Recombinant FLAG-tagged macaque FIX expressed in HEK 293 cells was efficiently secreted from the cells and detected by both a polyclonal antibody against human FIX and anti-FLAG antibody M2. However, its coagulation activity was significantly decreased compared with wild-type macaque FIX, macaque FIX T262A and human FIX expressed in HEK 293 cells (data not shown). These data suggest that the conformation of FLAG-tagged macaque FIX is altered. It is also possible that the FLAG sequence is immunogenic in macaques. Therefore, macaque FIX T262A may be closer to wild-type macaque FIX than tagged macaque FIX.

The mice that had high macaque FIX T262A expression survived more than 1 year without any events such as a sudden death. However, effect of over expression of FIX on the thrombogenecity may need to be studied carefully and precisely with the AAV vectors carrying the mouse FIX gene, since there may be a significant species difference in interaction of coagulation factors. Macaques with high FIX expression by the vector used in this study might also be good models for studying the effect of supra-physiological FIX level on the coagulation system. These may be the future studies.

In conclusion, macaque FIX T262A bound to the specific anti-human FIX monoclonal antibody 3A6, was efficiently expressed after gene transfer to the liver *in vivo*, and was quantified by the 3A6-based ELISA in the presence of wild-type macaque FIX. Macaque FIX T262A may have advantages over human FIX for studying the long-term expression of transgene-derived FIX in macaques. Therefore, macaque FIX T262A may be useful as a tool for FIX gene transfer studies in macaques.

Conflict of interest statement

The authors declare that they had no affiliation with or financial involvement in any organization or entity with a direct financial interest in the subject matter or materials discussed in the manuscript.

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Impact of acute cellular rejection on coagulation and fibrinolysis biomarkers within the immediate post-operative period in pediatric liver transplantation

Mimuro J, Mizuta K, Kawano Y, Hishikawa S, Hamano A, Kashiwakura Y, Ishiwata A, Ohmori T, Madoiwa S, Kawarasaki H, Sakata Y. Impact of acute cellular rejection on coagulation and fibrinolysis biomarkers within the immediate post-operative period in pediatric liver transplantation.
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Abstract: We studied restoration of the coagulation and fibrinolysis system in pediatric patients following liver transplantation and biomarkers of blood coagulation and fibrinolysis for suspecting the occurrence of acute cellular rejection. Coagulation activity recovered rapidly within two days following transplantation, but it took approximately 21–28 days for full recovery of the coagulation and fibrinolysis factors synthesized in the liver. PAI-1 levels were significantly higher in patients at the time of acute cellular rejection compared with levels after control of AR, and levels on days 14 and 28 in patients without AR. Plasma protein C and plasminogen levels at the time of rejection were significantly lower than those on day 14 in patients without AR. Statistical analysis suggested that an increase in plasma PAI-1 at a single time point in the post-operative period is a reliable marker among the coagulation and fibrinolysis factors for suspecting the occurrence of acute cellular rejection. These data suggested that appropriate anticoagulation may be required for 14 days after liver transplantation in order to avoid vascular complications and measurement of plasma PAI-1 levels may be useful for suspecting the occurrence of acute cellular rejection in pediatric patients following liver transplantation.

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Key words: liver transplant rejection – coagulation – fibrinolysis – plasminogen activator inhibitor 1

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Vascular thrombosis and immunological rejection of the transplanted liver in patients undergoing liver transplantation are frequent and serious post-operative complications (1). The majority of coagulation factors, factors regulating coagulation, and fibrinolysis factors are synthesized in the liver, and plasma coagulation factor levels may therefore fall for a short period after transplantation, but may return to normal levels upon regeneration of the grafted liver. Anastomosis of the vascular system of the grafted liver and the recipient vessels is carried out during liver transplantation, and anticoagulants are commonly administered for a period of time following surgery. However, restoration of the coagulation and fibrinolysis system following liver transplantation in pediatric patients has not been well studied (2, 3). In addition, the

Abbreviations: ADAMTS13, a disintegrin-like and metalloprotease with thrombospondin type 1 motif 13; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AR, acute rejection; AST, aspartate aminotransferase; ELISA, enzyme linked immunosorbent assay; LDH, lactate dehydrogenase; NAR, no acute rejection; PAI-1, plasminogen activator inhibitor 1; PELD, pediatric model for end-stage liver disease; PT-INR, prothrombin time-international normalized ratio; sES, soluble E-selectin; TM, thrombomodulin; vWF, von Willebrand factor; γ-GTP, γ-glutamyl transpeptidase.

thrombogenic state after liver transplantation is not well understood. We performed a single center study to investigate the coagulation and fibrinolysis system and the relationship between coagulation markers and acute cellular rejection following liver transplantation from living-related donors.

Materials and methods

Patients and study protocol

Sixty-three pediatric patients with liver failure due to biliary atresia (n = 59), ornithine transcarbamidase deficiency (n = 2), or Wilson's disease (n = 2) underwent living-related liver transplantation from April 2001 to March 2006 and were enrolled in this study. Most of the patients with biliary atresia had previously undergone hepatic porta-jejunostomies.

Description of patients

The patients were classified into two patient groups: one with acute cellular rejection (group AR, n = 24) and one with no acute cellular rejection (group NAR, n = 39). The diagnosis of acute cellular rejection was made by liver biopsy. There were no significant differences between group AR and group NAR in terms of age, gender, basal diseases, or the use of calcineurin inhibitors (data not shown). The PELD scores (AR, 13.0 ± 7.8; NAR, 15.1 ± 9.5), the amount of blood loss (AR, 85.0 ± 127.8 mL/kg; NAR, 125.8 ± 176.0 mL/kg), the amount of total blood transfusion (AR, 162.1 ± 109.1 mL/kg; NAR, 161.8 ± 170.8 mL/kg), the amount of plasma transfusion (AR, 64.1 ± 49.1 mL/kg; NAR, 94.2 ± 89.1 mL/kg), the cold ischemic time of graft liver (AR, 149.75 ± 126.4 min; NAR, 121.1 ± 69.1 min), and the warm ischemic time of graft liver (AR, 64.9 ± 18.1 min; NAR, 65.1 ± 13.3 min) upon operation were not significantly different between group AR and group NAR. Patients with severe infections or major bleeding episodes at the time of blood sampling for analysis were excluded from the analysis.

Immunosuppression and anticoagulation protocols

The standard protocol for immunosuppression was as follows. Both methylprednisolone and a calcineurin inhibitor (tacrolimus or cyclosporine) were used for immunosuppression. Intravenous administration of methylprednisolone (20 mg/kg) was started during the operation and the dosage was tapered to 3 mg/kg on day 1 and to 0.5 mg/kg on day 7 after liver transplantation. A calcineurin inhibitor was infused intravenously after transplantation and the blood concentration of tacrolimus or cyclosporine was adjusted to 18–20 ng/mL or 200–300 ng/mL till day 7 after liver transplantation, respectively. Intravenous injection of calcineurin inhibitor and methylprednisolone were converted to oral administration of these regimens after patient's oral intake had been fully confirmed and the blood concentration of tacrolimus or cyclosporine was adjusted 10–15 ng/mL or 100–150 ng/mL, respectively. The methylprednisolone dose was tapered to 0.06 mg/kg on day 30. Post-operative anticoagulation was performed with intravenous administration of dalteparin (low molecular weight heparin) at the dose of 2 U/kg/h, nafamostat mesilate (serine protease inhibitor with anticoagulant activity) at the dose of 0.1 mg/kg/h, and prostaglandin E1 at the dose of 0.01 μg/kg/min till day 7 after transplantation. Anticoagulation was continued with intravenous administration of heparin (unfractionated heparin) at the dose of 8 U/kg/h from day 8 to day 21 after liver transplantation.

Blood sample collection and analysis

All samples were obtained from patients with informed consent, according to the Declaration of Helsinki. Routine laboratory tests including complete blood counts, coagulation tests, blood chemistry analysis, and urinalysis were performed, and biomarkers of blood coagulation and fibrinolysis, i.e., PAI-1, TM, ADAMTS13, and sES were measured before and after liver transplantation on days 1, 3, 7, 10, 14, 21, and 28. Blood sampling was performed on days 35 and 49 in some patients. These were quantified using commercially available ELISAs (Mitsubishi Chemical Medience Co., Tokyo, Japan; Diaclone, Tepnel Research Products & Services, Cedex, France) (4, 5). The plasma activity levels of plasminogen and protein C were quantified using laboratory test kits (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA). Rationale for measurements of these biomarkers are as follows. PT-INR is currently used worldwide as a coagulation test to monitor the effects of anticoagulants such as coumarin in patients at risk of thrombosis. Protein C is a vitamin K-dependent protein synthesized in the liver that functions as an important regulatory factor for coagulation (6). Plasminogen is the zymogen of plasmin, a key enzyme in fibrinolysis, and is also synthesized in the liver (7). Therefore, plasma protein C and plasminogen levels were thought to be good markers for the restoration of the coagulation and fibrinolysis system following liver transplantation. Levels of these markers might correlate with protein synthesis in the liver, thereby reflecting regeneration of the graft liver. Additionally, measurement of these factors may also be important for patient management, because deficiency of protein C and type II plasminogen deficiency are thought to increase the risk of thrombosis (7, 8). The fibrin degradation product level, determined by the monoclonal antibody specific for degradation products of cross-linked fibrin, is a biomarker for the presence of a thrombus and is used to diagnose venous thrombosis and disseminated intravascular coagulation, however, the fibrin degradation product level may be affected by the presence of blood clots in the extravascular spaces (e.g., the peritoneal cavity), and may therefore not accurately reflect the thrombogenic state in the post-operative period. Thus, the soluble fibrin level was used to assess the thrombogenic state during the post-operative period following liver transplantation. PAI-1 is a primary regulator of fibrinolysis that is synthesized mainly in endothelial cells. Plasma PAI-1 levels change significantly in various pathological conditions (4). ADAMTS13 is the vWF cleaving protease that plays an important role in vWF multimer processing (9). It is synthesized in liver stellate cells and the liver is thought to be the primary source of ADAMTS13 in the circulation (9–11). In addition to the liver stellate cells, vascular endothelial cells in other organs may also be able to synthesize ADAMTS13 (12), and ADAMTS13 mRNA has been detected in the liver, kidneys and lungs in mice (13). ADAMTS13 deficiency results in platelet thrombus formation in the circulation, resulting in the development of a typical thrombotic microangiopathy (9). It is possible that ADAMTS13 deficiency might occur after liver transplantation, and plasma ADAMTS13 levels in patients were

therefore quantified following transplantation. TM, an important regulator of blood coagulation, is synthesized in vascular endothelial cells and is used as a marker of vascular injury (6). The sES level has been used as a marker for endothelial cell dysfunction (14). For example, the sES level is increased in systemic infections such as sepsis.

Diagnosis of acute cellular rejection

The diagnosis of acute cellular rejection was made by liver biopsy and was evaluated using the rejection activity index (3) scores (1, 15, 16). Patients suspected of suffering from acute cellular rejection because of deterioration of liver function (increased serum levels of bilirubin, AST, ALT, ALP, LDH, and γ-GTP compared with previous levels) were subjected to ultrasonography-guided liver biopsy. The liver biopsy specimens were examined for the presence of acute cellular rejection. Patients diagnosed with acute cellular rejection were subjected to intensive immunosuppressive therapy with intravenous methylprednisolone. Mycophenolate mofetil and/or OKT3 were also administered in some patients. Plasma samples obtained before starting administration of the intensive immunosuppressive regimens were evaluated in the following studies.

Statistical analysis

Statistical analyses were performed using SPSS software (SPSS Inc., Tokyo, Japan). Student *t*-tests were used to compare the mean values between groups. Multiple logistic regression analysis was used to investigate the association between biomarkers of blood coagulation and fibrinolysis and the occurrence of acute cellular rejection. p-values <0.05 were considered statistically significant.

Results

Analysis of the coagulation and fibrinolysis system following liver transplantation

Changes in mean values of coagulation tests in patients without acute cellular rejection, vascular complications, or severe infections are shown in Fig. 1. The coagulation activity after liver transplantation was assessed by measuring prothrombin time (PT-INR). The mean PT-INR value rose to approximately 1.8 on day 1, but quickly fell again to <1.5 on day 2, and then normalized gradually. These data suggest that the coagulation activity rapidly recovered after transplantation, once the graft liver started to function.

The mean protein C level of patients before liver transplantation decreased to 57.5% of the normal level. This may have been due to the decreased synthesis of protein C in the liver because most patients had liver failure. The mean protein C level fell to approximately 50% of the normal level on day 1 post-transplantation, and then increased gradually, reaching ≥80% of the normal level by day 14. The mean plasminogen level changed in a similar manner to protein C. By day 28, both protein C and plasminogen levels had returned to almost 90–100% of the normal levels. The nadir values of protein C and plasminogen on day 1 post-transplantation might

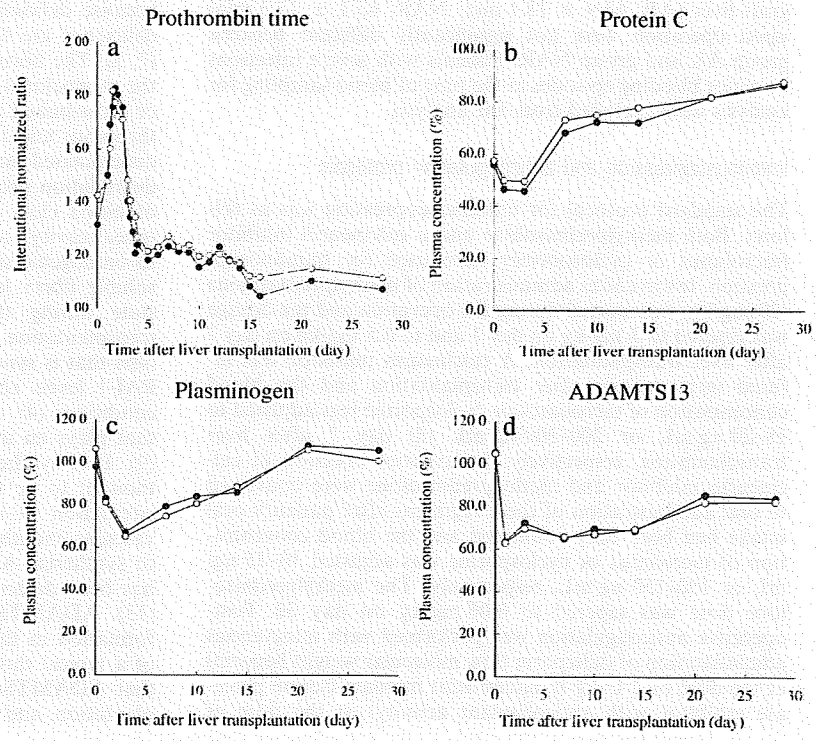

Fig. 1. Restoration of the coagulation and fibrinolysis system following liver transplantation. (a) The mean values of coagulation activity assessed by PT-INR and (b) plasma levels of protein C (normal range 67.1–129.0%), (c) plasminogen (normal range 85.0–120.0%), and (d) ADAMTS13 (normal range 100 ± 15%) of patients without complications (open circle) and of patients with acute cellular rejection (closed circle) are shown.

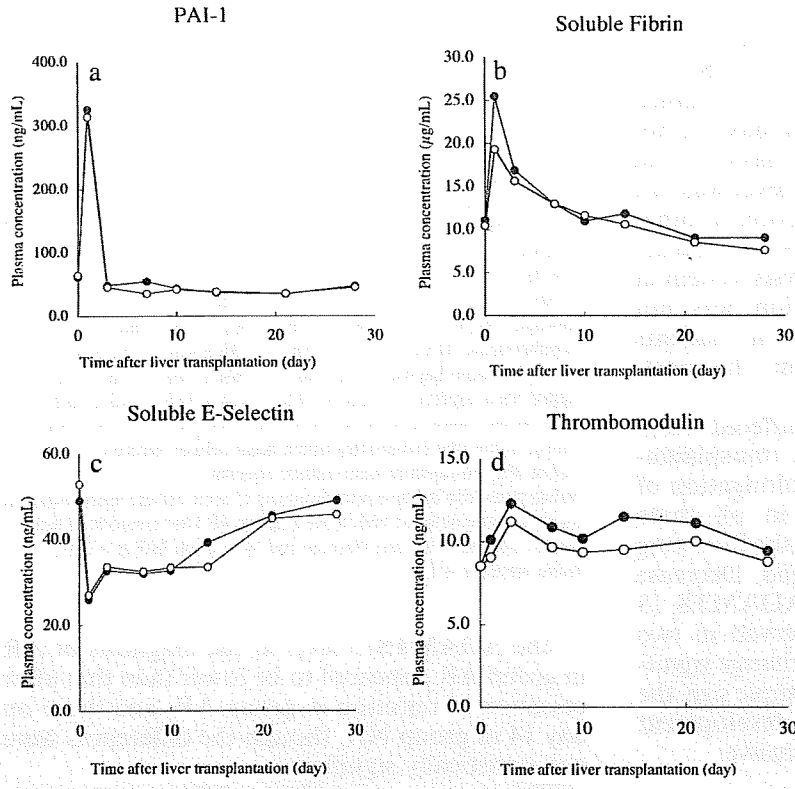

Fig. 2. Analysis of biomarkers of coagulation and fibrinolysis following liver transplantation. The mean plasma levels of PAI-1 (normal range 20–30 ng/mL), soluble fibrin (normal range: <7.5 μg/mL), sES (normal range: <37.5 ng/mL), and TM (normal range 4.46 ± 1.36 ng/mL) of patients without complications (open circle) and of patients with acute cellular rejection (closed circle) are shown.

be affected by plasma transfusion during and after surgery. These data suggest that the synthesis of coagulation factors in the graft liver may start on day 1, resulting in rapid recovery of coagulation activity, but it may take up to 14 days for recovery of the coagulation and fibrinolysis system to near normal levels, and 21–28 days for full restoration of the system after liver transplantation. These data also suggest that graft livers may regenerate to the appropriate size within four wk, though graft livers may vary in size depending on their recipients and donors. The average levels of the coagulation and fibrinolysis factors in patients with acute cellular rejection was not significantly different from those in patients without acute cellular rejection in the post-operative periods, but rate of restoration of the protein C and plasminogen levels on day 14 in group AR was slow.

Soluble fibrin levels in patients with no complications increased significantly on day 1 and then gradually decreased, normalizing by day 14 as shown in Fig. 2. These data suggest that the thrombotic state may continue for 14 days after liver transplantation, and that appropriate antithrombotic therapy may therefore be required during this period.

The mean plasma PAI-1 level was increased approximately 10-fold on post-operative day 1, compared with the normal level, but returned quickly to the normal level on day 3 after transplantation (Fig. 2). These data, together with the changes in the plasminogen level during the post-operative period (Fig. 1), suggest that fibrinolysis activity was suppressed on day 1 after liver transplantation.

The average plasma ADAMTS13 level decreased significantly on day 1 post-transplantation (Fig. 2), but the decrease was not as severe as that of protein C or plasminogen (Fig. 1). However, low levels of ADAMTS13 were maintained for 14 days after liver transplantation. These changes in plasma ADAMTS13 levels after liver transplantation did not parallel those of protein C or plasminogen (Fig. 1), reflecting the extrahepatic synthesis of ADAMTS13 and the possibility that ADAMS13 is synthesized not in hepatocytes, but in stellate cells in the liver. The plasma ADAMTS13 level fell to 28.4% of the normal level in one patient, but she showed no typical signs of thrombotic microangiopathy.

The TM level was increased on day 3 post-transplantation and remained at the upper limit of the normal range after day 7 (Fig. 2). The sES level was significantly increased in patients before liver transplantation (Fig. 2), which may be explained by the fact that many patients enrolled

in the study had undergone hepatic portajejunostomies and therefore had biliary tract infections before transplantation. The sES level was reduced post-transplantation, and remained almost within the normal range until day 14, but then was significantly increased on days 21 and 28 (Fig. 2). This increase in the sES level was not associated with the presence of infection or other disease states. The average changes of biomarkers of the coagulation and fibrinolysis system in patients with acute cellular rejection was not significantly different from those in patients without acute cellular rejection in the postoperative periods.

Three patients in this study suffered from hepatic artery thrombosis after liver transplantation, and an increased PT-INR (prolongation of prothrombin time) was detected in all three patients. Increase of plasma PAI-1 in the following samples of two patients was observed. Decrease of plasma protein C and plasma ADAMTS 13 in the following samples were observed in two patients. Other biomarkers did not change significantly. However, due to the small sample size, the predictive value of this test for the development of vascular complication was inconclusive.

Relationship between coagulation and fibrinolysis markers and acute cellular rejection

Patients were divided into two groups, group AR and group NAR, based upon the presence of acute cellular rejection as described above. The mean onset time of acute cellular rejection in group AR was on day 15 ± 8.7 after liver transplantation, while the mean time for data collection was on day 14 ± 7.9. Laboratory data and coagulation markers for each group at two time points were subjected to statistical analysis. Measurements taken immediately before the diagnosis of acute cellular rejection in group AR were compared with those taken after the cessation of rejection by intensive treatment with methylprednisolone in group AR, and those taken on days 14 and 28 in group NAR.

Statistical analysis of the mean levels of coagulation and fibrinolysis markers (Table 1) revealed that the PAI-1 level at the time of acute cellular rejection in group AR was significantly higher than that after cessation of rejection in group AR, and those on days 14 and 28 in group NAR (data for day 28 of group NR are not shown in Table 1).

The plasma protein C and plasminogen levels at the time of AR diagnosis in group AR were significantly lower than those on day 14 in group NAR.

Table 1. Coagulation and fibrinolysis biomarkers following liver transplantation

	Group AR (n = 24)		Group NAR (n = 39)
	Before*	After†	Day 14
PAI-1 (ng/mL)	79.3 ± 103.9‡	23.0 ± 10.7	38.5 ± 30.4
Plasminogen (%)	85.2 ± 22.8‡	99.4 ± 29.0	97.68 ± 13.8
Protein C (%)	65.7 ± 23.0‡	89.3 ± 37.9	87.2 ± 25.5
ADAMTS13 (%)	67.5 ± 24.1	77.8 ± 23.6	72.5 ± 17.4
ATIII (%)	96.3 ± 17.3	111.5 ± 57.4	99.3 ± 14.9
PT-INR	1.17 ± 0.21	1.08 ± 0.13	1.13 ± 0.13
Fibrinogen (mg/mL)	295.3 ± 116.4	296.3 ± 106.7	280.6 ± 74.0
Thrombomodulin (U/mL)	10.2 ± 3.8	10.8 ± 4.8	8.7 ± 5.2
Soluble E-selectin (μg/mL)	43.8 ± 16.7	46.4 ± 19.0	33.5 ± 17.2
Soluble fibrin (μg/mL)	13.57 ± 17.3	8.64 ± 14.9	10.2 ± 13.9

*Values at the time immediately before acute cellular rejection.
†Values after cessation of acute cellular rejection.
‡Values taken from the time point proximate to acute cellular rejection (before) are significantly different from those of group AR after cessation of acute cellular rejection (after) and those on day 14 in group NAR ($p < 0.01$). Values are mean ± s.d.

The ADAMTS13 level at the diagnosis of AR in group AR appeared to be lower than that after cessation of rejection in group AR, and those on day 14 in group NR, though the differences were not statistically significant.

There were no significant differences between the levels of other coagulation and fibrinolysis markers in patients at the time of rejection diagnosis and after cessation of acute cellular rejection in group AR, or the levels on days 14 and 28 in group NAR.

The changes of the coagulation and fibrinolysis factors and biomarkers before the diagnosis of acute cellular rejection by liver biopsy were studied. These biomarkers levels of samples obtained from the patients proximate to the diagnosis of acute cellular rejection (AR-proximate sample in Fig. 3) were compared with those obtained before the AR-proximate sample (earlier sample in Fig. 3). The PAI-1 level in the AR-proximate samples were significantly higher than that in the earlier samples. The mean values of protein C, plasminogen, and ADAMTS13 in the AR-proximate samples was expected to be higher than those in the earlier samples, but they were lower than the earlier samples though the differences were not statistically significant. The mean values of other biomarkers in the two time points were not significantly different.

Multiple logistic regression analysis was performed to identify the coagulation and fibrinolysis markers for suspecting the occurrence of acute cellular rejection. Absolute values of coagulation and fibrinolysis factors (protein C, plasminogen, ADAMTS13) synthesized in the liver

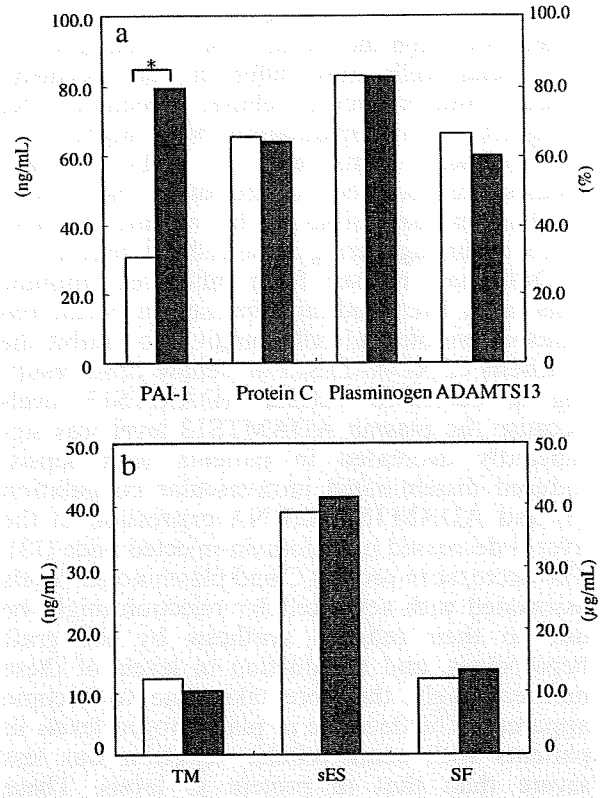

Fig. 3. Changes of coagulation and fibrinolysis factors and biomarkers in patients with acute cellular rejection. The mean plasma levels of coagulation and fibrinolysis factors and biomarkers obtained from patients with acute cellular rejection at two time points were shown. The AR-proximate samples (closed square) were obtained from the patients proximate to the diagnosis of acute cellular rejection. The earlier samples (open square) were obtained before the AR-proximate samples.

were difficult to ascertain using this method, and the changes in levels between time points were therefore analyzed. PAI-1 levels are independent of regeneration of the liver and an increase in plasma PAI-1 levels of >40 ng/mL at a single time point was therefore taken into account. Increases in soluble fibrin, TM, or sES since the previous time point, and above the normal range, were also taken into account. A summary of the multiple logistic regression analysis of coagulation and fibrinolysis markers is shown in Table 2. These data suggest that an increase in PAI-1 levels, and a decrease in protein C, plasminogen, or ADAMTS13 levels, were independently related to the occurrence of acute cellular rejection. Other markers were not related to the occurrence of acute cellular rejection (Table 2). Among these markers, an increase in plasma PAI-1 levels was observed in almost 80% of the patients in group AR.

Table 2. Multiple logistic regression analysis of biomarkers

	Odds ratio	Confidence interval	p-value
PAI-1	17.91	4.89–64.36	<0.001
ADAMTS13*	6.40	1.85–22.03	0.003
Protein C*	4.58	1.14–18.28	0.027
Plasminogen*	7.86	1.23–49.83	0.02
Soluble fibrin†	0.60	Not applicable	0.60
Soluble E-selectin†	0.60	Not applicable	0.65
Thrombomodulin†	0.421	Not applicable	0.42

*Decrease of marker values from the previous time point was adopted.
†Increase of marker values from the previous time point was adopted.

Discussion

The prevention and treatment of vascular thrombosis and immunological rejection of the transplanted liver during the post-operative period is a keystone of patient management. The present study analyzed the coagulation and fibrinolysis system following liver transplantation in pediatric patients to identify biomarkers for suspecting the occurrence of acute cellular rejection.

The present study suggests that the coagulation activity recovered rapidly once the graft liver started functioning, and that the graft liver might regenerate to the appropriate size in 21–28 days, with coincident full recovery of the coagulation and fibrinolysis system in pediatric patients undergoing liver transplantation. The present study also suggests that the hypercoagulable state persisted for 14 days after surgery, and that appropriate anticoagulation may therefore be required at least for 14 days post-transplant, even in the absence of any apparent vascular complications.

Recent advances in the management of patients with liver transplants have improved the clinical outcome of these patients. Adjustments in the doses of immunosuppressive drugs such as calcineurin inhibitors, based on their blood concentrations, are widely conducted after liver transplantation. However, immunological rejection of the transplanted liver still develops in a certain ratio of these patients, even when the blood calcineurin inhibitor concentration is within the appropriate therapeutic range (17, 18). A variety of methods for evaluating immune cell activation have been proposed as a basis for adjusting immunosuppressive therapy, and these have been shown to be useful for assessing the level of immunosuppression (19–22). Intensive treatment of acute cellular rejection with high dose methylprednisolone, with or without other medicines such as OKT3, is usually effective, though the prediction and rapid diagnosis of AR may be important for its effective treatment. In

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.

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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

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

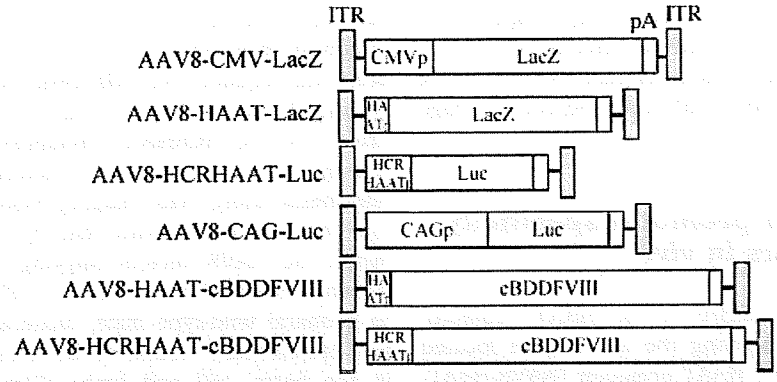

Figure 1. Schematic representation of AAV8 vectors. AAV8 vectors used in the present study are represented schematically. The promoter, the enhancer/promoter complex, or the enhancer/promoter/intron complexes, the genes for expression, and the polyadenylation signal sequence (pA) were flanked by two 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).

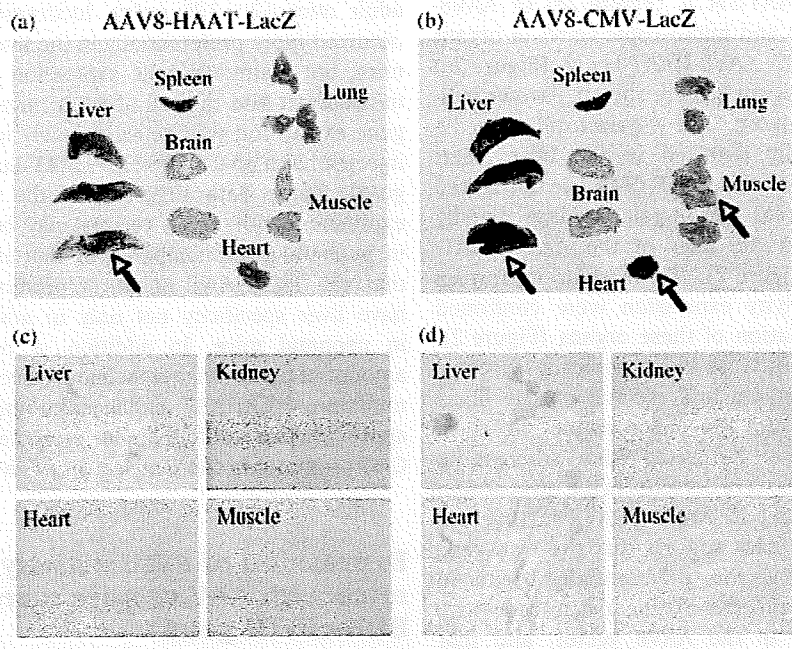

Figure 2. Lac Z gene expression in mice injected with AVV8 vectors. Expression of the Lac Z gene in vector-injected mice was analysed by X-gal staining. Macroscopic views of organs of mice injected with AAV8-HAAT-Lac Z (a) or with AAV8-CMV-Lac Z (b) and microscopic views of organs of mice injected with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

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].

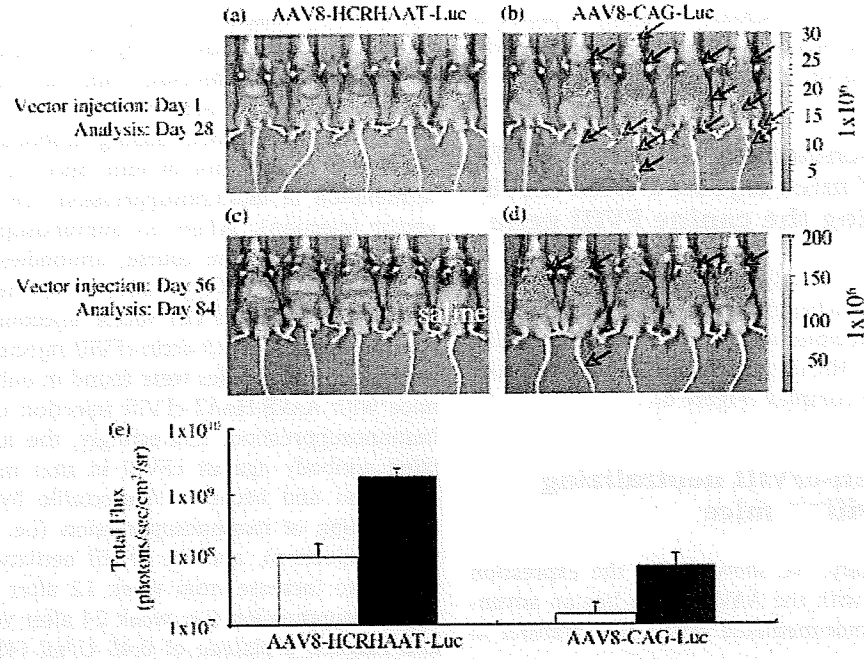

Figure 3. *In vivo* expression of luciferase gene driven by the HCRHAAT enhancer/promoter complex. Wild-type mice injected with AAV8-HCRHAAT-Luc or the AAV8-CAG-Luc on day 1 after birth (a, b) or on day 56 after birth (c, d) were analysed for expression of the luciferase gene using an *in vivo* imaging 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).

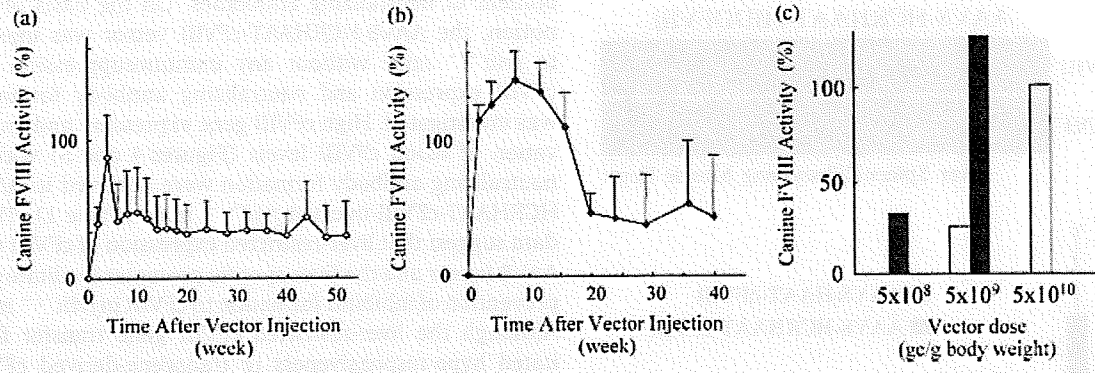

Figure 4. Expression of canine FVIII in *fviii*$^{-/-}$ mice injected with AAV8 vectors carrying the canine FVIII gene. The canine FVIII levels in *fviii*$^{-/-}$ mice injected with 5×10^{10} gc/g body weight of AAV8-HAAT-cFVIII (a) or 5×10^{9} gc/g body weight of AAV8-HCRHAAT-cFVIII (b) are shown. Values 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

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).

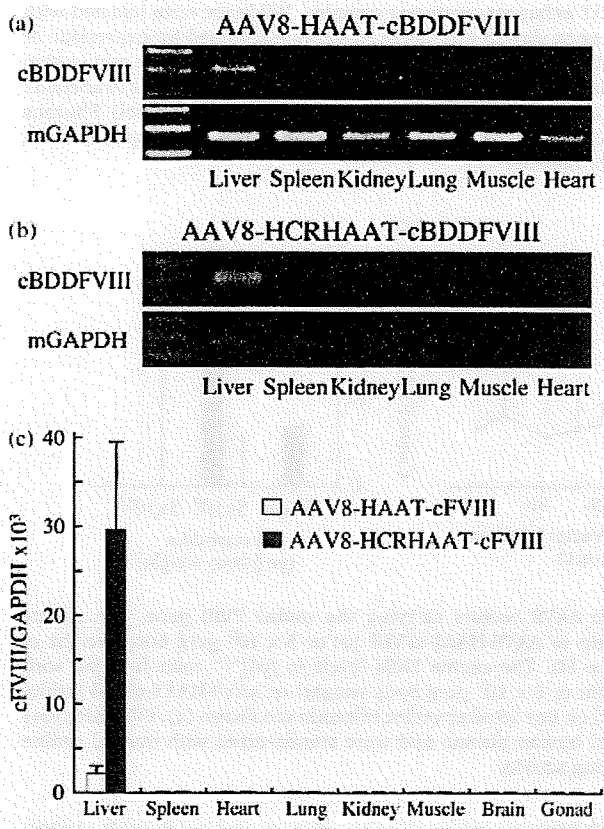

Figure 5. Analysis of transcripts of the canine FVIII transgene in organs of mice injected with AAV8 vectors carrying the canine FVIII gene. The transcripts of canine FVIII transgene in organs of fviii$^{-/-}$ mice injected with 5×10^9 gc/g body weight AAV8-HAAT-cFVIII (a) or 5×10^9 gc/g body weight 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

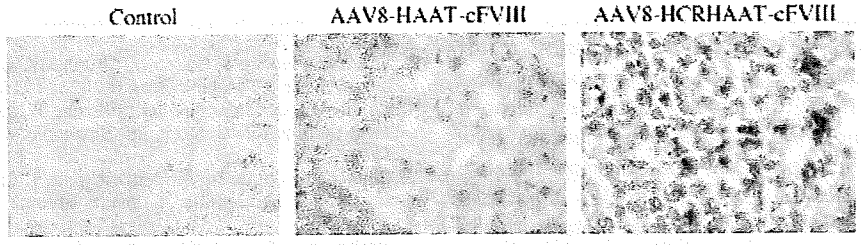

Figure 6. Immunohistochemistry of canine FVIII in the liver of mice injected with AAV8 vectors carrying the canine FVIII gene. Canine FVIII in the liver of *fviii*$^{-/-}$ mice injected with 5 x 10^9 gc/g body weight of AAV8-HAAT-cFVIII or 5 x 10^8 gc/g body weight AAV8-HCRHAAT-cFVIII was detected by 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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Short Report

A convenient enzyme-linked immunosorbent assay for rapid screening of anti-adeno-associated virus neutralizing antibodies

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Abstract

Background: Recombinant adeno-associated virus vectors based on serotype 2 (AAV-2) have become leading vehicles for gene therapy. Most humans in the general population have anti-AAV-2 antibodies as a result of naturally acquired infections. Pre-existing immunity to AAV-2 might affect the functional and safety consequences of AAV-2 vector-mediated gene transfer in clinical applications.

Methods: An enzyme-linked immunosorbent assay (ELISA) method was developed using microwell plates coated with intact particles of recombinant AAV-2 vectors, and horseradish peroxidase-conjugated anti-human immunoglobulin G (HRP-IgG). Neutralizing antibody titres were analysed by assessing the ability of serum antibody to inhibit transduction into HEK293 cells of AAV vectors that express β-galactosidase.

Results: Anti-AAV-2 antibodies were detected by ELISA in two of 20 healthy subjects. The positivity criterion (optical density >0.5) in ELISA corresponded to the cut-off value (320-fold dilution of serum) in the AAV-2 neutralization assay. Influences of interfering substances were not observed.

Conclusion: This ELISA method may be useful for rapid screening of anti-AAV-2 neutralizing antibodies in candidates for gene therapy.

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Introduction

Adeno-associated virus (AAV) is a small single-stranded DNA virus within the parvovirus family.[1,2] Among more than 100 genotypes of primate AAV, serotype 2 (AAV-2) is the most studied and was the first to be engineered for vector development. Recombinant AAV-2 vectors efficiently transduce both dividing and non-dividing cells and provide long-term gene expression without significant toxicity. Growing numbers of clinical trials have been conducted using AAV-2 vectors to combat various diseases. However, one major problem is the high prevalence of anti-AAV-2 antibodies in the human population. More than 90% of adults demonstrate antibodies that cross-react with one or more AAV serotypes, although markedly fewer (18–32%) show neutralizing antibodies (nAb).[3,4] Pre-existing immunity to AAV-2 may block transduction and intensify the innate response to vector administration, leading to a poor outcome of gene therapy. Thus, measurement of the anti-AAV-2 nAb titre is necessary.

Methods

Recombinant AAV-2 vectors were produced by the triple transduction method as described previously.[5] In brief, HEK293 cells were transfected with the following three plasmids: pAAV2-Rep/vp (containing the AAV-2 *rep* and *cap* genes), pAd (containing the adenovirus genome) and pW1 (containing the β-galactosidase-expression cassette). After three days of incubation, the transfected cells were frozen and thawed, and the recombinant AAV-2 vector particles that were released were purified by two sequential CsCl density gradient centrifugations.

Serum samples from healthy adults were purchased from Advanced BioServices LLC, (Reseda, CA, USA). AAV-specific antibodies were detected using an enzyme-linked immunosorbent assay (ELISA). Ninety-six-well microtitre plates (Invitrogen, Carlsbad, CA, USA) were coated with 0.5 μg (1.4×10^8 vector genomes [vg]) of AAV-2 vector particles per well. After blocking with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), the plates were washed with 2% sucrose. Serum samples diluted at 1:1000 with PBS/0.1% BSA were added to each well (100 μL/well). The plates were incubated for 1 h at room temperature (RT) and washed three times with PBS/0.05% Tween 20. A solution containing 1 μg/mL horseradish peroxidase-conjugated anti-human immunoglobulin G (HRP-IgG; self-prepared using a heterobifunctional