. L. & ZWILLING, E. (1964). Regeneration of the apical ectodermal ridge of the chick limb bud. *Devl Biol.* **9**, 38–55.

THÉVENET, A. (1981). Wound healing of the integument in the 5-day chick embryo. *Archs Anat. micros. Morph. exp.* **70**, 227–244.

ZWILLING, E. (1964). Development of fragmented and of dissociated limb bud mesoderm. *Devl Biol.* **9**, 20–37.

ZWILLING, E. (1968). Morphogenetic phases in development. *Devl Biol.* **2**, 184–207.

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