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Vascular regression during the formation of the free digits in the avian limb bud: a comparative study in chick and duck embryos

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SUMMARY

The pattern and structure of the blood vessels of the interdigital spaces of the leg bud have been studied by means of Indian ink injections and transmission electron microscopy in the chick and duck embryos. The results show that in the chick the interdigital necrotic process responsible for the freeing of the digits is followed by regression of the blood vessels. In the webbed foot of the duck, the interdigital necrotic processes are not followed by vascular regression. Transmission electron microscopic studies show that both in the chick and in the duck, interdigital blood vessels are immature structures lacking basal lamina. Dead cells of presumably endothelial origin were detected in the lumen of the regressing blood vessels of the chick but not in the duck. However, the intensity of this cell death process does not appear to be high enough to account by itself for the disappearance of the interdigital blood vessels. The possible relationships between interdigital mesenchymal cell death and vascular regression are discussed.

INTRODUCTION

The development of the hand and foot plate of birds, reptiles and mammals having free digits involves the regresssion of the interdigital tissue. Interdigital mesenchymal cell death (Saunders & Fallon, 1967; Pautou, 1975; Fallon & Cameron, 1977; Hinchliffe, 1982) and disruption of the ectodermal tissue (Hurle & Colvee, 1982) have been reported as the main mechanisms accounting for the disappearance of the interdigital tissue. However, the mechanism of interdigital tissue regression cannot be explained by a simplistic spontaneous disintegration of the tissue. There is now evidence of a mutual dependence between the regressive changes in the mesenchymal and ectodermal components of the interdigit (Hurle & Fernandez-Teran, 1983, 1984). A precocious disruption of the ectodermal tissue

Key words: Vascular regression, cell death, limb development.

of the limb induced in chick embryos by administration of Janus green, results in the absence of interdigital mesenchymal cell death, thus leading to formation of syndactylous limbs (Fernandez-Teran & Hurle, 1984). These results suggest that the morphogenesis of free digits cannot be adequately explained without a previous extensive analysis of the tissue changes taking place during the morphogenetic process.

In addition to mesenchymal and ectodermal tissue, capillary-like blood vessels constitute a major tissue component of the early developing limb bud (Romanoff, 1960; Hinchliffe & Johnson, 1980). They appear to develop from the endothelium of the aorta and large veins of the trunk by a sprouting and growing process rather than by a local transformation of the mesenchymal cells (Wilson, 1983), and the pattern of their arrangement seems to depend on an epithelial–mesenchymal interaction in a way similar to the determination of the proximodistal limb outgrowth (Feinberg & Saunders, 1982; Feinberg, Repo & Saunders, 1983). Several reports have been published suggesting that blood vessels might play an important role in limb morphogenesis by producing appropriate local microenvironments for cell differentiation (Caplan & Koutropas, 1973; Jargiello & Caplan, 1983). However, although interdigital blood vessels must be lost during interdigital tissue regression, a detailed analysis of the vascular changes during this process has not yet been made. This is not surprising since the study of the vascular system is one of the aspects of developmental biology which has been largely overlooked (Wolpert, 1983).

In the present paper we have studied the changes in the interdigital blood vessels during the normal regression of the interdigital tissue by means of Indian ink injections and transmission electron microscopy. In an attempt to correlate the possible vascular changes with other interdigital degenerative processes, we have compared the development of the interdigital vascular pattern of the chick foot, which has free digits, with that of the webbed foot of the duck.

MATERIALS AND METHODS

The interdigital blood vessels of the foot of white Leghorn chick embryos from day 7 to day 10 of incubation (stages 31 to 36 of Hamburger & Hamilton, 1951), and Pekin duck embryos ranging from day 9 to day 12 of incubation were studied by Indian ink injections and transmission electron microscopy.

To examine the pattern of the blood vessels the embryos were injected through the vitelline vessels with a sonicated solution of Indian ink at 37 °C. The leg buds of the embryos were then excised and fixed in formalin, dehydrated in ethanol, cleared in xylene and observed under the binocular dissecting microscope.

For transmission electron microscopy, 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. 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 or a Jeol 100 S electron microscope.

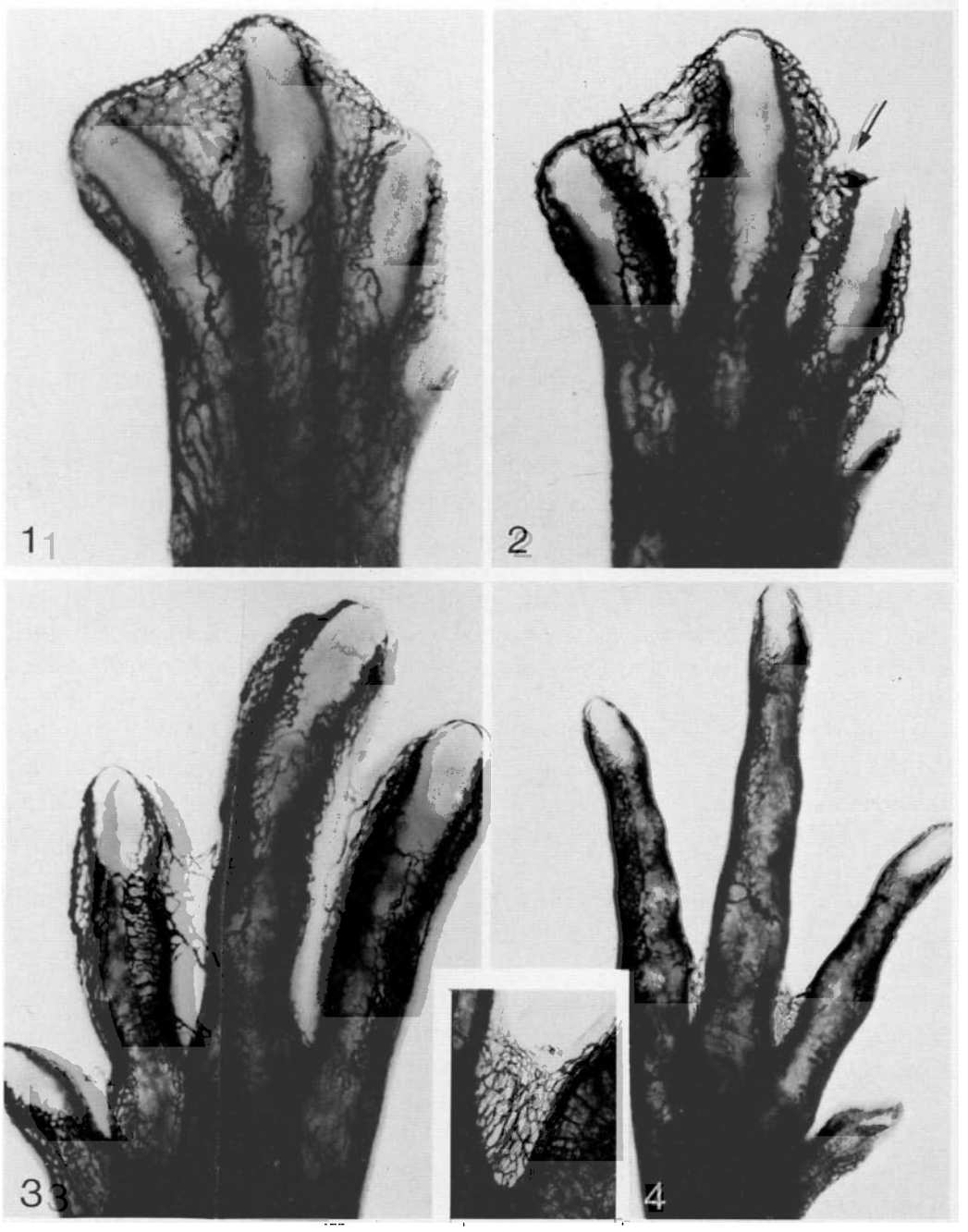
RESULTS

Chick embryo

At day 7 of incubation the digital arrays and the interdigital spaces are clearly recognizable in the chick embryo leg bud. Examination of the vascular injections reveals in each interdigital space a complex network of capillary-like blood vessels connected laterally with the main digital vessels and distally with the marginal sinus located under the regressing apical ectodermal ridge (AER) (Fig. 1). This vascular pattern is similar in all the interdigital spaces although the marginal sinus appears better developed in the third interdigital space. As development progresses, changes in the interdigital vascular pattern become evident. By day 8 of incubation, the marginal sinus shows irregularities and discontinuities at the interdigit I–II and II–III (Fig. 2). Small avascular patches can also be recognized within the interdigital spaces at this stage (Fig. 2). By day 8·5 of incubation, the interdigital vascular pattern shows dramatic changes. In all the interdigital spaces blood vessels are now scarce showing prominent avascular zones. As can be seen in Fig. 3 there are only a few blood vessels which cross the interdigit transversely connecting the main digital vessels. By day 9 of incubation, the digits are mostly free (Fig. 4). There is only a small triangular zone of interdigital tissue at the base of the digits. As can be seen in Fig. 4 blood vessels are now again abundant. They appear forming loops at the margin of the interdigital tissue with no marginal sinus recognizable (Fig. 4).

At day 7 of incubation the interdigital blood vessels display capillary-like morphology under the electron microscope (Fig. 5). The endothelial cells lining the lumen are elongated with a prominent nuclear region. Within the cytoplasm mitochondria, endoplasmic reticulum (mainly rough), free ribodomes and Golgi complexes are found. Plasmalemmal vesicles are scarce or absent. Cell junctions consisted mainly of cytoplasmic interdigitations showing occasionally a zonula adherens. A well-defined basal lamina was never present, and cell contacts between endothelial cells and the neighbouring mesenchyme were very abundant.

At days 8–8·5 of incubation, when interdigital mesenchymal cell death reaches the maximum intensity, healthy blood vessels can be observed in areas in which most of the cells were dead (Fig. 6). The structure of the endothelial cells at this stage was similar to that of previous stages although cytoplasmic autophagic vacuoles can be occasionally observed in the perinuclear zones (Fig. 7). In some instances blood vessels with degenerating cells projecting into the lumen of the vessel were observed (Fig. 8). These degenerating cells usually established contacts with the endothelial cells lining the lumen, suggesting that they are endothelial in origin. As can be seen in Fig. 9, the degenerating cells may occlude, at least partially, the lumen of the vessels. Discontinuities of the endothelial wall of the regressing vessels were never observed, thus discarding the possibility that



Figs 1–4.

the dead mesenchymal cells are eliminated into the blood stream. In some instances mesenchymal cells appear grouped together, forming structures which resemble rupturing blood vessels but possible intermediate stages of a process of vascular wall disintegration were never observed.

From day 9 of incubation degenerating endothelial cells are no longer observed and blood vessels display the same morphology of earlier stages without a well-defined basal lamina.

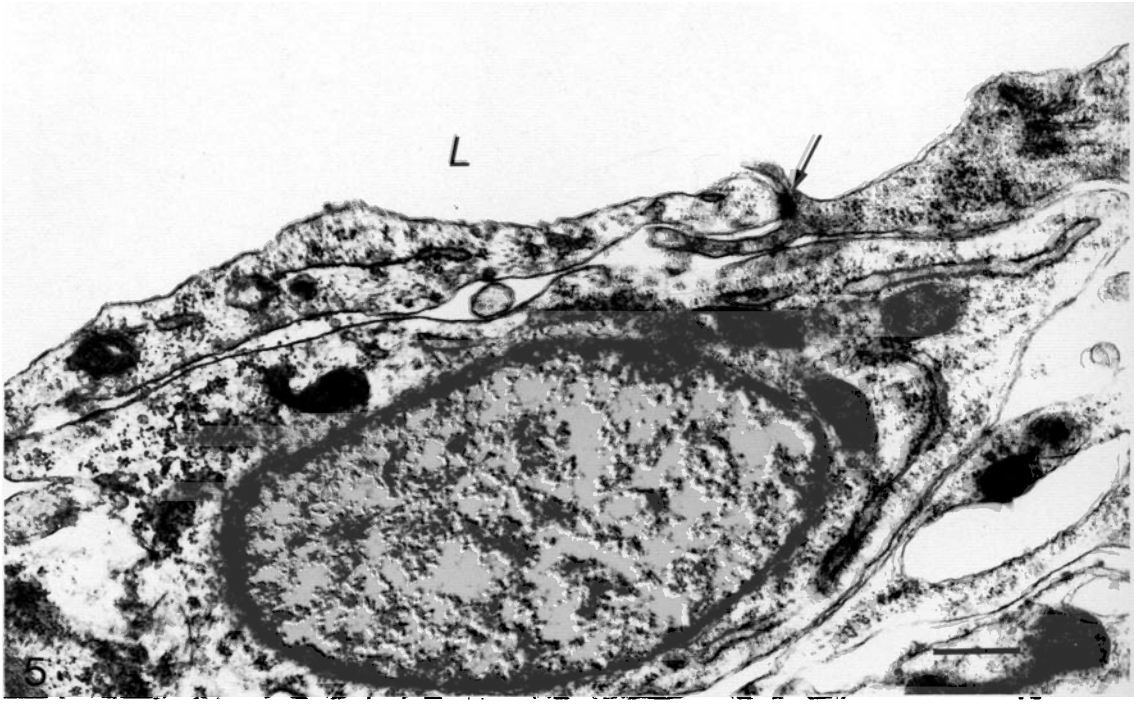


Fig. 5. Transmission electron micrograph showing the morphology of the interdigital capillaries at day 7 of incubation. Note the absence of basal lamina. Arrow shows a zonula adherens. Capillary lumen (*L*). Scale bar equals 0·5 μm.

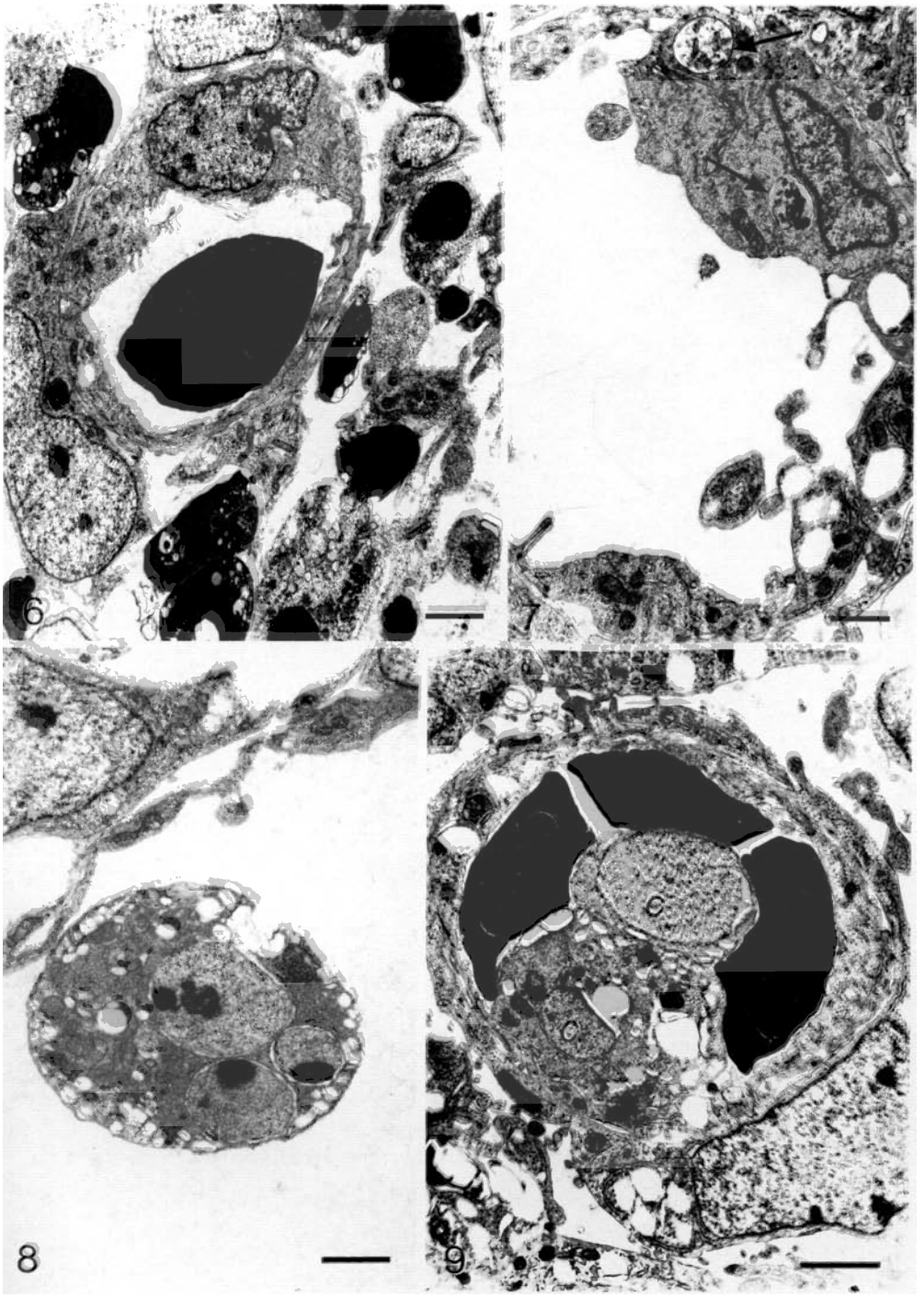
Figs 1–4. Embryonic chick leg buds at days 7 (1), 8 (2), 8·5 (3) and 9 (4) of incubation after Indian ink injection.

Fig. 1. At day 7 of incubation the interdigital vascular bed is very rich in blood vessels.

Fig. 2. At day 8, small irregularities of the interdigital vascular bed is observed in the interdigits II–III and III–IV (arrows).

Fig. 3. At day 8·5 the number of interdigital blood vessels appears drastically reduced in all the interdigital spaces.

Fig. 4. At day 9 the digits appear already free and in the definitive interdigital tissue blood vessels are again abundant; (inset) detailed view of the interdigit III–IV at day 9 of incubation showing the definitive arrangement of the interdigital blood vessels.



Figs 6–9.

Duck embryo

The interdigital vascular pattern of the duck leg at day 9 of incubation after Indian ink injection is similar to that of the chick at day 7. As can be seen in Fig. 10, the interdigital spaces displayed a very well-developed network of blood vessels with a prominent marginal sinus. At day 10 of incubation interdigital commissures produced by degeneration of the marginal interdigital tissue (Hurle & Colvee, 1982) are observed. Indian ink injections at this stage reveal that the density of blood vessels appears only reduced in the interdigit I–II (Fig. 11). The marginal sinus is also prominent at the margin of the foot plate. At day 11 of incubation interdigital vessels remain prominent in the interdigits II–III, III–IV. The only change to be mentioned at this stage is the progressive disappearance of the marginal sinus (Fig. 12).

Under the electron microscope the endothelial cells of the blood vessels of the duck show a morphology similar to that of the chick (Fig. 13). They have abundant mitochondria, free ribosomes and cisternae of endoplasmic reticulum and scarce plasmalemmal vesicles. As observed in the chick, a fully developed basal lamina was never observed in the stages studied. We did not observe degenerating cells in any of the stages studied.

DISCUSSION

As might be expected the elimination of the interdigital tissue of the chick involves the regression of the interdigital blood vessels while in the developing webbed foot of the duck the vessels do not regress. Two aspects of our results merit detailed discussion. (1) What kind of relationship, if any, is there between vascular regression and the structural changes of the remaining tissues of the interdigit? (2) How is vascular regression achieved?

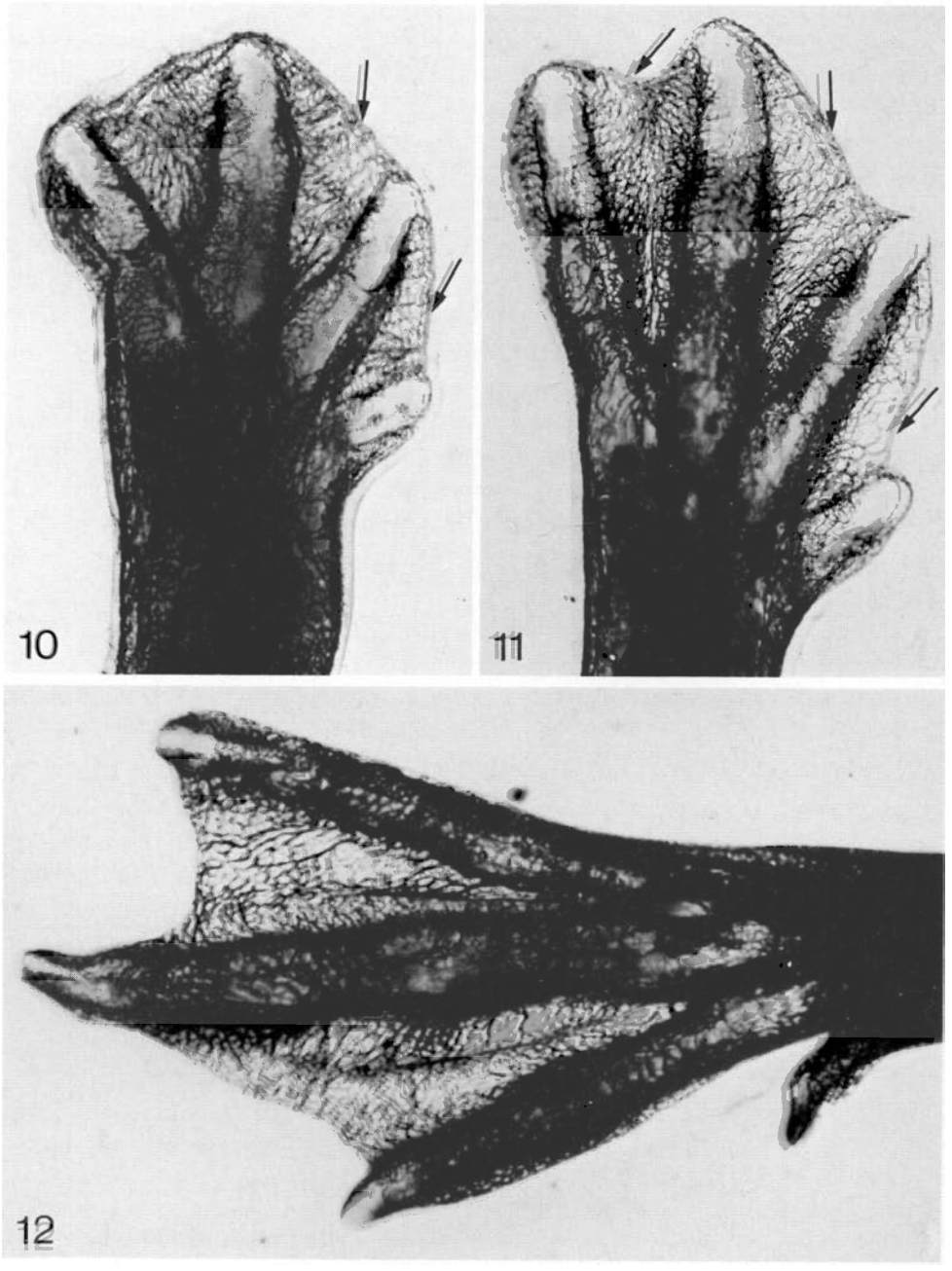
In the chick, interdigital vascular regression is not a precocious phenomenon at all; it commences at day 8–8·5. At this stage the other tissue components of the

Fig. 6. Transmission electron micrograph of the interdigital tissue of the chick limb at day 8 of incubation. Note the healthy appearance of the vascular endothelial cell in an area in which most of the surrounding mesenchymal cells are necrotic. Scale bar equals 2 μm.

Fig. 7. Transmission electron micrograph of an interdigital blood vessel at day 8·5 of incubation. Autophagic vacuoles (arrows) are present in the perinuclear zone of the endothelial cell. Note also the abundance of cell processes projecting into the lumen of the vessel. Scale bar equals 1 μm.

Fig. 8. Interdigital blood vessel showing a dead cell projecting into the lumen of the vessel. Day 8·5 of incubation. Scale bar equals 4 μm.

Fig. 9. Interdigital blood vessel occluded with erythrocytes and a dead cell (*c*). Note the absence of discontinuities in the walls of the vessel. Scale bar equals 2 μm.



Figs 10–12. Duck leg buds at day 9 (Fig. 10), 10 (Fig. 11) and 11 (Fig. 12) of incubation after Indian ink injection. With the exception of the first interdigital space no reduction in the density of the interdigital vascular bed can be detected. The marginal sinus (arrows) is prominent at days 9 and 10 but it disappears by day 11.

interdigit show an advanced stage of degeneration. In the interdigital mesenchymal tissue, cell death commences at day 7 (24 h earlier than vascular regression) reaching maximum intensity at day 8 (Hurle & Colvee, 1982). The ectodermal tissue of the interdigit shows flattening of the AER by day 7 while by day 8·5 ruptures of the basal lamina and detachment of epithelial cells into the amniotic sac are already taking place (Hurle & Fernandez-Teran, 1983). These facts rule out the possible involvement of blood vessels in the genesis of tissue regression. Furthermore the lack of vascular regression concomitant with the onset of mesenchymal cell death in the chick and during the process of interdigital cell death in the duck suggests that the hypothetical factor which triggers interdigital cell death is specific for mesenchymal cells, not affecting cells of other lineages.

From our studies we cannot discard the possibility that vascular regression is regulated by factors other than locally generated ones. As far as this question is concerned, changes in the limb circulatory pattern might modify blood flow to the interdigital spaces, thus inducing vascular regression. However, in studies carried out both *in vivo* and *in vitro* vascular development appears closely related to local changes in the extracellular milieu (Folkman & Haudenschild, 1980; Taylor & Folkman, 1982; Feinberg & Beebe, 1983; Montesano, Orci & Vassalli, 1983). While promoting and inhibiting angiogenic factors are well documented (Kuettner

Fig. 13. Low magnification electron micrograph showing an interdigital blood vessel of a duck embryo at day 10 of incubation. Note the presence of contacts between the endothelial cells and the mesenchymal cells (arrow). Scale bar equals 2 μm.

& Pauli, 1983; Simpson, Fraser & Thompson, 1983), vascular regression and the mechanisms which control it remain far less understood. If we assume that vascular regression is controlled by local factors, the chronological sequence of events in the developing interdigit of the chick indicates that vascular regression is secondary to changes produced at advanced stages of disintegration of the interdigital tissue. However, the absence of vascular regression in the interdigits of the webbed foot of the duck rules out a direct relationship between mesenchymal cell death and vascular regression. Fig. 5 is very illustrative of this hypothesis. In addition, during earlier stages of limb development, blood vessels also disappear in the zones in which the anlage of the phalangeal cartilages are formed (Latker, Feinberg & Beebe, 1983) and this process does not involve a previous tissue degeneration. These facts suggest that the intense reduction in the number of healthy cells available in the surrounding tissue might be more important than the degeneration itself. Studies on experimental models for angiogenesis have revealed that newly formed capillaries undergo regression when the angiogenic stimulus is removed (Ausprunk, Falterman & Folkman, 1978). Based on these results, to explain vascular regression it can be speculated that immature blood vessels are labile structures which require the presence of maintaining factors produced by the surrounding tissue. The disappearance of such hypothetical factors, due to the decrease of the cells responsible for their production or to modifications in the composition of the products produced by the cells in the course of tissue differentiation, would lead to vascular regression. It appears important to remark that the regressing blood vessels are immature structures lacking basal lamina. The existence of a basal lamina is regarded as a sign of vascular differentiation and its absence is indicative of a great plasticity of the developing capillary bed (Cliff, 1963; Schoefl, 1963; Glaser *et al.* 1983).

The mechanism of vascular disintegration is difficult to clarify only on the basis of morphological analysis. Studies on different models of vascular regression have reported stasis of blood, endothelial cell lysis, disruption of the vessels walls and subsequent removal of the degenerating tissue as the main mechanisms accounting for vascular regression (see Aunsprunk *et al.* 1978). Our observations revealed the presence of dead cell in the lumen of the regressing vessels of the chick suggesting that some endothelial cells die in the course of vascular regression. As has been pointed out above, the lack of discontinuities of the endothelial walls of the vessels and the existence of contacts between these degenerating cells and the healthy endothelial cells strongly suggest that they are of endothelial origin. However since the endothelial cells at these stages do not display distinctive morphological features the possibility that the dead cells were mesenchymal cells taken into the vessels cannot be ruled out. In any case the number of dead cells observed into the blood vessels in contrast with the intensity and speed of vascular regression suggests that other mechanisms may be also involved in vascular regression. In our study extravasated blood cells were not observed. This indicates that blood flow must be arrested prior to the disintegration of the blood vessels.

REFERENCES

AUSPRUNK, D. H., FALTERMAN, K. & FOLKMAN, J. (1978). The sequence of events in the regression of corneal capillaries. *Lab. Invest.* **38**, 284–294.

CAPLAN, A. I. & KOUTROUPAS, S. (1973). The control of muscle and cartilage development in the chick limb: The role of differential vascularization. *J. Embryol. exp. Morph.* **29**, 571–583.

CLIFF, W. J. (1963). Observations on healing tissue: a combined light and electron microscopic investigation. *Phil. Trans. R. Soc. Lond.* B, *biol. Sci.* **246**, 305–325.

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**, 485–489.

FEINBERG, R. N. & BEEBE, D. C. (1983). Hyaluronate in vasculogenesis. *Science* **220**, 1177–1179.

FEINBERG, R. N., REPO, M. A. & SAUNDERS, J. W. (1983). Ectodermal control of the avascular zone of the peripheral mesoderm in the chick embryo. *J. exp. Zool.* **226**, 391–398.

FEINBERG, R. N. & SAUNDERS, J. W. (1982). Effects of excising the apical ectodermal ridge on the development of the marginal vasculature of the wing bud in the chick embryo. *J. exp. Zool.* **219**, 345–354.

FERNANDEZ-TERAN, M. A. & HURLE, J. M. (1984). Syndactyly induced by Janus green B in the embryonic chick leg bud: a reexamination. *J. Embryol. exp. Morph.* **84**, 159–175.

FOLKMAN, J. & HAUDENSCHILD, C. (1980). Angiogenesis in vitro. *Nature* **288**, 551–556.

GLASER, B. M., KALEBIC, T., GARBISA, S., CONNOR, T. B. JR. & LIOTTA, L. A. (1983). Degradation of basement membrane components by vascular endothelial cells: role in neovascularization. In *Development of the Vascular System. Ciba Foundation Symposium 100* (ed. J. Nugent & M. O'Connor), pp. 150–158. London: Pitman.

HAMBURGER, V. & HAMILTON, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49–92.

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

HINCHLIFFE, J. R. & JOHNSON, D. R. (1980). *The Development of the Vertebrate Limb.* London: Oxford University Press.

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.

HURLE, J. M. & FERNANDEZ-TERAN, M. A. (1984). Fine structure of the interdigital membranes during the morphogenesis of the digits of the webbed foot of the duck embryo. *J. Embryol. exp. Morph.* **79**, 201–210.

JARGIELLO, D. M. & CAPLAN, A. I. (1983). The establishment of vascular- derived micro-enviroments in the developing chick wing. *Devl Biol.* **97**, 364–374.

KUETTNER, K. E. & PAULI, B. U. (1983). Inhibition of neovascularization by a cartilage factor. In *Development of the Vascular System. Ciba Foundation Symposium 100* (ed. J. Nugent & M. O'Connor), pp. 163–170. London: Pitman.

LATKER, C. H., FEINBERG, R. N. & BEEBE, D. C. (1983). Localized vascular regression during limb morphogenesis in the chick embryo. *Anat. Rec.* **205**, 107 A.

MONTESANO, R., ORCI, L. & VASSALLI, P. (1983). In vitro rapid organization of endothelial cells into capillary-like networks is promoted by collagen matrices. *J. Cell Biol.* **97**, 1648–1652.

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

ROMANOFF, A. L. (1960). The avian embryo. New York: McMillan.

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.

SIMPSON, J. G., FRASER, R. A. & THOMPSON, W. D. (1983). Angiogenesis and angiogenesis factors. In *Progress in Applied Microcirculation vol. 1* (ed. M. Messmer & F. Hammersen), pp. 71–85. Basel: Karger.

SCHOEFL, G. I. (1963). Studies on inflamation. III. Growing capillaries: their structure and permeability. *Virchows Arch. Pathol. Anat. Physiol. Klin. Med.* **337**, 97–141.

TAYLOR, S. & FOLKMAN, J. (1982). Protamine is an inhibitor of angiogenesis. *Nature* **297**, 307–312.

WILSON, D. (1983). The origin of endothelium in the developing marginal vein of the chick wing-bud. *Cell Differentiation* **13**, 63–67.

WOLPERT, L. (1983). Chairman's Introduction. In *Development of the Vascular System. Ciba Foundation Symposium 100* (ed. J. Nugent & M. O'Connor), pp. 1–3. London: Pitman.

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