Effect of Hypoxia on Stromal Precursors from Rat Bone Marrow at the Early Stage of Culturing

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 143, No. 4, pp. 386-389, April, 2007
Original article submitted December 6, 2006

We studied the effect of hypoxia on stromal precursors from rat bone marrow. Under hypoxic conditions the cultures were characterized by low heterogeneity, the percent of large spread morphologically different cells decreased. Hypoxia increased proliferative activity of cells, had no adverse effect, and decreased the percent of cells expressing hemopoietic markers.

Key Words: *stromal precursors; mesenchymal stem cells; hypoxia*

Bone marrow stromal cells, or mesenchymal stem cells (MSC), are residents of the bone marrow (BM) stroma. This small cell population is characterized by high proliferative activity, self maintenance in an undifferentiated state, and ability to differentiate into various cell types under the effect of certain stimuli [2,3,5,11,12]. The ability of MSC to differentiate at least into mesenchymal cells underlies their reparative properties.

Hypoxia plays a key role in various pathophysiological processes and tissue reparation. Reduced O_2 concentration affects MSC viability and proliferative activity, which modulates reparative processes in the tissue. Experiments on MSC culturing under hypoxic conditions showed the necessity of studying various aspects of MSC function in the presence of low O_2 concentration [7,10].

Here we studied the effect of normobaric hypoxia (5% O_2) on proliferation, viability, and phenotypic characteristics of MSC from rat BM at the early stage of culturing.

MATERIALS AND METHODS

We used αMEM (Biolot) containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum (Gibco), culture flasks and Petri dishes (Nunc), phosphate buffered saline (20 mM, Gibco) for cell washing; 0.02% trypsin with 0.05% EDTA (Gibco) for cell harvesting.

Immunophenotyping of MSC was performed on an Epics XL cytofluorometer (Beckman Coulter) using monoclonal antibodies (BD Bioscience) against CD90, CD44, CD54, CD73, CD45, and CD11b markers according to manufacturer's instructions.

Cell viability was estimated by the method of flow cytofluorometry using AnnexinV-FITC Kit (Immunotech). Morphological characteristics of MSC were evaluated under an Axiovert 25 phase contrast microscope (Zeiss) connected to a digital camera. Proliferative activity of MSC cultures was determined by cell counting in randomly selected fields of view (1 mm^2) before and after 4-day culturing under normoxic or hypoxic conditions.

Six independent experiments were performed (5-15 fields of view in each experiment).

The significance of differences was estimated by nonparametric Mann—Whitney test.

Hypoxia of the culture medium was induced in a sealed chamber (Stem Cell Technologies) by delivering a gas mixture of 95% N_2 and 5% CO_2 until O_2 concentration in the medium was brought to 5%. O_2 content and gas pressure were monitored using chamber sensors. Control cells were maintained in a CO_2 incubator under standard normoxic conditions (5% CO_2).

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0007-4888/07/1434-0411

TABLE 1. Effect of Hypoxia on MSC Viability (*M*±*m*)

Experimental conditions	Cell count, %		Total percent of damaged cells*
	apoptotic (AnnV+)	necrotic (PI)	
Normoxia	9.7±1.2	3.6±1.2	13.4±0.8
Hypoxia	4.5±1.3	1.3±0.3	5.8±1.3

Note. *Means for 3 cultures. Here and in Table 2: for measuring each parameter 5000-10,000 cells were analyzed.

The study was conducted with passages 1-4 of MSC cultures isolated from diaphyses of thigh bones and shin bones of young outbred rats [9].

RESULTS

Primary cultures of adherent precursors from rat BM consisted of cells of the same type and actively proliferate. However, after subculturing the population of MSC became heterogeneous and contained several types of cells differing in morphological characteristics and proliferative activity [1,6,9]. The culture of MSC included the following cells: actively proliferating spindle-shaped or triangular cells with homogeneous cytoplasm; actively proliferating and spread fibroblast-like cells with large nucleus; and slowly proliferating and highly spread cells that had a polygonal, oval, or irregular shape and heterogeneous cytoplasm. Proliferative activity in most cultures decreased by the 4th passage, slowly dividing and spread cells predominated. Further subculturing abolished this so-called "proliferative inhibition", proliferative activity increased and remained at a high level, which led to predominance of morphologically similar cells in the culture. Experiments were performed on MSC cultures of early passages (up to the 4th passage), *i.e.* before attaining proliferative inhibition.

Morphological characteristics of MSC were studied during culturing under various conditions. Hypoxia stimulated the formation of islets from small cells with optically homogeneous cytoplasm. These cells were in close contact with each other and formed colonies. The number and size of colonies were higher under hypoxic conditions (Fig. 1). Hypoxia was also accompanied by a decrease in the ratio of large (60-200 μ), spread, and slowly dividing cells with different size and various morphological characteristics. Hence, hypoxia decreased heterogeneity of MSC cultures and increased the percent of small cells (20-60 μ) of a similar type. It can be hypothesized that low proliferative activity of large spread cells in the culture reflects the severity of damage under conditions of oxidative stress during normoxic culturing (21% O_2). The decrease in O_2 concentration probably reduces the severity of oxidative stress, which affects the ratio of large spread cells in the culture ("mature" MSC) [9]. The inhibition of proliferation and morphological characteristics of these cells reflect the severity of damage during oxidative stress.

Hypoxia significantly increased proliferative activity of precursor cells, which is consistent with published data [4,7,10]. The number of cells increased by 7 and 2.1 times during culturing under hypoxic and normoxic conditions, respectively. Cell count in normoxic cultures increased to a different degree, which probably reflects individual characteristics of animals used as BM donors, specific features of culturing stages, and nonspecificity of the method of MSC isolation (impossibility to obtain the standard population of cells during each sampling procedure). Activation of MSC proliferation during hypoxia probably results from the induction of some kinase cascades (*e.g.*, SAP-kinase signal pathways JNK and p38), which are usually activated in response to stress and stimulate cell proliferation [13].

The percent of apoptotic and necrotic cells in hypoxic cultures was 2-fold lower than in normoxic cultures (Table 1), *i.e.* the stimulating effect of hypoxia on MSC proliferation at the early stage of

TABLE 2. Effect of Hypoxia on Expression of Surface Markers in MSC (%, *M*±*m*)

Experimental conditions	CD90	CD45	CD54	CD44	CD73	CD11b
Normoxia	100	0.11	98.3	99.7	88.2	2.37
Hypoxia	100	0.02	98.7	99.4	81.9	0.79

Note. Data for a second-passage culture.

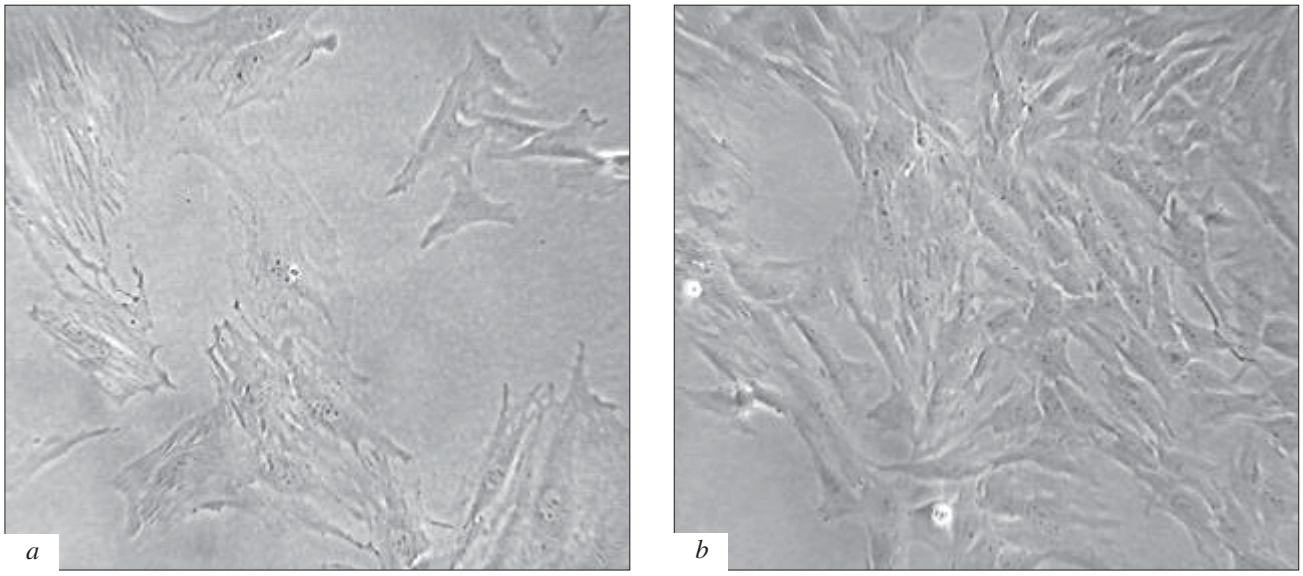

Fig. 1. Morphology of MSC after 4-day culturing under normoxic (*a*) and hypoxic (*b*) conditions.

culturing is associated with the absence of its damaging effect on cells. BM is an *in vivo* natural medium for MSC. O_2 concentration in BM does not exceed 5%, which can explain the cytoprotective role of hypoxia at the early stage of culturing (*i.e.* before the appearance of a stable population of proliferating cells resistant to novel conditions). On the other hand, hypoxia can mediate the formation of some antiapoptotic proteins [8].

Immunophenotyping of cultures under normoxic and hypoxic conditions showed that hypoxia had little effect on the expression of surface markers typical of MSC, but decreased the number of cells expressing hemopoietic markers (which are present in small amounts), thus stimulating the formation of immunophenotypically homogeneous population of precursor cells (Table 2).

Our results show that 4-day culturing at 5% O_2 has a stimulatory effect on MSC from rat BM. This effect is observed at the early stage of culturing and manifested in changed ratio of morphologically different cells in the culture, stimulation of cell proliferation, absence of damage and/or development of the protective effect on cells, and decrease in the ratio of cells expressing hemopoietic markers.

This work was supported by the Program of the Department of Biological Sciences (Russian Academy of Sciences) and Federal Agency of Science and Innovations (contract No. 02.467.11.3005).

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