Endometrial Mesenchymal Stem Cells Isolated from the Menstrual Blood

R. A. Musina, A. V. Belyavski*, O. V. Tarusova, E. V. Solovyova, and G. T. Sukhikh

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Stem cells from the endometrium isolated during menstrual bleeding were characterized. By their main surface markers, differentiation potential, and morphological signs these cells belong to mesenchymal stem cells. Specific features of this cell type are high clonogenic activity and low capacity to adipocyte differentiation.

Key Words: *mesenchymal stem cells; endometrium*

Mesenchymal stem cells (MSC) are present in virtually all organs and tissues. No basic differences between the cells isolated from different tissues were detected. Certain specificity of MSC isolated from different sources can be explained by the presence of progenitor cell fractions at different stages of differentiation.

Low-invasive methods for material collection for subsequent isolation of stem cells (SC) are preferred for clinical use. The most easily accessible sources of MSC and SC are fatty tissue, umbilical blood, and placenta.

We detected SC in the menstrual blood. The menstrual blood contains fragments of the endometrium detached during bleeding. This tissue forms *de novo* every month in the females, and hence, it is young tissue. Biologically it is intended for active participation in the gestation process; some extraembryonic organs develop from it, for example, placenta. The endometrium has two layers: basal and functional. The structure of the functional (surface) layer depends on ovarian hormones and undergoes deep restructuring during the menstrual cycle. The uterine mucosa is lined with a one-layer prismatic epithelium. The uterine mucosa lamina propria is formed by loose fibrous connective tissue. Some cells of the connective tissue develop into large round decidual cells with glycogen lumps and lipoprotein incorporations in the cytoplasm. It is logical to hypothesize that the endometrium discharged with menstrual blood and formed by connective tissue contains (in addition to other elements) SC. This study was undertaken to verify this hypothesis.

MATERIALS AND METHODS

The study was carried out on menstrual blood. Donors of blood were healthy, mean age 28 years (25-45 years). All participants in the study gave consent to use of the material with research purposes. A total of 30 blood specimens collected from 10 donors were studied. Observations were carried out for 4 years.

Stem cells were isolated no later than 24 h after material collection.

The mononuclear fraction of blood cells was isolated under standard conditions in Ficoll gradient. An aliquot of the blood was diluted with an equal volume of PBS, applied onto Ficoll (Ficoll-Paque, Pharmacia), and centrifuged at 400*g* for 30 min at 10°C. The median cell fraction was collected, washed in PBS, centrifuged at 200*g* for 10 min at ambient temperature, erythrocytes were lyzed

V. I. Kulakov Research Center of Obstetrics, Gynecology, and Perinatology; *V. A. Engelgardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia

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with 160 mM NH_4Cl, washed, and inoculated (6×10^8/cm^2) onto clean plastic (Costar). Tissue fragments were washed in PBS, crushed, placed into 0.1% collagenase solution (30 min, 37°C). The enzyme was inactivated with DMEM supplemented with 10% fetal bovine serum (FBS; Gibco), centrifuged at 200*g* for 10 min at ambient temperature, washed several times in PBS, and erythrocytes were lyzed, if necessary, under conditions identical for the blood. Fragments of lyzed tissues were removed by filtration through 100-μ Nylon filters (Millipore). The cells were precipitated (10^6/cm^2) on clean plastic. The medium was replaced after 24 h.

Culturing of MSC was carried out under identical conditions in DMEM (Gibco) with low glucose content (1 g/liter) with 10% FBS (Gibco), 2 mM glutamine, 1% penicillin and streptomycin. The cells were cultured under standard conditions at 37°C and 5% CO_2. The medium was replaced every 3-4 days. After attaining 80% confluence, the cells were harvested with 0.25% trypsin and EDTA and reinoculated (6-10 cell/cm^2). After passage 1, a portion of cells was frozen with 10% dimethylsulfoxide (DMSO) and 30% FBS in liquid nitrogen. Cytofluorometric analysis was carried out after 2 and 5 passages and after long culturing (2 years, 12 passages). The samples were analyzed on a FACSCalibur cytofluorometer (Becton Dickinson) in the logarithmic fluorescent scale using CellQuest software. Fluorescence was stimulated by Innova-90-6 argon laser (Coherent). The cells were characterized using a kit of direct monoclonal antibodies: FITC-labeled CD44, CD90, CD34, CD45; APC-labeled CD105/Endoglin; Cy2-labeled second anti-murine antibodies (Beckman Coulter, Coltak). Before the analysis on a flow cytofluorometer, the cells were harvested with 0.25% trypsin and EDTA, washed in PBS with 0.1% FBS. Cell aliquots (10^6 cells) were then incubated in the above buffer with monoclonal antibodies; after staining with indirect antibodies, the cells were washed in PBS with 0.1% FBS and incubated for 30 min with second antibodies. Dead cells were excluded from the analysis by propidium iodide (PI) staining and by diffusion parameters. The data were processed using WinMDI 2.8 software.

Cell differentiation was carried out by the standard methods. After 2 passages MSC were reinoculated (15-20 cell/cm^2) and cultured in DMEM with 4 g/liter glucose, 2 mM glutamine, 1% antibiotic/antimycotic, 10% FBS during 3 weeks with the following reagents:

- for adipogenic differentiation: 0.5 mM isobutylmethylxanthine (IBMX), 1 μM dexamethasone, 10 μM insulin, 200 μM indomethacin;
- for osteogenic differentiation: 0.1 μM dexamethasone, 50 μM ascorbate-2-phosphate, 10 mM β-glycerolphosphate.

After 3-6-week culturing, differentiated cells were washed twice in PBS and fixed. Adipocytes were fixed in 4% formaldehyde (60 min at ambient temperature) and then incubated in 0.5% Oil Red

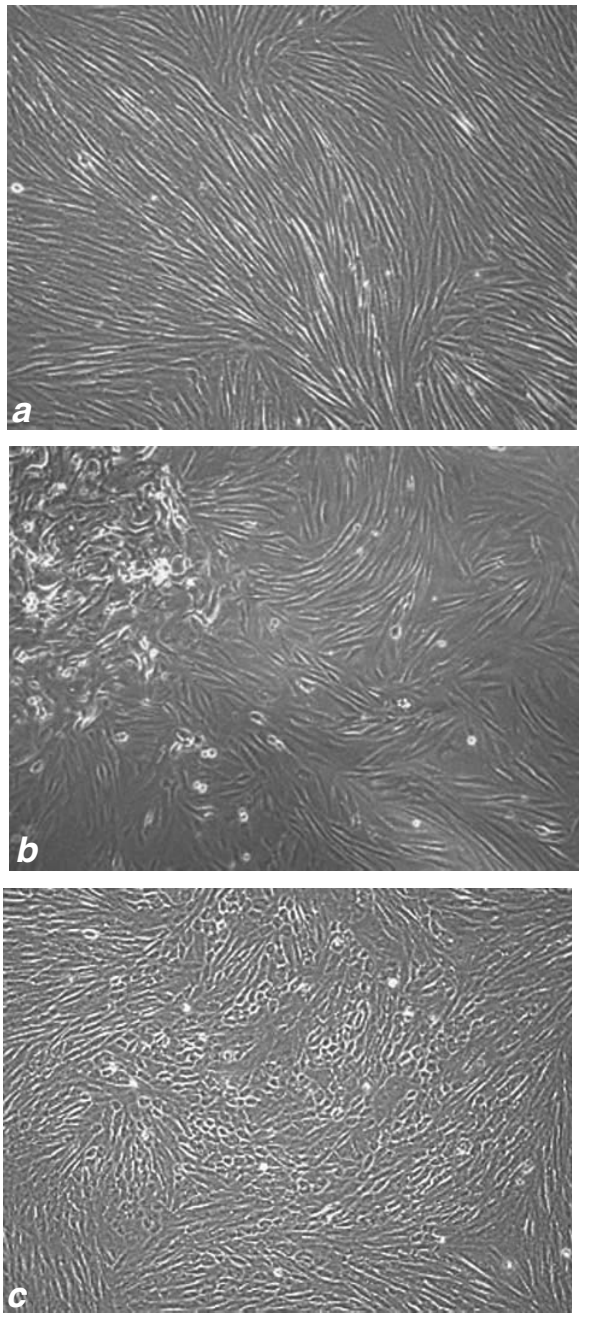

Fig. 1. Endometrial cells. *a*) MSC monolayer; *b*) two types of cells in endometrial cell cultures, passage 1; *c*) possible epithelio-mesenchymal transformations in compact monolayer of endometrial MSC.

solution for 20 min. Osteoblasts were fixed in ice-cold 70% ethanol for 1 h and then stained with Alizarin red S (40 mM, pH 4.1) for 10 min. Stained cells were analyzed by phase contrast microscopy (Olympus CK 40M) using ImageScope Lite software.

Proliferative activity of MSC was evaluated by the population doubling rate during the period of exponential growth, which was calculated by the formula: $\log N_1/\log N_2$, where N_1 is the initial cell count in confluent monolayer and N_2 initial number of cells inoculated before monolayer formation.

RESULTS

The morphology of menstrual blood MSC was typical of MSC. The MSC population in general is more uniform by cell size (10-100 μ) and shape, in comparison with cells derived from other sources (fat, bone marrow, skin). Cells of 50 to 70 μ in size with typical fibroblast-like shape predominated. A characteristic feature of these cells is smaller "curls" in the monolayer (Fig. 1, *a*). Small colonies of round cells were seen in some cultures at the early stages of culturing (before passage 1). During culturing they died and were replaced by typical MSC (Fig. 1, *b*). Proliferative activity of cells varied even in the same patients and the number of cells isolated from menstrual blood was also variable. It seems that these differences can be explained by female status during 1 month. For example, sometimes we failed to isolate SC from menstrual blood of women with respiratory diseases. The same was true for stress situations. Proliferative activity tended to decrease with prolongation of cell culturing. Cells from older patients exhibited significantly lower proliferative activity after long (more than 1 year) culturing in comparison with the cells from young patients. Cells from patients aged over 40 years could be cultured for 15-18 months, after which their growth was sharply inhibited, while cells from patients aged under 30 years could be cultured during 4 years and longer.

After formation of a compact monolayer, the MSC isolated from menstrual blood often sharply changed their phenotype; fibroblast-like colonies were replaced by smaller round epithelioid cells (Fig. 1, *c*). Presumably, we observed the effect of epithelio-mesenchymal transformations in culture.

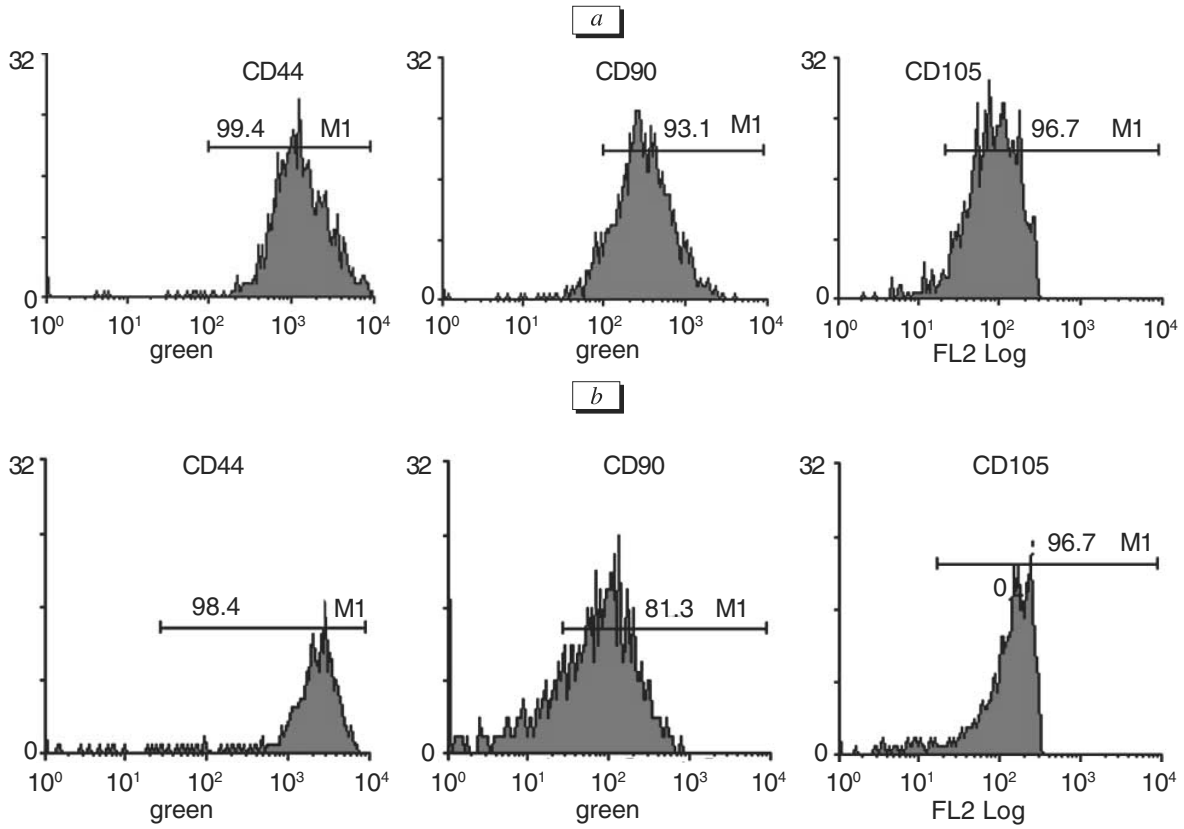

Fig. 2. Cytofluorometric analysis of expression of CD44, CD90, CD105 surface markers in endometrial MSC. *a*) patient N: cells cultured during 5 weeks (2 passages); *b*) patient T: long culturing (2 years; 12 passages).

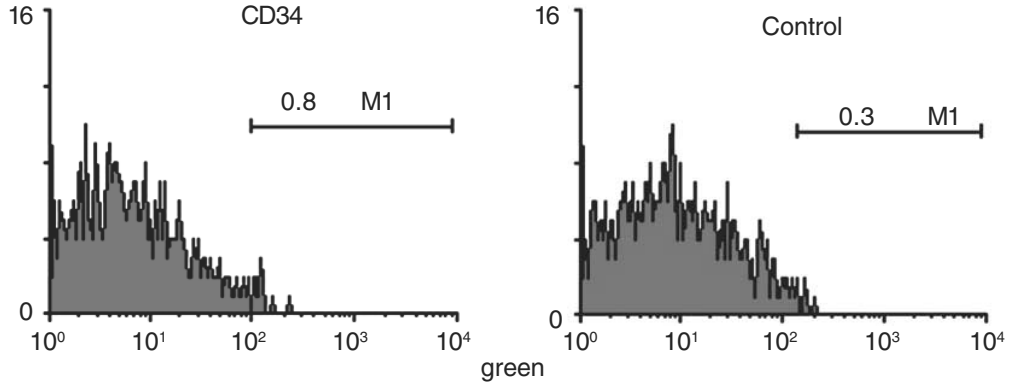

Fig. 3. Expression of negative marker (CD34; cytofluorometric analysis) in endometrial MSC and basal values.

This picture was observed not in all patients and only after formation of a very compact monolayer. After reinoculation, these cells reacquired a fibroblast-like shape typical of MSC.

The rate of endometrial MSC growth was evaluated visually. Lag period for all cultures was 24-48 h. The growth rate of all MSC types depends primarily on the quality of the initial material. Specimens of endometrial MSC analyzed in our study exhibited proliferative activity comparable to that of MSC derived from fatty tissue and skin and much higher in comparison with MSC from the umbilical blood and bone marrow.

Basal expression of surface markers did not exceed 2%. The expression of CD44, CD90, and CD105 was very high in all studied cells (81.3-99.4%, Fig. 2, *a*) and virtually did not change during culturing (Fig. 2, *b*). The expression of CD34 and CD45 markers did not exceed basal values (Fig. 3).

Comparison of MSC from different sources showed that specific feature of endometrial MSC was low capacity to differentiate into adipocytes in comparison with the bone marrow, fatty tissue, and skin cultures. This difference consisted in delayed start of differentiation (the cells started differentiating only by the end of week 3 of culturing) and low level of differentiated cells (<30% by the end of week 5 in culture, Fig. 4, *a*). By differentiation into osteoblasts, endometrial MSC virtually did not differ from cultures originating from other sources (Fig. 4, *b*).

The hypothesis that the endometrium contains stem progenitor cells responsible for regeneration and maintenance of cyclic changes in the female organism was put forward many years ago. However, only recently these cells were isolated and characterized. The discovery of these cells is a basic result, which can be essential for the solution of gynecological problems associated with many diseases [1,2]. Stromal cells of the endometrium with $CD13^+$ phenotype were described by the Japanese scientists. They were isolated from biopsy specimens obtained in resection of the uterus for myoma [3]. Differentiation of certain cells isolated from human endometrial biopsy specimens into chondrocytes was first demonstrated by American scien-

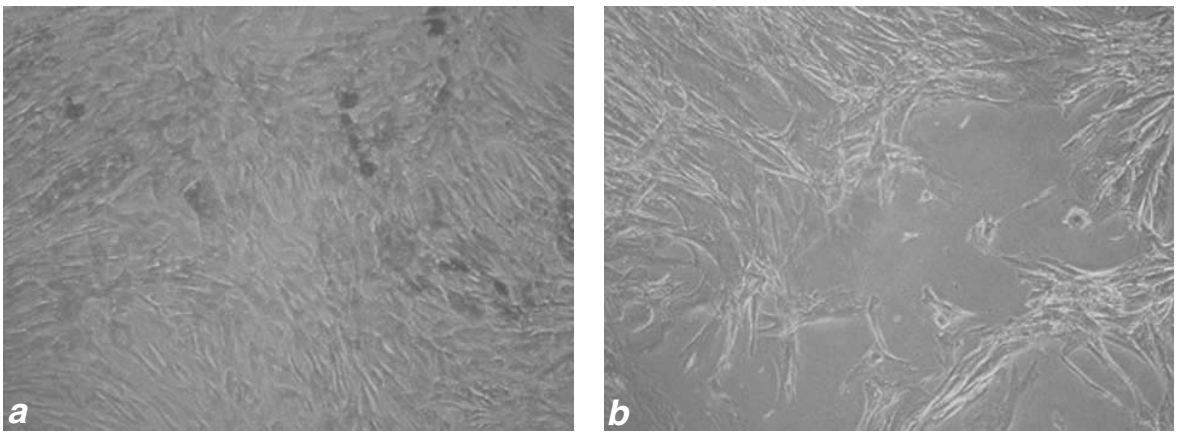

Fig. 4. Differentiation of endometrial MSC. *a*) adipogenic; *b*) osteogenic.

tists, which fact confirmed the presence of MSC fraction in the endometrium [5]. A group of scientists from the USA isolated SC from the menstrual blood and characterized them by surface markers and differentiation potential. These cells expressed CD9, CD29, CD41a, CD44, CD59, CD73, CD90, and CD105, retained karyotype stability during long-term culturing, exhibited much higher proliferative potential than umbilical blood MSC, and differentiated into 9 cell lines: cardiomyocytes, epithelium, nerve cells, myocytes, endothelium, pancreatocytes, hepatocytes, adipocytes and osteoblasts [4].

Our data confirm the presence of stem progenitor cells in the menstrual blood. Hence, the menstrual blood can be regarded as one of the most easily available sources of MSC with all characteristic features of these cells.

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