Chapter from the book *Vasculogenesis and Angiogenesis - from Embryonic Development to Regenerative Medicine*.

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1. Introduction

The first functioning system during embryonic development is the cardiovascular system; the development of other organs and systems is in strict dependence to the emergence and development of blood vessels. Although the interest in the structure and functioning of the vascular system has existed since antiquity, there are still gaps in knowledge of the vascular system development issues. The process of blood vessel formation plays an important role during prenatal development; in the postnatal life, with few exceptions, this process is, normally, poorly expressed. Usually, an augmentation of postnatal vasculogenesis and angiogenesis is associated with pathological situations: healing wounds, certain degenerative diseases (rheumatoid arthritis, diabetic retinopathy, psoriasis) or malignant growth and metastasis. In this context, it becomes imperative to understand the mechanisms and factors involved in tumor angiogenesis. Mainly, tumor angiogenesis repeats - at least in some aspects - embryonic stages of vascular development. Most studies related to embryonic vascular system development were performed on embryos from other species, and / or cell cultures. Detailed morphogenesis studies, demonstrated the existence of differences between the primitive circulatory system in fish and mammals. In 2004, Ginis and collaborators have conducted a comparative study on cultures of human embryonic versus mice endothelial cells. The authors demonstrated the existence of species-specific differences that are related not only to quantitative aspects, but also to the existence of different signaling pathways. Even when using the same signaling pathways in cells from different species, different members of the same family were involved. Studies on human embryonic tissues, from the points of view stated above, are very rare and controversial; for example, the morphogenesis of embryonic vessels and, the temporal sequence for the onset of smooth muscle actin expression are very well studied and characterized in birds and mice, but not in humans.

2. Material and methods

We investigated the blood vessel morphology, and distribution in human embryos, of different gestational ages. Since the only direct microscopic argument for active angiogenesis is endothelial cell proliferation, we also investigated this parameter, in order to evaluate vasculogenesis versus angiogenesis.
2.1 Materials
This study was conducted on five, seven, and twenty-four weeks human embryos; whole embryo specimens were obtained by therapeutic abortions, after signed patient consent, according to the Ethic Comity guidelines, and in compliance with the Helsinki Declaration of Human Rights.

2.2 Methods
The specimens were fixed in buffered formalin and embedded in paraffin according to the usual histological technique. Five-micrometer thick sections were stained with a routine Hematoxylin - Eosin method for morphological diagnosis. Additional sections were prepared for immunohistochemistry, as follows: microwave pH 6 citrate buffer antigen retrieval was performed, followed by endogenous peroxidase inhibition with 3% hydrogen peroxide. In pursuing our objectives, first we investigated the morphology of embryo-fetal vessels by double immunostaining: we used as endothelial marker CD34 (clone QBEnd 10, dilution 1:25), and as a perivascular cell marker- smooth muscle actin (SMA, ready to use, clone 1A4 antibody); the cell nuclei were stained with modified Lille’s hematoxylin. To assess endothelial cell proliferation, we used another double immunostaining: CD34/Ki-67 (ready to use, clone MIB1 antibody). After incubation with the primary antibody, the LSAB system was applied; the final reaction product was, for the first antibody, visualized in brown, with diaminobenzidine, and, for the second antibody, in red, with aminoethyl carbazole. The entire immunohistochemical technique was performed with DakoCytomation Autostainer. The slides were mounted with an aqueous medium, and examined with a Nikon Eclipse 600 microscope; images were taken, and processed with Lucia G system. On each slide we evaluated the expression/coexpression of the above mentioned markers in embryo-fetal vessels.

3. Results
We assessed embryonic blood vessels in both developing organs, and surrounding mesenchyme; the results varied with the embryo’s gestational age, and the staining method.

3.1 Hematoxylin-Eosin staining
As a general remark, this type of staining allowed us to identify with certainty, only vessels with developing/obvious lumen; the vascular islands and vascular cords were less noticeable. Embryonic tissue examination revealed the presence of blood vessels of different sizes, with different degrees of maturation, in all cases. In five and seven weeks old embryos, the mesenchymal tissue contained small blood vessels with relatively large lumens, thin walls, occasionally surrounded by perivascular cells. In the nervous organs, such small blood vessels were present in the subependymal space, at the boundary between white and gray matter, and in piamater (Fig. 1).
In five weeks embryos, vessels were extremely rare in the mesenchyme of developing organs, such as esophagus, compared to the peripheral (subperidermal) mesenchyme, and were represented by vascular islands and cords, and small vessels, with thin walls (Fig. 2). Only on rare occasions we found mature vessels, with a well structured wall; some of these vessels had emerging endothelial cords (Fig. 3).
Fig. 1. Cross sections of seven weeks embryo spinal cord, HE staining. In the subependymal space there are very small vessels, recognizable by the presence of megalocytes (subfig. a – arrow, X100). If at the white-gray matter border, there are small vessels with/without narrow lumen (subfig. b, X100), in the meningeal membranes there are vessels with larger lumen, that have perivascular cells attached to their endothelial cells (subfig. c, X400).

Fig. 2. Five weeks embryo mesenchyme, HE staining. We frequently identified vascular islands, cords, and vessels with lumen, in the same microscope field (subfig. a, X100); sometimes we found concentrations of branched and anastomozed vessels, that presented budding phenomenon (subfig. b, X100)
In seven weeks embryos, the vessels were more numerous in the mesenchyme of the already differentiated organs as the lungs and heart, than in the peripheral mesenchyme. Unlike vessels in other areas of mesenchyme, lung vessels—located close to the bronchi—were characteristically elongated, and their inner tip already presented perivascular cells (Fig. 4.a, X200). Again we observed the coexistence, in the same microscope field, of vascular islands, cords, and vessels with large lumen (Fig. 4.b, X200).

Fig. 3. Blood vessel with arterial morphology, that has in its upper part a cord-like cell proliferation (HE, X400).

Fig. 4. Seven weeks embryo lung, HE staining. Next to a bronchus in the canalicular phase of development rests an elongated blood vessel that has perivascular cells (subfig. a, X200). Further from the bronchi, the vascular islands coexist with blood vessels with wide lumen (surfing. b, X200).

3.2 CD34 / SMAct

Blood vessel classification was made according to the criteria described by Gee and his colleagues (2003). We classified as immature vessels those items that showed no obvious
lumen, and were positive only for CD34; intermediate vessels reacted positive for CD34, showed no perivascular cells (negative reaction for SMA), but showed obvious lumen; mature vessels showed lumen lined by CD34 positive cells, doubled by SMA positive perivascular cells.

In five weeks old embryos, the expression of the two markers depended on the studied area. Thus, in the mesenchyme adjacent to differentiating tissues/organs, the CD34 positive isolated cells, vascular cords (Fig.5) and intermediate CD34 positive/ SMAc negative vessels were predominant. Mature blood vessels positive for both CD34 and SMAc, were detected in the undifferentiated mesenchyme, away from the developing organs; the SMA expression was weak and inconstant (Fig.6).

![Fig. 5. Isolated CD34 positive cells, and CD34 positive/ SMAc negative vascular cords were the most frequent vascular structures found in the mesenchyme of five weeks old embryos (CD34/SMAc, X400).](image)

![Fig. 6. In five weeks embryos only axial vessels begin to acquire pericytes- positive for SMAc (CD34/ SMAc, X1000).](image)

In seven weeks old embryos, the aspects encountered in earlier stages were maintained, with the exception of larger vessels that had an almost complete investment of SMAc positive perivascular cells (Fig.7).
In the large blood vessel, with a more complex wall structure, the CD34 positive endothelial cells are almost completely surrounded by SMAct positive cells (X400).

In 24 weeks old fetus we studied the expression of CD34/SMAct in two locations: lungs –as a representative for parenchyma organs-, and esophagus –representative for hollow organs. The lung vessels, from the large ones that accompany the bronchi, to the small, interalveolar capillaries, were all positive for both markers; this means that at 24 weeks, the lung vessels are of mature type (Fig.8). There were some exceptions: at the periphery of the lung parenchyma, immediately under the pleura, we identified cord-like vascular structures positive only for the endothelial marker (immature vessels).

Fig. 7. In the large blood vessel, with a more complex wall structure, the CD34 positive endothelial cells are almost completely surrounded by SMAct positive cells (X400).

Fig. 8. Large lung blood vessel (right) with CD34 positive endothelium, and a thick SMAct positive muscle layer; note the presence of numerous CD34 positive cells (probably fibroblasts) in the outer adventitia. In the lung parenchyma, the interalveolar capillaries are CD34/ SMAct positive (X200).
In the esophageal wall, the reaction for the two markers was positive in all layers, but with different pattern from one layer to another. In the mucosa of the fetal esophagus immature, CD34 positive /SMAct negative, cord-like vascular structures, and intermediate vessels were observed (Fig. 9, subfigure a). The blood vessels of the submucosa had a larger lumen with a more complex wall structure; the proportion of CD34 positive/ SMAct positive vessels versus CD34 positive/ SMA negative ones was approximately equal. Also, the vessels in this layer tended to come in pairs: one immature vessel accompanied by one intermediate/mature vessel (Fig. 9, subfigure b). In the connective tissue of the muscular layer, blood vessels were mainly of immature type (CD34 positive /SMA negative), with a marked tendency for branching (Fig. 9, subfigure c). In contrast, blood vessels in adventitia were mostly mature vessels, positive for both endothelial and smooth muscle actin markers (Fig. 9, subfigure d).

Fig. 9. Esophagus of 24 weeks fetus. Subfigure a: vascular cord with the tendency to form lumen, and small CD34 positive/SMAct negative vessels, in the mucosa (X400). Subfigure b: pairs of vessels were the larger one, with a more irregular outline is incompletely surrounded by SMAct positive cells (X400). Subfigure c: blood vessels undergoing remodeling, and as a consequence, the reaction for SMAct is inconstantly positive (X200). Subfigure d: the small blood vessels in the adventitia are completely invested with SMAct positive cells (X200).
3.3 CD34 / Ki67
In the five weeks old embryos the reaction for the proliferative marker Ki67 was positive in mesenchymal cells, but negative in all vascular structures identified with CD34 (Fig. 10). In the peripheral mesenchyme of seven weeks old embryos, Ki67 was inconstantly positive in the immature and intermediate vessels; only the peripheral cells of the vascular islands were positive for both CD34 and Ki67 (Fig. 11, subfigure a). In larger vessels, on their outer circumferences, we observed cells with an intense positive reaction for Ki67 (Fig. 11, subfigure b). These cells were most likely perivascular cells in the process of attaching themselves to the vascular wall. In the vascular structures located in the mesenchyme surrounding developing organs, the endothelial cells lining larger arterial, or venous blood vessels, were CD34 positive/Ki67 negative (Fig. 12). At the same gestational age, even if we found vascular structures both inside, and at the periphery of the developing central nervous system, only those at the periphery were positive for Ki67 (Fig. 13).

Fig. 10. In five weeks embryos, endothelial cells nuclei are negative for Ki67; the only positive cells are the mesenchymal cells (subfigure b), and the nuclei of some perivascular cells (CD34/Ki67, X400).

Fig. 11. Subfigure a: in the central vascular island all cells are positive for CD34, but only those at the periphery are Ki67 positive; the reaction for Ki67 is also positive in some mesenchymal cells. Subfigure b: both endothelial and perivascular cells are positive for Ki67 (X1000).
Fig. 12. Large blood vessels with stabilized wall structure. Only few perivascular cells, and some cells in the mesenchyme are positive for Ki67 (X100, X200).

We also investigated CD34/Ki67 coexpression in the lungs and esophagus of 24 weeks fetuses. In the central zone of the lung parenchyma, in the walls of large blood vessels, occasionally, we encountered endothelial cells that expressed both CD34 and Ki67 (Fig. 14, subfigure a). On the other hand, in the subpleural parenchyma, the reaction for Ki67 was positive in most of the small blood vessels (capillaries) (Fig. 14, subfigure b). In the esophagus sections we detected endothelial cell proliferation in the blood vessels of lamina propria and submucosa (Fig. 14, subfigure c). A particular aspect was represented by the subepithelial capillaries, whose endothelial cells were negative for Ki67.

Fig. 13. Vascular cord positive for Ki67 at the periphery of central nervous system in the seven weeks embryos (CD34/Ki67, X200).
Fig. 14. Subfigure a, b: Fetus lungs: positive reaction for both CD34 and Ki67 in an isolated endothelial cell of a venous vessel, and in most endothelial cells lining the capillaries (X400). Subfigure c: esophagus wall- Ki67 was positive in the basal layer of the lining epithelium, and in the endothelial cells lining the blood vessels of lamina propria and submucosa.

4. Discussions

In the human species, endothelial cells can be detected in the yolk sac and in the embryo since the 24th day of gestation (Carmeliet, 2000). In the 35 days embryo, in endothelial cells and their precursors, CD34 is uniformly expressed (Tavian et al, 1999). Our results coincided with the literature, for the five weeks embryos; the reaction for CD34 was positive in all immature - isolated cells and vascular cords -, and intermediate vessels. But blood vessel maturation involves, besides endothelial cells, perivascular cells and extracellular matrix. The addition of perivascular cells (pericytes, smooth muscle cells) stabilizes the vascular wall by limiting the proliferation and migration of endothelial cells (Conway et al, 2001). The results that we’ve obtained have varied depending on the gestational age; in embryos
aged 5-7 weeks, the response for CD34 was constantly positive in the endothelial cells; the perivascular reaction for SMA was inconstantly positive, with a discontinuous pattern. At this age, next to developing organs, immature and intermediate vessels predominated. Gerecht-Nir (2004) indicates as the time of occurrence of a positive SMA reaction, the gestational age of four weeks, and the presence of mature type arterial vessels – with several layers of SMA positive perivascular cells–, in seven weeks embryos. On our slides we observed the existence of topographic-related differences in the maturation degree of the blood vessels: immature blood vessels next to differentiating organs, and mature ones, even with emerging cords from their walls, in the peripheral mesenchyme. These aspects suggest a more active vasculogenesis in the mesenchyme surrounding developing organs, and the onset of angiogenesis. Blood vessel maturation became more rapid with tissue and organ differentiation. In the fetal mesenchymal tissue, mature vessels were the most numerous; in the developing organs, vascular morphology was organ dependent. The prevalence of vasculogenesis over angiogenesis in five to seven weeks embryos is also supported by our findings regarding the coexpression of CD34 and Ki67; our data showed that there were no proliferating endothelial cells in five weeks embryos, and in seven weeks embryos, proliferation of endothelial cells was inconstant. Our results support also the fact that lung arteries are formed by vasculogenesis while pulmonary capillaries are formed by angiogenesis (Hall et al, 2000).

5. Conclusions

The coexistence of vascular islands, vascular cords, and vessels with lumen, in the same microscopic field, in five to seven weeks human embryos, reflects the dynamic nature of vasculogenesis at this gestational age. The coexistence of budding vessels suggests the early onset of angiogenesis; nevertheless the rarity of this phenomenon indicates the prevalence of vasculogenesis over angiogenesis at this gestational age. Further development of the vascular tree depends on the topographic location, and type of organ.

6. References


Vasculogenesis is the process of new blood vessel formation during embryonic development of the cardiovascular system. This is followed by formation of a vascular tree and finally the cardiovascular system with the myriad of blood vessels that nourish all tissues and organs. Angiogenesis, on the other hand is the process by which new blood vessels take shape from existing blood vessels by “sprouting” of endothelial cells thus expanding the vascular tree. Both scenarios are based on activation, migration, proliferation and maturation of unique precursor cells. The study of blood vessel formation is an essential component of embryonic development, congenital malformations, degenerative diseases, inflammation and cancer and thus has widespread appeal to the biomedical field. Moreover, scientists are now harnessing this information for the purpose of building living blood vessel substitutes for replacement of diseased arteries and veins. This book highlights novel advances in the field of vasculogenesis and angiogenesis, including embryogenesis and development, regulation of progenitor cells, cancer and blood vessel regeneration. We consider this book a good initial source of information for graduate students, medical students and scientists interested in the intricacies of blood vessel formation, maturation, disease and replacement.

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