EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

Proliferative Activity of Microvessels and Angiogenesis in Eutopic Endometrium in Patients with Peritoneal Endometriosis

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Comparative analysis of the density of microvessels and angiogenic activity in eutopic endometrium during different phases of the menstrual cycle in patients with peritoneal endometriosis showed that peritoneal form of endometriosis is a paracrine disease with dysregulation of angiogenesis processes in the eutopic endometrium. Excessive angiogenesis in this condition promotes implantation of eutopic endometrium on the peritoneum. Angiogenesis in eutopic endometrium can be maintained due to high concentrations of vascular endothelial growth factor A in the peritoneal fluid.

Key Words: *peritoneal endometriosis; angiogenic growth factors; fibroblast growth factor; microvessel density and proliferative density; eutopic endometrium*

Endometriosis is a prevalent gynecological pathology (7-50% cases) in women of reproductive age [1].

Recent studies of endometriosis are focused on angiogenesis and angiogenic growth factors. Some aspects of angiogenesis in ectopic endometrium are described [9,13].

Eutopic endometrium in patients with endometriosis differs appreciably from that in healthy women by its structure, proliferative activity, concentration of adhesive molecules, cytokines, growth factors, and gene expression [12].

Angiogenesis (formation of new vessels from already existing) is a cyclic physiological process for the endometrium accompanying menstrual cycle. Activity of these changes can be evaluated by assaying angiogenic growth factors: vascular endothelial growth factor (VEGF), its receptors (VEGF-R1 and VEGF-R2), basic fibroblast growth factor (bFGF), density of microvessels, and their proliferative density. The increase in these parameters can be recorded in patients with increased angiogenic activity of eutopic endometrium; these findings can confirm changes in the eutopic endometrium promoting direct (upon contact with the peritoneum) and indirect (through increase of angiogenic activity of the peritoneal fluid) implantation and development of heterotopies.

We compared the density of microvessels and angiogenic activity in the eutopic endometrium during different phases of the cycle in patients with the peritoneal form of endometriosis.

MATERIALS AND METHODS

The study was carried out in patients of reproductive age. Endometrial specimens were collected from the same patients (from 11 during the proliferative and from 17 during the secretory phases of the cycle). None of the patients included in the study received hormones for at least one month before surgery. The

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dissemination of disease was evaluated using American Fertility Society classification (AFS, 1985) and corresponded to stages II-III in all cases.

Control group consisted of 14 patients, who underwent tubal sterilization or laparoscopic intervention for benign ovarian tumors. The intervention was carried out during the proliferative phase of the cycle in 6 patients and during the secretory phase in 8. The diagnosis of peritoneal endometriosis in this group was rejected after thorough examination of the visceral and parietal peritoneum for detection of heterotopic endometrioid foci. Internal endometriosis was excluded in these patients after ultrasonic examination of the uterus. The phase of menstrual cycle was determined by day 1 of the last menses, ultrasonic findings, steroid hormone assay in the peripheral blood, and morphological examination of the endometrium. All patients gave informed consent to the use of blood and endometrial biopsy specimens for the study.

Endometrial tissue samples were collected. Each sample was divided into 2 parts, one for morphological study and the other was immediately frozen in fluid nitrogen and stored until examination. According to pathomorphological study, all specimens were endometrial tissue.

Blood for analysis was collected under standard conditions from the cubital vein after overnight fast on the day of intervention. Peritoneal fluid was collected during laparoscopic intervention strictly adhering to the regulations for preventing its contamination by blood. Peritoneal fluid specimens were put into sterile tubes and centrifuged at 1500*g* for 10 min at 10°C. Peritoneal fluid supernatant, similarly as serum samples, was stored at -70°C until analysis.

Analysis of VEGF-A and its receptors (VEGF-R1 and VEGF-R2) in the stroma, glandular epithelium, and microvessels of eutopic endometrium were analyzed in different phases of the cycle by histochemical method (according to a standard protocol). The results were analyzed by digital processing and Image-Pro^R Plus software and scored: no reaction (0), 0.1-33.3% (1); 33.3-66.6% (2), 66.6-99.9% (3). The percentage of the reaction was estimated from the maximum staining in a sample. Monoclonal antibodies to VEGF-A and its receptors (VEGF-R1 and VEGF-R2) were used for visualization.

The content of progesterone and 17β-estradiol was measured by enzyme immunoassay using Amerlite Assay kit (Immulite, Diagnostic Products Corporation).

VEGF-A and bFGF in the serum and peritoneal fluid were measured by ELISA (R&D Systems).

Proliferative activity of microvessels was evaluated by microvessel density and proliferative density, which were studied under standard conditions by histochemical method; the data were digitized using Image Pro^R Plus software and expressed in mm^2. Monoclonal antibodies to CD31 were used for vessel visualization, monoclonal antibodies to Ki-67 (DAKO A/S, Denmark) for evaluation of the number of proliferating endotheliocytes [14].

The results were analyzed using SPSS 11.5.1 or Statistica 6.0 software. The significance of differences was evaluated using paired or unpaired Student's *t* test and Wilcoxon's test. The results were presented as means and standard error/standard deviation (M±SE/SD). The differences between the groups were considered significant at $p<0.05$.

The study was carried out at Research Center of Obstetrics, Gynecology, and Perinatology, Russian Academy of Medical Sciences, and Department of Mother and Child Health, University of Uppsala (Sweden).

RESULTS

Analysis of clinical data revealed no intergroup differences by patient age and reproductive history.

The mean serum level of 17β-estradiol in patients with endometriosis was within the normal depending on the cycle phases (257.8±43.4 pmol/liter during the proliferative and 284.2±44.6 pmol/liter during the secretory phase of the cycle, *vs.* 411.0±161.1 and 322.5±78.5 pmol/liter, respectively, in controls). Progesterone content in patients with endometriosis was 5.0±0.6 nmol/liter during the proliferative phase and 19.5±3.3 nmol/liter during the secretory phase, *vs.* 2.9±0.9 and 32.1±2.5 nmol/liter, respectively, in patients without endometriosis. No significant differences between 17β-estradiol and progesterone levels in different phases of the cycle were detected in patients of two groups. Progesterone content was significantly higher ($p<0.01$) during the secretory phase than during the proliferative phase in both groups.

Microvessel density and proliferative density of the eutopic endometrium was studied in the control group and in patients with peritoneal endometriosis (Table 1). No appreciable differences between the groups were detected.

The mean values of proliferative density of microvessels in the control and main groups were virtually the same (Table 1).

Comparative analysis of microvessel density and proliferative density in eutopic endometrium in the two groups in different phases of the cycle revealed no appreciable differences during the proliferative phase of the menstrual cycle (Table 1). Microvessel density and proliferative density were significantly higher during the secretory phase of the cycle in patients with peritoneal endometriosis compared to the control group

TABLE 1. Microvessel Density and Proliferative Density in Eutopic Endometrium in the Control Group and in Patients with the Peritoneal Form of Endometriosis (M±SD, mm²)

Group	CD	CPD	Menstrual cycle phases			
			proliferative		secretory	
			CD	CPD	CD	CPD
Control	228.9±72.7	0.69±0.23	192.1±37.9	0.25±0.07^{xo}	243.9±80.8	0.85±0.43
Patients with peritoneal endometriosis	315.2±96.5	0.91±0.56	152.8±25.2	0.28±0.11^{xo}	452.8±170.1^{*+}	1.76±0.10*

Note. CD: microvessel density; CPD: microvessel proliferative density. $p<0.05$ *compared to control group; $^{+}$compared to CD during proliferative phase; ^{x}compared to CPD; ^{o}compared to CPD during secretory phase of the cycle.

and significantly surpassed the mean values for this group and the values during the proliferative phase of the cycle. In controls, only proliferative density of microvessels increased significantly during the secretory phase of the cycle in comparison with the mean values of this parameter for this group and with the values during the proliferative phase of the cycle.

The expression of VEGF-A and its VEGF-R1 and VEGF-R2 receptors in the stroma, glandular epithelium, and microvessels of eutopic epithelium in different phases of the cycle were compared in the groups (Table 2). VEGF-A expression in the glandular epithelium significantly increases during the secretory phase of the cycle in patients with endometriosis in comparison with controls. In controls, expression of VEGF-R1 and VEGF-R2 in stromal cells in both phases of the cycle was significantly higher than patients.

These data also attest to enhanced expression of VEGF-A and its receptors (VEGF-R1 and VEGF-R2) in the glandular epithelium in comparison with the stroma in both groups of patients in different phases of the cycle. No appreciable increase of VEGF-A expression in the glandular epithelium compared to the stroma was detected during the secretory phase in the control group.

Measurements of VEGF-A and bFGF levels (Table 3) indicate significantly higher levels of both parameters during the proliferative phase in the peritoneal fluid of control patients, bFGF level being significantly higher during the secretory phase as well. The levels of VEGF-A in the serum and peritoneal fluid of control patients were virtually the same.

The level of VEGF-A in the peritoneal fluid of patients with endometriosis was higher than in blood serum in both phases of the cycle. These values were significantly higher in endometriosis patients during the secretory phase of the cycle than in control patients (Table 3).

TABLE 2. Histochemical Evaluation of Expression of VEGF-A and Its Receptors (VEGF-R1 and VEGF-R2) in Eutopic Endometrium Stroma, Glandular Epithelium, and Microvessels during Different Phases of the Menstrual Cycle in Patients with Peritoneal Endometriosis (M±SD, points)

Marker, group	Proliferative phase			Secretory phase		
	stromal cells	glandular epithelium	microvessels	stromal cells	glandular epithelium	microvessels
VEGF-A						
control	1.2±0.3	2.3±0.3	0.19±0.20	0.5±0.3^{+}	0.5±0.3^{+}	0.17±0.19
endometriosis	0.8±0.1	2.0±0.2^{oo}	0.30±0.19	0.5±0.1	1.5±0.2^{*oo}	0.40±0.15
VEGF-R1						
control	1.2±0.2	2.0±0.3^{o}	1.25±0.70	1.2±0.2	2.0±0.2^{o}	1.19±0.60
endometriosis	0.4±0.2*	1.8±0.2^{oo}	1.14±0.52	0.5±0.2*	1.8±0.2^{oo}	1.3±0.4
VEGF-R2						
control	1.2±0.2	2.4±0.6	1.57±0.5	1.2±0.2	2.5±0.2	1.27±0.17
endometriosis	0.7±0.2*	1.8±0.2^{oo}	1.83±0.40	0.6±0.2*	1.8±0.2^{**oo}	1.94±0.21**

Note. *$p<0.01$, **$p<0.05$ compared to the control; $^{+}p<0.01$ compared to glandular epithelium during proliferative phase; $^{o}p<0.001$, $^{oo}p<0.01$ compared to stromal cells during proliferative phase.

TABLE 3. Serum and Peritoneal Fluid VEGF-A and bFGF Levels in Patients with Peritoneal Endometriosis during Different Phases of the Menstrual Cycle (M±SE, pg/ml)

Angiogenesis marker, cycle phase		Control group		Endometriosis patients	
		serum	peritoneal fluid	serum	peritoneal fluid
VEGF-A	proliferative	79.4±41.9	222.2±34.3*	117.4±18.3	217.6±35.3*
	secretory	120.9±14.5	124.7±17.3	109.1±36.0	281.9±64.7*+
bFGF	proliferative	3.5±1.0	61.1±5.8*	9.8±2.1++	43.6±9.4*
	secretory	4.3±1.4	43.8±15.0*	9.5±3.3	26.5±12.1

Note. *$p<0.05$ compared to serum in the same group; +$p<0.05$, ++$p<0.005$ compared to the control group.

The level of bFGF in the peritoneal fluid of endometriosis patients was significantly higher than in the serum during the proliferative phase of the cycle, its values in the serum being significantly higher than in control patients.

The results indicate that hormone levels in patients with peritoneal endometriosis and controls were comparable. Progesterone level was higher during the secretory phase in both groups, but this parameter increased 11-fold in the control group and only 3.9 times in the main group. The absence of appreciable increase in progesterone level during the proliferative phase in endometriosis patients can have an negative impact on endometrial growth suppression.

Despite the fact the growth and regression of endometrium is mainly regulated by estrogens and progesterone, the role of sex steroids in angiogenesis regulation in the endometrium remains unclear. The majority of scientists failed to prove the involvement of sex steroids in endometrial angiogenesis [5]. According to other data, the hormone effects should be distinguished and differentiated from other effects by their impact for initiation of endometriosis development and maintenance of the existing condition [8].

The study of endometriosis development in monkeys showed that the initiation of disease did not depend on estrogens or progesterone, but interactions between these hormones were obligatory for the existence of heterotopies [7].

The data on changes in angiogenesis in the eutopic endometrium are very different [3].

Our results indicate that microvessel density and proliferative density in patients with peritoneal endometriosis is significantly higher during the secretory phase of the cycle in comparison with the controls and is significantly higher than the mean values of these parameters during the proliferative phase in the same patients.

In ectopic foci of adenomyosis microvessel density is significantly increased in comparison with the endometrium of the same patients and with the controls [14]. However, we found no published data on the proliferative density of microvessels and on microvessel density and proliferative density in eutopic endometrium in peritoneal endometriosis.

Our data indicate high angiogenic activity of eutopic endometrium in peritoneal endometriosis, which agrees with published data on adenomyosis [11].

The presence of these changes leads to development of a sort of angiogenic potential, which creates conditions for implantation of eutopic endometrium in its retrograde transmission into the peritoneal cavity during menstruation. Extraction of VEGF-A into the peritoneal fluid from the endometrium can maintain the angiogenic potential and hence, further existence of heterotopies on the peritoneum or can stimulate the development of new ectopic foci.

According to our findings, expression of VEGF-A significantly increased in the glandular epithelium of endometriosis patients in comparison with the controls only during the secretory phase of the cycle. The expression of VEGF-R1 and VEGF-R2 in stromal cells during both phases of the cycle was decreased in patients with the peritoneal form of endometriosis *vs.* the controls.

No significant differences between the expression of VEGF-A and VEGF-R1 in vessels during different phases of the cycles and between the groups were detected. On the other hand, expression of VEGF-R2 in vessels was significantly higher during the secretory phase of the cycle.

Enhanced expression of VEGF-A and bFGF was recorded during the proliferative phase in control patients. The content of bFGF in the peritoneal fluid increased during the secretory phase as well. The content of VEGF-A in the peritoneal fluid of patients with endometriosis was significantly higher than in the serum during both phases of the cycle.

The results indicate increased angiogenic activity of the peritoneal fluid, which leads to increase of the chemotactic activity of the abdominal cavity macrophages and hence, the "vicious circle" of endomet-

riosis development is closed [10]. Hence, it was demonstrated *in vitro* that this activation of angiogenesis is realized through the paracrine mechanisms in the abdominal cavity and hence, through peritoneal fluid, promoting the development of endometriosis [12].

Activation of angiogenesis in ectopic endometrium can be due to local hypoxia [4]. Changes in α-hydroxybutyrate dehydrogenase and LDH activities indicated pronounced hypoxia in the eutopic endometrium in patients with the peritoneal form of endometriosis.

The main paracrine disorders in angiogenesis in patients with peritoneal endometriosis include several stages. Physicochemical factors, hypoxia, gene mutations, viruses modulate human genome and VEGF family genes, leading to changes in the architectonics of angiogenesis regulation. According to our findings, these changes increased expression of VEGF-A in endometriosis against the background of decreased expression of VEGF-R1 during the secretory phase of the cycle in glandular epithelial cells in comparison with physiological changes. Transduction of VEGF signal in paracrine regulation is aimed at activation of vascular growth at the expense of increased level of VEGF-R2 in vessels and decreased levels of VEGF-R1 and VEGF-R2 in endometrial stromal cells. Realization of this process consists in vascular growth and increased density and proliferative activity of microvessels. Excessive angiogenesis in eutopic endometrium creates conditions for its further growth in retrograde transmission into the abdominal cavity. The production of angiogenic factors, including VEGF-A, by the ectopic endometrium leads to their accumulation in the peritoneal fluid, maintenance of excessive angiogenesis in the pelvis and growth of heterotopies.

Hence, peritoneal endometriosis is a disease with objective signs of paracrine disorders in the regulation of angiogenesis processes in eutopic endometrium. Angiogenesis in this condition is excessive, promoting implantation of eutopic endometrium on the peritoneum. The maintenance of angiogenesis in ectopic endometrium is realized also due to high concentrations of VEGF-A in the peritoneal fluid. Drug therapy aimed at reduction of microvessel growth in the eutopic endometrium will modulate excessive angiogenesis and, presumably, this therapy will change the views and the results of traditional treatment of the peritoneal form of endometriosis.

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