RESEARCH ARTICLE

Recombinant Sendai virus provides a highly efficient gene transfer into human cord blood-derived hematopoietic stem cells

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Hematopoietic stem cells (HSCs) are a promising target for gene therapy, however, the low efficiencies of gene transfer using currently available vectors face practical limitations. We have recently developed a novel and efficient gene transfer agent, namely recombinant Sendai virus (SeV), and we have here characterized SeV-mediated gene transfer to human cord blood (CB) HSCs and primitive progenitor cells (PPC) using the jelly fish green fluorescent protein (GFP) gene. Even at a relatively low titer (10 multiplicity of infections), SeV achieved highly efficient GFP expression in CB CD34⁺ cells ($85.5 \pm 5.8\%$), as well as more immature CB progenitor cells, CD34⁺AC133⁺ ($88.2 \pm 3.7\%$) and

CD34⁺CD38[−] ($84.6 \pm 5.7\%$) cells, without cytokines prestimulation, that was a clear contrast to the features of gene transfer using retroviruses. SeV-mediated gene transfer was not seriously affected by the cell cycle status. In vitro cell differentiation studies revealed that gene transfer occurred in progenitor cells of all lineages (GM-CFU, $73.0 \pm 11.1\%$; BFU-E, $24.7 \pm 4.0\%$; Mix-CFU, $59 \pm 4.0\%$; and total, $50.0 \pm 7.0\%$). These findings show that SeV could prove to be a promising vector for efficient gene transfer to CB HSCs, while preserving their ability to reconstitute the entire hematopoietic series.

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Introduction

Gene therapy targeting hematopoietic stem cells (HSCs) holds promise to treat a number of genetic and acquired diseases. HSCs are an ideal target on this purpose, because these progenitors can reconstitute the entire hematopoietic system in a recipient during a lifetime. Successful application of stem-cell-based gene therapies to treat blood system disorders requires an efficient gene delivery system, long-term reconstitution of hematopoiesis from transduced HSCs, and stable expression of the therapeutic gene(s) in the affected blood cell lineages. Although a number of experimental studies have been done for gene transfer to HSCs, a major limitation of currently available gene transfer vectors is the low gene transfer efficiency.

We recently developed a novel viral vector for efficient gene transfer, namely recombinant Sendai virus (SeV), through which there is an efficient gene transfer into several systems, including airway epithelial cells,¹ vascular tissue,² skeletal muscle,³ as well as synovial cells.⁴ SeV, a member of the family *Paramyxoviridae*, has a non-segmented negative-strand RNA genome and

makes use of sialic acid residue on surface glycoprotein or asialoglycoprotein present on most cell types as a receptor.⁵ As SeV uses a cytoplasmic transcription system, it can mediate gene transfer to a cytoplasmic location.⁶ There are technical advantages in the use of recombinant SeV as gene therapy vector. First, the activity of SeV particles is stable and can be easily concentrated to high titers, which is in clear contrast to the features of retroviral vectors. Second, and most importantly, the modalities of target cell processing and viral transduction are technically non-demanding and feasible in clinical situations that require transduction into large numbers of target cells.

Since characteristics of gene transfer with SeV to HSCs have not been documented yet, we analyzed the efficiency and transgene expression in SeV-mediated gene transfer to cord blood (CB) hematopoietic precursor and progenitor cells to test whether SeV could overcome current limitations in gene transfer for HSC.

Results

Transduction efficiency in CB CD34⁺ cells

We first evaluated the efficiency of recombinant SeV vector-mediated gene transfer to CB CD34⁺ cells. Evaluated based on green fluorescent protein (GFP)

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Figure 1 (a) SeV-mediated, dose-dependent GFP gene transfer to human CB-derived CD34⁺ cells. After enrichment of CD34⁺ cells using microbeads bound to anti-human CD34 antibody, the vector solution with SeV-GFP at each titer was simply added to the cell suspension. Forty-eight hours later, the cells were subjected to flow-cytometric analyses. The bar graph indicates the dose-dependent increase in gene transfer efficiency, and the efficiency almost reached its peak at 10 multiplicity of infections (MOI=10). Data from four CB samples, each in triplicate, are shown as mean $\pm$ standard deviation values. ** $P < 0.01$. * $P < 0.05$. NS: not significant. (b) Flow-cytometric analysis indicating SeV-mediated, efficient gene transfer to immature CD34⁺ precursors (CD34⁺AC133⁺, CD34⁺CD38⁻ and CD34⁺CD81⁺ cells). Forty-eight hours after gene transfer at MOI=10 with SeV-GFP to each subpopulation, the cells were subjected to flow-cytometric analysis. The experiment was done in triplicate, with consistent results. (c) Effect of vector exposure time on SeV-mediated gene transfer into enriched CB CD34⁺ cells. Enriched CB CD34⁺ cells were exposed to SeV-GFP (MOI=10) by adding vector solution, and the cells were washed twice with fresh media after the respective incubation time. Forty-eight hours after the addition of vector solution, cells were subjected to flow-cytometric analysis. Data, each in triplicate, are shown as mean $\pm$ standard deviation values.

expression, using flow cytometry on day 2, CB CD34⁺ cells showed a dose-dependent, highly efficient GFP gene transduction rate with a plateau at the titer of 10 multiplicity of infections (MOI) and over (Figure 1a). Transduction in the following experiments was thus done at the titer of 10 MOI. As shown in Figure 1b, highly efficient expression of transgene on day 2 was observed not only in CB CD34⁺ cells ($85.5 \pm 5.8\%$), but also in more immature CB progenitors, CD34⁺AC133⁺

($88.2 \pm 3.7\%$) and CD34⁺CD38⁻ ($84.6 \pm 5.7\%$) cells, and multilymphoid progenitor, CD34⁺CD81⁺ cells ($87.3 \pm 4.7\%$).

Next, we evaluated the effect of vector exposure time on SeV-mediated gene transfer efficiency at the titer of MOI=10. The GFP gene expression on day 2 was not markedly affected by vector-cell interaction time, and even a 30-min exposure led to a GFP expression level comparable to that seen with a 48-h exposure (Figure 1c).

Figure 2 Colony assays for human cord blood-derived CD34⁺ cells treated by SeV-GFP. Representative fluorescence microscopic data on GFP expression of colonies from CB CD34⁺ cells. All cell lineages, including GM-CFU (upper series) and BFU-E (middle series) as well as Mix-CFU (bottom series), showed efficient GFP expression.

Transduction of CB-derived CFUs

To evaluate the gene transfer potential of the SeV vector to colony-forming CB progenitors, colony assays were done for CB CD34⁺ cells exposed to SeV. GFP expression on day 14 was analyzed in colonies derived from committed CFUs. Highly efficient GFP expression was observed in progenitor cells of all lineages (GM-CFU, $73.0 \pm 11.1\%$; BFU-E, $24.7 \pm 4.0\%$; Mix-CFU, $59 \pm 4.0\%$; Total, $50.0 \pm 7.0\%$) (Figure 2 and Table 1). Although the total number of colonies derived from SeV-treated CB CD34⁺ cells tended to be smaller ($P=0.09$) than those from control CB CD34⁺ cells, the numbers of GM-CFU, BFU-E and Mix-CFU colonies were almost the same (Table 1), thereby suggesting that the transduction procedure did not significantly impair growth of these colonies.

Efficiency of transduction relative to cell cycle status

To investigate the dependency of transduction efficiency with SeV on the cell cycle phases, we analyzed the

Table 1 Quantitative analysis of GFP expression in colony-forming cells (CFCs) from cord blood CD34⁺ cells by progenitor colony assay

		Total	GM-CFU	BFU-E	Mix-CFU
		GFP ⁺ /total (%)	GFP ⁺ /total (%)	GFP ⁺ /total (%)	GFP ⁺ /total (%)
CB1	SeV+	83/198 (41.9)	51/72 (70.8)	16/81 (19.8)	3/5 (60)
	SeV-	0/270	0/72	0/98	0/10
CB2	SeV+	138/258 (53.5)	75/90 (83.3)	36/123 (29.3)	6/11 (54.5)
	SeV-	0/320	0/150	0/110	0/6
CB3	SeV+	124/224 (55.4)	75/100 (75)	16/66 (24.2)	5/8 (62.5)
	SeV-	0/260	0/110	0/95	0/13

SeV+: transfected by SeV-GFP. SeV-: control.

Numbers of colonies per 2000 cells.

distribution of transduced CB CD34⁺ cells in each subcompartment of the cell cycle. Cell cycle fractionation was defined by three-color flow-cytometric analysis of transduced CB CD34⁺ cells stained with 7-AAD and Ki-67. We found that GFP was expressed in all phases of the cell cycle: $22.64 \pm 6.12\%$ of G₀, $78.32 \pm 6.83\%$ of G₁, and $83.05 \pm 2.73\%$ of S/G₂/M CB cells (Figure 3a). Similar results were obtained in CB CD34⁺ cells treated with aphidicolin (Figure 3b).

Effect of SeV-mediated gene transfer on HSCs proliferation. To evaluate the proliferative activity of transduced CB CD34⁺ cells, short-term liquid cultures were observed after exposure to the SeV vector. As shown in Figure 4a, the growth of CB CD34⁺ cells exposed to the SeV vector was dose-dependently inhibited with no significant difference among MOI=5, 10 and 100. When cell viabilities on days 2, 6 and 10 were compared between SeV-treated and control CB CD34⁺ cells, SeV-treated cells showed the lowest viability on day 6 (Figure 4b). Annexin V-positive rates of CB CD34⁺ cells treated with SeV at MOI=10 and control CB CD34⁺ cells were 10% and 2% respectively, on day 4 (data not shown). Absolute numbers of four subpopulations (CD34⁺GFP⁺, CD34⁺GFP⁻, CD34⁻GFP⁺ and CD34⁻GFP⁻ cells) in SeV-treated CB CD34⁺ cells at days 2, 6 and 10

Figure 3 Relationship between cell cycle status and efficiency of transduction with SeV. The percentages of cells with GFP expression in G₀, G₁, and S/G₂/M phases of the cell cycle are shown as mean $\pm$ standard deviation values. A representative flow-cytometric data from three experiments is presented: (a) No treatment; (b) progression of transduced cells from G₁/S boundary to S phase was blocked by aphidicolin treatment.

Figure 4 Effect of SeV infection on the proliferative activity of cord blood CD34⁺ cells. CD34⁺ cells were transduced with GFP gene by SeV vector at titers 0, 5, 10 and 100 MOIs: (a) the total cell number; (b) viability (MOI=0, 5, 10) and (c) absolute number of four subpopulations (CD34⁺GFP⁺, CD34⁺GFP⁻, CD34⁻GFP⁺ and CD34⁻GFP⁻ cells) were counted at each time point (MOI=10). Data from four cord blood samples, each in triplicate, are shown as mean $\pm$ standard deviation values.

are shown in Figure 4c. Although the proliferation of CD34⁺GFP⁻ cells was prominent, CD34⁺GFP⁺ cells continued to grow during the culture period.

Discussion

In the present study, we demonstrated that SeV vector efficiently transferred the GFP gene to CD34⁺ cell and CD34⁺ cell subpopulations derived from CB (Figures 1a and b). Transduction efficiency was not seriously affected by the cell cycle status of the CB. In addition, efficient and stable gene expression was observed in clonogenic erythroid, myeloid or mixed progenitor cells (Figure 2 and Table 1). These data show the efficient gene transfer potential of SeV to quiescent cells as well as dividing

cells, including HSCs and various differentiated hematopoietic lineages.

Non-dividing CD34⁺ cells are considered to include the most long-term subsets of HSCs.⁷ The quiescent stage of CD34⁺ cells is important because induction of cell proliferation is associated with a loss of the potential to reconstitute hematopoiesis and with changes in the expression of cellular receptors.^{8–10} In this regard, application of retroviruses to HSCs is limited, because efficient gene transfer is only seen in actively replicating target cells. In a retrovirus system, the fraction of dividing cells needs to be expanded before transduction by prestimulation with cytokines in order to increase the transduction efficiency of HSCs up to 40%.^{11–13} In the present study, we found that efficient gene transfer with SeV (>80%) was attained without prestimulation of CD34⁺ cells with cytokines (Figure 1a). Furthermore, the high expression of the GFP gene (25%) in G₀ compartment of CD34⁺ cells seen with SeV-mediated gene transduction (Figures 3a and b) was comparable to that noted in lentivirus-mediated gene transfer.¹⁴ These results suggest that resting progenitors, long-term culture-initiating cells (LTC-IC) and SCID-repopulating cell (SRC), are easily transduced by the SeV vector with minimal loss of their potential to repopulate, because they are highly enriched in the G₀ compartment.^{7, 15} In this regard, the SeV vector is a suitable vehicle for transferring genes into HSCs.

From a clinical point of view, in contrast to retrovirus and lentivirus vectors, SeV-mediated gene transfer to CB CD34⁺ cells requires only simple procedures. As shown in Figure 1c, CD34⁺ cells were efficiently transduced by simply adding SeV vector solutions at the titer of MOI=10 with only brief contact time, as noted with other cell types.¹² This simplicity in gene transfer procedures using SeV is also advantageous for clinical application to CB HSCs with their capacity to reconstitute the entire hematopoietic system.

The potential cytotoxicity of SeV to the target cell remains to be a concern, because SeV induces cytopathic effects in some cells, including CV-1 cells.¹⁶ In the present study, growth inhibition of transduced CD34⁺ cells was observed in our short-time liquid suspension culture. Since SeV-induced apoptosis might be possible in some permissive cells through activation of caspase 3 and caspase 8,¹⁷ we here assessed the accumulation of annexin V. As the annexin V-positive rate of SeV-treated CD34⁺ cells (10%) was higher than that of control CD34⁺ cells (2%) on day 4 and SeV-treated cells showed the lowest viability on day 6 (Figure 4b), the induction of apoptosis appears to contribute, probably in part, to decreased cell number. To minimize the damage of transduced cells, development of an SeV vector lacking cytotoxicity-associated genes such as C gene might be needed. In contrast, the formation of progenitor colonies, such as GM-CFU, BFU-E and Mix-CFU, in semi-solid culture was not significantly affected after transduction with SeV (Figure 2 and Table 1). The discrepancy may be explained by the possibility that CD34⁺ cells infected with SeV at the very early stage of hematopoietic differentiation was less sensitive to growth inhibition than those infected thereafter. Therefore, it is necessary to evaluate *in vivo* survival, proliferation and transgene expression of CB CD34⁺ cells transduced with SeV.

In summary, although further *in vivo* reconstruction studies are needed, the SeV vector is an important candidate for gene transfer to HSCs due to its potential for superior gene transfer into HSCs.

Materials and methods

Construction of SeV-GFP

SeV-GFP was constructed, as described.^{18,19} In brief, 18 bp of spacer sequence 5'-(G)-CGGCCGAGATCTT-CACG-3' with a *NotI* restriction site were inserted between the 5' non-translated region and the initiation codon of the nucleoprotein (N) gene. This cloned SeV genome also contains a self-cleaving ribosome site from the antigenomic strand of the hepatitis delta virus. The entire cDNA coding *jelly fish* enhanced GFP was amplified by PCR, using primers with a *NotI* site and new sets of SeV E and S signal sequence tags for an exogenous gene, then inserted into the *NotI* site of the cloned genome. The entire length of the template SeV genome, including exogenous genes, was arranged in multiples of six nucleotides (the so-called 'rule of six').²⁰ Template SeV genome with an exogenous gene and plasmids encoding N, P, and L proteins (plasmid pGEM-N, pGEM-P, and pGEM-L) was complexed with commercially available cationic lipids, then cotransfected with vaccinia virus vT7-3 into CV-1 or LLMCK cells.²¹ Forty hours later, the cells were disrupted by three cycles of freezing and thawing and injected into the chorioallantoic cavity of 10-day-old embryonated chicken eggs. Subsequently, the virus was recovered and the vaccinia virus was eliminated by a second propagation in eggs. Virus titer was determined by hemagglutination assay, using chicken red blood cells,²² and the virus was stored at -80°C until use.

CB cells samples

Heparinized CB samples were obtained from umbilical cord veins of four full-term human newborns without hereditary disorders or hematological abnormalities and were analyzed within 24 h. All samples were collected after written informed consent was obtained from the parents.

CD34⁺ cell selection and gene transfer

CB CD34 cells were labeled with hapten-conjugated monoclonal antibodies (mAbs) against human CD34 (clone QBEnd/10), followed by an anti-hapten antibodies (Abs) coupled with microbeads. The bead-positive cells (CD34 cells) were enriched on positive-selection columns set in a magnetic field. Flow-cytometric analysis of purified cells using a different clone of FITC-conjugated anti-CD34 mAb showed more than 95% purity. Purified CD34⁺ cells were then suspended at 2 to 4 × 10⁵ cells/ml in 2.5-cm tissue culture dishes in serum-free Iscove's Modified Dulbecco's medium (IMDM) containing 30% fetal bovine serum (FBS), 10 ng/ml of stem cell factor (SCF, Kirin Brewery Company, Tokyo, Japan), interleukin 6 (IL-6, Kirin Brewery Co), and thrombopoietin (TPO, Kirin Brewery Co). In all experiments, gene transfer was carried out by adding various amounts of vector solution to the media. Cells were then incubated at 37°C in a 5% CO₂ incubator, and collected for cell count and flow-cytometric analysis at different

time points, as indicated in Figures 1 and 4. To evaluate the effect of vector exposure time on SeV-mediated gene transfer, cells were washed twice with fresh media after treatment with SeV at MOI=10. Except for this experiment, the washing after transduction was omitted for purposes of simplification.

Colony assays

To determine the number of erythroid, myeloid, mixed lineage progenitors, 2000 of the transduced cells were plated in 35-mm tissue culture dishes containing 1 mL of 0.88% methylcellulose-based semi-solid culture medium supplemented with 30% FBS, 50 U/ml of interleukin 3 (IL-3, Kirin Brewery Co.), 50 ng/ml of SCF, 50 ng/ml of granulocyte-macrophage-colony-stimulating factor (GM-CSF, Kirin Brewery Co.), and 10 U/ml of erythropoietin (EPO, Kirin Brewery Co.). After 14 days of incubation at 37°C in a 5% CO₂ incubator, colony-forming units-granulocyte-macrophage (CFU-GM), burst-forming units-erythroid (BFU-E), colony-forming units-granulocyte erythrocyte monocyte macrophage (CFU-Mix) colonies were enumerated and GFP-expressing colonies were identified, using a fluorescence microscope.

Flow-cytometric analysis

Transduced cells were stained with appropriate amounts of phycoerythrin (PE)- and phycoerythrin 5.1(PC-5)-conjugated mAbs at 4°C for 20 min. All mAbs were obtained from Coulter-Immunotech (Miami, FL, USA). The cells were washed twice and suspended in phosphate-buffered saline (PBS). Flow-cytometric analysis was done using EPICS XL (Beckman-Coulter, Hialeah, FL, USA). A total of at least 10 000 events were analyzed for each sample. Gates were set according to forward and side scatters. Cell viability and annexin V-positive rate were assessed by 7-AAD staining and by annexin V-FITC (MBL, Nagoya, Japan) staining, respectively.

For cell cycle analysis, cells were analyzed for Ki-67 expression and DNA content as described by Jordan *et al.*²³ but with minor modifications. Briefly, cells incubated at 37°C in a 5% CO₂ incubator for 48 h after transduction were washed and resuspended in 1-ml PBS containing 0.4% formaldehyde. After 30 min incubation at 4°C, 1 ml of PBS containing 0.2% Triton X-100 was added and cells were left overnight at 4°C. Cells were then washed twice in PBS containing 1% bovine serum albumin (BSA) and stained with PE-conjugated anti-Ki-67 (clone MIB-1; Immunotech, West-brook, ME, USA) for 60 min at 4°C. Isotype-matched mAb control was used in parallel. Finally, cells were washed and resuspended in PBS containing 1% BSA and 5 µg/ml 7-aminoactinomycin-D (7-AAD; Sigma). After 3 h of incubation on ice, samples were run on flow cytometer using FL-1 and FL-2 channels for Ki-67 and 7-AAD, respectively. To block the cells at G1/S boundary, transduced cells were treated with 2 mg/ml of aphidicolin (Sigma Chemical Co, St Louis, MO, USA) at 37°C in a 5% CO₂ incubator for 48 h.

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