Expression of VEGF and VEGF-R3 Receptor by Placental Endothelial Cells in Health and Gestosis

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We carried out a comparative analysis of changes in VEGF secretion and expression of VEGF-R3 receptor by placental endothelial cells in health and gestosis and of changes in VEGF-R3 expression by EA.hy926 human endothelial cells during culturing with supernatants conditioned by placental explants from women with normal pregnancy and patients with gestosis. Reduced secretion of VEGF and expression of VEGF-R3 by placental endothelial cells in gestosis can be caused by functional deficiency of the endothelial cells and low viability of endothelial cells.

Key Words: *gestosis; endothelial cells; vascular endothelial growth factor*

Vasculo- and angiogenesis are the central processes in the development of the placenta. As one of the main functions of the placenta is realization of the contact between maternal and fetal blood vessels, the formation of placental vascular bed determines the efficiency of gestation processes, as the quality of villous vascularization and effective functioning of blood vessels determine vital activity of the fetus [2]. A frequent cause of spontaneous abortion is vascular pathology leading to disorders in the microcirculation, placentation, and eventuating in miscarriage. The function of endothelial cells (EC), expression of adhesion molecules, secretion of cytokines, growth factors, and other molecules by these cells, and EC permeability for soluble factors and molecules largely determine the effective formation and functioning of the placenta, normal course of pregnancy and labor. Placental cells are regulated by a great spectrum of cytokines and molecules, forming a balanced network and determining the formation of the placenta and its functioning at different stages.

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Differentiation of mesenchymal precursor cells into hemangioblasts, their further differentiation into EC are regulated by vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and their receptors (VEGF-R and FGF-R). The most important factor for the development of placental network and maintenance is VEGF. It belongs to a family of vascular growth factors, including isoforms VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and PlGF. These factors serve as ligands for the corresponding receptors: VEGF-A, VEGF-B, and PlGF serve as ligands for VEGF-R1 (Flt-1); VEGF-A, VEGF-C, VEGF-D, and VEGF-E are ligands for VEGF-R2 (KDR); and VEGF-C and VEGF-D serve as ligands for VEGF-R3 (Flt4) [8]. Vascular endothelial growth factor is essential for degradation of the basal membrane, fibrin, and interstitial matrix of EC, for EC proliferation, formation of new capillary tubules and new basal membrane, thus regulating all stages of angiogenesis in the placenta [4,5,7].

Impairment of VEGF secretion in the placenta and of its receptors expression can disorder the angiogenesis processes in the placenta, leading to placental insufficiency and augmenting EC dysfunction [13,14]. Expression of VEGF and its re-

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ceptors in the placenta in health and disease is described [6,12]. However, no models for *in vitro* reproduction of the processes in the placenta and evaluation of placental EC function were developed.

We compared changes in VEGF secretion and expression of VEGF-R3 in placental EC in health and gestosis and analyzed the changes in the expression of VEGF-R3 by EA.hy926 human EC, cultured with supernatants obtained by culturing of placental explants from women with normal pregnancy and gestosis.

MATERIALS AND METHODS

Ten normal human placentas (control) and 10 placentas from pregnant patients with gestosis were studied. All deliveries were carried out by cesarean section. Placental explants were fixed in formalin for subsequent immunohistochemical analysis of VEGF and VEGF-R3 expression. Other explants from the same placentas were cultured in DMEM with 10% serum for 24 h, after which supernatants were collected and frozen at -20°C. The content of VEGF in the supernatants was measured by flow cytofluorometry using BD Cytometric Bead Array (BD) and FACStrack cytofluorometer (BD).

Human EC strain EA.hy926 was derived by hybridization of HUVEC primary endothelial cell strain with A-549 lung carcinoma cells in 1983 by Dr. C. J. Edgel (University of North Caroline, USA). The EA.hy926 strain reproduces the main morphological, phenotypical, and functional characteristics of the endothelium. The cells were cultured in DMEM with 10% inactivated FCS (ICN), 50 μg/ml gentamicin sulfate, 2 mM L-glutamine, and HAT (ICN). The EA.hy926 cells were cultured with the resultant supernatants for 24 h, after which were fixed in formalin, and cytological analysis was carried out after hematoxylin and eosin staining; in addition, immunohistochemical analysis of VEGF-R3 expression was carried out.

Immunohistochemical analysis was carried out with monoclonal mouse antibodies to VEGF-R3 (1:50; Novocastra) and VEGF (1:20; BD Biosciences Pharmingen) according to a single-stage protocol with the antigen demasking (by high-temperature treatment of tissue) in 0.01 M citrate buffer (pH 7.6). A universal kit of biotin-treated anti-mouse and anti-rabbit immunoglobulins served as the second antibodies. The reaction was visualized using avidin complex with biotinilated peroxidase (ABC kit) with subsequent development of horseradish peroxidase by diaminobenzidine (Novocastra). The data were analyzed under a Nikon Eclipse 400 microscope using an image analysis system and Morphology 4.0 software.

RESULTS

Immunohistochemical study of placental preparations showed that the expression of VEGF-R3 by the syncytiotrophoblast (ST) cells and villous vascular EC of the placentas from patients with gestosis was significantly lower (expression area 2.31± 0.47%) than the expression of VEGF-R3 by ST cells and villous vascular EC of placentas from healthy pregnant women (expression area 4.88± 1.1%; $p<0.01$; Fig. 1).

The expression of VEGF by ST cells and placental villous vascular EC in patients with gestosis was significantly lower (expression area 0.58± 0.13%) that VEGF expression by ST cells and placental villous vascular EC in healthy pregnant women (expression area 3.11±0.49%; $p<0.01$).

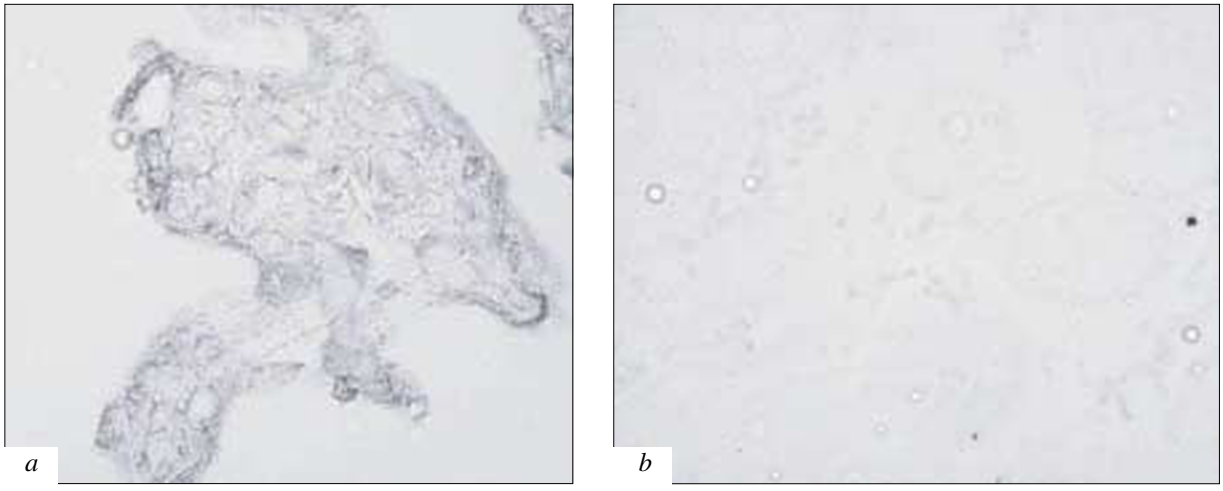

Fig. 1. Expression of VEGF-R3 by ST cells and villous vascular EC of placentas from healthy pregnant women (*a*) and patients with gestosis (*b*). Immunohistochemical staining, ×400.

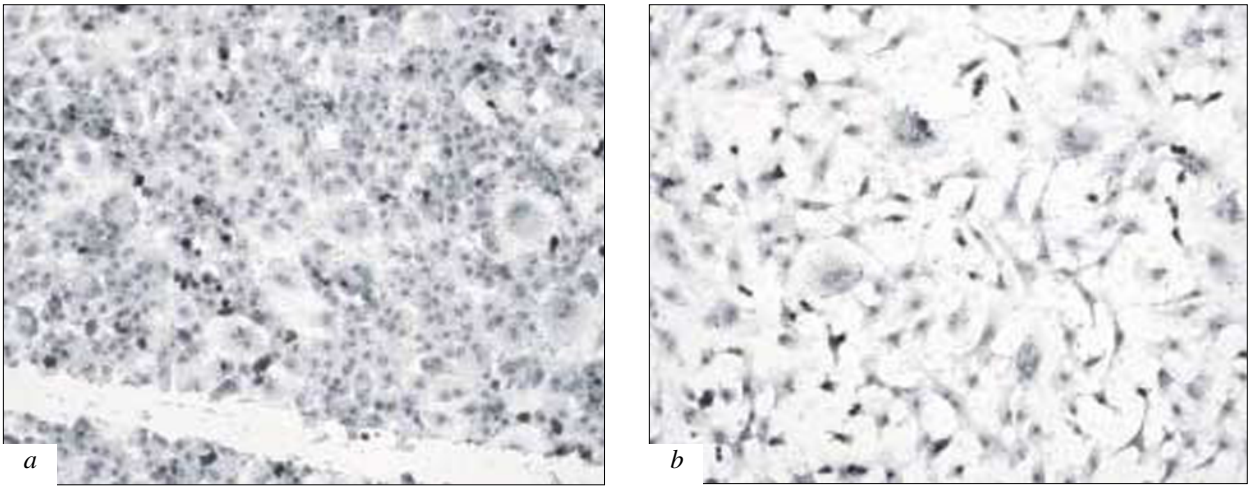

Fig. 2. Human EC EA.hy926 strain after 24-h culturing with supernatants obtained by culturing of placental explants from healthy pregnant women (*a*) and patients with gestosis (*b*). Hematoxylin and eosin staining, ×100.

Comparison of the expression areas for VEGF-R3 and VEGF in the studied groups revealed more intensive expression of VEGF-R3 in comparison with VEGF expression in the gestosis group ($p<0.05$). No appreciable differences in the expression of these markers were detected in the control group.

No VEGF was detected in the supernatants after culturing of placental explants from healthy pregnant women or patients with gestosis in DMEM with 10% serum during 24 h. This can be due to the fact that VEGF is secreted by placental cells at late terms of gestation in trace amounts (which is confirmed by the data of immunohistochemical analysis of placentas in our study) and immediately binds to target cells.

For comparison with the possible *in vivo* situation, we cultured EA.hy926 cells with supernatants obtained by culturing of placental explants from healthy pregnant women and patients with gestosis. Cytological study of EA.hy926 cultures stained with hematoxylin and eosin showed that the number of EA.hy926 cells incubated with supernatants obtained by culturing of placental explants from patients with gestosis decreased in a visual field, the cells were deformed, their nuclei were elongated and hyperchromatic. Morphological characteristics of EA.hy926 cells, incubated with supernatants obtained by culturing of placental explants from healthy pregnant women, did not change (Fig. 2).

Immunohistochemical study of EA.hy926 cell cultures showed that the cells incubated in common culture medium were characterized by low spontaneous expression of VEGF-R3 (expression area 2.15±0.01%). The expression of VEGF-R3 by EA.hy926 cells, incubated with supernatants obtained by culturing of placental explants from patients with gestosis, was significantly lower (expression area 4.89±1.01%) than VEGF-R3 expression by EA.hy926 cells, incubated with supernatants obtained by culturing of placental explants from healthy pregnant women (expression area 11.7±0.52%; $p<0.05$; Fig. 3).

One of the main functions of VEGF in the placenta at late terms of gestation is maintenance of high viability of EC and stabilization of the vascular bed. Our data obtained *in situ* and *in vitro* indicate reduction of EC viability in gestosis (presumably because of low expression of VEGF-R3 by these cells) and low secretion of VEGF by ST cells and villous vascular EC of placentas from patients with gestosis. Low expression of VEGF-R3 by placental EC in gestosis and by EA.hy926 cells, incubated with supernatants obtained by culturing of placental explants from patients with gestosis, can be explained by intensive loss of these receptors from the cell surface. This effect was described for VEGF-R1. The loss of VEGF-R1 led to accumulation of the secretory form of sVEGF-R1 in the serum of patients with gestosis and reduction of VEGF-R1 expression in the placenta, but did not lead to reduction of serum concentration of VEGF in patients with gestosis [11,14]. It seems that the secretory forms sVEGF-R1, sVEGF-R2, or sVEGF-R3 competitively bind VEGF-A, VEGF-B, or VEGF-C in the placenta, which leads to EC dysfunction, reduces cell viability, and impairs the development of the placental vascular network.

In addition, low expression of VEGF-R3 and low secretion of VEGF by EC can be explained by the inhibitory effect of various cytokines secreted by the microenvironment cells in the placenta. The concentrations of pro- and antiangiogenic factors are balanced in normal placenta, due to which the

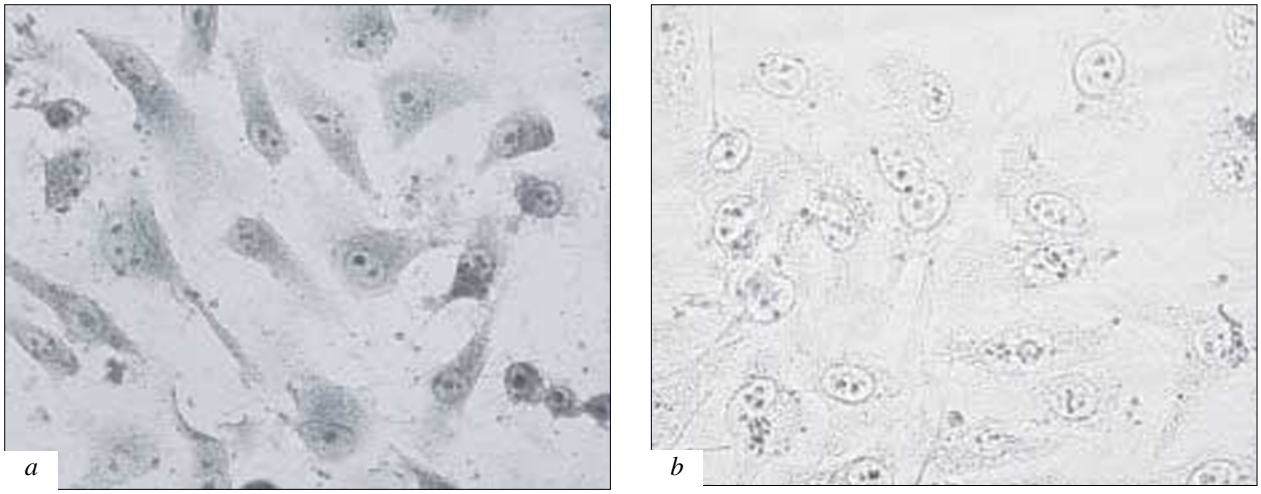

Fig. 3. Expression of VEGF-R3 by EA.hy926 cells after 24-h culturing with supernatants obtained by culturing of placental explants from healthy pregnant women (*a*) and patients with gestosis (*b*). Immunohistochemical staining, ×400.

vascular network develops normally and is stable. Imbalance resultant from hypoxia, infection, or EC dysfunction disorders the angiogenesis processes, placentation, and leads to spontaneous abortions. The placental macrophages are believed to play the key role in the production of cytokines and other factors in the placenta [9,10]. These cells can significantly modify the balance of cytokines and growth factors in the EC microenvironment, thus modifying their functions. Moreover, the factors secreted by placental macrophages also modify the trophoblast and decidual tissue cells, which, in turn, secrete cytokines, modifying the EC functions. The concentrations of proangiogenic factors in placental tissues are significantly reduced in gestosis, while the concentrations of antiangiogenic factors are increased. Antiangiogenic factors, such as TNF-α, secreted by placental macrophages, suppress proliferation and migration of EC [1,3]. Presumably, high content of antiangiogenic and proinflammatory factors in supernatants obtained after culturing of placental explants from patients with gestosis was responsible for reduced viability and low expression of VEGF-R3 by EA.hy926 human EC.

The proposed method for evaluating the status of EC can be used for *in vitro* simulation of *in vivo* processes in the placenta, which will help to detect the etiology and pathogenesis of placental failure and gestosis. The proposed model can also be used for trials of drugs intended for improvement of endothelial function.

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