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THE CHORIOALLANTOIC CAPILLARY PLEXUS OF THE CHICKEN EGG:

A MICROVASCULAR CORROSION CASTING STUDY

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Abstract

The chorioallantoic membrane of the avian egg serves as the principal organ of respiratory gaseous exchange for the embryo until close to hatching. It lies closely apposed to the inner shell membrane and contains an extremely dense capillary plexus supplied by the allantoic blood vessels. This study applied the microvascular corrosion casting technique to investigate the three-dimensional arrangement of the plexus at various stages of incubation. Casts were produced between days 6 and 14 of incubation, and their appearances were compared with those obtained from traditionally sectioned material and from freeze-cleaved specimens.

By day 6 the capillary network was remarkably profuse but showed considerable regional variation in vessel density. In some areas there were only short capillary buds whereas in other areas fusion had taken place so that a true plexus was formed. By day 10 the capillaries had become confluent to such a degree that the cast consisted of a thin sheet of resin perforated only by an array of small irregularly shaped orifices. These corresponded closely in size to the intervening columns of chorionic epithelial cells seen in the sectioned material.

It is clear from the appearances of the casts that the capillary surface density becomes maximal at approximately day 10 of incubation. From then on in incubation any increase in the diffusing capacity of the chorioallantoic membrane must be the result of either an increase in its overall surface area, or a decrease in the thickness of the air-blood barrier.

Key Words: Scanning electron microscopy, microvascular corrosion casting, chorioallantois, chorioallantoic membrane, chicken egg.

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Introduction

The chorioallantoic membrane of the avian egg lies immediately below the shell, being closely applied to the fibres of the inner shell membrane. It is formed when the extra-embryonic mesoderm covering the allantoic sac comes into contact and fuses with the mesoderm lining the undersurface of the chorion. This occurs early in the fifth day of incubation (Romanoff, 1960). Subsequent development is rapid and by the twelfth day the trilaminar membrane has extended over virtually the entire inner aspect of the shell (Fitze-Gschwind, 1973; Freeman and Vince, 1974). The blood vessels originally associated with the allantois proliferate equally rapidly, and by an early stage create a dense network of arteries and veins within the mesodermal layer (Figure 1). From this network of vessels arises an extremely delicate and intricate capillary plexus, which comes to be intimately associated with the overlying chorionic epithelial cells. The establishment of this plexus is of great physiological importance, for the chorioallantoic membrane acts as the principal organ of respiratory gaseous exchange of the chick until close to hatching. Oxygen, contained within the air spaces located between the interlacing fibres of the inner shell membrane, diffuses across the chorionic cells and is taken up by the erythrocytes circulating within this capillary plexus (Figure 2).

The architecture of this plexus will therefore be an important determinant of the diffusing capacity of the membrane. It is now well-established that as incubation proceeds the capillaries indent the chorionic epithelium, thus reducing the air-blood diffusion distance (Fitze-Gschwind, 1973; Wangensteen and Weibel, 1982). However despite several studies on the morphology of the plexus (e.g., Leeson and Leeson, 1963; Sethi and Brookes, 1971; Narbaitz, 1977), there is still no adequate description of its three-dimensional appearance. As Narbaitz (1977) pointed out this is principally because most work has been based on histological sections taken perpendicular to the membrane, with the result that the capillaries have been seen only in some variation of transverse or longitudinal section. To overcome this limitation Narbaitz

viewed sections taken parallel to the surface of the membrane, and concluded that the vascular arrangement is best described as a single blood sinus, the lumen of which is interrupted only by cylindrical cell columns connecting the floor to the roof. Whilst this technique provided considerable new insight into the structure of the plexus, it was limited by the relatively small size of the area that could be examined at any one time. Furthermore material from only one period of incubation (day 15) was studied, and by this stage of development it is generally agreed that the plexus has adopted its final configuration.

It was therefore decided to apply the microvascular corrosion casting technique to the chorioallantoic membrane, as part of a larger investigation into the factors influencing the rate of development of the capillary plexus. Casts were produced between days 6 and 14 of incubation, and the appearances were correlated with those obtained from traditionally sectioned material and from freeze-cleaved specimens.

Materials and Methods

Fertile eggs of the domestic chicken (Gallus domesticus) were incubated both in ovo and in shell-less conditions. For the in ovo cultures eggs were incubated under humidified normobaric atmospheric conditions at 37.5 °C and turned twice a day. The shell-less cultures were prepared according to the method of Dunn et al. (1981), and were incubated under identical conditions. Material for histological sectioning and freeze-cleaving was obtained from the in ovo preparations, whereas microvascular corrosion casts were produced from both in ovo and shell-less cultures.

Sectioned Material

On days 6, 8, 10, and 14 of incubation small areas of the shell were gently removed and the embryo anaesthetised by injection with Nembutal. Fixative solution consisting of 1.5% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M PIPES buffer with 0.1% picric acid, 2 mM calcium chloride and 15 $g l^{-1}$ sucrose (pH 7.2, osmolality 710 $mOsm.kg^{-1}$) was then applied to the chorioallantoic membrane in situ (Hayat, 1986). After 1 h samples of the membrane were removed, along with the attached shell membranes in the more advanced eggs, and placed in 0.1 M PIPES buffer made isotonic with sucrose. Following secondary fixation in 1% osmium tetroxide for 30 min the samples were pre-embedded in agar and dehydrated through ascending concentrations of ethanol. Following brief immersion in 100% acetone the agar blocks were embedded in Taab resin.

Thick sections (1 μm) prepared for light microscopy were stained with 1% methylene blue/Azure II, whereas thin sections for transmission electron microscopy were stained with uranyl acetate and lead citrate. These were viewed in a Philips EM 300 operating at 80 kV.

Freeze-cleaved Material

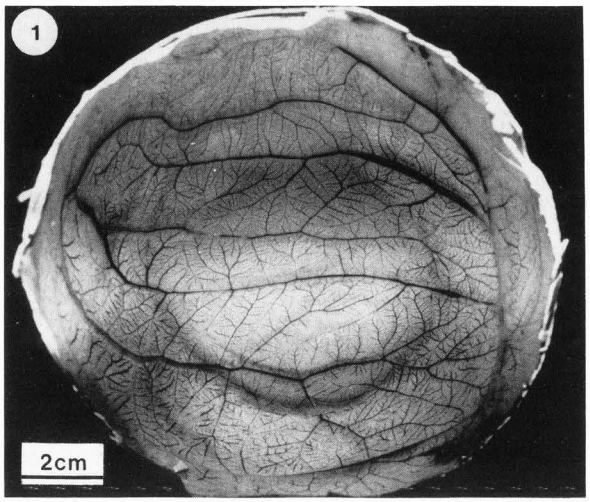
Freeze-cleaving of the shell and chorioallantoic membrane was carried out on eggs from day 10 onwards, for it is only after this stage of development that the chorioallantois fuses extensively with the inner shell membrane.

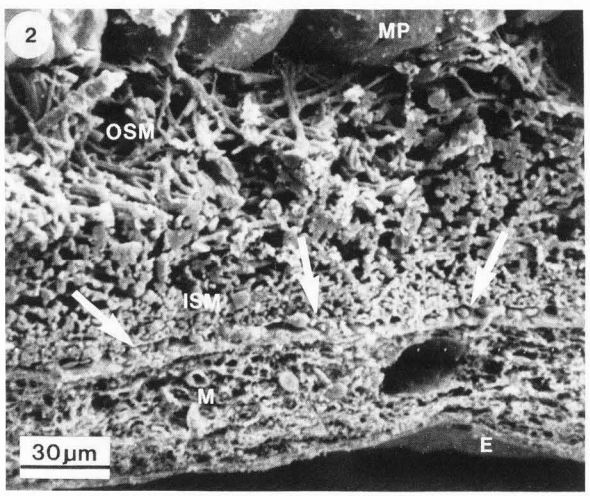
Eggs were fixed as above, and after 1 h small areas (approximately 1 cm^2) of the shell with the chorioallantoic membrane still attached were removed and dehydrated through ascending concentrations of ethanol. From 100% ethanol the specimens were frozen in liquid nitrogen at -192 °C and cleaved by striking with a chilled razor-blade. The pieces were thawed in 100% ethanol and then gradually rehydrated in 0.1 M PIPES buffer. Following secondary fixation in 1% osmium tetroxide for 30 min the samples were dehydrated through ascending concentrations of acetone and critical point dried. After being mounted on stubs they were coated with 20-40 nm of gold in a Polaron E 5000 Cool Sputter coater and viewed in a JEOL JSM 35CF SEM operating at 15 kV.

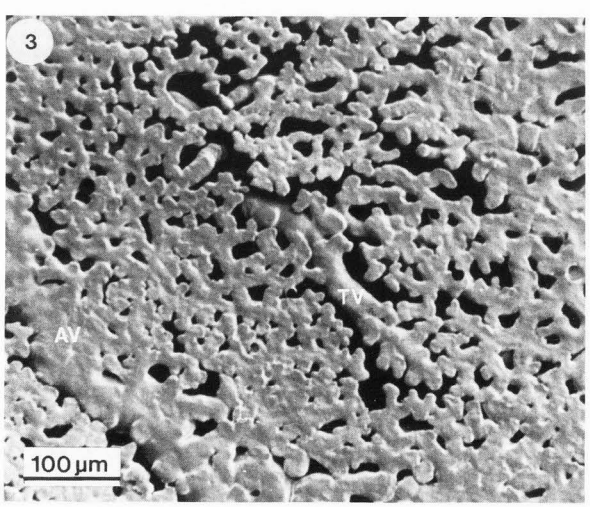
Microvascular Corrosion Casts

Microvascular corrosion casts were produced from 5 eggs on each of days 6, 8, 10, 12 and 14 of incubation, using a methacrylate resin prepolymerised to a viscosity of 3-5 centipoises by the method of Gannon (1981). Shell-less cultures were used on days 6 and 8, whereas in ovo preparations were used on the remaining days. The resin was prepared by adding 2 g of 2,4-dichlorobenzoyl peroxide (50% in silicone oil)(Polysciences cat. no. 0441) to 100 ml of methyl methacrylate monomer (inhibitor HQ 60 ppm)(Polysciences cat. no. 0834). This was dispensed into 14 ml vials and exposed to ultra-violet light for 60 min. To each 14 ml of pre-polymerised monomer were added 6 ml of hydroxypropyl methacrylate (Polysciences cat. no. 0730), 0.2 g of benzoyl peroxide (77% water wet)(Polysciences cat. no. 3968) and 0.6 ml of N,N-dimethylaniline (Polysciences cat. no. 0231).

Once a small area of shell had been removed from the in ovo preparations the injection technique was the same as that for the shell-less cultures. The embryos were first anaesthetized with Nembutal and then a loose ligature was placed around one of the allantoic arteries on the inner limb of the allantoic sac in order to tether the vessel. Using a dissecting microscope and a micromanipulator the vessel was cannulated with either a 30 G or a 25 G needle depending on the stage of incubation. The resin was then injected by gentle finger pressure from a syringe until the membrane was cleared totally of fetal blood. After initial hardening of the resin the cast was fully polymerised by standing in a 50 °C water-bath for at least 12 h. Maceration was performed using 10% potassium hydroxide solution at room temperature, and was generally completed within 12 h. Following washing in double-distilled water the casts were air-dried, mounted on stubs and coated with 20-40 nm of gold. They were viewed in a JEOL 35CF SEM operating at 10 kV.







Results

Day 6

On day 6 of incubation the vascularised area of the chorioallantois was approximately 5.3 cm^2 (SD = 1.92, n = 8), and the membrane was apposed to, but not fused with, the inner shell membrane.

Corrosion casting revealed the extremely delicate yet remarkably profuse nature of the capillary network at this stage of development (Figure 3). One of the most striking features was the considerable variation in density of the capillaries between different regions of the membrane. In some areas, often close to a larger vessel, the capillaries were relatively sparse and comprised only short arcades with numerous short side-branches (Figure 4). In other areas development was more advanced, for the branches had begun to meet and fuse so that the network took on a more plexiform appearance (Figure 5). Nonetheless, there were still many blind-ending capillary buds, and relatively large irregular spaces (10-20 µm diameter) within the meshwork.

One explanation for this variation might have been incomplete filling of the vascular system with the casting medium. This was not thought to be the case however, since similar variations in capillary size and density were observed in the sectioned material viewed under the light microscope (Figure 6a). Again

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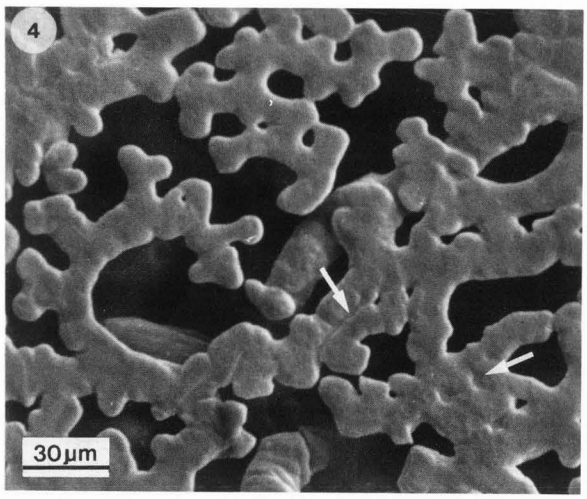
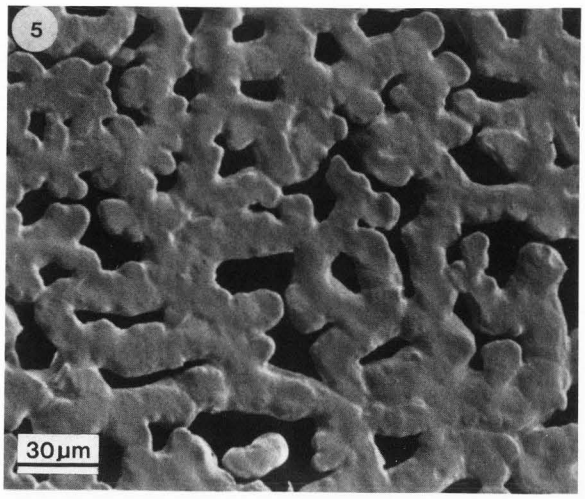
Figure 1. Photograph of the inner aspect of a shell on day 14 of incubation showing the interdigitating network of allantoic arteries and veins. From this dense network arises the chorioallantoic capillary plexus, which is responsible for respiratory gaseous exchange between the embryo and the atmospheric gas contained within the inner shell membrane.

Figure 2. Freeze-cleaved specimen from day 14 showing the pathway for respiratory gaseous exchange. Numerous pores penetrate the calcareous shell and open between the mammillary processes (MP) on its inner aspect. After passing down these pores the oxygen diffuses through the gas-filled spaces between the loose fibres of the outer shell membrane (OSM), and the more densely packed fibres of the inner shell membrane (ISM). It is then taken up by the erythrocytes circulating within the capillary plexus of the chorioallantoic membrane (arrowed). Beneath this is the extra-embryonic mesoderm (M), containing a larger allantoic blood-vessel and lined by the allantoic epithelium (E).

Figure 3. Day 6. Low power view of the cast from the shell-side illustrating the regional variation in capillary density that occurs at this stage of development. A large allantoic vessel (AV) runs under the plexus in the bottom left-hand corner of the frame, whereas a smaller terminal vessel (TV) ends by dividing into a number of capillary buds.


4
30µm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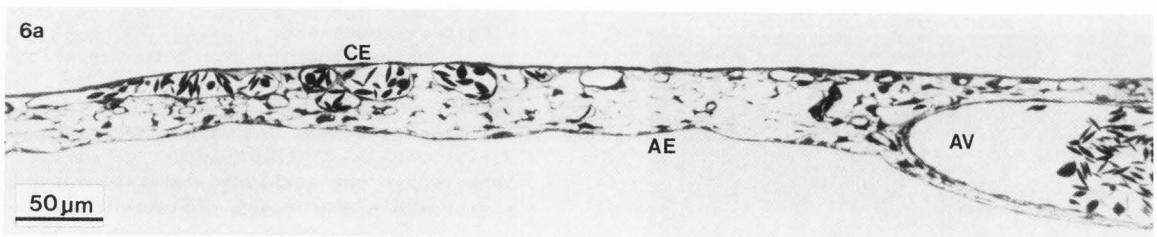
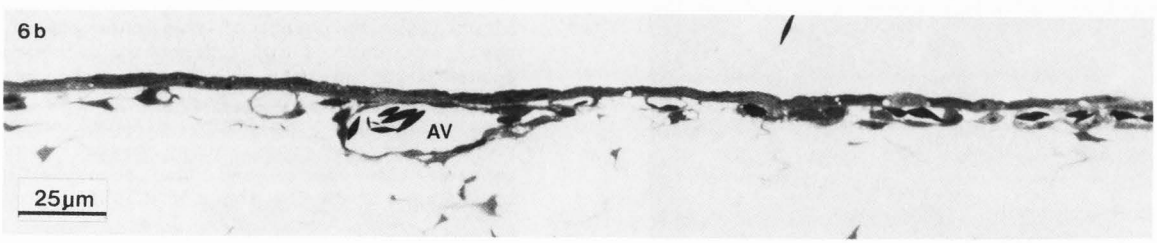
30µm


6a
CE
AE
AV
50µm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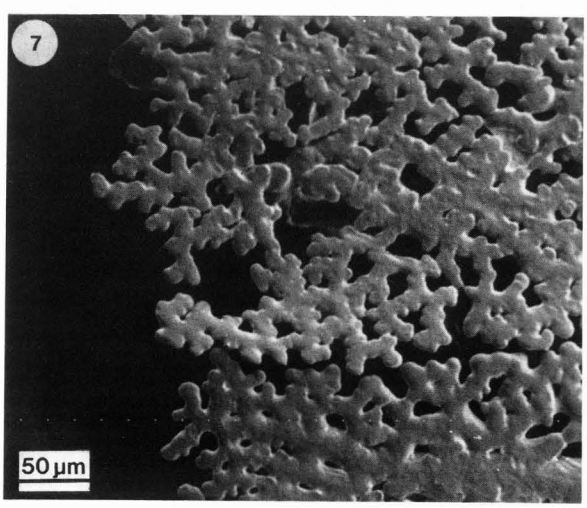
AV
25µm


7
50µm

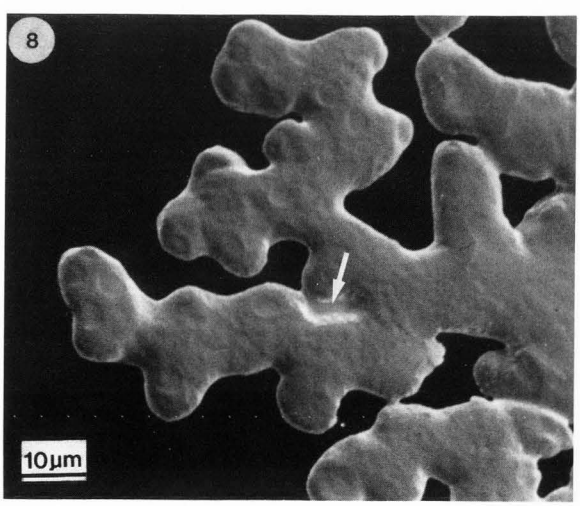

8
10µm

Figure 4. Day 6. Higher power view of Figure 3 showing a region with relatively few capillaries. Numerous short capillary buds are present but many have yet to unite and so form a plexus. The thin layer of resin occasionally seen filling the interstice between adjacent capillaries (arrowed) was thought to be indicative of exudation under a high injection pressure.

Figure 5. Day 6. Higher power view of a more mature area of the cast where the capillary buds are joining to form a true plexus. Large irregular spaces still separate many of the capillaries.

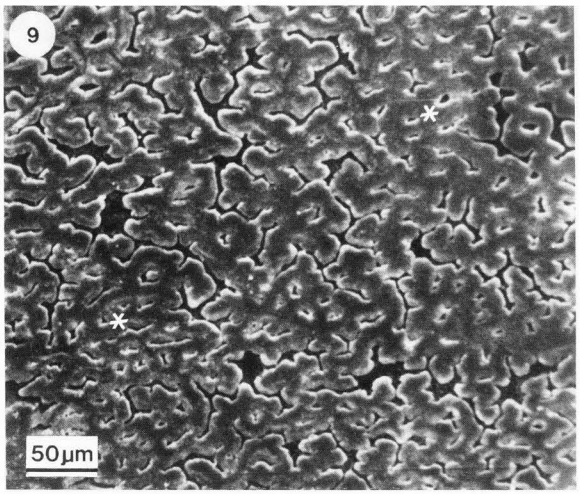
Figure 6. (a) Day 6. Photomicrograph of the chorioallantoic membrane demonstrating the regional variation in capillary density that occurs. To the left the capillaries are large and closely spaced whereas towards the allantoic vessel (AV) on the right the capillaries are of smaller calibre and less prominent. CE chorionic epithelium; AE allantoic epithelium.
(b) Day 8. Regional variations in capillary density are still present at this stage of incubation, for as shown here the capillaries are less frequent and of smaller calibre in the vicinity of the allantoic vessels (AV).

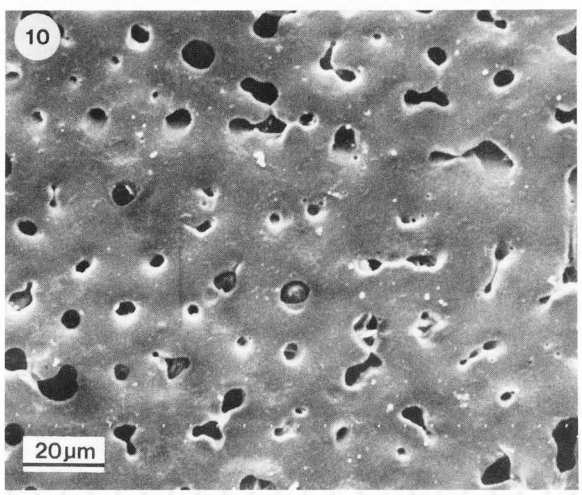
Figure 7. Day 6. Photomicrograph of the edge of the vascularised area of the chorioallantois illustrating the proliferation of capillary sprouts at the free margin (left).

Figure 8. Day 6. Higher power view of Figure 7 showing the capillary sprouts in more detail. Again slight leakage of resin (arrowed) indicates adequate injection pressure. It is believed the shallow depressions on the surface of the cast represent indentations caused by the nuclei of the endothelial cells.

Figure 9. Day 8. The capillary plexus is considerably denser at this stage of incubation, and the vessels display a greater degree of fusion than shown previously. In some areas (asterisked) this has occurred to such an extent that a sheet of resin has been produced, perforated only by a series of irregularly shaped orifices.

Figure 10. Day 10. The capillary fusion seen on day 8 is now more advanced, with the result that the cast consists of a continuous sheet of resin perforated by an array of orifices. These correspond closely in size and frequency to the intervening cell columns seen in the sectioned material.





capillary development was least prolific in the vicinity of the larger allantoic vessels. In addition there were occasional instances in the corrosion casts where a thin layer of resin appeared to be filling the interstice between adjacent capillaries (examples arrowed in Figure 4). These were considered to be equivalent to the "plastic strips" seen in other systems when exudation of resin occurs as a result of high injection pressure.

The larger allantoic blood vessels were generally hidden from view, but as they approached the plexus they could be seen to give off numerous capillary side-branches which became confluent with the main capillary network. The allantoic vessels then terminated by branching into a number of capillaries or capillary buds, an example of which is illustrated in the centre of Figure 4. It was not possible to determine however, whether a particular vessel was an arteriole feeding, or a venule draining the plexus.

At the margin of the vascularised area the cast displayed a proliferating edge of capillary buds, which were in the process of elongating and uniting together to form a more definite plexus (Figure 7). Again the appearance of the cast at higher power confirmed that the injection pressure had been sufficient to ensure complete filling of the vessels (Figure 8). Examination of the sectioned material under the light microscope confirmed that the

superficial capillary plexus stopped abruptly at the margin, but that the deeper plexus of larger vessels continued within the mesoderm, onto the inner limb of the allantoic sac.

Day 8

By day 8 of incubation the vascularised area of the chorioallantois had expanded to 25.9 cm^2 (SD = 3.9, n = 10), and it was beginning to form attachments to the inner shell membrane.

The corrosion casts revealed a considerably denser and more uniform capillary network, although regional variations were still apparent. Close to the major vessels fusion of most of the capillaries had taken place, although there were still many examples of blind-ending buds separated by irregularly shaped spaces (Figure 9). In these regions the cast thus retained some of the plexiform nature exhibited at day 6. In other areas however, more complete fusion of adjacent capillaries had taken place with the result that a thin sheet of resin was created, punctured only by irregular slit-like orifices (examples asterisked in Figure 9). The overall cast thus consisted of a patchwork of these two configurations.

The expansion of the surface area of the capillaries was confirmed by light microscopy, and in general the distribution of the vessels appeared more uniform than at day 6. However it was still noticeable that capillary profiles were less frequent and of smaller diameter in the vicinity of the supplying allantoic vessels, than in the intervening areas (Figure 6b). Most of the capillary profiles appeared ellipsoidal to a varying degree, and since very few circular examples were observed it was concluded that the majority of the vessels had indeed expanded laterally rather than simply been cut in oblique section.

Day 10

By day 10 of incubation the vascularised area of the chorioallantois had expanded to 45.6 cm^2 (SD = 5.4, n = 9), and it was now firmly attached over most of its extent to the inner shell membranes.

Viewing the corrosion cast revealed that the changes observed at day 8 had become more widespread and that the sheet-like areas of resin now predominated (Figure 10). Irregularly shaped apertures still perforated the cast at regular intervals, and these corresponded closely in size to the cell columns observed by freeze-cleaving and TEM (Figure 11).

Freeze-cleaving provided a clear impression of the nature of the air spaces between the fibres of the inner-shell membrane, and the relationship between the inner-shell membrane and the chorioallantoic membrane at this stage of incubation (Figure 12). The air-blood barrier was still relatively thick, but the cell columns intervening between the capillaries could readily be identified.

Transmission electron microscopy confirmed these findings, and revealed the manner in which the capillaries were just beginning to take up their "intraepithelial" position (Figure 13). Rather than lying beneath the epithelium, as in the early stages of incubation, the capillaries were starting to indent the epithelium, enwrapped by a layer of basement membrane. As a result the spaces between adjacent capillaries were filled not by mesenchyme, but by cells of the chorionic epithelium. In this way the cell columns were forming, but as yet there was no clear differentiation into "capillary-covering" and "villus-cavity" cells.

Day 14

By day 14 the vascularised area of the chorioallantois had extended to 63.2 cm^2 (S.D. = 3.5, n = 10), over virtually the entire inner aspect of the shell.

The corrosion casts showed little change from day 10, and revealed a uniform and extremely dense vascular network (Figure 14). There was no difference in either the size or the frequency of the apertures within the cast compared with the pattern seen at day 10.

Freeze-cleaving revealed that by now the capillaries had deeply indented the chorionic epithelium, so that the thickness of the

Figure 11. Day 10. Higher power view of the cast showing the variation in shape and size of the orifices.

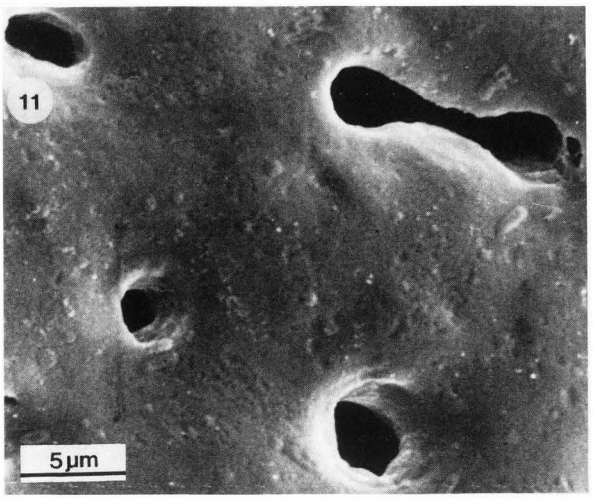
Figure 12. Day 10. Freeze-cleaved specimen showing the relationship of the chorioallantoic membrane to the inner shell membrane (ISM) at this stage of incubation. The air-blood barrier is still relatively thick. On the left a cell column (C) extending between two capillaries has been fractured, whereas another (arrowed) can be seen within the lumen of the capillary plexus.

Figure 13. Day 10. Transmission electron micrograph showing that the capillaries are beginning to take up an "intraepithelial" position at this stage of incubation. A complete layer of basement membrane is always interposed between the endothelial cells and the cells of the chorionic epithelium (CE) as the capillaries indent the latter. ISM inner shell membrane.

Figure 14. Day 14. The cast differs little from that at day 10, for the orifices are of the same size and frequency.

Figure 15. Day 14. Freeze-cleaved specimen showing that by now the capillaries have deeply indented the chorionic epithelium, with the result that the thickness of the air-blood barrier is greatly reduced.

Fig. 16. Day 14. Transmission electron micrograph showing the relationships between the fibres of the inner shell membrane (ISM), the processes of the "capillary-covering" cells (CC) and the capillary endothelial cells. Note that a complete layer of basement membrane (arrowed) still separates the capillaries from the chorionic epithelium.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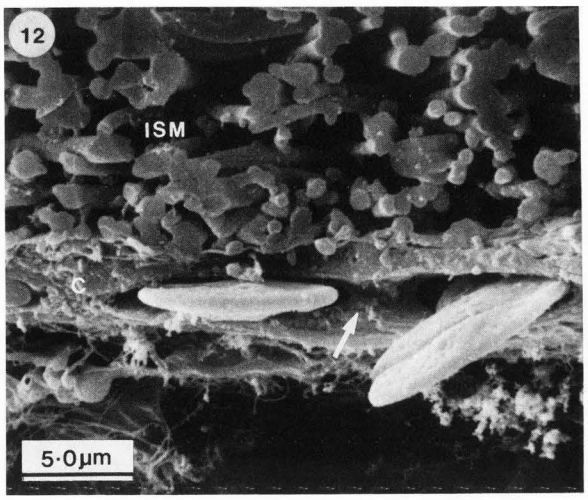
5µm


12
ISM
C
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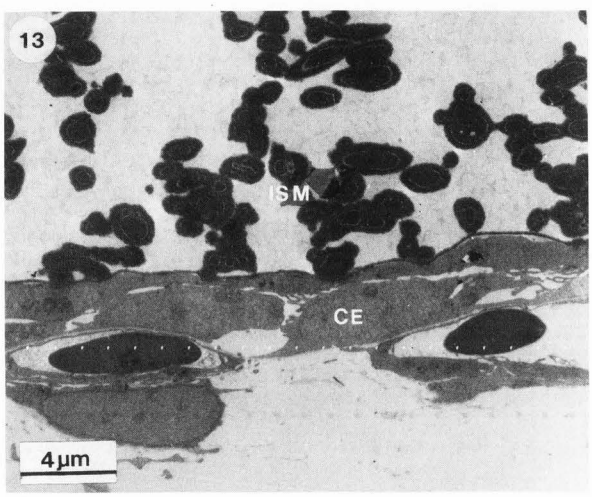
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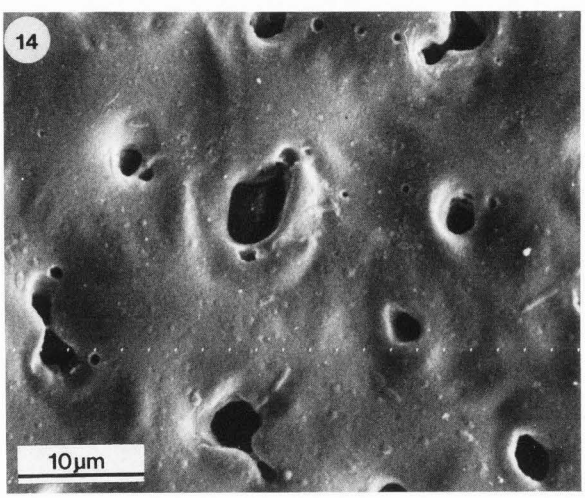
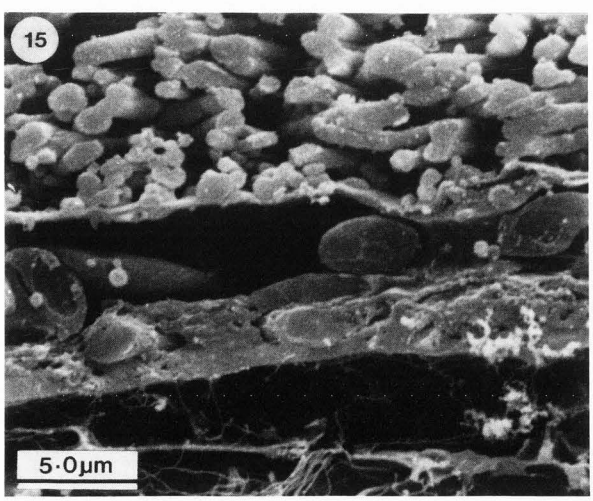
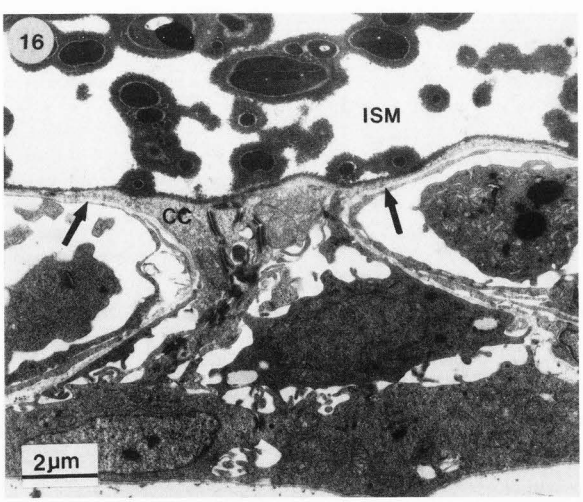
ISM
CE
4µm


14
10µm


15
5·0µm


16
ISM
CC
2µm

air-blood barrier was greatly reduced (Figure 15). The extreme delicacy of this barrier could readily be appreciated using this technique.

Transmission electron microscopy again confirmed these findings and provided more cytoplasmic detail of the cell columns (Figure 16). Both "capillary-covering" and "villus-cavity" cells could be identified within the chorionic epithelium, the "villus-cavity" cells being typified by the presence of apical microvilli and numerous mitochondria within their cytoplasm. Thin processes of the "capillary-covering" cells could be seen extending over the capillaries, interposed between the endothelial cells and the fibres of the inner shell membrane. A complete basement membrane separated the endothelial cells from the "capillary-covering" cells at all points. The diameters of the capillaries and of the intervening cell columns again corresponded closely to the measurements of the microvascular cast (compare Figure 14 with Figure 16).

Discussion

Technical Aspects of the Corrosion Casting Technique

Although the microvascular corrosion casting technique has been employed to investigate the vasculature of the developing chick embryo (Dollinger and Armstrong, 1974; Brigham and Rosenquist, 1982), this is believed to be the first application of the technique to the chorioallantoic membrane.

In view of the extreme delicacy of the vascular system contained within this membrane, particularly in the early stages of incubation, the casting procedure was restricted to simple injection of the resin. It was felt that flushing of the erythrocytes with a perfusate or pre-fixation of the endothelium as advocated by some authors (Lametschwandtner et al., 1984; Schraufnagel and Schmid, 1988), might inadvertently result in rupture of capillaries or dislodgement of the cannula. In practice the resultant casts appeared well-formed, with the individual capillaries adequately distended.

Systolic blood-pressure in the chick is generally very low during incubation, rising from approximately 2 mm Hg on day 6 to around 8 mm Hg on day 10 (Freeman and Vince, 1974). The injection pressure of the resin almost certainly exceeded these figures, and indeed as illustrated there were examples of extravasation of the resin in the specimens from days 6 and 8. Nonetheless such extravasations serve to provide an internal standard that the injection pressure had at least been sufficient to ensure full dilatation of the network (Hodde and Nowell, 1980). One can thus feel reassured that the blind-ending capillary buds observed were genuine features rather than artifacts created by incomplete filling. Further confirmation was provided by the close correlation between the appearances of the microvascular casts with those of the sectioned material.

Significance of the Observations

The differentiation of the chorioallantoic membrane, including the development of its vascular network, has been the object of a number of studies (e.g., Leeson and Leeson, 1963; Ganote et al., 1964; Sethi and Brookes, 1971; Coleman and Terepka, 1972; Fitze-Gschwind, 1973; Narbaitz, 1977). The application of the microvascular corrosion-casting technique considerably extends these studies, for it permits a rapid and unambiguous assessment of the three-dimensional configuration of the vessels over wide areas. By performing the technique on various days of incubation we have been able to follow the development of the network from the stage of prolific capillary buds to the formation of an exceptionally dense meshwork of vessels covering virtually the entire inner aspect of the shell.

Over the years a number of terms have been used to describe this network, which Narbaitz (1977) concluded is best considered as "a single blood sinus". We have avoided the use of the terms "sinus" and "sinusoid" for two reasons. Firstly, these terms are usually applied to vessels with irregular lumina, that lack an adventitia or basal lamina and which are lined by phagocytic macrophage-like cells interspersed amongst the endothelial cells. Although Leeson and Leeson (1963) considered the network to be lined by chorionic ectodermal cells, many other studies have confirmed the presence of a complete endothelium supported by a basal lamina, which becomes interposed between the endothelial and chorionic cells as the capillaries indent the epithelium (Ganote et al., 1964; Sethi and Brookes, 1971; Wangensteen and Weibel, 1982). Secondly, true sinusoids, such as those in the liver, develop initially as large venous spaces which are subsequently broken up into a number of anastomosing channels by the proliferation of cell columns (Le Gros Clark, 1971). This is contrary to the situation with regard to a capillary plexus, where a number of discrete capillaries unite to form an interconnecting network.

The various appearances of the corrosion casts readily confirm that, on the basis of these definitions, the vasculature of the chorioallantois constitutes a true capillary plexus during the early stages of incubation (days 6-8). Further proliferation and fusion of the capillaries results in the formation of a vascular network of exceptional density from the mid-point of incubation onwards. However, in order to avoid any misconceptions as to its structure or mode of development, we advocate the use of the term "capillary plexus" to describe the state of this network throughout incubation, rather than the alternatives of "sinus" or "sinusoid".

The Capillary Plexus as an Organ for Respiratory Exchange

One of the principal functions of the chorioallantoic membrane is to act as the organ of respiratory exchange for the embryo until close to hatching. The diffusing capacity (D)

of the membrane for oxygen is given by the equation:-

$$D = \frac{K \times (Sa + Sb)}{2 \times Th} \quad (1)$$

where K is Krogh's constant, Sa and Sb the surface areas in contact with the air and the fetal blood, respectively, and Th the harmonic mean thickness of the air-blood barrier (Wangensteen and Weibel, 1982). The rate of development of the capillary plexus is therefore of considerable physiological importance.

Bisonnette and Metcalfe (1978) demonstrated that the resistance offered to gas diffusion by the chorioallantoic membrane decreased three-fold between days 10 and 14 of incubation, greatly facilitating oxygen exchange. It was not possible in the present study to obtain reliable estimates of the capillary surface area from the casts because of technical inconsistencies in their production. Nonetheless, it is apparent from the illustrations that the surface area of the plexus per unit area of the membrane is effectively maximal as early as day 10 of incubation. Although there will be a further small increase in the overall surface area of the chorioallantoic membrane between days 10 and 14, it is clear that the reduction in resistance noted by Bisonnette and Metcalfe (1978) must be largely due to the decrease in the thickness of the air-blood barrier that occurs over this period (Fitze-Gschwind, 1973).

The corollary of this observation is that from day 10 onwards the chorioallantoic membrane has no scope to increase its diffusing capacity by further enlargement of the capillary surface area. This fits with the physiological evidence, for whereas incubation of eggs in a hyperoxic environment results in a reduction in the diffusion capacity of the membrane (Temple and Metcalfe, 1970), incubation under the stress of hypoxia cannot increase it (Metcalfe et al., 1979).

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References

Bissonnette JM, Metcalfe J (1978) Gas exchange of the fertile hen's egg: components of resistance. Respir. Physiol. 34, 209-218.

Brigham LE, Rosenquist GC (1982) Acrylic vascular casts of Hamburger-Hamilton stage 19-21 chick embryos. Teratology 25, A29-A30.

Coleman JR, Terepka, AR (1972) Fine structural changes associated with the onset of calcium, sodium and water transport by the chick chorioallantoic membrane. J. Membrane Biol. 7, 111-127.

Dollinger RK, Armstrong PB (1974) Scanning electron microscopy of injection replicas of the chick embryo circulatory system. J. Microscopy 102, 179-186.

Dunn BE, Fitzharris TP, Barret BD (1981) Effects of varying chamber construction and embryo pre-incubation age on survival and growth of chick embryo in shell-less culture. Anat. Rec. 199, 33-43.

Fitze-Gschwind V (1973) Zur entwicklung der chorioallantoismembran des huhnchens. (The development of the chorioallantoic membrane of chickens). Adv. Anat. Embryol. Cell Biol. 47, 7-51.

Freeman BM, Vince MA (1974) Development of the Avian Embryo, Chapman and Hall, London, 119-159.

Gannon BJ (1981) Preparation of microvascular corrosion casting media: procedure for partial polymerisation of methyl methacrylate using ultraviolet light, in: Scanning Electron Microscopy in Cell Biology and Medicine, Tanaka K, Fujita T (eds.), Excerpta Medica, Amsterdam, 227-233.

Ganote CE, Beaver DL, Moses HL (1964) Ultrastructure of the chick chorio-allantoic membrane and its reaction to inoculation trauma. Lab. Invest. 13, 1575-1589.

Hayat MA (1986) Basic Techniques for Transmission Electron Microscopy, Academic Press, London, 1-55.

Hodde KC, Nowell JA (1980) SEM of micro-corrosion casts. Scanning Electron Microsc. 1980;II: 89-106.

Lametschwandtner A, Lametschwandtner U, Weiger T (1984) Scanning electron microscopy of vascular corrosion casts - technique and applications. Scanning Electron Microsc. 1984;II: 663-695.

Leeson TS, Leeson CR (1963) The chorioallantois of the chick. Light and electron microscopic observations at various times of incubation. J. Anat. 97, 585-595.

Le Gros Clark WE (1971) The Tissues of the Body, Clarendon Press, Oxford, 186-228.

Metcalfe J, Bissonnette JM, Bowles RE, Matsumoto JA, Dunham SJ (1979) Hen's eggs with retarded gas exchange. I. Chorioallantoic capillary growth. Respir. Physiol. 36, 97-101.

Narbaitz R (1977) Structure of the intra-chorionic blood sinus in the chick. J. Anat. 124, 347-354.

Romanoff AL (1960) The Avian Embryo, Macmillan, New York, 1041-1140.

Schraufnagel DE, Schmid A (1988) Microvascular casting of the lung: vascular lavage. Scanning Microscopy, 2, 1017-1020.

Sethi N, Brookes M (1971) Ultrastructure of the blood vessels in the chick allantois and chorioallantois. J. Anat. 109, 1-15.

Temple GF, Metcalfe J (1970) The effects of increased incubator oxygen tension on capillary development in the chick chorioallantois. Respir. Physiol. 9, 216-233.

Wangensteen D, Weibel ER (1982) Morphometric evaluation of chorioallantoic oxygen transport in the chick embryo. Respir. Physiol. 47, 1-20.

Discussion with Reviewers

S.A. Dilly: You used both *in ovo* and shell-less membranes; why did you do this and did you detect any differences?
Authors: Shell-less culture allows for easy access to the chorio-allantoic membrane and to the associated allantoic blood vessels, and this is very important when dealing with the earlier stages of development when the structures are particularly fragile. However by day 10 of incubation the membrane has generally covered the open surface of the culture dish and then begins to grow downwards over the plastic film supporting the egg. After this stage of development therefore *in ovo* preparations offer the best prospect of obtaining casts from extensive and yet flat areas of the membrane. We did not perform comparisons between the two techniques, but Dunn and Fitzharris (1979) noted no difference in the pattern of vascularisation in their comparative study.

S.A. Dilly: The diffusing capacity of the membrane depends on the surface area in contact with air, surface area in contact with blood and the thickness of the air/blood barrier.
a) Can you provide values for the air/blood barrier at different incubation times?
b) Some of your photomicrographs suggest that the capillaries are flattened at day 6 and day 8 to present a larger surface area for exchange. Do your observations support this?
Authors: We are at present estimating values for the air/blood barrier at different incubation times and under different oxygen levels from traditionally sectioned material. Figures for normal development were provided by Fitze-Gschwind (1973). It is most likely that the capillaries do become flattened during development for it becomes increasingly difficult to find a circular profile within the plexus after day 8. The elliptical profiles seen thereafter cannot all be explained on the basis of tangential sections.

C.R. Leeson: What is the nature of the contact and fusion between the allantoic sac and the mesoderm of the inner shell membrane, referred to in the Introduction and Results?
Authors: This relationship was not investigated in the present study, but descriptions of the development of the extra-embryonic membranes are available in the classic texts of Romanoff (1960) and Freeman and Vince (1974).

C.R. Leeson: The authors state that "it was not possible to determine however, whether a particular vessel was an arteriole feeding, or a venule draining the plexus". I recognise this but would like to ask: Could it be that capillaries in relation to allantoic arterioles feeding the capillary plexus are of smaller diameter than those that are draining from the plexus into allantoic venules?
Authors: It is possible that such differences exist but it is doubtful whether the corrosion-casting technique would be a suitable method with which to investigate the question. Uncertainties regarding injection pressure and pressure drops due to resin viscosity must raise doubts about the validity of measurements performed on casts, particularly when one is trying to identify fairly small changes in vessel calibre. *In vivo* microscopy would perhaps be a more appropriate technique to clarify this point.

D.E. Schraufnagel: The chorioallantoic membrane has been used as a model organ system for assaying angiogenesis. Can what you have learned in this study be applied by those using this model to study angiogenesis?
Authors: The point most relevant to those studying angiogenesis is that the capillary plexus takes on a mature form relatively early in incubation, and so any experimental manipulation of the system must take account of this fact.

D.E. Schraufnagel: In Figures 4 and 8 you show that plastic has transuded outside the vascular space and suggest that this may result from excess syringe pressure. There is now a substantial literature about the "leaky" basement membranes of developing vessels. I feel the resin transudation is a problem only in angiogenic conditions and uncorrelated with the syringe pressure. Would you agree?
Authors: This is a phenomenon we had not considered and indeed it could well be the case, particularly since we did not fix the endothelial cells prior to resin injection due to the delicacy of the cannulation procedure.

Additional Reference

Dunn BE, Fitzharris TP (1979) Differentiation of the chorionic epithelium of chick embryos maintained in shell-less culture. Develop. Biol. 71, 216-227.