Specific Interaction of Cultured Human Mesenchymal and Hemopoietic Stem Cells under Conditions of Reduced Oxygen Content

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We studied the effect of reduced oxygen content (5%) on the phenotype and functional activity of cultured human mesenchymal stem cells. The expression of main immunophenotypic markers for mesenchymal stem cells (CD13, CD29, CD44, CD73, CD90, CD105, and HLA-I) remained practically unchanged under conditions of hypoxia. The expression of cell adhesion molecules (CD54 and CD106) increased during coculturing of mesenchymal stem cells and hemopoietic stem cells. These changes were accompanied by increased production of hemopoietins (interleukin-6 and interleukin-8) and enhanced colony-forming capacity of hemopoietic stem cells. Coculturing of mesenchymal stem cells and hemopoietic stem cells during hypoxia was followed by increased formation of hemopoietic islets and intensive production of interleukin-6, interleukin-8, and vascular endothelial growth factor (compared to cultures under normoxic conditions).

Key Words: *mesenchymal stem cells; hemopoietic stem cells; umbilical blood; coculturing; reduced oxygen concentration*

Bone marrow (BM) mesenchymal stem cells (MSC) are characterized by self-maintenance, active proliferation, recirculation in the organism, and differentiation into mesodermal cells. They provide hemopoietic microenvironment for hemopoietic stem cells (HSC). Direct contact between the microenvironment and hemopoietic cells is required for normal hemopoiesis [6,12]. Membrane contacts are required for the transfer of regulatory information and essential substances, migration and homing of precursor cells in specific sites of the hemopoietic tissue, and transport of hemopoietic growth factors in a biologically available form [7,16].

Hypoxia activates the processes of hemopoiesis and differentiation of hemopoietic cells [3,10]. However, little is known about the regulatory role of reduced oxygen content in BM in the cell-cell interaction during hemopoiesis. Here we studied the ability of MSC to maintain hemopoiesis during coculturing of these cells and HSC from human umbilical blood under conditions of normoxia (20% O_2) and reduced oxygen content (5% O_2).

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MATERIALS AND METHODS

Isolation and culturing of MSC. Experiments were performed on human BM MSC. The isolation and major manipulations with these cells were described elsewhere [2]. DMEM medium containing 1 g/liter glucose (Biolot), 10% fetal bovine serum (FBS, HyClone, Defined), 1 mM sodium pyruvate, 2 mM L-glutamine, 25 mM HEPES (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (Biolot) was used as a culture medium. The cells were reinocu-

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lated after attaining subconfluent density. The cultures were washed with Hanks balanced salt solution to remove the culture medium. They were incubated in a mixture of 0.05% trypsin and 0.02% EDTA (Gibco) at 37°C for 1-2 min. The effect of the enzyme was neutralized by adding the culture medium with 10% FBS after cell detachment form the sublayer. The cell suspension was centrifuged at 400*g* for 10 min. The pellet was resuspended in a freshly prepared culture medium and put in flasks (25 and 75 cm^2, Nunc) at a density of 5000 cells/cm^2.

Cell culturing under conditions of hypoxia. The conditions of prolonged hypoxia (5% O_2, 5% CO_2, and 90% N_2) were produced in a MSO-175M multigas incubator (Sanyo). Short-term experiments were performed in a hermetic incubation chamber (Stem Cell Technologies). The chamber was inflated with a gas mixture (95% N_2 and 5% CO_2) and maintained in a thermostat at 37°C. Oxygen content and pressure of the gas medium in the chamber were monitored by internal sensors.

Studying the expression of surface markers for MSC. Comparative phenotypic analysis of cells was performed on a FACS Calibur (Becton Dickinson) or Epics XL (Beckman Coulter) flow cytometer using fluorochromic (FITC or phycoerythrin) conjugates of monoclonal antibodies to the following surface markers: CD13, CD29, CD34, CD36, CD44, CD45, CD54. CD71, CD73, CD90, CD105, CD106, CD146, CD117, HLA-ABC, and HLA-DR (Becton Dickinson). The cultures were washed in Dulbecco's phosphate buffered saline (DPBS) and removed with trypsin-EDTA (similarly to reinoculation). The cells were pelleted by centrifugation at 400*g* for 5 min, resuspended in DPBS with 0.1% bovine serum albumin (DPBS-BSA), and divided into aliquots (according to the number of markers, 100,000 cells per sample). After repeated centrifugation, the excess amount of DPBS-BSA was removed to achieve a final suspension volume of 100 μl. Incubation with antibodies to study antigens was performed on ice in darkness for 30 min. The cell pellet was washed by centrifugation in DPBS-BSA, resuspended in a flow fluid (FACSFlow, Becton Dickinson), and analyzed (10,000 events per sample). The degree of nonspecific binding was estimated with the corresponding isotypic standards.

Study of cytokine production. Qualitative study of cytokine production by cultured cells was performed with samples of the culture medium frozen at -30°C. Culture media with the same content of serum were used as the control. Samples of the conditioned and control media were defrosted and clarified by centrifugation at 1000*g* for 10 min immediately before the study. Cytokine titers in culture medium samples were measured by the standard (Immunoassay Kit, BioSource Int., Inc.) and fluorometric enzyme immunoassay (FlowCytomix human Th1/Th2 11 plex, Bender MedSystems Inc.) according to manufacturer's recommendations. In the first case, cytokine concentration was estimated from standard calibration dilutions. In the second case, the concentrations of 11 cytokines were measured simultaneously by means of flow cytometry (FACS Calibur) with the corresponding software.

Coculturing of MSC and HSC. For MSC and HSC coculturing, cryopreserved samples of umbilical blood cells isolated by the method of sedimentation were used [4]. The cells were obtained from the Bank of Stem Cells (KrioTsentr, Moscow). Before initialization of cultures, umbilical blood cells were defrosted in a water bath at 37°C and washed with physiological saline containing 2.5% human serum albumin (Baxter) and 10% rheopolyglucin to remove the cryoprotecting agent. Nucleated cells were pelleted by centrifugation and resuspended in the culture medium. The concentration was brought to 10^6 cells/ml. MSC were cultured until monolayer formation. The suspension of umbilical blood cells was added to flasks during medium replacement. The medium with nonadherent cells was removed and replaced with a freshly prepared medium on days 2-3 of culturing. In the follow-up period, this medium was replaced at 2-3-day intervals. The formation of hemopoietic islets was estimated visually by phase contrast microscopy or after staining of fixed cultures with neutral red.

Detection of colony-forming units (CFU). In the standard test for colony formation, umbilical blood cells were cocultured with MSC and incubated in a commercial semisolid medium from methylcellulose and growth factors (MethoCult, StemCell Technologies Inc.). The number of colonies was evaluated by phase contrast microscopy. Umbilical blood cells that were not cultured with MSC served as a control.

The results were analyzed by MS Excel software.

RESULTS

Culturing of MSC under conditions of normoxia and hypoxia was accompanied by the formation of adhesive cell monolayer with fibroblast-like morphology and small number of processes.

Flow cytometry was performed to evaluate the immunophenotype of MSC after culturing under normoxic conditions. The cells were not stained for HSC markers (CD34, CD45, and CD117), but expressed a considerable number of specific antigens

TABLE 1. Comparative Study of the Phenotype of Passage 2 MSC during Culturing under Conditions of Normoxia and Hypoxia*

CD, %	O_2	
	20%	5%
CD13	96	96
CD29	100	100
CD34	0	0
CD44	100	100
CD45	0	0
CD36	5	5
CD54	40	44
CD90	100	100
CD71	31	28
CD73	100	100
CD146	16	17
CD117	0	0
HLA-I	100	99
CD106	45	37

Note. Normoxia, 20% O_2; hypoxia, 5% O_2. *Exposure for 24 h.

(CD13, CD29, CD44, CD73, CD90, CD105, and HLA-I; Table 1). Antigen expression was similar in cultures exposed to hypoxia for 24 and 48 h (Tables 1 and 2). The ratio of CD106-expressing cells (VCAM-1) decreased after culturing of MSC under conditions of reduced oxygen content (Tables 1 and 3).

The majority of parameters for surface antigen expression in MSC remained practically unchanged after coculturing with HSC. The exceptions were adhesion molecules ICAM-1 (CD54) and VCAM-1 (CD106). Table 3 shows that the ratio of cells expressing ICAM-1 and VCAM-1 increases after coculturing of MSC and HSC under conditions of normoxia (from 32 to 46%; and from 56 to 77%, respectively) and hypoxia (from 26 to 54%; and from 45 to 53%, respectively). HLA-I is a marker of major histocompatibility complex, which plays an important role in autoimmune processes. HLA-I expression on the surface of MSC was high (75%) during culturing at various concentrations of O_2, but significantly decreased after coculturing with HSC (to 14 and 31% under conditions of normoxia and hypoxia, respectively).

We studied the production of cytokines interleukin-12p70 (IL-12p70), interferon-γ (IFN-γ), IL-2, IL-10, IL-8, IL-6, IL-4, IL-5, IL-1β, tumor necrosis factor-α (TNF-α), and TNF-β. IL-6 at high concentration (400 pg/ml) was found in conditioned media of MSC cultures after exposure to normoxia and hypoxia for 7 or 14 days (Fig. 1). Conditioned media of hypoxic MSC were characterized by low production of IL-8 (10 pg/ml). They contained trace amounts of IL-2, IL-5, IL-1β, TNF-α, and TNF-β. Coculturing of MSC and HSC under conditions of normoxia and hypoxia was accompanied by a significant increase in the production of IL-6 and IL-8 (by tens of times). These cytokines were not detected in the media conditioned by HSC.

The production of vascular endothelial growth factor (VEGF) was studied by means of solid-phase enzyme immunoassay after incubation of MSC under conditions of normoxia or modified oxygen content for 7 and 14 days. Cytokine concentration was compared in samples of incubation media. VEGF production after culturing under hypoxic conditions was higher compared to that observed during normoxia (Fig. 2). Coculturing of MSC and HSC for 7 days was not accompanied by an increase in the production of VEGF. However, VEGF production was shown to increase by 1.5 times after 14-day coculturing under hypoxic conditions. VEGF was not detected in conditioned media of HSC after culturing under conditions of normoxia and reduced oxygen content.

Coculturing of MSC and HSC was accompanied by the formation of hemopoietic islets. They consisted of leukocyte-like cells and erythroid islets with several rings of erythroblasts at various stages of differentiation (Fig. 3). Larger and more numerous hemopoietic islets were formed after co-

TABLE 2. Comparative Study of MSC Phenotype at Various Passages under Conditions of Normoxia and Hypoxia*

CD, %	Passage					
	4th		5th		6th	
	N	H	N	H	N	H
90	81	93	99	98	96	96
54	57	46	10	10	17	19
HLA-I	99	100	97	96	96	94
105	100	100	96	95	93	96

Note. N, normoxia (20% O_2); H, hypoxia (5% O_2). *Exposure for 24 h.

culturing of cells under hypoxic conditions. In some samples, the layer of MSC was completely covered by these islets on day 14 of the study.

After coculturing with MSC for 7 or 14 days, HSC cultured in a semisolid medium under normoxic and hypoxic conditions formed numerous colonies (as differentiated from the cells cultured on a plastic surface of culture flasks, Fig. 4). Hypoxia had little effect on the formation of cell colonies.

Adhesion properties of microenvironmental cells contribute to the regulation of functional activity of hemopoietic precursor cells. The group of membrane proteins (various classes of cell adhesion molecules; and class I and II histocompatibility antigens) plays a major role in the association of hemopoietic and stromal cells [8,11,17]. MSC constitute the stroma of BM and are involved in the formation of the hemopoietic microenvironment [12]. Therefore, studying the immunophenotype of MSC is of particular importance.

The major immunophenotypic characteristics of MSC remained unchanged after culturing under conditions of reduced oxygen content (5%). However, coculturing was accompanied by a significant increase in the expression of cell adhesion molecules (*e.g.*, CD54 and CD106). Our findings show a close relationship between the mechanisms that mediate the regulatory effect of these systems.

Apart from intercellular and cell-matrix adhesion molecules, the cell-cell interaction is provided by various mediators that mainly produce a local short-distance effect. Cytokines and growth factors have a major role in the regulation of cell-cell interactions. Cytokine production by MSC was studied during culturing under various conditions. We showed that the secretion of hemopoietins IL-6 and IL-8 increases by tens of times during coculturing of various cells. Previous studies revealed that IL-6 stimulates proliferation of T lymphocytes and erythroid precursors and activates hemopoiesis. IL-8 is a major activator of cell adhesion molecules, which contributes to the interaction of neutrophils with endothelial cells and subendothelial matrix proteins. These cytokines belong to a group of "early" hemopoietins. Hemopoietins alone or in combination with other factors have a stimulatory effect on proliferation and differentiation of early hemopoietic precursor cells or induce division of G_0-phase HSC. The simultaneous production of

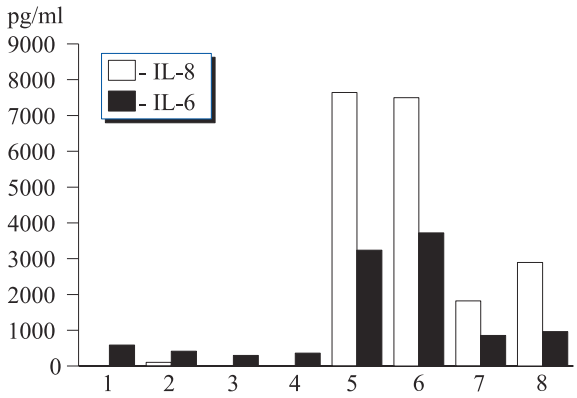

Fig. 1. Production of IL-6 and IL-8 by MSC under conditions of normoxia or hypoxia and after coculturing with HSC. Here and in Fig. 2: 7 days, normoxia (*1*); 7 days, hypoxia (*2*); 14 days, normoxia (*3*); 14 days, hypoxia (*4*); 7 days, coculturing during normoxia (*5*); 7 days, coculturing during hypoxia (*6*); 14 days, coculturing during normoxia (*7*); 14 days, coculturing during hypoxia (*8*).

TABLE 3. Comparative Study of MSC Phenotype after Culturing under Conditions of Normoxia or Hypoxia and Coculturing with Human Umbilical Blood HSC*

CD, %	Normoxia	Hypoxia	MSC+HSC	
			normoxia	hypoxia
CD13	99	98	99	99
CD29	98	96	97	92
CD34	1	1	1	0
CD44	99	99	97	99
CD54	32	26	46	54
CD73	98	91	98	99
CD90	98	98	97	97
CD106	56	45	77	53
CD117	1	1	0	1
HLA-I	75	62	14	31
HLA-II	10	6	7	8

Note. *Exposure for 14 days.

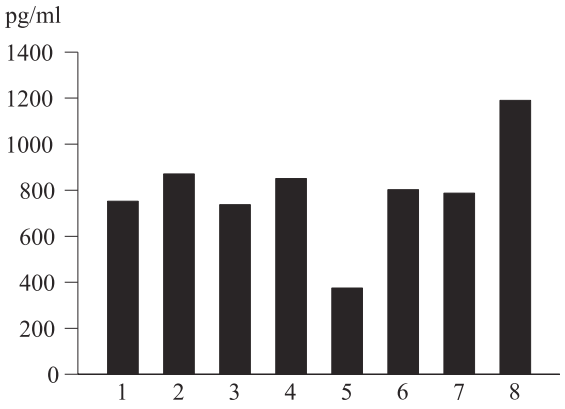

Fig. 2. Production of VEGF by MSC under conditions of normoxia or hypoxia and after coculturing with HSC.

cytokines by BM cells probably contributes to the maintenance and differentiation of MSC in the culture. These changes are associated with an increase in the secretion of hemopoiesis-stimulating agents. Secretory activity under hypoxic conditions was higher than during normoxia. Direct or indirect stimulation (via the interaction with adherent cells) of hemopoietic precursor cells depends on functional activity, but not on the number of microenvironmental cells. The function of these cells can be modified and varies in a wide range.

The observed differences are probably related to activation of fine regulatory mechanisms for intracellular oxygen homeostasis. They play a major role in the induction of the cellular systemic response. Published data show that cell hypoxia induces a mosaic adaptive reaction, which is mediated by transcription factor HIF-1α (hypoxia-inducible factor-1α) [5,9,14,15]. HIF-1α expression is associated with the intracellular oxygen state and has a modulatory effect on activity of several genes. The expression of these genes is essential for the maintenance of cellular and tissue homeostasis, anaerobic glycolysis, induction of erythropoiesis, and increase in blood flow (vasodilation and angiogenesis) [1,13]. Much attention was paid to this problem. However, the molecular mechanisms of HIF-1α activation, target genes for this factor, and HIF-1α binding sites in genomic sequences remain unknown.

We conclude that MSC play an important role in the hemopoietic microenvironment. This function of MSC is provided by a direct cell-cell interaction with hemopoietic cells and production of hemopoietic growth factors (particularly, under conditions of reduced oxygen content).

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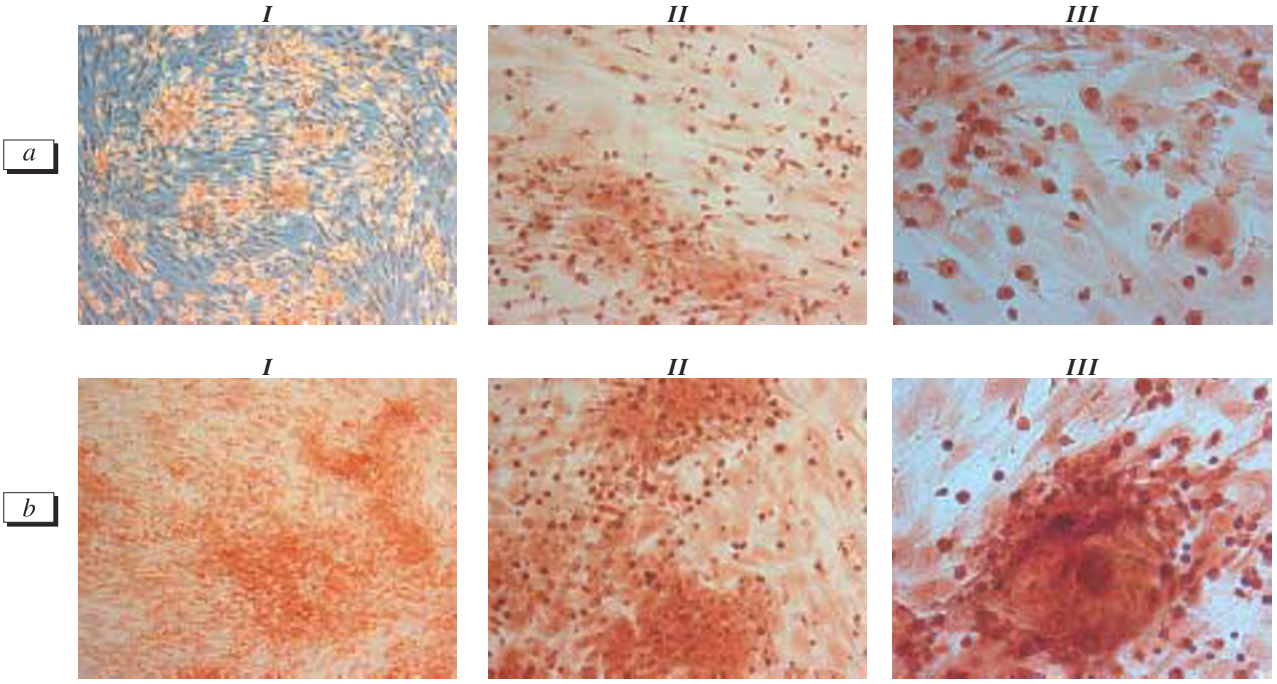

Fig. 3. Coculturing of MSC and HSC under conditions of normoxia (*a*) and hypoxia (*b*). Exposure for 14 days. Neutral red staining. *I*, ×100; *II*, ×200; *III*, ×400.

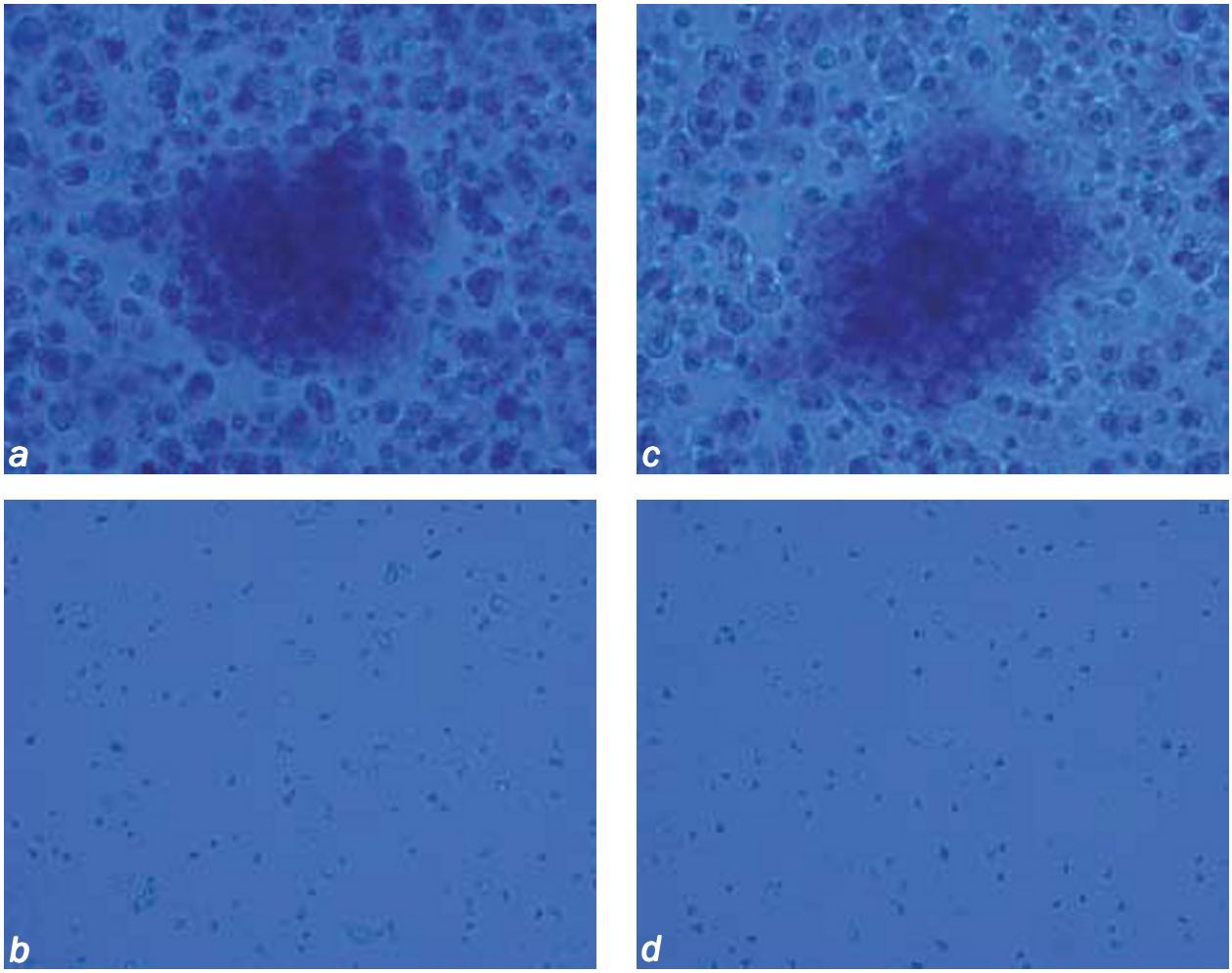

Fig. 4. Formation of HSC colonies in a semisolid medium under conditions of normoxia and hypoxia (*400). HSC+MSC, normoxia (*a*); HSC non-cocultured with MSC, normoxia (*b*); HSC+MSC, hypoxia (*c*); HSC non-cocultured with MSC, hypoxia (*d*). Exposure for 14 days. Methylene blue staining (May-Grunwald method).

REFERENCES

1. S. P. Osinskii, L. D. Gumenyuk, D. S. Osinskii, and S. P. Merentsev, *Onkologiya*, **8**, No. 1, 33-37 (2006).
2. Yu. A. Romanov, A. N. Darevskaya, N. V. Merzlikina, and L. B. Buravkova, *Kletochn. Tekhnol. Biol. Med.*, No. 3, 158-163 (2005).
3. D. C. Chow, L. A. Wenning, W. M. Miller, and E. T. Papoutsakis, *Biophys. J.*, **81**, No. 2, 675-684 (2001).
4. P. Denning-Kendall, C. Donaldson, A. Nicol, *et al.*, *Exp. Hematol.*, **24**, No. 12, 1394-1401 (1996).
5. E. A. Griffiths, S. A. Pritchard, I. M. Welch, *et al.*, *Eur. J. Cancer*, **41**, No. 18, 2792-2805 (2005).
6. C. L. Hardy and J. J. Minguell, *Exp. Hematol.*, **21**, No. 2, 283-288 (1993).
7. C. L. Hardy and J. J. Minguell, *Scanning Microsc.*, **7**, No. 1, 333-341 (1993).
8. M. Kobayashi, M. Imamura, T. Uede, *et al.*, *Stem Cells*, **12**, No. 3, 316-321 (1994).
9. T. Kurokawa, M. Miyamoto, K. Kato, *et al.*, *Clin. Cancer Res.*, **11**, 1583-1590 (2005).
10. J. A. Latiuppa, E. T. Papoutsakis, and W. M. Miller, *Exp. Hematol.*, **26**, No. 9, 835-843 (1998).
11. J. P. Levesque, D. I. Leavesley, S. Niutta, *et al.*, *Exp. Med.*, **181**, No. 5, 1805-1815 (1995).
12. M. K. Majumdar, M. Keane-Moore, D. Buyaner, *et al.*, *J. Biomed. Sci.*, **10**, No. 2, 228-241 (2003).
13. K. Nakanishi, S. Hiroi, S. Tominaga, *et al.*, *Clin. Cancer Res.*, **11**, 1583-1590 (2005).
14. D. L. Ramirez-Bergeron and M. C. Simon, *Stem Cells*, **19**, No. 4, 279-286 (2001).
15. G. L. Semenza, *Crit. Rev. Biochem. Mol. Biol.*, **35**, 71-203 (2000).
16. P. J. Simmons, A. Zannettino, S. Gronthos, and D. Leavesley, *Leuk. Lymphoma*, **12**, No. 5-6, 353-363 (1994).
17. J. G. Wilson, *Acta Haematol.*, **97**, No. 1-2, 6-12 (1997).