GENETICS

Expression of Thrombospondin-1 Gene mRNA and Protein in the Placenta in Gestosis

Yu. V. Ostankova, Ya. S. Klimovskaya, O. A. Gorskaya, A. V. Kolobov, I. M. Kvetnoi, S. A. Selkov, and D. I. Sokolov

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The expression of TSP-1 gene mRNA and TSP-1 protein in the placental tissue was studied during normal pregnancy and in gestosis. The formation of placental tissue in normal gestation was associated with expression of TSP-1 gene mRNA and of TSP-1 protein. Gestosis was associated with inflammatory reaction in the placenta characterized by increased counts of lymphocytes and macrophages in the villous stroma and involution degenerative changes in tissue. Disorders in placental villi maturation and branching in gestosis were paralleled by hyperexpression of TSP-1 gene mRNA by placental cells and hyperexpression of TSP-1 protein predominating in the stromal elements of terminal villi and near villous vessels.

Key Words: *thrombospondin; placenta; gestosis; macrophages; angiogenesis*

Gestosis is one of the most important problems of modern obstetrics, which ranks third among the causes of maternal mortality [1,2]. Despite numerous studies, the etiology and pathogenesis of gestosis remain not quite clear. Generalized endothelial dysfunction and disorders in the formation of placental tissue underlie the pathogenesis of gestosis [1,4]. Angiogenesis is an obligatory component of placenta development. This process is associated with apoptosis of some trophoblast cells and their subsequent replacement by new cells. Disorders in the production of factors regulating angiogenesis and apoptosis are the cause of abnormal development of the placental vascular network.

Thrombospondin-1 (TSP-1), a glycoprotein with antiangiogenic effect, is a factor inducing apoptosis of endothelial cell [10]. This factor is present in the platelets, is produced by tissue macrophages, and was found in low concentrations in blood plasma [3]. Thrombospondin-1 reacts with at least 9 receptors on endothelial cells: α3β1, α4β1, α6β1, α9β1, and αvβ3 integrins [11], LRP (LDL receptor-related protein) receptor, calreticulin-related receptor, heparan sulfate proteoglycanes, CD36 and CD47 [6]. Through these receptors TSP-1 regulates adhesion, proliferation, migration, and viability of endothelial cells [9], suppresses the effects of bFGF and VEGF towards endothelial cells by modifying all angiogenesis stages *in vitro* and *in vivo* [13].

Disorders in the formation of the vascular network in the placental tissue in gestosis can result from hyperproduction of antiangiogenic factors, *e.g.* TSP-1.

We studied the expression of TSP-1 gene mRNA and TSP-1 protein in placental tissue in normal pregnancy and gestosis.

MATERIALS AND METHODS

Ten placentas from women at weeks 38-39 of uneventful pregnancy (control) and 15 placentas from wom-

D. O. Ott Institute of Obstetrics and Gynecology, North Western Division of the Russian Academy of Medical Sciences, St. Petersburg, Russia. ***Address for correspondence:*** corbie@hotmail.ru. D. I. Sokolov

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en with gestosis of medium severity at weeks 38-39 (main group) were studied. The age of women varied from 18 to 37 years (mean 31.6±4.2 years). All women were primigravidae. Women with diabetes mellitus receiving insulin therapy, with hydramnios, oligoamnios, urogenital infections, acute infections or exacerbations of chronic infections, essential hypertension, and organic diseases of the circulatory system were excluded from the study. The groups of pregnant women were comparable by age and obstetrical history. Cesarean section was carried out in all cases. Fragments of the placentas were fixed in 10% neutral formalin for subsequent immunohistochemical analysis of TSP-1 expression in serial sections. Fragments of the same placentas for isolation of mRNA were plunged in guanidinthiocyanate buffer and stored at -20°C.

Immunohistochemical analysis was carried out using mouse monoclonal antibodies to TSP-1 (1:50, Novocastra) by a single-step protocol with the antigen demasking (high-temperature treatment of the tissue) in 0.01 M citrate buffer (pH 7.6). A universal kit of biotinilated antimouse and antirabbit immunoglobulins (Novocastra) served as second antibodies. Visualization was carried out by the avidin complex with biotinilated peroxidase (ABC-kit) with subsequent development of horseradish peroxidase with diaminobenzidene (Novocastra). The data were analyzed under a Nikon Eclipse 400 microscope and processed by computer analysis of microscopic images and Morphology 5.0 software (Videotest).

The expression of TSP-1 mRNA in placental tissue was evaluated by the qualitative PCR and real time PCR. mRNA was isolated by the protocol suggested by Chomczhynski *et al.* with Trizol kit (Invitrogen) with an original modification [5]. Interleukin-6 served as the internal control (high expression of its mRNA has been demonstrated in gestosis and normal pregnancy). Specific primers, probes, and amplification conditions were selected for qualitative PCR and real time PCR. Reverse transcription was carried out with MMulV reverse transcriptase and specific primer. Calibration curve was plotted using a series of the control group placental cDNA dilutions.

The quality of the reaction was evaluated by standard parameters. Threshold fluorescence was evaluated on the basis of the mean fluorescence of all samples for cycles 3-15. The expression levels were normalized by the comparative Ct method, after which specimens of the placentas from women with gestosis were compared with the corresponding parameters of the placentas from healthy women. The significance of differences was evaluated by precise two-tailed Fisher's test with Yates correction for paired comparisons with the control group and by the χ^2 test with Yates correction for paired comparisons and Bonferroni correction for multiple comparisons with the control group [8]. Odds ratio (OR) was used in case of significant differences.

RESULTS

The presence of TSP-1 gene cDNA (and hence, expression of mRNA) was detected by qualitative PCR in the placental tissues from healthy women and from women with gestosis. The fluorescence in UV light of TSP-1 gene mRNA in the placentas of women with gestosis was significantly brighter in comparison with the control.

The study of TSP-1 mRNA expression by real time PCR showed significantly higher ($p<0.03$) expression of mRNA in the placentas from women with gestosis in comparison with control placentas (Fig. 1). The increase in TSP-1 mRNA expression in women with gestosis attests to the involvement of this gene product in the pathogenesis of this condition. According to estimated OR, the risk of gestosis development was associated with the expression of TSP-1 gene mRNA: OR=7.8, $p<0.03$, df=1, Cl=1.162-52.377; in other words, the risk of this disease development in women with high expression of the studied gene mRNA was 7.8 times higher than in women with normal expression of this gene.

Morphological studies of the placentas in the control group showed predominance of terminal villi coated with unilamellar syncytiotrophoblast with a moderate number of capillaries. In gestosis (weeks 38-39), maturation and branching of the villi were disordered: the intermediate immature villi predominated, a pronounced fibroblastic reaction was seen in the villous stroma with reduction of the vascular network in the intermediate and terminal villi. Moderate involutional degenerative changes (fibrinoid in the

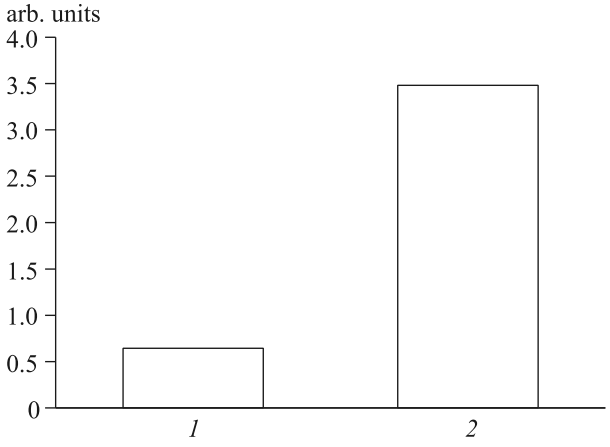

Fig. 1. Mean expression of TSP-1 gene mRNA in placental tissue in normal pregnancy (*1*) and gestosis (*2*).

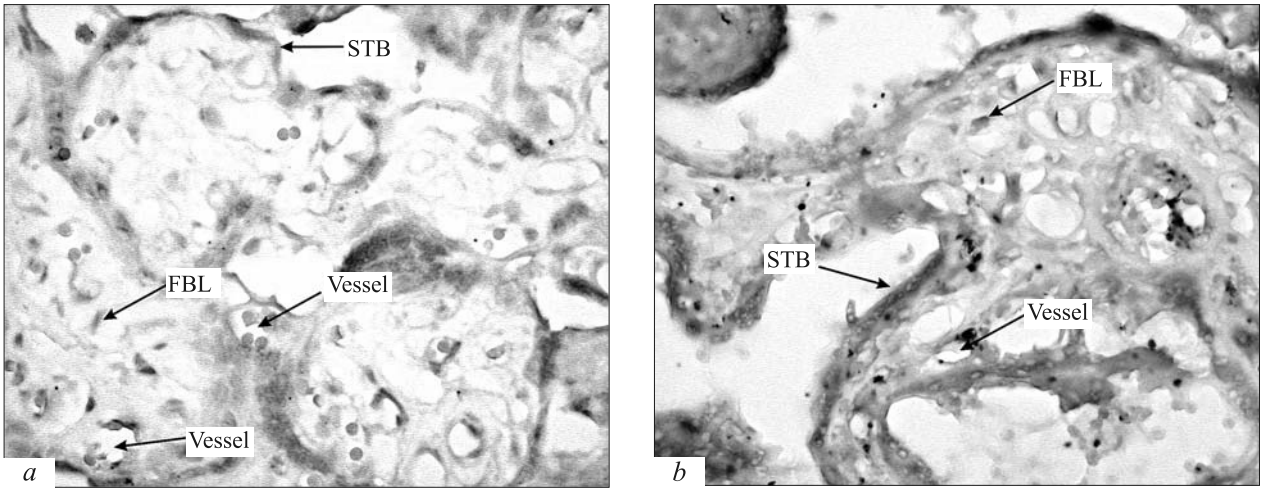

Fig. 2. Expression of TSP-1 in placental tissue during weeks 38-39 of normal pregnancy (*1*) and in gestosis (*b*), ×400. FBL: fibroblast; CTB: syncytiotrophoblast.

intervillous space, calcinosis of the villi and intervillous space), mononuclear infiltration with predominating lymphocytes were seen. Immunohistochemical analysis showed expression of TSP-1 protein in the placentas, this expression being significantly higher in gestosis (the expression area 0.61±0.05%, $p<0.05$; Fig. 2, *b*) than in normal pregnancy (expression area 0.08±0.01%; Fig. 2, *a*). The expression of TSP-1 protein in the placentas of control women was presented by solitary positively stained sites in stromal elements located near the vessels in intermediate villi, while in the main group it was diffuse and predominated in the stromal elements of terminal villi (Fig. 2). In the presence of pronounced involution degenerative changes in the placental tissue, the expression of TSP-1 protein was detected in the fibrinoid.

The expression of TSP-1 mRNA and production of this protein in the placenta in normal pregnancy attest to an important role of this factor in the regulation of angiogenesis processes in mature placenta. One of the most important sources of TSP-1 in the placenta are placental macrophages [12]. Normally, macrophages initiate angiogenesis, proliferation, migration, and differentiation of endothelial cells by secreting VEGF and bFGF. They also complete the angiogenesis processes by switching over to the secretion of antiangiogenic factors (TSP-1, TNF-α), inducing apoptosis in endothelial cells [12]. The presence of TSP-1 in the placental tissue at the end of normal pregnancy ensures completion of angiogenesis processes and promotes stabilization of the vascular bed, which we noted when evaluating the placental morphology.

Importantly that the counts of lymphocytes and macrophages in placental tissue were negligible in the 3rd trimester of normal gestation, while in gestosis the counts of these cells in villous stroma increased significantly. Initiation of inflammatory reaction in placental tissue in gestosis could contribute to disorders in the balanced cytokine system determining the physiological development of the placenta and stimulate production of antiangiogenic factors, *e.g.* TSP-1, in the placenta. Hyperexpression of TSP-1 gene mRNA and TSP-1 protein in placental tissue in gestosis compared to those in normal pregnancy reflected previously described disorders in the regulation of the placental vascular network formation [7] and intensification of the cytokine reactions towards placental cells. As the main producers of TSP-1 in placental tissue are placental macrophages [12], the detected hyperexpression of TSP-1 near the placental fetal vessels was most likely due to macrophage infiltration in the course of inflammatory process in gestosis. These data indicated a shift of the secretion profile of placental cells towards antiangiogenic factors, leading to disorders of angiogenesis processes in the placenta.

Hence, the production of TSP-1 by placental cells in the course of normal pregnancy is essential for completion of the placental structures formation. Gestosis is associated with realization of inflammatory reaction in placental tissue, characterized by increased counts of lymphocytes and macrophages in villous stroma and moderate involutional degenerative processes. Disturbances in maturation and branching of placental villi in gestosis are associated with hyperexpression of TSP-1 gene mRNA by placental cells and with hyperexpression of TSP-1 protein predominating in the terminal villi stromal elements and near villous vessels.

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