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RESEARCH ARTICLE

Expression of human coagulation factor VIII in adipocytes transduced with the simian immunodeficiency virus agmTYO1-based vector for hemophilia A gene therapy

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We demonstrate that transduction of adipocytes with a simian immunodeficiency virus agm TYO1 (SIVagm)-based lentiviral vector carrying the human coagulation factor VIII gene (SIVhFVIII) resulted in expression of the human FVIII transgene in vitro and in db/db mice in vivo. Cultured human adipocytes were transduced with the SIVagm vector carrying the GFP gene in a dose-dependent manner and transduction of adipocytes with SIVhFVIII resulted in efficient expression of human coagulation factor VIII (hFVIII; 320$\pm$39.8 ng/10^6 adipocytes/24 h) in vitro. Based upon successful transduction of adipocytes by SIV vectors carrying the lacZ gene in vivo in mice, the adipose tissue of db/db *mice was transduced with SIVhFVIII. There was a transient appearance of human FVIII in mouse plasma (maximum 1.8 ng/ml) on day 11 after the injection. Transcripts of human FVIII transgene and human FVIII antigen also were detected in the adipose tissue by RT-PCR and immunofluorescence, respectively, on day 14. Emergence of anti-human FVIII antibodies 14 days after the injection of SIVhFVIII may explain the disappearance of human FVIII from the circulation. These results suggest that transduction of the adipocytes with vectors carrying the human FVIII gene may be potentially applicable for gene therapy of hemophilia A.*
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Keywords: *adipocyte; simian immunodeficiency virus vector; hemophilia*

Introduction

Hemophilia A is an inherited X-linked lifelong bleeding disorder caused by abnormality in the coagulation factor VIII (FVIII) gene.[1,2] The genetic abnormalities result in deficiency of FVIII, which in turn creates a bleeding diathesis, such as life-threatening intracranial bleeding and bleeding in joints and muscles. Hemophilias occur as mild, moderate, or severe, depending on the blood FVIII level of 6% or more, 2–5%, or 1% or less. The current standard therapy is intravenous (i.v.) injection of human plasma-derived FVIII or recombinant FVIII. Aside from certain specific situations, such as preoperative factor coverage, i.v. infusion of FVIII is usually used to treat acute bleeding episodes and prophylactic FVIII i.v. infusion is not recommended. However, maintaining 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. Recombinant FVIII products are now commercially available, but may not be completely free from pathological substances such as prions or as yet unknown viruses. In this regard, gene therapy is being explored as the next generation therapy for hemophilia patients.[1,2]

Adipocytes are terminally differentiated and nondividing cells. They not only store excess energy in the form of fat but also synthesize and secrete a variety of biologically active molecules such as leptin, adiponectin, cytokines, and plasminogen activator inhibitor-1 to the circulation.[3] Subcutaneous adipose tissues are readily accessed for vector administration. In addition, the adipose tissue can be removed surgically if necessary. These characteristics present attractive features of adipocytes for therapeutic gene therapy. In the present study, we use SIVagmTYO1-based vectors to show that SIVagmTYO1vectors can transduce adipocytes *in vitro* and *in vivo*, resulting in therapeutic gene expression, an expression mode that may be applicable to hemophilia gene therapy.

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Results

Transduction of adipocytes with SIVeGFP and SIVhFVIII in vitro

To assess the *in vitro* transduction efficiency of adipocytes with the SIV vector, human adipocytes were cultured in the presence of increasing concentrations of SIVeGFP for 48 h. After transduction, cells were washed and incubated in medium for 72 h. As shown in Figure 1a (phase-contrast view), cells containing intracellular lipid droplets represent the typical adipocyte morphology. The fluorescent microscopy image showed that eGFP was expressed in lipid droplet-containing adipocytes (Figure 1b). Flow cytometry analysis (Figure 1c) of these cells showed that eGFP expression in human adipocytes increased in a dose-dependent manner. Approximately 62% of adipocytes were efficiently transduced with SIVeGFP at MOI 5×10^3 vg/cell (100 transduction units/cell). We also assessed human FVIII production in the transduced human adipocytes. Cells were incubated in the presence of increasing concentrations of SIVhFVIII. The supernatants were harvested after various incubation times and human FVIII antigen levels were quantified by ELISA. FVIII production from the human adopocytes started on day 5 and increased in a dose- and time-dependent manner (Figure 2). After

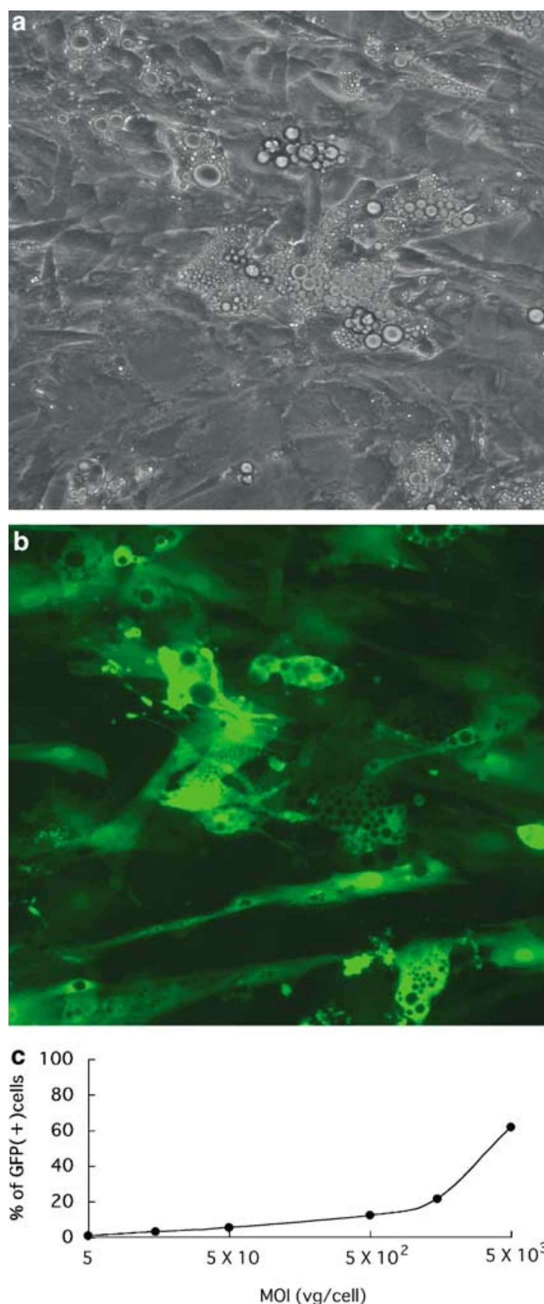

Figure 1 Transduction of human white adipocytes with SIVeGFP. Human white adipocytes (6.7×10^4cells/well) were incubated with increasing concentrations of SIVeGFP for 48 h. After incubation, cells were washed with PBS and incubated in media for 72 h (a, phase contrast). Expression of eGFP was visualized by fluorescence microscopy (b) and flow cytometry (c). The percentages of transduced cells expressing eGFP are shown (c) (mean, $n = 2$).

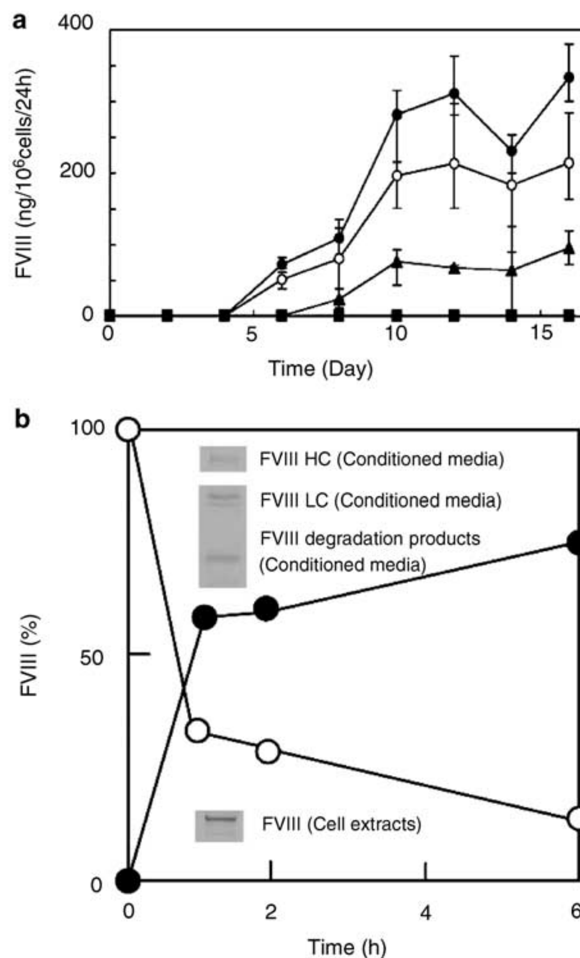

Figure 2 Transduction of human white adipocytes by SIVhFVIII. (a) Human white adipocytes (6.7×10^4 cells/well) were incubated with increasing concentrations of SIVhFVIII (■, 2×10^2 vg/cell; ▲, 6.5×10^2 vg/cell;ˆ, 2×10^3 vg/cell; ●, 6.5×10^3 vg/cell). After 48 h, cells were washed with PBS and cultured for 14 days. Supernatants were harvested and human FVIII antigen quantified by ELISA, as described in Materials and methods (mean±s.d., $n = 3$). (b) Pulse-chase experiments for FVIII production in human adipocytes (5×10^5 cells) were carried out on day 7 after transduction with SIVhFVIII (6.5×10^3 vg/cell). Amounts of [^{35}S]-labeled FVIII in the conditioned media (●) and in the cell extracts ˆ() were quantified as described in Materials and methods. Insets show the pertinent portion of the autoradiography of [^{35}S]-labeled FVIII in the cell extracts after pulse labeling and [^{35}S]-labeled FVIII species (heavy chain (HC); light chain (LC); degradation products) in the conditioned media after 1 h chase incubation.

transducing cultured human adipocytes with SIVhFVIII at MOI 6.5×10^3 vg/cell, 320 ± 39.8 ng of human factor VIII was produced from 10^6 adipocytes during a 24 h *in vitro* incubation. To study the secretion of FVIII from transduced adipocytes, pulse-chase experiments were performed. Approximately 53% of human FVIII, expressed in cultured adipocytes, was secreted from adipocytes during 1 h incubation periods, suggesting that adipocytes could secrete expressed FVIII efficiently. FVIII molecules identified in the conditioned media consisted of the heavy chain, the light chain, and degraded FVIII products, and the presence of these FVIII species in the conditioned media were consistent with the previous report.[4]

Transduction of the adipose tissue by SIVlacZ in vivo

To explore the possibility that the SIV vector can transduce adipocytes *in vivo*, SIVlacZ vectors were injected into the subcutaneous adipose tissue of 8-week-old *db/db* mice. Wild-type mice have adipose tissue in the mesenterium and peritesticular regions, but they are generally lean and do not have enough subcutaneous adipose tissues for vector injection. NOD/SCID mice are used frequently for gene therapy research because of their immunodeficiency, but they also have little subcutaneous adipose tissues. Among several types of obese mice used for metabolic disease research, *db/db* mice are well characterized, obese, and diabetic. They become obese by accumulating fat in the subcutaneous and visceral adipose tissues after 4 weeks of age. Thus, *db/db* mice are appropriate for studying *in vivo* transgene expression from subcutaneous adipose tissue. At 2 weeks after the injection, the adipose tissues were excised and processed for detection of β-galactosidase activity. As shown in Figure 3a, the adipose tissue was stained blue homogenously after the X-gal staining in the macroscopic view. In the histology sections of the adipose tissue, β-galactosidase activity was detected in the adipocytes of *db/db* mice (Figure 3b, c). These data suggest that SIV vectors are capable of transducing adipocytes *in vivo* in mice.

Plasma human FVIII levels in SIVhFVIII-injected db/db mice

To evaluate *in vivo* production of human VIII from adipocytes, SIVhFVIII was injected into subcutaneous adipose tissues of *db/db* mice. Mouse plasma was obtained on days 0, 4, 7, 11, 14, 18, 21, and 25 after vector injection and human FVIII levels in mouse plasma were quantified by an ELISA that recognizes only human FVIII. Plasma human FVIII levels (closed circle) increased to 1.8 ng/ml on day 11, but human FVIII antigen was not detectable in the circulation on day 14 after vector injection (Figure 4). Since *db/db* mice are immunocompetent, they may develop antibodies to human FVIII expressed *in vivo*. To explore the possibility that disappearance of human FVIII from the mouse circulation was caused by the presence of antibody against human FVIII in *db/db* mice, a solid-phase EIA for detection of *db/db* mouse antibody to human FVIII was carried out. As shown in Figure 4, anti-human FVIII antibodies (triangle) were detected in *db/db* mouse plasma obtained on day 14 and the levels increased gradually to 6.9 μg/ml by day 25.

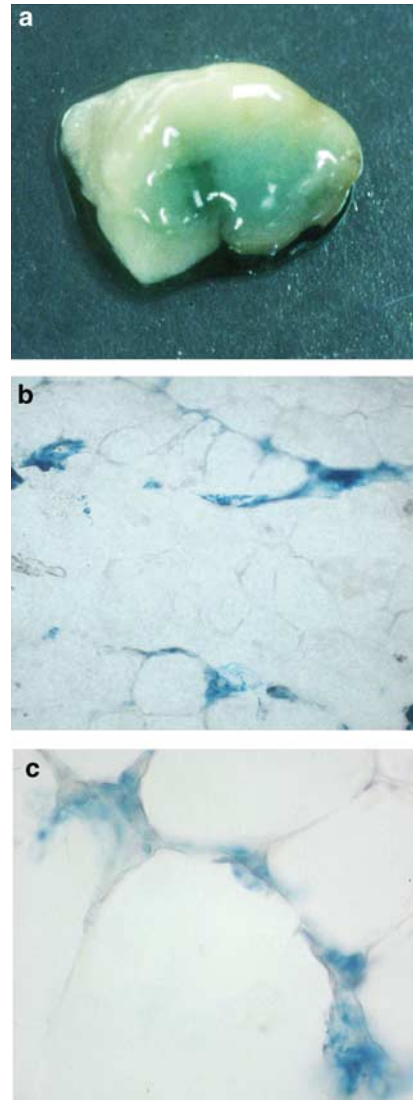

Figure 3 *Transduction of the adipose tissues of db/db mouse by SIVlacZ. The SIVlacZ vector (5×10^7) was diluted in PBS and was injected into the subcutaneous adipose tissues of db/db mice. The subcutaneous adipose tissues were excised on day 14 after injection. Tissues were processed for detection of β-galactosidase activity, as described in Materials and methods. The adipose tissue (macroscopic view) was homogenously stained blue (a). β-galactosidase activity was observed in the adipocytes, as reflected by the blue staining in the histology sections (b, c).*

Detection of FVIII transcripts in the adipose tissue of db/db mice

To assess the expression of transgenes in the adipose tissue of *db/db* mice, the adipose tissues were excised on day 14 after injection and subjected to reverse transcription-polymerase chain reaction (RT-PCR) analysis for detection of human BDD-FVIII transcripts using human FVIII or mouse GAPDH-specific primers. As shown in Figure 5, human FVIII transcripts were detected in the adipose tissue from the SIVhFVIII-injected mice, but not in the adipose tissue from SIVlacZ-injected mice. Mouse GAPDH transcripts were detected in both RNA preparations. These data suggest that the human FVIII transgene was expressed in the mouse adipose tissue.

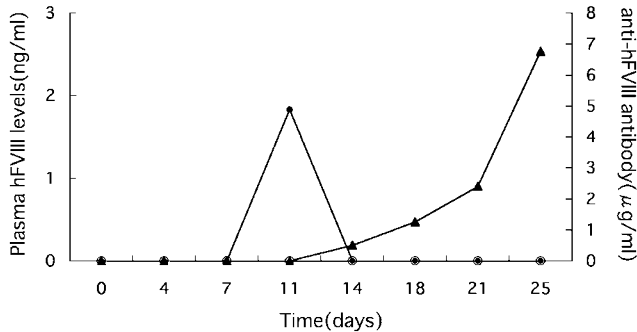

Figure 4 Plasma human FVIII levels in SIVhFVIII-injected db/db mice. Peripheral blood was obtained from SIVhFVIII-injected mice or the SIVlacZ-injected control mice on days 0, 4, 7, 11, 14, 18, 21, and 25 after injection. Human FVIII concentrations in plasma of db/db mice who received SIVhFVIII (closed circle) or SIVlacZ (open circle) injection were determined by ELISA (mean, $n=2$). Anti-human FVIII antibodies present in mouse plasma (closed triangle) were quantified by the solid phase EIA as in described in METHODS.

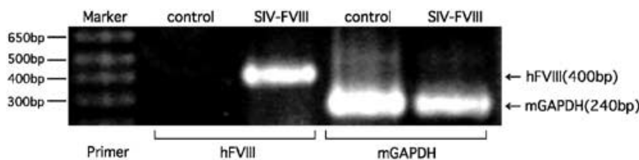

Figure 5 RT-PCR analysis of adipose tissue-derived RNA. RNA was isolated from the murine adipose tissue on day 14 after vector injection. A measure of 100 ng of RNA was subjected to RT-PCR with specific primer pairs for the human BDD-FVIII transcript (human FVIII) or for the mouse GAPDH transcript (mouse GAPDH). Amplified products were analyzed on 2% agarose gels followed by ethidium bromide staining (control, RNA isolated from the SIVlacZ injected adipose tissue; SIVhFVIII, RNA isolated from the SIVhFVIII-injected adipose tissue).

Detection of human FVIII expressed in the adipose tissue of db/db mouse

Mouse adipose tissues were processed for detection of human FVIII antigen, and imaged by immunofluorescence (Figure 6). Human FVIII was observed in adipocytes isolated from SIVhFVIII-injected mice (left), but not in cells from mice that received the SIVlacZ vector (right). These data confirm the notion that the human FVIII was produced from the SIVhFIII-transduced cells *in vivo*.

Discussion

The subcutaneous adipose tissue has attractive features for genetic therapy, such as easy accessibility, active biosynthesis and secretion behavior, and high vascularity.[3] However, adipocytes are terminally differentiated and nondividing cells with a characteristic phenotype expression. A variety of viral and nonviral vectors are used to transduce cells *in vitro* and *in vivo* for gene delivery, but it is still rather difficult to transduce nondividing cells efficiently. Adenoviral vectors are able to transduce nondividing cells including adipocytes,[5,6] but transgene expression by adenoviral vectors is thought to be transient and they are highly immunogenic. In contrast, pseudotyped lentiviral vectors are versatile enough to stably transduce various types of cells including nondividing cells, and were not as immunogenic as the adenoviral vectors.[7–10] But transduction of adipocytes with lentiviral vectors had not been studied yet. There are safety concerns in utilizing HIV-1-based lentivirus 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 nonpathogenic to both their natural hosts and to experimentally inoculated Asian macaques.[11] Additionally, due to the use of contaminated blood products, some hemophilia patients are HIV-1 carriers. If an HIV-1-based vector is administered 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 SIVagm TYO1 strain may be a better vehicle for hemophilia gene therapy because SIVagm TYO1 has less than 60% genomic sequence similarity to HIV-1.

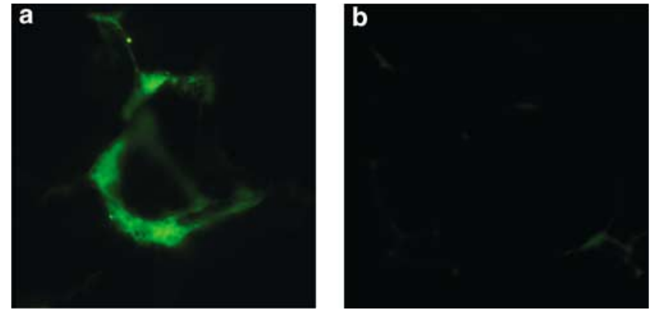

Figure 6 Detection of human FVIII antigen in mouse adipose tissue. The adipose tissue of SIVhFVIII injected db/db mice (a) or SIVlacZ-injected mice (b) were subjected to immunofluorescence staining for human FVIII antigen. Tissue sections were incubated with sheep anti-human FVIII polyclonal antibodies. The bound antibodies were detected by Alexa-Fluor488-conjugated secondary antibody and visualized using a fluorescence microscope (E800, Nikon Co Ltd, Tokyo, Japan).

We have shown that SIV vectors carrying the eGFP, the lac Z, or a therapeutic gene can transduce cultured human adipocytes *in vitro* and mouse adipose tissue *in vivo*. Transduction of the human adipocytes by the SIVeGFP vector *in vitro* was dose-dependent and appeared to be efficient. Production of human FVIII (320 ± 39.8 ng/10^6cells/24 h) from transduced adipocytes at MOI 6.5×10^3 vg/cell *in vitro* was considerable and efficient, raising the possibility of achieving therapeutic levels of plasma FVIII in mice if 10^6 adipocytes were transduced by SIVhFVIII efficiently *in vivo*. Thus, SIVhFVIII 4×10^9 vg was injected to the mouse subcutaneous adipose tissue. Human FVIII was detected in the mouse plasma and this human FVIII level was approximately 1–2% of the normal FVIII level of normal human subjects. The FVIII levels achieved in mice were relatively low, but such increase of the FVIII level would develop clinical effects in hemophilias such as decrease

of bleeding episodes and of the use of FVIII concentrates. Data on clinical trials of hemophilia A gene therapy support this notion.[12] However, this human FVIII level in mouse plasma was lower than that expected from the observed *in vitro* production rate. One contributing factor may be the shorter half-life of human FVIII in mice compared to humans. The half-life of injected human FVIII in mice is approximately 1 h,[13] whereas the half-life in hemophilia patients is closer to 8–12 h. Another possibility may be the inefficient transduction of adipocytes *in vivo* because the vector-containing solution might not diffuse throughout the adipose tissues, resulting in less adipocytes being exposed to the viral vectors. It is also possible that transduction of mouse adipocytes by the SIV vector is less efficient than that of human cells. Production of human FVIII from differentiated 3T3-L1 cells, transduced with the SIVhFVIII vector, was significantly lower than that from human adipocytes (not shown). The observation that anti-human FVIII antibodies were present in the mouse plasma obtained on day 14 and subsequently increased to 6.9 μg/ml by day 25 suggested that human FVIII was rapidly cleared from the mouse circulation by the formation of immune complexes after day 14. Thus, human FVIII was secreted into the circulation and was recognized by the *db/db* mouse immune system. The notion that human FVIII was synthesized in the adipocyts *in vivo* is also supported by detection of human FVIII gene transcripts and products in the adipocytes. In conclusion, adipose tissue is easily accessible and is an appropriate target for gene delivery. Recombinant SIV vectors may be applicable for adipocyte-targeted gene therapy for hemophilia A.

Materials and methods

Cell culture

Human white adipocytes prepared in 24-well culture plates were purchased from Zen-Bio Inc. (Research Triangle Park, NC, USA). These cells were differentiated from preadipocytes isolated from the adipose tissue of 49-year-old healthy subjects undergoing liposuction surgery with informed consent. The cells were shown to express leptin, CCAAT/enhancer-binding protein α, peroxisome proliferator-activated receptor γ, and STATs.[14,15] Human adipocytes were cultured in DMEM/HAM F10 medium supplemented with 3% fetal bovine serum, 15 mM HEPES, biotin (33 μM), pantothenate (17 μM), insulin (100 nM), dexamethazone (1 μM), penicillin 100 U/ml, streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml) (Zen-Bio Inc.). 3T3-L1 cells (ATCC) were cultivated in DMEM containing 10% FBS. Differentiation of 3T3-L1 cells to adipocytes was carried out in the medium containing dexamethasone, insulin, and 1-methyl-3 isobutylxantine, as described previously.[16]

Production of SIVagm vectors

Human FVIII cDNA spanning the entire coding region was a generous gift from Dr JA van Mourik (VU University Medical Centre).[17] As the B domain is excised from other FVIII domains upon activation by thrombin and is not essential for coagulation activity of FVIIIa, coding for the B domain was deleted from the human FVIII cDNA by PCR-based mutagenesis (BDD FVIII cDNA), as described previously.[18] The deletion was confirmed by sequencing. The characteristics and production of SIVagm vector used in this study were described previously.[19] Self-inactivating SIVagm vectors are pseudotyped with vesicular stomatitis virus glycoprotein G (VSVG). We constructed a series of gene transfer vectors to express the eGFP gene, the lacZ gene, and the hBDDFVIII gene driven by the cytomegalovirus (CMV) promoter. To produce SIV vectors, 293T cells were transfected with the packaging vector, the gene transfer vector, and pVSVG (Clontech), as described previously.[19] Transduction units of SIVeGFP were determined by infection of SIVeGFP to 293T cells, followed by determination of eGFP expression by FACS analysis. RNA dot blot analysis was performed to quantify the amount of SIVagmTYO1vector genome of vector preparations. When SIVhFVIII was produced in 293T cells, SIVeGFP also was prepared simultaneously and the transduction unit of SIVeGFP and the amount of vector genome were determined. The SIVeGFP preparation was used as the standard to estimate the transduction units of SIVhFVIII.

In vitro culture and transduction of human adipocytes

For transduction, increasing concentrations of the SIV vector were added to human adipocyte cell monolayers in 24-well culture plates, and the cells were incubated at 37°C for 48 h in the presence of 5% CO_2. After incubation, the medium was harvested and changed, according to the manufacturer's instruction. SIVeGFP-transduced adipocytes were analyzed for eGFP expression by fluorescence microscopy and flow cytometry, and the conditioned medium of SIVhFVIII-transduced adipocytes was harvested and subjected to FVIII ELISA. To study the secretion of FVIII from adipocytes transduced with SIVhFVIII, pulse-chase experiments were performed. SIVhFVIII-transduced adipocytes were cultured for 30 min in methionine-deficient DMEM (GIBCO-Invitrogen Japan, Tokyo, Japan) containing 3700 kBq/ml [^{35}S]-methionine (NEN Life Science Products, Inc., Boston, MA, USA), and then cultured in the complete medium (DMEM). After various incubation time periods, conditioned medium and cell extracts were prepared. To isolate [^{35}S]-labeled FVIII molecules, the conditioned media and cell lysates were subjected to immunoprecipitation using sheep polyclonal antibodies against human FVIII and protein A-coupled Sepharose CL-4B (Amersham Pharmacia Biotech, UK). [^{35}S]-labeled FVIII was analyzed by SDS-PAGE followed by autoradiography, and quantified using an image analyzer BAS 2000 (Fujifilm, Tokyo, Japan), as described.[20]

Mice

Experimental *db/db* mice (C57BL/KsJ-db/db) are well characterized obese and diabetic mice caused by the genetic abnormality of the leptin receptor gene,[21] and were purchased from Japan SLC Inc. (Hamamatsu, Japan). The *db/db* mice were kept in a clean P3-level experimental room, and were maintained on a sterile diet and given autoclaved water.

Transduction of mouse adipose tissues by SIV vectors

SIV vectors carrying either the lacZ gene or the hFVIII gene were diluted in PBS and injected into the subcutaneous adipose tissue of the mice. Peripheral

blood (100 μl) was collected from mouse tail veins into tubes containing heparin. Platelet poor plasma was prepared by centrifugation of the peripheral blood at 1000 g for 15 min, and subjected to FVIII ELISA. Some mice were killed on day 14 after the injection for detection of transcripts and products of the transgenes.

Enzyme immunosorbent assay (ELISA) for human FVIII antigen

Since human FVIII clotting activity could not be quantified directly in the *db/db* mice because of the presence of endogenous murine FVIII, human FVIII expressed in *db/db* mice was quantified by a human FVIII-specific ELISA, as described previously.[22] Briefly, 96-well microtiter plates (Costar, Cambridge, MA, USA) were coated with 1 μg/ml mouse monoclonal antibody to human FVIII (Chemo-Sero Institute, Kumamoto, Japan). 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, human FVIII bound to the plates was detected with sheep anti-human FVIII polyclonal antibodies (Cedarlane Laboratories Ltd, Homby, Ontario, Canada) and horseradish peroxidase-conjugated rabbit anti-sheep IgG.

Detection of anti-human FVIII mouse antibody

Microtiter plates were coated with purified human FVIII in PBS for 16 h. After blocking with 5% casein, FVIII-coated microtiter plates were incubated with mouse plasma at 10–1000-fold dilutions or monoclonal antibodies raised against human FVIII as the standard. Mouse IgG bound to human FVIII was detected by HRP-conjugated anti-mouse IgG, followed by incubation with HRP substrate ABTS (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA).

Detection of β-galactosidase and human FVIII in the mouse adipose tissue

Mouse adipose tissues were obtained from *db/db* mice. The adipose tissues were fixed with 2% paraformaldehyde in PBS for 5 min, washed with PBS, and then 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 for 1 h. The tissues were again washed, incubated with PBS containing sucrose (10–30%), and frozen with OTC compound (Tissue-Tek, Miles Inc., Elkhart, IN, USA) in dry ice/ethanol. Tissue sections were made at −35°C and attached to poly-lysine-coated glass slides. For the immunofluorescence study, the adipose tissues were fixed with 4% paraformaldehyde in 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 −35°C and attached to polylysine-coated glass slides. For the detection of human FVIII, tissue sections were blocked with 1% bovine serum albumin in PBS. Samples were incubated with polyclonal anti-human FVIII 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 human FVIII by fluorescence microscopy.

Detection of the BDD-FVIII transcript by RT-PCR

RNA was isolated from the adipose tissue by the acid-guanidine method and was 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 in a 50 μl reaction mixture containing 5 U of Taq polymerase, 10 mmol/l Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$, and 100 μM dNTPs in the presence of specific primer pairs (200 nM) designed to amplify the DNA fragments derived from the transcript of the BDD-FVIII transgene. 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 after agarose gel electrophopresis, and by sequencing. The primer sequences for human FVIII are ATTGGAG-CACAGACTGACTT and ATATGGTATCATCATAGTCA (400 bp). Primers for mouse GAPDH were purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

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