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Growth factors increase retroviral transduction but decrease clonogenic potential of umbilical cord blood CD34+ cells

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Abstract

Background and Objective. **The feasibility of gene marking or gene therapy protocols making use of purified CD34+ cells greatly depends on the efficiency of their stable transduction. The great potential of umbilical cord blood as a source of CD34+ cells combined with the availability of advanced cell purification procedures prompted us to evaluate whether incubation with growth factors might influence the type of cells effectively transduced by retroviral vectors.**

Design and Methods. **Isolated, at least 95% pure, CD34+ cells were infected with the LXSN murine retrovirus carrying the neomycin-resistance gene. Different schedules of CD34+ cell infection were performed with or without incubation for different times in the presence of interleukin-3 (IL-3), interleukin-6 (IL-6) and stem cell factor (SCF). Efficiency of transduction was evaluated by clonogenic assays, semiquantitative PCR and RT-PCR analyses performed either immediately or after 7 day expansion of CD34+ cells in liquid culture in the presence of erythropoietin (EPO), IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF).**

Results. **The results obtained indicated that the amount of transduced cells increased with the lenght of incubation with growth factors, either before or during infections. However, different types of cells were transduced depending on the duration of stimulation and infection. Thus, following one week culture of CD34+ cells in the presence of EPO, IL-3 and GM-CSF the clonogenic potential was affected dyshomogeneously. Precisely, with a single 3-hour infection performed after 12 hours of stimulation with growth factors, the clonogenic potential of the transduced cells greatly increased after one week in culture. In contrast, with a 48 hour infection, the transduced cells completely lost their clonogenic potential after one week in culture.**

Interpretation and Conclusions. **These results demonstrate that a reasonably high transduction efficiency of purified CD34+ cells can be achieved with short schedules of incubation/infection in the absence of stroma or extracellular matrix.**

Key words: retrovirus-mediated gene transfer, CD34+ cells, umbilical cord blood, PCR, hematopoietic growth factors

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Gene therapy approaches have opened up new perspectives in the treatment of a variety of genetic and acquired disorders, such as cancer and AIDS.[1,2] Hematopoietic stem cells are an attractive target for gene therapy because, in principle, genetic modification of a small number of cells could provide a person with stable populations of genetically modified cells.[3] Studies in animal models have shown that retrovirally-marked hematopoietic progenitor cells contribute to the replenishment of all major lineages and a small number of these cells (10^6/kg) can effectively repopulate the entire system.[4-6] In addition, CD34+ progenitor cells can be easily isolated from bone marrow (BM) or blood, genetically modified *ex vivo* and returned to the patient after their functional status has been verified. However, for most practical purposes, a number of requirements for gene transfer have to be met when choosing the appropriate vector. Adenoviral transduction of primitive human hematopoietic cells[7-9] has proven to be very efficient, but this approach is not suitable for correction of any clinical situation where stable integration of the transferred gene is required. Virus integration is necessary to achieve subsequent transmission of the gene to the progeny and obtain its long-lasting expression. Murine retroviruses can integrate in cellular DNA but retroviral transduction may be poorly efficient. Several approaches have been tested to overcome this limitation. Co-cultivation of stem cells with packaging cell lines greatly increased gene transfer efficiency,[10-13] but this approach is not clinically applicable. Stromal cell layers[13-16] and extracellular matrix[17,18] can enhance gene transfer, but generation of feeder layers is cumbersome and expensive. Increase in transduction efficiency has been reported following cell stimulation with growth factors but induction of proliferation, necessary to achieve infection, is coupled with differentiation and loss of self-renewal capacity. Therefore, the nature and timing of growth stimuli are of critical importance to induce cell cycling[19,20] without losing pluripotentiality. Various studies have addressed this point in murine CD34+ cells[21,22] and in human progenitor cells isolated from bone marrow,[14-15,23] peripheral blood (PB),[16,24-26] umbilical cord blood (CB)[27-29] or from both PB and CB.[30] However, in many cases, stromal support was provided to enhance transduction. Since CB-CD34+ cells differ from PB- or BM-CD34+

cells as to frequency and immunophenotype,[27] it is conceivable that their transduction can also be modulated differently. In this study, we investigated whether CB-derived CD34+ progenitor cells could undergo efficient retroviral transduction in stroma free culture. Enrichment via positive selection, which allows volume reduction and increased gene transfer efficiency,[31] allowed us to start with CD34+ cells that were at least 95% pure. Different schedules of infection and growth factor stimulation were tested and the results obtained indicate that retroviral transduction efficiency depends mainly on the length of the stimulation time rather than on the number and length of the infection procedures. However, by increasing the time of stimulation and/or infection, cells with lower clonogenic potential were transduced. A short infection after a short stimulation seems to be the best combination to achieve a consistent transduction of CB-derived CD34+ cells with reasonable clonogenic potential.

Materials and Methods

Chemicals and growth factors

Geneticine sulphate (G-418) was from Gibco-BRL (Life Technologies, Paisley, UK) and was used at 1.6 mg/mL. Erythropoietin (EPO), interleukin-6 (IL-6), interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were purchased from Genzyme (Cambridge, MA, USA), while stem cell factor (SCF) was a generous gift from Amgen (Thousands Oaks, CA, USA).

CD34+ cell selection

CB samples were collected in preservative free sodium heparin during normal full-term deliveries. Since the umbilical cords were expected to be discarded, informed consent was deemed unnecessary. Whole blood samples were diluted 1:4 in phosphate buffered saline (PBS) and low density mononuclear cells were isolated by Ficoll-Hypaque gradients (Lymphoprep, Nicomed Pharma, Oslo, Norway).

Cells were washed twice in PBS containing 0.5% bovine serum albumin and 5 mM ethylendiaminetetracetate. An aliquot of cells was taken for the evaluation of total cell number and the percentage of CD34+ cells. CD34+ cells were selected using the Miltenyi Mini-Macs Immunomagnetic Separation system (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) and the isolation kit used according to the procedure recommended by the manufacturer, as previously described.[32] Cell number, viability, morphology and immunophenotype were evaluated from an aliquot. Surface expression of CD34, CD33 and CD38 was analyzed by indirect immunofluorescence and cytofluorimetric analysis. The monoclonal antibody and the FITC-conjugated goat anti-mouse immunoglobulin used as second step reagents were from Becton Dickinson (Becton Dickinson, S. Josè, CA, USA). Samples were analyzed with a FACScan (Becton Dickinson).

Retroviral vector

The LXSN retrovirus was produced in the amphotropic Am12*env*+ packaging cell line[33] containing the LXSN vector,[34] which carries the neomycin phosphotransferase gene and confers resistance to G-418. Cells were grown in Dulbecco's modified medium (DMEM) supplemented with 10% fetal calf serum. Virus-containing supernatant was titrated using an NIH3T3 assay[35] and stored at –80°C. Virus titer (5×10^6 PFU/mL) was checked during the course of the study and found to be unmodified.

Infection/transduction

Purified CD34+ cells, resuspended at 1×10^5/mL in medium containing or not IL-3 (10 ng/mL), IL-6 (50 ng/mL) and SCF (50 ng/mL), according to Nolta *et al.*,[15] were infected for the indicated time with the LXSN retrovirus at a multiplicity of infection (m.o.i.) of 10 PFU/cell for the indicated time. Eight μg/mL hexadimethrine bromide (Polybrene, Sigma, St. Louis, MO, USA) were added to enhance virus-cell contact. A mock-infection was always performed in parallel under identical conditions. At the end of the infection, cells were washed twice and CFU-GM and BFU-E clonogenic assays were performed, immediately or after expansion for one week in the presence of EPO (3 U/mL), IL-3 (100 ng/mL) and GM-CSF (10 ng/mL).

Cell culture and clonogenic assays

Freshly isolated, infected and mock-infected CD34+ cells were analyzed in triplicate cultures for colony formation immediately or after one week expansion in liquid culture as described above. Cells at 5×10^3/mL were inoculated in semisolid medium containing 0.9% methylcellulose (BFU-E, erythroid progenitors) or 0.6% agar (CFU-GM, myeloid progenitors) in Iscove's modified Dulbecco's medium enriched with human AB pooled pretested serum. CFU-GM were grown in the presence of GM-CSF (100 ng/mL), SCF (50 ng/mL) and IL-3 (100 ng/mL) while BFU-E in the presence of EPO (2 IU/mL), SCF (50 ng/mL), IL-3 (100 ng/mL) and GM-CSF (10 ng/mL).

Efficiency of retroviral transduction was calculated by dividing the number of colonies grown in the presence of G-418 for the number of colonies grown in the absence of G-418. Mock-infected cells were cultured in identical manner. Cultures were incubated in humidified atmosphere of 5% CO_2 at 37°C for 14 days and colonies of more than 40 cells were scored under an inverted microscope at a 40× magnification.

Polymerase chain reaction (PCR)

DNA was extracted following standard procedures from equal numbers of infected and mock-infected cells (10^5) after expansion in liquid culture for one week under the conditions described above. DNA was then amplified in a 50 μL reaction with the primers specific for the α-globin chain or the

neomycin phosphotransferase gene (neo) in a DNA thermal cycler (Perkin-Elmer, Norwalk, CT, USA). The sequences of the primers were the following: 5'-CTGAAGCGGGAAGGGACT-3' (neoF), 5'-GGC-CACAGTCGATGAATC-3' (neoR), corresponding to position 2319-2678 of the LXSN sequence;[34] 5'-GAT-GCACCCACTGGACTCCT-3' (α-globin F), 5'-CCATTGTTGGCACATTCCGG-3' (α-globinR) corresponding to position 24-883 of the human α-globin chain sequence.[36] The amplification profiles were 1 min 94°C, 1 min 55°C, 1 min 72°C for 40 cycles and 1 min 94°C, 1 min 52°C, 1 min 30 sec 72°C for 30 cycles, respectively. The expected sizes of the amplification products were 1.9 Kb (α-globin) and 359 bp (neo). Amplified products were analyzed in a 1.5% agarose gel run in TBE buffer (TBE is 90 mM Tris-HCl pH 8,3; 90 mM boric acid; 2 mM EDTA) and stained with ethidium bromide. Semiquantitative PCR analysis was performed by densitometric analysis of the neo and α-globin bands (Gel Blot Program, Ultraviolet Products, Cambridge, UK).

RNA extraction and RT-PCR analysis

Total RNA extraction was made according to Chomczyski and Sacchi[37] from equal amounts of cells (10^6). One μg of total RNA, resuspended in sterile water, was retrotranscribed with oligo d(T) by means of a commercial kit used according to the procedure suggested by the manufacturer (Clontech, Palo Alto, CA, USA). At the end of the synthesis, the cDNA was diluted to 100 μL and then 10 μL were separately amplified with the neo or α-globin primers as described above.

Results

Purification of CB CD34⁺ cells and transduction with LXSN vector

CD34⁺ progenitor cells were enriched from the mononuclear cell fraction by immunomagnetic bead selection. The purity of the CD34⁺ fraction was higher than 95% (median 98%, range 95-99.5%) as assessed by cytofluorimetric analysis. Clonogenic assays performed immediately before the different infection schedules showed that CD34⁺ cells did consistently form myeloid and erythroid colonies (CFU-GM median 201, range 72-395; BFU-E median 278, range 75-433).

A certain interindividual variation in the number of CFU-GM and BFU-E colonies was observed, as already described by others.[38]

In all the experiments, purified CD34⁺ cells were infected with the same viral stock, whose titer was 5×10^6 PFU/mL, at a m.o.i. of 10 PFU/cell. Infections were performed at 37°C for the indicated time, ranging from 3 to 72 hours. At the end of infection, the percentage of CD34⁺ cells was re-evaluated by cytofluorimetric analysis. In all cases it was found to be unmodified (median 98%, range 94-99.8). Of these cells 40% (range 21-60%) were CD33⁺ and 94% (range 91-98%) were CD38⁺.

Evaluation of transduction efficiency

In all the experiments, infected and mock-infected cells were tested for clonogenicity in the presence or absence of 1.6 mg/mL G-418. This concentration was chosen after performing a titration curve with mock-infected cells cultured under the same conditions used in the following experiments. Lower G-418 doses allowed the growth of colonies in an inverse dose-dependent fashion, whereas no colony outgrew in the presence of 1.6 mg/mL G-418 (data not shown). In the effort to understand whether the growth factors present before and/or during infection might affect the nature of the transduced cells, clonogenic assays were carried out immediately after the infection or following one week expansion in liquid culture. In the presence of EPO, IL-3 and GM-CSF for one week pluripotential progenitor cells were driven to differentiate, as shown by the reduction in the percentage of CD34⁺ cells, median 40% (range from three different experiments 25-45%) and by the shift in the expression of the CD33 antigen to a mean of 90% (range 80-95%). Consistent with the latter finding, the absolute number of CFU-GM and BFU-E colonies derived from mock-infected cells, grown in the absence of G-418, decreased. Precisely, the CFU-GM median was 80 (range 35-155) and the BFU-E median was 55 (range 12-92). Transduction efficiency was thus expressed as the mean number of G-418-resistant colonies divided by the mean number of colonies grown in the absence of G-418×100 (variability within triplicate cultures never exceeded 7%). In order to understand whether the amount of transduced cells varied according to the different schedules, transduction efficiency was evaluated in all the different experimental conditions tested, by performing PCR analysis on equal number of infected cells grown for one week in liquid culture as described above. A semiquantitative estimate of transduction was thus obtained by densitometric scanning of the neo-specific amplification product corrected for the intensity of the α-globin-specific amplification product. Mock-infected CD34⁺ cells and a stably transduced cell line were used as negative and positive controls, respectively.

Efficiency of transduction in freshly isolated CD34⁺ CB cells

Since it was previously reported that about 10% of partially purified (62%), freshly isolated, CD34⁺ CB-derived progenitor cells could be transduced in the absence of stimuli,[28] we first investigated whether highly purified CD34⁺ cells could also be transduced in the absence of growth factors. Cells were thus infected for 3 hours at 37°C after culture for 12 hours in the presence or absence of IL-3, IL-6 and SCF, as described by Nolta *et al.*[15] This cytokine cocktail was previously shown to provide the best support for CB-derived CD34⁺ cell transduction. The results are summarized in Table 1. When clonogenic assays were performed immediately after infection, with CD34⁺ cells cultured

Table 1. Efficiency of neomycin-resistance gene transduction into freshly isolated CD34+ progenitor cells.

*pre-infection stimuli**	*percentage of neo-resistant colonies*			
	before°		*after#*	
	expansion			
	CFU-GM	*BFU-E*	*CFU-GM*	*BFU-E*
–	2.4	0.4	18	1.4
+	2.8	1.0	53	1.3

**12 hours in the presence of SCF, IL-3 and IL-6, see text for details. Infection was for 3 hours at 37°C; °clonogenic assay performed immediately at the end of the infection; #clonogenic assay performed after one week of expansion in liquid culture containing EPO, IL-3 and GM-CSF, see text for details.*

Table 2. Effect of number and length of infection on transduction efficiency of CD34+ cells.

*infection (no. x hours)**	*percentage of neo-resistant colonies*			
	before°		*after#*	
	expansion			
	CFU-GM	*BFU-E*	*CFU-GM*	*BFU-E*
1×3	2.6	1.2	49	1.3
1×24	4.9	2.1	4.2	1.8
3×24	15.8	4.9	1.5	0
1×72	15.6	5.2	0	0

**Cells were kept for 12 hours in the presence of SCF, IL-3 and IL-6 before the infection. Infections were performed for the indicated time in medium containing the three cytokines. Cytokines were replenished at each cycle of infection; °clonogenic assay performed immediately at the end of the infection; #clonogenic assay performed after one week of growth in liquid culture containing EPO, IL-3 and GM-CSF, see text for details.*

Table 3. Effect of length of prestimulation with IL-3, IL-6 and SCF on transduction efficiency of CD34+ cells.

*pre-stimuli (hours)**	*percentage of neo-resistant colonies*			
	before°		*after#*	
	expansion			
	CFU-GM	*BFU-E*	*CFU-GM*	*BFU-E*
12	3.7	1.9	3.4	1.1
24	6.4	2.6	2.4	0
36	16.7	5.2	0	0
48	24.1	3.7	0	0

**Cells were kept in the presence of SCF, IL-3 and IL-6, see text for details, before the infection. Infection was performed for 24 hours in medium containing the three cytokines; °clonogenic assay performed immediately at the end of the infection; #clonogenic assay performed after one week of growth in liquid culture containing EPO, IL-3 and GM-CSF, see text for details.*

in the absence of stimuli, a 2.4% and a 0.4% transduction efficiency were obtained for myeloid and erythroid progenitors, respectively. A similar efficiency was achieved upon infection of CD34+ cells preincubated for 12 hours with the cytokine cocktail. When the clonogenic assays were carried out after one week expansion of CD34+ cells in liquid culture with EPO, IL-3 and GM-CSF, the efficiency of transduction greatly increased, reaching 18% and 1.4% without and 53% and 1.3% with IL-3, IL-6 and SCF pre-infection stimulation, respectively. The quite low percentage of G-418-resistant BFU-E colonies was in line with a previous report.[26]

Effect of the number and length of infections on transduction efficiency

We next investigated whether a significant increase in efficiency could be achieved either by a longer time of infection or by sequential infections with fresh virus-containing medium. The results of these experiments are summarized in Table 2. In all the samples, 12 hours prestimulation with IL-3, IL-6 and SCF was performed and infection was carried out in medium containing the same cytokines. Clonogenic assays performed immediately after infection showed that the number of G-418 resistant colonies increased according to the total length of infection. Superimposable results were obtained either with a single 72 hour or with three sequential 24 hour infections. In contrast, when the clonogenic assays were performed after one week expansion in liquid culture with EPO, IL-3 and GM-CSF, the percentage of transduced colonies was found to decrease as a function of the total length of infection. As indicated above, cytofluorimetric analysis performed before these second clonogenic assays indicated a reduction in the percentages of CD34+ cells. Moreover, for both infected and mock-infected cells, the absolute number of colonies grown in the absence of G-418 decreased. However, semiquantitative PCR analysis performed on these samples, and shown in Figure 1, demonstrated that the amount of transduced cells increased with the length of infection. A similar intensity of neo-specific product was obtained either with a single infection of 72 hours or with three infections of 24 hours performed sequentially.

Effect of length of growth factor-stimulation on transduction efficiency

To verify whether the results described above were due to the growth factors present in the medium during the infection rather than to the infection *per se*, samples were incubated in IL-3, IL-6 and SCF-containing medium for different lengths of time before being subjected to a single infection lasting 24 hours. The results of these experiments are summarized in Table 3 (clonogenic assays) and shown in Figure 2 (semiquantitative PCR analysis). A pattern very similar to that previously shown was found. Both the

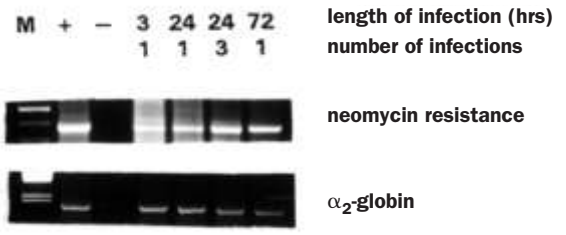

Figure 1. PCR analysis of neomycin-resistance gene in CD34⁺ cells subjected to different schedules of infection. Purified CD34⁺ cells were kept for 12 hours in the presence of SCF, IL-3 and IL-6 before the infection. Infection(s) were performed for the indicated time in medium containing the three cytokines. At the end of infection cells were grown for one week in liquid culture medium containing EPO, IL-3 and GM-CSF before extraction of DNA was performed. DNA derived from equal amount of cells was then separately amplified, as described under M&M, for neo (upper section) and α-globin (lower section) genes. ϕχ 174 RF DNA Hae digest was used as molecular weight marker (left).
+: positive control, DNA extracted from a G-418-resistant cell line; –: negative control.

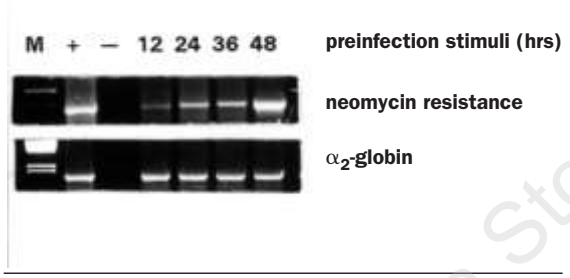

Figure 2. PCR analysis of neomycin-resistance gene in CD34⁺ cells subjected to different length of stimulation with SCF, IL-3 and IL-6 before infection. Infection was for 24 hours in medium containing the three cytokines. At the end of infection cells were subjected to the same procedure as that described in the legend to Figure 1.

percentages of infected cells and the intensity of neo-specific amplification products increased with longer stimulations. However, with more than 72 hours of infection/stimulation, no G-418 resistant colonies were found when the clonogenic assays were performed after one week growth in liquid culture with EPO, IL-3 and GM-CSF, further supporting the previous findings. The possibility that these results were due to the inability to express the inserted gene rather than to the decreased clonogenic potential of the transduced cells deserved consideration. RT-PCR analysis was thus performed on the same cell fractions whose PCR analysis is shown in Figure 2. As shown by the comparison of the bands in Figure 3 with those in Figure 2, the level of expression of the neo gene paralleled the amount of the gene integrated in the cellular DNA.

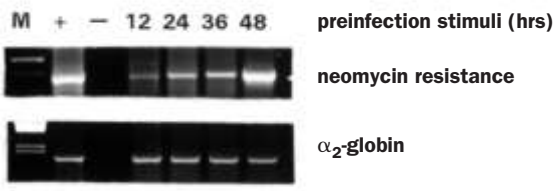

Figure 3. RT-PCR analysis of neomycin-resistance gene of the same samples as those described in the legend to Figure 2.

Discussion

A safe and efficient procedure of gene transfer into human hematopoietic progenitor cells is a prerequisite to develop successful clinical protocols for the treatment of genetic and acquired diseases. Several gene marking studies and a few therapeutic trials with gene transduced BM-, PB-mobilized or CB-derived progenitor cells have been already started (see refs. #1 and #2 for a review). These studies have emphasized the need for better definition of the steps involved in gene transfer into self-renewing, repopulating stem cells.

The purpose of the present study was to determine how growth factors and/or infection influence, in the absence of stroma or extracellular matrix, the transduction efficiency of CB-derived CD34⁺ progenitor cells. Previous studies, performed with PB- or BM-derived progenitor cells,[25,30] suggested that transduction efficiency increased with the number and length of infections. This conclusion was partially confirmed in our experiments, by both clonogenic assay and PCR analysis, where the percentage of neo-resistant cells increased with the duration of infection. However, a similar increase occurred prolonging the time of growth factor stimulation before a single 24 hour infection. Since in our and in the above mentioned studies, infection(s) was performed in the presence of IL-3, IL-6 and SCF, it is conceivable that the observed increase in transduction efficiency was due to the growth factors rather than to the infection *per se*. Our observation that, for a given time of infection, the efficiency of CD34⁺ cell transduction was unaffected by repeated changes of the virus-containing medium could probably be explained by the fact that the purification procedure produced a virus to target cell ratio (10 PFU/CD34⁺ cell) more than adequate to infect all the cycling cells.

The main result of the present study is the remarkable difference in transduction efficiency measured by the two consecutive clonogenic assays. In fact, when CD34⁺ cells were incubated for only 12 hours with IL-3, IL-6 and SCF and infected for 3 hours, the transduced cells proliferated and retained the clonogenic potential so that the transduction efficiency evaluated

in the two CFU-GM assays increased from 2.8% to 53%. By prolonging the time of incubation with cytokines, either before or during infection, the absolute number of transduced cells increased, regardless of the renewal of virus particles, as demonstrated by the PCR and RT-PCR assays, but the clonogenic potential of the transduced cells progressively decreased. In fact, transduction efficiency dropped to about 4% when the total time of prestimulation and infection reached 36 hours and was completely lost with a total time of 72 hours (Table 3). That is, an inverse correlation was observed between the total time of prestimulation plus infection and the clonogenic potential of the transduced cells. The results from the PCR and RT-PCR, performed on the totality of infected cells, clearly demonstrated that the inability of transduced cells to form colonies after expansion in liquid culture did not depend on the lack of the inserted gene or of its expression. Taken together, the results of these experiments suggest that the clonogenic potential of the transduced cells was influenced by changes in the duration of the infection/stimulation times. When this time was brief, transduced cells replicated more quickly than the untransduced ones, while the opposite occurred when this time was prolonged. Although these findings cannot be easily explained, they may help unravel the differences between the results obtained *in vitro* and those obtained *in vivo* either in clinical trials[1,2] or in reconstituting marrow SCID mouse models.[39-41] In the latter studies, the percentage of marked cells was never higher than 1%, while up to 80-90% of transduction efficiency was reached *in vitro*.[25,30] Recently, Larochelle *et al.*[39] using a repopulating marrow NOD/SCID mouse model, demonstrated that true progenitor cells were different from long-term culture (LTC-IC) proliferating cells. To explain the discrepancies between *in vivo* and *in vitro* results several authors[1,26,29,41] had already suggested that LTC-IC assays, in addition to being time-consuming and cumbersome, might be inadequate to measure the transduction efficiency of repopulating cells precisely. The type of transduced cells able to repopulate *in vivo* was found to depend on the nature of the stimulation[26,29] and the highest percentage of long-lasting retrovirally-marked cells found in humans was obtained with a very short (6 hours) infection performed without cytokine stimulation[42] or stromal support.[41] Our data demonstrate that by changing the total time of infection and growth factor stimulation, different types of cells were transduced. With a 3 hour infection without prestimulation, a rather consistent transduction was achieved suggesting that the growth factors present during infection were sufficient to induce cycling of progenitor cells. Whether endogenously produced growth factors[43] or those present in the serum or induced by other medium constituents also contributed to this finding can not be ruled out at present. In conclusion, the results presented here demonstrate that reasonably high transduction of CB-derived $CD34^+$ cells was achieved without co-cultivation with packaging cell line, stromal support and without loss of clonogenic potential.

Contributions and Acknowledgments

MVC wrote the paper and formulated the design of the study with the contribution of MP, VP and PGM, did RT-PCR assays and supervised both the production of virus stock and PCR assays. FS performed infections and PCR assays. MP supervised both $CD34^+$ cell purification and clonogenic assays, which were performed by PBi with the contribution of FM. PBo produced and titered the virus stock. EF carried out umbilical cord blood explants. VP was responsible for conception of the study and interpretation of results. PGM was responsible for funding as well as for conception of the study and interpretation of results. The authors wish to thank Amgen for the gift of SCF, Dr. D. Miller for LXSN vector and Genetix Pharmaceuticals for the Am12env packaging cell line.

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Disclosures

Conflict of interest: none.

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References

1. Dunbar CE. Gene transfer to hematopoietic cells: implications for gene therapy of human disease. Ann Rev Med 1996; 47:11-20.
2. Brenner MK. Gene transfer to hematopoietic cells. N Engl J Med 1996; 335:337-9.
3. Karlsson S. Treatment of genetic defects in hemopoietic cell function by gene transfer. Blood 1991; 78:2481-92.
4. Jordan CT, Lemischka IR. Clonal and systemic analysis of long term hematopoiesis in the mouse. Genes Dev 1990; 4:220-31.
5. Capel B, Hawley RG, Mintz B. Long- and short-lived murine haematopoietic stem cell clones individually identified with retroviral integration markers. Blood 1990; 75:2267-82.
6. Keller G, Snodgrass R. Life span of multipotential haematopoietic stem cells *in vivo*. J Exp Med 1990; 171: 1407-11.
7. Neering SJ, Hardy SF, Minamoto D, Spratt SK, Jordan CT. Transduction of primitive human hematopoietic cells with recombinant adenovirus vectors. Blood 1996; 88:1147-55.
8. Mannoni P, Bregni M, Tabilio A. European concerted action on human hematopoietic stem cell: gene transfer into hematopoietic stem cells. Haematologica 1996; 81:186-9.
9. Tosi P, Pellacani A, Visani G, Ottaviani E, Tura S. Adenoviral-mediated gene transfer can be accomplished in human myeloid cell lines and is inhibited by all-trans retinoic acid-induced differentiation. Haematologica 1997; 82:387-91.

10. Dick JE, Kamel-Reid S, Murdoch B, Doedens M. Gene transfer into normal haematopoietic cells using *in vitro* and *in vivo* assays. Blood 1991; 78:624-34.
11. Fink JK, Correll PH, Perry LK, Brady RO, Karlsson S. Correction of glucocerebrosidase deficiency after retroviral mediated gene transfer into hematopoietic progenitor cells from patients with Gaucher disease. Proc Natl Acad Sci USA 1990; 87:2334-8.
12. Cournoyer D, Scarpa M, Mitani K, et al. Gene transfer of adenosine deaminase into primitive human haematopoietic progenitor cells. Hum Gene Ther 1991; 2: 203-11.
13. Sutherland HJ, Lansdorp PM, Henckelman DH, Eaves AC, Eaves CJ. Functional characterization of individual human haematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layer. Proc Natl Acad Sci USA 1990; 87:3584-8.
14. Moore KA, Deisseroth AB, Reading CL, Williams DE, Belmont JW. Stromal support enhances cell-free retroviral vector transduction of human bone marrow long-term culture-initiating cells. Blood 1992; 79:1393-9.
15. Nolta JA, Smogorzewska EM, Kohn DB. Analysis of optimal conditions for retroviral-mediated transduction of primitive human haematopoietic cells. Blood 1995; 86:101-10.
16. Ward M, Pioli P, Ayello J, et al. Retroviral transfer and expression of the human multidrug resistance (MDR) gene in peripheral blood progenitor cells. Clin Cancer Res 1996; 2:873-6.
17. Moritz T, Patel VP, Williams DA. Bone marrow extracellular matrix molecules improve gene transfer into human haematopoietic cells via retrovirus vector. J Clin Invest 1994; 93:1451-7.
18. Moritz T, Dutt P, Xiaio X, et al. Fibronectin improves transduction of reconstituting haematopoietic stem cells by retroviral vectors: evidence of direct viral binding to chymotryptic carboxy-terminal fragments. Blood 1996; 88:855-62.
19. Agrawal YP, Agrawal RS, Sinclair AM, et al. Cell-cycle kinetics and VSV-G pseudotyped retrovirus-mediated gene transfer in blood-derived CD34+ cells. Exp Hematol 1996; 24:738-47.
20. Di Ianni M, Casciari C, Ciurnelli R, et al. Beta-galactosidase transduced T lympocytes: a comparison between stimulation by either PHA and IL-2 or a mixed lymphocyte reaction. Haematologica 1996; 81:410-7.
21. Bodine DM, Karlsson S, Nienhuis AW. Combination of IL-3 and IL-6 preserves stem cell function in culture and enhances retrovirus-mediated gene transfer into haematopoietic stem cells. Proc Natl Acad Sci USA 1989; 86:8897-901.
22. Luskey BD, Rosenblatt M, Zsebo K, William DA. Stem cell factor, IL-3 and IL-6 promote retroviral-mediated gene transfer into murine hematopoietic stem cells. Blood 1992; 80:396-402.
23. Hughes PFD, Thackher JD, Hogge D, et al. Retroviral gene transfer to primitive normal and leukemic hematopoietic cells using clinically applicable procedures. J Clin Invest 1992; 89:1817-24.
24. Cassel A, Cottler-Fox M, Doren S, Dunbar CE. Retroviral-mediated gene transfer into CD34-enriched human peripheral blood stem cells. Exp Hematol 1993; 21:585-91.
25. Valtieri M, Schirò R, Chelucci C, et al. Efficient transfer of selectable and membrane reporter genes in hematopoietic progenitor and stem cells purified from human peripheral blood. Cancer Res 1994; 54:4398-404.
26. Elwood NJ, Zogos H, Willson T, Begley CG. Retroviral transduction of human progenitor cells: use of granulocyte colony-stimulating factor plus stem cell factor to mobilize progenitor cells *in vivo* and stimulation of Flt3/Flk-2 ligand *in vitro*. Blood 1996; 88:4452-62.
27. Lu L, Xiao M, Clapp DW, Li ZH, Broxmeyer, HE. High efficiency retroviral mediated gene transduction into single isolated immature and replatable CD34+ hematopoietic stem/progenitor cells from human umbilical cord blood. J Exp Med 1993; 178:2089-96.
28. Shi YJ, Shen RN, Lu L, Broxmeyer HE. Comparative analysis of retroviral-mediated gene transduction into CD34+ cord blood haematopoietic progenitors in the presence and absence of growth factors. Blood Cells 1994; 20:517-24.
29. Plavec I, Voytovich A, Moss K, et al. Sustained retroviral gene marking and expression in lymphoid and myeloid cells derived from transduced haematopoietic progenitor cells. Gene Ther 1996; 3:717-24.
30. Bertolini F, Battaglia M, Corsini C, et al. Engineered stromal layers and continuous flow culture enhance multidrug resistance gene transfer in haematopoietic progenitors. Cancer Res 1996; 56:2566-72.
31. von Kalle C, Kiem HP, Goehle S, et al. Increased gene transfer into human hematopoietic progenitor cells by extended *in vitro* exposure to pseudotyped retroviral vector. Blood 1995; 84:2890-7.
32. Pasino M, Biglino P, Mori PG, et al. Isolation, characterization and *ex vivo* expansion of CD34+ cells from umbilical cord blood. Cancer Res Ther Control 1997; in press.
33. Markowitz D, Goff S, Bank A. Construction and use of a safe and efficient amphotropic packaging cell line. Virology 1988; 167:400-6.
34. Miller AD, Rosman GJ. Improved retroviral vectors for gene transfer and expression. Biotechniques 1989; 7:980-8.
35. Botwell DDL, Johnson GR, Kelso A, Cory S. Expression of genes transferred to haematopoietic cells by recombinant retroviruses. Mol Biol Med 1987; 4:229-36.
36. Dodè C, Rajagopal K, Lamb J, Rochette J. Rapid analysis of $\alpha^{3.7}$ thalassemia and $\alpha\alpha\alpha^{anti3.7}$ triplication by enzymatic amplification analysis. Br J Haematol 1992; 82: 105-11.
37. Chomczynski P, Sacchi N. Single-step method of RNA isolation by guanidinium-thiocyanate-phenol-chloroform extraction. Anal Biochem 1982; 162:156-9.
38. Lim FT, van Wisen L, Willemze R, Kanhai HH, Falkenburg JH. Influence of delivery on numbers of leukocytes, leukocyte subpopulations and haematopoietic progenitor cells in human umbilical cord blood. Blood Cells 1994; 20:547-59.
39. Larochelle A, Vormoor J, Hanenberg H, et al. Identification of primitive human haematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implication for gene therapy. Nature Med 1996; 2: 1329-37.
40. Li KJ, Dilber MS, Abedi MR, et al. Retroviral-mediated gene transfer into human bone marrow stromal cells: studies of efficiency and in vivo survival in SCID mice. Eur J Haematol 1995; 55:302-6
41. Emmons RVB, Dore S, Zujesky J, et al. Retroviral gene transduction of adult peripheral blood or bone marrow-derived CD34+ cells for six hours without growth factors or autologous stroma does not improve marking efficiency assessed in vivo. Blood 1997; 89:4040-6.
42. Brenner MK, Rill DR, Holladay MS, et al. Gene marking to determine whether autologous marrow infusion restores long-term haemopoiesis in cancer patients. Lancet 1993; 342:1134-7.
43. Behringer D, Kresin V, Henschler R, Mertelsman R, Lindemann A. Cytokine and chemokine production by CD34+ haemopoietic progenitor cells: detection in single cells. Br J Haematol 1997; 97:9-14.