this regard, the timely suspicion of acute cellular rejection using laboratory markers is a key indicator of the need for liver biopsy. Fluorescent-activated cell sorting analysis of CD25, CD28, and CD38 expression in peripheral lymphocytes is considered to be useful, not only for evaluation of the degree of immunosuppression, but also for the prediction of acute allograft cellular rejection (22). The present study showed that four coagulation and fibrinolysis markers, i.e., increase in PAI-1, decrease in protein C, decrease in plasminogen, and decrease in ADAMTS13, might be used as markers for suspecting the occurrence of acute cellular rejection. Statistical analysis suggested that an increase in the plasma PAI-1 level was the most reliable and sensitive marker for acute cellular rejection. Protein C, plasminogen, and ADAMTS13 are all synthesized in the liver, and their levels may therefore depend on the size and regeneration of the graft liver, and their plasma levels at any given time point might thus be less reliable as predictors of acute cellular rejection. PAI-1 is synthesized mainly in the vascular endothelial cells and its plasma level was elevated on day 1 after liver transplantation, and had returned to pretransplant levels after day 3. An increased plasma PAI-1 level at a single time point after day 1, together with a deterioration in liver function, may therefore be adopted as a predictive marker for acute cellular rejection.

Acute cellular rejection is characterized by portal inflammation, bile duct inflammation, and subendothelial cell inflammation (15, 16). Recent studies have suggested that not only T-cells, but also B-cells, are involved in acute cellular rejection, and cytokines and chemokines may also play roles in this process (23). As shown in a previous report, Toll-like receptor signaling through MyD88 may be involved in acute allograft rejection, indicating that toll-like receptors may be activated in the transplant setting causing inflammatory cytokine release (24). Therefore, the increase in PAI-1 levels seen during acute cellular rejection may be accounted for by immune cell-derived cytokine/chemokine activation of, and inflammation of, sinusoid-endothelial and portal vein endothelial cells. An increased PAI-1 level has previously been shown to be predictive for veno-occlusive disease developing after bone marrow transplantation (25), and this mechanism is thought to be responsible for busulfan-related toxic injury of sinusoidal endothelial cells (26, 27). The increase in plasma PAI-1 levels in patients with allograft cellular rejection is not as high as that seen in veno-occlusive disease, suggesting that the mechanisms and the outcomes of these PAI-1 increases may differ. Although the mechanisms of activation of endothelial cells may differ in veno-occlusive disease and in acute cellular rejection after allograft liver transplantation, both might result in increased plasma levels of PAI-1. Further studies are required to determine the precise mechanism responsible for the increase in PAI-1 levels occurring during acute cellular rejection.

Cytokines released from infiltrated immune cells in the liver, and inflammation in portal and sinusoid endothelial cells, might also inhibit the synthesis of ADAMTS13 in stellate cells, resulting in decreased plasma ADAMTS13 levels because the plasma ADAMTS13 level was significantly decreased in patients with sepsis-induced disseminated intravascular coagulation (5) and ADAMTS13 mRNA expression in the liver is decreased in endotoxin-injected mice (13). The decrease in protein C and plasminogen levels associated with acute cellular rejection might be due to their reduced synthesis by the graft hepatocytes, and a reduction in levels of these markers might therefore take time to become apparent. The decrease in plasminogen levels in patients with acute cellular rejection was less severe than that in protein C levels. These differences may be due to differences in the plasma half-lives of these molecules.

In conclusion, we have performed a comprehensive analysis of the coagulation and fibrinolysis system in pediatric patients undergoing orthotopic liver transplantation. Coagulation activity was quickly normalized by two days after liver transplantation. However, it took for 21–28 days for full restoration of the coagulation and fibrinolysis system. The post-operative thrombogenic state continued for approximately 14 days. PAI-1 may be used as predictive markers for acute cellular rejection in pediatric patients. These findings might also be applicable to adult liver transplant patients, though this needs to be confirmed by future prospective studies.

Acknowledgments

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RESEARCH ARTICLE

Liver-restricted expression of the canine factor VIII gene facilitates prevention of inhibitor formation in factor VIII-deficient mice

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Abstract

Background Gene therapy for hemophilia A with adeno-associated virus (AAV) vectors involves difficulties in the efficient expression of factor VIII (FVIII) and in antibody formation against transgene-derived FVIII.

Methods AAV8 vectors carrying the canine B domain deleted FVIII (cFVIII) gene under the control of the ubiquitous β-actin promoter, the liver-specific human α1 anti-trypsin promoter (HAAT) and the liver-specific hepatic control region (HCR) enhancer/human α1 anti-trypsin promoter complex (HCRHAAT) were used for the expression of cFVIII in FVIII deficient (*fviii*$^{-/-}$) mice.

Results Addition of the hepatic control region enhancer element to the HAAT promoter successfully augmented HAAT promoter activity without loss of liver-specificity *in vivo*. Using this enhancer/promoter complex, a high cFVIII transgene expression was achieved, resulting in increased blood cFVIII activities to more than 100% of the normal canine FVIII levels in *fviii*$^{-/-}$ mice at a 1 : 10 lower dose of the AAV8 vector carrying the cFVIII gene driven by the HAAT promoter. Under short-term immunosuppression, neutralizing antibodies against cFVIII developed in only one out of six mice when the HAAT promoter was used for cFVIII expression, whereas all the mice developed neutralizing antibodies against cFVIII when the β-actin promoter was used for cFVIII expression. No neutralizing antibodies against cFVIII developed in *fviii*$^{-/-}$ mice that received the AAV8 vector carrying the cFVIII gene driven by the HCRHAAT enhancer/promoter complex without immunosuppression.

Conclusions These data suggest that AAV8 vector-mediated liver-restricted cFVIII gene expression is sufficient for immune hypo-responsiveness to transgene-derived cFVIII in *fviii*$^{-/-}$ mice. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords adeno-associated virus vector; factor VIII; gene therapy; hemophilia A

Introduction

Hemophilia A is an inherited X-linked bleeding disorder caused by abnormalities in the coagulation factor VIII (FVIII) gene. The genetic

abnormalities result in FVIII deficiency, which in turn creates bleeding diathesis, such as life-threatening bleeding in the brain or harmful bleeding in joints and muscles. The current standard therapy involves the intravenous injection of monoclonal antibody-purified coagulation factor concentrates from plasma or recombinant coagulation factors. Hemophiliacs are not free from the risks of life-threatening intracranial bleeding and harmful bleeding. Therefore, gene therapy enabling the prevention of such bleeding by a sustained elevation of coagulation factor levels provides the next generation therapy for hemophilia [1–6]. Indeed, clinical trials for hemophilia gene therapy have recently been conducted, although with limited success [4–10]. Compared with gene therapy for hemophilia B, gene therapy for hemophilia A has been accompanied by difficulties involved in the efficient expression of FVIII because of the large size of the FVIII gene and the low expression of the FVIII gene in the full-length FVIII cDNA form. Recent studies have seen the development of new vectors and strategies [11–17]. Among the viral vectors, recombinant adeno-associated virus (AAV) vectors are preferred for gene therapy because they can transfer genes to nondividing cells, leading to the long-term expression of transgenes, and no pathological effects of wild-type AAV have been reported [1–7,11,18]. Because of the size limitation of genes carried on AAV vectors, the use of AAV vectors for hemophilia A gene therapy has not been as successful as that for hemophilia B. Recently, modification of the FVIII gene and the development of new AAV serotype vectors has allowed us to carry the FVIII gene on AAV vectors [15,19,20]. We previously demonstrated that canine B domain-deleted FVIII (BDDFVIII) could be expressed in skeletal muscles and liver using AAV1 vectors and AAV8 vectors, respectively, and the minimum β-actin promoter. In addition to the sustained expression of FVIII, tissue-specific expression of FVIII may also be helpful for hemophilia gene therapy to avoid adverse reactions. In the present study, we examined the possibility of liver-specific FVIII gene transfer in FVIII deficient (*fviii*$^{-/-}$) mice using the AAV serotype 8 vector carrying the canine FVIII (cFVIII) gene, which is located downstream of three different types of promoter/enhancer complex. We show that elevated liver-specific expression of this transgene can be achieved with AAV8 vectors carrying the therapeutic gene under the control of the minimum human α1-antitrypsin (HAAT) promoter in combination with the minimum hepatic control region (HCR) enhancer element *in vivo*. This can be advantageous when aiming to avoid the formation of neutralizing antibodies against the transgene product for long-term expression.

Materials and methods

Vector construction

Two DNA segments encoding the 5′ flanking region (−272 to +25; −168 to +25) of the HAAT gene were amplified by polymerase chain reaction (PCR) to obtain the 297 bp and 193 bp HAAT promoters. These DNA fragments contained the hepatic nuclear factor 1 responsive element. The minimum enhancer element (+24 to +186) of the HCR of the human apolipoprotein E gene [21–23] was also amplified by PCR. DNA fragments of the cytomegalovirus (CMV) promoter and the growth hormone intron 1 of p1.1c (Avigen Inc., Alameda, CA, USA) were replaced with the 297 bp HAAT promoter to generate p1.1HAAT. Similarly, DNA fragments of the CMV promoter and the growth hormone intron 1 of p1.1c were replaced with the minimum HCR enhancer element and the 193 bp HAAT promoter to generate p1.1HCRHAAT. Constructions of p1.1 CAG and p1.1β have been described previously [20,24]. DNA fragments encoding the canine BDDFVIII cDNA or the luciferase gene were placed downstream of the promoter sequences of p1.1HAAT or p1.1 HCRHAAT to produce plasmid vectors p1.1HAAT-cFVIII, p1.1HAAT-Luc, p1.1HCRHAAT-cFVIII and p1.1HCRHAAT-Luc, respectively. Similarly, the DNA fragment encoding the Lac Z gene was placed downstream of the promoter sequences of p1.1 HAAT to produce p1.1HAAT-Lac Z. P1.1β-Lac Z has been described previously [20]. The DNA fragment spanning the CMV promoter, the LacZ gene and the polyadenylation signal sequence of the pAAV2 CMV-Lac Z plasmid (Stratagene, La Jolla, CA, USA) was replaced by the DNA fragment spanning the HCRHAAT promoter, the cFVIII gene and the SV40 polyadenylation signal sequences of p1.1HCRHAAT-cFVIII to make pAAV2-HCRHAAT-cFVIII. Plasmids pAAV2-HCRHAAT-Luc, pAAV2-HAAT-LacZ, pAAV2-CAG-Luc and pAAV2-HAAT-cFVIII were made in a similar manner, respectively.

AAV vector production

The vector production system was kindly supplied by Avigen Inc. The AAV vectors were packaged with the AAV8 capsid by pseudotyping. The chimeric packaging plasmid for AAV8 capsid pseudotyping was a generous gift from Dr James M. Wilson (Division of Medical Genetics, Department of Medicine, University of Pennsylvania, PA, USA) [19]. The DNA fragments harboring the cFVIII gene, the luciferase gene or the Lac Z gene located downstream of the different promoters and flanked by AAV2 inverted terminal repeats (ITRs) were packaged by triple plasmid transfection of human embryonic kidney 293 (HEK 293) cells, which were kindly supplied by Avigen Inc., with the chimeric packaging plasmid (AAV2 rep/AAV8 cap), the adenovirus helper plasmid pHelper (Stratagene) and gene transfer plasmid vectors, as described previously [20,24]. For virus vector purification, the DNase-treated (Benzonase, Merck Japan, Tokyo, Japan) viral particle containing samples were subjected to two rounds of cesium chloride (CsCl)-density gradient ultracentrifugation in HEPES-buffered saline (pH 7.4) in the presence of 25 mM ethylenediaminetetraacetic acid, at 21 °C, as previously

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described [20]. Titration of recombinant AAV vectors was carried out by quantitative dot-blot hybridization using ^{32}P-labeled probes [20,24] or by quantitative PCR using a real time PCR system (StepOnePlus; Applied Biosystems Japan, Tokyo, Japan). The primer sequences used for quantification of the AAV8 vector carrying the cFVIII gene were CCGATTATTGCTCAGTACATCCG and CAACTGTTGAAGTCACAGCCCA, and the probe sequence was FAM-CAACCCATTACAGCATCCGCAGCACT. DNase in the samples was heat-inactivated before the PCR reaction.

Animal experiments

C57BL/6 wild-type mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). FVIII-deficient mice (hemophilia A mice) with targeted destruction of exon 16 of the FVIIII gene were generously provided by Dr H. H. Kazazian Jr (University of Pennsylvania, PA, USA). J1 ES cells were used for targeted destruction of the FVIII gene and blastocysts derived from C57BL/6 mice were used to generate chimaeras [25]. 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. AAV8 vectors were injected into the cervical vein of mice under anesthesia. Cyclophosphamide (100 μg/body/day; Sigma-Aldrich Japan, Tokyo, Japan) and tacrolimus (12.5 μg/body/day; Fujisawa Pharmaceuticals Co., Tokyo, Japan) were given (subcutaneously) for 12 weeks to AAV8-HAAT-cFVIII-injected *fviii*$^{-/-}$ mice after vector injection for immunosuppression [20]. No immunosuppresssants were administered to AAV8-HCRHAAT-cFVIII-injected *fviii*$^{-/-}$ mice.

Immunohistochemistry study

Tissues of vector-injected mice were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h at 4 °C, incubated in PBS containing sucrose (10–30%), and frozen in OCT compound (Tissue-Tek, Miles Inc., Elkhart, IN, USA), in dry ice/ethanol. Sections were prepared from frozen tissues at −25 °C, and attached to polylysine-coated glass slides. For the detection of cFVIII, tissue sections were blocked with 1% casein in PBS containing 0.1% Triton-X 100 for 30 min at room temperature, and incubated with sheep polyclonal anti-human FVIII antibodies (Cedarlane Laboratories Ltd, Burlington, NC, USA) for 2 h at 37 °C. After washing in PBS, sections were incubated with biotin-conjugated rabbit anti-(sheep immunoglobulin G) antibody followed by the ABC reagent and a DAB kit (Vectastain ABC Elite kit; Vector, Burlingame, CA, USA) [20].

Analysis of the Lac Z gene expression in mouse tissues

To analyse LacZ gene expression in mice injected with AAV8 vectors carrying the Lac Z gene, mice were irrigated with saline followed by PBS containing 2% paraformaldehyde and then mouse tissues were fixed in 2% paraformaldehyde in PBS for 5 min and washed with PBS. Portions of mouse tissues were directly suspended in PBS containing 1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 0.01% Na deoxycholate, 0.1% Triton X-100, at 25 °C for 1 h. The rest of the mouse tissues were incubated further in PBS containing sucrose (10–30%), and frozen in OCT compound (Tissue-Tek) in dry ice/ethanol. Sections were prepared from frozen tissues at −25 °C, attached to polylysine-coated glass slides, incubated in PBS containing 1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 0.01% Na deoxycholate, 0.1% Triton X-100, at 25 °C for 1 h.

Analysis of luciferase gene expression in mice

For *in vivo* bioluminescence imaging analysis, luciferin (150 μg per 100 μl/g body weight) was given to the mice injected with the AAV8 vector carrying the luciferase gene under anesthesia with isoflurane. Mice were subjected to direct imaging analysis and to quantification of photons transmitted through the mouse skin using IVIS Imaging Systems and Living Image software (Xenogen Co., Alameda, CA, USA). Photons in the area corresponding to the living mouse liver were quantified and expressed as photons/s/cm^2/sr.

Determination of cFVIII and cFVIII gene transcripts in mice

AAV8 vectors carrying the canine FVIII gene driven by the HAAT promoter or the minimum HCRHAAT enhancer/promoter complex were injected into the cervical vein plexus of 8-week-old *fviii*$^{-/-}$ mice under anesthesia. Blood was drawn from the cervical vein plexus and mixed with 1 : 10 volume of 3.8% sodium citrate periodically. Platelet-poor plasma was prepared and canine FVIII levels in mouse plasma were quantified by the activated partial thromboplastin time (APTT) method using FVIII-deficient plasma and standardized with normal canine plasma. Quantification of cFVIII transgene transcripts was performed by quantitative reverse transcriptase (RT)-PCR. RNA was isolated from mouse organs using an RNeasy Protect isolation kit (Qiagen Inc., Valencia CA, USA). DNase I-treated (Amplification grade; Invitrogen, Carlsbad, CA, USA) and heat-treated RNA samples were subjected to RT-PCR. The quantities of cFVIII transcripts were standardized against those of the GAPDH transcripts [20]. Immunohistochemistry for canine FVIII was carried out

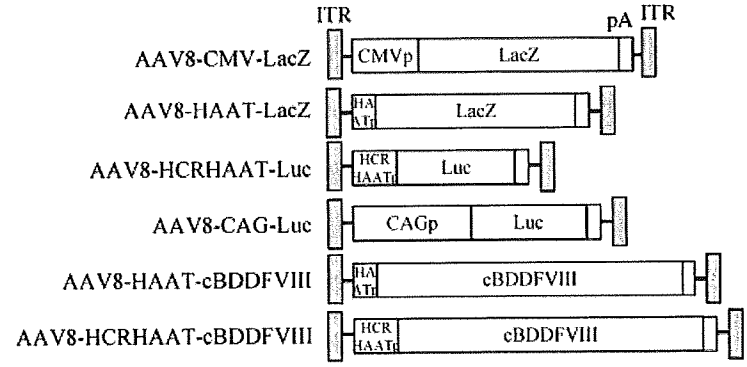

Figure 1. Schematic representation of AAV8 vectors. AAV8 vectors used in the present study are represented schematically. The promoter, the enhancer/promoter complex, or the enhancer/promoter/intron complexes, the genes for expression, and the polyadenylation signal sequence (pA) were flanked by two AAV2 ITR sequences. CMV, CMV promoter/the growth hormone gene intron 1 complex (1 kb); CAG, the CMV enhancer, β-actin promoter, and growth hormone intron 1 enhancer/promoter/intron 1 complex (1.7 kb); HAAT, the human α1 antitrypsin promoter (297 b); HCRHAAT, the hepatic control region of apolipoprotein E gene (163 b) and the human α1 antitrypsin promoter (193 b) complex; cBDDFVIII, canine B domain deleted FVIII cDNA (4.4 kb).

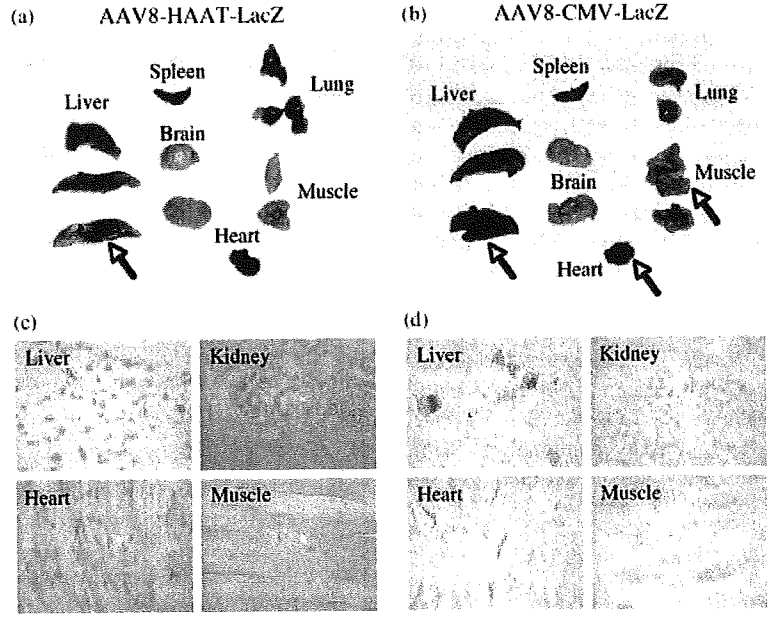

Figure 2. Lac Z gene expression in mice injected with AVV8 vectors. Expression of the Lac Z gene in vector-injected mice was analysed by X-gal staining. Macroscopic views of organs of mice injected with AAV8-HAAT-Lac Z (a) or with AAV8-CMV-Lac Z (b) and microscopic views of organs of mice injected with AAV8-HAAT-Lac Z (c) or with AAV8-CMV-Lac Z (d) are shown. Arrows indicate positive X-gal staining.

using sheep anti-(human FVIII) polyclonal antibodies, as previously described [20].

Determination of neutralizing antibody titer against cFVIII

Analysis of neutralizing antibodies against cFVIII developed in mice was performed by the Bethesda method using FVIII deficient plasma and normal canine plasma, as previously described [20].

Results

Construction of the AAV vectors

AAV8 vectors used in the present study are represented schematically (Figure 1). The lengths of the AAV8-HAAT-cFVIII and AAV8-HCRHAAT-cFVIII were 5.15 kb and 5.2 kb, respectively. The vector isolation efficiencies of AAV8-HCRHAAT-cFVIII (5.2 kb) and of AAV8-HAAT-cFVIII (5.15 kb) after purification by the two rounds density gradient ultracentrifugation of CsCl were 1.68×10^4 vector genome copies (gc)/cell and 1.87×10^4 vector

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gc/cell (the average of two preparations), respectively, whereas the average vector isolation efficiency of AAV8 vectors carrying the human factor IX gene (4.3 kb) by the same procedure was 3.68×10^4 vector gc/cell (average of three experiments).

Analysis of HAAT promoter specificity with AAV8 vectors *in vivo*

To study the cell specificity of the HAAT promoter *in vivo*, AAV8 vectors carrying the Lac Z gene located downstream of the 297 b HAAT promoter (AAV8-HAAT-Lac Z) or the CMV promoter/growth hormone intron 1 (AAV8-CMV-LacZ) complex were injected into the cervical vein of C57BL/6 mice (5×10^9 gc/g body weight), and expression of the Lac Z gene was analysed by detecting β-galactosidase activity by staining mouse tissues with X-gal. Macroscopic views of organs from mice injected with AAV8-HAAT-Lac Z (Figure 2a) or AAV8-CMV-Lac Z (Figure 2b) and microscopic views of organs from mice injected with AAV8-HAAT-Lac Z (Figure 2c) or AAV8-CMV-Lac Z (Figure 2d) are shown. Arrows indicate positive X-gal staining. The β-galactosidase activity was macroscopically detected in the liver, heart and skeletal muscles of the AAV8-CMV-Lac Z-injected mice (Figure 2), whereas the β-galactosidase activity was solely detected in the liver of the AAV8-HAAT-LacZ-injected mice (Figure 2). These data in respect of β-galactosidase activity expression were confirmed by microscopic examination of these organs (Figure 2). β-galactosidase activities were microscopically detected in hepatocytes, myocardium and skeletal muscle fibers in a similar manner to the macroscopic views of organs of AAV8-CMV-Lac Z-injected mice, whereas no β-galactosidase activities were detected in the myocardium or skeletal muscle fibers in AAV8-HAAT-LacZ-injected mice (Figure 2). These data suggest that the transgene expression with AAV8 vectors preferentially occurs in the liver, but is also affected by the tissue specificity of the promoter used in the AAV8 vector, and that the hepatocyte specificity of the HAAT promoter facilitates liver-restricted transgene expression with the AAV8 vector.

Transgene expression by the HCRHAART enhancer/promoter complex with AAV8 vectors

The DNA fragments spanning the HAAT promoter located downstream of the HCR of apolipoprotein E gene have been shown to express genes in the liver very efficiently [18,26,27]. We could also express human factor IX in mice at approximately 6–7 U/ml (18–21 μg/ml) using the AAV8 vectors carrying the human factor IX gene driven by the HCR enhancer (325 b)/HAAT promoter (297 b) complex (data not shown). However, the DNA fragments used in these studies were too large to enable the FVIII gene to be carried on AAV vectors. Thus, we utilized the minimum HCR enhancer element and the minimum HAAT promoter sequence for FVIII gene expression with AAV8 vectors. Tissue-specific expression of the luciferase gene driven by the HCRHAAT enhancer/promoter complex was quantified by analysing photons from mice under anesthesia using IVIS Imaging Systems (Xenogen Co.) and was compared with that by the CAG promoter. When the AAV8 vectors carrying the luciferase gene driven by the CAG promoter (2×10^9 gc/g) were injected to neonatal wild-type mice, luciferase gene expression was preferentially found in the liver, but also detected in the heart, tail and limbs (Figure 3). By contrast, luciferase gene expression was restricted to the liver in the neonatal mice, with injection of the AAV8 vectors carrying the luciferase gene driven by the minimum HCRHAAT enhancer/promoter complex (2×10^9 gc/g) (Figure 3). When the AAV8 vectors carrying the luciferase gene driven by the CAG promoter were injected into adult mice (2×10^9 gc/g), luciferase gene expression occurred more preferentially in the liver than in neonatal mice, but luciferase gene expression was still observed in the heart and the tail of adult mice. Again, luciferase gene expression was detected solely in the liver of mice injected with the AAV8-HCRHAAT-Luc vector (2×10^9 gc/g). These data suggest that the extrahepatic gene expression with AAV8 vectors may be relatively broad in neonatal mice compared to that in adult mice, and that the HCRHAAT enhancer/promoter complex may have liver specificity not only in adult mice, but also in neonatal mice. In addition, a comparison of the amount of photons from the mouse liver suggests that the minimum HCRHAAT enhancer/promoter complex had approximately ten-fold higher promoter activity than the CAG promoter in the mouse liver *in vivo*.

Expression of FVIII activity in *fviii*$^{-/-}$ mice with AAV vectors carrying the BDD cFVIII gene

FVIII clotting activity levels in hemophilia A mice after intravenous injection of AAV8-HAAT-cFVIII increased dose-dependently on day 28, achieving therapeutic FVIII levels (approximately 0.3 U/ml; 30% of the normal canine FVIII level) and normal FVIII levels in *fviii*$^{-/-}$ mice with the AAV8-HAAT-cFVIII at doses 5×10^9 gc/g and 5×10^{10} gc/g (Figure 4), respectively. FVIII clotting activity levels in *fviii*$^{-/-}$ mice after intravenous injection of AAV8-HCRHAAT-cFVIII were increased dose-dependently on day 28, achieving therapeutic canine FVIII levels (0.32 U/ml) and normal canine FVIII levels (1.45 U/ml) in *fviii*$^{-/-}$ mice with AAV8-HCRHAAT-cFVIII at doses of 5×10^8 gc/g and 5×10^9 gc/g, respectively (Figure 4), indicating that the high cFVIII activity level was achieved with AAV8-HCRHAAT-cFVIII at 1 : 10 of the dose of the AAV8-HAAT-cFVIII and the AAV8-β-actin-cFVIII [20].

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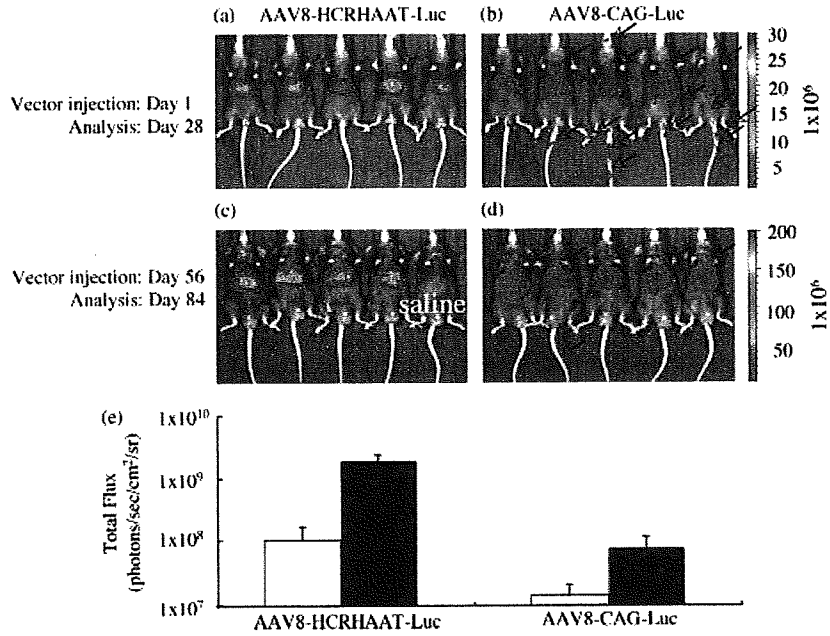

Figure 3. *In vivo* expression of luciferase gene driven by the HCRHAAT enhancer/promoter complex. Wild-type mice injected with AAV8-HCRHAAT-Luc or the AAV8-CAG-Luc on day 1 after birth (a, b) or on day 56 after birth (c, d) were analysed for expression of the luciferase gene using an *in vivo* imaging system on day 28 or on day 84, respectively. Photons detected through the mouse skin were visualized (a–d). Significant luminescence was detected at positions corresponding to the liver. Arrows indicate extrahepatic luminescence. No luminescence signal was detected in the nonvector injected mouse (saline, saline-injected mice, control). Photons transmitted through the skin of mice transduced with AAV8-HCRHAAT-Luc or with AAV8-CAG-Luc on day 1 (open square) or on day 56 (closed square) after birth were quantified 28 days after vector injection (e).

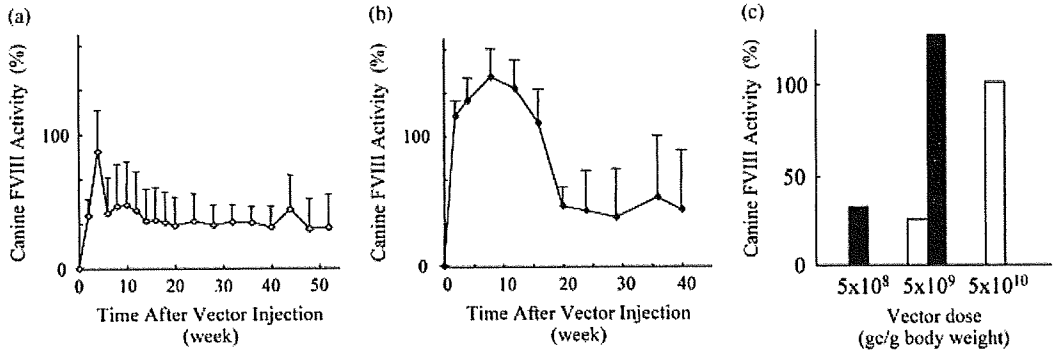

Figure 4. Expression of canine FVIII in *fviii*$^{-/-}$ mice injected with AAV8 vectors carrying the canine FVIII gene. The canine FVIII levels in *fviii*$^{-/-}$ mice injected with 5×10^{10} gc/g body weight of AAV8-HAAT-cFVIII (a) or 5×10^{9} gc/g body weight of AAV8-HCRHAAT-cFVIII (b) are shown. Values represent the mean ± SD. The canine FVIII levels in *fviii*$^{-/-}$ mice injected with AAV8-HCRHAAT-cFVIII (black bars; doses of 5×10^{8} gc/g body weight or 5×10^{9} gc/g body weight) or AAV8-HAAT-cFVIII (white bars; doses of 5×10^{9} gc/g body weight or 5×10^{10} gc/g body weight) on day 28 after vector injection are shown (c). FVIII activities were determined by the one-step APTT method using FVIII deficient human plasma and were standardized with normal canine plasma. One unit canine FVIII/ml represents 100% canine FVIII clotting activity.

Analysis of transcripts of canine FVIII transgene in organs of *fviii*$^{-/-}$ mice injected with AAV8 vectors carrying the canine FVIII gene

Analysis of cFVIII transcripts in vector-injected mice suggests that the cFVIII gene was specifically expressed in the liver (Figure 5) and no significant amount of cFVIII transcripts were detected by RT-PCR or quantitative RT-PCR in other organs of mice injected with AAV8-HCRHAAT-cFVIII or AAV8-HAAT-cFVIII. These data confirm that the expression of the cFVIII gene by the HAAT promoter or the HCRHAAT enhancer/promoter complex was liver specific. Transcript levels of the cFVIII transgene in the liver of AAV8-HCRHAAT-cFVIII injected mice were approximately ten-fold higher than in AAV8-HAAT-cFVIII-injected mice at the same vector dose. These data are in accordance with the cFVIII levels in the vector-injected

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mice, suggesting that the HCRHAAT enhancer/promoter complex had ten-fold higher transgene expression activity than the HAAT promoter *in vivo*.

Immunohistochemistry of canine FVIII in the liver of mice injected with AAV8 vectors carrying the canine FVIII gene

Immunohistochemistry analysis confirmed that cFVIII was efficiently expressed in hepatocytes of mice injected with a low dose of AAV8 vectors carrying the cFVIII gene under the control of the HAAT promoter or the HCRHAAT enhancer/promoter complex (Figure 6).

Analysis of anti-cFVIII neutralizing antibody in *fviii*$^{-/-}$ mice

In our previous study, we showed that the expression of the cFVIII gene with the AAV8 vector has an advantage over AAV1 vector-mediated cFVIII gene transfer to the skeletal muscles in terms of the immune reaction to the transgene product [20]. No neutralizing antibody development was observed until 12 weeks after vector injection of AAV8-β-actin-cFVIII under immunosuppression [20]. However, neutralizing antibodies against cFVIII developed in four out of four mice at 12 weeks after termination of immunosuppression (i.e. week 24 after vector injection). When no immunosuppressants were given throughout the course, antibodies against cFVIII were formed in six of eight mice 4–20 (mean 12.8) weeks after AAV8-β-actin-cFVIII vector injection (Table 1). By contrast to the AAV8-β-actin-cFVIII-injected *fviii*$^{-/-}$ mice, neutralizing antibodies were found in only one out of six mice with AAV8-HAAT-cFVIII injection under the same immunosuppression. Interestingly, the level of neutralizing antibody against cFVIII in that mouse gradually decreased and became undetectable by week 8 after termination of immunosuppression (i.e. week 20 after vector injection), and the cFVIII activity in the mouse started to increase from week 12 after termination of immunosuppression (i.e. week 24 after vector injection) and reached a plateau of 0.45 U/ml (45% of the normal canine FVIII level) by week 24 after termination of immunosuppression. Therapeutic levels of cFVIII in other AAV8-HAAT-cFVIII-injected *fviii*$^{-/-}$ mice were sustained for more than 40 weeks without immunosuppression (i.e. week 52 after vector injection) (Figure 4 and Table 1). These data lead us to speculate that the extrahepatic expression of cFVIII gene might correlate with the development of neutralizing antibodies. On the basis of this notion, the AAV8-HCRHAAT-cFVIII vector was injected to *fviii*$^{-/-}$ mice without any immunosuppression and cFVIII expression and neutralizing antibody formation was investigated. High cFVIII gene expression and an elevation of blood cFVIII levels (Figures 4 and 5) without neutralizing antibody formation were achieved in AAV8-HCRHAAT-cFVIII-injected *fviii*$^{-/-}$ mice (Table 1). These data suggest that liver-restricted expression of cFVIII with AAV8 vector and the liver-specific promoter facilitates the prevention of inhibitor formation to cFVIII in *fviii*$^{-/-}$ mice. Although the liver-restricted cFVIII gene transfer facilitated hypo-responsiveness to transgene-derived cFVIII, antibody formation against AAV8 capsid developed in the mice with AAV8-HCRHAAT-cFVIII injection in a similar manner to that in mice receiving other AAV8 vectors (data not shown).

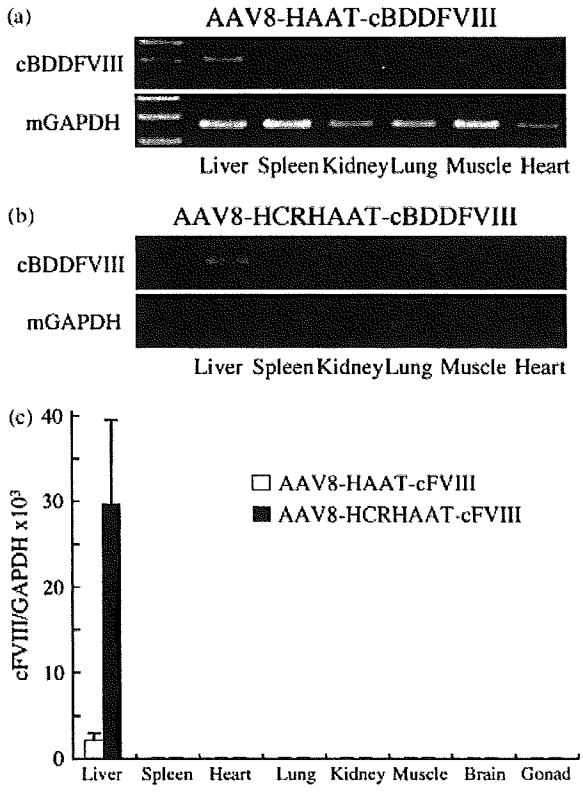

Figure 5. Analysis of transcripts of the canine FVIII transgene in organs of mice injected with AAV8 vectors carrying the canine FVIII gene. The transcripts of canine FVIII transgene in organs of fviii$^{-/-}$ mice injected with 5×10^9 gc/g body weight AAV8-HAAT-cFVIII (a) or 5×10^9 gc/g body weight AAV8-HCRHAAT-cFVIII (b) were detected by RT-PCR and were quantified using real-time PCR. The quantity of canine FVIII transgene transcripts was standardized with GAPDH transcripts (c) (AAV8-HAAT-cFVIII, white bar; AAV8-HCRHAAT-cFVIII, black bar).

Discussion

Various serotypes of AAV vectors have been developed, and each AAV serotype has its own tropism [11]. However, the tropism of an AAV serotype is not completely specific for a certain type of cell and transgene expression in target cells and organs may also be affected by the tissue specificity of the promoter used in the AAV vectors. Ubiquitous promoters, such as the CMV promoter and the CAG promoter, have been used in early studies of gene therapy; however, the use of a tissue-specific promoter for

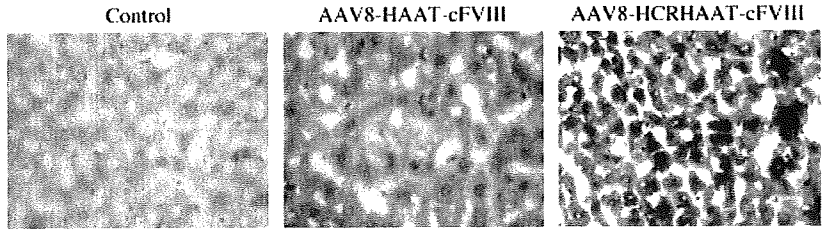

Figure 6. Immunohistochemistry of canine FVIII in the liver of mice injected with AAV8 vectors carrying the canine FVIII gene. Canine FVIII in the liver of *fviii*$^{-/-}$ mice injected with 5×10^9 gc/g body weight of AAV8-HAAT-cFVIII or 5×10^8 gc/g body weight AAV8-HCRHAAT-cFVIII was detected by immunohistochemistry with sheep anti-human FVIII polyclonal antibodies, as described in the Materials and methods. Positive staining is brown. As a control, liver sections obtained from *fviii*$^{-/-}$ mice without vector injection were simultaneously processed with the same antibody solution as the control.

Table 1. Neutralizing antibody against cFVIII in vector injected *fviii*$^{-/-}$ mice

Vector	AAV8-β-cFVIII	AAV8-β-cFVIII	AAV8-HAAT-cFVIII	AAV8-HCRHAAT-cFVIII
Immunosuppression*	Yes, 12 weeks	No	Yes, 12 weeks	No
CFVIII activity on day 56 after vector injection (%)	77.6 ± 21.3 (mean ± SD)	79.8 ± 81.8 (mean ± SD)	87.5 ± 30.6 (mean ± SD)	127.0 ± 17.1 (mean ± SD)
Neutralizing antibody formation (*n*)	4/4	6/8	1/6	0/6
Inhibitor titer (Bethesda U/ml)	10.7 ± 0.5 (mean ± SD)	18.5 ± 13.6 (mean ± SD)	4.0	Not detected
Spontaneous regression of neutralizing antibody	No	No	Yes	Not applicable

*Cyclophosphamide and tacrolimus were injected to mice after vector injection for 12 weeks.

cell-specific expression of a transgene is required to avoid undesirable effects. One such side-effect is the formation of antibody against the transgene product. In particular, a serious concern in hemophilia A gene therapy is the formation of antibody against transgene-derived FVIII. Liver-specific expression of transgene products upon gene transfer is attractive with regard to immune tolerance induction to the transgene products [18,28–30]. Indeed, AAV vector-mediated gene transfer to the liver has been shown to have a reduced pro-inflammatory risk compared to lentivirus vector-medicated gene transfer [18,31]. In addition, AAV8 vectors and AAV9 vectors do not express transgenes in the spleen [18]. On the basis of these notions, we developed an AAV8 vector carrying the cFVIII gene driven by the HAAT promoter or the HCRHAAT enhancer/promoter complex, and investigated the expression of cFVIII in *fviii*$^{-/-}$ mice.

Canine FVIII is a xenoantigen to mice; therefore, mice might develop neutralizing antibodies to cFVIII if cFVIII is expressed in mice. Indeed, *fviii*$^{-/-}$ mice developed neutralizing antibodies against cFVIII, even under immunosuppression, when the cFVIII gene driven by the β-actin promoter was expressed in skeletal muscles using the AAV1 vector [20]. However, when the cFVIII gene, driven by the same promoter, was transduced to *fviii*$^{-/-}$ mice using the AAV8 vector, no neutralizing antibodies against cFVIII developed in vector-injected *fviii*$^{-/-}$ mice under the same immunosuppression, suggesting that AAV8 vector-mediated FVIII gene transfer to the liver was advantageous over AAV1 vector-mediated gene transfer to the skeletal muscle in terms of neutralizing antibody formation against the transgene product cFVIII. However, the AAV8 vector-mediated cFVIII gene transfer with the β-actin promoter was not sufficient to prevent neutralizing antibody formation against transgene-derived cFVIII, as shown in the present study (Table 1). The present study demonstrated that extrahepatic expression of the transgene might function to develop neutralizing antibodies to cFVIII in *fviii*$^{-/-}$ mice. The minimum β-actin promoter, a part of the CAG promoter, had a significant promoter activity in HEK293 cells and was approximately one-half to one-third of that of the CAG promoter [20]. By contrast, the activities of the HAAT promoter and the HCRHAAT enhancer/promoter complex used in the present study were almost the same as the promoter-less control vector in HEK293 cells (not shown), suggesting that leaky gene expression of the HAAT promoter and the HCRHAAT enhancer/promoter complex in nonhepatocyte cells can be minimized. In addition, the leaky expression of the Lac Z gene driven by the HAAT promoter or of the luciferase gene driven by the HCR/HAAT promoter was not apparent *in vivo* (Figures 2 and 3). On the basis of this notion, we attempted to express cFVIII with AAV8-HCRHAAT-cFVIII in *fviii*$^{-/-}$ mice without immunosuppression to determine whether liver-restricted expression of cFVIII is sufficient for hypo-responsiveness of inhibitor (antibody) formation to cFVIII. In this experiment, none of the mice injected with the AAV8-HCRHAAT-cFVIII developed neutralizing antibodies against canine FVIII for up to 10 months without immunosuppression. Taken together, these data suggest that the liver-restricted transgene expression would be effective to reduce the immune reaction to transgene-derived canine FVIII. Immune tolerance induction to the transgene product is one of the key issues of gene therapy for genetic disease caused by a

single gene abnormality and has been extensively studied in a mouse hemophilia B model by expressing factor IX with viral vectors [18,28–30]. Hypo-responsiveness to transgene product FVIII including immune tolerance induction may be more important for hemophilia A gene therapy than for hemophilia B gene therapy because approximately 21–32% of severe hemophilia A patients develop inhibitors (alloantibody) against therapeutically injected FVIII, whereas inhibitors against factor IX form in approximately 9% of severe hemophilia B patients upon factor IX infusion. A variety of approaches for induction of hypo-responsiveness to FVIII including immune tolerance have been shown to be effective [32–34]. In this regard, liver-restricted expression of FVIII using the AAV8 vector together with the liver-specific promoter might be an alternative gene transfer approach for this purpose.

The vector doses required for the increase of the cFVIII activity level to 0.4–1.2 U/ml in *fviii*$^{-/-}$ mice suggested that the AAV8-HCRHAAT-cFVIII vector was approximately ten-fold more potent than both the AAV8-HAAT-cFVIII and the AAV8-β-actin-cFVIII vectors. Expression of the transgene may be mainly driven by the internal promoter used in the AAV vector; however, it is still possible that transgene expression is affected by the presence of the ITR and the A/D sequences because these elements may function as cis-acting elements in human cells, thereby interfering with the regulated downstream gene expression cassette [35,36]. In the context of minimizing nonspecific transgene expression with AAV vectors, a reduction of vector doses for gene transfer is also important and can be achieved using the AAV8 vector carrying the therapeutic gene driven by the HCRHAAT enhancer/promoter complex to avoid an undesirable immune reaction to the transgene product. This efficient cFVIII expression in FVIII deficient mice could be achieved by the use of this enhancer promoter complex [21,22,27], the removal of the DNA segment coding the FVIII B domain from the FVIII gene [37,38], and the high liver transduction efficiency of the AAV8 vector [11,15,39].

The site of extrahepatic expression of canine FVIII contributing to inhibitor formation has not been determined. One possibility is the expression of FVIII in skeletal muscles [40]. This remains the subject of future studies.

Acknowledgements

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Association of Asn221Ser mutation in tissue factor pathway inhibitor-β with plasma total tissue factor pathway inhibitor level

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Tissue factor pathway inhibitor (TFPI) is an anticoagulant protease inhibitor that inhibits the tissue factor-initiated blood coagulation cascade reactions. Based on these anticoagulant functions of TFPI, we hypothesized that genetic variations in *TFPI* may alter the TFPI expression or impair the anticoagulant function and could predispose persons to deep vein thrombosis (DVT). This study was undertaken to examine whether the genetic polymorphisms in *TFPI* are associated with the plasma TFPI levels and risk for DVT. We sequenced the entire coding regions of *TFPI* in 175 Japanese DVT patients and identified 12 genetic variants, including one missense mutation, Asn221Ser. The missense mutation occurred at the site presumably attached to the glycosylphosphatidylinositol anchor in the TFPI-β form. The allele frequency of the mutant Ser-coding allele of the Asn221Ser mutation was 8% in the Japanese general population consisting of 1684 individuals. The Asn221Ser mutation was significantly associated with the total TFPI levels (Asn/Asn, $n = 108$, total TFPI = 56.57 ± 0.88 ng/ml (mean ± SD) vs. Asn/Ser + Ser/Ser, $n = 16$, total TFPI = 63.44 ± 2.28 ng/ml, $P = 0.0058$). The genotype was not associated with the free TFPI level. This Asn221Ser mutation was not associated with DVT. Thus, the Asn221Ser mutation occurring in the TFPI-β form was associated with the total TFPI level, but not a risk for DVT. The absence of the putative glycosylphosphatidylinositol anchor in TFPI-β under pathological conditions remains to be studied. *Blood Coagul Fibrinolysis* 20:22–26 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: deep vein thrombosis, glycosylphosphatidylinositol, tissue factor pathway inhibitor

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Introduction

Tissue factor pathway inhibitor (TFPI) is a protease inhibitor with three tandem Kunitz-type inhibitor domains, which inhibits the tissue factor-initiated blood coagulation cascade reactions by forming a complex with factor VIIa/tissue factor via the Kunitz-1 domain and with factor Xa via the Kunitz-2 domain [1–3].

The human TFPI gene resides on the long arm of chromosome 2 and has been originally reported as consisting of 9 exons [4–6]. Later on, an alternative spliced form, TFPI-β, was identified. Therefore, the human TFPI gene is now known to have 10 exons separated by 9 introns. Mature TFPI-α consists of 276 amino acid residues. An alternatively spliced form, TFPI-β, has a β-form specific C-terminal region encoded by exon 8 instead of the Kunitz-3 domain and the C-terminal region encoded by exons 9 and 10. The C-terminal region specific to the β-form directs the attachment of a putative glycosylphosphatidylinositol (GPI) anchor at Asn221, resulting in the formation of GPI-anchored TFPI-β [7–10]. TFPI-β thus formed is supposed to stay on the endothelial cell surface. Most TFPI-α are present in plasma, but some also stay on the endothelial cell surface through the heparan sulfate-binding sites on their Kunitz-3 domain and C-terminal region. TFPI binding to endothelium is considered to be important for the regulation of the initial phase of the coagulation cascade [1–3]. Although TFPI-α consisted 80% of TFPI on the cell surface, a recent study [11] suggests that TFPI-β was responsible for most of the cellular inhibitory activity for factor VIIa/tissue factor.

Animal models suggest the link of TFPI dysfunction or deficiency with a prothrombotic phenotype [12–15]. Mice lacking exon 4 of the *Tfpi* gene that encoded the

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Kunitz-1 domain ($Tfpi^{K1delta}$) showed embryonic lethality through hemorrhage due to a consumptive coagulopathy [12]. The lethal hemorrhagic phenotype for mice carrying the factor $VII^{-/-}$ genotype has been rescued by having $Tfpi^{K1delta}$ alleles [13]. Mice with a combined heterozygous *Tfpi* deficiency and homozygous apolipoprotein E deficiency ($Tfpi^{+/K1delta}$/$apoE^{-/-}$) exhibited a more pronounced atherosclerosis and thrombosis than mice with $Tfpi^{+/+}$/$apoE^{-/-}$ [14]. The $Tfpi^{K1delta}$ allele conferred the lethal thrombotic phenotype to mice homozygous for the factor V Leiden mutation [15].

Based on these anticoagulant functions of TFPI, we hypothesized that genetic variations within the *TFPI* gene may alter the TFPI expression or impair the anticoagulant function and could predispose persons to deep vein thrombosis (DVT). To test this hypothesis, we sequenced the entire coding region of the *TFPI* gene, including the TFPI-β-specific exon 8, in unrelated patients with objectively confirmed DVT for genetic variation and identified one novel missense mutation, Asn221Ser. This missense mutation occurred at an amino acid residue presumably attached with the GPI anchor in the TFPI-β form. By genotyping this mutation in a Japanese general population, we assessed the effects of the genotypes on the plasma TFPI levels and compared the genotype prevalence of the mutation in DVT patients with that in population-based controls. Finally, we found that the Asn221Ser mutation increased in the total TFPI levels but did not confer a genetic risk for DVT in our Japanese population. The Asn221Ser mutation was observed at a significant prevalence in the population, and the mutant TFPI-β was presumably not modified with the GPI anchor. Thus, functional analysis of the mutant form is needed to clarify the loss of the membrane anchoring of TFPI-β.

Materials and methods

Deep vein thrombosis patient group and general population

One hundred and seventy-five DVT patients were registered by the Study Group of Research on Measures for Intractable Diseases working under the auspices of the Ministry of Health, Labor and Welfare of Japan, as described previously [16,17]. The patients consisted of the previously enrolled 161 Japanese DVT patients and additional 14 DVT patients. Diagnosis of DVT was made by ultrasonography, radioisotope venography, and magnetic resonant imaging angiography. As the controls, a general population randomly selected from Suita city residents, the Suita Study, was used. The study design of the Suita Study has been described previously [18,19]. There were no exclusion criteria for the control individuals. A total of 1684 participants were included. The protocol of this study was approved by the Ethical Review Committee of each institute. Only those who gave written informed consent for genetic analyses were included in this study.

Direct DNA sequencing of tissue factor pathway inhibitor in deep vein thrombosis patients

We sequenced all 10 exons and flanking regions and 651 bp of the upstream region of exon 1 in *TFPI* in 175 DVT patients. The method of direct sequencing was described previously [20]. Information on the primers and PCR conditions is available upon request. The obtained sequences were examined for the presence of mutations using NAMIHEI (version 1.0; Mitsui Knowledge Industry, Tokyo, Japan) and Sequencher software (version 4.0; Gene Codes Corporation, Ann Arbor, Michigan, USA), followed by visual inspection [21]. We have adopted the mutation nomenclature recommendation, wherein the A of the ATG of the initiator Met codon is denoted nucleotide +1, and the initial Met residue is denoted amino acid +1 [22].

Genotyping of general population

The Asn221Ser (c.662A>G) genotyping was performed by the TaqMan allele discrimination method [21] using the primers 5′-CCACAGTGTTAAACATATAAAGATGACTCACA/5′-AACATGGATGCATGAATGCAGAAG and the probes 5′-VIC-CCGCATTCTTCCAAC (the wild-type Asn coding allele)/5′-FAM-CGCACTCTTCCAAC (the mutant Ser coding allele).

Measurement of plasma antigen levels of total and free tissue factor pathway inhibitor

The total TFPI antigen level was measured by the Total TFPI ELISA kit (Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) [23]. The kit consisted of a rabbit anti-TFPI polyclonal antibody immobilized to the microplate well and a horseradish peroxidase-conjugated monoclonal antibody that can recognize the specific conformation formed between the Kunitz-1 and Kunitz-2 domains. The plasma antigen levels of free TFPI have been previously measured [24]. The antigen level of free TFPI was measured by a sandwich enzyme immunoassay method using the Kunitz-3 domain-specific monoclonal antibody [25]. The results were expressed as means ± one SD.

Statistical analysis

Pair-wise linkage disequilibrium between two polymorphisms was evaluated by determining r^2 with SNPAlyze software (version3.1; Dynacom, Mobara, Japan). The χ^2 test was used to compare the observed genotype frequencies with the Hardy–Weinberg equilibrium prediction. Comparisons between the case and control groups were analyzed by the χ^2 test using genotype and allele frequencies as independent variables. Association analyses in each sex of genotypes with plasma total and free TFPI levels were performed through logistic regression analysis considering the potential confounding

Table 1 List of genetic mutations identified in *TFPI* gene in 175 Japanese patients with deep vein thrombosis

Genomic	cDNA	Region	Amino acid change	Allele1 homo	Allele 1/2	Allele 2 homo	Total	Allele 1 frequency	Allele 2 frequency	Surrounding sequence	dbSNP ID
g.-51095C>T	c.-363-538C>T	promoter		66	78	31	175	0.60	0.40	ATTGT[C/T]TCACT	
g.-50983C>T	c.-363-426C>T	promoter		87	71	17	175	0.70	0.30	AAAAG[C/T]TTATT	rs10931292
g.-296G>A	c.-2-294G>A	intron2		110	59	6	175	0.80	0.20	TCAGC[G/A]TTTAC	rs2192824
g.-254T>C	c.-2-252T>C	intron2		173	2	0	175	0.99	0.01	ATATT[T/C]TCAAA	
g.6743G>A	c.174G>A	exon4	Ala58	174	1	0	175	1.00	0.00	AAGGC[G/A]GATGA	
g.19653T>C	c.628+8T>C	intron7		164	11	0	175	0.97	0.03	AAGAA[T/C]CTTGT	
g.19796_19799 GTTTdel	c.628+151_154 GTTTdel	intron7		139	29	7	175	0.88	0.12	AAATT[GTTT/---]AAGAC	rs8176613
g.24999A>G*	c.662A>G	exon8	Asn221Ser	149	25	1	175	0.92	0.08	GAAGA[A/G]TGCGG	rs7586970
g.25244T>C	c.847+60T>C	intron8		174	1	0	175	1.00	0.00	AAAAA[T/C]GAATA	
g.35804T>C	c.629-33T>C	intron8		149	25	1	175	0.92	0.08	TCACA[T/C]ATGGC	rs8176592
g.36030T>C	c.808+14T>C	intron9		173	2	0	175	0.99	0.01	GATAC[T/C]CTTCC	
g.37312T>A	c.1293+101T>A	3'flanking		128	45	2	175	0.86	0.14	TATAG[T/A]ATTCT	

Alleles 1 and 2 are major and minor alleles, respectively. * c.662A>G for the large scale genotyping by the TaqMan genotype discrimination method.The A of the ATG of the initiator Met codon is denoted nucleotide +1, and the initial Met residue is denoted amino acid +1, as recommended by the Nomenclature Working Group. The nucleotide sequence (GenBank Accession ID: NC_000002.10) was used as a reference sequence. c.-363-538C>T and c.-363-426C>T were previously designated as −399C>T and −287T>C, respectively.Linkage disequilibrium: c.662A>G and c.629-33T>C showed an r^2 of 1.00 and c.629-33T>C and c.1293+101T>A showed an r^2 of 0.22.

risk variable of age. Differences with P values less than 0.05 were considered statistically significant.

Results

Genetic polymorphisms in tissue factor pathway inhibitor gene in Japanese deep vein thrombosis patients

We sequenced all exons and flanking regions and 651 bp of the upstream region of exon 1 in *TFPI* in 175 DVT patients (Table 1). We identified 12 genetic variations, including c.662A>G in TFPI-β-specific exon 8 that encoded a missense mutation causing Asn221 to be replaced by Ser (Asn221Ser). This missense mutation occurred at the site presumably modified by the GPI anchor in the TFPI-β form. Among the 175 DVT patients, 25 were heterozygous carriers for the mutant Ser221-coding allele and one was homozygous. The allele frequency for the Ser221-coding allele was 0.077 in the DVT patients. This mutation, c.662A>G, was in complete linkage disequilibrium with c.629-33T>C.

Genotyping of Asn221Ser mutation in the general population and association with the plasma levels of total and free tissue factor pathway inhibitor and with deep vein thrombosis

We genotyped the Asn221Ser mutation in the general population consisting of 1684 individuals using the TaqMan allele discrimination method. The genotype was followed in the Hardy–Weinberg equilibrium ($P=0.6894$). The minor G-allele frequency of c.662A>G (Asn221Ser) in the general population was 0.079. Next, we examined the effects of the genotype on the plasma TFPI levels. We found that the plasma total TFPI level was significantly elevated in individuals with the mutant Ser-coding allele (Asn/Asn, $n=108$, total TFPI $=56.57\pm0.88$ ng/ml (mean ± SD) vs. Asn/Ser + Ser/Ser, $n=16$, total TFPI $=63.44\pm2.28$ ng/ml, $P=0.0058$; Table 2). This association was not found in men when the population was divided by sex. A free TFPI level was not associated with the genotype. Finally, we examined the association of this genotype with DVT. The allele frequency of the Ser221-coding allele in the general population group was not significantly different from that in the DVT patient group ($P=0.888$; Table 3). The genotype frequency of the Asn221Ser mutation was not statistically different between the DVT patient group and the general population group. Thus, the Asn221Ser mutation in *TFPI* was not associated with DVT.

Discussion

In the present study, we resequenced 350 alleles of *TFPI* in 175 unrelated Japanese patients with objectively confirmed DVT for genetic variation and identified 12 genetic variations, including one missense mutation, Asn221Ser. We genotyped this missense mutation in the general population and examined the association of its genotype with plasma TFPI levels. We found that the Ser-coding allele was associated with the increased plasma level of total TFPI but not associated with the

Table 2 Association of plasma free and total tissue factor pathway inhibitor levels with Asn221Ser mutation

	Combined			Women			Men		
	n	Free TFPI[a] (ng/ml)	Total TFPI[a] (ng/ml)	*n*	Free TFPI[a] (ng/ml)	Total TFPI[a] (ng/ml)	*n*	Free TFPI[a] (ng/ml)	Total TFPI[a] (ng/ml)
Asn/Asn	108	16.0 ± 0.4	56.6 ± 0.9	47	15.1 ± 0.6	55.1 ± 1.2	61	16.7 ± 0.6	57.8 ± 1.3
Asn/Ser+Ser/Ser	16	16.3 ± 1.1	63.4 ± 2.3	6	13.4 ± 1.2	64.7 ± 3.3	10	17.7 ± 1.4	62.5 ± 3.2
P		0.783	0.006		0.342	0.007		0.522	0.164

TFPI, tissue factor pathway inhibitor. [a]Age adjusted.

Table 3 Numbers and genotype frequencies of *TFPI* c.662A>G mutation (Asn221Ser) in the control and deep vein thrombosis groups

	General population, number (%)	DVT group, number (%)
Genotypes*		
AA	1425 (84.6)	149 (85.1)
AG+GG	259 (15.4)	26 (14.9)
Total	1684 (100)	175 (100)
Allele frequency**		
A allele	3101 (92.1)	323 (92.3)
G allele	267 (7.9)	27 (7.7)
Total	3368 (100)	350 (100)

DVT, deep vein thrombosis; OR, odds ratio; TFPI, tissue factor pathway inhibitor. Comparisons between the DVT and the control groups were analyzed using a χ^2 test with the genotypes as independent variables (indicated by *P* and OR). *OR = 0.960 (0.620–1.486) Pearson $P = 0.855$: $\chi^2 = 0.033$, Fisher $P = 0.913$. **OR = 0.971 (0.643–1.466) Pearson $P = 0.888$: $\chi^2 = 0.020$, Fisher $P = 1.000$.

free TFPI level. Finally, we found that the missense mutation did not show the association with DVT.

TFPI is present in plasma as well as on the endothelium [1–3]. The anticoagulant function of TFPI on the endothelium is thought to be physiologically important. TFPI-α binds to heparin-like glycosaminoglycan on the endothelium through both the Kunitz-3 domain and the C-terminal tail [26], and TFPI-β is thought to bind to endothelium through its unique C-terminal GPI anchor [7–10]. The functional mutation, Asn221Ser, occurred at the putative site for the GPI anchor, and the Ser221 bearing mutant was presumed not to be GPI anchored. Therefore, it is a reasonable assumption that the mutant TFPI-β is released from the cell surface to the plasma, resulting in the increase in the plasma total TFPI antigen level.

There are several reports on the association of the genetic polymorphisms with the plasma TFPI levels. A polymorphism present in intron 7, named −33T>C in intron 7, identified in the French population, has been reported to show the association with plasma total TFPI levels and DVT [27,28]. Individuals with the CC genotype showed higher total TFPI levels than those with the TT genotype. The age-adjusted odds ratio for DVT associated with the CC vs. the TT genotype was 0.6. This polymorphism, −33T>C in intron 7, is c.629-33T>C in our study. By means of intensive DNA resequencing, we found that c.629-33T>C was in complete linkage disequilibrium with the Asn221Ser mutation ($r^2 = 1.0$; Table 1). The distance between the two is 10 806 bp. Thus, we considered −33T>C in intron 7 to be a mere marker and the Asn221Ser mutation to be a real functional variant for the increased plasma total TFPI level.

Several missense mutations, including Pro151Leu (Pro179Leu in the nomenclature of the initial Met as +1) and Val264Met (Val292Met in the nomenclature of the initial Met as +1) in the *TFPI* gene have been reported in the Europeans [29–37]; however, these mutations were not identified in the present study. The Pro151Leu mutation has been identified in 1.2% of German DVT patients [29] and 0.2% of German blood donors [30]. The mutation has been identified in 2.0% of Spanish DVT patients [31]. There were no heterozygotes for this mutation in the 211 UK DVT patients [32]. Thus, the sample size for the resequencing of *TFPI* in the present study may not be high enough to identify this mutation in our DVT population. The Val264Met mutation has been identified in 4.9% of the control French population [36], so if it were present in the Japanese population with the same frequency, we would have detected it in the present study. It is now well known that there are ethnic differences in the genetic background of thrombophilia. Factor V Leiden mutation and prothrombin G20210A mutation are established risk factors predominantly found in Caucasian population [38], and protein S K196E mutation is a risk factor in Japanese population [39]. The Val264Met mutation in the *TFPI* gene might be an ethnic specific genetic variation.

In this study, we revealed the presence of the Asn221Ser mutation in *TFPI* that presumably confers defects on the GPI anchor attachment. This mutation did not show the association with DVT, but influenced the plasma TFPI levels. Ser-coding allele-bearing individuals would have low anticoagulant potency on the endothelium. Thus, it may confer the predisposed prothrombotic phenotype under some pathophysiological conditions such as disseminated intravascular coagulation and restenosis after angioplasty. Further studies are needed to clarify the thrombotic risks predisposed by this mutation.

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The authors declare no conflict of interest in connection with the submitted article.

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ORIGINAL ARTICLE

Induction of factor VIII-specific unresponsiveness by intrathymic factor VIII injection in murine hemophilia A

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Summary. *Background:* Hemophilia A is a congenital bleeding disorder caused by a deficiency of coagulation factor VIII. Approximately 30% of hemophilia A patients develop inhibitors against FVIII following replacement therapy. We have reported that neonatal exposure of FVIII antigen can induce antigen-specific immune tolerance by interferon-γ (IFN-γ)-dependent T-cell anergy in hemophilia A mice. *Objective:* The thymus plays crucial roles in self-tolerance, with negative selection of self-reactive effector T cells and positive selection of self-reactive regulatory T cells. We investigated the possibility of the induction of antigen-specific immune tolerance by intrathymic injection of FVIII in hemophilia A mice. *Methods:* Hemophilia A mice were injected with recombinant FVIII into the thymus under real-time high-resolution image guidance. *Results:* Anti-FVIII inhibitory antibody titers in mice challenged with intravenous administration of FVIII were significantly lower in mice (n = 22) that had received thymic FVIII injection than in mice (n = 18) without thymic injection (9.4 ± 2.3 vs. 122.5 ± 27.6 BU mL^{-1}, respectively, P = 0.00078). The CD4$^+$ T cells from thymic-injected mice could not proliferate or produce interleukin (IL)-2, IL-12 and IFN-γ in response to FVIII. The CD4$^+$CD25$^+$ T cells generated from thymic-treated mice but not from naïve mice efficiently suppressed the *in vitro* proliferative response of CD4$^+$ T cells and blocked the *in vivo* development of anti-FVIII antibodies in the adoptive transfer. *Conclusion:* These data suggest that intrathymic administration of FVIII could result in immune tolerance by induction of FVIII-specific regulatory T cells.

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Introduction

Hemophilia A is an X-linked hereditary bleeding disorder caused by deficiency in coagulation factor VIII [1]. Plasma-derived or recombinant FVIII is sufficiently available to permit its use for primary prophylaxis to avoid bleeding in patients with severe hemophilia A. A major complication of hemophilia A treatment is the development of neutralizing antibodies against the infused FVIII [2]. We have previously demonstrated that exposure to FVIII antigen within 24 h of birth induces antigen-specific immune tolerance by interferon (IFN)-γ-dependent T-cell anergy in hemophilia A mice [3].

The thymus plays a major role not only in the development of self-tolerance but also in acquired tolerance in autoimmunity and organ transplantation [4,5]. There are two mechanisms in the thymus to establish a self-tolerance system, consisting of negative selection of self-reactive effector T cells, and positive selection of self-regulatory CD4$^+$CD25$^+$ T cells [6,7,4,8]. The CD4$^+$CD25$^+$ T cells are known to be weakly reactive to antigenic stimulation and able to mediate suppression of CD25$^-$ naïve T cells [9,10]. In the thymus, CD4$^+$CD25$^+$ T cells are detected during the fetal period in humans, and during the perinatal period in mice [11]. The CD4$^+$CD25$^+$ T cells may be responsible for the translation of tolerance from an antigen-inoculated thymus to a mature but naïve peripheral immune system [12] [13]. Injection into the thymus of organs or cells has been successful in the induction of T-cell-mediated immunologic tolerance [14,15,16]. Allogenic grafts of pancreatic islets had better survival when grafted in the thymus, and provided long-term protection against spontaneous autoimmune diabetes if grafted during the early period after birth [17]. The expression or presentation of nominal antigen in the thymus might lead to enhanced deletion of autoreactive T cells or to induction of a number of antigen-specific regulatory T cells. In this study, we investigated the possibility of the

induction of antigen-specific immune tolerance by intrathymic injection of FVIII in hemophilia A mice. Our study may open new perspectives for the manipulation of FVIII-specific tolerance in the thymus of hemophilia A patients.

Materials and methods

Hemophilia A mice

FVIII-deficient mice (B6; $_{129}S_4$-$F8^{tm1Kaz}$/J) with targeted destruction of exon 16 of the FVIII gene were previously described and kindly provided by H. H. Kazazian Jr (University of Pennsylvania, Philadelphia, PA, USA) [18]. The experimental protocol was approved by the institutional Animal Care and Concern Committee of Jichi Medical University.

Intrathymic injection under the real-time high-resolution imaging system

Hemophilia A mice, 1–3 days old, were anesthetized by inhalation with 2.5% isoflurane in the anesthesia unit (Univentor, ZTN 08, Malta), and were imaged with a 30–50-MHz mechanical sector transducer with 50-μm axial and 115-μm lateral resolution (Vevo 770; Visualsonic Inc., Toronto, Canada). Two-dimensional real-time imaging of the thymus was accomplished with a 12 × 12-mm field of view and an optimal depth of 12.5 mm. When a cross-section with the thymus was located, a glass microcapillary needle (Becton Dickinson, San Jose, CA, USA) was placed at the parasternal area on the chest in the ultrasound imaging plane. Under real-time image guidance, the thymus was punctured with the needle, and 0.05 U g^{-1} body weight (BW) of highly purified, albumin-free preparations of recombinant FVIII (Kogenate FS; Bayer Healthcare, Leverkusen, Germany) or 0.005 U g^{-1} BW of human albumin (Sigma-Aldrich, St Louis, MO, USA) was injected precisely using a microinjector remote control system (Fig. 1A,B). Mice were then stimulated with intravenous FVIII (0.05 U g^{-1} BW) every 2 weeks, from 10 to 18 weeks of age. Blood samples were obtained 2 weeks after each of the injections from the jugular vein, and were added at a 9 : 1 (v/v) ratio to 0.38% sodium citrate; plasma was then separated by centrifugation. The plasma samples were subsequently stored at − 80 °C until further analysis.

Assay for FVIII inhibitors

FVIII inhibitor levels were measured according to the Bethesda methods. In brief, mouse plasma (50 μL) was incubated with 50 μL of normal pooled human plasma at 37 °C for 2 h. Residual human FVIII activity was measured in a one-stage assay using 50 μL of FVIII-deficient human plasma (Kokusai-Shiyaku, Kobe, Japan) and a 50-μL sample from the previous incubation. Samples were mixed with 100 μL of phospholipid activator, incubated at 37 °C for 3 min, and then mixed with 100 μL of 20 mmol L^{-1} $CaCl_2$. Clotting times were measured with a coagulometer (CA-500; Sysmex, Kobe, Japan). Coagutrol N

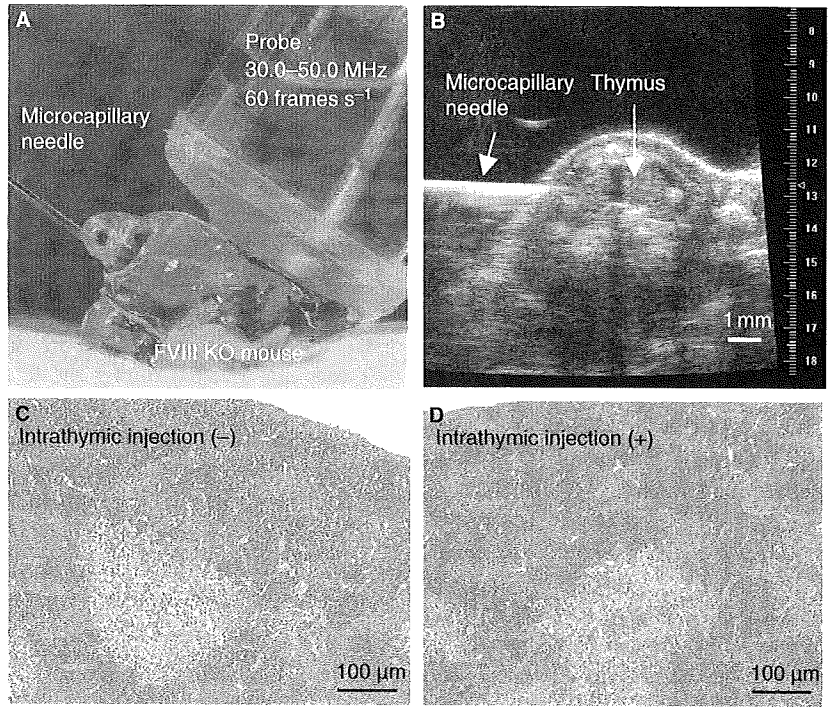

Fig. 1. Intrathymic injection of FVIII antigen using a high-resolution ultrasound system. Under real-time imaging of the thymus (A), a glass microcapillary needle was placed at the parasternal area and used to puncture the thymus (B). (C) The thymic sections were analyzed with hematoxylin and eosin staining 5 days after without (left panel) or with (right panel) thymic injection of FVIII in hemophilia A mice.

(Kokusai-Shiyaku) was diluted with Owren's Veronal Buffer to produce a standard curve of FVIII activity. The measurements were made in the linear portion of the response range.

Anti-FVIII IgG measurements

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

Determination of IgG subclass of anti-FVIII antibodies

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

Tetanus immunization of FVIII-deficient mice

Mice were injected intraperitoneally with 1 Limit of flocculation of tetanus toxoid (TT) vaccine (Takeda Chemical Industries, Tokyo, Japan). Plasma samples were obtained after 3 weeks, and anti-TT antibody titers were determined by ELISA as previously described. In brief, microtiter plates were coated with 5 μg mL^{-1} formaldehyde-inactivated tetanus toxin, *Clostridium tetanii* (Calbiochem, Darmstadt, Germany), for 16 h at 4 °C. After washing and blocking with Tris-buffered saline containing 5% BSA, mouse plasma samples were added to the wells and incubated for 2 h at 37 °C. After washing with 0.5% BSA in PBS containing 0.05% Tween-20, 100 μL of HRP-conjugated goat anti-mouse IgG was added for 1 h at 37 °C. Then, the peroxidase substrate was added and the absorbance at 405 nm was measured.

Cell preparation

Mice $CD4^+$ T cells were prepared by depletion of non-$CD4^+$ T cells with the autoMACS cell sorting system (Miltenyi Biotech GmbH, Bergish Gladbach, Germany), according to the manufacturer's instructions. $CD4^+CD25^+$ T cells were isolated by $CD25^+$ positive selection from $CD4^+$ T cells with magnetic cell sorting, using a CD4CD25 Isolation Kit (Miltenyi Biotech). The purity of sorted $CD4^+CD25^+$ T cells was confirmed to be more than 85% by flow cytometric analysis. Antigen-presenting cells were prepared from mice splenocytes by depletion of T cells using the magnetic sorting system with anti-CD90 (Thy1.2)-conjugated microbeads (Miltenyi Biotech), followed by irradiation with a single dose of 20 Gy (Gamma Cell; Norton International, ON, Canada), to prevent nonspecific proliferative responses during the *in vitro* FVIII stimulation assay.

Flow cytometric analysis

Cells from teased organs were labeled in PBS containing 1% BSA and 2 mmol L^{-1} EDTA at 4 °C for 30 min in the dark under continuous agitation. The following antibodies were used for phenotypic analysis: allophycocyanin-labeled anti-CD25 IgG (PC61.5; eBioscience, San Diego, CA, USA), fluorescein isothiocyanate-conjugated anti-CD4 IgG, phycoerythrin (PE)-labeled anti-CD45 IgG (30-F11; BD Pharmigen, Franklin Lakes, NJ, USA), and forkhead family transcription factor (Foxp3)–PE IgG (eBio7979; eBioscience), used according to the manufacturer's instructions. Isotype-matched irrelevant antibodies (BD Pharmigen) were used as controls. At least three events were analyzed on a FACS Aria (Becton Dickinson).

Proliferation assay with [^{3}H]thymidine incorporation

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

Cytokine assays

Splenocytes were incubated in 24-well plates at 1.0×10^6 cells per well in the absence or presence of 3 nmol L^{-1} human recombinant full-length FVIII (Kogenate FS) at 37 °C in 5% CO_2. Production of the cytokines interleukin (IL-2), IL-4, IL-12 and IFN-γ by $CD4^+$ T cells derived from each mouse was analyzed at 72 h with the ELISA kits (Biotrak ELISA System; Amersham Biosciences, Piscataway, NJ, USA), according to the manufacturer's instructions. In addition, levels of IL-10 were measured at 96 h by the ELISA system (Biotrak ELISA System).