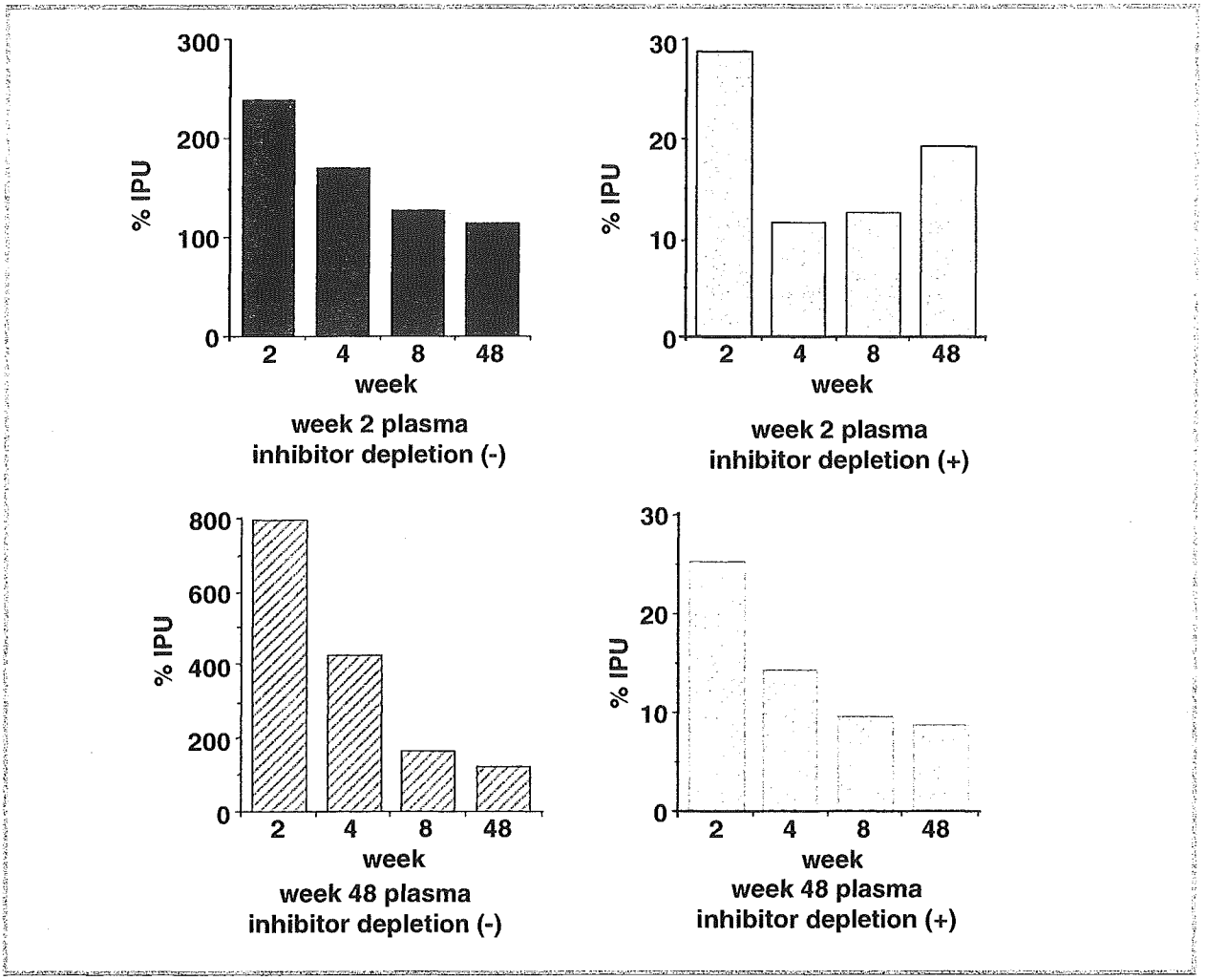

Figure 5. Alteration of anti-FVIII antibody-neutralization activity with the course of ITI. Samples were from a patient in whom ITI was incompletely successful. Anti-FVIII antibodies were depleted from sample plasma using FVIII-Sepharose beads. Sample plasma obtained at weeks 2 and 48 with/without anti-FVIII antibody depletion was mixed with plasma obtained at weeks 2, 4, 8 and 48. IP titers are expressed as a percent, taking the control (mixing buffer A instead of sample plasma) as 100%. Although sample plasma without anti-FVIII antibody depletion increased IP titer in both cases of adding week 2 and week 48 plasmas, anti-FVIII antibody depletion from sample plasma resulted in a reduction of the IP titer. Anti-FVIII antibody-depleted week 2 plasma decreased IP titer in plasma samples in a time-independent manner, while anti-FVIII antibody-depleted week 48 plasma decreased IP titer in a time sequence.

results imply that anti-idiotypic antibodies existed in plasma before and during ITI from patients in whom ITI was incompletely successful or unsuccessful.

Alterations of anti-idiotypic antibodies during ITI

Addition of anti-FVIII antibody-depleted plasma reduced IP titer in all cases (Figure 5), whereas addition of week 2 or week 48 untreated plasma increased IP titer compared to that of each control to which buffer A had been added. Anti-FVIII antibody-depleted week 2 plasma maximally reduced IP titer of week 4 plasma. Meanwhile, anti-FVIII antibody-depleted week 48 plasma reduced IP titer of week 2, 4, 8, and 48 plasma samples, in a time sequence.

Discussion

Idiotype refers to the assembly of antigenic determinants which are located in the variable regions of antibodies or antigen-specific receptors of T cells. Each idiotype contains the determinants that are specific to the antibody molecule or T-cell receptor, which is called *idiotope*. Corresponding anti-idiotypic antibodies can develop against an immunogenic idiotope. Idiotypes and anti-idiotypic antibodies are natural components of immune responses, and exert a regulatory role which maintains the homeostasis of the immune system. These interactions between idiotypes and anti-idiotypic antibodies constitute an idiotypic network. Immunological tolerance might be found in the subtle equilibrium

between anti-FVIII and corresponding anti-idiotypic antibodies. Since normal individuals produce both anti-FVIII and corresponding anti-idiotypic antibodies,[25,26] the cause of the tolerance to FVIII is not limited to deletion of self-reactive B and T cells.[27] It might thus be fruitful to investigate whether an idiotypic network plays a role in establishing and maintaining tolerance.

The immunoprecipitation method used in the present study, originally used to measure levels of all anti-FVIII antibodies in plasma samples, is highly sensitive and is independent of inhibitor activity measurable by the Bethesda method. By testing plasma samples from patients in clinical trials, 5% to 10% of patients without a detectable inhibitor by the Bethesda method have a significant immune response which ranges from 3-fold to 100-fold above the background.[28,29] It has been reported that the half-life of transfused FVIII is reduced as the anti-FVIII antibody concentration detected by an ELISA, which is as sensitive as the IP assay, increases in non-inhibitor patients with hemophilia A.[30] This study has a limitation, but indicates that some of the inhibitor-negative patients may have symptom exacerbation as a result of a rise of anti-FVIII antibodies.

In the present study, in order to elucidate whether plasma from patients after ITI contained anti-idiotypic antibodies, simple mixing studies were performed using the immunoprecipitation method. In the previous studies the detection method generally used was a solid-phase ELISA. We developed a novel liquid phase blocking IP method, which should provide more reliable results. Anti-FVIII antibody-neutralization activity was observed in plasma from patients in whom ITI was completely successful, but not in plasma from those in whom ITI was incompletely successful or unsuccessful, suggesting the presence of anti-FVIII antibody-neutralization factor in plasma from the completely successfully ITI treated patients. The second assays revealed that IgG-depleted plasma from patients with completely successful ITI contained no anti-FVIII antibody-neutralization factor. Furthermore, the third assays proved the presence of anti-idiotypic antibodies even in plasma samples that showed positive inhibitor titer. These results suggest that anti-FVIII antibody-neutralization factor is an IgG; i.e., the anti-FVIII antibody-neutralization factor would be anti-idiotypic antibodies against anti-FVIII antibodies, and anti-idiotypic antibodies exist at any time in any hemophilic patients who receive FVIII replacement therapy.

The last mixing studies using one patient's plasma samples taken at different time points provided the possibility to examine the affinity maturation of IgG anti-idiotypic antibodies. Anti-FVIII antibody-depleted plasma obtained during an early period of ITI (week 2 plasma) reduced IP titer in plasma samples in a time independent manner. On the other hand, the anti-FVIII antibody-neutralization activity of anti-FVIII antibody-free plasma obtained at completion of ITI increased over time. These results indicate that neutralization activity of anti-idiotypic antibody increases over the course of ITI. Affinity maturation of IgG antibodies in adaptive immune responses is a well-accepted mechanism to improve effector functions of IgG within 2 weeks to several months after antigen encounter. Idiotypic suppression function is a driving force for diversification and maturation of the antigen-induced response.[31] Further studies will be necessary to clarify the role of affinity maturation of anti-idiotypic antibodies in ITI.

Our observations suggest that, in patients whose IP titer was suppressed by ITI, anti-idiotypic antibodies shifted the immune system toward the steady-state equilibrium that prevented alloimmunity in hemophilia A patients with inhibitors. The present study thereby contributes to a better understanding of the association of anti-idiotypic antibodies with ITI.

YS was responsible for the conception and design of the study, for acquisition, analysis and interpretation of data and for drafting the article. IT, KF and KY were responsible for acquisition, analysis and interpretation of data. MS and AY were responsible for conception of the study, enrolling patients and interpretation of data. All authors drafted or critically revised the manuscript, and all authors approved the final version of the manuscript. The authors reported no potential conflict of interest.

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Effectiveness of factor VIII infusions in haemophilia A patients with high responding inhibitors

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Summary. We report here the efficacy of factor VIII (FVIII) infusions in two haemophiliacs with inhibitors using clot waveform analysis on the MDA® II system, which was possible to detect very low levels of FVIII activity < 1.0 U dL^{-1}. In the presence of type 1 inhibitors at the level of 6.2 (patient 1) and 14.4 (patient 2) Bethesda Units mL^{-1}, 3.2 and 6.5 U dL^{-1} of FVIII:C remained 30 min after the infusion of FVIII (100 U kg^{-1}), respectively. Moreover, 0.9 U dL^{-1} of FVIII:C remained 24 h after infusion in patient 2. In both cases, these changes were reflected by qualitative improvement in the aPTT clot waveform and quantitative changes in the minimum value of the second derivative of the aPTT waveform (Min2) that reflects clot acceleration. These results suggest that FVIII infusion may be continued with clinical benefit in some haemophiliacs with high responding inhibitors. Furthermore, the haemostatic response may be monitored accurately and efficiently by clot waveform analysis.

Keywords: clot waveform analysis, haemophilia A, high responders, immune tolerance induction, inhibitor

Introduction

Alloantibodies to factor VIII:C (FVIII:C) develop in approximately 20–30% of patients with severe haemophilia A who have been treated with FVIII replacement therapy [1,2]. These patients can be divided into two groups: high responders, demonstrating an anamnestic inhibitor response after exposure to FVIII and low responders who show no significant inhibitor response after challenge with factor VIII [3]. Replacement therapy with FVIII concentrates in patients with inhibitors often becomes ineffective, thus complicating clinical management. Therefore, they are usually treated with (activated) prothrombin complex concentrates [(A)PCCs] or recombinant factor VIIa, i.e. FVIII bypassing therapy [4,5]. Alternatively, immune tolerance induction (ITI) may be attempted to reduce or eradicate the inhibitor activity [6–8]. Several investigators have reported that tolerance may be achieved in approximately 76–89% of patients with inhibitors [6–8]. The reduction in inhibitor level was associated with a decrease in the number of bleeding episodes and an improved quality of life. Unfortunately, some high responders do not respond sufficiently to ITI. Inhibitor levels in such patients often fluctuate for long periods of time. As the dose of FVIII infused regularly does not appear to neutralize the inhibitor sufficiently, it might be presumed that it would also be haemostatically ineffective in these patients. Nevertheless, clinical evaluation frequently suggests a subjective improvement in the severity of the disease even when ITI is not completely successful. This suggests the possibility that the regular infusion of FVIII might decrease the number of bleeding episodes in high responders. Clearly it would be useful to document this subjective clinical assessment of improvement with objective measurements of the changes in plasma levels of FVIII:C achieved following FVIII infusion. Unfortunately, monitoring of the very low levels of FVIII:C achieved in such circumstances has been limited by the sensitivity of the FVIII:C assay systems available. Recently, however, we have demonstrated that using the MDA® II Hemostasis system (bioMérieux, Durham, NC,

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USA), a photo-optical automatic coagulation analyzer, very low levels of FVIII can be measured relatively accurately [9]. Moreover, due to the systems capability of performing clot waveform analysis, it is useful for the evaluation of dynamic changes in blood coagulation [10,11]. Consequently, qualitative as well as quantitative comparisons may be made both between patients and within a patient during the response to therapeutic intervention [9]. In this study, we examined whether this approach may be used to document objectively the clinical efficacy of FVIII infusion in two high responder patients, who appeared to respond insufficiently to ITI treatment as assessed by their inhibitor response.

Materials and methods

Patients

Patient 1 was diagnosed as having severe haemophilia A when he experienced recurrent subcutaneous haematomas at 4 months of age. An FVIII:C inhibitor (1.2 BU mL^{-1}) was detected for the first time at 13 months. The maximum inhibitor level recorded before ITI was attempted was 5.0 BU mL^{-1} and was 2.0 BU mL^{-1} immediately prior to its initiation. At the age of 9, ITI was attempted with the intermediate dose regimen of our facility: a daily administration of 4000 U (100 U kg^{-1}) of recombinant FVIII (Recombinate™; Baxter Healthcare Corporation, Westlake, CA, USA) for 3 weeks, followed by an infusion four times a week. The maximum inhibitor level after ITI was 152.0 BU mL^{-1}. The inhibitor level had fluctuated for 3 years since ITI was initiated. Although the increases in the inhibitor titre were seen at 14 and 36 months without any clinical events such as infection, the regular infusion of FVIII was continued. Infusion studies were performed 5, 19 and 33 months after the initiation of ITI (Fig. 1a).

Patient 2 was diagnosed as having severe haemophilia A when he experienced recurrent subcutaneous haematomas and purpura 4 months after birth. A Factor VIII:C inhibitor (3.2 BU mL^{-1}) was detected for the first time at 23 months. The maximum inhibitor level recorded before ITI was attempted was 62.0 BU mL^{-1} and was 3.9 BU mL^{-1} immediately prior to its initiation. At the age of 9, ITI was attempted with a daily administration of 4000 U (100 U kg^{-1}) of recombinant FVIII (Recombinate™) for 3 weeks, followed by infusion three times a week. The maximum inhibitor level after ITI was 17.4 BU mL^{-1}. The inhibitor activity had not fallen below 4 BU mL^{-1} for 2 years since ITI was initiated.

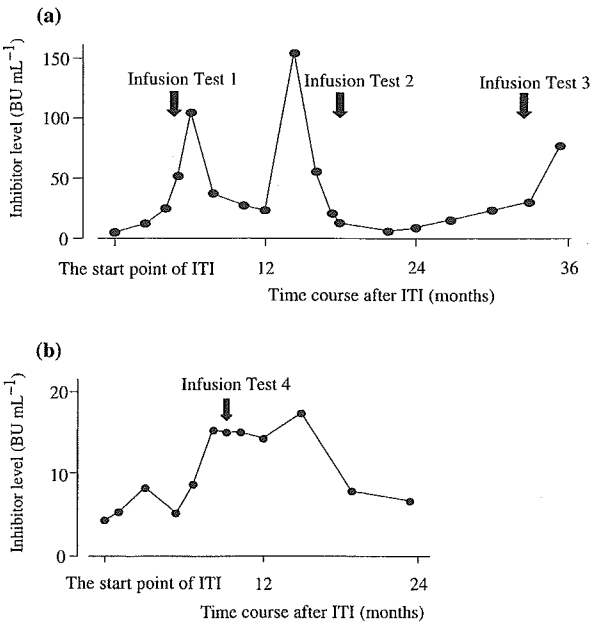

Fig. 1. Inhibitor levels after the initiation of ITI. (a) Patient 1, (b) patient 2. Closed arrows represent the dates of each infusion test. BU, Bethesda Unit; ITI, immune tolerance induction.

An infusion study was performed 9 months after the initiation of ITI (Fig. 1b).

Informed consent for the study was obtained from the patients and their parents.

Blood sample preparation

Nine volumes of whole blood were withdrawn into 1 volume of 3.8% (w/v) trisodium citrate solution. Platelet-poor plasma was separated by centrifugation at 2500 *g* for 15 min. The plasma obtained was frozen immediately in ethanol solution cooled by dry ice and stored at −70 °C until the time of study.

FVIII:C inhibitor assay and characterization

Inhibitor assays were performed using the Bethesda method as described by Kasper *et al.* [12]. The mode of FVIII:C inactivation was determined by reaction kinetics between FVIII:C and the inhibitors as described by Biggs *et al.* [13,14]. The FVIII-specific IgG subclass of the alloantibodies was determined by enzyme-linked immunosorbent assay as previously described [15]. Immunoblot analysis was performed as previously described [16].

Activated partial thromboplastin time (aPTT) test

The aPTT was performed on the bioMérieux MDA® II Hemostasis System using MDA® reagents (Platelin®

LS, Platelin® LS $CaCl_2$) supplied by the manufacturer (bioMérieux). Verify® 1 (bioMérieux) calibrated against the international standard was used as the normal control plasma.

FVIII:C assay

FVIII:C assay was performed by the one-stage clotting method on the MDA® II system using FVIII:C-deficient plasma (bioMérieux) as the substrate [17]. The standard curve was prepared using the Verify® 1 reagent in serial doubling dilutions from 1:10 to 1:5120. The patient (test) plasmas were diluted to 1:10. One hundred units (U) of FVIII:C was defined as the amount of FVIII:C activity present in 1 dL of normal plasma (Verify® 1). The detection limit of this assay system was 0.2 U dL^{-1} [9].

aPTT waveform analysis

Clot waveform analysis of the optical data from the aPTT test was performed as described by Braun *et al.* [11]. The waveform recorded was automatically processed mathematically by the MDA® II system, using algorithms built into the software. The data obtained were accessed using waveform information and export research tools (WIT®/WET®) provided by bioMérieux.

The aPTT and the minimum value of the second derivative (Min2) of the aPTT waveform, i.e. the percentage change in light transmission (T) over time (S^2) reflecting the acceleration in fibrinogen to fibrin conversion at the point in time that clotting is initiated, was measured in all cases.

Infusion studies

The studies were performed in both patients before and following one of their scheduled FVIII infusions during their ITI maintenance protocol, i.e. 100 U kg^{-1} of recombinant FVIII (Recombinate™) four times weekly in patient 1 and three times weekly in patient 2. Blood samples for the determination of aPTT, Min2 and FVIII:C assay were taken from the patients at each time point before and after the bolus infusion of 100 U kg^{-1} of FVIII; three time points (pre-, 30 min and 24 h postinfusion) for infusion test 1and 2 in patient 1, and infusion test 4 in patient 2, whereas six time points (pre-, 15 min, 30 min, 1 h, 6 h and 24 h postinfusion) for infusion test 3 in patient 1. Neither patient had received FVIII concentrates or (A)PCC for 48 h before the initiation of each infusion test.

Results

Characterization of inhibitors

The antibodies in both patients were considered to be type 1 inhibitors with complete inactivation of FVIII:C characterized by the reaction kinetics between FVIII:C and the inhibitors. The main IgG subclass of patient 1's inhibitor was IgG_4 with some IgG_1 and IgG_2 subclasses, whereas in patient 2 the antibodies were predominantly of the IgG_4 subclass. Immunoblot analysis revealed that in patient 1 the antibodies recognized both the heavy (44 kDa fragment) and light chains (72 kDa fragment) of FVIII, whereas in patient 2 the antibodies recognized only the heavy chain (44 kDa fragment). Thus, both sets of inhibitors showed the typical patterns of FVIII alloantibodies.

Infusion studies

In patient 1, separate infusion studies were performed 5, 19 and 33 months after ITI was initiated when the inhibitor levels were 50.0, 6.2 and 27.0 BU mL^{-1}, respectively (Fig. 1a). In the first study (infusion test 1), there was no improvement in FVIII:C level, Min2 and the aPTT waveform after the infusion of FVIII (Table 1, Fig. 2b). In the second study (infusion test 2), when the inhibitor level was 6.2 BU mL^{-1}, 3.2 U dL^{-1} of FVIII:C was detected

Table 1. Coagulation tests before and after FVIII infusion.

	Patient 1						Patient 2			
	Infusion test 1			Infusion test 2			Infusion test 4			
	Before	30 min after	24 h after	Before	30 min after	24 h after	Before	30 min after	24 h after	Normal control (mean ± SD)
aPTT (s)	119.9	119.9	121.0	109.3	68.7	105.8	132.3	52.2	64.8	29.2 ± 1.2
Min2	10.0	10.0	9.8	11.0	56.0	14.0	9.0	93.0	62.0	218 ± 21
FVIII:C (U dL^{-1})	<0.2	<0.2	<0.2	<0.2	3.2	<0.2	<0.2	6.5	0.9	
Inhibitor titre (BU mL^{-1})	50.0			6.2			14.4			

aPTT, activated partial thromboplastin time; BU, Bethesda unit.

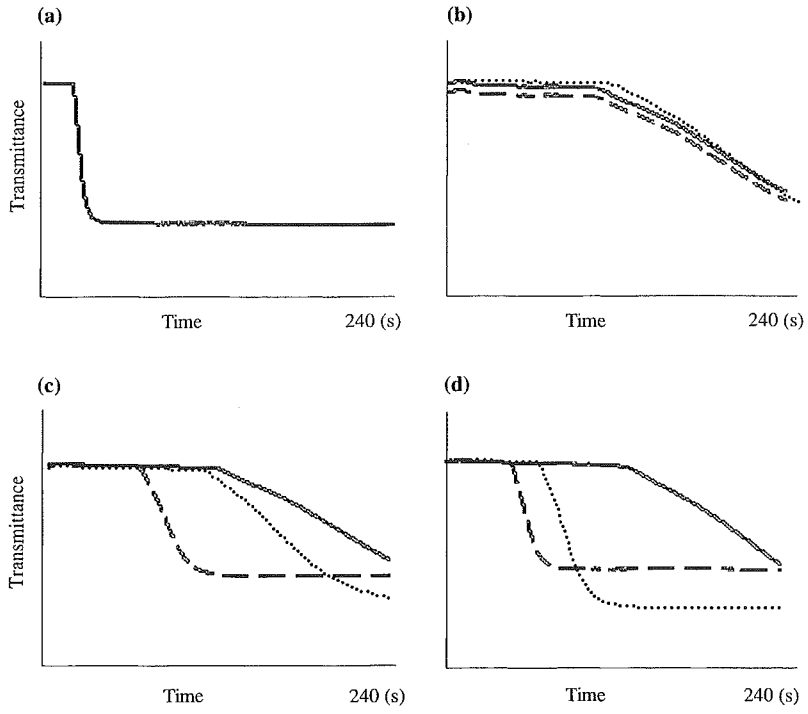

Fig. 2. aPTT clot waveforms observed in patients 1 and 2 pre- and post-FVIII infusion (a) Normal control, (b) patient 1 (infusion test 1); (c) patient 1 (infusion test 2); (d) patient 2 (infusion test 4). The MDA® II Hemostasis system plots and exports a hard copy of a continuous tracing of the changes in light transmission over time that occur during the performance of the aPTT. The aPTT waveforms obtained from a single test before (solid line), 30 min after (broken line) and 24 h after (dotted line) the infusion of 100 U kg^{-1} of FVIII into each patient are shown.

Table 2. Coagulation tests in the time course study in patient 1 after FVIII infusion.

	Infusion test 3 in patient 1						Normal control
	Before	15 min after	30 min after	1 h after	6 h after	24 h after	(mean ± SD)
aPTT (s)	120.4	67.2	68.0	69.6	77.1	112.7	29.2 ± 1.2
Min2	11.5	62.5	60.0	57.5	49.0	19.5	218 ± 21
FVIII:C (U dL^{-1})	< 0.2	2.4	2.3	1.9	1.0	< 0.2	
Inhibitor titre (BU mL^{-1})	27.0						

aPTT, activated partial thromboplastin time; BU, Bethesda unit.

30 min postinfusion with the significant improvement in Min2 and the aPTT waveform (Table 1, Fig. 2c). In the time course study (infusion test 3), when the inhibitor level was 27.0 BU mL^{-1}, the detection of FVIII:C and the improvement of Min2 were observed for at least 6 h postinfusion (Table 2).

In patient 2, only one infusion study (infusion test 4) was performed 9 months after ITI was initiated when the inhibitor level was 14.4 BU mL^{-1} (Fig. 1b). The FVIII:C levels 30 min and 24 h postinfusion were measured to be 6.5 and 0.9 U dL^{-1}, respectively. The improvement of Min2 and the aPTT waveform were observed 30 min after, even 24 h after the infusion of FVIII (Table 1, Fig. 2d).

Clinical evaluation

After the initiation of regular infusions of FVIII for ITI treatment, the number of bleeding episodes decreased in patient 1 from 26–35 to five to eight episodes over a 1-year period. Similarly, in patient 2, the number of bleeding episodes observed over a year decreased from 13–18 to six to seven episodes per year. The increased inhibitor level at 14 and 36 months in patient 1 seemed to have no adverse effect on bleed frequency.

Discussion

When no significant fall in the inhibitor level is observed in high responders 2–3 years after the initiation of ITI, it is generally considered necessary to abandon conventional FVIII concentrate replacement therapy in favour of expectant FVIII bypassing therapy for bleeding episodes as and when they occur [3]. Nonetheless, some investigators have advocated continuing with conventional replacement therapy albeit at significantly increased dosage to achieve the

desired haemostatic effect [18]. Studies of these two patients confirmed our previous experience that indeed the clinical status of such patients, as assessed by the incidence of bleeding episodes, might be improved by the regular infusion of FVIII despite the persistence of relatively high levels of alloantibodies to FVIII:C. However, on this occasion we were also able to document that the improvement, measured by relatively subjective clinical assessment, was associated with objective improvement in their coagulation status measured *ex vivo*. This was assessed qualitatively using clot waveform analysis and quantitatively using the aPTT, Min2 and one-stage FVIII:C assay on the MDA® II Hemostasis system.

In patient 1, when his inhibitor level was 50.0 BU mL^{-1} and probably rising (Fig. 1a), infusion of 4000 U (100 U kg^{-1}) of recombinant factor VIII was not associated with change in any of the measured parameters 30 min after the infusion. However, 14 months later when the inhibitor level was substantially lower (6.2 BU mL^{-1}) (Fig. 1a), infusion of the same dose of FVIII was associated with normalization of his clot waveform profile (Fig. 2), substantial but incomplete correction of the aPTT and Min2 and a measurable level of FVIII:C in the one-stage assay. In the time course study, when the inhibitor level was 27.0 BU mL^{-1} (Fig. 1a), the improvement of the aPTT and Min2 and the detection of FVIII:C had been observed for up to 6 h postinfusion. These observations are of particular importance as the level observed, moved him from the severe to moderate category of severity of haemophilia A at least 6 h following the infusion of FVIII. Theoretically, this would be expected to be associated with a reduction in the bleeding episodes experienced, which was in fact observed. These observations in patient 1 during the second and third infusion tests were generally mirrored by the observations in patient 2. Moreover, in this patient one-stage FVIII:C assay detected a concentration of FVIII (0.9 U dL^{-1}) 24 h postinfusion. Very low levels of FVIII:C < 1.0 U dL^{-1} might remain to be neutralized completely in the presence of some type 1 inhibitors. Using the MDA® II assay system allowed the evaluation of very low levels of FVIII:C < 1.0 U dL^{-1} [9]. Again, this level is at the cut-off between the severe and moderate categories of severity and is in line with the improved clinical status of this patient.

[1]The neutralized amount of FVIII was calculated by the following formula: body weight (kg) × 20 × inhibitor titre (BU mL^{-1})

On theoretical grounds, the amount of FVIII that could be neutralized in patients 1 (infusion tests 2 and 3) and patient 2 (infusion test 4) would be approximately 5000, 21 600 and 11 500 U, respectively.[1] In these cases, we have continued regular infusion of 4000 U FVIII not for neutralization of the inhibitors, but for ITI treatment, resulting in the decrease in bleeding episodes by the prophylactic effect. Analysis of the inhibitor epitopes revealed that the antibodies in patient 1 recognized both A2 and C2 domains of FVIII, whereas the antibodies in patient 2 recognized A2 domain. Both A2 and C2 domains are common binding regions for FVIII alloantibodies [3]. The difference in response observed in patient 1 following the three infusion tests may be important. When the inhibitor level was 50.0 BU mL^{-1}, the infusion was ineffective and the coagulation parameters were unchanged, whereas 3.2 and 2.3 U dL^{-1} of FVIII:C was detected 30 min after the infusion of FVIII in the presence of 6.2 and 27.0 BU mL^{-1} of inhibitor, respectively. These data suggest that the response may depend on the inhibitor level. This in turn may reflect the development of anti-idiotypic or non-neutralizing antibodies during ITI treatment, which may be interfering with the binding of the inhibitors with FVIII infused. Clearly, more detailed studies are needed to confirm this hypothesis.

In conclusion, our studies confirm that the regular infusion of FVIII might still be a therapeutic option in high responders, whose inhibitor level is < 30 BU mL^{-1}. They also demonstrate further evidence that clot waveform analysis provides an important tool in measuring objectively *ex vivo* changes in coagulation status that are otherwise inaccessible [9]. In patients such as those described here it is clearly important to correlate perceived clinical improvement with objective tests of haemostatic function wherever possible. The tests described are simple to perform and provide relatively immediate guidance to the prescribing doctor as to the feasibility of continuing with conventional therapy. Further and more controlled clinical study of this approach would appear to be justified.

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Successful Gene Transfer Using Adeno-Associated Virus Vectors into the Kidney: Comparison among Adeno-Associated Virus Serotype 1–5 Vectors in vitro and in vivo

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Key Words

β-Galactosidase · Dependovirus · Epithelial cell · Gene therapy, adeno-associated virus serotype 1–5 vectors · Viral vectors, gene therapy

Abstract

Background/Aim: Gene transfer into the kidney has great potential as a novel therapeutic approach. However, an efficient method of gene transfer into the kidney has not been established. We explored the transduction efficiency of renal cells in vitro and in vivo using adeno-associated virus (AAV) serotype 1–5 vectors encoding the β-galactosidase gene. ***Methods:*** In the in vitro study, rat kidney epithelial cell line NRK52E cells were transfected with AAV serotype derived vectors. In the in vivo study, AAV serotype derived vectors were selectively injected into the kidney using a catheter-based gene delivery system in rats and mice mimicking the clinical procedure. The efficiency of gene expression was histologically evaluated on the basis of the β-galactosidase expression. ***Results:*** AAV serotype 1, 2, and 5 vectors transduced in rat kidney epithelial cell line NRK52E cells in vitro, whereas AAV serotype 3 or 4 vectors showed no transduction. In addition, the kidney-specific injection of AAV serotype 2 vectors successfully transduced in tubular epithelial cells, but not in glomerular, blood vessel, or interstitial cells in vivo, whereas the rest of the serotypes showed no transduction. ***Conclusion:*** Since kidney-specific gene delivery via the renal artery by catheterization is highly feasible in humans, these findings provide useful information for promising strategies in renal gene therapy.

Introduction

Gene transfer into the kidney has a great potential as a novel therapeutic approach for renal diseases. However, the ability to pursue gene therapy for renal diseases is substantially limited, because there is no efficient and adequate local gene delivery system into the kidney and because of the potential adverse effects on renal functions

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associated with systemic gene therapy. A few reports have been published on gene delivery into the kidney using recombinant virus vectors in experimental models [1, 2]. The direct injection of retrovirus vectors into the kidney has been reported to transduce in tubular epithelial cells, but not in glomerular or vascular cells [3]. Because retrovirus vector mediated gene transduction requires target cell replication, the use of retrovirus vectors in renal gene therapy is still restricted. The adenovirus vector is an alternative vector system that results in high gene transduction in both dividing and nondividing cells. Several investigations demonstrated that adenovirus vectors were successfully transduced in renal cells [4–6]. However, the clinical application of adenovirus vectors also has been limited by cytotoxicity, immune responses, and short-term transgene expression.

Adeno-associated virus (AAV) vectors have a number of attractive features for the clinical application of renal gene therapy: no cytotoxicity, the ability to transduce in both dividing and nondividing cells, low immune responses, and long-term transgene expression [7–9]. When the experiment was started, there were six primary isolates of AAV (AAV serotypes 1–6) [8, 10–14], although the cap sequence of serotype 6 is strongly related to that of serotype 1 [8]. Two novel serotypes were recently isolated from monkeys [15]. The distinct AAV serotypes were shown to have different tropisms for the cells and tissue. For instance, the AAV serotype 1 vectors could efficiently transduce the skeletal muscles [16], whereas AAV serotype 5 vectors could efficiently transduce liver [17] and central nervous system [18]. Although these differences in the tropism might be due to the receptors and processes during AAV transduction, the precise mechanisms are unknown. In terms of AAV vector mediated gene transfer into the kidney, it is not known whether any of the serotypes of AAV vectors can achieve transduction. In the present study, we explored the transduction efficiency of renal cells in vitro using AAV serotype 1–5 vectors. To achieve efficient gene transduction in vivo, we developed a catheter-based injection system that can be inserted into the kidney of rats and mice and tested these distinct AAV serotype vectors.

Materials and Methods

Cell Culture and Reagents

Normal rat kidney cell lines (NRK52E and ACTT-CRL1571) displaying a tubuloepithelial phenotype [19] were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were plated onto 60-mm dishes (Falcon, Franklin Lakes, N.J., USA) at a density of 3×10^6 and cultured at 37°C in 5% carbon dioxide and 95% air. The primary antibody against aquaporin-1 (AQP-1) was prepared with rabbit immunization, targeting for rat AQP-1 (234–247; Arg-Ser-Ser-Asp-Phe-Thr-Asp-Arg-Met-Lys-Val-Trp-Thr-Ser). The reagents were obtained from Sigma (St. Louis, Mo., USA) unless otherwise indicated.

Experimental Animals

Twelve male Lewis rats (initial body weight 110–150 g), originally purchased from Japan SLC (Shizuoka, Japan) and maintained at our animal center, and 10 male BALB/c mice (initial body weight 27–33 g), purchased from CLER Japan (Tokyo, Japan), were used in this study. The animals had free access to standard chow and drinking. All experiments in this study were performed in accordance with the Jichi Medical School Guide for Laboratory Animals.

Preparation for AAV Vectors

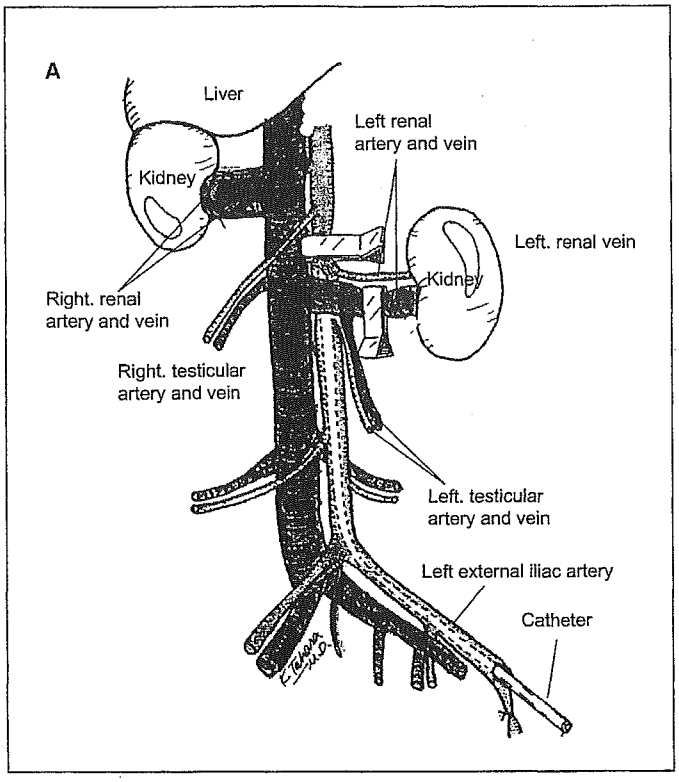
AAV vectors encoding the β-galactosidase (β-gal) gene were produced based on plasmid transfection [10]. Briefly, subconfluent 293 cells were cotransfected with AAV vector plasmid, AAV helper plasmid, and adenovirus helper plasmid by a calcium phosphate precipitation method. Recombinant AAV was harvested by three cycles of freezing/thawing. The vector solution was then purified twice on a gradient. The vector titer was determined by quantitative dot-blot hybridization of DNase-treated stocks.

Detection of β-Gal Expression

Detection of the β-gal expression was described previously [20, 21]. Briefly, samples of the kidney from rats and mice were embedded in OCT compound (Miles Laboratories, Elkhart, Ind., USA), frozen in liquid nitrogen, and cut into thin (10–20 μm) sections. The sections were fixed with 0.2% glutaraldehyde for 5 min at room temperature, washed three times in 0.1 *M* of phosphate-buffered saline (PBS; pH 7.4) for 5 min, transferred to X-gal staining solution [1 mg/ml of 5-bromo-4-chloro-3-indolyl-β-*D*-galactopyranoside, 2 m*M* $MgCl_2$, 5 m*M* potassium hexacyanoferrate [III], 5 m*M* potassium hexacyanoferrate [II] trihydrate] at 37°C for 2 h, and then counterstained with kernechtrot solution. To detect β-gal expression in cultured cells, the cells were washed with PBS three times, fixed with 0.2% glutaraldehyde for 5 min at room temperature, washed with PBS three times, and reacted with β-gal staining solution.

Immunohistochemistry

Fresh-frozen kidney sections were fixed with 100% acetone for 20 min at room temperature, washed, and transferred to X-gal staining solution as described above. Using a standard avidin-biotin complex technique, the sections stained with X-gal were blocked with 1.5% H_2O_2 for 10 min, 5% bovine serum albumin/5% horse serum for 20 min, and avidin/biotin (Vector Laboratories, Burlingame, Calif., USA) and incubated for 2 h with rabbit anti-rat AQP-1 antibody (dilution 1:1,000), followed by biotin-conjugated antirabbit IgG (Rockland Immunochemicals, Gilbertsville, Pa., USA, dilution 1:250) for 1 h and horseradish peroxidase-labeled streptavidin (Vector Laboratories; dilution 1:200) for 30 min. Immunoreactive cells were detected with 3,3′-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan). To estimate the transduction efficiency, X-gal-positive cells of AQP-1-positive cells were quantified using a grid point counting method. The percentages of cells double positive for both X-gal and AQP-1 were calculated.



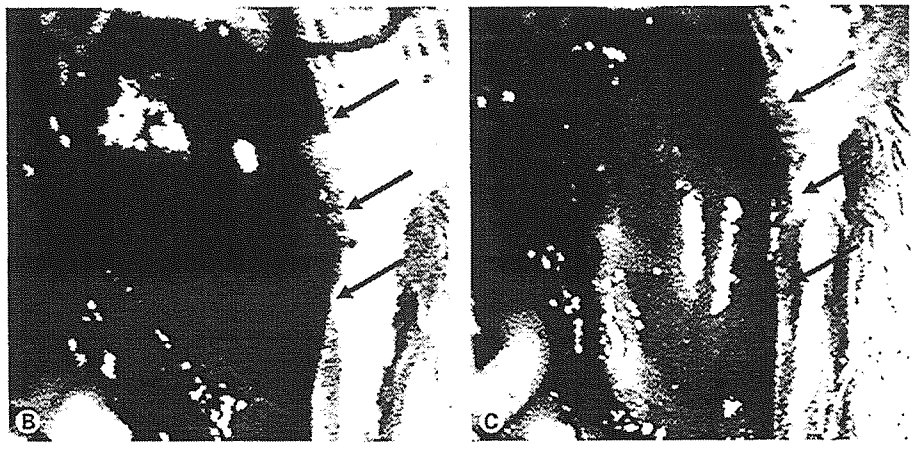


Fig. 1. Procedure for catheter-based gene delivery into the kidney. **A** A 2-Fr flexible catheter is inserted via rat left iliac artery and abdominal aorta, and the tip is placed into the left renal artery. After the aorta is clamped just above the left renal artery, AAV solution is injected after saline to wash out blood through the catheter. The left renal vein then is clamped for 10 min. **B**, **C** The color of the kidney was dark-red before (**B**; arrows) and pale after (**C**; arrows) injection of AAV solution into mice.

In vitro Gene Transduction in NRK52E Cells

The cells were plated on 24-well dishes (Falcon) at 5×10^4/well. After incubation for 16 h, 1.5×10^{10} vector genomes of each of the AAV serotype 1–5 vectors were added to each well (3×10^5 particles/cell). The cells were cultured for 48 h, and the β-gal gene expression was evaluated histologically.

In vivo Gene Transduction into the Kidney

Gene delivery was performed with each of the AAV serotype 1–5 vectors (5.0×10^{11} and 1.0×10^{11} vector genomes in rats and mice, respectively). To achieve efficient gene delivery into the kidney in vivo, we developed a catheter-based gene delivery system for use in rats and mice that mimicked the clinical procedure. The flexible Solo-Cath catheter (2 and 1 Fr for rats and mice, respectively; Solomon Scientific, Plymouth Meeting, Pa., USA) was inserted via the left iliac artery and the abdominal aorta, and the tip was placed beneath the left artery (fig. 1A). After the aorta was clamped just above the left renal artery, 1 ml (rats) or 0.5 ml (mice) of AAV vector solution was injected after 1–2 ml of saline to wash out blood via the catheter. The left renal vein then was clamped for 10 min. Catheter and clip were removed. Figures 1B and C show the color of the kidney before and after the injection of AAV vector solution into the left kidney of mice. The color of the injected kidney changed from dark-red to pale. Histological analysis confirmed that no injury occurred

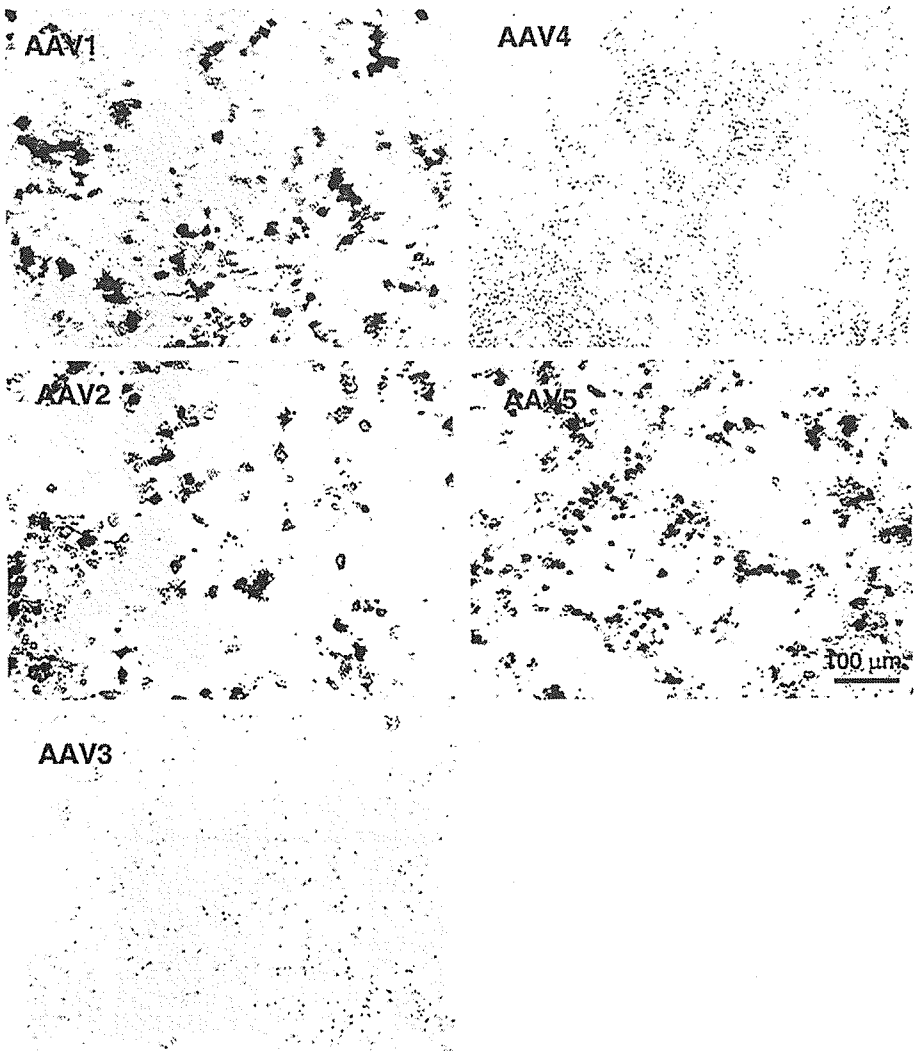


Fig. 2. In vitro transduction of NRK52E cells by AAV serotype derived vectors. NRK52E cells were transduced with AAV serotype 1–5 vectors for 48 h, and the β-gal expression was evaluated. Efficient gene transduction is observed in the cells transduced with AAV serotype 1, 2, and 5 vectors. The results are representative of two independent experiments.

in associationwith this procedure in the injected kidney. Each serotype of AAV vectors was delivered into 2 rats and mice each which were sacrificed on day 14 to evaluate which serotype of AAV vectors had the ability for gene transduction. To investigate long-term gene transduction, additional 2 rats were transduced by AAV2 vectors and sacrificed on day 28.

Results

In vitro Transduction by AAV Serotype Derived Vectors in NRK52E Cells

To evaluate the efficiency of AAV serotype derived vectors for gene transfer into renal cells, we used rat epithelial cell line NRK52E cells as an in vitro model. The cells were exposed to AAV serotype derived vectors encoding the β-gal gene for 48 h, and the β-gal expression was evaluated. The AAV serotype 1, 2, and 5 vectors efficiently transduced these cells (fig. 2). These observations suggest that AAV vectors are useful for gene delivery in renal cells in vitro and that the transduction efficiency might differ among AAV serotypes.

In vivo Gene Transduction by Distinct AAV Serotype Derived Vectors into the Kidney

The vector solutions were selectively injected into rat kidneys using the procedure as described in Materials and Methods, and the animals were sacrificed and the β-gal expression evaluated 14 days after gene delivery. The injection of AAV serotype 2 vectors showed β-gal expression, whereas the rest of the serotypes showed no expres-

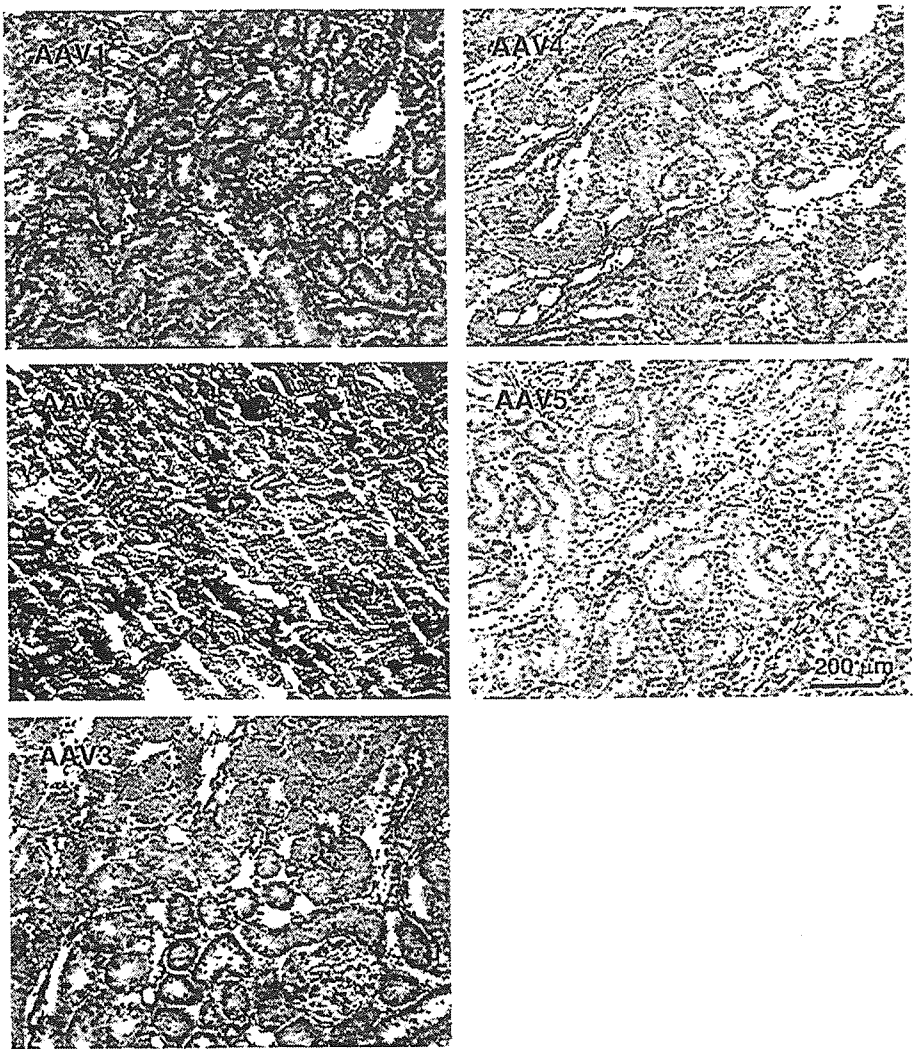


Fig. 3. In vivo transduction by AAV serotype derived vectors into the rat kidney. Solutions with AAV serotype 1–5 vectors encoding the β-gal gene were selectively injected into rat kidney. The animals were sacrificed on day 14, and the kidney sections were evaluated for β-gal expression. Injection of AAV serotype 2 vectors resulted in gene transduction, whereas AAV serotype 1, 3, 4, and 5 vectors showed no transduction. The results represent two independent experiments.

sion (fig. 3). Transduction was observed in cortical tubular epithelial cells, but not in glomeruli, endothelial cells, smooth muscle cells, or interstitial cells. We further confirmed the transduction efficiency with AAV serotype derived vectors into the murine kidney. Consistent with the experimental findings in rats, only AAV serotype 2 vectors showed β-gal expression in the tubular epithelial cells in the kidneys of mice, whereas the other serotypes showed no expression (fig. 4).

We next evaluated the long-term gene transduction by AAV serotype 2 vectors and demonstrated β-gal expression in the rat renal cortex 28 days after gene delivery (fig. 5A). No β-gal expression was observed in extrarenal organs, including liver or skeletal muscle. Immunohistochemical analysis revealed coexpression of β-gal with a proximal tubule marker, AQP-1 [22], suggesting AAV serotype 2 vector mediated gene transduction into the proximal tubular cells. The proportions of gene transduction were 3.2 and 4.0% of the proximal tubular cells.

Discussion

The two major findings of this study are that, in vitro, the AAV serotype 1, 2, and 5 vectors transduced gene expression in kidney epithelial cells, whereas AAV serotype 3 and 4 vectors showed no transgene expression; in vivo, only the AAV serotype 2 vectors transduced gene expression in the proximal tubule of kidney when the vectors were selectively injected into the kidney of rats and

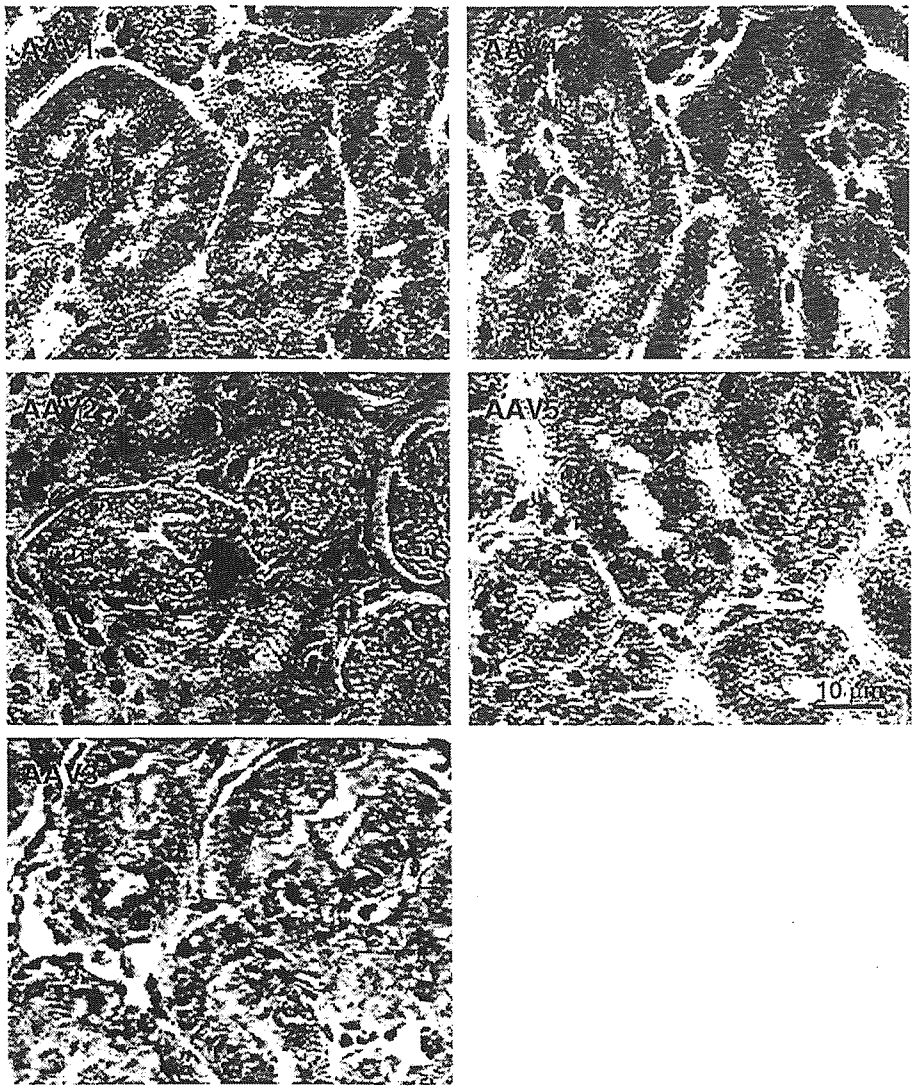


Fig. 4. In vivo gene transduction by distinct AAV serotype vectors into the mouse kidney. Transduction of AAV vectors and evaluation of β-gal expression in mice are essentially described in the figure 1 legend. Consistent with the experiments in rats, the injection of AAV serotype 2 vectors showed gene transduction in the kidney of mice, whereas AAV serotype 1, 3, 4, and 5 vectors showed no transduction. The results are representative of two independent experiments.

mice using a catheter-based procedure. These findings suggest that the transduction efficiency might differ among AAV serotype vectors and that kidney-specific gene delivery using the AAV serotype 2 vectors has the potential for renal gene therapy. The difference in the relative transduction efficiency was not due to a difference in the potency of the serotype-derived vectors, because the capacity of each of the vector stocks was tested and confirmed in vitro and in vivo (data not shown).

Gene transfer into the kidney has a great potential as a novel therapeutic approach. However, efficient gene transduction to the kidney has not been established. Adenovirus-mediated gene delivery is a feasible strategy for gene transduction in the kidney, because it transduces efficient gene expression in the kidney in several experimental models [4–6]. However, there are substantial limitations associated with the clinical application of adenovirus vectors. First, adenovirus vectors show short-term expression (weeks to months) of the transduced gene, because adenovirus does not integrate into the host genome. Second, adenovirus vector mediated gene transduction may cause adverse inflammatory and immunologic responses. Moreover, the risk of recombination with wild-type and generation of replicative mutant virus has not been eliminated.

In contrast to adenovirus vectors, AAV vectors have a number of attractive features for the clinical use of gene therapy. Recombinant AAV vectors are safe, nonpatho-

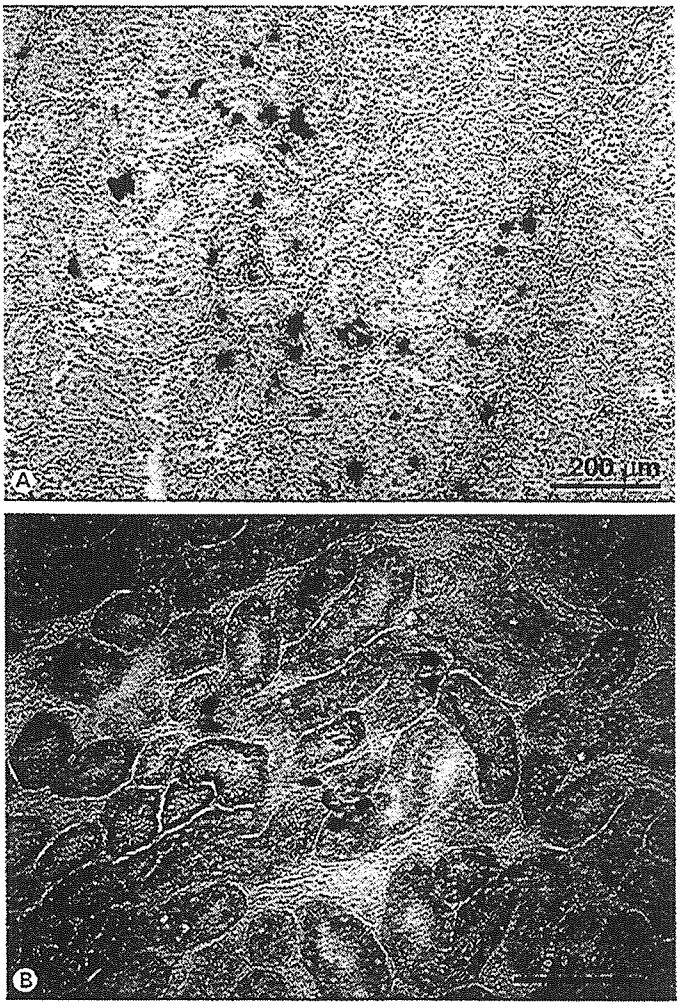


Fig. 5. In vivo long-term transduction by AAV serotype 2 vectors into the rat kidney. Solutions with AAV serotype 2 vectors encoding the β-gal gene were selectively injected into rat kidney, and the rats were sacrificed on day 28. The kidney sections were evaluated for β-gal expression (**A**) and immunohistochemical staining with anti-rat AQP-1 antibody (**B**).

genic, and nonreplicating according to the life cycle of the wild-type virus [23, 24]. Further, unlike the reports using adenovirus vectors, AAV vectors can give rise to long-lasting gene expression without an obvious immune response [9]. An increasing number of reports demonstrated that AAV vectors can transduce into various cell types. Regarding the kidney, the usefulness of the AAV vectors was demonstrated in vitro [25] and in vivo [26]. AAV serotype 2 was used in both studies, because it was representative of AAV and because other serotypes were not focused at that time. However, it is now possible to compare the efficiency of gene transfer into the kidney by using distinct AAV serotype vectors. We, therefore, evaluated the usefulness of AAV serotype 1–5 vectors in renal cells in vitro and in vivo. Interestingly, AAV serotype 1, 2, and 5 vectors transduced renal epithelial cell lines in vitro, whereas only AAV serotype 2 vectors transduced the tubular epithelial cells. Consistent with our findings, AAV serotype 2 vector mediated delivery of reporter genes into tubular epithelial cells was shown in the mouse kidney. Of note, only the tubular epithelial cells, but not the glomerular or vascular cells, were transduced following in vivo AAV serotype 2 vector administration. This means that the tubular epithelial cells are highly susceptible to AAV serotype 2, but the reason for this phenomenon is unclear. Thus far, heparan sulfate as a primary receptor [27] and fibroblast growth factor receptor [28] and αvβ5 integrin [29] as coreceptors have been identified for AAV serotype 2. Therefore, the distribution of these molecules might explain the difference in transduction efficiency. Further investigations are required to determine the precise mechanisms of AAV-mediated gene transfer and to promote gene therapy approaches using AAV vectors in the kidney.

To establish a safe and clinically relevant approach, we developed the catheter-based method of AAV vectors to achieve an organ-specific gene delivery. Alternative approaches for kidney gene delivery have been also demonstrated. Lipkowitz et al. [26] reported that intraparenchymal injection of AAV serotype 2 vectors in the mouse kidney could transduce into tubular epithelial cells. Furthermore, successful gene delivery from renal vein or ureter into the kidney using naked DNA or HVJ liposomes was reported as other approaches for kidney-targeted gene transfer [2, 30, 31]. Since the approach developed in this study mimicked the clinical procedure and might be more physiological than alternative approaches, we believe that this procedure could be useful for future clinical applications.

In summary, we demonstrated that AAV vectors could transduce β-gal gene expression in renal cells in vitro and in vivo. In particular, the catheter-based direct gene delivery of AAV serotype 2 vectors caused successful gene transduction into the kidney tubular epithelial cells of rats and mice. The procedure demonstrated in this study has clinical advantages, because it seems to allow organ-specific gene delivery in the kidney. Since kidney-specific gene delivery via catheterization of the renal artery is highly feasible in humans, our findings provide useful information for promising strategies in renal gene therapy.

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GENE THERAPY FOR ORGAN GRAFTS USING RAPID INJECTION OF NAKED DNA: APPLICATION TO THE RAT LIVER

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Background. **We developed a nonviral gene transfer method using rapid injection of naked DNA targeting the liver and applied it in a rat model of liver transplantation.**

Methods. **Inbred Dark Agouti and Lewis rats were used. To test the efficacy and adverse effects of systemic or local (catheter-based) injection, different volumes of phosphate-buffered saline containing naked DNA encoding β-galactosidase (lacZ) were injected. Luciferase expression was followed by non-invasive imaging, and a cytotoxic T-lymphocyte antigen 4-immunoglobulin (CTLA4Ig) protein was tested functionally by allogenic heart transplantation. Gene transfer was then tested in rat auxiliary liver transplantation (ALT) and orthotopic liver transplantation (OLT). The timing of gene transfer was evaluated in the auxiliary liver transplantation model, and OLT was performed using a liver graft to which luciferase or the CTLA4Ig gene was transferred 2 days before.**

Results. **LacZ was expressed extensively in a volume-dependent manner; however, a large volume often induced recipient death. After local delivery of CTLA4Ig cDNA to the liver, survival of Dark Agouti heart grafts lengthened with increased CTLA4Ig se-**

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rum levels. Liver grafts injected with naked DNA at the time of donation did not survive, but livers grafted 2 days after gene transfer survived. Successful expression of luciferase and production of CTLA4Ig were finally confirmed in the rat that underwent OLT.

Conclusions. **We successfully applied a nonviral hydrodynamic gene transfer method to the rat liver and showed its potential in liver grafting. The high incidence of graft failure when this procedure is performed on the day of organ donation is a potential limitation that needs to be overcome in clinical application.**

Genetic modification of a liver allograft before transplantation using ex vivo gene transfer offers considerable scope in modifying rejection and promoting tolerance to the graft (*1–5*). Moreover, the liver is an important target organ for gene therapy because it plays a major role in metabolism and protein production. Recent studies have reported highly potent gene transduction in liver grafts using viral vectors (*2, 5–7*). However, the clinical application of virus vectors for gene transduction is limited because of cytotoxic and immune responses (*8, 9*). There may be added complications in patients treated with systemic immunosuppressive agents after transplantation.

Several recent studies have shown that the intravascular delivery of plasmid DNA through the vein is an effective method for transfecting hepatocytes in rodents. Systemic injection through a tail vein with a large volume of plasmid DNA solution can achieve effective gene transfer into the liver (*10–16*).

We recently focused on a novel method of gene transfer to the graft organ using an organ-selective injection technique (*17*). In the present study, we tested a protocol of rapid injection of naked DNA into the donor hepatic vein. Marker genes of LacZ and luciferase and the cytotoxic T-lymphocyte antigen 4-immunoglobulin (*CTLA4Ig*) gene were tested, and the approach was applied to auxiliary liver transplantation (ALT) and orthotopic liver transplantation (OLT).

MATERIALS AND METHODS

Rats

Inbred Lewis ($RT1^l$) and Dark Agouti (DA) ($RT1^a$) rats, weighing 180 to 250 g and originally purchased from Charles River Japan, Inc. (Yokohama, Japan) and CLEA Japan, Inc. (Tokyo, Japan), respectively, were used. Rats were maintained in our animal center. All experiments were performed in accordance with the "Jichi Medical School Guide for Laboratory Animals."

Plasmid Constructs

The firefly luciferase (*Photinus pyralis*) expression plasmid pGL3 was obtained from Promega (Madison, WI). The β-galactosidase expression plasmid pCAG-LacZ and the *CTLA4Ig* expression plasmid pCAG-CTLA4Ig were amplified in the DH5 α strain of *Escherichia coli*, and large-scale preparation of plasmid DNA was performed by the alkaline lysis method. Closed circular plasmid DNA was then purified twice by equilibrium centrifugation in CsCl-ethidium-bromide gradients. DNA concentrations were measured by ultraviolet absorption at 260 nm.

Experiment I: Evaluation of Rapid Injection of Naked DNA into the Systemic Circulation or Selectively into the Liver

We used a pCAG-LacZ marker gene to test gene expression in the rat liver after systemic injection of a large volume of solution containing naked DNA. Either 125 or 250 μg of pCAG-LacZ was diluted in five volumes of phosphate-buffered saline (PBS) (10%, 7.5%, 6.25%, 5.0%, or 2.5% of body weight) and injected into the dorsal penile vein of Lewis rats, weighing 180 to 200 g, using a 21-G needle and a 20-mL syringe. The injection took no longer than 12 sec. We evaluated the adverse effects of injection and monitored the number of rats that died or stopped breathing immediately after injection. We attempted resuscitation of all rats with apnea using artificial ventilation. Surviving rats were killed, and their livers were excised 3 days after transfection and fixed in 0.2% glutaraldehyde solution before staining with β-gal solution to detect pCAG-LacZ expression.

Peripheral blood was taken at 1, 3, 5, and 7 days to determine the levels of serum aspartate aminotransferase (AST) and alanine aminotransferase as indices of liver damage. As a control, peripheral blood from normal Lewis rats without any treatment was taken (n=4).

Changes in systemic blood pressure and central venous pressure (CVP) during systemic injection of the large-volume solution were determined. The rats were administered ether anesthesia, and 22-G and 14-G Teflon tubes (Surflo, Terumo Co., Ltd., Tokyo, Japan) were used to cannulate the femoral artery and the cervical vein, respectively, and connected to a pressure transducer (Life Kit, Nihon Koden, Tokyo, Japan) to measure systemic blood pressure and CVP.

To reduce the adverse effects induced by systemic injection of large-volume solutions, we developed a method of selectively injecting the liver using a catheter, that is, catheter-based rapid injection of large-volume solution with naked DNA (CB-RILV). After cannulation using a 0.5-mm silicon tube into the inferior vena cava (IVC) from the right common iliac vein, the supra-hepatic IVC was cross-clamped with a Satinsky clamp, and a 4–0 silk suture was wrapped around the infrahepatic IVC and microclipped. Then, four volumes of PBS (i.e., 1.5%, 2.0%, 2.5%, and 3.0% of rat body weight) diluted with 125 μg pCAG-LacZ were rapidly injected.

Rats transfected with LacZ were killed 3 days later, and LacZ expression was evaluated by β-gal staining.

Experiment II: Application of the CTLA4Ig Gene to Rat Liver Using the CB-RILV Method

Adult male Lewis and DA rats, weighing 200 to 250 g, served as recipients and donors, respectively. Two days before transplantation, 125 μg or 250 μg of pCAG-CTLA4Ig in a fixed volume of 2.5% body weight PBS was rapidly injected into the recipient liver, as described previously. DA heart allografts were transplanted into the cervical space of the Lewis recipient using the cuff technique reported previously (*18*). Recipients were divided into three groups of six animals each: group 1, untreated control recipients; group 2, recipients injected with 125 μg of pCAG-CTLA4Ig; and group 3, recipients injected with 250 μg of pCAG-CTLA4Ig. Cardiac pulsation was assessed daily, and graft cardiac arrest was defined as the graft survival time. The serum CTLA4Ig level in the recipient peripheral blood obtained on days 0, 2, 5, and 7 and every week thereafter was measured by enzyme-linked immunosorbent assay. Recipients whose grafts rejected were killed for histologic study of the grafts.

Experiment III: Application to Rat Liver Transplantation

To assess the liver condition, we performed ALT by using a modified method previously reported (*19*). Donor Lewis rats were transfected with pCAG-CTLA4Ig or pGL3 using the CB-RILV method 2 days before or 1 day before ALT; 125 μg of pCAG-CTLA4Ig or pGL3 was injected into the donor liver using the CB-RILV method.

Finally, we tested a rat OLT model using luciferase or the *CTLA4Ig* gene as an expression marker or soluble product, respectively. OLT was performed with hepatic artery reconstruction using our previously reported method (*20*).

Luciferase expression within the donor liver after ALT or OLT was determined using a non-invasive living image acquisition IVIS system (Xenogen Inc., Alameda, CA). Serum CTLA4Ig levels in rats that received a pCAG-CTLA4Ig-transfected liver were measured 2, 5, 7 14, 21, and 28 days after transplantation.

Detection of LacZ Expression (β-gal Staining)

The rats were killed and the livers were excised 3 days after transfection. Samples were fixed in 0.2% glutaraldehyde solution and stained with β-gal, and LacZ expression was observed as described previously (*21, 22*).

Detection of Serum CTLA4Ig Level by Enzyme-Linked Immunosorbent Assay

CTLA4Ig levels in serum were assayed by enzyme-linked immunosorbent assay. A 96-well microtiter plate (MICROTEST Flat Bottom, FALCON, Becton Dickinson Labware, Franklin Lakes, NJ) was coated with anti-mouse CTLA4Ig antibody (purified mouse anti-human monoclonal antibody, 555851, PharMingen, San Diego, CA) and incubated for 12 hr at 4°C with 50 μL of the solution in each well. After washing with PBS containing 0.1% Tween, 50 μL of the serum sample was added to each well. The plate was incubated for 2 hr at room temperature. The secondary antibody, anti-human IgG1 Fc conjugated with horseradish peroxidase (MH1715, Dai Nihon Seiyaku, Osaka, Japan) diluted to 1:100 with PBS, was added to each well and incubated for 1 hr at room temperature. Each well was washed, and a 50-μL solution of ortho-phenylenediamine dihydrochloride (P-8412, Sigma Chemical Co., St. Louis, MO) solution diluted to 3 mg/mL with PBS and 0.15 μL/mL H_2O_2 was added to each well. After 2 min, 50 μL of 1 M H_2SO_4 was added to each well, and the absorption values at 492 nm were obtained with the Microplate Reader. The CTLA4Ig concentration was quantified by comparing it with the absorbance value of the control recombinant human CTLA4Ig sample.

In Vivo Luciferase Imaging

Expression of the luciferase gene was evaluated using the non-invasive IVIS system (Xenogen Inc.) without killing the animals. This system collects photons of light emitted from tissue using a cooled charged-couple device camera. After isoflurane anesthesia was induced, the rats that were transfected with pGL3 or rats that received a graft liver transfected with pGL3 were injected intraperitoneally with 150 mg/kg body weight n-Luciferin potassium salt (Xenogen Inc.) dissolved to 15 mg/mL with PBS. Fifteen minutes later, the levels of light emitted from the bioluminescent liver were measured. An integration time of 1 min was used for image acquisition of the luminescence. The data were digitized and displayed on a monitor in the Living Image System (Xenogen Inc.). Signal intensity was quantified as the sum of the detected photons.

RESULTS

Lac Z Expression in the Rat Liver and Adverse Effects of Systemic Injection of a Large-Volume Solution Containing Naked DNA

We observed the expression of 125 μg of pCAG-LacZ after systemic rapid injection of a gene solution diluted in more than 6.25% of body weight volume of PBS. When pCAG-LacZ was diluted in PBS less than 5.0%, LacZ was poorly expressed. After 10% of body weight volume of PBS was injected, four of five rats (80%) stopped breathing, and three rats (60%) died. When the injected volume of PBS was lower than 6.25% of body weight, no rats stopped breathing or died. The serum AST level transiently increased 1 day after rapid PBS injection and then decreased to a normal level 3 days after injection. The peak serum AST level after injection of a 7.5% of body weight volume of PBS was approximately 400 to 510 IU/mL, and after injection of a 5.0% volume it was less than 145 IU/mL (Table 1). The normal AST and alanine aminotransferase levels obtained from separate Lewis rats without any treatment (n=4) was 78.2±10.6 IU/mL and 51.5±5.8 IU/mL, respectively (mean±standard deviation).

To clarify the hemodynamic change after systemic injection of a large-volume solution, systemic arterial pressure and CVP were measured. The CVP during the injection of 6.25% of body weight volume of PBS was transiently elevated to greater than 32 mm Hg and continued to increase more than 15 mm Hg. At the same time, the mean systemic blood pressure in the rats rapidly decreased to less than 60 mm Hg, and the heart rate also decreased. Several minutes were needed for the CVP, the systemic blood pressure, and the heart rate to recover.

Because of these adverse effects, we developed a catheter-based method to rapidly deliver a large volume of naked DNA solution (CB-RILV method) into the rat liver. Expression of LacZ after catheter-based rapid injection of 125 μg cDNA diluted in 2.5% of body weight PBS was tested, and the results are shown in Figure 1. Efficient LacZ expression in the liver was observed 3 days after catheter-based injection of 125 μg DNA diluted in 2.5% of body weight volume of PBS. However, liver damage associated with this method was not significantly different between the group that received 2.5% of body weight volume group (serum AST and alanine aminotransferase were ~270 IU/mL and 150 IU/mL 1 day after injection, respectively) and the other groups (data not shown).

Production of CTLA4Ig Protein for the Rat Liver Induced by the CB-RILV Method

To test whether the secreted serum CTLA4Ig works effectively, we transplanted DA cardiac allografts into Lewis recipients transfected with pCAG-CTLA4Ig. The cardiac graft survival times are shown in Table 2. In groups 2 and 3, three of six grafts showed prolonged survival compared with the

TABLE 1. Adverse effects of systemic injection of various volumes of solution with the naked LacZ gene

Injection volume (%)	No. rats	Mortality (%)	Apnea (%)	Liver damage[a]	
				AST	ALT
10	5	3/5 (60)	4/5 (80)	949	1,039
7.5	4	0/4 (0)	2/4 (50)	458	506.5
6.25	5	0/5 (0)	0/5 (0)	NT	NT
5.0	6	0/6 (0)	0/6 (0)	132.5	93.5
2.5	4	0/4 (0)	0/4 (0)	110	58
–[b]	4	—	—	78.2	51.5

[a] Peripheral blood from surviving rats was drawn on days 1, 3, 5, and 7. The peak values are indicated and expressed as the mean.
[b] Data of peripheral blood from normal rats without any treatment were shown.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; NT, not tested.