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FINE STRUCTURE AND ORGAN CULTURE OF CHICK EMBRYO DORSAL AORTA

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Abstract

Embryonic blood vessels have not been grown in organ culture, in a way which might easily submit them to studies of vascular organogenesis. The chick embryo dorsal aorta is easily accessible and relatively simple to explant to culture. Its organ culture may provide a model for wounding and repair of the intima and/or media and provide a model for studies of growth (or maintenance) and differentiation. Fresh dorsal aorta and its organ culture from the chick embryo was characterized morphologically by light microscopy scanning (SEM) and transmission electron microscopy (TEM) from days 4 to 18. Explants were incubated up to 4 days at 37.5°C in normal atmosphere in media including whole chick embryo extract (EE) diluted to 20%. The normal fine structure by SEM shows that as the embryo ages the endothelial cells of the aorta tend to elongate, their microvilli lengthen and increase in size and number up to about 11 days, after which they decrease. TEM also demonstrates an increased differentiation of smooth muscle cells as embryonic age advances. The best culture medium was EE_{20}-Tyrode's. Most of the explants from 14 and 17 days cultured in EE_{20}-Tyrode's contracted into small balls of tissue, endothelial side out. None of these explants showed any significant cellular degeneration. None of the explants survived in EE_{20}-DMEM. Addition of insulin to each medium had no effect on the explants. Intimal cells resembling myofibroblasts were observed in the contracted explants. Using a simple culture condition the chick embryo dorsal aorta can be sustained in organ culture for at least 4 days. The most suitable age for organ culture studies is 9 days (or perhaps somewhat younger); however, it is the most difficult to obtain.

KEY WORDS: organ culture, dorsal aorta, chick embryo, endothelium, myoblast, myofibroblast, transmission electron microscopy, scanning electron microscopy, contraction, microvilli.

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Introduction

Establishment of a suitable but simple organ culture system for major embryonic vessels such as the dorsal aorta would be a major breakthrough for future studies of vascular organogenesis. Organ culture of a full thickness piece of a vessel wall could lend the developing intima and media to separate testing for developmental dependencies. An organ culture model would also allow observations of the vessel wall and the nutritional and biochemical requirements for growth and differentiation.

Some fine structural studies have previously been performed on the very early events of development of blood vessels (Meier, 1980; Hirakow and Hiruma, 1981; Poole and Coffin, 1988). Although one study has been made of the developing aortic arch of the chick (Simone-Santoro and Renda, 1971), a search of the literature reveals no such studies on normal stages of dorsal aorta development in the chick embryo.

There have been previous reports of organ culture of chick embryo dorsal aorta. However, in those reports (Conti et al., 1968a,b,; Conti and Cappelli, 1968) whole sections of dorsal aorta were cultured from 2 to 7 days. Such cultures do not lend themselves to observation in vitro of the intimal surface, nor to potential manipulation of same. They indicate that segments of ten days or younger from the chick embryo did not survive. They also state that segments of the aorta from embryos older than 10 days differentiated in culture according to their cranial or caudal location in the embryo.

Buck (1977, 1979) has reported on the organ culture of the aorta from rats, which was maintained up to 15 days. However, these tissues were grown in a complex medium with fetal calf serum and required CO_2 incubation.

Kim (1984) has also grown aortae from adult rats in organ culture in a study of calcification. Gottleib and Boden (1984) have studied adult porcine aortae in organ culture for endothelial response to injury.

As a first step toward determining if the chick embryo dorsal aorta could eventually

constitute a model for studies of organogenesis we report here initial morphological studies of its growth periods and its viability and response in organ culture.

Materials and Methods

Segments of the dorsal aorta were obtained from 4 to 18 day old chick embryos. The 4 day old embryo was the earliest we could study due to its limited size and accessibility to dissection. Specimens which were dissected for morphological studies were fixed in full strength Karnovsky's fixative (Karnovsky, 1965) and dehydrated through graded alcohols. All tissues studied by light microscopy were processed to paraffin. Sections were stained by hematoxylin and eosin, and by Masson's trichrome method. Those specimens reserved for study by scanning electron microscopy (SEM) were washed in sodium cacodylate buffer, then run through graded alcohols, 50% – absolute, after which they were placed in the chamber of a Tousimis Samdri-790 Critical Point Dryer. The intermediate fluid is ethanol and the transitional fluid is liquid CO_2. The specimens were coated with approximately 300 A of gold in a Polaron Sputter Coater, model #5100, using Argon gas. The samples were then examined in an ETEC autoscan. With the exception of the 4 day old aorta, all vessels were split lengthwise to expose the intimal surface. Because the 4 day old aorta was so small the whole dorsal region was fixed and processed (dried) after which the top half of the aorta was removed. The effort was made to characterize the proximal and distal portions separately in each case.

Those specimens reserved for transmission electron microscopy (TEM) study were embedded in EPOX 812 (Ladd). Sections were cut with a diamond knife, stained with uranyl acetate and lead citrate and studied in a Philips EM 300 transmission electron microscope.

For the organ culture studies, all procedures were conducted under sterile conditions. Segments of the aorta from the early embryo (5 days) were obtained by submersing the whole embryo in chick Ringer's solution. This embryo was then dissected off of the yolk and washed in fresh Ringer's, after which the dorsal region of the body was cut from the embryo and the dorsal aorta exposed. In all other cases the embryo was removed from the egg, washed and cleaned free of yolk and membranes and laid in a glass petri dish. The anterior wall was opened with a pair of small fine tipped surgical scissors and the rib cage opened and laid back laterally. The descending dorsal aorta was identified from the junction of the subclavian artery in the superior position and branches of the renal arteries in the inferior position. The dorsal aorta was always filled with blood and thereby easily recognized. The dorsal aorta was freed from the posterial abdominal wall by undermining the vessel with number 13 beading needles until a suitably long segment could be released from the wall transected and transferred to fresh chick Ringers. The 9 day aorta was decidedly more difficult to undermine than the earlier or later stages. It appears to have a stronger continuity with the posterior wall. This strong adherence seems relieved in the older embryos. Usually two segments of the descending aorta could be obtained by this procedure. After appropriately dissecting the descending aorta one beading needle was very gently inserted into the lumen of one segment until it was completely on the shaft (Fig. 1). With the tip of the other needle the piece of aorta was then split lengthwise after which it was peeled off of the needle. The piece was then transferred onto a stainless steel platform of the organ culture dish by a disposable pipette and flattened out under a dissecting microscope endothelium side up. Usually these pieces measured approximately two to three mm. in width and five to six mm. in length (Fig. 2). Where possible, proximal pieces were identified versus the distal ones.

In all 106 pieces of descending aorta were organ cultured. These were distributed in the following way: 8 came from 5 day embryos, 10 from 9 days, 36 from 14 days, 40 from 17 days, and 12 from 18 days of embryonic age.

Four different culture mediums were used, each of which included 100% reconstituted chick embryo extract (Difco) diluted in the final medium to 20% (EE_{20}). The four media included: 1) EE_{20}-Tyrode's balanced salt solution, 2) EE_{20}-Dulbecco's Minimal Essential Medium (DMEM), 3) EE_{20}-Tyrode's with insulin (5 mgm/ml) and 4) EE_{20}-DMEM with insulin (5 mgm/ml). Sixty-two explants were cultured in EE_{20}-Tyrode's, 29 explants in EE_{20}-DMEM, 10 explants in Tyrode's with insulin and 5 in DMEM with insulin.

The organ culture dish received five ml of distilled water on a cellulose ring in the outer moat. The dishes were covered and placed in a standard convection type incubator for up to 4 days at 37.5°C. CO_2 flow was not necessary for a short term study of up to 5 days incubation because the culture medium pH was not compromised within that length of time. Four days was chosen for culture because from previous studies (Kischer, 1967) it is known to be an optimum time within which good viability is expressed, as well as differentiation, before any change of medium is necessary, which might introduce another variable. Observations were made on the culture dishes daily by a dissecting microscope and eventually all explants were harvested and fixed for analysis by light, scanning or transmission electron microscopy.

Results

Fine Structure of Normal Stages

Scanning Electron Microscopy: As the chick embryo dorsal aortic endothelium differentiates the cells are observed to demonstrate an increasing size and number of microvilli up to
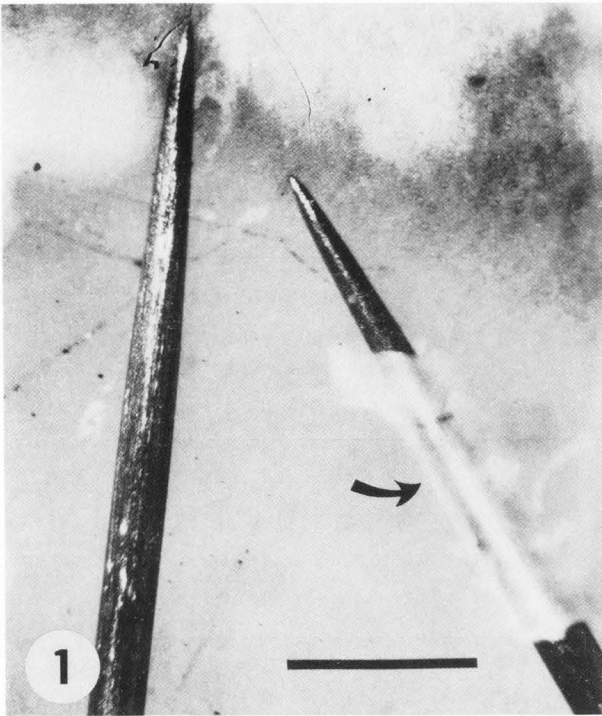
Fig. 1. An intact segment of the chick embryo dorsal aorta (DA) has been sleeved over one beading needle (➜). The tip of the other needle is used to slice open the vessel. Bar = 1 mm.
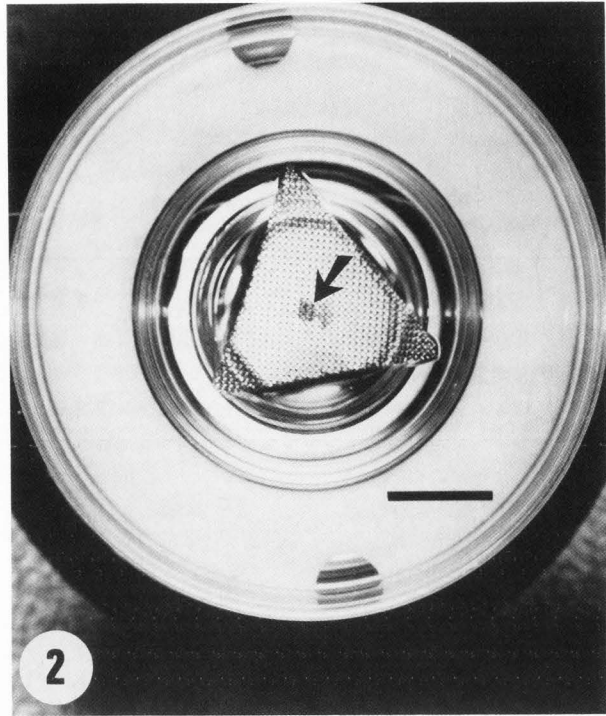

Fig. 2. An explant from 9 day DA (➜) on the stainless steel platform within the Falcon organ culture dish. Bar = 10 mm.

11 days (Figs. 3, 4 and 5, 6). Thereafter the microvilli seem to decrease up to 18 days (Figs. 7, 8 and 9, 10). However, the microvilli, in size or number, were not quantified. The endothelial cells appear to elongate with age and become more confluent. A further finding illustrates a difference between the proximal and distal portions of the dorsal aorta. This was clearer in the later stages, but not so in the earlier ones. For example, a reduction in number and size of the microvilli appears to be advanced in the proximal portions compared to the distal ones (Figs. 11 and 12). By SEM observation there does not appear to be a substantial change in the surface vesicles.

Transmission Electron Microscopy: The early stage of the aortic intima (5 days) shows endothelial cells with a moderate number and variety of organelles, such as mitochondria and rough endoplasmic reticulum (Fig. 13). The basal lamina appears incomplete and often is seen two to three cell profiles below the surface cell (BL). The cells of the tunica media have not yet differentiated into smooth muscle cells, but some of the cells demonstrate areas reflective of actomyosin structures (A). As development proceeds, the endothelial cells appear to be somewhat more pleomorphic;

however, the fine structure does not appear to change significantly. The basal lamima appears more pronounced. By late development (16 days) more areas of actomyosin filaments are observed in the cells in the media than in earlier stages (Fig. 14). There is the suggestion that this transition occurs preemptively in a luminal to abluminal gradient. Collagen appears to increase but does not approach adult proportions up to 18 days. The undulations of the intercellular spaces seen in Fig. 14 suggest a differentiating conformity to an internal elastic membrane, which has not yet fully formed.

Organ Culture

The results of the organ culture experiments are summarized in TABLE I.

The 8 explants of 5 day old dorsal aorta did not survive. All 10 explants of 9 day chick embryo dorsal aorta survived. All were harvested after three days incubation and processed for microscopical analysis. All of these explants were grown in EE_{20}-Tyrode's. Six of those explants showed minimal contraction while 4 explants remained flat on the platform. The contraction phenomenon was observed pervasively in explants cultured from older chick embryos and resulted in the explant rounding up into a small ball of tissue endothelium side out on the stainless steel platform (Fig. 15). This contrasted sharply
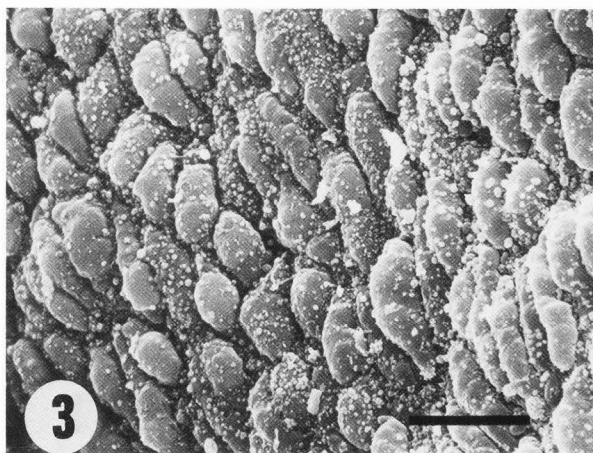
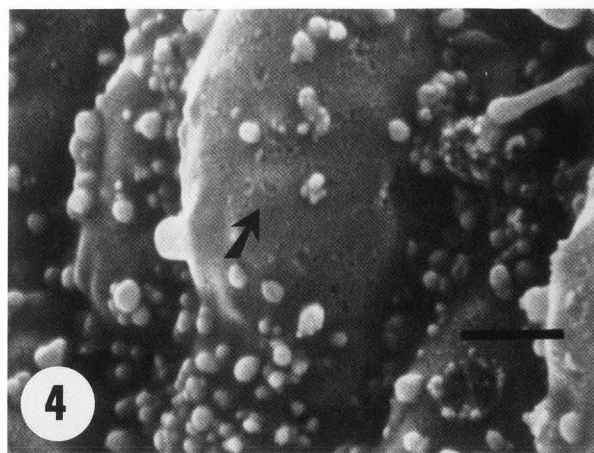Fig. 3. SEM of intimal surface from 5 day distal DA. Small, rounded microvilli on endothelial cells. Bar = 10 μm.

Fig. 4. Surface of endothelial cell from Figure 3. Surface vesicles (➤). Bar = 1 μm.

Fig. 5. SEM of endothelial surface of 11 day distal DA. Numerous microvilli. Cells beginning to elongate. Bar = 10 μm.
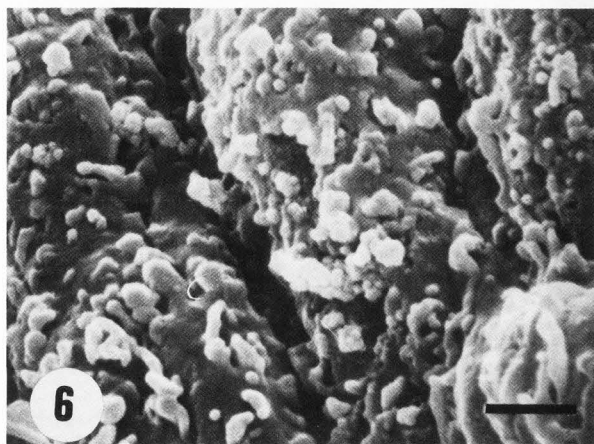

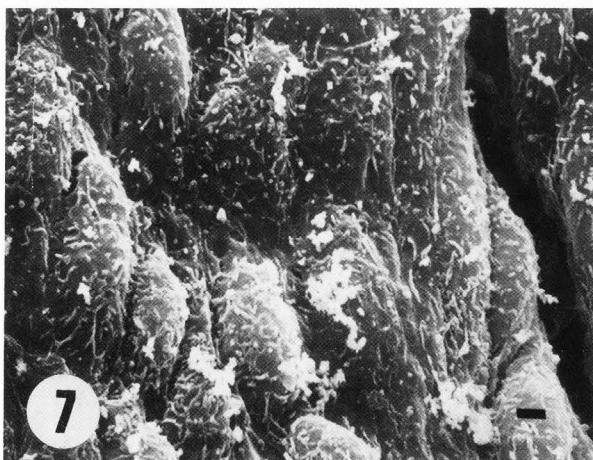Fig. 6. Surface of endothelial cells from Figure 5. Microvilli of varying size. Bar = 1 μm.

Fig. 7. SEM of intimal cells of 15 day distal DA, note reduction and lengthening of microvilli. Bar = 1 μm.

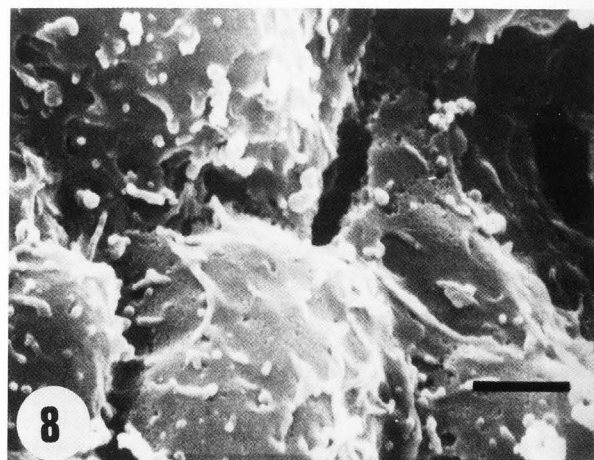

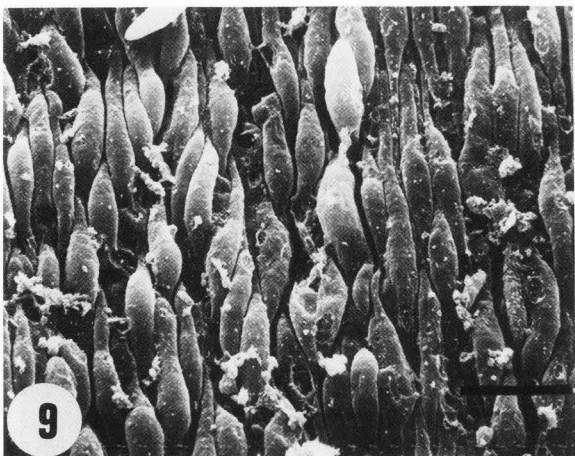Fig. 8. Surface of endothelial cell from figure 7. Bar = 1 μm.

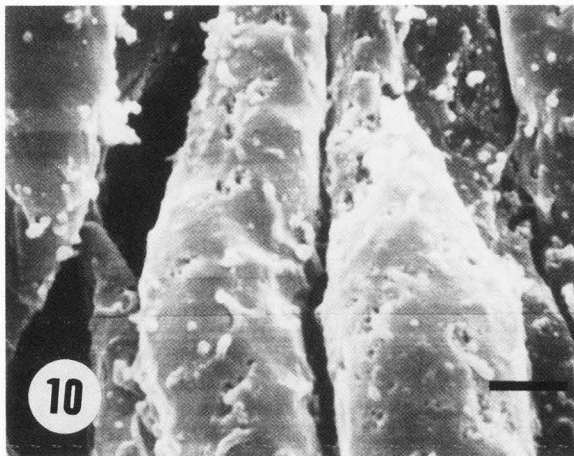Fig. 9. SEM of intimal cells from 18 day distal DA. Elongated cells with fewer microvilli. Bar = 10 μm.

Fig. 10. Surface of endothelial cell from Figure 9. Bar = 1 μm.
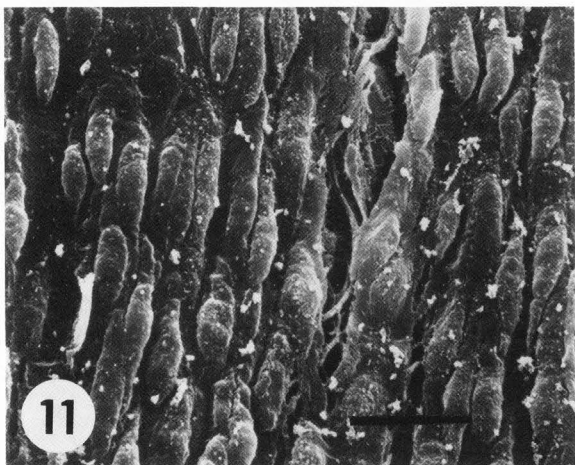

Fig. 11. SEM of endothelium of 16 day DA, proximal portion. Note paucity of microvilli. Bar = 10 μm.
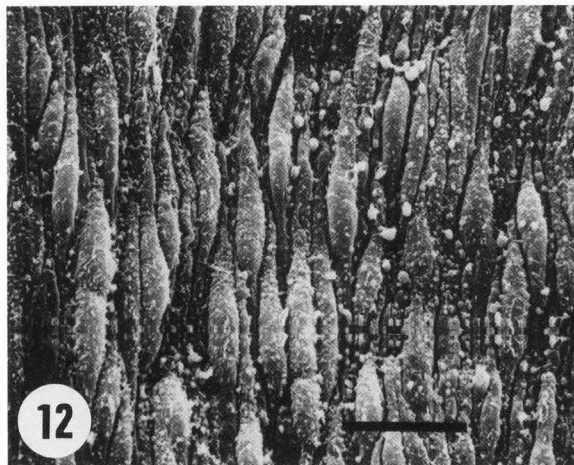

Fig. 12. SEM of endothelium of 16 day DA, distal portion. Note increased numbers of microvilli compared with Figure 11. Bar = 10 μm.

with those explants which remained flat (Fig. 16). Nine of twelve explants cultured from 14 day chick embryos contracted in EE_{20}-Tyrode's. All of these explants remained viable. In contrast none of the explants cultured in DMEM were contracted, but none survived.

All explants cultured from 17 day chick embryo dorsal aorta were contracted in the EE_{20}-Tyrode's, but remained viable. None of the explants were contracted in the DMEM, but none survived.

None of the explants cultured from eighteen day chick embryo contracted. Most of these explants demonstrated cell death or degeneration. Similarly, none of the explants from 18 day chick embryo cultured in DMEM survived.

TABLE I
Days of Embryonic Age of Dorsal Aorta Cultured

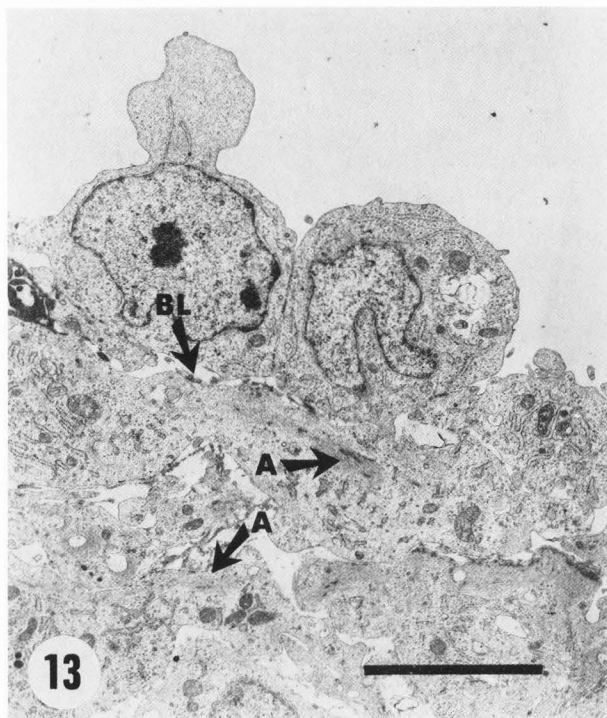| Medium used	5d	9d	14d	17d	18d
Tyrode's	8	10	12	25	7
survived	0	10	12	25	1
contracted/flat	--	6/4	9/3	25/0	0/1
DMEM	--	--	9	15	5
survived			0	0	0
Tyrode's & Insulin	--	--	10	--	--
survived			10		
contracted/flat			6/4		
DMEM & Insulin	--	--	5	--	--
survived			0		

Fig. 13. TEM of 5 day chick embryo DA. RER present in endothelial cells. Discontinuous basal lamina (BL ➡). Patches of microfilaments in media cells (A ➡). Bar = 5 μm.
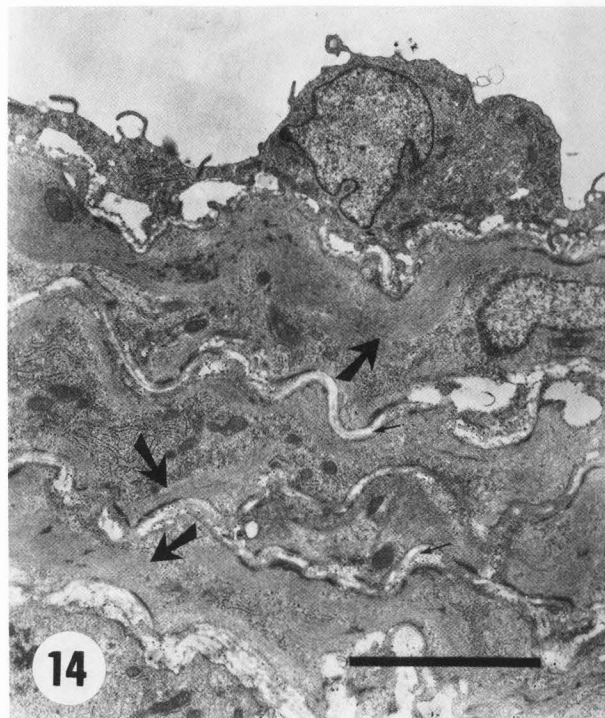
Fig. 14. TEM of 16 day chick embryo DA. Advance in differentiation of media cells. Increase in microfilament bundles (➡). Intercellular spaces (→). Bar = 5 μm.

Although insulin was added to the Tyrode's and to the DMEM media in the case of 14 day chick embryo there was no demonstrable effect as viewed by our methods of analysis.

Light Microscopy. Explants from all selected ages and each medium type were fixed and processed for study by light microscopy. Cross sections of the explants were viewed by hemotoxylin and eosin (H and E) and Masson's trichrome stains. Samples of the freshly harvested aorta were compared at the equivalent growth stage with the explants. The tunica media of the descending aorta from fresh-fixed 17 day chick embryo demonstrates very little staining of collagen by the Masson's trichrome method (Fig. 17). This appears consistent with the TEM analysis.

Scanning Electron-Microscopy. The luminal surface from all of the contracted or rounded up explants demonstrate no microvilli and no clear delineations between cells. Nevertheless this surface is intact and by SEM shows no degenerative changes (Fig. 18). Those explants which remained flat during incubation show typical endothelial cell surfaces (Fig. 19). However, these surfaces reveal reduced or absent microvilli (Fig. 20, compare with Fig. 6). In some instances the endothelial cells are separated producing cellular gaps or

spaces. Beneath the endothelial layer one can see uni-directionally aligned fibrous material draped over rugae. This layer could be subintimal elastic tissue or collagen draped over smooth muscle or potential smooth muscle cells (Fig. 21). In the cases of those explants which remain flat the edges show endothelial cells at various orientations to the long axis of the explant (Fig. 22).

Transmission Electron-Microscopy. The examination of the contracted explants by TEM confirms a viable endothelium virtually devoid of microvilli (Fig. 23), but which contains much rough endoplasmic reticulum and which is underlain with a discontinuous basal lamina. Some areas of endothelium resemble myofibroblasts with attendant microtendons (Fig. 24). In the case of an actively contracting or rounding up explant examined after one or two days of incubation the edge of the explant demonstrates by thin section several small profiles of cells, as if they are actively migrating across the wound edge (Fig. 25, compare with Fig. 22).

The cells of the media show good uni-directional alignment and are undoubtedly myoblastic, but at this time during embryogenesis they seem more fibroblastic in appearance (Fig. 26). These cells are compact
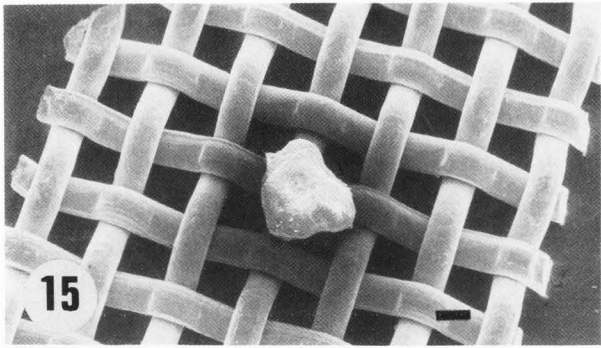
Fig. 15. SEM of 14 day explant contracted after 4 days of culture in EE_{20}-Tyrodes. Endothelium sill out. Bar = 200 μm.
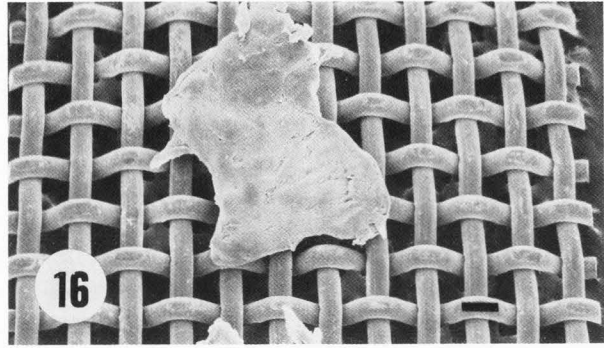

Fig. 16. SEM of 9 day explant still flat after 3 days in culture in EE_{20}-Tyrodes. Bar = 200 μm.
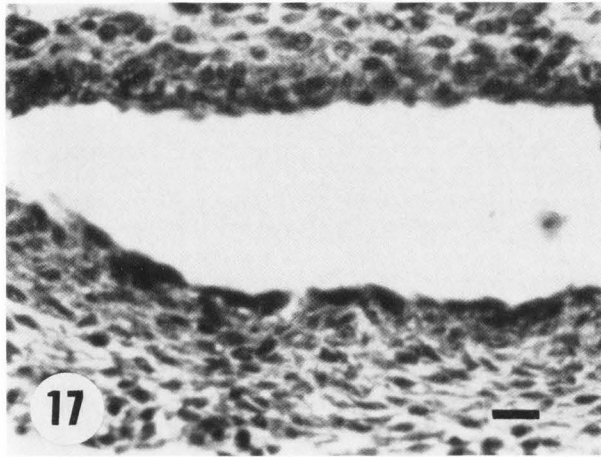
Fig. 17. Tissue section of 17 day DA freshly fixed from chick embryo. RBC caught in the lumen. Little or no collagen observed. Masson's Trichrome stain. Bar = 10 μm.
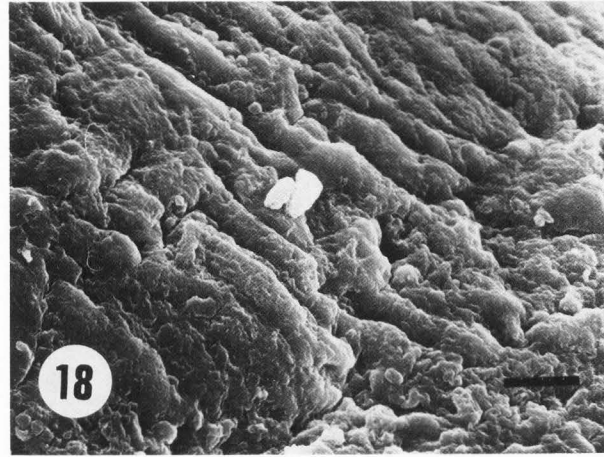
Fig. 18. SEM of intimal surface of 14 day explant contracted after 4 days of culture in EE_{20}-Tyrodes. No microvilli observed. Bar = 10 μm.

Fig. 19. SEM of 9 day explant cultured for 3 days in EE_{20}-Tyrodes. Intimal surface showing reduced microvilli and occasional rents and tears. Bar = 5 μm.

Fig. 20. Surface of endothelium from figure 19. Microvilli present but are reduced in size. Compare with figure 6. Bar = 1 μm.
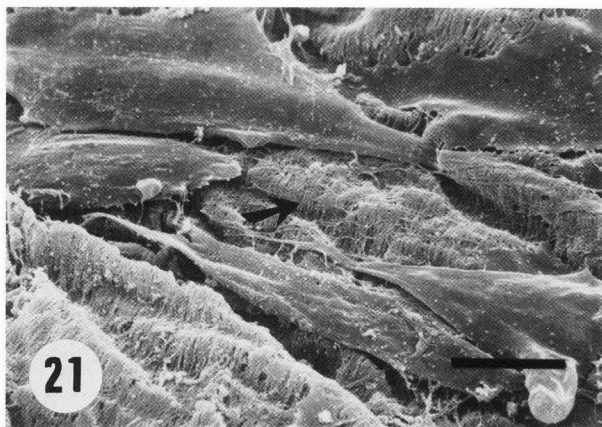
Fig. 21. SEM of flat explant from 9 day DA cultured in EE_{20}-Tyrodes. Gap in intimal surface (➡). Note ribbed appearance of subintimal layer. Bar = 10 μm.
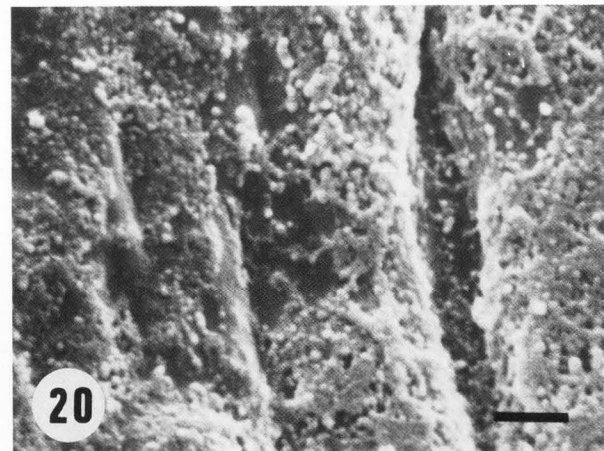

Fig. 22. SEM of edge of explant from 9 day DA grown in EE_{20}-Tyrodes for 3 days. Note some cells in transverse orientation to others. Bar = 10 μm.

Fig. 23. TEM of contracted explant from 14 day DA grown for 4 days in EE_{20}-Tyrodes. Endothelial cells with much RER. Incomplete basal lamina (➡). Bar = 5 μm.
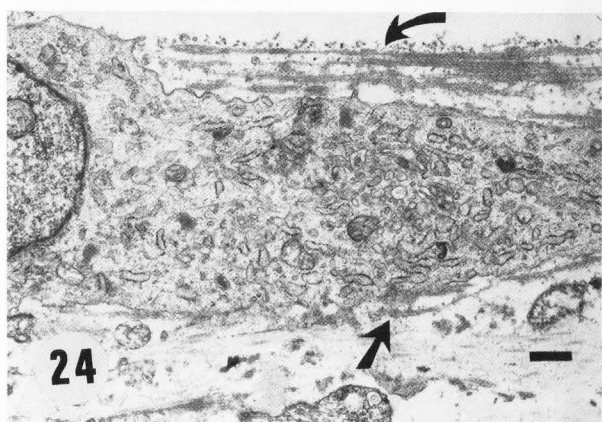
Fig. 24. TEM of endothelium from explant of 17 day DA grown in EE_{20}-Tyrodes for 3 days. Note microtendons of endothelial cells (➡) and basal lamina (➡). Bar = 5 μm.
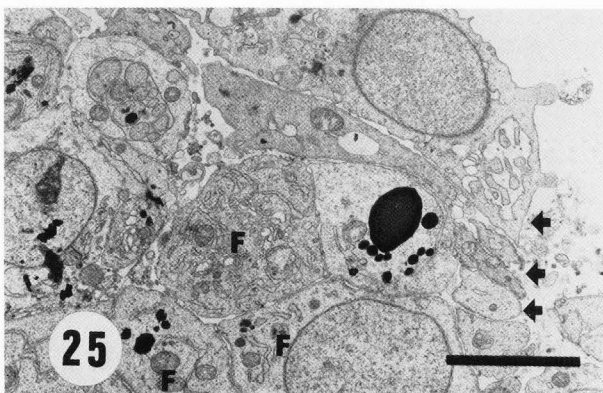
Fig. 25. TEM of edge (E) of contracted explant from 14 day DA grown in EE_{20}-Tyrodes for 3 days. Note many profiles of fibroblast-like cells (F), and presumed endothelial cells (◄). Compare with figure 22. Bar = 5 μm.
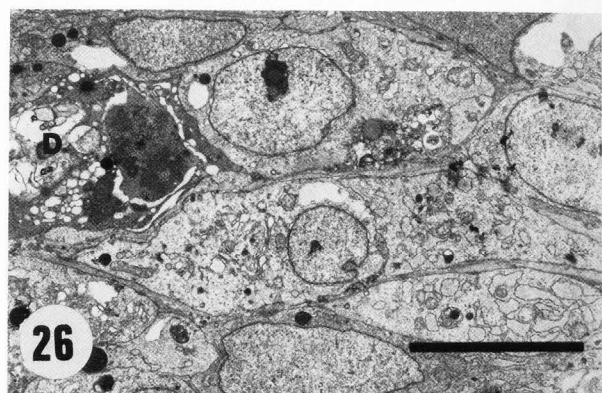
Fig. 26. TEM of media from 14 day DA grown in EE_{20}-Tyrodes for 3 days. Media cells show unidirectional alignment but no strong features of myocytes. Degenerating cells (D). Bar = 5 μm.

but collagen fibrils are usually observed where intercellular spaces are present.

Discussion

It is clear from this study that chick embryonic dorsal aorta (descending aorta) can be successfully organ cultured starting with embryonic day 9, by using a simple medium consisting of 20% chick embryonic extract and Tyrode's balanced salt solution. It is also clear that a specially designed medium such as DMEM is not only unsuitable but may contain ingredients which may be toxic to at least this embryonic tissue. A further observation indicates that addition of insulin to the culture medium neither improves nor compromises the maintenance or growth of the blood vessel in organ culture.

The fine structure study of the normal stages of the dorsal aorta was designed to provide a morphological basis by which to compare the morphology of the explants and assess viability. With the exception of the loss of microvilli on the surface the fine structure of the explants compares very favorably with the normal stages. Those explants which showed beginning contraction among the nine day age and those which were fully contracted from the 14 day age were judged to be from the distal portion of the dorsal aorta.

Among those viable explants which remained flat (9 and 14 days) a few areas of the intimal surface demonstrated intercellular spaces or gaps between the endothelial cells. A similar kind of phenomenon was observed by Buck (1977, 1979) in his organ cultures of descending thoracic aorta from young rats. Pexieder (1981) demonstrated intercellular openings in embryonic endocardium in several species, but states they have not been reported in SEM studies of typical blood vessels. The size of the reported openings are somewhat less than observed in this study, which were observed principally in the cultured vessels. A later study by Buck (1979) followed regenerative events after endothelial stripping of the intima. Apparently, denuded areas of the intima were not recovered by proliferation of new endothelial cells from previously persisting endothelial cells. Rather, the new covering of the intima appeared to arise by a proliferation of smooth muscle cells from the subendothelium. In the present study from the chick embryo we did not observe an indication that the intercellular spaces were being repopulated by any cell. However by transmission electron microscopy it appeared there was considerable proliferation of the cells in the tunica media. These cells were certainly not differentiated as yet into smooth muscle cells although some intracellular structures resembled patches of actomyosin filaments.

An unexpected result of this study was the rounding up of the explants from 9 through 17 days chick embryo age, much more pronounced at 14 and 17 days. Such a phenomenon has previously been observed in the case of organ culture of chick embryonic skin (Kischer, 1973). This phenomenon suggests that there should be a significant population of myofibroblasts within the intima and media of the vessel explant. Their identification is not difficult because their microtendons are easily identified (Ryan et al. 1974). Indeed, examination by transmission electron microscopy does show their presence but in numbers somewhat less than there should be to account for the extreme contraction observed. Therefore, we cannot account fully for this result at present but certainly our observations form the basis for further and more detailed studies.

The edge of the explant behaves as a wound edge in that we see profiles of many cells as though there is an active proliferation (see Figs. 22 and 24). This conforms to Buck's observations (1977) which described transverse oriented cells at the cut margins of explants.

The cells of the tunica media within the explant are capable of further differentiation, as suggested by the TEM findings. Additionally, many mitotic figures were observed amongst the media cells.

The maintenance of the endothelium and the demonstrated capacity for growth and differentiation of the tunica media of the chick embryo descending aorta explanted to organ culture suggests that this vessel may be a good candidate to explore further as a model for studies of organogenesis and possibly injury and repair to blood vessels. It is further suggested that the best age to explore more fully this possibility would be 9 days or perhaps somewhat younger.

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Discussion with Reviewers

T.J. Poole: DMEM is normally used with 5% carbon dioxide for buffering. Could the death of the explants be due to the basic pH of this medium when cultures are in air?
Authors: The DMEM used contained HEPES buffer and the bicarbonate component even though the cultures were not gassed. The medium also contained phenol red. The pH was 7.4, the same as for the Tyrode's medium. At no time up to the maximum of 4 days in culture did the red indicator change color.

T.J. Poole: Is the disappearance of microvilli in culture a normal consequence of aging or a response to culture conditions?
Authors: It appears to be both. As indicated by the normal stages, the number and size diminish and the shape changes as hatching approaches. Organ culture seems to accelerate their disappearance.

T.J. Poole: What makes the younger dorsal aortae more suitable for organ culture?
T. Pexieder: Is 8th/9th embryonic day really the best age for dorsal aorta explantation or is it a time period when it is for you

technically feasible?
Authors: From the standpoint of potential use as a model for vascular studies, the younger aged aortae would provide more stages of differentiation for study. Further, the younger stages do not contract as much as the older ones. This would allow for more manipulation in potential studies which would not be complicated by the contraction phenomenon. However, the younger stages are more difficult to dissect away from the embryo than the older ones because of a difference in their continuity with the posterior abdominal wall.

R. Hirakow: Have you some definitive morphological criteria to distinguish "fibroblast," "myofibroblast" and "myoblast"?
Authors: Traditionally, the myofibroblast cell type is distinguished from the fibroblast through electron microscopy. Both contain moderate to large amounts of rough endoplasmic reticulum; but, the myofibroblast also contains patches of contractile filaments laced with cytoplasmic dense bodies (similar to smooth muscle cells). Additionally, this cell demonstrates extracellular microtendons, apparently used for anchoring in the extracellular matrix. The myoblast contains actin and myosin filaments in increasing amounts in its course towards differentiation into a smooth muscle cell.

R. Hirakow: What are the "attendant microtendons"?
Authors: Microtendons were first described by Ryan et al. (1974, text reference). They were identified by electron microscopy as "basal lamina-like arrays of fine filaments," extending into the ground substance. Singer and Paradiso (Cell, 16:675-685, 1979) demonstrated these filaments were mediated by fibronectin with intracellular actin filaments.

R. Hirakow: When you removed the dorsal aorta from the embryo with beading needles, to what extent was the aorta injured by such manipulation? Did you check it?
Authors: All of our specimens were removed, cut and dissected with the beading needles. We had no evidence of persistent injury to the aorta because of this procedure. The only "injury" therefore, would be at the cut edges. Checks on persistent damage would have been done (and were the results of our procedures) by examining the normal stages and the cultured vessels by light, scanning and transmission microscopy.

T. Pexieder: Do you realize that the Karnovsky's fixative is strongly hypertonic and that, in the absence of osmium tetroxide postfixation, you did not abolish the osmotic reactivity of your tissues?
Authors: Karnovsky's fixative is, indeed, hypertonic, registering about 1100 milliosmols. However, this fixative has been used for many years and traditionally relied

upon for excellent fixation properties as well as for long term storage of tissues. It is quite stable in its properties. Some leaching of low molecular weight proteins, and perhaps small ions may occur while stored. However, the combination of paraformaldehyde and glutaraldehyde for maximal fixation is probably superior to any other fixative available, except for contemporary cryofixation procedures. In fact, postfixation with osmium tetroxide is often undesirable and may account for significant protein loss and altered morphology (see Hyatt, M.A., Fixation for Electron Microscopy, Academic Press, 1981, pp. 110-111 and 148-149).

T. Pexieder: Please give more details on the flattening of the explants and how you can keep them flat.
Authors: The explants are flattened by carefully touching the edges of the tissue, endothelium side up, to the bars of the stainless steel platform (the grid). This is all done through a sizeable drop of BSS transferred to the grid within which is the tissue. The edges will stick lightly with gentle pressure. Immediately, the drop of BSS is aspirated off with a disposable pipette. All of this procedure is done under the dissecting microscope.

T. Pexieder: Please comment on the abundance of swollen microvilli on your SEM micrographs.
Authors: Only one micrograph suggests swollen microvilli, figure 3. In fact, these microvilli are hardly swollen, being of a diameter virtually the same as those in other stages. However, they are round instead of elongated as in figure 8. In figures 13 and 14, both TEMs of surface endothelial cells, there are profiles of surface extensions of varying size and conformation. There are no obvious "swollen" characteristics to those cells or their extensions.

T. Pexieder: How did you take into account the age-dependent osmolarity variations of the chick embryo internal milieu (cf. Romanoff, 1967)?
Authors: The reference is to Romanoff's book, "Biochemistry of The Avian Embryo" (Interscience, New York). This book does not have osmolarity measurements, as such, listed. However, such items as freeze point depression of the whole embryo (not for the blood) and for other fluids, such as allantoic fluid, are listed and correlated with time of development. Since osmolality is plotted as a function of freeze point depression one can make reasonable assumptions pertaining thereto. Osmolality, therefore, would increase with age of the embryo. Additionally, protein concentration of blood increases with development. We did not control for an intrinsic changing osmotic property of the vessel wall with increase in embryonic age. How much osmotic "shock" the cells from different aged vessels might undergo while being exposed to the same culture medium is unknown. However, by the parameters tested, there does not seem to be significant evidence of osmotic differences.

T. Pexieder: In figures like figure 5 how can you differentiate between endothelial cell elongation and changes due to the vessel wall contraction?
Authors: Figure 5 is from a normal development stage. Thus, vessel wall contraction at this point would come about from contractile cells, such as smooth muscle cells, of which none are present at this stage.

T. Pexieder: The endothelial cells in Fig. 20 have the same morphology as that of necrotic endocardium from prostaglandin induced abortions (1981, text reference). Do you still think they are normal?
Authors: Our figure 20 does not show necrosis or erosions. It shows a reduction in size and fusion of microvilli. Therefore, it does not compare with figure 16a, page 243 of your article. In fact, many of our cultures in DMEM appeared similar to the necrotic endocardium you are referring to.