THE JOURNAL OF GENE MEDICINE
J Gene Med 2004; 6: 1049–1060.
Published online 1 June 2004 in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/jgm.609

RESEARCH ARTICLE

Sustained transgene expression by human cord blood derived CD34$^+$ cells transduced with simian immunodeficiency virus agmTYO1-based vectors carrying the human coagulation factor VIII gene in NOD/SCID mice

Jiro Kikuchi[1]
Jun Mimuro[1,2]*
Kyoichi Ogata[1]
Toshiaki Tabata[3]
Yasuji Ueda[3]
Akira Ishiwata[1]
Konzoh Kimura[1]
Katsuhiro Takano[1]
Seiji Madoiwa[1,2]
Hiroaki Mizukami[4]
Yutaka Hanazono[4]
Akihiro Kume[4]
Mamoru Hasegawa[3]
Keiya Ozawa[2,4]
Yoichi Sakata[1,2]

[1]*Division of Cell and Molecular Medicine, The Center for Molecular Medicine, Jichi Medical School, Tochigi-ken 329-0498, Japan*

[2]*Hematology Division of Department of Medicine, Jichi Medical School, Tochigi-ken 329-0498, Japan*

[3]*DNAVEC Research Inc., Ibaraki-ken 305-0003, Japan*

[4]*Division of Genetic Therapeutics, The Center for Molecular Medicine, Jichi Medical School, Tochigi-ken 329-0498, Japan*

*Correspondence to: Jun Mimuro, Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical School, Tochigi-ken 329-0498, Japan.
E-mail: mimuro-j@jichi.ac.jp

Received: 16 September 2003
Revised: 12 February 2004
Accepted: 22 March 2004

Abstract

Background Gene therapy is being studied as the next generation therapy for hemophilia and several clinical trials have been carried out, albeit with limited success. To explore the possibility of utilizing autologous bone marrow transplantation of genetically modified hematopoietic stem cells for hemophilia gene therapy, we investigated the efficacy of genetically engineered CD34$^+$ cell transplantation to NOD/SCID mice for expression of human factor VIII (hFVIII).

Methods CD34$^+$ cells were transduced with a simian immunodeficiency virus agmTYO1 (SIV)-based lentiviral vector carrying the enhanced green fluorescent protein (eGFP) gene (SIVeGFP) or the hFVIII gene (SIVhFVIII). CD34$^+$ cells transduced with SIV vectors were transplanted to NOD/SCID mice. Engraftment of transduced CD34$^+$ cells and expression of transgenes were studied.

Results We could efficiently transduce CD34$^+$ cells using the SIVeGFP vector in a dose-dependent manner, reaching a maximum ($99.6 \pm 0.1\%$) at MOI of 5×10^3 vector genome/cell. After transducing CD34$^+$ cells with SIVhFVIII, hFVIII was produced (274.3 ± 20.1 ng) from 10^6 CD34$^+$ cells during 24 h *in vitro* incubation. Transplantation of SIVhFVIII-transduced CD34$^+$ cells ($5–10 \times 10^5$) at a multiplicity of infection (MOI) of 50 vector genome/cell into NOD/SCID mice resulted in successful engraftment of CD34$^+$ cells and production of hFVIII (minimum 1.2 ± 0.9 ng/mL, maximum 3.6 ± 0.8 ng/mL) for at least 60 days *in vivo*. Transcripts of the hFVIII gene and the hFVIII antigen were also detected in the murine bone marrow cells.

Conclusions Transplantation of *ex vivo* transduced hematopoietic stem cells by non-pathogenic SIVhFVIII without exposure of subjects to viral vectors is safe and potentially applicable for gene therapy of hemophilia A patients.

Keywords hemophilia; gene therapy; simian immunodeficiency virus; hematopoietic stem cell

Introduction

Hemophilia A is an inherited X-linked lifelong bleeding disorder caused by abnormality in the coagulation factor VIII (FVIII) gene [1]. The genetic abnormalities result in deficiency of FVIII, which in turn creates a bleeding

phenotype, such as life-threatening intracranial bleeding and bleeding in joints and muscles. Hemophilias occur as mild, moderate, or severe phenotypes, depending on the blood FVIII level of 6% or more, 2–5%, or 1% or less [1,2]. Current standard therapy is intravenous (i.v.) injection of human plasma-derived FVIII or recombinant FVIII. Aside from certain specific situations such as pre-operative factor coverage, i.v. infusion of FVIII usually is used to treat acute bleeding episodes. However, maintenance of blood FVIII levels to more than 5% of the normal FVIII concentration may result in significant clinical improvement. Furthermore, if one can increase FVIII levels to more than 1% in severe hemophilia patients, they may have significantly fewer bleeding episodes and improved quality of life. Additionally, in the past, infection with hepatitis B and C viruses or human immunodeficiency virus (HIV) in hemophilia patients was a tragic result of contaminated blood-derived commercial products. In this regard, gene therapy is being explored as the next generation therapy for hemophilia patients. Hemophilia is considered suitable for gene therapy for the following reasons: (1) treatment is feasible through replacement of a normal copy of the factor VIII gene; (2) increase of factor VIII levels to just 1% or more of the normal level may provide significant clinical improvement; and (3) gene therapy offers the potential for more sustained and less expensive treatment than the current standard therapy [2].

Hematopoietic stem cells are thought to be highly desirable targets for gene therapy because of their capability for self-renewal and multi-lineage differentiation [3]. Non-obese diabetic severe combined immunodeficient (NOD/SCID) mice are well-characterized immunodeficient mice obtained by transferring the SCID mutation onto the NOD background. They lack functional T and B cells and diminished natural killer (NK) and macrophage activities and, thus, they are thought to be suited for transplantation of hematopoietic stem cells. Human hematopoietic stem cells can be engrafted and generate their progeny in NOD/SCID mice, and virally transduced hematopoietic stem cells have also been transplanted successfully into NOD/SCID mice [4–6].

Retroviral vectors including lentiviral vectors are now widely used to transduce hematopoietic stem cells [4–6]. These vectors can integrate transgenes into the target cell genome, allowing transmission of the transgenes to daughter cells *in vivo* [7]. Lentiviral vectors transduce hematopoietic stem cells more efficiently than the classical retroviral vectors. However, there are safety concerns in utilizing HIV-1-based lentiviral vectors for gene therapy clinical trials. In this regard, simian immunodeficiency virus agmTYO1 (SIVagmTYO1)-based vectors are of particular interest. SIVagmTYO1 is an HIV-related lentivirus isolated from the African green monkey and shown to be non-pathogenic to both their natural hosts and to experimentally inoculated Asian macaques [8,9]. Additionally, due to the use of contaminated blood products, some hemophilia patients are HIV-1 carriers. If an HIV-1-based vector is administrated to such patients, the replication-competent lentivirus particles carrying the therapeutic gene may be generated by homologous recombination between the recombinant HIV vector and the wild-type HIV genome. The packaging signal in the HIV vector sequence may be another factor contributing to production of replication-competent lentivirus particles. From this perspective, then, a SIV vector based on the SIVagmTYO1 strain may be a better vehicle for hemophilia gene therapy because SIVagmTYO1 has less than 60% genomic sequence similarity to HIV-1. We have developed SIVagmTYO1-based vectors that can transduce various cells, including hematopoietic stem cells. While early studies have indicated the lack of factor VIII secretion by hematopoietic cells [10], recent studies have shown the ability of hematopoietic cell lines [11] and stem cells [12,13] to secrete detectable amounts of factor VIII upon lentiviral transduction. In the present study, we use human cord blood derived $CD34^+$ (CB-$CD34^+$) cells and SIVagmTYO1-based vectors to show that high FVIII expression levels are achieved in $CD34^+$ cells transduced with the SIV vector *in vitro* and that transplantation of SIVagmTYO1 vector-transduced hematopoietic stem cells may be used for hemophilia gene therapy *in vivo*.

Materials and methods

Cell lines, medias and cytokines

Recombinant human thrombopoietin (TPO) and stem cell factor (SCF) were kindly supplied by Kirin Brewery Co. (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) and Iscove's modified Dulbecco's medium (IMDM) were purchased from Invitrogen Japan (Tokyo, Japan) and fetal bovine serum (FBS) from Hyclone (Logan, UT, USA). Human embryonal kidney 293T (HEK293T) cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM containing 10% FBS.

$CD34^+$ cell purification

Cord blood was drawn from umbilical cords of normal full-term deliveries after obtaining informed consent. Mononuclear cells were separated by density gradient centrifugation using Lymphoprep tubes (Dai-ichi Pharmaceutical, Tokyo, Japan) after depletion of phagocytes using silica particles (Immuno Biological Laboratories, Gunma, Japan). The mononuclear cells were suspended in phosphate-buffered saline (PBS) at $3–5 \times 10^7$ cells/ml and mixed with Dynabeads M-450 CD34 (Dynal AS, Oslo, Norway) at a bead-to-cell ratio of 1 : 1. After incubation at 4 °C for 30 min with gentle rotation, the cell-beads suspension was placed in a DYNAL MPC (magnetic particle concentrator) to collect the Dynabeads M-450 CD34-rosetted cells. The rosetted cells were incubated with DETACHaBEAD CD34 (Dynal) at 37 °C for 15 min to release $CD34^+$ cells from the beads. The purity of $CD34^+$

cells was evaluated by flow cytometry using a FACScan (Becton Dickinson) and approximately 95% of the separated cells were CD34-positive.

Production of SIVagm vectors

Human FVIII cDNA spanning the entire coding region was a generous gift from Dr. J. A. van Mourik. Because the B domain is excised from other FVIII domains upon activation by thrombin and is not essential for coagulation activity expression of FVIIIa, the human FVIII cDNA was subjected to PCR-based mutagenesis to delete most of the fragment that encodes the FVIII B domain (BDD FVIII cDNA) as described previously [14]. The characteristics and production of SIVagm vector used in this study have been described previously [9,14]. Self-inactivating SIVagm vectors are pseudotyped with vesicular stomatitis virus glycoprotein G (VSVG). We constructed gene transfer vectors to express the hBDDFVIII gene and eGFP gene driven by the cytomegalovirus (CMV) promoter. To produce SIV vectors, HEK293T cells were transfected with the packaging vector, the gene transfer vector, and pVSVG (Clontech) as described previously [9,14]. Transduction units of SIV vectors carrying the eGFP gene (SIVeGFP) were determined by infection of SIVeGFP to HEK293T cells followed by determination of eGFP expression by FACS analysis. Since determination of transduction units of SIV vectors carrying the human BDDFVIII cDNA (SIV hFVIII) was difficult compared with SIV eGFP, RNA dot blot analysis was performed to quantify the amount of SIV vector genome (vg) of vector preparations. To detect replication-competent SIV particles in cells infected with SIV vectors, HEK 293T cells were cultivated in media containing SIV eGFP or SIV hVIII at a multiplicity of infection (MOI) of 100 vg/cell. RNA was isolated from the supernatants of vector-infected cells on day 6 after infection using an RNA isolation kit (QIAamp viral RNA mini kit; QIAGEN). Detection of the gag gene and the pol gene required for virus replication in the RNA preparation was carried out by reverse transcription-polymerase chain reaction (RT-PCR) using the gag-specific primers (5′-GTC CTA GAC ATT AGG CAG GGA CCT-3′, 5′-TTT TGC CCC CAT CCA CCG TCC ATA-3′) and the pol-specific primers (5′-CAG AAA TTC AAA AGA AGG AAA AGC A-3′, 5′-CTT CTT GGG AGG TAA AGT TAG GCC CA-3′), respectively.

In vitro culture and transduction of human CB-CD34$^+$ cells

The cells were cultivated at 5×10^5 cells/mL in IMDM supplemented with 1% bovine serum albumin (BSA), 10 μg/mL bovine pancreatic insulin, 200 μg/mL human transferrin (BIT 9500; StemCell Technologies, Vancouver, Canada), 40 μg/mL human low-density lipoproteins (Chemicon International), 10^{-4} mol/L 2-mercaptoethanol, SCF (100 ng/mL) and TPO (50 ng/mL) at 37 °C with 5% CO_2 in 48-well tissue culture plates (Falcon, Lincoln Park, NJ, USA) [15]. For transduction, SIV vector ($0.25–5 \times 10^{10}$ vg/mL) was added to the cell suspension, and the plates were incubated at 37 °C in the presence of 5% CO_2. After incubation, SIVeGFP-transduced CD34$^+$ cells were analyzed for eGFP expression by flow cytometry and the conditioned medium of SIVhFVIII-transduced CD34$^+$ cells was harvested and subjected to FVIII enzyme-linked immunosorbent assay (ELISA).

Mice

Experimental NOD/SCID mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). The NOD/SCID mice were kept in a clean experimental room and were maintained on a γ-irradiated sterile diet and given autoclaved, distilled water containing 1 μg/mL of neomycin sulfate after transplantation [16]. Peripheral blood was drawn into EDTA-containing tubes from mouse tail veins and platelet-poor plasma was prepared by centrifugation. Platelet-rich plasma was also prepared from 1 mL of mouse blood upon sacrifice. Platelets were collected from platelet-rich plasma by centrifugation and were extracted in 50 μL of 0.5% Triton X-100/PBS. Bone marrow cells and bone marrow blood were collected from femurs by irrigation with PBS. After centrifugation, bone marrow cells were subjected to fluorescence-activated cell sorting (FACS) analyses and the supernatants were subjected to ELISA for quantification of human FVIII. The bone marrow volume of a mouse femur preparation was estimated to 10 mm^3 since it was approximately one-third of the volume of the femur calculated by the expression of 1 mm (radius)$^2 \times \pi \times 10$ mm (length).

Transplantation of CB-CD34$^+$ cells into NOD/SCID mice

Xenograft transplantation of the CB-CD34$^+$ cells was performed according to methods previously described [16]. Briefly, $5–10 \times 10^5$ CB-CD34$^+$ cells were transduced with SIVagm vector and injected into 6- to 12-week-old NOD/SCID mice through the tail vein after sub-lethal irradiation with 350–360 cGy of γ-ray (^{60}Co, Gamma Cell; Nortion International, Kanata, ON, Canada). For internal controls, NOD/SCID mice were injected with non-transduced (mock) CB-CD34$^+$ cells using identical procedures. Because NK cell activity of NOD/SCID mice is not completely impaired, 400 μL of PBS containing 20 μL of anti-asialo GM1 antiserum (Wako; Osaka, Japan) were injected intraperitoneously to the recipient mice immediately before the cell transplantation to delete NK cells [17]. Anti-asialo GM1 antiserum injection was carried out once a week after transplantation of CD34$^+$ cells. Peripheral blood (100 μL) was collected from mouse tail veins into tubes containing EDTA. Platelet-rich plasma (PRP) was prepared by centrifuging whole blood at 200 *g*

for 10 min. After collection of PRP, platelet-poor plasma was prepared by centrifugation at 400 *g* for 5 min, and subjected to FVIII ELISA. Peripheral leukocytes were prepared after disrupting red blood cells using Lysis buffer (155 mM NH_4Cl, 10 mM NH_4HCO_3, 0.1 mM EDTA, pH 7.4). Leukocytes and platelets were subjected to flow cytometric analysis using the FACScan. Mice were sacrificed on day 60 after transplantation. Bone marrow cells were drawn from femurs and the spleen cells were also collected. These cell suspensions were filtered through sterile 40-μm cell strainers (#2340; Falcon) to get rid of clumps and clots [16]. Cells and platelets were processed for flow cytometric analysis and the immunofluorescent analysis (see below). Platelet-poor plasma prepared from peripheral blood and the supernatant of bone marrow cell suspension were subjected to the FVIII ELISA assay.

Flow cytometric analysis of transplanted human cells in NOD/SCID mice

Surface markers on human hematopoietic cells reconstituted in peripheral blood, bone marrow cells, and spleen cells of NOD/SCID mice were analyzed by flow cytometry as described [18]. Briefly, after depletion of erythrocytes using Lysis buffer, mouse peripheral white blood cells, bone marrow cells, spleen cells, and platelets were incubated on ice for 30 min with a series of fluorescence-labeled monoclonal antibodies (Dako Japan, Tokyo, Japan) to human cluster of differentiation (CD) antigens in 100 μL of PBS containing 5% FBS. The presence of human hematopoietic cells was determined by detection of cells positively stained with phycoerythrin-cyanine 5-succinimidyl ester (PE-Cy5)-conjugated anti-human CD45. Successful engraftment of human hematopoietic cells was defined by the presence of at least 1% of human $CD45^+$ cells in peripheral blood or bone marrow of NOD/SCID mouse 60 days after transplantation [17,18]. Specific subsets of human hematopoietic cells were quantified by gating human CD45-positive cells and detection of surface antigens with fluorescein isothiocyanate isomer-1 (FITC)-conjugated anti-human CD3 and CD33 or R-phycoerythrin(RPE)-conjugated anti-human CD14, CD19, and CD34. Platelets, separated from the peripheral blood or the bone marrow, were detected with RPE-conjugated anti-human CD41.

ELISA for hFVIII antigen

Since human FVIII (hFVIII) clotting activity could not be quantified directly in the NOD/SCID mice because of the presence of endogenous murine FVIII in the plasma, hFVIII expressed in NOD/SCID mice was quantified by a hFVIII-specific ELISA as described previously [14,19]. Briefly, 96-well microtiter plates (Costar, Cambridge, MA, USA) were coated with 1 μg/mL mouse monoclonal antibodies to hFVIII (Chemo-Sero Therapeutic Institute, Kumamoto, Japan) [19]. After blocking with 5% casein in PBS, mouse plasma samples or pooled normal human plasma in Tris-buffered saline (TBS) containing 0.1% Tween 20, 1% casein were added. After 16 h incubation at 4 °C, hFVIII bound to the plates was detected with sheep anti-hFVIII polyclonal antibodies (Cedarlane Laboratories Ltd, Homby, ON, Canada) and horseradish peroxidase-conjugated rabbit anti-sheep IgG. Monoclonal antibody-purified hFVIII was kindly provided by the Chemo-Sero Therapeutic Institute and was used as the standard. Normal pooled platelet-poor plasma was also used as the standard. The ELISA could specifically detect hFVIII in mouse plasma as low as 0.5 ng/mL or FVIII in 300-fold diluted normal human plasma [14].

Immunofluorescence microscopy

Bone marrow cells were attached to glass slides using a Cytospin3 (Shandon, ThermoShandon, Inc., Pittsburgh, PA, USA), fixed with 4% paraformaldehyde in PBS and blocked with 1% BSA and 1% donkey serum in PBS. Samples were incubated with polyclonal anti-hFVIII antibody at 4 °C for 16 h. After washing in PBS, cells were incubated with donkey anti-sheep IgG antibody conjugated with Alexa488 (Molecular Probes, Eugene, OR, USA) at 4 °C for 16 h for visualization of hFVIII by fluorescent microscopy as described previously [14].

Detection of the BDD-FVIII transcript by RT-PCR

Total cellular RNA was isolated from 10^5 cells by the acid-guanidine method [20] and were reverse-transcribed to cDNA using reverse transcriptase (Superscript; Invitrogen Japan, Tokyo, Japan) and oligo-(dT) primers in a 20 μL mixture (QIAGEN Japan, Tokyo, Japan) after DNase I (Amplification grade, Invitrogen) treatment. Subsequent PCR-amplification was carried out with 1 μL of cDNA solution (corresponding to 5000 cells) in a 50 μL reaction mixture containing 5 units of Taq polymerase, 10 mmol/L Tris-HCl (pH 8.5), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$ and 100 μmol/L dNTPs in the presence of specific primer pairs (200 nmol/L) designed to amplify the DNA fragments derived from the transcript of the BDD-FVIII transgene [14]. Each PCR cycle consisted of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The PCR products were analyzed by agarose gel electrophoresis. Authenticity of PCR products was confirmed by their molecular sizes on agarose gel electrophoresis, and their sequences. The primer sequences are as follows: hFVIII: sense, 5′-ATT GGA GCA CAG ACT GAC TT-3′; antisense, 5′-ATA TGG TAT CAT CAT AGT CA-3′ (400 bp); human GAPDH: sense, 5′-TGA TGA CAT CAA GAA GGT GGT GAA G-3′; antisense, 5′-TCC TTG GAG GCC ATG TGG GCC AT-3′ (240 bp); mouse GAPDH: sense, 5′-GCA GTG GCA AGT GGC AAA GTG GAG ATT-3′; antisense, 5′-TGA GTG GAG TCA TAC TGG AAC ATG-3′ (88 bp)

Results

SIV vectors

SIV vectors carrying the human BDDFVIII cDNA (SIVh-FVIII) used in this study have been shown previously to transduce cells efficiently *in vitro* and *in vivo* [14]. No PCR-amplified fragments for the SIV gag gene or the SIV pol gene were detected by RT-PCR in the supernatants of vector-infected 293T cells *in vitro* (not shown), suggesting that no replication-competent virus particles were generated in the vector-infected cells.

Transduction of CB-CD34$^+$ cells with SIVeGFP and SIVhFVIII

To assess the *in vitro* transduction efficiency of CB-CD34$^+$ cells with the SIV vector, CB-CD34$^+$ cells were cultured in the presence of increasing concentrations of SIVeGFP for 48 h or in the presence of the fixed concentration of SIVeGFP for various incubation times. After incubation, expression of eGFP in CB-CD34$^+$ cells was analyzed by flow cytometry. As shown in Figure 1A, eGFP expression in CB-CD34$^+$ cells increased in a dose-dependent manner. 99.6 ± 0.1% of CB-CD34$^+$ cells were efficiently transduced with SIVeGFP at MOI of 5×10^3 vg/cell (100 TU/cell). Half-maximal expression of eGFP was achieved when cells were incubated with SIVeGFP at MOI of 50 vg/cell (1TU/cell). When CB-CD34$^+$ cells were cultured in the presence of SIVeGFP at MOI of 50 vg/cell (1TU/cell), expression of eGFP increased with time, reaching a maximum at 48 h incubation, followed by a gradual decline at 72 h (Figure 1B). The gradual decline in eGFP expression may be due to either pseudotransduction or reduction of cell viability. We also assessed hFVIII production in CB-CD34$^+$ cells *in vitro*. CB-CD34$^+$ cells were incubated in the presence of increasing concentrations of SIVhFVIII and the supernatants were harvested after 48 h incubation and were subjected to ELISA for the hFVIII antigen. FVIII production in the CB-CD34$^+$ cells increased in a dose-dependent manner, reaching a maximum at 274.3 ± 20.1 ng/10^6 cells/24 h at MOI of 5×10^3 vg/cell (Figure 1C).

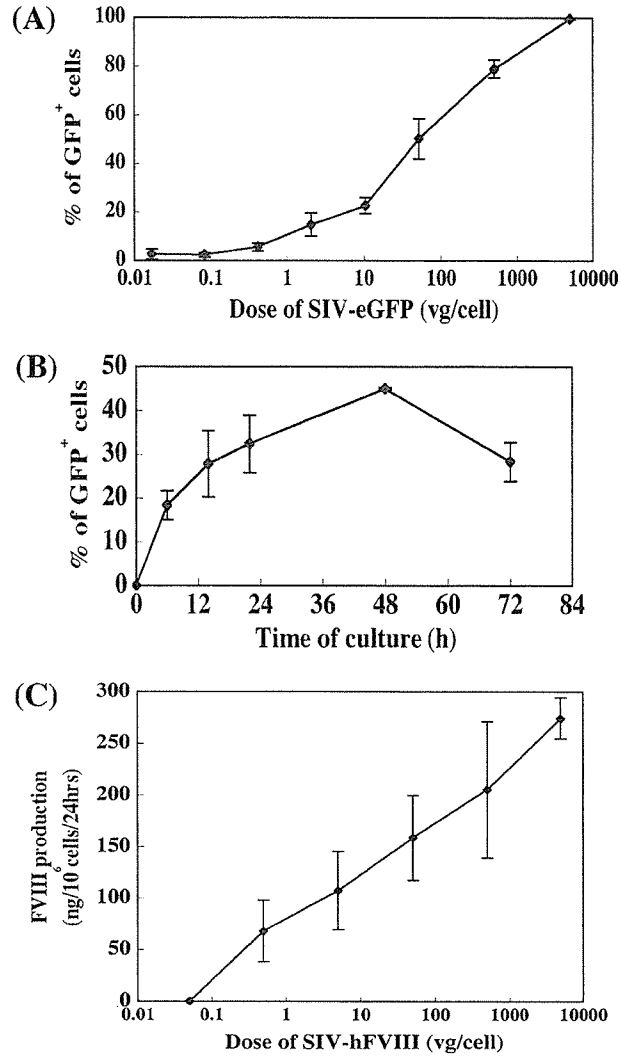

Figure 1. Transduction of CB-CD34$^+$ cells by SIVeGFP and SIVhFVIII. CB-CD34$^+$ cells (5×10^5 cells/mL) were incubated with increasing concentrations of SIVeGFP for 48 h (A) or with a fixed concentration (MOI, 50 vg/cell) of SIVeGFP for various times (B). After incubation, expression of eGFP was analyzed by flow cytometry. The percentages of transduced cells expressing eGFP are shown (mean ± SD, n = 3). CB-CD34$^+$ cells (5×10^5 cells/mL) were incubated with increasing concentrations of SIVhFVIII and supernatants were harvested after 24 h incubation and subjected to the FVIII ELISA for quantification of the hFVIII antigen (mean ± SD, n = 3)

Determination of human CB-CD34$^+$ cell-derived white blood cells in NOD/SCID mice

CB-CD34$^+$ cells were transduced with the SIVhFVIII vector in the presence of TPO and SCF, because transduction efficiency is enhanced when cells are induced to enter the cell cycle [21]. Reduced cell viability after transduction was observed due to exposure with SIV in the absence of cytokines and even in the presence of cytokines at high MOIs (data not shown). Reduction of cell viability observed during transduction may well be due to cytotoxicity of VSVG. *Ex vivo* expanded CB-CD34$^+$ cells are reportedly less potent for engraftment *in vivo* [22]. Therefore, we transduced cells with an MOI of 50 vg/cell for 24 h in the presence of cytokines and then transplanted them into NOD/SCID mice. 5×10^5 of the transduced CB-CD34$^+$ cells (+/− hFVIII) suspended in 400 μL of IMDM were injected intravenously (i.v.) into NOD/SCID mice after sub-lethal irradiation as described in Methods. Peripheral white blood cells were obtained from recipient mice and were subjected to flow cytometry to confirm engraftment of the human cells. Figure 2 shows the percentage of human CD45$^+$ cells in peripheral white blood and bone marrow cells of NOD/SCID mice after

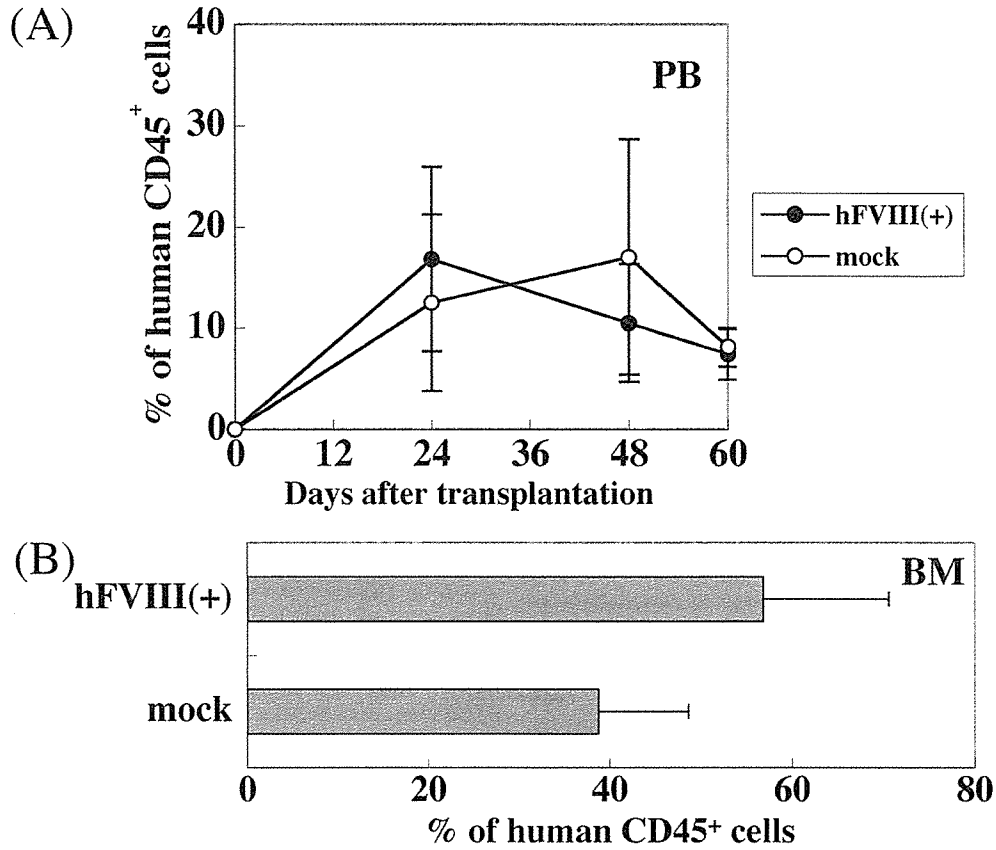

Figure 2. Presence of human CD45$^+$ cells in peripheral white blood and bone marrow cells of NOD/SCID mice after transplantation of human CB-CD34$^+$ cells. Peripheral blood was obtained from recipient mice on days 24, 48, and 60 after transplantation of CB-CD34$^+$ cells. White blood cells derived from transplanted human cells were quantified by detecting human CD45$^+$ cells by flow cytometry. The percentages of human CD45$^+$ cells in peripheral blood white blood cells (PB) from the NOD/SCID mice who received SIVhFVIII (closed circles) and mock (open circles) transduced CB-CD34$^+$ cells (A) and those in bone marrow cells (BM) collected on day 60 (B) are shown (mean ± SD, n = 3)

transplantation. The percentages of human CD45$^+$ cells in mouse peripheral white blood cells were 16.8 ± 9.1% on day 24, 10.5 ± 5.8% on day 48 and 7.4 ± 2.5% on day 60 after transplantation (Figure 2A). When the mice were sacrificed on day 60, the percentage of human CD45$^+$ cells in bone marrow cells was 56.9 ± 9.9%(Figure 2B). These data suggested that the transplanted human cells were well engrafted in the NOD/SCID mice.

Plasma hFVIII levels in NOD/SCID mice after transplantation

Mouse plasma was obtained on days 1, 6, 24, 48 and 60 after transplantation and human FVIII (hFVIII) levels in mouse plasma were quantified by the hFVIII-specific ELISA. Plasma hFVIII levels rose to 3.0 ± 1.2 ng/mL on day 1 and reached a maximum at 3.6 ± 0.8 ng/mL on day 6 after transplantation. hFVIII levels gradually decreased by 24 days, but continued a low-level basal production of at least 1.2 ng/mL for 60 days after transplantation (Figure 3A). Since the normal hFVIII concentration in human plasma is 100–200 ng/mL [23], levels of hFVIII in plasma of NOD/SCID mice which received transduced cell transplantation were approximately 1–3% of the normal hFVIII. However, hFVIII levels in the bone marrow of recipient mice were 13.4 ± 6.5 ng/mL (Figure 3B), suggesting that those in recipient mouse bone marrow were considerably higher than the plasma levels. Control animals that received mock-transduced human CB-CD34$^+$ cells did not yield any detectable hFVIII in their plasma or the bone marrow.

Expression of CD markers in engrafted human cells from peripheral blood, spleen, and bone marrow

To analyze populations of human cells in CB-CD34$^+$ cell-engrafted NOD/SCID mice, bone marrow cells and spleen cells were isolated at 60 days after transplantation and analyzed for the expression of human lineage-specific markers by flow cytometry. Figure 4 shows a typical analysis of bone marrow cells from a mouse engrafted with SIVhFVIII-transduced CB-CD34$^+$ cells. From this mouse bone marrow, 42.8% of mouse bone marrow cells were positive for human CD45. In the human CD45$^+$ cell fraction, human CD34, CD19, CD3, CD14, or CD33 positive cells were 22.9, 65.1, 0.1, 14.8, and 13.2%, respectively. On average, human CD45$^+$

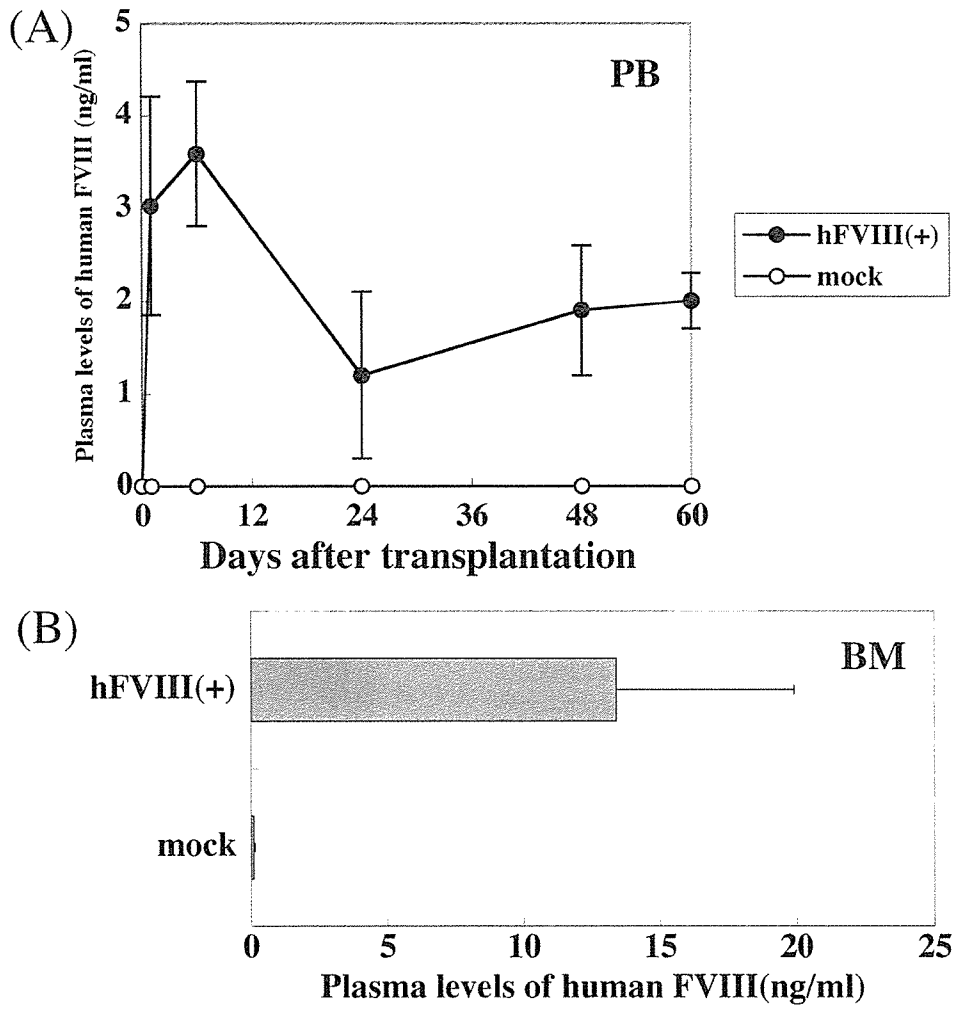

Figure 3. Plasma hFVIII levels in transplanted NOD/SCID mice. Peripheral blood was obtained from recipient mice on days 1, 6, 24, 48, and 60 after transplantation of CB-CD34$^+$ cells. Human FVIII concentrations in plasma (PB) of the NOD/SCID mice which received SIVhFVIII (closed circles) or mock (open circles) transduced CB-CD34$^+$ cells (A) and the hFVIII concentrations of the bone marrow preparation (BM) obtained on day 60 (B) are shown (mean ± SD, n = 3)

cells account for 56.9 ± 9.9% of mouse bone marrow cells. The average percentage of human cells expressing human CD34, CD19, CD33, or CD14 markers in the human CD45$^+$ cell fraction was 22.6 ± 3.7, 54.9 ± 7.9, 6.0 ± 0.8, or 10.3 ± 1.0%, respectively. No human CD3$^+$ T cells were detected in the mouse bone marrow samples. CD41$^+$ human platelets were found in not only 0.32 ± 0.06% of the peripheral blood platelets, but also in 16.4 ± 5.8% of the bone marrow platelets. Transduction of human CD34$^+$ cells by SIV vectors did not affect expression of lineage-specific markers in reconstituted peripheral blood cells, bone marrow cells, or spleen cells.

Detection of FVIII transcripts in bone marrow cells of NOD/SCID mice

To assess the expression of genes in engrafted bone marrow cells of NOD/SCID mice, bone marrow cells were harvested at 60 days after injection and subjected to RT-PCR analysis for detection of human BDD-FVIII transcripts. Total RNA extracted from bone marrow cells was subjected to PCR amplification using human FVIII, human GAPDH, or mouse GAPDH specific primers. As shown in Figure 5A, hFVIII transcripts were detected in bone marrow cells from the mice injected with hFVIII gene-transduced CB-CD34$^+$ cells (lanes 4, 5), but not detected from mock CB-CD34$^+$ cells (lanes 6, 7). Similarly, the hFVIII transcripts were observed in spleen cells and peripheral blood cells from mice engrafted for FVIII production, whereas the mock-transduced mice were negative. The transcripts of human GAPDH were detected in all cells (Figure 5B) derived from recipient mice, but not those from NOD/SCID mice without CD34$^+$ cell transplantation (Figure 5B, lane 3). Also the transcripts of mouse GAPDH were detected in all cells derived from NOD/SCID mice (Figure 5C, lanes 3–11). Human GAPDH transcripts were not detected in the liver, the lung, or the kidney (data not shown).

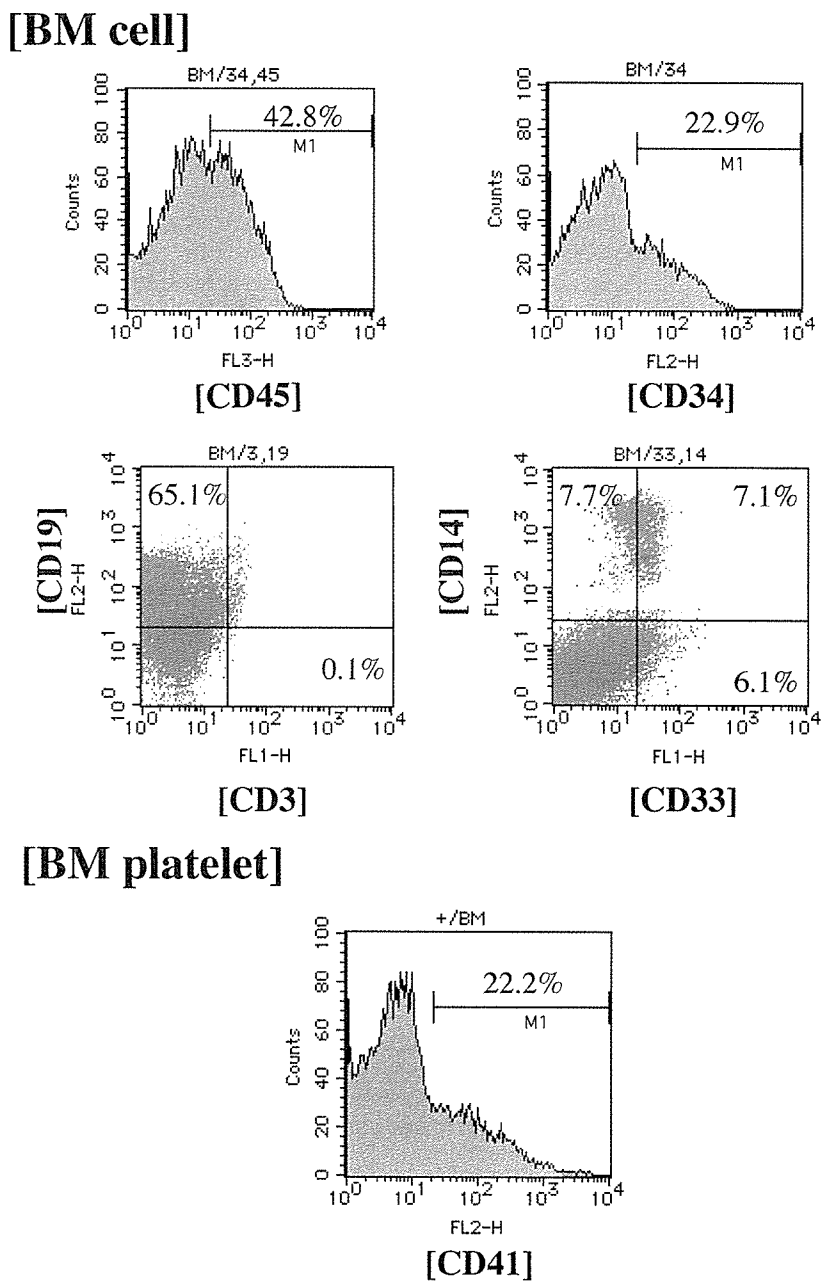

Figure 4. Expression of lineage markers in human CD45$^+$ cells and detection of human CD41$^+$ platelets in bone marrow cells. Expression of CD3, CD19, CD33, CD14, and CD34 in the human CD45$^+$ cells isolated from the bone marrow of a recipient mouse were analyzed by flow cytometry. Human platelets were detected by staining with RPE-conjugated anti-human CD41. The figure shows typical histograms of CD antigen expression on bone marrow cells obtained from NOD/SCID mouse on day 60 after transplantation with hFVIII-transduced CB-CD34$^+$ cells

Detection of hFVIII expressed in bone marrow cells and platelets of NOD/SCID mice

To detect hFVIII molecules in engrafted human-derived cells, mouse bone marrow cells were processed for detection of FVIII antigen in tissues using immunofluorescence. As shown in Figure 6, hFVIII was detected in bone marrow cells isolated from mice injected with SIVhFVIII-transduced CB-CD34$^+$ cells (Figure 6B), but not in cells from mice who received the mock-transduced CB-CD34$^+$ cells (Figure 6A). These data confirm the notion that the hFVIII was produced from the SIVhFIII-transduced cells. Upon sacrifice, platelets were collected from 1 mL of peripheral blood of recipient mice and extracted with 0.1 mL PBS containing Triton X-100 (0.5%). The platelet extracts were subjected to ELISA for quantification of hFVIII. We could detect 2 ng of hFVIII in platelets derived from 1 mL of recipient mouse peripheral blood.

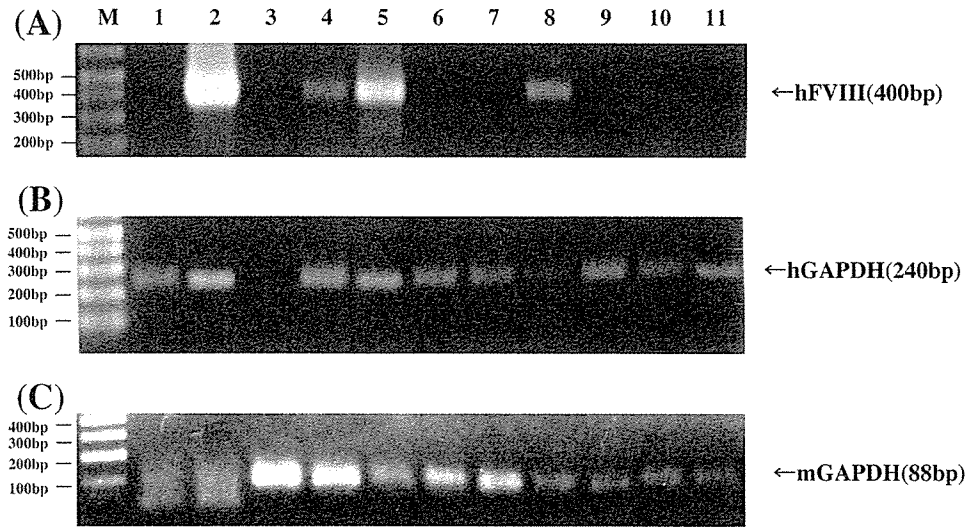

Figure 5. RT-PCR analysis of bone marrow cells. On day 60 after transplantation, RNA obtained from 5000 bone marrow cells was subjected to RT-PCR analyses with specific primer pairs for the human BDD-FVIII transcript (A), the human GAPDH transcript (B), and the mouse GAPDH transcript (C). PCR-amplified products were analyzed on 2% agarose gels followed by ethidium bromide staining (1, non-transduced CD34$^+$ cells; 2, SIVhFVIII-transduced CD34$^+$ cells; 3, NOD/SCID bone marrow; 4 and 5, recipient FVIII-transduced bone marrow cells; 6 and 7, mock-transduced bone marrow cells; 8, recipient spleen cells; 9, mock spleen cells; 10, recipient peripheral white blood cells; 11, mock peripheral white blood cells)

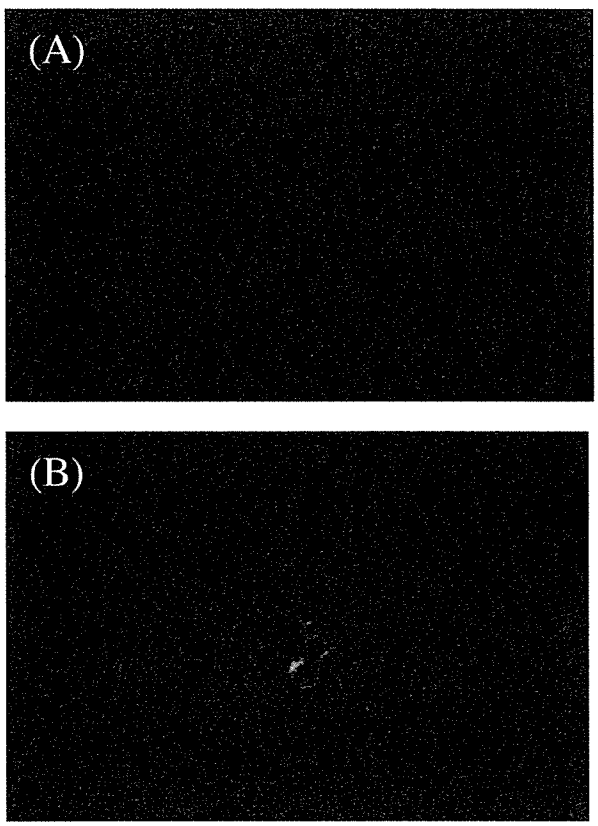

Figure 6. Immunofluorescent microscopy of hFVIII in bone marrow cells of the NOD/SCID mice. Bone marrow cells obtained from mock mice (A) or from recipient mice (B) were attached to glass slides using a Cytospin3. After fixation with 4% paraformaldehyde and washing with PBS, cells were incubated with sheep anti-hFVIII polyclonal antibodies. Bound antibodies were detected by AlexaFluor488-conjugated secondary antibody and visualized using a fluorescence microscope (E800; Nikon Co. Ltd., Tokyo, Japan)

Discussion

We have shown that SIV vectors carrying either eGFP or a therapeutic gene can transduce isolated CB-CD34$^+$ cells efficiently and that these cells can be transplanted into NOD/SCID mice successfully. We achieved high-level expression of human FVIII (hFVIII) in CB-CD34$^+$ cells transduced with SIVhFVIII *in vitro*, although VSVG-pseudotyped SIV vectors affected cell viability at high MOIs. Successful engraftment of SIV vector-transduced and FVIII-producing human CB-CD34$^+$ cells into NOD/SCID mice and the relatively low but effective expression level of the hFVIII antigen *in vivo* for 60 days were also achieved.

Hematopoietic stem cells are of considerable interest for gene therapy because of their self-renewal ability. Many reports have shown that hematopoietic stem cells are present in the CD34$^+$ cell fraction in humans and engraftment of human hematopoietic stem cells to mice is achievable by transplantation of CD34$^+$ cells into NOD/SCID mice. Previous reports showed that viral vector-transduced CB-CD34$^+$ cells could be engrafted in NOD/SCID mice [4–6]. However, *ex vivo* transduction of the FVIII gene to hematopoietic stem/progenitor cells by retroviral vector followed by transplantation into lethally irradiated normal or hemophiliac mice did not result in FVIII expression in the plasma, despite efficient engraftment of transduced cells [11–13]. Similarly, subcutaneous implantation of murine or human fibroblasts or bone marrow stromal cells transduced with hFVIII using retroviral vectors into immunodeficient mice resulted in long-term persistence of the engineered host cells *in vivo*, but no or only transient FVIII expression in plasma [24–26]. These disappointing results may well be

a result of inefficient transduction of the hematopoietic stem cells by the retroviral vectors.

Lentiviral vectors have been developed to overcome this inefficiency [7], with resultant transduction and integration of therapeutic genes into the target genome [4–6]. The commonly used lentiviral vectors are derived from HIV-1 or HIV-2 and endeavors have been made to make lentiviral vectors much safer [27]. However, the pathogenicity of HIV-based lentiviral vectors to humans is not clear especially in HIV carriers. Therefore, use of these vectors raises safety issues. The safety of HIV-derived vectors ultimately must be proven, but remains difficult because of the limited availability of animal models of HIV-induced diseases. The SIV lentiviral vectors used in this study were derived from SIVagmTYO1, non-pathogenic to its natural hosts or to experimentally infected Asian macaques [8], and no replication-competent virus particles were detected in vector-infected cells *in vitro*. Furthermore, the risk of development of replication-competent lentivirus particles in HIV carrier patients may be significantly lower than that for the HIV-1-based vectors because of the low sequence homology between HIV-1 and SIVagmTYO1. In this regard, SIVagmTYO1-based vectors have an advantage regarding safety issues and clinical application of gene therapy. To our knowledge, this is the first report of stable production of human FVIII in mice by hematopoietic cells reconstituted from CB-CD34$^+$ cells transduced with SIV vectors.

In the current study, the transduction efficiency of the CB-CD34$^+$ cells with the SIV vectors (Figure 1) was comparable to HIV-based lentiviral vectors [4]. Production of hFVIII (274.3 ± 20.1 ng/10^6 cells/24 h) by transduced CB-CD34$^+$ cells *in vitro* was considerable, raising the possibility of achieving therapeutic levels of plasma FVIII in the mice. After engraftment of transduced cells in NOD/SCID mice, plasma FVIII levels were maximal at 3.6 ± 0.8 ng/mL on day 6 after transplantation, declining gradually to a sustained level of 1.2 ng/mL for at least 60 days. The hFVIII production was observed in plasma and at the gene level in bone marrow cells. The hFVIII levels in plasma were lower than expected based on the *in vitro* production rate. One contributing factor may be the shorter half-life of hFVIII in mice compared with humans. The half-life of injected hFVIII in mice is approximately 1 h, whereas the half-life in hemophilia patients is closer to 8–12 h [25]. Chao *et al.* showed low-level production of hFVIII in immunocompetent C57BL/6 mice expressing a hFVIII inhibitor or in NOD/SCID mice without a detectable inhibitor. In these studies, FVIII levels increased after 10 months in both mice populations at the disappearance time of the inhibitor in the C57BL/6 mice [28]. Thus, secreted hFVIII was likely degraded in the NOD/SCID mice, analogous to that observed in the immunocompetent mice, resulting in low circulating levels of FVIII. It is also possible that the number of FVIII-producing cells decreased gradually after transplantation. We incubated CB-CD34$^+$ cells with SIVhFVIII at MOI of 50 vg/cell and approximately 50% of the CB-CD34$^+$ cells were transduced. These cells consist of hematopoietic stem cells and hematopoietic progenitor cells. During the early period after transplantation, the transduced cells produced hFVIII, as reflected by the relatively high level of plasma hFVIII in mice on days 1 and 6 after transplantation. After day 6, transduced human hematopoietic progenitor cells may have differentiated to progeny cells, which would be liberated from the bone marrow and cleared. Thus, the number of hFVIII-producing cells might be decreased after day 6 as they are derived solely from transduced human hematopoietic stem cells, their progeny and differentiated cells. The expected result would be declining plasma FVIII levels in the later periods of post-transplantation. It is also possible that silencing of the CMV promoter *in vivo* could have occurred, that in turn reduced FVIII production. The FVIII levels achieved in mice in this study were relatively low but such an increase of the FVIII level would develop clinical effects in hemophilias such as decrease of bleeding episodes and of use of FVIII concentrates. Data on clinical trials of hemophilia A gene therapy support this notion [29,30]. Therefore, we think that the FVIII levels achieved in this study were relatively low but an increase of FVIII to these levels would develop clinical improvement in severe hemophilia patients.

The replication mechanism of HIV-1 has been extensively studied in host cells. HIV-1 has the unique property among retroviruses to replicate in non-dividing cells. This property enables HIV-1 and other lentiviral-based vectors to transduce non-dividing hematopoietic stem cells. The central DNA flap of HIV-1 is thought to function as a cis-determinant of HIV-1 DNA nuclear import and to play a crucial role for lentiviral vector nuclear import and gene transduction of hematopoietic stem cells [31,32]. The SIVagm vectors used in this study were developed essentially according to the HIV-1-based vectors. Although SIVagm vectors are self-inactivating type vectors, the central DNA flap has not been included in the vectors as yet. We were able to transduce CD34$^+$ cells using SIVagm vectors efficiently *in vitro*, as shown in Figure 1, but FVIII production was decreased after transplantation. Thus, transgene integration into the CD34$^+$ cell genome by the SIVagmTYO1 vector might not occur efficiently. Currently, we are redesigning the SIVagmTYO1 vector to include the DNA flap. Use of such third-generation SIVagmTYO1 vectors will be of interest in future studies.

Analyses of lineage marker expression on the hematopoietic cells from the bone marrow and spleen of NOD/SCID mice confirmed engraftment and hematopoiesis of the transduced cells in the mice. Furthermore, we demonstrated CD41$^+$ platelets in the peripheral blood and the bone marrow, indicating that the human megakaryocytic progenitors could differentiate and mature to produce platelets in the mice. In fact, 2 ng of human FVIII were detected by ELISA in the platelet extracts derived from 1 mL of recipient mouse peripheral blood. These data also suggest that this is another advantage of transplantation of FVIII-producing hematopoietic stem cells, since the FVIII can be stored in platelets. These

platelets circulate in blood and secrete FVIII upon platelet activation in the vicinity of bleeding, so that local FVIII concentrations may be higher than that in the circulation.

We demonstrated efficient transduction of CB-CD34$^+$ cells by a SIV vector carrying the human FVIII gene. Taking advantage of their self-renewal and multi-lineage differentiation capabilities, transplantation of *ex vivo* engineered CB-CD34$^+$ cells enabled their engraftment in NOD/SCID mice, transgene expression, and human FVIII production. Because of xenograft transplantation, we transplanted human CD34$^+$ cells to NOD/SCID mice with myeloablation by irradiation. However, this gene therapy strategy can be potentially applicable to clinical studies because autologous transplantation of genetically transduced hematopoietic stem cells can be achieved with non-myeloablative conditioning [33]. Transplantation of *ex vivo* SIV vector-transduced CD34$^+$ cells without exposure of subjects to viral vectors is a useful approach with potential clinical application for gene therapy of hemophilia patients.

Gene therapy of human ADA-SCID and X-SCID by autologous transplantation of genetically modified hematopoietic stem cells has been shown to be very effective [33,34]. However, a leukemia-like disorder emerged in two X-SCID patients who received retrovirally mediated common γ(γc) gene transfer to hematopoietic stem cells [35]. This disorder appeared to be caused by insertion of the vector-derived γc gene in the LMO2 gene [35]. Similar to retrovirus vector-mediated gene transfer, random integration of the transgene to hematopoietic stem cell genomes takes place upon transduction by lentiviral vectors. Thus, vector-derived DNA insertion to such a leukemia-linked gene LMO2 can also happen in SIV vector-mediated FVIII gene transfer to hematopoietic stem cells. The SIV vector used in this study is designed to be a self-inactivating type vector to minimize activation of genes in the vicinity of the integration site. Thus FVIII gene transfer to hematopoietic stem cells by SIVhFVIII may be much safer than retrovirally mediated γc gene transfer to hematopoietic stem cells for X-SCID gene therapy. However, the risk of the inactivation of tumor suppressor genes still remains. Therefore, attempts such as the use of a regulated and cell-specific promoter, reduction of multiple insertion of the transgene into a single cell, and incorporation of a suicide gene into the vector should be studied to reduce the risk of development of an unpredictable disorder in the future.

Acknowledgements

We thank Dr. J. A. van Mourik for the full-length FVIII cDNA, Dr. Koichiro Tsuji, the Institute of Medical Science, the University of Tokyo, and Dr. Mika Wada, the Department of Pediatrics, Nihon University of Medicine, for advice on transplantation of human CB-CD34$^+$ cells into NOD/SCID mice. We also thank Ms. Fumino Muroi and Ms. Kou Kosaka for technical assistance with the human FVIII ELISA assay and purification of CB-CD34$^+$ cells. This work is supported in part by Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare and Grants-in-aid for Scientific Research from the Ministry of Education and Science. This manuscript was edited by IdEst, Inc.

References

1. Hoyer LW. Hemophilia A. *N Engl J Med* 1994; **330**: 38–47.
2. Kay MA, High K. Gene therapy for the hemophilias. *Proc Natl Acad Sci U S A* 1999; **96**: 9973–9975.
3. Kume A, Hanazono Y, Mizukami H, Urabe M, Ozawa K. Hematopoietic stem cell gene therapy: a current overview. *Int J Hematol* 1999; **68**: 227–233.
4. Miyoshi H, Smith KA, Mosier DE, Verma IM, Torbett BE. Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vector. *Science* 1999; **283**: 682–686.
5. Woods NB, Fahlman C, Mikkola H, *et al.* Lentiviral gene transfer into primary and secondary NOD/SCID repopulating cells. *Blood* 2000; **96**: 3725–3733.
6. Scherr M, Battmer K, Blomer U, *et al.* Lentiviral gene transfer into peripheral blood-derived CD34$^+$ NOD/SCID-repopulating cells. *Blood* 2002; **99**: 709–712.
7. Naldini L, Blomer U, Gallay P, *et al.* In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 1996; **272**: 263–267.
8. Honjo S, Narita T, Kobayashi R, *et al.* Experimental infection of African green monkeys and cynomolgus monkeys with a SIVAGM strain isolated from a healthy African green monkey. *J Med Primatol* 1990; **19**: 9–20.
9. Nakajima T, Nakamaru K, Ido E, Terao K, Hayami M, Hasegawa M. Development of novel simian immunodeficiency virus vectors carrying a dual gene expression system. *Hum Gene Ther* 2000; **11**: 1863–1874.
10. Hoeben RC, Einerhand MP, Briet E, van Ormondt H, Valerio D, van der Eb AJ. Toward gene therapy in haemophilia A: retrovirus-mediated transfer of a factor VIII gene into murine haematopoietic progenitor cells. *Thromb Haemost* 1992; **67**: 341–345.
11. Tonn T, Herder C, Becker S, Seifried E, Grez M. Generation and characterization of human hematopoietic cell lines expressing factor VIII. *J Hematother Stem Cell Res* 2002; **11**: 695–704.
12. Kootstra NA, Matsumura R, Verma IM. Efficient production of human FVIII in hemophilic mice using lentiviral vectors. *Mol Ther* 2003; **7**: 623–631.
13. Tiede A, Eder M, von Depka M, *et al.* Recombinant factor VIII expression in hematopoietic cells following lentiviral transduction. *Gene Ther* 2003; **10**: 1917–1925.
14. Ogata K, Mimuro J, Kikuchi J, *et al.* Expression of human coagulation factor VIII in adipocytes transduced with the simian immunodeficiency virus agmTYO1-based vector for haemophilia A gene therapy. *Gene Ther* 2004; **11**: 253–259.
15. Furukawa Y, Kikuchi J, Nakamura M, Iwase S, Yamada H, Matsuda M. Lineage-specific regulation of cell cycle control gene expression during haematopoietic cell differentiation. *Br J Haematol* 2000; **110**: 663–673.
16. Ueda T, Tsuji K, Yoshino H, *et al.* Expansion of human NOD/SCID-repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6, and soluble IL-6 receptor. *J Clin Invest* 2000; **105**: 1013–1021.
17. Yoshino H, Ueda T, Kawahata M, *et al.* Natural killer cell depletion by anti-asialo GM1 antiserum treatment enhances human hematopoietic stem cell engraftment in NOD/Shi-scid mice. *Bone Marrow Transplant* 2000; **26**: 1211–1216.
18. Ma F, Wada M, Yoshino H, *et al.* Development of human lymphohematopoietic stem and progenitor cells defined by expression of CD34 and CD81. *Blood* 2001; **97**: 3755–3762.
19. Yonemura H, Sugawara K, Nakashima K, Nakahara Y, Hamamoto T, Mimaki I. Production of recombinant human factor VIII by co-expression of the heavy and light chains. *Protein Eng* 1993; **6**: 669–674.
20. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156–159.
21. Sutton RE, Reitsma MJ, Uchida N, Brown PO. Transduction of human progenitor hematopoietic stem cells by human immunodeficiency virus type 1-based vectors is cell cycle dependent. *J Virol* 1999; **73**: 3649–3660.

22. Xu R, Reems JA. Umbilical cord blood progeny cells that retain a CD34+ phenotype after ex vivo expansion have less engraftment potential than unexpanded CD34+ cells. *Transfusion* 2001; **41**: 213–218.
23. VandenDriessche T, Vanslembrouck V, Goovaerts I, *et al.* Long-term expression of human coagulation factor VIII and correction of hemophilia A after in vivo retroviral gene transfer in factor VIII-deficient mice. *Proc Natl Acad Sci U S A* 1999; **96**: 10379–10384.
24. Hoeben RC, Fallaux FJ, Van Tilburg NH, *et al.* Toward gene therapy for hemophilia A: long-term persistence of factor VIII-secreting fibroblasts after transplantation into immunodeficient mice. *Hum Gene Ther* 1993; **4**: 179–186.
25. Dwarki VJ, Belloni P, Nijjar T, *et al.* Gene therapy for hemophilia A: production of therapeutic levels of human factor VIII in vivo in mice. *Proc Natl Acad Sci U S A* 1995; **92**: 1023–1027.
26. Chuah MK, Van Damme A, Zwinnen H, *et al.* Long-term persistence of human bone marrow stromal cells transduced with factor VIII-retroviral vectors and transient production of therapeutic levels of human factor VIII in nonmyeloablated immunodeficient mice. *Hum Gene Ther* 2000; **11**: 729–738.
27. Naldini L, Verma IM. Lentiviral vectors. *Adv Virus Res* 2000; **55**: 599–609.
28. Chao H, Walsh CE. Induction of tolerance to human factor VIII in mice. *Blood* 2001; **97**: 3311–3312.
29. Roth DA, Tawa NE Jr, Proper JA, *et al.* The factor VIII transkaryotic therapy study group. Nonviral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A. *N Engl J Med* 2001; **344**: 1782–1784.
30. Powell J, Ragni MV, White GC, *et al.* Phase 1 trial of FVIII gene transfer for severe hemophilia A using a retroviral construct administered by peripheral intravenous infusion. *Blood* 2003; **102**: 2038–2045.
31. Zennou V, Petit C, Guetard D, Nerhbass U, Montagnier L, Charneau P. HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* 2000; **101**: 173–185.
32. Sirven A, Pflumio F, Zennou V, *et al.* The human immunodeficiency virus type-1 central DNA flap is a crucial determinant for lentiviral vector nuclear import and gene transduction of human hematopoietic stem cells. *Blood* 2000; **96**: 4103–4110.
33. Aiuti A, Slavin S, Aker M, *et al.* Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* 2002; **28**: 2410–2413.
34. Hacein-Bey-Abina S, Le Deist F, Carlier F, *et al.* Sustained correction of X-linked severe combined immunodeficiency by *ex vivo* gene therapy. *N Engl J Med* 2002; **346**: 1185–1193.
35. Hacein-Bey-Abina S, von Kalle C, Schmidt M, *et al.* A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* 2003; **348**: 255–256.

Leukemia & Lymphoma, November 2004 Vol. 45 (11), pp. 2349–2351

Taylor&Francis
healthsciences

Severe Hepatitis and Complete Molecular Response Caused by Imatinib Mesylate: Possible Association of Its Serum Concentration with Clinical Outcomes

SATORU KIKUCHI[a], KAZUO MUROI[a,b,*], SATOKO TAKAHASHI[a], CHIZURU KAWANO-YAMAMOTO[a,b], MASAAKI TAKATOKU[a], AKIRA MIYAZATO[a], TADASHI NAGAI[a], MASAKI MORI[a,b], NORIO KOMATSU[a] and KEIYA OZAWA[a,b]

[a]*Division of Hematology, Department of Medicine, Jichi Medical School, Minamikawachi, Japan;* [b]*Division of Cell Transplantation and Transfusion, Jichi Medical School, Minamikawachi, Japan*

(Received 10 June 2004)

A 40-year-old female with chronic myelogeneous leukemia (CML) in the chronic phase was treated with imatinib mesylate (STI571) because of interferon resistance. She achieved complete cytogenetic response but not complete molecular response 3 months after STI571 administration. Six months later, she developed severe liver damage without evidence of actively infectious hepatitis A, B, C, G, E, TT virus, Epstein-Barr virus or cytomegalovirus. A significant serum level of STI571 (107 ng/ml) was detected, although she had not taken the drug for 6 days. Liver biopsy demonstrated massive hepatic necrosis, consistent with drug-induced hepatitis. She achieved complete molecular response, although she did not take STI571 for 47 days after the development of hepatitis. These results suggest that both hepatitis and molecular response were associated with the serum STI571 concentration.

Keywords: STI571; Serum concentration; Hepatitis; Molecular response

INTRODUCTION

STI571, an inhibitor of BCR-ABL tyrosine kinase, shows clinical activity in the treatment of CML in the chronic phase and less activity in the treatment of CML in blastic transformation [1–4]. Although STI571 has been well tolerated in clinical trials, various non-hematological adverse effects such as nausea, fluid retention, edema, muscle cramps and rash, most of which are mild, have been reported [5]. Recently, it has been reported that 3 female patients with CML treated with STI571 developed severe hepatitis [6,7]. We report a CML patient demonstrating both severe hepatitis and complete molecular response caused by STI571.

CASE REPORT

A 40-year-old female was diagnosed as having Philadelphia chromosome (Ph1)-positive CML in the chronic phase in December 1995 and received interferon soon after the diagnosis. Four months after interferon therapy, major cytogenetic response (Ph1, 1/29 cells) was obtained. Complete or major cytogenetic response was maintained from April 1996 to October 2000 by interferon alone. In November 2002, Ph1 in the bone marrow increased to 23/24 cells, although the interferon dose was increased. Therefore, STI571 at a daily dose of 400 mg started on January 2, 2003. At this time, there were no biochemical abnormalities including those of liver enzymes detected. The clinical course was uneventful and she had no adverse effects except slight leukocytopenia and anemia. On March 26, 2003, she achieved complete cytogenetic response in the bone marrow again. Although BCR-ABL messages were still detected by RT–PCR assay, 400 mg of STI571 was continuously given. On May 21, slight increase in aspartate aminotransferase (AST, 96U/l; normal range, 11–30) and alanine aminotransferase (ALT, 152 U/l; normal range, 4–30) but not total bilirubin (0.86 mg/dl; normal range, 0.29–1.03) was noticed. From June 5 to 7, she took STI571 but immediately vomited it. Between June 8 to 10, she did not take STI571 because of nausea. On June 11, she was admitted because of nausea and general fatigue (Fig. 1).

*Corresponding author. Address: Division of Cell Transplantation and Transfusion, Jichi Medical School, Minamikawachi, Tochigi 329-0498, Japan. Tel.: 81-285-44-2111. Fax: 81-285-44-5087. E-mail: muroi-kz@jichi.ac.jp

ISSN 1042-8194 print/ISSN 1029-2403 online
DOI: 10.1080/10428190412331272721

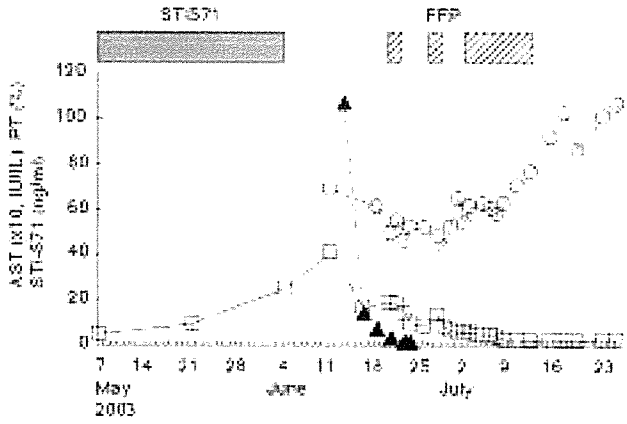

FIGURE 1 Clinical course. Open circles, open squares and filled triangles indicate percentage of prothrombin time, serum aspartate aminotransferase level and serum STI571 concentration, respectively. FFP, fresh frozen plasma.

Laboratory examination demonstrated increased levels of AST (406 U/l), ALT (559 U/l) and total bilirubin (2.7 mg/dl) and prolongation in prothrombin time (PT) (14.0 s, 69%; normal range, 10.4–12.2 s). The peripheral blood showed a hemoglobin level of 11.4 g/dl, a platelet count of 121×10^9/l, and a white blood cell count of 4.7×10^9/l with 57% neutrophils, 3% eosinophils, 1% basophils, 6% monocytes and 33% lymphocytes. Serological tests for IgM anti-hepatitis A virus (HAV) antibody, HBV surface antigen, HCV antibody and HEV antibody were negative. HGV, TT virus, HBV, HCV, cytomegalovirus and Epstein-Barr virus were not detected by the PCR method. Autoantibodies including antimitochondrial antibody and antismooth muscle antibody were negative. Ultrasonography of the abdomen showed nonspecific liver damage without gallstones. Computed tomography of the abdomen showed low-density lesions in the periportal areas of the liver, suggesting hepatitis.

She was suspected of having STI571-induced hepatitis [6], although drug lymphocyte-stimulating test for STI571 was negative. Residual STI571 in serum was demonstrated by liquid-chromatography tandem mass spectrometry [8] as follows: 107.0 ng/ml on June 13, 13.8 ng/ml on June 16, 7.4 ng/ml on June 18 and 3.6 ng/ml on June 20. STI571 had been discontinued since admission, however, total bilirubin increased to the maximum level of 9.98 mg/dl on June 27 and production of blood coagulation factors was impaired to 46% of PT, 39% of antithrombin III activity, 32% of protein C activity, and 139 mg/dl of fibrinogen on June 22. A total of 72 units of fresh frozen plasma were infused to replace coagulation factors produced in the liver. On July 23, percutaneous liver biopsy demonstrated severe centrolobular hepatic necrosis without evidence of veno-occlusive disease, consistent with drug-induced hepatitis [7]. Bone marrow aspiration the next day showed continuous complete cytogenetic response by conventional karyotypic analysis and there were no detectable BCR/ABL messages on RT–PCR. The patient was discharged on June 26 after recovery of hepatic function. On October 15, 2003, she still maintained normal blood counts with normal differential. A rechallenge test of STI571 has not been performed.

DISCUSSION

Our patient showed 2 important events during STI571 administration. One is drug-related liver toxicity. STI571 is not only metabolized by the cytochrome P450 enzymes, CYP3A4, CYP2C9 and CYP2D6 but also competitively inhibits CYP3A4 [9]. Drugs and foods inhibiting CYP3A4 such as erythromycin, clarithromycin, itraconazole and grapefruit juice increase the serum concentration of STI571 and lead to enhance STI571 toxicity in patients concurrently taking both STI571 and 1 of the CYP3A4-inhibiting agents [9]. Our patient took STI571 alone but not any agents, foods or supplements including herbs that affect CYP3A4. We do not know whether severe liver damage caused by STI571 is the result of immunologic idiosyncrasy (hypersensitivity reaction) or injury from a toxic metabolite (metabolic idiosyncrasy) [10]. Recently, Gambacorti-Passerini *et al.* have reported the pharmacokinetic analysis of STI571 in CML patients [11]. STI571 plasma concentrations were measured in 8 CML patients treated with 400 mg of the drug. After administration on day 1, peak concentration (C_{max}) of STI571 was achieved between 1 and 3 h; then in all of the patients, the drug was slowly cleared from plasma, being still detectable at 24 h. In 11 patients with CML treated with 400 mg, C_{max}, area under the curve in a 24-h period (24-h AUC) and half-life were 2.35 ± 1.0 μg/ml, 24.66 ± 8.5 μg.h/ml, and 12.5 ± 2.4 h, respectively. Similar analysis in healthy subjects treated with 400 mg of STI571 were shown in the CSTI571B2102 study conducted by Novartis Pharmaceuticals [12]: C_{max}, 1.56 ± 0.29 μg/ml; 24-h AUC, 16.30 ± 3.48 μg.h/ml; and half-life, 16.7 ± 3.1 h. Since a significant serum level of STI571 was detected 7 days after cessation of the drug administration in our patient, metabolic idiosyncrasy of STI571 in the liver is suggested. Interestingly, in our patient and others [6,7], severe liver damage caused by STI571 involved females in all cases. Therefore, we should carefully monitor STI571 administration to female patients with CML.

Another important finding in our patient is complete molecular response following severe liver damage caused by STI571. Before sustaining liver damage, the patient had achieved complete cytogenetic response but not complete molecular response by STI571. Complete molecular response was obtained followed by severe liver damage, although STI571 had not been given to the patient since the development of liver damage. As discussed above, severe liver damage in our patient was suggested to be associated with increased serum concentration of STI571. The achievement of complete molecular response may also have been caused by the

increased serum concentration of STI571. It is necessary to clarify the relationship between serum STI571 concentrations and clinical outcomes in CML patients.

Acknowledgments

The authors wish to thank doctors in the Division of Gastroenterology for useful suggestion on drug-induced hepatitis.

References

[1] Druker, B.J., Talpaz, M., Resta, D.J., Peng, B., Buchdunger, E., Ford, J.M., *et al.* (2001) "Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia", *The New England Journal of Medicine*, **344**, 1031–1037.

[2] Sawyers, C.L., Hochhaus, A., Feldman, E., Goldman, J.M., Miller, C.B., Ottmann, O.G., *et al.* (2002) "Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study", *Blood*, **99**, 3530–3539.

[3] Kantarjian, H., Sawyers, C., Hochhaus, A., Guilhot, F., Schiffer, C., Gambacorti-Passerini, C., *et al.* —International STI571 CML Study Group. (2002) "Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia", *The New England Journal of Medicine*, **346**, 645–652.

[4] Kantarjian, H.M., Cortes, J., O'Brien, S., Giles, F.J., Albitar, M., Rios, M.B., *et al.* (2002) "Imatinib mesylate (STI571) therapy for Philadelphia chromosome-positive chronic myelogenous leukemia in blast phase", *Blood*, **99**, 3547–3553.

[5] Hensley, M.L. and Ford, J.M. (2003) "Imatinib treatment: Specific issues related to safety, fertility, and pregnancy", *Seminars in Hematology*, **40**(Suppl 3), 21–25.

[6] Ohyashiki, K., Kuriyama, Y., Nakajima, A., Tauchi, T., Ito, Y., Miyazawa, H., *et al.* (2002) "Imatinib mesylate-induced hepatotoxicity in chronic myeloid leukemia demonstrated focal necrosis resembling acute viral hepatitis", *Leukemia*, **16**, 2160–2161.

[7] James, C., Trouette, H., Marit, G., Cony-Makhoul, P. and Mahon, F.X. (2003) "Histological features of acute hepatitis after imatinib mesylate treatment", *Leukemia*, **7**, 978–979.

[8] Parise, R.A., Ramanathan, R.K., Hayes, M.J. and Egorin, M.J. (2003) "Liquid chromatographic-mass spectrometric assay for quantitation of imatinib and its main metabolite (CGP 74588) in plasma", *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, **791**, 39–44.

[9] O'Brien, S.G., Peng, B., Dutreix, C., Mehring, G., Milosavljev, S., Gapdeville, R., *et al.* (2001) "A pharmacokinetic interaction of glivec and simvastatin, a cytochrome 3A4 substrate, in patients with chronic myeloid leukemia", *Blood*, **98**, 141a (Abstract).

[10] Goodman, Z.D. (2002) "Drug hepatotoxicity", *Clinics in Liver Disease*, **6**, 381–397.

[11] Gambacorti-Passerini, C., Zucchetti, M., Russo, D., Frapolli, R., Verga, M., Bungaro, S., *et al.* (2003) "Alpha1 acid glycoprotein binds to imatinib (STI571) and substantially alters its pharmacokinetics in chronic myeloid leukemia patients", *Clinical Cancer Research*, **9**, 625–632.

[12] www.eudra.org/humandocs/PDFs/EPAR/glivec/241801en6.pdf

Topoisomerase inhibitors enhance the cytocidal effect of AAV-HSVtk/ganciclovir on head and neck cancer cells

TAKEHARU KANAZAWA[1,4], HIROAKI MIZUKAMI[1], HIROSHI NISHINO[2], TAKASHI OKADA[1], YUTAKA HANAZONO[1], AKIHIRO KUME[1], KEN KITAMURA[5], KEIICHI ICHIMURA[2] and KEIYA OZAWA[1,3]

[1]Division of Genetic Therapeutics, Center for Molecular Medicine, Departments of [2]Otolaryngology, and [3]Hematology, Jichi Medical School, 3311-1 Yakushiji Minamikawachi, Tochigi 329-0498; [4]Department of Otolaryngology, Faculty of Medicine, University of Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215; [5]Department of Otolaryngology, School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima Bunkyo, Tokyo 113-8519, Japan

Received March 8, 2004; Accepted May 3, 2004

Abstract. Adeno-associated virus (AAV) is a non-pathogenic virus with a single-strand DNA genome. AAV vectors have several unique properties suited for gene therapy applications. However, an obstacle to their application is a low efficiency of transgene expression, mainly due to a limited second-strand synthesis. Previously, we reported that γ-rays enhanced the transduction efficiency and cytocidal effect of AAV vector harboring the herpes simplex virus-thymidine kinase (AAVtk) and ganciclovir (GCV) system. In the present study, we investigated whether topoisomerase inhibitors (etoposide and camptothecin) enhance the AAV vector-mediated transgene expression and the killing effect by AAVtk/GCV system. The enhancement of transgene expression was observed in a concentration-dependent manner on human laryngeal carcinoma cells (HEp-2 cells) and HeLa cells. Southern analysis confirmed that etoposide enhanced the double-strand synthesis of the AAV vector genome in HEp-2 cells and HeLa cells. The cells were efficiently killed by AAVtk/GCV system, as expected. More importantly, both etoposide and camptothecin augmented the cytocidal effect of the AAVtk/GCV system. These findings suggest that the combination of AAV-mediated suicide gene therapy and treatment with topoisomerase inhibitors may have synergistic therapeutic effects in the treatment of cancers.

Introduction

Advanced head and neck cancers exhibit a high mortality rate despite aggressive treatments involving surgery, radiotherapy, and chemotherapy. Patients often present with locally advanced conditions and the long-term survival rates have not improved appreciably over the past several decades. Current treatments for the advanced stages have shown little success, because tumors cannot be eradicated with an acceptable toxicity. One alternative strategy that has shown promise in the treatment of cancer is a gene therapy. Several virus vectors have been explored for the experiments of head and neck cancers, including retroviral (1), adenoviral (2) and adeno-associated virus (AAV) vectors (3). AAV is a non-pathogenic virus with a single-strand DNA genome (4,5). AAV vectors have emerged as a useful alternative to other vectors (6), and AAV have been evaluated in preclinical and clinical models for cystic fibrosis (7), Parkinson's disease (8) and hemophilia B (9). AAV vectors have a broad host range and can transduce head and neck cancer cells (3). However, an obstacle to their application is a low transgene expression efficiency, mainly due to limited second-strand synthesis (10,11). Genotoxic stresses such as chemotherapeutic agents, UV, heat shock, γ-ray irradiation have been reported to enhance the second-strand synthesis of the AAV vector genome and improve the transgene expression (12-15). Thus, an AAV vector encoding a suicide gene would kill target cells more efficiently when combined with other therapeutic agents. One well-studied suicide gene is the herpes simplex virus type-1 thymidine kinase (HSVtk)/ganciclovir (GCV) (16,17). Although the HSVtk/GCV enzyme/prodrug system has been shown to be effective for controlling tumor growth in animal models, it is difficult to eradicate cancer cells by the HSVtk/GCV system alone and tumors may recur after termination of the prodrug therapy (18-21). Thus, other therapeutic modalities, such as combination therapies, are under development (22,23). In our previous study, we demonstrated that γ-ray irradiation enhance AAV-mediated transgene expression and augmented the antitumor activity of HSVtk/GCV system on human head and neck cancer xenografts (24). Although radiotherapy has been one of the most valuable treatments for advanced head and neck cancer, chemotherapy with topoisomerase inhibitors has also been utilized. In this study, we explored the possibility of combining suicide gene therapy using the AAV vector with topoisomerase inhibitors, frequently used as chemotherapeutic agents, to enhance cytotoxicity, thereby providing a more effective means to control tumor growth.

Correspondence to: Dr Keiya Ozawa, Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, 3311-1 Yakushiji Minamikawachi, Tochigi 329-0498, Japan
E-mail: kozawa@ms.jichi.ac.jp

Key words: adeno-associated virus vector, herpes simplex thymidine kinase, head and neck neoplasms, topoisomerase inhibitor

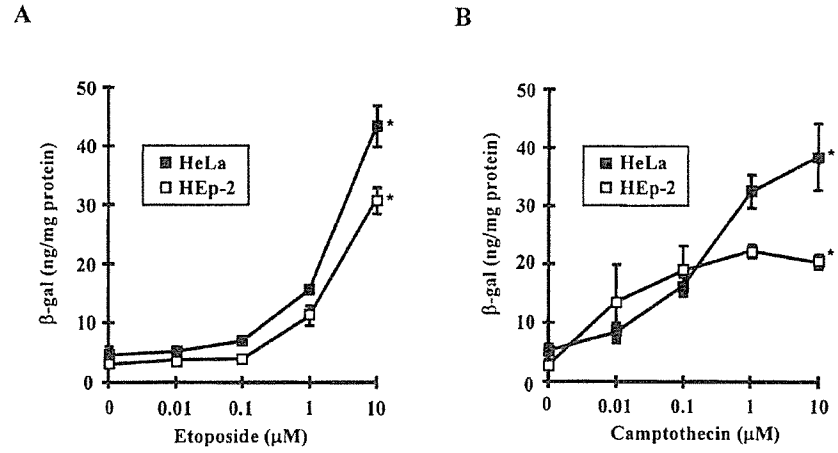

Figure 1. Effect of topoisomerase inhibitors on AAV vector-mediated transgene expression. HeLa cells (closed square) or HEp-2 cells (open square) were transduced with $1x10^3$ particles/cell of AAVLacZ 12-h pre-treatment with topoisomerase inhibitors. (A), The cells were pre-treated with etoposide at concentrations ranging from 0 to 10 μM. (B), The cells were pre-treated with camptothecin at concentrations ranging from 0 to 10 μM. Thirty-six hours after transduction, the expression levels of LacZ were assayed by using the ß-gal ELISA kit. Data were statistically analyzed by one-way ANOVA (*P<0.01).

Materials and methods

Cell lines. HeLa cells, 293 cells and the human laryngeal carcinoma HEp-2 cell line (a gift from the Cell Resource Center for Biomedical Research, Tohoku University), were cultured in DMEM/F12 (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Irvine Scientific, Santa Ana, CA) at 37°C in 5% CO_2.

Plasmids. The plasmid pAAVLacZ contains the CMV promoter, human growth hormone first intron, *Escherichia coli* LacZ gene, and SV40 early polyadenylation sequence between two inverted terminal repeats. A 1.8-kb DNA fragment encoding the HSVtk gene was obtained by double digestion with *Hin*cII and *Pvu*II of plasmid M2 (25) (a gift from Dr Y. Mishina, Yokohama City University, Japan) and subcloned into the pAAVLacZ in place of the LacZ gene (pAAVtk). pH19 is an AAV helper plasmid harbouring rep/cap sequences, and an adenovirus helper plasmid plAd5 contains the adenovirus early genes; E2a, E4, and VA.

AAV vector production. AAV vectors were produced based on the plasmid transfection (26). Briefly, subconfluent 293 cells were cotransfected with AAV vector plasmid, pH19, and plAd5 by a calcium phosphate-precipitation method. The cells were harvested and the recombinant AAV particles were released by three cycles of freeze/thaw. The vector solution was then purified through CsCl gradient twice as described previously (3). The vector titer was determined by a quantitative dot blot hybridization of DNase-treated stocks.

Transduction efficiency of HeLa or HEp-2 cells with AAV vectors. One day before transduction, $1x10^5$ cells were plated onto 3.5-cm dishes in triplicate. The cells were transduced with different amounts of AAVLacZ. Thirty-six hours after transduction with AAVLacZ, the amount of ß-galactosidase was quantitated by using the ß-gal ELISA kit (Boehringer-Mannheim, Hilden, Germany).

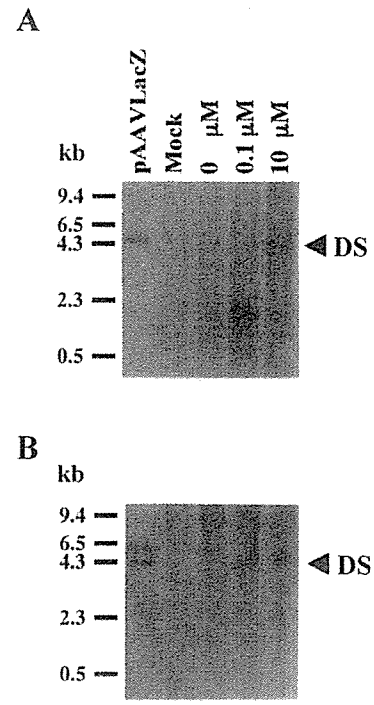

Figure 2. Second-strand synthesis of AAVLacZ genome after etoposide treatment in HeLa cells or HEp-2 cells. HeLa cells (A) or HEp-2 cells (B) were transduced with $1x10^4$ particles/cell of AAVLacZ following pre-treatment with 0, 0.1, 10 μM of etoposide. Two days later, total DNA was isolated. After mung bean nuclease treatment, the DNA samples were loaded on 1% agarose gels, transferred onto nylon membranes, and then hybridized with a radiolabeled CMV-specific probe. Signals were detected by using an imaging analyzer. Lane 1, a 4.7-kb fragment derived from pAAVLacZ; lane 2, mock-transduced; lanes 3-5, AAVLacZ-transduced following pre-treatment with etoposide (0, 0.1, 10 μM) treatment.

The enhancement of transgene expression by topoisomerase inhibitor treatment. The topoisomerase inhibitors used in this study were etoposide (Wako Pure Chemical, Osaka, Japan) and camptothecin (TopoGEN, Inc., Columbus, USA). Stock solutions of etoposide (10 mM) and camptothecin (10 mM)

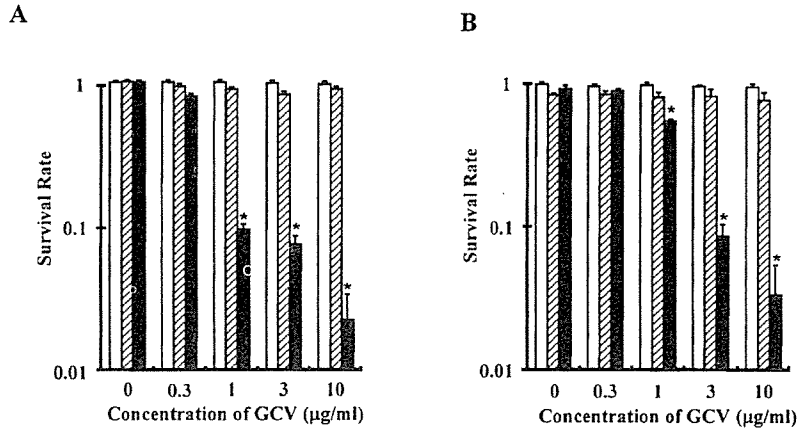

Figure 3. Survival of HeLa cells and HEp-2 cells upon AAVtk/GCV in terms of GCV concentration. HeLa cells (A) or HEp-2 cells (B) were mock-transduced (open bar), transduced with $3x10^5$ particles/cell of AAVLacZ (hatched bar), or AAVtk (closed bar). Twenty-four hours after transduction, the cells were exposed to different concentrations of GCV. Data were analyzed by two-way ANOVA. Asterisks mean that the data obtained for AAVtk-transduction were significantly different from those with or without transduction of AAVLacZ ($P<0.01$).

in dimethyl sulfoxide were stored at -20°C and diluted into PBS for use in experiments. HeLa cells and HEp-2 cells were treated with topoisomerase inhibitors for 12 h, washed twice with PBS, and then transduced with $1x10^3$ particles/cell of AAVLacZ. Thirty-six hours after transduction, we measured the amount of ß-galactosidase with the ß-gal ELISA kit (Boehringer-Mannheim).

Analysis of the second-strand synthesis of the vector genome. HeLa cells and HEp-2 cells grown in 10-cm dishes ($1x10^6$/dish) were transduced with $1x10^4$ particles/cell of AAVLacZ following pre-treatment with etoposide. Two days later, total DNA was isolated with the DNA Extractor WB kit (Wako Pure Chemical). Genomic DNA (40 μg) digested with 80 units of mung bean nuclease (Takara, Tokyo, Japan) were resolved on 1% agarose gels, transferred to nylon membranes (Hybond N^+; Amersham, Buckinghamshire, UK) in 50% formamide, 6X SSC, 0.5% sodium dodecyl sulfate, 5X Denhart's solution, and 100 μg/ml of denatured salmon sperm DNA at 42°C overnight. The membranes were washed, and then analyzed by using an image analyzer (BAS-1500, Fuji, Tokyo, Japan).

GCV treatment. The cells were plated and transduced with AAVtk at the dose of $1x10^5$ particles/cell. Twenty-four hours after transduction with AAV vectors, culture media were replaced by fresh media containing various concentrations of GCV ranging from 0 to 10 μg/ml. After a 7-day incubation in the presence of GCV, surviving cells were counted. The survival rate was calculated from the ratio of the number of cells not treated with GCV. To evaluate the synergistic effect of the AAVtk/GCV system and topoisomerase inhibitor treatment, the cells were transduced with AAVtk following either etoposide (0.1 μM, 10 μM) or camptothecin (0.01 μM, 1 μM) treatment.

Results

Effect of topoisomerase inhibitor on AAV-mediated transgene expression. The transduction efficiency of HEp-2 cells was almost as high as that of HeLa cells. The amount of ß-gal in HeLa cells were 220 μg/mg proteins, when the cells were transduced with $1x10^4$ particles/cell of AAVLacZ (24). Both etoposide and camptothecin have shown to increase the transduction efficiency with AAV vectors, mainly by accelerating the rate of leading strand synthesis of the AAV vector genome. In Fig. 1, etoposide and camptothecin treatment significantly increased LacZ expression in HeLa cells and HEp-2 cells in a concentration-dependent manner (one-way ANOVA: $P<0.01$).

Topoisomerase inhibitors enhance the second-strand synthesis of the AAV genome in HeLa and HEp-2 cells. To examine whether the second-strand synthesis of the AAV vector genome occurs more efficiently in the topoisomerase inhibitors treated-cells, HeLa cells and HEp-2 cells were treated with 0, 0.1, or 10 μM of etoposide, and then transduced with $1x10^4$ particles/cell of AAVLacZ. Forty-eight hours after transduction, total DNA was isolated, treated with mung bean nuclease, and then loaded on 1% agarose gels. After transfer to nylon membranes, signals corresponding to the AAVLacZ genomes were detected (Fig. 2). Mung bean nuclease was used to digest the single-strand DNA and to clearly visualize the double-stranded replicative form (DS) of the AAV vector genome. The DS was almost equal to 4.7-kb fragment derived from pAAVLacZ in size. At the concentration of 10 μM, in both HeLa and HEp-2 cells, the intensity of signal corresponding to the DS increased significantly, suggesting that the augmented transgene expression was associated with the conversion of the AAV vector genome to the double-stranded form.

The killing of HeLa and HEp-2 cells in terms of GCV concentration. Fig. 3 shows the killing effect of various concentration of GCV on HeLa cells (Fig. 3A) and HEp-2 cells (Fig. 3B) transduced with $3x10^5$ particles/cell of AAVtk (closed bar). When the AAVtk-transduced-cells were treated with 1 μg/ml of GCV, 90% of HeLa cells and 47% of HEp-2 cells were killed. As the concentration of GCV was increased, surviving cells were reduced and 98% of HeLa cells and 96% of HEp-2 cells were killed by the exposure to 10 μg/ml of GCV. This killing rate was significantly higher than that in

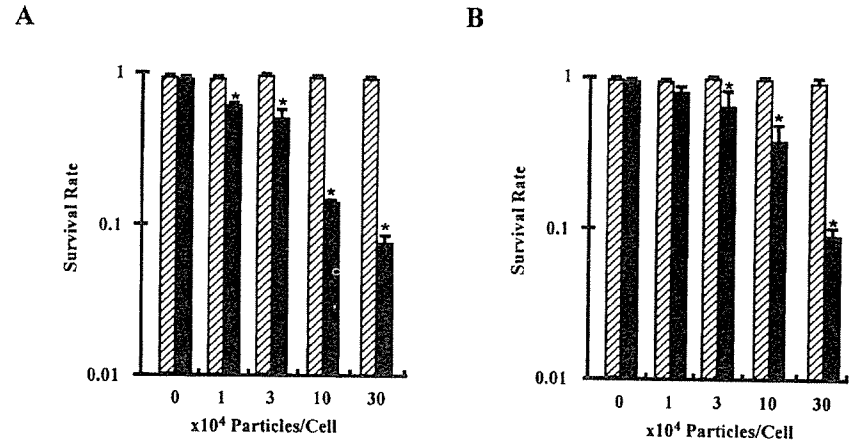

Figure 4. Survival of HeLa cells and HEp-2 cells upon AAVtk/GCV in terms of AAV doses. HeLa cells (A) or HEp-2 cells (B) were, transduced with various doses of AAVLacZ (hatched bar), or AAVtk (closed bar). Twenty-four hours after transduction, the cells were exposed to 3 μg/ml of GCV. Data were analyzed by two-way ANOVA. Asterisks mean that the data obtained for AAVtk-transduction were significantly different from those with or without transduction of AAVLacZ ($P<0.01$).

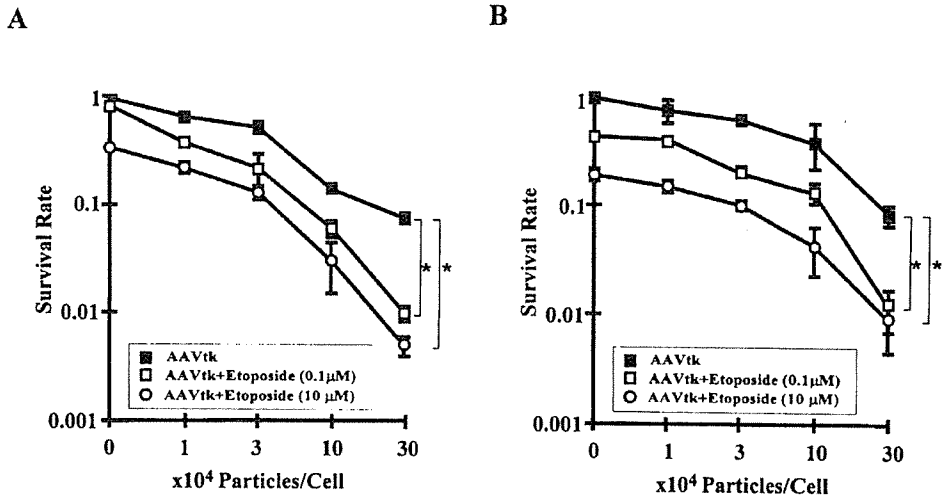

Figure 5. Enhancement of the cytocidal effect of AAVtk by etoposide. HeLa (A) or HEp-2 cells (B) were transduced with various concentrations of AAVtk with or without etoposide treatment. Twenty-four hours after transduction, the cells were treated with 3 μg/ml of GCV. Closed squares: AAVtk-transduced cells. Open squares: AAVtk-transduced with etoposide treatment (0.1 μM). Open circles: AAVtk-transduced cells with etoposide treatment (10 μM). Asterisks mean that the AAVtk-transduced and etoposide treated cells were significantly different from the AAVtk-transduced and non-treated cells ($P<0.01$).

AAVLacZ-transduced cells (hatched bar) or mock-transduced cells (open bar) (two-way ANOVA: $P<0.01$).

The killing of HeLa and HEp-2 cells in terms of AAV vector dose. Fig. 4 shows the killing effect of GCV (3 μg/ml) on HeLa cells and HEp-2 cells transduced with various doses of AAVtk. When HeLa cells were transduced with $3x10^4$ particles/cell of AAVtk, 50% of the cells were killed upon exposure to GCV. As the dose of AAVtk was increased, the number of surviving cells was reduced. This killing rate was significantly higher than that in the case of AAVLacZ-transduced cells, as expected (Fig. 4A). Similarly, AAVtk-transduced HEp-2 cells were killed by exposure to GCV, which was significantly higher than the killing rate in AAVLacZ-transduced cells (Fig. 4B) (two-way ANOVA: $P<0.01$).

Enhanced cytocidal effect of the AAVtk/GCV system by topoisomerase inhibitors (etoposide or camptothecin). To investigate whether etoposide treatment enhances the killing effect of AAVtk/GCV, HeLa (Fig. 5A) and HEp-2 cells (Fig. 5B) were transduced with various doses of AAVtk following pre-treatment with 0.1 μM or 10 μM etoposide, and then cultured in GCV (3 μg/ml). When HeLa cells were transduced with $3x10^4$ particles/cell of AAVtk, 53% of the 0.1 μM treated cells and 84% of the 10 μM treated cells were killed by the addition of GCV. When HeLa cells were transduced at $3x10^5$ particles/

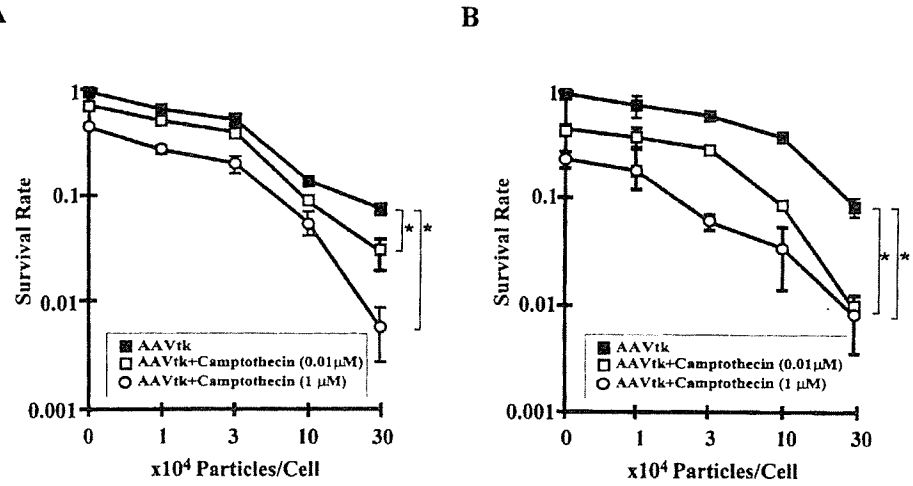

Figure 6. Enhancement of the cytocidal effect of AAVtk by camptothecin. HeLa (A) or HEp-2 cells (B) were transduced with various doses of AAVtk with or without camptothecin treatment. Twenty-four hours after transduction, the cells were treated with 3 μg/ml of GCV. Closed squares: AAVtk-transduced cells. Open squares: AAVtk-transduced with camptothecin treatment (0.01 μM). Open circles: AAVtk-transduced cells with camptothecin treatment (1 μM). Asterisks mean that the AAVtk-transduced and camptothecin treated cells were significantly different from the AAVtk-transduced and non-treated cells ($P<0.01$).

cell, etoposide enhanced the killing effects of AAVtk/GCV system by 21-fold. Etoposide treatment also enhanced the killing effects on HEp-2 cells by 5-fold. The enhancement by etoposide treatment was calculated from the ratio of 10 μM-treated survival rate to non-treated survival rate. These results show that etoposide treatment enhances the killing effects of AAVtk/GCV system significantly (two-way ANOVA: $P<0.01$). Similarly, we examined whether camptothecin treatment enhanced the killing effect of AAVtk/GCV. When HeLa cells were transduced at $3x10^5$ particles/cell, the combined therapy with camptothecin and AAVtk/GCV killed 12-fold HeLa cells (Fig. 6A) and 9-fold HEp-2 cells (Fig. 6B), compared with AAVtk/GCV system alone. These results show that camptothecin treatment also enhances the killing effects of AAVtk/GCV system significantly (two-way ANOVA: $P<0.01$).

Discussion

The application of AAV vectors to gene therapy for head and neck cancer is limited by their low transduction efficiency. Our previous studies showed the enhancement of AAV vector-mediated transgene expression and killing effect of AAVtk/GCV on the head and neck cancer cells by γ-ray (3). In this study, we demonstrated that topoisomerase inhibitors enhanced AAV vector-mediated transgene expression and cytocidal effect of AAVtk/GCV on target cells. The enhanced transgene expression and killing effect can be explained by the higher conversion efficiency of AAV vector genome to double-stranded form. In fact, we demonstrated that topoisomerase inhibitors augmented the second-strand synthesis and transgene expression on the cancer cells. Topoisomerase inhibitors have been utilized as chemotherapeutic agents. Topoisomerases are known to catalyze the reversible breakage and rejoining of DNA to check the DNA unwind during replication (27). Although etoposide and camptothecin inhibit different enzymes, both can induce similar DNA repair function.

It is unclear what kind of host DNA polymerases require for the conversion of single-stranded vector genomes to double-stranded molecules. According to the study that compared the transduction efficiency of dividing cells to non-dividing cells (13), DNA repair synthesis or other activities associated with DNA repair require transduction rather than replicative DNA synthesis. Some unspecified DNA repair mechanism activated by topoisomerase inhibitors may contribute to enhance the second-strand synthesis of AAV vector genome.

Since the mechanism of enhancement of transgene expression has vital significance for the use of AAV vectors, several studies have focused on this topic. Qing *et al* (28) reported that dephosphorylation of the single-stranded D sequence-binding protein facilitated second-strand synthesis of the AAV vector genome. Sanlioglu *et al* (29) reported that the enhancement of AAV vector transduction by UV and adenovirus E4orf6 correlated with induction of two distinct molecular conversion pathways, and UV led to increased abundance of circular AAV vector genome. However, the precise mechanisms by which genotoxic agents facilitate second-strand synthesis of AAV vector genome remains unknown.

Several studies reported that AAV vectors were useful for the treatment of cancers in model experiments. Kunke *et al* (30) showed that expressing the antisense of human papillomavirus early gene effectively killed the tumors derived from cervical cancer cells. In suicide gene therapy, HSVtk-expressing AAV vectors have been reported in the application to several kinds of cancer. The AAV vectors expressing HSVtk and interleukin 2 effectively killed glioma cells implanted into brains of nude mice (31). The expression of HSVtk driven by a liver-specific promoter via AAV vectors in tumors experimentally produced by implantation of hepatocellular carcinoma cells successfully retarded the tumor progression (32). We previously demonstrated the enhancement of the cytocidal effect of AAVtk/GCV system by γ-ray *in vitro*