Infection of Stromal and Hemopoietic Precursor Cells with Lentivirus Vector *In Vivo* and *In Vitro*

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We developed a method for gene transfer into mesenchymal stromal cells. Lentivirus vector containing green fluorescent protein gene for labeling stromal and hemopoietic precursor cells was obtained using two plasmid sets from different sources. The vector was injected into the femur of mice *in vivo* and added into culture medium for *in vitro* infection of the stromal sublayer of long-term bone marrow culture. From 25 to 80% hemopoietic stem cells forming colonies in the spleen were infected with lentivirus vector *in vivo* and *in vitro*. Fibroblast colony-forming cells from the femoral bones of mice injected with the lentivirus vector carried no marker gene. The marker gene was detected in differentiated descendants from mesenchymal stem cells (bone cavity cells from the focus of ectopic hemopoiesis formed after implantation of the femoral bone marrow cylinder infected with lentivirus vector under the renal capsule of syngeneic recipient). In *in vitro* experiments, the marker gene was detected in sublayers of long-term bone marrow cultures infected after preliminary 28-week culturing, when hemopoiesis was completely exhausted. The efficiency of infection of stromal precursor cells depended on the source of lentivirus. The possibility of transfering the target gene into hemopoietic precursor cells *in vivo* is demonstrated. Stromal precursor cells can incorporate the provirus *in vivo* and *in vitro*, but conditions and infection system for effective infection should be thoroughly selected.

Key Words: *hemopoietic stem cells; stromal microenvironment; lentivector; ectopic hemopoiesis focus; long-term bone marrow culture*

Gene transfer into cells of various organs is the main task and aim of gene therapy. Lentivirus vectors can transduce dividing and silent target cells [16]. This system was used for gene introduction *in vivo* into renal [6], endothelial [1], retinal vascular [13], hemopoietic [11,16], and other cells. Gene transduction by lentivectors was used for the treatment of tumors [7], hereditary diseases [5], and AIDS [15]. As mesenchymal stromal cells are sources of pluripotent precursor cells for tissue engineering, attempts were made at gene transduction in these cells *in vitro* [2,10,12].

The hierarchy of mesenchymal stem cells (MSC) remains not studied. It is known that mesenchymal stromal cells capable of adipogenic, fibroblast, chondrogenic and osteogenic differentiation are stem cells proper or their closest descendants [7]. In addition to these cells, polypotent precursors forming fibroblast colonies in culture (CFUf) are distinguished [9]. True MSC capable of self-maintenance and differentiation and forming ectopic hemopoiesis foci after transfer of the bone marrow cylinder under the renal capsule of syngeneic animals were

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demonstrated for mice. Individual labeling of MSC is essential for studies of hierarchy of these cells and their developmental potential. Introduction of the marker gene into MSC is a difficult problem, because MSC, similarly as the majority of the stromal microenvironment cells, divide much more rarely than hemopoietic stem cells. Moreover, no universal methods for obtaining and culturing of mouse MSC were developed up to the present time. Foreign genes were introduced into human, porcine, simian, and rat mesenchymal stromal cells only by *ex vivo* methods.

Now we tried to label mouse MSC *in vivo* and *in vitro* using lentivirus vectors. This is essential for studies of the hierarchy of the mesenchymal cell compartment and for the development of methodological approaches to the use of MSC in gene therapy.

MATERIALS AND METHODS

The study was carried out on 9-26-week-old (CBA× C57BL/6)F_1 female mice. A needle No. 21 was injected into the femoral bone through the knee joint and a small volume of the bone marrow was aspirated with a dry syringe. The bone marrow was removed from the needle cone with a cotton turunda, after which a Hamilton syringe needle was inserted into the needle and 20 μl suspension of viral particles in serum-free medium (ICN) with 1% BSA (Gibco) was injected. Hamilton syringe was removed from the needle and inserted again to pump the air through the needle.

Splenic CFU (CFUs) were analyzed by Till and McCulloch's method [14]. Bone marrow CFUf (10^6) were placed into a 25-cm^2 plastic flask in 5 ml a-MEM (ICN) with 20% FCS (HyClone) and 5 ng/ml basic fibroblast growth factor (a gift from M. E. Gasparian, Cand. Biol. Sc., Laboratory of Protein Engineering, Institute of Organic Biochemistry). After 14 days, the resultant fibroblast colonies were stained with 0.1% crystal violet in 20% methanol and counted under an inverted microscope. In some cases DNA was isolated from the pool of colonies for the analysis of provirus integration. Long-term bone marrow culture (LBMC) was maintained as described previously [14]. The focus of ectopic hemopoiesis from the bone marrow or LBMC sublayer was obtained by the standard method.

For LBMC infection with the lentivirus vector the culture medium was completely removed from the flasks and viral particles were applied onto the cell layer in a dose of 10^7 cells per flask in 3 ml complete nutrient medium with 4 μg/ml polybrene (Sigma). After 16 h the medium was replaced with 10 ml complete nutrient medium.

Third-generation lentivectors containing eGFP marker gene were obtained using phCMVC-VSV-G(R861), pGpur(R1246), pMDLg/pRRE, and pRSV Rev plasmids [4], kind gifts from laboratory headed by Prof. J. Dick (University of Toronto) with permission from Prof. L. Naldini (Milan) and from Prof B. Fehse (University Hospital Eppendorf). Viral stocks were obtained by calcium phosphate transfection of plasmids in 293T or PhoenixGP cells (gift from Prof. B. Fehse). Virus titer was determined using NIH3T3, HeLa, 293T, and PhoenixGP cells. Green cells carrying the marker protein were counted on a flow cytofluorometer (Becton Dickinson). The virus vector for injection to mice was concentrated 100-fold by centrifugation (3 h at 20,000 rpm).

PCR analysis of DNA for detection of eGFP gene was carried out with the following primers: forward EGFP-w1 (5'-ATGGTGAGCAAGGGCGAGGA-3') and reverse EGFP-C1 (5'-AGACGTTGTGGCTGTTGTAG-3') using which a 454 b.p. fragment was synthesized. Primers complementary to Smc gene were used for internal control (to confirm the presence and integrity of the analyzed DNA): SmcVD 5'-GAGAGGAGGATCTTGGACCT-3' (forward) and SmcUR 5'-CACCGACGGTCCTTGCAGAT-3' (reverse) using which a 360 b.p. fragment was synthesized from a gene copy located in X chromosome. Both pairs of primers were used in the multiplex variant, each in a concentration of 0.5 μM. Smc primers could also be used in a concentration of 0.125 μM. A total of 32-36 PCR cycles were carried out under the following conditions: 1 min at 94°C (denaturation), 1 min at 62°C (annealing) and 2 min at 72°C (synthesis). The resultant fragments were analyzed by electrophoresis in 2% agarose gel.

The data were statistically analyzed by Student's *t* test.

RESULTS

Mice were injected with 10^5 viral particles in 20 μl into each femur; the viral particles were obtained by using vector from the laboratory headed by J. Dick (the virus was titered on HeLa cells). After 6 weeks, the bone marrow from one femoral bone was explanted in LBMC. In parallel with this, 10^6 cells from the same bones were inoculated in the test for evaluation of CFUf concentration. The bone marrow cylinder from the other femoral bone was explanted under the renal capsule of syngeneic animals. Cells from the crural bone marrow (40,000 cells) were injected to lethally irradiated mice for the analysis of labeled CFUs. DNA for PCR ana-

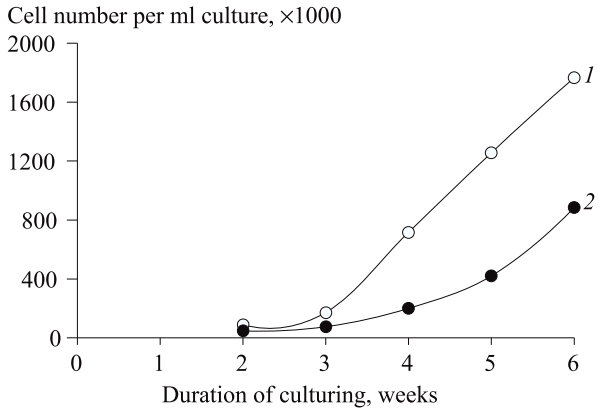

Fig. 1. Cumulative cell production in LBMC, infected with lentivector. *1*) control; *2*) virus.

lysis for evaluation of the provirus integration was isolated from the remaining cells (Table 1).

Viral particles were injected directly into the mouse femur, but high percent (43.8±12.2%) of labeled CFUs was found after 6 weeks in another hemopoietic territory, crural bones. It is noteworthy that the marker gene could remain undetected in the total fraction of the crural bone marrow cells by PCR analysis, which could be explained by low incidence of labeled early hemopoietic precursors in the bone marrow insufficient for the sensitivity of the selected PCR conditions. Injection of the lentivirus vector did not modify CFUs concentration in the bone marrow and was not toxic for recipients. We should speak about the efficiency of hemopoietic precursor cells infection with great care, because hemopoietic cells constantly divide, actively migrate, and the life span of the majority of precursors is short. However, local injection of just little amount of viral particles led to the appearance of numerous labeled precursors on another hemopoietic territory. This fact can be used for short-term gene therapy with direct introduction of the target gene in hemopoietic precursors omitting the *ex vivo* infection phase.

The bone marrow from the femoral bones of infected mice was inoculated into LBMC, used for evaluation of CFUf concentration, and transplanted to syngeneic mice under the renal capsule. All analyzed fibroblast colonies (more than 70 from each mouse) were not labeled. Provirus incorporation was detected after 12 weeks in half of adherent LBMC cell sublayers.

After transplantation of the bone marrow cylinder under the renal capsule of syngeneic animals, the stroma in the forming focus of ectopic hemopoiesis was from the donor and the hemopoietic cells belonged to the recipient. The foci were isolated under sterile conditions and the cells from them were inoculated in LBMC, the CFUf concentration was evaluated, and DNA was isolated from the bone shell. The volume of material was insufficient, and hence, DNA from bone shell was pooled. The marker gene was detected in it. On the other hand, neither fibroblast colonies from the foci, nor LBMC sublayers contained the provirus. These data indicate very low efficiency of infection of MSC and CFUf descending from them by the lentivector used.

Lentivirus infection of LBMC *in vitro* suppressed hemopoiesis in the culture (Fig. 1). The summary cell production decreased more than 2-fold within 6 weeks. Only 0.5% cells expressing the marker gene were detected in the suspension fraction of the cultures 1 and 2 weeks after lentivirus infection. After 6-week culturing (by the moment of the sublayer implantation under the renal capsule), 25% CFUs from the suspension fraction carried eGFP gene. The resultant ectopic hemopoiesis foci were again explanted in LBMC for obtaining stromal sublayers carrying minimum admixture of hemopoietic cells. No marker gene in sublayers were detected by PCR analysis. This indicates very low efficiency of *in vitro* MSC infection with this lentivirus.

The lentivector offered by Dr. B. Fehse proved to be more effective (virus stock titer 10^8). The sublayer cells were removed with trypsin 4 weeks after infection of old non-hemopoietic 28-week culture. The number of cells in the control culture sublayers was $1.2\pm0.03\times10^6$, in sublayers of virus-infected cultures 3.3×10^5. It seems that this lentivirus vector is toxic for LBMC cells, similarly as the previous one. The number of cells in infected

TABLE 1. Parameters of Provirus Integration in Hemopoietic Precursors (CFUs) and Bone Marrow Cells

Mouse No.	Number of CFUs per 10^5 crural bone marrow cells	Labeled CFUs, %	Integration in total crural bone marrow cells
1	46.7±2.0	80	—
2	35.8±2.7	35	—
3	39.2±1.0	28.6	+
4	35.8±2.7	31.6	+

sublayers dropped 4-fold, but the survivors retained proliferative activity and their effective label was detected by PCR analysis. The sublayer cells were inoculated in 96-well plates (3000 and 4300 cells per well). After 1 week, the sublayer cells were removed, cells from 5 wells were pooled and analyzed by the PCR for detecting the marker gene. All analyzed 12 pools carried the marker gene. In the experiment with lentivirus vector provided by Prof. J. Dick the marker was detected in just 2 of the 4 studied sublayers, this indicating the significance of selection of the lentivirus vector for effective gene transfer into stromal cells. After passages of the sublayer cells, the gene was detected in 8 of 32 DNA samples isolated from cells which underwent 3 passages, this indicating that intact cells replaced the infected ones.

Hence, MSC and stromal microenvironment cells can be labeled with lentivirus vectors *in vivo* and *in vitro*, but the efficiency of their infection will be significantly lower than that of hemopoietic cells.

Obviously, the efficiency of systems making use of lentivirus vectors for gene transfer into hemopoietic and stromal cells varies greatly and its components should be thoroughly selected, particularly for MSC.

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