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Efficacy and Safety of Long-term Prophylaxis in Severe Hemophilia A Dogs Following Liver Gene Therapy Using AAV Vectors

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Developing adeno-associated viral (AAV)–mediated gene therapy for hemophilia A (HA) has been challenging due to the large size of the *factor VIII* (*FVIII*) complementary DNA and the concern for the development of inhibitory antibodies to FVIII in HA patients. Here, we perform a systematic study in HA dogs by delivering a canine *FVIII* (cFVIII) transgene either as a single chain or two chains in an AAV vector. An optimized cFVIII single chain delivered using AAV serotype 8 (AAV8) by peripheral vein injection resulted in a dose-response with sustained expression of FVIII up to 7% (n = 4). Five HA dogs administered two-chain delivery using either AAV8 or AAV9 *via* the portal vein expressed long-term, vector dose–dependent levels of FVIII activity (up to 10%). In the two-chain approach, circulating cFVIII antigen levels were more than fivefold higher than activity. Notably, no long-term immune response to FVIII was observed in any of the dogs (1/9 dogs had a transient inhibitor). Long-term follow-up of the dogs showed a remarkable reduction (>90%) of bleeding episodes in a combined total of 24 years of observation. These data demonstrate that both approaches are safe and achieve dose-dependent therapeutic levels of FVIII expression, which supports translational studies of AAV-mediated delivery for HA.

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INTRODUCTION

Hemophilia A (HA) is an X-linked bleeding disorder characterized by deficiency in factor VIII (FVIII), a key component in the coagulation cascade.[1] Current treatment for HA is by protein replacement either in response to bleeds or as a preventative therapy. Prophylaxis in children with severe HA requires three to four injections per week; however, this approach does not prevent breakthrough bleedings that occur at times when FVIII levels are subtherapeutic. A recent study demonstrated that children with severe HA receiving prophylactic treatment with recombinant FVIII exhibit fewer joint bleeds and less joint damage than those on high-dose protein therapy on demand.[2] However, there is no consensus yet on the long-term impact of prophylaxis and whether adults would benefit from similar regimens. Moreover, differences in the underlying joint status and pharmacoeconomics further complicate the management of the disease in adult populations.

Therapeutic strategies based on cell or gene therapy for the management of HA have aimed at sustained expression of therapeutic levels of FVIII that provides the benefits of prophylaxis without the pitfalls of replacement therapy. Collective experience by our and other groups demonstrate the potential of adeno-associated virus (AAV) vectors to ensure sustained expression of clotting factors either by liver- or skeletal muscle–directed strategies.[3–6] Although the levels of FVIII required for therapeutic effect are 50-fold lower than for factor IX (FIX), there are two major differences from a gene therapy perspective. The first is the limited packaging capacity of AAV for the large *FVIII* complementary DNA and the second is the high risk of immune responses to the neotransgene that is anticipated with protein-based therapy and potentially with any novel therapy for HA.

The canine HA model mimics the severe human disease at molecular and phenotypic levels and thus provides an ideal model for preclinical studies.[7] Studies in mouse models have demonstrated efficient liver transduction of AAV serotype 8 (AAV8) or AAV9.[5,8–11] We have previously reported some initial studies using AAV8 and AAV9 (ref. 5) and another group has reported the use of AAV8 in HA dogs.[6] In the canine HA model, we reported that coadministration of AAV8 or AAV9 encoding separately the heavy and light chains of the cFVIII was safe and resulted in long-term, dose-dependent expression of therapeutic levels of cFVIII.[5] However, whether the expression of relatively large amounts of nonfunctional protein could affect the immune responses to protein replacement with FVIII protein and/or induce damage of the target tissue, as reported in other models of FVIII expression,[12] raised safety concerns. Jiang *et al.*[6] further reported that sustained expression of cFVIII as a single chain by AAV vectors from serotypes 2, 6, and 8 was only effective in some HA dogs and there was no clear vector-dose dependency on

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FVIII expression, a fact not observed for FIX expression in large animal models of hemophilia B.[13–15]

The limitations of these two studies were the small numbers of animals for each vector serotype, variable vector dosage, the use of two different strains of dogs and two different delivery approaches. Here, we report a study in HA dogs in which we systematically compare AAV vectors encoding the cFVIII gene by a single- or two-chain approach by hepatic or noninvasive peripheral vein delivery. We demonstrate that both strategies are efficacious, prevent >90% of spontaneous bleeding episodes, and have no evidence for toxicity. In addition, there is no evidence of long-term immune responses to FVIII that was sustained upon challenges with recombinant cFVIII. These data will form the basis for clinical studies in humans with severe HA.

RESULTS

Single-chain cFVIII delivery in HA mice

Previously, we described a 5.6-kb single-chain B-domain-deleted cFVIII (cFVIII-BDD) construct that cannot package efficiently into an AAV vector resulting in poor vector yield.[10] We sought to improve the packaging efficiency of the cFVIII-BDD constructs by minimizing the regulatory elements in order to shorten the cFVIII transgene construct. Ten novel single-chain constructs (**Supplementary Figure S1**) utilizing the insulin-like growth factor–binding protein promoter (IGFBP) or the hepatic control region-human α-1 antitrypsin enhancer-promoter (HCR-hAAT) were systematically compared *in vitro* in HepG2 cells and by hydrodynamic infusion in HA mice (**Supplementary Figure S2**). AAV vectors were generated from the three cFVIII-BDD constructs that were <5.3 kb (**Figure 1a**) and expressed cFVIII at levels comparable to those observed with our 5.6 kb cFVIII-BDD construct. HA mice were treated with 5×10^{10} vector genomes/mouse of the AAV8-cFVIII-BDD constructs and compared to the original 5.6 kb construct (**Figure 1b**). The cFVIII constructs with the IGFBP and HCR-hAAT promoters are comparable in terms of maximum FVIII expression levels that can be achieved. These constructs are both 5.2 kb, our goal for minimizing the transgene size, and gave similar yields of vector (data not shown). Thus, we concluded that either the IGFBP or HCR-hAAT promoter would be a good candidate for translating to larger animal models.

Correction of the HA phenotype after single-chain delivery of FVIII in HA dogs

Since the IGFBP and HCR-hAAT promoter constructs expressed similar levels of cFVIII-BDD in the mouse studies (**Figure 1**) and the hAAT promoter has been used clinically in phase I clinical studies for AAV-FIX,[15,16] the HCR-hAAT-cFVIII construct was selected for studies in the HA dog (**Figure 2a**). No significant differences were observed between AAV8 and AAV9 in our previous studies;[5] thus, we used AAV8 to deliver the single-chain cFVIII by peripheral vein infusion. Data in mice and nonhuman primates suggest that peripheral vein delivery of AAV8 for FIX expression is comparable to hepatic delivery.[5,15] Thus, we focused on the use of AAV8 by intravenous injection to simplify the clinical applicability of this approach. In contrast to portal vein delivery, peripheral vein infusion is minimally invasive, well-tolerated and does not require protein treatment at the time of vector

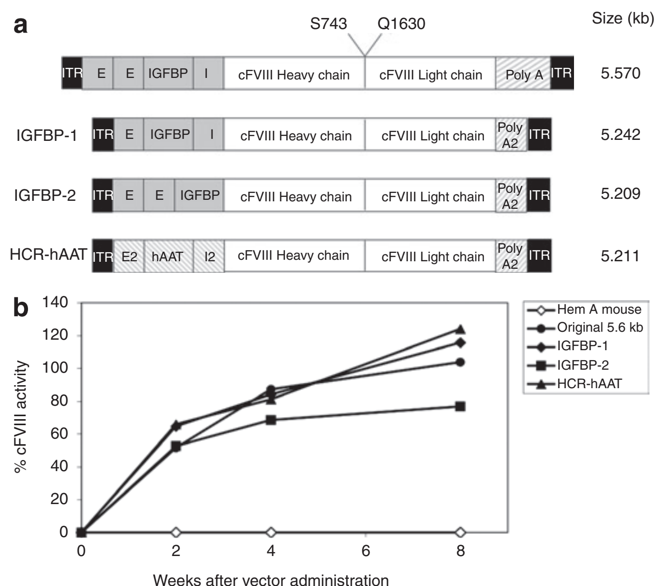

Figure 1 Comparison of canine factor VIII (cFVIII) gene constructs in hemophilia A mice. (**a**) The three best performing constructs in terms of expression in the hydrodynamic studies in hemophilia A mice (**Supplementary Figures S1** and **S2**) were selected for adeno-associated virus (AAV) production. Two of these constructs utilized the insulin-like growth factor binding protein promoter/enhancer[10] (IGFBP-1 and IGFBP-2) while one used a minimal hepatic control region-human α-1 antitrypsin enhancer/promoter (HCR-hAAT)[27]. The IGFBP single-chain gene constructs were derived from the original 5.6 kb construct that used the IGFBP promoter (184 bp), two α1-microglobulin/bikunin enhancers (E; 100 bp) and β-globin intron (I; 175 bp). The HCR-hAAT promoter constructs utilize a shortened version of the hepatic control region of the human apolprotein ExxC-1 gene locus (E2) and a 65-bp SV40 intron (I2). The 5.6 kb construct used a 263-bp SV40 polyadenylation signal, while all of the 5.2 kb constructs used a 134-bp SV40 polyadenylation signal sequence. (**b**) Hemophilia A mice were treated with 5×10^{10}vg/mouse of the AAV8-cFVIII-BDD constructs and compared to the original 5.6 kb construct. FVIII activity was determined by Coatest assay. At the 8-week time point, the levels of cFVIII activity as determined by Coatest were 104 ± 21 for the original 5.6 kb construct, 116 ± 17 for the IGFBP-1, 77 ± 30 for IGFBP-2, and 124 ± 24 for the HCR-hAAT construct. These results were confirmed by aPTT (data not shown). There was no significant difference between the expression levels of the IGFBP and HCR-hAAT promoters in the constructs (unpaired *t*-test; two-tailed $P = 0.45$). However, as previously reported,[35,36] the exclusion of the intron resulted in lower levels of expression relative to the other constructs. IGFBP[10] or a minimal HCR-hAAT[27] were used to generate the cFVIII single-chain constructs. The IGFBP single-chain gene constructs were derived from the original 5.6 kb construct (Construct A) that used the insulin-like growth factor binding protein (IGFBP) promoter (184 bp), two α1-microglobulin/bikunin enhancers (E; 100 bp) and β-globin intron (I; 175 bp) with an SV40 polyadenylation signal (Poly A1; 263 bp). The HCR-hAAT promoter constructs (Construct E and J) utilize a shortened version of the hepatic control region of the human apolprotein ExxC-1 gene locus (E2). A 65-bp SV40 intron (I2) and a 134-bp SV40 polyadenylation (Poly A2) sequence is also used. ITR, inverted terminal repeats.

infusion. Dogs M06 and M50 receiving the high vector dose (4×10^{13} vector genomes/kg) had a mean cFVIII activity of 7.8 ± 2.7 and 3.1 ± 1.5% of normal, respectively (**Table 1**; **Figure 3a**). At low dose (2×10^{13} vector genomes/kg) cFVIII plateau levels were 0.7 ± 0.5 and 1.7 ± 0.8% for dogs L51 and M66, respectively. As the dogs were not treated with cFVIII protein at the time of the vector infusion, cFVIII activity could be assayed immediately following vector administration and was detected within 1 day

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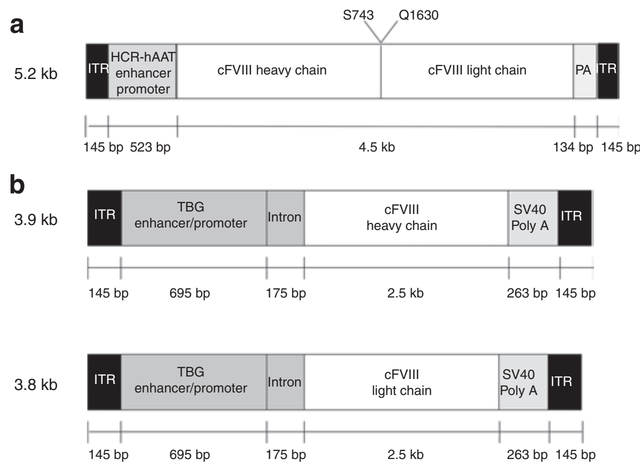

Figure 2 Canine factor VIII (cFVIII) adeno-associated virus (AAV) gene constructs delivered to hemophilia A dogs. (**a**) The single-chain construct includes the 4.5 kb domain-deleted cFVIII driven by the shortened version of hepatic control region (HCR) of human apolprotein ExxC-1 gene locus (192 bp) and the human α-1 antitrypsin promoter (266 bp) with an SV40 intron (65 bp). An SV40 polyadenylation signal (PA) is used (134 bp). The AAV2 inverted terminal repeats (ITRs) (145 bp) flank the transgene construct. The total vector size is 5.2 kb. (**b**) The two-chain delivery AAV vector approach for FVIII utilizes two separate AAV vectors to deliver the cFVIII complementary DNA (cDNA). One construct contains the 2.5 kb heavy-chain and the second construct contains the 2.4 kb light-chain cDNA. Both constructs use the thyroxine-binding globulin gene promoter/enhancer (TBG; 695 bp) fused to an intron (175 bp) and an SV40 polyadenylation signal (Poly A; 263 bp). The AAV2 inverted terminal repeats (ITRs; 145 bp) flank each transgene construct. The total vector size is 3.9 kb for the heavy chain and 3.8 kb for the light chain.

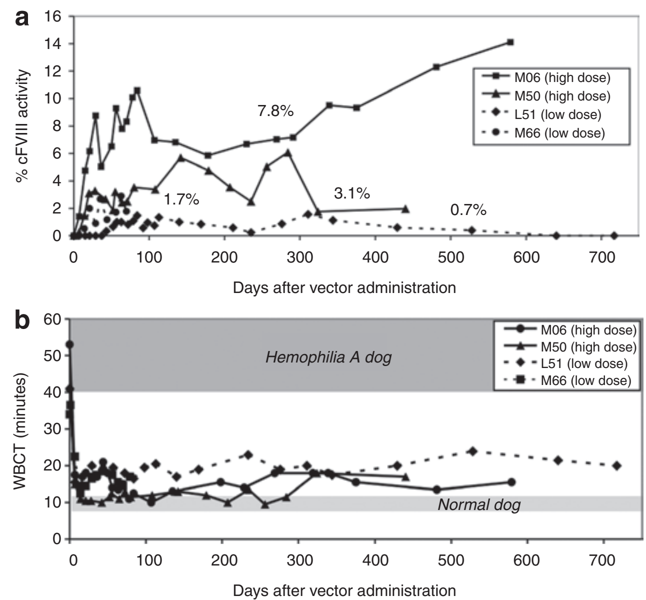

Figure 3 Canine factor VIII (cFVIII) activity after single chain delivery of cFVIII using adeno-associated virus 8 (AAV8) and AAV9 in hemophilia A (HA) dogs. (**a**) Coatest assay to determine cFVIII activity after single-chain delivery of cFVIII using AAV8 and AAV9. The solid lines represent dogs administered the high dose (4×10^{13}vg/kg), while the dashed lines represent dogs administered the low dose (2×10^{13} vg/kg). The % activity labeled next to each data line is the average activity from all the time points analyzed. (**b**) WBCT after single chain delivery of cFVIII using AAV8 and AAV9. The dark gray–shaded region is the range of values for untreated HA dogs (>40 min), while the light gray–shaded region represents the normal dog values (8–12 minutes). WBCT, whole-blood clotting time.

after vector administration by shortening of the whole-blood clotting time (WBCT), and by Coatest within 7 days after vector administration. The protein was functional based on the shortening of the WBCT to 15–21 minutes (normal dog WBCT is 8–12 minutes; **Figure 3b**). The cFVIII heavy- and light-chain antigen levels in the circulation of the single-chain dogs were equivalent (**Table 1**; **Figure 4a**) and were consistent with the activity levels. Furthermore, we used thromboelastography for assessing the functional parameters of expressed FVIII (**Supplementary Figure S3**).

Long-term expression after two-chain delivery of cFVIII in HA dogs

Our earlier studies in HA mice and dogs demonstrated that two-chain delivery of cFVIII using AAV8 and AAV9 efficiently targeted liver and resulted in therapeutic levels of circulating FVIII.[5] In this study, we expanded the dose cohorts to include additional HA dogs administered AAV8 and AAV9 expressing cFVIII to demonstrate long-term dose-dependent expression of the transgene. A total of five HA dogs were administered AAV-cFVIII *via* the portal vein using two-chain delivery. One AAV8-treated dog (F24) and one AAV9-treated dog (H19) were previously described[5] but long-term follow-up and additional analyses are presented here.

Two male dogs were coadministered AAV8-TBG-HC and AAV8-TBG-LC (**Figure 2b**). F24 was administered a high dose of 1.25×10^{13} vector genomes/vector/kg and Linus was administered a low dose of 6.0×10^{12} vector genomes/vector/kg. We observed dose-dependent expression of cFVIII as determined by Coatest assay. These dogs expressed a mean value of 5.7 ± 2.3% (F24) and 1.8 ± 0.8% (Linus; **Figure 5a**; **Table 1**). Canine FVIII was functional as noted by shortening of the WBCT to near-normal levels (10–15 minutes) that remained consistent throughout the study (**Figure 5b**).

The dogs that were coadministered AAV9-TBG-HC and AAV9-TBG-LC received a similar vector dosage (**Figure 5**; **Table 1**). Canine FVIII levels for the dog Woodstock were comparable to those of AAV8-injected dogs in the high-dose cohort. However, in the second high-dose dog (J60), the expression levels were fivefold higher (**Figure 5a**). This dog had an unanticipated surgical complication that required splenectomy within minutes of vector administration; whether this modified the AAV9 vector delivery to the liver is unknown. The dog administered the low dose (H19) expressed 0.7 ± 0.6% activity that was 1.7% at the final time point analyzed. Taken together, these data demonstrate dose-dependent sustained expression of cFVIII following hepatic delivery of AAV8 and AAV9 vectors.

To address a potential heavy- and light-chain imbalance, we generated a series of monoclonal and polyclonal antibodies specific to cFVIII and developed a cFVIII enzyme-linked immunosorbent assay (ELISA) for each chain.[17] In contrast to single-chain delivery, we observed that these dogs expressed higher levels of antigen than would be predicted based on activity (**Figure 4b**). In fact, we detected more than four- to eightfold higher levels of the light chain than heavy chain in the circulation. The levels

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Table 1 Hemophilia A dogs administered two- or single-chain delivery of cFVIII

FVIII delivery approach	AAV serotype	Vector dose	Route of administration	Hemophilia A dog	Gender	Age at time of vector administration (years)	Weight (kg)	Number of days evaluated	FVIII activity		Mean cFVIII antigen levels (ng/ml)[a]		Ratio of light chain/ heavy chain	Mean WBCT (min)[a]
									Peak (%)	Mean (%)[a]	Heavy chain	Light chain		
Two-chain	AAV-8	1.25×10^{13} vg/v/kg	Portal vein	F24	M	1.3	21.6	797	8.4	5.7 ± 2.3	10.3 ± 2.7	41.9 ± 17.7	4.0	12.7 ± 1.9
		6×10^{12} vg/v/kg	Portal vein	Linus	M	4.0	17.7	>1552	2.1	1.8 ± 0.8	9.8 ± 3.3	62.9 ± 15.4	6.4	17.9 ± 2.6
	AAV-9	1.25×10^{13} vg/kg	Portal vein	Woodstock	M	2.9	20	>1576	3.4	2.4 ± 0.8	10.4 ± 2.3	71.4 ± 22.6	6.9	16.6 ± 2.3
			Portal vein	J60[b]	F	1.2	15.2	>1170	15.6	10.3 ± 4.1	14.7 ± 8.8	73.6 ± 24.5	5.0	12.3 ± 2.1
		6×10^{12} vg/v/kg	Portal vein	H19	M	1.0	18.5	>2063	2.0	0.7 ± 0.6	7.8 ± 2.6	65.1 ± 14.4	8.3	16.9 ± 2.4
Single-chain	AAV-8	4×10^{13} vg/kg	IV	M06	M	0.5	20.1	>579	9.3	7.8 ± 2.7	4.8 ± 1.5	4.5 ± 1.2	0.9	15.5 ± 2.7
			IV	M50	M	0.6	16.8	>440	3.2	3.1 ± 1.5	4.7 ± 1.5	2.2 ± 1.2	0.5	12.0 ± 2.0
		2×10^{13} vg/kg	IV	L51	M	0.4	13.7	>717	1.5	0.7 ± 0.5	4.0 ± 2.1	2.3 ± 1.9	0.6	19.1 ± 2.4
			IV	M66	M	1.3	21.6	>80	2.9	1.7 ± 0.8	3.1 ± 0.5	4.1 ± 2.7	1.3	16.7 ± 2.7

Abbreviations: AAV, adeno-associated virus; cFVIII, canine factor VIII; IV, intravenous; WBCT, whole-blood clotting time.
[a]Mean ± SD. The mean FVIII activity was calculated based on the mean of all data points from day 7 after vector administration onward, including time points that had no detectable FVIII activity. [b]J60 had a splenectomy at the time of vector infusion due to surgical complications.

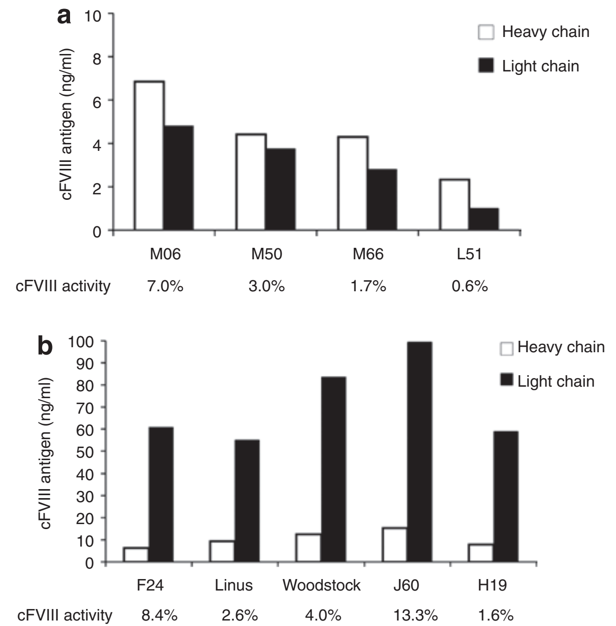

Figure 4 Canine factor VIII (cFVIII) antigen levels after adeno-associated virus–mediated delivery of cFVIII in hemophilia A (HA) dogs. (**a**) Enzyme-linked immunosorbent assay (ELISA) to detect cFVIII heavy-chain (white bars) and light-chain (black bars) antigen levels at day 85 after single-chain delivery of cFVIII. cFVIII activity for each dog at this time point is indicated. HA dogs have no detectable antigen and normal dogs have 80–120 ng/ml. (**b**) ELISA to detect cFVIII heavy-chain (white bars) and light-chain (black bars) antigen levels at day 85 after two-chain delivery of cFVIII. cFVIII activity for each dog at this time point is indicated.

of heavy chain correlated closely with the activity in contrast to those of light chain (**Table 1**). The findings were similar for dogs injected with AAV8 or AAV9 vectors and demonstrated that the light chain is more abundant than the heavy chain in the circulation after two-chain AAV-FVIII delivery as predicted by previous results in the HA mouse model.[10,18]

Immunological challenges with recombinant cFVIII-BDD in AAV-injected HA dogs

In terms of safety, we looked for any evidence of an immune response to FVIII throughout the course of the study. Notably, before vector administration, these dogs had been exposed to plasma-derived cFVIII or recombinant cFVIII-BDD and none of these dogs had any evidence for an immune response to cFVIII; thus, they did not appear to be prone to inhibitor development. No inhibitors to cFVIII were detected in any of the two-chain dogs or in the high-dose cohort of the single-chain dogs. To further exclude the possibility of non-neutralizing antibodies, we established an ELISA to detect IgG antibodies specific to cFVIII and assessed plasma samples from the dogs for antibodies simultaneously with cFVIII activity measurements.[17] After vector administration, no IgG1 or IgG2 antibodies to cFVIII were detected in any of these dogs. The only dog that had evidence of an immune response to cFVIII was a single-chain dog treated at the low dose (L51; **Figure 6**). Importantly, this dog had transient IgG2 antibodies to FVIII until day 52 with a Bethesda titer detectable until day 37 (**Figure 6a**). No IgG1 antibodies to cFVIII were detected. FVIII activity was not detectable until day 43 when a rise in FVIII

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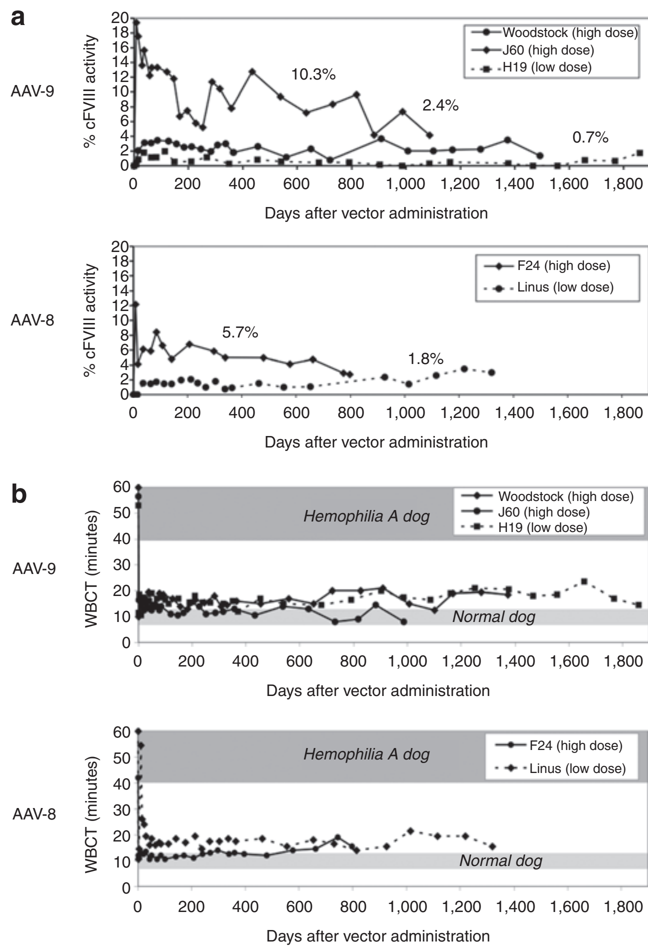

Figure 5 Canine factor VIII (cFVIII) activity after two-chain delivery of cFVIII using adeno-associated virus 8 (AAV8) and AAV9 in hemophilia A (HA) dogs. (**a**) Coatest assay to determine cFVIII activity after two-chain delivery of cFVIII using AAV8 and AAV9. The solid lines represent dogs administered the high dose (1.25×10^{13} vg/v/kg) while the dashed lines represent dogs administered the low dose (6×10^{12} vg/v/kg). The % activity labeled next to each data line is the average activity from all the time points analyzed. (**b**) WBCT after two-chain delivery of cFVIII using AAV8 and AAV9. The dark gray–shaded region is the range of values for untreated HA dogs (>40 minutes), while the light gray–shaded region represents the normal dog values (8–12 minutes). WBCT, whole-blood clotting time.

expression in L51 coincided with the disappearance of anti-cFVIII antibodies (**Figure 6b**). Notably, the WBCT for L51 was shortened to 15–20 minutes throughout the study.

The sustained transgene expression and the absence of inhibitors to FVIII in most of the dogs (8/9) suggest that the AAV delivery of cFVIII is not immunogenic. To further test this hypothesis, we challenged seven dogs with repeated injections of purified recombinant cFVIII protein[17] at 1–5 years after gene transfer. No anti-cFVIII IgG or neutralizing antibodies were detected in the dogs, including the dog L51 that had transient anti-cFVIII antibodies immediately following vector administration. The cFVIII activity was consistent with activity levels before the challenge. In addition, we determined that the recovery of the protein 15 minutes after infusion was 70 ± 12%, which was similar to observations in naive dogs.[17] Together, we formally exclude the presence of antibodies to cFVIII.

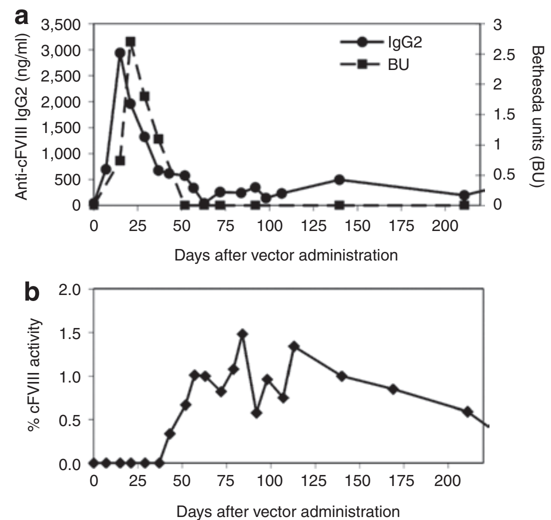

Figure 6 Transient immune response to canine factor VIII (cFVIII) observed in L51 after administration of adeno-associated virus 8 (AAV8)-cFVIII. (**a**) Anti-cFVIII IgG2 antibodies were detected by a cFVIII-specific IgG2 enzyme-linked immunosorbent assay. No IgG1 antibodies were detected. Bethesda assay detects FVIII neutralizing antibodies. (**b**) Canine FVIII activity was determined by Coatest assay.

Clinical outcomes after AAV vector administration

HA dogs typically have five to six spontaneous bleeding episodes per year that require treatment.[19] In total, the nine HA dogs were followed for 297 months (**Table 2**). During this time period, we would expect 123 spontaneous bleeding episodes but we observed a total of only seven spontaneous bleeding episodes. Two additional bleeding episodes were due to hemostatic challenge caused by a fighting incident and a foot injury. These bleeding episodes in response to a hemostatic challenge such as trauma are consistent with observations in patients who express 1–5% of normal FVIII levels and require treatment under these circumstances. Notably, one bleeding episode occurred early after vector administration when the FVIII activity was not detectable. Two of the nine documented bleeding episodes in treated dogs were in high-dose animals, while seven of the nine episodes were in low-dose animals. Two untreated HA dogs that were followed concurrently with the dogs in our study had 12 bleeding episodes in 36 months. Overall, >90% of the expected bleeding episodes were prevented.

At the time of vector administration and throughout the study, clinical blood chemistries were followed (**Supplementary Table S1**). During the first week following vector administration, the two of the five dogs that were administered the two-chain vector *via* the portal vein in an open surgical procedure had mild elevations in liver function tests (alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase). In addition, several dogs had low platelet counts and/or hemoglobin levels, perhaps due to blood loss and/or the fluids administered at the time of surgery. No clinical sequelae were noted (e.g., fever, inappetance, nausea). Thus, acute liver toxicity was observed that was not long term. Notably, none of the dogs treated with the single-chain vectors *via* an intravenous infusion had any changes in blood chemistries. There was no evidence of chronic liver toxicity in any of the dogs.

Table 2 Frequency of bleeding episodes after AAV vector administration

FVIII delivery approach	AAV vector dose	Hemophilia A dog	Number of months evaluated	Bleeding episodes			
				Spontaneous	Due to trauma	Total	Expected[a]
Two-chain	High	F24	26	0	0	0	11
		Woodstock	53	1	0	1	22
		J60	39	0	0	0	16
	Low	Linus	51	3[b]	1	4	21
		H19	68	1	0	1	28
Single-chain	High	M06	19	1	0	1	8
		M50	14	0	0	0	6
	Low	L51	24	1	0	1	10
		M66	3	0	1	1	1
Total			**297 months**	**7**	**2**	**9**	**123**
Untreated controls	NA	HA-1	24	8	0	8	10
		HA-2	12	4	0	4	5
Total			**36 months**	**12**	**0**	**12**	**15[a]**

Abbreviations: AAV, adeno-associated virus; FVIII, factor VIII; HA, hemophilia A; NA, not applicable.
[a]Expected is five bleeding episodes per year. [b]One bleed occurred early after vector administration when FVIII activity was not detectable (0%).

DISCUSSION

Early phase I clinical trials using AAV vectors for the treatment of genetic diseases including hemophilia B hold therapeutic promises for HA. However, the development of AAV-based approaches for HA that reach therapeutic levels in large animal models of HA has been challenging. In this study, we demonstrate long-term dose-dependent expression of therapeutic levels of FVIII in HA dogs using either a single- or two-chain delivery approach. Overall, we conclude that both approaches exhibit comparable, but not identical, efficacy and safety profiles. The use of novel species-specific reagents offers us the opportunity to characterize in detail the expression levels and immune responses to the neotransgene.

Therapeutic levels of FVIII were observed in all of the HA dogs treated with either the single- or two-chain delivery approach. However, only the high-dose cohorts had therapeutic levels of FVIII that were sustainable (**Table 1**). These dogs had a mild phenotype based on their FVIII activity and clinical phenotype. The low-dose cohorts were on the threshold of a moderate phenotype with levels of FVIII that were 0.5–2%. These FVIII levels also correlated with clinical observations that most of the bleeding episodes occurred in the dogs treated at low AAV doses (**Table 2**).

FVIII antigen level analysis found equivalent amounts of light and heavy chain in the circulation after single-chain delivery that correlate closely with the FVIII activity. The two-chain approach relies on cellular transduction by both the heavy- and light-chain vectors to generate biologically active FVIII. We observed that the light chain is 4–8 times more abundant than the heavy chain, which is a moderate imbalance compared to remarkable differences in the two chains reported in HA mice.[18] In the dogs, the amount of FVIII activity correlated closely with the amount of heavy chain in the circulation and probably represents cells that are cotransduced. As FVIII is a heterodimer, this suggests that in the two-chain approach, the heavy chain circulates predominantly in complex with the light chain. In contrast, the excess amount of circulating light chain may be due to its inherent stabilization by von Willebrand Factor[20,21] or differences in the cellular processing and/or secretion of the two chains.[18] Nevertheless, both the single- and two-chain approaches result in biologically functional protein.

Notably, no long-term evidence for inhibitors to FVIII was observed in any dogs. However, a transient neutralizing antibody to the transgene that was observed in one dog spontaneously resolved within 2 months. There are several hypotheses to explain why this dog (L51) developed an antibody to FVIII. One hypothesis is that this dog may have been prone to development of inhibitors. L51 was a member of a pedigree that includes several litters of inhibitor prone dogs, a subcolony of HA dogs at the Chapel Hill dog colony that is currently being characterized.[22] L51 did not have any evidence for anti-cFVIII antibodies before entering into this study. In fact, this dog had been administered recombinant cFVIII protein three times in an earlier study[17] before vector administration. Another hypothesis is that the levels of the FVIII antigen were too low (<1%). In the single-chain approach, the activity and antigen levels are equivalent. In contrast, in the two-chain approach, the activity levels were low but antigen levels were more than fivefold higher than activity. We speculate that high antigen levels may avoid immune responses to the transgene even in the absence of activity as seen in patients with mutations, which result in a cross-reacting material positive phenotype. Although we did observe a chain imbalance in the two-chain approach, this did not have any measurable detrimental effects on phenotypic correction or immune response. Furthermore, the transient nature of the antibodies observed in L51 followed by sustained expression of FVIII in the absence of antibodies suggests that tolerance to FVIII may occur with continuous expression of FVIII after AAV administration in the liver. Supportive evidence comes from studies in hemophilia B mice and nonhuman primates, which demonstrated that hepatic delivery of AAV-FIX induces tolerance to FIX that is dependent on regulatory T cells.[23–25] Overall, the risk of inhibitor development in these dogs is very low in contrast to the 20–30% risk in patients after protein therapy.

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Because none of the AAV-treated dogs had any evidence for an immune response to either plasma-derived or recombinant cFVIII-BDD before AAV delivery, we challenged the dogs by repeated injection of recombinant cFVIII protein after vector injection. None of the dogs developed an immune response to FVIII, which is consistent with our previous report in naive HA dogs challenged with the same cFVIII protein.[17] Notably, a dog with no detectable FVIII activity (H19) and another dog with no detectable FVIII antigen or activity (L51) at the time of the protein challenges did not develop antibodies to FVIII. Collectively, the data suggest that AAV-cFVIII does not increase the risk of antibody formation to cFVIII in noninhibitor prone dogs compared to protein replacement. Furthermore, these data suggest that new baseline levels provided by AAV-FVIII can be achieved without complicating further infusion of the protein such as may be required to prevent bleeding during surgical procedures or trauma-related bleeding episodes.

The disease phenotype improved significantly when compared to untreated dogs followed in parallel with the experimental group or by estimating the expected number of bleeds per dog. We determined that >90% of the bleeds were prevented, corresponding to a frequency of 0.4 bleeds per year per dog. These data are in agreement with the rates of 0.47 per year per person bleeding in HA boys undergoing intensive primary prophylaxis and in contrast to those boys on therapy on demand (rates of 4.9 per year per person), *i.e.* in response to bleeds.[2] Further objective studies on the joint status in AAV-treated HA dogs and controls will be important to define whether there is an impact on the underlying arthropathy.

One goal of this study was to understand whether the single- or two-chain approach demonstrated an advantage for consideration in translation to clinical application. Therapeutic levels of FVIII can be achieved with both approaches with similar total vector dosage using intravenous or portal vein delivery. In terms of immune responses to FVIII, it is notable that even at low antigen levels, the immune response to FVIII was only transient and resolved spontaneously within a relatively short period of time as demonstrated by L51. In both approaches, there was no evidence of an immune response to FVIII even when activity is <1% of normal. In an ongoing study, we have demonstrated that two-chain AAV delivery induces tolerance to FVIII in dogs that have inhibitors to FVIII before vector administration.[22] The administration of single chain vector in these dogs may be a more stringent challenge for determining the ability of that vector to induce tolerance to FVIII. Each AAV vector carries a risk of insertional mutagenesis[26] thus in the single chain delivery the risk of insertional mutagenesis for particles that would not produce functional FVIII would be avoided, theoretically favoring this strategy.

Ongoing phase I clinical trials for AAV-mediated delivery of FIX for hemophilia B will provide insight as to how to move forward with gene therapy for HA. One trial uses AAV2-hFIX with transient immune suppression to avoid immune responses to AAV capsid, while a second trial uses AAV8 to deliver a self-complementary vector encoding FIX.[15,16] Recently, data in one subject of the latter trial showed sustained expression of FIX at levels of 2% of normal (3 months follow-up, ongoing observation) without immune suppression (A. Davidoff, St. Jude Children's Research Hospital, personal communication, 9 September 2010). It will be important to define whether cytotoxic T lymphocyte responses to AAV capsid, which present cross-reactivity among distinct serotypes, will also hamper the safety of this strategy as the dose-escalation study progresses. Thus, it will be important to determine whether immune suppression will be required for AAV8 delivery. The major limitation of this study is the relatively high vector dose required to achieve therapeutic levels of FVIII. If the effectiveness of the immune suppression on the immune response to AAV is vector dose–dependent, then several options for lowering the vector dose can be explored for FVIII including the use of self-complementary vectors to deliver the two-chain approach,[27] introduction of tyrosine mutations in the AAV capsid to augment the transduction efficiency,[28] and the use of codon-optimized FVIII. In summary, in the canine model of HA we determined that AAV-FVIII liver gene therapy is safe and feasible. The long-term follow-up of sustainable expression of a nonimmunogenic transgene that improves the disease phenotype in a dose-dependent manner fulfills the basic requirements to support clinical application.

MATERIALS AND METHODS

cFVIII transgene constructs. Two-chain cFVIII constructs were previously described.[10] Single-chain cFVIII constructs were modified from the previously described construct (**Supplementary Materials and Methods**).[10]

AAV vector production. Recombinant AAV vectors were produced by triple transfection and purified by CsCl-gradient centrifugation as previously described.[29] After purification, endotoxin testing was performed by Endochrome-K (Charles River Laboratories, Wilmington, MA) or Limulus Amebocyte Lysate gel-clot method (QCL-1000; Bio Whittaker, Walkersville, MD). Vector titers were obtained by Taqman PCR (Applied Biosystems, Foster City, CA). The two-chain AAV vectors were generated at the Penn Vector Core at The University of Pennsylvania and the single-chain vectors were generated in the Children's Hospital of Philadelphia Research Vector Core.

AAV vector administration in HA mice. Male HA exon 16 knockout mice[30,31] on a C57BL/6/129Sv background or HA exon 16/CD4 knockout mice were generated in our laboratory. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. AAV vectors were administered to 6- to 8-week-old male mice (n = 5 mice/vector) at a volume of 200 µl of phosphate-buffered saline/5% sorbitol *via* tail-vein injection. Plasma samples were collected in 3.8% sodium citrate by tail sectioning.

AAV vector administration and recombinant protein challenges in HA dogs. The HA dogs are maintained at the University of North Carolina, Chapel Hill. All procedures in the dogs were approved by the Institutional Animal Care and Use Committee at the University of North Carolina. All of the dogs in the study were male except for J60. The two-chain AAV vectors (AAV-cFVIII-HC and AAV-cFVIII-LC) were coadministered *via* the portal vein as previously described[5] using 6×10^{12} and 1.25×10^{13} vector genomes/vector/kg. The animals were treated with normal dog plasma (150–200 cc normal dog plasma) before and immediately following vector administration. For intravenous administration of the single-chain AAV vector (2×10^{13} and 4×10^{13} vector genomes/kg), the vector was infused *via* the saphenous vein at a total volume of 10 ml/kg phosphate-buffered saline. This procedure did not require FVIII treatment. Hematologic and biochemical analysis of blood and serum samples were performed in a clinical pathology laboratory. For the immunologic challenges, the dogs were challenged with 2.5 µg/kg (25 IU/kg) of cFVIII protein one time per week for four consecutive weeks. Plasma samples were drawn before and 15 minutes after each protein infusion for FVIII activity, antibody and

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recovery analysis. Recovery analysis was determined by subtracting the baseline FVIII activity from the activity 15 minutes after vector infusion to determine the contribution of the recombinant protein to the activity measurements. The percent recovery of the protein was calculated based on the amount of infused recombinant protein (25 IU/kg).

cFVIII antigen, activity, and antibody assays. Normal canine plasma was used as a standard for the quantification of the cFVIII activity using the Chromogenix Coatest SP4 FVIII (Diapharma, Lexington, MA). WBCT was performed as previously described.[32] Thromboelastography was performed using citrated whole blood drawn and tested within 2 minutes after collection using thromboelastography 5000 Thromboelastograph Analyzer (Haemoscope, Niles, IL) according to the manufacturer's instructions. The first 3 ml is discarded and 360 µl is placed per cup after premixing with 1 ml with kaolin. Thromboelastography recordings are allowed to proceed for ~90 minutes.[33] cFVIII heavy- and light-chain antigens were detected using a cFVIII-specific enzyme-linked immunosorbent assay (ELISA).[17] Anti-cFVIII antibodies were detected by Bethesda assay[34] or by cFVIII-specific IgG1 and IgG2 antibodies by ELISA.[17] IgG ELISA and cFVIII activity measurements were performed simultaneously on the same plasma samples.

Statistical analysis. An unpaired two-tailed Student's *t*-test was used for statistical analysis, with $P < 0.05$ indicating statistical significance.

SUPPLEMENTARY MATERIAL

Figure S1. Single chain cFVIII expression constructs.
Figure S2. Hydrodynamic delivery of pAAV-cFVIII gene constructs.
Figure S3. Thromboelastography of HA dogs after single chain delivery of cFVIII.
Table S1. Clinical blood chemistries after vector administration.
Materials and Methods.

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