Exp Oncol 2008
30, 2, 121–128

Experimental ONCOLOGY

INFLUENCE OF MESENCHYMAL STEM CELLS DERIVED FROM BONE MARROW OF CHILDREN WITH ONCOHEMATOLOGICAL DISEASES ON PROLIFERATION AND SELF-RENEWAL OF HEMATOPOIETIC PROGENITOR CELLS *IN VITRO*

Ya. Isaikina*, T. Shman

Belarusian Research Centre for Pediatric Oncology and Hematology, Minsk, Belarus

Aim: **To test the ability of cultured mesenchymal stem cells (MSCs) from bone marrow (BM) of children with oncohematological diseases after chemotherapy and radiation to support proliferation and self renewal of hematopoietic cells *in vitro*. *Methods:* BM samples of 8 patients and 9 healthy children-donors used for MSCs preparation applying techinque of expansion *in vitro*. $CD34^+$ cells were isolated from BM of donors. The ability of MSCs to maintain hematopoietic stem cells (HSCs) proliferation was tested in semisolid methylcellulose medium and in liquid long-term-culture (LTC) medium. *Results:* The presence of MSCs derived from BM of patients in methylcellulose medium induced 2-fold increase of the number of commited myeloid progenitors without cytokines, 7-fold increase together with growth factors and 14-fold increase of the amount of earlier pluripotent hematopoietic precursor cells (CFU-GEMM) compared to expansion of HSCs without MSCs and cytokines. The presence of MSCs layer of patients in liquid LTC medium significantly promoted the hematopoietic cells proliferation rate, measured on 7th, 14th and 21st day. The total number of cells was multiplied 161.2-fold on 21st day as compared to 116.4-fold without MSCs layer. In the presence of MSCs layer, we detected the increase of proportion of bipotent CFU-GM precursors from 4% to 11% and pluripotent CFU-GEMM precursors from 0.1% to 0.6% in population of HSCs. In both types of experiments the capacity of patients' MSCs to support HSCs proliferation and self renewal was the same as for healthy donors' MSCs. *Conclusion:* In this study, MSCs were isolated from BM of children with malignancies after high-dose chemotherapy or radiation. The ability of these MSCs to maintain hematopoiesis *in vitro* was tested. It was shown that co-transplantation of autologous MSCs is a good way to improve hematopoietic stem cells engraftment and reduce a period of granulocytopenia after autologous HSCs transplantation in case of insufficient $CD34^+$ cell number in autologous transplant.**

Key Words: **mesenchymal stem cells, oncohematological diseases, hematopoiesis, hematopoietic progenitors.**

High-dose chemotherapy alone and sometimes together with radiotherapy with following autologous transplantation of hematopoietic stem cells (HSCs) is used for treatment of patients with advanced stages or relapses of malignant diseases. However, reconstitution of neutrophil and platelet cell number after autotransplantation of HSCs is delayed for about 5% of patients. In most cases it could be explained by low dose of transplanted $CD34^+$ cells per kg of patient's body weight [1, 2].

Dimitri et al observed that the number and quality of mobilized peripheral blood stem cells (PBSC) was low in patients that received multiple rounds of chemotherapy and grafts with low numbers of HSCs. Poor quality of HSCs was the cause of graft failure upon their autologous infusion [3].

Received: February 5, 2008.

*Correspondance: Fax: 265-42-22
E-mail: yaninai@mail.ru

Abbreviations used: BFU-E – burst-forming units erythroid; BM – bone marrow; GF – growth factors; CFU-G – colony forming units – granulocyte; CFU-GEMM – colony forming units – granulocyte-erythroid-monocyte-megakaryocyte; CFU-M – colony forming units – macrophage; CFU-GM – colony forming units – granulocyte and macrophage; HBSS – Hanks Balanced Salt Solution; HSCs – hemopoietic stem cells; IL-3 – interleukin-3; IMDM – Iscove Modified Dulbecco Medium; LTC-IC – long-term-culture initiating cell; MNCs – mononuclear cells; MSCs – mesenchymal stem cells; PBC – peripheral blood stem cells; rhGM-CSF – recombinant human granulocyte-macrophage colony-stimulating factor; rhSCF – recombinant human stem cell factor; SCT – stem cells transplantation; HPC – hematopoietic progenitor cell.

Recently, some approaches were suggested to solve this problem. One of them is a generally accepted procedure of repeated attempts of PBSC collection or aspiration of considerable volume of bone marrow (BM) in case of insufficient $CD34^+$ cell number in transplant at the time of primary collection of PBSC. Another approach includes expansion of hematopoietic stem cells *ex vivo*. This is an attractive method to improve hematological recovery or reduce graft size. However, although clinical trials indicated that total number of $CD34^+$ cells and primitive stem cells in PBSC can be successfully expanded *in vitro*, hematopoietic recovery by using such grafts is not improved [4, 5]. This could be explained by the reduction of stem cells ability to home to the bone marrow after incubations with several cytokine combinations, which leads to the loss of their ability to repopulate the hematopoietic system of irradiated recipients [6].

At the present time, there is another highly attractive approach to reduce the hematopoiesis reconstitution period after transplantation of HSCs — the simultaneous autotransplantation of mesenchymal stem cells (MSCs). Mesenchymal stem cells are precursors of stromal stem cells, osteoblasts, adipocyts, endothelial cells, which form regions of hematopoietic inductive environment in bone marrow, thus, supporting the production of leucocytes, red cells and platelets [7, 8]. From one hand, their impact on hematopoiesis precursors is realized by secretion of soluble cytokines, chemokines, peptides, mediators and hormones. From the other hand, MCSs form the extra-

cellular matrix from collagen, fibronectin and laminin molecules, which provide adhesion of hematopoietic cells. Stromal cells continuously synthesize colony stimulating factor (CSF), interleukin-6 (IL-6), IL-1, IL-7, IL-8, SCF, Flt-3-ligand, GM-CSF, thrombopoietin, insulin-like growth factor, transforming growth factor (TGF), thus, supporting the maintenance of the determined level of blood cells number. IL-1 is the main cytokine production inductor, and TGF is able to inhibit hematopoiesis [9]. The ability of MSCs to support hematopoiesis *in vitro* has been shown by many authors in simultaneous cultivation of MSCs and HSCs [10]. The capacity of stromal cells to support the expansion of primitive hematopoietic progenitors for more than 10 weeks was revealed [11].

Until recently, practically all of the investigations of MSCs effect on hematopoietic precursor cells proliferation and self-renewal were carried out by using stromal cells layer derived from bone marrow MSCs of healthy donors. Therefore, it is important to study functional potential of mesenchymal stem cells derived from bone marrow of children, who are the candidates for HSCs transplantation after high dose chemotherapy. We investigated the ability of cultured MSCs from patients' BM to support proliferation and self-renewal of hematopoietic cells *in vitro.* It can be of great importance for creation of therapeutical strategy of autologous MSCs application for hematopoiesis support and neutropenia period reduction after HSCs autologous transplantation for children with insufficiency of $CD34^+$ cell number in graft.

MATERIALS AND METHODS

Materials. Bone marrow samples of 8 patients with IV stage of non-Hodgkin's lymphoma, Hodgkin disease and Ewing sarcoma have been used. Protocol of treatment of these patients included autologous transplantation of HSCs. Comparative analysis has been performed by evaluation of bone marrow samples of 9 children-donors of hemopoietic stem cells for allogenic transplantation.*

BM cell preparation. Samples of BM were obtained from allogenic or autologous bone marrow transplants. Mononuclear cells (MNCs) were isolated by centrifugation on a Ficoll-Hypaque gradient (density 1.077 g/ml) (Sigma, USA) at 400 g for 25 min, washed twice in Hanks Balanced Sold Solution (HBSS) (Sigma, USA).

$CD34^+$ cells separation. $CD34^+$ cells were derived from bone marrow of healthy donors. The procedure of $CD34^+$ cells processing from fraction of bone marrow mononuclear cells was carried by means of Human CD34 Selection Kit for positive selection (StemCell Technologies, Canada) according to manufacturer's protocol.

Human mesenchymal cells preparation. Mononuclear cells were suspended in Iscove Modified Dulbecco Medium (IMDM) with 10% fetal bovine serum (FBS) (Sigma, USA) at concentration of 1×10^6/ml and were transferred to tissue culture flask (Sarstedt, Germany) at density $0.5 \times 10^6/cm^2$. Cells were cultured at 37 C with 5% CO_2, in humidified atmosphere. When cells achieved 80–90% of confluence, the adherent layer was washed with HBSS to remove residual FBS and was detached with 0.25% trypsin-EDTA solution (Sigma, USA). Then MSCs were washed in IMDM with 10% fetal bovine serum and $0.6–2 \times 10^6$ cells were transferred to a new 25 sm^2 flask (I passage). When ~ 90% confluent layer was produced, the manipulation was repeated again. For evaluation of mesenchymal cells influence on hematopoietic cell growth we used the same MSCs from II–IV passages for the healthy donors' and patients' preparations.

Long-term culture hematopoietic and mesenchymal stem cells. Mesenchymal cells were seeded at concentration of 0.5×10^5/well into 24-wells plates. Cells were cultured in IMDM-medium supplemented with 10% FBS overnight at 37 °C in 5% CO_2. Monolayer of cells was treated with 2% glutaraldehyde or irradiated at 30 Gy to stop proliferation of mesenchymal cells. Then, purified donors' $CD34^+$ cells (1×10^4/ml) were added to the wells. Cells were cultured in IMDM supplemented with 15% FBS, 2 mM L-glutamine and 2-mercaptoethanol in presence and without a cocktail of recombinant human cytokines. We used following growth factors (GF): IL-3 (20 ng/ml), IL-6 (20 U/ml), stem cell factor SCF (50 ng/ml), Flt-3-ligand (100 ng/ml). Cells were incubated for 21 days in standard conditions with weekly replacement of half of the medium. Cells were counted and their viability was analyzed at 7th, 14th and 21st day.

Semisolid assay of clonogenic progenitors in the different conditions. Pluripotent and line-committed hematopoietic precursor cells form different types of colonies or «colony-forming units» (CFU) in short-term culture. Myeloid line-committed precursors form colony-forming units granulocyte (CFU-G), CFU — macrophage (CFU-M); bypotential precursor forms CFU — granulocyte-macrophage (CFU-GM); pluripotential hematopoietic precursor forms CFU- granulocyte — erythroid-monocyte-megakaryocyte (CFU-GEMM) and erythroid precursor forms burst-forming units erythroid (BFU-E). Experiments were performed in six parallel variants. Initially, $CD34^+$ cells were suspended at a concentration 1×10^4 cells/ml in 3 ml complete methylcellulose medium (1% methylcellulose in IMDM, 30% fetal bovine serum, 1% albumin, 2 mM L-glutamine) without and with presence of colonystimulating growth factors: 10 ng/mL recombinant human IL-3 (rhIL-3); 10 ng/mL recombinant human granulocyte macrophage — CSF (rhGM-CSF); 50 ng/mL recombinant human stem cell factor (rhSCF); 3 U/mL human erythropoietin (Epo). Further, each of the two variants of cells in methylcellulose medium were plated (1×10^4 cells/well in 1 ml) in presence and without MSC layer derived from bone marrow of patients and from bone marrow of donors as a control in 6 wells of 24-wells plastic microplates. Plates were incubated at 37 °C with 5% $C0_2$ and ⩾ 95% humidity for 21 days. Number of CFU-G, CFU-M,

* Patients provided written consent to perform the study, and research was approved by the Ethic Committee of the Research Center.

CFU-GM, CFU-GEMM and BFU-E were scored macroscopically at 21st day.

Statistical analysis. Statistical processing of data was performed with the statistical package STATISTICA, Ver 6.0. (Stat Soft Inc, USA). Mann — Whitney U test was used to evaluate significant differences between samples of patients and donors. Wilcoxon matched pairs test was used to evaluate the fold increase of cells cultured under different condition.

RESULTS

Effect of MSCs on maintaining of CD34⁺ cells proliferation in semisolid medium. Research was performed using MSCs derived from bone marrow of 8 patients and 9 healthy donors and hematopoietic CD34⁺ cells selected from samples of healthy donors. Evaluation of clonogenic progenitors content was carried out after cultivation of CD34⁺ cells in methylcellulose medium for 21 days in each of 6 variants: hematopoietic cells as the negative control, hematopoietic cells with MSCs of donors, hematopoietic cells with MSCs of patients, hematopoietic cells with growth factors (GF), hematopoietic cells with GF and MSCs of donors, and hematopoietic cells with GF and MSCs of patients. All types of myeloid line colonies, i. e. CFU-G, CFU-M, CFU-GM were termed "myeloid progenitors". The level of variability in myeloid progenitor cells number under different cultural conditions was relative to myeloid progenitor's number in negative control (Fig. 1, *a*). Analysis of results showed relative increase in myeloid progenitors number when CD34⁺ cells were cultured in the presence of GF alone, MSCs alone or GF and MSCs simultaneously. The presence of both patients' and donors' MSCs in methylcellulose medium resulted in 1.86 ± 0.25-fold ($p < 0.05$) and in 1.85 ± 0.3 ($p < 0.05$) — fold increase in the number of myeloid progenitors cells respectively as compared with negative control, while adding only growth factors in methylcellulose medium increased CFU-GM number 3.3 ± 0.62-fold. Hematopoietic stem cells proliferated more intensively in the simultaneous presence of GF and MSCs in growth medium. Myeloid progenitors number on average was 7.31 ± 1.24-fold higher in the presence of patients' MSCs ($p < 0.01$) and 7.18 ± 1.05-fold higher in the presence of donors' MSCs as compared with the negative control ($p < 0.01$). The significant difference between impacts of patients and donors' MSCs on proliferation of the myeloid precursor cells was not detected.

Influence of both patients' and donors' MSCs on the earlier pluripotent hematopoietic precursor cells proliferation was evaluated CFU-GEMM number in each of 6 variants. The Fig. 1, *b* presents relative increase in CFU-GEMM number in case of GF alone or GF and MSCs simultaneously added with CD34⁺cells. We didn't observe any CFU-GEMM number expansion when CD34⁺ cells were cultured in the presence of MSC without GF. Maximum increase in CFU-GEMM number was detected when GF and MSCs were added simultaneously as compared with variants of MSCs alone (15.9 ± 7.3 *vs* 0.9 ± 0.4 for donors ($p < 0.05$) and 14.07 ± 6.9 *vs* 0.9 ± 0.2 for patients ($p < 0.05$)) or GF alone (15.9 ± 5.9 *vs* 8.2 ± 3.1 from donors ($p < 0.05$) and 14.07 ± 6.9 *vs* 5.33 ± 2.16 from patients ($p < 0.05$)).

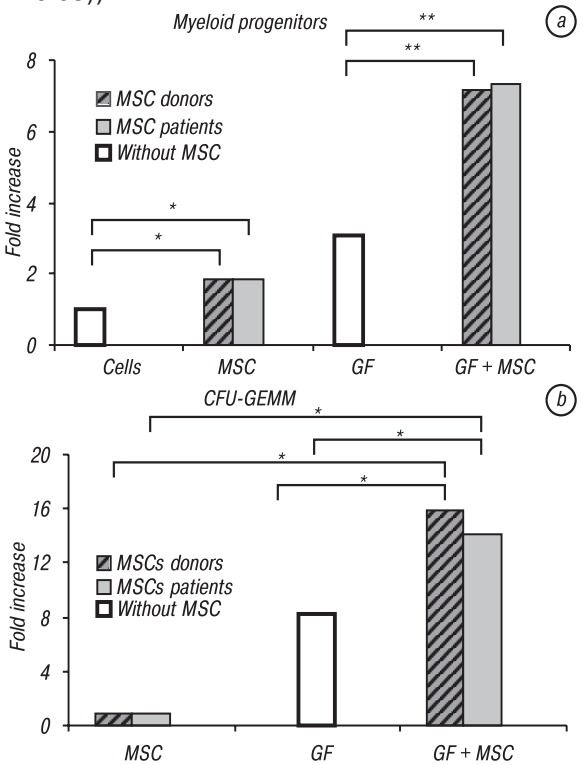

Fig. 1. Effect of MSC derived from BM of patients with oncohematological diseases on proliferation of CD34⁺ cells in semisolid medium. MSCs derived from donors' BM served as a control. 1 x 10^4 of CD34⁺ cells of healthy donors were cultured in methylcellulose medium under different conditions: culture medium alone; with GF, with patients' MSCs; with GF and patients' MSCs; with donors' MSCs; with GF and donors' MSCs. Cells were cultured for 21 days and number of CFU was scored. Each column represents the fold increase of progenitors observed in six separate variants, relative to number of progenitors grown without GF and MSCs: *(a)* Fold increase of total number of CFU-G + CFU-M + CFU-GM and termed here as "myeloid progenitors"; *(b)* Fold increase of pluripotential precursors cells (CFU-GEMM). Wilcoxon matched pairs test was used for comparison of fold cells increase inside each group (*$p < 0.05$, **$p < 0.01$)

Mann — Whitney U test was used to compare the effect of donors' and patients' MSCs on fold increase of hematopoietic cells' numbers. It confirmed that MSCs derived from patients' BM with statistical validity had the same supporting effect on the proliferation of the pluripotent hematopoietic precursor cells (CFU-GEMM) as donors' MSCs.

Influence of MSCs on maintaining of CD34⁺ cells proliferation in liquid long-term cell culture. In order to investigate the influence of MSCs on hematopoietic cells expansion in liquid complete growth medium, two experiments with four cultivation variants were performed: 1) (n = 8) — only CD34⁺ cells, CD34⁺ cells on MSCs layer of healthy donors, CD34⁺ cells with growth factors, CD34⁺ cells with growth factors on MSCs layer of healthy donors; 2) (n = 8) — only CD34⁺ cells, CD34⁺ cells on MSCs layer of patients, CD34⁺ cells with GF, CD34⁺ cells with GF on MSCs layer of patients. For both experiments, CD34⁺ cells were isolated from

bone marrow of healthy donors' samples, and the initial concentrations of these cells were 1 x 10^4 for each cultivation variants. Kinetics of $CD34^+$ cells proliferation for different variants is presented on Fig. 2. The absolute cells numbers from 2 variants of both experiments, when MSCs layer was absent and $CD34^+$ cells were grown only in culture medium, or $CD34^+$ cells were grown in the presence of GF alone, were integrated (n = 16 in every case). Results represented the mean absolute cells number ± SD of six different experiments on 7th, 14th, and 21st day of culturing. We did not observe significant difference in cells number when $CD34^+$ cells were cultivated in serum-containing culture medium, and the total amount of cells was 0.8 ± 0.07 x 10^4, 0.55 ± 0.03 x 10^4 and 0.5 ± 0.01 x 10^4 on 7th, 14th and 21st day, respectively. The presence of both donors' and patients' MSCs layer without GF had no effect on significant differences of absolute number of growing cells during 21 days. Total amount of cells in the presence of donors MSCs was 1.5 ± 0.6 x 10^4, 1.1 ± 0.2 x 10^4 and 0.8 ± 0.2 x 10^4 on 7th, 14th and 21st day, respectively, and in the presence of patients' MSCs was 2.1 ± 0.7 x 10^4, 2.75 ± 1.7 x 10^4 and 2.6 ± 1.1 x 10^4 on 7th, 14th and 21st day, respectively. The presence of cytokines in culture medium significantly increased the absolute number of cells from 1 x 10^4 to 15.5 ± 2.5 x 10^4, to 52 ± 11.3 x 10^4 and to 130.5 ± 10.3 x 10^4 on day 7, 14 and 21 respectively, $p < 0.05$. The increase of total cells number when $CD34^+$ cells were cultured in the presence of both donors' and patients' MSCs and cytokines was the most evident, and total number of cells in the presence of donors' MSCs increased from 1 x 10^4 to 24 ± 4.2 x10^4, to 94 ± 7.7 x 10^4 and to 195 ± 24.7 x 10^4 on day 7, 14 and 21 respectively, $p < 0.01$ and with patients' MSCs from 1 x 10^4 to 14 ± 1.1 x 10^4, to 85 ± 2.8 x 10^4 and to 161 ± 7.3 x 10^4 on day 7, 14 and 21, respectively, $p < 0.01$. No statistical difference was found between effects of patients' and donors' MSCs of on hematopoietic stem cells expansion.

To compare the functional ability of donors' and patients' MSCs to support proliferation of $CD34^+$ cells, we calculated the fold increase of cells number (cells number at the output/cells number initial) for each cultivation variant (Fig. 3). The greatest fold increase was observed when $CD34^+$ cells were cultured in medium containing GF complex on 7th day, with further increase on 14th and then 21st day. Active proliferation was typical for $CD34^+$ cells in any cultivation variant. The cells number of $CD34^+$ cells from healthy donors with GF alone increased from 20.3-fold to 74.6-fold and to 155.3 fold on 7th, 14th and 21st day, respectively, and for patients from 11.1-fold to 36.0-fold and to 116.4-fold on day 7th, 14th and 21st, respectively. While culturing cells with both GF and healthy donors MSCs layer, hematopoietic cells number increased from 26.0-fold to 102.3-fold and to 213.5-fold on day 7th, 14th and 21st, respectively, and both GF and patients' MSCs layer — from 13.9-fold to 85.3-fold and to 161.2-fold on 7th, 14th and 21st day, respectively.

On 7th, 14th and 21st day, the number of cultured cells in the presence of both GF and MSCs layer was significantly greater when compared with GF alone, $p < 0.05$, both for healthy donors' and patients' MSCs. The increase of the number of hematopoietic cells in the presence of donors' MSCs layer compared with ab-

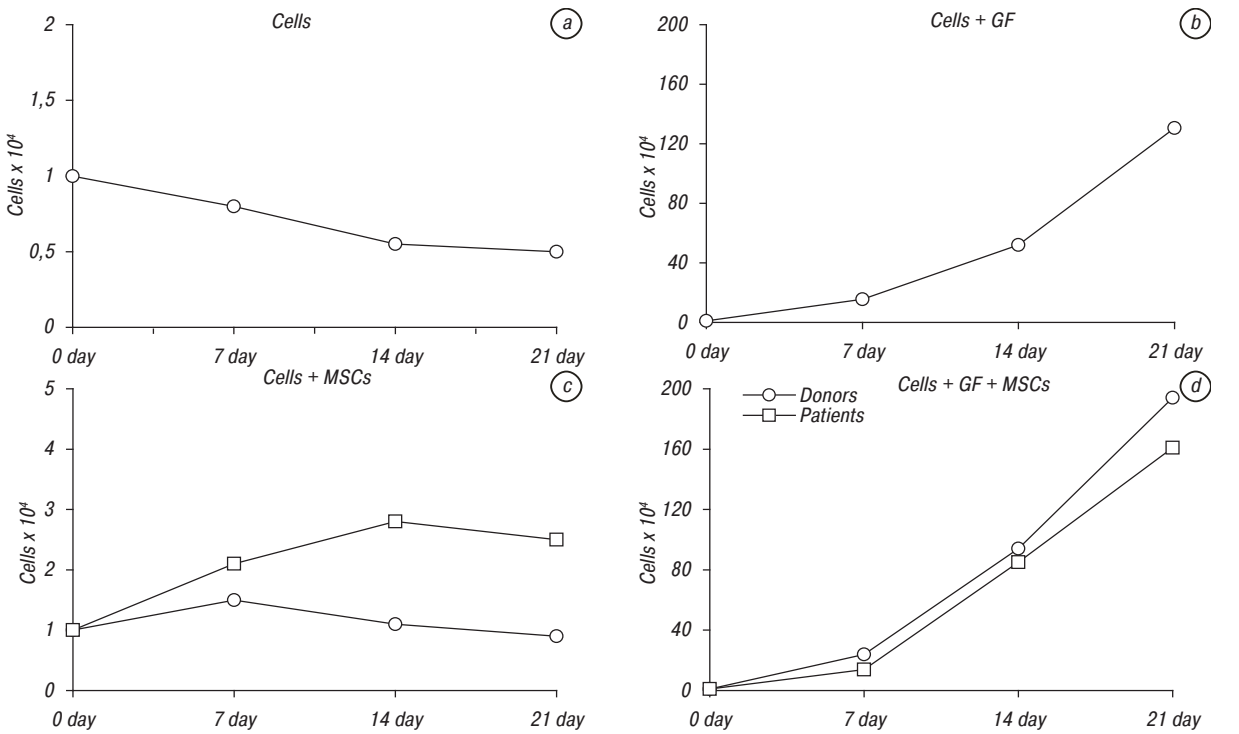

Fig. 2. Effects of several cytokines, and donors' and patients' MSCs on *ex vivo* expansion of hematopoietic stem cells. BM $CD34^+$ cells were cultured in four cultivation variants: *(a)* Hematopoietic cells in serum-containing culture medium; *(b)* $CD34^+$ cells with GF. $CD34^+$ cells on feeder layer of donors' and patients' MSCs; *(c)* $CD34^+$ cells with growth factors on feeder layer of donors' and patients' MSCs; *(d)* The results represent the mean cells number ± SD of 6 different experiments on days 7, 14 and 21. □ MSCs of patients; ○ MSCs of healthy donors

sence of MSCs layer was 26 *vs* 20.3-fold, respectively, ($p < 0.01$) on 7th day, 102.3 *vs* 74.6-fold ($p < 0.01$) on 14th day, and 213.5 *vs* 155.3-fold on 21st day. Culturing of $CD34^+$ cells with patients' MSCs layer also demonstrated the significantly greater increase of hematopoietic cells number on 7th, 14th and 21st day as compared with absence of MSCs layer: 13.9 *vs* 11.1-fold, respectively, ($p < 0.05$) on 7th day, 85.5 *vs* 36.1-fold ($p < 0.05$) on 14th day and 161.2 *vs* 116.4-fold ($p < 0.01$) on 21st day.

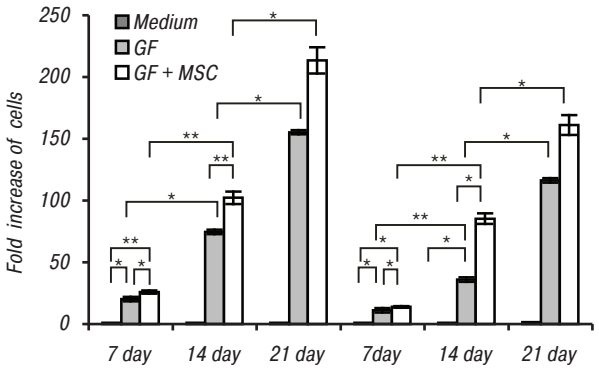

Fig. 3. MSCs layer significantly increased proliferation of $CD34^+$ cells cultured in medium with GF in comparison with $CD34^+$ cells grown in culture medium or cultured in the presence of GF alone. After 7th, 14th and 21st days cells were harvested, and the fold increase was calculated. For statistical analysis, Mann — Whitney U test was used to compare the effect of donors MSCs and MSCs of patients on fold increase of hematopoietic cells ($*p < 0.05$, $**p < 0.01$)

Statistical analysis (Mann — Whitney U test) comparing the fold increase in the number of cells for two independent groups of donors and patients didn't detect statistically valid difference between the effects on hematopoietic cells proliferation of co-cultured for 3 weeks donors' MSCs and patients' MSCs derived from BM of patients with oncohematological diseases.

Influence of MSCs on changing of the cell population profile after CD34⁺ cells expansion. MSCs influence on changing of $CD34^+$ cells population profile after cells expansion was studied by culturing cells in liquid LTC medium in presence of GF alone or combined with MSCs for 3 weeks, using clonogenic assay progenitors in methylcellulose.

Morphological analysis of colony forming cells (CFC) demonstrated the existence of pluripotent hematopoietic precursor cells CFU-GEMM, bypotential precursors — CFU-GM and committing precursors CFU-G, CFU-M, BFU-E. All types of colony-forming units (CFU) were scored and results presented in Table. A mean total of 579 CFC/10^4 $CD34^+$ cells was observed in initial samples: 31% of cells (185/10^4 $CD34^+$ cells) corresponded to CFU-G, 15% (89/10^4 $CD34^+$ cells) to CFU-M, 4% (23/10^4 $CD34^+$ cells) to CFU-GM, 49% (262/10^4 $CD34^+$ cells) to BFU-E, and 0.1% (0.6/10^4 $CD34^+$ cells) to multipotent progenitor cells (CFU-GEMM). A mean total of 252 CFU/10^4 $CD34^+$ cells was observed after expansion $CD34^+$ cells in cultural medium in the presence of GF alone upon a 3-week culturing period. CFU comprised 32% of CFU-G, 62% of CFU-M, 5.2% of CFU-GM, 0.3% of CFU-GEMM. When $CD34^+$ cells were grown in the presence of the GF together with MSCs layer, the spectrum of CFU was: 40% of CFU-G, 48% of CFU-M, 11% of CFU-GM, 0.6% of CFU-GEMM.

Table. Effect of MSCs on population profile of hematopoietic progenitors throughout a 3-week culturing period

CD34+ cells	Progenitor cells per 1 x 10^4 $CD34^+$ cells plated (mean ± SEM)				
	CFU-G	CFU-M	CFU-GM	CFU-GEMM	BFU-E
Initial cells (n = 9)	185 ± 73	89 ± 39	23 ± 17	0.6 ± 0.3	282 ± 119
Expansion with GF (n = 7)	80.5 ± 35	155.5 ± 53	13 ± 6	0.7 ± 0.36	ND
Expansion with GF+MSCs (n = 7)	113 ± 32	135.5 ± 64	31 ± 12	1.6 ± 0.7	ND

ND – not detected.

Thus, our findings indicated that the dominant populations of hematopoietic precursors proliferated *in vitro,* in initial samples and cultured for 21 days, were committing precursors CFU-G and CFU-M. The proportion of CFU-G/CFU did not differ before and after $CD34^+$ cells expansion for 21 days without MSCs layer (32% *vs* 32%) or with MSCs layer (32% and 40%). The proportion of CFU-M/CFU increased after $CD34^+$ cells expansion for 21 days without MSCs layer from 15% to 62% and with MSCs layer from 15% to 48% compared to $CD34^+$ cells initial.

The proportion of immature CFU-GM and pluripotent CFU-GEMM precursors to total CFU increased after hematopoietic stem cells cultivation without MSCs layer and in the presence of MSCs compared to initial samples: for CFU-GM from 4% to 5% and from 4% to 11%, respectively, and for CFU-GEMM from 0.1% to 0.3% and from 0.1% to 0.6%, respectively.

Relative changes of progenitors number in $CD34^+$ cell population after expansion for 3 weeks in presence MSCs layer to number of corresponding compartment of CFU in initial samples of $CD34^+$ cells is shown on Fig. 4. Relative changes of CFU-GM and CFU-GEMM number after cultivation both with MSCs and without MSCs did not differ significantly from number of CFU-GM and CFU-GEMM in initial population of $CD34^+$ cells. Though number of CFU-GM and CFU-GEMM were greater approximately by 2.5-fold after expansion in the presence of MSCs layer than without MSCs ($p < 0.05$). BFU-E wasn't detected in any analyzed sample after expansion of $CD34^+$ cells under the described culturing conditions for three weeks, while BFU-E number consisted 49% (281.6 ± 159.1) in initial population of $CD34^+$ cells.

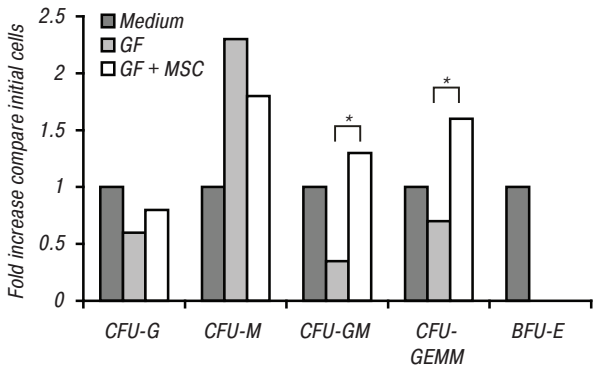

Fig. 4. Relative changes of hematopoietic progenitors number after expansion of $CD34^+$ cells for 21 days in presence of MSCs layer. Corresponding compartments of CFU in initial samples of $CD34^+$ cells recognized as 1 (n = 9; $*p < 0.05$)

DISCUSSION

In this work we examined the potential of MSCs derived from the bone marrow of children with oncohematological diseases, treated by high-dose polychemotherapy and radiotherapy, to support hematopoietic progenitors proliferation *in vitro*. It is important for creation of a therapeutical strategy of application of autologous MSCs for neutropenia period reduction after HSCs autologous transplantation for children with insufficient number of $CD34^+$ cells in graft.

In some publications it was demonstrated that BM stroma greatly damaged after high-dose chemotherapy or radiotherapy [12, 13]. Patients stromal cells cultured for 4–5 weeks after conditioning regimen, including busulphan and cyclophosphamide, are able to give a monolayer only in 20% of cases in comparison with 80% shown for healthy donors [14]. However, we used procedure of MSCs isolation and expansion from BM of children with malignant neoplasms, and increased initial number of cells for more than 3×10^3-fold, as it was demonstrated in our previous work [15].

The ability of MSCs to support hematopoiesis *in vitro* has been shown by many authors in experiments under the conditions of simultaneous cultivation of MSCs and HSCs [16–18].

Dexter et al were among the first researchers, who studied the influence of stromal layer on proliferation of BM cells. They had shown that stromal cells supported hematopoietic cells proliferation and creation of colony-forming unit spleen (CFU-S), while CFU-S were absent when hematopoietic cells were cultivated without stromal layer [19].

Some studies examined the capacity of cultivated MSCs to support human hematopoiesis *in vitro.* They revealed that not only committed cells but also primitive hematopoietic precursors are able to proliferate under the contact with MSCs. Thus, hematopoietic precursors maintained the ability not only to differentiate but also to self-replicate [20]. Wagner et al verified that primitive fractions of HPC had much stronger adherence to BM-derived MSCs than their more differentiated counterparts, and that LTC-IC frequency was higher in the adherent fraction than in the nonadherent fraction of $CD34^+$ cells [21].

Koller et al and Breems et al demonstrated that stromal cells significantly increased the number of progenitor and primitive stem cells after expansion of BM-derived $CD34^+$ cells for 2 weeks in presence of IL-3, SCF, GM-CSF, and erythropoietin. Cultivation *ex vivo* reduced the ability of $CD34^+$ cells to produce progenitors in LTC without stromal soluble factors or in presence of stromal cells. The best expansion of CFU and LTC-IC and optimal maintenance of graft quality were observed when PBSC were cultured in stroma-contact [3, 22–24]. On the other hand, Verfaillie et al demonstrated significantly worse recovery of LTC-IC from cultures, in which progenitors were grown in contact with stroma. Also, researchers have shown that direct contact with stroma inhibited proliferation of LTC-IC even in case of glutaraldehyde-fixed stroma, which was no longer capable for producing growth inhibitory or stimulatory cytokines [25]. At the same time, Harvey et al showed that direct cell-to-cell contact between HSCs and urogenital ridge derived stromal cells increased the CFU-C numbers 5 times compared with non-contact co-culture [26].

Our observations of hematopoietic stem cells expansion in semisolid medium without growth cytokines *in vitro* testified that the presence of MSCs derived from BM of patients with oncohematological disease increased the number of committing myeloid progenitors approximately 2-fold ($p < 0.05$). The most intensive proliferative activity of progenitor cells was shown in the presence of patients' MSCs and GF (SCF, GM-CSF, IL-3, and EPO). In this case, we detected 7-fold increase in the number of myeloid progenitors and 14-fold in the amount of earlier pluripotent hematopoietic precursor cells (CFU-GEMM) compared with hematopoietic stem cells expansion in methylcellulose medium without MSCs and cytokines. These findings are in line with data obtained for hematopoiesis supporting function of MSCs derived from BM of healthy donors, and with statistical validity reveal that supporting effect on proliferation of pluripotent hematopoietic precursor cells (CFU-GEMM) of patients' MSCs did not differ from supporting effect of MSCs from healthy donors.

In the present work we also studied the effect of MSCs layer from patients' BM on maintaining of hematopoietic stem cells proliferation in liquid LTC medium containing a combination of several cytokines. We revealed that the presence of MSCs layer significantly promoted the rate of hematopoietic cells proliferation on 7th, 14th and 21st days, and total cells number was multiplied 161.2-fold on 21th day as compared with 116.4-fold in the absence of MSCs layer. Similar data was obtained when $CD34^+$ cells were grown on donors' MSCs layer, and the cells number increased 213.5-fold in the presence of MSCs layer compared to 155.3-fold without MSCs.

Interestingly, our study of hematopoietic stem cells proliferation kinetics under various culturing conditions demonstrated that the presence of both patients' and donors' MSCs layers without GF didn't have significant influence on expansion of hematopoietic cells within a 3 week. At the same time, we showed that MSCs from BM of both oncohematological patients and healthy donors didn't inhibit proliferation of committed progenitors and primitive stem cells even if $CD34^+$ cells were cultivated in the absence of growth factors, whereas in case of growing $CD34^+$ cells without cytokines and MSCs we observed a loss of hematopoietic cells. This fact confirmed the role of MSCs in prevention of HSCs apoptosis [27, 28].

It is very important that when $CD34^+$cells were cultured both in semisolid medium or in liquid LTC medium the capacity of MSCs derived from BM patients with oncohematological diseases to support proliferation and self renewal of hematopoietic precursor cells was the same as of MSCs derived from bone marrow of healthy donors.

In the present work we studied the cell population profile upon 3 weeks of $CD34^+$ cells expansion in LTC

medium. Cells were cultivated with cytokines alone or both cytokines and MSCs. We observed that MSCs presence did not alter the population of committed CFU-G and CFU-M precursors as compared with initial population of $CD34^+$ cells. The significant increase in proportion of more primitive bipotent precursors (CFU-GM) and pluripotent (CFU-GEMM) precursors in the population of hematopoietic cells after $CD34^+$ cells expansion in the presence of MSCs layer is a very important finding. Altogether, obtained results are in line with conclusions made by other researchers that stromal cells support self-renewal of progenitor compartment but they do not alter the differentiation and proliferation of mature cells [11].

It is important that in our study of precursor cells population grown for 3 weeks from isolated $CD34^+$ cells we observed a complete disappearance of erythroid progenitors independently of MSCs layer presence, whereas other authors, who studied kinetics of different hematopoietic progenitor cells in standard long-term dextertype cultures revealed a loss of significant amount of erythroid progenitors in 5 weeks and the complete absence of these cells in 7 weeks. The probable reason of disappearance of erythroid progenitors in our study could be in the high amount of cytokines, such as Flt-3-ligand, in the cultural medium, which provided the shift towards myelopoiesis.

In summary, we explored functional potential of MSCs derived from BM of children with malignancies after high-dose chemotherapy or radiation to maintain hematopoiesis *in vitro* and came to the conclusion that efficacy of patients' MSCs to support the proliferation and self-renewal of hematopoietic cells was not different from MSCs derived from bone marrow of healthy donors. In our study all experiments with MSCs derived from BM of patients with oncological disorders and BM of donors were performed in identical conditions, and both types of MSCs were derived and expanded using common protocol. Moreover, autologous MSCs of patients who were the candidates for HSCs transplantation, similarly to donors'MSCs, greatly increased the number of more primitive bipotent myeloid and pluripotent precursors, while co-cultivated with hematopoietic stem cells. Thus, we consider that co-transplantation of autologous MSCs is good way to improve hematopoietic stem cells engraftment and reduce a period of granulocytopenia after autologous HSCs transplantation in case of insufficient $CD34^+$ cell number in autologous transplant.

REFERENCES

1. **Bielski M, Yomtovian R, Lazarus HM, Rosenthal N.** Prolonged isolated thrombocytopenia after hematopoietic stem cell transplantation: morphologic correlation. Bone Marrow Transplant 1998; **22**: 1071–6.

2. **Dercksen MW, Rodenhuis S, Dirkson MKA, *et al.*** Subsets of $CD34^+$ cells and rapid hematopoietic recovery after peripheral-blood-stem cell transplantation. J Clin Oncol 1995; **13**: 1922–32.

3. **Breems DA, Blokland EAW, Siebel KE, *et al.*** Stroma-contact prevents loss of hematopoietic stem cell quality during *ex vivo* expansion of $CD34^+$ mobilized peripheral blood stem cells. Blood 1998; **91**: 111–7.

4. **Brugger W, Heimfeld S, Berenson RJ, *et al.*** Reconstitution of hematopoiesis after high-dose chemotherapy by autologous progenitor cells generated *ex vivo*. N Eng J Med 1995; **333**: 283–7.

5. **Williams SF, Lee WJ, Bender JG, *et al.*** Selection and expansion of peripheral blood $CD34^+$ cells in autologous stem cell transplantation for breast cancer. Blood 1996; **87**: 1687–91.

6. **Van der Loo JC, Ploemacher RE.** Marrow- and spleen-seeding efficiencies of all murine hematopoietic stem cell subsets are decreased by preincubation with hematopoietic growth factors. Blood 1995; **85**: 2598–606.

7. **Reyes M, Lund T, Lenvik T, *et al.*** Purification and *ex vivo* expansion of postnatal human marrow mesodermal progenitor cells. Blood 2001; **98**: 2615–25.

8. **Prockop DJ.** Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997; **276**: 71–4.

9. **Charbord P.** Stromal Support of Hematopoiesis. In: S Sell, ed. Stem Cells Handbook. Totowa, New Jersey: Humana Press Inc, 2004; 143–54.

10. **Ogawa M.** Differentiation and proliferation of hematopoietic stem cells. Blood 1993; **81**: 2844–53.

11. **Friedrich C, Zausch E, Sugrue SP, Gutierrez-Ramos JC.** Hematopoietic Supportive Functions of Mouse Bone Marrow and Fetal Liver Microenvironment: Dissection of Granulocyte, B-Lymphocyte and Hematopoietic Progenitor Support at the Stroma Cell Clone Level. Blood 1996; **87**: 4596–606.

12. **Migliaccio AR, Migliaccio G, Johnson G, *et al.*** Comparative analysis of hematopoietic growth factors released by stromal cells from normal donors or transplanted patients. Blood 1990; **75**: 305–12.

13. **O'Flaherty E, Sparrow R, Szer J.** Bone marrow stromal function from patients after bone marrow transplantation. Bone Marrow Transplant 1995; **15**: 207–12.

14. **Tauchmanovà L, Serio B, Puente A, *et al.*** Long-lasting bone damage detected by dual-energy X-ray absorptiometry, phalangeal osteosonogrammetry, and *in vitro* growth of marrow stromal cells after allogeneic stem cell transplantation. J Clin Endocrinol Metab 2002; **87**: 5058–65.

15. **Isaikina Y, Kustanovich A, Svirnovski A.** Growth kinetics and self-senewal of human mesenchymal stem cells derived from bone marrow of children with oncohematological diseases during expansion *in vitro*. Exp Oncol 2006; **28**: 146–51.

16. **Punzel M, Liu D, Zhang T, *et al.*** The symmetry of initial divisions of human hematopoietic progenitors is altered only by the cellular microenvironment. Exp Hematol 2003; **31**: 339–47.

17. **Robinson SN, Ng J, Niu T, *et al.*** Superior *ex vivo* cord blood expansion following co-culture with bone marrow-derived mesenchymal stem cells. Bone Marrow Transplant 2006; **37**: 359–66.

18. **Yamaguchi M, Hirayama F, Murahashi H, *et al.*** *Ex vivo* expansion of human UC blood primitive hematopoietic progenitors and transplantable stem cells using human primary BM stromal cells and human AB serum. Cytotherapy 2002; **4**: 109–18.

19. **Dexter TM, Testa NG.** Differentiation and proliferation of hemopoietic cells in culture. Methods Cell Biol 1976; **14**: 387–405.

20. **Xie CG, Wang JF, Xiang Y, *et al.*** Cocultivation of umbilical cord blood CD34+ cells with retro-transduced hMSCs leads to effective amplification of long-term culture-initiating cells. World J Gastroenterol 2006; **12**: 393–402.

21. **Wagner W, Wein F, Roderburg C, *et al.*** Adhesion of hematopoietic progenitor cells to human mesenchymal stem cells as a model for cell-cell interaction. Exp Hematol 2007; **35**: 314–25.

22. **Koller MR, Palsson MA, Manchel I, Palsson B.** Long-term culture-initiating cell expansion is dependent on frequent medium exchange combined with stromal and other accessory cell effects. Blood 1995; **86**: 1784–93.

23. **Koller MR, Manchel I, Brott DA, Palsson Bø.** Donor-to-donor variability in the expansion potential of human bone marrow cells is reduced by accessory cells but not by soluble growth factors. Exp Hematol 1996; **24**: 1484–93.

24. **Breems DA, Blokland EA, Ploemacher RE.** Stroma-conditioned media improve expansion of human primitive hematopoietic stem cells and progenitor cells. Leukemia 1997; **11**: 142–50.

25. **Verfaillie CM, Catanzaro P.** Direct contact with stroma inhibits proliferation of human long-term culture initiating cells. Leukemia 1996; **10**: 498–504.

26. **Harvey K, Dzierzak E.** Cell-cell contact and anatomical compatibility in stromal cell-mediated HSC support during development. Stem Cells 2004; **22**: 253–8.

27. **Breems DA, Blokland EA, Neben S, Ploemacher RE.** Frequency analysis of human primitive haematopoietic stemcell subsets using a cobblestone area forming cell assay. Leukemia 1994; **8**: 1095–04.

28. **Kadereit S, Deeds LS, Haynesworth SE, *et al.*** Expansion of LTC-ICs and Maintenance of p21 and BCL-2 Expression in Cord Blood CD34+/CD38– Early Progenitors Cultured over Human MSCs as a Feeder Layer. Stem Cells 2002; **20**: 573–82.

29. **Mayani H, Gutierrez-Rodriguez M, Espinoza L, *et al.*** Dexter-type long-term cultures established from human umbilical cord blood cells. Stem Cells 1998; **16**: 127–35.

ВЛИЯНИЕ МЕЗЕНХИМАЛЬНЫХ СТВОЛОВЫХ КЛЕТОК ИЗ КОСТНОГО МОЗГА ДЕТЕЙ С ОНКОЛОГИЧЕСКИМИ И ГЕМАТОЛОГИЧЕСКИМИ ЗАБОЛЕВАНИЯМИ НА ПРОЛИФЕРАЦИЮ И СПОСОБНОСТЬ К САМОПОДДЕРЖАНИЮ КЛЕТОК — ПРЕДШЕСТВЕННИКОВ ГЕМОПОЭЗА *IN VITRO*

Цель: **исследовали способность культивируемых *in vitro* мезенхимальных стволовых клеток (МСК), выделенных из костного мозга детей со злокачественными новообразованиями, получавших в качестве лечения высокодозовую полихимиотерапию, поддерживать гемопоэз *in vitro*. *Материалы и методы:* МСК выделяли и наращивали из проб костного мозга 8 пациентов и 9 здоровых доноров детского возраста. $CD34^+$ клетки выделяли из проб костного мозга доноров. Были поставлены эксперименты по совместному культивированию МСК и $CD34^+$ клеток в среде метилцеллюлозы и в среде для долгосрочного культивирования с добавлением цитокинов и при их отсутствии. *Результаты:* в метилцеллюлозной среде наличие МСК пациентов увеличивало количество коммитированных миелоидных предшественников в 2 раза, МСК совместно с ростовыми факторами стимулировало пролиферацию этого типа клеток в 7 раз, а полипотентных предшественников — в 14 раз. При долгосрочном культивировании с цитокинами на слое МСК общее количество ГСК на 21-й день возростало в 161,2 раза. При пролиферации $CD34^+$ клеток в среде с МСК содержание бипотентных КОЕ-ГМ повысилось с 4 до 11%, а полипотентных КОЕ-ГЭММ — с 0,1 до 0,6%. В экспериментах по экспансии CD34+ клеток результаты при использовании в качестве фидерного слоя МСК пациентов или МСК доноров статистически не отличались. *Выводы:* проведенные исследования позволяют сделать вывод о том, что МСК из костного мозга пациентов со злокачественными новообразованиями обладают достаточным функциональным потенциалом для пролиферации и самоподдержания ГСК. Ко-трансплантация аутологичных МСК при аутотрансплантации ГСК с низким содержанием $CD34^+$ клеток в трансплантате может являться перспективной для сокращения периода восстановления гемопоэза в ранний посттрансплантационный период.**

Ключевые слова: **мезенхимальные стволовые клетки, злокачественные новообразования, гемопоэз, предшественники гемопоэза.**