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Characteristics of Multipotent Mesenchymal Stromal Cells Isolated from the Endometrium and Endometriosis Lesions of Women with Malformations of the Internal Reproductive Organs

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We isolated and characterized cell cultures from eutopic endometrium and endometriotic lesions of women with malformations of the internal reproductive organs. The cells had fibroblast-like shape and intensively expressed CD90, CD73, CD105, CD44, CD146, and CD117 and were capable of induced adipogenic and osteogenic differentiation *in vitro*. The obtained cultures exhibited properties of multipotent mesenchymal stromal cells; at the same time, they demonstrated *in vitro* immunophenotypic differences from cell cultures of eutopic and ectopic endometrium of women without developmental abnormalities, which suggests their functional difference. The cells from eutopic endometrium and from ectopic endometriotic lesions can be used as the model for studying of the etiology and pathogenesis of endometriosis and for testing new drugs for this specific group of patients. Markers CD90 and CD117 were identified as promising molecules for the development of minimally invasive diagnostics of endometriosis based on cell cultures from eutopic endometrium.

Key Words: *endometriosis; endometrium; multipotent mesenchymal stromal cells; immunophenotype; malformations of the internal reproductive organs*

Endometriosis affects 10% women of reproductive age and 30-50% women with pain syndrome and infertility [8]. Uterovaginal malformations are detected in 4.3-6.7% women of reproductive age and 8% women with infertility [8]. Endometriosis is observed in 77% cases of malformations of the internal reproductive organs associated with retrograde menstruations and in 37% cases of malformations with undisturbed menstrual blood outflow [1].

Etiology and pathogenesis of endometriosis remain poorly studied. Implantation theory of endometriosis implies transport of endometrial cells from the uterine cavity through the fallopian tubes into the peritoneal cavity and adjacent organs in patients with congenital disturbances of menstrual blood outflow. The retrograde menstruation theory is very popular, but cases of endometriosis in prepubertal girls, in postmenopausal women, in women with uterine agenesis, and extremely rare cases of endometriosis in men attest to more intricate etiopathogenetic mechanisms of this disease [3].

During the last decade, stem cells of the endometrium are actively studied as the source of its regeneration [15]. The study of cell culture from eutopic endometrium revealed expression of phenotypic surface markers of adult stem cells (CD146, CD73, and CD90) and transcription factors that play a role in the maintenance of their pluripotency (Oct4, NANOG, KLF4, and Sox2) [3]. It was hypothesized that regeneration of the endometrium is mainly determined by multipotent mesenchymal stromal cells (MMSC) [7,11].

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Of particular interest is to study cell cultures from the endometrium and endometriosis lesions in women with congenital uterovaginal malformations, especially associated with impaired menstrual blood outflow that contradicts the theory of retrograde menstruation as the common cause of endometriosis development.

The purpose of this study was to describe and compare immunophenotype of cell cultures isolated from eutopic and ectopic endometrium in women with congenital uterovaginal malformations with and without endometriosis and to compare them with similar cell cultures from the same sources from the women without congenital abnormalities.

MATERIALS AND METHODS

Our prospective cohort study included 42 women (age 18-42 years) with malformations of the internal reproductive organs, patients of V. I. Kulakov Research Center of Obstetrics, Gynecology, and Perinatology. The patients were divided into two groups: group 1 consisted of 17 patients with impaired and 4 patients with normal menstrual blood outflow combined with endometriosis and group 2 included 16 patients with impaired and 5 patients with normal menstrual blood outflow without endometriosis. The inclusion criteria were documented uterovaginal malformations and external genital endometriosis, reproductive age, strong motivation to restore reproductive function, and signed informed consent for participation in the study. Exclusion criteria were severe concomitant extragenital pathology, malignant tumors and acute inflammatory diseases of reproductive organs, gender disorders, and anorectal malformations. Analysis of constitutional features, infectious diseases, hereditary diseases, concomitant extragenital pathology, and surgical interventions revealed no statistically significant differences between the groups.

The obtained biological specimens were divided into 3 groups: group 1a — eutopic endometrium from patients with external genital endometriosis, group 1b — ectopic endometrium from endometriosis lesions (1a and 1b are the main group), group 2 — endometrium of patients without external genital endometriosis (control group).

Eutopic endometrial tissue was sampled during hysteroscopy and diagnostic curettage of the uterus, ectopic endometrial tissue was isolated from endometriotic lesions excised during laparoscopy.

The material for the study was taken during the first phase of the menstrual cycle [4]. The tissue samples under sterile conditions were placed in 50-ml conical centrifuge tubes (Costar) with 20 ml PBS and within 20-30 min transferred to the laboratory in a special container. The specimens were washed with PBS, minced with scissor and scalpel, and incubated in 0.07% collagenase IA (Sigma-Aldrich) at 37°C for 30 min. The cell suspension was centrifuged at 2000 rpm, the pellet was suspended in DMEM/F-12 (PanEco) containing 10% fetal calf serum (HyClone), 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine (PanEco). The isolated cells were transferred to culture flasks (seeding density $3\text{-}5\times10^3$ nucleated cells/cm^2). The medium was changed every 3 days. After attaining confluence, the cultures were passaged.

The morphology of cell cultures was assessed on microphotographs of randomly selected fields of view under a Nikon Eclipse TS100 phase-contrast microscope.

Immunophenotype of passage 1 cells was determined by using monoclonal antibodies labeled with FITC, PE, or APC to antigens CD14, CD31, CD34, CD44, CD45, CD73, CD90, CD105, CD117, CD146, CD26, CD106, CD54, CD86, CD200, HLA-DR, HLA-ABC, and Cytokeratin-7 (BD Pharmingen) on a FACSCalibur (BD) flow cytofluorometer according to manufacturer's guide. FITC-, PE- and APC-labeled IgG of corresponding classes were used as isotypic control.

The results were statistically processed using Microsoft Excel 10.0 software; significance of differences between the groups was evaluated using nonparametric Mann—Whitney and Kruskal—Wallis tests at $p\leq0.05$.

RESULTS

Passage 1 cultures enzymatically isolated from eutopic and ectopic endometrium of women with malformations of the internal reproductive organs primarily consisted of fibroblast-like cells (length 60-100 μ) with an admixture of polygonal cells with a size of ~50 μ (Fig. 1).

Passage 1 cultures intensively expressed surface markers of MMSC CD73, CD90, and CD105 (Table 1). The level of CD73 expression was >90% and did not differ in the studied groups of cultures; expression of CD90 and CD105 considerably varied and significantly differed in cell cultures from ectopic and eutopic endometrium (Table 1). It is noteworthy that the number of CD105$^+$ cells in cultures from eutopic endometrium of patients with congenital malformations in group 1a and group 2 was considerably higher than in cultures of eutopic endometrium from women with endometriosis without malformations: 65.4, 68.0, and 16.2%, respectively; the expression of CD105 in group 1b was significantly lower than in groups 1a and 2. The expression of CD90 in cell cultures from eutopic endometrium of women with malformations without endometriosis was similar to that in women without malformations and with endometriosis: 43.4 and 50.7%, respectively (Table 1) [6]. It should be

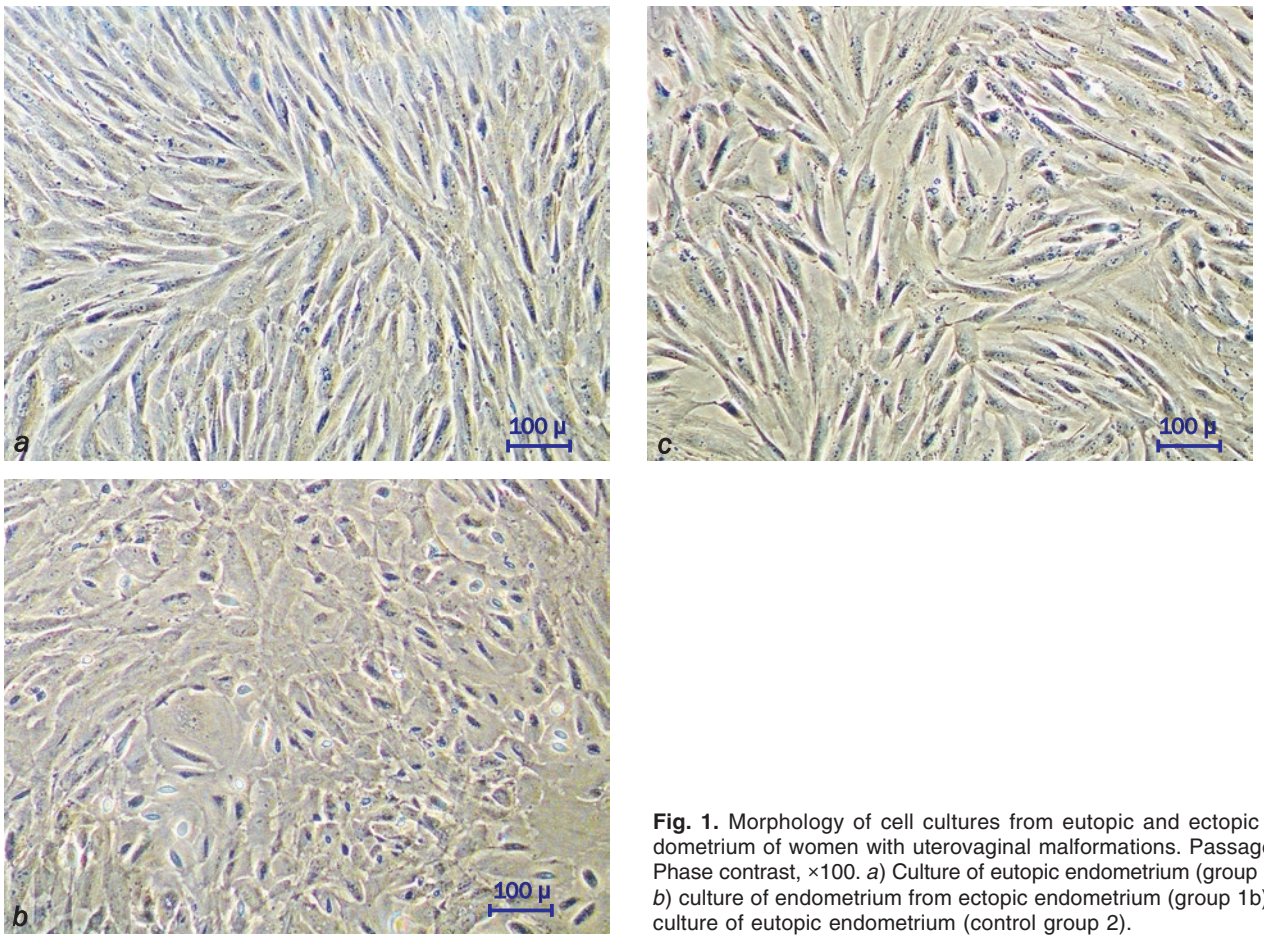

Fig. 1. Morphology of cell cultures from eutopic and ectopic endometrium of women with uterovaginal malformations. Passage 1. Phase contrast, ×100. *a*) Culture of eutopic endometrium (group 1a), *b*) culture of endometrium from ectopic endometrium (group 1b), *c*) culture of eutopic endometrium (control group 2).

noted that CD90 expression in passage 1 cultures from eutopic endometrium of women with malformations and endometriosis was significantly lower than in cultures of ectopic endometrium of the same women and eutopic endometrium from women with malformations and without endometriosis (Table 1). This suggests that this marker can be used for the development of minimally invasive diagnosis of endometriosis at the early stages based on Pipelle sampling of eutopic endometrium and for characteristic of the cultured cells. HLA-ABC expression in group 1b cultures was significantly lower than in groups 1a and 2 (Table 1).

Expression of HLA-DR, CD14, CD45, CD31, CD86, and CD106 in the studied cultures was negligible. In contrast to the previous report on low expression of CD200 in cell cultures from eutopic endometrium of patients with endometriosis without malformations [5], we did not detect the expression of this marker. It should be noted that some cultures of group 1a contained high number of cells expressing SSEA-4 (95%), while in most cultures of group 1b and group 2, this marker was not detected. There are published reports that MMSC from the adipose tissue, mammary gland, and bone marrow expressed SSEA-4 at appreciable levels [16,20], but in cell cultures from the endometrium [22] and fallopian tubes [13], this marker was not detected. We found no published descriptions of cell cultures isolated from the endometrium and endometriosis lesions of women with congenital uterovaginal malformations.

According to previous studies [14], adhesion of endometrial tissue to the peritoneal mesothelium is mediated by cell adhesion molecules integrins or CD44 (integrated cellular glycoprotein, hyaluronic acid receptor). Stromal cells from eutopic endometrium of women with endometriosis demonstrated changed integrin profile compared to cells from the endometrium of healthy women [14]. It was shown that stromal cells of ectopic endometrium intensively expressed glycoprotein CD44 involved in cell—cell interactions, adhesion, and migration. Moreover, mesothelial peritoneal cells secreted glycosaminoglycans. Some researchers believe that the interaction of glucosamine with CD44 plays an important pathogenetic role in adhesion of endometrial cells to mesothelial cells [12].

We found that MMSC derived from eutopic and ectopic endometrium of patients with external genital endometriosis also expressed CD44, and this expression was significantly higher in cell cultures from endometriosis lesions (Table 1).

TABLE 1. Immunophenotypic Characteristics (%) of Passage 1 Cell Cultures Derived from Eutopic (Group 1a) and Ectopic (Group 1b) Endometrium of Women with Congenital Reproductive System Malformations

Marker	Median	Interquartile range	Min-Max	*P*
CD73				
group 1a	95.6	89.8-99.2	78.4-99.9	P_1=0.83
group 1b	98.5	92.8-99.2	52.7-99.7	P_2=0.93
group 2	98.1	92.4-99.3	90.2-99.9	P_3=0.48
CD90				
group 1a	30.2	21.4-50.5	15.1-77.8	P_1=0.00
group 1b	72.3	63.6-85.6	30.0-93.9	P_2=0.00
group 2	43.4	32.3-67.2	10.4-74.8	P_3=0.08
CD105				
group 1a	65.4	34.2-84.6	1.9-91.1	P_1=0.00
group 1b	19.6	11.7-32.5	1.0-80.5	P_2=0.00
group 2	68.0	25.3-90.7	2.4-91.9	P_3=0.78
HLA-ABC				
group 1a	98.5	95.50-99.60	94.50-99.80	P_1=0.00
group 1b	89.3	87.00-93.40	46.20-98.60	P_2=0.00
group 2	98.8	98.20-99.20	97.3-100.0	P_3=0.34
Cytokeratin				
group 1a	1.3	0.80-8.70	0.07-48.90	P_1=0.09
group 1b	9.7	1.82-47.45	0.61-65.80	P_2=0.04
group 2	2.2	0.52-13.10	0.16-46.80	P_3=0.88
CD117				
group 1a	42.5	28.50-52.20	18.20-62.60	P_1=0.02
group 1b	66.5	43.30-69.50	34.50-87.70	P_2=0.01
group 2	18.2	7.92-78.40	7.91-78.50	P_3=0.20
CD146				
group 1a	33.8	27.75-44.15	3.70-92.90	P_1=0.00
group 1b	2.0	0.56-8.10	0.07-91.20	P_2=0.00
group 2	37.8	18.30-42.50	5.70-82.00	P_3=0.59
CD26				
group 1a	25.6	18.60-54.50	10.80-59.90	P_1=0.00
group 1b	82.5	77.20-88.20	7.50-95.20	P_2=0.00
group 2	31.2	21.80-36.50	7.83-56.40	P_3=0.67
CD44				
group 1a	96.5	95.70-97.70	82.30-98.70	P_1=0.00
group 1b	98.1	97.20-99.30	84.00-99.70	P_2=0.01
group 2	99.2	98.00-99.50	81.70-99.60	P_3=0.43
CD54				
group 1a	92.7	73.15-99.90	40.1-100.0	P_1=0.22
group 1b	84.7	60.50-94.05	52.60-99.50	P_2=0.32
group 2	98.4	84.65-98.65	69.40-99.78	P_3=0.67
All groups, CD34	2.0	0.64-4.54	0.23-9.70	

Note. P_1: comparison between the three groups (Kruskal—Wallis test); P_2: comparison of groups 1a and 1b; P_3: comparison of groups 1a and 2 (Mann—Whitney test).

In [18], expression of Oct-4 and C-kit (CD117) proteins was compared in eutopic and ectopic endometrium of women with severe endometriosis and in healthy women. The number of stem cells expressing Oct-4 was significantly higher in ectopic endometrium than in eutopic, in women suffering from endometriosis, and in the endometrium of healthy women. Enhanced expression of c-kit, protooncogene and marker of adult stem cells, was observed in ectopic endometrium in comparison with eutopic endometrium in women with endometriosis and in healthy women. These findings suggest that the population of undifferentiated stem cells can play an important role in the maintenance and growth of ectopic endometrial cells.

In addition, we analyzed the expression of C-kit protein in the endometrium of patients with endometriosis and in healthy women, as well as in endometriosis lesions in the pelvic peritoneum, ovaries, and rectum [21]. The expression of C-kit in eutopic endometrium of patients with endometriosis was higher than in the endometrium of healthy women. The expression of C-kit was significantly higher in rectal endometriosis lesions; no significant differences between the expression of this protein in endometriosis lesions on the small pelvis peritoneum and ovaries were found. It was concluded that C-kit is involved in the pathogenesis of external genital endometriosis. A positive correlation between the expression of C-kit and degree of invasion of endometriotic heterotopias was also noted.

In our study, CD117 expression in cell cultures from ectopic endometrium was significantly higher than in MMSC from eutopic endometrium of both patients with endometriosis and control women. Moreover, CD117 expression in cell cultures from eutopic endometrium of women with endometriosis significantly surpassed that in patients without endometriosis with congenital uterovaginal malformations (Table 1). The expression of this marker varied in a wide range and was significantly higher in both eutopic and ectopic endometrium of patients with malformations in comparison with cell cultures from patients without malformations. In cell cultures from eutopic endometrium of patients with congenital malformations, CD117 expression was significantly lower than in cell cultures from eutopic and ectopic endometrium of patients without malformations (Table 1) [5,6]. Hence, CD117 is a promising marker for the development of early minimally invasive diagnosis of endometriosis based on culturing of cells from eutopic endometrium.

CD146 (MCAM, melanoma cell adhesion molecule) is considered a marker of endothelial cells. MCAM acts as a receptor for alpha laminin 4, a matrix molecule widely presented within the vascular wall. MCAM is stably expressed in cells of the vascular wall, including endothelial cells, smooth muscle cells, and pericytes. The function of CD146 is poorly studied. CD146 is now considered as a marker of mesenchymal stromal cells from different sources; the function of this molecule can be associated with cell multipotency: CD146 expression is more intensive on MMSC with high differentiation potential [19]. CD146 is an essential specific marker of endometrial MMSC [10]. In our study, CD146 expression in MMSC from eutopic endometrium of women with malformations of the external reproductive organs was significantly higher than in MMSC from ectopic endometrium; at the same time, CD146 expression in MMSC from eutopic endometrium did not depend on the presence or absence of endometriosis (Table 1). CD146 expression in cells from ectopic endometrium of women with malformations was appreciably lower than in cell cultures of patients without malformations (Table 1) [5], while expression of this marker in cells from eutopic endometrium was higher in women with malformations in comparison with patients without malformations and with endometriosis and in comparison with the control group (Table 1) [6].

Expression of cytokeratin in cells from ectopic endometrium was significantly higher than in cells from eutopic endometrium (Table 1). This is consistent with previously obtained data on cell cultures from patients without malformations [5,6]. The presence of a population of cytokeratin-expressing cells reflects the presence of epithelial cells in passage 1 MMSC cultures.

The observed differences in the expression of important cell markers CD90, CD105, cytokeratin, CD117, CD44, and CD146 suggest that cells from eutopic and ectopic endometrium of patients with congenital malformations of the internal reproductive organs represent a special cell population phenotypically different from cells from the same source derived from women without malformations. The revealed phenotypic characteristics of cell cultures from patients with malformations of the internal reproductive organs associated and endometriosis confirm the hypothesis about the development of ectopic and eutopic endometrium from germ cell of the gonadal ridges [17].

To prove plasticity of the isolated cultures, induction of their osteogenic and adopoigenic differentiation was studied [5,6]. All studied cultures were capable of directed differentiation, while spontaneous differentiation of cultures in the absence of differentiating factors was not detected.

Our findings suggest that the analyzed cell cultures from eutopic and ectopic endometrium of patients with uterovaginal malformations met the minimal criteria of multipotent mesenchymal stromal/stem cells: adhered to plastic, expressed typical surface markers CD90, CD105, and CD73, did not express CD45, HLA-DR,

CD34, and CD14, and were capable of adipogenic and osteogenic differentiation *in vitro* [9]. These cultures were similar to cultures isolated from eutopic and ectopic endometrium of patients without malformations and demonstrated immunophenotypic differences that can be attributed to functional differences of MMSC from different sources. The revealed differences allow us to consider this cell population as a promising model for the development and testing of new drugs aimed at suppression of the growth and spread of endometriosis, as well as for the study of the etiology and pathogenesis of endometriosis in women with uterovaginal malformations.

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