Immunosuppressive Effects of Multipotent Mesenchymal Stromal Cells in Cultures with Different O_2 Content in the Medium

A. N. Gornostaeva, E. R. Andreeva, I. V. Andrianova, and L. B. Buravkova

Translated from *Kletochnye Tekhnologii v Biologii i Meditsine*, No. 2, pp. 92-96, April, 2011
Original article submitted December 8, 2010

We studied the effects of multipotent mesenchymal stromal cells isolated from the adipose tissue on proliferation and viability of immunocompetent cells at different concentration of O_2 (5 and 20%) in culture medium. It was shown that co-culturing with multipotent mesenchymal stromal cells 3-fold reduced proliferative index of phytohemagglutinin-activated lymphocytes, while their viability remained unchanged and did not depend on partial oxygen pressure in the medium. These findings suggest that low O_2 concentration in tissues will not affect immunosuppressive properties of multipotent mesenchymal stromal cells, which is very important for their application in regenerative medicine.

Key Words: *multipotent mesenchymal stromal cells; immunocompetent cells; low partial oxygen pressure; co-culturing; proliferative index*

Multipotent mesenchymal stromal cells (MMSC) can be isolated from various adult tissues, including BM and adipose tissue. These cells are very important for clinical practice due to their capacity to differentiate into different cell types and high proliferative and paracrine activities. Immunomodulatory properties of these cells were also reported: inhibition of proliferation and attenuation of lymphocyte activation *in vitro* and suppression of transplant rejection reaction *in vivo* [1,10,14]. MMSC are characterized by low expression of co-stimulatory and HLA-molecules and therefore they are less sensitive to cytotoxic influences than other cell types, which makes possible transplantation of allogeneic MMSC.

MMSC can interact with immune system cells at high (systemic circulation) and low (target tissues) pO_2. The interaction of MMSC and lymphocytes *in vitro* is usually studied at standard O_2 (20%); however, it is known that O_2 content in tissues varies from 1 to 8%. Low O_2 content in culture medium can modify various characteristics of MMSC-precursors (proliferation, differentiation, viability) [2,6,9]. In light of this, it was interesting to find out whether the suppressive effect of MMSC depends on pO_2.

Here we studied the effect of MMSC on proliferation and viability of immunocompetent cells *in vitro* at different O_2 concentration in culture medium.

MATERIALS AND METHODS

MMSC were isolated from human adipose tissue by a routine method [19] with some modifications [2]. The isolated cells were transferred to Petri dishes and cultured in α-MEM either under standard conditions with 5% CO_2+95% air (20% O_2), 37°C, 100% humidity (N-MMSC), or in a Sanyo multigas incubator at 5% O_2 and 5% CO_2 (Hyp-MMSC). The cells were subcultured after attaining 70-80% confluence. Passage 2 cells were used for the experiments.

Blood mononuclear cells (BMC) were isolated from the peripheral blood of volunteers on a density

Institute of Biomedical Problems, Russian Academy of Sciences, Russia. ***Address for correspondence:*** Buravkova@imbp.ru. L. B. Buravkova

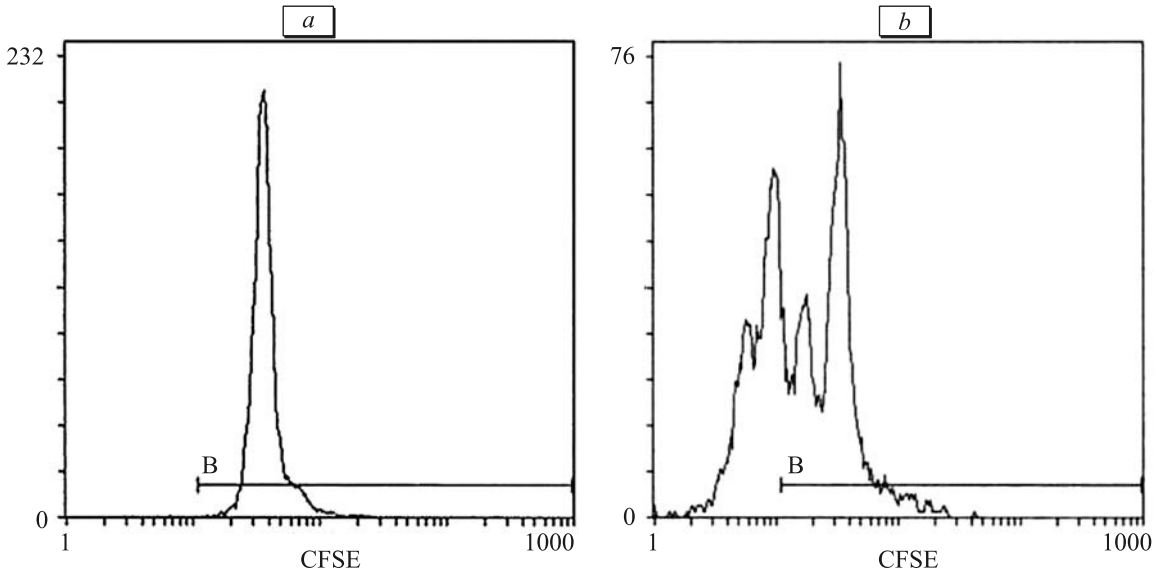

Fig. 1. Diagram of CFSE fluorescence intensity distribution in human lymphocytes. *a*) BMC, 0 h incubation; *b*) BMC, 72 h incubation.

gradient (ρ=1.077, Histopaque, Sigma). For lymphocyte activation, phytohemagglutinin was added to the culture medium in a concentration of 10 μg/ml (PHA-BMC).

Cell viability was evaluated using AnnexinV-FITC–Propidium Iodide kit (Immunotech). The cells were stained routinely and analyzed on a Beckman Coulter EPICS XL flow cytofuorometer.

For evaluation of proliferative activity, some cells were stained with CFSE (CFSE-BMC) in a concentration of 5 μmol/liter as described previously [17]. Analysis on Beckman Coulter EPICS XL flow cytofluorometer yields a histogram of cell distribution by fluorescence intensity (Fig. 1). After each division, the content of the dye in daughter cells and fluorescence intensity decreased 2-fold. The peak with maximum fluorescence intensity corresponded to lymphocytes not entering the division cycle (Fig. 1, *a*), while cells passing one or more divisions were presented by peaks with lower intensity shifted to the left along the X axis (Fig. 1, *b*).

For evaluation of cell-cell interaction between MMSC and lymphocytes we analyzed the effect of MMSC on proliferative activity of PHA-preactivated BMC in mixed lymphocyte culture (Table 1).

Passage 1 N-MMSC and Hyp-MMSC attaining 80% confluence were harvested by trypsinization, washed with PBS (Dulbecco), and suspended in RPMI-1640 containing 5% inactivated FCS, 100 μg/ml streptomycin, and 10 μg/ml PHA. Then, MMSC were mixed 1:10 with BMC. After 72 h, BMC and BMC+PHA suspensions were sampled and proliferative index and viability of immunocompetent cells were analyzed on a Beckman Coulter EPICS XL cytofluorometer.

The cells were analyzed under a Leica DMIL microscope equipped with attachments for phase-contrast and fluorescent microscopy and video camera for image grabbing and digitalization. The observations were performed immediately after the start of the experiment and after 24 and 72 h. For more detailed visualization of cell-cell interaction during immediate content of MMSC with lymphocytes, DIC microscopy was performed using an Olympus IX81 microscope.

Significance of differences was evaluated using nonparametric Mann–Whitney test.

TABLE 1. Scheme of Experiment with Mixed Lymphocyte Culture

Non-activated BMC		Activated BMC		Non-activated BMC with MMSC		Activated BMC with MMSC	
BMC, 72 h in culture, RPMI-1640		PHA-BMC, 72 h in culture, RPMI-1640		BMC+MMSC 1:10 MMSC/BMC, 72 h in co-culture, RPMI-1640		PHA-BMC+MMSC 1:10 MMSC/PHA-BMC, 72 h in co-culture, RPMI-1640	
20% O_2	5% O_2	20% O_2	5% O_2	20% O_2	5% O_2	20% O_2	5% O_2

RESULTS

Immediately after the start of co-culturing, the mixed culture looked like a suspension of bright CFSE-stained BMC and unstained MMSC. After 24 h, MMSC adhered to plastic, the intensity of BMC fluorescence decreased (Fig. 2). Some cells adhered to MMSC (Fig. 2, *a*). Lymphocytes were clearly visualized by fluorescent microscopy by the presence of green fluorochrome in the cytoplasm (Fig. 2, *b*). After 72 h, MMSC were well flattened, the number of aggregates of activated lymphocytes increased. DIC-microscopy showed MMSC adherent to plastic, lymphocytes adherent to MMSC, and BMC suspended in culture medium

Proliferative activity of lymphocytes was evaluated after 72-h culturing. Proliferative index of non-activated BMC was low (0.5-2%), while after PHA activation this parameter increased to 5-33% depending on lymphocyte donor. Co-culturing with MMSC reduced the relative content of proliferating PHA-BMC (1-28%). Proliferative index varied in a wide range, therefore, for comparison of the data obtained in different experiments the results were presented as the ratio of proliferative indexes PHA-BMC and PHA-BMC+MMSC. The effects of interaction were evaluated at 20 and 5% O_2 in culture medium. Co-culturing of lymphocytes and MMSC led to an about 3-fold decrease in proliferative index compared to the control (by 2.8 and 3.2 times at O_2 concentration of 20 and 5%, respectively). Suppression of proliferative activity of PHA-BMC did not depend on O_2 concentration in culture medium. The relative content of viable cells among non-activated BMC was considerably higher than in activated cells at both 20 and 5% O_2, respectively (Fig. 3).

Lymphocyte culturing under conditions of reduced pO_2 did not affect the relative content of viable cells compared to lymphocytes cultured at 20% O_2. Co-culturing with MMSC also had no significant effect on BMC viability (Fig. 3).

We showed that co-culturing of BMC and MMSC in a medium with standard O_2 content (20%) suppressed proliferation of PHA-activated lymphocytes. This confirms the results of other researchers [3,5,7,8,13]. A possible mechanism of this effect is inhibition of the expression of cyclin D2, which leads to suppression of lymphocyte proliferation by MMSC via induction of cell cycle arrest in G_0 phase [7].

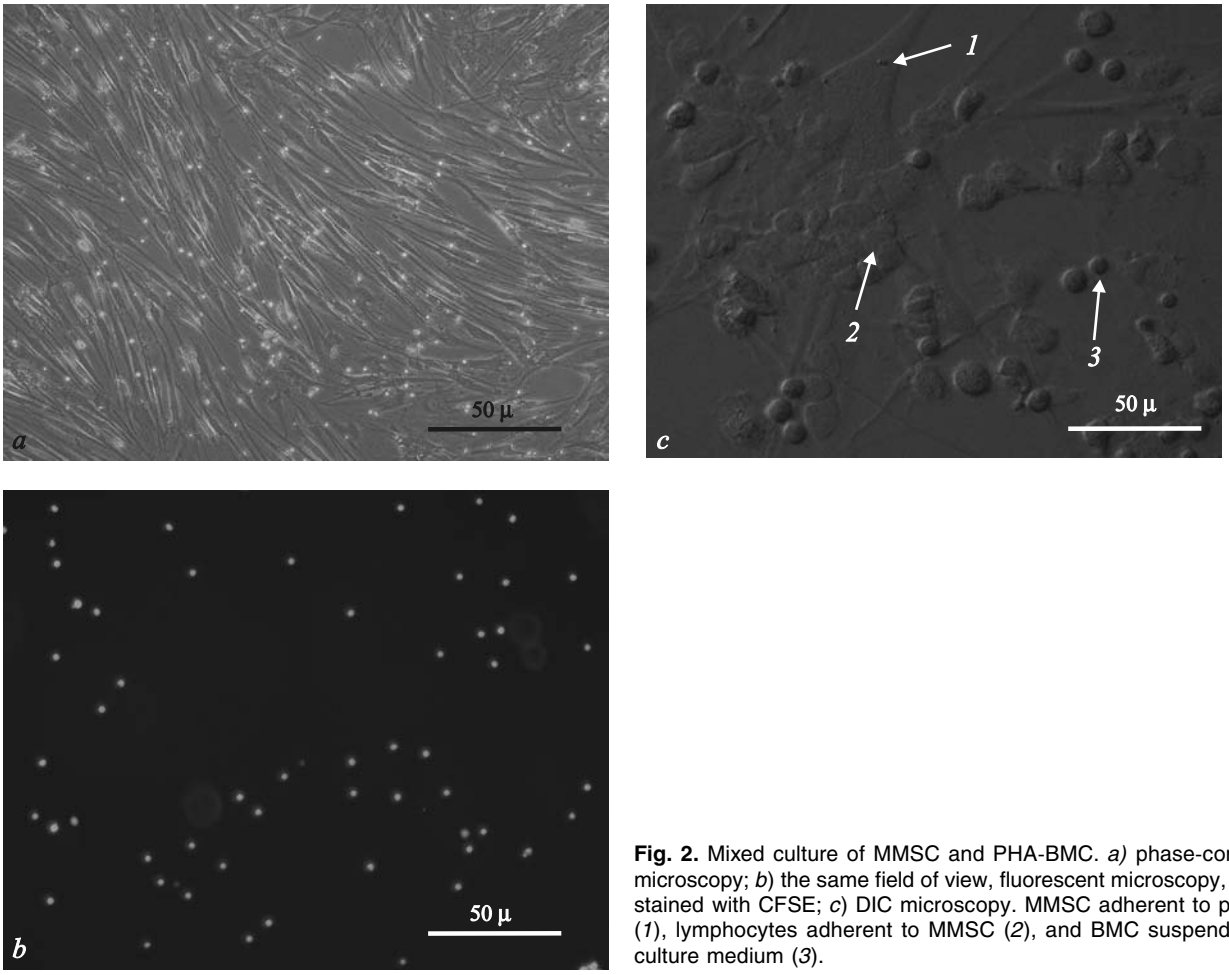

Fig. 2. Mixed culture of MMSC and PHA-BMC. *a)* phase-contrast microscopy; *b*) the same field of view, fluorescent microscopy, BMC stained with CFSE; *c*) DIC microscopy. MMSC adherent to plastic (*1*), lymphocytes adherent to MMSC (*2*), and BMC suspended in culture medium (*3*).

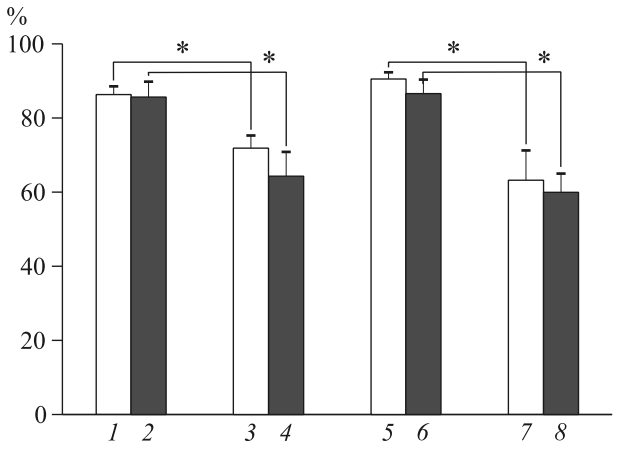

Fig. 3. Percent of viable cells among BMC and PHA-BMC after 72 h culturing at 20%: BMC (*1*), PHA-BMC (*3*), BMC+MMSC (*5*), PHA-BMC+MMSC (*7*) and 5% O_2: BMC (*2*), PHA-BMC (*4*), BMC+MMSC (*6*), PHA-BMC+MMSC (*8*). Open bars: 20% O_2; dark bars: 5% O_2.

MMSC can interact with immune system cells in systemic circulation and in target tissues, *i.e.* at high and low pO_2, respectively. We found that proliferation of lymphocytes in mixed culture with MMSC equally decreased at 5 and 20% O_2. Hence, the reduced O_2 content did not affect the immunosuppressive properties of MMSC.

There are controversial data on proliferative activity of lymphocytes at different pO_2. It was shown that hypoxia (2% O_2, 40 h) suppressed lymphocyte proliferation via inhibition of the synthesis of mitotic cyclins A and B [15]. The expression of the gene encoding potassium channel protein also decreased by 20% under hypoxic conditions (1% O_2, 24 h), which also inhibited lymphocyte proliferation [5]. According to other reports, hypoxia (5% O_2, 24 h) increases proliferation of lymphocytes stimulated with concanavalin A or PWM mitogen by 30-50% [12]. In our study we revealed no effect of low O_2 content on proliferative activity of PHA-stimulated lymphocytes.

The relative content of viable cells among activated lymphocytes was considerably lower than among non-activated cells, which is typically observed after *in vitro* induction with mitogens. Co-culturing with MMSC did not change BMC viability. Culturing at low pO_2 (5% O_2) did not change the percent of viable cells, which agrees with previous data [1]. Other investigators revealed increased viability of lymphocytes under condition of hypoxia [12,15] and induction of apoptosis in T lymphocytes under conditions of reduced O_2 content [16]. It is known that MMSC maintain viability of both non-activated [4] and activated lymphocytes [18]. These findings attest to multidimensional effect of hypoxia depending on its severity and duration.

Thus, proliferation of T cells *in vitro* during co-culturing with MMSC decreased irrespective of pO_2 (5% or 20% O_2), while viability of immunocompetent cells remained unchanged. Moreover, our findings suggest that the degree and pattern of immunosuppressive effect depend on both donor of MMSC and donor of BMC. That is why administration of MMSC *in vitro* should be preceded by analysis of the interaction of allogeneic MMSC and immunocompetent cells of the recipient *in vitro*. The model of mixed lymphocyte culture can be used as the test system for this study.

The study was supported by Program of Fundamental Studies, Department of Biological Sciences of the Russian Academy of Sciences, and grant NSh-4592.2010.4.

REFERENCES

1. L. B. Buravkova and E. R. Andreeva, *Fiziol. Cheloveka,* **36**, No. 5, 1-11 (2010).
2. L. B. Buravkova, O. S. Grinakovskaya, E. R. Andreeva, *et al., Thitologiya,* **51**, No. 1, 5-11 (2009).
3. V. S. Sergeev, *Klet. Tekhnol. Biol. Med*., No. 2, 39-41 (2005).
4. F. Benvenuto, S. Ferrari, E. Gerdoni, *et al., Stem Cells*, **25**, No. 7, 1753-1760 (2007).
5. L. Conforti, M. Petrovic, D. Mohammad, *et al*., *J. Immunol.*, **170**, No. 2, 695-702 (2003).
6. C. Fehrer, R. Brunauer, G. Laschober, *et al., Aging Cell*, **6**, No. 6, 745-757 (2007).
7. S. Glennie, I. Soeiro, P. J. Dyson, *et al., Blood*, **105**, No. 7, 2821-2827 (2005).
8. C. Gotherstrom, *Transplantation*, **84**, No. 1, 35-37 (2007).
9. W. L. Grayson, F. Zhao, B. Bunnell, *et al., Biochem. Biophys. Res. Commun*., **358**, No. 3, 948-953 (2007).
10. B. J. Jones and S. J. McTaggart, *Exp. Hematol.*, **36**, No. 6, 733-741 (2008).
11. M. Krampera, L. Cosmi, R. Angeli, *et al., Stem Cells*, **24**, No. 2, 386-398 (2005).
12. J. A. Krieger, J. C. Landsiedel, and D. A. Lawrence, *Int. J. Immunopharmacol.,* **18**, No. 10, 545-552 (1996).
13. K. Le Blanc, C. Tammik, K. Rosendahl, *et al., Exp. Hematol.*, **31**, No. 10, 890-896 (2003).
14. M. Najar, R. Rouas, G. Raicevic, *et al*., *Cytotherapy*, **11**, No. 5, 570-583 (2009).
15. A. Naldini and F. Carraro, *J. Cell Physiol*., **181**, No. 3, 448-454 (1999).
16. J. Sun, Y. Zhang, M. Yang, *et al*., *Cell. Mol. Immunol.,* **7**, No. 1, 77-82 (2010).
17. D. Suva, J. Passweg, S. Arnaudeau, *et al., J. Cell Physiol*., **214**, No. 3, 588-594 (2008).
18. A. Uccelli, L. Moretta, and V. Pistoia, *Eur. J. Immunol.*, **36**, 2566-2573 (2006).
19. P. A. Zuk, M. Zhu, H. Mizuno, *et al., Tissue Eng*., **7**, No. 2, 211-228 (2001).