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Characteristics of Multipotent Mesenchymal Stromal Cells Isolated from Human Endometrium and Endometriosis Lesions

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Cell cultures isolated from endometriosis lesions by enzymatic dissociation consisted of fibroblast-like cells expressing CD90, CD73, and CD105; cell viability in these cultures was >90%, but this parameter decreased by passage 3. Zero passage cultures contained 10-25% epithelial cells expressing cytokeratin-7, but by passage 2, the cultures became more homogeneous and epithelial cells disappeared. The proportion of proliferating cells and population doubling level increased from passage 1 to passage 3. The cultures from the endometrium were induced to adipogenic and osteogenic differentiation *in vitro*. The cultures derived from ectopic endometrium have properties of multipotent mesenchymal stromal cells that exhibited *in vitro* similarities and differences from cell cultures from eutopic endometrium, which allows using this cell model for the search and testing of new drugs and technologies aimed at suppression of the growth and spread of endometriosis lesions.

Key Words: *endometriosis; endometrium; multipotent mesenchymal stromal cells; immunophenotype; proliferation*

Endometriosis is a chronic gynecological condition characterized by benign growth of the tissue morphologically and functionally similar to the endometrium outside the uterus. Endometriosis affects 10% women worldwide and is detected in 35-50% women with infertility [1]. The causes of endometriosis remain unknown. The most prevalent theory explains the etiology of this condition by retrograde flow of menstrual blood [11] into the pelvic cavity followed by attachment of endometrial cells and formation endometriosis lesions. Other pathogenetic factors include genetic predisposition, changes in cellular immunity, hormonal shifts, metaplasia of the peritoneal mesothelium into endometrial epithelium, and abnormalities of embryogenesis with pathological existence of residual paramesonephric (Mullerian) ducts and activation of their growth on the peritoneum and in other organs; effects of environmental factors cannot also be excluded [2]. Endometriosis lesions are characterized by cell invasion and abnormal cell growth that can be associated with activity of stem cells. The existence of stem/progenitor cells in the endometrium is beyond doubt; regeneration of the endometrium is apparently provided by multipotent mesenchymal stromal cells (MMSC) [5]. These cells are probably responsible for the growth and development of endometriosis lesions [13]. Comparison of MMSC cultures from eutopic endometrium and endometriosis lesions and analysis of their functional similarities and differences seem to be interesting. This will allow application of this cell model for the search and testing of new drugs and therapies aimed at suppression of the growth and proliferation of endometriosis lesions.

Here we analyzed immunophenotypic characteristics, viability, proliferation potential, and cell cycle phase distribution of MMSC isolated enzymatically

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from human endometriosis lesions and compared them with cultures of eutopic endometrium.

MATERIALS AND METHODS

Specimens of ectopic endometrium (endometriosis lesions) were collected under sterile conditions during laparoscopic surgeries from patients admitted at V. I. Kulakov Research Center of Obstetrics, Gynecology, and Perinatology and promptly transferred to sterile 50-ml conic centrifuge tubes (Costar) with 20 ml PBS. The tubes were placed in a special transportation container and delivered to the laboratory within 30-40 min. 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 and 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 at seeding density of $3\text{-}5\times10^3$ nucleated cells/cm^2. The medium was changed every 3 days; the cultures were passaged before confluence.

The morphology of cell cultures was assessed by microphotographs of randomly selected fields of view under an Eclipse TS100 phase-contrast microscope (Nikon).

Cell immunophenotype was determined during passages 0-2 using monoclonal antibodies labeled with FITC, phycoerythrin (PE), or allophycocyanin (APC) to antigens CD14, CD31, CD34, CD44, CD45, CD73, CD90, CD105, CD117, CD146, CD200, HLA- DR, HLA-ABC, and cytokeratin-7 (BD Pharmingen) on a FACSCalibur flow cytofluorometer (Becton Dickinson) according to manufacturer's guide. FITC-, PE- and APC-labeled IgG of the corresponding class were used as the isotypic control. We examined 21 cultures of ectopic endometrium.

In cultures of passages 1 and 3, proliferative activity of cells was evaluated by analyzing microphotographs of at least 5 fields of view in each flask taken under a Nikon Eclipse TS100 phase contrast microscope. The cells were counted using Sigma ScanPro 5.0 Image Analysis Software (SPSS Inc.) and the number of population doublings (PD) was calculated by the formula $PD=\log_2 N/N0$, where N0 and N are the initial and final cell counts [14].

The fractions of cells at the early and late stages of apoptosis and the fraction of necrotic cells were analyzed using FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen). The distribution of cells at different stages of the cell cycle was evaluated by PI staining. This method was also used for evaluation of the percent of cells at the late stages of apoptosis characterized by DNA degradation and appearance of an additional peak with fluorescence intensity below the G_1-peak (Sub-G_1) on the histogram. Analysis was performed on a FACSCalibur flow cytofluorometer according to manufacturer's instruction.

The results were statistically processed using Microsoft Excel 2003 software; significance of differences was evaluated using nonparametric Mann–Whitney test at $p\leq0.05$.

RESULTS

Passage 1 cultures enzymatically isolated from the ectopic endometrium mainly consisted of 60-120-μ fibroblast-like cells with an admixture of polygonal cells with a diameter of ~60 μ (Fig. 1).

Cytofluorometry showed that even primary cell culture (zero passage) was characterized by intensive expression of surface markers characteristic of MMSC (CD90, CD73, and CD105; Table 1). The expression of CD105 in ectopic endometrium starting from zero passage appreciably surpassed that in eutopic endometrium [3] and slightly increased from passage to passage. The number of cells expressing HLA-ABC in cultures from ectopic endometrium was significantly lower than in cultures from eutopic endometrium; this parameter slightly decreased during passaging and its range also decreased. CD73 expression on cells of ectopic endometrium increased during passaging and reached almost 100% by passage 2. In cultures from endometriosis lesions, expression of CD44 participating in cell adhesion, binding with hyaluronic acid, and signaling increased from zero to second passage and the range of this parameter decreased; in contrast, expression of this marker in cells from eutopic endometrium decreased by passage 2. CD146 (MCAM) expression in zero passage cells from endometriosis lesions greatly varied, but the range of this parameter substantially decreased by passage 2 and dropped below the values observed in cultures of eutopic endometrial cells. Zero passage cultures derived from ectopic endometrium contained from 2 to 40% cytokeratin-expressing epithelial cells, but these cells practically disappeared by passage 2, which increased culture homogeneity as it was observed in eutopic endometrial cell cultures. Cultures from endometrial lesions by passages 1 and 2 practically did not contain monocytes, hematopoietic precursors, and endothelial cells expressing CD14, CD34, CD45, CD31, and HLA-DR.

The presence of cells expressing CD200 in cultures from ectopic endometrium (the fraction of these cells in zero passage cultures varied from 0 to 36%, mean 13.3%), a marker involved in the maintenance of immunological tolerance in myeloid cells [8] is worthy of note; expression of this marker decreased

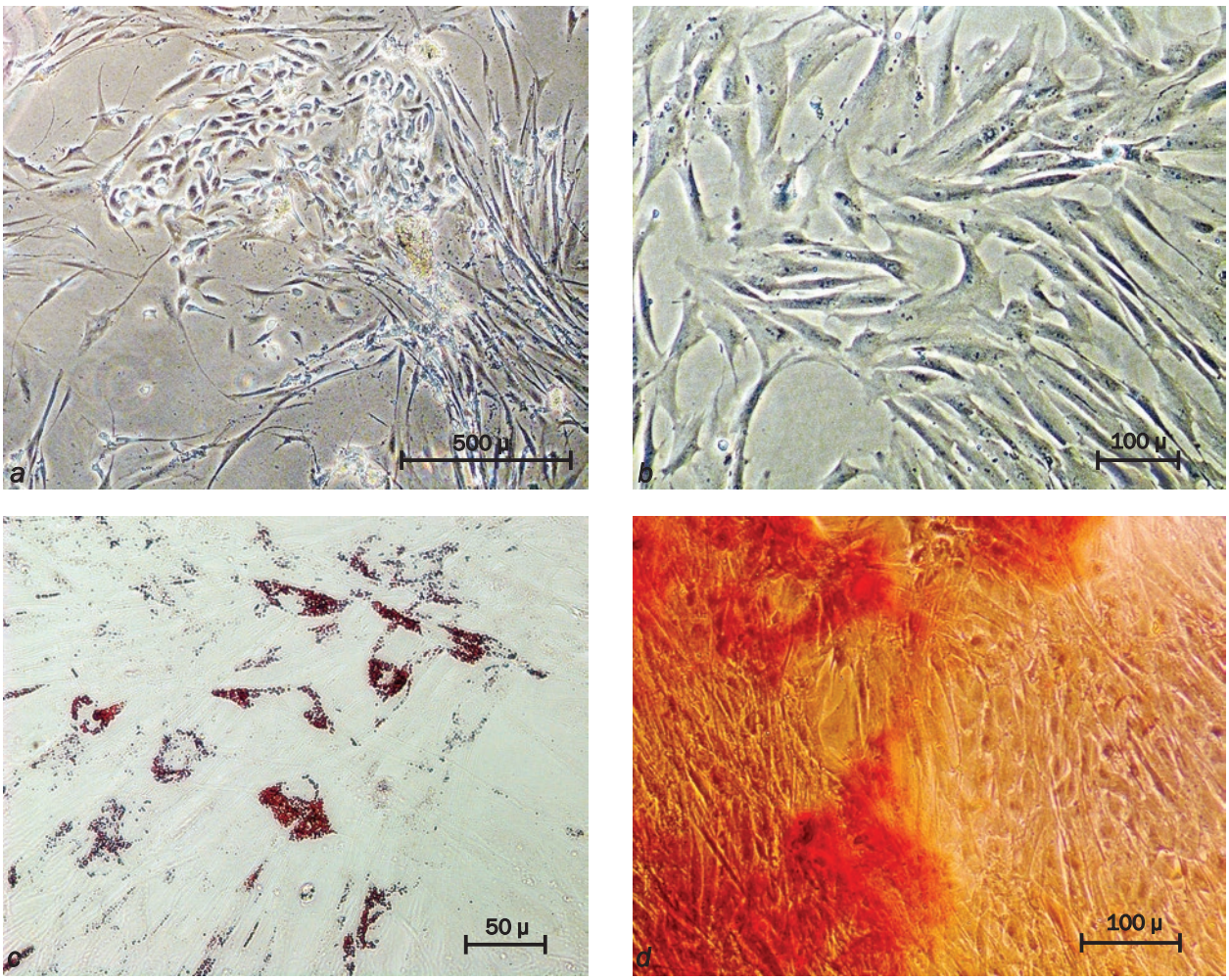

Fig. 1. Morphology and differentiation of cells isolated from ectopic endometrium. Phase contrast. Staining with Sudan III (*c*) and alizarin red (*d*). ×40 (*a*), ×100 (*b*, *d*), ×200 (*c*). *a*) Passage 0; *b*) passage 2; *c*) adipogenic differentiation; *d*) osteogenic differentiation.

during the next passages. Considerably higher expression of CD200 in passage 1 cultures from ectopic endometrium in comparison with cultures from eutopic endometrium could not be explained by the presence of lymphocytes or endothelial cells, because the fraction of $CD45^+$ and $CD31^+$ cells in these cultures is very low.

CD200 expression is a negative prognostic factor in patients with multiple myeloma and acute myeloid leukemia; CD200 was also reported to reduce the production of Th-1 cytokines *in vitro* in solid tumors (including melanoma, ovarian carcinoma, and renal cell carcinoma [12]). It is known that MMSC from different tissues often express CD200 [10,15], while expression of CD200R receptor is restricted to myeloid cells and binding of CD200 with CD200R leads to suppression of many immune system cells, in particular, macrophages [7]. Previous studies [10] have demonstrated that bone marrow MMSC from different donors expressed CD200 at different levels (7.50-52.5%), while MMSC from the umbilical cord virtually did not express this marker. $CD200^+$ MMSC from the bone marrow suppressed TNF secretion by IFN-γ-stimulated macrophages in co-culture, while $CD200^-$ MMSC from the umbilical cord produced no such effect. It was reported [16] that placental MMSC of fetal origin more intensively expressed CD200 (71%) than MMSC of maternal origin (~2%) and that the first group of cells were more potent in stimulating angiogenesis *in vitro* and immunosuppression *in vivo*; the authors attribute this difference to CD200 expression. All these data attest to an important role of CD200 in the interaction with immune system cells. Different expression of this marker in MMSC from different sources can reflect their different immunological activity. We found no reports describing expression of CD200 on MMSC of eutopic and ectopic endometrium.

The expression of receptor to stem cell factor, c-kit proto-oncogene (CD117), in ectopic endome-

TABLE 1. Immunophenotyping of Cell Cultures (%) Derived from Ectopic Endometrium on Passages 0, 1, and 2 (*M*±*SD*)

Marker	Passage		
	0	1	2
CD90	65.2±25.3	62.5±21.6	59.9±9.8
CD105	49.6±40.3	57.2±24.8	54.7±20.0
CD73	84.8±11.3	93.9±7.1	97.4±2.9
HLA–ABC	48.0±50.2	52.8±31.7	41.3±23.7
CD117	26.3±25.2	31.8±31.5	27.7±26.6
CD44	48.7±25.3	71.2±20.3	69.8±3.9
CD146	47.6±40.7	11.9±17.9	6.0±4.9
CD26	18.5±10.4	34.5±20.9	27.1±16.7
CD54	31.1±10.4	28.2±9.5	25.1±8.3
Cytokeratin-7	15.6±11.1	11.4±10.3	3.1±2.6
CD200	13.3±18.5	8.1±8.9	2.1±2.1
HLA-DR	4.6±4.6	1.3±0.6	0
CD34	2.9±1.2	0	–
CD45	2.1±1.8	0	–
CD31	3.8±1.5	1.5±0.7	0
CD106	1.9±1.0	3.9±6.3	1.7±1.8
CD10	15.5±10.6	15.1±13.4	10.3±5.8
CD14	3.0±3.2	4.8±4.9	0

Note. "–", not determined.

trium cells greatly varied in zero passage cultures (2.7-51.2%); neither the mean (~30%), not the range of this parameter changed during culturing at passages 1 and 2. The same was true for cells from eutopic endometrium [3]. It can be assumed that expression of CD117 is associated with individual physiological characteristics of cells from different donors and is very important judging from the tendency to maintain this level during culturing. However, the specific role of this protein for stromal cells remains to be elucidated.

The reports describing immunophenotypic and functional properties of primary cell cultures from ectopic endometrium are extremely rare; immunohistochemical or PCR studies of endometriosis lesions per se predominate. Our findings generally agree with the previous data [6,9], but cultures isolated by us from ectopic endometrium contained less CD90-, CD105-, and CD44-positive cells; most cells expressed CD73 during both passages 1 and 2. Minor differences in these standard markers can be explained by differences in isolation procedures: in the cited studies, epithelial cells and aggregates were removed by passing the cell suspension through filters with different pore diameters (100, 70 and 40 μ) and stromal cells were analyzed starting from passage 2, while early passages were not studied. Moreover, expression of informative markers such as CD14, CD31, CD117, CD200, HLA-DR, and HLA-ABC in cell cultures from ectopic endometrium was not measured in the above studies. In report [9], weak expression of cytokeratin (<3.8%) was revealed in the population of stromal cells from eutopic endometrium [9] despite removal of epithelial cells by filtration, whereas in our study, cell cultures from both eutopic and ectopic endometrium did not express cytokeratin starting from passage 2 [3]. Thus, cell cultures used in our study contained no cytokeratin-expressing cells starting from passage 2, *i.e.* homogeneity of cultures increased during culturing and removal of epithelial cells is unnecessary.

The viability of cultured cells was studied using specific fluorescent dyes by assessing the percentage of apoptotic, necrotic, and live cells in passages 1 and 3 cultures. In passage 1 cultures from ectopic endometrium, live cells (Ann^-/PI^-) constituted 95.0±4.6% of the total cell number, but this parameter significantly decreased by passage 3 (84.6±9.0%); the proportion of

TABLE 2. Fractions of Cells (%) in Different Phases of the Cell Cycle in MMSC Cultures from Ectopic Endometrium (*M*±*SD*)

Passage	Sub-G1	G0/G1	S/G2/M
1	1.8±1.3	83.0±8.5	15.3±6.2
2	4.9±3.8	78.7±7.1	16.5±4.5
3	3.7±2.8	76.5±8.9	22.6±5.7
4	3.5±1.3	77.0±6.8	18.6±5.5

early apoptotic cells (Ann^+) increased from 4.4±2.3 to 10.10±4.92%. The percent of necrotic cells (PI^+) during passages 1 and 3 constituted 7.1±2.1 and 7.5±3.4%, respectively. Interestingly, the fractions of necrotic (PI^+) and early apoptotic cells (Ann^+) in cultures of ectopic endometrium was significantly higher than in cultures from eutopic endometrium [3], but the fraction of late apoptotic cells (Ann^+/PI^+) in the cultures from ectopic endometrium was significantly lower than in cultures from eutopic endometrium (0.7±0.4 and 2.1±1.7%, respectively, during passage 1).

The population doubling level in cultures from ectopic endometrium increased with increasing the fraction of proliferating cells (Table 2) by passage 3: (2.6±1.1% during passage 1 and 3.0±1.3% during passage 3; $p \leq 0.05$). The fraction of G0/G1 phase cells decreased and the fraction of late apoptotic cells (Sub-G1) increased (Table 2). Moreover, population doubling level for cultures from ectopic endometrium was 1.4-fold higher than for the population of eutopic endometrial cells (2.3±1.1 and 2.2±1.7%, respectively, for passage 1 and passage 3 cultures, respectively). This is consistent with published report [9], where cumulative population doubling level in passage 4 culture of endometrial cells was 1.3-fold higher than in the population of endometrial cells.

To prove plasticity of isolated cultures, they were induced to osteogenic and adopoigenic differentiation as described previously [3]. No spontaneous differentiation in cultures in the absence of differentiation factors was observed.

Thus, we can conclude that ectopic endometrium at the early stages of culturing formed mixed cultures containing epithelial and mesenchymal cells, which was confirmed by the presence of cytokeratin-positive cells (10-25%, mean 15%), but culture homogeneity increased after passaging. Passage 2 cultures contained practically no epithelial cells. Cell viability decreased during culturing, while the fraction of S/G2/M-phase cells and proliferation index increased after passaging.

Thus, the analyzed cell cultures met the minimal criteria of MMSC: adhered to plastic, expressed typical surface markers CD90, CD105, and CD73, did not express CD34, CD45, HLA-DR, and CD14, and were capable of adipogenic and osteogenic differentiation [4]. The cultures have similarities and differences from MMSC cultures derived from eutopic endometrium, which could be due to functional differences of MMSC from different biomaterials. The detected differences of MMSC cultures from ectopic and eutopic endometrium suggest that this cell model can be used for the search and testing of new drugs aimed at suppression of the growth and spreading of endometriosis lesions.

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