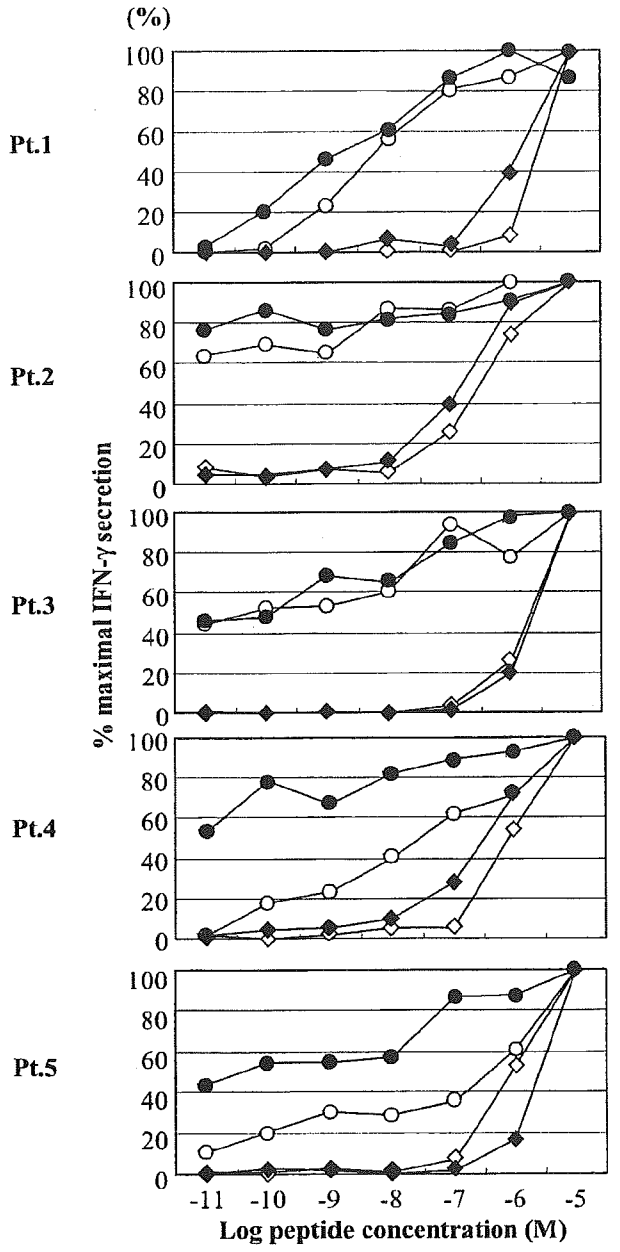

FIG. 4. Functional avidity assay. The reactivity of peptide-specific cells in PBMCs from five patients against log-fold dilutions of peptide was determined. Symbols: ○, wild type. ●, 2F; ◇, 5C; ◆, 2F5C.

Epitope presentation from native Nef protein. Strong selection for Nef138-10(2F) in the presence of CTLs with high in vivo functional avidity against the peptide prompted us to examine the processing and presentation of the Nef138-10 CTL epitope from the native protein. Native Nef proteins containing wild-type or variant CTL epitopes were expressed in an HLA-A*2402-positive-T-cell line (KWN-T4) via SeV. CTL epitope presentation was examined by two CTL clones established from A24-positive patients outside these cohorts. Although the two CTL clones were established by stimulation with the wild-type peptide (Nef138-10), they killed the target cells pulsed with Nef138-10(2F) peptides almost as well as the wild type (Fig. 5A and B). Both CTL clones efficiently killed the target cells expressing either wild-type Nef or Nef with −1T substitution in the flanking region (−1T2Y5T). However, these CTL clones failed to kill the target cells infected with vectors expressing Nef138-10(2F) with (−1T2F5T) or without (−1I2F5T) the −1T substitution in the flanking region. As expected, the CTL clones did not kill the target cells infected with a vector coding Nef138-10(2F5C), a nonbinding mutant (−1I2F5C) (Fig. 5A and B). Western blot analysis revealed that Nef proteins with wild-type or variant CTL epitopes were expressed abundantly in the target cells. Taken together, these data indicate that a Y-to-F substitution within the CTL epitope itself but not the −1T substitution in the flanking region resulted in the poor antigen presentation against CTL, which resulted in the escape.

DISCUSSION

We showed a significantly higher prevalence of a stereotypic amino acid substitution [Nef138-10(2F)] at an A24-restricted CTL epitope in Nef among A24-positive Japanese hemophiliacs compared with A24-negative counterparts. The origin of their HIV-1 infection was from the plasma collected and processed in Western countries where HLA-A*2402 was less prevalent (19). It is inferred that Nef138-10(2F) might be rare in a population where HLA-A*2402 is not prevalent but that it has a selective advantage in the presence of HLA-A*2402. Our findings with Australians are consistent with this notion. Although we examined only two HIV-1-infected A24-positive Caucasian Australians, both had Nef138-10(2F). On the other hand, Nef138-10(2F) was rare in A24-negative Australians. Japanese and Australians are distinctly different in the frequency of HLA-A*2402 within their respective populations (allele frequency of HLA-A24 is 35.1 and 7.8%, respectively) (19). Nef138-10(2F) was also positively selected among Japanese patients who were infected through USI. Interestingly, we detected Nef138-10(2F) frequently among A24-negative Japanese who were infected through USI. The result suggests that HIV-1 that went through selective pressure by HLA-A*2402 is actually circulating among the Japanese population because of the high prevalence of HLA-A24. Although we showed the reversion of Nef138-10(2F) to the wild type, it occurred very slowly over years, allowing the horizontal spread via sexual contact. In this study, we showed that HIV-1 with Nef138-10(2F) is actually a CTL escape mutant. Although the stereotypic Y-to-F substitution occurred at an anchor residue, Nef138-10(2F) peptide did bind to HLA-A*2402 heavy chain with almost the same efficiency as did the wild type (Fig. 3). This result is consistent with the algorithm prediction of the published binding motif (http://hiv-web.lanl.gov/content/immunology/motif_scan/motif.html). When native Nef proteins with or without a substitution were overexpressed in the A24-positive target cells via SeV, the Y-to-F substitution at the second position of the CTL epitope virtually abolished the killing by the CTL clones. The substitution in the flanking region did not affect the killing substantially. Therefore, the mechanism for the CTL escape appeared to reside in the processing of native Nef proteins and subsequent antigen presentation rather than HLA binding. A proteosomal cleavage

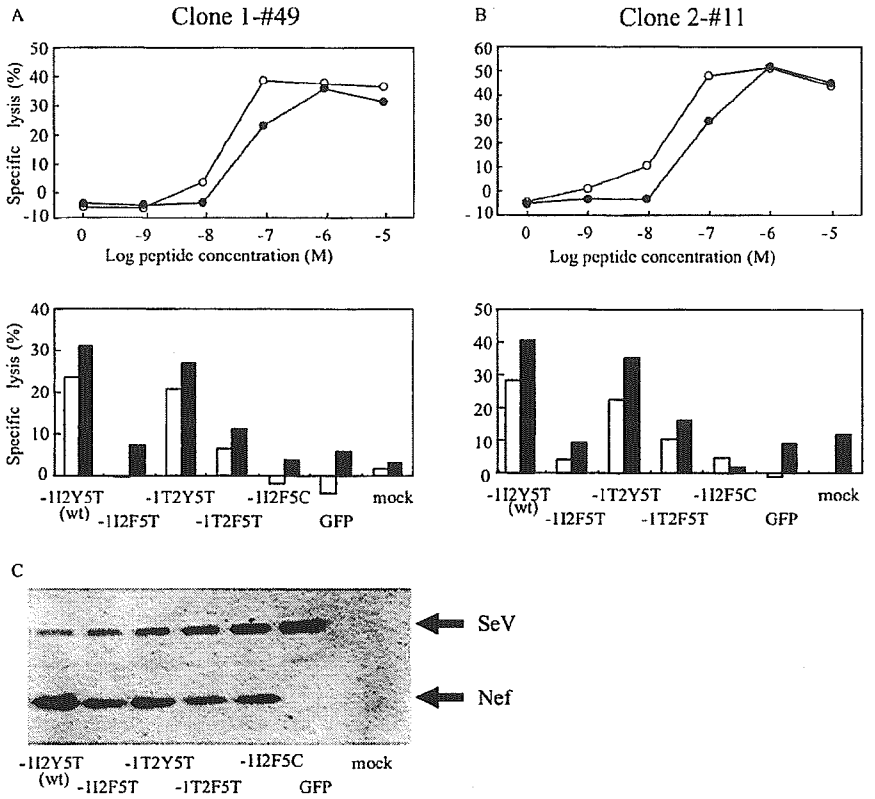

FIG. 5. Killing activity of clone 1-#49 (A) against KWN-T4 target cells pulsed with log-fold dilutions of peptide (top) and expressing native Nef proteins containing wild-type sequences (−1I2Y5T), a Y-to-F substitution at the second position of the CTL epitope (−1I2F5T), an I-to-T substitution at the −1 flanking position (−1T2Y5T), double substitutions at the −1 and second positions (−1T2F5T), and double substitutions at the second and fifth positions (−1I2F5C) (bottom). The effector-versus-target ratio was 1:1 (□) or 2.5:1 (■) in panel A and 1:1 (□) or 4:1 (■) in panel B. Killing activity against KWN-T4 cells infected with control vector expressing green fluorescent protein (GFP) and mock infected (mock) are also shown. (C) Western blot analysis of intracellular expression of various Nef mutants in KWN-T4 target cells. KWN-T4 target cells expressing native Nef proteins containing wild-type sequences (−1I2Y5T), a Y-to-F substitution at the second position of the CTL epitope (−1I2F5T), an I-to-T substitution at the −1 flanking position (−1T2Y5T), double substitutions at the −1 and second positions (−1T2F5T), and double substitutions at the second and fifth positions (−1I2F5C) were examined. KWN-T4 cell lysates infected with control vector expressing GFP and mock infected are also shown. An aliquot (3 μg) of the same KNW-T4 target cells used for the killer assay in the upper panel was used for the Western blot. Symbols: ○, Nef138-10; ●, Nef138-10(2F).

prediction program, NetChop (23), suggested the possibility that the Y-to-F substitution in the second position creates a new cleavage site at the fifth T residue in the CTL epitope. Proteolytic cleavage within the epitope could be the cause of poor antigen presentation.

Although we could not show the process of positive selection for Nef138-10(2F), Nef138-10(2F5C), and Nef138-10(5C), the high prevalence of Nef138-10(2F) in A24-positive patients and the reversion in A24-negative patients suggested that one point mutant, Nef138-10(2F), was selected first, and then two or three point mutants, Nef138-10(2F5C), evolved. Once the T-to-C amino acid substitution at the fifth position is acquired, the binding capacity of the CTL epitope to the HLA-A*2402 heavy chain is abolished (Fig. 3), and the Y-to-F substitution at the second position may become dispensable even in the presence of HLA-A*2402.

In our cohort of patients, Nef138-10(2F) accompanied a −1T substitution in the flanking region very frequently. We observed sequential reversion in the CTL epitope and flanking region at least in one patient with an A24-negative background. As of 11 October 2003, the HIV-1 sequence database showed that the 2F substitution (74 sequences) accompanied the −1T substitution frequently (64.9%) but accompanied the wild-type residue (I) only rarely (9.5%). On the other hand, the wild-type residue (Y) in the second position of the CTL epitope (195 sequences) accompanied wild-type (I) residue more frequently (57.4%) than the −1T substitution (20.5%). Although the function of the region surrounding Nef138-10 has not been elucidated, there seems to be a compensation between these two residues.

In simian immunodeficiency virus infection, CTLs with high functional avidity select for escape variants (29). However, we found CTLs with high functional avidity not only against the wild type but also against Nef138-10(2F) in five patients studied. It is not known how these CTLs against Nef138-10(2F) are maintained in vivo. Very recently, new insights into the exogenous pathway for antigen presentation to CTLs have been elucidated (15, 17). Cross presentation by professional antigen-

presenting cells such as dendritic cells may be responsible. Our study underlines the difficulties in evaluating the effective CTL responses in vivo by CTL assays in which peptides are used, such as ELISPOT.

For example, a CTL escape variant of Epstein-Barr virus was demonstrated in a highly A11-positive population in New Guinea (9). HLA-restricted CTL responses appear to be driving HIV-1 evolution at a population level (20). As far as we know, this is the first direct demonstration of horizontal transmission of CTL escape mutants of HIV-1 at a population level. We previously reported stereotypic amino acid substitutions in HIV-1 at some CTL epitopes restricted by HLA-B35 (21). Stereotypically selected HIV-1 may become dominant through transmission where certain HLA types are highly prevalent. Recently, a rare HLA supertype was shown to have a selective advantage for the prognosis of HIV-1 infection (34). In a population with less diverse HLA types, such as that of Japan, HLA types may have a large impact on HIV-1 evolution and escape. Our study may prove to have important implications for understanding viral pathogenesis and vaccine development.

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Influence of single-nucleotide polymorphisms in the multidrug resistance-1 gene on the cellular export of nelfinavir and its clinical implication for highly active antiretroviral therapy

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Protease inhibitors (PIs) such as nelfinavir (NFV) suppress HIV replication. PIs are substrates of P-glycoprotein (P-gp), the product of the multidrug-resistance-1 (*MDR1*) gene. Three single-nucleotide polymorphisms (SNPs) are present in exons of the *MDR1* gene: *MDR1* 1236, *MDR1* 2677 and *MDR1* 3435. We speculated that these genetic polymorphisms affected PI concentration in the cell. To verify this hypothesis, we first genotyped these SNPs in 79 Japanese patients by the SNaPshot method and found incomplete linkage disequilibrium between the SNPs. Because the SNP at *MDR1* 3435 has been reported to be associated with P-gp expression, we evaluated the effect of that SNP on the export of NFV from HIV-positive patients' lymphoblastoid cell lines by measuring time-dependent decrease in the amount of intracellular NFV by high-performance liquid chromatography. We found the intracellular concentration of NFV in lymphoblastoid cell lines (LCLs) with the homozygous T/T genotype at *MDR1* 3435 were higher than that with C/C genotype with statistical significance. This suggests that the activity of P-gp in patients' LCL cells with the *MDR1* 3435 T/T genotype was lower. In a retrospective study we evaluated the effect of the SNPs on CD4 cell count recovery in response to antiretroviral treatment with PIs, and obtained statistically significant evidence that suggested marginal association of the SNP at *MDR1* 1236 but not at *MDR1* 2677 or *MDR1* 3435. As *in vitro* results were not consistent with the clinical evaluation, clinical importance of *MDR1* genotyping for antiretroviral therapy remains to be investigated in a larger, case-controlled study.

Introduction

Antiretroviral therapy with HIV protease inhibitors (PIs) in combination with reverse transcriptase inhibitors dramatically improved the prognosis of patients infected with HIV-1. However, some patients fail to achieve the maximal virological suppression. We speculate that such failure is partly because PIs do not accumulate in lymphocytes in their active free forms in a concentration high enough to inhibit viral replication [1,2], although the intracellular active PI levels have, to the best of our knowledge, not yet been determined. The activity of P-glycoprotein (P-gp), the product of the multidrug resistance-1 (*MDR1*) gene, appears to affect intracellular PI concentration, because PIs such as nelfinavir (NFV) are substrates of P-gp [2]. P-gp is a glycosylated membrane protein belonging to the ATP-binding cassette superfamily of membrane transporters. P-gp is expressed in many tissues and cell types including intestinal epithelial cells and lymphocytes, where it acts as an energy-dependent exporter [3–9]. The *MDR1* is polymorphic and at least three single-nucleotide polymorphisms (SNPs) have been identified in the exons in a healthy Japanese population [10] as well as in other ethnic groups [6]. *MDR1* 1236 and *MDR1* 3435 are silent mutations in exons 12 and 26 [3,11], respectively, whereas *MDR1* 2677 is a substitution mutation in exon 21 [11]. Reportedly, the SNP at *MDR1* 3435 is associated with the amount and activity of P-gp protein both *in vitro* and *in vivo* [3,12]. In addition, individuals with the T/T genotype at *MDR1* 3435 were found to express less P-gp in lymphocytes and in intestinal epithelial cells [3,13] and showed lower efflux of rhodamine from natural killer (NK)

cells than those with the C/C genotype [13]. According to these observations, *MDR1* polymorphisms seem to affect the intracellular PI concentration and the outcome of antiretroviral treatment. However, the role of *MDR1* 3435 SNP in the response to antiretroviral therapy is still controversial [12,14].

The objective of this study was to evaluate the effect of three *MDR1* SNPs on the intracellular concentrations of NFV and to evaluate the impact of those SNPs on virological and immunological response to antiretroviral treatment, including NFV and PIs. We genotyped the SNPs in 79 Japanese patients and compared the velocity of NFV efflux among selected patients' lymphoblastoid cell lines (LCLs) with different *MDR1* 3435 genotypes. We also analysed the viral loads and CD4 cell counts after initiation of antiretroviral treatment with prescriptions with PIs including NFV in 21 patients.

Materials and methods

Patients

A total of 79 HIV-positive Japanese patients were enrolled in this study. These patients attended a hospital AIDS clinic at the Institute of Medical Science, University of Tokyo (IMSUT). The patients provided their written informed consent to participate in the study and to supply blood samples for DNA analysis and cell culture. Of the 79 patients, 21 receiving highly active antiretroviral therapy (HAART) including PIs were divided into three groups: 11 patients receiving HAART with NFV, four patients receiving HAART with indinavir (IDV) and six patients receiving HAART with saquinavir (SQV) or lopinavir/ritonavir (LPV/RTV). CD4 cell counts and HIV-RNA of plasma were analysed for 9 months after the initiation of the antiretroviral treatment. The study has been approved by the ethics committee of IMSUT.

Single-nucleotide polymorphisms

We typed three single-nucleotide polymorphisms (SNPs) at *MDR1* 1236 (exon 12), *MDR1* 2677 (exon 21) and *MDR1* 3435 (exon 26) by polymerase chain reaction (PCR) followed by ABI PRISM SNaPshot Multiplex Kit (PE Biosystems, Foster City, Calif., USA) [15]. Information on primers and conditions for PCR was obtained at http://snp.ims.u-tokyo.ac.jp [10].

Cells and determination of uptake and efflux of NFV

Peripheral blood mononuclear cells (PBMCs) were separated from patients' whole blood with Ficoll-Conray gradient centrifugation. LCLs were obtained by transforming PBMCs with Epstein-Barr virus (EBV), which was obtained from cell-free supernatants of EBV-producing B95–8 cell lines [16]. LCLs were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, Mo., USA) supplemented with 10% heat-inactivated fetal calf serum.

To determine the time course of NFV uptake into LCL cells, LCL cells (1×10^6/10 ml, counted with a haematocytometer) were incubated at 37°C in a medium containing 10 μM NFV. Cells were harvested by centrifugation at 1500 ×*g* for 5 min at 4°C and immediately frozen at −80°C until high-performance liquid chromatography (HPLC) analysis. To determine the velocity of NFV efflux from LCL cells, these patients' LCL cells were incubated at 37°C in a medium containing 10 μM NFV for 3 h. The cells were then quickly washed twice with 10 ml ice-cold phosphate-buffered saline and cultured in 10 ml NFV-free medium for up to 3 h. After an interval, aliquot cells were harvested by centrifugation at 1500 ×*g* for 5 min at 4°C and immediately frozen at −80°C until HPLC analysis.

Reverse transcription-PCR (RT-PCR)

For quantification of *MDR1* transcript, RNA from 1×10^7 LCL cells was isolated using Trizol reagents (Invitrogen Corp, Carlsbad, Calif., USA). First strand cDNA was obtained by using ReverTra Ace (Toyobo, Osaka, Japan) with 1 μg of total RNA. cDNA was subjected to PCR. Information on primers and conditions for PCR was obtained as previously described [17]. We used human glyceraldehyde 3-phosphate dehydrogenase mRNA as a positive control.

Determination of intracellular concentration of NFV by HPLC

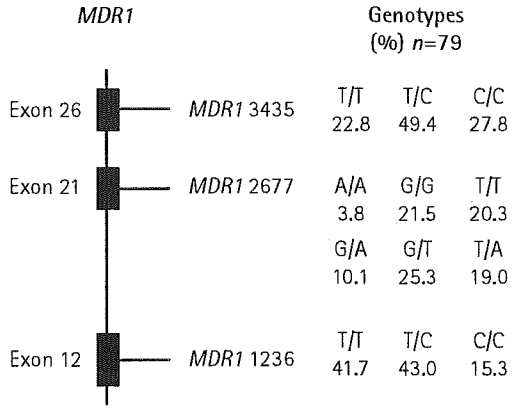
The patients' frozen LCL cells were extracted with 1.5 ml of ethanol. The extracts were then clarified by centrifugation at 2050 ×*g* for 10 min at 4°C. The ethanol extracts were evaporated at 30°C and dissolved in 180 μl of mobile phase, which was a mixture of phosphate buffer (containing 50 mM KH_2PO_4 and 50 mM Na_2HPO_4; pH 5.63) and acetonitrile (1:1, v:v) [18]. The amounts of NFV were measured using a Sensyu Pack ODS C_{18} column (5 μm particle size; 150×4.6 mm, Sensyu Scientific Co, Tokyo, Japan) at a flow rate of 1.5 ml/min by HPLC (Shimadzu Co, Tokyo, Japan). The UV detection wave length was 220 nm and efavirenz (EFV) was used as an internal standard. The lower limits of detection and quantification were 20 ng (30.1 pmole)/10^6 cells, and the calibration range was 20–2000 ng (30.1–3010 pmole/10^6 cells).

Results

We typed the three SNPs at *MDR1* 1236 (exon 12), *MDR1* 2677 (exon 21) and *MDR1* 3435 (exon 26) in DNA samples from 79 HIV-positive Japanese patients

(Figure 1). We found that it was consistent with the Hardy–Weinberg principle (Tables 1 and 2). Furthermore, in all possible two-way comparisons of the three SNPs at *MDR1* 1236 (exon 12), *MDR1* 2677 (exon 21, excluding the genotypes containing G) and *MDR1* 3435 (exon 26), we found significant linkage disequilibrium between *MDR1* 2677 A (T) and *MDR1* 1236 C (T), *MDR1* 2677 A (T) and *MDR1* 3435 C (T), and *MDR1* 1236 C (T) and *MDR1* 3435 C (T), respectively.

Figure 1. Frequency of SNPs in *MDR1*



The SNPs at *MDR1* 1236, *MDR1* 2677 and *MDR1* 3435 were typed by the SNaPshot method. Genotype frequencies at each site are shown as percentage among 79 HIV-infected Japanese patients. The thin vertical line at left represents the *MDR1* gene on human chromosome 7. The closed boxes represent exons 12, 21 and 26.

Table 1. Hardy–Weinberg principle at *MDR1* 1236 (*n*=79)

	T/T	T/C	C/C
Observed number of patients	33	34	12
Expected number of patients	31.7*	36.7†	10.6‡

p: Frequency for the T allele $\frac{33\times2+34}{2\times79}=0.633$

q: Frequency for the C allele 1–p=0.367

*$79\times p^2=31.7$

†$79\times2pq=36.7$

‡$79\times q^2=10.6$

Table 2. Hardy–Weinberg principle at *MDR1* 3435 (*n*=79)

	T/T	T/C	C/C
Observed number of patients	18	39	22
Expected number of patients	17.8*	39.4†	21.8‡

p: Frequency for the T allele $\frac{18\times2+39}{2\times79}=0.475$

q: Frequency for the C allele 1–p=0.525

*$79\times p^2=17.8$

†$79\times2pq=39.4$

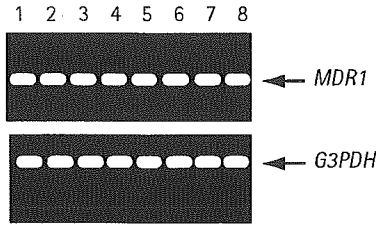
‡$79\times q^2=21.8$

Reportedly, *MDR1* 3435 T/T genotype was associated with lower expression of P-gp in leukocytes [13] so we hypothesized that the genotype was also associated with slower cellular export of NFV in patients' lymphocytes. To investigate this, we first established LCLs by immobilizing selected patients' PBMCs with EBV. We selected eight patients' LCLs with *MDR1* 3435 C/C (*n*=4) and T/T (*n*=4) and verified similar levels of *MDR1* in these LCLs by RT-PCR (Figure 2). We observed little variation in *MDR1* transcripts.

We found that uptake of NFV was rapid into LCLs reaching a steady-state within 5 min (Figure 3). We studied eight patients' LCLs with *MDR1* 3435 T/T and *MDR1* 3435 C/C to compare the steady-state intracellular concentration of NFV after 3 h incubation in a medium containing 10 μM NFV. The intracellular concentrations of NFV in LCLs with *MDR1* 3435 T/T and C/C genotypes were 2593 μM and 2411 μM, respectively (*n*=4), with no statistical difference. We calculated these values by hypothesizing that the LCLs are ideal spheres (10 μm diameter) and that NFV distributes uniformly in the cell.

We then compared NFV efflux from those LCLs with different genotypes at *MDR1* 3435. Before measuring export of NFV, LCLs were cultured with NFV to a saturated level. These NFV-loaded cells were transferred to NFV-free medium and cultured for 3 h with intermittent sampling of cell aliquots. We compared the efflux of NFV from the eight patients' LCLs with *MDR1* 3435 T/T and C/C (*n*=4 each), which had been verified to express *MDR1* mRNA by

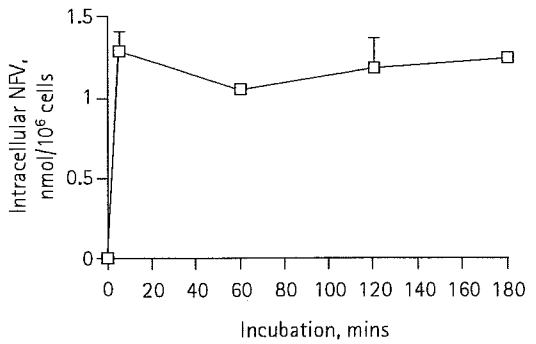
Figure 2. *MDR1* mRNA expression in LCLs



We selected eight patients' LCLs with *MDR1* 3435 C/C (lanes 1–4) and T/T (lanes 5–8) and measured the expression of *MDR1* mRNA. Total cellular RNA from LCLs was subjected to RT-PCR with primer sets for *MDR1* and *G3PDH* transcripts. Aliquots were subjected to agarose gel electrophoresis. The genotypes at *MDR1* 1236, 2677 and 3435: lanes 1 and 2, (T/T, G/G, C/C); lane 3, (T/C, G/A, C/C); lane 4 (C/C G/A C/C); lane 5 (T/T, G/T, T/T); and lanes 6–8 (T/T, T/T, T/T).

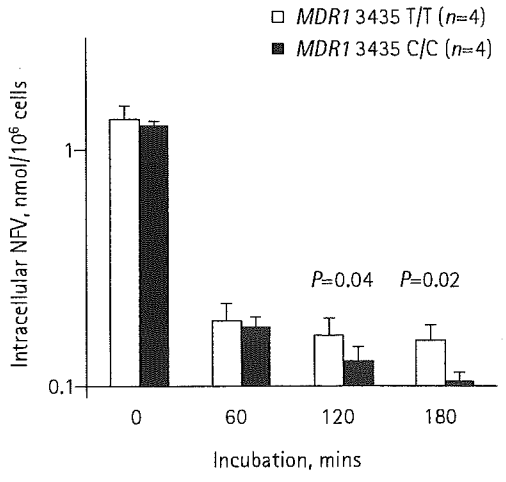
RT-PCR (Figure 2). The concentration of intracellular NFV in LCLs with the homozygous T/T genotype at *MDR1* 3435 was higher than in those with C/C genotype at 120 min and 180 min. This difference was statistically significant (P=0.04 and 0.02, respectively, Mann–Whitney U-test, Figure 4). This meant the NFV efflux in patients' LCL cells with the *MDR1* 3435 T/T genotype was slower than that with C/C genotype. Thus, we suspect the activity of P-gp in patients' LCLs with the *MDR1* 3435 T/T genotype is lower than that with the C/C genotype.

Figure 3. A typical time course of NFV uptake



LCL cells (1×10^6/10 ml) were incubated in medium containing 10 μM of NFV. Cells were harvested at 0, 5, 60, 120 and 180 min and assayed for intracellular NFV by HPLC. The horizontal axis shows the incubation time in min. The vertical axis shows the intracellular amount of NFV per 10^6 cells. The error bars represent the standard deviations.

Figure 4. NFV efflux from patients' LCLs



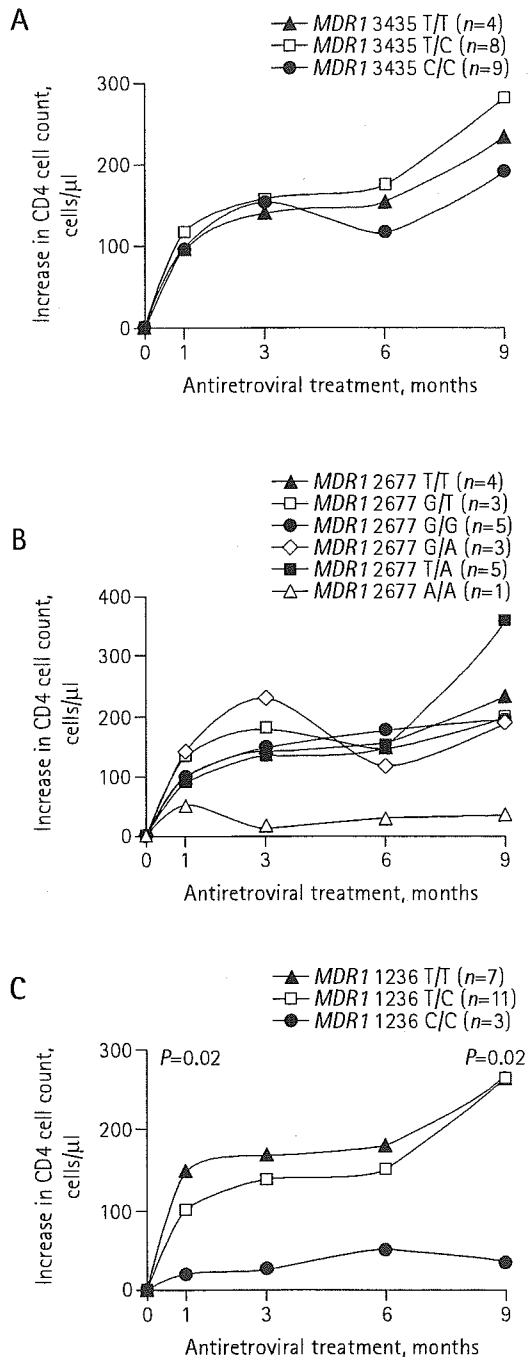
LCL cells were incubated in medium containing 10 μM of NFV for 3 h. Cells were then washed and cultured in NFV-free medium. Intracellular concentration of NFV was determined at 0, 60, 120 and 180 min by HPLC. The horizontal axis shows the incubation time in min. The vertical axis shows the intracellular amount of NFV per 10^6 cells. We selected eight patients (described in the legend to Figure 2) and examined the velocity of NFV efflux from those cells. The intracellular concentration of NFV was measured several times in all patients' LCLs, and data were similar in every test. The error bars represent the standard deviations.

To examine the influence of *MDR1* 3435 genotypes on the response to treatment, we assessed increase in CD4 cell counts and viral suppression in 21 patients after initiation of HAART. At first, we hoped to analyse data obtained from a group of patients receiving NFV alone as a PI, but could not, due to the small number of NFV-receiving patients. Thus, we carried out the analysis in those patients receiving PIs including NFV (n=11), indinavir (n=4) and saquinavir/lopinavir/ritonavir (n=6). CD4 cell counts before treatment were similar among patients with various genotypes. Patients with various genotypes at *MDR1* 3435 showed similar changes in CD4 cell counts (Figure 5A) and viral suppression (Figure 6A) during 9 months of HAART. We found patients with the *MDR1* 1236 T/T showed higher increase in CD4 cell counts at 1 month (148 cells/μl) and 9 months (264 cells/μl) after initiation of therapy than those with *MDR1* 1236 C/C (20 cells/μl and 34 cells/μl, respectively) (Figure 5C). We suspected that *MDR1* 1236 T/T was associated with a higher rate of recovery of CD4 cell counts for patients receiving HAART with PI. We did not find differences in rates of viral suppression among the patients with various *MDR1* 1236 genotypes (Figure 6C). We did not observe a statistical difference in CD4 cell counts or viral loads among patients with different *MDR1* 2677 genotypes (Figures 5B and 6B).

Discussion

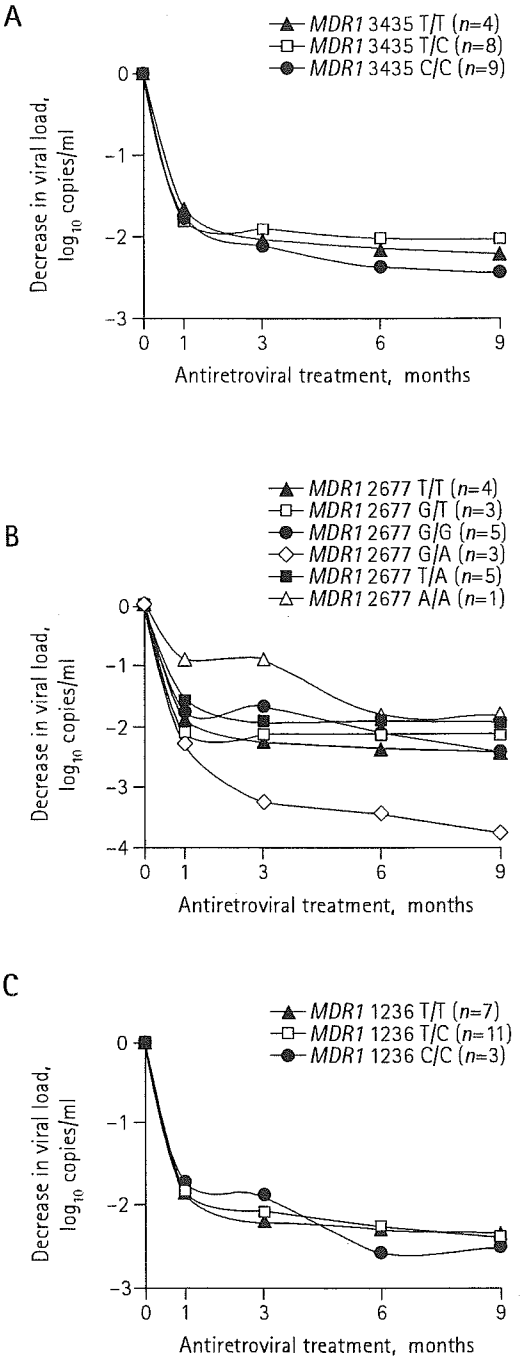
In this study, we genotyped three SNPs at *MDR1* 1236 (exon 12), *MDR1* 2677 (exon 21) and *MDR1* 3435 (exon 26) (Figure 1) in 79 HIV-positive Japanese patients and found incomplete linkage disequilibrium – as has also been reported in other ethnic groups [6]. We found that genotype frequencies of the SNPs at *MDR1* 1236 (exon 12) and *MDR1* 3435 (exon 26) in this population were in Hardy–Weinberg equilibrium. This suggested that the studied population was precisely genotyped and unbiased in terms of the *MDR1* gene. We compared the activity of P-gp among patients' LCLs with different *MDR1* 3435 genotypes by measuring NFV efflux from the cultured LCL cells by HPLC. We found that the intracellular concentration of NFV in LCLs with the homozygous T/T genotype at *MDR1* 3435 was higher than in those with the C/C genotype at 120 min and 180 min. This difference was statistically significant (P=0.04 and 0.02, respectively; Mann–Whitney U-test; Figure 4). In contrast, in the retrospective evaluation of 21 HIV-positive patients

Figure 5. Increase in CD4 cell count among patients with the various genotypes of *MDR1* during antiretroviral treatment



We assessed increase in CD4 cell counts among 21 patients. Every subject had CD4 cell counts and viral loads at months 0, 1, 3, 6 and 9. **(A)** *MDR1* 3435: T/T (▲); C/C (●); T/C (□). (B) *MDR1* 2677: T/T (▲); G/G (●); G/T (□); G/A (◇); T/A (■); A/A (△). (C) *MDR1* 1236: T/T (▲); C/C (●); T/C (□). The vertical axis shows the increase in CD4 cell count during treatment. *P* values were calculated by the Mann-Whitney U-test.

Figure 6. Suppression of viraemia among patients with various genotypes of MDR1 after antiretroviral treatment



We assessed suppression of viraemia among the same 21 patients as described in the legend to Figure 5. **(A)** *MDR1* 3435: T/T (▲); C/C (●); T/C (□). (B) *MDR1* 2677: T/T (▲); G/G (●); G/T (□); G/A (◇); T/A (■); A/A (△). (C) *MDR1* 1236: T/T (▲); C/C (●); T/C (□). The vertical axis shows decrease in viral load. Values are shown as $\log_{10}$ copies/ml plasma.

receiving PIs, we failed to observe a statistical difference in CD4 cell counts and viral suppression among patients with different *MDR1* 3435 SNPs (Figures 5A and 6A). Furthermore, we found that patients with the *MDR1* 1236 T/T genotype showed a greater increase in the CD4 cell counts during HAART therapy with PI at months 1 and 9 than patients with the *MDR1* 1236 C/C genotype (Figure 5C). The contribution of genetic variations in the *MDR1* gene to the patients' clinical characteristics, if any, seems very complicated and thus is difficult to evaluate in a straightforward manner.

As the steady-state intracellular concentration of NFV was about 250 times higher than that in the medium (10 μM), the uptake of NFV seems active rather than passive. However, these *in vitro* data depart from what has been observed in *in vivo* measurements of NFV in patients [19,20] presumably due to the presence of alpha(1)-acid glycoprotein to which NFV binds in plasma [21]. Furthermore, this discrepancy may also be due to the differential distribution of NFV among tissues rather than in free artificial medium. Therefore, our *in vitro* data should be considered as such, that is, *in vivo* lymphocytes may be unlikely to have this high intracellular to extracellular concentration ratio (250:1).

We observed an association of slower efflux of NFV *in vitro* with the T/T genotype at *MDR1* 3435. In fact, P-gp has been found to export PIs from lymphocytes and reduce their anti-HIV activity *in vitro*, and its low activity has been found to be associated with the T/T genotype at *MDR1* 3435 [13]. As the SNP at *MDR1* 3435 is a silent mutation, one possible explanation for this association is that the T/T genotype at *MDR1* 3435 renders *MDR1* mRNA unstable in the cell. Another possible explanation for the association is that *MDR1* 3435 SNP is in linkage disequilibrium with the SNPs at *MDR1* 1236 (exon 12) and *MDR1* 2677 (exon 21), the latter of which is a substitution mutation. This amino acid substitution from the *MDR1* 2677 SNP may be responsible for the observed difference (Figure 4) [11]. Another possible explanation is that *MDR1* 3435 SNPs are in linkage disequilibrium with a polymorphism(s) elsewhere in the genome that modifies *MDR1* expression or function [3,12].

Although an *in vitro* study showed that the velocity of NFV efflux in patients' LCLs with the *MDR1* 3435 T/T genotype was slower than that with the C/C genotype, we failed to observe a statistical difference in CD4 cell counts and viral suppression among patients with different *MDR1* 3435 genotypes (Figures 5A and 6A). Four equally possible accounts seem to explain this discrepancy. Firstly, since the C/C genotype at *MDR1* 3435 is also correlated with higher expression of P-gp in intestinal epithelial cells that adsorb PIs, the *MDR1* 3435 C/C is likely to be associated with higher absorption of PIs and higher PI concentration in plasma [12,22]. The higher plasma levels of NFV in 3435 C/C patients in one study [12] is puzzling and as yet not fully understood. Secondly, the sample size (n=21) in this study may be too small to evaluate CD4 cell counts or viral suppression in a statistical way. Thirdly, since the enrolled patients received different treatment combinations of PIs and reverse transcription inhibitors during antiretroviral therapy, the clinical evaluation was not normalized. Finally, because LCLs – immobilized B cells – but not CD4+ T cells were used in this study, the function of P-gp in a setting of HIV-1 infection may not have been accurately tested. In contrast to the *MDR1* 3435, we observed a marginal but statistically significant association of the *MDR1* 1236 SNP with the CD4 cell count increase although this SNP is a silent mutation. To our knowledge, this clinical association of *MDR1* 1236 with statistical significance is unprecedented, although its clinical significance remains to be investigated. In conclusion, a large-scale and case-controlled study would be required to test whether SNPs of *MDR1* affect the clinical course during antiretroviral therapy with PIs and the prognosis of infected patients.

Acknowledgements

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REGULAR ARTICLE

Phenotype correction of hemophilia A mice with adeno-associated virus vectors carrying the B domain-deleted canine factor VIII gene☆

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KEYWORDS
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Abstract Adeno-associated virus (AAV) vectors carrying the B domain-deleted canine FVIII (BDD cFVIII) gene utilizing the β-actin minimum promoter (167b) pseudotyped with serotype 1 (AAV1-β-actin-cFVIII) and serotype 8 (AAV8-β-actin-cFVIII) were developed to express cFVIII in hemophilia A mice. FVIII clotting activities measured by the APTT method increased in hemophilia A mice with intramuscular injection of AAV1-β-actin-cFVIII in a dose-dependent manner. Therapeutic FVIII levels (2.9±1.0%) in hemophilia A mice with the AAV1-β-actin-cFVIII dose of 1×10^{12} gc/body were achieved, suggesting partial correction of the phenotype with AAV1-β-actin-cFVIII vectors. FVIII clotting activity levels in hemophilia A mice with intravenous injection of AAV8-β-actin-cFVIII also were increased dose-dependently, achieving therapeutic FVIII levels (5–90%) in hemophilia A mice with the AAV8-β-actin-cFVIII doses of $1–3 \times 10^{11}$ gc/body and supernormal FVIII levels (180–670%) in hemophilia A mice with the AAV8-β-actin-cFVIII dose of 1×10^{12} gc/body. Transduction of the liver with AAV8-β-actin-cFVIII is superior to transduction of skeletal

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muscles with AAV1cFVIII regarding the FVIII production and antibody formation. These data suggested that both AAV1 and AAV8 vectors carrying the FVIII gene utilizing a minimum promoter have a potential for hemophilia A gene therapy.

Introduction

Hemophilia A is an inherited X-linked life-threatening bleeding disorder caused by abnormalities in the factor VIII (FVIII) gene that lead to deficiency of FVIII and bleeding diathesis. Hemophilia is considered suitable for gene therapy because it is caused by a single gene abnormality and therapeutic coagulation factor levels may well be in a wide range (5–100%) without strict gene regulation [1–3]. Gene therapy is expected to provide an alternative to current FVIII supplemental therapy because it may be able to prevent lethal intracranial bleeding episodes and provide a good quality of life without bleeding. Among a variety of vectors, Adeno-associated virus (AAV) vectors are thought to be ideal for transfer of therapeutic genes since they are derived from non-pathogenic viruses and have been demonstrated to provide sustained transgene expression in non-dividing cells with little toxicity, although delivery of the FVIII gene using AAV vectors is limited by their small packaging capacity [4].

The dual AAV vector system that utilized two AAV2 vectors separately carrying the FVIII heavy chain gene and the FVIII light chain gene was successful for expressing functional FVIII molecules and correction of phenotypes of hemophilia A mice [5]. A recent report has shown that construction of single AAV vectors carrying the 4.5-kb B domain-deleted (BDD) canine FVIII (cFVIII) gene can be packaged in AAV vectors using the 543-base (b) DNA fragments composed of the insulin-like growth factor binding protein (IGBP) promoter, an enhancer element, and an intron, although the packaging efficiency for incorporation of the cFVIII gene into the AAV vectors was low [6].

The liver would appear to be the appropriate target organ for transduction because FVIII is physiologically synthesized in this organ. However, if any adverse reaction to the therapy occurs, removal of the liver would be an unacceptable solution. In fact, minor liver dysfunction upon AAV2 vector injection into the hepatic artery was reported in clinical trials for hemophilia B gene therapy, whereas no liver dysfunction was observed upon injection of the same vector into the skeletal muscles in the same series of clinical trials [7,8]. Although the precise mechanisms of these phenomena have not yet been elucidated, the T cell response to viral capsid was thought to be one of the causes of the liver injuries [8]. In this respect, surgically removable organs such as skeletal muscles may well be the alternative target organs.

To explore the possibility that skeletal muscles transduced with AAV vectors could produce FVIII resulting in increase of FVIII levels in the circulation, we packaged the BDD FVIII gene in AAV vectors using the same promoter and compare production of FVIII in the skeletal muscles and in the liver that transduced with AAV vectors. We developed AAV vectors carrying the BDD cFVIII gene utilizing the β-actin minimum promoter (167b) and tried to express canine FVIII in hemophilia A mice. Recent studies on recombinant AAV vectors have shown that AAV serotypes have tropism, suggesting that a specific AAV serotype vector can be used for gene delivery to certain organs [3,6,9]. AAV serotype 1 (AAV1) may be the best AAV serotype for transduction of skeletal muscles and the AAV serotype 8 (AAV8) is superior to other AAV serotypes for transduction of the liver [6,9], thus in this study, we constructed AAV1 and AAV8 vectors carrying the BDD cFVIII gene and studied efficacies of these vectors for FVIII transgene expression, long term transgene expression, and neutralizing antibody formation to the transgene products in the hemophilia A mice.

Materials and methods

Vector construction

The full-length human FVIII (hFVIII) cDNA was a generous gift from Dr. J.A. van Mourik (Blood Coagulation, Sanquin, Amsterdam, Netherlands) and the human B domain deleted (BDD) FVIII (hFVIII) cDNA was generated by PCR-based mutagenesis as described [10,11]. The canine FVIII (cFVIII) cDNA was a generous gift from Dr. Brownlee (Chemical Pathology Unit, University of Oxford, UK) and the BDD canFVIII cDNA also was generated by PCR-based mutagenesis. The intervening amino acid sequence of the heavy chain and the light chain of BDD cFVIII was $RSFS^{743}$-Q^{1630}NPPVSK. The CAG promoter is a chimeric promoter, composed of the CMV enhancer, the chicken β-actin promoter,

and an intron, was derived from pCAGGS [12]. The chicken β-actin minimum promoter (−155~+12, 167 b) was generated by PCR, cloned in pCR2.1 TOPO (Invitrogen), and sequenced. Plasmid vector p1.1c, composed of the CMV promoter, human growth hormone gene intron 1, and the SV40 polyadenylation signal sequences, was kindly supplied by Avigen Inc. The DNA fragments spanning the CMV promoter and the human growth hormone intron of p1.1c were replaced with the CAG promoter, the phosphoglycerokinase 1 (PGK1) promoter, or the β-actin minimum promoter DNA fragments to make plasmid p1.1CAG, p1.1PGK1, or p1.1β-actin, respectively. The DNA fragments encoding the BDD hFVIII cDNA or the BDD cFVIII cDNA were cloned in the downstream of the respective promoter sequences of these plasmids to make p1.1CMV-hFVIII, p1.1CAG-hFVIII, p1.1PGK1-hFVIII, p1.1β-actin-hFVIII, and p1.1β-actin-cFVIII, respectively. The Lac Z gene was cloned in the downstream of the β-actin promoter to make plasmid p1.1β-actin Lac Z. DNA fragments spanning the promoter, the LacZ gene, and the poly adenylation signal sequence of pAAV2 Lac Z (Stratagene) were replaced with DNA fragments spanning the β-actin promoter, the BDD cFVIII cDNA, and the SV40 poly adenylation signal sequences of p1.1β-actin-cFVIII to make the gene transfer vector pAAV2-β-actin-cFVIII in which these DNA fragments were flanked by ITR sequences of AAV serotype 2 (AAV2) as described previously [9,13]. The gene transfer vector pAAV2-β-actin-Lac Z equipped with AAV2 ITRs was also constructed. 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, Philadelphia, PA) [6]. The packaging plasmid composed of the AAV2 rep gene and the cap gene derived from AAV1 for AAV1 capsid pseudotyping was described previously [9].

AAV vector production

Viral vectors were packaged with AAV1 or AAV8 capsid by pseudotyping. The FVIII gene or the Lac Z gene located in the downstream of the β-actin minimum promoter and flanked by AAV2 ITRs was packaged by triple plasmid transfection of human embryonic kidney 293 (HEK 293) cells, kindly supplied by Avigen Inc., with the chimeric packaging plasmid, the adenovirus helper plasmid pHelper (Strategene, La Jolla, CA), and gene transfer plasmid vectors (pAAV2-β-actin-cFVIII or pAAV2-β-actin-Lac Z) as described previously [9,13]. For virus vector purification, the DNAse (Benzonase, Merck Japan, Tokyo, Japan)-treated virus particle containing samples were subjected to two rounds of iodixyanol-density gradient ultracentrifugation in HEPES-buffered saline (pH 7.4) in the presence of 25 mM EDTA at 21 C as described [9]. Titration of recombinant AAV vectors was carried out by quantitative dot-blot hybridization using the ^{32}P-labeled probes [9,13].

Analysis of the ß-actin minimum promoter activity

Expression of hFVIII in HEK293 cells transfected with plasmid vectors p1.1β-actin-hFVIII, p1.1CMV-hFVIII, p1.1CAG-hFVIII, and p1.1PGK1-hFVIII by the calcium phosphate coprecipitation method was studied to show that the β-actin minimum promoter had a enough FVIII expression activity. After incubation with the DNA containing media for 6 h, HEK 293 cells were incubated further in DMEM/HAM F-12 media supplemented with 10% fetal bovine serum for 48 h at 37 C in the presence of 5% CO_2. FVIII clotting activities in the conditioned media of HEK 293 cells harvested after 48 h incubation were quantified by the activated partial thromboplastin time (APTT) method using FVIII deficient plasma. FVIII activities were expressed as the percentages of normal control plasma.

Animal experiments

FVIII-deficient mice (Hemophilia A mice) with targeted destruction of exon 16 of the FVIIII gene were previously reported by Bi et al. [14] and generously given to us by Dr. H. H. Kazazian Jr. (University of Pennsylvania, Philadelphia, PA). J1 ES cells were used for targeted destruction of the FVIII gene and blastcysts derived from C57BL/6 mice were used to generate chimaeras [14]. C57BL/6 wild-type mice were purchased from SLC Inc. Mice were maintained in a standard lighting condition in a clean room. All surgical procedures were carried out in accordance with guidelines approved by the institutional Animal Care and Concern Committee at Jichi Medical School [15]. Male hemophilia A mice and male wild-type C57BL/6 mice were used in the experiments. Blood was drawn from the cervical vein plexus of mice and mixed with 1 / 10 volume of 3.8% sodium citrate, and then platelet-poor plasma was prepared by centrifugation. AAV8 vectors were injected intravenously into the cervical vein plexus while AAV1 vectors were injected directly to the skeletal muscles of lower extremities of mice under anesthesia with isoflurene [15]. Cyclophosphamide (100 μg/body/day, SIGMA-ALDRICH Japan, Tokyo, Japan) and tachrolimus (12.5 μg/body/day, Fujisawa Pharmaceuticals Co., Tokyo, Japan) were

given (s.c.) to mice daily after vector injection as the immunosuppressant.

Analysis of cFVIII expression in mice

FVIII activities were measured by the activated partial thromboplastin time (APTT) method used for determination of plasma FVIII activities of hemophilia patients utilizing human FVIII-deficient plasma as described [15]. Antigen levels of cFVIII in mouse plasma were determined by ELISA (Asserachrom FVIIIC: Ag, Diagnostica Stago, Parsipanny, NJ) as described [6]. Analyses of neutralizing antibodies against cFVIII developed in mice were performed by the Bethesda method as described using FVIII deficient plasma and normal canine plasma. Detection of the transcripts of cFVIII transgene was performed by RT-PCR [10,11]. RNA was isolated from the mouse organs using an RNA isolation kit (RNeasy Protect kit; Qiagen Inc., Valencia, CA). DNAse I (Amplification grade, Invitrogen, Carlsbad, CA)-treated and heat-treated RNA samples were subjected to RT-PCR using a pair of primers (sense: 5′-GTTGGAGCACAAACTGACTTCC-3′, antisense: 5′-CAATTGAGAAGGTGTCATCATACTC-3′) for cFVIII and an RT-PCR kit (SuperScript One-Step RT-PCR System, Invitrogen). PCR amplification (25–30 cycles) for cFVIII was performed as described [10,11]. A primer pair for mouse GAPDH mRNA (R&D Systems, Inc., Minneapolis, MN) was used instead of cFVIII primers in the control RT-PCR experiments. For detection of cFVIII molecules in mouse tissues by immunohistochemistry, the skeletal muscles and the liver were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h at 4 °C, incubated with PBS containing sucrose (10–30%), and then frozen in the presence of OCT compound in dry ice/ethanol. Sections were prepared from frozen tissues at −25 °C and attached to poly-lysine coated glass slides. For detection of FVIII, tissue sections were blocked with 1% rabbit serum in PBS containing Triton-X 100 (0.1%) and incubated with sheep polyclonal anti-human FVIII antibodies (Cedarlane Laboratories Ltd, Homby, Ontario, Canada) at 4 °C for 16 h [10,11]. After washing in PBS, sections were incubated with biotin-conjugated rabbit anti-sheep IgG antibody followed by the ABC reagents (Vectastain ABC Elite kit; Vector, Burlingame, CA) and a DAB kit (Vector).

Detection of ß-galactosidase in mouse tissue

To analyze Lac Z gene expression in mice injected with AAV-β-actin-Lac Z, mouse tissues were fixed with 2% paraformaldehyde in PBS for 5 min, washed with PBS, incubated with PBS containing sucrose (10–30%), and frozen with OTC compound (Tissue-Tek; Miles, Inc., Elkhart, IN) in dry ice/ethanol. Sections, prepared from frozen tissues at −25 °C and attached to poly-lysine coated glass slides, were 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 [10]. Some sections further were processed for the Feulgen reaction (red purple) to visualize nuclei.

Results

Expression of FVIII by the ß-actin minimum promoter

We studied expression of hFVIII in HEK293 cells transfected with plasmid vectors p1.1β-actin-hFVIII, p1.1CMV-hFVIII, p1.1CAG-hFVIII, and p1.1PGK1-hFVIII. FVIII clotting activities detected in the conditioned medium of HEK293 cells are shown in Fig. 1. Expression of FVIII driven by the β-actin minimum promoter (167 b) in the 293 cells was approximately 1/3–1/2 of that by the CMV promoter (1 kb) or the CAG promoter (1.7 kb). Although the β-actin minimum promoter is weaker than the CMV promoter and the CAG promoter, it

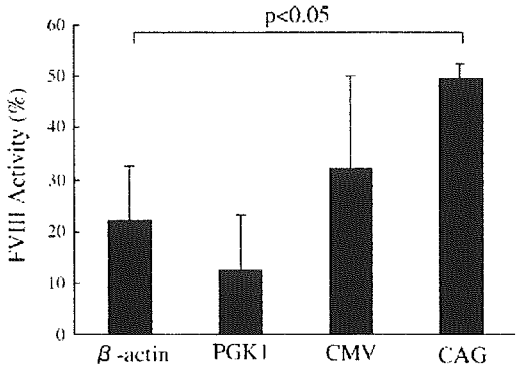

Figure 1 FVIII expression by the β-actin minimum promoter in vitro. FVIII clotting activities expressed in the conditioned media of HEK 293 cells transfected with p1.1CMV-FVIII, p1.1 CAG-FVIII, p1.1 PGK1-FVIII, or p1.1 β-actin-FVIII after 48 h incubation are shown. FVIII clotting activities in the conditioned media were quantified by the APTT method using FVIII deficient plasma and FVIII clotting activities were expressed as the percentages of normal control plasma. There were no FVIII activities in the conditioned media of HEK 293 cells with mock transfection. There was a significant difference of FVIII activity levels in the conditioned media of HEK 293 cells transfected with p1.1 CAG-FVIII and those in the conditioned media of HEK 293 cells transfected with p1.1 β-actin-FVIII ($n=4$, Student's t-test, $p<0.05$).

was stronger than the PGK1 promoter (515 b) by 1.2-fold in terms of FVIII expression activity. Since the β-actin minimum promoter was stronger than the PGK1 promoter and was short enough to construct 5.1-kb AAV vectors carrying the BDD FVIII cDNA, we used the β-actin minimum promoter to produce AAV vectors carrying the BDD FVIII gene.

Expression of Lac Z gene by the ß-actin minimum promoter in vivo

To confirm that the β-actin minimum promoter can express a transgene in vivo, AAV vectors carrying the Lac Z gene located in the downstream of the β-actin minimum promoter (AAV1-β-actin-Lac Z, AAV8-β-actin-LacZ) were injected to wild-type mice and expression of the Lac Z gene was studied by X-gal staining. When AAV1-β-actin-Lac Z was injected to the skeletal muscles of lower extremities of wild-type mice, Lac Z gene expression was observed in muscle fibers as shown in Fig. 2A. No apparent Lac Z gene expression was observed in other organs in the AAV1-β-actin-Lac Z injected mice (not shown), suggesting that transgene expression in other organs was minimum. Lac Z gene expression of mice with intravenous injection of AAV8-β-actin-Lac Z mainly was observed in the liver as shown in Fig. 2B. Lac Z gene expression also was observed in other organs including the heart, lung, and skeletal muscles in accordance with the previous report [16]. The liver could be transduced with intravenously injected AAV8-β-actin-Lac Z almost as efficiently as intraportally injected vectors (not shown).

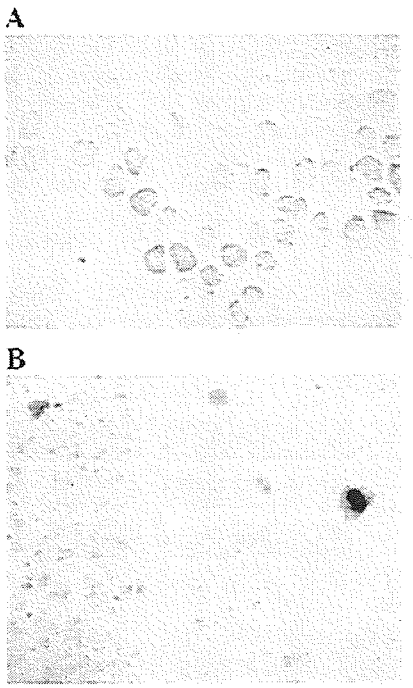

Figure 2 Expression of the Lac Z gene in mice transduced with AAV vectors carrying the Lac Z gene located downstream of the β-actin minimum promoter. X-gal staining of the skeletal muscles of mice with intramuscular injection of AAV1-β-actin-Lac Z (A) and of the liver of mice with intravenous injection of AAV8-β-actin-Lac Z (B) is shown.

Expression of FVIII by AAV vectors carrying the BDD cFVIII gene

AAV1-β-actin-FVIII vectors were injected into skeletal muscles of hemophilia A mice and AAV8-β-actin-FVIII vectors were intravenously injected into the cervical vein plexus of hemophilia A mice. FVIII clotting activities of citrated plasma drawn from mice were measured by the APTT method using FVIII-deficient human plasma.

FVIII clotting activities in mouse plasma increased on days 14 and 28 after AAV1 vector injection. The increase of FVIII clotting activities on day 28 after injection was dose-dependent. The FVIII activity levels in peripheral blood increased to $2.9 \pm 1.0\%$ in hemophilia A mice with the AAV1-β-cFVIII dose of 1×10^{12} gc/body (Fig. 3), suggesting partial correction of the phenotype with AAV1-β-cFVIII vectors. After these periods, FVIII activities decreased to the basal levels of mice before vector injection. FVIII antigen levels increased in parallel with levels of FVIII activity, confirming expression of cFVIII transgene in mice (not shown). Analyses for antibody against transgene products showed that neutralizing antibodies developed in 4 out of 6 tested mice by week 12 after vector injection, although the antibody titers were not high (Table 1). The RT-PCR analysis and the immunohistochemistry study suggested the presence of the transgene transcripts and products in the vector-injected muscles, suggesting that decrease of FVIII levels may be accounted for by the presence of neutralizing antibody to cFVIII.

FVIII clotting activity levels in hemophilia A mice with intravenous injection of AAV8-β-actin-cFVIII also were increased dose-dependently on day 28, achieving therapeutic FVIII levels (5–90%) in hemophilia A mice with the AAV8-β-actin-cFVIII doses of $1-3 \times 10^{11}$ gc/body and supernormal FVIII levels (180-670%) were achieved in hemophilia A mice with the AAV8-β-actin-cFVIII dose of 1×10^{12} gc/body (Fig. 4). These data on AAV8 vector-transduced FVIII expression were almost comparable with the results of the previous study using the single AAV8 vector carrying the BDD cFVIII gene [6], suggesting that β-actin minimum promoter almost

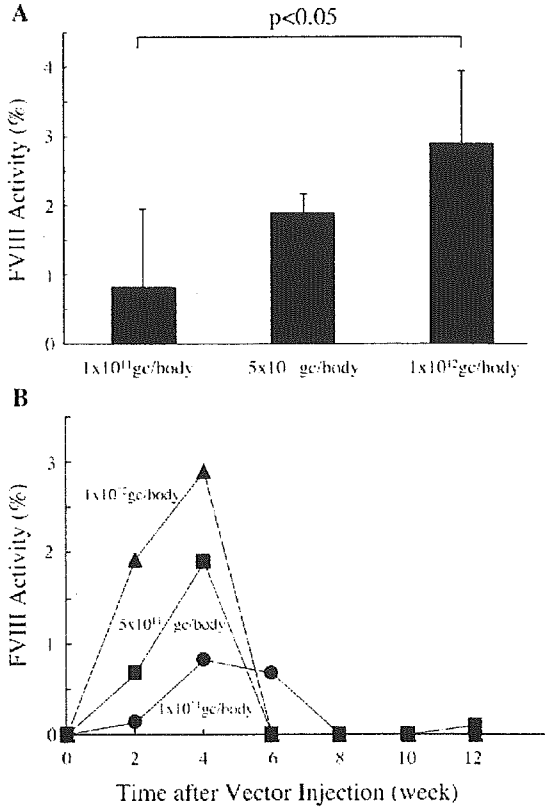

Figure 3 FVIII levels in plasma of hemophilia A mice after intramuscular injection of AAV1-β-actin-cFVIII. FVIII clotting activity levels expressed in plasma of hemophilia A mice ($n=4$) on day 28 after intramuscular injection of AAV1-β-actin-cFVIII are shown in panel A. Activity levels of cFVIII in peripheral blood of hemophilia A mice with injection of AAV1-β-actin-cFVIII (circles, 1×10^{11} gc/body: squares, 5×10^{11} gc/body: triangles, 1×10^{12} gc/body) are shown in panel B.

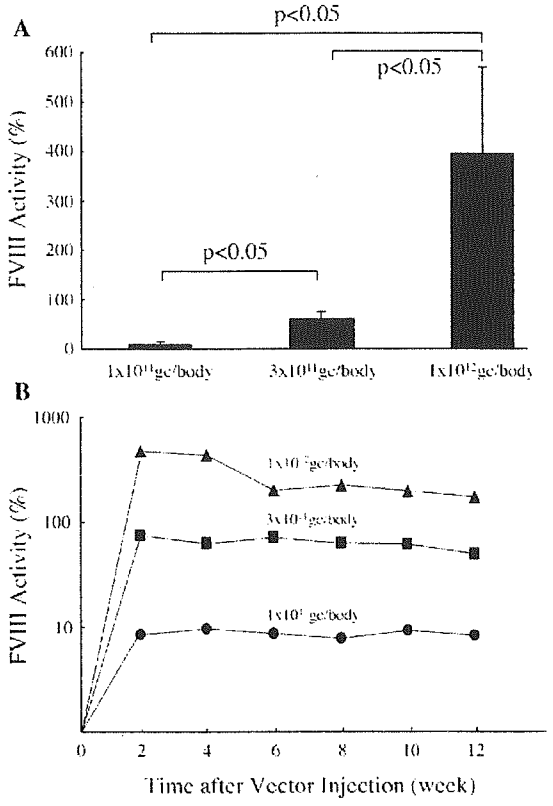

Figure 4 FVIII levels in plasma of hemophilia A mice after intravenous injection of AAV8-β-actin-cFVIII. FVIII clotting activity levels expressed in plasma of hemophilia A mice ($n=4$, each group) on day 28 after intravenous injection of AAV8-β-actin-cFVIII are shown in panel A. Activity levels of cFVIII in peripheral blood of hemophilia A mice ($n=4$, each group) with injection of AAV8-β-actin-cFVIII (circles, 1×10^{11} gc/body; squares, 3×10^{11} gc/body; triangles, 1×10^{12} gc/body) are shown in panel B.

worked as efficiently as the chimeric IGBP promoter complexes. High-level expression of FVIII in the vector-injected hemophilia A mice was sustained for more than 12 weeks. No apparent neutralizing antibody developed during the 12-week period after vector injection (Table 1). FVIII antigen levels also increased in parallel with FVIII activity levels, confirming expression of the cFVIII transgene in mice (not shown). The antigen levels of cFVIII determined by the ELISA for human FVIII were approximately 1/5 of the FVIII activity levels determined by the APTT method. Analyses for cFVIII transcripts suggested that the cFVIII gene mainly was expressed in the liver (Fig. 5) together

Table 1 Neutralizing antibodies against cFVIII developed in hemophilia A mice

	Inhibitor positive mouse	Bethesda units/mL
AAV1cFVIII	4/6 (66.7%)	9.4 ±9.5[a]
AAV8cFVIII	0/9 (0%)	Not detected

[a] Neutralizing antibodies detected by week 12 after vector injection.

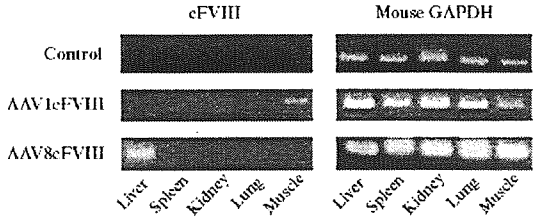

Figure 5 Analysis for cFVIII transcripts in mice. The RT-PCR analyses for the transcripts derived from the cFVIII gene (cFVIII) of RNA isolated from hemophilia A mouse organs without (control) or with intramuscular injection of AAV1-β-actin-cFVIII vectors (AAV1cFVIII) or intravenous injection of AAV8-β-actin-cFVIII vectors (AAV8cFVIII) are shown. For the control, the RT-PCR analysis for mouse GAPDH (Mouse GAPDH) of RNA isolated from hemophilia A mice with or without injection of AAV-β-actin-cFVIII vectors was performed simultaneously.

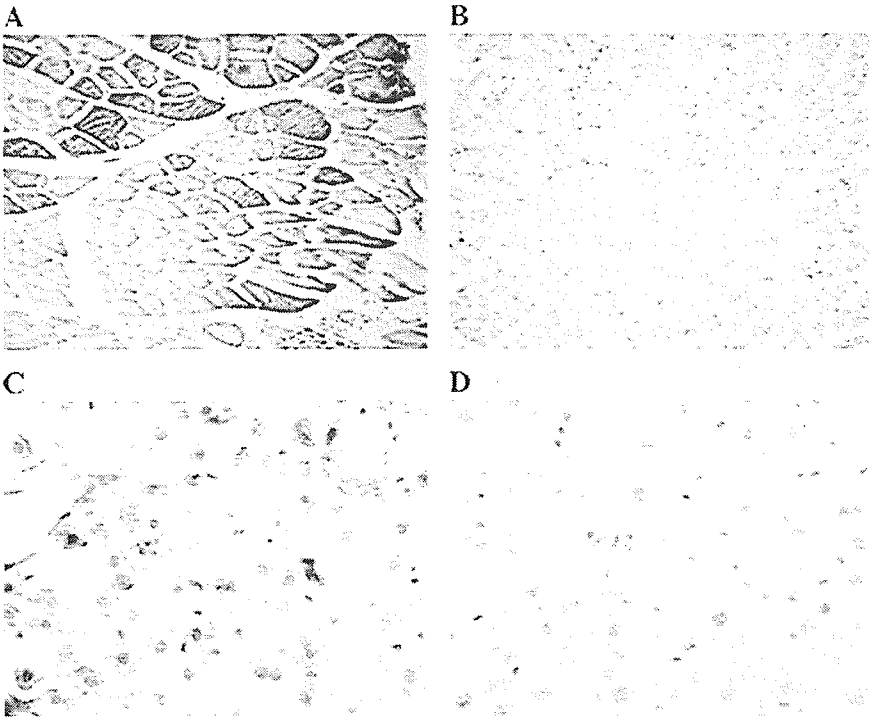

Figure 6 Immunohistochemical Analysis for cFVIII transgene products. Immunohistochemistry for cFVIII of the skeletal muscles of hemophilia A mice with intramuscular injection of AAV1-β-actin-cFVIII vectors (A) and the liver of hemophilia A mice with intravenous injection of AAV8-β-actin-cFVIII vectors (C) is shown (positive stain: brown). For the control, sections of the skeletal muscles (B) and the liver (D) obtained from hemophilia A mice without vector injection were processed simultaneously with anti-FVIII antibodies.

with the traceable expression in the heart, lung, and spleen (not shown). In accordance with the data on cFVIII transcripts, cFVIII molecules were immunohistochemically detected in the skeletal muscles of AAV1-β-actin-cFVIII injected mice and in the liver of mice with intravenous injection of AAV8-β-actin-cFVIII (Fig. 6).

Discussion

Because of the size and nature of the FVIII gene (cDNA), there were difficulties in hemophilia A gene therapy compared with gene therapy for hemophilia B. These difficulties were solved by efforts of many investigators that allowed use of a modified FVIII gene such as BDD FVIII cDNA, improved vector systems, and new strategies. Based upon these studies, a few clinical trials of hemophilia A gene therapy were conducted [17–19]. Increase of FVIII activities in the circulation and clinical improvements were observed in patients who received vector injection or transplantation of genetically modified cells. However, long-term expression of FVIII from the transgenes was not achieved in these studies. Thus, reexamination of the vector systems, the target organs for transduction, and the promoters may be required.

The recombinant AAV vectors are thought to be one of the better vectors in terms of its capability to transduce non-dividing cells and long-term transgene expression, although delivery of the FVIII gene using AAV vectors were limited by its small packaging capacity [4]. The dual AAV vector system utilizing separate AAV2 vectors independently carrying the FVIII heavy chain gene and the FVIII light chain gene could express functionally active FVIII [5]. However, there was an imbalance in the expression levels of the FVIII heavy chain and FVIII light chain, suggesting that over-expressed free FVIII light chain molecules might be more immunogenic than the native molecules. The BDD FVIII gene could be packaged in AAV2 or AAV8 vectors in the previous studies and these vectors could efficiently transduce the liver with intraportal injection of the vectors [6]. Transduction of the liver with peripheral vein injection of AAV8 vectors was as efficient as portal vein injection of vectors, although that of AAV2 vectors was not [6].

The liver would appear to be the appropriate target organ for transduction because FVIII is physiologically synthesized in this organ, so FVIII

synthesis in hepatocytes and its subsequent secretion into the circulation may be warranted. However, if any adverse reaction to the therapy occurs, removal of the liver would be an unacceptable solution. In fact minor liver dysfunction upon AAV2 vector injection into the hepatic artery was reported in clinical trials for hemophilia B gene therapy. In this respect, surgically removable organs such as skeletal muscles may well be the alternative target organs. AAV1 vector-based transduction of the skeletal muscles has beneficial characteristics of removing the transgenes. This is the first report of sufficient expression of FVIII in the skeletal muscles transduced with AAV vectors and suggests that skeletal muscle-directed FVIII expression has a potential for hemophilia A gene therapy.

Compared with synthesis and secretion of FVIII into the circulation from the liver, transport of sufficient FVIII into the circulation from the skeletal muscle fibers is not assured. Based upon our data, it is apparent that transduction of the liver with AAV8-β-actin-cFVIII is superior to transduction of skeletal muscles with AAV1-β-actin-cFVIII regarding FVIII production. The difference between FVIII levels in the peripheral blood of these vector-injected mice may be due to how the transduced cells secrete FVIII molecules into the circulation. Hepatocytes actively secrete a variety of molecules including FVIII into the circulation. Since recombinant cFVIII is in a BDD form, its expression in and secretion from hepatocytes is expected to be better than native FVIII [20], accounting for the high cFVIII expression in mice with intravenous injection of AAV8 vectors carrying the cFVIII gene though cFVIII expressing hepatocytes were not abundant. Although muscle fibers are surrounded by capillaries, transport of recombinant FVIII molecules from muscle fibers to capillaries would not be as efficient as that from hepatocytes.

In terms of the immune reaction to transgene products, muscle stem cells have been shown to function as antigen-presenting cells, suggesting that expression of the transgene by the ubiquitous promoter in the skeletal muscles might lead to development of antibodies against the transgene products if there is no immune tolerance to the transgene products [21]. This was confirmed by Wang et al. [22]. Neutralizing antibody formation was observed in 66.7% of mice with AAV1cFVIII injection even with administration of immunosuppressant, while it was not observed in mice with AAV8-β-actin cFVIII injection by week 12 after vector injection, supporting the potential advantage of AAV8 vector-based transduction of the liver over the muscle-directed transduction by AAV1 vectors.

Each vector system has advantages and disadvantages in these respects. We may need to confirm the results obtained in hemophilia mice using dogs and non-human primates that genetically are more close to humans because there may be differences in transduction efficiency of various serotypes between mice and humans [23]. Taken together, we may need to perform a comparative study using another animal models such as hemophilic dogs and non-human primates that are more genetically close to humans than mice to address these questions. Additionally, use of tissue-specific promoters to minimize neutralizing antibody formation may be a better strategy for expressing transgenes in a tissue- and organ-specific manner. These experiments will be performed in future studies.

In conclusion, our data suggested that both AAV1 and AAV8 vectors carrying the FVIII gene utilizing a minimum promoter have the potential for hemophilia A gene therapy. Our present studies have provided important insight about selecting the appropriate target for delivery of the therapeutic genes and the vector system for the hemophilia A gene therapy.

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