表2　年齢による生体内回収率の違い

年齢	生体内回収率 (%)	変動係数 (%)	第Ⅸ因子活性上昇値 (U/dL per IU/kg)	変動係数 (%)
16歳未満	44.2±8.9 (72)	20.0	0.88±0.18 (72)	20.1
16歳以上	61.3±16.8 (100)	26.1	1.23±0.34 (100)	27.3

生体内回収率および第Ⅸ因子活性上昇値：平均±標準偏差（16歳以上の値を100%とした時の%）

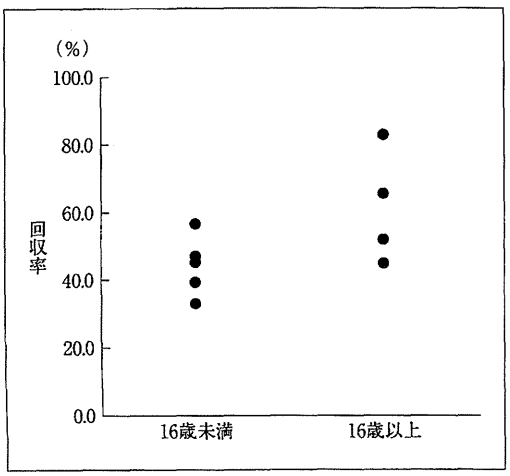

図1　年齢による生体内回収率の違い

また，第Ⅸ因子活性上昇値（IVR）は，16歳未満が0.88±0.18（U/dL per IU/kg）であり，16歳以上が1.23±0.34（U/dL per IU/kg）であった。この結果は，生体内回収率の結果とよく一致している。

3．体重，Hct値およびBMIと生体内回収率の相関

体重と生体内回収率の相関について検討した結果〔**図2**-(a)〕，有意な正の相関が確認された（P＝0.048，決定係数R^2＝0.448）。体重は，年齢により増加することから，年齢と生体内回収率の相関についても検討したが，有意な相関はなかった（P＝0.105）。

次に，Hct値と生体内回収率の相関を確認した結果〔**図2**-(b)〕，有意な相関はなかった（P＝0.564）。また，BMIと生体内回収率の相関を検討した結果〔**図2**-(c)〕，有意な正の相関が確認された（P＝0.002，決定係数R^2＝0.805）。

このように，生体内回収率は，体重およびBMIと正の相関が認められたことから，体重やBMIは第Ⅸ因子製剤の回収率を左右する重要な因子である可能性が示唆された。

4．同一患者の生体内回収率

4例の患者で個人の生体内回収率を比較した結果，0～23カ月の期間で回収率の個人の変動係数は，1.6～21.0%と大きく異なる場合も確認された（**表3**）。同じ月内で測定された生体内回収率は比較的近い値を示したが，12カ月以上の間隔がある場合には回収率が大きく異なっていた。したがって，患者の日常管理として，生体内回収率を測定する場合には1年に1回程度の実施が必要と考えられ，その結果を基に適切な投与量を設定することが患者のQOLの維持・向上に重要である。

なお，大きな手術を実施する場合には，事前に生体内回収率の測定を実施することは十分な止血管理を実施するという手術管理上の必要性ばかりでなく，不必要かつ過剰な投与を避けるという観点からも重要である。

5．ノバクトM静注用と生体内回収率

今回の検討は，第52回日本小児血液学会総会（2010年12月）において発表した内容[11]から血漿由来第Ⅸ因子製剤であるノバクトM静注用のデータのみを抽出し，その後の臨床データを加え，再度解析をした。また，学会発表時には，製剤の表示値（規格値）で生体内回収率等を測定したが，今回の検討では製剤の実測値で求めた。

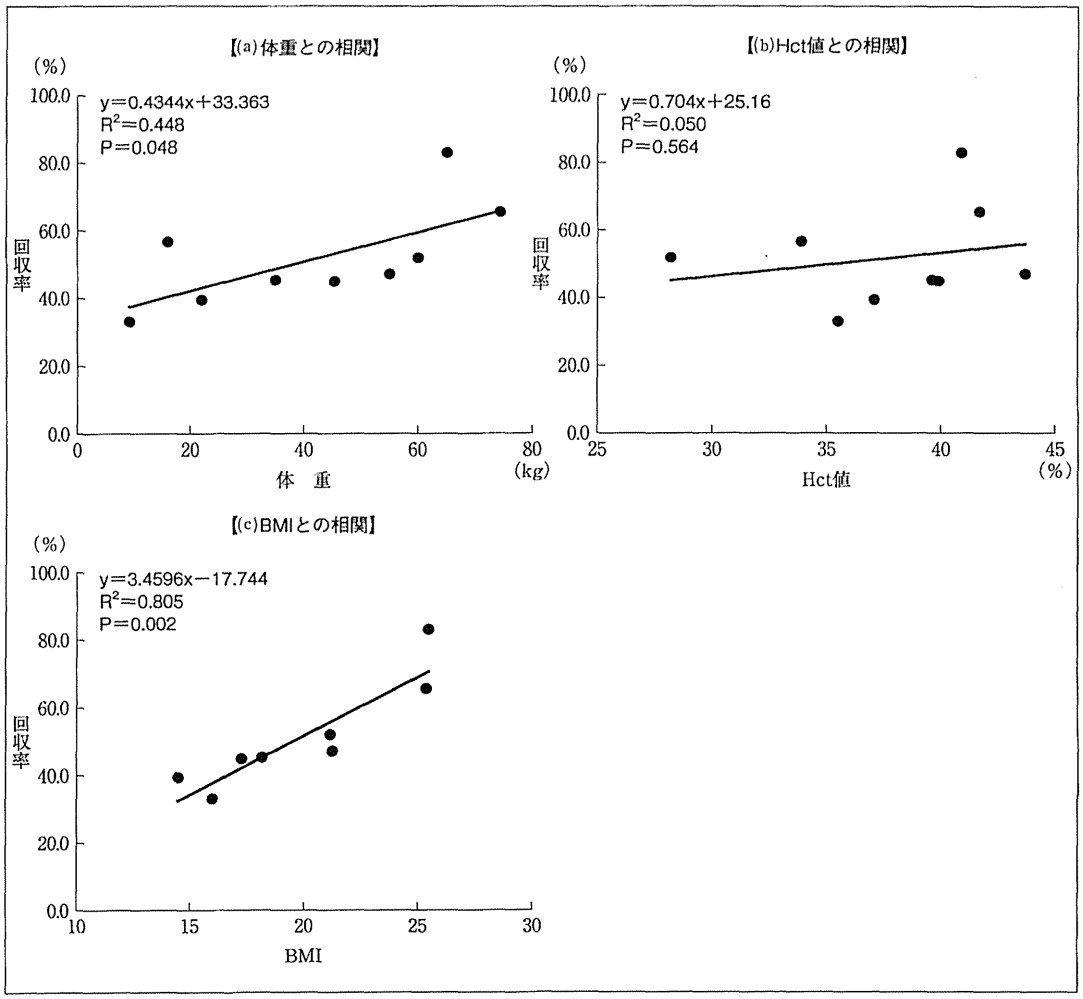

図2　体重，Hct値およびBMIと生体内回収率の相関

旧来のノバクトMの生体内回収率は，臨床試験[12]の成績として平均84.3%と報告されている。しかし，当時のノバクトMは，第Ⅸ因子活性が50単位/mL（倍/mL）の製剤であり，現在の第Ⅸ因子国際標準品と測定方法で評価すると，第Ⅸ因子活性が80国際単位/mL（IU/mL）に相当する。このため，実質的に投与された第Ⅸ因子量は，1.6倍多いことになる。臨床試験の生体内回収率は国際単位で評価すると，見かけ上の投与が増加することに伴い，旧来の生体内回収率の平均84.3%を1.6で除した値である平均52.7%になる[13]。この値は，今回の検討した生体内回収率（平均51.8%）とよく一致している。

ま　と　め

治療歴のある血友病B患者において血漿由来モノクローナル抗体精製血液凝固第Ⅸ因子製剤（ノバクトM静注用）の薬物動態について検討した。

1）生体内回収率は，51.8±15.0%であり，第Ⅸ因子活性上昇値（IVR）は1.04±0.30（U/dL per IU/kg）であった。

2）年齢による生体内回収率の違いを検討

表3　同一患者の生体内回収率

患者番号	測定間隔（カ月）	生体内回収率（%）	平均	標準偏差	変動係数（%）
1	0	51.7	44.2	6.6	14.9
	4	41.7			
	23	39.3			
2	0	44.6	39.4	5.9	14.9
	0	40.5			
	12	33.0			
8	0*	53.1	52.5	0.9	1.6
	0	51.9			
9	0	76.3	66.5	13.9	21.0
	14	56.6			

*：出血時の測定

した結果，16歳未満の回収率は44.2±8.9%であったのに対し，16歳以上の回収率は61.3±16.8%であり，両者は大きく異なっていた。

3）体重とBMIは，生体内回収率と有意な正の相関が確認されたが，Hct値は回収率と有意な相関がなかった。

4）今回の検討では，生体内回収率に個人差があること，更に同じ患者でも回収率に変動があることが確認されたので，個々の患者で回収率を適宜測定し，その結果を基に適切な投与量を設定することが重要である。

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13) ノバクト®M静注用400単位・800単位・1600単位 添付文書（2012年2月改訂）

Minimizing the Inhibitory Effect of Neutralizing Antibody for Efficient Gene Expression in the Liver With Adeno-associated Virus 8 Vectors

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Neutralizing antibodies (NAbs) against adeno-associated viruses (AAVs) are known to interfere with AAV vector-mediated gene transfer by intravascular delivery. Evading the inhibitory effects of antibodies against AAV vectors is necessary for efficient transfer of therapeutic genes clinically. For this purpose, we tested the efficacy of saline flushing in order to avoid contact of vectors with NAbs present in blood. Direct injection of the AAV8 vector carrying the *factor IX* (*FIX*) gene into the portal vein of macaques using saline flushing achieved transgene-derived FIX expression (4.7 ± 2.10–10.1 ± 5.45% of normal human FIX concentration) in the presence of NAbs. Expression was as efficient as that (5.43 ± 2.59–12.68 ± 4.83%) in macaques lacking NAbs. We next tested the efficacy of saline flushing using less invasive balloon catheter-guided injection. This approach also resulted in efficient expression of transgene-derived FIX (2.5 ± 1.06–9.0 ± 2.37%) in the presence of NAbs (14–56× dilutions). NAbs at this range of titers reduced the efficiency of transduction in the macaque liver by 100-fold when the same vector was injected into mesenteric veins without balloon catheters. Our results suggest that portal vein-directed vector delivery strategies with flushing to remove blood are efficacious for minimizing the inhibitory effect of anti-AAV antibodies.

Received 21 August 2012; accepted 14 November 2012; advance online publication 18 December 2012. doi:10.1038/mt.2012.258

INTRODUCTION

Gene and cell therapies are expected to be the next generation of therapies for a variety of inherited diseases. Hemophilia is thought to be an ideal target disease for these approaches as it is caused by a genetic abnormality in the factor VIII gene for hemophilia A, or the factor IX (FIX) gene for hemophilia B.[1–7] The current strategy of hemophilia gene therapy involves inducing expression of the normal coagulation factor gene or transplanting cells expressing the respective coagulation factor. The liver is normally the primary target of gene transfer for coagulation factors since the majority of these coagulation factors are synthesized in the liver with appropriate post-translation modifications before secretion into the circulatory system.

Substantial effort has been applied to express coagulation factor genes using various vector types. Among the viral vectors, recombinant adeno-associated virus (AAV) vectors are preferred for therapeutic gene transfer *in vivo* because they reside in the episome and rarely integrate into genomes. However, retrovirus vectors including lentivirus vectors require integration into the host cell genome.[6,7] In addition, AAV vectors can transfer genes to nondividing cells and allow long-term expression of transgenes in these cells.

Clinical trials for hemophilia gene therapy have recently been conducted using various types of vectors.[4–11] These trials were designed based upon data obtained from mouse models of hemophilia and hemophiliac dogs and proved to be more efficient in these models than for humans. Species differences between humans and these other animal models might partially account for the results observed. Therefore, gene transfer studies in non-\ human primates may well predict the efficacy of gene transfer in humans. Indeed, *FIX* gene transfer studies using a new type of vector have been conducted in rhesus macaques.[12,13] The results from these studies provided the basis for recent hemophilia B gene therapy clinical trials employing an AAV8 vector.[13–16] Gene transfer in mice using AAV vectors results in excellent transduction efficiency. This is especially so for AAV8 vector-mediated gene transfer in the mouse liver;[12–14,17] however, the efficacy of AAV8 vectors is modest in macaques.[13]

There are also difficulties associated with *FIX* gene expression when using AAV8 vectors in nonhuman primates. Growing evidence suggests that the presence of neutralizing antibodies (NAbs) against AAV8, due to previous natural infection by wild-type AAV, significantly inhibits transduction in the macaque liver. It is likely that antibodies against one serotype of AAV cross-react with other AAV serotypes.[18] A hemophilia B gene therapy

J.M., H.M., S.H., and T.I. contributed equally to this work.

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Table 1 Expression of macaque T262A in nonhuman primates with AAV8-HCRHAAT-macFIXT262A

Macaque number	Age	Vector dose (vg/kg)	Route of vector injection	FIX T262A concentration (%)	Vector genome copies in liver tissue (vg/diploid genome)	Anti-AAV8 NAb titer
#14	5.7	1×10^{12}	Mesenteric vein	0.02 ± 0.019	0.1	56×
#17	5.8	1×10^{13}	Mesenteric vein	0.13 ± 0.081	0.4	56×
#24	6.6	1×10^{12}	Mesenteric vein	0.09 ± 0.048	0.5	14×
#28	7.8	5×10^{12}	Saphenous vein	12.68 ± 4.83	38.2	Negative
#30	2.9	5×10^{12}	Saphenous vein	5.43 ± 2.59	48.2	Negative
#31	2.9	5×10^{12}	Saphenous vein	7.64 ± 2.32	49.6	Negative

Abbreviations: AAV, adeno-associated virus; FIX, factor IX; HAAT, α1-antitrypsin; HCR, hepatic control region.
The concentration of macaque FIX T262A is expressed as a percentage of normal human plasma FIX concentration; anti-AAV8 neutralizing antibody (NAb) titer is expressed as the final dilution of the test serum in the assay; vector genome (vg) copies in liver cells were determined by quantitative PCR and expressed as copy numbers per cell.

clinical study using an AAV8 vector was successfully conducted in hemophilia B patients negative for pre-existing antibodies against AAV8.[15] Because of the high prevalence of AAV infection in humans,[18] evading NAbs against this virus is an important hurdle to overcome before AAV8 vectors can be routinely and effectively employed for therapies.

The aim of our study was to develop an administration method of AAV8 vectors that assisted in minimizing the inhibitory effect of NAbs against AAV in macaques that were already seropositive for AAV8 antibodies.

RESULTS

The AAV8 vector carrying the macaque *FIX T262A* gene located downstream of the liver-specific chimeric promoter consisted of an enhancer element of hepatic control region (HCR) of the *ApoE/C-I* gene and the 5′ flanking region of the *α1-antitrypsin* (HAAT) gene (AAV8-HCRHAAT-macFIXT262A). This vector was used to express mutant macaque FIX containing a single amino acid substitution of Thr to Ala at the position 262 (macaque FIX T262A) in the following experiments. Macaque FIX T262A but not wild-type macaque FIX could be bound to human FIX-specific monoclonal antibody 3A6, thereby macaque FIX T262A expressed in macaques with AAV8-HCRHAAT-macFIXT262A could be precisely quantified by an enzyme immunoassay with 3A6.[17] The amino acid sequence of macaque FIX is highly homologous to the human FIX amino acid sequence. Twelve amino acid residues of human FIX are different at corresponding positions of macaque FIX, while only one amino acid of macaque FIX T262A is different from wild-type macaque FIX. Expression of macFIX T262A in a macaque would mimic a situation where normal human FIX is expressed in a hemophilia B patient with a missense mutation in the *FIX* gene.

Results corresponding to the expression of macaque FIX T262A following injection of AAV8HCRHAATmacFIXT262A can be seen in **Table 1**. When AAV8HCRHAATmacFIXT262A (5×10^{12} vector genome copies (vg)/kg) was injected into the saphenous veins of three AAV8 NAb-negative macaques (#28, #30, #31), expression of macFIX T262A in the therapeutic range (>5% of normal FIX concentration) was achieved. However, injection of the same vector (1×10^{12}–1×10^{13} vg/kg) into the mesenteric vein branches of AAV8 NAb-positive macaques (#14, #17, #24; inhibitory titers: 14–56×) resulted in subtherapeutic levels (<0.2%) of macFIX T262A expression. The amount of vector DNA in the liver of AAV8 NAb-positive macaques was ~1% of that seen in AAV8 NAb-negative macaques (**Table 1**). These data suggest that low titers of NAbs against AAV8 significantly inhibit transduction even when the vector is injected into the mesenteric vein branches. In addition, only short period of time may be required for NAbs in the blood to neutralize the AAV8 vector since the blood of the mesenteric vein rapidly goes to the liver through the portal vein after gathering with the blood from other viscera.

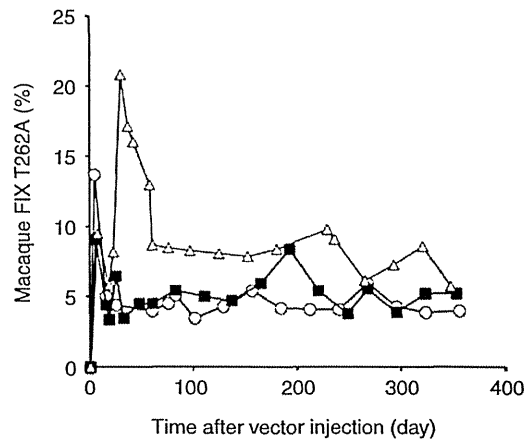

Figure 1 Expression of FIX T262A in macaques after direct vector injection into portal veins. Macaques (n = 3) were subjected to direct injection of AAV8 vector into the portal vein. Concentrations of FIX T262A in macaque plasma samples (macaque #26, open triangles; #27, open circles; #29, closed squares) were measured by ELISA. AAV, adeno-associated virus; ELISA, enzyme-linked immunosorbent assay; FIX, factor IX.

Evading AAV8 NAbs could be achieved by ensuring the AAV8 vector and NAbs do not come into physical contact with each other in the blood. Blood enters the liver from the hepatic artery and portal vein. The hepatic artery accounts for ~20–30% of blood flow, while the portal vein supplies the remaining blood flow to hepatocytes.[19,20] Blood from the portal vein and hepatic artery are eventually mixed in the sinusoids of the liver; however, the blood from the portal vein mainly supplies hepatocytes. Therefore, direct injection of AAV8 vectors into the portal vein branch was

Table 2 Expression of macaque T262A in nonhuman primates with direct, and balloon catheter-guided vector (AAV8-HCRHAAT-macFIXT262A) injection into the portal vein

Macaque ID	Age	Vector dose (vg/kg)	Injection method to portal vein branch	FIX T262A concentration (%)	Vector genome copies in liver tissue (vg/diploid genome)	Anti-AAV8 NAb titer
#26	10.1	5×10^{12}	Direct	4.7 ± 2.10	77.9	28×
#27	7.4	5×10^{12}	Direct	10.1 ± 5.45	28.5	14×
#29	11.0	5×10^{12}	Direct	5.3 ± 1.40	64.3	14×
#37	7.5	5×10^{12}	Catheter-guided	9.0 ± 2.37	61.1	14×
#38	10.7	5×10^{12}	Catheter-guided	3.2 ± 1.21	13	56×
#42	7.7	5×10^{12}	Catheter-guided	2.5 ± 1.06	15.3	14×

Abbreviations: AAV, adeno-associated virus; FIX, factor IX; HAAT, α1-antitrypsin; HCR, hepatic control region; Nab, neutralizing antibody.
FIX T262A concentration is expressed as a percentage of normal human plasma FIX concentration; anti-AAV8 NAb titer is expressed as the final dilution of the test serum in the assay; vector genome (vg) copies in liver cells were determined by quantitative PCR and expressed as copy numbers per cell.

investigated to determine whether saline flushing to remove blood from the portal vein just before injection of the vector would diminish the inhibitory effects of anti-AAV8 NAbs. Three macaques (#26, #27, #29; inhibitory titers: 14–28×) were directly injected with vector (5×10^{12} vg/kg) into the left portal vein after flushing saline to remove blood (**Supplementary Table S1**). Expression of transgene-derived FIX (macaque FIX T262A) increased to therapeutic levels with the AAV8 vector carrying the macaque *FIX T262A* gene and persisted for greater than 1 year in the three macaques (**Figure 1**). Average FIX and vector genome levels in macaque liver tissues are presented in Table 2. Compared with the results of vector injection to the mesenteric vein of NAb-positive macaques #14, #17, and #24 (Table 1), the levels of macaque FIX T262A in the circulation of the macaques #26, #27, and #29 that received vector injection directly to the left portal vein with flushing to remove blood, were increased to therapeutic levels with significant amounts of vector genome detected (Table 2).

Blood chemistry analysis and liver biopsies were conducted following administration of the vector to determine whether there were any adverse effects induced by the injection. Moderate increases in liver enzymes, such as transaminases, were observed just after injection of the vector (**Supplementary Figure S1**). However, no significant pathological changes were seen in liver biopsy samples taken on days 14, 28 or 48 (data not shown). We did not observe an increase in the number of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling)-positive hepatocytes in the liver biopsy specimens (data not shown).

The direct injection of the AAV8 vector into the left portal vein branch with saline flushing to remove blood from the portal vein just before injection of the vector was effective to minimize the inhibitory effects of anti-AAV8 NAbs. Therefore, we explored the possibility of utilizing a balloon catheter to perform the vector injection into the portal vein branch with saline flushing to remove blood, taking the concern about the safety of the procedures into consideration. Using a microballoon catheter, we injected the vector into the left portal vein of three anti-AAV8 antibody-positive macaques (#37, # 38, #42; inhibitory titers: 14–56×) (Table 2, **Supplementary Table S2**). Fluorography in macaque #37 representing angiography of the portal vein branch is shown in **Figure 2** and **Supplementary Video S1**. Increase of FIX T262A to therapeutic levels was achieved in the three macaques (#37, # 38, #42),

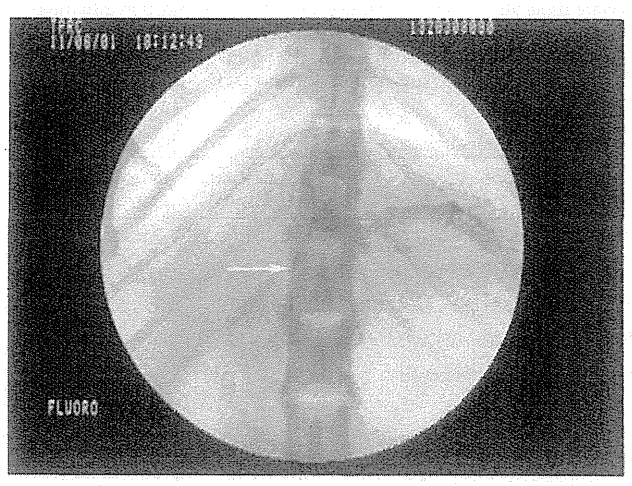

Figure 2 Fluorography in macaque #37. A balloon catheter was inserted into the portal vein of macaque #37 and contrast medium injected before vector administration. The left portal vein branches can be visualized. The arrow (white) indicates the tip of the catheter. See **Supplementary Video S1** which also recorded inflation of the balloon before the vector administration and deflation of the balloon after the administration.

and macaque FIX T262A expression in the circulation persisted (**Figure 3**, Table 2). The two portal vein vector delivery methods were successful in expressing macaque FIX T262A with the AAV8 vector in NAb-positive macaques (Table 2). The data suggest that the gene transfer efficiency using the catheter-guided vector injection method is similar to that of the direct vector injection into the portal vein branch with flushing.

Blood chemistry analysis and liver biopsies were conducted following injection of the vector. Increases in the levels of liver enzymes just after injection of the vector were not observed, suggesting that the ischemic effect of the temporary occlusion of the left portal vein branch was minimum compared with that of the direct vector injection procedure. Moderate increases in transaminases were observed following the vector injection, but did not persist (**Supplementary Figure S1**). Although the cause of the changes in the liver enzymes was not elucidated, no animals showed pathological changes, including histology of liver biopsy samples (data not shown).

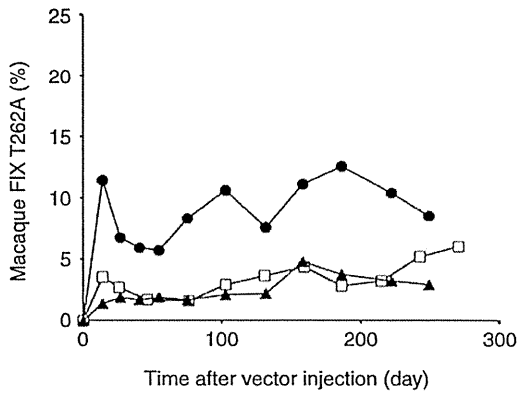

Figure 3 Expression of FIX T262A in macaques following balloon catheter-guided vector injection into portal veins. Three macaques (n = 3) were subjected to balloon catheter-guided vector injection into the portal vein. Concentrations of FIX T262A in macaque plasma samples (#37, closed circles; #38, open squares; #42, closed triangles) were measured by ELISA. ELISA, enzyme-linked immunosorbent assay; FIX, factor IX.

Table 3 Vector injection rate

Route of injection (macaque number)	Elapsed time (seconds)	Rate of injection (vg/kg/second)
Mesenteric vein (#14, #17, #24)	5	2×10^{11}–2×10^{12}
Saphenous vein (#28, #30, #31)	5	1×10^{12}
Portal vein (direct) (#26, #27, #29)	8–10	5×10^{11}
Portal vein (catheter) (#37, #38, #42)	15–22	2.3×10^{11}–3.3×10^{11}

Abbreviation: vg, vector genome.

Vector injection rates of the four different vector injection procedures are listed in Table 3 for comparison. The vector injection rates of the portal vein-directed strategies were similar to those of bolus vector injection into the saphenous vein and the mesenteric vein. Thus, the effect of vector injection speed on the transduction efficiency of the vector was thought to be minimal.

DISCUSSION

There are many features that make recombinant AAV vectors attractive for transferring therapeutic genes into target organs, and many vectors have been tried for the treatment of various diseases.[6,7,11,15,21–23] However, lines of evidence suggest that NAbs against AAV interfere with AAV vector-mediated gene transfer by intravascular vector delivery.[7,23–26] A clinical gene therapy trial for hemophilia B using a self-complementary AAV8 vector carrying the FIX gene has been conducted and reported to be successful.[15] However, even the self-complementary AAV8 vector failed to express FIX in a subject with a relatively high anti-AAV8 antibody titer compared with other subjects with no or lower antibody titers.[15]

According to the previous reports on the prevalence of NAbs against various AAV serotypes in normal subjects, the seropositivity against AAV8 is 15–30%, which is lower than that against AAV2 (50–60%), although the technical details of the NAb assay is different.[27,28] These reports have also demonstrated that the antibody titer against AAV8 is generally lower than for AAV2. Our data suggest that a low titer of NAbs against AAV8 can interfere with transduction even if the vector is injected into the mesenteric vein. Therefore, the use of another serotype vector such as AAV5 vector could be the next approach for this type of gene therapy because of the divergence in capsid sequence of AAV5 from other AAV serotypes.[13,16] Although the prevalence of NAbs against AAV5 is much lower than those against AAV1 and AAV2, and the prevalence of NAbs against AAV5 is comparable to or even lower than that against AAV8 in humans,[25,27] it is possible that subjects of gene therapy may contain cross-reactive NAbs against various AAV serotypes.

Another approach for evading NAbs against AAV could be the use of chemically or genetically modified AAV variants. Such variants could include AAV vector mutants with amino acid substitutions, or chimeric AAV vectors made by serotype shuffling.[23] Approaches that enable evasion of NAb inhibitory effects are necessary if researchers and clinicians wish to effectively apply AAV vectors for gene therapy because of NAb cross-reactivity.

An alternative approach for overcoming the inhibitory effect of NAb against AAVs is to develop a vector injection method. In the current study, two portal vein vector delivery strategies were employed that ensured that the AAV8 vector and NAbs do not come into physical contact with each other in the blood. These strategies were investigated using macaques whether the strategies could efficiently transduce hepatocytes with the AAV8 vector in the presence of NAbs. The first approach was the direct injection of AAV8 vectors into the portal vein branch after flushing with saline to remove blood. This strategy proved to be successful for the vector expressing FIX T262A in anti-AAV8 antibody-positive macaques. Since there are safety concerns about the direct vector injection method, injection of the vector into the portal vein using a balloon catheter was investigated. The catheter-guided vector injection may be less invasive than the direct vector injection into the portal vein branch because exfoliation of hepatic hilum is not required. In addition, fine surgical skills, such as manipulation of the hepatic hilum and suturing the venotomy site of portal vein after the direct vector injection without causing stenosis, are required for the direct vector injection method into the left portal vein. Obviously, catheterization from the mesenteric vein branch is required for the balloon catheter-guided vector injection method but insertion of a catheter into the portal vein from a branch of the mesenteric vein is not difficult for a cardiologist and a radiologist familiar with angiography. In addition, suturing the venotomy site of the mesenteric vein branch is easier and safer than suturing the venotomy site of portal vein, and the ischemic effect of this procedure was expected to be less than that of the direct vector injection into the portal vein branch. Taken together, these studies suggested that both the direct vector injection into the left portal vein and the balloon catheter-guided vector injection into the left portal vein were similarly effective for hepatocyte transduction with the AAV8 vector in the presence of low titer NAbs but the balloon catheter-guided vector injection method into the left portal vein was thought to be safer than the direct vector injection into the left portal vein.

Considering that the antibody titer against AAV8 was generally lower than that against AAV2 and that NAbs at low titers could interfere with the AAV8 vector-mediated gene transfer to the liver significantly, we selected macaques with low NAb titers

for the portal vein vector delivery strategies. However, the impact of the presence of high titer NAbs on the efficacy of these methods was not studied. Thus, the extent of AAV8 NAb titer, for that this approach is effective, needs to be investigated in the future.

In conclusion, we have provided the basis for an alternative approach for gene transfer to the liver that minimizes the deleterious effects of anti-AAV NAbs. Our result might expand the potential of the AAV vector-mediated gene delivery for medical application.

MATERIALS AND METHODS

AAV vector production. Construction of pAAV2-HCRHAAT-macFIX T262A and production of AAV8 carrying the macaque FIX T262A gene (AAV8HCRHAATmacFIXT262A) has been previously described.[17] Briefly, DNA fragments harboring the *macFIXT262A* gene located downstream of the chimeric promoter consisted of an enhancer element of HCR of the human ApoE/C-I gene and the 5′ flanking region of the human HAAT gene (HCRHAAT promoter), and the SV40 polyadenylation signal sequence flanked by AAV2 inverted terminal repeats in pAAV2-HCRHAAT-macFIX T262A. The genes were packaged by triple plasmid transfection of human embryonic kidney 293 cells (Avigen, San Diego, CA) to generate AAV8-HCRHAAT-macFIXT262A, with the chimeric packaging plasmid (AAV2 rep/AAV8 cap), and the adenovirus helper plasmid pHelper (Stratagene, La Jolla, CA), as previously described.[17] The chimeric packaging plasmid for AAV8 capsid pseudotyping[29] was constructed by inserting the synthetic AAV8 *Cap* gene (Takara Bio, Otsu, Shiga, Japan) downstream of the AAV2 Rep gene of pHelp19. For virus vector purification, the DNase (Benzonase; Merck Japan, Tokyo, Japan)-treated viral particles containing samples were subjected to two rounds of cesium chloride-density gradient ultracentrifugation in HEPES-buffered saline (pH 7.4) supplemented with 25 mmol/l EDTA at 21 °C, as previously described.[17] Titration of recombinant AAV vectors was carried out by quantitative PCR using a real-time PCR system (StepOnePlus; Applied Biosystems Japan, Tokyo, Japan).[17] AAV8HCRHAATmacFIXT262A was previously shown to express macaque FIXT262A in mice efficiently.[17] Human FIX could be expressed in macaques and detected, however, macaques developed antibody against human FIX under certain experimental conditions. Only one amino acid residue at position 262 was humanized in macaque FIX T262A for detection with the human FIX-specific monoclonal antibody.

Animals. Cynomolgus macaques were bred and maintained at the Tsukuba Primate Research Center (Ibaraki, Japan). The animal experiments using macaques were performed at the Tsukuba Primate Research Center according to the guidelines of the Institutional Animal Care and Concern Committees at Jichi Medical University and the Tsukuba Primate Research Center. The use of macaques in animal experiments was approved by the Animal Care and Concern Committees. All surgical procedures were carried out under anesthesia, with vital signs and electrocardiogram monitoring conducted in accordance with the stipulated guidelines. Male macaques with low NAb titers (<56×) were used in this study.

Vector injection from peripheral and mesenteric vein. Injection of AAV8 vector to a saphenous vein (peripheral vein) was performed under intramuscular anesthesia. Injection of the AAV8 vector into a terminal branch of the superior mesenteric vein was carried out with laparotomy under anesthesia with isoflurane and electrocardiogram monitoring.

Direct portal vein vector injection with saline flushing. Direct injection of the vector solution into the left portal vein was carried out after induction of general anesthesia with isoflurane and sterilization. A right subcostal incision (5 cm) was made through the skin and the subcutaneous tissue. The abdominal cavity was explored and the soft tissue of hepatic hilum was exfoliated surgically, then the main portal vein and its right and left branches were exposed. The main portal vein was cannulated with a plastic cannula type 20G needle (Surflo; Terumo, Tokyo, Japan), which was advanced into the left portal vein branch. The left and right portal vein branches were then clamped with vascular forceps. After flushing the left portal vein with saline, the vector solution was injected, and then a second saline solution, for flushing, was injected. Volumes of solutions used in the experiments were determined by taking a standard liver volume, a hepatic vascular bed volume, and effects of solutions on the systemic circulation into consideration.[19,30] A standard liver volume of a macaque was estimated with the formula (standard liver volume = 706.2 × body surface area + 2.4)[31] and the vascular bed volume of the liver was estimated to 25–30% of the standard liver volume.[19] A hepatic vascular bed volume can increase to 60% of the liver volume upon infusion of solutions and this may function as a reservoir and reduce the effects of the solutions on the systemic circulation.[20,32,33] The forceps were then removed immediately and the venotomy site was closed with an 8-0 prolene suture.

Catheter-guided vector injection to the portal vein with saline flushing. Balloon catheter-guided injection of the vector into the left portal vein of AAV8 antibody-positive macaques was carried out after the induction of general anesthesia. A 5-cm right paramedian incision was made through the skin and subcutaneous tissue. The abdominal cavity was carefully entered, with a part of the ileum identified and brought out through the incision. A peripheral branch of the superior mesenteric vein was cannulated with a plastic cannula type 20G needle (Surflo; Terumo). A temporary occlusion microcatheter (Iliguman 3.3F; Fuji System, Tokyo, Japan) was advanced into the left portal vein using a guide-wire (run through 0.014 (0.36 mm); Terumo) under a fluoroscope. The positions of the catheter and the balloon were confirmed by imaging with contrast medium. Blood flow in the left portal vein was occluded with a silicone balloon catheter and 40 ml of saline, followed by the AAV8 vector solution, and another 20 ml of saline was injected sequentially through the microcatheter. Volumes of solutions used in the experiments were determined as above with taking the result of the experiment of direct vector injection to the left portal vein branch into consideration. Following deflation of the balloon, the microcatheter was withdrawn and the peripheral venotomy ligated. The abdominal wall was then closed in layers.

Analysis of macaque FIX T262A expression in macaques. Macaque FIX T262A was bound to 3A6, a human FIX-specific monoclonal antibody for analyses. An enzyme-linked immunosorbent assay (ELISA) for the detection of macaque FIX T262A was carried out using 3A6, as previously described.[17,34]

NAb assay. An assay for the detection of anti-AAV8 NAbs was performed as previously reported, with some modifications.[35,36] Briefly, 5×10^4 2V6.11 cells/well were seeded in the wells of 96-well culture plates. Ponasterone A was added to the culture media the day before transduction to induce expression of the E4 gene. On the day of transduction, 10 µl of serum (undiluted, or subject to serial twofold dilutions) was incubated with the vector (AAV8-CMV-LacZ, 5×10^7 vg/10 µl) at 37 °C for 1 hour, and this mixture was added to a culture well. Sucrose was added to the culture medium such that the final concentration was 125 mmol/l. The culture medium was removed after a 48-hour incubation, and β-galactosidase activity quantified with a β-Gal assay kit (Invitrogen, Carlsbad, CA). Briefly, o-nitrophenyl-β-D-galactopyranoside was added to cell lysates, incubated for 30 minutes, and color change quantified with a microplate reader (Benchmark Plus; Bio-Rad, Hercules, CA). If β-galactosidase activity was inhibited with a test sample that contained more than 50% of control fetal bovine serum, it was judged as positive for neutralizing capacity. The inhibitory titer of the serum sample was expressed as the highest final dilution in the culture medium that showed inhibitory activity.

Quantitation of AAV8 vector DNA in macaque tissue. Quantitation of AAV8 vector DNA in macaque tissues was performed using quantitative

PCR assays using a StepOnePlus instrument (Applied Biosystems Japan). DNA was isolated from macaque tissues using a DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA) and subjected to PCR using primers 5′-GAT AAC TGG GGT GAC CTT GG-3′ and 5′-GCC TGG TGA TTC TGC CAT GA-3′, and Cybergreen reagent (Applied Biosystems Japan).

SUPPLEMENTARY MATERIAL

Figure S1. Changes in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in macaques.
Table S1. Direct vector injection into the portal vein of macaques.
Table S2. Balloon catheter-guided vector injection into macaques.
Video S1.

ACKNOWLEDGMENTS

This study was supported by Grants-in-Aid for Scientific Research (20591155, 21591249, and 21790920) and the Support Program for Strategic Research Infrastructure from the Japanese Ministry of Education, Culture, Sports, Science and Technology; and Health, Labor, and Science Research Grants for Research on HIV/AIDS and Research on Intractable Diseases from the Japanese Ministry of Health, Labor, and Welfare. This work was done in Shimotsuke, Tochigi and Tsukuba, Ibaraki, Japan. The authors declared no conflict of interest.

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Thrombosis Research 131 (2013) 444–449

Contents lists available at SciVerse ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Overexpression of factor VII ameliorates bleeding diathesis of factor VIII-deficient mice with inhibitors

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ARTICLE INFO

Article history:
Received 21 November 2012
Received in revised form 26 February 2013
Accepted 12 March 2013
Available online 6 April 2013

Keywords:
Factor VII
Adeno-associated virus vector
Hemophilia A
Gene therapy

ABSTRACT

Introduction: Factor VIII (FVIII) treatment for hemophilia A has difficulties in correcting bleeding diathesis in the presence of inhibitors.
Materials and Methods: An adeno-associated virus type 8 (AAV8) vector containing the factor VII (FVII) gene or the activated factor VII (FVIIa) gene was used to investigate the therapeutic effect of FVII or FVIIa overexpression in FVIII-deficient mice with inhibitors.
Results: Following repeated human FVIII injection, FVIII-deficient mice developed anti-human FVIII antibodies that cross-reacted with mouse FVIII. High transgene expression of murine FVII or murine FVIIa was achieved using the AAV8 vector and resulted in increased blood FVII activity greater than 800% of normal murine FVII levels in vector-injected FVIII-deficient mice. Thromboelastography analysis showed significant improvements in clotting time, clot formation time, α angle, and mean clot firmness in AAV8 vector-injected FVIII-deficient mice with inhibitors. Overexpression of FVIIa ameliorated the bleeding phenotype of FVIII-deficient mice with inhibitors and significantly increased the survival rate after tail clipping. In addition, overexpression of FVII increased the survival rate of FVIII-deficient mice with inhibitors after tail clipping though it was not as efficient as FVIIa overexpression.
Conclusions: These data suggest that FVII overexpression is an alternative strategy for the treatment of hemophilia A with inhibitors.

Introduction

Hemophilia A is an inherited X-linked bleeding disorder caused by abnormalities of the coagulation factor VIII (FVIII) gene. The current standard therapy for hemophilia A is intravenous injection of recombinant FVIII or plasma-derived FVIII concentrates. Prophylactic administration of FVIII concentrates is effective in preventing harmful bleeding. However, severe hemophilia A patients develop antibodies against FVIII (inhibitors) upon frequent infusion of FVIII concentrates. Gene therapy enables the prevention of life-threatening bleeding in the brain and harmful bleeding in joints by sustained elevation of coagulation factor levels and provides next generation therapy for hemophilia [1,2]. Indeed, clinical trials for hemophilia A and B have been conducted with a variety of gene therapy vectors [3–7]. The current strategy of gene and cell therapy for hemophilia A is the transfer of the normal FVIII gene *in vivo* or transplantation of cells expressing FVIII. However, this strategy may not work for hemophilia A with inhibitors. Thus, an alternative gene transfer approach for hemophilia A with inhibitors could be the overexpression of activated factor VII (FVIIa) [8], which is effective for reducing bleeding diathesis of hemophilia B mice and hemophilia A and B dogs [8,9]. In addition, ectopic expression of FVIIa in platelets reduced bleeding in hemophilia A mice [10]. In the current study, we investigated the forced expression of the mouse FVIIa gene or the FVII gene by an adeno-associated virus type 8 (AAV8) vector in FVIII-deficient (F8KO) mice in the presence of inhibitors against mouse FVIII to determine the therapeutic effect of overexpression of FVII using a genetic approach for hemophilia A with inhibitors.

Materials and Methods

Vector Construction

The characteristics and activity of the liver specific chimeric enhancer/promoter complex, consisting of an enhancer element of the hepatic

Abbreviations: FVII, Factor VII; FVIII, Factor VIII; AAV, adeno-associated virus; F8KO, Factor VIII knock out; HCR, hepatic control region; HAAT, human alpha-1 antitrypsin; CMV, cytomegalovirus; SV40, simian virus 40; PCR, polymerase chain reaction; PT, prothrombin time; APCC, activated prothrombin complex concentrate; FX, Factor X; FIX, Factor IX; FXII, Factor XII.

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http://dx.doi.org/10.1016/j.thromres.2013.03.007

control region (HCR, +1– + 325) of the human *Apo E/C-I* gene and the 5' flanking region of the human *α1-antitrypsin* (HAAT, −275– + 25) gene, (HCRHAAT promoter), were described previously [11,12]. The mouse FVII gene (cDNA) and mouse FVIIa gene were cloned in our laboratory as reported previously [10]. A DNA fragment encoding the murine FVII gene (cDNA) or murine FVIIa gene (cDNA) was inserted downstream of the HCRHAAT promoter of p1.1HCRHAAT [11,12] to produce p1.1HCRHAAT-mFVII or p1.1HCRHAAT-mFVIIa. A DNA fragment spanning the cytomegalovirus (CMV) promoter, the LacZ gene, and the polyadenylation signal sequence of the pAAV2 CMV-Lac Z plasmid (Strategene, La Jolla, CA, USA) was replaced by a DNA fragment spanning the HCRHAAT promoter, the FVII gene and the simian virus 40 (SV40) polyadenylation signal sequences of p1.1HCRHAAT-mFVII to produce pAAV2-HCRHAAT-mFVII. The gene transfer plasmid vector pAAV2-HCRHAAT-mFVIIa was constructed as for p1.1HCRHAAT-mFVII.

AAV Vector Production

The vector production system was kindly supplied by Avigen Inc. (San Diego, CA, USA). AAV vectors were packaged with the AAV8 capsid by pseudotyping [11,12]. The chimeric packaging plasmid for AAV8 capsid pseudotyping was synthesized as described previously [12]. DNA fragments harboring the mouse FVII gene or the mouse FVIIa gene located downstream of the HCRHAAT promoter and flanked by AAV2 ITRs were packaged by triple plasmid transfection of human embryonic kidney 293 (HEK 293) cells, kindly supplied by Avigen Inc., with the chimeric packaging plasmid (AAV2 rep/AAV8 cap), and the adenovirus helper plasmid pHelper (Stratagene, La Jolla, CA, USA), as described previously [11–13]. For virus vector purification, DNase (Benzonase, Merck Japan, Tokyo, Japan)-treated viral particle-containing samples were subjected to two rounds of cesium chloride-density gradient ultracentrifugation in HEPES-buffered saline (pH 7.4), in the presence of 25 mM EDTA, at 16 °C, as described previously [11,12]. Titration of recombinant AAV vectors was performed by quantitative polymerase chain reaction (PCR) using a real time PCR system (StepOnePlus, Applied Biosystems, Tokyo, Japan).

Animal Experiments

C57BL/6 wild-type mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). FVIII-deficient (F8 knock out, F8KO) mice with a targeted destruction of exon 16 of mouse *F8* were reported previously by Bi et al. [14], and generously provided by Dr. H. H. Kazazian Jr. (University of Pennsylvania, Philadelphia, PA, USA) [11,13]. Mice were maintained under standard lighting conditions in a clean room. All surgical procedures were carried out in accordance with the guidelines of the institutional Animal Care and Concern Committee of Jichi Medical University.

Inhibitor Generation in FVIII-deficient Mice

F8KO mice were repeatedly injected with human FVIII concentrates (Kogenate FS, Bayer Yakuhin Ltd, Tokyo, Japan) according to the method described by Madoiwa [15] with modifications. Briefly, F8KO mice (16-weeks-old) were injected with 0.05 U/g of human FVIII concentrates (Kogenate) once a week, 4 times and blood was drawn after immunization. Mouse plasma samples were subjected to the FVIII inhibitor assay (Bethesda method) using human FVIII-deficient plasma (Thrombocheck Factor VIII, Sysmex, Kobe, Japan) [15]. Cross-reactivity of FVIII inhibitors raised against human FVIII to mouse FVIII was determined with normal mouse plasma and mouse FVIII-deficient plasma obtained from untreated F8KO mice. Briefly, plasma obtained from F8KO mice was mixed with plasma from wild-type mice (C57/B6) for 2 hours and remaining FVIII activity was quantified using FVIII-deficient plasma.

Determination of Mouse FVII Activity in Mice

The AAV8 vector (5×10^{13} vg/kg) carrying either the mouse FVII gene (AAV8-mFVII) or the mouse FVIIa gene (AAV8-mFVIIa) was injected to the cervical vein plexus of 20-week-old F8KO mice under anesthesia. Blood was drawn from the cervical vein plexus and mixed with 1/10 volume of 3.8% sodium citrate 4 weeks after the vector injection. Platelet-poor plasma was prepared and FVII levels in mouse plasma were quantified by the prothrombin time (PT) method using prothrombin time reagent (Thrombocheck PT, Sysmex, Kobe, Japan) and FVII-deficient plasma (Sysmex, Kobe, Japan) and standardized to normal mouse plasma. Since the plasma of hemophilia A mice has normal mouse FVII activity, the baseline FVII activity of the plasma obtained was determined before vector injection. The plasma obtained from vector-injected mice was diluted and subjected to measurement of FVII activity.

Thromboelastography Analysis

Thromboelastography analysis of mouse blood was performed using a ROTEM apparatus (Pentapharm GmbH) as previously described [10]. Briefly, blood samples containing 0.38% sodium citrate were prepared 4 weeks after vector injection and analyzed using a ROTEM apparatus with the star-TEM reagent (Pentapharm GmbH) according to the manufacturer's instructions.

Tail Clipping Test

Mice were subjected to tail clipping under anesthesia 7 weeks after vector injection. Tails of mice were excised with surgical scalpels 2 cm proximate from the tail ends. Then mice were observed under standard conditions for 24 hours to determine the rate of mortality.

Statistical Analysis

Student's *t*-test, Welch's *t*-test and Chi-square test were used for statistical analysis using software Statcel Ver.3 for Excel. *P* values less than 0.05 were considered statistically significant.

Results

Inhibitor Development in F8KO Mice After Human FVIII Injection

Human FVIII is immunogenic in mice and repeated injection of human FVIII concentrates results in antibody development. As shown in Fig. 1, F8KO mice receiving repeated injection of human FVIII concentrates developed antibodies that cross-reacted with mouse FVIII. The inhibitory titer of the antibody against mouse FVIII (inhibitors) was sufficiently high to inhibit mouse FVIII. There was no significant difference between inhibitor titers in AAV8-mFVII-injected and AAV8-mFVIIa-injected F8KO mice. Thus, these mice could be used to study genetic approaches for hemophilia A with inhibitors.

Expression of Mouse FVII and Mouse FVIIa in F8KO Mice with Inhibitors

The AAV8 vector (5×10^{13} vg/kg) carrying either the mouse FVII gene or the mouse FVIIa gene was injected to F8KO mice with inhibitors against mouse FVIII, to investigate whether the vector could express high levels of FVII or FVIIa in mice. The mean level of FVII activity increased to more than 800% of the baseline in F8KO mice receiving AAV8-mFVII (Fig. 2). The levels of FVII activity in AAV8-mFVIIa injected mouse plasma were similar to those for AAV8-mFVII-injected F8KO mice. There was no significant difference between the FVII activity levels of AAV8-mFVII-injected F8KO mice and AAV8-mFVIIa-injected F8KO mice.

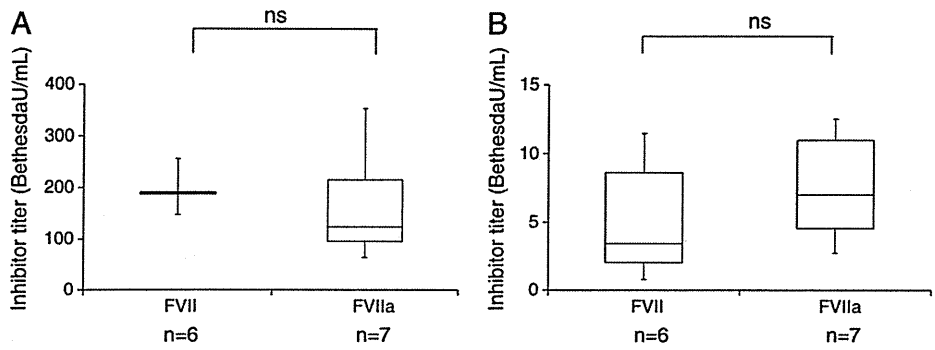

Fig. 1. Inhibitory titers of anti-FVIII antibodies. Inhibitory activity against human FVIII (A) and mouse FVIII (B) in F8KO mice are shown. Plasma samples were diluted in buffer, incubated with normal human plasma or wild-type mouse plasma for 2 hours and subjected to the APTT assay using FVIII-deficient plasma. Inhibitory titers were calculated according to the Bethesda method. Values are shown as Whisker Box plots. There were no significant differences between the FVII levels of AAV8-mFVII-injected mice (n = 6) and AAV8-mFVIIa-injected mice (n = 7) (Student's *t*-test). ns: not significant.

Treatment Efficacy of Overexpressed FVII or FVIIa

Treatment efficacy of overexpressed FVII and FVIIa by the respective AAV8 vectors was studied in F8KO mice with inhibitors by thromboelastography analysis and a tail-clipping test (Figs. 3, 4 and Table 1). The representative thromboelastograms of AAV8-mFVIIa-injected mice were comparable to wild-type mice except for the clotting time (Fig. 3). Thromboelastography analysis of blood from AAV8-mFVII-injected mice showed that the parameters and thromboelastogram improved but that the changes were to a lesser extent compared with AAV8-mFVIIa-injected mice. Thromboelastography analysis showed that the clot formation time, maximum clot firmness and α angle improved in F8KO mice with AAV8-mFVIIa injection and were similar to those of wild-type mice. All thromboelastography parameters except for the clotting time of FVIIa-overexpressed F8KO mice were similar to those of wild-type mice. Although the clotting time was shortened in FVIIa overexpressed F8KO mice, it was still prolonged relative to wild-type mice. The thromboelastography parameters of FVII-overexpressed F8KO mice were inferior to those of FVIIa-overexpressed mice. The improvement of the thromboelastography parameters from FVII-overexpressed F8KO mice was apparent. However, the *in vivo* effect of FVII or FVIIa overexpression might be less than expected from the thromboelastography analysis. Therefore, these mice were subjected to tail clipping challenge 7 weeks after vector injection to investigate whether overexpression of FVII or FVIIa could ameliorate the bleeding diathesis of F8KO mice. As reported previously, all F8KO mice that did not receive vector injection (n = 7) died within 24 hours of tail clipping regardless of the presence of inhibitors against mouse FVIII. The survival rate of AAV8-mFVIIa-injected mice (n = 7) was 85.7% and 50.0% for AAV8-mFVII-injected mice (n = 6), suggesting that overexpression of FVII and of FVIIa significantly ameliorated bleeding diathesis of F8KO mice with inhibitors.

The FVII activities in AAV8-mFVII-injected F8KO mice and AAV8-mFVIIa-injected mice were 515% (18 weeks after vector injection) and 488% (29 weeks after vector injection), respectively.

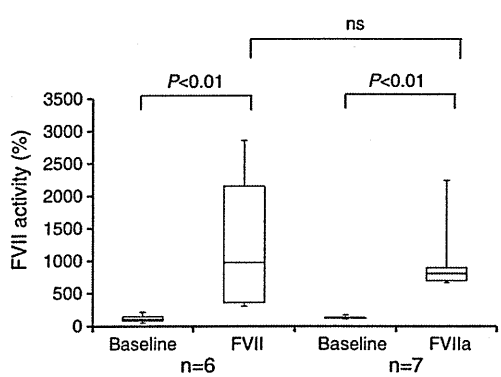

Fig. 2. Mouse FVII and mouse FVIIa expression in F8KO mice after injection of AAV8 vectors. FVII activity levels in plasma from F8KO mice 4 weeks after vector injection (FVII, AAV8-mFVII-injected mice (n = 6); FVIIa, AAV8-mFVIIa-injected mice (n = 7) were quantified. The baseline FVII activity levels (baseline) of these mice were also measured simultaneously. Values are shown as Whisker Box plots. The differences between values were analyzed by Student's *t*-test and *p* values are shown. ns: not significant.

Discussion

Inhibitor (antibody against infused FVIII) development in hemophilia A patients interferes with FVIII treatment. Thus, the next generation therapy for hemophilia A using a genetic approach to force expression of the normal FVIII gene or transplanting cells expressing FVIII may be applicable to hemophilia A patients without inhibitors, but may not be effective in correcting bleeding diathesis in hemophilia A patients with inhibitors. The incidence of inhibitor development in hemophilia A is much higher than for hemophilia B, suggesting that inhibitor development in hemophilia A patients is a serious problem that interferes with therapy [16]. In the medical practice setting, bleeding events of hemophilia A patients with inhibitors can be treated with activated prothrombin complex concentrate (APCC) or recombinant FVIIa. Therefore, forced expression of FVIIa is an alternative approach for hemophilia gene therapy when the recipients have inhibitors as described previously [8]. Treatment with overexpression of FVIIa in hemophilia B mice using an AAV2 vector was effective for ameliorating the bleeding phenotype of hemophilia B mice [8]. This was also shown in hemophilia A and B dogs treated with AAV8 vectors carrying the canine FVIIa gene [9]. In the current study, we explored the possibility of ameliorating bleeding diathesis of hemophilia A mice with inhibitors by overexpressing FVII or FVIIa using an AAV8 vector carrying the respective gene.

Inhibitors against human FVIII develop by repeated infusion of human FVIII concentrates to FVIII-deficient mice. This inhibitor is thought to be an antibody against the xenoantigen (human FVIII) but it also cross-reacts with mouse FVIII (Supplementary Fig. S1). Therefore, it could act as an alloantibody against mouse FVIII in F8KO (hemophilia A) mice. Titers of the inhibitor against mouse FVIII were approximately 1/40–1/20 of that against human FVIII by the Bethesda assay but high enough to inhibit mouse FVIII activity. Thereby, hemophilia A mice with inhibitors generated by repeated injection of human FVIII concentrates could be used as a model of hemophilia A with inhibitors.

We overexpressed FVII or FVIIa in hemophilia A mice with inhibitors against mouse FVIII, using an AAV8 vector. Levels of FVIIa in mice are thought to be comparable to the therapeutic levels of

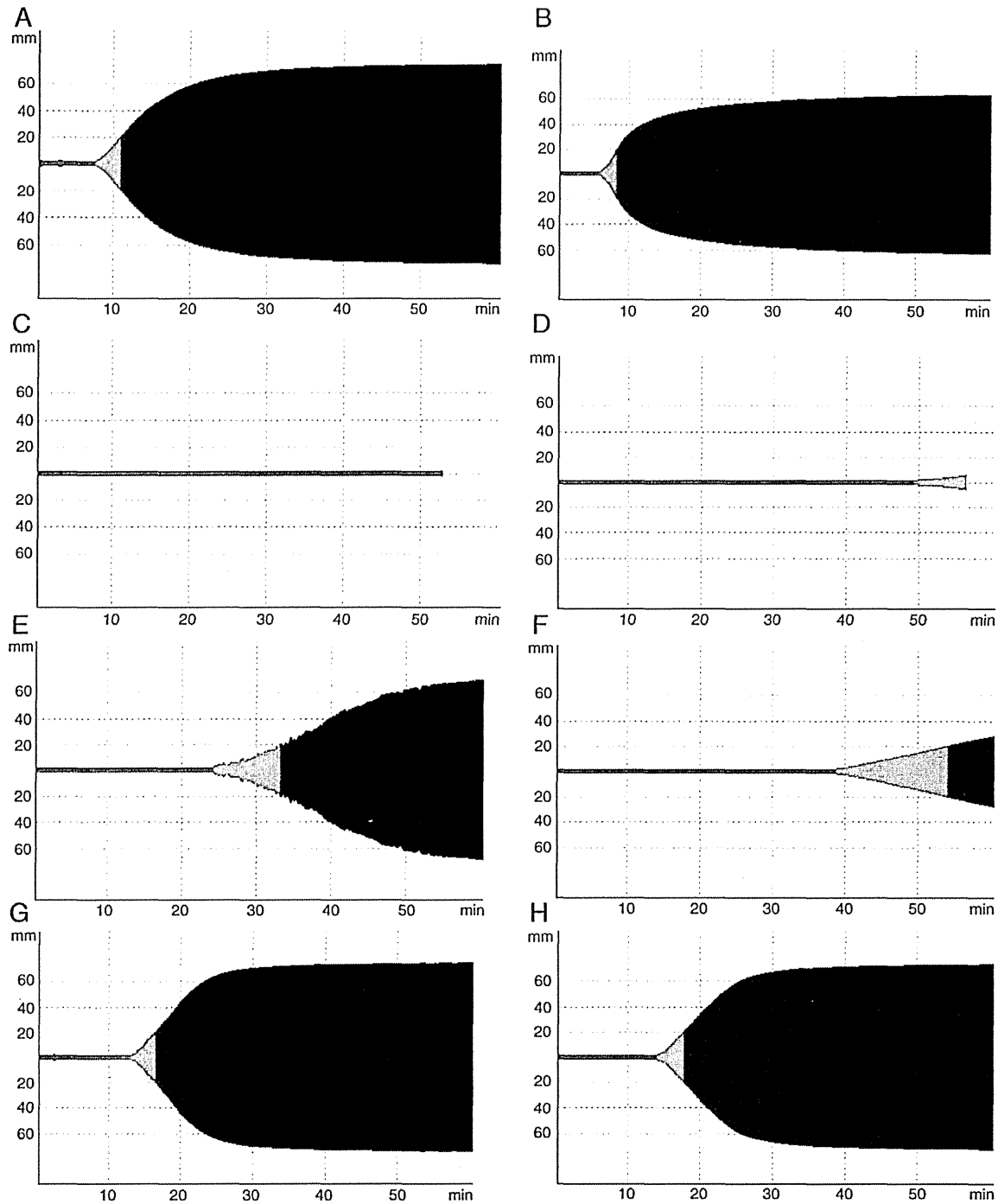

Fig. 3. Thromboelastography analysis. Mouse blood obtained from wild-type mice (A, B), F8KO mice (C, D), AAV8-mFVII-injected F8KO mice with inhibitors (E, F), or AAV8-mFVIIa-injected mice with inhibitors (G, H) was analyzed using a ROTEM delta. The representative thromboelastograms of these mice are shown.

recombinant human FVIIa concentrates in hemophilia A patients with inhibitors to stop bleeding. The therapeutic effect of overexpression of FVII or FVIIa shown in the thromboelastography analysis was confirmed by tail clipping challenge *in vivo*. The overexpression of FVIIa by an AAV8 vector significantly increased the survival rate of hemophilia A mice with inhibitors. The therapeutic effect of FVII overexpression was also significant in the tail clipping challenge in hemophilia A mice with inhibitors. The survival rate of FVII-overexpressed F8KO mice after tail clipping was higher than untreated F8KO mice and lower than FVIIa-overexpressed F8KO mice. Although the survival rate was lower than that for mice with FVIIa overexpression, the difference between these survival rates was not statistically significant.

When hemophilia patients were administered the anti-virus drug ribavirin for the treatment of hepatitis C, these patients bled less frequently [17]. Increased levels of FVII in these patients might account for reduced bleeding diathesis [18], suggesting that high expression levels of FVII in hemophilia patients with or without inhibitors

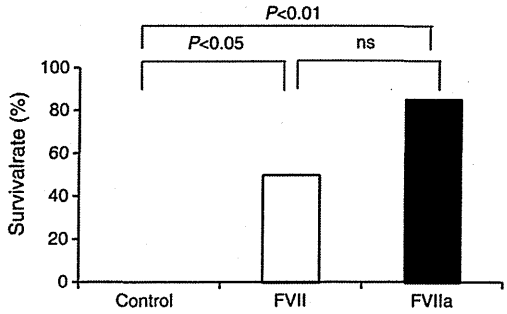

Fig. 4. Survival rates of F8KO mice after tail clipping. The survival rates of F8KO mice are shown. All wild-type mice survived challenge by tail clipping (data not shown), while all F8KO mice (n = 7) died after tail clipping regardless of the presence of inhibitors against FVIII. Significant numbers of mouse FVII overexpressing F8KO mice with inhibitors (n = 6) and mouse FVIIa overexpressing F8KO mice with inhibitors (n = 7) survived challenge by tail clipping. The differences between values were analyzed by Chi-square test and *p* values are shown. ns: not significant.

might ameliorate bleeding. Our results were consistent with this previous report. The therapeutic effect of forced FVII expression using an AAV vector was previously studied in FVII deficient (F7KO) mice [19]. In this animal model, forced FVII expression protected postnatal hemorrhage and improved the survival of vector-injected F7KO mice. The mechanism of therapeutic effect of forced FVII expression in F7KO mice was similar to that of forced FVIII expression in F8KO mice. The current study showed a therapeutic effect of FVII overexpression on bleeding diathesis in F8KO mice.

The hypothetical mechanism of ameliorating bleeding diathesis of F8KO mice by FVII overexpression could be explained as follows. FVII is converted to FVIIa mainly by activated factor X (FXa), and possibly by activated factor IX (FIXa), activated factor XII (FXIIa), thrombin, and FVIIa [20–23]. The physiological role of FVII activating protease in blood coagulation is still unknown [24]. When bleeding occurs, FVIIa binds to tissue factor and activates FX, FIX, and possibly FVII [25]. When FVII is present at a high concentration due to AAV8 vector-induced FVII overexpression, subsequently generated FXa, FIXa, and FVIIa activate FVII and produce a large amount of FVIIa that could in turn bind to tissue factor at the bleeding site and accelerate coagulation. However, this may be less efficient than the amplification of the coagulation cascade by the FIXa/FVIIIa pathway. Enhanced hemostasis with FVII overexpression might differ from FVIIa overexpression. When FVIIa is overexpressed, the FVIIa concentration in the circulation is high and FVIIa in complex with tissue factor activates FX and FIX. Compared with FVIIa overexpression, the FVIIa concentration in the circulation may not be such high when FVII is overexpressed, but the local FVIIa concentration at the bleeding site might be high due to amplification effects as described above. This might account for the increased survival of F8KO mice with FVII overexpression after tail clipping although the improvement of ROTEM parameters with FVII was modest.

Studies to test the safety of overexpression of FVIIa demonstrated it was related to the early death of mice [26,27]. This was apparent in mice with overexpression of an FVIIa variant that had a higher coagulation activity [26]. This adverse event was attributed to the thrombogenicity of the overexpressed FVIIa or its variant. Therefore, continuous overexpression of FVIIa might have a higher risk of adverse events than intermittent administration of FVIIa. The safety of overexpression of FVIIa in dogs was previously shown using molecular markers [9]. However, no pathological examination was conducted, so the thrombogenicity of FVIIa overexpression in the canine model is not clear. Overexpression of human FVII with an AAV8 vector was studied in monkeys and human FVII overexpression continued up to 28 weeks after vector injection without toxicity [19]. Although further studies are required to determine the safety of continuous FVII overexpression regarding thrombogenicity, we demonstrated a therapeutic effect of FVII overexpression in a mouse model of hemophilia A with inhibitors. Our future studies will investigate the safety of FVII overexpression in hemophilia mice and wild type mice in comparison with FVIIa overexpression. In addition to the genetic approach, FVII variants with a longer plasma half-life or drugs that enhance FVII gene expression might be an alternative therapeutic approach for hemophilia patients with inhibitors in clinical settings.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.thromres.2013.03.007.

Table 1
Changes in thromboelastography parameters following the over expression of mFVII and mFVIIa.

	CT	CFT	MCF	α angle
Wild type	255 ± 91.8 (n = 4)	175.5 ± 25.1 (n = 4)	64.3 ± 1.0 (n = 4)	60.5 ± 2.1 (n = 4)
F8KO	2726 ± 409.7 (n = 8)	ND	ND	ND
FVII	1698 ± 403.0 (n = 4)	1132 ± 572.0 (n = 5)	33 ± 35.9 (n = 5)	28.5 ± 10.1 (n = 4)
FVIIa	751.6 ± 139.4 (n = 5)	183 ± 36.0 (n = 5)	74.4 ± 1.7 (n = 5)	59.2 ± 4.6 (n = 5)

ND: values were not determined because CFT, α angle, and MCF could not be measured in most control F8KO mouse blood samples.
CT: clotting time, CFT: clot formation time, MCF: maximum clot firmness.
Wild-type: wild-type mice, F8KO: F8 knock out (FVIII-deficient) mice, FVII: FVII over expressing F8KO mice, FVIIa: FVIIa over expressing F8KO mice.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology; Health and Labour Science Research Grants for Research from the Japanese Ministry of Health, Labour and Welfare; and the Support Program for Strategic Research Platform from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Conflict of Interest Statement

The authors confirm no conflicts of interest to declare.

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HUMAN GENE THERAPY 24:283–294 (March 2013)

DOI: 10.1089/hum.2012.162

Genetically Modified Adipose Tissue-Derived Stem/Stromal Cells, Using Simian Immunodeficiency Virus-Based Lentiviral Vectors, in the Treatment of Hemophilia B

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Abstract

Hemophilia is an X-linked bleeding disorder, and patients with hemophilia are deficient in a biologically active coagulation factor. This study was designed to combine the efficiency of lentiviral vector transduction techniques with murine adipose tissue-derived stem/stromal cells (mADSCs) as a new method to produce secreted human coagulation factor IX (hFIX) and to treat hemophilia B. mADSCs were transduced with simian immunodeficiency virus (SIV)-hFIX lentiviral vector at multiplicities of infection (MOIs) from 1 to 60, and the most effective dose was at an MOI of 10, as determined by hFIX production. hFIX protein secretion persisted over the 28-day experimental period. Cell sheets composed of lentiviral vector-transduced mADSCs were engineered to further enhance the usefulness of these cells for future therapeutic applications in transplantation modalities. These experiments demonstrated that genetically transduced ADSCs may become a valuable cell source for establishing cell-based gene therapies for plasma protein deficiencies, such as hemophilia.

Introduction

HEMOPHILIA IS a congenital bleeding disorder that is attributed predominantly to a hereditary lack of biologically active coagulation factor VIII (FVIII) or factor IX (FIX). Worldwide, 105 to 160 per million of the male population suffer from this disease (Bolton-Maggs and Pasi, 2003). Current standard therapy is generally provided after the onset of bleeding episodes and relies on the infusion of FVIII or FIX concentrates. Unfortunately, these treatments are expensive, limiting access to this type of therapy for a majority of patients with hemophilia (Pipe *et al.*, 2008). Thus, alternative molecular and cellular methods are needed for the treatment of hemophilia. Studies have shown that even a small increase in clotting activity (~1–2%) over normal levels can improve the bleeding from severe to mild-to-moderate (Bolton-Maggs and Pasi, 2003), representing a dramatic improvement in quality of life by reducing the need for immediate clotting factor injections to prevent uncontrolled bleeding.

Cell-based therapies have received a great deal of attention as a next-generation therapeutic approach for hemophilia (Oh *et al.*, 2006; Follenzi *et al.*, 2008; Kasuda *et al.*, 2008; Tatsumi *et al.*, 2008a,b; Ohashi *et al.*, 2010). There has been enormous interest in the transplantation of stem cells to produce clotting factors (Chuah *et al.*, 2004; Oh *et al.*, 2006; Coutu *et al.*, 2011). Some types of stem cells can be readily isolated from human patients with minimal invasiveness (Lin *et al.*, 2008), such as adipose tissue-derived stem/stromal cells (ADSCs, also known as adipose tissue-derived mesenchymal stem cells, AT-MSCs) (Zuk *et al.*, 2001; Li *et al.*, 2011b). ADSCs are actively proliferative *in vitro* and are multipotent, with the potential to differentiate into mesodermal, endodermal, and ectodermal lineages (Lee *et al.*, 2004; Peister *et al.*, 2004; Wang *et al.*, 2004; Seo *et al.*, 2005; Aurich *et al.*, 2007; Banas *et al.*, 2007; Liu *et al.*, 2007). These cells would be an ideal autologous source of stem cells with the potential to reduce the need for immunosuppression after reimplantation back into patients.

In terms of hemophilia, native ADSCs need to be genetically modified to produce and secrete FVIII or FIX because ADSCs do not naturally express coagulation factors. A multitude of genetic approaches (Anjos-Afonso *et al.*, 2004; Haleem-Smith *et al.*, 2005; Oh *et al.*, 2006; Talens-Visconti *et al.*, 2006; Sugii *et al.*, 2010; Coutu *et al.*, 2011; Li *et al.*,

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2011a,b) have been applied to exogenously produce clotting factors.

In this study, we used simian immunodeficiency virus (SIV) lentiviral vectors derived from SIVagmTYO-1, a simian immunodeficiency virus strain isolated from African green monkeys (Nakajima *et al.*, 2000). The SIV vector has been reported to have a different safety profile compared with other lentiviral vectors, in that this strain lacks the ability to become pathogenic in its natural host, African green monkeys, and in experimentally inoculated Asian macaques (Honjo *et al.*, 1990; Nakajima *et al.*, 2000; Kikuchi *et al.*, 2004; Ogata *et al.*, 2004). SIV also has low sequence homology to the HIV genome (Jin *et al.*, 1994). Therefore, SIV vectors are assumed to hold low or almost no risk of causing homologous recombination that generates a replication-competent virus, even in circumstances in which the SIV vector coexists with HIV in the same cells inside a patient. The SIV vector is likely safer than other vectors and maintains the inherent ability to integrate into both proliferating and non-proliferating cells (Coffin *et al.*, 1997; Walther and Stein, 2000; Li and Lu, 2009), making it an ideal vector to provide persistent expression of exogenous genes and possibly making it advantageous for application in future clinical studies.

Another approach to increasing the level of engraftment of transplanted cells at local sites is to engineer functional tissues. Our laboratory has established a cell sheet-engineering technology using temperature-responsive culture dishes that are grafted with a temperature-responsive polymer, poly (*N*-isopropylacrylamide) (PIPAAm) (Kikuchi and Okano, 2005; Yang *et al.*, 2005). This technology allows us to recover monolithic cell sheets without any enzymatic digestion and has already been applied to regenerative medicine (Nishida *et al.*, 2004; Obokata *et al.*, 2011). To establish a tissue engineering-based treatment modality with murine ADSCs (mADSCs) for hemophilia, this method was applied to create a contiguous cell sheet of vector-transduced mADSCs.

Materials and Methods

Mice

C57BL/6J male mice (8 weeks old) were purchased from a commercial vendor (CLEA Japan, Tokyo, Japan). All animal procedures were conducted in accordance with the institutional guidelines of the Animal Care Committee of Tokyo Women's Medical University (Tokyo, Japan).

Preparation of mouse ADSCs

Adipose tissues were isolated from the inguinal region in the mice, minced with forceps, and enzymatically digested with 0.1% type I collagenase (17100-017; Invitrogen/Life Technologies, Carlsbad, CA) at 37°C for 1 hr. The stromal–vascular fraction (SVF) was collected by centrifugation at $700\times g$ for 5 min and washed twice. The SVF was resuspended with Dulbecco's modified Eagle's medium (DMEM)–F12 (11320-033; Invitrogen/Life Technologies) supplemented with 10% fetal bovine serum (FBS, 04110101; Japan Bio-Serum, Hiroshima, Japan) and GlutaMAX-I supplement (35050-061; Invitrogen/Life Technologies). This medium is referred to in text as "basic medium." The SVF was plated on PRIMARIA tissue culture-treated dishes (35-3803; BD Biosciences, Franklin Lakes, NJ) and cultured at 37°C in a 5% CO_2 incubator. The medium was aspirated and changed 3 days after plating. Adherent proliferating cells were trypsinized for subculturing (defined as passage 1) approximately 7–8 days after plating. The subcultured cells were defined as mADSCs.

Flow cytometry

mADSCs at passage 2 were suspended and incubated with an Fc blocker (553141), followed by antibodies: fluorescein isothiocyanate-conjugated CD29 (CD29–FITC; 555005), phycoerythrin-conjugated CD44 (CD44–PE; 553134), CD90.2–PE (553005), CD31–PE (553373), CD45–PE (553081), isotype control–PE (553930), and isotype control–FITC (553960). All antibodies were obtained from BD Biosciences and the catalog numbers are shown in parentheses. The cells were analyzed with a flow cytometer (Gallios; Beckman Coulter, Brea, CA).

Osteogenic differentiation of mADSCs followed by alkaline phosphatase assay and alizarin red S staining

mADSCs (passage 2) were replated at 1×10^4 cells/cm^2 in a 6-well plate for staining with alizarin red S, and 3.3×10^4 cells/cm^2 were plated for an alkaline phosphatase (ALP) assay using minimum essential medium (MEM) α with GlutaMAX-I (32571; Invitrogen/Life Technologies) supplemented with 10% FBS. Twenty-four hours after cell plating, differentiation was initiated by incubating the cells with osteogenic differentiation medium: MEM α GlutaMAX-I with 10% FBS, containing β-glycerophosphate disodium salt hydrate (G9891; Sigma-Aldrich, St. Louis, MO) at 10 mmol/liter, ascorbic acid (323-44822; Wako, Osaka, Japan) at 50 μmol/liter, dexamethasone (Dex) (BG08A; Fuji-Seiyaku, Tokyo) at 100 nmol/liter; or commercially available osteogenic differentiation medium (hMSC osteogenic BulletKit, PT-3002; Lonza Japan, Tokyo, Japan). The osteogenic differentiation medium was changed every 3–4 days. Seven days after osteogenic induction, the ALP assay was performed on mADSCs, using a LabAssay ALP kit (291-58601; Wako) according to the manufacturer's instructions. Four weeks after osteogenic induction, mADSCs were fixed with 4% paraformaldehyde (PFA) (100412; Muto-kagaku, Tokyo, Japan), washed with purified water (Synthesis A10; Millipore, Billerica, MA), and stained at room temperature for 10 min with alizarin red S (011-01192; Wako) at 10 g/liter.

Adipogenic differentiation of mADSCs and oil red O staining

mADSCs (passage 2) were replated at 3×10^4 cells/cm^2 with DMEM–F12 supplemented with 10% FBS and GlutaMAX-I (basic medium) and cultured until confluency. The basic medium was replaced with adipogenic differentiation medium: basic medium supplemented with isobutylmethylxanthine (IBMX) (I7018; Sigma-Aldrich) at 0.5 mmol/liter, indomethacin (095-02472; Wako) at 100 μmol/liter, Dex at 500 nmol/liter, and insulin (Wako) at 10 μg/ml. Seventy-two hours later, the adipogenic differentiation medium was removed and replaced with basic medium containing insulin (10 μg/ml). This latter medium was changed every 3–4 days. Two weeks after adipogenic induction, the mADSCs were fixed with 10% formalin, washed with phosphate-buffered saline (PBS) and isopropanol (Wako), and subsequently

stained with oil red O solution (09091; Muto-kagaku) at room temperature for 10–20 min.

Preparation and titering of replication-defective SIV lentiviral vectors

Self-inactivating (SIN) simian immunodeficiency virus (SIV) vectors were used in this study as previously described (Ogata *et al.*, 2004). Enhanced green fluorescent protein (EGFP) or the hFIX minigene (hFIX cDNA containing the first intron) were cloned 3′ of the cytomegalovirus (CMV) promoter. The SIV vector was pseudotyped with the G glycoprotein of the vesicular stomatitis virus (VSV-G) envelope, and the vector titers were determined as previously described (Nakajima *et al.*, 2000; Ogata *et al.*, 2004).

SIV lentiviral vector transduction of mADSCs in vitro

mADSCs were plated on a 6-well tissue culture plate at 4000 cells/cm^2 with DMEM–F12 basic medium (2 ml/well). Twenty-four hours after plating, the medium was replaced with basic medium (1 ml/well) containing lentiviral vectors (at increasing multiplicities of infection [MOIs]) in the presence of Polybrene (hexadimethrine bromide, H-9268; Sigma-Aldrich) at 8 μg/ml. Twenty-four hours after the vectors were introduced, fresh basic medium (1 ml/well) was added without Polybrene. Afterward, the transduced cells were propagated with basic medium to perform ELISAs or clotting assays as described below. To induce osteogenic differentiation or adipogenic differentiation, the medium was changed to the appropriate differentiation medium (see previously) 96 hr after vector transduction, and the cells were cultured and assessed as described previously.

ELISA

The culture medium in a 6-well tissue culture plate was replaced with fresh medium (1 ml/well) 24 hr before medium collection. The culture medium was harvested and frozen at −80°C until assessment by ELISA. hFIX ELISA was performed with an Asserachrom IX:Ag kit (630423; Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

Clotting assay

The culture medium was changed to fresh medium containing menaquinone (vitamin K_2) (V-9378; Sigma-Aldrich) 24 hr before medium collection. Normal plasma (Coagtrol N, 13490; Sysmex, Hyogo, Japan) was mixed with FIX-deficient plasma (15450; Sysmex) and the clotting time was measured at 37°C to obtain a standard curve for calibration. Subsequently, the clotting time of culture medium mixed with FIX-deficient plasma was measured with a semiautomatic coagulation analyzer (KC4 Delta; Trinity Biotech, Wicklow, Ireland).

mADSC sheets

mADSCs (passage 2 or 96 hr after vector transduction) were replated on an UpCell temperature-responsive culture dish (CellSeed, Tokyo, Japan), at a density of 4.5×10^4 cells/cm^2. After 5 days of culture at 37°C in 5% CO_2, the temperature was changed from 37 to 20°C to initiate detachment of the mADSC cell sheet from the culture surface. The mADSCs were harvested as a monolayer cell sheet after an ~20-min incubation at 20°C. Fluorescence images of EGFP-transduced mADSC sheets detaching from the temperature-responsive culture dishes were captured with an OV110 imager (Olympus, Tokyo, Japan). For ELISA, hFIX-transduced mADSC sheets were harvested and reattached onto collagen type IV-coated tissue culture dishes (Asahi Glass, Tokyo, Japan). After 24 hr of culture, the medium was collected for ELISAs to detect hFIX.

Transmission electron microscopy

Transmission electron microscopy (TEM) of mADSC sheets was performed by Tokai Electron Microscopy Analysis (Aichi, Japan). mADSC sheets were fixed with a solution comprising 2% PFA, 2% glutaraldehyde (GA) in 0.1 *M* PBS, and then incubated in 2% GA in 0.1 *M* PBS (supplied by the company).

Statistical analysis

Data are presented as means ± standard deviation (SD). Group-wise comparisons were made by one-way analysis of variance (ANOVA) followed by the Tukey (HSD) post-hoc test, using SPSS statistics software (PASW statistics, version 18; SPSS, Chicago, IL). A value of $p<0.05$ was considered statistically significant.

Results

Characterization of mADSCs

Figure 1A presents an optical microphotograph of mADSCs at passage 2. The cell-surface protein profiles of mADSCs were analyzed by flow cytometry. mADSCs at passage 2 expressed the mesenchymal stem cell markers (Mitchell *et al.*, 2006) CD29 (99.7%), CD44 (95.4%), and CD90.2 (92.6%) (Fig. 1B). mADSCs were negative for CD31. A small population of the cells were CD45 positive (9.3%), but this may be due to contamination with hematopoietic cells.

The mADSCs was verified as bipotent, demonstrating the ability to differentiate into their osteogenic and adipogenic lineages. Osteogenic induction increased ALP activity (Fig. 1C) and calcium accumulation as visualized by alizarin red S staining (Fig. 1D right). No calcium deposition was found in the noninduced condition (Fig. 1D left). Adipogenic differentiation was observed by the presence of lipid droplets detected by oil red O staining (Fig. 1E right). A few lipid droplets were observed in the noninduced condition (Fig. 1E left), suggesting that some mADSCs have the ability to spontaneously differentiate along the adipogenic lineage. These results confirmed that the mADSCs isolated in this study possessed the same characteristics as those of previously reported ADSCs (Liu *et al.*, 2007).

Efficiency of lentiviral transduction of mADSCs

Figure 2A depicts the organization of the replication-defective SIV lentiviral vector, showing the location of the cytomegalovirus promoter (CMVprom) and the gene of interest (GOI). The GOIs used in this study were EGFP (SIV-EGFP) and human FIX (SIV-hFIX). To determine the optimal dose for transducing mADSCs using SIV lentiviral vectors, mADSCs at passage 2 were infected with SIV-EGFP at

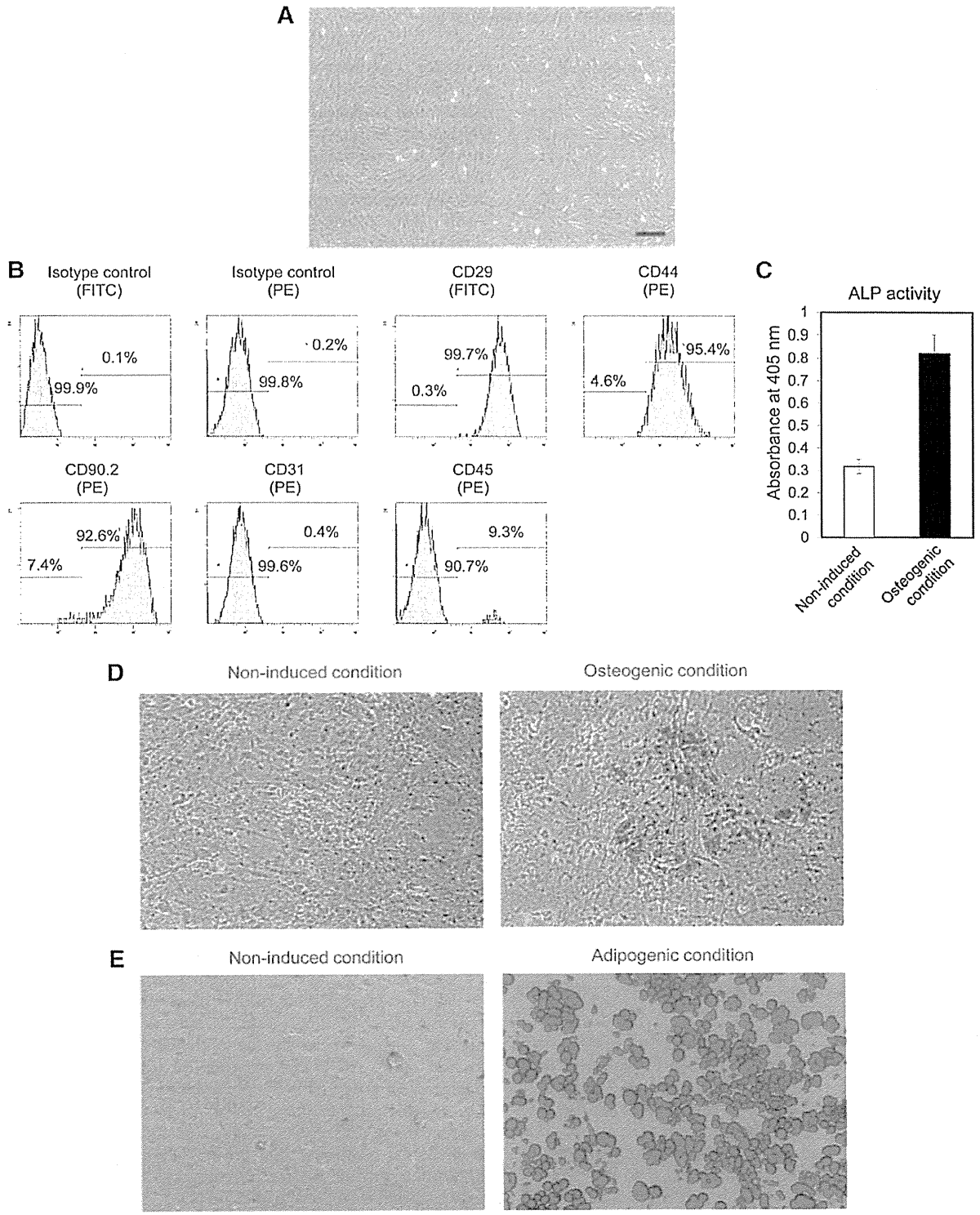

FIG. 1. Confirmation of the characteristics and bipotency of mouse adipose tissue-derived stem/stromal cells (mADSCs). **(A)** A microphotograph of mADSCs at passage 2, showing their spindle-like morphology. Scale bar: 50 μm. **(B)** Characterization of mADSCs by flow cytometry. mADSCs of passage 2 expressed the mesenchymal stem cell markers CD29, CD44, and CD90.2 and were negative for CD31 and CD45. FITC, fluorescein isothiocyanate; PE, phycoerythrin. **(C–E)** The osteogenic and adipogenic differentiation of mADSCs was verified by an alkaline phosphatase (ALP) assay and alizarin red S staining (markers of osteogenesis), and by oil red O staining (marker of adipogenesis), respectively. **(C)** The ALP assay was performed on day 7 of the culture of osteogenically differentiated mADSCs (n=3). **(D)** Alizarin red S staining was performed after 4 weeks of osteogenic induction culture conditions. The stain in the photograph of the induced condition indicates calcium deposition in the cells. **(E)** Oil red O staining was performed 2 weeks after the initiation of adipogenic induction culture conditions. Color images available online at www.liebertpub.com/hum