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DEPARTMENT II

COPOTOIU LARISA - CRISTINA

PhD THESIS

VARIABILITY IN EXPRESSION OF VASCULAR AND LYMPHTIC MARKERS IN HUMAN EMBRYONIC TISSUES AND FETAL APPENDAGES

- AB S T R A C T -

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INTRODUCTION

The morphofunctional complexity of the placenta, a temporary organ, has stimulated in recent decades numerous investigations resulting in important acquisitions regarding the role of the placenta in embryo-fetal development and in the genesis of some disease manifestations in prenatal life.

During pregnancy, the placenta fulfills the most diverse functions, receiving and processing the metabolic needs of the product of conception. Knowing the main aspects of placental morphophysiology brings us closer to understanding the pathological manifestations of this organ.

Placental diseases have as a substrate increased or over-limited demands, hypofunctional structural regressions, non-physiological adaptations, retention or transfer noxes from mother to fetus. Correlation of clinical and structural data in the organogenesis and organopathogenesis of the placenta allows us, along with the establishment of a more truthful diagnosis, to apply an appropriate therapy that is so useful for the safety of the fetus and the mother. For this purpose, the obstetrician must know the nature and significance of the lesions, functional deviations at the level of the placental organ, in order to integrate and interpret them in the complex of clinical-biological manifestations of pregnancy.

Embryonic tissue and embryonic annexes have always been a controversial microscopic aspect in terms of the existence and functionality of certain histological structures in fetal development but also in the maternal-fetal interrelationship. The morphological aspects of the placental structures are sometimes neglected even in high-risk or prematurely terminated pregnancies and this has a negative impact on the subsequent elucidation of the causes that lead to these unwanted events. Embryonic organogenesis necessarily includes the development of vascular networks adapted to each individual organ. The collection of human embryos is extremely difficult, they usually come from terminations of pregnancy on demand or determined by various pathological conditions. The immunohistochemical and molecular microscopic analysis of the precursor stages of embryonic development involves the extraction of the whole embryo, an extremely difficult maneuver to perform under the conditions of pregnancy embodiment techniques.

The main objectives of this research were:

(1) Clinical epidemiological analysis of cases; (2) Conventional microscopic analysis and through digital image analysis techniques of the development of embryonic vascular networks in weeks 5-7 in different primordia of embryonic organs; (3) Identification of the

expression of lymphatic markers (podoplanin) in the umbilical cord and placental structures and the characterization of the positive structures observed in these tissues; (4) Analysis of podoplanin gene expression in embryo-fetal structures by applying RNAscope techniques to umbilical cord and placenta sections.

Keywords: podolanin; placental villi; decidua; normal human placenta

THE GENERAL PART

Embryonic tissue and embryonic annexes have always been a controversial microscopic aspect in terms of the existence and functionality of certain histological structures in fetal development but also in the maternal-fetal interrelationship. The morphological aspects of the placental structures are sometimes neglected even in high-risk or prematurely terminated pregnancies and this has a negative impact on the subsequent elucidation of the causes that lead to these unwanted events. Embryonic organogenesis necessarily includes the development of vascular networks adapted to each individual organ. The collection of human embryos is extremely difficult, they usually come from terminations of pregnancy on demand or determined by various pathological conditions. The immunohistochemical and molecular microscopic analysis of the precursor stages of embryonic development involves the extraction of the whole embryo, an extremely difficult maneuver to perform under the conditions of pregnancy embodiment techniques.

The vascular network represents a key point in the embryonic development of all embryonic organs and represents a critical moment with heterogeneous cellular components that will later determine the normal or pathological development of the embryonic organs. Vascular networks in different organs develop differently. The mechanisms underlying the development of vascular networks are incompletely described in human embryonic tissues due to the strict ethical complications that apply to the use of human embryonic tissues. The mathematical models of development of embryonic vascular networks are heterogeneous from one organ to another and are the basis of the application of morphometric studies of the formation of vascular networks. In addition to the limited microscopic aspects described in the literature regarding embryonic vascular features, even less data are available regarding studies performed on embryonic appendages, namely the umbilical cord and placenta. The umbilical cord represents a particular form of connective tissue that includes partially differentiated mesenchymal cells, a particular gelatinous extracellular matrix and large vessels. Data on the existence of small-caliber blood capillaries dispersed in the mucous connective tissue of the umbilical cord as well as the existence of lymphatic structures in the umbilical cord are highly controversial issues at this time in the specialized literature. Data on the

existence of umbilical vessels other than the great vessels have recently been published in the literature and have demonstrated the existence of lymphatic vessels that have a caudal trajectory along the umbilical artery. However, despite all this the expression of lymphatic markers in the umbilical cord is very little studied being reported in only 3 articles in the literature. Furthermore, molecular analyzes such as tissue gene identification (in situ hybridization or RNAscope) are not found in the specialized literature applied to fetal appendages.

The pathogenesis in different placental pathologies is not clear, some risk factors are well known, such as previous caesarean section, curettage, uterine surgery, advanced maternal age and multiparity (80).

Angiogenesis disorders and generalized inflammation are the dominant symptoms in various pregnancy-related pathologies, such as preeclampsia and uterine growth restriction.

In this study we hypothesize that different diseases associated with pregnancy have the same angiogenic and lymphangiogenic profile of the placenta, the only difference being the degree of exacerbation of the lesions.

Based on the above, they derived a series of objectives of the present study that tried to elucidate the lesser-known aspects of microscopy and molecular analysis of embryo-fetal structures in their early and late stages of development.

The present study approaches the fetal embryo both from a clinical point of view and from a microscopic and molecular point of view. The statistical analysis of the cases included in the study was later completed with clinical correlations. The study of embryonic structures was limited to the early stages of embryonic development, weeks 5-7 of embryonic development.

THE SPECIAL PART

The special part includes 6 chapters: 2 introductory (motivation, materials and methods), and 2 immunohistochemical studies with distinct purposes: Combining RNAscope, Immunohistochemistry (IHC) and Digital Image Analysis to Assess Podoplanin (PDPN) Protein and PDPN_mRNA Expression on Formalin-Fixed Paraffin-Embedded Normal Human Placenta Tissues and Evaluation of Vasculogenic Factors in the Developing Embryo at Weeks Five and Seven with a Special Focus on CD133 and TIE2 Markers.

The workgroup included 100 placenta and adjacent umbilical cord from which we select 30 placentas from term pregnancies. Inclusion criteria: placenta from term pregnancy with live fetuses and no maternal/fetal disease associated with pregnancy.

The expression and function of podoplanin (PDPN) in the normal human placenta has been debated in placental evaluation. This study emphasizes the importance of a multimodal approach of PDPN expression in normal human placentas. A complete examination is performed using immunohistochemistry, RNAscope and automated Digital Image examination (DIA) interpretation. QuPath DIA-based analysis automatically generated the stromal and histological scores of PDPN expression for immunohistochemistry and RNAscope stains. The umbilical cord's isolated fibroblasts and luminal structures expressed PDPN protein and PDPN_mRNA. RNAscope detected PDPN_mRNA upregulation in syncytial placental knots trophoblastic cells, but immunohistochemistry did not certify this at the protein level. The study found a significant correlation between the IHC and RNAscope H-Score ($p = 0.033$) and Allred Score ($p = 0.05$). A successful multimodal strategy for PDPN assessment in human placentas confirmed PDPN expression heterogeneity in the full-term human normal placenta and umbilical cord at the protein and mRNA level. In placental syncytial knots trophoblastic cells, PDPN showed mRNA overexpression, suggesting a potential role in placenta maturation.

Human embryo vasculogenesis (blood vessel development starting from endothelial precursors) includes the ability of mesenchymal cells and pluripotent stem cells to differentiate into endothelial cells. Quantification of endothelial progenitor cells is difficult to assess during the early steps of human embryo development due to several factors, especially due to the paucity of human embryo tissue which is usually discarded after early-stage pregnancy abortive methods. CD133 (Promimin-1) is a general marker of progenitor cells, but combined with other endothelial markers such as CD34, it may identify endothelial progenitor cells during embryonic development. CD34 immunohistochemistry was previously performed by our team to identify human embryo capillaries and comparatively assess microvessel density between different human embryonic tissues. TIE2 is an angiopoietin receptor strongly involved in the newly formed blood vessel maturation due to its expression in some mesenchymal precursors for future pericytes. CD34 assesses the presence of endothelial cells but its single use does not evaluate the endothelial progenitor state as CD133 may do nor vessel maturation as TIE2 may do. Data about the dynamics of CD133/TIE2 expression in the early stages of human embryo development are scarce. Hence, in this study, we aimed to comparatively assess the dynamic of CD133+ endothelial precursors and TIE2 expression on five and seven-week-old human embryonic tissues with a special emphasis on their expression on embryonic vascular beds.

CD133 and TIE2 immunohistochemistry was performed on five and seven-week-old human embryonic tissues followed by their quantification using the Qu Path digital image analysis (DIA) automated method.

CD133 and TIE2 showed divergent patterns of expression during the initial phases of human embryonic development, specifically in the vascular endothelium of tiny capillaries. The expression of CD133 in endothelial cells lining the perfused lumen gradually decreased from five to seven-week-old embryos. It remained expressed with greater intensity in cells located at the tip of the vascular bud that emerged into pre-existing capillaries. TIE2 was much more specific than CD133, being restricted to the level of the vascular endothelium; therefore, it was easier to quantify using digital image analysis. The endothelium of the embryonic aorta was an exception to the divergent expression, as CD133 and TIE2 were consistently co-expressed in the seven-week-old embryo. The Qu Path DIA assessment increased the accuracy of CD133 and TIE2 evaluation, being the first time they were quantified by using automated software and not manually.

High heterogeneity of CD133 and TIE2 was observed between five and seven-week-old embryonic tissues as well as between different embryonic regions from the same gestational age. The unique finding of CD133/TIE2 co-expression persistence inside aortic endothelium needs further studies to elucidate the role of this co-expression.

GENERAL CONCLUSIONS AND PERSONAL CONTRIBUTIONS

1. Podoplanin is expressed both at the protein and gene levels in the human placenta at term and in the umbilical cord. The accuracy of determining the presence of podoplanin in the placenta is increased, and the debates about its existence in the human placenta and umbilical cord are lessened, thanks to the combined method of evaluating podoplaninexpression by RNAscope and immunohistochemistry. The distribution and arrangement of podoplanin-positive structures differs in the fetal, maternal, and umbilical cord placentas at term. Podoplanin-positive cells resemble stars at term and have a number of extensions that join together to create PDPN-positive networks. Podoplanin-positive cells frequently exhibited a propensity for intracytoplasmic vacuolization and fusion of these vacuoles, resulting in the outlines of structures resembling vessels with a separate lumen. Red blood cells were absent from the podoplanin-positive vascular-like structures, indicating the presence of lymphatic vessels in the placenta and umbilical cord at term.

2. The decidual cells showed strong gene and protein expression of PDPN. The distribution of PDPN in all decidual cells was strictly submembrane, giving it a unique feature at the protein level.
3. The placental nodules from the full-term placenta had the highest Allred scores and H_Score. This feature, which has never been reported before, implies that podoplanin plays a role in the development of the fetal placenta.
4. The distinct expression of TIE2 and CD133 in the vascular endothelium of both big aortic-type arteries and tiny embryonic capillary-type vessels during the early phases of human embryonic development. The embryonic aortic endothelium is an exception to this divergent expression, as evidenced by the seven-week embryo's continuous co-expression of TIE2 and CD133. It is impossible to determine the role of CD133 and TIE2 co-expression at the aortic endothelium of the human embryo due to the paucity of information surrounding this co-expression in the literature.
5. A theory that has already been accepted at the level of the aortic endothelium in the aorto-gonado-mezodermic area but has not yet been described at the level of the thoracic aorta is that this co-expression supports the hemogenic role of the embryonic aorta endothelium, which gives endothelial cells the ability to differentiate both in endothelial precursor and hematopoietic precursors.