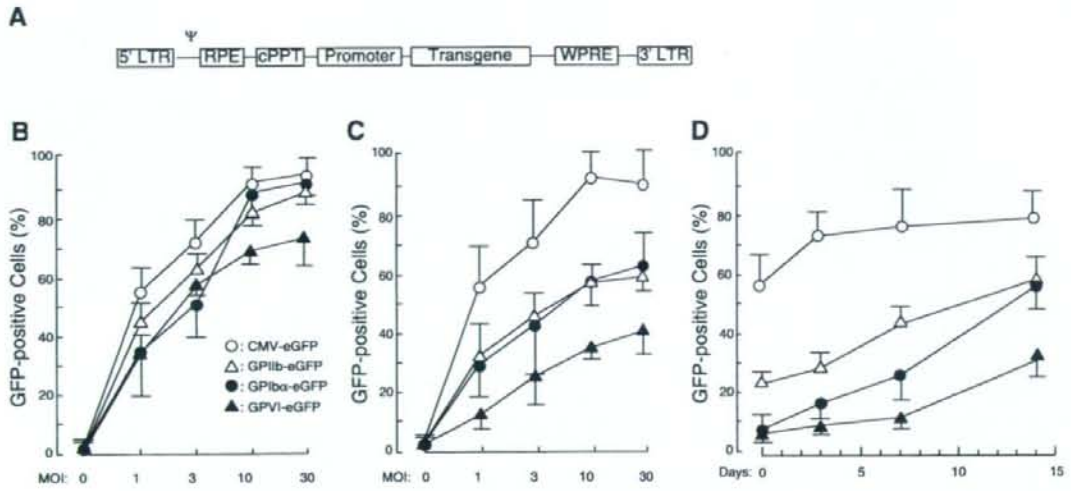

Figure 2. Expression of eGFP in UT-7/TPO cells and $CD34^+$-derived megakaryocytes transduced with SIV vectors carrying the *eGFP* gene driven by the CMV, GPIIb, GPVI, or GPIbα promoter. *A)* A schematic diagram of SIV constructs used in this study is shown. UT-7/TPO (*B*) and $CD34^+$-derived megakaryocytes (*C*) were infected with SIV-*CMV*-eGFP (open circle) SIV-GPIbα-eGFP (closed circle), SIV-GPIIb-eGFP (open triangle), or SIV-GPVI-eGFP (closed triangle) at indicated MOI. Expression of eGFP in cells was analyzed by flow cytometry. Columns and error bars are mean ± SD (n=3). *D)* $CD34^+$-derived megakaryocytes at day 0, 3, 7, and 14 after start of differentiation were transduced with SIV-*CMV*-eGFP (open circle) SIV-GPIbα-eGFP (closed circle), SIV-GPIIb-eGFP (open triangle), or SIV-GPVI-eGFP (closed triangle) at MOI of 30. Columns and error bars are mean ± SD (n=3).

earlier stage of megakaryopoiesis. Furthermore, the GPIbα compared with the GPIIb promoter seemed to work during a later phase of megakaryocyte maturation, and the percentages of eGFP expression did not change after differentiation (day 14 in Fig. 2*D*). eGFP expression driven by the CMV promoter was not affected by megakaryocyte differentiation (Fig. 2*D*). We selected the GPIbα promoter as the platelet-specific promoter for *in vivo* experiments because the promoter activity of GPIbα was the strongest in differentiated megakaryocytes (Fig. 1) and the promoter drove in the later phase of megakaryopoiesis (Fig. 2).

Establishment of efficient transduction of KSL cells with SIV vector

We next optimized the transduction protocol for KSL cells by using an SIV vector containing the *eGFP* gene driven by the CMV promoter. The transduction efficiency of eGFP in cultured KSL cells reached 60–80% (**Fig. 3*A***). The plateau value for transduction was observed with a MOI of 10–30. One day (24 h) after incubation with the viral vector was sufficient to achieve the efficient expression of the transduced gene (Fig. 3*B*). eGFP expression then gradually declined (Fig. 3*B*); the decrease might have been due to the reduction in cell viability, because PI-positive cells (dead cells) increased with time (data not shown). Although lentiviral vectors can express transgenes for long periods even in the absence of integration in $CD34^+$ cells (23), KSL cells could not maintain eGFP expression for long periods *in vitro*. Accordingly, we cultured KSL at an MOI of 30 for 24 h and transplanted the cells into recipient mice in the following experiments.

Preferential eGFP expression in platelets using SIV vectors harboring GPIbα promoter *in vivo*

To compare the strength and the specificity of the CMV and GPIbα promoters and to assess eGFP transduction by SIV vectors *in vivo*, KSL cells transduced with SIV-CMV-eGFP or SIV-GPIbα-eGFP were transplanted to recipient mice (Ly5.2). One hundred thousand cultured KSL cells (Ly5.1) transduced with SIV-CMV-eGFP or SIV-GPIbα-eGFP (MOI of 30) were transplanted together with 5×10^5 competitor cells (Ly5.2). When KSL cells transduced with SIV-CMV-eGFP were transplanted, eGFP expression was observed in 35–45% of $CD45^+$ cells and 7–11% of platelets in peripheral blood (**Fig. 4*A*** and ***B***). Interestingly, transduction of the SIV vector harboring the GPIbα promoter resulted in efficient gene marking to platelets (16–27%); however, only marginal eGFP expression was observed in $CD45^+$ and red blood cells (Fig. 4*A*, *B*). We next analyzed bone marrow cells from transplanted mice using specific markers to identify macrophages, granulocytes, B lymphocytes, T lymphocytes, and erythroblasts. Whereas eGFP was expressed in these lineages of cells of mice that received KSL cells transduced with SIV-CMV-eGFP, the GPIbα promoter drove just marginal eGFP expression in these cell lineages, confirming the specificity of its activity in megakaryocytes and platelets *in vivo* (Fig.

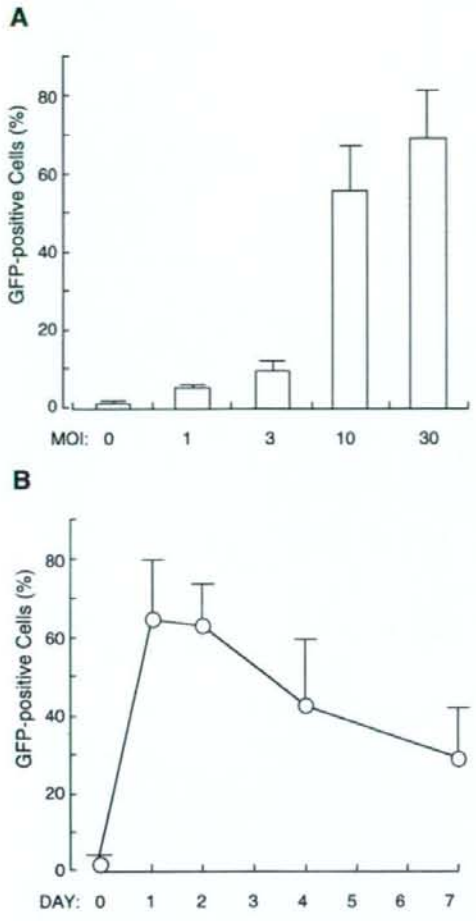

Figure 3. Transduction of KSL cells with SIV-*CMV*-eGFP. Cultured KSL cells were transfected with increasing concentrations of SIV-*CMV*-eGFP for 24 h (*A*) or with a fixed concentration (MOI=30) for various incubation times (*B*). After indicated incubations, expression of eGFP in KSL cells was analyzed by flow cytometry. Percentages of transduced cells expressing eGFP are shown. Columns and error bars are mean ± SD (n=3).

4*C*). We next performed a second bone marrow transplantation using marrow cells obtained from mice that had been transplanted 4 months earlier. As shown in Fig. 4*D*, eGFP expression driven by the CMV and GPIbα promoters in hematopoietic cells was sustained after the second stem cell transplantation, indicating that the promoter maintains transgene expression during differentiation of hematopoietic stem cells.

Expression of hFVIII and phenotypic correction in hemophilia A mice transplanted with KSL cells transduced with SIV-GPIbα-hFVIII

To determine whether platelet-directed gene therapy enables sustained expression of FVIII, we constructed two SIV-based vectors containing *hBDD-FVIII* cDNA under the control of either the CMV (SIV-CMV-hFVIII) or GPIbα promoter (SIV-GPIbα-hFVIII). We transplanted mice with 1×10^5 transduced KSL cells after lethal γ-irradiation. We first analyzed the presence of the *hFVIII* gene transcripts in organs of the transplanted recipients at 3 months after transplantation. Analyses for *hFVIII* transcripts driven by the CMV promoter revealed that the *hFVIII* gene was expressed mainly in bone marrow and to a lesser extent in the spleen (**Fig. 5*A***, *middle panel*). Interestingly, *hFVIII* mRNA was predominantly found in both the spleen and bone marrow in the recipients of KSL cells transduced with SIV-GPIbα-hFVIII (Fig. 5*A*, *lower panel*). To quantify the mRNA expression concentration in each organ, vector-specific WPRE expression was measured by real-time quantitative RT-PCR. As expected, based on the results from RT-PCR, bone marrow and spleen are the major sites for mice transplanted with KSL cells transduced with SIV vectors (Fig. 5*B*). Furthermore, we examined vector integration into the genome of bone marrow hematopoietic cells after transplantation and detected that 0.19–2.3 vector copies/genome were integrated in cells of transplanted mice (CMV promoter: 1.07±0.95; n=4; GPIbα promoter: 0.98±0.62; n=4). In accordance with the data on *hFVIII* transcripts, hFVIII molecules were immunohistochemically detected in bone marrow and the spleen in both types of transduced mice (**Fig. 6**). Interestingly, cells expressing GPIbα concurrently expressed hFVIII in bone marrow obtained from mice transduced with SIV-GPIbα-hFVIII (Fig. 6*A*).

Finally, we evaluated whether platelet-specific gene transduction using SIV-GPIbα-hFVIII resulted in phenotypic correction of FVIII-deficient hemophilia A mice. The plasma FVIII antigen concentration with or without platelet activation was measured in transplanted FVIII-deficient mice at 30 and 60 days after transplantation. We detected FVIII activity in the transplanted mice in which 1–2% correction was noted in the plasma of mice transplanted with KSL cells transduced with SIV-GPIbα-hFVIII (**Fig. 7*A***). When platelets were stimulated with collagen and PMA, the plasma FVIII concentration increased to 2–3.5% (Fig. 7*A*). The mortality rate after tail clipping was significantly improved in transduced mice (Fig. 7*B*). Furthermore, ectopically expressed hFVIII levels had not attenuated, and the appearance of inhibitor against hFVIII was not detected in mice transplanted with KSL cells transduced with SIV-GPIbα-hFVIII at day 60 after the transplantation (data not shown). We simultaneously performed transplantation experiments using SIV-*CMV*-hFVIII. Plasma levels of hemophilia A mice transplanted with KSL transduced with SIV-CMV-hFVIII were 3–6% after the transplantation, and phenotypic correction was also observed, as reported previously (19, 24, 25).

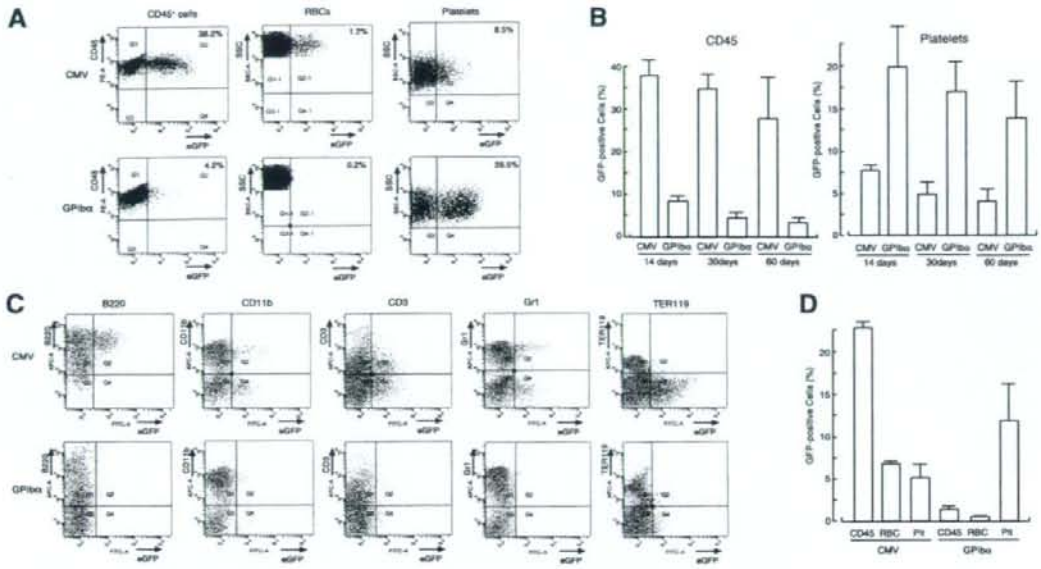

Figure 4. Effect of promoter differences on eGFP expression in blood cells *in vivo*. Cultured KSL cells were transduced with SIV-*CMV*-eGFP or SIV-GPIbα-eGFP at a MOI of 30. Each irradiated mouse received 100,000 transduced cells together with 5×10^5 unfractionated whole marrow cells. *A)* Representative flow cytometry analyses of eGFP-positive cells in $CD45^+$ lymphocytes and granulocytes, red blood cells (RBCs), and platelets in peripheral blood are shown. *B)* Percentages of eGFP-positive cells in $CD45^+$ cells (*left*) and platelets (*right*) 14, 30, and 60 days after transplantation are shown. Columns and error bars are mean ± SD (n=5 per group). *C)* 60 days post-transplantation, bone marrow cells were stained using antibodies to detect B lymphocytes (B220), T lymphocytes (CD3), granulocytes (Gr1), macrophages (CD11b), and erythroblasts (TER119). GFP-positive cells in each lineage cells are measured by flow cytometry. Data represent 3 experiments. *D)* Flow cytometric analyses of $CD45^+$ cells, RBCs, and platelets in peripheral blood obtained from mice 30 days after second bone marrow transplantation. Columns and error bars mean ± SD (n=5 per group).

DISCUSSION

In this study, we examined the gene transduction of platelets and megakaryocytes by using an SIV lentiviral vector harboring a platelet-specific promoter *in vivo*. Since the strategy of using platelets as potential targets for producers of transgene products has already been proposed in transgenic mice (3, 4), it was shown that it is possible to apply this strategy to correct hemorrhagic disorders including hemophilia by efficient platelet-directed gene transduction *in vivo*. However, detailed comparisons of platelet-specific promoters and the efficiency of the transduction of transgenes *in vivo* have not previously been reported. In our system, the transduction of hematopoietic stem cells with an SIV lentiviral vector resulted in the expression of the transgene

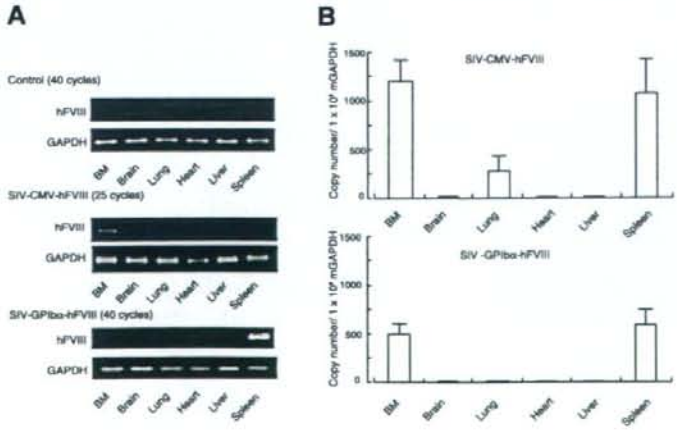

Figure 5. Expression of hFVIII in organs obtained from mice transplanted with hFVIII-transduced KSL cells. *A)* KSL cells not transduced (control) or transduced with SIV-*CMV*-hFVIII or SIV-GPIbα-hFVIII vector were injected into lethally irradiated CL57/B6 mice as described in Materials and Methods. RT-PCR analyses for the transcripts derived from the *hFVIII* gene in the indicated organs are shown. For control, RT-PCR for mouse GAPDH of RNA was performed simultaneously. Data represent 4 experiments. *B)* mRNA expression derived from SIV vectors was quantified by real time quantitative RT-PCR as described in Materials and Methods. Columns and error bars are mean ± SD (n=4 per group).

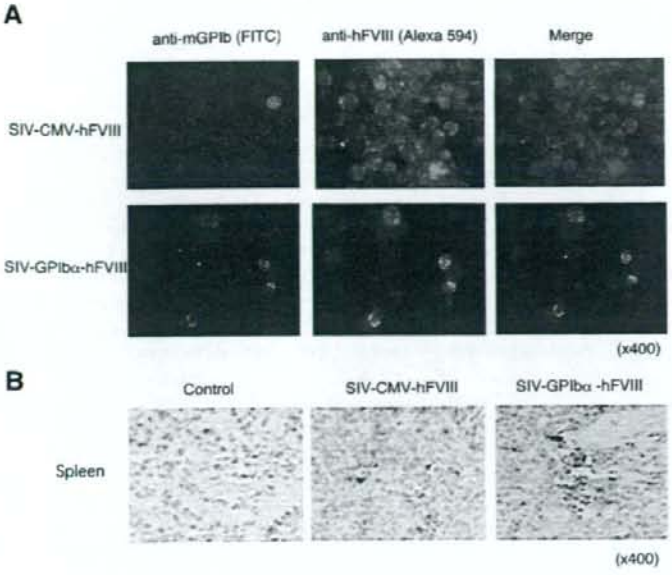

Figure 6. Expression of hFVIII in bone marrow and spleen in transplanted mice. *A)* Isolated bone marrow cells from mice transplanted with KSL cells transduced with SIV-*CMV*-hFVIII or SIV-GPIbα-hFVIII were immunostained for mouse GPIbα (*left*) and hFVIII (*middle*); 2 images are overlapped in right column, showing that in SIV-GPIbα-hFVIII-transduced bone marrow cells, GPIbα and FVIII expression overlapped. *B)* Immunohistochemistry for hFVIII in spleen of each transplanted mice (positive stain: brown). For control, sections of spleen obtained from mice transplanted with KSL cells without vector infection were processed simultaneously with anti-FVIII antibodies. Original magnification ×400.

in ≈20% of platelets and also resulted in a phenotypic correction of hemophilia A mice, suggesting that platelet-targeting gene therapy has the potential for further clinical applications. This is a first study to achieve a phenotypic correction of a coagulation abnormality such as hemophilia A by using platelet-directed gene transduction.

Megakaryocytes have a finite life span of ≈10–21 days (26); therefore, hematopoietic stem cells are a more practical target than megakaryocytes for genetic transfer to establish long-term expression of a target protein in platelets. Because lentiviruses are capable of infecting certain types of quiescent cells, there has been significant interest in the application of lentivirus-derived vectors to the transduction of hematopoietic cells; indeed, it has been shown that lentiviral vectors can efficiently transduce hematopoietic stem cells (27). We used the SIV lentiviral system for efficient platelet-targeting gene transduction because of its probable safety. The SIV lentiviral system was derived from SIVagmTYO1 and is nonpathogenic to its natural host and to experimentally infected Asian macaques (16). Replication-competent virus particles were not detected in vector-infected cells, and the risk of development of replication-competent lentivirus particles in HIV carrier patients may be significantly lower than that for the HIV-based vectors (19). Accordingly, SIV vectors have a safety advantage for clinical applications of gene therapy.

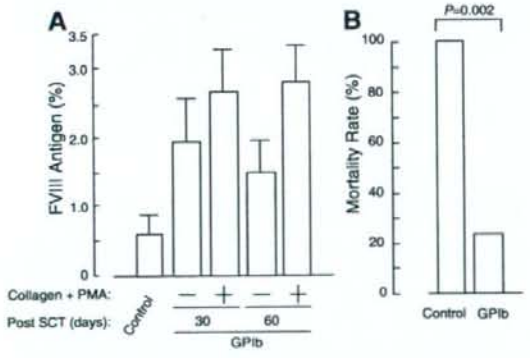

Figure 7. Phenotypic correction of hemophilia A mice by platelet-targeting gene delivery. *A)* Blood from FVIII-deficient mice transplanted with KSL cells transduced with SIV-GPIbα-hFVIII was stimulated without or with 50 μg/ml of collagen and 1 μM PMA for 15 min. After centrifugation, platelet-poor plasma was obtained, and hFVIII antigen levels were measured by ELISA. Columns and error bars are mean ± SD (n=4 per group). *B)* Mortality rate within 24 h after tail clipping in mice transplanted with KSL cells transduced with control or SIV-GPIbα-hFVIII (n=10 for control; n=8 for GPIbα). Mortality rate was statistically evaluated by a χ^2 test.

Most reported studies have used the GPIIb promoter for megakaryocyte- and platelet-specific gene transduction. We used the GPIbα promoter as a platelet-specific promoter in this study because the promoter activity of GPIbα was more potent than that of GPIIb in UT-7/TPO and $CD34^+$-derived megakaryocytes. Another reason we selected this platelet-specific promoter was that the GPIbα promoter works at a late stage of megakaryopoiesis. Although the *GPIIb* gene is expressed in platelets and megakaryocytes, it is an early gene for megakaryopoiesis (28). In conditional knockout mice in which the thymidine kinase gene was driven by the GPIIb promoter, the administration of gancyclovir led to a dramatic reduction in the platelet count (29). In bone marrow, erythroid and myeloid progenitors were also affected, which indicated the presence of *GPIIb* in progenitor cells (29). Indeed, 18% of human $CD34^+$

hematopoietic stem cells already expressed GPIIb, and so the appearance of GPIb was markedly delayed as compared with that of GPIIb, indicating that GPIb is a later marker of megakaryocytic maturation. Platelet-targeting gene therapy using the GPIbα promoter was therefore expected to allow more specific and restricted expression of gene products in platelets than that using the GPIIb promoter.

Another important finding here was that the eGFP gene driven by the CMV promoter showed significantly decreased expression in platelets, despite the high transduction efficiencies of $CD45^+$ cells *in vivo*. Generally, the reduction of transgene expression caused by a shortened protein half-life is even more pronounced in terminally differentiated blood cells (30). The decreased expression might have been mediated by the down-regulation of the transgene during differentiation; the stability of the encoded protein is at least as relevant for the expression of a transgene as the choice of the promoter or *cis*-elements influencing RNA processing in differentiated cells (30). In this context, the use of the GPIbα promoter, which drives expression in late megakaryocyte differentiation, might be important for gene transduction of terminally differentiated anucleate platelets.

Our strategy of platelet-directed gene transduction has potential for not only inherited platelet disorders (such as Glanzmann's thrombasthenia and Bernard-Sourlier syndrome) but also other hemorrhagic disorders. Hemophilia A is an X chromosome-linked bleeding disorder caused by defects in the *FVIII* gene and affecting ≈1:5000 males (31). Hemophilia is considered suitable for gene therapy because it is caused by a single gene abnormality and therapeutic coagulation factor levels may well vary over in a broad range (5–100%; ref 31). Although sustained therapeutic expression of FVIII has been achieved in preclinical studies using a wide range of gene transfer technologies targeted at different tissues (32), emergence of neutralizing Ab often limits their clinical applications (33). The targeting of hematopoietic stem cells is not an exception. Although lentiviral FVIII gene transduction of hematopoietic stem cells is able to produce therapeutic levels of FVIII (19, 24, 25, 34), the emergence of neutralizing antibodies to FVIII has resulted in decreased levels of FVIII activity (34). Platelet-directed gene therapy for hemophilia A has a possible advantage for therapeutic applications, because the use of the platelet-specific system may limit the development of inhibitors by preventing the expression of FVIII in antigen presenting cells. Furthermore, 10–30% of populations with hemophilia A develop inhibitors to infusion products, which leads to the disruption of coagulation and severe bleeding (31). Under these conditions, platelet-directed gene therapy of hemophilia A is very attractive because platelets could specifically store the protein in the bloodstream and then specifically release it at sites of thrombus formation, thereby minimizing the influence of any circulating inhibitors. For further clinical application, the long-term observations are required to substantiate long-term *in vivo* gene expression because our observation periods were limited in this study.

During the course of this study, the therapeutic expression of GPIIb/IIIa in GPIIIa-deficient mice using HIV-lentivirus vector containing GPIIIa cDNA under the control of the GPIIb promoter was reported (35). That study used a heterogeneous population of bone marrow cells as a source for stem cell transplantation and gene transduction. We demonstrated efficient transduction of KSL murine hematopoietic cells by a SIV vector harboring the GPIbα promoter and phenotypic correction of hemophilia A mice. Primitive KSL cells are a nearly homogeneous population, and a single KSL cell frequently can provide long-term multilineage engraftment of lethally irradiated mice (36). Targeting of primitive hematopoietic stem cells is thought to be a safer approach, because the number of transduced cells needed for reconstitution is much lower than that needed when using a heterogeneous bone marrow population. The development of leukemia in two children with severe combined immunodeficiency disease who were transplanted with retroviral vector-transduced bone marrow cells caused renewed concern about the risks associated with the integration of proviral sequences into chromosomal DNA (37). One way to possibly reduce the risks of insertional mutagenesis would be to use transduction protocols that minimize the total number of genetically modified cells (38). From this aspect, our procedure using KSL cells transduced with SIV lentiviral system is a practical approach for platelet-specific gene modification in clinical applications.

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REGULAR ARTICLE

Phenotype correction of hemophilia A mice with adeno-associated virus vectors carrying the B domain-deleted canine factor VIII gene☆

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Abstract Adeno-associated virus (AAV) vectors carrying the B domain-deleted canine FVIII (BDD cFVIII) gene utilizing the β-actin minimum promoter (167b) pseudotyped with serotype 1 (AAV1-β-actin-cFVIII) and serotype 8 (AAV8-β-actin-cFVIII) were developed to express cFVIII in hemophilia A mice. FVIII clotting activities measured by the APTT method increased in hemophilia A mice with intramuscular injection of AAV1-β-actin-cFVIII in a dose-dependent manner. Therapeutic FVIII levels (2.9 ± 1.0%) in hemophilia A mice with the AAV1-β-actin-cFVIII dose of 1×10^{12} gc/body were achieved, suggesting partial correction of the phenotype with AAV1-β-actin-cFVIII vectors. FVIII clotting activity levels in hemophilia A mice with intravenous injection of AAV8-β-actin-cFVIII also were increased dose-dependently, achieving therapeutic FVIII levels (5–90%) in hemophilia A mice with the AAV8-β-actin-cFVIII doses of $1–3 \times 10^{11}$ gc/body and supernormal FVIII levels (180–670%) in hemophilia A mice with the AAV8-β-actin-cFVIII dose of 1×10^{12} gc/body. Transduction of the liver with AAV8-β-actin-cFVIII is superior to transduction of skeletal

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muscles with AAV1cFVIII regarding the FVIII production and antibody formation. These data suggested that both AAV1 and AAV8 vectors carrying the FVIII gene utilizing a minimum promoter have a potential for hemophilia A gene therapy.

Introduction

Hemophilia A is an inherited X-linked life-threatening bleeding disorder caused by abnormalities in the factor VIII (FVIII) gene that lead to deficiency of FVIII and bleeding diathesis. Hemophilia is considered suitable for gene therapy because it is caused by a single gene abnormality and therapeutic coagulation factor levels may well be in a wide range (5–100%) without strict gene regulation [1–3]. Gene therapy is expected to provide an alternative to current FVIII supplemental therapy because it may be able to prevent lethal intracranial bleeding episodes and provide a good quality of life without bleeding. Among a variety of vectors, Adeno-associated virus (AAV) vectors are thought to be ideal for transfer of therapeutic genes since they are derived from non-pathogenic viruses and have been demonstrated to provide sustained transgene expression in non-dividing cells with little toxicity, although delivery of the FVIII gene using AAV vectors is limited by their small packaging capacity [4].

The dual AAV vector system that utilized two AAV2 vectors separately carrying the FVIII heavy chain gene and the FVIII light chain gene was successful for expressing functional FVIII molecules and correction of phenotypes of hemophilia A mice [5]. A recent report has shown that construction of single AAV vectors carrying the 4.5-kb B domain-deleted (BDD) canine FVIII (cFVIII) gene can be packaged in AAV vectors using the 543-base (b) DNA fragments composed of the insulin-like growth factor binding protein (IGBP) promoter, an enhancer element, and an intron, although the packaging efficiency for incorporation of the cFVIII gene into the AAV vectors was low [6].

The liver would appear to be the appropriate target organ for transduction because FVIII is physiologically synthesized in this organ. However, if any adverse reaction to the therapy occurs, removal of the liver would be an unacceptable solution. In fact, minor liver dysfunction upon AAV2 vector injection into the hepatic artery was reported in clinical trials for hemophilia B gene therapy, whereas no liver dysfunction was observed upon injection of the same vector into the skeletal muscles in the same series of clinical trials [7,8]. Although the precise mechanisms of these phenomena have not yet been elucidated, the T cell response to viral capsid was thought to be one of the causes of the liver injuries [8]. In this respect, surgically removable organs such as skeletal muscles may well be the alternative target organs.

To explore the possibility that skeletal muscles transduced with AAV vectors could produce FVIII resulting in increase of FVIII levels in the circulation, we packaged the BDD FVIII gene in AAV vectors using the same promoter and compare production of FVIII in the skeletal muscles and in the liver that were transduced with AAV vectors. We developed AAV vectors carrying the BDD cFVIII gene utilizing the β-actin minimum promoter (167b) and tried to express canine FVIII in hemophilia A mice. Recent studies on recombinant AAV vectors have shown that AAV serotypes have tropism, suggesting that a specific AAV serotype vector can be used for gene delivery to certain organs [3,6,9]. AAV serotype 1 (AAV1) may be the best AAV serotype for transduction of skeletal muscles and the AAV serotype 8 (AAV8) is superior to other AAV serotypes for transduction of the liver [6,9], thus in this study, we constructed AAV1 and AAV8 vectors carrying the BDD cFVIII gene and studied efficacies of these vectors for FVIII transgene expression, long term transgene expression, and neutralizing antibody formation to the transgene products in the hemophilia A mice.

Materials and methods

Vector construction

The full-length human FVIII (hFVIII) cDNA was a generous gift from Dr. J.A. van Mourik (Blood Coagulation, Sanquin, Amsterdam, Netherlands) and the human B domain deleted (BDD) FVIII (hFVIII) cDNA was generated by PCR-based mutagenesis as described [10,11]. The canine FVIII (cFVIII) cDNA was a generous gift from Dr. Brownlee (Chemical Pathology Unit, University of Oxford, UK) and the BDD canFVIII cDNA also was generated by PCR-based mutagenesis. The intervening amino acid sequence of the heavy chain and the light chain of BDD cFVIII was $RSFS^{743}$-Q^{1630}NPPVSK. The CAG promoter is a chimeric promoter, composed of the CMV enhancer, the chicken β-actin promoter,

and an intron, was derived from pCAGGS [12]. The chicken β-actin minimum promoter (−155-+12, 167 b) was generated by PCR, cloned in pCR2.1 TOPO (Invitrogen), and sequenced. Plasmid vector p1.1c, composed of the CMV promoter, human growth hormone gene intron 1, and the SV40 polyadenylation signal sequences, was kindly supplied by Avigen Inc. The DNA fragments spanning the CMV promoter and the human growth hormone intron of p1.1c were replaced with the CAG promoter, the phosphoglycerokinase 1 (PGK1) promoter, or the β-actin minimum promoter DNA fragments to make plasmid p1.1CAG, p1.1PGK1, or p1.1β-actin, respectively. The DNA fragments encoding the BDD hFVIII cDNA or the BDD cFVIII cDNA were cloned in the downstream of the respective promoter sequences of these plasmids to make p1.1CMV-hFVIII, p1.1CAG-hFVIII, p1.1PGK1-hFVIII, p1.1β-actin-hFVIII, and p1.1β-actin-cFVIII, respectively. The Lac Z gene was cloned in the downstream of the β-actin promoter to make plasmid p1.1β-actin Lac Z. DNA fragments spanning the promoter, the LacZ gene, and the poly adenylation signal sequence of pAAV2 Lac Z (Stratagene) were replaced with DNA fragments spanning the β-actin promoter, the BDD cFVIII cDNA, and the SV40 poly adenylation signal sequences of p1.1β-actin-cFVIII to make the gene transfer vector pAAV2-β-actin-cFVIII in which these DNA fragments were flanked by ITR sequences of AAV serotype 2 (AAV2) as described previously [9,13]. The gene transfer vector pAAV2-β-actin-Lac Z equipped with AAV2 ITRs was also constructed. The chimeric packaging plasmid for AAV8 capsid pseudotyping was a generous gift from Dr. James M. Wilson (Division of Medical Genetics, Department of Medicine, University of Pennsylvania, Philadelphia, PA) [6]. The packaging plasmid composed of the AAV2 rep gene and the cap gene derived from AAV1 for AAV1 capsid pseudotyping was described previously [9].

AAV vector production

Viral vectors were packaged with AAV1 or AAV8 capsid by pseudotyping. The FVIII gene or the Lac Z gene located in the downstream of the β-actin minimum promoter and flanked by AAV2 ITRs was packaged by triple plasmid transfection of human embryonic kidney 293 (HEK 293) cells, kindly supplied by Avigen Inc., with the chimeric packaging plasmid, the adenovirus helper plasmid pHelper (Strategene, La Jolla, CA), and gene transfer plasmid vectors (pAAV2-β-actin-cFVIII or pAAV2-β-actin-Lac Z) as described previously [9,13]. For virus vector purification, the DNAse (Benzonase, Merck Japan, Tokyo, Japan)-treated virus particle containing samples were subjected to two rounds of iodixyanol-density gradient ultracentrifugation in HEPES-buffered saline (pH 7.4) in the presence of 25 mM EDTA at 21 C as described [9]. Titration of recombinant AAV vectors was carried out by quantitative dot-blot hybridization using the ^{32}P-labeled probes [9,13].

Analysis of the ß-actin minimum promoter activity

Expression of hFVIII in HEK293 cells transfected with plasmid vectors p1.1β-actin-hFVIII, p1.1CMV-hFVIII, p1.1CAG-hFVIII, and p1.1PGK1-hFVIII by the calcium phosphate coprecipitation method was studied to show that the β-actin minimum promoter had an enough FVIII expression activity. After incubation with the DNA containing media for 6 h, HEK 293 cells were incubated further in DMEM/HAM F-12 media supplemented with 10% fetal bovine serum for 48 h at 37 C in the presence of 5% CO_2. FVIII clotting activities in the conditioned media of HEK 293 cells harvested after 48 h incubation were quantified by the activated partial thromboplastin time (APTT) method using FVIII deficient plasma. FVIII activities were expressed as the percentages of normal control plasma.

Animal experiments

FVIII-deficient mice (Hemophilia A mice) with targeted destruction of exon 16 of the FVIII gene were previously reported by Bi et al. [14] and generously given to us by Dr. H. H. Kazazian Jr. (University of Pennsylvania, Philadelphia, PA). J1 ES cells were used for targeted destruction of the FVIII gene and blastcysts derived from C57BL/6 mice were used to generate chimaeras [14]. C57BL/6 wild-type mice were purchased from SLC Inc. Mice were maintained in a standard lighting condition in a clean room. All surgical procedures were carried out in accordance with guidelines approved by the institutional Animal Care and Concern Committee at Jichi Medical School [15]. Male hemophilia A mice and male wild-type C57BL/6 mice were used in the experiments. Blood was drawn from the cervical vein plexus of mice and mixed with 1 / 10 volume of 3.8% sodium citrate, and then platelet-poor plasma was prepared by centrifugation. AAV8 vectors were injected intravenously into the cervical vein plexus while AAV1 vectors were injected directly to the skeletal muscles of lower extremities of mice under anesthesia with isoflurene [15]. Cyclophosphamide (100 μg/body/day, SIGMA-ALDRICH Japan, Tokyo, Japan) and tachrolimus (12.5 μg/body/day, Fujisawa Pharmaceuticals Co., Tokyo, Japan) were

given (s.c.) to mice daily after vector injection as the immunosuppressant.

Analysis of cFVIII expression in mice

FVIII activities were measured by the activated partial thromboplastin time (APTT) method used for determination of plasma FVIII activities of hemophilia patients utilizing human FVIII-deficient plasma as described [15]. Antigen levels of cFVIII in mouse plasma were determined by ELISA (Asserachrom FVIIIC: Ag, Diagnostica Stago, Parsipanny, NJ) as described [6]. Analyses of neutralizing antibodies against cFVIII developed in mice were performed by the Bethesda method as described using FVIII deficient plasma and normal canine plasma. Detection of the transcripts of cFVIII transgene was performed by RT-PCR [10,11]. RNA was isolated from the mouse organs using an RNA isolation kit (RNeasy Protect kit; Qiagen Inc., Valencia, CA). DNAse I (Amplification grade, Invitrogen, Carlsbad, CA)-treated and heat-treated RNA samples were subjected to RT-PCR using a pair of primers (sense: 5'-GTTGGAGCACAAACTGACTTCC-3', antisense: 5'-CAATTGAGAAGGTGTCATCATACTC-3') for cFVIII and an RT-PCR kit (SuperScript One-Step RT-PCR System, Invitrogen). PCR amplification (25–30 cycles) for cFVIII was performed as described [10,11]. A primer pair for mouse GAPDH mRNA (R&D Systems, Inc., Minneapolis, MN) was used instead of cFVIII primers in the control RT-PCR experiments. For detection of cFVIII molecules in mouse tissues by immunohistochemistry, the skeletal muscles and the liver were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h at 4 C, incubated with PBS containing sucrose (10–30%), and then frozen in the presence of OCT compound in dry ice/ethanol. Sections were prepared from frozen tissues at −25 C and attached to poly-lysine coated glass slides. For detection of FVIII, tissue sections were blocked with 1% rabbit serum in PBS containing Triton-X 100 (0.1%) and incubated with sheep polyclonal anti-human FVIII antibodies (Cedarlane Laboratories Ltd, Homby, Ontario, Canada) at 4 C for 16 h [10,11]. After washing in PBS, sections were incubated with biotin-conjugated rabbit anti-sheep IgG antibody followed by the ABC reagents (Vectastain ABC Elite kit; Vector, Burlingame, CA) and a DAB kit (Vector).

Detection of ß-galactosidase in mouse tissues

To analyze Lac Z gene expression in mice injected with AAV-β-actin-Lac Z, mouse tissues were fixed with 2% paraformaldehyde in PBS for 5 min, washed with PBS, incubated with PBS containing sucrose (10–30%), and frozen with OTC compound (Tissue-Tek; Miles, Inc., Elkhart, IN) in dry ice/ethanol. Sections, prepared from frozen tissues at −25 C and attached to poly-lysine coated glass slides, were incubated in PBS containing 1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 0.01% Na deoxycholate, 0.1% Triton X-100 at 25 C for 1 h [10]. Some sections further were processed for the Feulgen reaction (red purple) to visualize nuclei.

Results

Expression of FVIII by the ß-actin minimum promoter

We studied expression of hFVIII in HEK293 cells transfected with plasmid vectors p1.1β-actin-hFVIII, p1.1CMV-hFVIII, p1.1CAG-hFVIII, and p1.1PGK1-hFVIII. FVIII clotting activities detected in the conditioned medium of HEK293 cells are shown in Fig. 1. Expression of FVIII driven by the β-actin minimum promoter (167 b) in the 293 cells was approximately 1/3–1/2 of that by the CMV promoter (1 kb) or the CAG promoter (1.7 kb). Although the β-actin minimum promoter is weaker than the CMV promoter and the CAG promoter, it

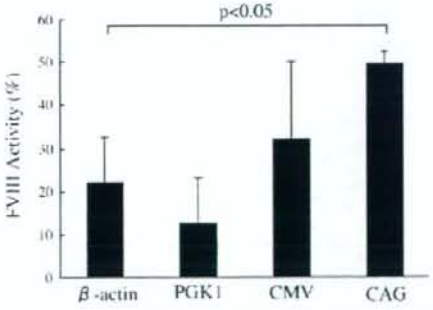

Figure 1 FVIII expression by the β-actin minimum promoter in vitro. FVIII clotting activities expressed in the conditioned media of HEK 293 cells transfected with p1.1CMV-FVIII, p1.1 CAG-FVIII, p1.1 PGK1-FVIII, or p1.1 β-actin-FVIII after 48 h incubation are shown. FVIII clotting activities in the conditioned media were quantified by the APTT method using FVIII deficient plasma and FVIII clotting activities were expressed as the percentages of normal control plasma. There were no FVIII activities in the conditioned media of HEK 293 cells with mock transfection. There was a significant difference of FVIII activity levels in the conditioned media of HEK 293 cells transfected with p1.1 CAG-FVIII and those in the conditioned media of HEK 293 cells transfected with p1.1 β-actin-FVIII ($n=4$, Student's t-test, $p<0.05$).

was stronger than the PGK1 promoter (515 b) by 1.2-fold in terms of FVIII expression activity. Since the β-actin minimum promoter was stronger than the PGK1 promoter and was short enough to construct 5.1-kb AAV vectors carrying the BDD FVIII cDNA, we used the β-actin minimum promoter to produce AAV vectors carrying the BDD FVIII gene.

Expression of Lac Z gene by the ß-actin minimum promoter in vivo

To confirm that the β-actin minimum promoter can express a transgene in vivo, AAV vectors carrying the Lac Z gene located in the downstream of the β-actin minimum promoter (AAV1-β-actin-Lac Z, AAV8-β-actin-LacZ) were injected to wild-type mice and expression of the Lac Z gene was studied by X-gal staining. When AAV1-β-actin-Lac Z was injected to the skeletal muscles of lower extremities of wild-type mice, Lac Z gene expression was observed in muscle fibers as shown in Fig. 2A. No apparent Lac Z gene expression was observed in other organs in the AAV1-β-actin-Lac Z injected mice (not shown), suggesting that transgene expression in other organs was minimum. Lac Z gene expression of mice with intravenous injection of AAV8-β-actin-Lac Z mainly was observed in the liver as shown in Fig. 2B. Lac Z gene expression also was observed in other organs including the heart, lung, and skeletal muscles in accordance with the previous report [16]. The liver could be transduced with intravenously injected AAV8-β-actin-Lac Z almost as efficiently as intraportally injected vectors (not shown).

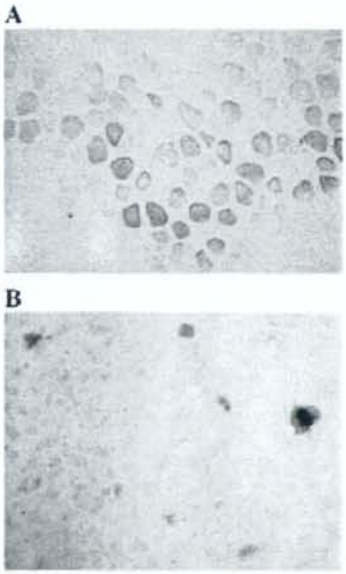

Figure 2 Expression of the Lac Z gene in mice transduced with AAV vectors carrying the Lac Z gene located downstream of the β-actin minimum promoter. X-gal staining of the skeletal muscles of mice with intramuscular injection of AAV1-β-actin-Lac Z (A) and of the liver of mice with intravenous injection of AAV8-β-actin-Lac Z (B) is shown.

Expression of FVIII by AAV vectors carrying the BDD cFVIII gene

AAV1-β-actin-FVIII vectors were injected into skeletal muscles of hemophilia A mice and AAV8-β-actin-FVIII vectors were intravenously injected into the cervical vein plexus of hemophilia A mice. FVIII clotting activities of citrated plasma drawn from mice were measured by the APTT method using FVIII-deficient human plasma.

FVIII clotting activities in mouse plasma increased on days 14 and 28 after AAV1 vector injection. The increase of FVIII clotting activities on day 28 after injection was dose-dependent. The FVIII activity levels in peripheral blood increased to $2.9 \pm 1.0\%$ in hemophilia A mice with the AAV1-β-actin-cFVIII dose of 1×10^{12} gc/body (Fig. 3), suggesting partial correction of the phenotype with AAV1-β-actin-cFVIII vectors. After these periods, FVIII activities decreased to the basal levels of mice before vector injection. FVIII antigen levels increased in parallel with levels of FVIII activity, confirming expression of cFVIII transgene in mice (not shown). Analyses for antibody against transgene products showed that neutralizing antibodies developed in 4 out of 6 tested mice by week 12 after vector injection, although the antibody titers were not high (Table 1). The RT-PCR analysis and the immunohistochemistry study suggested the presence of the transgene transcripts and products in the vector-injected muscles, suggesting that decrease of FVIII levels may be accounted for by the presence of neutralizing antibody to cFVIII.

FVIII clotting activity levels in hemophilia A mice with intravenous injection of AAV8-β-actin-cFVIII also were increased dose-dependently on day 28, achieving therapeutic FVIII levels (5–90%) in hemophilia A mice with the AAV8-β-actin-cFVIII doses of $1-3 \times 10^{11}$ gc/body and supernormal FVIII levels (180–670%) were achieved in hemophilia A mice with the AAV8-β-actin-cFVIII dose of 1×10^{12} gc/body (Fig. 4). These data on AAV8 vector-transduced FVIII expression were almost comparable with the results of the previous study using the single AAV8 vector carrying the BDD cFVIII gene [6], suggesting that β-actin minimum promoter almost

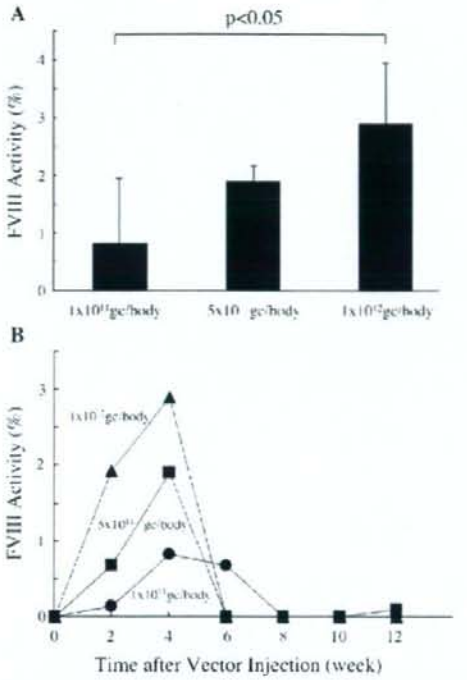

Figure 3 FVIII levels in plasma of hemophilia A mice after intramuscular injection of AAV1-β-actin-cFVIII. FVIII clotting activity levels expressed in plasma of hemophilia A mice ($n=4$) on day 28 after intramuscular injection of AAV1-β-actin-cFVIII are shown in panel A. Activity levels of cFVIII in peripheral blood of hemophilia A mice with injection of AAV1-β-actin-cFVIII (circles, 1×10^{11} gc/body; squares, 5×10^{11} gc/body; triangles, 1×10^{12} gc/body) are shown in panel B.

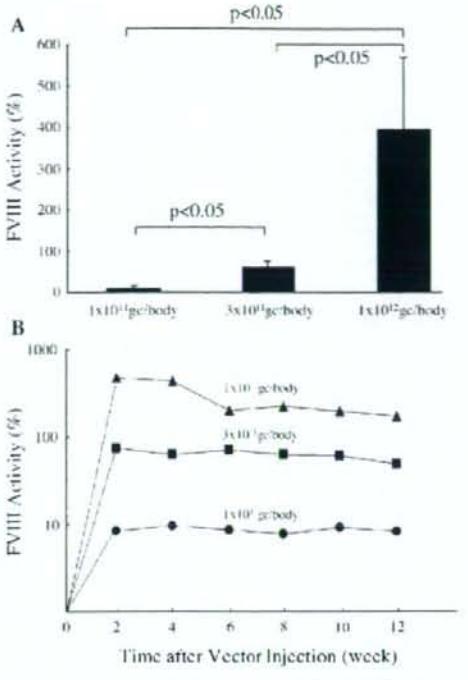

Figure 4 FVIII levels in plasma of hemophilia A mice after intravenous injection of AAV8-β-actin-cFVIII. FVIII clotting activity levels expressed in plasma of hemophilia A mice ($n=4$, each group) on day 28 after intravenous injection of AAV8-β-actin-cFVIII are shown in panel A. Activity levels of cFVIII in peripheral blood of hemophilia A mice ($n=4$, each group) with injection of AAV8-β-actin-cFVIII (circles, 1×10^{11} gc/body; squares, 3×10^{11} gc/body; triangles, 1×10^{12} gc/body) are shown in panel B.

worked as efficiently as the chimeric IGBP promoter complexes. High-level expression of FVIII in the vector-injected hemophilia A mice was sustained for more than 12 weeks. No apparent neutralizing antibody developed during the 12-week period after vector injection (Table 1). FVIII antigen levels also increased in parallel with FVIII activity levels, confirming expression of the cFVIII transgene in mice (not shown). The antigen levels of cFVIII determined by the ELISA for human FVIII were approximately 1/5 of the FVIII activity levels determined by the APTT method. Analyses for cFVIII transcripts suggested that the cFVIII gene mainly was expressed in the liver (Fig. 5) together

Table 1 Neutralizing antibodies against cFVIII developed in hemophilia A mice

	Inhibitor positive mouse	Bethesda units/mL
AAV1cFVIII	4/6 (66.7%)	9.4 ± 9.5[a]
AAV8cFVIII	0/9 (0%)	Not detected

[a] Neutralizing antibodies detected by week 12 after vector injection.

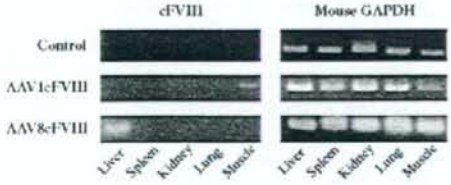

Figure 5 Analysis for cFVIII transcripts in mice. The RT-PCR analyses for the transcripts derived from the cFVIII gene (cFVIII) of RNA isolated from hemophilia A mouse organs without (control) or with intramuscular injection of AAV1-β-actin-cFVIII vectors (AAV1cFVIII) or intravenous injection of AAV8-β-actin-cFVIII vectors (AAV8cFVIII) are shown. For the control, the RT-PCR analysis for mouse GAPDH (Mouse GAPDH) of RNA isolated from hemophilia A mice with or without injection of AAV-β-actin-cFVIII vectors was performed simultaneously.

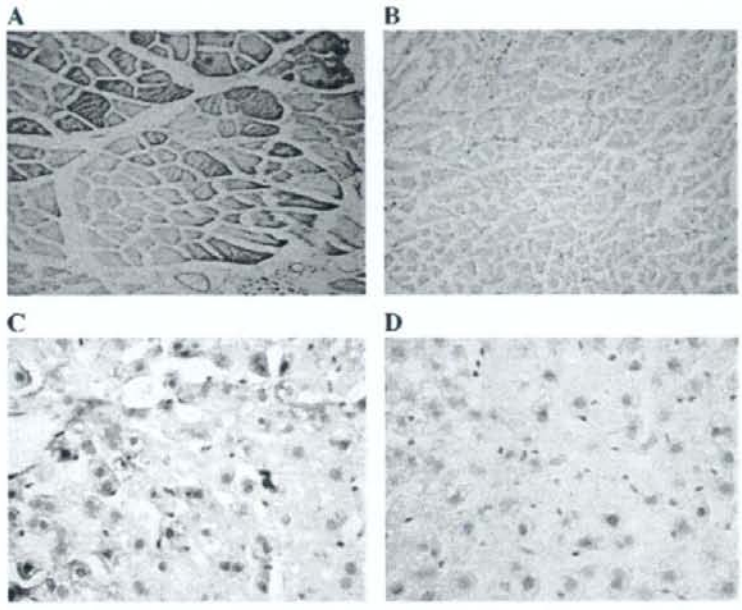

Figure 6 Immunohistochemical Analysis for cFVIII transgene products. Immunohistochemistry for cFVIII of the skeletal muscles of hemophilia A mice with intramuscular injection of AAV1-β-actin-cFVIII vectors (A) and the liver of hemophilia A mice with intravenous injection of AAV8-β-actin-cFVIII vectors (C) is shown (positive stain: brown). For the control, sections of the skeletal muscles (B) and the liver (D) obtained from hemophilia A mice without vector injection were processed simultaneously with anti-FVIII antibodies.

with the traceable expression in the heart, lung, and spleen (not shown). In accordance with the data on cFVIII transcripts, cFVIII molecules were immunohistochemically detected in the skeletal muscles of AAV1-β-actin-cFVIII injected mice and in the liver of mice with intravenous injection of AAV8-β-actin-cFVIII (Fig. 6).

Discussion

Because of the size and nature of the FVIII gene (cDNA), there were difficulties in hemophilia A gene therapy compared with gene therapy for hemophilia B. These difficulties were solved by efforts of many investigators that allowed use of a modified FVIII gene such as BDD FVIII cDNA, improved vector systems, and new strategies. Based upon these studies, a few clinical trials of hemophilia A gene therapy were conducted [17–19]. Increase of FVIII activities in the circulation and clinical improvements were observed in patients who received vector injection or transplantation of genetically modified cells. However, long-term expression of FVIII from the transgenes was not achieved in these studies. Thus, reexamination of the vector systems, the target organs for transduction, and the promoters may be required.

The recombinant AAV vectors are thought to be one of the better vectors in terms of its capability to transduce non-dividing cells and long-term transgene expression, although delivery of the FVIII gene using AAV vectors were limited by its small packaging capacity [4]. The dual AAV vector system utilizing separate AAV2 vectors independently carrying the FVIII heavy chain gene and the FVIII light chain gene could express functionally active FVIII [5]. However, there was an imbalance in the expression levels of the FVIII heavy chain and FVIII light chain, suggesting that over-expressed free FVIII light chain molecules might be more immunogenic than the native molecules. The BDD FVIII gene could be packaged in AAV2 or AAV8 vectors in the previous studies and these vectors could efficiently transduce the liver with intraportal injection of the vectors [6]. Transduction of the liver with peripheral vein injection of AAV8 vectors was as efficient as portal vein injection of vectors, although that of AAV2 vectors was not [6].

The liver would appear to be the appropriate target organ for transduction because FVIII is physiologically synthesized in this organ, so FVIII

synthesis in hepatocytes and its subsequent secretion into the circulation may be warranted. However, if any adverse reaction to the therapy occurs, removal of the liver would be an unacceptable solution. In fact minor liver dysfunction upon AAV2 vector injection into the hepatic artery was reported in clinical trials for hemophilia B gene therapy. In this respect, surgically removable organs such as skeletal muscles may well be the alternative target organs. AAV1 vector-based transduction of the skeletal muscles has beneficial characteristics of removing the transgenes. This is the first report of sufficient expression of FVIII in the skeletal muscles transduced with AAV vectors and suggests that skeletal muscle-directed FVIII expression has a potential for hemophilia A gene therapy.

Compared with synthesis and secretion of FVIII into the circulation from the liver, transport of sufficient FVIII into the circulation from the skeletal muscle fibers is not assured. Based upon our data, it is apparent that transduction of the liver with AAV8-β-actin-cFVIII is superior to transduction of skeletal muscles with AAV1-β-actin-cFVIII regarding FVIII production. The difference between FVIII levels in the peripheral blood of these vector-injected mice may be due to how the transduced cells secrete FVIII molecules into the circulation. Hepatocytes actively secrete a variety of molecules including FVIII into the circulation. Since recombinant cFVIII is in a BDD form, its expression in and secretion from hepatocytes is expected to be better than native FVIII [20], accounting for the high cFVIII expression in mice with intravenous injection of AAV8 vectors carrying the cFVIII gene though cFVIII expressing hepatocytes were not abundant. Although muscle fibers are surrounded by capillaries, transport of recombinant FVIII molecules from muscle fibers to capillaries would not be as efficient as that from hepatocytes.

In terms of the immune reaction to transgene products, muscle stem cells have been shown to function as antigen-presenting cells, suggesting that expression of the transgene by the ubiquitous promoter in the skeletal muscles might lead to development of antibodies against the transgene products if there is no immune tolerance to the transgene products [21]. This was confirmed by Wang et al. [22]. Neutralizing antibody formation was observed in 66.7% of mice with AAV1cFVIII injection even with administration of immunosuppressant, while it was not observed in mice with AAV8-β-actin cFVIII injection by week 12 after vector injection, supporting the potential advantage of AAV8 vector-based transduction of the liver over the muscle-directed transduction by AAV1 vectors.

Each vector system has advantages and disadvantages in these respects. We may need to confirm the results obtained in hemophilia mice using dogs and non-human primates that genetically are more close to humans because there may be differences in transduction efficiency of various serotypes between mice and humans [23]. Taken together, we may need to perform a comparative study using another animal models such as hemophilic dogs and non-human primates that are more genetically close to humans than mice to address these questions. Additionally, use of tissue-specific promoters to minimize neutralizing antibody formation may be a better strategy for expressing transgenes in a tissue- and organ-specific manner. These experiments will be performed in future studies.

In conclusion, our data suggested that both AAV1 and AAV8 vectors carrying the FVIII gene utilizing a minimum promoter have the potential for hemophilia A gene therapy. Our present studies have provided important insight about selecting the appropriate target for delivery of the therapeutic genes and the vector system for the hemophilia A gene therapy.

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Adipose Tissue as a Novel Target for *In Vivo* Gene Transfer by Adeno-Associated Viral Vectors

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ABSTRACT

Traditionally, skeletal muscle and liver are the preferred target organs for gene transfer to supply a transgene product into the systemic circulation. In this respect, adipose tissue presents a number of attractive features. However, adipose tissue transduction *in vivo* has not been feasible by conventional methods. To solve this issue, we tested the utility of excipients in adeno-associated virus (AAV) vector-mediated gene transfer and found that Pluronics are suitable for this purpose. In a histological analysis of adipose tissue in *db/db* mice, Pluronic F88 showed the greatest augmentative effect on β-galactosidase expression in combination with the AAV1 vector. When the vector encoding mouse erythropoietin (Epo) was used in the same manner, increased plasma Epo concentrations were observed (230 ± 80 versus 58 ± 14 mU/ml). Moreover, the plasma Epo concentration returned to the normal level after the surgical removal of transduced adipose tissue. No damage was observed in the transduced tissue. Our results indicate that the proposed method is safe and efficient for gene transfer into adipose tissues, thus providing an alternative for supplemental gene therapy.

OVERVIEW SUMMARY

Adipose tissue holds promise as an alternative depot organ in gene transfer approaches. However, no efficient method of gene transfer into adipose tissue *in vivo* has been established. In this study, we explored the utility of excipients to augment gene transfer into the adipose tissue of mice and found that Pluronic F88 was useful for this purpose when combined with AAV serotype 1 vectors. The improvement was also demonstrated with vectors encoding murine erythropoietin, and the mice became polycythemic. Moreover, after removing transduced adipose tissue, plasma erythropoietin levels returned to normal, which suggests the unique advantage of this method.

INTRODUCTION

IN SUPPLEMENTAL GENE THERAPY, skeletal muscle and the liver have been the preferred targets for gene transfer to supply transgene products into the systemic circulation. However, adipose tissue presents a number of attractive features. Adipose tissue can be found throughout the body and is easily accessible for vector injection. Increasing evidence supports the notion that adipocytes are designed to secrete numerous factors into the systemic circulation (Mohamed-Ali *et al.*, 1998). Further, the majority of adipocytes are considered to be nondividing, which is suitable for achieving long-term expression of transferred genes by the use of nonintegrating vectors such as adeno-associated virus (AAV) vectors (Russell and Kay, 1999). Moreover, the transduced tissue can be safely removed when unexpected events occur, thus adding a unique feature to safety considerations. However, adipose tissue transduction has not been feasible through conventional methods, and few studies have investigated its efficacy *in vivo* (Nagamatsu *et al.*, 2001; Ogata *et al.*, 2004). To overcome these limitations and develop a more practical method, we tested the usefulness of excipients for gene transfer. To achieve efficient and widespread gene transfer, it is essential to assure that the vectors stay within the target tissue for a certain period of time. For this purpose, nonionic surfactants are promising because they have low toxicity and unique features that help stabilize the membrane (American Pharmaceutical Association [AphA] and Royal Pharmaceutical Society of Great Britain [RPSGB], 1986). Moreover,

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improved gene delivery with Pluronic compounds was demonstrated in various applications (Kabanov and Alakhov, 2002; Kabanov *et al.*, 2005). Therefore, we tested the effects of these compounds on *in vivo* gene transfer targeting adipose tissue.

MATERIALS AND METHODS

Cells and plasmids for AAV vector preparation

HEK293 cells, a human embryonic kidney cell line, were maintained as described in a previous report (Fan *et al.*, 1998). Murine erythropoietin (Epo)-encoding plasmids have already been described by Mochizuki *et al.* (2004). AAV vectors of serotype 1 through 5, which encode LacZ or Epo and have a cytomegalovirus (CMV) promoter, were prepared by transient transfection, purified and quantitated as described previously (Matsushita *et al.*, 1998; Mochizuki *et al.*, 2004). Usually, the stocks of AAV vectors contained approximately $1\text{-}2 \times 10^{10}$ genome copies/μl.

In vitro *assessment of potential toxicity of surfactants*

A panel of Pluronics was provided by Asahi Denka (Tokyo, Japan). Tween 80 was obtained from Sigma-Aldrich (St. Louis, MO). The surfactants were dissolved in distilled H_2O to prepare stock solutions at a concentration of 20%. To assess the potential compatibility, we added various concentrations of these surfactants to cultured 293 cells at the time of confluency. After 24 hr, the cells were examined microscopically. The highest concentrations of the surfactants that did not produce any deleterious effect on these cells were tested. The effect of the surfactants on AAV vector capsids was also examined by including a 10% surfactant in the vector stocks (AAV-LacZ with serotype 2 capsid, 2×10^{10} VG/μl) for 24 hr; subsequently, the vector solutions were added to the cultured 293 cells in a 96-well plate at a dose of 2×10^4 VG/cell. The final concentration of the surfactants within the culture medium was 0.05%. Two days later, the infectivity was assessed by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining (Fan *et al.*, 1998).

Analysis of gene expression and of enhancing effect in vivo

For the *in vivo* experiments, the AAV vector solutions containing various concentrations of Pluronic F68, F88, and Tween 80 were injected into the subcutaneous adipose tissues of C57BLKS/J *db/db* mice (Japan SLC, Hamamatsu, Japan) at the age of 10 to 12 weeks. At the time of injection, the average weight of the animals was more than 50 g, and the subcutaneous adipose tissues were well developed. The AAV-CMV-LacZ vectors with various serotypes (serotype 1–5) were tested at a dose of 6×10^{10} VG/body. Two weeks after injection, the mice were killed, and their adipose tissues were enucleated, stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), cut into pieces, and then frozen with Tissue-Tek optimal cutting temperature (O.C.T.) compound (Sakura Finetek, Torrance, CA) in dry ice–ethanol, as described (Ogata *et al.*, 2004). When the tissue blocks were sliced for microscopic examination, they were also evaluated macroscopically and photographed. A densitometric analysis of the blocks was performed in order to substantiate the difference in the efficiency of transduction of adipose tissue under various injection conditions. In practice, five areas of the corresponding blocks were chosen and quantified, using Image Gauge software (version 3.0; Fuji Photo Film, Tokyo, Japan). After subtracting the background value, these numbers were analyzed and the statistical significance was evaluated. Experiments were performed with AAV-Epo vectors under conditions optimized with the AAV-LacZ vectors. Later, experiments at a higher vector dose (2×10^{11} VG/body) were included. On the basis of the preliminary experiments, the volume of injection was optimized as 100 μl/body (50 μl per lobe) and kept constant thereafter. Whole blood was collected from the tail vein every 2 weeks. Plasma Epo concentrations were quantified with an enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics, Mannheim, Germany). Complete blood counts were performed with a PC-608 particle counter (Erma, Tokyo, Japan).

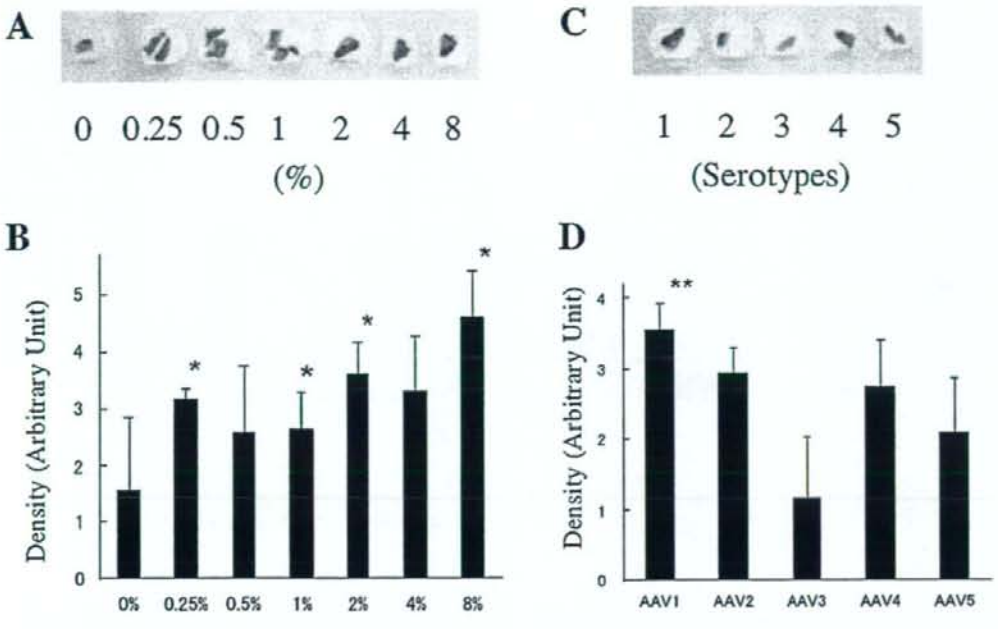
Detection of murine Epo within adipose tissue of db/db *mice*

Adipose tissues were obtained from *db/db* mice at the time of sacrifice or later by operation. For the immunofluorescence study, adipose tissues were fixed and frozen in the presence of the O.C.T. compound in dry ice–ethanol, following which they were reacted with goat anti-human Epo (Santa Cruz Biotechnology, Santa Cruz, CA) and donkey anti-goat IgG conjugated with Alexa 488 (Invitrogen Molecular Probes, Eugene, OR) at 4°C for 16 hr to visualize murine Epo by fluorescence microscopy, as described in a previous report (Ogata *et al.*, 2004).

Detection of murine Epo transcript within adipose tissue by reverse transcription-polymerase chain reaction

Total RNA was isolated from adipose tissue with an RNeasy lipid tissue kit (Qiagen, Hilden, Germany) and was converted to

FIG. 1. Distribution of LacZ expression within adipose tissues. AAV-CMV-LacZ vectors of various serotypes (serotypes 1–5) were tested ($n = 3$ for each serotype) at a dose of 6×10^{10} VG/body. Two weeks after injection, adipose tissues were removed, stained with X-Gal, and resected in pieces. Tissues were frozen with Tissue-Tek and then cut into 20-μm-thick slices for microscopic analysis. Blocks were also evaluated macroscopically, photographed, and analyzed by densitometry. (**A**) Representative blocks of adipose tissue injected with various concentrations of Pluronic F88. (**B**) Densitometric analysis of the corresponding blocks with various concentrations of Pluronic F88. Asterisks indicate statistical significance ($p < 0.05$) compared with values of tissues without F88 (0% data). (**C**) Blocks of adipose tissue injected with serotypes of AAV-LacZ vectors with 2% F88. (**D**) Densitometric analysis of blocks corresponding to the serotypes of AAV-LacZ vectors. Double asterisks indicate statistical significance ($p < 0.05$) relative to values obtained with the rest of the serotypes. Microscopic analysis of adipose tissue sections transduced with AAV1-LacZ without excipients is shown at low (**E**) and high (**F**) magnifications. Adipose tissues transduced with AAV1-LacZ in the presence of 2% Pluronic F88 are shown at low (**G**) and high (**H**) magnifications.


A
0 0.25 0.5 1 2 4 8
(%)
C
1 2 3 4 5
(Serotypes)
B
Density (Arbitrary Unit)
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D
Density (Arbitrary Unit)
AAV1 AAV2 AAV3 AAV4 AAV5
E
F
G
H

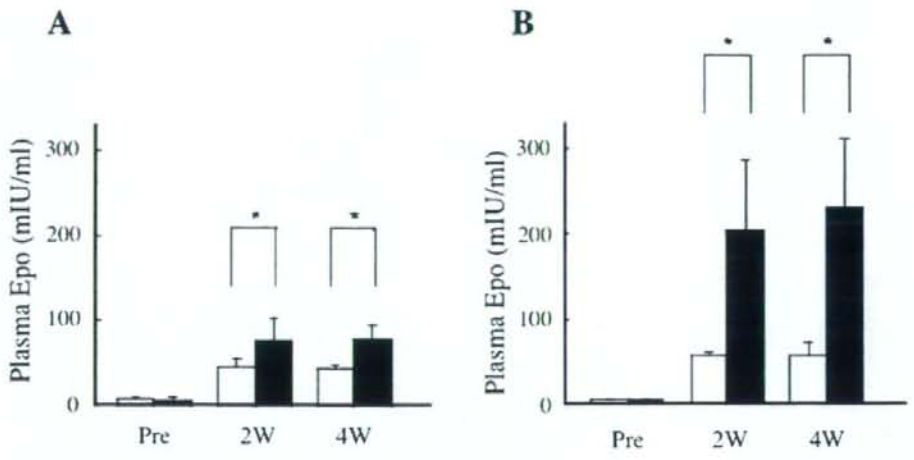

FIG. 2. Plasma Epo concentrations in *db/db* mice after injection of AAV1-Epo vectors into adipose tissue at a dose of (**A**) 6×10^{10} or (**B**) 2×10^{11} VG/body. Open and solid columns represent groups without and with 2% Pluronic F88, respectively, at the time of vector injection into adipose tissue. Each column and error bar indicate, respectively, the mean and SD of the group ($n = 5$). Asterisks indicate significance ($p < 0.05$).

cDNA with reverse transcriptase (SuperScript; Invitrogen, Carlsbad, CA) and oligo(dT) primers in a 20-μl mixture after DNase I (amplification grade; Invitrogen) treatment according to the manufacturer's instructions. Subsequent polymerase chain reaction (PCR) amplification was carried out with 1 μl of cDNA solution in a 50-μl reaction mixture containing 5 units of *Taq* polymerase, 10 m*M* Tris-HCl (pH 8.5), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, and 100 μM dNTPs in the presence of specific primer pairs (200 n*M*) designed to amplify DNA fragments derived from the transcript of the mouse Epo transgene. Each PCR cycle involved denaturation at 94°C for 15 sec, annealing at 56°C for 30 sec, and extension at 72°C for 30 sec. The PCR products were analyzed by agarose gel electrophoresis. The authenticity of the PCR products was confirmed by observing their molecular sizes after agarose gel electrophoresis and by sequencing (ABI PRISM 310 genetic analyzer; Applied Biosystems, Foster City, CA). The primer sequences for mouse Epo were 5'-GTG CAG AAG GTC CCA GAC TGA GTG A-3' and 5'-TTG GCG TAG ACC CGG AAG AGC TTG-3'. The primers for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Clontech Laboratories (Palo Alto, CA).

Removal of transduced tissue

AAV1-Epo vector solution (2×10^{11} VG/body) including 2% Pluronic F88 was injected into the subcutaneous adipose tissues of *db/db* mice. After 4 weeks, the adipose tissues were removed by standard surgical techniques under anesthesia. Plasma Epo concentrations were followed up 2 weeks thereafter. Four animals were analyzed and monitored.

Data and statistical analysis

Data are presented as means ± SD and were analyzed by Student *t* test after confirming an insignificant difference in variance between groups. $p < 0.05$ was considered statistically significant.

RESULTS

Screening of excipients in vitro

First, *in vitro* screening experiments were performed using nonionic surfactants. We tested Pluronic F68, F88, L72, P85, and Tween 80 for their effects on cell culture. Pluronic F68 and F88 were innocuous to cultured 293 cells at concentrations of up to 10 and 0.3%, respectively. The rest of the surfactants showed deleterious effects on the cultured cells when included at concentrations of 0.1% or higher. Next, we incubated AAV2-LacZ vectors with up to 10% Pluronic F68 and F88 solutions for 1 hr and checked their infectivity in 293 cells at 1×10^5 VG/cell. There were no differences in the infectivity of the vectors treated with these excipients, as assessed by X-Gal staining (data not shown).

Assessment of LacZ expression by use of Pluronics

On the basis of general safety data and *in vitro* experiments, we selected Pluronic F68, Pluronic F88, and Tween 80 as candidates for vector injection into *db/db* mice. Because preliminary experiments indicated the usefulness of Pluronic F88 combined with the AAV1 vector, we tested the usefulness of F88 at various concentrations. Comparison of blocks showed that there appeared to be an augmentation of LacZ expression with increasing concentration (Fig. 1A). The enhanced expression was substantiated by densitometric analysis of the blocks (Fig. 1B). On the basis of the result, we compared the usefulness of serotypes 1 through 5 combined with 2% Pluronic F88. The result showed that AAV1 was the most suitable serotype to trans-

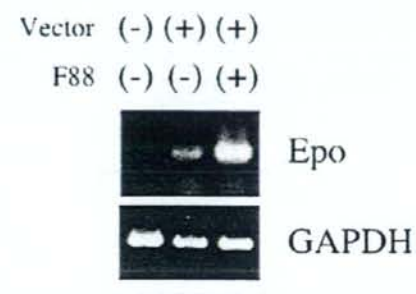

FIG. 3. Transgene expression from adipose tissue excised 2 weeks after vector injection. Immunofluorescence micrographs obtained with an antibody against Epo are shown. Adipose tissues without vector injection (**A**), or injected with AAV1-Epo vectors in the absence (**B**) or presence (**C**) of 2% Pluronic F88, were analyzed in the same fashion. (**D**) Results of RT-PCR, using mRNA extracted from adipose tissue, are shown. Lanes 1, 2, and 3 correspond to the adipose tissues shown in (**A**), (**B**), and (**C**), respectively.

duce adipose tissue *in vivo* (Fig. 1C and D). Enhancement of LacZ expression was also observed microscopically in the presence of Pluronic F88 (Fig. 1G and H) when compared with that of the vector alone (Fig. 1E and F).

Assessment of murine erythropoietin expression by use of Pluronic F88

On the basis of the findings obtained with LacZ vectors, we used vectors encoding murine erythropoietin (Epo) to demonstrate the enhancement of Epo concentration in a more quantitative manner. Increased plasma Epo concentration was observed 2 and 4 weeks after vector injection, and there was enhancement of Epo in the presence of 2% Pluronic F88 at a dose of 6×10^{10} VG/body (Fig. 2A). At a higher dose of 2×10^{11} VG/body, the enhancement effect was more prominent (230 ± 80 versus 58 ± 14 mU/ml at 4 weeks; Fig. 2B).

Assessment of transgene expression within adipose tissue

In the histological analysis of transduced tissues, a significant enhancement of Epo expression was observed by immunofluorescence when 2% F88 was included in the vector solution (Fig. 3A–C). Results of reverse transcription (RT)-PCR also showed enhanced expression of murine Epo in the presence of 2% F88 (Fig. 3D). Tissue damage or cellular infiltrates were not observed in the transduced adipose tissues throughout the histological evaluation (data not shown).

Effect of removal of transduced tissue

Bilateral lobes of abdominal adipose tissue were selected as a target for transduction. Plasma Epo concentrations were monitored after injection of AAV1-Epo vector (2×10^{11} VG/body) with 2% F88. After 4 weeks of observation, both lobes of the adipose tissue were removed *en bloc*, using standard surgical techniques. A significant decrease in plasma Epo concentration was observed 2 weeks after operation, with a return to the baseline level (Fig. 4). In addition, mice after removal of transduced tissue showed extended survival compared with "non-operated" mice. The survival period of *db/db* mice is summarized in Table 1.

DISCUSSION

In this study, we demonstrated the advantages of using excipients in adipose tissue transduction with AAV vectors. In practice, Pluronics have been widely used as excipients, including for administration to humans (APhA and RPSGB, 1986). Generally, one of the most frequent complications associated with administering a surfactant *in vivo* is hemolysis. With regard to this, it is noteworthy that Pluronics have a membrane-protecting effect on erythrocytes and that Pluronic F68 has long been used as a drug to prevent hemolysis and thrombotic events during extracorporeal circulation (Wright *et al.*, 1963). Further, their efficacy for use in the treatment of vasoocclusive disease in sickle cell anemia is currently being evaluated (Gibbs and Hagemann, 2004). Pluronics are also used in