The Chick Embryo Chorioallantoic Membrane in the Study of Angiogenesis and Metastasis

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Contents

Introduction

As pointed out by Auerbach in 1991 “Perhaps the most consistent limitation to progress in angiogenesis research has been the availability of simple, reliable, reproducible, quantitative assays of the angiogenesis response.”

In vitro angiogenesis assays, based on endothelial cell cultures or tissue explant, focus on isolated endothelial cell functions (e.g., endothelial cell proliferation, migration, or invasion) and do not examine the coordination of cell functions required for a successful angiogenic response (Jain et al., 1997; Auerbach et al., 2000).

Although in vitro angiogenesis assays have been useful for identification of potential molecular targets to block endothelial cell responses and preliminary screening of novel pharmacological agents, they frequently cannot be correlated with in vivo angiogenesis measurements. This is most likely the result of the complex and multiple cellular mechanism evoked during new blood vessel formation in vivo. In vitro assays cannot be considered conclusive and the activity of a compound must be confirmed in other assays of increasing complexity, including in vivo assays of angiogenesis, angiogenic-dependent tumor growth, and metastasis.

In vivo angiogenesis assays examine the entire spectrum of molecular and cellular processes. However, these in vivo assays are not only expensive and technically difficult to perform but also require substantial amounts of test compound and mostly rely on selective morphometric analysis for quantification (Jain et al., 1997; Auerbach et al., 2000). Because of these limitations, current drug development strategies for identification and testing angiogenesis inhibitors depend principally on the use of in vitro systems.

Currently, novel angiogenesis-targeted therapies lack in vivo screening models suitable for objective, quantitative preclinical testing, making it difficult to obtain a dose–response analysis and estimate therapeutic doses before initiating clinical trials.

The development of inhibitors of angiogenesis relies on a range of preclinical assays that mimic the various steps of the angiogenic cascade. Knowledge of the mechanism of action of the tested compound will dictate the choice of assay. Alternatively, the behavior of the compound in different assays may indicate the mechanism of action.

In vivo assays are usually unsuitable for the quantitative screening of a large number of compounds, as they are often complex, expensive, and may require specific surgical skills. Nonetheless, they are always required to confirm ultimately the activity of a potential drug.

The classical assays for studying angiogenesis in vivo include the hamster check pouch, the rabbit ear chamber, the rodent dorsal skin and air sac, the iris and avascular cornea of the rodent eye (Ribatti and Vacca, 1999), and the chick embryo chorioallantoic membrane (CAM). Several models have been introduced, including subcutaneous implantation in rodents of various three-dimensional substrates, including a polymer sponge (Andrade et al., 1987); Matrigel, a laminin-rich mixture of basement membrane components (Passaniti et al., 1992); and a polyvinyl alcohol foam disk covered on both sides with a Millipore filter (disk angiogenesis system) (Fajardo et al., 1998). Finally, zebrafish (*Danio rerio*) embryos may represent a suitable model to study the mechanisms of angiogenesis and angiosuppression during development (Nicoli and Presta, 2007).

The CAM of the developing chick embryo is an extraembryonic membrane mediating gas and nutrient exchanges until hatching. The main function of the CAM is to serve as the respiratory organ for the embryo. It also plays a role in the storage of excretions, electrolyte transport (sodium and chloride) from the allantoic sac, and mobilization of calcium from the shell to start bone mineralization.

Since the CAM has a very dense capillary network, it is commonly used to study in vivo both angiogenesis and antiangiogenesis in response to different factors. During 2000–2009, over 700 publications have used the chick embryo CAM as a model system to study angiogenesis and antiangiogenesis (NCBI, Pub Med). The CAM, particularly that of the White Leghorn, is the most widely used. The CAM of the Japanese quail has also been used. The quail-derived endothelium expresses a unique marker which can be identified using the QH1 antibody.

Chapter 1
Chorioallantoic Membrane Vasculature

1.1 Chorioallantoic Membrane and Its Embryological Origin

Chick embryo development lasts 21 days before hatching. There are four extraembryonic membranes of the chick: the yolk sac, the amnion, the serosa, and the allantois. The serosa of the chick is occasionally called chorion; the term chorion, however, is more frequently applied to the composite layer formed by the fusion of the allantois and the serosa.

The allantois of the chick embryo appears at about 3.5 days of incubation as an evagination from the ventral wall of the endodermal hind gut. During the fourth day, it pushes out of the body of the embryo into the extraembryonic celom. Its proximal portion lies parallel and just caudal to the yolk sac. When the distal portion grows clear of the embryo it becomes enlarged. The narrow proximal portion is known as the allantoic stalk and the enlarged distal portion as the allantoic vesicle. Fluid accumulation distends the allantois so that its terminal portion resembles a balloon in entire embryos.

The allantoic vesicle enlarges very rapidly from the fourth to the tenth day of incubation. In this process, the mesodermal layer of the allantois becomes fused with the adjacent mesodermal layer of the chorion to form the CAM. A double layer of mesoderm is thus created: its chorionic component is somatic mesoderm and its allantoic component is splanchnic mesoderm. In this double layer an extremely rich vascular network develops and is connected with the embryonic circulation by the allantoic arteries and veins. Immature blood vessels, lacking a complete basal lamina and smooth muscle cells, scattered in the mesoderm grow very rapidly until day 8 and give rise to a capillary plexus, which comes to be intimately associated with the overlying chorionic epithelial cells and mediates gas exchange with the outer environment. At day 14, the capillary plexus is located at the surface of the ectoderm adjacent to the shell membrane. Rapid capillary proliferation continues until day 11; thereafter, the endothelial cell mitotic index declines rapidly, and the vascular system attains its final arrangement on day 18, just before hatching (Ausprunk et al., 1974).

The allantoic (umbilical) artery after emerging from the embryonic abdominal wall branches into two primary chorioallantoic arteries and the CAM is drained by

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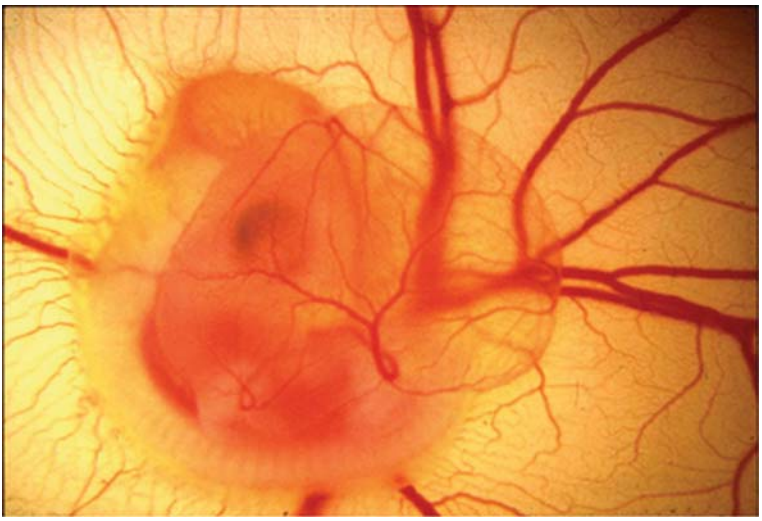

Fig. 1.1 Allantoic sac of a 5-day embryo showing in ovo distribution pattern of allantoic vessels (reproduced from Ribatti, 2008)

a single chorioallantoic vein (Fig. 1.1). The allantoic artery passes out along the stalk of the allantois to the inner wall of the allantoic sac, where it divides into two strong branches, one running cephalic and the outer caudal to the margins of the sac where they pass over to the outer wall. The allantoic vein runs in the inner wall and passes over the CAM near to the sero-amniotic connection. Fuchs and Lindenbaum (1988) described six or seven generations of branches of the allantoic artery. The first five or six are located in a plane parallel to the CAM surface and deep to the vein, which has a similar distribution. The fifth and sixth generations of blood vessels change the direction, passing almost vertically in the two-dimensional capillary plexus. In the outer wall the arteries and veins branch and interdigitate in the deeper portions of the mesoderm, and end in an extraordinary fine-meshed capillary network interspersed with the ectodermal cells (Fig. 1.2).

From day 6 to day 14, the third-order vessels do not increase significantly in number, while the number of first- and second-order vessels is increased. Moreover, between day 6 and day 10 intercapillary distances are substantially reduced, while between days 10 and 14, they remain constant. Finally, the average length of the first-, second-, and third-order microvessels are significantly reduced by day 14: this finding is consonant with the interpretation that consecutive branching of respective vessel order might serve to increase the total number of vessels, while simultaneously the length of each microvessel with the expanding network decreases (De Fouw et al., 1989).

This circulation and the position of the allantois immediately subjacent to the porous shell confer a respiratory function on the highly vascularized CAM. Three-dimensional analyses of the CAM microcirculation have revealed an architecture

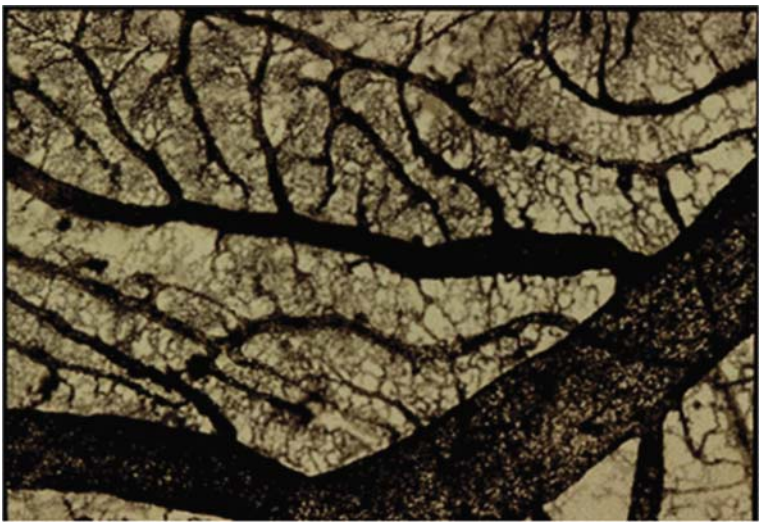

Fig. 1.2 A macroscopic picture of the CAM vascular tree after intravenous India ink injection at 14 days of incubation. Note the extreme complexity of the vascular architecture (reproduced from Ribatti, 2008)

similar to that of the mature pulmonary microvasculature (Fuchs and Lindenbaum, 1988).

In addition to the respiratory interchange of oxygen and carbon dioxide, the allantois also serves as a reservoir for the waste products excreted by the embryo, mostly urea at first and chiefly uric acid, later. CAM is also involved in the mobilization of calcium from the shell to start bone mineralization.

1.2 Morphology of Chorioallantoic Membrane Blood Vessels

On day 4, all CAM vessels have the appearance of undifferentiated capillaries. Their walls consist of a single layer of endothelial cells lacking a basal lamina (Ausprunk et al., 1974). Between days 4.5 and 5.0 CAM microvascular endothelial cells are thicker, have few plasmalemmal vesicles, and contain large vacuoles (Fig. 1.3) (Rizzo et al., 1995a).

By day 8, the CAM displays small thin-walled capillaries with a luminal diameter of 10–15 μm beneath the chorionic epithelium and other vessels with a diameter of 10–115 μm in the mesodermal layer, whose walls have a layer of mesenchymal cells surrounding the endothelium and are completely wrapped by a basal lamina together with the endothelial cells (Ausprunk et al., 1974).

On days 10–12, the capillaries resemble those in the 8-day membrane and are now near the surface of the chorionic epithelium (Fig. 1.4). The capillary walls remain simple in structure, containing endothelial cells and a few mesenchymal cells