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Characteristics of Multipotent Mesenchymal Stromal Cells Isolated from Human Endometrium

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Cell cultures isolated from human endometrium by enzyme digestion consisted of highly viable fibroblast-like mesenchymal cells expressing CD90, CD73, and CD105. During passage 1, the cultures contained a small fraction of cytokeratin-7$^+$ epithelial cells that disappeared by passage 2. The cultures from the endometrium could be induced to adipogenic, osteogenic and chondrogenic differentiation *in vitro*. These findings suggest that human endometrium is a convenient source of biomaterial for minimally invasive isolation of cultures that exhibit typical morphology and immunophenotypic profile of resident multipotent mesenchymal stromal cells retain high viability *in vitro*.

Key Words: *endometrium; multipotent mesenchymal stromal cells; immunophenotype; viability*

Human endometrium is a multicomponent system comprising various cell types (surface and glandular epithelium, stromal cells, blood and vascular cells). Abnormal proliferation of the endometrium leads to severe gynecological diseases, such as endometrial hyperplasia or hypoplasia and endometrial cancer. Endometrial lesions (especially in the basal layers) can lead to the formation of cicatrices and intrauterine synechiae and to the development of Asherman syndrome (with secondary amenorrhea) and eventuate in infertility and pregnancy loss. Multipotent mesenchymal stromal cells (MMSC) isolated, among other sources, from the endometrium and menstrual blood [8], have great potential for reparation of damaged tissue. Due to high differentiation potential, these cells can be committed towards mesenchymal cells: osteogenic, adipogenic, and chondrogenic. MMSC are characterized by low immunogenicity, self-maintenance capacity, and high proliferative potential and are promising for cell therapy of various pathological conditions, *e.g.* related to endometrium failure. There are no reports about spontaneous transformation of human endometrial MSC during long-term culturing [3], which is very important for the use of this material in cell therapy, because large cell mass required for this procedure is generated by long-term culturing. The safety of transplantation of endometrial MSC was tested on experimental animals and no signs of toxicity were observed [4]. These findings confirm the prospects of using endometrial MSC for replacement therapy [1].

In the present study, we evaluate immunophenotypic profile, viability, and differentiation potential of mesenchymal cell cultures from human endometrium isolated by the enzymatic method.

MATERIALS AND METHODS

The study was approved by Ethical Committee of V. I. Kulakov Research Center of Obstetrics, Gynecology, and Perinatology. Specimens of the endometrium were collected under sterile conditions during diagnostic pipelle endometrial biopsy sampling from patients aged 22-30 years admitted at V. I. Kulakov Research Center of Obstetrics, Gynecology, and Perinatology. Specimens of subcutaneous fat (3-5 ml) were collected into a sterile syringe from the incision during abdomi-

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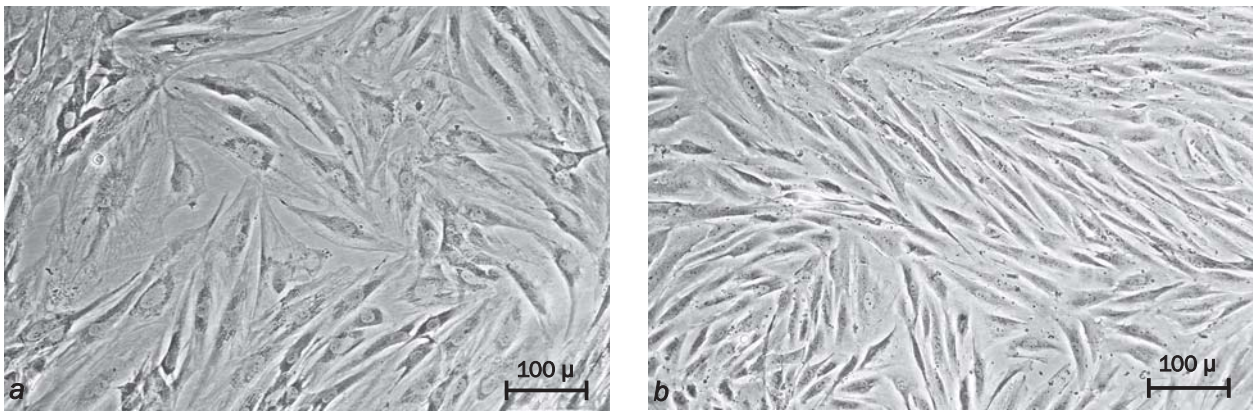

Fig. 1. Morphology of cells isolated from human adipose tissue (*a*) and endometrium (*b*). Passage 1. Phase contrast, ×200.

nal surgery for gynecological diseases in women aged 22-30 years. All patients signed informed consent.

The collected samples from the vacuum aspirator (pipelle) and the syringe were transferred to a 50-ml conical centrifuge tubes (Costar). The tubes were placed in a special transportation container and delivered to the laboratory within 30-30 min. The samples were washed with PBS, minced, and incubated in 0.07% collagenase IA (Sigma-Aldrich) at 37°C for 90 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 3-5×10^3 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 by microphotographs of randomly selected fields of view under an Eclipse TS100 phase-contrast microscope (Nikon).

Immunophenotyping of cells of the first and second passages was performed routinely using FITC-, PE-, or APC-labeled monoclonal antibodies 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 (BD) flow cytofluorometer according to manufacturer's guide. FITC-, PE- and APC-labeled IgG of the corresponding classes were used as the isotypic control. We analyzed 23 cultures from the endometrium and 21 cultures from the adipose tissue.

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) on a FACSCalibur flow cytofluorometer according to manufacturer's guide.

Plasticity of isolated MMSC from the endometrium was assessed as described elsewhere [2] on passage 2 cultures. After fixation of cultures, the wells were washed twice with distilled water and the cells were stained with 2% alizarin red (pH 4.2) for 2-3 min (osteogenic differentiation), 1% alcian blue in 0.1N HCl for 30 min (chondrogenic differentiation), or 0.2% Sudan III for 10 min (adipogenic differentiation). Then, the wells were washed with distilled water and stained cells were visualized under a light microscope.

The data were processed statistically using Microsoft Excel. Significance of differences between the groups was evaluated using nonparametric Mann–Whitney *U* test at $p \leq 0.05$.

RESULTS

Morphological analysis of cell isolated by enzyme digestion of the endometrium and adipose tissue samples showed relative homogeneity of their population composition. Passage 1 cultures derived from the endometrium consisted mainly of elongated fibroblast-like cells (60-120 μ) and contained minor admixture (5-10%) of polygonal cells (~60 μ) (Fig. 1). Cultures of the adipose tissue consisted of larger (70-200 μ) fibroblast-like cells.

Cytofluorometry showed that passage 1 cultures derived from the endometrium carried surface differentiation markers CD90, CD73, and HLA-ABC (Table 1); marker CD105 also typical of mesenchymal cells was detected on a minor fraction of cells. Despite intensive expression of CD73 and HLA-ABC, surface markers CD90 and CD105 were detected on only 60 and 20% cells, respectively. Passage 1 cultures also contained a small population of cytokeratin$^+$ cells: from 1 to 10% in cultures from different donors. The cultures virtually did not contain monocytes and hematopoietic progenitor cells, which was seen from very low or no expression of CD14, CD34, CD45, and HLA-DR markers. Minor expression of CD200 in passage 1 cultures as well the presence of a small amount of CD31$^+$ cells can be explained by admixture of endothelial cells that disappeared by passage 2.

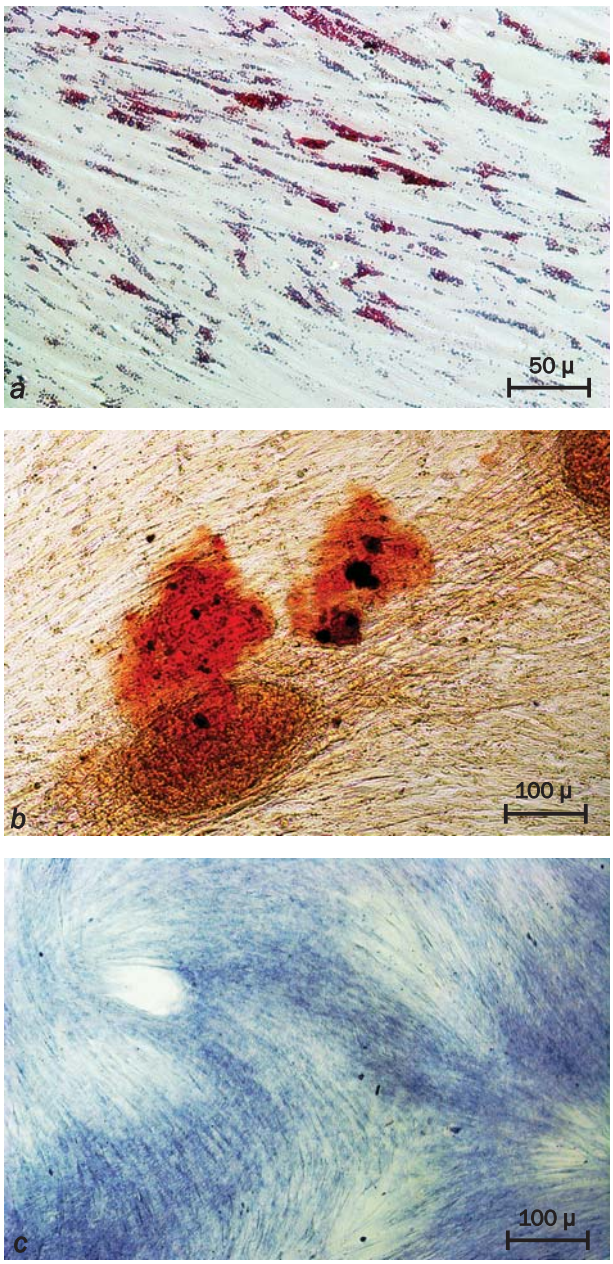

Fig. 2. Differentiation of MMSC from the endometrium towards adipogenic (*a*), osteogenic (*b*), and chondrogenic (*c*) lineage cells. Staining with Sudan III (*a*), alizarin red (*b*), and alcian blue (*c*). Phase contrast, ×200 (*a*); ×100 (*b*, *c*).

During passage 2, the expression of CD90 and CD73 remained unchanged, while expression of CD105 increased from 16 to ~50%. The expression of HLA-ABC and CD146 slightly decreased by passage 2. Practically all epithelial cells expressing cytokeratin disappeared. The expression of CD44 marker also decreased in passage 2 cultures. These findings generally agree with previous data [9], but in contrast to cultures described in the above report, cultures of endometrial cells isolated by us contained lower number of $CD90^+$ cells and almost all cells expressed CD73 during passages 1 and 2. In another report [13], almost all cells derived from the endometrium carried markers CD90, CD73, CD105, and CD146. These differences can be due to additional sorting of stromal cells on magnetic beads coated with antibodies to antigens of epithelial cells (BerEP4, EpCAM) and leukocytes (CD45) used to remove unwanted cells. After enzymatic isolation applied in our study, cells expressing cytokeratin were practically not detected by the second passage, *i.e.* culture homogeneity increased during culturing.

Expression of receptors for growth and transforming factors is an important sign attesting to the presence of stem and progenitor cells in the obtained cultures. The product of *c-kit* gene (CD117) is a receptor for stem cell factor (SCF), a protooncogen. c-kit protein is present on the surface of hematopoietic and other undifferentiated cells. SCF binding promotes cell survival, their proliferation, and differentiation (expression of c-kit decreases with increasing the degree of cell differentiation); it is also involved in mobilization of progenitor cells. Enhanced expression of this marker was detected in various types of malignancies and in epithelial cells in endometriosis foci [11]. In our experiments, cell cultures isolated from the endometrium were characterized by medium levels of CD117 expression during passages 1 and 2, while the degree of expression of this marker in different cultures significantly varied (from 6.6 to 78.9%). These differences can be explained by functional characteristics of

TABLE 1. Immunophenotypical Characteristics of Cell Cultures Derived from the Endometrium on Passages 1 and 2 (*M*±*SD*)

Surface marker	Relative content of cells, %	
	passage 1	passage 2
CD90	50.7±13.8	52.3±12.5
CD105	16.2±3.7	47.2±11.1
CD73	96.1±1.8	97.7±2.2
CD44	99.2±0.8	64.4±13.3
HLA-ABC	98.9±0.7	71.3±13.1
CD146	24.7±11.1	20.5±16.4
CD117	31.8±22.5	34.1±24.9
Cytokeratin-7	6.8±5.5	0.6±0.2
CD31	1.3±0.8	0
CD200	1.5±1.5	0
CD45	1.5±0.5	0
HLA-DR	0	0
CD14	0	0
CD34	0	0

TABLE 2. Viability of Cells in Cultures from the Endometrium and Adipose Tissue on Passages 1 and 2 (*M*±*SD*)

Passage	Cell source	Percent of aberrant cells			
		Ann^+	PI^+	$AnnV^+/PI^+$	$AnnV^-/PI^-$
1	Endometrium	1.2±0.2	3.1±0.4	5.2±1.1	90.5±1.1
	Adipose tissue	2.4±0.8	1.8±0.8	8.4±1.4	87.5±1.4
2	Endometrium	1.6±0.4	2.8±1.1	2.7±0.7	92.9±1.5
	Adipose tissue	1.6±0.4	1.3±0.1	2.2±1.0	95.0±0.8

Note. $AnnV^+$, cells at the early stage of apoptosis; $AnnV^+/PI^+$, cells at the late stage of apoptosis; PI^+, necrotic cells; $AnnV^-/PI^-$, viable cells.

cultures of endometrial cells from different patients. It has been previously demonstrated by the immunohistochemical method that this protein is present in stromal cells of the endometrium throughout the life and its expression increases during pregnancy [5]. However, other researchers also using immunohistochemical methods have shown that c-kit was present mainly in epithelial cells of the endometrium and was practically absent in stromal cells [11]. In the rat model of chronic ischemia of the endometrium, gradual decrease in the expression of c-kit gene mRNA and intensification of apoptosis in endometrial stem cells were demonstrated by real-time PCR [10]. In addition, researchers have used CD117, in particular, for isolation of cardiac and renal stem cells [7.12]; in a feline model of cardiomyopathy, intracoronary administration of autologous CD117-expressing MMSC more effectively improved animal status than autologous cardiac stem cell [14]. All these facts confirm the important role of CD117 stem cell, but specific functional role of this marker of mesenchymal cells for the positive effects of cell therapy remains unclear. This question requires further studies.

The viability of isolated cells was evaluated by the percentage of damaged and live cells in culture stained with specific fluorescent dyes. Annexin V specifically binds with phosphatidylserine on the cell surface during its apoptotic death, while propidium iodide penetrates through the damaged membrane of necrotic cells and binds with DNA. Combined staining ($AnnV^+/PI^+$) indicates that the cells are at a late stage of apoptosis, because propidium iodide penetrates through the damaged membrane. Thus, comparative analysis of the ratio of damaged and live cells in passage 1 cultures from the endometrium and adipose tissue showed that cells derived from the endometrium exhibited higher viability (Table 2). During both the first and second passages, live cells constitute more than 90% of these cultures. The number of cells entering apoptosis in cultures from the endometrium during both passages was higher than in adipose tissue cultures, but the differences were insignificant. In general, cell viability in cultures from these sources remained high (≥87%).

For evaluation of the plasticity of isolated cultures, their osteogenic, adipogenic, and chondrogenic differentiation was induced. The dynamics of differentiation of the analyzed cultures could be visually traced only for adipogenic differentiation. After 1-week culturing with inductors of adipogenic differentiation, solitary cells containing lipid vacuoles were seen under a microscope without fixation and staining. After 2-week culturing under these conditions, the number of cells with lipid vacuoles increased. After the end of culturing, the cultures were fixed and stained with Sudan III; lipid vacuoles were found in 80-90% cells (Fig. 2). Osteogenic differentiation of the analyzed cells was difficult to detect visually. For detection of calcium phosphate accumulation in cultures from the endometrium during differentiation, the cells were stained with alizarin red after the end of culturing. Chondrogenic differentiation capacity of the studied cultures was evaluated by their capacity to synthesize proteoglycans; the latter were detected by specific alcian blue staining. In none of the experiments, spontaneous differentiation of initial cultures was detected in culture medium without differentiation factors after staining.

The results suggest that the studied cultures isolated from the endometrium were characterized by the capacity to differentiate *in vitro* towards adipogenic, osteogenic and chondrogenic lineage cells typical of cells of mesodermal origin. This differentiation in the presence of certain inducers suggests that cells in these cultures represent MMSC.

Thus, adherent culture isolated from the endometrium by the enzymatic method at the early stages of culturing represented mixed culture of epithelial and mesenchymal cells, which was seen from the presence of the admixture of cytokeratin-expressing cells (up to 10%). Their viability and expression of typical mesenchymal stromal cells markers increased during *in vitro* culturing and the cultures became more homogeneous.

The analyzed cell cultures meet the minimal criteria of MMSC: adhesion to plastic; expression of typical surface markers CD90, CD105, and CD73; lack of CD34, CD45, HLA-DR, and CD14; and capacity to differentiate towards mesodermal lineage cells (adipogenic, osteogenic, and chondrogenic) [6]. Thus, we can conclude that MMSC from human endometrium can be a convenient and readily available source for low-invasive preparation of multipotent MSC for the use in cell therapy and regenerative medicine.

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