Proliferative Potential of Multipotent Mesenchymal Stromal Cells from Human Bone Marrow

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We studied the capacity of multipotent mesenchymal stromal cells isolated from human bone marrow (BM) to long-term passaging, cloning, and re-cloning. Initial multipotent mesenchymal stromal cells and cells after gene labeling were studied. Multipotent mesenchymal stromal cells were obtained from donors (13-59 years) and cultured for 7 passages. Third generation lentivector was used for delivery of green fluorescent protein marker gene. The procedure of infection revealed reduced proliferative potential of multipotent mesenchymal stromal cells from elder donors. Hierarchy of precursor cells differing by their proliferative potential was demonstrated in the culture of multipotent mesenchymal stromal cells. Three categories of multipotent mesenchymal stromal cells were identified: mature cells incapable of proliferation (75.7±2.4% population) and cells with low and high proliferative potential (17.6±2.1 and 6.7±0.3%, respectively). The relative content of these cells insignificantly differed from passage to passage. The efficiency of cloning also remains stable, but re-cloning capacity sharply decreased after passage 3 and completely disappeared in multipotent mesenchymal stromal cells after cryopreservation. Thus, cultured multipotent mesenchymal stromal cells represent a heterogeneous and hierarchically organized population and the characteristics of this population depend of the duration of culturing and age of BM donor. This should be taken into account when using multipotent mesenchymal stromal cells in clinical practice.

Key Words: *multipotent mesenchymal stromal cells; lentivector; proliferative potential; heterogeneity of cell population*

Homeostasis of BM is maintained by two types of SC: hemopoietic and mesenchymal SC. The hierarchical organization of the hemopoietic system [2] based on proliferative potential and differentiation capacity of hemopoietic SC allows quantitative characterization of precursor cells. The hierarchy of MSC is still poorly studied. *In vivo* experiments on mice demonstrated the existence of several categories of mesenchymal precursor cells differing by their capacity to transfer hemopoietic microenvironment and form clones in culture [1].

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Human MSC are studied *in vitro*. Human MSC are defined as plastic-adherent self-maintaining cells capable of differentiation into all lineages of this tissue under appropriate conditions [11]. However, their capacity to self-maintenance has not been proved yet. In light of this, the International Society for Cellular Therapy proposed to term these cells as multipotent mesenchymal stromal cells (MMSC), but not mesenchymal stem cells [6]. It was demonstrated that first passage MMSC represent a heterogeneous population of cells differing by their proliferative potential [8]. MMSC expanded in culture over many passages are now actively used in clinical practice [3]. For comprehensive characterization of MMSC, thorough analysis at a single cell level throughout the culturing period should be carried out.

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In the present study an attempt was undertaken to clone MMSC and to analyze the proliferative potential of obtained clones over 7 passages. We analyzed initial MMSC and MMSC genetically labeled using a lentivirus carrying green fluorescent protein gene. It was demonstrated that MMSC differed by their proliferative potential and represent a heterogeneous hierarchically organized cell population.

MATERIALS AND METHODS

BM samples were obtained from 17 healthy donors (8 men and 9 women) aged 13-59 years (median 31 years).

All BM samples were obtained for allogeneic transplantation at the Department of High-Dose Therapy of Hemoblastoses and Bone Marrow Transplantation, Hematological Research Center; informed consent was obtained from all donors.

Nucleated cells from BM cell suspension were isolated in α-MEM medium (ICN) with 0.1% methylcellulose (Sigma) and cultured at a seeding density of $2.5\text{-}3\times10^6$ cells per 25-cm^2 flask (T25) in α-MEM medium supplemented with 10% FCS (HyClone), 2 mM L-glutamine (ICN), 100 U/ml penicillin (Ferane), and 50 μg/ml streptomycin (Ferane). After attaining confluence, the cells were harvested using 0.25% trypsin solution in physiological saline (Sigma) with 0.02% EDTA (ICN) and subcultured at a seeding density of 4×10^3 cells per 1 cm^2 flask bottom. MMSC were cultured for 6-7 passages.

For evaluation of proliferative potential, MMSC at each passage were cloned in 96-well plates (1 cell per well) in α-MEM medium (ICN) with 10% FCS (HyClone) and 5 ng/ml basic fibroblast growth factor (kindly provided by M. E. Gasparyan, Laboratory of Protein Engineering, M. M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences). The number of clone-forming MMSC was determined using Poisson formula. After attaining confluence in the well, MMSC were transferred into 24-well, and then into 6-well plates and T25 flasks. Similarly, re-cloning was carried out using confluent MMSC sublayer from 96-well plate as the cell source.

The number of mitoses passed by the cell was determined as $\log_2$ (cell count in T25 or well of 96-well plate).

Osteogenic and adipogenic differentiation capacities of clones were analyzed using standard differentiation media [13].

For determining the concentration of fibroblast colony-forming units (CFU-F) in BM, 10^6 nucleated cells were placed in a plastic T25 flask in 5 ml α-MEM medium (ICN) with 20% FCS (HyClone). After 14 days, the formed fibroblast colonies were stained with 0.1% crystal violet in 20% methanol and counted under an inverted microscope.

For infection of MMSC with lentiviral vector, the medium was removed and viral particles were applied onto the cell layer (10^7 per flask in 3 ml α-MEM medium with 10% FCS and 8 μg/ml polybrene, Sigma). After 6 h the medium was replaced with 10 ml complete nutrient medium.

Lentiviral vector of the third generation LeGo containing marker gene of green fluorescent protein was obtained using phCMVC-VSV-G (R861), pGpur(R1246), pMDLg/pRRE, and pRSV Rev plasmids (kindly provided by K. Weber, Prof. B. Fehse, K. Stocking, and R. Tsien). Viral stocks were obtained by calcium-phosphate transfection of plasmids into PhoenixGP cells (kindly provided by Prof. B.Fehse). The concentration of viral particles was increased 100-fold by centrifugation at 18,000 rpm for 3.5 h. The titer of the virus was determined on 293T and PhoenixGP cells. The number of cells carrying the marker protein was determined on a flow cytofluorometer (Beckton Dickinson).

Gene expression was evaluated using quantitative Taqman real-time PCR with reverse transcription (RT-PCR).

For RNA isolation, standard protocol was used with minor modifications. Denaturation solution was added directly into the culture flasks (after preliminary washout with phosphate buffer), then the flask content was collected and further procedures were performed according to standard protocol [4]. Reverse transcriptase (M-MLV, Promega) was used for synthesis of primary DNA chains after hybridization of mRNA with poly-T primers according to manufacturer's instructions. The presence of the required gene was detected using specific primers and probes. All PCR were carried out on an Applied Biosystems device.

Gene expression was standardized for each sample by the expression of house-keeping *ACTB* (β-actin) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) genes. Restriction was performed in the following regimen: preliminary denaturation at 95°C for 10 min; than cyclic denaturation at 95°C for 20 sec, hybridization with primers and elongation at 60°C for 60 sec. All experiments were performed in triplicates.

The data were processed statistically using Student's *t* test.

RESULTS

Proliferative potential of unlabeled MMSC and MMSC labeled using a lentiviral virus carrying green fluorescent protein gene was studied. MMSC from 6 donors (4 women and 2 men) did not proliferate after infection, while in non-infected cultures of the same

cells the total cell production did not significantly differ from the median for all donors (3.737×10^9 cells, Table 1). The age of all these donors was above the median (31 years). The mean age of these women was 45.3±5.3 years, and the age of the two men was 46 and 59 years.

No correlations between cumulative cell production in MMSC cultures, donor age, and concentration of CFU-F in BM samples were revealed. Some authors reported that the concentration of CFU-F in BM and proliferative potential of stromal cells decreased with age [3,12], while others did not reveal such changes [7]. However, stress influences, *e.g.* infection with lentiviral vector *in vitro*, can modulate age-related changes in MMSC. After infection, MMSC can be divided into 2 groups: MMSC passing 1-2 passages with subsequent proliferation arrest (group 1) and MMSC passing more that 4 passages (group 2). Donor's age in groups 1 and 2 was 47.7±4.0 and 24.8±3.3, respectively (p=0.001). Thus, the decrease in the proliferative potential of MMSC after stress exposure is seen even in this small sample.

Expression of insulin-like growth factor 1 (IGF1) in stromal cells of mouse BM increases with age, which impairs regulatory functions of hemopoietic microenvironment. This affects the properties of hemopoietic stem cells [9]. Analysis of the expression of IGF1 in MMSC of passages 1-2 from 32 donors (age 13-59 years) revealed no correlation with age. Nevertheless, a tendency towards the increase in IGF1 expression was seen in MMSC passing no more that 2 passages after infection (group 1) in comparison with that in group 2 MMSC (relative expression levels 49.6±30.4 and 24.6±9.0, respectively). At the same time, MMSC with higher proliferative potential (group 2) demonstrated a tendency towards the increase in relative level of expression of bone morphogenetic protein 4 (BMP 4; 0.9±0.4 and 2.4±1.2, respectively) responsible for proliferation of mesenchymal stromal cells and inducing division of hemopoietic stem cells [10]. These findings attest to negative correlation between the proliferative potential of MMSC and donor's age.

For evaluation of proliferative potential, labeled MMSC from 6 donors were cloned from single cells over 7 passages. The number of cells in confluent

TABLE 1. Characteristics of BM Donors and MMSC Obtained from These Donors

Donor No.	Sex	Age	CFU-F $\times10^6$	Number of passages after infection	CCP		REL	
					labeled MMSC, $\times10^6$	over 5 passages, $\times10^6$	BMP4	IGF1
75	F	24	3.3	6	1791	4878	0.4	87.3
76	F	13	31.3	4	987	1193	12.6	1.0
77	F	16	23	4	1396	1952	0.5	17.1
82	F	31	8.7	1	0.18	N.s.	0.01	12
83	M	59	9.7	1	0.09	2148	2.9	73.4
84	M	27	23.3	7	2689	4077	1.4	13.3
85	F	45	25.6	1	0.1	2772	0.1	192.8
86	F	49	50.0	1	0.09	3305	1.3	12.0
87	M	22	121.3	9	2218	3397	2.1	34.3
88	M	38	8.7	4	517	830	0.5	0.5
89	M	41	48.7	7	7393	12898	0.4	5.7
90	M	46	10.5	2	467	630	0.5	5.8
91	F	56	8.3	1	0.13	4834	0.4	2.1
93	F	15	19	6	7323	13505	3.3	0.2
98	M	43	34	6	8764	11275	0.1	56.7
100	F	16	14	7	6791	22213	0.5	30.2
103	M	18	90	7	32297	38895	n.s.	n.s.

Note. CCP: cumulative cell production; REL: relative expression level; BMP4: bone morphogenetic protein; N.s.: differences are not significant.

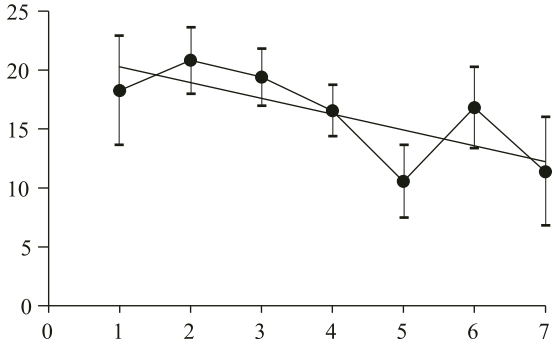

Fig. 1. Mean number of clones per 60 cells. Abscissa: passage number; ordinate: number of clones. Straight line shows the trend.

monolayers in T25 flasks was about 556±48×10^3 and slightly varied from passage to passage. The percent of labeled cells was also stable: 60.1±4.3%. About 180-240 cells were cloned at each passage, *i.e.* 1/3000 of labeled population was studied in these experiments. The mean efficiency of cloning for passages 4-7 was 0.34±0.03, *i.e.* every third cell formed a clone. The mean number of clones formed by MMSC decreased from passage to passage (Fig. 1), which attests to a decrease in proliferative potential of MMSC.

After attaining confluence in the well of 96-well plate, MMSC were transferred into 24-well, and then into 6-well plates and T25 flasks. The cells not forming the clone were considered "mature" (they constituted 75.7±2.4% MMSC population). We accepted that cells not forming confluent monolayer in 96-well and 24-well plates possessed low proliferative potential (LPP; 17.6±2.1%) and cells forming confluent monolayer in 6-well plates and T25 flasks had high proliferative potential (HPP; 6.7±0.3%). The number of clones with HPP was 2-3-fold lower that clones with LPP (Fig. 2, *a*). The percent of LPP clones among all proliferating MMSC was 71.4±1.9% (Fig. 2, *b*). Two populations can be distinguished among MMSC with HPP: cells attaining confluence in 6-well plate, *i.e.* passing ≥16 mitoses and cells attaining confluence in T25 flask, i.e. passing ≥18 mitoses. These cells constituted 2.9±0.4 and 3.8±0.7% of the total MMSC population, respectively.

It was demonstrated for each passage that clones attaining confluence in T25 flasks were capable of osteo- and adipogenic differentiation under the action of the appropriate inductors.

Cloned MMSC from 4 donors were re-cloned from wells of 96-well plate after attaining confluence after 2 and 3 weeks (rapidly and slowly proliferating clones, respectively). It was found that rapidly proliferating clones contained greater number of clonogenic precursors that slowly proliferating ones (24.7±11.7 and 7.3±3%, respectively). The number of clonogenic precursors correlated with the size of the initial clone (number of cells in the clone 2583±810 and 916±340 and number of clonogenic precursors in the clone 24±9 and 5±4, respectively; correlation coefficient 0.94). The existence of correlation between the size of the initial clone and the number of clonogenic precursors in the given clone attests to MMSC hierarchy.

The re-cloning capacity of MMSC clones decreased from passage to passage. The content of clonogenic precursors during passages 1, 2, 3, and 4 was 24.7±11.7, 17.3±6.1, 15.8±1.5, and 4.4±2.5%.

MMSC after cryopreservation were incapable of re-cloning.

Thus, MMSC aged during culturing and gradually lost highly proliferating clonogenic precursors. Despite the fact that MMSC can survive cryopreservation and can proliferate with the formation of a large cell mass, clonogenic precursors irreversibly lost the proliferative potential. Moreover, the number of MMSC with HPP decreased during culturing, which attests

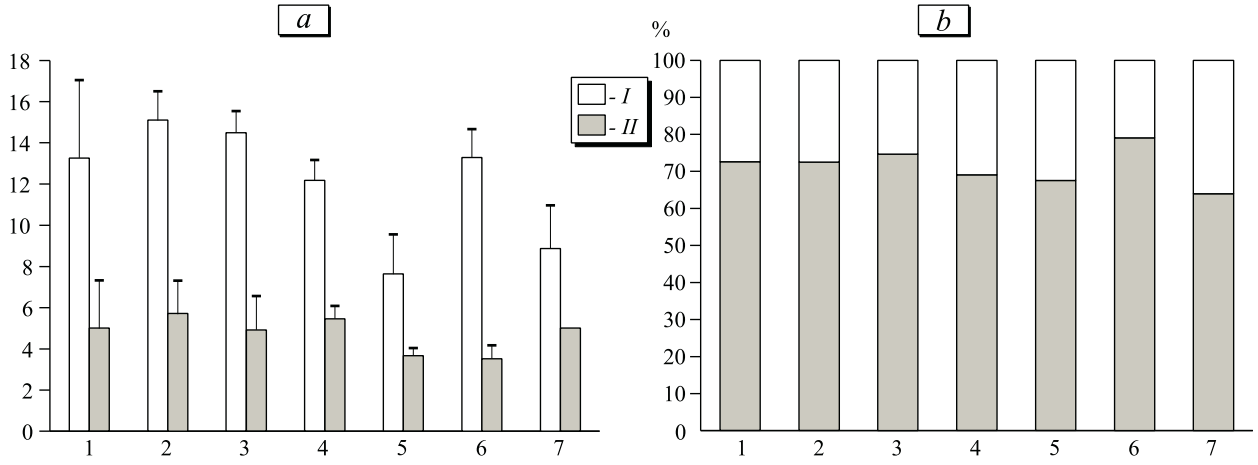

Fig. 2. Characteristics of MMSC clones. *a*) mean number of clones with LPP (*I*) and HPP (*II*; per 60 cells). Abscissa: passage number; ordinate: number of clones. *b*) ratio of clones with LPP (*I*) and HPP (*II*; standardized for 1 plate): abscissa: passage number; ordinate: percent of clones.

to exhausting of this population due the absence of true MSC or their inability of self-maintenance under conditions of cell culture.

These findings confirm hierarchical organization in the population of cultured human MMSC [8]. MMSC differ by their proliferative potential. At least 3 cell populations can be distinguished: mature non-proliferating cells and cells with LPP and HPP. Proliferative potential of MMSC decreases with increasing donor's age and duration of culturing. These data should be taken into account when using MMSC for therapeutic purposes and especially for gene therapy and regenerative medicine.

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