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Study of Cytokine Profile and Angiogenic Potential of Peritoneal Fluid in Patients with External Genital Endometriosis

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The concentrations of proinflammatory cytokines (IL-1β, IL-6), vascular endothelium growth factor, tumor growth factor-β, and insulin-like growth factor-1 were measured in the peritoneal fluid of patients with external genital endometriosis and healthy women by enzyme immunoassay. The effect of peritoneal fluid from patients with external genital endometriosis on proliferative activity of EA.Hy926 human endothelial cells was evaluated by the method based on the analysis of cell cycle by flow cytometry. The concentrations of IL-1β, IL-6, and insulin-like growth factor-1 were increased in patients with endometriosis in comparison with healthy women. The peritoneal fluid from patients with endometriosis (but not from healthy women) significantly increased mitotic activity of endothelial cells and exhibited high angiogenic potential, which can promote implantation and growth of endometrial transplants. Presumably, insulin-like growth factor-1 stimulates this process.

Key Words: *endometriosis; angiogenesis; growth factors; insulin-like growth factor*

The etiology and pathogenesis of external genital endometriosis (EGE) were extensively studied during the entire twentieth century, but even today the data on the mechanisms of this disease remain contradictory. The implantation theory is acknowledged by the greatest number of scientists; according to this theory, the main factor promoting dissemination of endometrial cells and emergence of ectopic foci is "retrograde menstruation". Menstrual blood reflux is a common thing occurring in all women during menses, but not all women develop endometriosis [1]. Processes in the peritoneal cavity leading to the development or regression of endometrioid focus play a special role in the development of EGE. The peritoneal fluid (PF) of EGE patients is characterized by high levels of activated macrophages and cytokines produced by them (TNF-α, IL-1β, IL-6, IL-8) [9-11]. Presumably, local inflammatory reaction creates favorable conditions for the development of endometrioid transplants. The formation and functioning of endometrial transplant are largely determined by the degree of vascularization. Stimulation of angiogenesis in endometriosis foci is realized at a local level and is regulated by angiogenic growth factors and cytokines secreted by peritoneal macrophages, endometrioid heterotopy cells, free endometrial cells, and endothelial cells (EC).

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Angiogenesis is regulated by numerous cytokines, growth factors, patterns of EC interactions with each other, with components of extracellular matrix, and with microenvironment. Initiation, course, and completion of angiogenesis depend on the balance of pro- and antiangiogenic factors in EC microenvironment. Changes in the balance of cytokines and other factors in EC microenvironment underlie the development of cancer, coronary atherosclerosis, stroke, arthritis, and retinopathy. Angiogenesis includes several successive stages. Each stage is controlled by cytokines and growth factors released by EC and microenvironmental cells.

Macrophages regulate capillary growth and formation of granulation tissue during chronic inflammation and wound healing. They initiate angiogenesis by releasing proangiogenic factors stimulating proliferation, migration, and differentiation of EC and stimulate the growth and development of vascular network. Macrophages also produce antiangiogenic factors terminating angiogenesis. Impaired switch over from the pro- to antiangiogenic phenotype can lead to uncontrolled angiogenesis. Macrophages can actively improve EC viability or cause apoptosis of these cells.

The PF of EGE patients is characterized by high content of vascular endothelium growth factor (VEGF), fibroblast growth factor, insulin-like growth factor-1 (IGF-1), and tumor growth factor-β (TGF-β) [8,13,15]. The concentration of fibroblast growth factor in EGE does not differ from that in healthy women [15]. TGF-β detected in high concentrations in PF of EGE patients [14] is characterized by anti-inflammatory activity, inhibits the production of IL-1, IL-2, TNF-α, and other cytokines, and suppresses EC proliferation. The data on the local production of growth factors in endometriosis are contradictory. Presumably, imbalance in the content of PF cells and proinflammatory and angiogenic factors produced affects proliferation of EC in EGE and promotes the development of the disease.

We measured the concentrations of proinflammatory cytokines (IL-1β and IL-6) and growth factors (VEGF, IGF-1, TGF-β) in PF of EGE patients and healthy women and evaluated the effect of PF from EGE patients on proliferative activity of human EC.

MATERIALS AND METHODS

Twenty-seven women with EGE aged 29.6±5.6 years were examined. The diagnosis was confirmed by endoscopic data and histological analysis of operation material. Control group consisted of 12 women aged 31-42 years undergoing surgical sterilization. PF was collected during laparoscopic surgery.

The production of IL-1β and IL-6 was evaluated by enzyme immunoassay using Protein Contour test systems, VEGF using Cytoimmune Sciences Inc. system, TGF-β1 using a kit from Biosource Internation, and IGF-1 by a kit from Diagnostic System Lab. Inc. The results were evaluated on a Multiscan MC 344 microplate photometer (Labsystems).

EA.Hy926 cells were cultured in DMEM/F12 supplemented with 10% FCS, 50 μg/ml gentamicin sulfate, 2 mM L-glutamine, and HAT (ICN).

Proliferative activity of EC was evaluated by analyzing the cell cycle by flow cytometry. EA.Hy926 cells were cultured in DMEM/F12 with 1 or 10% FCS with DNA synthesis inhibitor aphidicolin (6 μM) for 24 h at 37°C and 5% CO_2 and then for 18 h in a fresh portion of the same medium without aphidicolin. PF from EGE patients (n=10) or control PF from healthy women (n=10) were added into some wells with 1% FCS. After the end of culturing the cells were stained with propidium iodide. Cell cycle was evaluated on a FACStrack cytofluorometer using ModFit 3.0 software (Becton Dickinson). EA.Hy926 cells were incubated in a medium with 1% FCS without aphidicolin, which led to initiation of cell cycle and DNA synthesis. Preincubation of EA.Hy926 cells in the medium with 1 or 10% FCS and DNA synthesis inhibitor aphidicolin synchronized the cell culture at the cell cycle G1/S interphase (Fig. 1, *a*). DNA synthesis was resumed after removal of aphidicolin from the medium (Fig. 1, *b*). The percentage of cells starting DNA production in the presence of 1 or 10% FCS in this case differed significantly from the control (culturing with aphidicolin for 46 h; Table 1). The data were statistically processed using Student's t test.

RESULTS

Peritoneal fluid from EGE patients contained high levels of proinflammatory cytokines IL-1β (24.3 pg/ml) and IL-6 (1869.4 pg/ml) surpassing their concentrations in the control group (13.9 and 27.9 pg/ml, respectively; $p<0.05$). IL-1 and IL-6 differently regulate the life span and proliferation of EC. Endogenous IL-1β shortens the life span of EC, promotes degradation of EC basal membrane, and initiates angiogenesis. IL-6 indirectly initiates angiogenesis through induction of VEGF expression. Proinflammatory cytokines can activate the secretion of growth factors by EC and peritoneal macrophages.

We found no VEGF and TGF-β1 in PF from EGE patients, presumably because many cytokines

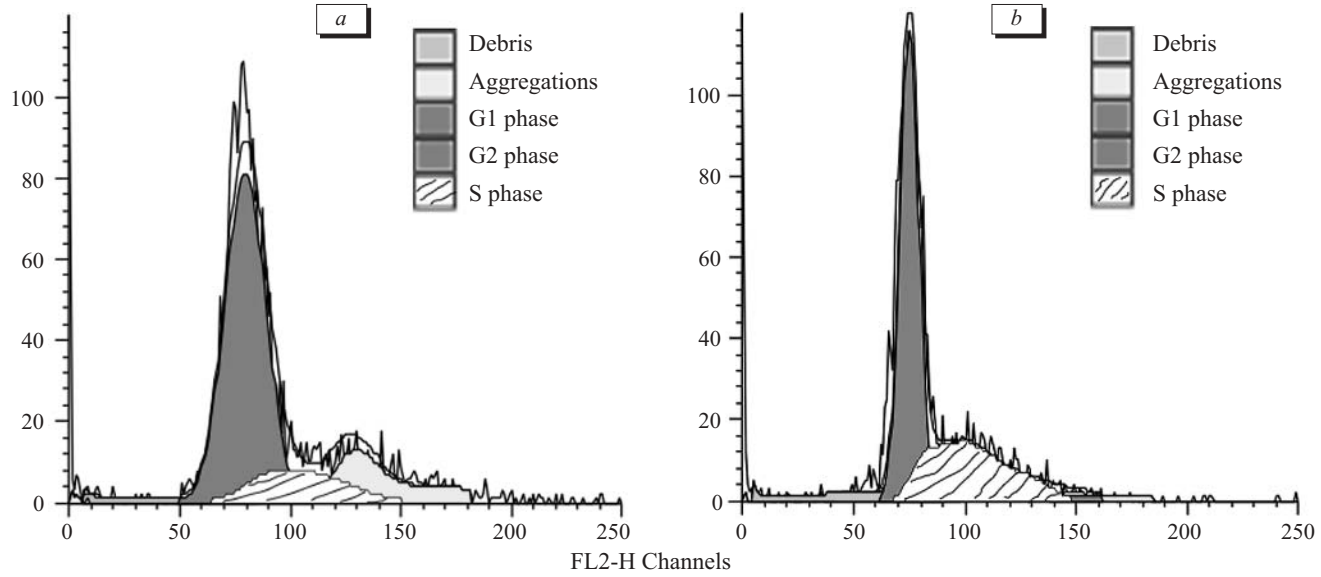

Fig. 1. EA.Hy926 cell cycle. *a*) preliminary culturing of EA.Hy926 cells in medium with 1% FCS and aphidicolin inhibited DNA synthesis and led to synchronization of cell culture at the G1/S cell cycle interphase; *b*) resumption of DNA synthesis after removal of aphidicolin from the medium.

and growth factors are released upon direct contact of the effector and target cells. Fibroblast growth factors, TGF-β, VEGF, and cytokines can be stored in the extracellular matrix and released after its degradation, thus becoming angiogenesis factors. The absence of TGF-β1 in PF from EGE patients can also be explained by active phase of angiogenesis, during which the cytokine balance is shifted towards the proangiogenic profile. PF samples from EGE patients are characterized by high angiogenic activity.

Peritoneal fluid from healthy women did not modify EC proliferation, while PF from EGE patients significantly increased the percentage of cells passing from G1/S cell cycle phase into M/G2 phase ($p<0.01$), this indicating intensification of EC mitotic activity (Table 1).

High angiogenic potential of PF can be due to IGF-1, whose concentration in PF from EGE patients (6384.6 pg/ml) significantly surpassed that in controls (5184.5 pg/ml).

IGF-1 belongs to IGF protein family, including IGF-1, IGF-2 proteins, their receptors IGF-1R and IGF-2R, and a complex of IGF-1-binding proteins (IGFBP). IGF-1 is produced by almost all tissues, including monocytes, macrophages, EC, and malignant tumor cells, which attests to its involvement into tumor growth. IGF-1 is a mediator of cell growth, differentiation, and transformation [12]; it realizes its effects through IGF-1R receptor [7]. The effects

TABLE 1. Effect of PF on EC Proliferation (*M*±*m*)

Conditions of EC culturing	Number of cells in cell cycle phase, %		
	G0/G1	S	M/G2
Without aphidicolin, 42 h, 1% FCS	67.4±2.2	28.3±2.5	4.2±1.2
With aphidicolin, 42 h, 1% FCS	80.3±5.6	17.1±2.1	2.5±1.1
With aphidicolin, 24 h, then 18 h without aphidicolin			
1% FCS	65.0±2.3	32.0±2.4	2.21±1.10
10% FCS	55.9±3.4	43.8±2.2	1.25±1.10
Aphidicolin, 24 h, then 18 h with PF from			
healthy women, 1% FCS	71.3±5.2	28.39±3.50	1.2±1.1
EGE patients, 1% FCS	57.4±2.4	38.8±2.3	5±1.3

of IGF-1 are modulated by IGFBP. The circulating endocrine form of IGF-1 secreted by liver cells under the control of growth hormone is highly affine for IGFBP. Impaired synthesis and secretion of IGFBP increases the risk of cancer development. Autocrine or paracrine forms of IGF-1 are characterized by low affinity for IGFBP and are secreted by other cells.

Endothelial cells express IGF-1 and IGF-1R [3] and therefore can be subjected to endocrine, autocrine, and paracrine stimulation by IGF-1. The expression of IGF system components in EC is regulated by TGF-β1 and VEGF [6]. IGF-1 stimulates absorption of neutral amino acids, glucose, DNA synthesis, stimulates the proinflammatory and vasodilating responses of EC, regulates migration and angiogenesis, activates migration and proliferation of EC and formation of vascular tubules *in vitro* [3,4]. IGF-1 induces VEGF secretion, promoting vascular growth in malignant diseases. IGF-1 is involved in angiogenesis associated with inflammatory processes [2,5].

Hence, PF from EGE patients is characterized by proangiogenic potential due to the presence of proinflammatory cytokines (IL-1β and IL-6) and IGF-1. These factors can be responsible for initiation and long-term maintenance of angiogenesis processes, leading to emergence and growth of ectopic endometrioid foci. IGF-1 can initiate and maintain local angiogenesis in endometrioid focus in the absence of one of the main angiogenic factors (VEGF) in PF. The absence of angiogenesis inhibitor TGF-β1 in PF from EGE patients seems to indicate a shift of the pro- to antiangiogenic factors balance towards the proangiogenic profile. The method for evaluation of the proliferative potential of PF can be used for diagnosis, choice of therapy, and evaluation of its efficiency.

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