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A SIMULTANEOUS *EX VIVO* MODEL OF EMBRYOGENESIS : II. VASCULOGENESIS

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Abstract : Vasculogenesis was simultaneously studied with embryogenesis in *in ovo* chick embryo culture, which was harvested at 40 hours. Endodermal cells and vascular endothelial cells were studied using a new combination of stains, immunohistochemistry (for nuclei and basement membrane) and NADPH-diaphorase activity in whole-mounts, paraffin sections and etched semithin sections. The model can be used for the study of developmental process of blood vessels as well as embryonic physiology of blood vessels vis-a-vis organogenesis in response to different angiogenic agents, drug trials, cancer therapy by angiostatic chemicals/ radiations and toxins. Considering that vasculogenesis/angiogenesis as one of the fundamental phenomena in physiology, pathophysiology, toxicology and pharmacology of developmental sciences, the model in developing embryo is presented.

Key words : *in ovo* culture plastic sections NADPH-diaphorase whole mounts IHC endothelial cells

INTRODUCTION

The phenomenon of capillary formation has been considered as a basic event in reproduction, development, angiogenic diseases, tumour and repair. Embryonic microvasculature is so far studied in foetus or in neonates of chick and mammals for the study of neural transplant (1–5). Embryonic microvasculature in general has also been studied for various aspects in different animals in central nervous system (6–12). Literature survey reveals that the studies of embryonic microvasculature development were either carried out for the neural transplants/grafts or the late stage embryonic study of specific brain regions/ blood-brain-barrier. Hence in all these studies, development of vasculature was studied at the particular stage, when the initial capillary formation has already occurred. In fact, in neither of these studies, early development of capillaries (earliest possible stage along with organogenesis) has been studied. The present study was conducted to use chick embryo as a model for the development of the blood vessels vis-a-vis organogenesis. In an earlier article scopes and avenues of the organogenesis model have been presented (5a). The present article puts forward the morphological data of a (simultaneous) model for the earliest

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vasculogenesis, which can be utilized for a wide variety of studies. Positive technical aspects of the model are that it is easy to standardize, highly reproducible and allows a good range of experimental manipulations. It also resolves the ethical problems of handling big laboratory animals. Thus for the initial information regarding the phenomenon of early vasculogenesis vis-a-vis organogenesis the model can be readily utilized. The report suggests the use of the model at the preliminary stages of drug-trials, which can subsequently be tested in the mammalian system.

METHODS

Blood vessels harvested from 40 hours old *in ovo* cultured chick (Leg horn; *Gallus gallus*) embryos (the total number of embryos used for the standardization and experiment varied from 120–150). Time and conditions of cultures are as described in the preceding article. Blood vessels are fixed *in situ* using formalglutaraldehyde(4°C). In some experiments thicker vessels were microdissected out and perfused with fixatives for better results (especially for plastic section). In addition to routine H & E, a new combination of stains was tried on the extraembryonic blood vessels (as described in preceding article). For the paraffin sections, the blood vessels were mounted in a flat condition as well as rolled to get a consistency (whichever was suitable). Haematoxylin with either eosin, pyronin or PAS were used for staining. Semithin sections for light microscopy were done at the 1μ thickness in spurr or araldite (EM Sciences). Etching of plastic sections and subsequent staining were done as mentioned in the preceding article. Whole mount immunohistochemistry of post perfused and fixed blood vessels were done for laminin (polyclonal) and Ki 67 (monoclonal) (Dil 1 : 100; DAKO USA) using ABC kit (Vector Laboratories USA) with diaminobenzedine (DAB) (0.5 mg/ml of DAB and 0.01% H_2O_2) as chromogen. Details of the IHC procedure is mentioned in the preceding article (5a). Haematoxylin was used as counter stain. Whole mount NADPH-diaphorase staining was performed as described in the preceding article. Since zona opaca and zona pellucida are elliptical in shape, their area were determined from lesser and greater diameters (from camera lucida drawings) using Olympus microscope (camera lucida factor 9.9). Parameters determined are zona opaca area, zona pellucida area, zona opaca index (obtained from the ratio of zona opaca area and body length), zona pellucida index (obtained from the ratio of zona pellucida area and body length). Mean ± S.E.M. were used for the computation of data. Students 't' test was applied to get significance levels of the differences between means. To review the vascular map, photographs of the embryo were taken in cultured conditions after injecting 0.2% trypan blue subblastodermaly. DAB, NBT, NADPH, trypan blue and other AR grade laboratory reagents were procured from Sigma USA. Routine histological stains were obtained from the local sources. Molarity, percentage and the sources of other chemicals were mentioned in the preceding article.

RESULTS

Subblastodermal injections of 0.2% trypan blue to *in ovo* embryos show the circulation map at 40 hours of development

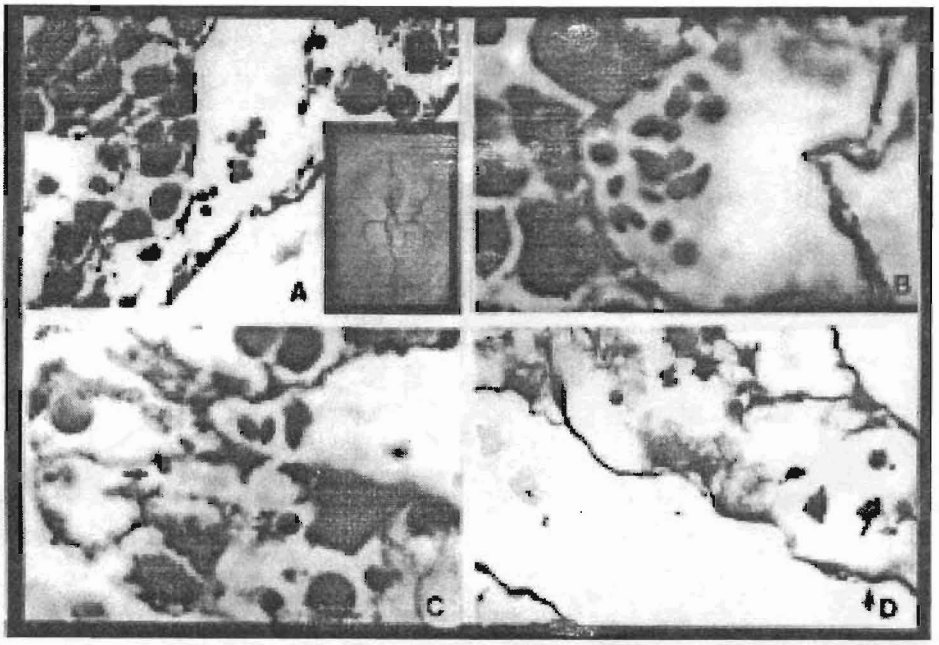


Fig. 1 : Photomicrograph of paraffin (A, B, C) and semithin plastic (D) sections of 40 hours old embryos stained using H & E (A), pyronin and H (B, D), and PAS and H (C). Photograph A [40 x10] and B [100 x 10] show zona pellucida (inset A : embryo in *in ovo*); similarly C [100 x 10]: zona opaca (arrow at empty fat droplets, open arrow at dark granular inclusions); D [100 x 10]: endothelium (arrow at the presumptive basement membrane).

(Fig. 1A inset). In the whole mounts of the blood vessels from 40 hour-old embryos two distinct regions are identified: (i) thin layers of vessels with serosal covering that immediately encircles the growing embryo. This region is zona pellucida. (ii) outside zona pellucida lies the thick-endodermal-cell-rich opaque zone of blood vessels called zona opaca. Zonae are elliptical in shape covering the zona vascularis, which embodies sinus terminalis. The extraembryonic tissue is made up of (i) inner endodermal cell layer immediate to yolk (ii) outer splanchnic mesoderm which differentiates into blood vessels, blood islands and mesenchymal cells (extraembryonic coelom lies between splanchnic and somatic mesoderm) and (iii) outer serosal layer.

With the new combination of stains the zona pellucida, zona opaca and their transitional zone are well-visualized (Fig. 1C and 1E, Ref. 1). The thinner vessels of zona pellucida are stained more differentially by indigocarmine while zona opaca blood vessels are more prominently lined by dark brown to red staining endodermal cells (Fig. 1E [arrow], Ref. 5a). Blood cells in clumps tend to take PAS positive colour. A typical transitional zone is shown in Fig. 1C (5a). Table I shows the parameters of the extraembryonic regions.

Blood vessels

The mesoderm-derived blood vessels around 40 hours of incubation contain

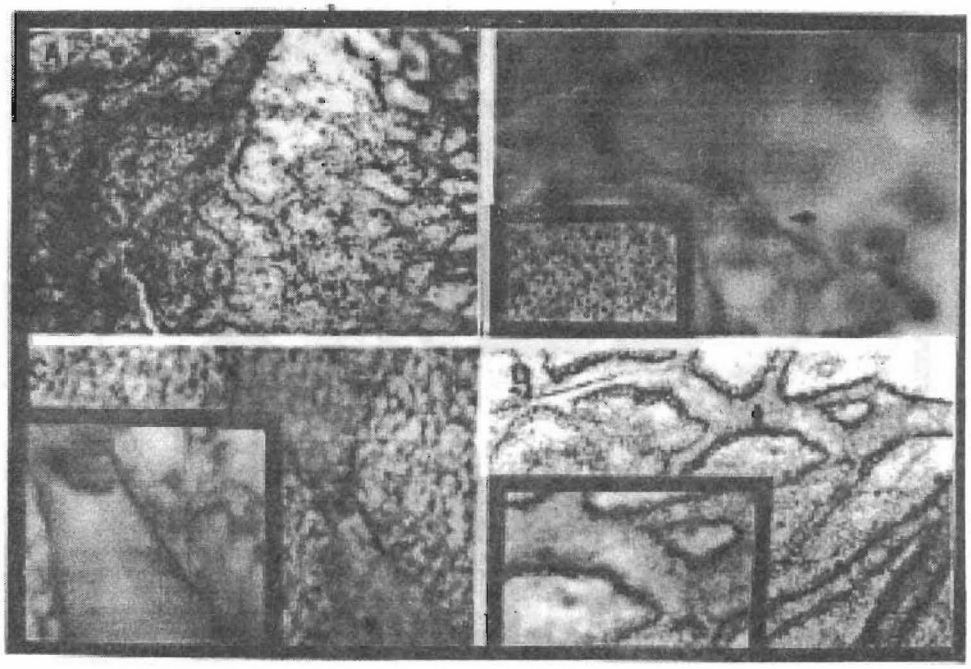


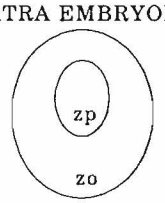
Fig. 2: Photomicrograph of whole mounts (A, B, C, D) of the embryos stained for NADPH-diaphorase activity (A), laminin antigen (B,D) and Ki 67 antigen (C). Photograph A [6.3 x 6.3] shows both zona opaca (arrow) and pellucida (open arrow); similarly B [100 x 10]: zona pellucida (arrow at the presumptive basement membrane deposition, inset [20 x 10]; C [40 x 10]: zona pellucida (arrow at Ki 67 positive nuclei, inset [100 x 10]); D [6.3 x 6.3]: zona opaca (arrow at laminin positive endothelium, inset [10 x 10]).

endothelial linings. In paraffin sections (Fig. 1A, 1B and 1C), blood cells are observed only inside the endothelial encasements. In semithin sections the spindle shaped endothelial nucleus and its thin film of cytoplasm is observed (Fig. 1D). Part of the serosal layer is seen on the other side of blood vessels. Presumptive basement membrane can be poorly identified at one area (Fig. 1D, arrow). However, the vessel is seen incompletely closed. Immunohistochemical staining for Ki 67 in whole-mounts shows endothelial cell nuclei as dots in low magnification (Fig. 2C, arrow). In high magnification (inset) they look like pearls (spindle shaped) in the string. In contrast, endothelium apears (when stained for laminin) like a continuous line in low magnification (Fig. 2D, arrow) as well as in high magnification (Fig. 2D, inset). However, in oil immersion objective (100x) laminin is shown to stain the presumptive basal lamina (Fig. 2B). Interestingly, the staining appears discontinuous (Fig. 2B, arrow). Inset of the same picture shows laminin staining in positive controls from different area of the same specimen.

Blood vessels associated endodermal cells

In zona pellucida, blood vessels appear characteristically thinner with cuboidal endodermal cells rich in PAS positivity. The endodermal cells have eccentric nucleus and histochemically highly reactive

TABLE I : Quantification of Extraembryonic regions from animals harvested at forty-hours of *in ovo* culture

Diagramatic representation	*Items*	*Description*	*Values Mean ± S.E.M.*
EXTRA EMBRYONIC  AREAS	Zona opaca (*Zo*)	Elliptical Area	82.64 mm^2±9.85 (25)
	Zona Pellucida (*Zp*)	Elliptical Area	17.38 mm^2±1.58 (25)
	Zona opaca Index	[*Zo**Bl*]	13.29 mm±1.01 (25)
	Zona Pellucida Index	[*Zp**Bl*]	2.785 mm±0.23 (25)

homogeneous cytoplasmic substances (that react with PAS, alcian blue, safranin, DAB, picric acid, toluidin blue, eosin, pyronin and Biebrich scarlet acid fuchsin). In paraffin sections, they appear strongly positive for most of the stains (results not shown). Figure 1A shows blood vessels of this area with adjacent endodermal cells containing the yolk drops. The characteristic feature of these cuboidal endodermal cells is that these are loosely arranged around blood vessels as compared to zona opaca (compare Fig. 1B & 1C) although they appear intensely dark stained. On the other hand, endodermal cells of zona opaca are columnar in structure (Fig. 1C) which are arranged tightly along the blood vessels. They contain fatty substances that give empty appearance in paraffin sections (Fig. 1C, arrow). The other featues of the cytosolic material of these cells are the presence of pleomorphic granular drops. In paraffin sections, conspicuous membrane bound dark granular structures are also seen in zona opaca endodermal cells (Fig. 1C, open arrow).

When both zonae are stained for the NADPH-diaphorase activity, they show certain histochemical differentiations. The diaphorase activity is seen in the opaca vessels as well as adjacent endodermal cells (Fig. 2A, arrow) in contrast to zona pellucida where vessels only are positive (Fig. 2A, open arrow).

DISCUSSION

Development of endothelium during embryogenesis occurs via both vasculogenesis (endothetial cells originating from the progenitor cell types) and angiogenesis (new capillary is born from existing vessels) in contrast to adults where angiogenesis is the rule (13). Mobbs and McMillan (14) found that in chick, the mesodermal layer differentiates into blood vessels when studied between stage 11 and 15 of Hamburger and Hamilton (15). Since stage 11 corresponds to 34 hours and stage 15 corresponds to 48 hours of incubation, and our study is restricted to the initial 40 hours of development, we consider vasculogenesis to be under operation (16). At this hour the primary circulation (extra embryonic circulation to yolk sac) is established (14, 17). However, like others we also found it difficult to identify arteries or veins except by the direction of the blood flow following the subblastodermal injection of dye (14).

The study of vasculogenesis has been done either on CAM or in shell-less cultures (18–22). Recently Hirashima et al studied the proliferation, differentiation and cell-cell adhesion of endothelial cell progenitors *in vitro* (23). In this study, the cellular events that occur during vasculogenesis have been studied using vascular endothelial-cadherin, platelet-endothelial cell adhesion molecule-1 and vascular endothelial growth factor.

Here we report the results of the study at 40 hours of development (between stage 11 and 15) which is earlier as compared to both CAM study (mostly starts from day 6) and shell-less cultures (starts from stage 15–23 corresponding to 2–3 days of incubation). For the zonae of vasculature, indices were chosen to be a better representation of body length of the embryos on which the areas of the zonae are dependent. The ratio of zonal areas (zona opaca area : zona pellucida area) is 4.75 which is comparable to the ratio of zonal indices (4.77).

The differences in the staining pattern of the zonae (using the new combination of stains) is attributed to the endodermal cells associated with the vasculogenesis. The high-stained character of zona opaca endodermal cells can be explained by their high content of cytoplasmic materials. Since the endoderm of avian yolk sac abuts directly on the yolk mass (nutrient store) and is primarily involved in transport of yolk to extraembryonic (as well as embryonic) circulation, the study of the endodermal cells associated to vasculogenic areas is justified (14). These cells contain both lipoid and protein materials (14). Yolk-granules and cells of zona opaca have low density lipid, phosvitin, α, β-lipovitellin, calcium, iron, phospholipid, phosphoprotein, polysaccharides which can explain and match their highly staining nature (including PAS positivity) with their function (actively absorbing region of yolk sac in contrast to zona pellucida (see 14, 16). Presence of empty appearance (vaculated) of endodermal cells in paraffin section in our study confirms the inclusion of lipid materials as observed by others (16). The respective cuboidal and columnar structure of zona pellucida and zona opaca endodermal cells with their pleomorphic/granular drops in this study are also in confirmation with another study that correlates their function (absorption) with morphological features (high endocytotic activity) (14). Since zonae are metabolically highly active areas, we studied NADPH-diaphorase activity in these regions. To our knowledge, we are the first to report diaphorase activity in early blood vessels and associated endodermal cells (zona opaca). It is interesting to note that the enzyme activity matches the metabolic status of zona opaca endodermal cells.

Proliferation is one of the characteristic features of blood vessel development (see 13). In order to know the status of the endothelial cells, blood vessels in whole-mount have been stained for Ki 67 which shows the proliferative nature of endothelial nucleus. Although the blood vessels in whole-mounts and paraffin sections mostly appear continuous, in a few areas of semithin sections, the vascular endothelium is discontinuous. However, we have not found such a discontinuity as often as Mobbs and McMillian (14). Since basal lamina is the important component of basement

membrane formation in capillary endothelium, we stained the whole-mount of vessels for laminin. Antisera for Ki 67 and laminin were used in chick because of their highly conserved nature as mentioned by several authors (24–26). Laminin antibody has already been used for immunoreactivity in chick embryos by Ribatti et al (27). In addition, both positive and negative controls were run during standardization and experiments (28–29). Negative controls were run using buffer in place of primary antibody in positive control slides and the test samples.

In oil immersion objectives, the laminin staining appears incomplete and discontinuous as observed by others (14). In fact, the basal lamina is better observed in laminin stain than semithin sections. Allen and Wilson ultrastructurally identified the lack of basement membrane in 3 day old area vasculosa vessels, a capillary character similar to the tumour associated angiogenesis (22). Our results of incomplete and discontinuous laminin deposition as well as its rare and poor morphological appearance in the presumptive basement membrane area (in semithin sections) explains the similar lack of basement membrane encouraging such a model for use in study of tumour associated angiogenesis.

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