どウイルスタンパク質の他、さらに、宿主のHMG-I(Y)タンパク質などが共存しているという報告が散見されるもののその報告者とは異なる研究者による厳密な検証はなされていないので、真偽は不明である。さらに、メカニズムは不明であるが、この巨大核酸タンパク質複合体（PIC）は細胞質から細胞核に移行し、細胞核内で宿主ゲノムDNAに組み込み（狭義の組み込み反応）を起こしてプロウイルスとなる。この一連の過程はレトロウイルスに特徴的な反応なので、この過程を特異的に阻害することは理論上可能である。そのような制御が可能になれば、治療に大いに役立つであろう。　本研究は、このDNAへの変換以降のステップのメカニズムを明らかにする事を第一の目的とする。

血友病Bにおける変異遺伝子を相補するために野生型IX因子遺伝子のcDNAを肝幹細胞に導入し長期的に野生型凝固因子を発現させることを考えた。凝固因子は肝臓で合成されるので、最も自然な遺伝子治療戦略であると考えたからである。そのようなベクターをC型肝炎ウイルス（HCV）をもとにして開発することを目的とする。C型肝炎ウイルスは(+)一本鎖RNAをゲノムとして有し、慢性肝炎を起こす。肝細胞にＲＮＡゲノムが長期にわたって維持される特徴がある。まず凝固因子遺伝子の代わりに検出が容易なネオマイシン耐性遺伝子または緑色蛍光タンパク質（GFP）遺伝子を遺伝子導入のマーカーとして利用して実験を行うことにした。

B. 研究方法と結果

まずHIVをベースにしたベクターを作製した。ベースとして採用したのはHXNベクター（島田隆博士より供与を受けた）である。HIV-1の構造から、5'のLTR、3'のLTR、gag,の5'末端領域以外の *gag* のほぼ全部、*pol*, *env* が欠失している。かわりに、Tkプロモーターでドライブされるneomycin耐性（*neo*）遺伝子が組み込まれている。従ってこのベクターが組み込まれた細胞はG418に耐性となる。HXNのTkプロモーターのすぐ上流に178塩基のHIVのウイルス中央多プリン配列(cPPT)または、それを含みさらにそれよりも長い282塩基のcPPTを挿入したベクターを作製し，遺伝子導入効率（出現するG418耐性細胞コロニー形成能）、組み込み効率、核移行高効率を調べた。282塩基長のcPPT領域を有するHXNベクターではそうでないHXNベクターに比べて遺伝子導入効率が5%程度にまで低下した。178塩基長のcPPT領域を有するHXNベクターではそうでないHXNベクターに比べて遺伝子導入効率が逆に200%程度にまで上昇した。ベクターのcDNAの核移行の程度を調べた。282塩基長であれ178塩基長であれcPPT領域を有するHXNベクターとそうでないHXNベクターで核移行の程度は同じであった。組み込み効率は282塩基長のcPPT領域を有するHXNベクターでのみ低下していた。

T7 RNA polymeraseを用いる

in vitro 転写系を用いてHCV レプリコンRNA を作製した。C 型肝炎ウイルス（HCV）によって劇症肝炎を起こした患者から脇田らが樹立したJFH1 株をもとにネオマイシン耐性（*neo*）遺伝子でコア・エンベロープ遺伝子を置き換えた。このレプリコン RNA を試験管内転写によって合成しヒト肝癌 Huh7 細胞に電気的にトランスフェクトした。1ug 当たり１００―５００個程度の G418 耐性コロニーが出現した。このコロニーを継代しても少なくとも２ヶ月は G418 耐性であった。ネオマイシン耐性遺伝子を GFP 遺伝子に変更した場合、緑色蛍光を発する細胞は１週間は認められるもののそれ以降には認められなくなった。さらに、*neo* 遺伝子にGFP遺伝子を融合させてマーカーとしたときにはG418 耐性細胞コロニーはまったく出現しなかった。

（倫理面での配慮）
該当しない

C. 考察

cPPT を挿入した HIV ベクターの遺伝子導入効率はその cPPT の長さあるいは、cPPT の近傍領域の配列に依存する場合があることを示した。cPPT は核移行効率に関わるというよりも核移行後のなんらかのステップに重要であることを明らかにした。cPPT の近傍領域には HIV ベクターの RNA 量に関わる働きのあることも明らかにした。今後は血友病治療遺伝子を trasngene とした場合の組み合わせで調べねばならないだろう。

また、*neo* 遺伝子を有する HCV レプリコンは長期にわたって細胞で維持された。しかし、さらに長期の維持と発現が可能かは不明である。またさらに、GFP 遺伝子を有する HCV レプリコンは維持されない。この理由は不明であるが、RNA の配列が HCV レプリコンの複製に toxic に働いている可能性があると思われる。

D. 結論

HIV の組み込みに cis に関わる領域のあることを明らかにした。

E. 健康危険情報

該当なし

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研究成果の刊行物・別刷

Journal of Thrombosis and Haemostasis, **2**: 275–280

ORIGINAL ARTICLE

Specific detection of human coagulation factor IX in cynomolgus macaques

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Summary. After screening for species-specific antihuman factor (F)IX monoclonal antibodies, we found that antibody 3A6 did not bind to cynomolgus FIX. The 3A6 epitope was found to include Ala262 of human FIX. The 3A6 antibody was used as a catching antibody in an enzyme immunoassay (EIA) for specific detection of human FIX in cynomolgus macaque plasma. No significant increase of substrate hydrolysis was observed when EIA buffer containing cynomolgus macaque plasma was subjected to the 3A6-based EIA. Addition of up to 30% cynomolgus macaque plasma or canine plasma to the assay did not alter detection of human FIX. Three cynomolgus macaques were injected with human FIX ($10\,\mathrm{U\,kg^{-1}}$; i.v.) and the circulating human FIX was quantified in the macaque plasma. The FIX level in the circulation increased to $470 \pm 37.6\,\mathrm{ng\,mL^{-1}}$ at 1 h after the injection and gradually decreased to $1.79 \pm 1.1\,\mathrm{ng\,mL^{-1}}$ by day 5, which is approximately 0.06% of the normal human plasma FIX concentration. These data suggest that the cynomolgus macaque can be used as a primate model for studying hemophilia B gene therapy by transduction of macaque organs with vectors to express human FIX *in vivo* and detection of human FIX using the 3A6 monoclonal antibody.

Keywords: cynomolgus macaque, factor IX, hemophilia B.

Introduction

Hemophilia B is an X-linked, hereditary life-long bleeding disorder caused by genetic abnormality of the coagulation factor (F)IX gene. The genetic abnormalities result in deficiency of FIX, 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 patient blood FIX level of ≥6%, 2–5%, or ≤1%. Although recombinant FIX products are available in the USA, current standard therapy in Japan is still intravenous (i.v.) injection of heat-treated and monoclonal antibody (mAb)-purified FIX concentrates from plasma. Aside from certain specific situations, such as preoperative factor coverage, i.v. infusion of FIX concentrates is usually used to treat acute bleeding episodes and prophylactic FIX i.v. infusion is not recommended in Japan.

Notably, maintenance of blood FIX levels of ≥2% of the normal plasma FIX concentration may result in significant clinical improvement. Unfortunately, in the past, infection with hepatitis B and C viruses or human immunodeficiency virus (HIV) in hemophilia patients was a tragic result of contaminated blood-derived commercial products. After introduction of heat treatment, detergent treatment, and/or mAb isolation of FIX, the risk of viral infection by commercial products was significantly reduced, but not eliminated. Recombinant FIX products are now commercially available in the USA, but also may not be completely free of pathological substances such as prions or as yet unknown viruses. In this context, gene therapy is being explored as the next generation therapy for hemophilia [1,2]. To develop gene therapy technology, a good animal model is necessary. Hemophilia B mice (FIX knock-out mice) and hemophilia B dogs are available and have been used to study gene therapy approaches for hemophilia B. Based upon promising results gathered in these animals after receiving intramuscular injection of type 2 AAV vectors carrying the FIX gene [3–6], clinical trials for hemophilia B by transduction of skeletal muscles with these vectors were initiated, but have had limited success [7,8]. Vector doses of 1.8×10^{12} vector genome $(\mathrm{vg})\,\mathrm{kg^{-1}}$ yielded plasma FIX levels of >1% in mice, whereas the same vector dose yielded circulating FIX levels of 0.2–0.4% in dogs. In humans, no significant increase of FIX levels was observed with the same vector dose. One possible explanation of the differences in these results is that the transduction

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efficiency of skeletal muscles by type 2 AAV vectors in humans is different from that in these animals. Since this vector dose was the highest dose used in the clinical trial, the possibility that vector doses were not high enough for FIX expression in hemophilia B patients was most likely [8]. In this regard, a primate model may be required to mimic more closely the human situation. Anti-FIX mAbs were screened for their inability to bind simian FIX. One antibody was found and forms the basis for an enzyme immunoassay (EIA) and quantification of human FIX in primates down to 1.7 ng mL^{-1}, or 0.06% of the normal plasma FIX concentration.

Materials and methods

Murine monoclonal antibodies

Murine mAbs raised against human FIX were developed by standard procedures. Quantification of human FIX with mAb 3A6 was reported previously [9]. JKIX-1 is a murine mAb that binds to human FIX in the presence of calcium [10]. JKIX-1 was labeled with NHS-biotin (Vector Labs, Burlingame, CA, USA) according to the manufacturer's instructions.

Enzyme immunoassay and Western blotting for FIX

Microtiter plates were coated with 3A6 in PBS (1 μg mL^{-1}) for 16 h at 4 °C. After blocking with 5% casein in PBS, samples containing FIX (wild-type human FIX or FIX mutants; see below) were incubated in Tris-buffered saline pH 7.4 (TBS) containing 1% casein and 0.1% Triton X-100 at 37 °C for 2 h. After washing with TBS containing 0.1% Triton X-100, bound FIX antigen was detected with sheep antihuman FIX polyclonal antibody (Cedarlane Labs Ltd, Hornby, Ontario, Canada) followed by horseradish peroxidase (HRP)-labeled antisheep IgG (Vector Labs). JKIX-1 based EIA was also carried out in a similar manner using buffer containing 5 mM $CaCl_2$ except during the plate-coating step. Western blotting for FIX was carried out as described previously [11] except for detection of HRP-labeled antibodies bound to polyvinylidene fluoride membrane using chemiluminescent reagent ECL+ (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). For quantifying human FIX in cynomolgus macaque, macaque plasma diluted in TBS containing Triton X-100 (0.1%) and casein (1%) was added to 3A6-coated microtiter plates and 3A6-bound FIX was detected by biotin-labeled JKIX-1 followed by HRP-conjugated streptavidin (Vector Labs).

Expression of human FIX and mutant FIX in CHO-K1 cells

Human FIX cDNA was a generous gift from Dr G. G. Brownlee (Chemical Pathology Unit, University of Oxford, Oxford, UK). Human FIX cDNA was directionally cloned into the pcDNA3 expression vector (Invitrogen Japan, Tokyo, Japan) to make plasmid pcDNAFIXWT. This was subjected to site-directed mutagenesis to construct pcDNAFIX/G226D/V227A (G226 to D and V227 to A), pcDNAFIX/A262T (A262 to T), and pcDNAFIX/L321S (L321 to S), for expression of FIX mutants with the indicated amino acid substitutions. To determine the epitope of mAb 3A6, FIX mutants were expressed in CHO-K1 cells cultivated in HAMF-12 media in the presence of vitamin K (10 μg mL^{-1}). CHO-K1 cells (5×10^6) in 0.8 mL of Dulbecco's PBS were incubated with 20 μg of pcDNAFIX/WT, pcDNA-FIX/G226D/V227A, pcDNAFIX/A262T, or pcDNAFIX/L321S, on ice for 15 min and subjected to electroporation at 300 V (25 μF) using a Gene Pulser (BioRad Labs, Hercules, CA, USA). To make stable transfectants that express wild-type human FIX and FIX mutants, cells were cultured in the presence of geneticin (250 μg mL^{-1}) (Gibco-Invitrogen Japan, Tokyo, Japan). Cloned geneticin-resistant cell lines were selected for FIX expression by the JKIX-1-based EIA and selected clones were cultured in the presence of vitamin K (10 μg mL^{-1}). Coagulation activities of recombinant wild-type FIX and FIX mutants expressed in CHO-K1 cells were determined using FIX-deficient plasma (Dade Behring, Marburg, Germany) and an automated coagulometer model CA-500 (Sysmex, Tokyo, Japan). Recombinant FIX antigen concentration in the conditioned medium was determined with the two different EIAs as described above.

Detection of human FIX in cynomolgus macaque

Human plasma was mixed with increasing concentrations of cynomolgus macaque plasma in EIA buffer and subjected to the EIA as described to see if macaque FIX inhibited human FIX binding to 3A6. Human FIX concentrates (10 U kg^{-1}) (Christmassin M®; Mitsubishi Pharma Co., Tokyo, Japan) were injected intravenously into three cynomolgus macaques. After injection, peripheral blood was drawn and citrated plasma was collected at indicated time periods. Concentrations of human FIX in cynomolgus macaque plasma were determined using 3A6 as a capture antibody with detection by biotin-conjugated JKFIX-1 as described above.

Binding of mAb 3A6 to deglycosylated FIX and canine FIX

Conditioned media of Chinese hamster ovary (CHO) cells secreting wild-type FIX and mutant FIX A262T were incubated in the absence or presence of N-glycosidase F (10 U mL^{-1}) (Roche Diagnostics GmbH, Mannheim, Germany) and analyzed for binding of 3A6 to deglycosylated wild-type FIX and deglycosylated FIX A262T by Western blotting [12]. We also studied the effect of canine FIX on binding of 3A6 to human FIX by the EIA. Human plasma (1 : 100 dilution) in the buffer containing increasing concentrations (0–30%) of canine plasma obtained from a beagle dog was subjected to the 3A6-based EIA as described above to see if the presence of canine FIX decreased binding of human FIX to mAb 3A6.

Immunohistochemical study of the cynomolgus macaque liver

A small part of the liver was obtained from a cynomolgus macaque under anesthesia with ketamine hydrochloride. Biopsy specimens were fixed with 4% paraformaldehyde in PBS at 4 °C

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for 2 h, 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 liver tissues at −25 °C, attached to poly lysine-coated glass slides and subjected to immunohistochemistry by the standard ABC method using mouse mAb 3A6, sheep polyclonal antibodies to human FIX, control IgG (normal sheep IgG, normal mouse IgG), biotin-conjugated second antibodies and streptavidin–biotin complex reagents (Vectarstain ABC Elite kit; Vector Labs), and diaminobenzidine (DAB). Sections were counterstained with Meyer's hematoxylin solution to visualize nuclei.

Results

After screening for a species-specific mAb that recognizes solely human FIX, we found that mAb 3A6 could distinguish human FIX from macaque FIX. We developed an EIA for human FIX using 3A6 and biotin-labeled JKIX-1. The assay is sensitive to 1 ng mL^{-1} purified human FIX and detects FIX antigen in human plasma at $1:3 \times 10^3$ dilution. No increase in substrate hydrolysis was observed when macaque plasma or canine plasma was added to the microtiter plates instead of human plasma. Furthermore, addition of cynomolgus macaque plasma to human plasma did not influence the substrate hydrolysis of human plasma containing samples (Fig. 1), nor did the presence of canine plasma in the buffer influence data of the EIA for human FIX.

Macaque FIX is highly homologous to human FIX with 97% amino acid similarity [13]. Since 3A6 binding is not dependent on divalent cations and binds to the catalytic domain of human FIX after RVV XCP treatment under reducing conditions on Western blotting (not shown), the 3A6 epitope was deemed to be probably a linear sequence. The amino acid sequence of cynomolgus macaque FIX is identical to rhesus macaque FIX [14]. Amino acid residues Gly226, Val227, Ala262, or Leu321 of human FIX were targeted as residues in the 3A6 epitope based upon the sequence similarity among human FIX, macaque FIX, and mouse FIX. Thus, these residues were substituted with Asp, Ala, Thr, or Ser, as these are the corresponding residues in macaque FIX. Because Gly226 and Val227 are positioned sequentially, site-directed mutagenesis was carried out to substitute these two residues with Asp and Ala in the same molecule. Conditioned media of stably transfected CHO-K1 cells were harvested and binding of 3A6 to wild-type FIX and FIX mutants was analyzed by the 3A6-based EIA, the JKIX-1-based EIA, and by Western blotting. As shown in

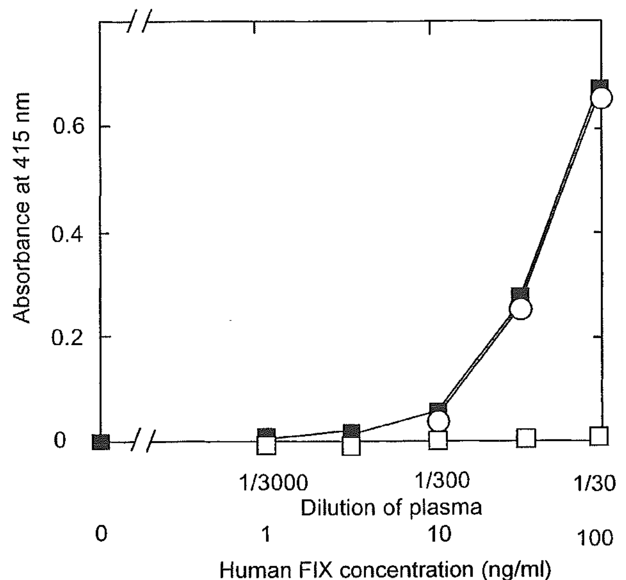

Fig. 1. Effect of macaque plasma on human factor (F)IX detection by 3A6-based enzyme immunoassay. Human plasma (■) or macaque plasma (□) diluted 30–3000-fold was incubated in microtiter plates coated with 3A6 antibody. Human plasma was diluted 30–300-fold with buffer containing 30% macaque plasma (○). Bound FIX was detected by biotin-labeled JKIX-1 followed by horseradish peroxidase-conjugated streptavidin as described in Methods.

Table 1 and Fig. 2, 3A6 bound to wild-type FIX, to FIX G226D/V227A and to FIX L321S, but failed to bind to FIX A262T. Human FIX does not have an oligosaccharide side chain at Asn260, but macaque FIX may have N-linked carbohydrates at this position because of formation of the N-linked glycosylation consensus sequence Asn-X-Thr. Mutant FIX A262T could also have an extra oligosaccharide side chain at Asn260. To study the possibility that the potential extra oligosaccharides linked to Asn260 of FIX A262T directly interfere with binding of mAb 3A6 to FIX A262T, the conditioned media of CHO cells secreting FIX A262T were treated with N-glycosidase F which can remove N-linked oligosaccharide side chains, including complex type carbohydrates, from glycoprotein and analyzed for 3A6 binding by Western blotting. As shown in Fig. 2B, 3A6 bound to wild-type FIX and deglycosylated FIX (lower molecular weight form) but did not bind to FIX A262T even after removal of carbohydrate side chains. Thus, the potential glycosylation at Asn260 may not be essential for no cross-reactivity of mAb 3A6 to FIX A262T and to macaque FIX, although it

Table 1 Concentration and activity of recombinant human factor (F)IX expressed in CHO-K1 cells

	Wild- type FIX	FIX G226D/V227A	FIX A262T	FIX L321S
3A6-based EIA, %	17.7	7.6	0	8.8
JKIX-1-based EIA, %	16.5	7.5	5.0	9.5
Clotting activity, %	16.3	1.8	3.4	2.9
Specific activity	0.99	0.24	0.68	0.30

Recombinant FIX antigen determined by enzyme immunoassay (EIA) is expressed as a percentage of the FIX concentration of control plasma. Specific activities are calculated by dividing the clotting activity of the conditioned medium by the antigen concentration determined by JKIX-1-based EIA.

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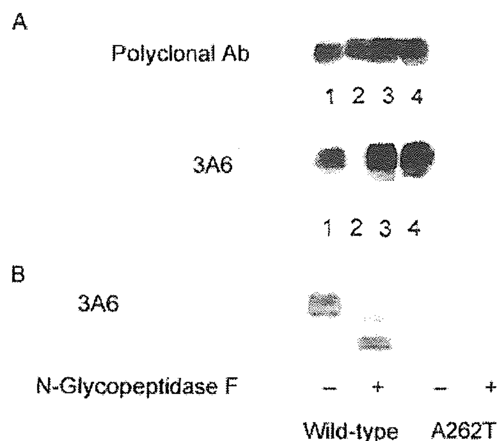

Fig. 2. Western blot analysis of factor (F)IX mutants. (A) Conditioned media of CHO-K1 cells secreting FIX G226D/V227A (lane 1), FIX A262T (lane 2), FIX L321S (lane 3) and wild-type FIX (lane 4) were transferred to polyvinylidene fluoride (PVDF) membrane after SDS–PAGE under reducing conditions and incubated with either polyclonal antibodies to human FIX or monoclonal antibody 3A6. Bound antibodies were detected with horseradish peroxidase (HRP)-labeled antisheep IgG or HRP-labeled antimouse IgG, respectively, followed by chemiluminescent reagents, as described in Materials and methods. (B) Chinese hamster ovary (CHO) cell-conditioned media containing wild-type FIX (wild-type) or FIX A262T (A262T) incubated in the absence (−) or presence (+) of N-glycopeptidase F were transferred to PVDF membrane after SDS–PAGE and analyzed for binding of 3A6 to recombinant FIX as above.

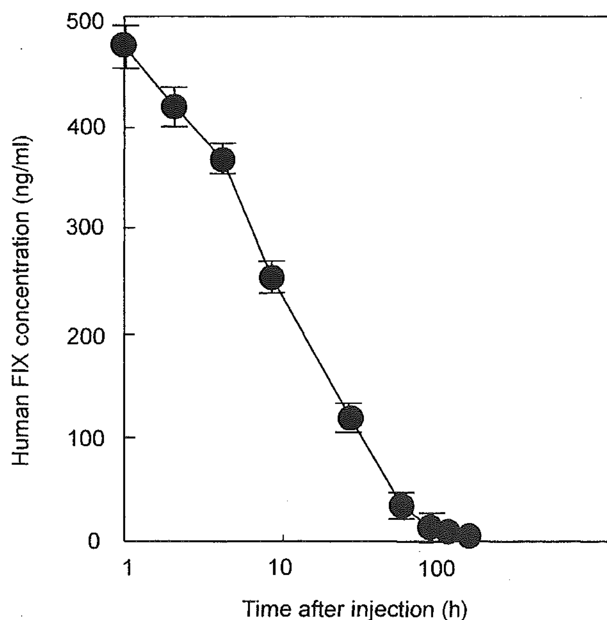

Fig. 3. Quantification of human factor (F)IX in cynomolgus macaque. FIX concentrates ($10\,U\,kg^{-1}$), used to treat hemophilia patients, were injected intravenously into three cynomolgus macaques. After injection, peripheral blood was drawn and platelet-poor plasma was prepared. Human FIX levels in macaque plasma was determined by enzyme immunoassay using 3A6 and biotin-labeled JKIX-1. Data represent mean ± SD ($n = 3$).

may affect the interaction of 3A6 with FIX A262T and with macaque FIX indirectly. The presence of canine plasma in the buffer did not decrease human FIX binding to 3A6 in the EIA (not shown), supporting this possibility. The coagulation activities of the FIX mutants were also determined to explore the possibility that these FIX mutants developed severe conformational changes in the catalytic domain by these amino acid substitutions. The specific activities (clotting activity of the conditioned medium divided by the antigen concentration determined by JKIX-1-based EIA) of recombinant wild-type FIX, FIX G226D/V227A, FIX A262T, and FIX L321S were 0.98, 0.24, 0.68, and 0.3, respectively (Table 1). These observations demonstrated that the amino acid substitutions did affect coagulation activity. However, FIX A262T did retain about 70% of the activity relative to wild-type FIX, suggesting that conformational effects were minimized and that loss of 3A6 binding to FIX A262T was due primarily to epitope alteration.

In order to detect and quantify human FIX in the cynomolgus macaque, $10\,U\,kg^{-1}$ human FIX was injected intravenously into three animals and the concentration of human FIX in their plasma was quantified with the 3A6-based EIA (Fig. 3). Human FIX plasma levels increased to $470 \pm 37.6\,ng\,mL^{-1}$ by 1 h after the injection, then gradually decreased with a half-life of 10 h. When FIX is injected intravenously into hemophilia B patients, circulating FIX levels decrease rapidly (α phase $t_{1/2}$, 4–5 h) then slowly (β phase $t_{1/2}$, 23–28 h) [15]. The rapid decrease of FIX is though to be due to distribution into extravascular space. Since human FIX levels in macaques at early time points after injection were not measured, our data may not accurately discriminate the rapid-phase and the slow-phase disappearance of human FIX in macaques. The difference between $t_{1/2}$ of intravenously injected human FIX in macaques in our experiments and that in the previous study [16] may be accounted for by the difference in FIX doses. On day 4, plasma human FIX levels fell to $4.2 \pm 2.8\,ng\,mL^{-1}$ (0.14% of the normal human FIX concentration) and were reduced further to $1.79 \pm 1.1\,ng\,mL^{-1}$ (0.06% of the normal human FIX concentraion) on day 5, indicating that the 3A6-based EIA was able to detect human FIX levels as low as 0.1% of the normal human FIX concentration.

To confirm that mAb 3A6 does not bind to cynomolgus macaque FIX *in vivo*, an immunohistochemical study was carried out. As shown in Fig. 4, positive immunostaining was observed when the frozen sections of the macaque liver were incubated with sheep polyclonal antibodies to human FIX. However, no immunostaining was observed in the liver sections incubated with 3A6.

Discussion

A wide variety of disorders are caused by genetic abnormality, giving rise to the potential of gene therapy as the next generation of therapeutics for many diseases. To establish gene therapy technology, a good animal model is required. Advances in developmental biotechnology have allowed us to create a variety of mouse disease models, transgenic mice and knock-out

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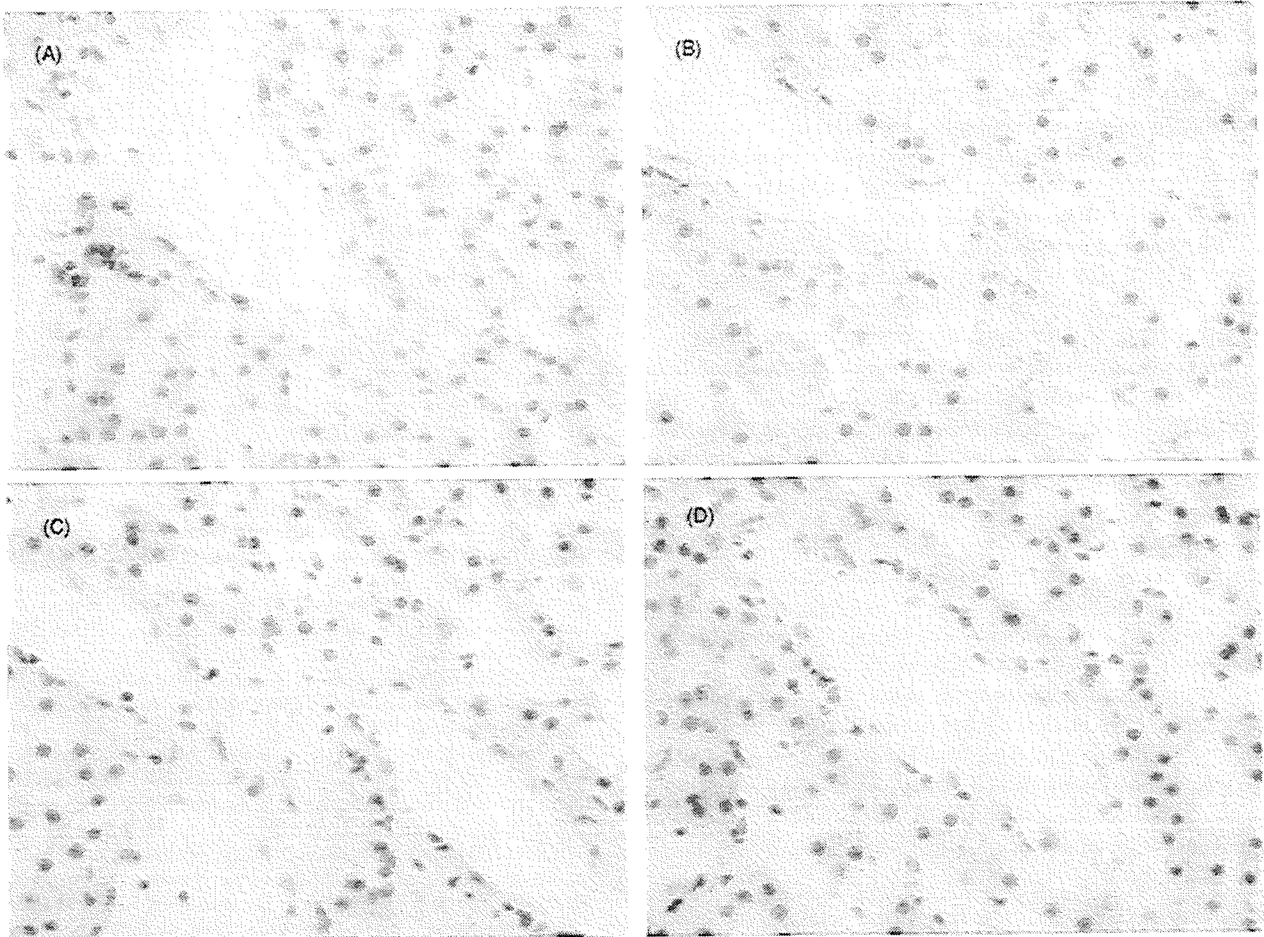

Fig. 4. Immunohistochemical study of the macaque liver. Frozen sections of the macaque liver on glass slides were incubated with monoclonal antibody 3A6 (A), normal mouse IgG (B), sheep polyclonal antibodies to human factor IX (C), or normal sheep IgG (D) and bound antibodies were detected by the avidin–biotin complex method as described in Materials and methods.

mice. However, there are obvious species differences between humans and mice, making it difficult under some circumstances to extrapolate data obtained in mice to human patients. Hemophilia B mice (FIX knock-out mice) and hemophilia B dogs have been used to study gene therapy approaches for treatment of hemophilia B [3–6]. Better animal models may be required, however, because there may be significant differences in transduction efficiency of skeletal muscles with AAV2 vectors between mice and dogs. Primates are used successfully as models in other disease applications, but there are as yet no hemophilic primates available. If one can distinguish human molecules from primate molecules *in vivo*, primates may be used for hemophilia gene therapy research, despite the fact that the genetic abnormality is not indigenous to the species.

Rhesus macaques are proposed to be a good primate model for studying hemophilia B gene therapy because of the amino acid sequence similarity between human FIX and macaque FIX and low immunogenicity of human FIX to rhesus macaque [13]. However, quantification of the human FIX expressed was difficult due to cross-reactivity of the rabbit anti-FIX antibodies. Rhesus macaques also developed antibodies to human FIX upon receiving viral vectors carrying the human FIX gene despite the high amino acid sequence homology [17]. A human FIX-specific EIA was developed using macaque antihuman FIX antibodies and EIA quantified human FIX levels in macaque plasma at 30 ng mL^{-1} (1% of the normal FIX level). The amino acid sequence of cynomolgus macaque FIX is identical to that of rhesus macaque FIX [14], raising the possibility that distinguishing the recombinant human molecule expressed in cynomolgus macaques *in vivo* from the endogenous macaque FIX molecule may be difficult. Thus, seven antihuman FIX mAbs available in our facilities were screened for their inability to bind to simian FIX. The 3A6 antibody did not bind to macaque FIX and an EIA was developed with this antibody to quantify human FIX in macaque plasma. The EIA was approximately 20-fold more sensitive than that used in the previous study [13], detecting human FIX at 1.79 ng mL^{-1}, or 0.06% of the normal plasma levels in cynomolgus macaques. The advantage of mAb 3A6 was also confirmed by the data that 3A6 did not react with macaque FIX in the liver by immunohistochemistry (Fig. 4).

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Type 2 AAV vectors were initially considered for expression of transgenes in skeletal muscle, although there appears to be a significant difference in transduction efficiency of skeletal muscles with AAV vectors among different AAV serotypes. In mice, type 1 AAV vectors appear to be superior to other AAV serotypes, since types 3 and 5 AAV vectors are less potent than the type 1 AAV vector but have better skeletal muscle transduction efficiency than type 2 AAV vectors [18]. It remains possible that the transduction efficiency of human skeletal muscle by different AAV vectors differs from that in mice and dogs. It is also possible that the transduction efficiency of other organs, such as the liver, by AAV vectors has significant species specificity.

The cynomolgus macaque is native to southern Asia and has been used as a simian model in medical research, such as in Parkinson's disease. As reported previously, human FIX could be immunogenic to rhesus macaques transduced with adenoviral vectors or AAV vectors carrying the human FIX gene and to cynomolgus macaques that received repeated subcutaneous injections of human FIX in the presence of Freund's adjuvant [13,15]. Thus, as far as antibodies to human FIX develop in macaques during transduction with vectors carrying the human FIX gene, the long-term study for human FIX expression is impossible, but if antibody development to expressed human FIX is suppressed, macaques may mimic the human situation more closely and provide a more accurate assessment of viral vector transduction efficiencies, and we may be able to evaluate the efficacy of therapeutic FIX gene construction and select appropriate promoters, vectors, and organs for transgene expression, taking advantage of monoclonal antibody 3A6.

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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±39.8 ng/10⁶ 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

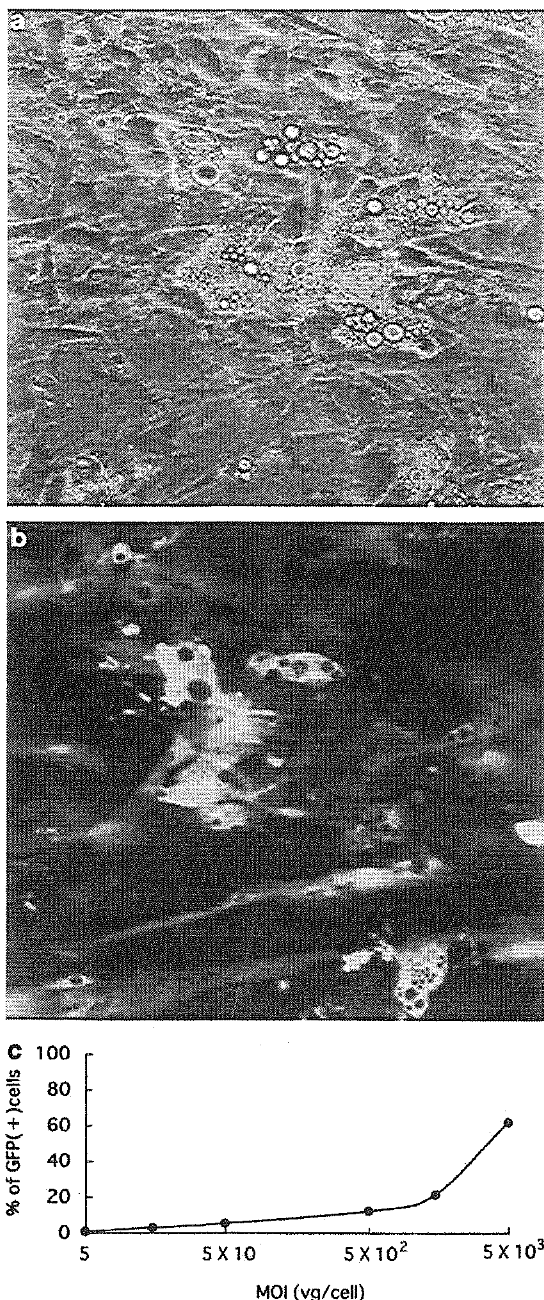

Figure 1 Transduction of human white adipocytes with SIVeGFP. Human white adipocytes (6.7×10^4cells/well) were incubated with increasing concentrations of SIVeGFP for 48 h. After incubation, cells were washed with PBS and incubated in media for 72 h (a, phase contrast). Expression of eGFP was visualized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

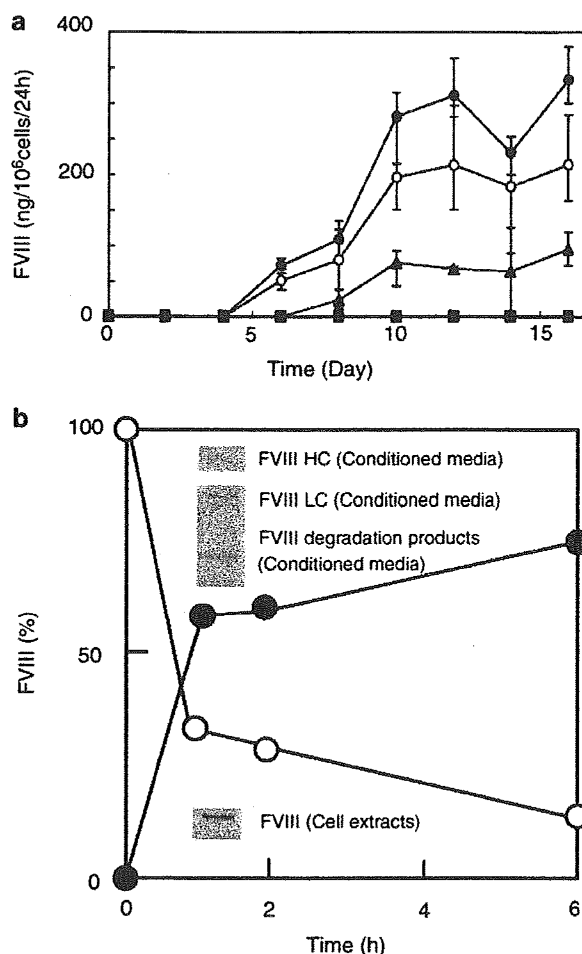

Figure 2 Transduction of human white adipocytes by SIVhFVIII. (a) Human white adipocytes (6.7×10^4 cells/well) were incubated with increasing concentrations of SIVhFVIII (■, 2×10^2 vg/cell; ▲, 6.5×10^2 vg/cell; ˆ, 2×10^3 vg/cell; ●, 6.5×10^3 vg/cell). After 48 h, cells were washed with PBS and cultured 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 () 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.

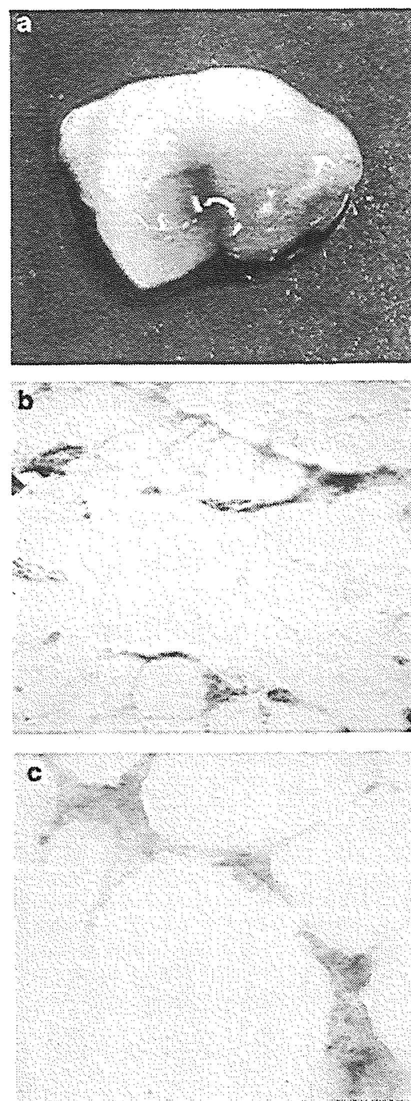

Figure 3 Transduction of the adipose tissues of db/db mouse by SIVlacZ. The SIVlacZ vector (5×10^7) was diluted in PBS and was injected into the subcutaneous adipose tissues of db/db mice. The subcutaneous adipose tissues were excised on day 14 after injection. Tissues were processed for detection of 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).

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.

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.

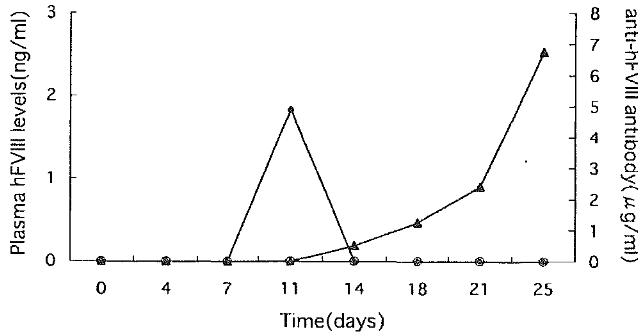

Figure 4 *Plasma human FVIII levels in SIVhFVIII-injected db/db mice. Peripheral blood was obtained from SIVhFVIII-injected mice or the SIVlacZ-injected control mice on days 0, 4, 7, 11, 14, 18, 21, and 25 after injection. Human FVIII concentrations in plasma of db/db mice who received SIVhFVIII (closed 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.*

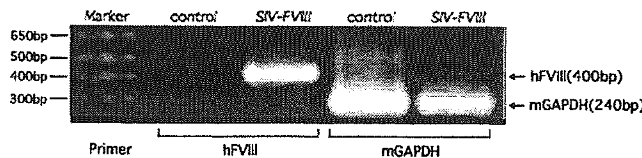

Figure 5 *RT-PCR analysis of adipose tissue-derived RNA. RNA was isolated from the murine adipose tissue on day 14 after vector injection. A measure of 100 ng of RNA was subjected to RT-PCR with specific primer pairs for the human BDD-FVIII transcript (human FVIII) or for the mouse GAPDH transcript (mouse 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.

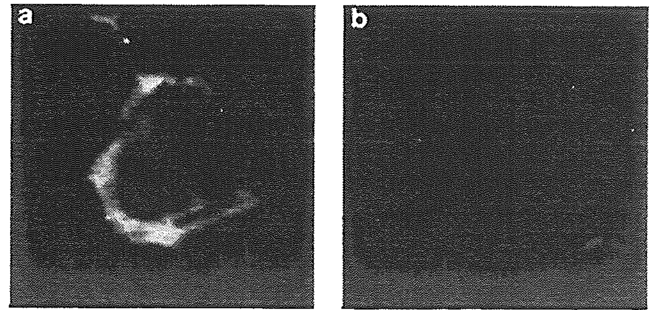

Figure 6 *Detection of human FVIII antigen in mouse adipose tissue. The adipose tissue of SIVhFVIII injected db/db mice (a) or SIVlacZ-injected mice (b) were subjected to immunofluorescence staining for human FVIII antigen. Tissue sections were incubated with sheep anti-human FVIII polyclonal antibodies. 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