Chapter 5

Angiogenesis-Related Factors in Early Pregnancy Loss

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Abstract

The habitual loss of early pregnancy is one of the major problems of obstetrics nowadays, provided that the cause of more than 50% of all early pregnancy losses is unknown. Adequate angiogenesis is one of the main indicators of proper formation of placental system, making the basis of fetal life support. The objective description of angiogenesis in physiological development of pregnancy and in pathological conditions is complicated by the difficulties in obtaining and characterizing placental tissue in early pregnancy. Thus, angiogenesis-related factors are promising indicators to characterize angiogenesis in pregnancy. This chapter draws attention to alteration in angiogenesis-related factors in peripheral blood of patients with habitual early pregnancy losses. Investigation of factors (vascular endothelial growth factor (VEGF), sFlt-1, sKDR, metalloproteinase (MMP)-2, MMP-9, tissue inhibitor (TIMP)-1, TIMP-2 and placental growth factor (PLGF)), which specifically and nonspecifically regulate angiogenesis in pregnancy, was performed in the most significant terms for placentogenesis: 6 weeks, 7–8 weeks and 11–14 weeks of pregnancy. It was found that in a missed abortion there was a significant imbalance of angiogenesis-related factors compared with normal pregnancy. These results reflect a disturbance of angiogenesis in a missed abortion and point to the importance of the studied factors in the pathogenesis of early pregnancy losses.

Keywords: Angiogenesis, angiogenic factors, pregnancy, angiogenesis inhibitors, matrix metalloproteinases, pro- and antiangiogenic factors ratio, VEGF/VEGF-R1, VEGF/VEGF-R2, MMP-9/TIMP-1

1. Introduction

Adequate formation of uteroplacental and fetal placental blood flow is the determining factor of physiological pregnancy and fetal development. Successful uterine-placental vascular morphogenesis and embryonic morphogenesis of fetal blood system are the basis of these processes. There are two stages of vascular morphogenesis: vasculogenesis—primary formation and development of blood vessels *de novo* from committed mesodermal cells—and angiogenesis—formation of new blood vessels from existing vascular structures, which reflect the formation of vascular system of a fetus and placenta during pregnancy [1–4].

"Early pregnancy" period includes several time intervals when the most significant for angiogenesis events occur, determining further course and outcome of pregnancy. During gestation up to 6 weeks, the primary fetal circulatory system and placental bed with the development of villi are formed, and extensive vascularization of placental villous tree occurs. The 6–8th weeks of pregnancy are marked by the start of transition to the placental circulation, as well as by the most expressed invasion of extravillous trophoblast into maternal spiral arteries (first wave of trophoblast invasion). The period of 11–13 weeks is considered to be borderline and is characterized by the completion of embryogenesis, the starting period of fetal development, fading of the first wave of trophoblast invasion and further increase in the volume of uteroplacental blood flow [4–8].

During trophoblast invasion into endometrium, interactions with components of the extracellular matrix, which is mediated by cell adhesion molecules, occur. Proteinases participate in the degradation of extracellular matrix and cell migration deep into the myometrium through the uterine spiral arteries. Cytotrophoblast cells change their phenotype from epithelial to endothelial, producing a large number of soluble factors contributing to the development of the vasculature [9, 10].

Growth factors play the key role in vessels formation. They serve as cell mitogens, as attractants in the formation of vascular architectonics, and most important, as morphogens. The main regulators of angiogenesis are members of the family of vascular endothelial growth factor (VEGF). In addition to direct activators of angiogenesis, there is a large group of factors, whose effect on angiogenesis is nonspecific. It includes matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) [11–14].

Decidual NK-cells in early pregnancy, at the stage preceding the invasion of trophoblast into the maternal arteries, produce VEGF, placental growth factor (PLGF) and matrix metalloproteinases (MMPs), in particular MMP-2 and MMP-9 [15–17]. These MMPs are collagenases of IV type which specifically hydrolyze the collagen of basement membranes and thereby facilitate cell invasion through the basement membranes and stimulate angiogenesis [18–21]. Decidual NK-cells are the main source of MMP-2 and TIMP-2 from the group of tissue inhibitors of matrix metalloproteinases (TIMPs) [22–24].

Trophoblast also produces factors regulating processes of vessels formation. Particularly, MMP-9, TIMP-1, TIMP-2 and TIMP-3 are produced by the cells of extravillous trophoblast. Villous cytotrophoblast cells and invasive endovascular trophoblast produce MMP-2

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which is considered a key regulator of cell invasion in early gestation (up to 8 weeks), according to some authors [17, 19].

Production of anti-angiogenic factors is an integral part of the normal angiogenesis. As a result of the molecular dialog during vascularization, production of inhibitors serves as a hamper for excessive trophoblast invasion, as well as an obstacle for the further development of the vascular bed and for vascularization of pathologically changed tissue sites. Angiogenic factors are specifically expressed in endothelium and in the placenta during pregnancy. These include receptors VEGF-R1 (Flt-1), VEGF-R2 (Flk-1, KDR) and VEGF-R3 (Flt-4). Soluble forms of these receptors are able to bind growth factors in circulation, slowing or blocking angiogenesis [25].

Humoral factors involved in vascular formation processes are more accessible for research in maternal circulation, and change of their content in mother's blood reflects changes in the content of these factors in the fetal blood circulation and tissues. In this regard, a complex study of angiogenesis-related factors and their ratios is crucial for understanding and predicting vascular morphogenesis disorders during pregnancy.

2. Characteristics of the groups and study design

The prospective study included 66 patients with early stage pregnancy.

The control group consisted of 20 patients with normal pregnancy. All the patients had one or two previous pregnancies uneventfully completed at term.

The main subgroup A consisted of 16 patients with the history of miscarriage and current threatening miscarriage. All patients from the main subgroup A gave live births at term. The main subgroup B included 30 patients whose pregnancy ended as «missed abortion».

In patients of the control group and of the main subgroup A, the study of angiogenesis-related factors in peripheral blood samples was performed within 6 weeks, at 7–8 weeks and at 11–14 weeks of pregnancy. In the main subgroup B, the investigation was conducted at the time of diagnosis of «missed abortion» (8 patients were examined before 6 weeks, 14 patients—at 7-8 weeks and 8 patients—at 11-14 weeks).

The criteria for inclusion into the main groups were as follows: history of two and more early pregnancy losses, no childbirth in current marriage, singleton natural pregnancy.

Exclusion criteria were endometriosis, POS, uterine fibroids, induced pregnancy and/or extragenital conditions (diabetes mellitus, psoriasis and systemic autoimmune diseases, and malignancies), FV L and FII G20210A gene mutations, and activation of bacterial viral infections. Groups were matched for the patient's age.

Diagnosis of recurrent miscarriage was established according to the International Classification of Diseases 10th Edition (ICD-10-CM). Pregnancy was diagnosed based on the ultrasonography study and HCG.

The samples of peripheral blood for investigation of angiogenesis-related factors were obtained from the cubital vein not later than 5 days after ultrasonic detection of cessation of pregnancy development in the patients of the main subgroup B in case of intact chorion (absence of retroplacental and/or retro amniotic hematomas, vaginal tract bleedings). Prior to investigation, the serum was stored at the temperature of «-80°C».

3. Methods

Determination of serum levels of VEGF, VEGF-R1 (sFlt-1), VEGF-R2 (sKDR), MMP-2, MMP-9, TIMP-1, TIMP-2 and PLGF was performed by enzyme-linked immunosorbent assay (ELISA) using standard test systems: Bender MedSystems GmbH (Austria) and R&D Systems (USA). The optical density was measured using the plate reader BioTek (USA) at the wavelength of 450 nm. Construction of the calibration curve and calculation of the concentrations of VEGF, sFlt-1, sKDR, MMP-2, MMP-9, TIMP-1, TIMP-2 and PLGF were performed by linear regression equation in logarithmic coordinates.

The statistical processing was carried out using statistical analysis package for Microsoft Office Excel 2007 and software package Statistica for Windows 7.0, Statsoft Inc. (USA). Control of the normality of obtained parameters in studied groups was performed with Shapiro-Wilk W test. The significance of differences of mean values of the measured parameters was evaluated using unpaired t-test with different dispersions. Differences were considered significant at a significance level $p < 0.05$.

4. Results

Results of the study of angiogenesis-related factors are presented in **Tables 1** and **2**.

4.1. Time course of angiogenesis-related factors in patients with normal pregnancy

It was found that VEGF had the maximum concentration in blood at terms up to 6 weeks of gestation: Contents of this factor was significantly higher (more than 10 times) compared with further points of study. Concentration of PLGF at terms up to 6 weeks and at 7–8 weeks remained unchanged but increased more than two times by 11–14 weeks; this is consistent with the published data [24]. This trend in changing of VEGF and PLGF during early pregnancy probably provides pro-angiogenic effect because under the decrease in VEGF, PLGF is able to synergistically enhance angiogenesis promoted by VEGF [26].

Level of sVEGF-R1 was minimal at the starting point of the study but significantly increased reaching maximum values at 11-14 weeks. In contrast, the concentration of sVEGF-R2 was decreased only at 7-8 weeks of pregnancy. It is known that VEGF interacts with receptors VEGF-R1 and VEGF-R2, while VEGF-R1 is the only receptor for PLGF [27]. These factors compete for binding with VEGF-R1. Under increased secretion of VEGF and PLGF, this leads to exhaustion of VEGF-R1 and predominance of VEGF-R2 in circulation. PLGF is also supposed to be able to replace VEGF in the VEGF/VEGF-R1 complex, activating the

Factor (u.m.)	Groups	Factor's values in terms of study		
		Up to 6 weeks	7–8 weeks	11–14 weeks
VEGF (pg/ml)	Control	100.5 ± 24.5	5.5 ± 1.3#	8.9 ± 3.2#
	Main subgroup A	202.7 ± 82.3	5.9 ± 0.9#	4.2 ± 1.2#
	Main subgroup B	49.6 ± 28.6	4.7 ± 1.9	3.2 ± 0.4
VEGF-R1 (ng/ml)	Control	0.4 ± 0.1	1.1 ± 0.2#	1.7 ± 0.4#
	Main subgroup A	0.9 ± 0.1*	1.4 ± 0.1*#	1.7 ± 0.1#•
	Main subgroup B	0.4 ± 0.1**	5.8 ± 2.6*#	0.5 ± 0.2*,**•
VEGF-R2 (ng/ml)	Control	9.4 ± 1.9	4.9 ± 0.6#	9.3 ± 1.5•
	Main subgroup A	8.4 ± 1.9	10.6 ± 0.4*	10.5 ± 0.6
	Main subgroup B	12.5 ± 2.0	11.6 ± 1.5*	12.5 ± 2.0
PLGF (pg/ml)	Control	12.2 ± 1.4	13.2 ± 2.5	28.9 ± 4.1#•
	Main subgroup A	7.5 ± 0.6*	9.9 ± 0.8#	22.5 ± 2.5#•
	Main subgroup B	8.5 ± 0.7*	9.5 ± 2.0	8.8 ± 2.4*,**

*Comparison with the control group;
**comparison with the main subgroup A;
#the differences within one group in comparison with the data obtained before 6 weeks.
•the differences within one group in comparison with data obtained at 7-8 weeks (# < 0.05).

Table 1. Blood levels of pro- and antiangiogenic factors of the first trimester of pregnancy.

Factor (u.m.)	Groups	Factor's values in terms of study		
		up to 6 weeks	7–8 weeks	11–14 weeks
MMP-2 (ng/ml)	Control	330.6 ± 31.1	331.8 ± 13.2	364.2 ± 30.9
	Main subgroup A	415.3 ± 21.9*	411.1 ± 30.0*	392.6 ± 34.8
	Main subgroup B	526.8 ± 16.0*,**	378.7 ± 33.7#	271.8 ± 21.9*, **#•
MMP-9 (ng/ml)	Control	556.5 ± 71.4	379.6 ± 50.1#	558.8 ± 92.8
	Main subgroup A	537.2 ± 78.6	500.1 ± 51.1	578.9 ± 109.0
	Main subgroup B	296.1 ± 132.6	403.6 ± 79.3	278.0 ± 67.7*,**
TIMP-1 (ng/ml)	Control	435.6 ± 31.0	357.5 ± 26.1#	388.5 ± 23.9
	Main subgroup A	440.3 ± 62.1	458.8 ± 34.1*	344.0 ± 42.3•
	Main subgroup B	321.4 ± 27.9*	403.9 ± 48.9	342.0 ± 27.4
TIMP-2 (ng/ml)	Control	22.6 ± 2.1	19.4 ± 1.5	21.0 ± 1.5
	Main subgroup A	23.3 ± 2.4	21.2 ± 2.6	29.4 ± 4.1*
	Main subgroup B	30.4 ± 1.4*,**	25.3 ± 3.0*	18.1 ± 1.3**#•

*Comparison with the control group;
**comparison with the main subgroup A;
#the differences within one group in comparison with the data obtained before 6 weeks.
•the differences within one group in comparison with data obtained at 7-8 weeks ($p < 0.05$).

Table 2. Blood levels of matrix metalloproteinases and their tissue inhibitors in the first trimester of pregnancy.

expression of VEGF-R2 [28]. According to the published data, VEGF-R2 is a key receptor in angiogenesis [29] although its affinity to VEGF is quite lower than VEGF-R1 [30].

It is known that normally pro-angiogenic molecules are less than their inhibitors; nevertheless, intensive vascularization of placenta and of fetal developing organs occurs in pregnancy. This situation can be described as "pro-angiogenic state." However, it is quite difficult to quantify it, as the described models of angiogenic factors-receptor interactions do not give guide on the factor/inhibitor ratio, allowing to specify the pro-angiogenic state or block of angiogenesis [26, 31]. Therefore, trends observed in normal pregnancy may serve as references for challenging cases.

Blood levels of MMP-2 and TIMP-2 vary insignificantly in the studied terms of normal pregnancy. Significant decrease in MMP-9 and TIMP-1 concentration was found at 7–8 weeks compared with the terms before 6 weeks of pregnancy.

Normally, MMPs blood level is insignificant. Soluble forms of MMPs are present in blood as inactive proenzymes and transfer into active forms after propeptide cleavage under the impact of activation factors including inhibitors. Constant concentrations in the system "protease-antiprotease" (MMP-2/TIMP-2) may indicate extracellular matrix remodeling and vascular morphogenesis which intensively go at these terms; degradation of interstitial collagens and basement membrane collagens under the participation of these factors is obvious.

4.2. Time course of angiogenesis-related factors in patients of the main subgroup A

Blood levels of PLGF and sVEGF-R1 in patients of main subgroup A significantly increased with gestational age. In contrast, concentration of VEGF was maximum at the starting point of the study but significantly decreased (more than 30 times) by 7-8 weeks and then remained at the same level till 11–14 weeks of gestation.

Lack of significant differences in dynamics of MMP-2, MMP-9, TIMP-2 and sVEGF-R2 was observed during entire pregnancy. Concentration of TIMP-1 significantly decreased by 11–14 weeks.

Thus, in the main subgroup A, we found significant fluctuations of certain soluble factors. For several factors (VEGF, PLGF, MMP-2, TIMP-2), trends of this levels alterations at the same study points coincided with those observed during normal pregnancy; there was no statistically significant differences between the groups and the most studied terms. At the same time, for sVEGF-R1, sVEGF-R2, MMP-9 and TIMP-1, the trends were significantly different from the control group. However, these differences do not seem sufficient to cause dramatic consequences in pregnancy. Probably for successful outcome of pregnancy, a balanced production of VEGF and PLGF, key angiogenic factors for pregnancy [14, 24, 32], is necessary, as well as the balance between MMP-2 and TIMP-2, because MMP-2 is the key regulator of trophoblast invasion [17, 19] especially in early pregnancy.

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4.3. Angiogenesis-related factors in the main subgroup B

4.3.1. Blood levels of angiogenesis-related factors in patients with missed abortion before 6 weeks of gestation

Missed abortion before 6 weeks was characterized by significant differences of the levels of angiogenesis-related factors: sVEGF-R1, PLGF, MMP-2, TIMP-1 and TIMP-2. Thus, with missed abortion, the level of PLGF was lower than in the control group; however, there was no significant difference with the main subgroup A. Blood level of sVEGF-R1 did not differ from the values of the control group. However, comparison with the main subgroup A showed that in complicated pregnancy which further accomplished with childbirth, level of sVEGF-R1 was higher than in missed abortion. These results may indicate the existence of subtle mechanisms of regulation in the system "ligand/receptor," where the most important aspect is not a certain level of material content of the molecule, but the impacts of the factors on each other.

The concentrations of MMP-2 in the main subgroups A and B were significantly higher than in the control group. The maximum level of MMP-2 in blood was detected in missed abortion. TIMP-1 and TIMP-2 levels in the control group and main subgroup A differed insignificantly; in the main subgroup B, the level of TIMP-1 was significantly lower and the level of TIMP-2 was significantly higher at these terms.

Thus, more distinct differences specific for missed abortion were found in the group of factors "protease-antiprotease." The content of the factors in the system MMP-2–TIMP-2 at this term is, probably, critical for pregnancy. It is known that MMP-2 and TIMP-2 are most important at early terms of gestation during trophoblast invasion into maternal tissues [19]. Constant expression of MMP-2 and TIMP-2 was detected in apical layer of the syncytiotrophoblast and decidual NK-cells [19]. The published data suggest a possibility of syncytium activation by MMP-2, and the participation of this molecule in the processing of paracrine factors synthesized by these cells [19, 21]. There is a probability of change in the balance of production of biologically active molecules determining the development of pregnancy, due to the excessive production of MMP-2 or TIMP-2.

Pregnancy-initiated angiogenesis is closely associated with tissue remodeling and vascularization. Changes occurring in the endometrium decidualization include as follows: infiltration of tissues by immune cells (uNK-cells and macrophages), extracellular matrix remodeling with cell invasion through the matrix and membrane, and lysis of muscle-elastic tissue elements. In this period, the most important processes are formation of the placental bed and vascularization of the villi [6, 8, 10]. It is known that embryos which stopped to develop at 3–5 weeks had vascular villi, large hydropic dystrophy of the villous stroma and no embryonic blood vessels [33–35]. The disbalance of angiogenic factors, causing autocrine and paracrine effects to each other and to the cells, leads to microenvironment that is not compatible with the development of pregnancy. Excessive protease activity under these conditions may also reflect degenerative processes in the uterus before abortion.

4.3.2. Blood levels of angiogenesis-related factors in patients with missed abortion at 7–8 weeks

Pathological changes specific for missed abortion at 7–8 weeks were reflected by the significant increase in VEGF-R1, VEGF-R2 and TIMP-2 levels compared with the control group.

The interval of 7–8 weeks of gestation is marked by the first wave of trophoblast invasion into the mother's arteries and the start of uteroplacental blood flow. Extravillous trophoblast has a high invasive potential and expresses VEGF, PLGF and VEGF-R1 [36]. The receptor VEGF-R2 is expressed by the cells of fetoplacental complex [36, 37]. It should be noted that the level of VEGF-R1 is five times higher and VEGF-R2 more than two times differ from these factor levels in the control group. According to the published data, soluble forms of VEGF-R1 and VEGF-R2 are able to block angiogenesis and adversely affect the migration and proliferation of endothelial cells [27, 38, 39]. However, comparison with the main subgroup A did not show any significant differences in the studied period. Therefore, excessive levels of soluble forms of VEGF-R1 and VEGF-R2 in maternal circulation are not the main cause of this pathology at this term. It is known that adequate angiogenesis of villous tree and chorionic villi are critical stage and condition for further development of the placenta and fetus [1–4]. Inadequate start of restriction of uterine arteries at these terms initiates a chain of troubles in the system mother-placenta-fetus.

The key process of pregnancy is cytotrophoblast invasion into uterine arteries. At the same time, there is arteriolar lumen expansion under the impact of metalloproteinases. Excess of tissue inhibitors at 7–8 weeks, probably, leads to incomplete trophoblast invasion and insufficient lumen expansion which under the increased secretion of VEGF-R1 and VEGF-R2 can result in vasospasm and enhanced vascular permeability-specific manifestations of increased contents of these factors [36, 40] which adversely affect the embryo development.

4.3.3. Blood levels of angiogenesis-related factors in patients with missed abortion at 11–14 weeks of pregnancy

Missed abortion detected at of 11–14 weeks was characterized by a significant decrease in sVEGF-R1, PLGF, MMP-2 and MMP-9 compared with the control group and main subgroup A. Level of TIMP-2 was significantly lower than in the main subgroup A, but close to the control group.

The end of the first trimester of pregnancy is marked by the start of the fetal period of intrauterine human development, fading of the first wave of trophoblast invasion and preparation for a second wave at 16-18 weeks of pregnancy. Significant decrease in soluble forms of VEGF-R1 and PLGF in the main subgroup B could evidence the role of these factors in the pathologic processes leading to pregnancy loss. PLGF is the main regulatory factor in the first trimester of physiological pregnancy [2, 24] that acts as paracrine regulator of decidual angiogenesis and autocrine regulator of trophoblast function [24]. The synergy of effects of PLGF and VEGF on angiogenesis manifests with the morphogenesis of more mature and stable vasculature [24, 27]. Its effect is more significant in angiogenesis than in vasculogenesis [2]. Besides inhibition of angiogenesis, soluble form of the receptor VEGF-R1 also provides “support,” and thus, the effect depends on the factor blood level. Probably in this situation, levels of sVEGF-R1 and PLGF are insufficient for adequate angiogenesis.

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4.4. Ratio of angiogenesis-related factors

Dynamic balance in the system ligand/receptor provides a state of the system which empowers the implementation of its function, if this system tends towards the harmonic balance. The equilibrium point in such systems is always moving, because ligand/receptor pairs, being complex systems, are influenced by a variety of factors. In terms of the dynamic balance theory, ligand's positions in this study were occupied by the factors which specifically and nonspecifically affect angiogenesis, while the receptor's positions were occupied by its inhibitors. Studied ligands and receptors have multiple substrate specificity, but because their activity is affected by other factors that are present in the bloodstream and were not included in this study, investigation of ligand/receptor pairs in terms of classical concepts is difficult. Moreover, current models describing interactions of VEGF family members with the receptors [26] as well as of the matrix metalloproteinase family members with the inhibitors [31] do not provide quantitative binding characteristics of the system receptor/ligand for these molecules. The situation is complicated by the different levels of expression of these factors by various cell types. Therefore, the use of any index or ratio characterizing the dynamic situation in the selected time interval as a pro-angiogenic or nonproangiogenic state does not seem possible. To describe such situations, "surrogate" indexes characterizing the ligand/receptor ratio may be most appropriate to define any process, particularly angiogenesis. Such ratios are used to calculate the risk of development of pregnancy complications, such as preeclampsia [41–44].

Changes of the ligand/receptor pair ratio (VEGF/VEGF-R1, VEGF/VEGF-R2, PLGF/VEGF-R1, MMP-9/TIMP-1, MMP-2/TIMP-2) in the studied groups are presented in **Figure 1**.

4.4.1. VEGF/VEGF-R1 and VEGF/VEGF-R2 ratios

VEGF/VEGF-R1 ratio (**Figure 1A**) in the control group before 6 weeks was significantly higher than in the main subgroups A and B (0.562; 0.178 and 0.0312, respectively). In the control group, we observed significant differences between the terms before 6 weeks of gestation and other terms. Interestingly, the pattern of the VEGF/VEGF-R1 ratio change in the main subgroup A had tendencies similar to the control group. In the main subgroup B, the values of ligand/receptor pairs were significantly low, except for the last study term (0.0086) compared with the control group (0.006) and the main subgroup A (0.0024).

VEGF/VEGF-R2 ratio changed in a similar way (**Figure 1B**). Before 6 weeks of pregnancy, the significant changes were noted only in the main subgroup B (0.0023), compared with the control group (0.020) and the main subgroup A (0.011).

Tendencies of changes of VEGF/VEGF-R2 were similar in the control and the main subgroup A, demonstrating at the same time significant differences at 7-8 weeks. The main subgroup B was characterized by minimal values of the ratio which significantly differed in terms before 6 and 7–8 weeks compared with the other groups, but do not change within group in all terms of pregnancy.

It is known that realization of different mechanisms of angiogenesis depends on the type of receptor interacting with VEGF. Activation of VEGF-R2 leads to stimulation of angiogenesis

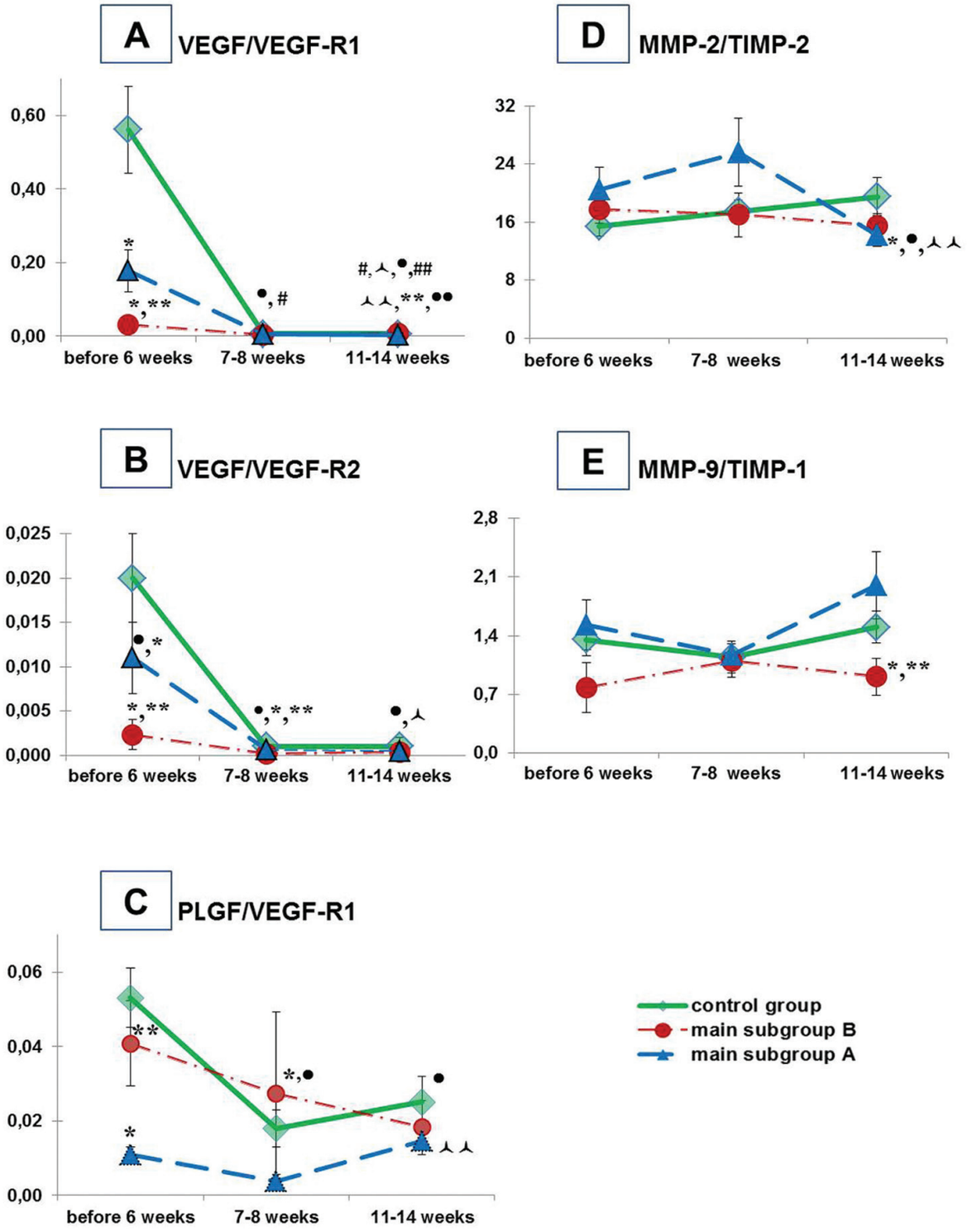

Figure 1. Factor/receptor (A, B, C) and factor/inhibitor (D, E) ratio in the blood of women at early terms of pregnancy. *Significant difference from the control group; **significant difference from the main subgroup A; ˙significant differences inside the control group in comparison with terms before 6 weeks; ¨significant difference inside the control group in comparison with terms 7-8 weeks; #significant difference inside the main subgroup A in comparison with terms before 6 weeks; ##significant difference inside the main subgroup A in comparison with terms 7–8 weeks; ⅄significant difference inside the main subgroup B in comparison with terms before 6 weeks; ⅄⅄significant difference inside the main subgroup B in comparison with terms 7–8 weeks ($p < 0.05$).

by triggering proliferation, migration, differentiation and inhibition of apoptosis of endothelial cells. Activated VEGF-R1 receptor stimulates intercellular interactions, branching of vascular network and regulates the trophoblast invasion into the spiral arteries [27]. Use of blocking antibodies for VEGF-R2 causes reduction in decidual angiogenesis and pregnancy loss in mice, while use of antibodies of similar effect for VEGF-R1 does not cause such effects [37]. In other studies, the lack of VEGF-R1 in experimental animals led to overdevelopment of disorganized vessels and clusters of endothelial cells, and the absence of VEGF-R2—to reduction in development of vasculature [2]. It has been demonstrated that interaction of VEGF/VEGF-R2 regulates the development of fetoplacental complex [27] and acts as a paracrine system in the processes of formation of primitive embryo vascular network [24, 29]. On the contrary, the formation of an active complex VEGF/VEGF-R1 mostly affects the processes of differentiation and migration of trophoblast and also regulates invasion [27].

4.4.2. PLGF/VEGF-R1 ratio

PLGF factor affects endothelium through specific binding with the receptor VEGF-R1 (**Figure 1C**). According to the published data, PLGF more impacts the processes of angiogenesis, than vasculogenesis; however, PLGF and VEGF-R1 also affect the mobilization of mesenchymal progenitors of endothelial cells, which are involved in vasculogenesis [2]. PLGF enhances angiogenesis acting synergistically with VEGF, and it is also able to replace VEGF in the complex with VEGF/VEGF-R1 releasing it for VEGF-R2 activation. It is also known that PLGF is a paracrine regulator of decidual angiogenesis and autocrine regulator of trophoblast's functions in differentiation and invasion [24] and also the main regulating factor in normal pregnancy in the first trimester [2, 24].

The ratio of PLGF/VEGF-R1 for each group of pregnant women had its own tendencies. There were significant differences between the main subgroup A and control group before 6 and at 7–8 weeks. However, there was notable misbalance of factors in ligand/receptor pair PLGF/VEGF-R1 manifesting with significant deviations from the average value of the factors ratio at 7-8 weeks of pregnancy in the main subgroup B. Moreover, low ratio values were noted in the ligand/receptor pair of the main subgroup A at all studied points. The reduced value of this ratio in the main subgroup A may be due to the changes in the dynamic system PLGF/VEGF-R1 (both toward the increase in sVEGF-R1 and toward the decrease in PLGF). Obtained ratio values for the given ligand/receptor pair for all groups at early terms of pregnancy may evidence the acceptable fluctuations of the values of the factors in this pair, which are nonsignificant for the development of pregnancy.

4.4.3. MMP-9/TIMP-1 and MMP-2/TIMP-2 ratio

Analysis of the obtained results showed no significant differences of ratios of free forms of MMPs and tissue inhibitors TIMPs (MMPs/TIMPs) between the groups at the most studied terms of pregnancy (**Figure 1D, E**). Significant differences were shown for the MMP-9/TIMP-1 ratio at 11-14 weeks for the main subgroup B (0.91) and the control group (1.48). The significant decrease in this ratio in the main subgroup B may evidence degradation processes and autolysis in missed abortion. The character of changes of the ligand/receptor ratio within

studied groups confirms some stable dynamic equilibrium of the factors concentrations. Probably, nonspecific effects of the factors in the studied ligand/receptor pairs on angiogenesis processes are of somewhat conservative nature comprising prevention of excessive protease activity, and sufficient for an adequate angiogenesis at the studied terms of pregnancy. However, use of these ratios is not informative to characterize the pathologic processes at studied terms in patients of these groups, excluding the ratio MMP-9/TIMP-1 at 11-14 weeks of pregnancy. The observed reduction in MMP-9/TIMP-1 at 11-14 weeks may serve as an alert of the development of critical events.

5. Conclusions

This study revealed the features of humoral systems regulating angiogenesis during physiological pregnancy and in patients with successful and unsuccessful perinatal outcomes. We found that deviations in the peripheral blood contents of angiogenesis-related factors: VEGF-R1, VEGF-R2, MMP-9 and TIMP-1 in patients with the history of missed abortion do not reflect critical for angiogenesis events in the first trimester of pregnancy. However, a significant disbalance of soluble factors, regulating angiogenesis, detected in patients with missed abortion shows that matrix metalloproteinases and their tissue inhibitors play the leading role in pregnancy losses before 6 and 7–8 weeks. Analysis of ligand/receptor ratios complements the obtained results, as we have found a significant decrease in the VEGF/VEGF-R1 and VEGF/VEGF-R2 ratios before 6 weeks of pregnancy despite the fact that there were no significant differences between individual molecules forming these pairs. The nature of VEGF/VEGF-R1 and VEGF/VEGF-R2 ratios alterations within the groups at the studied terms suggests the presence of a single mechanism that regulates interactions between VEGF and its receptors VEGF-R1 and VEGF-R2. In patients with pregnancy losses at 11-14 weeks, we found low concentrations of PLGF and sVEGF-R1 and also of MMP-2 and MMP-9, and reduction in MMP-2/TIMP-2 ratio, which are probably insufficient for an adequate angiogenesis at this term. Since an adequate angiogenesis is the determining factor for the development of pregnancy, early identification of criteria alerting about a trouble in fetoplacental system will also have diagnostic and prognostic value. Detection of the markers is especially important in cases of habitual pregnancy loss of unknown origin, because the disturbance of angiogenesis may be one of the causes of missed abortion. Taking into account difficulties with obtaining placental tissue at the studied terms of pregnancy, the angiogenesis-related factors may serve as unbiased indicators of placental angiogenesis. The obtained results allow to presume various mechanisms of pregnancy pathology at early terms and to demonstrate the possibility of using the analysis of ligand/receptor pairs to characterize the angiogenesis processes in early pregnancy.

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