Characteristics of Mesenchymal Stromal Precursor Cells Labeled with Lentiviral Vector in Long-Term Bone Marrow Culture

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Mouse mesenchymal stromal precursor cells were labeled with lentiviral vector in long-term bone marrow culture. We studied the fate of labeled cells in the stromal sublayer of the long-term bone marrow culture and in ectopic hemopoiesis foci formed from the labeled cultures. The incidence of labeled polypotent fibroblast CFU in sublayers of long-term bone marrow culture and in ectopic hemopoiesis foci formed from these sublayers under the renal capsule of syngeneic mice was also analyzed. It was shown that the marker gene was present in about 40% cells of the stromal sublayer and 30% fibroblast CFU and that effective gene transfer did not affect the total production of hemopoietic cells. The size of ectopic hemopoietic foci formed after implantation of labeled sublayers of the long-term bone marrow culture under the renal capsule did not differ from the control. Differentiated cells of the osseous shell in these foci carried the marker gene in 40% cases. Analysis of fibroblast CFU in these foci showed that despite the total concentration of fibroblast CFU was comparable to that in the bone marrow, the concentration of labeled fibroblast CFU was about 6%, which suggests that one more class of precursors probably exists in the hierarchy of stromal cells presumably between mesenchymal stem cells and fibroblast CFU. Our findings demonstrate the capacities of mesenchymal stem cells to self-maintenance and differentiation without loosing the marker gene integrated into the genome.

Key Words: *stromal mesenchymal cells; fibroblast colony-forming units; lentiviral vector; focus of ectopic hemopoiesis; long-term bone marrow culture*

All self-renewing tissues of the adult organism contain stem cells (SC). Differentiation potential of SC is described, but the hierarchy of precursors is determined not for all tissues. For instance, precursors of SC of hair follicle, sebaceous and sudoriferous glands, and interfollicular epidermis, skin SC carrying *Lgr6* marker gene were described in 2010 [11]. In the bone marrow, two types of SC co-exist: hemopoietic (HSC) and mesenchymal (MSC). The hierarchy of HSC is well studied [3,4], but little is known about the hierarchy of MSC. For mice, co-existence of true MSC was proven; they are capable of self-maintenance and differentiation and form the focus of ectopic hemopoiesis after transplantation of the bone marrow cylinder under the renal capsule of a syngeneic recipient [6]. It was found that fibroblast CFU (CFU-F) are polypotent descendants of MSC with lower proliferative potential incapable of hemopoietic microenvironment transfer [10]; inducible stromal precursors forming hemopoietic microenvironment in long-term bone marrow culture (LTBMC) and in foci of ectopic hemopoiesis are even more differentiated descendants [1]. MSC label-

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ing opens new possibilities for the studies of stromal SC. Under conditions of physiological stability, MSC are rarely dividing cells; therefore, lentiviral vector systems are a convenient tool for marker gene transfer into these cells.

Lentiviral vectors can transduce both dividing and resting target cells [12]. This system was used for *in vivo* gene introduction into renal, endothelial, retinal, hemopoietic, and other cells. Mesenchymal stromal cells are the source of pluripotent precursor cells for tissue engineering; some attempts at *in vitro* gene introduction into these cells were undertaken [7].

Here we labeled mouse MSC *in vitro* in adherent cell layer (ACL) of LTBMC using lentiviral vectors. The fate of labeled MSC and their descendants was studied after retransplantation of labeled ACL under the renal capsule of syngeneic recipients followed by the analysis of CFU-F and terminally differentiated SC. It was shown that this approach can be successfully used for the studies of mesenchymal cell hierarchy and for development of methods of MSC application in gene therapy.

MATERIALS AND METHODS

Experiments were performed on 9-26-week-old female $(CBA\times C57Bl/6)F_1$ mice.

LTBMC was maintained as described previously [8]. Bone marrow from one femur was placed into a 25-cm^2 flask in 10 ml complete culture medium of the following composition: 80% Fisher medium (ICN), 2 mM glutamine (ICN), 10^{-6} M hydrocortisone (Sigma), antibiotics (100 U/ml penicillin, 50 μg/ml streptomycin) and 20% serum ($^1/_3$ FCS, HyClone+$^2/_3$ equine serum, GibcoBRL). The cells were cultured at 33°C and 5% CO_2; the medium was half-replaced weekly, the removed cells were not returned.

For studies of cell properties, ACL was disintegrated with 0.025% trypsin (ICN), the cells were washed and cloned in 96-well plates (5-200 cells per well) in α-MEM medium (ICN) with 20% FCS (HyClone) and 5 ng/ml basic fibroblast growth factor (bFGF; 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 labeled CFU-F was determined using Poisson formula.

For the analysis of stromal precursors in the culture, the sublayer cells were harvested with a scraper (taking care of minimal separation of cell from each other) and implanted under the renal capsule of syngeneic mice as described previously [2].

For determining the concentration of CFU-F, 5×10^5 cells from the ectopic hemopoietic focus were placed in a 25-cm^2 plastic flask in 5 ml α-MEM medium (ICN) with 20% FCS (HyClone) and 5 ng/ml bFGF. After 14 h, the formed fibroblast colonies were stained with 0.1% crystal violet on 20% methanol and counted under an inverted microscope. In some cases, DNA was isolated from pooled colonied for the analysis of provirus integration.

For infection of LTBMC with the lentiviral vector, the medium was completely removed from the flasks and viral particles were applied onto the sublayers: 10^7 per flask in 3 ml α-MEM with 10% FCS and 8 μg/ml polybrene (Sigma). After 6 h the medium was replaced with 10 ml complete culture medium.

Lentiviral vectors of the third generation LeGo containing marker gene of green fluorescent protein eGFP and red fluorescent protein Cherry (C2) were obtained using phCMVC-VSV-G (R861), pGpur(R1246), pMDLg/pRRE, and pRSV Rev plasmids (kindly provided by Prof. B. Fehse, University Hospital Eppendorf, Hamburg, and R. Tsien, HMIM San Diego). 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 measured on a flow cytofluorometer (Becton Dickinson).

PCR analysis of DNA for the presence of eGFP gene was performed with primers EGFP-w1: 5'-ATG-GTGAGCAAGGGCGAGGA-3' (forward) and EGFP-C1: 5'-AGACGTTGTGGCTGTTGTAG-3' (reverse) wielding a 454-b.p. fragment. C2 gene was detected using primers Ch-D: 5'-ACC-CAG-GAC-TCC-TCC-CTG-CA-3' (forward) and Ch-com-R: 5'-CAC-ATA-GCG-TAA-AAG-GAG-CAA-C -3' (reverse) allowing the synthesis of a 559-b.p. fragment. PCR was performed under the following conditions: 1 min at 94°C, 1 min at 62°C, and 2 min at 72°C (32-36 cycles). The products were separated by electrophoresis in 2% agarose gel.

The data were processed statistically using Student *t* test.

RESULTS

Six hours after infection of LTBMC with the lentiviral vector, 43.30±10.98% ACL cells included the provirus and expressed the marker protein. Relative content of labeled cells increased from 15 to 87% (median 26.4%). Relative content of labeled cells did not correlate with the total number of cells in ACL. The mean number of cells in ACL was $(1.34\pm0.26)\times10^6$ and varied from 0.33×10^6 to 2.34×10^6 per flask (median 1.22×10^6). Infection of the culture with the virus did not affect the production of hemopoietic cells. The

total cell production in control and infected cultures did not significantly differ over 15 weeks after infection (Fig. 1).

The number of labeled CFU-F from ACL was lower than the number of labeled cells in 4 of 6 experiments (32.0±4.3%). The proportion between labeled cells in the sublayer and labeled CFU-F is present in Figure 2. The incidence of labeled CFU-F calculated by the Poisson formula varied from 1:11 to 1:690 sublayer cells. Since CFU-F are polypotent precursors of stromal microenvironment cells, these findings attest to their heterogeneity by the proliferative potential, *i.e.* by the capacity to form different number of cells. According to published data, only 7% mesenchymal precursor cells of human bone marrow exhibit high proliferative potential, 29% have low proliferative potential, while 26% can form clusters only [9].

Thus, the lentivirus infects both differentiated cells and precursor cells of the stroma, which stably express the marker protein *in vitro*. Some labeled CFU-F are capable of retransplantation. The cells from flasks with CFU-F from ACL LTBMC were harvested with trypsin and recloned into 25-cm^2 flasks (300,000 cells per flask). In 2 of 4 experiments, only clusters carrying the marker gene were formed and 4 and 27 labeled colonies were obtained in two other cases. These findings confirm stable maintenance of the marker gene during retransplantation *in vitro* and heterogeneity of CFU-F.

Implantation of ACL under the renal capsule of syngeneic mice leads to the formation of a focus of ectopic hemopoiesis, where hemopoietic cells originate from the recipient and stromal cells have donor origin. The size of the focus is determined by the content of nucleated cells and is directly proportional to the number of transplanted MSC capable of hemopoietic microenvironment transfer [2]. Labeling of stromal cells with lentivirus had no effect on the capacity of MSC to form hemopoietic microenvironment after implantation under the renal capsule in mice. The size of foci formed by infected ACL did not significantly differ from the size of foci formed by ACL of control cultures (2.05±0.54 and 2.8±0.7 nucleated cells, respectively). Only MSC are involved into the formation of ectopic hemopoiesis focus, while implanted mature cells are eliminated during the first few days after transplantation [5]. Labeled MSC differentiate into all elements of stromal microenvironment. For instance, labeled osteogenic cells were present in 54.16±11.68% osseous shells in these foci. Nucleated cells washed from the shells and carrying the marker gene were detected in 41.10±16.82% foci. These findings confirm preserved differentiation capacity of MSC carrying the alien gene. MSC carrying the marker gene are characterized by high proliferative potential, which

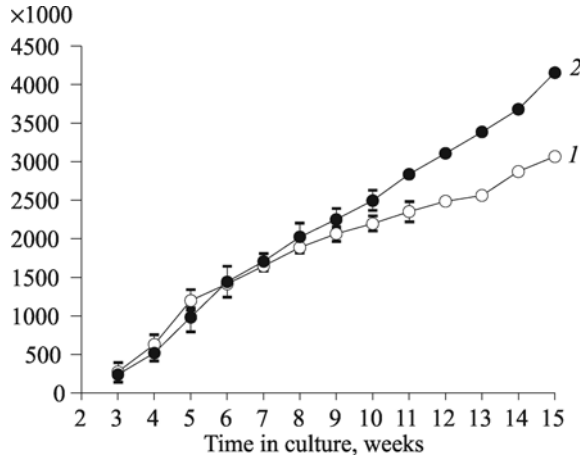

Fig. 1. Cumulative cell production in LTBMC infected with lentiviral vector (data of 5 experiments). Ordinate: cell number per 1 ml culture. *1*) control; *2*) virus.

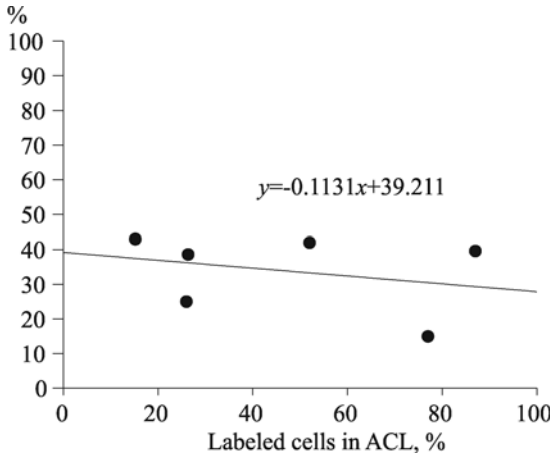

Fig. 2. Relationship between the relative number of labeled cells in ACL and relative number of labeled CFU-F. Ordinate: labeled CFU-F from ACL.

was demonstrated by the analysis of colonies formed by descendants of CFU-F from stromal cells of ectopic hemopoiesis focus. The concentration of CFU-F among stromal cells of ectopic hemopoiesis focus formed by ACL of labeled and control cultures was similar and did not differ from than in mouse bone marrow (Fig. 3). The relative content of labeled CFU-F was 6.16±2.97%. These labeled stromal precursor cells, descendants of infected MSC first forming the microenvironment in culture were transferred under the renal capsule, where they formed an ectopic hemopoiesis focus and then were again transferred into culture, where their descendants, CFU-F, formed labeled colonies. Considerable differences (by almost 5 times) in the relative content of labeled CFU-F among ACL cells and in ectopic hemopoiesis foci formed from these ACL, suggest that one more class of precursors can exist in the hierarchy of stromal cells. Its position in the hierarchy is between MSC and CFU-F, because

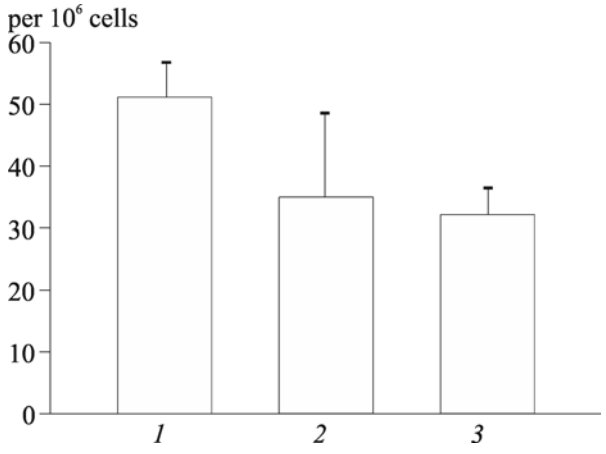

Fig. 3. Concentration of CFU-F in the bone marrow and ectopic hemopoiesis foci from ACL LTBMC. *1*) bone marrow; *2*) transplant from LTBMC; *3*) transplant from labeled LTBMC.

retransplantation does not reduce the relative content and proliferative potential of MSC [6]. Hence, the decrease in the relative content of labeled CFU-F in ectopic hemopoiesis focus compared to that immediately after infection of sublayer cells can be explained by the existence of a precursor incapable of microenvironment transfer, but labeled with the lentiviral vector, involved in the formation of ACL, and capable of differentiating into CFU-F, rather then the loss of MSC (because a focus of normal size is formed).

Our findings demonstrate the capacities of MSC to self-maintenance and differentiation without loosing the marker gene integrated into the genome. Both MSC and their descendants at different levels of stromal cell hierarchy carry the alien gene. It was shown that the marker gene was present in MSC, CFU-F, and mature stromal cells. Under conditions of physiological stability, all these cells are relatively long-living and infrequently dividing cells. Thus, it is evident that MSC are not only suitable, but also promising object for gene therapy.

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