

The importance of immunohistochemical evaluation of the vascular changes from the decidua and placenta in recurrent pregnancy loss

MARIA MAGDALENA MANOLEA¹⁾, OLGUȚA ALICE GAVRILĂ²⁾,
 FLORINA CARMEN POPESCU³⁾, LILIANA NOVAC¹⁾,
 GAROFIȚA OLIVIA MATEESCU³⁾

¹⁾Department of Obstetrics and Gynecology,
 University of Medicine and Pharmacy of Craiova

²⁾"Filantropia" Municipal Hospital, Craiova

³⁾Research Center for Microscopic Morphology and Immunology,
 University of Medicine and Pharmacy of Craiova

Abstract

The angiogenesis is a complex process, incompletely understood, regulated by various stimulating and inhibiting angiogenic factors. In the present study, we proposed to evaluate the angiogenic changes that occur in the cases with recurrent pregnancy loss comparing with a control group represented by women with requested abortion. The evaluation of the changes in the vascular bed was made by immunohistochemical methods, evaluating the answer of the curettage products to the mouse anti-human CD31 and CD34 monoclonal antibodies immunolabeling. The endothelial cells reaction to the CD31 antibody was different, very intense in the normal or slightly congestive vessels. The endothelial cells from the strongly congestive vessels had a light and scratchy reaction. We found intense positive reactions in the control group for CD34 in the vessels from the villous axis and also in the vessels from the spongy decidua. In the study group, we found light positive reaction in the vessels from the decidua situated in the proximity of the necrotic areas; we found a light positive reaction also in the vessels and mesenchymal fibroblasts from some chorionic villous axis.

Keywords: vasculogenesis, placental bed, recurrent miscarriage.

Introduction

Most embryonic loss, even 80% by some authors, occurs during early pregnancy [1]. The reason early pregnancy is such a critical period of gestation is probably because of the major developmental events that take place, including formation of the placenta, a process known as placentation. Although the human placenta is a transient organ that persists only nine months, the effects of this organ on the offspring remain for a lifetime [2]. Placentation includes extensive angiogenesis in maternal and fetal placental tissues, accompanied by an important increase in uterine and umbilical blood flows [3]. These events provide the developing conceptus with an optimal uterine environment to meet its metabolic demands and probably also influences the rate of physiological exchanges between the maternal and fetal systems later in pregnancy [4].

The angiogenesis is a complex process, incompletely understood, regulated by various stimulating and inhibiting angiogenic factors. There are few things known about the angiogenic process in a normal pregnancy. An abnormal spiral artery remodeling is associated with a pathological pregnancy. It is to be studied the importance of the interactions between the trophoblast and the vascular cells of the spiral arteries,

both in the normal and in the pathological pregnancy [5].

In the present study, we proposed to evaluate the angiogenic changes that occur in the cases with recurrent pregnancy loss comparing with a control group represented by women with requested abortion, from whom the informed consent was obtained, showing normal evolution of first trimester pregnancy in clinical and ultrasound assessment. The evaluation of the changes in the vascular bed was made by immunohistochemical methods, evaluating the answer of the curettage products to the mouse anti-human CD31 and CD34 monoclonal antibodies immunolabeling. The histological study of the placental bed allowed us to remark in all the cases comprised in the study group a chronic inflammatory infiltrate and in some cases even an acute inflammatory infiltrate.

Materials and Methods

We included in the present study 48 patients with a first trimester pregnancy, which presented in the Ist Obstetrics-Gynecology Clinic of "Filantropia" Municipal Hospital from Craiova, between 2009–2011. The study was approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova. Twenty-three cases that answered the next clinical and echographic criteria had been included in the study group: obstetrical

antecedents of spontaneous abortion or stopping development pregnancy; ultrasound diagnosis of an empty gestational sac or stopping development pregnancy; first trimester bleeding pregnancy; cooperating patients regarding the further observation.

The control group was represented by 25 women with requested abortion, from whom the informed consent was obtained, showing normal evolution of first trimester pregnancy in clinical and ultrasound assessment.

There has been made uterine curettage in all the cases after a complete preoperative evaluation and the obtained product was sent for histopathological and immunohistochemical evaluation.

The tissue fragments have been immediately fixed in solution of 10% neutral formalin and then processed by the classic method of wax embedding resulting paraffin blocks. These blocks were sectioned, at the paraffin microtome (Microm HM350) equipped with system of transfer of sections on water bath (STS, Microm), resulting sections with thickness of 4 μ m. The sections were then stained by the usual method with Hematoxylin–Eosin and then analyzed at the optic microscope, evaluating the vascular changes both in the decidua, and in the placental villi.

The immunohistochemical (IHC) study was realized on fine sections, of 3 μ m, achieved with the same equipment, which have been displayed on slides covered with poly-L-lysine, allowed to get dry at 37°C over night and then processed by the immunohistochemical (IHC) method as follows.

For the immunohistochemical (IHC) study, we used the immunostaining evaluation for the CD 31 and CD34 antigens with the specific antibodies. By the IHC technique, we were initially realized the antigenic recovery in the microwave, at 750 W, in appropriate solutions for each marker, for 20 minutes. Then, the sections were allowed to cool to room temperature and were incubated for 5 minutes in a solution of hydrogen peroxide. Then, the next step consisted in: washing in solution of phosphate buffer (PBS), incubating with the specific primary antibody for 30 minutes, the signal amplification with an EnVision (Dako, Bucharest, Romania) specific detection system and visualization of the antigen-antibody reaction by developing in the dark in a 3,3'-diaminobenzidine (DAB, Dako) solution. The nuclei counterstaining was realized by passing sections through a Hematoxylin solution, followed by clarification and mounting slides with Canada balsam.

In the present study, we evaluated the immunoreactions of the vascular markers mentioned above, using the following antibodies: anti-CD31, clone JC70A, Dako, 1:50 dilution, and anti-CD34, clone QBenD10, Dako, 1:100 dilution. For interpretation, we considered that the reaction was specific at membrane level in endothelial cells of vascular walls.

Results

The classic histological study showed for the control group a normal morphology with compact decidua, represented by large cells, with eosinophilic cytoplasm, and also sponge decidua with numerous glands of secretor type in a decidual transformed chorion. Adjacent, there were identified chorionic villi in different stages of

maturation, namely the mono and bitrophoblastic chorionic villi (Figure 1), lined by two layers of cells (chorion and syncytiotrophoblast) with conjunctive axis without vascular elements; tertiary villi lined by chorion- and syncytiotrophoblast) with conjunctive-vascular axis with vessels of various dimensions and here and there with Hofbauer cells. In seven cases, we noticed also the presence within the decidua of some histological elements suggestive for a chronic inflammatory process of reduced intensity.

The classic histological study of the placental bed allowed us to remark in all the cases comprised in the study group the presence of mono and bitrophoblastic chorionic villi, some with edema in the villous axis (Figure 2) and intervillous syncytial proliferations (syncytial buds). In 17 cases, we noticed a chronic inflammatory infiltrate (moderate chronic diffuse inflammatory infiltrate (Figure 3) in 12 cases and in five cases nodular inflammatory infiltrate with lymphocyte, plasma cells and macrophage type). In eight cases, we noticed an acute inflammatory infiltrate identified by the presence of neutrophil polymorphonuclear leukocytes. In four cases, we also noticed sponge decidua with fibrosis.

We found in the study group compact and sponge decidua (glands of secretor type), with an abundant vascularisation with small and large vessels, normal and dilated vessels both in the decidua and in the villous axis. The vascular changes were very pregnant and of different kinds: we found decidua with necrobiosis lesions and hemorrhagic areas in six cases; hyperemia, inflammation and vascular thrombosis were noticed in 21 cases both in large vessels and in small collapsed vessels. Intraluminal hemorrhages associated with acute inflammatory infiltrate were found in three cases.

Taking into account the fact that on the classic histology study we noticed vascular changes, we have proposed to point out the endothelial cells by immunohistochemical analysis with the monoclonal antibody CD31.

The endothelial cells reaction to the CD31 antibody was different, very intense in the normal or slightly congestive vessels. The endothelial cells from the strongly congestive vessels had a light and scratch reaction.

However, in the control group we noticed positive answers for the CD31 in the big and small vessels from the decidua, in the endothelial cells from the villous axis, in endothelial buds from secondary and tertiary villi (Figure 4). However, in the study group we found negative answers in 16 cases and in nine cases very light answers: in sponge decidua – rare vessels CD31-positive in the endothelial cells (Figure 5) and scratch positive in the dilated capillaries.

CD34 is a marker that allows us to appreciate the vascular density and it is strongly correlated with the angiogenesis.

In the control group, we found intense positive reactions in the vessels from the villous axis and also in the vessels from the sponge decidua; tertiary villi with numerous capillary vessels situated both in peripheral and in central area CD34-positive in endothelial cells (Figure 6).

In the study group, we found light positive reaction in the vessels from the decidua situated in the proximity

of the necrotic areas; we found a light positive reaction also in the vessels and mesenchymal fibroblasts from some chorionic villous axis. A high-positive immunostaining for CD34 was found in seven cases that presented vascular dilatations in sponge decidua

(Figure 7). We also noticed in six cases secondary villi with numerous small vessels CD34 positive situated predominantly in the peripheral area of the villi, circumferentially below the cytotrophoblast (Figures 8 and 9).

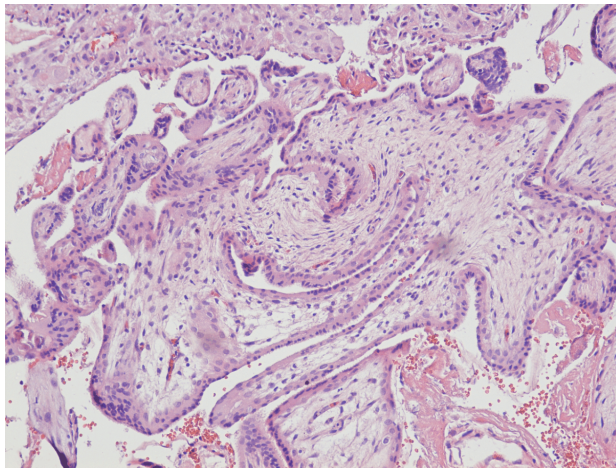


Figure 1 – Primary and secondary villi at the nidation site (HE stain, $\times 100$).

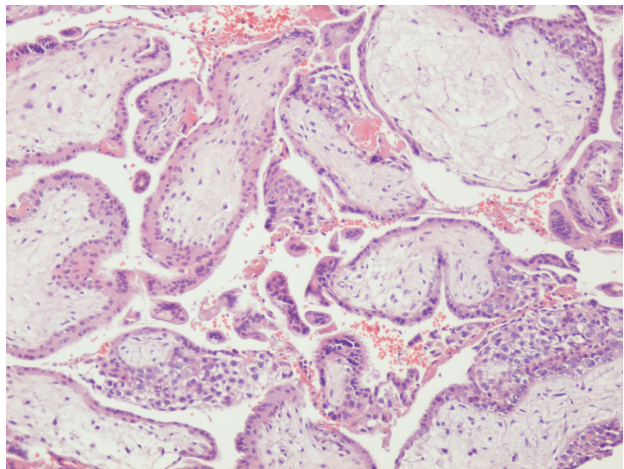


Figure 2 – Primary and secondary villi with inter-villous syncytial proliferation and edema in the villous axis (HE stain, $\times 100$).

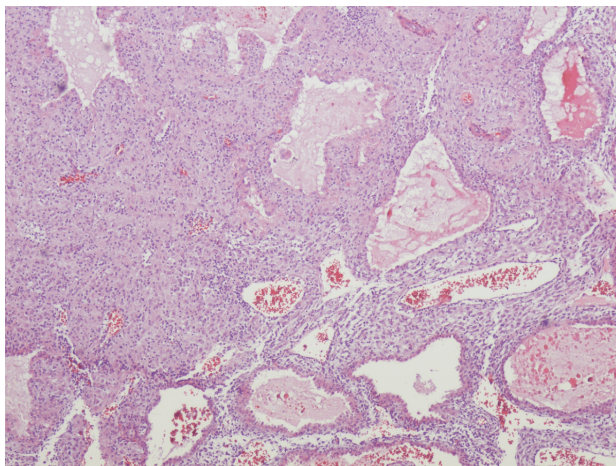


Figure 3 – Sponge decidua with dilated vessels, vascular hyperemia and moderate chronic diffuse inflammatory infiltrate (HE stain, $\times 40$).

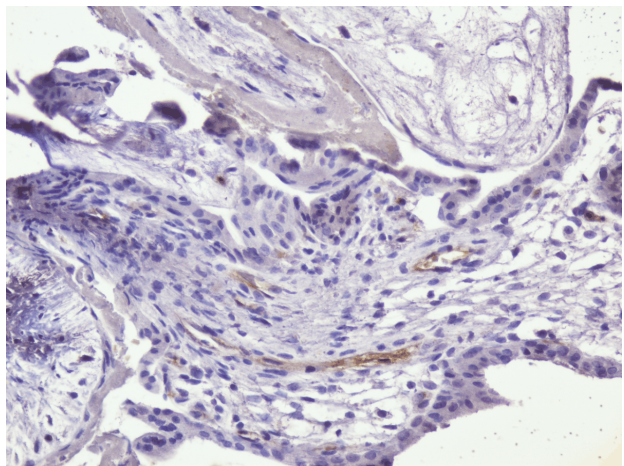


Figure 4 – Tertiary villi with capillary vessels CD31-positive (LSAB technique, $\times 200$).

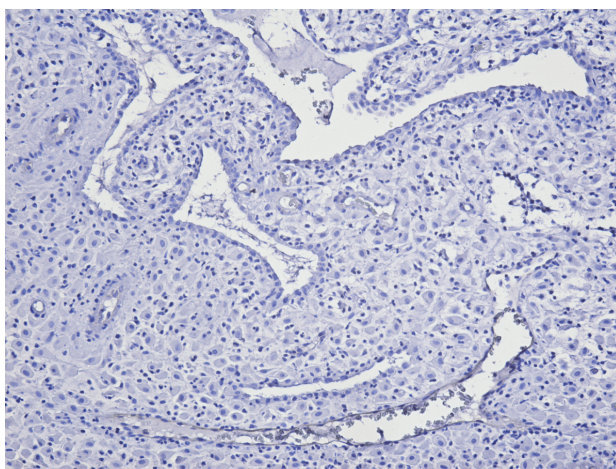


Figure 5 – Sponge deciduas: rare vessels CD31-positive in the endothelial cells (LSAB technique, $\times 100$).

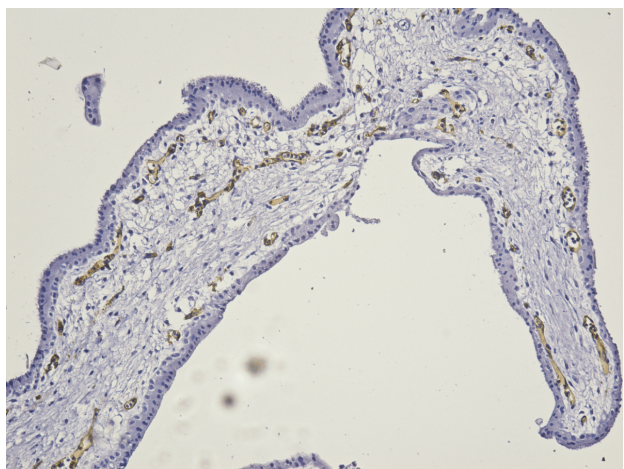


Figure 6 – Tertiary villi with cu numerous capillary vessels situated both in peripheral and in central area CD34-positive in endothelial cells (LSAB technique, $\times 100$).

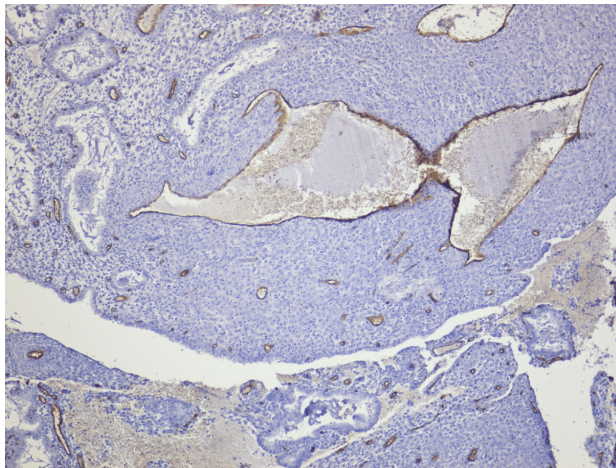


Figure 7 – *Sponge decidua with vascular dilatations: immunostaining high positive for CD34 (LSAB technique, $\times 40$).*

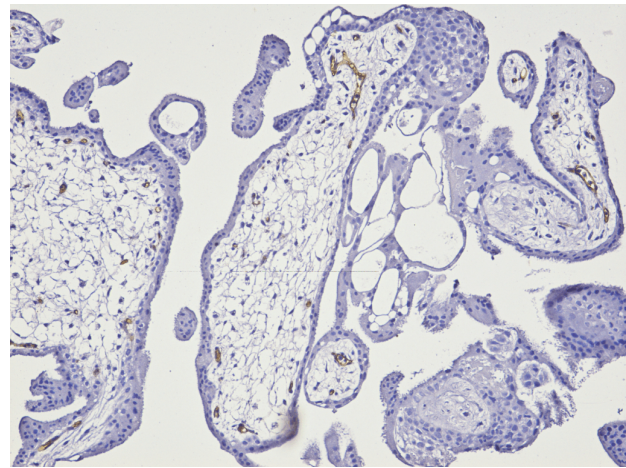


Figure 8 – *Secondary villi with numerous small vessels CD34-positive situated predominantly in the peripheral area of the villi (LSAB technique, $\times 100$).*

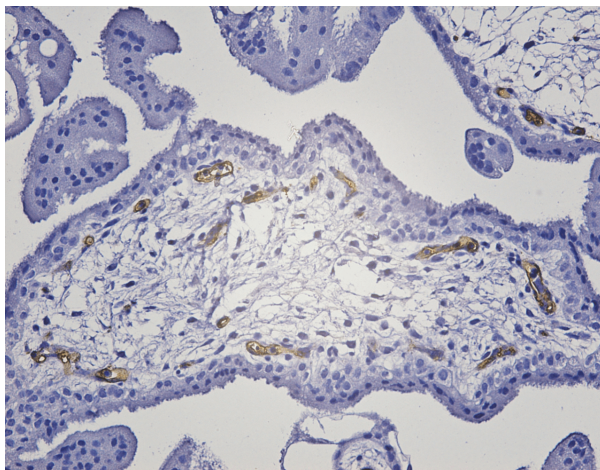


Figure 9 – *Detail of the precedent figure. Secondary villi with frequent small vessels situated circumferentially below the cytotrophoblast (LSAB technique, $\times 200$).*

Discussion

Early in normal placental development, extravillous cytotrophoblast invades the uterine spiral arteries of the decidua and myometrium. These invasive fetal cells replace the endothelial layer of the uterine vessels, transforming them from small resistance vessels to flaccid, high-caliber capacitance vessels. This vascular transformation allows the increase in uterine blood flow needed to sustain the fetus through the pregnancy [6].

Studies on normal chorionic villous vascularization, showed that first embryonic capillaries appears between days 18 and 20 post-conception [7].

The development of the chorionic villous vascular system seems to be mostly characterized by the maturation of luminized vessels from primitive hemangioblastic cell cords, and margination of vessels due to decrease of villous stromal area and the increase of the total vascular area [8].

Angiogenesis is characterized by increased vascular permeability, endothelial cell proliferation and migration,

and is regulated by various growth factors, including vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and angiopoietins (Ang), and proteases such as the membrane-type matrix metalloproteinases (MT-MMP) [9].

Disturbances in vascular development may play a role in the pathogenesis of miscarriages [10, 11].

Ultrasound studies showed significant reductions of the vascular parameters such as endometrial vascularization index, flow index, and vascularization flow index, as well as subendometrial vascularization index and vascularization flow index, for the patients with unexplained recurrent miscarriage in comparison to normal pregnancies patients [12, 13].

The CD31 antigen is a transmembrane endothelial cell adhesion molecule belonging to the immunoglobulin family [14]. The expression of CD31 is confined to the surface of circulating platelets, leukocytes, and the endothelial intercellular junction [15]. One of the roles of CD31 could be to modulate the endothelial permeability, thereby modulating leukocyte–endothelial transmigration [16].

In our study, we noticed positive answers for the CD31 in the control group in the big and small vessels from the decidua, in the endothelial cells from the villous axis, in endothelial buds from secondary villi. However, in the study group we found negative or very light answers in a few small vessels and scratch positive in the dilated capillaries.

The literature data support our findings. Haynes MK *et al.* (1997) found an intense immunostaining of CD31 in endothelium of early normal pregnancy decidua [17], while Vailhé B *et al.* (1999) observed a heterogeneous staining of CD31 in decidua from abortion, with some weakly stained vessels [18].

Endothelial cells are phenotypically heterogeneous. Therefore, they are also heterogeneous in their immunoreactivity, thus justifying the choice of a panel of different markers to assess the vascularization of a tissue [19].

CD34 is a transmembrane protein, with an extra-

cellular region made up of an amino-terminal mucin domain and a globular domain, which is related to immunoglobulin (Ig) domains. The pattern of expression of CD34 structure suggests that it plays an important role in early hematopoiesis [20]. The monoclonal antibody against CD34 could therefore provide advantages over currently available antibodies [8].

In our study, we found intense positive reactions in the control group for CD34 in the vessels from the villous axis and also in the vessels from the spongy decidua.

In the study group, we found light positive reaction in the vessels from the decidua situated in the proximity of the necrotic areas; we found a light positive reaction also in the vessels and mesenchymal fibroblasts from some chorionic villous axis.

The literature findings on this topic are inconsistent. Our data do not support the Vailhé B *et al.* (1999) study that did not detect a difference in vascularization between normal pregnancy decidua basalis and decidua basalis from abortions [18]. However, Plaisier M *et al.* (2009) found that the vessel density detected by CD34 was significantly lower in decidua parietalis and decidua basalis of first trimester spontaneous abortions compared with the controls [21]. They also found a variation of the vascularization pattern of missed abortion cases and matched controls.

Moreover, Vailhé B *et al.* (1999) observed a dramatic increase in vascularization in decidua parietalis from first trimester human spontaneous abortions suggesting that the vessels of decidua basalis are phenotypically different from the vessels of decidua parietalis [18].

In order to have a global view of the vascular changes occurring in the placental bed, immunohistochemistry findings may be related to innovative methods for the research of the vascular structures, such as 3D study using Confocal Laser Scanning Microscopy (CLSM). This innovative 3D technique visualizes 3D datasets as enlarged 3D holograms and provided detailed insight in the spatial arrangement of first trimester villous vascularization, the beginning of lumen formation within various junctions of hemangioblastic cords between five and seven weeks gestational age and in the gradual transition of vasculogenesis to angiogenesis [22].

✉ Conclusions

The histological and immunohistochemical findings of our study might indicate a correlation between decidua vascularization changes and the occurrence of miscarriages.

Our data suggest vascular disorders and support the results of other histological and ultrasound studies that postulate a link between recurrent miscarriage and the changes of the vascularization pattern in the placental bed.

Further investigations are needed to assess methods of quantifying aspects of angiogenesis in the placental bed and add new perspectives for vascular research.

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Corresponding author

Maria Magdalena Manolea, University Assistant, MD, PhD, Department of Obstetrics and Gynecology, University of Medicine and Pharmacy of Craiova, 2–4 Petru Rareș Street, 200349 Craiova, Romania; Phone +40251–522 458, Fax +40251–593 077, e-mail: manoleamaria@gmail.com

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