Evaluation of VEGF in placental bed biopsies from preeclamptic women by immunohistochemistry

T. Cirpan[1], *Assist. Prof.*; **F. Akercan**[1], *Assoc. Prof.*; **M.C. Terek**[1], *Assist. Prof.*; **M. Kazandi**[1], *Assoc. Prof.*; **H.T. Ozcakir**[2], *Assoc. Prof.*; **G. Giray**[3], *Assist. Prof.*; **S. Sagol**[1], *Prof.*

[1]*Department of Obstetrics and Gynecology, Ege University Faculty of Medicine, Izmir;* [2]*Department of Obstetrics and Gynecology;* [3]*Department of Histology and Embryology, Celal Bayar University Faculty of Medicine, Manisa (Turkey)*

Summary

Objective: The aim of the study was to determine VEGF protein with immunohistochemical staining in placental bed biopsies of preeclamptic pregnancies in comparison to normal controls. *Design*: Prospective cohort study. *Methods*: The placental bed biopsies were obtained from 12 patients with preeclapmsia and ten patients for a control group at the time of cesarean delivery. Tissue samples of the placental bed were examined for VEGF protein distribution with avidin-biotin-peroxidase immunohistochemistry. Two blinded histopathologists were asked to score each sample for the intensity of staining and the number of cells stained in a randomly selected HPF of each sample. The resulting "H-score" was computed as a product of intensity and percent of cells stained. *Results*: VEGF expression was significantly lower in both the myometrium and stroma of the preeclamptic group compared to the control group (77.2 ± 25.4 vs 134 ± 44.3, p = 0.007; 194.1 ± 20.7 vs 170.2 ± 17, p = 0.017, respectively). *Conclusion*: VEGF expression is significantly lower in placental bed biopsies of preeclamptic pregnancies.

Key words: VEGF; Preeclampsia; Placental bed.

Introduction

Preeclampsia is a multisystem disorder peculiar to human pregnancy. It occurs in 4-5% of all pregnancies and remains a leading cause of maternal and neonatal mortality and morbidity [1]. Characterized by new onset of proteinuria and hypertension after 20 weeks of pregnancy, preeclampsia can progress to seizures (eclampsia), stroke, renal failure, pulmonary edema, liver failure, and coagulopathy. The only effective treatment is delivery, which may result in substantial neonatal morbidity and mortality if the fetus is delivered before 30 weeks of gestation. The pathophysiology of this syndrome is not fully understood. Preeclampsia is currently believed to be a 2-stage disease. In the early stage suboptimal development of the placenta and a hemodynamic maladaptation to pregnancy exist. The first stage is characterized by shallow cytotrophoblast invasion of maternal spiral arterioles, resulting in placental insufficiency. At this stage maternal constitutional factors such as genetic and immunological factors and pre-existing vascular diseases may play a role. There are no maternal signs or symptoms during this stage. Due to this defective placentation, the hypoxic placenta releases soluble factors into the maternal circulation, which induce systemic endothelial dysfunction. This causes the second stage of the disease: the maternal syndrome. During this stage, hypertension and proteinuria, the clinical signs of preeclampsia, are manifested. This factor then results in the late vascular dysfunction characterized mainly by a generalized endothelial dysfunction, leading to the clinical syndrome of preeclampsia [2]. The placental bed, the area of the maternal uterus underlying the placenta, plays a key role in supporting placental function during gestation. There are angiogenic growth factors, such as vascular endothelial growth factor (VEGF), produced by the placenta and these factors are specific for vascular growth and differentiation. VEGF plays a major role in the mediation of angiogenesis, and hypoxia is a potent stimulus for VEGF gene expression [3,4].

The aim of the study was to determine VEGF with immunohistochemical staining in placental bed biopsies of patients with preeclampsia and to compare it with placental bed biopsies of normotensive pregnancies.

Materials and Methods

Placental bed biopsies were obtained from patients who were admitted to the Department of Obtetrics and Gynecology, Ege University Faculty of Medicine. Written informed consent was obtained from each patient. All biopsies from the center of the placental bed [5] were obtained with biopsy forceps in patients with preeclampsia (n = 12) and a control group (n = 10) at the time of cesarean delivery.

Women with preeclampsia were selected on the basis of the criteria described by the International Society for the Study of Hypertension in Pregnancy: diastolic blood pressure of ≥ 110 mm Hg on any occasion or ≥ 90 mm Hg on two separate occasions at least two hours apart and proteinuria of 1 g or 2+ sticks in two clean midstream urine samples taken at least four hours apart or 0.3 g in 24 hours or 1+ stick in a sample of specific gravity of < 1.03 and pH < 8, developing after 20 weeks of gestation and returning to normal values within three months after delivery [6]. In the study group there were no severe preeclampsia cases according to the American College of Obstetricians and Gynecologogists criteria [7]. The women selected for this

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investigation were previously healthy with uncomplicated pregnancies, except for preeclampsia in the study group. In particular, no women with chronic hypertension, renal disease, or diabetes were included. Women in both the preeclamptic and the control group were delivered by cesarean section. All pregnancies were singletons, and none of the women were in active labor at the time of cesarean delivery.

Cesarean section was performed according to obstetrical indications in both groups: non-reassuring fetal status (repeated decelerations and prolonged low variability on fetal heart rate tracings) (n = 4), cephalopelvic disproportion (n = 5), unfavorable cervical ripening (n = 3), repeat cesarean section (n = 3), breech presentation (n = 3), brow presentation (n = 1), transverse lie (n = 1), placenta previa (n = 2).

Placental bed biopsies were examined for VEGF distribution with avidin-biotin-peroxidase immunohistochemistry. The biopsies were fixed in formalin solution for a maximum of 24-48 hours. Samples were washed with water and soaked in a graded series of ethanol (60, 70, 80, 90, 100%). Then they were held in a solution of xylene for 90 minutes and embedded in paraffin at 60°C. Cross-sections (5 μm thick) were cut and prepared for both histochemical and immunohistochemical staining. Hematoxylin and eosin (H&E) staining was used for histological examination. For immunohistochemical staining, sections were first incubated at 60°C overnight and then incubated in xylene for 30 minutes. After washing with serial concentrations of ethanol (95, 80, 70, 60%), the sections were washed with distilled water and phosphate-buffered saline (PBS) for ten minutes. They were then held in 2% trypsin in Tris buffer at 37°C for 15 minutes and washed with PBS three times for five minutes. The limits of sections were drawn by a Dako pen (Dako, Glostrup, Denmark) and incubated in a solution of 3% H_2O_2 for 15 minutes to inhibit endogenous peroxidase activity. Then the sections were washed with PBS and incubated overnight at +4°C with primary antibodies: VEGF in a 1/200 dilution (Santacruz-sc-7269, CA). Afterwards they were washed three times for five minutes each with PBS, followed by incubation with biotinylated IgG and then with streptavidin-peroxidase conjugate (Zymed 85-9042, Lot no: 20570999, CA). After washing, three times for five minutes with PBS, sections were incubated with DAB substrate containing diaminobenzidine (Zymed 00-2020, Lot = 21074104,CA) for five minutes to detect immunoreactivity and then with Mayer's hematoxylin. Sections were covered with mounting medium and were analyzed on a light microscope with a BX 40 microscope (Olympus, Tokyo, Japan). Control samples were processed in an identical manner, but without the primary antibody step.

All of the immunostained sections were reviewed by the two histologists who were blinded to the groups. All the biopsies were "real placental bed" biopsies, i.e., they looked for the presence of trophoblasts as a quality control. Slides were examined under low power (x 4 objective) to identify regions containing VEGF staining of decidua (i.e., trophoblastic cells, decidual cells, inflammatory cells and spiral arteries) and myometrium in the placental bed. Five areas selected at random were scored, and in sections where all of the staining appeared intense, one random field was selected. The proportion of decidua (i.e., trophoblastic cells, decidual cells, inflammatory cells and spiral arteries) and myometrium in each selected field was determined by counting at high magnification. At least 100 cells were scored per x 40 field for each group. All sections were scored in a semiquantitative fashion, which considered both the intensity and percentage of cells staining at each intensity. Intensities were classified as 0 (no staining), +1 (weak staining), +2 (moderate staining), and +3 (very strong staining), whereas 10% groupings were used for the percentage of cells that stained positive. For each slide, a value designated H-SCORE was obtained by application of the following algorithm: H-SCORE = Σ (I x PC), where I and PC represent intensity and percentage cells that stain at each intensity, respectively, and corresponding H-SCOREs were calculated separately.

Data are expressed as mean ± standard deviations (SD). The differences in groups were analyzed by the Mann Whitney U-test. Pearson's correlation analysis was used to correlate the variables; a p value < 0.05 was considered statistically significant. The statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) Version 10.0 for Windows (SPSS Inc., USA).

Results

The demographic variables of the patients are summarized in Table 1. Apgar scores at 1 and 5 min, birth weights (g) and placental weights (g) are also summarized in Table 1. Seven out of 12 (58.33%) patients of the preeclamptic group and six out of ten (60%) patients of control group were nulliparous. Immunohistochemical staining of VEGF was significantly lower in both the myometrium and stroma of the preeclamptic group as compared to controls (77.2 ± 25.4 vs 134 ± 44.3, p = 0.007; 194.1 ± 20.7 vs 170.2 ± 17, p = 0.017, respectively) (Table 2).

Sections were composed of decidua along with myometrium and light microscopic examination of placental bed biopsies of the control group and preeclamptic group revealed very strong and moderate VEGF immunoreactivity, respectively. Moderate VEGF immunostaining in preeclamptic woman is shown in Figure 1.

Table 1. — *Demographic variables of the patients.*

	Control group (n = 10)	Preeclamptic group (n = 12)	*p*
Age	31.2 ± 5.9	28.8 ± 5.4	0.87
Gravida	1.9 ± 0.5	1.4 ± 0.9	0.28
Parity	0.6 ± 0.3	0.4 ± 0.3	0.72
Gestational week	38.1 ± 1.6	37.1 ± 2.2	0.51
Birth weight (g)	3235 ± 482	2937 ± 655	0.19
Placental weight (g)	485 ± 76	425 ± 97	0.16
Apgar score at 1 min	9.1 ± 0.4	8.2 ± 1.4	0.7
Apgar score at 5 min	9.0 ± 0.3	8.6 + 0.6	0.53

Table 2. — *Immunostaining scores for VEGF in the two groups.*

	Control group (n = 10)	Preeclamptic group (n = 12)	*p*
VEGF (myometrium)	134 + 44.3	77.2 + 25.4	0.007
VEGF (decidua)	194.1 + 20.7	170.2 + 17	0.017

VEGF: Vascular endothelial growth factor.

Discussion

VEGF is a disulphide-linked homodimeric glycoprotein that is selectively mitogenic for endothelial cells and appears to play a major role in the mediation of vasculogenesis and angiogenesis [8-13]. Hypoxia is a potent stimulus for induction of VEGF synthesis. Expression of the angiogenic growth factors such as VEGF was demon-

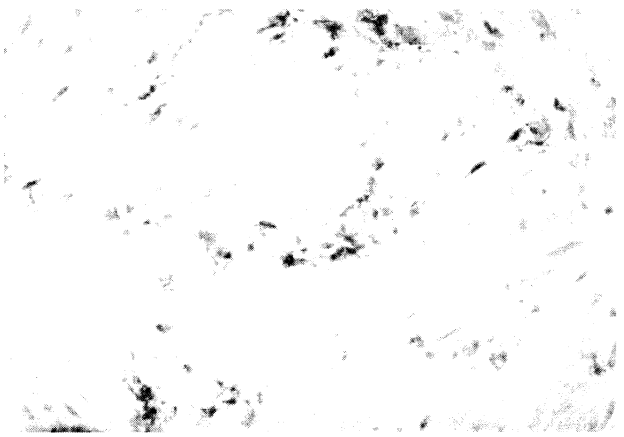

Figure 1. — VEGF immunostaining in a preeclamptic woman; the sections were composed of decidua along with myometrium. The myometrium revealed weak VEGF immunoreactivity, while the decidua revealed moderate VEGF immunoreactivity (hematoxylin-eosin stain, original magnification, 20 x).

strated in isolated human term cytotrophoblasts and in vitro differentiated syncytiotrophoblasts [14]. VEGF and other factors are likely to be involved in uterine vessel remodelling and in angiogenesis which occurs during the growth of the placenta throughout pregnancy [15]. Another important role has been postulated for VEGF in the regulation of trophoblast invasion, proliferation and differentiation [8].

In the rhesus monkey, Wang *et al.* [16] detected that there was immunostaining of VEGF in the placental villi and endometrial compartments including spiral arterial walls and the glandular epithelium. It means that VEGF and VEGF-receptors have a role in trophoblast invasion, maternal vascular transformation, and fetoplacental vascular differentiation and development during early pregnancy in the rhesus monkey. The predominant growth factor in the early placenta appears to be VEGF [17].

In normal pregnancies, successful placentation involves the development of a low-impedance uteroplacental circulation after trophoblast invasion and transformation of the maternal intramyometrial portion of the spiral arterioles. In pregnancies complicated by preeclampsia, trophoblast invasion of the spiral arterioles is abnormal, resulting in impaired uteroplacental perfusion. This may result in release of factors into the maternal circulation which are responsible for endothelial dysfunction, vasoconstruction and hypertension [18, 19].

There is accumulating evidence that deficient trophoblast invasion of the placental bed spiral arteries is crucial to the pathogenesis of preeclampsia and intrauterine growth restriction. However, the factors which regulate the process of trophoblast invasion remain unclear. Lash *et al.* [20] have investigated whether extravillous trophoblast invasion and motility are mediated by angiogenic growth factors such as VEGF. They stated that caution must be exercised before any extrapolation to the in vivo situation, however, it could be speculated that the increased motility in response to VEGF may be an initial response to attract trophoblast cells to the decidua, and that VEGF might then limit the degree to which trophoblast cells invade.

Taylor *et al.* [21] postulated that decreased placental growth factor production results in abnormalities of placental angiogenesis through direct and indirect effects on other vasculotropic growth factors.

Several investigations have shown that VEGF concentrations are increased in the plasma or serum of women with preganacies complicated by hypertensive disorders compared with normotensive women, although this has not been confirmed in other studies [1,22-30]. Livingston *et al.* [31] showed that women with severe preeclampsia had significantly lower plasma concentrations of both vascular endothelial growth factor and placental growth factor than did women with normotensive pregnancies. They stated that there was an abnormality of growth factor expression in the placenta during pregnancies complicated by preeclampsia.

Studies of the placental VEGF immunostaining in pregnancies complicated by more or less severe hypertensive disorders have reported a decrease of this factor, while in another study an increase was observed [23, 27, 32]. In a current study, Sgambati *et al.* [33] showed that levels of VEGF mRNA were higher in gestational hypertension cases and lower in cases of preeclampsia with HELLP syndrome with respect to the controls; in the cases of preeclampsia, the levels were the same as the controls. They concluded that in the pregnancies with gestational hypertension, an increase of VEGF might be a compensatory mechanism attempting to restore blood flow towards normal. They also showed that VEGF immunostaining was weak in the placentas of preeclamptic and HELLP patients and concluded that in more severe cases like preeclampsia and HELLP, there was probably also an attempt of compensation but only some components of the placenta seemed to be able to produce VEGF as shown by immunohistochemistry.

Tsatsaris *et al.* [34] investigated whether dysregulation in the VEGF family may explain the defective uteroplacental vascularization characterizing preeclampsia. They compared maternal plasma, placentas, and placental bed biopsies of pregnancies complicated by early onset severe preeclampsia or intrauterine growth retardation to normal pregnancies. The mRNA levels of VEGF-A, PlGF, and their receptors were quantified in placentas and placental beds. Levels of VEGF-A, PlGF, and soluble VEGF receptor (VEGFR) were assessed in maternal plasma. They concluded that in compromised pregnancies, elevated levels of VEGF-A and VEGFR-1 mRNAs may reflect the hypoxic status of the placenta, whereas membrane-bound VEGFR-1 was decreased in the placental bed of preeclamptic patients. Preeclampsia was associated with low levels of circulating PlGF and increased levels of total VEGF-A and soluble VEGFR-1. Free VEGF-A was undetectable in the maternal blood. Immunohistochemical studies revealed that VEGF-A and PlGF were localized in trophoblastic cells. Altogether, their results suggest two different pathophysiological mechanisms associated with preeclampsia. The first one is related to

an overproduction of competitive soluble VEGFR-1 that may lead to suppression of VEGF-A and PlGF effects. The second one is the down-regulation of its membrane bound form (VEGFR-1) in the placental bed, which may result in defective uteroplacental development.

Additionally, in the present study, it was demonstrated that immunostaining of angiogenic growth factor VEGF was significantly lower in the placental bed biopsies of preeclamptic pregnancies.

Common obstetrical complications manifest altered placental bed vascularity. Placental bed vascularization reflects a complex interaction of regulatory factors. Understanding the regulation of vascular growth in the placental bed will provide much needed insight into placenta-related vascular insufficiencies.

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Address reprint requests to:
T. CIRPAN, M.D.
Department of Obstetrics and Gynecology
Ege University Faculty of Medicine
Bornova, Izmir
35100 (Turkey)