Resistance of Rat Bone Marrow Mesenchymal Stromal Precursor Cells to Anoxia *In Vitro*

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We studied the effect of significantly reduced oxygen content (0% O_2 in gaseous phase) in culture medium on rat bone marrow mesenchymal stromal precursor cells. The cells retained their morphology and expression of characteristic markers during several days under these conditions; they were viable, proliferated, and some cultured cells were even capable of realizing the initial stages of osteogenic and adipogenic differentiation. Further culturing of precursor cells under conditions of anoxia led to activation of apoptosis in cultures and to progressive necrosis.

Key Words: *mesenchymal stromal precursor cells; hypoxia; anoxia*

We previously showed that 96-h hypoxia (5% O_2) *in vitro* stimulated rat bone marrow (BM) mesenchymal stromal precursor cells (MSC), which manifested in reduction of heterogeneity of cells in these cultures, stimulation of their proliferative activity, reduction of the percentage of damaged cells in culture; expression of markers specific of MSC remains unchanged under these conditions [1]. These data are in line with the results of other authors, demonstrating the possibility of stimulating the proliferation of undifferentiated cells under conditions of hypoxia [5,8,12,13]. One of the causes of this phenomenon can be reduced intensity of oxidative stress due to culturing of cells in medium more natural for MSC, residents of BM, where oxygen content did not exceed 5%. Hence, culturing under these conditions can be considered as approximation of normal conditions, but not a hypoxic exposure.

Now we studied the effect of a significant reduction of oxygen concentration (0% O_2 in gaseous phase) on rat BM MSC cultures.

MATERIALS AND METHODS

The following reagents were used in the study: α-MEM (ICN) with 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco), and 10% FCS (HyClone) for cell culturing; 20 mM phosphate buffer (Gibco) for cell washing; 0.02% trypsin with 0.05% EDTA (Gibco) for trypsinization; 4% paraformaldehyde (Merck) for cell fixation; 10^{-9} M dexamethasone (Sigma), 10 mM glycerol-2-phosphate (Sigma), and 0.2 mM 2-phospho-L-ascorbic acid (Fluka) for induction of osteogenic differentiation; 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine (Sigma), 10 μg/ml insulin (NovoNordisk) for induction of adipogenic differentiation of cells. The MSC immunophenotype was evaluated by flow cytofluorometry on an Epics XL cytofluorometer (Beckman Coulter) using monoclonal antibodies (BD Bioscience) to the studied markers. Cell viability was evaluated using AnnexinV-FITC Kit (Immunotech). The morphological characteristics of MSC were studied in an Axiovert 25 phase contrast microscope with image analysis system (Zeiss); cell proliferation was evaluated by counting cells by means of Sigma ScanPro 5 software in fixed visual fields chosen at random (8-30) within an area of 1 mm^2. The initial stages of osteogenic differentiation were analyzed by activity of alkaline phosphatase detected by Alkaline Phosphatase Kit (Sigma-Aldrich). Adipogenic differentiation was evaluated by accumulation of lipid droplets (detected with Oil Red stain, Sigma) in the cytoplasm. The studies were carried out on MSC culture passages 1-4. The cells were isolated

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from the femoral diaphyses of young outbred rats as described previously [4].

Low oxygen level in culture medium was created in a hermetic chamber (Stem Cell Technologies) blown through with the gas mixture (95% N_2, 5% CO_2) to O_2 concentration of 0%, recorded by the pickups in the chamber. It was assumed that oxygen concentrations in gaseous and liquid phases were leveled after several hours of incubation [2]. The chamber was placed into a thermostat for cell incubation at 37°C. Control cells were incubated under standard normoxia conditions in a CO_2 incubator (5% CO_2) at 37°C.

The significance of differences was evaluated using nonparametric Mann—Whitney's test and Student's *t* test.

RESULTS

Anoxia virtually did not influence the morphology of MSC cultures. The percentage of cells carrying CD90, CD54, CD44, CD29 (more than 95%), CD45, CD11b (less than 0.6%) molecules, and the type of expression of these molecules were the same in cultures incubated in normoxia and anoxia. A slight reduction of the percentage of positive cells under conditions of anoxia was observed only for CD73 marker (72% in normoxia and 67% in anoxia).

Culturing of MSC under conditions of anoxia for 96 h led to a negligible or zero stimulation of cell proliferation, which increased just 1.2 times on average in comparison with the cultures incubated under conditions of normoxia (Fig. 1). The absence of cell proliferation inhibition or its deceleration in anoxia disagree with the results of experiments demonstrating cell cycle arrest under conditions of anoxia [3,6,7]. For some cells, proliferation inhibition in media with low oxygen content is explained by mechanisms of inactivation of the enzymes maintaining the production of nucleic acids and subsequent inhibition of DNA replication. Moreover, it was shown to take place only under conditions of stringent reduction of oxygen level (0.01%) or anoxia, but not at oxygen concentrations of 0.1-1% [7]. Cell proliferation arrest due to blockade of replication during anoxia was also confirmed by a drop of bromodeoxyuridine incorporation into cells [6]. It was reported that NHIK 3025 cells were capable of passing into the cell cycle S stage at a very low oxygen concentration in the medium (0.01-0.13%), but the completion of DNA synthesis under these conditions was impossible [3].

The division of rat BM MSC under conditions of anoxia created in the gaseous phase seems to indicate their special resistance to very low oxygen concentrations, because trace oxygen remains in the pericellular layer of the medium after it is forced out from the fluid.

Evaluation of viability of MSC cultured under conditions of normoxia and anoxia for 96 h showed a trend opposite to the previously revealed protective antiapoptotic effect of 5% oxygen content [1]. Despite virtually the same summary count of damaged cells in normoxia and anoxia, the content of cells starting apoptosis in normoxic cultures was significantly (2-fold) lower than in anoxic cultures. This indicated activation of apoptosis processes at the initial stages of exposure to anoxia (Fig. 2). Presumably, association of anoxia with apoptosis is justified, because significant reduction of partial oxygen pressure increases the incidence of point mutations, and accumulation of point mutations under these conditions can be prevented by apoptotic cell death [9].

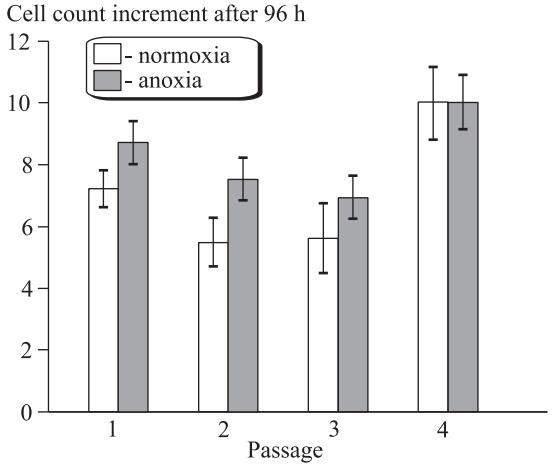

Fig. 1. Proliferation of rat BM MSC cultured over 96 h under conditions of normoxia and anoxia (mean multiplicity of cell count increment in randomly selected fixed visual fields; summary data of 7 experiments).

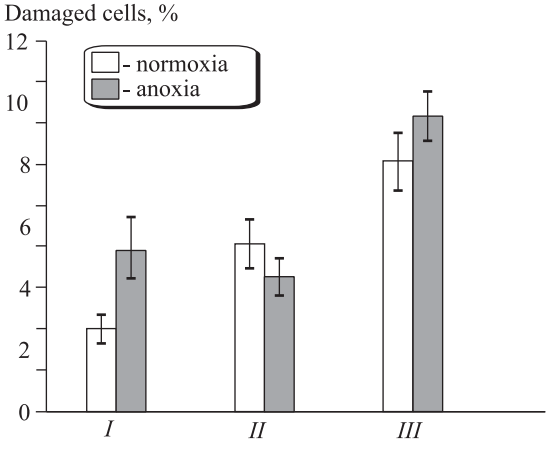

Fig. 2. Viability of rat BM MSC cultured for 96 h under conditions of normoxia and anoxia: mean percentage of apoptotic (*I*), necrotic (*II*), damaged (*III*) cells in different cultures during passages 1-4. Summary data of 12 measurements are presented; each includes analysis of 5000-10,000 cells.

Apoptosis under conditions of low oxygen content in the medium is caused by different mechanisms. The key role is played by active oxygen forms, JNK kinase, and cytochrome release from the mitochondria, mediated, in turn, by various factors, including p53. Induction of p53 can result from stabilization of HIF-1α protein, the key transcription factor functioning in hypoxia and capable of triggering other mechanisms of apoptosis, not depending on p53 [9].

The trend to antiapoptotic effect of hypoxia and the proapoptotic effect of anoxia are in good agreement with the results of experiments demonstrating that the maximum stabilization of HIF is observed at oxygen level below 5% [10]. Antiapoptotic effect of hypoxia at this oxygen concentration can be realized through signal routes not depending on HIF.

Longer exposure of cells to anoxia led to a significant reduction and loss of their viability (Fig. 3). The main mechanism of cell death in this case was necrosis, though the percentage of apoptotic cells also significantly increased.

Hence, the fate of the cell depends on the intensity and length of hypoxic exposure. These factors determine the interactions between various pro- and antiapoptotic signal pathways, regulating the balance of pro- and antiapoptotic proteins in the cell. Predominance of necrotic over apoptotic processes under conditions of long exposure of cells to anoxia sees to be natural: in contrast to necrosis, programmed cell death requires energy expenditures, and the variant of death depends (among other things) on the energy status of the cell [11].

Evaluation of the osteogenic differentiation potential of the rat BM MSC under conditions of anoxia showed cells with active alkaline phosphatase in the studied cultures, but the percentage of these cells was significantly lower than in normoxic and hypoxic cultures in case of both spontaneous and induced differentiation. Since longer culturing of MSC under conditions of anoxia led to a significant deterioration of their viability, further differentiation of precursor cells in the osteogenic direction became impossible.

Different effects of hypoxia (2% O_2) and anoxia (0.02% O_2) on osteogenic differentiation of human BM MSC and osteoblasts from mouse skull were demonstrated. Cell incubation in anoxia (but not hypoxia) led to inhibition of the expression of Runx2 protein, the key transcription factor in osteogenic differentiation processes. Inhibition of this transcription factor resulted in inhibition of the nodule formation and significant reduction of the formation of mineralized components of the bone matrix [14].

Evaluation of the adipogenic differentiation potential of precursor cells under conditions of anoxia showed that these MSC started differentiation into adipocytes. Fat droplets started accumulating in their cytoplasm, the cells acquired more globular shape and formed clusters, but further exposure under conditions of anoxia led to death of differentiating cells.

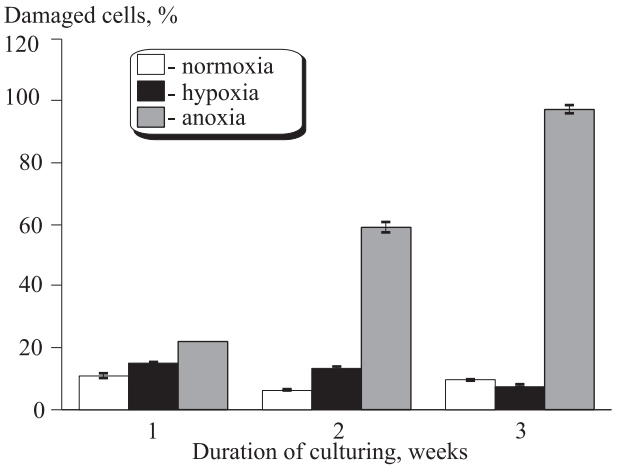

Fig. 3. Viability of rat BM MSC cultured under conditions of normoxia, hypoxia, and anoxia.

Hence, cells cultured during several days in a medium containing no oxygen retained their morphological characteristics, expression of characteristic surface markers, remained viable, and proliferated. The processes of their osteogenic and adipogenic differentiation were not completely blocked, this indicating high resistance of adult precursor cells to oxygen shortage in medium.

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