Translational Data from Adeno-Associated Virus-Mediated Gene Therapy of Hemophilia B in Dogs

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Abstract

Preclinical testing of new therapeutic strategies in relevant animal models is an essential part of drug development. The choice of animal models of disease that are used in these studies is driven by the strength of the translational data for informing about safety, efficacy, and success or failure of human clinical trials. Hemophilia B is a monogenic, X-linked, inherited bleeding disorder that results from absent or dysfunctional coagulation factor IX (FIX). Regarding preclinical studies of adeno-associated virus (AAV)-mediated gene therapy for hemophilia B, dogs with severe hemophilia B (<1% FIX) provide well-characterized phenotypes and genotypes in which a species-specific transgene can be expressed in a mixed genetic background. Correction of the hemophilic coagulopathy by sustained expression of FIX, reduction of bleeding events, and a comprehensive assessment of the humoral and cell-mediated immune responses to the expressed transgene and recombinant AAV vector are all feasible end points in these dogs. This review compares the preclinical studies of AAV vectors used to treat dogs with hemophilia B with the results obtained in subsequent human clinical trials using muscle- and liver-based approaches.

Introduction

THE SUCCESSES OF ADENO-ASSOCIATED VIRUS (AAV)-mediated gene therapy of hemophilia B in humans have been welcome and exciting advances.[1–6] Through detailed studies of the relatively small number of people with hemophilia B successfully treated with gene therapy to date, investigators have also identified barriers to more widespread applicability of this approach including preexisting antibodies to AAV, manufacturing challenges for large-scale production of AAV vectors, and the need for long-term follow-up to identify potential safety issues.[7] This review focuses on the role of dogs with hemophilia B in the preclinical muscle- and liver-based gene therapy studies that have progressed to human trials and discusses current research directions that target these barriers.

The ability to monitor correction of the hemophilic coagulopathy and the frequency of bleeding events is perhaps the strongest reason for using dogs with hemophilia B in preclinical studies.[8–11] Also, most of the dogs that have been used weighed 20 or more kilograms, and thus scaling up to humans is in the range ∼3- to 10-fold; as opposed to mice, which weigh ∼25 g, and constitute an ∼2800-fold scale-up. The size of dogs places demands on manufacturing that can slow progress if vector production is limiting. Next, dogs provide a relevant model for identifying the challenges involved with targeting gene therapy to skeletal muscle or liver in humans. For example, transducing discrete areas of skeletal muscle (within 0.5 cm of the injection site) or liver in dogs is likely to be more informative of the transduction of human tissues than the often widespread transduction in mice. Finally, most strains of mice are inbred whereas the available hemophilic dogs are generally outbred. Dogs thus more faithfully model the clinical situation, where immune responses to recombinant gene therapy vectors occur in the context of a highly heterogeneous human population.

Canine Hemophilia B

Severe canine hemophilia B (<1% factor IX [FIX] activity or antigen) recapitulates both the genotypes and phenotype that occur in humans with severe human hemophilia B.

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TABLE 1. RECOMBINANT ADENO-ASSOCIATED VIRUS-MEDIATED GENE THERAPY FOR CANINE AND HUMAN HEMOPHILIA B IN SKELETAL MUSCLE

	Mutation	M/F	Vector	Route	Vector genomes/kg	IS	Follow-up	FIX (ng/ml)	FIX activity (%)	Anti-FIX (BIU)
Canine Hemophilia B										
Herzog et al.[20,21]; Arruda et al.[22]										
E57	G→A G379E	M	AAV-1-CMV PK9	IM	2.4×10^{11}	Y	2.75 yr	104		2–8.5
E35	G→A G379E	F	AAV-1-CMV PK9	IM	1.0×10^{12}	N	9.5 mo	87		8–15
B45	G→A G379E	M	AAV-2-CMV-cFIX	IM	1.3×10^{11}	N	3.5 yr	2.6±0.7		n.d.
B46[b]	G→A G379E	M	AAV-2-CMV-cFIX	IM	1.1×10^{12}	N	14 mo	12±2		n.d.
B93	G→A G379E	M	AAV-2-CMV-cFIX	IM	3.4×10^{12}	N	3 yr	17±2		n.d.
B48	G→A G379E	F	AAV-2-CMV-cFIX	IM	3.0×10^{12}	N	3 yr	21±2		n.d. (IgG2+)
D32[b]	G→A G379E	M	AAV-2-CMV-cFIX	IM	5.6×10^{12}	Y	2.5 yr	40		n.d.
B85	G→A G379E	F	AAV-2-CMV-cFIX	IM	8.5×10^{12}	N	3.5 yr	69±6		≤6.8
D31	G→A G379E	M	AAV-2-CMV-cFIX	IM	8.5×10^{12}	N	3.25 yr	39		n.d. (IgG2+)
B14	G→A G379E	M	AAV-2-CMV-cFIX	IM	1.1×10^{13}	N	3 yr	69±6		≤24.5
Herzog et al.[16]										
Wilbur	5-bp del fs, C→T, 176X	M	AAV-2-CMV-cFIX	IM	1.0×10^{12}	Y	30 wk	34±9		IgG1 and IgG2
Wes[a]	5-bp del fs, C→T, 176X	M	AAV-2-CMV-cFIX	IM	1.1×10^{12}	N	30 wk	0		IgG1 and IgG2
Sauvignon	5-bp del fs, C→T, 176X	M	AAV-2-CMV-cFIX	IM	1.2×10^{12}	N	30 wk	0		IgG2
Clark[a]	5-bp del fs, C→T, 176X	M	0.5 mg cFIX protein IV	IV	n/a	N	30 wk	n/a		
Arruda et al.[27]										
F57	G→A G379E	M	AAV-2-CMV-cFIX	ILP	1.7×10^{12}	Y	27 mo	260±52	5.5±1.3	n.d.
H08	G→A G379E	M	AAV-2-CMV-cFIX	ILP	3.0×10^{12}	Y	8 mo	210±16	4.2±1.5	n.d.
D99	G→A G379E	F	AAV-2-CMV-cFIX	ILP	3.7×10^{12}	Y	39 mo	730±60	15±2	n.d.
E60	G→A G379E	F	AAV-2-CMV-cFIX	ILP	3.9×10^{12}	N	10 mo	0	<1	Yes
E59[b]	G→A G379E	F	AAV-2-CMV-cFIX	IV	2.9×10^{12}	Y	37 mo	31–78	1±0.4	n.d.
Arruda et al.[28]; Haurigot et al.[29]										
Low-dose										
I04	G→A G379E	M	AAV-2-CMV-cFIX	ATVRX	1.0×10^{12}	Y	5 yr	32±7		n.d.
I05	G→A G379E	M	AAV-2-CMV-cFIX	ATVRX	1.0×10^{12}	Y	5 yr	141±18		n.d.
J04	G→A G379E	M	AAV-2-CMV-cFIX	ATVRX	1.0×10^{12}	Y	3.5 yr	27±4		n.d.
Mid-dose										
H48	G→A G379E	M	AAV-2-CMV-cFIX	ATVRX	3.0×10^{12}	Y	3.75 yr	275±75		n.d.
H34	G→A G379E	F	AAV-2-CMV-cFIX	ATVRX	3.0×10^{12}	Y	5.25 yr	76±14		n.d.
I07	G→A G379E	F	AAV-2-CMV-cFIX	ATVRX	3.0×10^{12}	Y	4 yr	125±39		n.d.
J03	G→A G379E	M	AAV-2-CMV-cFIX	ATVRX	3.0×10^{12}	N	3.75 yr	81±13		n.d.
J62	G→A G379E	M	AAV-2-CMV-cFIX	ATVRX	3.0×10^{12}	N	3.25 yr	120		1.5
M14	G→A G379E	F	AAV-2-CMV-cFIX	IM	3.0×10^{12}	N	>2–4 mo	11±0.1		n.d.
M24	G→A G379E	F	AAV-2-CMV-cFIX	IM	3.0×10^{12}	N	>2–4 mo	10±0.1		n.d.
High-dose										
H24	G→A G379E	M	AAV-2-CMV-cFIX	ATVRX	8.5×10^{12}	Y	5.25 yr	20±3		n.d.
M25	G→A G379E	F	AAV-2-CMV-cFIX	ATVRX	8.5×10^{12}	Y	1 yr	23±3		n.d.

(continued)

TABLE 1. (CONTINUED)

	Mutation	M/F	Vector	Route	Vector genomes/kg	IS	Follow-up	FIX (ng/ml)	FIX activity (%)	Anti-FIX (BIU)
AAV-6-CMV-cFIX in dogs that had not received AAV2-cFIX										
M13		F	AAV-6-CMV-cFIX	ATVRX	3.0×10^{12}	Y	1yr	259±37		n.d.
M20		M	AAV-6-CMV-cFIX	ATVRX	3.0×10^{12}	Y	1yr	213±25		n.d.
AAV6-cFIX or AAV2-cFIX in dogs that had previously received AAV2-cFIX										
E59[b]	G→A G379E	F	AAV-6-CMV-cFIX	ATVRX	1.5×10^{12}	Y	4yr	259±50		n.d.
H27[b]	G→A G379E	F	AAV-6-CMV-cFIX	ATVRX	1.5×10^{12}	Y	3.5yr	89±47		n.d.
D32[b]	G→A G379E	M	AAV-2-CMV-cFIX	ATVRX	4.0×10^{12}	Y	2yr	<10		n.d.
B46[b]	G→A G379E	M	AAV-2-CMV-cFIX	ATVRX	4.0×10^{12}	Y	6mo	<10		n.d.
Finn et al.[31]										
N07	G→A G379E	M	AAV-6-cFIXR338L	ATVRX	2.62×10^{12}	Y	>5yr	0.65	5.5	n.d.
M55	G→A G379E	M	AAV-6-cFIXR338L	ATVRX	3.0×10^{12}	Y	>5yr	1.5	8	n.d.
M59	G→A G379E	M	AAV-6-cFIXR338L	ATVRX	3.0×10^{12}	Y	>5yr	0.35	3.5	n.d.
Human Hemophilia B										
Kay et al.[1]; Manno et al.[2]; Jiang et al.[3]										
Patient A	G→T R4L	M	AAV-2-CMV-hFIX	IM	2.0×10^{11}	N	3.7yr		1.4	n.d.
Patient B	G→C A352P	M	AAV-2-CMV-hFIX	IM	2.0×10^{11}	N	3.7yr		<1	n.d.
Patient C	G→C G114R	M	AAV-2-CMV-hFIX	IM	2.0×10^{11}	N	3.7yr		<1	n.d.
Patient D	T→C C18A	M	AAV-2-CMV-hFIX	IM	6.0×10^{11}	N	3.7yr		<1	n.d.
Patient E	G→A G184R	M	AAV-2-CMV-hFIX	IM	6.0×10^{11}	N	3.7yr		<1	n.d.
Patient F	T→C S110P	M	AAV-2-CMV-hFIX	IM	6.0×10^{11}	N	3.7yr		1	n.d.
Patient G	C→T R180W	M	AAV-2-CMV-hFIX	IM	1.8×10^{12}	N	3.7yr		<1	n.d.
Patient H	C→A P368T	M	AAV-2-CMV-hFIX	IM	1.8×10^{12}	N	3.7yr		<1	n.d.

[a]Combined mutations in FIX and pyruvate kinase genes.
[b]Denotes dogs that received more than one injection of AAV vector at different points in time.
BIU, Bethesda inhibitor units; bp, base pair; del, deletion; F, female; FIX, factor IX; fs, frameshift; IM, intramuscular; IS, immunosuppression (Y, yes; N, no); M, male; n/a, not applicable; n.d., not detected.

Inheritance occurs in an X-linked manner. Like their human counterpart, hemophilia B dogs exhibit bleeding into soft tissues and joints that is spontaneous and severe; without prompt treatment with FIX, the bleeds can be crippling or fatal.[10] The bleeding events are random but occur with a measurable frequency over time. Reduction in the frequency of annualized bleeding events can be used as a metric for judging success of a therapeutic intervention in these dogs.[11]

Two hemophilia B dog models with different mutations and immune responses to canine FIX have been used. First, the Chapel Hill strain has a missense mutation, a G-to-A substitution at nucleotide 1477, that results in the substitution of glutamic acid for glycine at position 379 in the catalytic (serine protease) domain of the molecule.[12] Amino acid 379 in canine factor IX corresponds to position 381 in human factor IX, an amino acid that has been rigorously conserved among the trypsin-like serine proteases throughout evolution. This mutation results in a complete lack of circulating factor IX in the affected animals.[13] This strain only rarely makes inhibitory antibodies in response to intravenous administration of canine FIX. A large number of missense mutations have been reported in human hemophilia B, and at least one occurs at the same location.[14] This patient has severe hemophilia B and was reported as not making inhibitory antibodies to FIX to date.

A second strain of hemophilia B dogs that has been used has a deletion mutation at nucleotides 772–777 and a C-to-T transition at nucleotide 777.[15] The mutation results in a premature stop codon at amino acid residue 146, which is just before the nucleotide sequence encoding the activation peptide. This strain has a propensity to develop inhibitory antibodies to canine FIX after intravenous administration of this protein.[16] Likewise, several deletion mutations have been reported in human hemophilia B and at least one occurs at the same location in association with moderate to severe hemophilia B.[17,18] The inhibitor status of humans with the homologous deletion mutation has not been reported. Immunologic challenges with canine FIX protein in these two strains of hemophilia B dogs, however, have shown that the risk of developing an inhibitory antibody to FIX is substantially higher in the null mutation when compared with missense mutation. Thus, the difference in inhibitor formation exhibited by these two models provides a stringent strategy for addressing the immune responses to FIX that are the major safety concern in the development of novel strategies for treating hemophilia B.

Skeletal Muscle-Based Gene Therapy for Hemophilia B

Rationale

Skeletal muscle-based gene therapy for hemophilia B was pursued for several reasons. First, target muscles were chosen that were easily accessible, would limit vector biodistribution, and that could be removed if gene transfer resulted in unanticipated adverse events. Because there was no prior experience with parenteral administration of AAV in humans, this latter issue was a potential concern. Fortunately, to date, muscle removal has not been necessary. Second, many people with hemophilia also have liver disease due to prior infections from contaminants in replacement products of human origin. The safety of administering AAV vectors to the liver in the presence of advanced liver

disease has not been established. For people with concurrent hemophilia B and liver disease, a muscle platform is potentially an attractive alternative for AAV-mediated gene therapy. Third, posttranslational modifications of FIX that are critical for activity, such as γ-carboxylation of the N-terminal glutamic acid residues, are supported by skeletal myocytes.[19] Two approaches for AAV-based gene transfer to skeletal muscle have been tried in dogs and are discussed below.

Direct intramuscular injection of AAV vectors

A series of studies have been reported in which AAV vectors were administered by direct intraskeletal muscle injection (Table 1).[16,20–22] AAV serotypes 1, 2, and 6 have been tested. In the hemophilia B dogs with the missense mutation that only rarely develop inhibitors to canine FIX, a series of findings helped identify a safe dose for the design of the clinical trial in humans. First, AAV-1 appeared to be associated with an increased risk of inhibitor formation to FIX, and cyclophosphamide did not prevent the antibody formation in the small number of animals tested.[22] In contrast, when AAV-2 was given by intramuscular injection, a threshold dose of up to 8.5×10^{12} vector genomes (VG)/kg distributed over several intramuscular sites at a single sitting appeared to be associated with transient or no inhibitor formation.[22] An especially important issue that became evident was that increasing the dose of vector injected at a single intramuscular site resulted in a higher frequency of inhibitory antibodies.[21] The development of AAV-1 vectors with more robust efficacy in transducing skeletal muscle in comparison with AAV-2 vectors did allow us to test whether decreasing the vector dose per site would be safe and efficacious. Even with reducing the vector dose per site by 5- to 20-fold, however, inhibitory antibodies to FIX were still detected. By comparison, in the hemophilia B dogs with the deletion mutation, neutralizing anti-FIX antibodies developed with intramuscular injection of the same AAV-2 vector at a dose of 1×10^{12} VG/kg.[16] Thus, with the intramuscular injections, the risk of forming anti-FIX inhibitory antibodies is influenced by the AAV serotype, the dose of vector and especially the dose per site, as well as the underlying mutation. The role of the serotype likely relates to the amount of FIX produced locally, whereas the role of the causative mutation relates to the degree of tolerance to FIX.

Although this compendium of information provided critical safety data needed for designing the clinical trials that used a muscle platform in humans as discussed below, it also became clear that the number of intramuscular injections needed to administer a therapeutic vector dose was too high to be practical clinically. Thus, different methods were needed for delivering therapeutic doses of AAV vector to skeletal muscle. Because skeletal muscle exhibits one of the highest densities of capillaries in the body, the use of intravascular delivery was considered an attractive alternative strategy for achieving a widespread area of muscle transduction.

Isolated limb perfusion and afferent transvenular retrograde extravasation

Two intravascular approaches for transduction of large amounts of skeletal muscle have been pursued in hemophilia

B dogs. First, using an approach initially developed for delivering chemotherapy to an isolated limb in humans[23–25] and gene therapy of large muscle groups in animal models of muscular dystrophy,[26] isolated limb perfusion (ILP) was performed by surgically isolating and cannulating the femoral artery and vein while a tourniquet was applied proximally.[27] Transient (6 weeks) immunomodulation with cyclophosphamide was given concurrently to prevent the development of anti-FIX antibodies. Notably, long-term (> 3 years) FIX expression with circulating levels of 4–15% was achieved (Table 1). These levels were substantially higher than what was observed with intravenous infusion of the same vector but without the ILP procedure. Omission of immune suppression was accompanied by inhibitory anti-FIX antibody formation. Immunohistochemistry of muscle biopsies confirmed that FIX expression was present in several muscle groups from the treated limb. This ILP procedure included administering papaverine for vasodilation and histamine to increase vascular permeability.[26] Because histamine is not currently Food and Drug Administration (FDA)-approved for use in humans and has proinflammatory effects, this procedure needed modification before translation to humans. These data, however, did provide proof-of-concept supporting the rationale for intravascular delivery of AAV vectors to skeletal muscle.

The second approach, afferent transvenular retrograde extravasation (ATVRX), is much simpler to perform. A tourniquet is applied at the hip to occlude blood flow and isolate the hind limb. Using a distal vein for access, the AAV vector is infused intravenously under pressure to increase vascular permeability.[28–30] As in the ILP approach, transient (6 weeks) immunomodulation with cyclophosphamide was also given to prevent the development of anti-FIX antibodies. Importantly, this approach produced widespread transduction of muscle and sustained, dose-dependent therapeutic levels of canine FIX up to 10-fold higher than those obtained by direct intramuscular injections. Although all animals developed a robust humoral antibody response to the AAV capsid, no T cell responses to the capsid antigen were detected by interferon (IFN)-γ enzyme-linked immunosorbent spot (ELISPOT).[29] Preliminary studies on interleukin (IL)-10 ELISPOT screening of lymphocytes showed reactivity to canine FIX-derived peptides, and restimulation of T cells *in vitro* in the presence of the identified canine FIX epitopes resulted in the expansion of $CD4^+FoxP3^+IL-10^+$ T cells. It is possible that these T cells may have suppressor activity and may contribute to modulation of the immune responses to the transgene product in AAV-mediated gene transfer, but additional studies are needed to confirm this idea.

The accumulating data on gene therapy for hemophilia B strongly suggest that lower AAV vector doses are less likely to induce an immune response regardless of the target organ.[4–6] The ATVRX procedure has also been used with a strategy to reduce vector dose while still expressing a therapeutic level of FIX, using the gain-of-function mutation FIX-R338L (Padua) that exhibits an 8- to 9-fold increase in specific activity of FIX.[31] To date, three hemophilia B dogs have received an AAV-6 vector containing canine FIX-R338L along with transient cyclophosphamide immunomodulation (Table 1). Neither anti-FIX antibodies nor T cell responses to FIX-R338L have been detected using a dose range of 2.6–3×10^{12} VG/kg. Remarkably, the net level of FIX activity (3.5–8%) is 8- to 9-fold higher in specific activity, as was

originally found in humans who inherited this gain-of-function mutation.[32] These dogs have not had any detectable thrombotic complications and have enjoyed a marked reduction in the frequency of bleeding events.

Several findings in these muscle-based recombinant AAV gene therapy studies deserve comment. First, multiple assays have been developed that provide a comprehensive assessment of the T and B cell-mediated immune responses to recombinant AAV vectors and the expressed FIX in dogs.[21,28,29] Second, transient immune modulation has been associated with circumventing immune responses that would otherwise limit the success of AAV-mediated gene transfer. Third, efficient muscle transduction can be achieved with the ATVRX procedure in hemophilia B dogs naive to recombinant AAV vectors and by using alternative AAV serotypes in hemophilia B dogs with high titers of neutralizing anti-AAV-2 antibodies from prior exposure in earlier gene transfer studies (Table 1).[28,29] At present, it is estimated that more than 40% of people with hemophilia B being screened as potential gene therapy candidates will be excluded on the basis of the presence of neutralizing antibodies to AAV-2 or other serotypes brought about by prior exposure to wild-type virus earlier in life. Delivery through an ATVRX procedure, in which the closed circulation is first flushed with normal saline, may make it possible to use skeletal muscle even in those subjects with neutralizing antibodies (although this has not yet been demonstrated experimentally). Fourth, canine FIX can be modified to recapitulate the human R338L gain-of-function mutation to help develop strategies for avoiding immune responses to AAV vectors by lowering the dose required for therapeutic expression.

Muscle-based gene therapy for human hemophilia B

To date, one muscle-based clinical trial has been conducted in hemophilia B and the AAV vector was administered by direct intramuscular injections (Table 1). This was the first trial of intramuscular injection of an AAV vector for any disease. This phase 1 safety study used the same AAV-2 CMV-FIX vector that had been tested in the hemophilia B dogs except that the transgene was human rather than canine.[1–3] Guided by the results in the hemophilia B dogs, patients with missense mutations were selected for study. As expected, based on the preclinical studies in dogs and other species (not discussed), expression levels were low (< 1% to 1%) but no serious adverse events have been reported with follow-ups ranging up to 4 years. Three important findings from the human study that were presaged by the preclinical studies in hemophilia B dogs were as follows: (1) intramuscular injection of AAV-FIX at doses up to 2×10^{12} VG/kg in humans was safe, with no evidence of formation of inhibitory antibodies to FIX or other toxicities; (2) the characteristics of skeletal muscle transduction by AAV-2 were similar in humans, dogs, and mice; and (3) local transgene expression appeared stable over a period of up to 10 years after vector injection.[3,33] As discussed previously, the number of intramuscular injections required for this approach to achieve clinically therapeutic levels of FIX will likely preclude further development of the direct intramuscular injection approach at this time. It is reasonable to suggest that the ATVRX procedure combined with the FIX-R338L gain-of-function variant or alternate serotypes of AAV vectors would be acceptable for

muscle-based gene transfer for hemophilia B subjects with severe underlying liver disease or high titers of neutralizing to recombinant AAV capsid antigens that would exclude them from being enrolled in liver-based gene therapy.

Liver-Based Gene Therapy for Hemophilia B

Rationale

Because FIX is normally produced in hepatocytes, the liver is a natural target for gene therapy of hemophilia B. AAV vectors with a natural tropism for the liver, inclusion of liver-specific promoters in expression cassettes, and multiple routes of administration have been used to drive expression of therapeutic levels of FIX. Another benefit of targeting the liver is that immune tolerance to antigens expressed from an AAV vector in the liver has been well documented.[34,35] This immune tolerance is due, at least in part, to the induction of antigen-specific regulatory T cells.[36,37] Such a scenario would be expected to decrease the likelihood of the formation of anti-FIX antibodies after liver-based gene therapy. As discussed below, an unexpected T cell-mediated immune response to recombinant AAV vector capsid proteins occurred in humans after liver-based gene therapy. Although this event initially threatened the success of gene transfer, fortunately, a short course of prednisolone, administered with appropriate timing, was associated with interruption of this immune response and preservation of FIX expression.

Portal and peripheral vein administration of AAV vectors for canine hemophilia B

Both the portal vein and peripheral vein routes of administration have been reported in hemophilia B dogs, using AAV vectors made from serotypes 2, 5, and 8 (Table 2).[38–42] Both strains of hemophilia B dogs were included and in all cases expression of FIX was detected. Only one dog exhibited transient anti-FIX antibodies, and this specific hemophilia B dog (Beech) had the null mutation in the FIX gene described previously, another genetic mutation resulting in pyruvate kinase deficiency that is associated with iron overload in the liver, and anti-phospholipid antibodies.[40] Also, in a separate study, dogs that previously had been given AAV-2 vectors by portal vein were subsequently given pseudotyped AAV-2/8 by repeat portal vein administration, and these retreated dogs exhibited higher levels of FIX expression (Table 2).[41] This finding raises the possibility that some human subjects may achieve higher levels of circulating FIX with repeat liver-based gene therapy using different serotypes of AAV or modifications of the AAV capsid proteins.

Liver-based gene therapy for human hemophilia B

The preclinical information in dogs suggested that the humoral immune response to FIX in liver-based gene therapy for hemophilia B would be acceptable for both missense and deletion mutations, and this has been the case so far in human subjects who have been infused with AAV vectors. In the first study, administration of the AAV-2 vector via the hepatic artery was well tolerated and expression levels were initially in a therapeutic range (Table 2).[4] The vector dose response that had been detected in hemophilia B dogs with liver-based gene therapy was also initially seen in these subjects. Unexpectedly, FIX expression declined to baseline over ~8 weeks, and was accompanied by a reversible and asymptomatic elevation in liver transaminases.[4] Over time, detailed investigations generated evidence to support the hypothesis that the transduced hepatocytes had been destroyed by a CD8$^+$ T cell-mediated immune response that targeted antigens of the AAV capsid.[43] Thus, this study showed that recombinant AAV-2 vectors could successfully transduce human hepatocytes *in vivo* as had been shown in other species, but that a modification of the protocol was necessary to achieve long-lasting expression in humans.

Armed with this information, a self-complementary AAV-8 vector was given via a peripheral vein to humans with hemophilia B[5,6] with a broader range of molecular defects than were included in the first liver-based trial (Table 2). With doses below 2.0×10^{12} VG/kg, the subjects exhibited stable expression of FIX in the range of 1.4–2.86% of normal FIX activity. With doses at or above 2.0×10^{12} VG/kg, four of six subjects developed elevations in liver transaminases and a reduction in FIX expression. Short-term administration of prednisolone was associated with interruption of the capsid-specific T cell response and prevention of further loss of FIX expression. Despite this need for immunomodulation at higher doses in these four subjects, persistent, long-term expression of FIX has now been documented. As in the first liver-based trial, no study subjects have developed inhibitory antibodies to FIX to date. The ranges of FIX expression levels are consistent with conversion of the hemophilia B phenotype from severe to mild or moderate. Indeed, this has been the case because most of these subjects have either reduced their dependence on prophylactic FIX infusions or stopped prophylaxis altogether. Spontaneous bleeding events have been infrequent to rare and, when they have occurred, bleeding has been easily managed with standard FIX replacement therapy. The follow-up in this liver-based trial is relatively short (~3 years), but the sustained expression of FIX that has been observed for >10 years in many hemophilia B dogs provides an encouraging outlook for a much longer period of benefit.

Discussion

The results of preclinical gene therapy studies in dogs with hemophilia B have helped investigators design safe and efficacious AAV-mediated gene therapy trials in people with hemophilia B. To date, the formation of inhibitory antibodies to FIX has been circumvented in both muscle- and liver-based human trials at least in part by using the results obtained from studies in dogs. Also, results showing long-term (years) expression of FIX from both muscle and liver have been successfully translated from dogs to humans. The reduction in frequency of bleeding events and use of factor IX products enjoyed by the successfully treated dogs has also been translated to the participants in the second liver-based trial. This improvement in bleeding phenotype was a secondary outcome of study design in humans but of primary importance to the field of gene therapy.

The T cell-mediated immune response to the AAV vector that occurred in the liver-based trials, however, was not predicted in preclinical studies in any species. The reasons for this apparent species-specific immune response to recombinant AAV gene therapy vectors for hemophilia B are

TABLE 2. RECOMBINANT ADENO-ASSOCIATED VIRUS-VECTOR MEDIATED GENE THERAPY FOR CANINE AND HUMAN HEMOPHILIA B IN LIVER

	Mutation	M/F	Vector	Route	Vector genomes/kg	IS	Follow-up	FIX (ng/ml)	FIX activity (%)	Anti-FIX (BIU)
Canine Hemophilia B										
Snyder et al.[39]										
B89	$G{\to}A$ G379E	M	AAV-2-MFG-cFIX	PV	1.8×10^{11}	N	8 mo	10→45		n.d.
B84	$G{\to}A$ G379E	F	AAV-2-MFG-cFIX	PV	2.3×10^{11}	N	8 mo	30→95		n.d.
Mount et al.[40]										
E34	5-bp del fs, $C{\to}T$, 176X	F	AAV-2-(ApoE)₄/hAAT-cFIX	PV	8×10^{11}	N	1 yr	262±92	5±2.5	n.d.
Brad	5-bp del fs, $C{\to}T$, 176X	M	AAV-2-(ApoE)₄/hAAT-cFIX	PV	1.2×10^{12}	N	1 yr	590±150	8.5±2	n.d.
Semillion	5-bp del fs, $C{\to}T$, 176X	M	AAV-2-(ApoE)₄/hAAT-cFIX	PV	1.6×10^{12}	N	1 yr	220±65	5±2.5	n.d.
Beech[a]	5-bp del fs, $C{\to}T$, 176X[a]	M	AAV-2-(ApoE)₄/hAAT-cFIX	PV	3.4×10^{12}	N	1 yr	≤2560	≤3	Yes
Wang et al.[41,42]										
C51	$G{\to}A$ G379E	F	AAV-2-LSP-cFIX	PV	3.7×10^{11}	N	1 yr	3.6±0.5		n.d.
C55	$G{\to}A$ G379E	M	AAV-2-LSP-cFIX-WPRE	PV	4.6×10^{12}	N	6 mo	218.1±26.5		n.d.
H12	$G{\to}A$ G379E	F	AAV-2/8 LSP-cFIX-W	PV	5.25×10^{12}	N	68 wk	468±109.6	9.4±2.2	n.d.
G43	$G{\to}A$ G379E	M	AAV-2/8 LSP-cFIX-W	PV	5.26×10^{12}	N	62 wk	1297.4±235.2	25.9±4.7	n.d.
D39	$G{\to}A$ G379E	F	1. AAV-2-LSP-cFIX-WPRE 2. AAV-2/8 LSP-cFIX-W	PV	2.8×10^{12} 9.28×10^{12}	N	3 mo 262 wk	31.7±3.2 785.4±61.6	15.7±1.2	n.d.
C52	$G{\to}A$ G379E	M	1. AAV-2-LSP-cFIX 2. AAV-2/5 LSP-cFIX-W	PV	2.8×10^{11} 2.3×10^{13}	N	1 yr 303 wk	2.9±0.7 798.5±84.4	16.0±1.7	n.d.
Harding et al.[38]										
F56	$G{\to}A$ G379E	M	AAV-2-LSP-βgb-cFIX-WPRE	IV	2.2×10^{12}	N	7 mo	<5		n.d.
G10	$G{\to}A$ G379E	M	AAV-2-LSP-βgb-cFIX-WPRE	IV	1.9×10^{13}	N	7 mo	30±4.84		n.d.
Human Hemophilia B										
Manno et al.[4]										
Subject A	R16X	M	AAV-2-hAAT-FIX	HA	8.0×10^{10}	N	12 mo		<1%	n.d.
Subject B	W310X	M	AAV-2-hAAT-FIX	HA	8.0×10^{10}	N	40 mo		<1%	n.d.
Subject C	—[b]	M	AAV-2-hAAT-FIX	HA	4.0×10^{11}	N	34 mo		<1%	n.d.
Subject D	R180Q	M	AAV-2-hAAT-FIX	HA	4.0×10^{11}	N	33 mo		<1%	n.d.
Subject E	W407X	M	AAV-2-hAAT-FIX	HA	4.0×10^{11}	N	15 mo		<1%	n.d.
Subject F[c]	G133E	M	AAV-2-hAAT-FIX	HA	2.0×10^{12}	N	31 mo		11%	n.d.
Subject G	R-4Q[c]	M	AAV-2-hAAT-FIX	HA	2.0×10^{12}	N	30 mo		3%	n.d.
Nathwani et al.[5,6]										
Patient 1	$G{\to}A$ E387K	M	scAAV2/8-LP1-hFIXco	IV	2.0×10^{11}	N	48 mo		2.17±0.83	n.d.
Patient 2	2-bp del fs	M	scAAV2/8-LP1-hFIXco	IV	2.0×10^{11}	N	44 mo		1.4±0.3	n.d.
Patient 3	$G{\to}T$ W215C	M	scAAV2/8-LP1-hFIXco	IV	6.0×10^{11}	N	42 mo		2.86±1.23	n.d.
Patient 4	$G{\to}A$ A309T	M	scAAV2/8-LP1-hFIXco	IV	6.0×10^{11}	N	41 mo		2.17±0.72	n.d.
Patient 5	$C{\to}T$ R180W	M	scAAV2/8-LP1-hFIXco	IV	2.0×10^{12}	Y	40 mo		3.56±1.65	n.d.
Patient 6	−52 del C	M	scAAV2/8-LP1-hFIXco	IV	2.0×10^{12}	Y	39 mo		7.21±2.92	n.d.
Patient 7	3 bp del fs	M	scAAV2/8-LP1-hFIXco	IV	2.0×10^{12}	Y	24 mo		5.00±0.63	n.d.
Patient 8	$C{\to}T$ T426I	M	scAAV2/8-LP1-hFIXco	IV	2.0×10^{12}	N	22 mo		6.67±1.50	n.d.
Patient 9	$C{\to}A$ A233D	M	scAAV2/8-LP1-hFIXco	IV	2.0×10^{12}	N	20 mo		5.23±1.54	n.d.
Patient 10	$G{\to}T$ G129X	M	scAAV2/8-LP1-hFIXco	IV	2.0×10^{12}	Y	16 mo		2.89±1.62	n.d.

[a] Deletion mutation in FIX, pyruvate kinase deficiency, and anti-phospholipid antibody.
[b] No mutation found in the coding region.
[c] Mutation in propeptide, position −4.
HA, hepatic artery; IV, intravenous; PV, portal vein.

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not completely understood. In humans, wild-type AAV infection is not associated with a recognized disease syndrome but seroepidemiology studies have suggested influence on reproductive outcomes.[44] In contrast, parvoviral infections in dogs are associated with a high mortality rate ($>90\%$) and the disease is extremely contagious and can be devastating in a colony setting.[45] Because of the dangerous nature of the canine parvoviral infection, most puppies are immunized between 4 and 6 weeks of age to prevent disease. Both canine parvovirus and AAV are members of the Parvoviridae family and share sequence identity.[46] Thus, it is possible that the immune response to canine parvoviral vaccination in puppies could influence recombinant AAV-mediated gene therapy in adult dogs.[47] The canine immune responses to recombinant AAV vectors have limited transgene expression in canine α_1-antitrypsin deficiency.[48] Also, a dog model of Duchenne muscular dystrophy showed an AAV capsid-mediated response and clearance of most but not all transduced cells over time.[49] Long-term expression, however, was achieved by short-term administration of antithymocyte globulin, cyclosporine, and mycophenolate mofetil.[50] At present, the essential practice of canine parvoviral immunization is a common denominator in these studies. How this vaccination program in puppies with developing immune systems influences the immune response to non-replicating recombinant AAV vectors of different serotypes in different disease processes in dogs with mature immune systems remains an ongoing area of investigation.[7,51]

The current level of success of gene therapy for hemophilia raises the question of how reimbursement will be structured for the gene therapy vectors that are approved and become licensed for clinical use.[52–54] This important issue will require considerable discussion, debate, and planning. Traditionally, medications are paid for when they are received. This approach has generally been accepted for treatment for rare diseases such as hemophilia, with the understanding that the cost, as with all new medications, is in part paying for the research required for safe and effective drug development. This approach has been less acceptable with new medications that, although highly effective,[55] target very large populations of affected patients. For example, the projected cost for treating the more than 3 million patients with hepatitis C in the United States with recently approved medications is $\sim\$85,000$ per patient.[56] In comparison, it is estimated that there are ~200 patients with lipoprotein lipase deficiency (LDLD) in Europe who would potentially be candidates for Glybera (alipogene tiparvovec), the first gene therapy drug to be approved. Glybera is anticipated to cost $1.4 million dollars.[57] Because the anticipated cure rates are high with these respective treatments for hepatitis C and LDLD, it is imperative to identify mechanisms for payment. An alternative payment plan that has been suggested for medications that are anticipated to have a high cost is to consider an annuity approach that would provide reimbursement on an annual basis if preagreed-on goals for successful treatment are achieved from year to year. To evaluate the potential feasibility and acceptability of such an approach, it will be essential to have preclinical data that reliably predict continued therapeutic success over such a time frame. Preclinical testing of gene therapy vectors in dogs with hemophilia B has accurately predicted successful long-term (>10 years) expression of FIX (K.A.H., V.R.A., H.H.S., M.A.K., and T.C.N., unpublished follow-up data on the dogs listed in Tables 1 and 2).[58] These preclinical data on long-term expression will likely become increasingly important in the discussions of how reimbursement for these new and highly effective medications will occur.

Another aspect of product development in which hemophilic dogs have proven valuable is observation for long-term or late side effects, especially for insertional mutagenesis events. Such events occurred with integrating vectors used for the X-linked SCID trials,[59] and the first cases of insertional mutagenesis leading to leukemia were reported 3 years after vector transduction. This is a delayed complication that could not be readily observed in mouse models where life expectancy is closer to 18 months. Data from a number of investigators have established that AAV vectors, although predominantly nonintegrating, do integrate at sites throughout the genome when administered at doses used in ongoing clinical studies.[60–62] The absence of hepatocellular carcinoma in dogs that have received relevant doses of recombinant AAV vectors and were subsequently surveyed for as long as a decade or more is thus reassuring in this respect.

As a result of the successful translation of gene therapy for hemophilia B from preclinical animal models to humans, at least three other groups are initiating AAV vector-based trials for hemophilia B including the FIX-R338L (Padua) discussed previously.[7,63] Also, attention is now being redirected to gene therapy for hemophilia A.[64] The number of available or eligible subjects with hemophilia A and hemophilia B may become a barrier to successful enrollment in a large number of trials. To meet these demands, both the hemophilia A[65,66] and hemophilia B dogs will continue to provide excellent models to test new gene therapy approaches for these rare bleeding disorders.[67]

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Author Disclosure Statement

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