MORPHOLOGY AND PATHOMORPHOLOGY

Low Level of O_2 Inhibits Commitment of Cultured Mesenchymal Stromal Precursor Cells from the Adipose Tissue in Response to Osteogenic Stimuli

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Mesenchymal stromal precursor cells from human lipoaspirate (lMSC) cultured at 5% O_2 formed 50% less mineralized matrix in response to osteogenic induction than cells cultured under standard conditions (20% O_2). After lMSC percultured at 5% O_2 were transferred to normoxic conditions (20% O_2), they produced the same amount of matrix as lMSC permanently cultured at 20% O_2. Hence, hypoxia inhibited the commitment of lMSC under the effect of osteogenic stimuli, which can be important in reparative and regenerative medicine.

Key Words: *fatty tissue mesenchymal stromal precursor cells; low oxygen content; osteogenic differentiation*

Mesenchymal stromal precursor cells (MSC), for example, originating from the adipose tissue (lipoaspirate, lMSC), are now regarded as a source of cell material for reparative and regenerative medicine [6]. These cells actively proliferate *in vitro* and are capable of osteogenic, adipogenic, chondrogenic, and, presumably, angiogenic differentiation [7,10,14]. The development of approaches to optimization of conditions for *in vitro* production of sufficient amount of lMSC for clinical use and the possibility of regulating the direction and rate of these cells differentiation are important for their effective clinical use.

It was previously shown that culturing of MSC isolated from different tissues stimulated their proliferative activity under conditions of hypoxia [1,2,4,8,12], due to which high volumes of cell material with stable characteristics could be obtained within a short period. Information about the differentiation status of the resultant MSC is essential for subsequent biomedical use of these cells.

We studied the effects of permanent culturing under conditions of low O_2 content (5%) on the capacity of human lMSC to differentiation in the osteogenic direction.

MATERIALS AND METHODS

The study was carried out on 5 human lipoaspirate specimens. The cells were isolated as described previously [14]. Directly after isolation the cells were transferred into culture flasks, part of which were cultured under standard conditions (5% CO_2+95% air; 20% O_2), 37°C, 100% humidity (normoxia) and the other part were incubated in a Sanyo multigas incubator at 5% O_2.

Immunophenotyping of the cells was carried out by the standard method on an Epics XL cytofluorom-

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eter (Beckman Coulter) according to manufacturer's instruction using FITC- or phycoerythrin- (PE; Immunoteck) labeled antibodies to CD31, CD34, CD117(c-kit), CD54, CD73, CD90, CD105, HLA-ABC markers. Antibodies (IgG) labeled with FITC or PE of respective classes served as the isotopic control for antibodies.

Osteogenic induction of passage 2 lMSC permanently cultured at 20 and 5% O_2 was carried out as described previously [10]. Differentiation was evaluated semiquantitatively after 3 weeks of induction by the area occupied by mineralized matrix, detected by alizarine red staining (Fig. 1). Analysis was carried out using Adobe Photoshop software on digital images of 10 visual fields (1 mm^2) selected at random. The criteria of evaluation were as follows: *a*: no mineralized matrix; *b*: its area is less than 30%; *c*: 30-60%; and *d*: 60-100% area is occupied by mineralized matrix.

Statistical analysis was carried out using Excel and Sigma Stat 3.5 software.

RESULTS

By morphology and expression of MSC-specific antigens, lMSC isolated in our study and cultured under standard conditions (20% O_2) and at low oxygen content (up to 5% O_2) did not differ from cells described by other authors [3,7,14]. The presence of CD54, CD73, CD90, CD105, CD106, and HLA-ABC antigens and the absence of markers characteristic of hemopoietic precursor cells indicated that the studied cells could be characterized as lMSC.

Normoxic (20% O_2) and hypoxic (5% O_2) lMSC did not form mineralized matrix without osteogenic stimuli in the culture medium. Addition of osteogenic inductors to lMSC culture medium resulted in the formation of the matrix after 21 days (Fig. 1). Mineralized matrix occupied about 80% of total area in lMSC culture grown under conditions of normoxia (20% O_2; Fig. 2). The area occupied by the matrix in lMSC cultures permanently incubated under conditions of

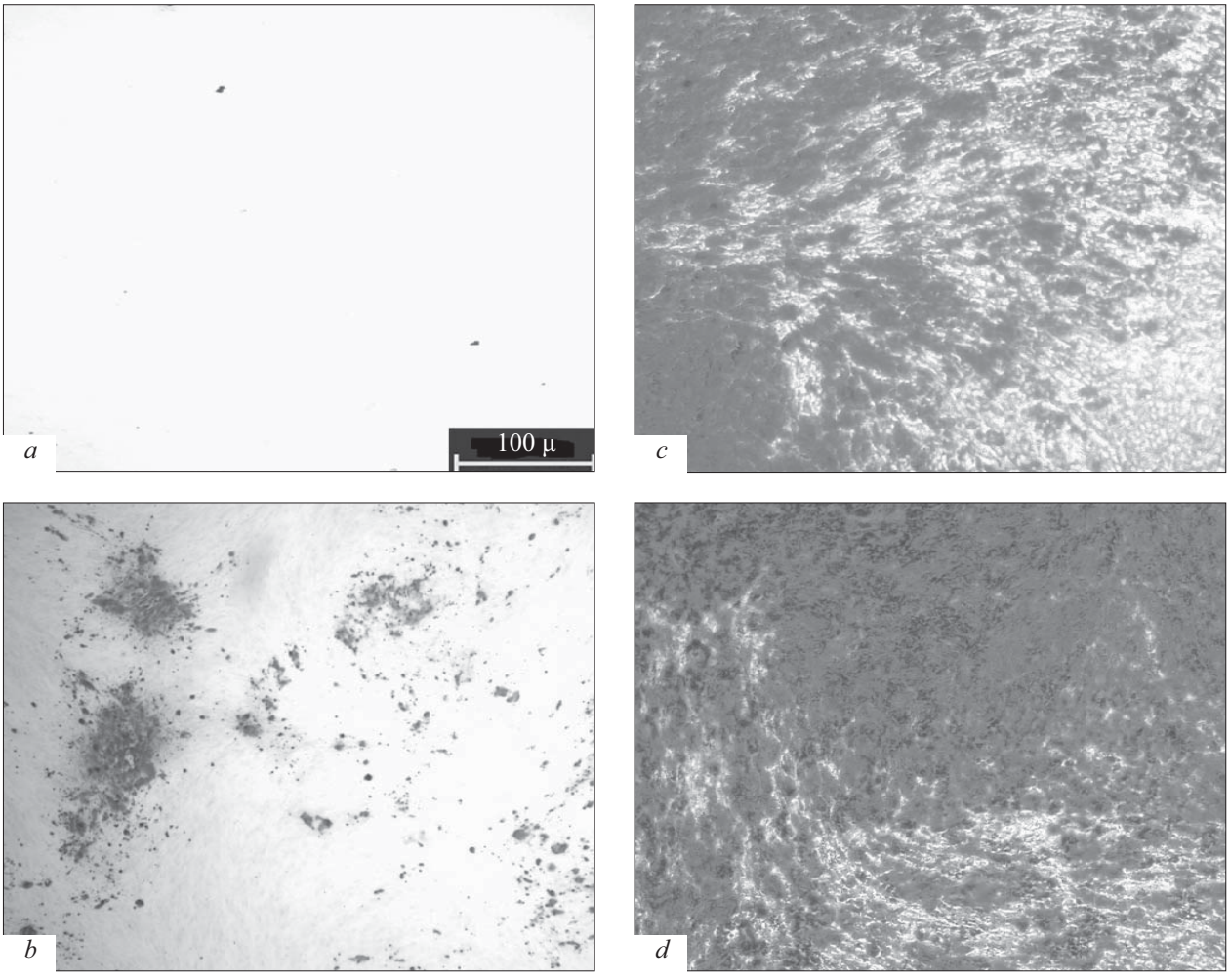

Fig. 1. Mineralized matrix in lMSC cultures after induction of osteogenic differentiation. Alizarine red staining. *a*) no mineralized matrix (0); *b*) less than 30% (1); *c*) 30-60% (2); *d*) 60-100% (3) of the area occupied by mineralized matrix.

hypoxia (5% O_2) was about 30% (Fig. 2). After osteogenic induction of normoxic lMSC transferred into hypoxic conditions, the area of the matrix was also about 30%. Opposite transfer (cells permanently cultured at 5% O_2 transferred into normoxia for the period of differentiation) led to enlargement of the mineralized matrix area to 75%. On the other hand, histochemical study of alkaline phosphatase activity after 7 days of osteogenic induction showed no appreciable differences between normoxic and hypoxic lMSC.

Hence, culturing of lMSC under conditions of hypoxia (5% O_2) decreases their capacity to osteogenic differentiation in comparison with lMSC cultured under standard conditions (20% O_2). Interestingly that transfer of lMSC permanently cultured at 5% O_2 to normoxic conditions (20% O_2) restored their capacity to differentiation, while transfer of lMSC from normoxia to hypoxia reduced their capacity to respond to osteogenic induction. Importantly that reversibility of hypoxic effect on the differentiation potential of human bone marrow MSC was previously demonstrated [8]. Hence, low content of O_2 in lMSC culture medium seems to be a factor inhibiting, but not abolishing the commitment of precursor cells.

The gaseous composition of the culture medium is an important parameter, determining the viability of cultured cells, the concentration of O_2 is one of the most conservative parameters during culturing. For example, oxygen content in this medium virtually always corresponds to O_2 content in the air. This concentration is not physiological, as it is known that oxygen content in lung alveoles is 14%, in arterial blood 12%, in venous blood 5.3%, and in interstitial fluid from 5 to 1%. Hence, the selected level of O_2 (5%) is close to its concentrations in body fluids. Similar concentrations (2-8% O_2) were used in other studies of the MSC differentiation potential in permanent culturing in hypoxic medium. The results of these studies of bone marrow MSC (bmMSC) were contradictory. Some studies demonstrated induction of osteogenic differentiation in bmMSC cultures [5,8], while other experiments detected reduction of the bmMSC osteogenic potential [4,12]. Hence, the results obtained in culture media of similar gaseous composition (O_2 content reduced to 2-8%, close to the described physiological level) with MSC originating from the same source (bone marrow) do not permit us to draw a universal conclusion on the impact of hypoxic culture medium for the direction of bmMSC differentiation vector.

The differentiation potentialities of MSC from human lipoaspirate cultured under conditions of hypoxia were described in only one report [7] demonstrating reduction of differentiation potential of these cells. These data completely coincide with our results. Another study [13] demonstrated reduction of osteogenic potential of mouse lMSC. One of the mechanisms of the detected effects can be the inhibitory effect of hypoxia on the expression of Runx2 transcription factor (one of the key activators of osteogenesis [11]).

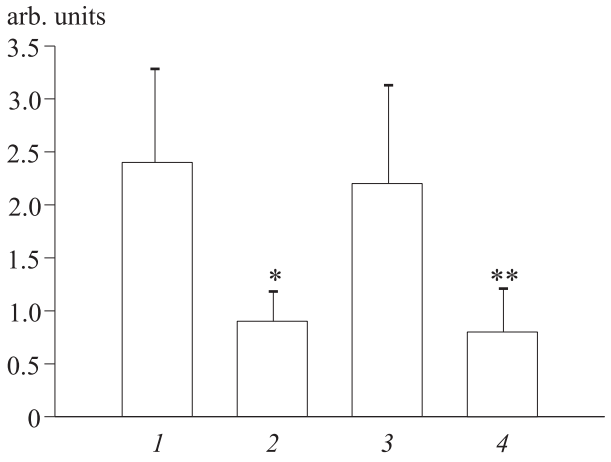

Fig. 2. Area occupied by mineralized matrix in lMSC cultures after osteogenic differentiation induction. *1*) lMSC permanently cultured at 20% O_2; *2*) lMSC permanently cultured at 5% O_2; *3*) lMSC, permanently cultured at 5% O_2, transferred into 20% O_2 conditions with subsequent osteogenic induction; *4*) lMSC, permanently cultured at 20% O_2, transferred into 5% O_2 conditions with subsequent osteogenic induction. $p<0.01$ compared to: **2*, ***3*. Results of 1 of 3 representative experiments are shown.

The findings of the present study are interesting not only as the data on the basic mechanisms of MSC functioning, but can be used in clinical practice. It was shown previously that the use of "hypoxic" protocol of lMSC culturing led to a 2-3-fold increase in the cell material production in comparison with the same duration of MSC culturing at 20% O_2 [1,2,4,8,12]. The present results demonstrate the possibility of maintaining the undifferentiated status of lMSC biomass under conditions of low oxygen content. These data can be important for reparative and regenerative medicine, when, depending on the procedure, cells differentiated in a certain direction (for example, in osteoplasty), or less committed precursor cells, capable of differentiation in different directions in the defect zone (in bone fractures), are needed.

Hence, the study demonstrated that low oxygen level in culture medium can serve as a factor regulating the rate of lMSC commitment, which can be important for further basic and applied studies in cell biology and physiology.

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