Apoptosis and Proliferative Activity in Endometrium during Peritoneal Endometriosis

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Apoptosis and proliferative activity of the glandular epithelium and endometrial stroma in patients with peritoneal endometriosis and in women of reproductive age without endometriosis were studied at various stages of menstrual cycle. Differences in the intensity of apoptosis were revealed manifesting in changed expression of proteins in the glandular epithelium of patients with endometriosis during the secretory phase of the menstrual cycle compared to normal. Proliferative activity of the glandular epithelium in the proliferative phase was higher than in the secretory phase. Our results suggest that the imbalance between apoptosis proteins in the endometrium plays a role in the pathogenesis of peritoneal endometriosis.

Key Words: *endometrium; endometriosis; proliferative activity; apoptosis*

Peritoneal endometriosis is a very prevalent pathology [1]. The population frequency of this disease in women of reproductive age reaches 10% and increases to 40-50% in infertile women and women with pelvic pain syndrome. The pathogenetic mechanisms of endometriosis remain unknown. An important role in the pathogenesis of peritoneal endometriosis is played by disturbances leading to enhanced implantation and survival of the endometrium in ectopic areas [8]. Viable endometrial cells in menstrual discharge are capable of growing under *in vivo* and *in vitro* conditions.

Homeostatic regulation of growth in any cell population reflects the balance between cell proliferation and death. Excessive growth of cells results from intensive proliferation, or insufficient level of cell death, or both oppositely directed processes. Previous studies revealed cell genes encoding protein synthesis with homologous amino acid sequence and are involved in the regulation of apoptosis. Inhibitors of this process include Bcl-2, Bcl-X_{long}, Bcl-w, and MCL-1, while Bax, Bak, Bcl-x_{short}, and Bad are activators of apoptosis. These genes stimulate synthesis of the corresponding proteins that form homodimeric and heterodimeric complexes and determine the ratio between inhibitors and activators of apoptosis ("rheostat of life") [2].

Here we studied proliferative activity and expression of proteins inhibiting or activating apoptosis in the endometrium of control-group patients and patients with peritoneal endometriosis during the proliferative and secretory phase of the menstrual cycle.

MATERIALS AND METHODS

We examined 56 patients of reproductive age (20-44 years, 31.0±0.88 years). The patients had primary or secondary infertility (n=26), pelvic pain syndrome (n=19), or both (n=11). Samples of the blood and endometrium were obtained during the proliferative (n=19) and secretory phase of the menstrual cycle (n=23). In patients with endometriosis hormonal therapy was discontinued not less than 1 month before surgery. The diagnosis was verified by morphological examination. The severity of peri-

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toneal endometriosis corresponded to stages II-III (classification of the American Fertility Society) [11]. There were no intergroup differences in the age of patients and clinical manifestations and severity of endometriosis.

The control group included 14 patients that were subjected to laparoscopic sterilization (ligation of Fallopian tubes) or surgery for benign ovarian neoplasms. Treatment was performed during the proliferative (6 patients) or secretory phase of menstrual cycle (8 patients). The diagnosis of peritoneal endometriosis in these patients was excluded after examination of the visceral and parietal peritoneum for endometrial heterotopias. We took into account day 1 of the last menstruation and results of ultrasonography. The phase of the cycle was verified by the concentration of steroid hormones in the peripheral blood and morphological signs of the endometrium.

Tissue samples were obtained during biopsy of the endometrium. Each sample was divided into 2 parts. Part 1 was subjected to morphological examination. Part 2 was immediately frozen in liquid nitrogen and stored until further analysis. Pathomorphological examination showed that all samples are presented by endometrial tissue.

Immunohistochemical study of protein inhibitors (Bcl-2, Bcl-x_1, and MCL-1) and activators of apoptosis (Bak and Bax) in normal endometrium was performed by the avidin-biotin-immunoperoxidase method. Mouse anti-Bcl-2 (Dako), rabbit anti-Bax and anti-Bak, and rabbit anti-MCL-1 and anti-Bcl-x_1 (Santa Cruz Biotechnol.) served as primary antibodies. The samples were stained with diaminobenzidine (Vector Laboratories) and poststained with hematoxylin. Samples of lymph nodes served as the positive control for Bcl-2, Bax, Bcl-x_1, and MCL-1 according to the manufacturer's recommendations. Human ovarian carcinoma cells were used as the positive control for Bak [7]. Tissue samples incubated with 2% solution of bovine serum albumin in phosphate buffered saline (instead of primary antibodies) served as the negative control [5]. The presence and intensity of staining were estimated in 5 randomly selected fields of view under a Nikon microscope (×400). Staining of endometrial components was expressed using a 4-point scale (0, no staining; 1, weak staining; 2, moderate staining; 3, intensive staining of endometrial structures).

Proliferative activity was determined by immunohistochemical detection of nuclear antigen in dividing Ki67 cells from the glandular epithelium and endometrial stroma. Antibodies against Ki67 (MIB-1, Immunotech, 1:300) served as primary antibodies. We estimated the total number of stained cells in 10 randomly selected fields of view (×400). The ratio between the count of positively stained cells and total number of nuclei in the stroma and glandular epithelium of the endometrium was taken as the index of proliferation.

The results were analyzed by means of SPSS for Windows software. Intergroup differences were significant at $p<0.05$.

RESULTS

Expression of apoptosis markers in the glandular epithelium of ectopic endometrium in patients far

TABLE 1. Expression of Bcl-2 Proteins in Endometrium of Patients with Endometriosis and Control Patients during Various Phases of the Menstrual Cycle (*M*±*SD*)

Marker	phase	Control group		Endometriosis	
		stroma	glandular epithelium	stroma	glandular epithelium
Bcl-2	proliferative	1.0±0.1	$1.8\pm0.5^{++}$	1.0±0.1	2.2 ± 0.7^{oo}
	secretory	1.0±0.1	$1.8\pm0.5^{++}$	1.1±0.3	$1.8\pm0.6^{*o}$
MCL-1	proliferative	1.2±0.3	$1.7\pm0.3^{++}$	1.0±0.1	1.7 ± 0.5^{o}
	secretory	1.0	$1.4\pm0.3^{++x}$	1.2±0.2	1.9 ± 0.3^{o}
Bcl-x_1	proliferative	1.0	$1.7\pm0.3^{+}$	1.1±0.1	2.2 ± 0.7^{ooo}
	secretory	1.0	$1.8\pm0.3^{++}$	1.0±0.1	1.8 ± 0.2^{ooo}
Bak	proliferative	1.5±0.5	$2.5\pm0.9^{++}$	1.1±0.3	2.3 ± 0.5^{ooo}
	secretory	1.4±0.5	$2.8\pm0.3^{++x}$	1.3±0.5	2.3 ± 0.4^{o}
Bax	proliferative	1.5±0.4	$2.5\pm0.4^{++}$	1.2±0.3	2.0 ± 0.6^{o}
	secretory	1.1±0.2	$2.0\pm0.3^{++}$	1.2±0.3	2.1 ± 0.6^{o}

Note. $^{*}p<0.05$ compared to proliferative phase; $^{+}p<0.01$ and $^{++}p<0.001$ compared to the stroma (control); $^{o}p<0.05$, $^{oo}p<0.01$, and $^{ooo}p<0.001$ compared to the stroma (endometriosis); $^{x}p<0.05$ compared to the glandular epithelium (endometriosis).

surpassed that in the stroma during both the proliferative and secretory phase of menstrual cycle (Table 1).

Endometriosis patients and controls significantly differed by MCL-1/Bax (0.9/0.7, p=0.03), Bcl-2/Bax (1.0/0.7, p=0.02), Bcl-2/Bak (1.1/0.7, p=0.05), and Bcl-x_l/Bax (1.1/0/7, p=0.04) in the glandular epithelium during the proliferative phase. Moreover, intergroup differences were revealed in MCL-1/Bax (0.9/0.7, p=0.04) during the secretory phase of the menstrual cycle.

Proliferative activity of the glandular epithelium in patients of both groups during the proliferative phase was higher compared to that observed during the secretory phase of the menstrual cycle (Fig. 1). No significant differences were revealed in the endometrial stroma during the proliferative and secretory phase of menstrual cycle. Proliferative activity was similar in patients with peritoneal endometriosis and controls. Proliferative activity of the glandular epithelium in the secretory phase was lower than in the proliferative phase. These differences probably resulted from the inhibitory effect of progesterone on proliferation, which was most significant by the end of the cycle. Ki67 expression was not detected in the glandular epithelium, but present in the stroma of 2 control women and 2 endometriosis patients on day 28 of the cycle.

Much attention was paid to studying apoptosis in animals and humans during various phases of menstrual cycle. Cyclic variations of this marker are characterized by activation of apoptosis by the end of the secretory phase and during menstrual phase. The intensity of apoptosis in the endometrium is maximum in the late secretory, menstrual, and early proliferative phase [6].

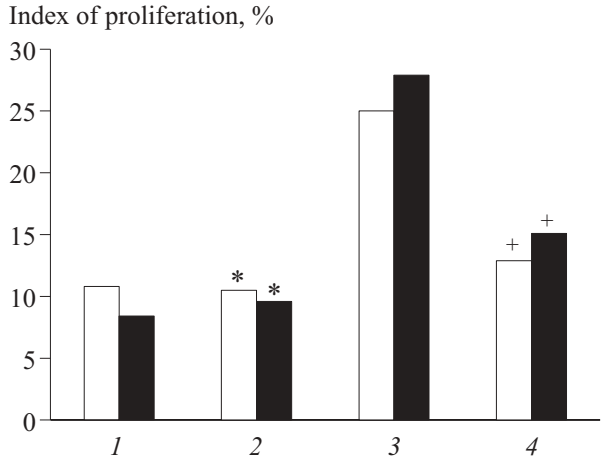

Fig. 1. Index of proliferation (%) in the endometrium of control patients and patients with endometriosis during various phases of the menstrual cycle. Stroma, proliferative phase (*1*); stroma, secretory phase (*2*); glandular epithelium, proliferative phase (*3*); glandular epithelium, secretory phase (*4*). Light bars, control; dark bars, endometriosis. p<0.05: *compared to the stroma; +compared to the proliferative phase.

Cyclic variations in the endometrium over the menstrual cycle are probably associated with changes in the ratio between activators and inhibitors of apoptosis in glandular epithelial cells [9]. Protein Bax is expressed in the glandular epithelium during the secretory phase and, more rarely, during the proliferative phase. Bak expression is mainly observed in the glandular epithelium of functional layer during the secretory phase of the cycle [10]. The increase in the expression of this protein corresponded to activation of apoptosis in the endometrium during the secretory phase of the cycle, which was estimated by *in situ* labeling of DNA.

The index of apoptosis in glandular endometrial cells from patients with endometriosis was higher than in control patients [3]. In normal endometrium the intensity of apoptosis was maximum during the late secretory, menstrual, and early proliferative phase of the menstrual cycle. However, cyclic variations in apoptosis were less significant in patients with endometriosis. Our results are consistent with published data that the intensity of spontaneous apoptosis in the endometrium of patients with endometriosis is lower compared to healthy women [4]. Study of ectopic endometrium revealed further decrease in the intensity of apoptosis in eutopic endometrium.

Our results reflect variations in the intensity of apoptosis in the endometrium of patients with peritoneal endometriosis. They are associated with not only expression of apoptosis proteins, but also changes in the ratio between activators and inhibitors of apoptosis. The observed imbalance is one of the pathogenetic factors for peritoneal endometriosis, which provides higher viability of endometrial cells compared to normal. Moreover, it maintains physiological activity of cells after implantation in ectopic regions.

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