

Cytokines, Including Stem Cell Factor Alone, Enhance Lentiviral Transduction in Nondividing Human LTCIC and NOD/SCID Repopulating Cells

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Hematopoietic stem cells (HSC) require extensive cytokine-mediated stimulation and proliferation for efficient transduction by oncoretroviral vectors. Since lentiviral vectors can transduce nondividing cells, the need for cytokine stimulation has been questioned. We studied HIV-based lentiviral transduction of human early hematopoietic progenitors from umbilical cord blood in the presence or absence of IL-3, IL-6, stem cell factor (SCF), and Flt-3L (36SF) or SCF alone and characterized the effects of these conditions on the stem cell phenotype. Gene transfer was significantly higher in the presence of 36SF in mass culture cells, CFC, LTCIC, and NOD/SCID repopulating cells (SRC). Transduction of primitive progenitor/stem cells was poor without cytokines, with only 12% LTCIC and 23% SRC transduced, compared to 59% in LTCIC and 81% in SRC with 36SF. SCF alone matched transduction rates of multiple cytokines with 70% in CFC. Cytokines prevented apoptosis, expanded CD34⁺ cell number, and maintained CFC and LTCIC frequencies. Cytokine stimulation increased transduction of nondividing Ara-C-resistant and aphidicolin-inhibited cells similar to dividing cells. These data suggest that cytokines enhance lentiviral transduction of HSC, without requiring cell division, and maintain the stem cell phenotype. SCF stimulation alone was sufficient for high level transduction.

Key Words: gene therapy, lentivirus, LTCIC, NOD/SCID

INTRODUCTION

Clinical application of gene transfer into human hematopoietic stem cells (HSC) has proved difficult using the current class of oncoretroviral vectors based on murine retroviruses, despite promising preclinical results. Oncoretrovirus requires cell division to gain access to genomic DNA for integration [1]. Since HSC exist in a resting state within the bone marrow and are only occasionally called upon to enter the cell cycle to contribute to hematopoiesis [2,3], transduction may be infrequent. Resting HSC have therefore been treated *ex vivo* with cytokine combinations for 3–5 days in an effort to stimulate cell division while trying to prevent loss of stem cells to differentiation. During cytokine treatment several transductions would be carried out to maximize the likelihood of “catching” a cell passing through the cell cycle for transduction. These extended culture periods with cytokines, while maximizing transduction, often result in a decrease in the total number of cells capable of hema-

topoietic reconstitution. In contrast, a short culture period with cytokines may not result in appreciable loss of HSC [4].

Lentiviral vectors based on the human immunodeficiency virus (HIV) incorporate mechanisms permitting infection of nondividing cells, an obvious advantage for transduction of HSC. These include nuclear localization signals on viral proteins matrix and integrase [5,6], components of the preintegration complex [7]. Vectors incorporating the central polypurine tract and central termination sequences (cPPT/CTS) of HIV show increased nuclear import [8].

Wild-type HIV is unable to infect quiescent T cells efficiently until the cells are stimulated to exit G₀ phase [9,10], and infection of primary monocytes/macrophages similarly occurs in the proliferating fraction [11,12]. More precisely, T cells and macrophages must progress into later stages of the G₁ phase of the cell cycle for efficient infection [13,14]. This is not due to the low nucleotide pools present in G₀-phase cells [13], implying that other

cellular events occurring in G_1 are necessary for productive infection.

Since lentiviral vectors hold promise for transducing nondividing HSC, it has been questioned whether any cytokine prestimulation is needed or desirable, given the experience with the extended stimulatory culture periods required for oncoretroviral transduction. Maintenance of HSC in a resting state by the absence of growth factors would theoretically prevent loss of repopulating ability. While it is true that transduction of human $CD34^+$ cells without cytokine stimulation has been observed by us and others [15–17], no systematic comparison of transduction with or without cytokine stimulation has been carried out on cells representing more primitive progenitors, including long-term culture initiating cells (LTCIC) or NOD/SCID repopulating cells (SRC). Neither has there been a characterization of the effects these transduction conditions have on the stem cell phenotype. We therefore set out to determine whether a short prestimulation period would enhance lentiviral transduction of HSC compared to no stimulation and the effects of these conditions on maintenance of progenitors. Furthermore, we analyzed transduction under a minimal cytokine exposure to stem cell factor (SCF). The effects of these cytokine treatments on the cell cycle and whether this influenced transduction were determined. Transduction was analyzed in mass culture, colony-forming cells (CFC), LTCIC, and SRC. We found that a short cytokine stimulation enhanced transduction at all progenitor levels and was necessary for efficient transduction of the more primitive cells. Stimulation with SCF alone could increase transduction equal to a more stimulatory cytokine cocktail. In addition, culture without cytokines resulted in rapid loss of progenitors compared to culture with cytokines. The increased transduction with prestimulation did not require progression through $S/G_2/M$ phases of the cell cycle, indicating that other signal transduction or cellular activation events are needed for efficient transduction of HSC.

RESULTS

Gene Transfer to Human Hematopoietic Progenitors

A cytokine prestimulation period has traditionally been used for oncoretroviral transduction of HSC with the goal of stimulating division without differentiation. Since HIV does not require cell division for transduction, we investigated the possible advantage of immediate transduction of human $CD34^+$ cells in the absence of cytokine stimulation and compared it to transduction after a 1-day prestimulation with cytokines. Even though we purified $CD34^+$ cells, the mass culture is heterogeneous in the distribution of early progenitors and is quite different from the mostly resting HSC population, which could lead to flawed conclusions about HSC transduction. Previous work by us and others has shown equivalent trans-

duction of mass culture with or without stimulation [8,15,16,18]. These studies are extended here to analysis of LTCIC and SRC, which more accurately approximate the true stem cell.

We transduced human $CD34^+$ cells from umbilical cord blood at a multiplicity of infection (m.o.i.) of 1 and cell density of $2-4 \times 10^5/\text{ml}$ with a lentiviral vector containing a green fluorescent protein (GFP) or a mutant methylguanine–DNA methyltransferase (P140K MGMT) marker transgene (LV-GFP or LV-P140K) either immediately after isolation in the absence of cytokines or after a 1-day culture period in medium containing IL-3, IL-6, SCF, and Flt-3L (36SF). After the transduction period, cells were transferred to fresh cytokine-containing medium for flow cytometric analysis 3–5 days later, plated in methylcellulose for a CFC assay, plated over feeder cells for an LTCIC assay, or transplanted into irradiated NOD/SCID recipients.

We analyzed the level of transduction of the mass culture by flow cytometric determination of transgene expression and $13 \pm 2\%$ of the cells were transduced without cytokine stimulation (range 8.2–20%) and $19 \pm 2\%$ with cytokine stimulation ($P = 0.002$; range 15–29%; Fig. 1). Transduction of committed progenitors at the CFC level, by PCR detection of transgene in individual colony-forming units (CFU), showed $39 \pm 5\%$ provirus positive without cytokines versus $78 \pm 4\%$ with cytokines ($P = 0.0001$), with ranges of 16–67 and 60–100%, respectively. The discrepancy between transduction by gene expression and by PCR can be partially explained by poor proportionate expression from the CMV promoter (unpublished observation).

Transduction of more primitive cells was considerably reduced without cytokine stimulation. When we analyzed transduction of CFC derived from LTCIC after 5 weeks of culture, we found a transduction level of $12 \pm 8\%$ without cytokines (range 0–26%), while $59 \pm 11\%$, with a range of 25–75%, were transduced with cytokine stimulation ($P = 0.03$; Fig. 1).

We also analyzed transduction of human CFU derived from bone marrow of NOD/SCID mice 6–8 weeks post-transplant. Analysis by PCR showed a gene transfer rate of $81 \pm 1\%$ (range 80–82%) if the $CD34^+$ cells were transduced with cytokine stimulation, while only $23 \pm 9\%$ (range 8.3–39%) were transduced in the absence of cytokines ($P = 0.003$; Figs. 1 and 2). Engraftment of cytokine-stimulated and unstimulated transduced cells transplanted into NOD/SCID mice was 4–68% and each displayed multilineage potential, as evidenced by flow cytometric detection of human $CD19^+$, $CD33^+$, and $CD34^+$ cells (data not shown).

Overall, these data reveal that efficient lentiviral transduction of primitive hematopoietic progenitors, in addition to more differentiated cells, is improved by cytokine stimulation. Transduction of the most primitive hematopoietic subsets, as represented by the LTCIC and SRC

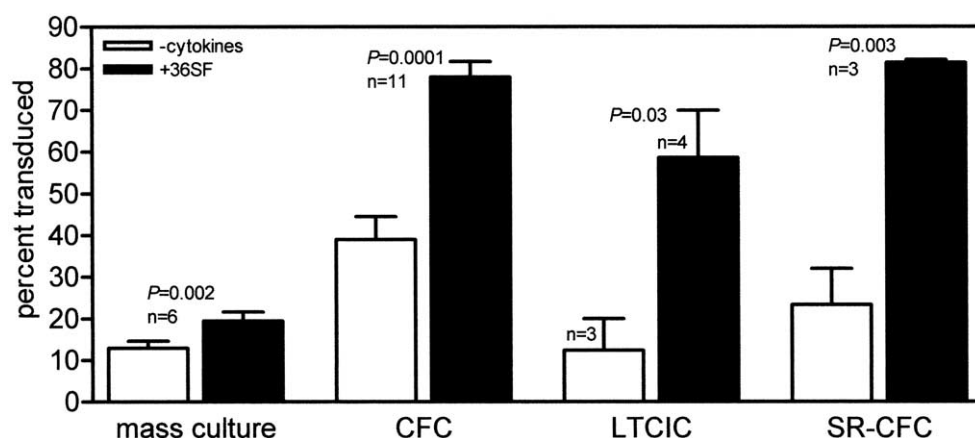


FIG. 1. Comparison of lentiviral transduction of CD34⁺ progenitors in the presence or absence of IL-3, IL-6, SCF, and Flt-3L. CD34⁺ cells were transduced either immediately after isolation in the absence of cytokines or after a 1-day prestimulation with 36SF. After 1 day of transduction, cells were further manipulated for analysis of mass culture, CFC, LTCIC, or SR-CFC (human CFC derived from the bone marrow of transplanted NOD/SCID mice). Transduction in the presence of 36SF was significantly higher in committed and uncommitted progenitors.

assays, was quite high when performed with cytokine stimulation and profoundly impaired when carried out in the absence of cytokines.

Maintenance of Progenitor Phenotype

Extended exposure of human CD34⁺ cells to cytokines results in loss of the stem cell phenotype [19]. It has been speculated that lentiviral vectors may allow efficient transduction in the absence of cytokines, but the effect of such transduction conditions on progenitors has not been evaluated. In addition, recent data suggest that a short cytokine exposure, or “resting” the cells after stimulation, may improve engraftment of HSC [20]. We therefore analyzed the effects of a short transduction without cytokines and prestimulation/transduction with cytokines on cell survival and maintenance of hematopoietic progenitors.

Analysis of cell viability over the culture periods showed that 36SF protected cells from apoptosis up to 48 h as determined by propidium iodide (PI) staining, while cells rapidly became apoptotic without cytokines, exhibiting massive cell death by 2 days (Fig. 3A). Under our transduction conditions, the 2-day total culture time with cytokines results in much greater cell survival (10 ±

2% apoptotic) than even a short, 1-day culture without cytokines (19 ± 4% apoptotic). As expected, SCF alone also enhanced survival over the 2-day culture period with 10 ± 1 and 15 ± 2% apoptotic at days 1 and 2, respectively (Fig. 3A).

CD34 is a marker for hematopoietic progenitors. We analyzed the absolute number of CD34⁺ cells in culture after 1 and 2 days without or with cytokines, respectively, and found that CD34⁺ cell numbers expanded 26 ± 19% over 2 days with 36SF, but culture without cytokines for 1 day resulted in a 30 ± 4% loss ($P = 0.02$; Fig. 3B). This implies a greater potential for repopulating capacity in the cytokine-containing cultures.

We then measured maintenance of hematopoietic progenitors by determining the frequency of CFC and LTCIC after 1 and 2 days of culture with or without 36SF. As can be seen in Fig. 3C, culture with cytokines resulted in maintenance of greater numbers of CFC than culture without cytokines ($P = 0.005$). The frequency of CFC in cytokine-containing transductions was essentially unchanged at 16 ± 1% after 2 days while transduction without cytokines resulted in a large drop in CFC after 1 day of culture, from 17 ± 1 to 8.3 ± 1.1%, that did not recover at 2 days. CFC frequency in SCF-alone cultures declined to 8.5% at day 1, but rebounded to 16% at day 2, in concordance with a 63% expansion of CD34⁺ cell numbers (data not shown). As a measure of primitive progenitor/stem cells, the frequency of LTCIC after each transduction culture was determined and it was found that cells transduced in medium containing 36SF contained 15 LTCIC per 10,000 cells and cells transduced in medium without cytokines contained 3 LTCIC per 10,000 cells, a reduction of 80% (Fig. 3D). This would indicate that the cytokine-containing cultures maintained early progenitors to a greater degree than the no-cytokine cultures, suggesting that without cytokine support, stem cells are quickly lost.

Taken together, these data show that prestimulation and lentiviral transduction for 2 days in the presence of IL-3, IL-6, SCF, and Flt-3L protects cells from apoptotic

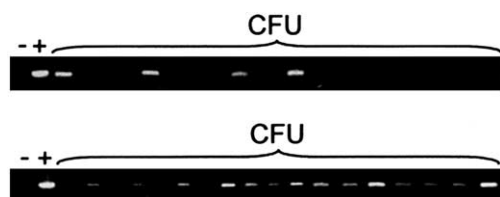


FIG. 2. PCR analysis of human CFU derived from transplanted NOD/SCID bone marrow. Human CFU were grown from NOD/SCID mice 6–8 weeks following transplantation with CD34⁺ cells transduced with LV-GFP in the absence (top) or presence (bottom) of 36SF. PCR analysis shows detection of the GFP transgene in 4 of 18 (top) and 14 of 18 (bottom) representative CFU. – and + represent negative (methylcellulose between CFU) and positive (transduced 293T cell extracts) PCR controls, respectively.

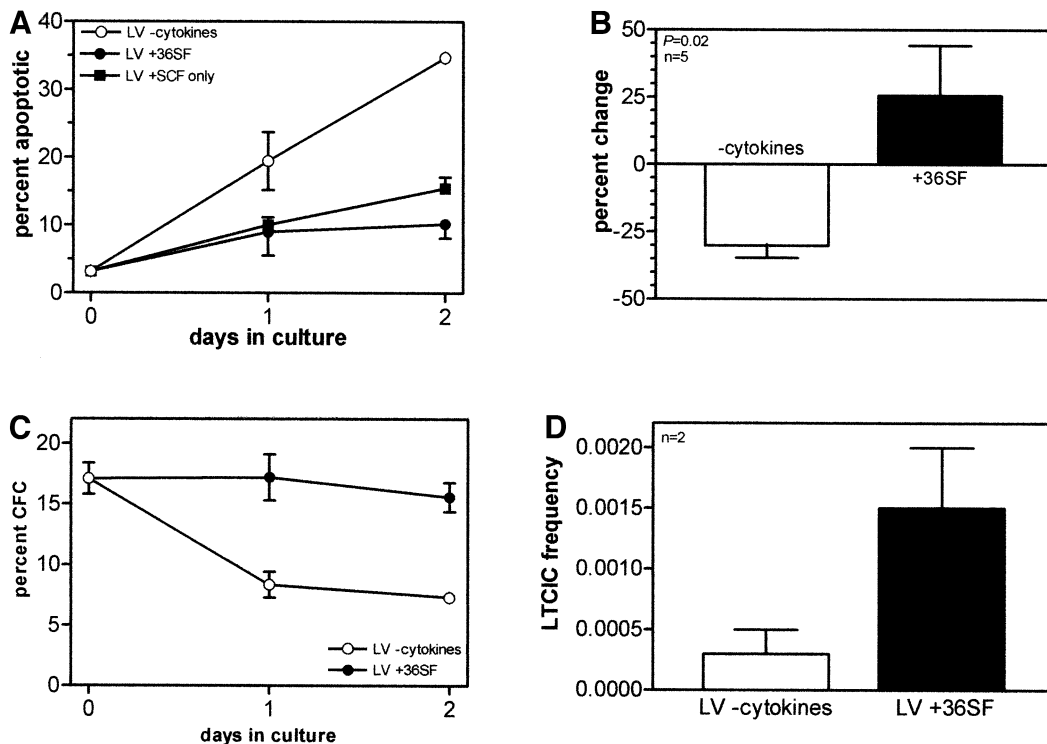


FIG. 3. Effects of culture on progenitor maintenance. Human cord blood CD34⁺ cells were cultured for up to 2 days in the presence or absence of 36SF or SCF alone. (A) The degree of apoptosis was determined at 1 and 2 days by flow cytometry after staining with PI. Culture with cytokines protected cells from apoptosis. Each point represents the average of two to four experiments. (B) The total number of CD34⁺ cells before and after either 1 day culture without cytokines or 2 days with 36SF was determined and percentage gain or loss was calculated. Cytokines caused expansion of CD34⁺ cells and their absence resulted in significant loss ($P = 0.02$). (C) The frequency of CFU formation in methylcellulose was determined before and after 1 and 2 days culture with or without 36SF or SCF alone and expressed as a percentage of total cells plated. CFC are maintained in cytokine culture. Each point represents the average of two to seven experiments. (D) The frequency of LTCIC after 1 day culture without cytokines or 2 days with 36SF was determined after 5 weeks long-term culture. The frequency of LTCIC was greater in 2-day cytokine cultures than in 1-day cytokine-free cultures ($n = 2$ paired experiments).

cell death and preserves a greater number of progenitor/stem cells than a short, 1-day transduction without cytokines. SCF alone has a similar capacity to maintain progenitors over the transduction culture period.

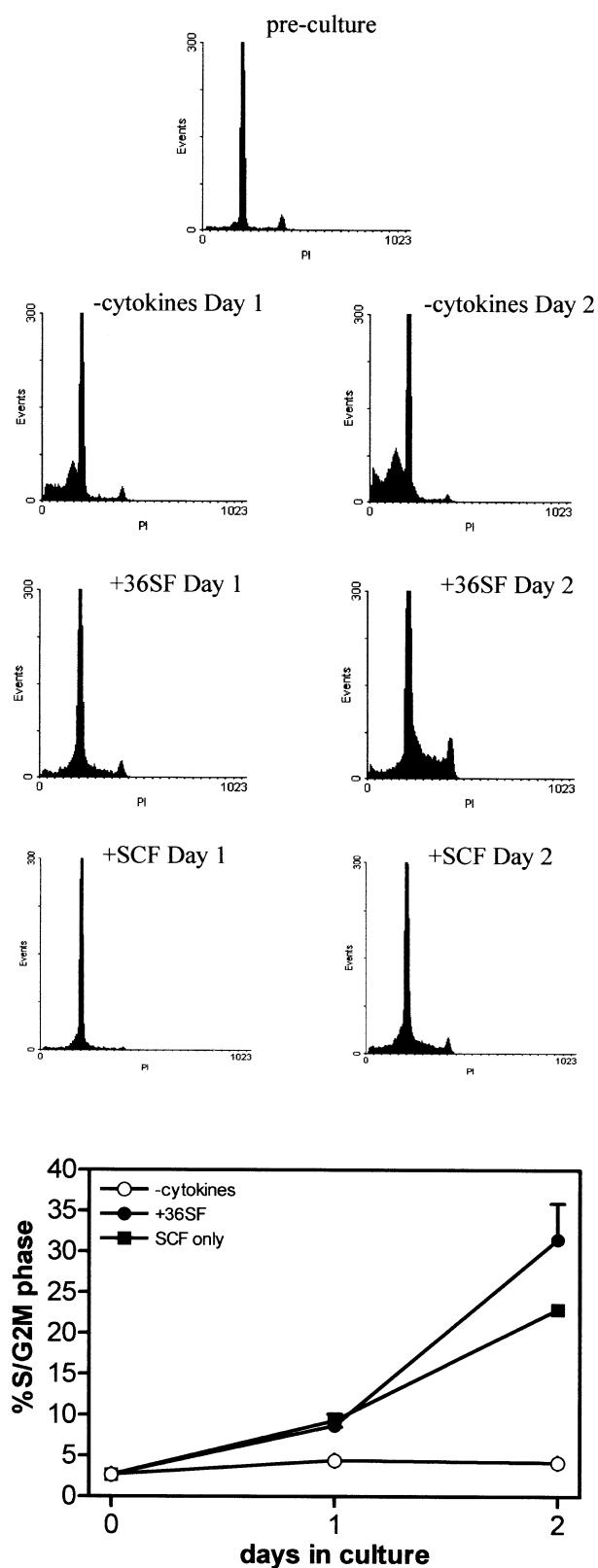
Cytokine-Induced Cell Cycle Progression

Cytokine stimulation of CD34⁺ cells induces cell division and can have effects on the progenitor phenotype and susceptibility to transduction. We therefore evaluated the effects that culture conditions had on the cell cycle distribution of human CD34⁺ cells by PI staining at 0, 1, and 2 days of culture with 36SF, SCF alone, or no cytokines. Freshly isolated cells were almost entirely (97%) in G₀/G₁ phases of the cell cycle and this was unchanged when cultured without cytokines for up to 2 days (Fig. 4). Culturing in the presence of 36SF resulted in a small increase in cells in S/G₂/M at 1 day, which was substantial by 2 days, reaching a maximum at day 4 of 43%. SCF alone induced S/G₂/M progression that was slightly lower than 36SF stimulation (Fig. 4). Quantitation of the proportions of cells in S/G₂/M at the end of the transduction period

showed that 4.2% of the unstimulated cultures, 36% of the 36SF-stimulated cultures, and 23 ± 1% of the SCF-alone cultures was in S/G₂/M (Fig. 4, bottom). These data show that with our cytokine mix of 36SF or SCF alone, cell cycle progression lags at 1 day, with a substantial increase occurring by 2 days.

Cell Cycle Progression and Transduction

It was not clear from our cell cycle data whether the increased transduction observed after cytokine stimulation was due to the increased cell cycle entry after the first day. An approach to determining whether transduction was a function of increased cell cycle entry involved selectively killing cells that were undergoing DNA synthesis. This was accomplished by treating stimulated cells with 2 μM arabinosylcytosine (Ara-C) for 2 h following the 1-day transduction period. Ara-C, a chain-terminating nucleoside analog, effectively kills cells undergoing DNA synthesis. Thirty-six percent of stimulated and transduced cells surviving Ara-C treatment showed GFP expression compared to 24% in the untreated cultures (Fig. 5, left).



Furthermore, although Ara-C treatment killed 40% of CFC (data not shown), surviving CFC had a transduction rate of 71% compared to 77% of untreated CFC (Fig. 5, right). Thus, most transduction occurred within 24 h of virus addition and cells entering S or G₂ phases were not preferentially transduced.

Lentiviral transduction of aphidicolin-treated human CD34⁺ cells was similarly used as a method to examine whether cytokine-induced cell cycle entry increased transduction. Aphidicolin was added to cultures at the beginning of a 1-day cytokine stimulation period, followed by lentiviral transduction at an m.o.i. of 1 for 1 day in the presence of 36SF and aphidicolin. Effective G₁ arrest is shown in Fig. 6A. Following transduction, cells were either plated in methylcellulose for CFC assay or further cultured in cytokine-containing medium for 4 days before analysis by flow cytometry.

Transduction as determined by GFP expression of stimulated but cell cycle-inhibited CD34⁺ cells was essentially equivalent to the uninhibited culture. We found 21% GFP⁺ cells in the aphidicolin-treated cultures and 22% GFP⁺ cells in the untreated cultures (Fig. 6B). In addition, stimulated CFC were transduced to 83% in the presence of aphidicolin and 86% in the absence of aphidicolin, both above the 29% transduction of unstimulated CFC (Fig. 6C). Extending aphidicolin treatment for 48 h after addition of virus gave identical results (data not shown).

Gene Transfer under Minimal Cytokine Conditions

Since transduction can be increased independent of cell cycle progression, we asked whether a minimal stimulation with SCF alone could increase transduction while maintaining progenitor survival. CD34⁺ cells were stimulated 1 day with 36SF or SCF alone and transduced with LV-GFP as above. Transduction was determined in mass culture by flow cytometry 4 days following transduction. We found that SCF-stimulated cultures were transduced as well as 36SF-stimulated cultures, to 22% by flow cytometry (Fig. 7, left). In two independent experiments, analysis of CFC transduction by PCR detection of provirus in CFU showed an average of 70% transduction whether stimulated with SCF alone or with 36SF (Fig. 7, right).

SCF-exposed cultures were protected from apoptosis, with 10 ± 1 and 15 ± 2% apoptotic at days 1 and 2, respectively (Fig. 3A). Entry into cell cycle was similar to that resulting from 36SF exposure at day 1, 9.3 ± 0.8% versus 8.7 ± 1.4%, and lower than 36SF at day 2, 23 ± 1% versus 32 ± 4%, respectively (Fig. 4). CFC frequency was

FIG. 4. Cell cycle analysis of *in vitro*-cultured CD34⁺ cells. (Top) Aliquots of human CD34⁺ cells were stained for cell cycle analysis with PI before and after 1- and 2-day culture in the presence or absence of 36SF or SCF alone. Representative histograms are shown. (Bottom) The proportions of CD34⁺ cells in S/G₂/M phases were quantitated using ModFit software. Each point represents the average of two to four experiments.

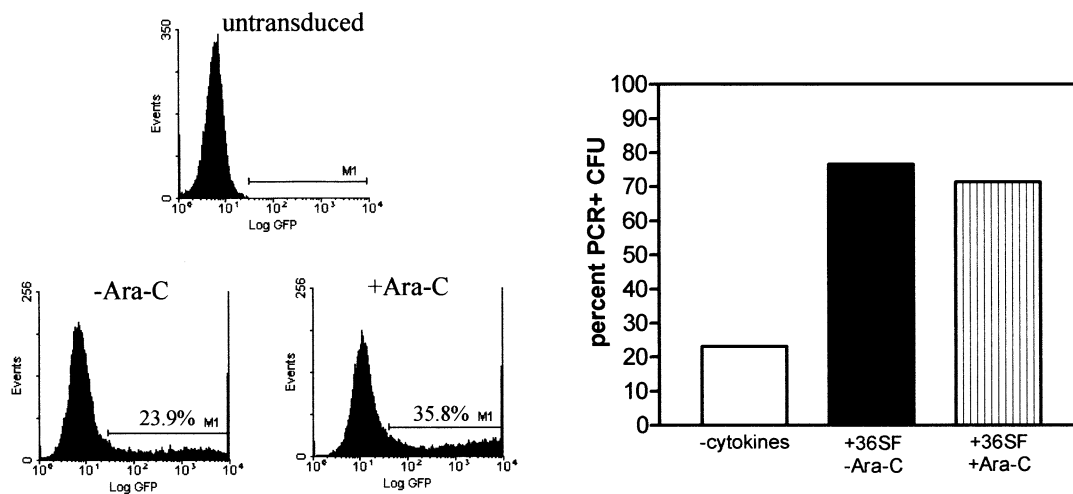


FIG. 5. Transduction of Ara-C-treated CD34⁺ cells. 36SF-stimulated and transduced cells were treated, or not, with 2 μ M Ara-C for 2 h following the 1-day transduction period. (Left) Flow cytometric detection of GFP expression in Ara-C-treated or untreated CD34⁺ cells. (Right) The proportion of transgene PCR positive CFU obtained after transduction under the specified conditions.

16% at the end of transduction, the same as it was before culture (data not shown). These data indicate that SCF alone may be sufficient to substitute for a 36SF cocktail, promoting progenitor survival and high lentiviral transduction.

DISCUSSION

In this study we show that lentiviral transduction of human hematopoietic progenitors is increased when transduction occurs after a 1-day prestimulation with 36SF or SCF alone, compared to a transduction immediately after isolation in the absence of cytokine stimulation. The difference was small, but significant, between the unstimulated and the 36SF-stimulated groups when the mass culture was analyzed (19% versus 13%, $P = 0.002$) and was larger at the CFC level (78% versus 39%, $P = 0.0001$). Transduction after SCF exposure resulted in gene transfer to 22% of mass culture and 70% of CFC. We and others have suggested that transduction of CD34⁺ cells in mass culture is equivalent with or without cytokines [8,15,18,21,22], but our results here using larger n demonstrate this is not the case. Some studies compared an immediate 24-h transduction without cytokines to that of immediate transduction with cytokines [8,18,22]. Our cell cycle data are suggestive that HSC are slowly stimulated out of a resting state during the first 24 h and this could account for cytokines having little effect in such a short culture period.

In contrast to other studies [8,22–24], we analyzed transduction using assays for primitive hematopoietic progenitors, since transduction analysis of committed progenitors may not be predictive of HSC transduction. Among the more primitive progenitor subsets, transduc-

tion of LTCIC or SRC without cytokine prestimulation was poor, achieving only 12 and 23% by PCR, respectively, compared to high gene transfer levels with cytokine stimulation of 57% to LTCIC and 81% to human CFU recovered from transplanted NOD/SCID mice. A profound resistance to lentiviral transduction of unstimulated human cells in primitive progenitor assays has not previously been documented.

Transduction of quiescent human CD34⁺ cells in mass culture has been shown to be poor in the absence of cytokines [23]. An apparent block in the early stages of the viral life cycle could not be overcome by cytokine treatment following transduction. These authors show data suggesting that transduced cells are more likely to express proliferating cell nuclear antigen, but our data with Ara-C-treated cells show no preference for S-phase transduction. Transducing in a cytokine cocktail that does not stimulate robust S-phase entry, such as SCF, may be an important distinction for designing optimal transduction culture conditions, since it has been reported that G₁-phase cord blood CD34⁺ cells have repopulating capacity similar to that of G₀ cells [25].

While the studies by Sutton *et al.* [23,24] have sought to characterize the apparent defect in lentiviral transduction of G₀-phase cells at a molecular level, our goal with these studies was to quantify differences in transduction using preclinical models of more primitive progenitors, such as LTCIC and NOD/SCID repopulating cell assays. We were then able to define these differences in the framework of the effects our specific culturing conditions had on progenitor maintenance and cell cycle progression, leading to the hypothesis that a minimal SCF treatment alone could be sufficient to enhance transduction. This work therefore has relevance to future clinical appli-

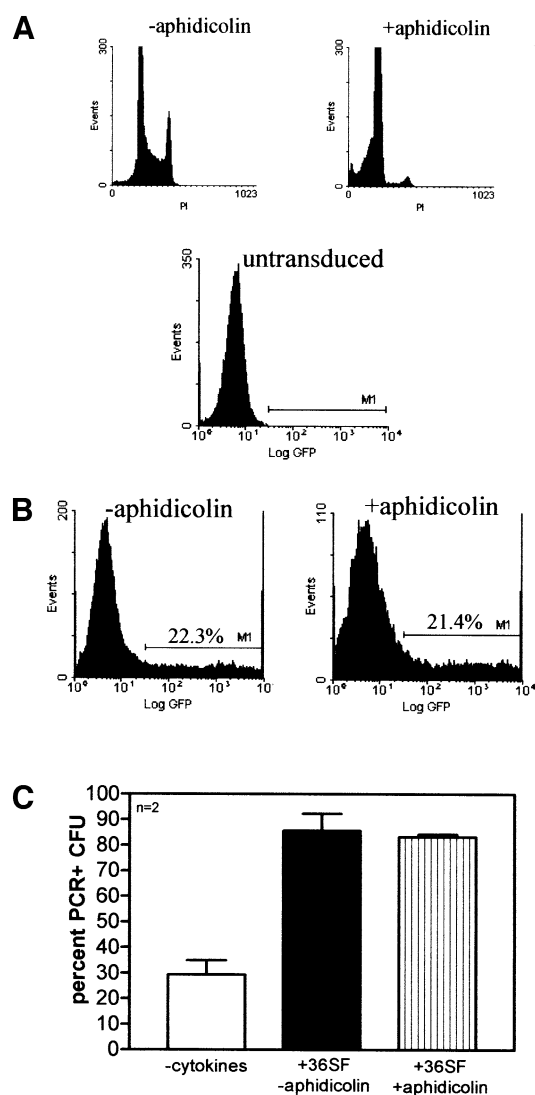


FIG. 6. Transduction of aphidicolin-treated CD34⁺ cells. 36SF-stimulated cells were treated with 2 μ g/ml aphidicolin beginning at isolation and lasting through the transduction period. (A) PI staining showing aphidicolin-arrested cells in G₁ phase. (B) Flow cytometric determination of GFP expression in CD34⁺ cells treated or untreated with aphidicolin. (C) PCR detection of the transgene in CFU obtained after CD34⁺ cell transduction under the specified conditions ($n = 2$).

cation of lentiviral gene therapy in that (1) HSC will require cytokines during transduction by lentiviral vectors and (2) a complex cocktail of cytokines, some of which are no longer clinically produced, is not needed to achieve sufficient levels of gene transfer.

We were able to show that although cytokines induce progression of a portion of CD34⁺ cells through the cell cycle (Fig. 4), this does not account for the increased transduction observed after cytokine prestimulation. Specifically, when Ara-C was used to eliminate replicating

cells following transduction, it was found that transduction of the nonreplicating CFC population, which escaped Ara-C killing, was slightly higher than the total, untreated CFC population, indicating that the replicating population was not preferentially transduced (Fig. 5B). In addition, aphidicolin-inhibited, cytokine-stimulated progenitors were transduced equally as well as cultures not treated with aphidicolin and better than unstimulated cultures (Fig. 6).

There may be two explanations for this observation. Data using wild-type HIV indicate that although it can infect nondividing cells, T cells, macrophages, and other cells, infection of cells in G₀ phase does not occur efficiently [9–12]. Cellular factors that contribute to nuclear translocation or integration may be inactive or absent in quiescent cells. It has been reported that progression into G_{1b} phase is required for productive infection [26]. It is likely that our culture period with cytokines stimulates cells into this phase, thus permitting transduction. This may also explain why others did not find a significant increase in transduction after a short transduction period with cytokines [8,18,22] since cells require time to progress from G₀ to G_{1b}.

In addition, stimulation with cytokines through their respective receptors activates signal transduction pathways that may function in one or more of the early events in the viral life cycle, from uncoating of the viral core to completion of integration. It has been learned from studies of the wild-type lentivirus that phosphorylation of components of the preintegration complex is important for nuclear translocation [27]. These signals could potentially be supplied during wild-type HIV infection by the coreceptors CXCR4 and CCR5, but supplied *in trans* during transduction with modified vectors through SCF or other cytokines that activate appropriate signal transduction pathways. However, when we treated producer cells and CD34⁺ cells with the MEK inhibitor PD98059, we did not observe any effect on mass culture transduction in the presence of 36SF (data not shown). The effect of signal transduction may be very virus, cell, and pathway specific, and further investigation into this area may yield useful insight into lentiviral transduction and HIV biology.

The purpose of including cytokines in gene therapy of HSC traditionally has been to increase retroviral transduction and expand the stem cell population. However, a more basic reason to include cytokines during any HSC transduction procedure is that they support the survival of primitive cells, as we have described here. While it is true that long-term culture of HSC with cytokines often results in loss of the stem cell phenotype, a shorter, 2-day culture period likely does not have such a dramatic detrimental effect. Recent data suggest that following a cytokine stimulation with a short period designed to rest the cells back into G₀/G₁ using SCF on fibronectin can have a positive impact on long-term engraftment in a primate

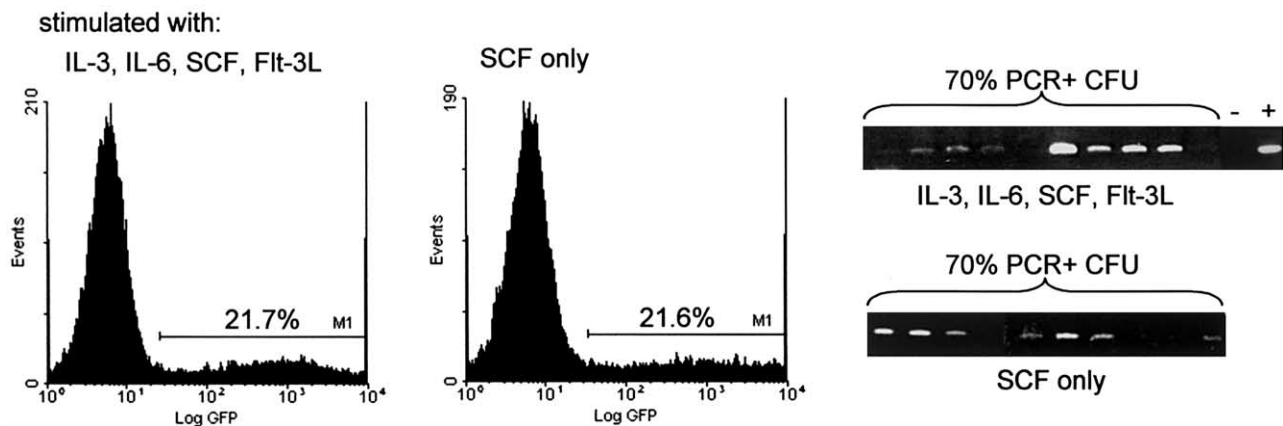


FIG. 7. Transduction after SCF exposure. Human CD34⁺ cells were stimulated 1 day with SCF alone or a cocktail of 36SF followed by transduction with LV-GFP. Transduction as assessed by (left) flow cytometric detection of GFP and (right) PCR detection of the transgene in CFU shows equivalent transduction. Representative of two independent experiments. – and + represent negative (H₂O) and positive (transduced 293T cell extracts) controls, respectively.

model [20]. Our short course of cytokine stimulation may accomplish similar results, and investigation of transduction under similar conditions may be a useful area of investigation.

In summary, lentiviral transduction of human hematopoietic progenitors is greater under cytokine stimulation than transduction in the absence of cytokines. This effect is magnified in more primitive progenitor populations. Stimulation with SCF alone increased transduction as much as 36SF. Inclusion of cytokines had a positive effect on maintenance of progenitors, compared to transduction without cytokines, which resulted in dramatic cell death and loss of progenitors. The cytokine effect did not require cell cycle progression, implying that other intracellular events are responsible for enhancing transduction. We would conclude that lentiviral transduction in the presence of cytokine support offers advantages in both transduction and stem cell maintenance, compared to a 1-day transduction in the absence of cytokines.

MATERIALS AND METHODS

Vector production. Second-generation packaging and self-inactivating lentiviral plasmids pHR'CMV.GFP.SIN-18, pCMVΔR8.91, and pMD.G were kindly provided by D. Trono (University of Geneva). For some experiments, a mutant methylguanine-DNA-methyltransferase (P140K MGMT) gene was used as a marker transgene. This was constructed by replacing the enhanced GFP gene of pHR'CMV.GFP.SIN-18 with P140K MGMT as described [15]. These vectors contained the cPPT/CTS, which was cloned into each exactly as described [8].

Lentiviral stocks were produced by transient cotransfection of the plasmids into 293T cells as follows: Cells (10.1×10^6) were seeded into 15-cm tissue culture dishes 24 h prior to transfection. Transfection was done in serum-free OptiMEM (Life Technologies) using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions and using plasmid quantities of 15.1 μg pHR'CMV.P140K.SIN-18 or pHR'CMV.GFP.SIN-18, 15.1 μg pCMVΔR8.91, and 5.0 μg pMD.G. At 20 h post-transfection, the transfection mix was replaced with 12.6 ml Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inacti-

vated fetal bovine serum (FBS; Cellgro) and 2 mM GlutaMAX (Life Technologies). Virus (LV-P140K or LV-GFP)-containing supernatant was collected 20–44 h posttransfection, filtered through a 0.2-μm filter, and stored at –80°C. Fresh virus was titered by assaying expression after transduction of 293T cells exposed to dilutions of virus in the presence of 5 μg/ml Polybrene. Expression of P140K MGMT was analyzed by flow cytometry after immunolabeling with the anti-AGT monoclonal antibody mT3.1 (Kamiya Biomedical, Seattle, WA) and an anti-mouse phycoerythrin (PE)-conjugated secondary antibody (Caltag Laboratories, Burlingame, CA), while expression of GFP was determined directly by flow cytometry (Coulter XL) after washing with Dulbecco's phosphate-buffered saline (DPBS). Unconcentrated titer was $5\text{--}7 \times 10^6$ /ml and the same virus lot (LV-GFP or LV-P140K) was used for all experiments.

Hematopoietic progenitor isolation and transduction. Human umbilical cord blood was obtained through the Comprehensive Cancer Center Stem Cell Facility from normal deliveries of mothers giving informed consent at Rainbow Babies and Children's Hospital (Cleveland, OH). Mononuclear cells were obtained by Ficoll-Paque (Pharmacia) density centrifugation and CD34⁺ cells were enriched using a Miltenyi MiniMACS CD34⁺ Isolation Kit (Miltenyi Biotech).

Following enrichment, CD34⁺ cells were either transduced immediately without addition of cytokines or cultured overnight in the presence of cytokines. Immediate transduction occurred in DMEM containing 10% heat-inactivated FBS and 2 mM GlutaMAX. CD34⁺ cells were suspended in medium to a density of $3\text{--}4 \times 10^5$ /ml, 5 μg/ml Polybrene was added, and LV-GFP or LV-P140K was added to achieve an m.o.i. of 1. No cytokines were added. Transduction occurred in six-well plates that were centrifuged (spinoculated) for 45 min at 600g at room temperature before incubation at 37°C in a 5% CO₂ humidified environment [15]. Transduction without cytokines was allowed to proceed for 1 day before further manipulation.

Cytokine-stimulated cells were cultured after isolation for 1 day in Iscove's medium containing 10% FBS, 2 mM GlutaMAX, 20 ng/ml IL-3 (kindly provided by Systemix), 20 ng/ml IL-6 (kindly provided by Systemix), 100 ng/ml SCF (kindly provided by Amgen), and 100 ng/ml Flt-3L (kindly provided by Immunex). After this 1-day prestimulation period, cells were transduced under the same conditions as described above except that IL-3, IL-6, SCF, and Flt-3L were included in the transduction medium. Further manipulation occurred 1 day after the start of transduction.

To inhibit cell cycle progression in cytokine-containing cultures, 2 μg/ml aphidicolin was added immediately after isolation and maintained in culture until after transduction, a total of 2 days. For Ara-C (Sigma) treatment, cells were treated with 2 μM Ara-C for 2 h following the 1-day transduction period.

Flow cytometry. GFP-expressing transduced cells in mass culture were analyzed 3–5 days posttransduction by flow cytometry after being washed with DPBS. P140K MGMT-expressing transduced cells in mass culture were analyzed after immunolabeling with the anti-AGT monoclonal antibody mT3.1 and an anti-mouse PE-conjugated secondary antibody.

Three-color flow cytometry was used to detect GFP⁺ human cells of various lineages in NOD/SCID bone marrow using anti-CD45–CyChrome and anti-CD19[–], -CD33[–], -CD34–PE antibodies (Pharmingen).

CFC assay. Following transduction, 1200–3000 cultured cells were plated in 4 ml methylcellulose (Stem Cell Technologies, Canada) containing 0.1 mM hemin (Sigma), 20 ng/ml IL-3, 100 ng/ml SCF, 100 ng/ml GM-CSF (Immunex), and 4 units/ml erythropoietin (Amgen, Inc.) and 1 ml was plated in each of three 35-mm tissue culture dishes. Hematopoietic CFU containing at least 100 cells were counted 13 days after plating, plucked, and processed for PCR analysis by digestion with proteinase K.

LTCIC assay. LTCIC assays were performed as described [28]. Briefly, following transduction, serial dilutions of 100–10,000 cells were plated in replicates in wells of a 96-well plate in which M2-10B4 feeder cells (ATCC) were previously placed and irradiated at 15 Gy. Cultures were maintained in LTCIC medium (Stem Cell Technologies) supplemented with 10^{–6} M hydrocortisone for 5 weeks with half-medium changes occurring weekly. At 5 weeks, cells were harvested by trypsinizing, suspending the entire cell contents of each well in 1 ml methylcellulose, and plating in a 24-well plate. CFU were counted and plucked for PCR analysis after 13 days. The LTCIC frequency was estimated assuming a Poisson distribution.

SRC assay. NOD/SCID mice were given 250 cGy radiation followed by transplantation of 2–7 × 10⁵ cells through the tail vein. In some cases, 5–10 × 10⁶ irradiated CD34[–] “accessory” cells were cotransplanted. Mice were maintained on water containing penicillin or Sulfatrim (Alpharma, Baltimore, MD) until analysis. Bone marrow was analyzed at 6–8 weeks posttransplantation. Cells were prepared for flow cytometry to detect CD45, CD19, CD33, and CD34 cells as described above. Bone marrow was plated in methylcellulose to generate human CFU for PCR analysis as described above.

Cell cycle analysis. Cell cycle distribution and apoptosis were determined by PI staining. Briefly, cells were fixed with methanol, RNA was digested using RNase A, and DNA was stained using a 200 μg/ml PI solution. Quantitation of G₀/G₁ and S/G₂/M cells was done using ModFit software (Verity Software House, Topsham, ME). Reported cell cycle percentages are after exclusion of apoptotic (sub-G₁) cells.

PCR. PCR was done on 13-day-old CFU after proteinase K digestion as previously described [15]. GFP-specific primers were upper, AGATCCGC-CACAACATCGAG, and lower, CCATGCCGAGAGTGATCCC. To detect P140K MGMT nested PCR was performed using HIV- and P140K MGMT-specific primers as previously described [15]. Negative controls were either water or blank methylcellulose from plates containing positive CFU.

Statistics. All values are reported as averages ± SEM. Statistical comparison of plus and minus IL-3, IL-6, SCF, and Flt-3L data was done using the unpaired *t* test, except for the mass culture and CFC transduction data, for which the paired *t* test was used. *P* values were generated with GraphPad Prism software (GraphPad Software, Inc.).

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