(University of Pennsylvania, Philadelphia, PA, USA) [14]. Adult mice aged 10 weeks and newborn mice, < 24 h after normal vaginal delivery (day 0), were used for this study. Individual mice were identified by tail markings. The experimental protocol was approved by the institutional Animal Care and Concern Committees of Jichi Medical School.

Neonatal injection of human FVIII

We intended to mimic the clinical situation in which hemophilic patients are infused intermittently with FVIII. Therefore, mice were immunized with i.v. recombinant human FVIII formulated with sucrose (0.05 U g^{-1} body weight; Kogenate FS; Baxter Healthcare, Glendale, CA, USA) [20,21]. Injections were performed under generalized anesthesia (2.5% isoflurene mixed with oxygen and nitrous oxide) using the jugular vein, a 30 G needle, and a zoom stereomicroscope (Nikon, Tokyo, Japan). This dose is comparable to that given to a severe hemophilia A patient with the goal of raising the patient plasma FVIII level to 100%. Blood samples were obtained 2 weeks after each of the injections from the jugular vein, and were added at a 9 : 1 (v/v) ratio to 0.38% sodium citrate and plasma was separated by centrifugation. The plasma samples were subsequently stored at − 80 °C until further analysis.

Assay for the human FVIII inhibitors

Coagulation inhibition was examined using a partial thromboplastin time-based FVIII clotting assay. All samples were diluted in Owren's Veronal Buffer (Dade Behring, Deerfield, IL, USA). Mouse plasma (50 μL) was incubated with 50 μL normal pooled human plasma at 37 °C for 2 h. Residual human FVIII was measured in a one-stage assay using 50 μL FVIII-deficient human plasma (Kokusai-Shiyaku, Kobe, Japan) and 50 μL sample from the previous incubation. Samples were mixed with 100 μL phospholipid activator, incubated at 37 °C for 3 min, followed by 100 μL of 20 mM $CaCl_2$. Clotting times were measured with a coagulometer (CA-500; Sysmex, Kobe, Japan). Coagutrol N (Kokusai-Shiyaku) was diluted with Owren's Veronal Buffer for a standard curve of FVIII activity.

AntiFVIII IgG measurements

AntiFVIII IgG concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) in microtiter wells (Nunc, Roskilde, Denmark) coated with 1 μg mL^{-1} recombinant human FVIII. After blocking with 5% bovine serum albumin (BSA) in PBS, serial dilutions of murine plasma were added at 4 °C for 16 h. Each well was washed with 0.5% BSA in PBS containing 0.05% Tween 20. Horseradish peroxidase (HRP)-conjugated goat antimouse IgG (Cappel, Aurora, OH, USA) was added at 37 °C for 1 h. ABTS Microwell substrate (KPL, Gaithersburg, MD, USA) was added and the absorbance at 405 nm was read. AntiFVIII antibody concentrations were estimated from the linear portion of a standard curve obtained using antihuman FVIII monoclonal antibodies (kindly provided from The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) that bind to FVIII.

Determination of IgG subclass of antiFVIII antibodies

Microtiter wells were coated with 1 μg mL^{-1} recombinant human FVIII in PBS for 16 h at 4 °C. After blocking with 5% BSA in PBS, serial dilutions of murine plasma were added for 2 h at 37 °C. The wells were washed with 0.5% BSA in PBS containing 0.05% Tween 20. The IgG subtypes of antiFVIII antibodies bound to immobilized human FVIII were determined by incubation with isotype-specific rabbit antimouse IgGs (Mouse Typer; BioRad, Hercules, CA, USA) for 1 h at 37 °C. After washing with 0.5% BSA in PBS containing 0.05% Tween 20, the wells were incubated with goat antirabbit HRP conjugate for 1 h at 37 °C. Substrate development was performed for 15 min at 25 °C using ABTS Microwell substrate as described above.

Immunoblotting of FVIII

To characterize murine antibodies against human FVIII, immunoblotting of thrombin cleavage products of FVIII was performed as described previously [22]. In brief, one unit of human thrombin was added to 50 units of recombinant human FVIII and incubated for 1 min at 37 °C. Reaction was stopped by addition of sodium dodecyl sulfate (SDS) sample buffer. The thrombin-digested recombinant human FVIII proteins were separated by 7.5–12.5% SDS polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions, and were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) by electroblotting. The membranes were cut into strips and incubated with 5% casein (Calbiochem, Darmstadt, Germany) for 16 h at 4 °C. Each strip was incubated with the appropriate dilution of plasma samples from the human FVIII-injected mice in 0.05 M Tris–HCl pH 7.4, 0.14 M NaCl (TBS) containing 0.5% casein and 0.05% Tween 20 for 2 h at 25 °C. After washing, the strips were incubated with HRP-conjugated goat antimouse IgG for 2 h at 25 °C and the strips were developed using aminoethylcarbazole (Sigma, St Louis, MO, USA) in 0.1 M sodium acetate pH 5.2 at 25 °C. AntiFVIII polyclonal antibody (Cedarlane, Ontario, Canada) and antiFVIII monoclonal antibodies, which specifically recognized heavy chain and light chain (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan), were used as controls.

Tetanus immunization in tolerant FVIII-deficient mice

Mice were injected intraperitoneally with 1 Lf of tetanus toxoid (TT) vaccine (Takeda Chemical Industries, Tokyo, Japan). Plasma samples were obtained after 3 weeks, and anti-TT antibody titers were determined by ELISA as previously described [23]. In brief, microtiter plates were coated with 5 μg mL^{-1} formaldehyde-inactivated tetanus toxin, *Clostridium tetanii* (Calbiochem), at 4 °C for 16 h. After washing and

blocking with TBS containing 5% BSA, mice plasma samples were added to the wells and incubated for 2 h at 37 °C. After washing with 0.5% BSA in PBS containing 0.05% Tween 20, 100 μL of HRP-conjugated goat antimouse IgG were added at 37 °C for 1 h. After washing, ABTS Microwell substrate was added and the absorbance at 405 nm was measured.

Preparation of splenocytes and proliferation assay

Four days after the final treatment of human FVIII, mouse splenocytes were isolated as described [24]. To measure T-cell proliferation, 1×10^5 cells per well were cultured with 0–3 nM human FVIII at 37 °C for 72 h in complete RPMI 1640 (Gibco BRL, Rockville, MD, USA). ^{3}H-thymidine was added (0.037 MBq well^{-1}; Amershan Bioscience, Uppsala, Sweden) at 37 °C for 18 h. The cells were harvested, and ^{3}H-thymidine incorporation was determined by scintillation counting (Top count; Packard, Meriden, CT, USA).

Cytokine assays

Splenocytes were incubated in 24-well plates at 10×10^5 cells per well with 2 nM of human recombinant FVIII for 24 h. Cytokine production was measured by ELISA (Biotrak ELISA System; Amersham Biosciences, Piscataway, NJ, USA) with 100 μL of culture supernatant as previously described [25]. Cytokine concentrations in culture supernatants were estimated by extrapolation from the linear portion of standard curves made from recombinant mouse interleukin-2 (IL-2; BD Biosciences, San Jose, CA, USA), interleukin (IL)-4, interleukin (IL)-10, or interferon (IFN)-γ as appropriate. For restoration of splenocyte responses, stimulation was conducted in the presence of 10 U mL^{-1} of mouse recombinant interleukin (IL)-12 or IFN-γ (BD Biosciences), as described previously [25,26].

Statistical analysis

Data are expressed as means ± SE. Statistical differences were determined using a two-tailed unpaired Student's *t*-test. A *P*-value of < 0.05 was considered to be significant.

Results

Induction of inhibitory antibodies against FVIII in adult hemophilia A mice

The immune response of hemophilia A mice toward FVIII was determined after i.v. injection of human FVIII (0.05 U g^{-1} body weight) into adult mice at 2-week intervals. Antihuman FVIII inhibitory antibodies were detected after the third injection in all mice (Fig. 1). Titers continued to increase and reached a plateau after the seventh injection (at 24 weeks of age). The IgG subclass of antiFVIII antibodies was mainly IgG1 in all mice at 20 weeks of age (Fig. 1, inset). IgG2a and IgG2b subclass antibodies were also detected at 50 weeks of age. These results are compatible with previous studies

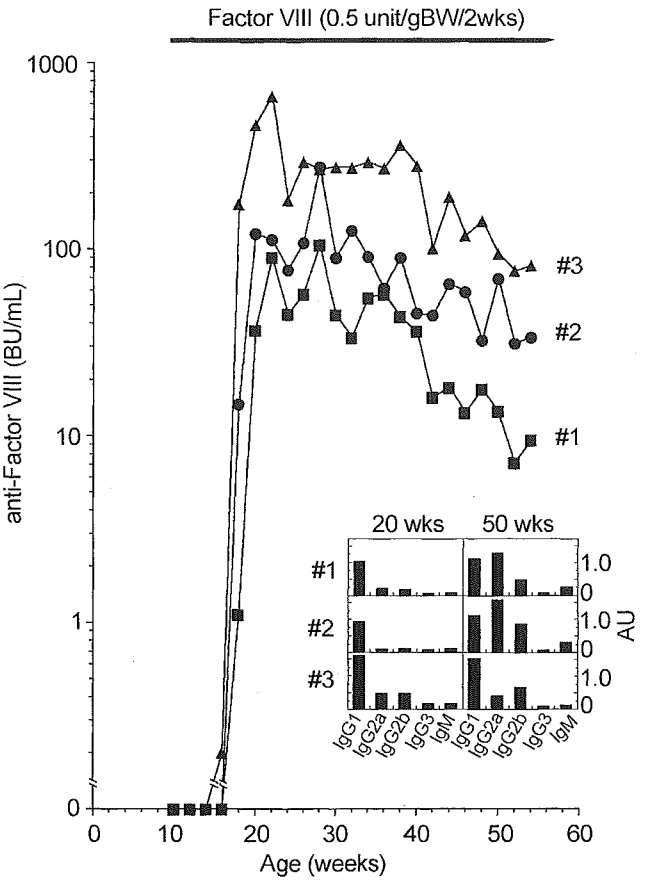

Fig. 1. Formation of inhibitory antibodies against human factor (F)VIII in hemophilia A adult mice. FVIII-deficient adult mice were injected intravenously with human FVIII (0.05 U g^{-1} body weight) at 10 weeks, followed by repeated injections at 2-week intervals until 56 weeks. The mice were bled on day 14 after each injection. The antiFVIII inhibitor titer was determined by Bethesda assay as described in Materials and methods. (Inset) AntiFVIII antibody titers of different IgG subtypes in hemophilia A adult mice with repeated FVIII treatments were measured by ELISA at 20 and 50 weeks. Data are expressed as the means of triplicate assays.

demonstrating that human FVIII is highly immunogenic in hemophilic mice [18], and that antibodies induced by human FVIII in a murine knockout model of hemophilia A were mainly of the IgG1 subclass [22]. Considering these results, we evaluated the immunogenic responses of hemophilia A mice against human FVIII after the fourth injection.

AntiFVIII immunoglobulin production is suppressed by neonatal i.v. injection in hemophilia A mice

All but one of the hemophilic mice treated with FVIII at 10, 12, 14 and 16 weeks developed high titers [196.4–448.2 Bethesda Units (BU) mL^{-1}] of antiFVIII inhibitory antibody (Fig. 2A). In contrast, 13 out of 14 mice intravenously injected within 24 h after birth (day 0) had undetectable antiFVIII inhibitory antibody levels (Fig. 2B). When mice were treated with FVIII on day 1 or day 3 after birth, high titers of antiFVIII inhibitors (> 50 BU mL^{-1}) developed after the final injection in one of 10 (10%) and in seven of 13 mice (54%), respectively (Fig. 3A).

Administration of supraphysiological doses of FVIII (0.1 and 0.5 U g^{-1} body weight; estimated at 200% and 1000% normal FVIII level, respectively) into neonatal mice on day 0, followed by injection with 0.05 U g^{-1} body weight of FVIII at 10, 12, 14, and 16 weeks resulted in high-titer antiFVIII inhibitory antibody in one out of nine and three of 17 animals, respectively (Fig. 3B). Thus, exposure to a physiological level of FVIII antigen on day 0 contributed to induction of neonatal tolerance to human FVIII.

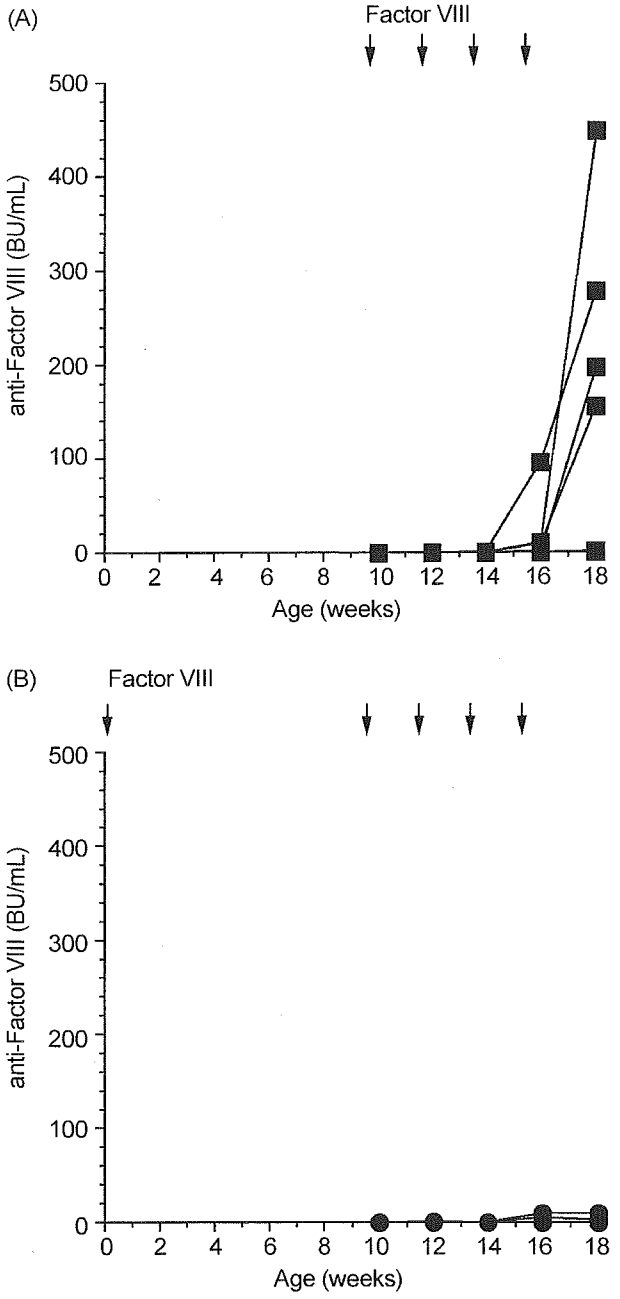

Fig. 2. Effect of neonatal intravenous injection of factor (F)VIII on antiFVIII inhibitory antibody formation in hemophilia A mice. (A) Adult mice ($n = 5$) were injected intravenously with human FVIII (0.05 U g^{-1} body weight) at 10, 12, 14, and 16 weeks. The mice were bled on day 14 after each injection. (B) Neonatal hemophilia A mice ($n = 14$) were given an initial i.v. injection of human FVIII (0.05 U g^{-1} body weight) within 24 h after birth, followed by repeated injections at 2-week intervals beginning 10 weeks after the first injection. The antiFVIII inhibitor titer was determined by Bethesda assay. Arrows indicate injection points.

FVIII-specific IgG formation is inhibited by neonatal i.v. injection in hemophilia A mice

High antiFVIII IgG titers developed after the fourth injection in four of five mice treated as adults (Fig. 4A). Thrombin cleavage products of complete human FVIII were incubated with plasma samples from these mice to characterize the

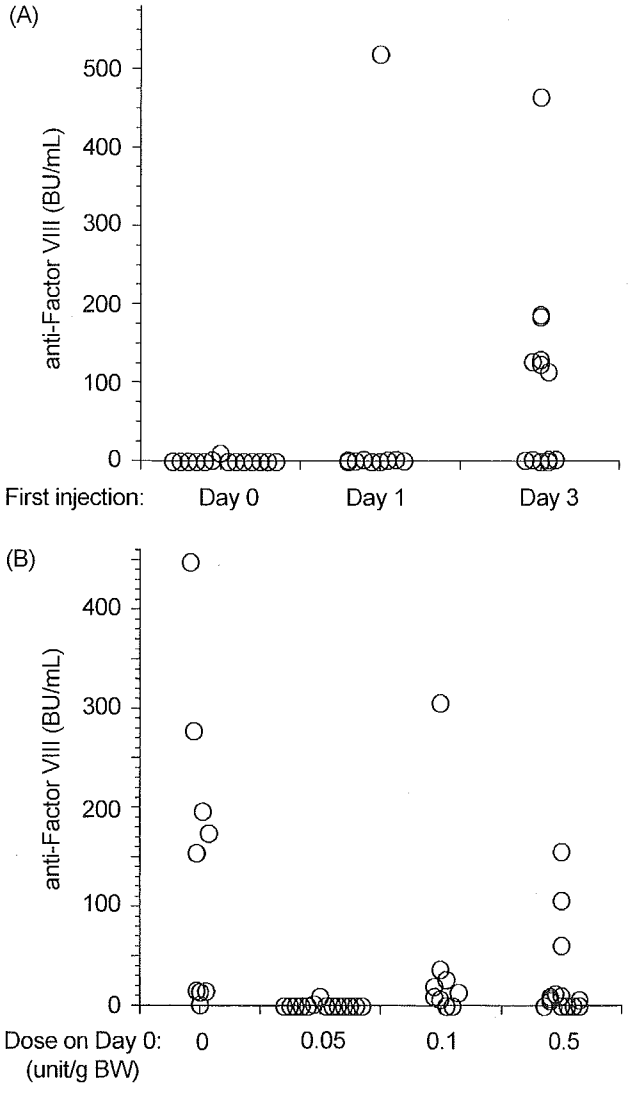

Fig. 3. Timing and dose effect of factor (F)VIII injection on antiFVIII inhibitory antibody formation. (A) The initial i.v. administration of FVIII was given within 24 h (day 0, $n = 14$), between 24 and 48 h (day 1, $n = 10$) and between 72 and 96 h (day 3, $n = 13$) after birth. Each mouse was followed by repeated injections at 10, 12, 14, and 16 weeks. The mice were bled on day 4 after the fifth treatment, and antiFVIII inhibitor titers were measured by Bethesda assay. (B) Zero unit g^{-1} body weight (saline control; $n = 9$), 0.05 U g^{-1} body weight ($n = 14$), 0.1 U g^{-1} body weight ($n = 9$), or 0.5 U g^{-1} body weight ($n = 17$) of FVIII were injected into neonatal mice on day 0, and then treated with 0.05 U g^{-1} body weight of FVIII at 10, 12, 14, 16 weeks. The mice were bled on day 4 after the fifth treatment, and antiFVIII inhibitor titers were measured by Bethesda assay.

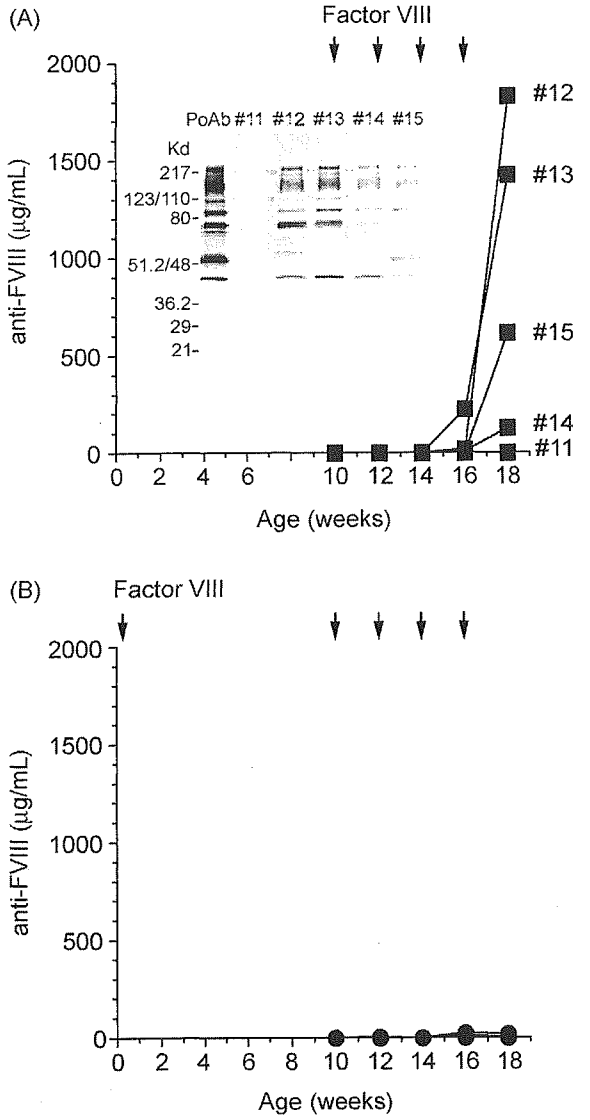

Fig. 4. Effect of neonatal injection of factor (F)VIII on FVIII-specific IgG formation in hemophilia A mice. (A) Adult mice ($n = 5$) were injected intravenously with human FVIII (0.05 U g^{-1} body weight) at 10, 12, 14, and 16 weeks. The mice were bled on day 14 after each injection. (Inset) Immunoblots of thrombin cleavage products of human FVIII incubated with plasma samples from FVIII-deficient mice that were bled on day 4 after the fourth administration of FVIII. (B) Neonatal hemophilia A mice ($n = 14$) were given an initial i.v. injection of human FVIII (0.05 U g^{-1} body weight) within 24 h after birth, followed by repeated injections at 2-week intervals beginning 10 weeks after the first injection. The FVIII-specific IgG was measured with ELISA as described in Materials and methods. Arrows indicate injection points.

antibody reaction sites. The immunoreactive sites were found in parts of 90-kDa heavy chain, as well as 90–210-kDa heavy chains, the 44-kDa heavy chain and 72-kDa light chain (Fig. 4A, inset). In contrast, none of the mice treated intravenously within the first 24 h of life developed IgG antibodies against FVIII (Fig. 4B). No reactivity against intact molecules or thrombin cleavage products of FVIII was found in neonatal treated animals (data not shown). When antiFVIII inhibitors were compared with antiFVIII IgG antibodies from 241 samples from hemophilia A mice that had been repeatedly injected, there was a high correlation between antiFVIII antibodies detected by the Bethesda assay and by ELISA ($\chi^2 = 0.732$, data not shown).

FVIII-specific T-cell response is blocked by neonatal exposure to FVIII antigen in hemophilia A mice

To evaluate whether neonatal administration of FVIII exerts a direct suppressive effect on FVIII-specific T cells, splenocytes obtained 4 days after the final injection were assayed for a T-cell proliferative response to FVIII in mice initially treated during the adult or neonatal period. There was a FVIII dose-dependent T-cell proliferative response of spleen cells from adult hemophilia mice (Fig. 5A). In contrast, no response was detected at any FVIII level with splenocytes from the neonatally treated mice. In both groups, there was no proliferative response of T cells at any dose of human albumin (Fig. 5B). Thus, neonatal injection of FVIII could be important for prevention of an immune response to FVIII injected during adulthood.

Tolerance induction of neonatal FVIII-deficient mice is FVIII specific

To determine whether the suppression of antibody for FVIII was specific, five mice were immunized intraperitoneally with TT vaccine 2 weeks after the last challenge with FVIII. Anti-TT antibody titers were measured by ELISA in mice plasma samples at day 21 after immunization (Fig. 6). The results demonstrated that neonatal FVIII-treated mice were able to mount a T-cell-dependent immune response to a different antigen and the antibody response was similar to that in adult treated animals. These results indicated that the suppression observed was FVIII specific.

Neonatal exposure to FVIII develops an IFN-γ-mediated splenic anergy upon challenge with FVIII during adult life

Mice that were injected with human FVIII on the day of birth, and subsequently challenged with FVIII as an adult, developed splenocytes that failed to proliferate and did not produce IL-4, IL-10, or IFN-γ in response to FVIII stimulation (Fig. 7A,C). However, the splenic cells secreted significant amounts of IL-2 (Fig. 7B). To address whether the defect in proliferation might be related to an IFN-γ deficiency, we stimulated the splenocytes with FVIII in the presence of IFN-γ or its inducer IL-12 and assayed for proliferation. As shown in Fig. 7, exogenous IFN-γ restored the proliferative response upon stimulation with FVIII. IL-12 was also able to restore T-cell proliferation and IFN-γ production, suggesting that this form of anergy might be IFN-γ dependent [26].

Discussion

Intravenous injection of recombinant human FVIII in FVIII knockout mice (exon 16 and 17 disruption) results in a

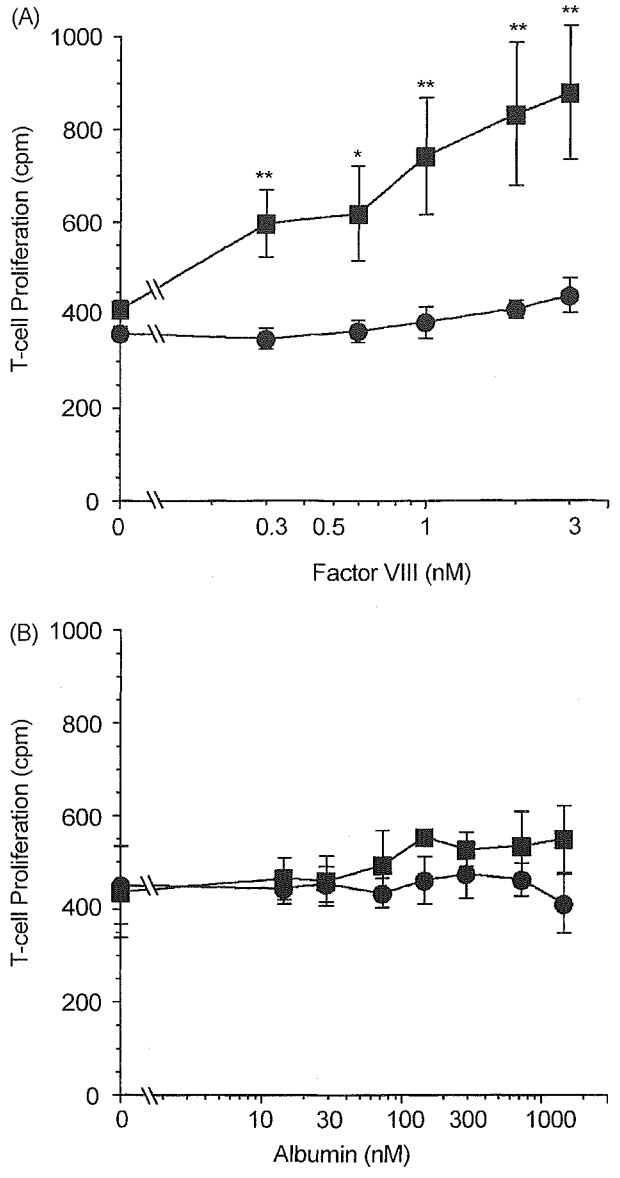

Fig. 5. Antifactor (F)VIII-specific T-cell proliferative response is blocked by neonatal FVIII administration. Adult hemophilia A mice (■, $n = 9$) were given i.v. administrations of human FVIII (0.05 U g^{-1} body weight) at 10, 12, 14, and 16 weeks. Neonatal hemophilia A mice (●, $n = 8$) were injected intravenously within 24 h after birth, and were repeatedly injected every 2 weeks beginning 10 weeks of the initial injection. Splenocytes were obtained 4 days after the final injection, and the amount of ^{3}H-thymidine incorporation was determined by scintillation counting as described in Materials and methods. The concentrations of FVIII (A) and albumin (B) in the cultures are indicated on the horizontal axis. Data are shown as the mean ± SD. $^*P < 0.05$; $^{**}P < 0.03$.

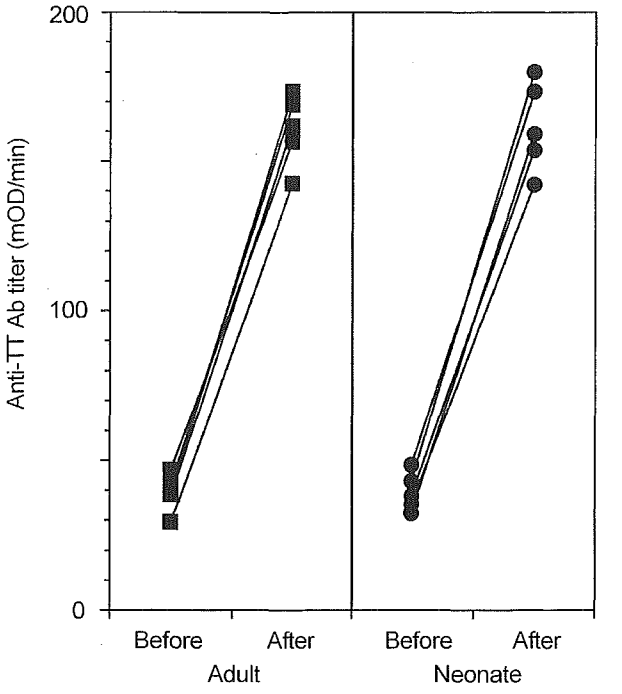

Fig. 6. Humoral immune response to tetanus toxoid. Adult hemophilia A mice (■, $n = 5$) were given i.v. administrations of human factor (F)VIII (0.05 U g^{-1} body weight) at 10, 12, 14, and 16 weeks. Neonatal hemophilia A mice (●, $n = 5$) were injected intravenously within 24 h after birth and were injected repeatedly every 2 weeks beginning 10 weeks after the first injection. Each mouse was injected intraperitoneally with one Lf unit of tetanus toxoid vaccine 2 weeks after the last challenge with FVIII. Plasma samples were obtained before and 3 weeks after the tetanus toxoid injection. Antitetanus toxoid antibody titers were measured by ELISA as described in Material and methods.

T-cell-dependent formation of antiFVIII antibodies, suggesting that the murine knockout model of hemophilia A might be useful for elucidating mechanisms of the formation of inhibitory responses [18,22]. In the current study, we repeatedly injected human FVIII into FVIII knockout adult mice (exon 16 disruption), and showed that all mice developed antiFVIII inhibitory antibodies (Fig. 1). AntiFVIII-specific antibodies detected by ELISA were mainly IgG1 at 20 and 50 weeks, suggesting that the immune response toward human FVIII in this model is regulated mainly by Th2 lymphocytes [27,28] as observed in clinical cases [22,29,30]. Furthermore, the IgG antibodies recognized the non-functional B domain, as well as functional domains of FVIII (Fig. 4A), suggesting that the immune response in these mice model is similar to that in hemophilia A patients [31,32].

Neonatally induced T-cell tolerance is attributed to several mechanisms, including clonal deletion of antigen-reactive T cells and suppressor cell development [11,33,34]. Pittmann and coworkers showed that a single dose of FVIII to neonatal Balb/cBy mice within 30 h after birth resulted in the induction of antigen-specific non-responsiveness [35]. Newborn mice were infused intraperitoneally with 5000-fold quantities of human FVIII, to approximate levels used to normalize FVIII levels in severe hemophilia A patients. The high antigen doses preferentially stimulated a type 2 cytokine response, whereas low doses of antigen induced a type 1 cytokine response [36–38]. In addition, enhanced type 2 and decreased type 1 responses are associated with neonatal tolerance to alloantigens [39]. However, Sarzotti and coworkers reported that inoculation of newborn mice with a high-dose antigen does not result in

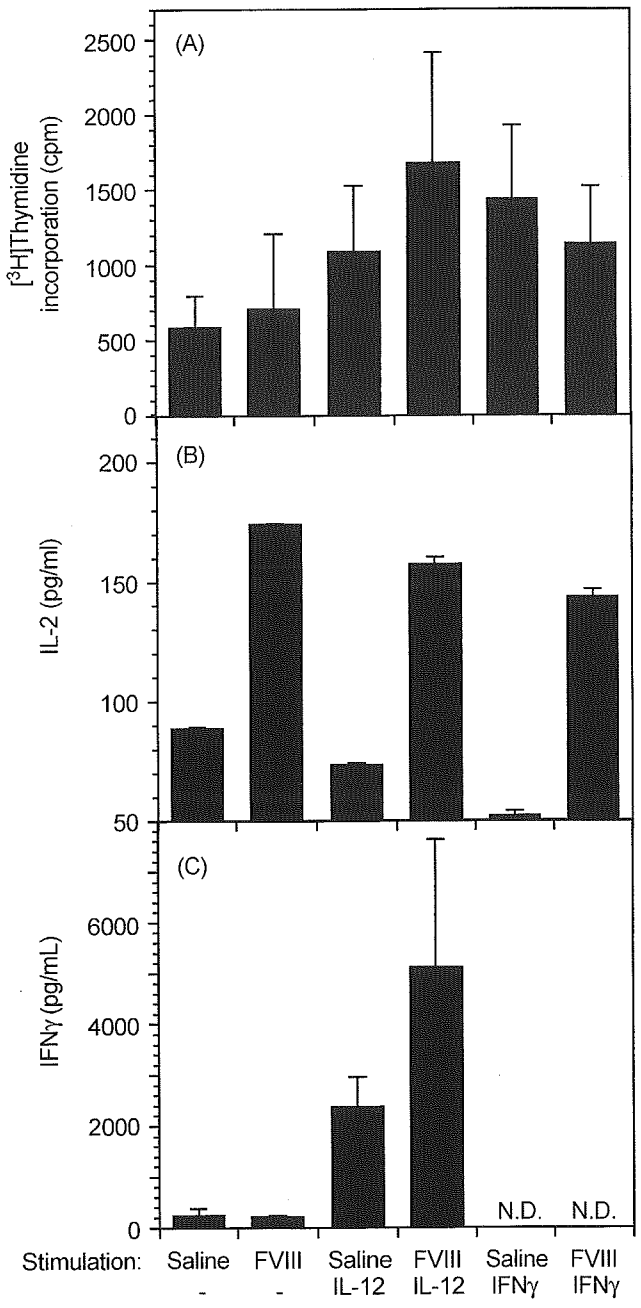

Fig. 7. The splenic T cells of factor (F)VIII-tolerized mice are restored by exogenous interferon (IFN)-γ or interleukin (IL)-12. Neonatal hemophilia A mice ($n = 3$) were injected intravenously with 0.05 U g^{-1} body weight of human FVIII within 24 h after birth, and were repeatedly injected at 10, 12, 14, and 16 weeks. Four days after the final injection, splenocytes (1×10^5 cells $well^{-1}$) were stimulated *in vitro* with 2 nM of human FVIII in the absence or presence of 10 U mL^{-1} of cytokines (IL-12 or IFN-γ), and proliferation (A), IFN-γ (B), and interleukin-2 (C) production were measured as described in Materials and methods. The values (cpm and pg mL^{-1}) represent the mean ± SD of individually tested mice and are representative of three experiments.

immunological non-responsiveness and the initial dose is critical in the development of immunity in newborns [12]. We showed that day 0 intravenous injection of 0.05 U g^{-1} body weight of FVIII was sufficient to block development of antiFVIII antibodies (Figs 2 and 4) and T-cell reactivity (Fig. 5). Conversely, one of nine mice and three of 17 mice developed high-titer antiFVIII inhibitory antibodies when day 0 mice were treated with 2-fold (0.1 U g^{-1} body weight) or 10-fold higher doses (0.5 U g^{-1} body weight) of FVIII, respectively (Fig. 3). These findings suggest that the day 0 exposure to a physiological level of FVIII antigen induces the neonatal immune tolerance.

We demonstrated that the tolerant mice immunized with TT developed high anti-TT antibody titers (Fig. 6). This result indicates that the tolerance to FVIII exposure from week 10 onward was antigen-specific unresponsiveness and the ability to mount a humoral immune response to other T-cell-dependent antigens remained intact in mice exposed to neonatal FVIII.

FVIII-deficient mice injected with FVIII at birth and subsequently challenged as adults failed to develop a T-cell proliferative response to FVIII, although these splenocytes produced significant amounts of IL-2 (Fig. 7). The splenocytes did not produce IFN-γ, but exogenous IFN-γ, as well as IL-12, restored splenic proliferation in an antigen-specific manner. Additionally, IL-12-treated T cells continued to secrete IL-2 and regained the ability to produce IFN-γ. This phenomenon may qualify as anergy with the distinction that the T cells produce IL-2, but are unable to produce IFN-γ, suggesting that this form of anergy may be IFN-γ mediated [25,40]. The fact that T cells produce IFN-γ when they regain proliferative capacity upon IL-12 treatment, a key cytokine for differentiation of T cells into effector Th1 cells, further justifies the involvement of IFN-γ in this form of unresponsiveness [41,42].

From clinical experience, patient age at initial treatment appears to influence inhibitor formation in severe hemophilia A [43,44]. It is reported that inhibitors developed in a hemophilia A neonate, but he had received FVIII concentrate on day 10 postpartum [45]. Recently, VandenDriessche and coworkers reported that i.v. injection of retroviral vectors carrying the human FVIII gene into 2–3-day-old newborn FVIII-deficient mice corrected the hemophilia A phenotype [46]. However, six of the 13 animals developed FVIII inhibitors (7–350 BU mL^{-1}). On the other hand, intravascular prenatal administration of an adenoviral vector carrying the human factor IX transgene induced immune tolerance in five of nine MF1 mice [47]. Considering these findings, the continuous presence of antigen in the blood stream might be essential for immune tolerance induction during the perinatal period.

It seems unlikely that i.v. injection of FVIII immediately after birth would be any more hazardous than if given by intraperitoneal or intramuscular administration for prophylactic purposes as well as immune tolerance induction [48]. Our findings indicate that neonatal tolerance can be induced in hemophilia A mice by i.v. injection of appropriate amounts of human FVIII within 24 h after birth. This immune tolerance model may provide a basis for new approaches toward prevention of inhibitor development during replacement therapy in hemophilic patients.

Acknowledgements

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References

1 Hoyer LW. Hemophilia A. *N Engl J Med* 1994; **330**: 38–47.
2 Scharrer I, Bray GL, Neutzling O. Incidence of inhibitors in haemophilia A patients—a review of recent studies of recombinant and plasma-derived factor VIII concentrates. *Haemophilia* 1999; **5**: 145–54.
3 Kreuz W, Becker S, Lenz E, Martinez-Saguer I, Escuriola-Ettingshausen C, Funk M, Ehrenforth S, Auerswald G, Kornhuber B. Factor VIII inhibitors in patients with hemophilia A: epidemiology of inhibitor development and induction of immune tolerance for factor VIII. *Semin Thromb Hemost* 1995; **21**: 382–9.
4 Ehrenforth S, Kreuz W, Scharrer I, Linde R, Funk M, Gungor T, Krackhardt B, Kornhuber B. Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs. *Lancet* 1992; **339**: 594–8.
5 Unuvar A, Warrier I, Lusher JM. Immune tolerance induction in the treatment of paediatric haemophilia A patients with factor VIII inhibitors. *Haemophilia* 2000; **6**: 150–7.
6 Colowick AB, Bohn RL, Avorn J, Ewenstein BM. Immune tolerance induction in hemophilia patients with inhibitors: costly can be cheaper. *Blood* 2000; **96**: 1698–702.
7 Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells. *Nature* 1953; **172**: 603–6.
8 Etlinger HM, Chiller JM. Maturation of the lymphoid system. I. Induction of tolerance in neonates with a T-dependent antigen that is an obligate immunogen in adults. *J Immunol* 1979; **122**: 2558–63.
9 Etlinger HM, Chiller JM. Maturation of the lymphoid system. II. Characterization of the cellular levels of unresponsiveness induced in neonates by a T-dependent antigen that is an obligate immunogen in adults. *J Immunol* 1979; **122**: 2564–70.
10 Forsthuber T, Yip HC, Lehmann PV. Induction of TH1 and TH2 immunity in neonatal mice. *Science* 1996; **271**: 1728–30.
11 Gammon G, Dunn K, Shastri N, Oki A, Wilbur S, Sercarz EE. Neonatal T-cell tolerance to minimal immunogenic peptides is caused by clonal inactivation. *Nature* 1986; **319**: 413–5.
12 Sarzotti M, Robbins DS, Hoffman PM. Induction of protective CTL responses in newborn mice by a murine retrovirus. *Science* 1996; **271**: 1726–8.
13 Dazzi F, Rosato A, Tison T, Vianello F, Radossi P. An animal model to explore the molecular basis of factor VIII (FVIII) inhibitor formation: evidence of anti-FVIII T-cell response and importance of administration route. *Thromb Haemost* 1995; **73**: 1026.
14 Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian HH Jr. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nat Genet* 1995; **10**: 119–21.
15 Bi L, Sarkar R, Naas T, Lawler AM, Pain J, Shumaker SL, Bedian V, Kazazian HH Jr. Further characterization of factor VIII-deficient mice created by gene targeting: RNA and protein studies. *Blood* 1996; **88**: 3446–50.
16 Hausl C, Maier E, Schwarz HP, Ahmad RU, Turecek PL, Dorner F, Reipert BM. Long-term persistence of anti-factor VIII antibody-secreting cells in hemophilic mice after treatment with human factor VIII. *Thromb Haemost* 2002; **87**: 840–5.
17 Wu H, Reding M, Qian J, Okita DK, Parker E, Lollar P, Hoyer LW, Conti-Fine BM. Mechanism of the immune response to human factor VIII in murine hemophilia A. *Thromb Haemost* 2001; **85**: 125–33.
18 Qian J, Borovok M, Bi L, Kazazian HH Jr, Hoyer LW. Inhibitor antibody development and T cell response to human factor VIII in murine hemophilia A. *Thromb Haemost* 1999; **81**: 240–4.
19 Connelly S, Andrews JL, Gallo AM, Kayda DB, Qian J, Hoyer L, Kadan MJ, Gorziglia MI, Trapnell BC, McClelland A, Kaleko M. Sustained phenotypic correction of murine hemophilia A by *in vivo* gene therapy. *Blood* 1998; **91**: 3273–81.
20 Yoshioka A, Shima M, Fukutake K, Takamatsu J, Shirahata A. Safety and efficacy of a new recombinant FVIII formulated with sucrose (rFVIII-FS) in patients with haemophilia A: a long-term, multicentre clinical study in Japan. *Haemophilia* 2001; **7**: 242–9.
21 Abshire TC, Brackmann HH, Scharrer I, Hoots K, Gazengel C, Powell JS, Gorina E, Kellermann E, Vosburgh E. Sucrose formulated recombinant human antihemophilic factor VIII is safe and efficacious for treatment of hemophilia A in home therapy—International Kogenate-FS Study Group. *Thromb Haemost* 2000; **83**: 811–6.
22 Reipert BM, Ahmad RU, Turecek PL, Schwarz HP. Characterization of antibodies induced by human factor VIII in a murine knockout model of hemophilia A. *Thromb Haemost* 2000; **84**: 826–32.
23 Rossi G, Sarkar J, Scandella D. Long-term induction of immune tolerance after blockade of CD40–CD40L interaction in a mouse model of hemophilia A. *Blood* 2001; **97**: 2750–7.
24 Kruisbeek AM, Shevach E. Proliferative assays for T Cell function. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, eds. *Current Protocols in Immunology*. Unit 3.12. New York: Wiley, 1996: 1–12.
25 Min B, Legge KL, Pack C, Zaghouani H. Neonatal exposure to a self-peptide-immunoglobulin chimera circumvents the use of adjuvant and confers resistance to autoimmune disease by a novel mechanism involving interleukin 4 lymph node deviation and interferon gamma-mediated splenic anergy. *J Exp Med* 1998; **188**: 2007–17.
26 Min B, Legge KL, Bell JJ, Gregg RK, Li L, Caprio JC, Zaghouani H. Neonatal exposure to antigen induces a defective CD40 ligand expression that undermines both IL-12 production by APC and IL-2 receptor up-regulation on splenic T cells and perpetuates IFN-gamma-dependent T cell anergy. *J Immunol* 2001; **166**: 5594–603.
27 Stevens TL, Bossie A, Sanders VM, Fernandez-Botran R, Coffman RL, Mosmann TR, Vitetta ES. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature* 1988; **334**: 255–8.
28 Mosmann TR, Coffman RL. Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Adv Immunol* 1989; **46**: 111–47.
29 Fulcher CA, de Graaf Mahoney S, Zimmerman TS. FVIII inhibitor IgG subclass and FVIII polypeptide specificity determined by immunoblotting. *Blood* 1987; **69**: 1475–80.
30 Kavanagh ML, Wood CN, Davidson JF. The immunological characterization of human antibodies to factor VIII isolated by immunoaffinity chromatography. *Thromb Haemost* 1981; **45**: 60–4.
31 Gilles JG, Arnout J, Vermylen J, Saint-Remy JM. Anti-factor VIII antibodies of hemophiliac patients are frequently directed towards nonfunctional determinants and do not exhibit isotypic restriction. *Blood* 1993; **82**: 2452–61.
32 Fulcher CA, de Graaf Mahoney S, Roberts JR, Kasper CK, Zimmerman TS. Localization of human factor FVIII inhibitor epitopes to two polypeptide fragments. *Proc Natl Acad Sci USA* 1985; **82**: 7728–32.
33 Nossal GJ. Cellular mechanisms of immunologic tolerance. *Annu Rev Immunol* 1983; **1**: 33–62.
34 Loblay RH, Fazekas de St Groth B, Pritchard-Briscoe H, Basten A. Suppressor T cell memory. II. The role of memory suppressor T cells in tolerance to human gamma globulin. *J Exp Med* 1983; **157**: 957–73.
35 Pittman DD, Alderman EM, Tomkinson KN, Wang JH, Giles AR, Kaufman RJ. Biochemical, immunological, and *in vivo* functional characterization of B-domain-deleted factor VIII. *Blood* 1993; **81**: 2925–35.
36 Bretscher PA, Wei G, Menon JN, Bielefeldt-Ohmann H. Establishment of stable, cell-mediated immunity that makes 'susceptible' mice resistant to *Leishmania major*. *Science* 1992; **257**: 539–42.
37 Pfeiffer C, Murray J, Madri J, Bottomly K. Selective activation of Th1- and Th2-like cells *in vivo*—response to human collagen IV. *Immunol Rev* 1991; **123**: 65–84.

38 Hosken NA, Shibuya K, Heath AW, Murphy KM, O'Garra A. The effect of antigen dose on CD4+ T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model. *J Exp Med* 1995; **182**: 1579–84.

39 Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989; **7**: 145–73.

40 Jenkins MK, Pardoll DM, Mizuguchi J, Quill H, Schwartz RH. T-cell unresponsiveness *in vivo* and *in vitro*: fine specificity of induction and molecular characterization of the unresponsive state. *Immunol Rev* 1987; **95**: 113–35.

41 Szabo SJ, Dighe AS, Gubler U, Murphy KM. Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J Exp Med* 1997; **185**: 817–24.

42 Van Parijs L, Perez VL, Biuckians A, Maki RG, London CA, Abbas AK. Role of interleukin 12 and costimulators in T cell anergy *in vivo*. *J Exp Med* 1997; **186**: 1119–28.

43 Lorenzo JI, Lopez A, Altisent C, Aznar JA. Incidence of factor VIII inhibitors in severe haemophilia: the importance of patient age. *Br J Haematol* 2001; **113**: 600–3.

44 Yee TT, Beeton K, Griffioen A, Harrington C, Miners A, Lee CA, Brown SA. Experience of prophylaxis treatment in children with severe haemophilia. *Haemophilia* 2002; **8**: 76–82.

45 Haya S, Lorenzo JI, Dasi MA, Aznar JA. Development of a factor VIII inhibitor in a newborn haemophiliac. *Haemophilia* 1998; **4**: 755–6.

46 VandenDriessche T, Vanslembrouck V, Goovaerts I, Zwinnen H, Vanderhaeghen ML, Collen D, Chuah MK. 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 USA* 1999; **96**: 10379–84.

47 Waddington SN, Buckley SM, Nivsarkar M, Jezzard S, Schneider H, Dahse T, Kemball-Cook G, Miah M, Tucker N, Dallman MJ, Themis M, Coutelle C. *In utero* gene transfer of human factor IX to fetal mice can induce postnatal tolerance of the exogenous clotting factor. *Blood* 2003; **101**: 1359–66.

48 Kulkarni R, Lusher JM. Intracranial and extracranial hemorrhages in newborns with hemophilia: a review of the literature. *J Pediatr Hematol Oncol* 1999; **21**: 289–95.

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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

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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 $CD34^+$ ($CB\text{-}CD34^+$) 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).

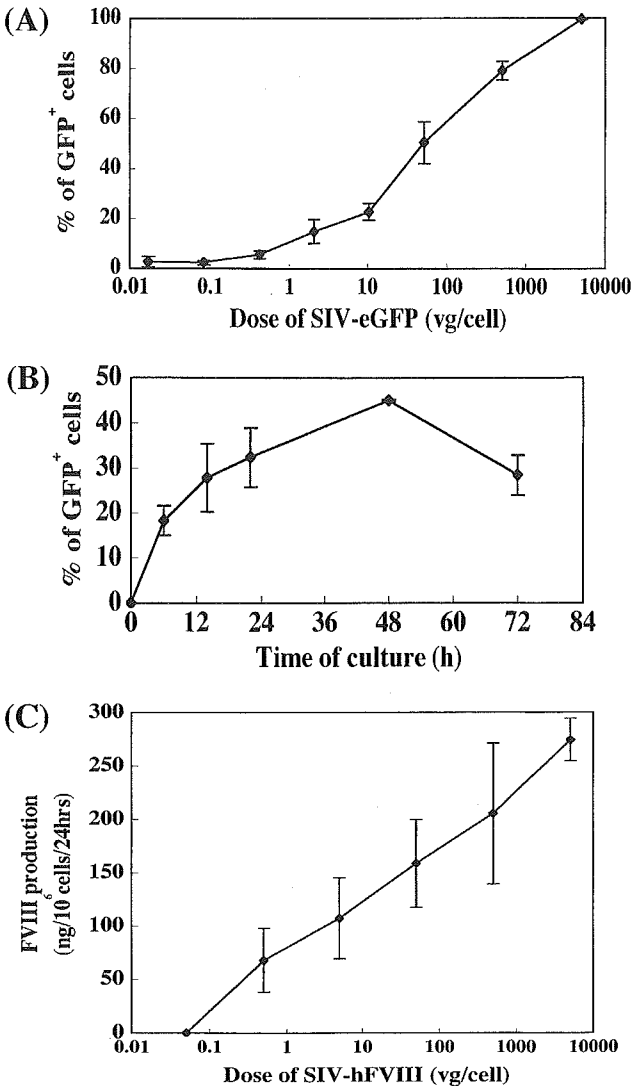

Figure 1. Transduction of CB-CD34$^+$ cells by SIVeGFP and SIVhFVIII. CB-CD34$^+$ cells (5×10^5 cells/mL) were incubated with increasing concentrations of SIVeGFP for 48 h (A) or with a fixed concentration (MOI, 50 vg/cell) of SIVeGFP for various times (B). After incubation, expression of eGFP was analyzed 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

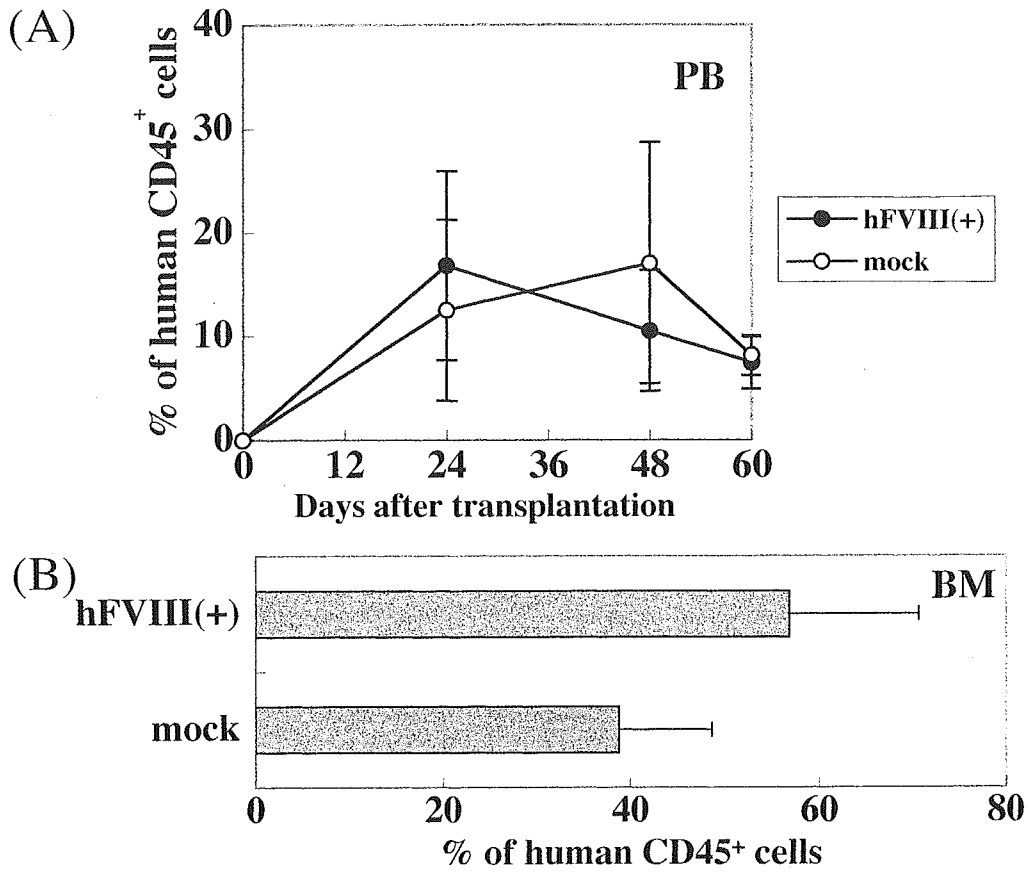

Figure 2. Presence of human CD45$^+$ cells in peripheral white blood and bone marrow cells of NOD/SCID mice after transplantation of human CB-CD34$^+$ cells. Peripheral blood was obtained from recipient mice on days 24, 48, and 60 after transplantation of CB-CD34$^+$ cells. White blood cells derived 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$^+$

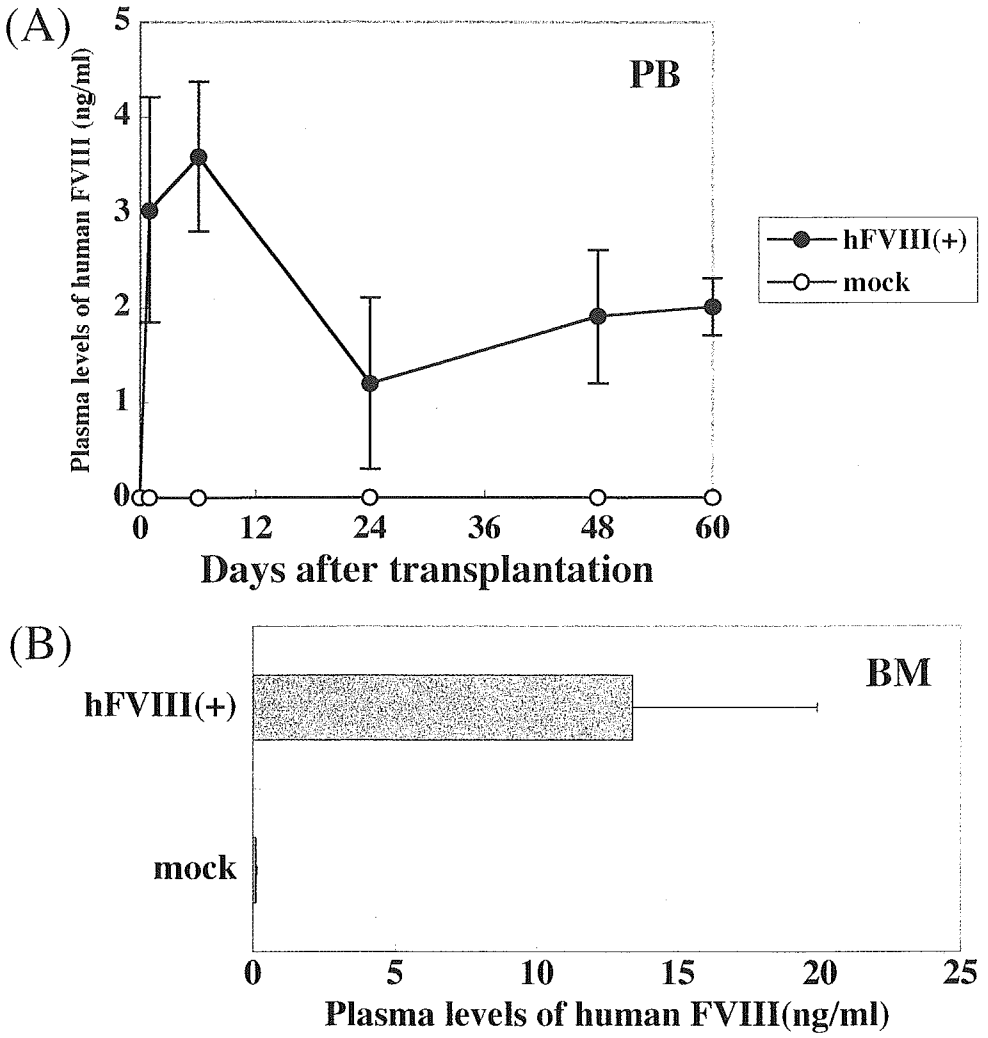

Figure 3. Plasma hFVIII levels in transplanted NOD/SCID mice. Peripheral blood was obtained from recipient mice on days 1, 6, 24, 48, and 60 after transplantation of CB-CD34$^+$ cells. Human FVIII concentrations in plasma (PB) of the NOD/SCID mice which received SIVhFVIII (closed circles) or mock (open 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).

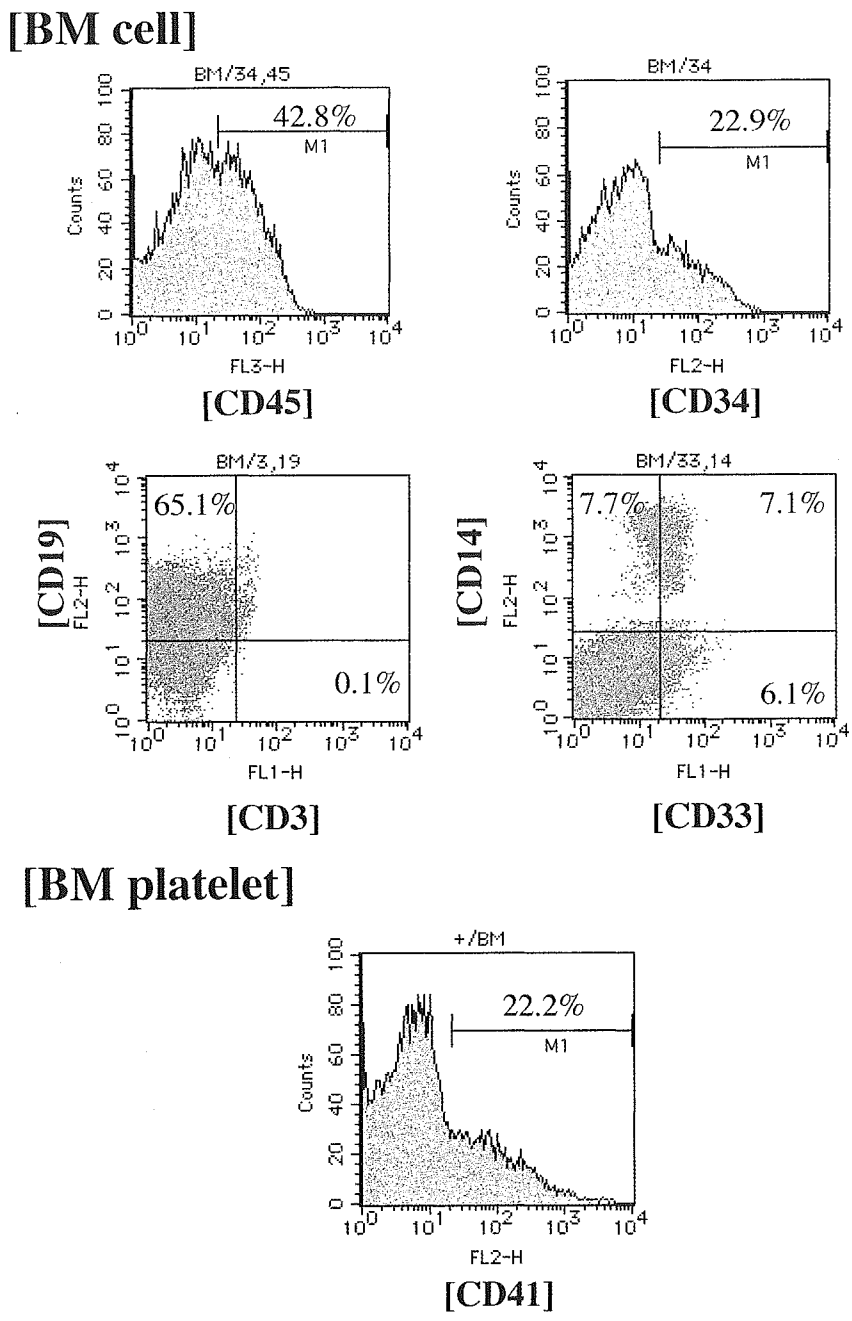

Figure 4. Expression of lineage markers in human CD45$^+$ cells and detection of human CD41$^+$ platelets in bone marrow cells. Expression of CD3, CD19, CD33, CD14, and CD34 in the human CD45$^+$ cells isolated from the bone marrow of a recipient mouse were analyzed by flow cytometry. Human platelets 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.

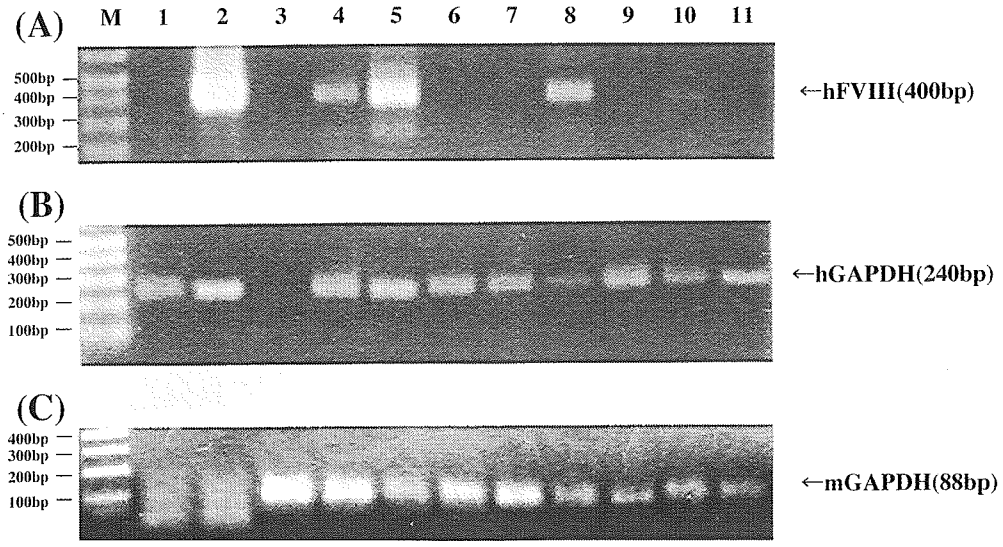

Figure 5. RT-PCR analysis of bone marrow cells. On day 60 after transplantation, RNA obtained from 5000 bone marrow cells was subjected to RT-PCR analyses with specific primer pairs for the human BDD-FVIII transcript (A), the human GAPDH transcript (B), and the mouse GAPDH transcript (C). PCR-amplified 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)

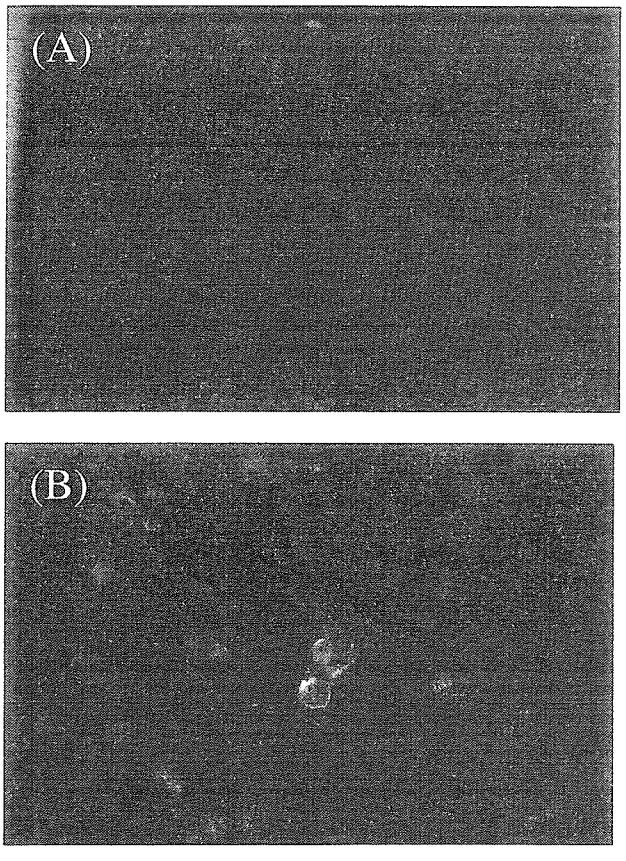

Figure 6. Immunofluorescent microscopy of hFVIII in bone marrow cells of the NOD/SCID mice. Bone marrow cells obtained from mock mice (A) or from recipient mice (B) were attached to glass slides using a Cytospin3. After fixation with 4% paraformaldehyde and washing with PBS, cells were incubated with 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.

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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.