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Spatial distribution of blood vessels in the chick embryo chorioallantoic membrane

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ABSTRACT The chick embryo chorioallantoic membrane (CAM) is a useful tool with which to study both angiogenesis and anti-angiogenesis in vivo. CAM vascular growth pattern - including the way through vessels fills the available space - can be quite easily described and quantified using image analysis procedures, in order to evaluate different parameters, including fractal dimension, lacunarity and non-fractal order-disorder parameters. In the present study, we further expanded this morphological description, by estimating an index expressing the degree of symmetry characterizing the CAM vascular tree structure in the course of the embryonic development. Moreover, a uniformity index was estimated quantitatively to characterize the space-filling features of the vessels, i.e. the degree of spatial uniformity of their distribution in the tissue.

KEY WORDS: *Angiogenesis, chorioallantoic membrane, spatial distribution vascular growth, vascular morphogenesis*

Introduction

Embryonic blood vessels patterns are characterized by the combination of deterministic and random processes. The chick embryo chorioallantoic membrane (CAM) is formed on embryonic day 3-4 (ED 3-4) by the fusion of the chorion and the allantois, and consists of three layers: ectoderm (from the chorion), mesoderm, and endoderm (from the allantois) (Ribatti *et al*., 2001). By ED16, the CAM has become so large that it covers most of the yolk sac, and is closely pressed against the shell membranes, enabling it to act as a gas-exchange organ, receiving oxygen and eliminating carbon dioxide through the pores in the shell (Romanoff and Evans, 1960).

During the development of the vasculature in the CAM at ED4, undifferentiated vessels are scattered in the mesoderm and grow rapidly until ED8, at which time primary vessels differentiate into an artery-venous system and a capillary network at the base of the chorion, which mediate gas exchange with the outer environment (Ribatti *et al*., 2001).

Six or seven generations of branches of the allantoic arteries are located in a plane closely parallel to the CAM undersurface, and deep to the location of the allantoic vein, which has a similar distribution (Fuchs and Lindenbaum, 1988). The 5th and 6th generation of blood vessels change direction, passing vertically in the two-dimensional capillary plexus. According to Schlatter *et al.,* (Schlatter *et al*., 1997), CAM vascularization undergoes three phases of development, with both sprouting angiogenic process and non-sprouting angiogenic process or intussusceptive microvascular growth (IMG). In the early phase (ED 5-7), multiple capillary sprouts invade the mesenchyme, fuse and form the primary capillary plexus. During the second (intermediate phase) (ED 8-12), sprouts are no longer present, since they have been replaced by tissue pillars, with a maximal frequency at ED 11. During the late phase (ED 13 and older), the growing pillars increase in size to form inter-capillary meshes more than 2.5 μm in diameter. Intravascular casting, coupled with serial sectioning for light and electron microscopy, and demonstration of pillars or use of confocal laser scanning microscopy, have provided indisputable evidence of the presence of trasluminal pillars, a typical morphological expression of IMG (Djonov *et al*., 2000).

Fibroblast growth factor-2 (FGF-2) is present in elevated amounts in the CAM from ED 6 to ED 18, maximal concentrations being observed between ED 10 and ED 14. Moreover, neutralizing antibodies to FGF-2 inhibit vessel growth, confirming a role of FGF-2 in vascularization of the CAM (Ribatti *et al*., 1995). Baum *et al.,* (Baum *et al*., 2010) reported an endogenous expression of vascular endothelial growth factor-A (VEGF-A) with two peaks at

Abbreviations used in this paper: CAM, chorioallantoic membrane; ED, embryonic day; FGF-2, Fibroblast growth factor-2; IMG, intussusceptive microvascular growth; TGFβ1, transforming growth factor beta 1; VEGF-A, vascular endothelial growth factor-A; VEGFR-2, VEGF receptor-2.

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ED 8–9 and 11–12. We have found two peaks in VEGF-A expression at ED 7 and ED 18 (Marinaccio *et al.*, 2013). By means of an *in situ* hybridization study, we have demonstrated that between ED 12 and ED 15, VEGF mRNA is expressed by chorionic epithelial cells, whereas VEGF receptor-2 (VEGFR-2) mRNA is expressed by endothelial cells of the capillary plexus beneath the chorion and by the endothelial cells of the mesodermal vessels (Ribatti *et al.*, 2002). A high expression level of hypoxia inducible factor 1 alpha (HIF-1α), VEGF and VEGF receptor-2 (VEGFR-2) at ED 11 correlates with a peak in angiogenic process at this stage (Baum *et al.*, 2010, Makanya *et al.*, 2016, Marinaccio *et al.*, 2013).

From the morphological standpoint, two specific aspects merit particular consideration. A first question is how the network grows during the angiogenic process. In this respect, vessel branching and network topology and complexity are significant features (Guidolin *et al.*, 2021, Kopylova *et al.*, 2018). A second question concerns the way the pattern of vessels fills the available space. This feature, indeed, contributes significantly to a better understanding of tissue perfusion and therefore of vascular network efficiency.

To devise morphometric methods aimed at providing a quantitative description of the aforementioned structural features of vascular morphogenesis, we previously investigated, by means of automatic image analysis procedures, different parameters, including fractal dimension, lacunarity, and non-fractal order-disorder parameters, including positional, topological and orientation order in the CAM vasculature (Guidolin *et al.*, 2004a). Overall, these data allowed us to describe the level of structural complexity exhibited by the vascular network in the CAM during development or following treatment with stimulatory or inhibitory factors (as, for instance, transforming growth factor-beta 1 (TGF-β1) or angiostatin respectively). In the present study, we further expanded this morphological description, by estimating an index expressing the degree of symmetry characterizing the CAM vascular tree structure in the course of the embryonic development. Moreover, a uniformity index was estimated to provide quantitative characterization of the space-filling features of the vessels, i.e. the degree of spatial uniformity of their distribution in the tissue.

Results

The values of the evaluated morphometrical parameters are provided in Table 1. In the considered time interval, the vascular tree significantly increased in size, as shown by the percent area it occupies in the tissue. Furthermore, an increase of about 56% in the uniformity index was observed, indicating a tendency of the vessels to assume a uniform spatial distribution in the tissue. This structural rearrangement appeared to be associated with an increased complexity of the vascular tree, as suggested by the fivefold increase observed in the number of branching points per squared millimeter and the significant increase exhibited by the fractal dimension. As indicated by the time course of the symmetry index, such a growth process seemed to lead to a geometry with an increasing presence of symmetry features.

TABLE 1

MORPHOMETRIC PARAMETERS

Parameter	ED7 (N= 20)	ED11 (N= 20)	ED15 (N= 20)
Area%	11.2 ± 0.2	21.4 ± 7.1	25.8 ± 1.5
Uniformity index (UI)	0.25 ± 0.02	0.35 ± 0.02	0.39 ± 0.04
Fractal dimension (D)	1.191 ± 0.006	1.397 ± 0.023	1.506 ± 0.029
Branching points (N/mm^2)	1.30 ± 0.08	3.09 ± 0.08	8.29 ± 0.54
Symmetry index (S_i)	0.605 ± 0.002	0.734 ± 0.023	0.827 ± 0.001

Values are mean ± sd

The order-disorder parameters proposed in this study permitted a more detailed description of the changes in the spatial arrangement of the vascular tree. With the aim of utilizing these parameters to verify their changes in experimental conditions, we conducted a preliminary study, testing in the CAM assay a classic pro-angiogenic factor - FGF-2 - and a classic anti-angiogenic factor – vinblastine - in the CAM assay, as previously described (Ribatti *et al.*, 1995, Vacca *et al.*, 1999). The preliminary results have demonstrated an increase of about 80% in the uniformity index after FGF-2 treatment with FGF-2, and a decrease of 35% after treatment with vinblastine.

Discussion

The avian CAM represents a useful tool for studying both angiogenetic and anti-angiogenetic processes (Guidolin *et al.*, 2004a). Indeed, it allows both intravascular and topical administration of factors and agents. Furthermore, it is a relatively rapid assay and can be adapted quite easily to study angiogenesis-dependent processes, such as tumor growth (Makanya *et al.*, 2015).

The morphology of the blood vessel pattern in the CAM during normal development and after application of factors or drugs can also be quite easily quantified by morphometric techniques and by computer-assisted image analysis methods. In this respect, basic parameters, such as vessel length density and endothelial proliferation intensity, were shown to be capable of capturing significant aspects of the effect of growth factors on the control of the local vascular development (Kurz *et al.*, 1995, Kurz *et al.*, 1998). Analysis of the network architecture based on the estimate of the number of branching points per unit area was used as an accurate measure of vascular network change through sprouting angiogenesis (Zudaire *et al.*, 2011) and, similarly, estimation of the pillar density provided an indication of vascular growth through intussusception (Makanya *et al.*, 2007). Although the growth of vessels does not seem to be fractal (Kurz *et al.*, 1998), fractal dimension is considered as a useful statistical index characterizing the complexity of the vascular network architecture in studies performed by using the CAM assay (Kirchner *et al.*, 1996). Fractal dimension alone, however, may not fully characterize all the aspects of the vascular bed complexity. As pointed out by Smith and collaborators (Smith *et al.*, 1996), a given value of D does not uniquely specify a structure morphology, and very different looking structures can have the same or very similar fractal dimension. For this reason, parameters capturing the level of order/disorder of the spatial arrangement of the investigated pattern have been suggested to integrate the information provided by the fractal dimension (Guidolin *et al.*, 2004a). In this context, a question of particular interest is how the network architecture forms during growth. Two structural aspects can be considered in this regard, namely how a main trunk divides into smaller branches, and how the branching occurs at different distances from the root point of a vascular tree. Morphometric approaches addressing this point were recently proposed by our group (Guidolin *et al.*,

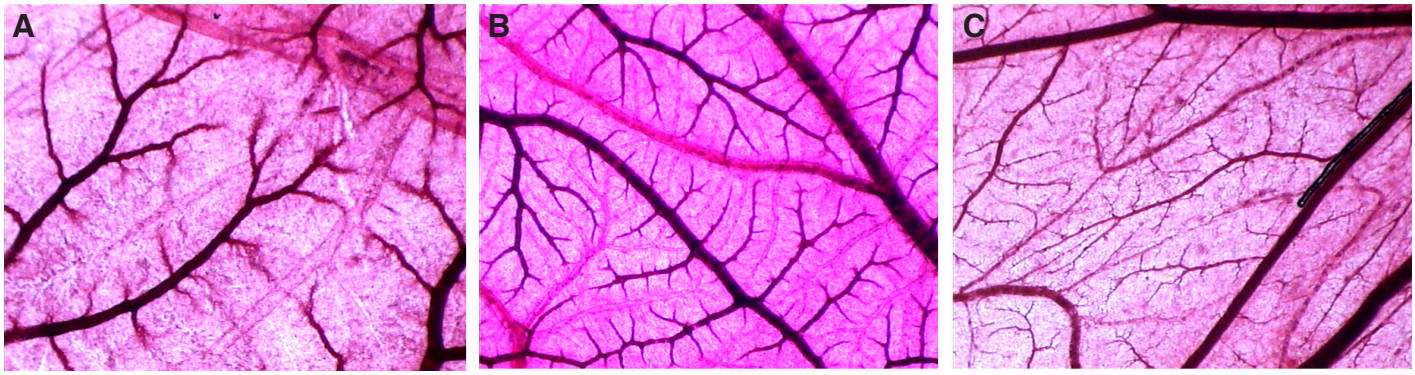

Fig. 1. Macroscopic features of the CAM vascular tree stained with paracarminium at the three developmental stages (A, ED7; B, ED11; C, ED15) investigated in this study. *Note the progressive increase in the complexity of the vascular architecture.*

2021), to allow a description not only of the overall structure of the vascular network (total length, number of vascular branches and branching points density), but also of the modality of vessel ramification, and of the spatial arrangement of the growing vascular tree (Guidolin *et al.*, 2020).

Morphological features receiving less attention in the analysis of a vascular network architecture are its symmetry properties. Mathematical models and simulations, however, suggest that the symmetry properties of a vascular tree may significantly affect not only the topology of the vasculature as a whole, but also its functional features (Kopylova *et al.*, 2018). In the present study, a possible computer-assisted image analysis approach to explore the presence of symmetries in CAM vessels has been proposed. It is based on a recent theoretical development by Bormashenko and collaborators (Bormashenko *et al.*, 2019) and provides a "symmetry index" indicating the overall degree of symmetry of the analyzed pattern. The suggested procedure, however, does not enable the identification of the single types of spatial symmetry characterizing the network, and this constitutes a major limitation of the method.

A characteristic with a major effect upon the functional properties of a vascular tree is the way it fills the available space, since this feature determines tissue perfusion. A parameter derived from fractal geometry, known as 'lacunarity' (Guidolin *et al.*, 2004a), has been suggested to provide this type of information. In a general sense, indeed, it can be considered as a measure of non-uniformity (Smith *et al.*, 1996), potentially useful to detect situations in which irregular spatial distribution of the vascular pattern occurs as a response to angiogenic or anti-angiogenic stimuli. Alternative proposed procedures (see (Guidolin *et al.*, 2021) for details) are based on methods derived from the field of point pattern statistics (Gelfand *et al.*, 2010). The distribution of the distances between branching points can indeed be used for testing the randomness of the spatial pattern they generate and the deviations of the spatial distribution of points from randomness. This topic is also addressed by the "uniformity index" explored in the present study as a tool to describe the spatial uniformity of the vessel distribution in CAM tissue. Based on conventional morphological operations usually available in image analysis software, it may represent a relatively simple method of characterizing this important morphological feature exhibited by a vascular pattern.

Materials and methods

Thirty fertilized White Leghorn chick eggs, staged according to Hamburger & Hamilton (HH) (Hamburger and Hamilton, 1951) stages, were incubated from the start of their embryogenesis in an incubator under conditions of constant humidity at a temperature of 37 °C. At ED3, a square window was opened in the egg shell after removal of 2–3 mL of albumen, in order to detach the developing CAM from the shell. The opening was closed with a glass of the same dimension as the square and the incubation continued. At ED 7, 11, and 15 of incubation, five CAM from each stage and from different eggs were cut, fixed, completely stained with paracarminium, and embedded in paraffin. Ten μm histological sections from each stage were cut according to a plane parallel to the CAM surface, mounted on a slide, photographed with an Olympus stereomicroscope and morphometrically analyzed.

Image analysis methods

Images obtained at different time steps (Fig. 1) underwent computer-assisted image analysis procedures to evaluate the morphological changes exhibited by the vascular tree during development. The Image J software, freely available at http://rsb.info.nih.gov/ij (Schneider *et al.*, 2012), was used to perform the analysis.

An example of the image analysis procedure is herewith reported. Briefly, after shading correction and contrast enhancement, an adaptive discrimination procedure (see [(Russ, 2011)), operating with a local threshold, was applied to select vessel profiles virtually exclusive of all background (Fig. 2 A). By using binary thinning procedures (Seul *et al.*, 2000) the skeleton of this image was then derived (Fig. 2 B) and pruned to remove eventually present small art factual branches. Vascular density was estimated from the first binary image by evaluating the area fraction covered by the vascular tree, while the binary skeleton was further processed to identify the points of branching (Guidolin *et al.*, 2004a, Russ, 2011). Their (x, y) coordinates were used to derive global indices of uniformity in space occupancy, morphological complexity and structural symmetry.

To characterize the degree of uniformity in space occupancy exhibited by the vascular tree, a uniformity index can be estimated according to a previously described procedure (Guidolin *et al.*, 2009). This index can be calculated from the coordinates of the branching points and can have any value between 1 (when the objects are distributed in a regular array) and 0 (when maximal clustering occurs).

To globally describe the morphological complexity in quantitative terms, the fractal dimension (D) can be a valuable parameter (Guidolin *et al.*, 2004b). It measures the rate of addition of structural detail with increasing magnification, scale, or resolution (Cutting and Garvin, 1987). D of the binary skeleton was estimated using the "box counting" method at multiple origins as indicated by Smith *et al.,* (Smith *et al.*, 1996).

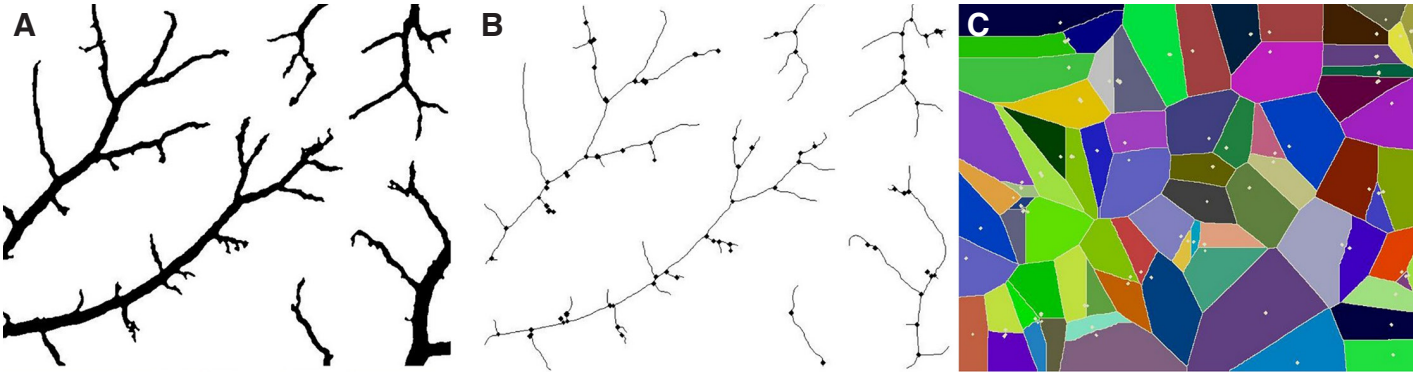

Fig. 2. Schematic illustration of the image analysis procedure. (A) *Binary image of the vascular tree. This image was used to estimate the area percentage covered by vessels.* **(B)** *Binary thinning procedures applied to the image A allow to derive the binary skeleton of the vascular tree and the identification of the branching points, identified as black dots.* **(C)** *Voronoi diagram associated with the distribution of branching points.*

Briefly, from grids of increasing size overlying the image, the number of boxes containing any pixel was counted. This number was recorded as a function of grid size and D was calculated as –1 times the slope of the regression line, from a plot of the log of size on the x-axis and the log of box count on the y-axis. To minimize grid location effects, the algorithm started from a number (10 in our case) of locations, generating a set of values for D. The average value over all locations was considered as the final estimate of D. The amount of branching, expressed as the number of branching points per unit area, was also estimated as a further index of the vascular tree complexity.

As discussed by Bormashenko and collaborators (Bormashenko *et al.*, 2019), the 2D pattern of branching points may also help in identifying the presence of symmetries in the vascular tree. This method starts with the construction of the Voronoi diagram of the pattern (Fig. 2 C). After measuring the lengths of the edges of that graph, the Voronoi entropy of the diagram can be defined as:

$$S = -\sum P_{Li} ln P_{Li}$$

where $P_L i$ is the probability of finding an edge with length L_i. If all the edges of the diagram are of different length, the Voronoi entropy is simply S=lnE (where E is the number of edges in the diagram), meaning that the pattern demonstrates no symmetry. If, instead, elements of symmetry are present, S will be lower than lnE. Thus, a global index of symmetry can be defined as

$$I_s = 1 - \frac{S}{lnE}$$

It will be 0 when the pattern does not exhibit elements of symmetry and it will increase as far as symmetry features are present.

Statistics

Within each considered time step, the obtained values for area percentage, uniformity index, complexity parameters and symmetry index were averaged to provide a representative value of each parameter characterizing the vascular tree at that time step. Differences between time steps were then statistically tested by one-way analysis of variance (ANOVA), followed by Dunnet's test for multiple comparisons vs. the initial time. The GraphPad Prism 3.0 statistical package (GraphPad Software Inc., San Diego, CA) was used for the analysis, and $p < 0.05$ was considered as the limit for statistical significance.

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