Multiyear therapeutic benefit of AAV serotypes 2, 6, and 8 delivering factor VIII to hemophilia A mice and dogs

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Introduction

Hemophilia A, a congenital deficiency or dysfunction of factor VIII (FVIII), is the most common severe inherited bleeding disorder in humans. Severe hemophilia A patients have less than 1% of normal FVIII activity, and suffer from spontaneous or traumatic joint and muscle hemorrhage, leading to a chronic painful and disabling arthropathy. Bleeding into body cavities or the brain can result in significant morbidity and mortality if not treated aggressively. Current treatment in the developed world, FVIII protein replacement, has established that restoring circulating FVIII levels above 1% of normal prevents most spontaneous bleeding, and levels above 5% are sufficient to improve the disease from a severe to a mild form. However, the limited worldwide supplies of both plasma-derived and recombinant FVIII, its short half-life in vivo (≈12 hours), and the high cost of treatment (> $150,000 per year) make gene therapy an attractive alternative to better manage and cure the disease.

Previously, we have shown that gene therapy with an AAV2 vector encoding a B-domain–deleted FVIII (cFVIII) cDNA under the control of a liver-specific promoter resulted in an average of 2% to 3% of normal canine FVIII activity in 2 hemophilia A dogs, providing preliminary support for the feasibility of this approach in humans. In order to further improve the efficacy of liver-targeted AAV-cFVIII, we explored the possibility of using alternative serotypes of AAV. We also assessed the duration of therapeutic benefit following a single injection of AAV-cFVIII in hemophilia A dogs. Since the isolation of AAV2, many different AAV serotypes have been isolated from human and nonhuman primate tissues. In comparison with the prototypic AAV2, AAV vectors pseudotyped with other serotypes show superior transduction efficiency in various tissues: AAV1 in muscle, pancreatic islets, heart, vascular endothelium, brain and central nervous system (CNS), and liver; AAV3 in Cochlear inner hair cells; AAV4 in brain; AAV5 in brain and CNS, lung, eye, arthritic joints, and heart; AAV6 in muscle, heart, and liver; AAV7 in muscle; and AAV8 in muscle, pancreas, heart, and liver. The tissue tropism of different AAV serotypes may permit targeting of AAV vectors to human disease. However, as most of these tissue-specific tropisms have been reported in the rodent, it is important to evaluate cross-species fidelity of differential targeting among serotypes in larger animal models.

In this report, we have compared the efficacy, gene transfer efficiency, and biodistribution of AAV-cFVIII vectors of serotypes 2, 5, 6, and 8 delivered by portal-vein injection in hemophilia A mice. Furthermore, since prior studies have demonstrated that the hemophilia dog model, compared with the mouse model, more accurately predicts the therapeutic outcomes in humans and other primates, we have determined the long-term efficacy and safety of AAV2-cFVIII, AAV6-cFVIII, and AAV8-cFVIII vectors in hemophilia A dogs, to assess whether dogs recapitulate the findings for different vector serotypes observed in mice. The potential application of alternative serotype AAV-FVIII vectors in humans...
not only may enhance the therapeutic efficacy, but also may diminish some neutralization by pre-existing anti-AAV2 antibodies that are prevalent in the human population.

**Materials and methods**

**Vectors**

The AAV B-domain–deleted canine FVIII vector construct was described previously. Briefly, the vector genome contains a minimal transferrin receptor promoter (202 nt), a synthetic intron (106 nt), the BDD-cFVIII cDNA (4367 nt), and a synthetic polyA sequence (46 nt), flanked by AAV2 inverted terminal repeats (ITRs, 145 nt). The vector genome with AAV2 ITRs was packaged into capsids from AAV2, and pseudotyped into AAV5, AAV6, and AAV8 by triple transfection in 293 cells. Vectors were purified by CsCl-gradient centrifugation, as described. Vector genomes were titrated by quantitative real-time polymerase chain reaction (PCR) using primers and probes specific to canine FVIII light-chain sequences, and a linearized plasmid DNA standard.

**Animal procedures**

Hemophilia A mice were obtained from H. Kazazian. AAV-cFVIII vectors were diluted in PBS to a final volume of 200 μL and injected into the portal vein of mice. Citrated mouse plasmas were isolated from 200 μL blood collected via the retro-orbital plexus at multiple time points. Upon termination of the study, mice were humanely killed and perfused with PBS, and portions of liver, spleen, kidney, lung, heart, and gonadal tissue were harvested and snap-frozen in liquid nitrogen for molecular analyses; additional liver samples were embedded in OCT for fluorescence in situ hybridization. Hemophilia A dogs were bred at Queens University and injected with AAV-cFVIII vectors following the procedure previously described. Briefly, prior to surgery, dogs were treated intravenously with 25 mg diphenhydramine hydrochloride and 100 mg hydrocortisone. The dogs were then infused with canine cryoprecipitate to raise their plasma FVIII levels to more than 50% of normal levels. A midline incision was made in the abdomen of the animal, and the AA V-cFVIII vector was administered via the portal vein using a 23-gauge butterfly catheter at a rate of 0.24 to 0.6 mL/kg per minute. Postoperatively, the dogs were treated with cryoprecipitate at 7 and 24 hours. For the liver biopsies, a similar presurgical schedule was followed. After a thoracic-to-abdomen incision was made to expose the liver, six 6-mm liver sections were removed from multiple sites and then further divided into two 3-mm sections. One section from each site was preserved in OCT and snap-frozen on dry ice, and the second was snap-frozen in liquid nitrogen. All animal procedures were reviewed by the institutional Animal Care Committees and were in accordance with the “Guide for the Care and Use of Laboratory Animals” and the Canadian Council for Animal Care.

**Coatest assay**

Canine FVIII activity in mouse and canine plasma was measured by the Coatest VIII:C assay from Chromogenix (DiPharma, West Chester, OH), following the manufacturer’s instructions, using BDD human FVIII (ReFacto; Genetics Institute, Cambridge, MA) serially diluted from 50 to 1.56 mU/mL in hemophilia A murine or canine plasma to generate a standard curve.

**Bethesda assay**

Neutralizing antibodies to canine FVIII in mouse and canine plasma were measured by the Bethesda assay, as described previously. The standard curve for residual canine FVIII activity was generated with serially diluted normal canine plasma in hemophilia A murine or canine plasma.

**Southern blotting analysis**

Liver genomic DNAs harvested from mice were digested with BamHI and BglII, and hybridized with 32P-labeled probes representing sequences of a 974-bp BamHI-BamHI and a 760-bp BglII-BglII fragment in the canine FVIII light- and heavy-chain coding region, respectively. The standards were digested in human plasma and quantified against a standard generated with linearized plasmid pAAV-cFVIII DNA digested with BamHI and BglII. Hybridization for canine liver genomic DNAs digested with BamHI was described previously. The standards were digested in mouse plasma and quantified against a standard generated with linearized plasmid pAAV-cFVIII DNA digested with BamHI.

**Fluorescence in situ hybridization (FISH)**

The steady-state levels of FVIII in mouse plasma achieved by AA V5 delivery were 83% of normal levels. A midline incision was made in the abdomen of the animal, and the AA V-cFVIII vector was administered via the portal vein using a 23-gauge butterfly catheter at a rate of 0.05 to 0.2 mL/kg per minute. Postoperatively, the dogs were treated with cryoprecipitate at 3 and 24 hours. For the liver biopsies, a similar presurgical schedule was followed. After a thoracic-to-abdomen incision was made to expose the liver, six 6-mm liver sections were removed from multiple sites and then further divided into two 3-mm sections. One section from each site was preserved in OCT and snap-frozen on dry ice, and the second was snap-frozen in liquid nitrogen. All animal procedures were reviewed by the institutional Animal Care Committees and were in accordance with the “Guide for the Care and Use of Laboratory Animals” and the Canadian Council for Animal Care.

**Results**

**Enhanced efficacy of AAV6 and AAV8 serotypes compared with AAV2 and AAV5 in hemophilia A mice**

First, we compared the efficacy of AAV5-cFVIII versus AAV2-cFVIII vectors at doses of 3 × 1011 and 6 × 1011 vg/mouse delivered by portal-vein injection in C57BL/6 hemophilia A mice. The steady-state levels of FVIII in mouse plasma achieved by AAV5 delivery were 83% ± 14 and 302 ± 58 mU/mL at the low and high dose (n = 4/dose group), respectively, over 12 weeks (Figure 1A). In comparison, AAV2-cFVIII, at the same doses, achieved steady-state FVIII levels of 1235 ± 76 and 2337 ± 134 mU/mL in hemophilia A mice (n = 16/group and n = 8/group, respectively) over 22 weeks, 8- to 14-fold higher than AAV5 (Figure 1A). The normal canine FVIII activity is approximately 7000 mU/mL when measured against a human FVIII standard.
resulted in the development of canine FVIII-neutralizing antibodies that prevented detection of FVIII activity in plasma (next section). In addition, we may have underestimated the steady-state FVIII level achieved by 3 \times 10^{11} \text{vg/mouse of AAV6-cFVIII due to the exclusion of mice that developed canine FVIII antibodies after treatment (next section). On the other hand, decreasing the dose of AAV6-cFVIII to 3 \times 10^{10} \text{vg/mouse (n = 6) was sufficient to achieve therapeutic levels of FVIII at 416 \pm 9 \text{mU/mL, which is equivalent to 5.9\% of normal canine FVIII activity (Figure 1B). Further reduction of dose to 1 \times 10^{10} \text{vg/mouse (n = 5), however, produced only subtherapeutic levels (<1\%) of FVIII at 65 \pm 4 \\
\text{mU/mL (Figure 1B).}}}

The efficacy of AAV8-cFVIII is roughly 2-fold greater than AAV6 at 1 \times 10^{11} \text{vg/mouse (P < .001; Figure 1C), since AAV8 achieved steady-state FVIII levels of 3138 \pm 194 \text{mU/mL (n = 6), in comparison with 1665 \pm 99 \text{mU/mL by AAV6 (n = 15). At 3 \times 10^{11} \text{vg/mouse, by week 2 the average FVIII level expressed from AAV8-cFVIII was 7818 \pm 957 \text{mU/mL (n = 6) versus 3675 \pm 570 \text{mU/mL from AAV6 (n = 15) (P = .008, Figure 1C). Long-term comparison with mice dosed with 3 \times 10^{11} \text{vg/mouse of AAV8-cFVIII was not feasible due to FVIII-neutralizing antibody formation (next section).}}}

### Development of canine FVIII-neutralizing antibodies following overexpression of canine FVIII in hemophilia A mice

None of the mice treated with AAV2-cFVIII or AAV5-cFVIII vectors developed any antibodies to canine FVIII; however, mice that received AAV6-cFVIII and AAV8-cFVIII had a high incidence of antibody formation. FVIII-neutralizing antibodies were detected in 32\% (7/22), 71\% (5/7), and 100\% (9/9) of mice dosed with AAV6-cFVIII at 3 \times 10^{11}, 6 \times 10^{11}, and 9 \times 10^{11} \text{vg/mouse, respectively; as well as 67\% (4/6) of mice that received 3 \times 10^{11} \text{vg/mouse of AAV8-cFVIII. Antibodies were detected at 2 to 6 weeks after vector injection, except for one mouse that did not develop FVIII antibodies until week 12. The presence of FVIII-neutralizing antibodies prevented measurement of FVIII activity in plasma; however, in cases where FVIII activity was detectable prior to the onset of FVIII-neutralizing antibodies, it always exceeded the normal canine level of 7000 \text{mU/mL (Figure 2A), consistent with the notion that overexpression of canine FVIII instead of vector dose induced canine FVIII antibodies. Nevertheless, the development of FVIII antibodies was not associated with loss of vector DNA, as shown by the lack of correlation between the neutralizing antibodies, as defined by Bethesda assay, and the copy numbers of canine FVIII DNA in liver as measured by Southern blotting analysis (Figure 2B). A Pearson test found no statistically significant correlation between Bethesda units and canine FVIII DNA copy numbers (r^2 = 0.012, P = .748). Also, the average canine FVIII DNA copy numbers are comparable in mice irrespective of their antibody status (ie, AAV6: 5.31 \pm 0.26 vs 5.45 \pm 0.93, and AAV8: 9.38 \pm 0.80 vs 7.44 \pm 0.35 in mice dosed with 3 \times 10^{11} \text{vg/mouse, with and without canine FVIII-neutralizing antibodies [Figure 2B]. Therefore, the neutralizing antibody development was not associated with T-cell–mediated destruction of transduced hepatocytes, which would result in the elimination of cells containing canine FVIII DNA in the liver.}}

### Differential liver transduction efficiencies by serotype variants of AAV-cFVIII vector in hemophilia A mice

To determine the gene transfer efficiency in liver by different serotypes of AAV-cFVIII vectors, mice were humanely killed upon

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**Figure 1. Relative efficacy of different serotypes of AAV-cFVIII delivered by portal-vein injection to hemophilia A mice.** Canine FVIII activity in mouse plasma was measured by Coatest and converted to percentage of normal canine FVIII activity (7000 \text{ mU/mL). Results presented are the mean and standard error of the mean (SEM). (A) Comparisons between mice dosed with 3 \times 10^{11} and 6 \times 10^{11} \text{vg/mouse of AAV5-cFVIII (n = 4/dose) or AAV2-cFVIII (n = 16 at 3 \times 10^{11} \text{vg/mouse and n = 8 at 6 \times 10^{11} \text{vg/mouse, respectively). (B) Comparisons between mice that received AAV2 and AAV6 vectors. AAV2-cFVIII was injected at 1 \times 10^{11} (n = 6), 3 \times 10^{11} (n = 16, from panel A), and 9 \times 10^{11} \text{vg/mouse (n = 6); AAV6-cFVIII was injected at 1 \times 10^{10} (n = 5), 3 \times 10^{10} (n = 6), 1 \times 10^{11} (n = 15), and 3 \times 10^{11} \text{vg/mouse (n = 15). (C) Comparisons between mice treated with 1 \times 10^{11} and 3 \times 10^{11} \text{vg/mouse of AAV6-cFVIII (n = 15/dose, from panel B) and AAV8-cFVIII (n = 6/dose). Results after week 2 from mice dosed with 3 \times 10^{11} \text{vg/mouse of AAV6-cFVIII are not shown due to prevalent canine FVIII-neutralizing antibody formation in this group.}}

In the next comparison between AAV2-cFVIII and AAV6-cFVIII vectors, 3 groups of mice (n = 6-16/group) received AAV2-cFVIII at 1 \times 10^{11}, 3 \times 10^{11}, and 9 \times 10^{11} \text{vg/mouse, and achieved steady-state FVIII levels of 806 \pm 51, 1201 \pm 72, and 3243 \pm 143 \text{mU/mL, respectively, over 30 weeks (Figure 1B). In comparison, 1 \times 10^{11} and 3 \times 10^{11} \text{vg/mouse of AAV6-cFVIII resulted in steady-state FVIII levels of 1665 \pm 99 and 3717 \pm 227 \text{mU/mL, respectively, in hemophilia A mice (n = 15/group), which are 2- to 3-fold higher than that by AAV2-cFVIII (P < .001) (Figure 1B). A higher dose of AAV6-cFVIII at 9 \times 10^{11} \text{vg/mouse achieved steady-state FVIII levels of 806/800}}

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termination of the study, and genomic DNAs were isolated from liver and analyzed by Southern blotting for canine FVIII DNA. A representative Southern blot is shown in Figure 3A, and the results are summarized in Table 1 and correlate remarkably with their differences in expressed FVIII activity. Specifically, AAV5 at doses of $3 \times 10^{11}$ and $6 \times 10^{11}$ vg/mouse, respectively, resulted in 8- to 15-fold less canine FVIII DNA copy numbers/diploid genome (0.08 ± 0.02 and 0.40 ± 0.20) compared with AAV2-transduced livers (1.24 ± 0.25 and 3.01 ± 0.41). In turn, AAV2 dosing led to 3- to 4-fold less FVIII DNA than that detected in AAV6-transduced livers (4.88 ± 0.58 and 9.79 ± 1.73, respectively). The gene transfer efficiency was further increased by about 2-fold in AAV8-transduced livers (1.95 ± 0.26 and 8.73 ± 0.81) relative to AAV6-transduced livers (0.74 ± 0.14 and 4.88 ± 0.58) at doses of $1 \times 10^{11}$ and $3 \times 10^{11}$ vg/mouse, respectively. Results from QPCR corroborate those from Southern blotting (Table 1).

To further assess the percentage of liver transduced by AAV-cFVIII, liver sections from mice dosed with $3 \times 10^{11}$ vg of either AAV6 or AAV2 vectors were analyzed by FISH for canine FVIII mRNA. As shown in Figure 3B, in contrast to the negative staining of either naive hemophilia A liver or AAV6-cFVIII–transduced liver hybridized with probes comprising sense sequences of canine FVIII, the antisense probe positively stained for canine FVIII mRNA in AAV6-cFVIII–treated mouse liver. On average, 26% ± 3% of liver cells were positive for canine FVIII mRNA in the mice (n = 4) treated with $3 \times 10^{11}$ vg AAV6, which is slightly higher than the 18% ± 4% in mice (n = 5) treated with the same dose of AAV2 (data not shown). However, this difference was not statistically significant ($P = 0.20$).

In addition, the biodistribution of the vector DNA was found predominantly in liver with a mean of $5.778 \pm 1.981$ copy/diploid genome at $3 \times 10^{11}$ vg/mouse of AAV6-cFVIII (Table 2), followed by $0.024 \pm 0.005$ copy/genome disseminated in spleen, $0.004 \pm 0.002$ in kidney, $0.002 \pm 0.001$ in both lung and heart, and $0.001$ in gonad. The detection limit of QPCR is 0.0003 copy/diploid genome.

**Long-term therapeutic benefit of AAV-cFVIII delivered to hemophilia A dogs**

Next, we determined the long-term efficacy and safety of the AAV-cFVIII vector in hemophilia A dogs in the colony at Queen’s University (Kingston, ON). In the Queen’s University hemophilia colony at Queen’s University at Queen’s University, 61% dogs treated with the same dose of AAV6-cFVIII (Table 2), followed by $0.024 \pm 0.005$ copy/genome disseminated in spleen, $0.004 \pm 0.002$ in kidney, $0.002 \pm 0.001$ in both lung and heart, and $0.001$ in gonad. The detection limit of QPCR is 0.0003 copy/diploid genome.

**Table 1. Hepatic gene transfer efficiency by different serotypes of AAV-cFVIII vectors in hemophilia A mice**

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<tr>
<th>AAV serotype</th>
<th>Vector dose, vg/mouse</th>
<th>No. of mice analyzed</th>
<th>Southern blot, mean ± SEM</th>
<th>QPCR, mean ± SEM</th>
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<td>5</td>
<td>$3 \times 10^{11}$</td>
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<td>0.08 ± 0.02</td>
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<td>8</td>
<td>$3 \times 10^{11}$</td>
<td>6</td>
<td>8.73 ± 0.81</td>
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ND indicates not determined.

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**Figure 2.** Formation of canine FVIII-neutralizing antibodies in hemophilia A mice. (A) Correlation between the overexpression of canine FVIII and subsequent presence of FVIII-neutralizing antibodies in mice in which FVIII activities were detectable before the onset of FVIII-neutralizing antibodies. The FVIII activities at weeks 2 and 6 after vector injection were determined by Coatest. The FVIII-neutralizing antibodies at weeks 2 and 6 were determined by Bethesda assay. (B) Lack of correlation between the levels of FVIII-neutralizing antibodies and transgene neutralizing antibodies at weeks 2 and 6 were determined by Bethesda assay. (C) Correlation between the overexpression of canine FVIII and subsequent neutralizing antibodies at weeks 2 and 6 were determined by Bethesda assay. (D) Correlation between the overexpression of canine FVIII and subsequent neutralizing antibodies at weeks 2 and 6 were determined by Bethesda assay.

**Figure 3.** Liver transduction efficiency by AAV6-cFVIII and AAV2-cFVIII in hemophilia A mice. (A) Southern blotting analysis. Genomic DNAs from AAV-treated mouse livers yield 2 positive bands from BamHI and BglI digestion, representing the canine FVIII light-chain (LC) and heavy-chain (HC), respectively. The standards are generated with digested plasmid DNAs spiked in liver genomic DNAs from naive hemophilia A mice. (B) RNA FISH for canine FVIII transcripts in liver sections of a mouse treated with $3 \times 10^{11}$ vg of AAV6-cFVIII (i-iii). (i) Section hybridized with a cocktail of canine FVIII antisense probes; (ii) same section counterstained with DAPI for nuclei; (iii) section hybridized with canine FVIII sense probes; and (iv) a naive mouse liver hybridized with canine FVIII antisense probes. Pictures were taken at 20 × magnification.
A dogs, the FVIII transcript is aberrantly spliced, resulting in a polyadenylated transcript lacking exons distal to 22 and terminating with a novel sequence element. This mutation is similar to the intron 22 inversion found in approximately 45% of severe hemophilia A patients. Phenotypically, these hemophilia A dogs are cross-reacting material negative (CRM-), have a circulating FVIII activity less than 1%, and have a propensity for spontaneous musculoskeletal bleeding requiring on average 5 infusions of cryoprecipitate per year.

Eight hemophilia A dogs were treated with AAV6-cFVIII vector via portal-vein injection (Table 3). Four dogs (Elisa, Angus, Vector, and Junior) received escalating doses of AAV2 from 6 x 10^{12} to 2.3 x 10^{13} vg/kg, 3 dogs (Alexis, Maurizio, and Morag) received either 1 x 10^{13} or 1.7 x 10^{13} vg/kg AAV6, and 1 dog (Floopers) was injected with 1 x 10^{13} vg/kg AAV8.

Except for Angus and Morag, all other dogs have expressed sustained therapeutic levels (> 1%) of FVIII activity as measured by Coatest assay for 44 to 171 weeks (the last time points assessed) (Figure 4A; Table 3). The highest peak level achieved was 598 mU/mL (8.5% canine FVIII level), in Alexis dosed with 1 x 10^{13} vg/kg AAV6, followed by 351 mU/mL (5.0%, Junior, 2.7 x 10^{13} vg/kg AAV2) and 312 mU/mL (4.5%, Floopers, 1 x 10^{13} vg/kg AAV8). Elisa, Vector, and Maurizio received 6 x 10^{12} vg/kg AAV2, 1.5 x 10^{13} vg/kg AAV2, and 1 x 10^{13} vg/kg AAV6, peaked at comparable levels of 269 mU/mL (3.8%), 256 mU/mL (3.7%), and 227 mU/mL (3.2%), respectively. The steady-state FVIII levels are roughly 60% of the peak levels except for Maurizio, who stabilized at about 80% of his maximal FVIII expression. Therefore, AAV-cFVIII–treated hemophilia A dogs have persistently expressed 2% to 5% of normal canine FVIII activity by Coatest, values that are consistent with results obtained by the 1-stage aPTT-based FVIII assay (data not shown). The therapeutic benefits were also shown by improved whole-blood clotting time (WBCT), from an average of 15.98 ± 0.79 minutes before treatment to the normal ranges of 2 to 6 minutes after treatment (Figure 4B; Table 3). Specifically, the average WBCTs from all time points measured after treatment for all 6 dogs are 5.22 ± 1.02 (Elisa), 5.85 ± 1.44 (Vector), 4.96 ± 0.85 (Junior), 4.21 ± 0.77 (Alexis), 4.57 ± 0.53 (Maurizio), and 4.69 ± 1.16 (Floopers).

To assess the gene transfer efficiency in liver, biopsies were obtained by open surgery either at the end of the study or at week 20 (Morag and Floopers), and the canine BDD-FVIII transgene DNAs were quantified by Southern blotting (Figure 5). From each dog, multiple biopsies were collected from different sites in the liver and were found to contain comparable amounts of canine BDD-FVIII DNA, with less than 2-fold variation. The exception was Elisa, in which canine BDD-FVIII DNA was undetectable in the left lateral lobe. On average, 1 x 10^{13} vg/kg AAV8 or AAV6 resulted in similar copy numbers of canine BDD-FVIII DNA per diploid genome of liver in Floopers (0.8 ± 0.1), Alexis (0.69 ± 0.06), and Maurizio (0.85 ± 0.28). Elisa, Vector, and Junior who were treated with AAV2 at 6 x 10^{12}, 1.5 x 10^{13}, and 2.7 x 10^{13} vg/kg contained 0.14 ± 0.13, 1.52 ± 0.28, and 0.8 copies of canine BDD-FVIII DNA per cell, respectively (Table 3).

Angus and Morag were treated with 1.5 x 10^{13} vg/kg AAV2 and 1 x 10^{13} vg/kg AAV6, respectively, and expressed only subtherapeutic levels of FVIII (Table 3). FVIII activity in Angus peaked at 64 mU/mL (0.91%) and remained at 38 ± 14 mU/mL, whereas in Morag the FVIII was below the detection limit of Coatest (23 mU/mL). The low copy number (0.05 ± 0.01) of canine BDD-FVIII DNA/cell detected in Morag’s liver indicates that the low levels of FVIII expression resulted, at least in part, from poor gene transfer efficiency (Figure 5; Table 3). No neutralizing antibodies to canine FVIII have been detected in either Angus or Morag (data not shown). However, despite their undetectable levels of circulating FVIII activities, Angus and Morag have

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The copy numbers of canine FVIII DNA per diploid genome as determined by QPCR. The limit of detection is 0.0003 copy/diploid genome.

*Individual mice dosed with 3 x 10^{11} vg AAV6-cFVIII.

8

| Animal name | SAV serotype | Sex | Age | Weight, kg | Dose, vg/kg | Duration of study, wk | Peak FVIII level, mU/mL | Steady-state FVIII level, mU/mL | WBCT after treatment, min± SEM | FVIII DNA copy/diploid genome in liver biopsy, * | FVIII DNA copy/diploid genome in liver biopsy, § |
|-------------|--------------|-----|-----|------------|-------------|------------------------|--------------------------|-------------------------------|---------------------------------|-------------------------------------------|
| Elisa§      | 2 F          | 7 m | 10  | 6 x 10^{13} | 171         | 269 (3.84)             | 174 ± 6 (2.49 ± 0.09) | 5.22 ± 1.02                  | 0.14 ± 0.13                     |
| Angus       | 2 M          | 1 y | 15.4| 1.5 x 10^{13}| 62          | 64 (0.91)              | 38 ± 5 (0.54 ± 0.07)   | 6.37 ± 1.02                  | ND                              |
| Vector      | 2 M          | 8 m | 15.2| 1.5 x 10^{13}| 96          | 256 (3.66)             | 149 ± 11 (2.13 ± 0.16)  | 5.85 ± 1.44                  | 1.52 ± 0.28                     |
| Junìor§     | 2 M          | 1 y | 12.8| 2.7 x 10^{13}| 139         | 351 (5.02)             | 225 ± 9 (2.31 ± 0.13)   | 4.96 ± 0.85                  | 0.80                         |
| Alexis      | 6 F          | 1 y | 8 m | 9.4      | 1.0 x 10^{13}| 73         | 598 (5.54)             | 331 ± 27 (4.73 ± 0.99)       | 4.21 ± 0.77                    | 0.69 ± 0.06                     |
| Morag       | 6 F          | 6 m | 10.5| 1.7 x 10^{12}| 46          | < 23 (< 0.33)          | < 23 (< 0.33)           | 6.29 ± 1.57                  | 0.05 ± 0.01                     |
| Maurizio    | 6 M          | 1 y | 2 m | 11.9     | 1.0 x 10^{13}| 44         | 227 (3.24)             | 175 ± 9 (2.50 ± 0.13)        | 4.57 ± 0.53                    | 0.85 ± 0.28                     |
| Floopers    | 8 F          | 1 y | 11.2| 1.0 x 10^{13}| 61          | 312 (4.46)             | 177 ± 16 (2.53 ± 0.23)   | 4.69 ± 1.16                  | 0.80 ± 0.10                     |

*FVIII activities in canine plasma were determined by Coatest and converted to percentage of normal canine FVIII activity of 7000 mU/mL. Pretreatment hemophilia A dogs had FVIII activity less than 1%.
†Average WBCT from all time points measured after treatment with duplicate measurements at each time point. Pretreatment WBCT was 14 to 17 minutes.
§Some data up to week 56 for Elisa and week 36 for Junior have been reported previously.
ND indicates not determined.

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shown clinical improvement. First, in comparison with their prior history of 6 bleeding episodes the year before the treatment, they have not experienced any spontaneous bleeding since the AAV treatment. Second, the WBCTs of Angus and Morag were decreased from 15.87 and 16.28 minutes before treatment to 6.37 and 1.02 and 6.29 and 1.57 minutes after treatment, respectively (Figure 6A). Third, clot formation was improved, as assessed in the TEG assay, which permits a more sensitive global assessment of homeostasis in the plasma. Whereas the pre-AAV plasma samples from these 2 hemophilia A dogs did not form detectable fibrin after more than 70 minutes, the TEG tracings for Angus and Morag showed “R” values of 23.8 and 31.3 minutes, clotting angles of 6.4 and 1.9 degrees, and maximal amplitudes of 6.3 and 2.4 mm, respectively, after AAV treatment (Figure 6B).

AAV-cFVIII vectors of serotypes 2, 6, and 8 induced no toxicity in hemophilia A dogs. Complete panels of serum chemistry and hematologic parameters have shown mostly normal values throughout the study, except for a slight increase (2-fold above the baseline) of alkaline phosphatase within the first week after surgery, and a self-resolving mild increase in creatine kinase in some cases, likely relating to intraoperative muscle damage (data not shown). H&E staining of liver biopsies has also shown healthy hepatic architecture; no inflammation, necrosis, or fibrosis; and no tumors (data not shown).

Discussion

Comparison of transduction efficiencies by different serotypes of AAV-cFVIII in murine liver

In this study, we have compared the efficiency of gene transfer, protein synthesis, and secretion when FVIII was delivered by AAV5, AAV2, AAV6, and AAV8 via portal-vein injection in hemophilia A mice. At doses of $1 \times 10^{11}$ to $6 \times 10^{13}$ vg/mouse, the transduction efficiency increased in the following order: AAV5 $<$ AAV2 $<$ AAV6 $<$ AAV8. Whereas there was an 8- to 14-fold improvement from AAV5 to AAV2 as determined by plasma FVIII activities, the differential changes observed with AAV2, AAV6, and AAV8 are moderate at about 2- to 3-fold stepwise, yet statistically significant as determined by the 2-tailed, unpaired t-test. However, we may have underestimated the efficacy mediated by AAV6 and AAV8 vectors at doses of $3 \times 10^{11}$ vg/mouse or higher since mice that developed FVIII-neutralizing antibodies were excluded from
the analysis. Therefore, it is important to note that the serotype differences were corroborated by both Southern blotting and QPCR analyses, which measured the copy numbers of canine FVIII DNAs in mouse livers. Both assays are independent of the development of canine FVIII-neutralizing antibodies.

Earlier studies of various serotypes of AAV vectors used for liver targeting are controversial with regard to the differences in the transduction efficiency among AAV1, 2, 5, 6, and 8.20,27,44,45 AAV1 is 97% homologous in its capsid amino acid sequences to AA6 studied here, and both serotypes have generally been found to be concordant in tissue tropism. The interstudy comparisons are complicated because of the choices of different transgenes; the choices of different expression cassettes, including upstream regulatory sequences if the transgene is the same; and the potency of vectors produced in different labs with different production and assay methods. In addition, the disagreement on the magnitude of differences between AA2 and other serotypes may partly result from differences in purification methods (ie, AA2 vectors purified by heparin-based column chromatography contain excess empty viral particles, whereas most serotypes purified by CsCl-gradient centrifugation are free from empty particles). Empty particles inhibit transduction efficiency of full particles, leading to a lower overall response.46 However, on the other hand, since it has been reported that CsCl may damage AAV particles under certain conditions,47 and since we do not have column-purified AA6 and AA8 vectors to compare, we cannot rule out the possibility that CsCl is more detrimental to AA6 and AA8 than AA2 particles, resulting in lower infectivity and thus lower efficacy than reported by other groups.30,48

Nevertheless, results from this study are comparable with those by Grimm et al36 and Sarkar et al.28 In the first study,36 AAV-FIX vectors of serotypes 1 to 6 were all purified by CsCl-gradient centrifugation by the same group that produced the AAV-cFVIII vectors studied in the present report. The authors showed that portal-vein injection of AAV-FIX resulted in 12- and 2-fold increases from AA5 to AA2 and from AA2 to AA6, respectively, as determined by circulating human FIX levels and QPCR of FIX transgene DNA (Table 16). In the second study,28 at doses of either $1 \times 10^{12}$ or $3 \times 10^{10}$ gc/mouse, AA8 B-domain–deleted FVIII vector resulted in a roughly 2-fold higher FVIII activity over AA2 as measured at 6 months and 15 months following portal- vein injection in hemophilia A mice.

Development of FVIII-neutralizing antibodies in hemophilia A mice

FVIII is more immunogenic than other coagulation factors such as FIX, since approximately 25% of hemophilia A and 3% of hemophilia B patients who have received protein replacement therapy develop inhibitors to FVIII or FIX, respectively, after repeated exposures. We also detected a high incidence of FVIII-neutralizing antibodies in hemophilia A mice treated with AAV-cFVIII, which is not fully attributable to the production of xenogeneic FVIII, because a previous study has shown this propensity to be independent of the species of origin of the transgene FVIII40; and overexpression of canine FIX did not result in inhibitor formation in C57BL/6 mice (same strain as our hemophilia A mice). On the other hand, our results strongly suggest that the formation of FVIII-neutralizing antibodies is a consequence of the expression of supraphysiologic levels of FVIII. Consistent with this notion, a minimal adenoviral vector expressing supraphysiologic levels of human FVIII also induced FVIII inhibitors as early as 2 weeks after intravenous injection in hemophilia A mice.50 In addition, an E1/E2a/E3-deficient adenoviral vector encoding canine FVIII induced FVIII inhibitors in hemophilia A dogs, and the titers of the FVIII inhibitors were proportional to the prior expression levels of canine FVIII.51 Furthermore, it was shown that in hemophilia A mice, whereas repeated administration of human FVIII to achieve sustained normal human levels (50 U/kg, equivalent to 10 μg/kg) induced anti-FVIII antibodies in most animals, a single injection of a 5- to 50-fold higher dose of human FVIII elicited a strong anti-FVIII IgG response within 14 days.52 However, it is important to note that in the present study, the development of FVIII-neutralizing antibodies does not lead to T-cell–mediated elimination of transduced hepatocytes, as evidenced by the persistence of transgene DNA in the livers of AAV-cFVIII–treated hemophilia A mice, which is in contrast to the marked innate immune response and associated inflammation/necrosis observed with adenoviral vectors.

On the other hand, since AAV-transduced FVIII-producing hepatocytes remain in the face of FVIII inhibitors, it is also possible that sustained FVIII expression may eventually lead to immune tolerance to FVIII. Indeed, one of the AAV2-cFVIII–treated hemophilia A dogs (Junior) developed FVIII inhibitors only transiently, peaking at 9 BU at 4 weeks after vector infusion and resolving by 9 weeks.5 In addition, Chao and Walsh53 also observed that human FVIII antigen levels were partially restored, coinciding with the disappearance of the anti-hFVIII inhibitors, 10 months after vector injection in 3 C57BL/6 mice. However, in the present study, mice that developed FVIII inhibitors had no detectable FVIII activities up to 34 weeks after vector infusion. The different outcome between the 2 mouse studies could be attributed to (1) an insufficient duration of follow-up, (2) the difference between hemophilia A versus normal mice, or (3) the difference in the FVIII exposure, supraphysiologic versus low levels (as in normal mice and Junior).

Long-lived therapeutic activity in the absence of toxicity by AAV-cFVIII in hemophilia A dogs

The most significant finding of this study is the demonstration of the long-term efficacy and safety of multiple AAV-cFVIII serotypes in hemophilia A dogs, greatly extending our initial pilot study that had short-term results using AAV2. Long-term efficacy lasting approximately 4 years suggests the potential for a cure in humans. Preclinical and clinical studies on AAV-FIX have also shown multicyear stable FIX expression in a hemophilia B canine model and predicted the therapeutic dose likely to be effective in humans (H.J., L.B.C., S.P.-W., T.L., D.N., J.A.V., S.Z., C.D.S., Jurg Sommer, Sharmila Vijay, Darren Warren, Federico Mingozi, Katherine A. High, G.F.P.; “Effects of transient immunosuppression on adeno-associated, virus-mediated, liver-directed gene transfer in rhesus macaques and implications for human gene therapy; submitted April 18, 2006).33,54-59

Of 8 hemophilia A dogs treated with AAV2-cFVIII, AAV6-cFVIII, and AAV8-cFVIII vectors at doses from $6 \times 10^{12}$ to $2.7 \times 10^{13}$ vg/kg, 6 dogs had sustained circulating FVIII activities at 2% to 5% for 1 to 4 years, with no evidence of a diminution in FVIII production. In addition to improved Coaest t and aPTT values, WBCT decreased from 14 to 17 minutes before treatment to normal at 4 to 6 minutes after treatment, and the spontaneous bleeding episodes were decreased from an average of 5 times per year before treatment to 0 after treatment. The remaining 2 dogs, Angus and Morag, who received 1.5 $\times 10^{13}$ and 1.7 $\times 10^{13}$ vg/kg AA2 and AA6, respectively, failed to express FVIII activity more than 1% of normal. Angus and Morag have no pre-existing
neutralizing titers against AAV2 and AAV6, respectively (data not shown). In addition, the low levels of FVIII activity in these dogs are not due to the development of FVIII-neutralizing antibodies, but correlate with poor gene transfer within the liver. However, despite subtherapeutic FVIII activity levels (<1%), evidence of improved homeostasis was detected. In addition to improved WBCT (6 minutes), both dogs no longer bled spontaneously. Furthermore, both dogs clearly showed improved global homeostasis after AAV therapy in the TEG assay. The TEG assay has a greater sensitivity than traditional clotting assays and allows the analysis of clot (fibrin) formation in real time, demonstrating that the rate of fibrin formation correlates to FVIII levels from 0.001% to 100%.60,61 In these studies, all 8 dogs have maintained clinical chemistry and hematologic parameters within normal limits. No abnormal histopathologic findings were found in liver biopsies.

As observed with AAV-FIX studies, the levels of FVIII expression achieved in mice did not scale up to dogs. Whereas I × 10^13 vg/kg (3 × 10^11 vg/mouse) of AAV2-cFVIII, AAV6-cFVIII, and AAV8-cFVIII achieved roughly 20%, 50%, and 110% of normal canine FVIII activity, respectively, in hemophilia A mice, the same dose of all 3 vectors resulted in only 2% to 5% of normal FVIII levels in hemophilia A dogs. Thus, the underestimate of efficacious doses seen in the transition from mice to dogs and nonhuman primates further supports the canine model as a predictor of effective doses in humans. Firm conclusions regarding differences in efficacy between AAV serotypes are not possible due to the small sample sizes; however, taken together, differences among AAV2, AAV6, and AAV8 do not appear to be substantial. This is notably different in comparison with AAV1, which consistently performs better than AAV2 in muscle in both mouse and dog models.62 It is possible that AAV1 has a tropism for skeletal myocytes irrespective of species, whereas the liver specificity of AAV6 and AAV8 does not cross species from murine to canine. Species-dependent tropism has been previously reported, for example, although AAV1 vector transduced murine pancreatic islets better than AAV2, the order was reversed in transduction of human islets.63 However, it is also possible that unlike easily accessible skeletal muscles, only a limited number of hepatocytes are accessible to AAV vectors due to inefficient delivery via either portal vein or hepatic artery; this may also explain the lack of vector dose response seen in hemophilia A dogs.

Future research should focus on improving the strength of the transgene promoter, which is minimal due to the large size of the B-domain–deleted FVIII gene, and on improving the efficiency of hepatic delivery, to achieve enhanced therapeutic benefit while minimizing the risk of potential toxicity or a cytotoxic T-cell immune response to vector capsid. Alternative serotypes such as AAV6 and AAV8 may partially circumvent the inhibition of transduction by low-level anti-AAV2 antibodies prevalent in humans.64

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References
Multiyear therapeutic benefit of AAV serotypes 2, 6, and 8 delivering factor VIII to hemophilia A mice and dogs

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