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Muscle Gene Therapy for Hemophilia

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Abstract

Muscle-directed gene therapy for hemophilia is an attractive strategy for expression of therapeutic levels of clotting factor as evident from preclinical studies and an early phase clinical trial. Notably, local FIX expression by AAV-mediated direct intramuscular injection to skeletal muscle persists for years. Development of intravascular delivery of AAV vector approaches to skeletal muscle resulted in vector in widespread areas of the limb and increased expression of FIX in hemophilia B dogs. The use of FIX variants with improved biological activity may provide the opportunity to increase the efficacy of these approaches. Studies for hemophilia A are less developed at this point, but utilizing transgenes that improve hemostasis independent of FIX and FVIII has potential therapeutic application for both hemophilia A and B. Continuous monitoring of humoral and T cell responses to the transgene and AAV capsid in human trials will be critical for the translation of these promising approaches for muscle gene therapy for hemophilia.

Keywords: AAV; Hemophilia; Skeletal muscle; Gene therapy; Immunology

Introduction

Preclinical studies on the intramuscular delivery of factor IX (FIX) by a recombinant adeno-associated viral (AAV) serotype 2 vector in hemophilia B (HB) dogs led to the first clinical trials on AAV-FIX for hemophilia B [1-3]. This trial demonstrated that the AAV2 delivery to skeletal muscle was safe, however, the levels of FIX in the circulation were not sufficient to improve the disease phenotype [2]. In a liver-directed clinical trial, therapeutic levels of FIX were observed in the high dose cohort. However, the duration of expression was transient due to immune responses to the AAV2 capsid [4]. More recently, sustained therapeutic levels of FIX have been observed after delivery of AAV8-FIX to the liver in hemophilia B patients and transient immunosuppression may be required for some subjects to prevent loss of the transgene expression due to AAV capsid-mediated cellular responses [5]. Therefore, hepatic-directed approaches in some patients remain as obstacles. Thus, ectopic expression of FIX such as using novel skeletal muscle-directed for hemophilia are being developed as alternative approaches. Viral and non-viral vectors have been tested for muscle-directed gene delivery. Early data in murine models showed that skeletal muscle also has the potential to express therapeutic levels of factor VIII for hemophilia A [6]. In this review we will focus on the use of AAV because of the extensive data in large animal models and from early phase clinical trials.

Considerations for Ectopic Expression of FIX

The synthesis of FIX in ectopic sites such as skeletal muscle offers several key advantages. First, many adult hemophilia B patients have acquired viral hepatitis thus concerns about gene transfer into patients with underlying liver disease would be alleviated. Second, skeletal muscle is well-preserved in hemophilia patients (i.e. the tissue is not diseased) even among those patients not on prophylaxis and most muscle fibers are maintained for the lifetime of the individual. In addition, the skeletal muscle is well-vascularized tissue allowing access of secreted proteins into the circulation. From a safety standpoint, the risk of germ line transmission is very low compared to systemic administration as required for liver delivery as observed in preclinical studies in rabbits and early phase clinical trials [2,4,7-9]. In terms of immunology, the administration of AAV into the skeletal muscle offers several advantages. Since the AAV delivery is not systemic, the exposure of AAV to the circulating neutralizing antibodies to the viral capsid is minimized resulting in efficient transduction of the tissue, as we noted in the clinical study for hemophilia B in which local expression of the transgene and/or viral gene copy numbers did not correlate with baseline neutralizing antibody titers to AAV-2 capsid. These findings were also documented in two trials using AAV-1 vectors. However, studies in mice on readministration of AAV vectors by IM route using the same serotype were unsuccessful but the use of alternate serotype by IM injection in fact increase transgene expression levels [10,11]. These apparent conflicting data could be in part to the nature of the neutralizing antibodies to AAV capsid, i.e. natural infection in humans with wild-type AAV-2 viruses and robust immunization by IM injection of AAV vector [12,13]. Furthermore, if $CD8^+$ T cell responses to the AAV capsid developed after the gene transfer (see below), the cytotoxicity to the muscle would result in less relative damage compared to liver toxicity where these patients have additional coagulation problems. However, there are limitations in the ectopic expression of FIX in terms of efficacy and safety as discussed below.

Synthesis of Biologically Active FIX in Myotubes

While FIX is normally synthesized by hepatocytes, other cell types including muscle fibers are capable of synthesizing functional FIX. Post-translational modifications can influence the coagulation activity, half-life, and immunogenicity of a protein. Thus, extensive biochemical studies to determine if myotubes could provide the necessary post-translational modifications was important to support development of a muscle-based gene transfer approach for a secreted clotting

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factor, FIX. Using a model system of human cultured myotubes transduced with recombinant AAV to deliver human FIX, the protein was purified from the medium for *in vitro* and *in vivo* biochemical characterization and pharmacokinetics [14]. The myotube synthesized FIX was secreted as a single-chain form that migrates similarly to the plasma-derived FIX on SDS-PAGE gel. N-terminal sequence analysis showed the correct removal of the signal sequence and propeptide of the mature protein. Gamma-carboxylation of the N-terminal glutamic acid residues was similar to plasma-derived FIX. Thus, the critical post-translational modifications for protein activity were efficiently performed by the skeletal muscle which resulted in a protein with specific activity comparable to the plasma-derived FIX (180 and 200 U/mg, respectively). However, the N-glycosylation pattern, serine phosphorylation and tyrosine sulfation were not identical to plasma derived FIX. These posttranslational modifications were also distinct from the plasma-derived FIX when compared to the recombinant FIX produced in CHO cells that has been used clinically for decades [15].

Studies showed that at high levels of FIX expression after increasing the amounts of AAV, the specific activity of the muscle derived FIX decreased. Because γ-carboxylase and furin/paired amino acid-cleaving enzyme (PACE) enzymes are present in skeletal muscle at 10% of the levels in liver, it is possible that expression of high FIX levels may saturate the enzyme activities in the muscle and the protein specific activity could be hampered. Although the muscle derived FIX is not identical to plasma derived FIX, the clotting activity and pharmacokinetics of muscle-derived FIX protein in hemophilia B mice were similar to the plasma-derived or clinically used recombinant FIX produced in CHO cells [14]. In other experimental models, the production of erythropoietin by AAV-muscle directed expression in non-human primates resulted in a protein with a distinct isoelectric migration pattern compared to the wild-type erythropoietin [16], suggesting abnormal posttranslational modifications yet remain to be determined. Thus, careful assessment of the transgene produced from ectopic sites, such as skeletal muscle, is critical for the determination of both safety and efficacy of a given approach.

Studies on AAV-based Gene Therapy for Skeletal Muscle Expression of Canine FIX in Dogs with Hemophilia B as a Platform for Translation Studies for Early Phase Clinical Studies

Our group showed that intramuscular (IM) injection of AAV-2 vector expressing FIX resulted in long-term expression of FIX in mice [1] and hemophilic dogs [3]. This work formed the basis for a clinical trial in which an AAV-FIX vector was administered by direct IM injection into skeletal muscle of adults with severe hemophilia B. We demonstrated that IM injection of AAV-FIX in humans was safe and resulted in local gene transfer and expression as judged by biopsy of injected muscle in all patients tested. However, at the doses administered, it did not result in sustained circulating levels >1% [2,17]. Notably, local evidence of gene transfer remains stable for ~4 years in one subject tested and for 10 years in another subject [18,19]. Preclinical and clinical studies showed limited spreading of AAV-2 transduction around the needle track injection site. Recent studies using 3D microscopy images confirmed these early findings [20]. Although access to skeletal muscle is easily performed by direct percutaneous IM injections, achievement of AAV therapeutic target doses in humans has proved impractical because of the large number (>300 sites) of injections required mainly to avoid unwanted immune responses to FIX. Thus, there is a fundamental interest in exploiting techniques that allow transduction of large numbers of muscle fibers without requiring hundreds of IM injections.

The major complication of the current therapy for hemophilia (intravenous infusion of clotting factors) is development of inhibitory antibodies [21,22]. A wealth of data in hemophilic mouse and dog models demonstrates that inhibitors also arise in the context of gene transfer for hemophilia [23-25]. Some of the factors that influence the risk of inhibitor formation in the setting of gene transfer include, in addition to the underlying mutation in the *F9* gene, the vector itself, the route of administration, the presence or absence of a tissue-specific promoter, and the vector dose, some of them will be discussed here. A complex interplay among these host- and vector-dependent factors may control the eventual outcome, so that, for example, an innate immune response to a virally-derived vector may promote an adaptive immune response to the transgene product.

In early studies, we assessed the risk for inhibitor formation in dogs treated with AAV-2 or AAV-1 vectors by IM, and all experiments reported below use a vector encoding canine FIX as the transgene [3,26,27]. The hemophilia B model from the UNC-Chapel Hill colony is characterized by a missense mutation in the canine *F9* gene [3]. The mutant protein resulted in a substitution of glyciine to a glutamic acid residue at position 379. RNA analysis showed normal FIX transcript and structural modeling suggested an aberrant conformational change that alters the hydrophobic properties of the mutant FIX. Notably, the plasma FIX protein antigen and activity was undetectable [28], this phenotype was further confirmed by a series of immunologic assays [29].

In dogs treated with AAV-2 vectors we determined that as the dose of vector increased to greater than 5.6×10^{12} vg/kg the likelihood of inhibitor formation also increases (Table 1). Notably, the dose per site is a strong predictor of the risk for inhibitor formation; among three animals that received comparable doses (8×10^{12} vg/kg), the dog (B14) that received a high dose/site (1.2×10^{13} vg/site) formed a long-lasting high titer inhibitory antibody, whereas the two that received a lower dose/site (2×10^{12} vg/site) as shown for Dogs B85 and D31, either failed to form an inhibitor or developed only a transient low titer inhibitor. Whether the dose dependence of risk of inhibitor formation resulted from increasing levels of FIX antigen and/or higher numbers of viral particles was not completely clear. There is growing evidence on the higher efficacy of AAV-1 in mice [30,31], thus one can produce similar amounts of circulating FIX using a much lower dose of vector [27]. We sought to use dogs to distinguish among these possibilities experimentally. In this context, Dog E35 received AAV-1 at a 4-fold lower dose/site and 8-fold lower dose/kg than the dose associated with inhibitor formation with AAV-2, however, high titers of inhibitor to FIX were detected two weeks post-injection and persisted for lifetime of animal. Previously, we have determined that transient immunosuppression with cyclophosphamide at the time of IM injection was an effective maneuver to prevent inhibitor formation to FIX in dogs injected with high doses of AAV-2 (see Dog D32) [26]. We therefore injected a second dog (E57) with AAV-1 under transient immunosuppression. Dog E57 received AAV-1 at 30-fold lower doses/site and 20-fold lower dose/kg than dog D32, yet E57 developed a long-lasting inhibitor after stopping cyclophosphamide while D32 did not. These data suggest that the amount of transgene product (antigen) synthesized locally is also a major factor promoting development of a harmful immune response. This conclusion is consistent with a body of data in the vaccine literature that correlates amount of antigen

Dog	AAV Serotype	Route	Vector genome per kg	Vector dose per site	Transient Immunosuppression	MaximumcF.IX level (ng/ml)	Anti-cF.IX Bethesda Unit
B45	AAV-2	IM	1.3×10^{11}	4.1×10^{10}	no	2.6	none
B48	AAV-2	IM	3.4×10^{12}	1.1×10^{12}	no	40	none
D32	AAV-2	IM	5.6×10^{12}	2.0×10^{12}	yes	40	none
D31	AAV-2	IM	8.5×10^{12}	2.0×10^{12}	no	39	none
B85	AAV-2	IM	8.5×10^{12}	2.1×10^{12}	no	70	6.8 BU, transient
B14	AAV-2	IM	1.1×10^{13}	1.2×10^{13}	no	30	≤ 24.5
E35	AAV-1	IM	1.0×10^{12}	5×10^{11}	no	87*	8-15 B.U.
E57	AAV-1	IM	2.4×10^{11}	6.8×10^{10}	yes	104	2-8.5 B.U.
D99	AAV-2	ILP	3.7×10^{12}	N/A	yes	730	none
F57	AAV-2	ILP	1.7×10^{12}	N/A	yes	260	none
H08	AAV-2	ILP	3.0×10^{12}	N/A	yes	210	none
E60	AAV-2	ILP	3.9×10^{12}	N/A	no	~500*	22 BU, long-lasting
H48/H34/I07	AAV-2	ATVRX	3.0×10^{12}	N/A	yes	76-275	no
J03	AAV-2	ATVRX	3.0×10^{12}	N/A	no	81	no
J62	AAV-2	ATVRX	3.0×10^{12}	N/A	no	120	1.5 BU, transient
M13	AAV-6	ATVRX	3.0×10^{12}	N/A	yes	259	no
M20	AAV-6	ATVRX	3.0×10^{12}	N/A	yes	213	no

Immunosuppression consisted of 6 weekly doses of cyclophosmphamide
*Maximum cF.IX levels detected before antibody formation to F.IX

Table 1: Canine FIX expression by AAV-mediated gene transfer to skeletal muscle in hemophilia B dogs.

delivered to an intramuscular site with strength of immune response [32]. This also has been observed in reports of neutralizing antibody formation to erythropoietin following expression of supraphysiological levels of erythropoietin (10 to 100,000-fold over the baseline) after IM injection of alternate serotypes of AAV vectors in macaques [33,34]. In addition, the safety parameters (i.e. vg/kg; vg/site) obtained with AAV-2 in a missense mutation were not safe in dogs with an early stop codon mutation and triggered immune responses to FIX [35]. These data in large and immunocompetent animal models provide a firm conclusion on the safety of AAV vectors that was observed also in murine models [36] but it was understated by others suggesting no immune responses to human FIX in mice following IM injection of AAV [30].

In addition to the safety concerns addressed earlier, there are efficacy considerations that might impose an upper limit on the amount of vector that could be injected into a single site such as: 1) skeletal muscle has limited capacity to execute essential post-translational modifications of FIX [14] and 2) vector uptake into target cells is receptor-mediated and could be saturable at high doses [20]. Thus, intravascular delivery of AAV is of great interest as a strategy to enhance the efficacy of gene therapy to the muscle.

Regional Intravascular Delivery of AAV-FIX to Skeletal Muscle in Hemophilia B Dogs

The development of vector delivery via intravascular route to muscle is highly attractive because skeletal muscle has one of the highest densities of capillary networks in the body. However, vector transport across the endothelium is a major obstacle for the transduction of a widespread area of the muscle. To overcome these limitations, we carried out studies in dogs in which we increased the capillary permeability by using chemical or hydrostatic strategies.

The permeability of the microvasculature in small animals suggests that with increasing body size, gravitational and inertial effects on the circulation resulted in progressive restriction on the fluid and solute flow across capillary wall [37]. Put simply, mice are intrinsically leaky compared to large animals, thus the clinical translation of data on intravascular delivery of AAV to skeletal muscle in mice could be hampered [38]. Moreover, data in humans showed that the width of the capillary basement membrane is increased in adults when compared to infants and children [39]. In this study, there was a correlation between the distance from the heart and the increase in the width of the capillary basement membrane. Thus, studies in adult animals are highly desirable for preclinical studies on intravascular delivery of AAV to muscle.

Stedman's group recently reviewed the development of these strategies and described in detail modifications required for small and large animal studies [40]. In our studies, the first method was the isolated limb perfusion (ILP) in which the vector was delivered through the femoral artery vessel in the presence of vasoactive drugs [41]. The second approach was the transvenular delivery in which the vector was injected through a superficial vein in the distal part of the hind limb under elevated hydrostatic pressure [42].

A potential method for widespread gene transfer to skeletal muscle is the ILP technique. ILP was initially developed as a method for delivering large doses of chemotherapy via femoral artery to the treatment of tumor-bearing limb, without systemic side-effects among adults [43]. However, a study in hamsters showed that AAV, with a diameter of ~20nm, delivery through the femoral artery failed to penetrate the intact skeletal muscle endothelial barrier [44]. To be used successfully as a method for gene transfer to skeletal muscle using an AAV vector via arterial delivery, ILP requires vasoactive drugs to facilitate vector delivery from the circulation to the target cell.

In our ILP study in dogs [41], a tourniquet was placed at the level of the proximal thigh to restrict the perfusion to a single limb. The femoral vessels were isolated and cannulated and papeverine was injected systemically for vasodilation. Vector-containing solution was mixed with histamine for increased capillary permeability and permitted to dwell for 15-20 min. After this procedure, the limb circulation was flushed with PBS to remove residual papaverine or histamine. The

procedure was well-tolerated, the animal was followed closely to detect arterial or venous thrombosis, edema, restriction to movement or pain. No complications were observed at early or late time points (>7 years, ongoing observations). In this study, we documented that random biopsy of the perfused limb showed transgene expression whereas in the IM injected muscle, expression was restricted to the injection site [41]. Biodistribution studies after 8 weeks post vector delivery revealed that vector DNA was not found in tissues harvested other than the perfused limb.

In a group of three HB dogs, ILP delivery of AAV-2-FIX at doses ranging from $1.7x10^{12}$ to $3.7x10^{12}$ vg/kg resulted in long-term expression of FIX at levels of 4-15% of normal. There was excellent correlation between antigen and activity levels that suggests that at this level of expression, FIX synthesized in skeletal muscle is fully biologically active. As further proof of this, it should be noted that, in the 74 months of cumulative observation of the three treated dogs, there have been only two bleeding episodes requiring treatment. The expected bleeding number based on observation of the colony over many years is ~5.5 episodes/12 months, or 34 over this period of observation in this ILP cohort group [45]. As a comparison for efficacy, we have shown a historical control in which a dog from the same colony was injected with the same vector (AAV-CMV-cFIX) at a comparable dose ($3.4x10^{12}$ vg/kg) by direct intramuscular injection, with resulting circulating FIX levels of <1%. Thus, by simply changing the vector delivery, FIX expression increased by 5-15-fold. However, we determined that transient immunosuppression (6 weeks total) is required to prevent antibody formation to FIX. We speculated that the combination of surgery and the use of pro-inflammatory drugs such as histamine might contribute to the unwanted immune response to FIX in the dog without immunosuppression (Dog E60, Table 1).

Despite of the successful outcome of ILP, these data better represent a proof-of-principle on the feasibility of achieving widespread gene delivery to skeletal muscle [41]. The limiting factors were the invasive nature of the procedure and the requirement for histamine. The notion of histamine as a permeabilizing agent derives from the data that histamine receptor activation modulates rapid and reversible junctions between adjacent endothelial cells. Emerging data on the mechanism and exact site of histamine action in the microvasculature showed the location of the histamine receptor was on the postcapillary venules, not at the capillaries per se [46]. At this location, the endothelial cell junctions do not show the extensive tight junctional architecture of the capillaries and they might exhibit low resistance and facilitate the transport from the lumen to the interstitium. These data provide the rationale for the second approach in which we tested the possibility to enhance vector delivery to skeletal muscle by increasing the hydrostatic pressure at the postcapillary level.

The approach consisted of the placement of an atraumatic tourniquet at the groin level, combined with pressurized flow of saline from a distal catheter. We restricted the infusion to a single limb, as described above for the ILP. In this model, AAV vector is infused by a non-invasive pressurized infusion of vector-containing solution through the superficial saphenous vein without surgical or pharmacological intervention as described by Dr. Stedman's group [37,40] known as afferent transvenular retrograde extravasation (ATVRX). This resulted in afferent flow through the valves within the major veins of the extremity but locally retrograde flow through the valveless venules. Thus, at the microcirculation the elevated hydrostatic pressure culminates with extravasation of vector across the endothelium and into the interstitium followed by widespread transduction of muscle as seen in rats and dogs [37]. An intravenous catheter was placed in the superficial saphenous vein under direct visualization. AAV vector was diluted in 0.9% saline at 20 ml/kilogram body weight and infused within 3 minutes under elevated pressure of 300 mmHg. At 15 minutes after vector delivery, the tourniquet was released. The procedure was well-tolerated and the animal returned to normal deambulation shortly after recovery from anesthesia [42,47].

These data showed therapeutic FIX transgene expression following delivery of AAV-2 or AAV-6 vectors through ATVRX to skeletal muscle in 18 HB dogs. This large cohort of animals includes those dogs naïve to AAV as well as dogs previously injected with AAV-2 vector [42,47]. The latter model, dogs developed NAB to AAV-2 capsid and thus mimic, to a certain extent, the majority of humans, the natural host of AAV-2, in which 80% are tested positive for this AAV serotype. In total, ATVRX proved a safe and efficacious vector delivery to skeletal muscle of HB dogs. In summary, the major findings of these studies were:

1. We identified the AAV-2 vector dose (3×10^{12} vg/kg) that resulted in circulating therapeutic FIX levels that are close to the doses already tested in early phase clinical trials using AAV vectors and prove safe. The improvement of the disease phenotype was remarkable whether assayed by standard functional clotting assays or by clinical observation of the reduced numbers of bleeding episodes (> 90% from the expected bleedings).
2. FIX antigen and clotting activity correlate well, which suggest that the muscle capacity of performing the complex posttranslational modifications of FIX up to 8% of normal was not saturated.
3. Transient immunosuppression was required to sustained expression of FIX without the risk of inhibitor formation. Although only one HB dog developed a low titer, transient inhibitory antibody (Table 1), the risk and benefit assessment of a short-term immunosuppression regimen is favorable for the safety of this strategy.
4. The maximum dose tolerated was identified (8.5×10^{12} vg/kg) since it was associated with formation of transient non-neutralizing antibody to the transgene even in the presence of transient immunosuppression.
5. The use of AAV serotype 6 by ATVRX provided a safe and efficacious alternative in naïve HB dogs.
6. Intravascular delivery of AAV-6 via ATVRX in the presence of high titers of NAB to AAV-2 capsid was effective at titers up to 1:100. This is significantly better than delivery to the liver, in which the presence of NAB titers to AAV-2 or AAV-8 at titers of 1:5 or higher prevent AAV-2 and AAV-8 gene transfer in non-human primates.
7. The safety profile in terms of biodistribution demonstrated show that the vector could be detected outside of the perfused limb. The conical shape of the dog upper thigh precludes optimal vascular isolation from the systemic circulation in contrast to the cylinder anatomy of the thigh musculature of non-human primates and humans. With continued improvements in catheter technology for humans, further improvement of the safety of this strategy is anticipated.
8. Short- and long-term follow-up showed no evidence of vascular damage or valve insufficiency.

Studies in non-human primates comparing IM and ATVRX-based strategies are in agreement with the data obtained in the hemophilia dog model on the superiority of the intravascular delivery [48]. Currently, there are no clinical studies using this delivery method using AAV vectors. However, planned studies by Phillip Moullier's group will be highly informative on the safety of this strategy [49]. Moreover, whether ATVRX-AAV is safe in dogs with early stop codon mutations remains to be determined. Another limitation of these studies is that dogs and non-human primates do not develop AAV capsid-mediated cellular immune responses as observed in human liver trials or by direct IM injection (see below) [50].

Together, these findings demonstrate that intravascular delivery to skeletal muscle allows long-term expression of therapeutic levels of FIX. Now we can take advantage of more potent AAV serotypes, while unwanted immune responses to the transgene can be avoided by short-term immunosuppression (total 6 weeks), a duration comparable to that of the successful AAV liver trial for hemophilia B (total 8 weeks) [5].

The Potential of Clinical Feasibility of the Delivery Method via ATVRX

Recently, Dr. Powers' group from UNC-Chapel Hill reported the first clinical trial on the delivery of saline to an isolated limb via ATVRX [51]. In this report, seven adult subjects with underlying muscle diseases (including Becker muscular dystrophy, limb-girdle muscular dystrophy among others) were enrolled. This was a dose escalation study of a single limb perfusion with 0.9% saline (no vector was injected) starting with 5%-20% of limb volume by placing an intravenous catheter into the saphenous vein. Here, the tourniquet was placed above the knee with inflated pressure fixed at 310 mmHg and the infusion rates of 80 ml/minute varied from 4 to 12 minutes. Total volume infused ranged from 200-975 ml. Overall the procedure was well-tolerated with no sustained severe adverse event. No major changes in muscle enzymes (as judged by serial measurements of serum creatine kinase) were observed. Careful assessment of the compartment pressure (muscle tissue pressure) and tissue oxygen saturation demonstrated a transient elevation of compartment pressures in only one subject. This individual received the largest volume (20% limb volume) and tissue pressure returned within the safe range within 80 minutes, and the follow-up showed no sequelae. Considering that hemophilia patients do not have remarkable underlying muscle disease, such as the subjects tested here, the safety profile is likely to be more favorable. Thus, the hemodynamic parameters in these studies support the translation of ATVRX for vector delivery to skeletal muscle for hemophilia.

FIX Variants

The ectopic expression of FIX has limitations that are based on the fact that the muscle is not the natural site of FIX synthesis. First, the skeletal muscle may be unable to adequately perform posttranslational modifications of FIX when it is expressed at high levels in a limited muscle area, as discussed earlier. Second, plasma-derived FIX binds to extracellular spaces specifically to the collagen IV [52]. Since skeletal muscle is rich in collagen IV, local expression of FIX may facilitate the attachment to the collagen IV and this could limit the amount of FIX that has access to the circulation [1], as seen in mice and further in dogs [41]. Thus, strategies to overcome these limitations were pursued by developing novel approaches based on of the use of FIX variants that have increased specific activity or less affinity for collagen IV. Together, these strategies would provide alternative molecules that may improve the efficacy of muscle-directed gene therapy.

In studies to identify the region of FIX or activated FIX that binds to collagen IV in extracellular spaces [52,53], it was found that mutation of residues 3-11 (known as the γ-carboxyglutamic acid domain/Gla domain) in the amino terminus of FIX were responsible for this binding [54]. Structure-function studies demonstrated that mutation of lysine to alanine at residue 5 (K5A) or valine to lysine at residue 10 (V10K) resulted in FIX variants that poorly bind to endothelial cells yet retained normal clotting activity. Introduction of these variants (K5A, V10K) into AAV-FIX for delivery into the muscle was hypothesized to improve the release of FIX into the circulation. Intramuscular injection of AAV-1-K5A resulted in circulating FIX levels that were 2-fold higher than the wild type while double variant of AAV-1-K5A/V10K improved the circulating levels of FIX 3-5-fold higher than wild type. Gene copy number analysis confirmed that the increased expression was due to a lower affinity of K5A/V10K for the extracellular spaces rather than variation in transduction efficiency [55].

Few variants of FIX that have increased specific activity have been identified. One variant was characterized by recombinant methods to locate residues of activated FIX that bind to activated FVIII. Mutation of arginine 338 to alanine (R338A) resulted in a protein with 2-3-fold higher clotting activity than wild type FIX [56]. In hemophilia B mice, intramuscular delivery of AAV-1-R338A-FIX resulted in 2-fold increase in specific activity of this variant compared to wild-type FIX [55]. However, once FIX levels were >10% of normal (>500ng/ml) the specific activity decreased, as suggested by early data in myotube in culture transduced by AAV-FIX [14]. In a tail clip assay, bleeding times for AAV-1-R338A-FIX treated mice were shorter than mice administered AAV-1-WT-FIX and notably, the blood loss was similar to that of normal mice. Moreover, the increased specific activity of R338A was also observed after AAV delivery into the liver where the specific activity was 6-fold better than wild type FIX.

With any novel therapeutic protein, the possibility of an immune response to the protein is a safety concern. In hemophilia B mice that are tolerant to FIX-WT, we observed that mice do not develop an immune response to FIX after intramuscular delivery of wild type or variant (R338A, K5A/V10K) forms of FIX. Thus, these variants do not appear to be more immunogenic than FIX-WT.

Recently a novel and promising FIX variant with increased specific activity was identified in a patient that presented with thrombosis with FIX clotting activity of 776% of normal with normal FIX antigen levels (92%). DNA analysis revealed that this patient had a point mutation in the *F9* gene causing an amino acid substitution of leucine to arginine at position 338 (R338L, named FIX-Padua) [57] and segregates in a typical X-linked pattern. Characterization of FIX-Padua in a recombinant expression system demonstrated that the specific activity was 390 ± 28U/mg compared to 45 ± 2.4 U/mg for wild type FIX. Further studies on AAV delivery of FIX-Padua in hemophilia B mice and dogs will provide the opportunity to determine if this variant will be more efficacious in the setting of gene transfer.

Overall, these FIX variants provide the opportunity to improve the efficacy of the intravascular delivery approaches to muscle with AAV-FIX. These variants may also minimize the burden imposed on the myotubes for post-translational modifications. To date, most of the studies using FIX variants have been restricted to direct IM injection of AAV in murine models. The development of clinically attractive intravascular delivery of AAV to skeletal muscle in HB dogs together with FIX variants increase the likelihood of an effective muscle-based strategy using vector dosing already tested in human hemophilia B liver trials.

Prospects of Skeletal Muscle Target Approaches to Treat Hemophilia A

Therapeutic approaches to treat hemophilia A are actively being developed such as expression of factor VIII (FVIII) in hepatocytes, platelet-specific, hematopoietic stem cells, and endothelial cells [58]. Muscle-directed studies in hemophilia A mice demonstrated that FVIII can be expressed and detected in the circulation following AAV delivery of FVIII by co administration of one vector expressing the FVIII heavy chain and one expressing the FVIII light chain. Functional FVIII was detected in the circulation up to 9% of normal levels [6]. This two chain delivery approach requires that the heavy chain and light chain be expressed in the same cell thus investigation of a muscle approach using recently developed single chain vectors may provide a more efficient strategy. Further studies that examine the ability of myotubes to accommodate the post-transcriptional modifications of FVIII will be critical to define the potential efficacy of a muscle-based strategy for hemophilia A.

Recently, variants of FIX that bypass the need of FVIII or FIX activity to establish hemostasis in hemophilia A mice have been described [59]. Normally, activated FIX assumes protease activity once it forms the tenase complex with activated FVIII and FX. However, these variants (FIX K265T and FIX V181I/K265T/I383V) allow FIX to become activated in the absence of FVIIIa. When these variants were delivered by non-viral approaches to hepatocytes in hemophilia A mice, the expression of the FIX variants shortened the clotting times when tested using *in vitro* and *in vivo* clotting assays. Furthermore, both FIX variants were effective at establishing hemostasis in the presence of inhibitory antibodies to FVIII. Since it remains challenging to treat hemophilia A patients after the onset of inhibitor formation, these FIX variants offer a new approach to improve hemostasis in these patients. The main safety concern would be the risk of spontaneous activation of coagulation that would lead to a potential risk for thrombosis but the use of the zymogen FIX (inactive form) of the protein and its high functional specificity that only bypasses FVIII are properties that would suggest a low pro-thrombotic risk. In addition, there is the potential for an immune response to the altered FIX protein.

Alternate approaches using other similar vitamin-K dependent proteins such as FVIIa [60] or FXa [61] using AAV vector to skeletal muscle to bypass FVIII to establish hemostasis in hemophilia A may also prove promising alternatives. Overall, skeletal muscle approaches to deliver FIX gene to treat hemophilia B provide a platform for future strategies to treat hemophilia A by muscle gene therapy.

Immune Responses to AAV Capsid after Intramuscular Gene Transfer

After a T cell response to AAV capsid was observed in a clinical trial for AAV delivery of FIX for hemophilia B using a liver-directed approach [4], studies to evaluate the immune responses to AAV capsid in animal models and human clinical trials have been ongoing. Humoral responses to AAV capsid in rodents, dogs, non-human primates are very similar to that of humans. In contrast, T cell-mediated cytotoxicity to the vector capsid and consequent destruction of the vector transduced cells has been, so far, restricted to humans. Importantly, dogs do not have detectable anti-AAV-2 antibodies prior to exposure to AAV vectors, however, anti-AAV antibodies to other serotypes (AAV-6, AAV-8 and AAV-9) have been observed in dog models [62,63]. In canine models prospective studies of IM or intravascular delivery in the presence or absence of immunosuppression did not detect a T cell response to AAV capsid [47]. In contrast, in another study using an IM approach for muscular dystrophy in a small group of dogs of a different strain, an immune response to AAV capsid was detected [64]. Whether data from animal models can predict immune responses to AAV capsid that may occur in humans remains to be determined.

Currently, the immune responses to AAV have been monitored during several clinical trials to obtain human data on these responses (reviewed in [50]). The development of a T cell mediated immune response to AAV capsid after direct intramuscular gene transfer (in skeletal muscle) has been observed in independent human clinical trials. In fact, the largest data set on capsid T cell responses is from muscle-directed AAV gene therapy trials. Four clinical studies using intramuscular delivery of AAV-1 revealed $CD8^+$ T cell responses to AAV capsid in some patients. These studies demonstrated that anti-AAV antibody titers cannot predict the T cell response to the capsid. Notably, the magnitude of the T cell response tends to correlate with the vector dose. These studies are limited by the small number of patients, the low vector dose and the ability to access only peripheral blood mononuclear cells rather than cells within the target tissue which are the most relevant to the immune response. Other factors may play a role in the response including the inflammation within the tissue prior to AAV delivery, MHC Class I expression levels within the target tissue and the immunomodulatory properties of the transgene.

Interestingly, even when transgene expression is maintained in the muscle after AAV delivery, cellular infiltrates have been detected in some animal models and in humans. It was hypothesized that the lymphocyte infiltrates may be directed at the AAV capsid and/or the transgene. However, the long-term expression of the transgenes did not support this hypothesis. Recent studies in a mouse model reported that the antigen specific $CD8^+$ T cells in AAV transduced skeletal muscle were associated with programmed cell death of these effector cells and thus these cells may undergo apoptosis [65]. Thus, the cellular infiltrates were immunologically silent providing no cellular destruction of the transduced cells, such as observed in the liver. This illustrates the complexity of the host immune response in the setting of gene transfer that is tissue as well as transgene specific.

Conclusions

Progress on AAV delivery of FIX to muscle has been active with improvements in (1) the delivery of AAV vector to skeletal muscle by the intravascular route; (2) the identification of FIX variants with enhanced activity; (3) extensive studies on the immune response to the transgene in preclinical models; (4) and observations from clinical trials on the feasibility of the delivery methods and the risks of AAV capsid-mediated $CD8^+$ T cells in muscle-directed strategies. These collective data will direct the next generation of trials for expression of FIX for hemophilia B. Studies for hemophilia A or hemophilia with inhibitors using factor VIII or using transgenes with bypassing activity are promising strategies for further development of muscle-based gene therapy.

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References

1. Herzog RW, Hagstrom JN, Kung SH, Tai SJ, Wilson JM, et al. (1997) Stable gene transfer and expression of human blood coagulation factor IX after intramuscular injection of recombinant adeno-associated virus. Proc Natl Acad Sci U S A 94: 5804-5809.

2. Manno CS, Chew AJ, Hutchison S, Larson PJ, Herzog RW, et al. (2003) AAV-

mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. Blood 101: 2963-2972.

3. Herzog RW, Yang EY, Couto LB, Hagstrom JN, Elwell D, et al. (1999) Long-term correction of canine hemophilia B by gene transfer of blood coagulation factor IX mediated by adeno-associated viral vector. Nat Med 5: 56-63.
4. Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, et al. (2006) Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat Med 12: 342-347.
5. Nathwani AC, Tuddenham EG, Rangarajan S, Rosales C, McIntosh J, et al. (2011) Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. N Engl J Med 365: 2357-2365.
6. Mah C, Sarkar R, Zolotukhin I, Schleissing M, Xiao X, et al. (2003) Dual vectors expressing murine factor VIII result in sustained correction of hemophilia A mice. Hum Gene Ther 14: 143-152.
7. Arruda VR, Fields PA, Milner R, Wainwright L, De Miguel MP, et al. (2001) Lack of germline transmission of vector sequences following systemic administration of recombinant AAV-2 vector in males. Mol Ther 4: 586-592.
8. Favaro P, Finn JD, Siner JI, Wright JF, High KA, et al. (2011) Safety of liver gene transfer following peripheral intravascular delivery of adeno-associated virus (AAV)-5 and AAV-6 in a large animal model. Hum Gene Ther 22: 843-852.
9. Schuettrumpf J, Liu JH, Couto LB, Addya K, Leonard DG, et al. (2006) Inadvertent germline transmission of AAV2 vector: findings in a rabbit model correlate with those in a human clinical trial. Mol Ther 13: 1064-1073.
10. Mendell JR, Rodino-Klapac LR, Rosales-Quintero X, Kota J, Coley BD, et al. (2009) Limb-girdle muscular dystrophy type 2D gene therapy restores alpha-sarcoglycan and associated proteins. Ann Neurol 66: 290-297.
11. Mingozzi F, Meulenberg JJ, Hui DJ, Basner-Tschakarjan E, Hasbrouck NC, et al. (2009) AAV-1-mediated gene transfer to skeletal muscle in humans results in dose-dependent activation of capsid-specific T cells. Blood 114: 2077-2086.
12. Petry H, Brooks A, Orme A, Wang P, Liu P, et al. (2008) Effect of viral dose on neutralizing antibody response and transgene expression after AAV1 vector re-administration in mice. Gene Ther 15: 54-60.
13. Riviere C, Danos O, Douar AM (2006) Long-term expression and repeated administration of AAV type 1, 2 and 5 vectors in skeletal muscle of immunocompetent adult mice. Gene Ther 13: 1300-1308.
14. Arruda VR, Hagstrom JN, Deitch J, Heiman-Patterson T, Camire RM, et al. (2001) Posttranslational modifications of recombinant myotube-synthesized human factor IX. Blood 97: 130-138.
15. Schaub R, Garzone P, Bouchard P, Rup B, Keith J, et al. (1998) Preclinical studies of recombinant factor IX. Semin Hematol 35: 28-32.
16. Lasne F, Martin L, de Ceaurriz J, Larcher T, Moullier P, et al. (2004) "Genetic Doping" with erythropoietin cDNA in primate muscle is detectable. Mol Ther 10: 409-410.
17. Kay MA, Manno CS, Ragni MV, Larson PJ, Couto LB, et al. (2000) Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. Nat Genet 24: 257-261.
18. Jiang H, Pierce GF, Ozelo MC, de Paula EV, Vargas JA, et al. (2006) Evidence of multiyear factor IX expression by AAV-mediated gene transfer to skeletal muscle in an individual with severe hemophilia B. Mol Ther 14: 452-455.
19. Buchlis G, Podsakoff GM, Radu A, Hawk SM, Flake AW, et al. (2012) Factor IX expression in skeletal muscle of a severe hemophilia B patient 10 years after AAV-mediated gene transfer. Blood 119: 3038-3041.
20. Xiao PJ, Li C, Neumann A, Samulski RJ (2012) Quantitative 3D Tracing of Gene-delivery Viral Vectors in Human Cells and Animal Tissues. Mol Ther 20: 317-328.
21. Astermark J, Santagostino E, Keith Hoots W (2010) Clinical issues in inhibitors. Haemophilia 5: 54-60.
22. Hay CR, Dimichele DM (2012) The principal results of the International Immune Tolerance Study: a randomized dose comparison. Blood 119: 1335-1344.
23. Arruda VR, Favaro P, Finn JD (2009) Strategies to modulate immune responses: a new frontier for gene therapy. Mol Ther 17: 1492-1503.
24. Cao O, Furlan-Freguia C, Arruda VR, Herzog RW (2007) Emerging role of regulatory T cells in gene transfer. Curr Gene Ther 7: 381-390.
25. High KA (2011) Gene therapy for haemophilia: a long and winding road. J Thromb Haemost 1: 2-11.
26. Herzog RW, Fields PA, Arruda VR, Brubaker JO, Armstrong E, et al. (2002) Influence of vector dose on factor IX-specific T and B cell responses in muscle-directed gene therapy. Hum Gene Ther 13: 1281-1291.
27. Arruda VR, Schuettrumpf J, Herzog RW, Nichols TC, Robinson N, et al. (2004) Safety and efficacy of factor IX gene transfer to skeletal muscle in murine and canine hemophilia B models by adeno-associated viral vector serotype 1. Blood 103: 85-92.
28. Evans JP, Brinkhous KM, Brayer GD, Reisner HM, High KA (1989) Canine hemophilia B resulting from a point mutation with unusual consequences. Proc Natl Acad Sci U S A 86: 10095-10099.
29. Herzog RW, Arruda VR, Fisher TH, Read MS, Nichols TC, et al. (2000) Absence of circulating factor IX antigen in hemophilia B dogs of the UNC-Chapel Hill colony. Thromb Haemost 84: 352-354.
30. Chao H, Liu Y, Rabinowitz J, Li C, Samulski RJ, et al. (2000) Several log increase in therapeutic transgene delivery by distinct adeno-associated viral serotype vectors. Mol Ther 2: 619-623.
31. Xiao W, Chirmule N, Berta SC, McCullough B, Gao G, et al. (1999) Gene therapy vectors based on adeno-associated virus type 1. J Virol 73: 3994-4003.
32. Zinkernagel RM (2000) Localization dose and time of antigens determine immune reactivity. Semin Immunol 12: 163-171.
33. Chenuaud P, Larcher T, Rabinowitz JE, Provost N, Cherel Y, et al. (2004) Autoimmune anemia in macaques following erythropoietin gene therapy. Blood 103: 3303-3304.
34. Gao G, Lebherz C, Weiner DJ, Grant R, Calcedo R, et al. (2004) Erythropoietin gene therapy leads to autoimmune anemia in macaques. Blood 103: 3300-3302.
35. Herzog RW, Mount JD, Arruda VR, High KA, Lothrop CD Jr (2001) Muscle-directed gene transfer and transient immune suppression result in sustained partial correction of canine hemophilia B caused by a null mutation. Mol Ther 4: 192-200.
36. Wang L, Dobrzynski E, Schlachterman A, Cao O, Herzog RW (2005) Systemic protein delivery by muscle-gene transfer is limited by a local immune response. Blood 105: 4226-4234.
37. Su LT, Gopal K, Wang Z, Yin X, Nelson A, et al. (2005) Uniform scale-independent gene transfer to striated muscle after transvenular extravasation of vector. Circulation 112: 1780-1788.
38. Wang Z, Zhu T, Qiao C, Zhou L, Wang B, et al. (2005) Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. Nat Biotechnol 23: 321-328.
39. Williamson JR, Vogler NJ, Kilo C (1971) Regional variations in the width of the basement membrane of muscle capillaries in man and giraffe. Am J Pathol 63: 359-370.
40. Petrov M, Malik A, Mead A, Bridges CR, Stedman HH (2011) Gene transfer to muscle from the isolated regional circulation. Methods Mol Biol 709: 277-286.
41. Arruda VR, Stedman HH, Nichols TC, Haskins ME, Nicholson M, et al. (2005) Regional intravascular delivery of AAV-2-F.IX to skeletal muscle achieves long-term correction of hemophilia B in a large animal model. Blood 105: 3458-3464.
42. Arruda VR, Stedman HH, Haurigot V, Buchlis G, Baila S, et al. (2010) Peripheral transvenular delivery of adeno-associated viral vectors to skeletal muscle as a novel therapy for hemophilia B. Blood 115: 4678-4688.
43. Eggermont AM, de Wilt JH, ten Hagen TL (2003) Current uses of isolated limb perfusion in the clinic and a model system for new strategies. Lancet Oncol 4: 429-437.
44. Greelish JP, Su LT, Lankford EB, Burkman JM, Chen H, et al. (1999) Stable restoration of the sarcoglycan complex in dystrophic muscle perfused with histamine and a recombinant adeno-associated viral vector. Nat Med 5: 439-443.
45. Nichols TC, Raymer RA, Franck HW, Merricks EP, Bellinger DA, et al. (2010) Prevention of spontaneous bleeding in dogs with haemophilia A and haemophilia B. Haemophilia 3: 19-23.
46. Simionescu M, Gafencu A, Antohe F (2002) Transcytosis of plasma macromolecules in endothelial cells: a cell biological survey. Microsc Res Tech 57: 269-288.

47. Haurigot V, Mingozzi F, Buchlis G, Hui DJ, Chen Y, et al. (2010) Safety of AAV factor IX peripheral transvenular gene delivery to muscle in hemophilia B dogs. Mol Ther 18: 1318-1329.

48. Toromanoff A, Cherel Y, Guilbaud M, Penaud-Budloo M, Snyder RO, et al. (2008) Safety and efficacy of regional intravenous (r.i.) versus intramuscular (i.m.) delivery of rAAV1 and rAAV8 to nonhuman primate skeletal muscle. Mol Ther 16: 1291-1299.

49. Toromanoff A, Adjali O, Larcher T, Hill M, Guigand L, et al. (2010) Lack of immunotoxicity after regional intravenous (RI) delivery of rAAV to nonhuman primate skeletal muscle. Mol Ther 18: 151-160.

50. Mingozzi F, High KA (2011) Immune responses to AAV in clinical trials. Curr Gene Ther 11: 321-330.

51. Fan Z, Kocis K, Valley R, Howard JF, Chopra M, et al. (2012) Safety and Feasibility of High-pressure Transvenous Limb Perfusion With 0.9% Saline in Human Muscular Dystrophy. Mol Ther 20: 456-461.

52. Wolberg AS, Stafford DW, Erie DA (1997) Human factor IX binds to specific sites on the collagenous domain of collagen IV. J Biol Chem 272: 16717-16720.

53. Cheung WF, van den Born J, Kuhn K, Kjellen L, Hudson BG, et al. (1996) Identification of the endothelial cell binding site for factor IX. Proc Natl Acad Sci U S A 93: 11068-11073.

54. Cheung WF, Hamaguchi N, Smith KJ, Stafford DW (1992) The binding of human factor IX to endothelial cells is mediated by residues 3-11. J Biol Chem 267: 20529-20531.

55. Schuettrumpf J, Herzog RW, Schlachterman A, Kaufhold A, Stafford DW, et al. (2005) Factor IX variants improve gene therapy efficacy for hemophilia B. Blood 105: 2316-2323.

56. Chang J, Jin J, Lollar P, Bode W, Brandstetter H, et al. (1998) Changing residue 338 in human factor IX from arginine to alanine causes an increase in catalytic activity. J Biol Chem 273: 12089-12094.

57. Simioni P, Tormene D, Tognin G, Gavasso S, Bulato C, et al. (2009) X-linked thrombophilia with a mutant factor IX (factor IX Padua). N Engl J Med 361: 1671-1675.

58. Sabatino DE, Nichols TC, Merricks E, Bellinger DA, Herzog RW, et al. (2012) Animal models of hemophilia. Prog Mol Biol Transl Sci 105: 151-209.

59. Milanov P, Ivanciu L, Abriss D, Quade-Lyssy P, Miesbach W, et al. (2012) Engineered factor IX variants bypass FVIII and correct hemophilia A phenotype in mice. Blood 119: 602-611.

60. Margaritis P, Roy E, Aljamali MN, Downey HD, Giger U, et al. (2009) Successful treatment of canine hemophilia by continuous expression of canine FVIIa. Blood 113: 3682-3689.

61. Ivanciu L, Toso R, Margaritis P, Pavani G, Kim H, et al. (2011) A zymogen-like factor Xa variant corrects the coagulation defect in hemophilia. Nat Biotechnol 29: 1028-1033.

62. Shin JH, Yue Y, Smith B, Duan D (2012) Humoral Immunity to AAV-6, 8, and 9 in Normal and dystrophic dogs. Hum Gene Ther 23: 287-294.

63. Rapti K, Louis-Jeune V, Kohlbrenner E, Ishikawa K, Ladage D, et al. (2012) Neutralizing antibodies against AAV serotypes 1, 2, 6, and 9 in sera of commonly used animal models. Mol Ther 20: 73-83.

64. Wang Z, Allen JM, Riddell SR, Gregorevic P, Storb R, et al. (2007) Immunity to adeno-associated virus-mediated gene transfer in a random-bred canine model of Duchenne muscular dystrophy. Hum Gene Ther 18: 18-26.

65. Velazquez VM, Bowen DG, Walker CM (2009) Silencing of T lymphocytes by antigen-driven programmed death in recombinant adeno-associated virus vector-mediated gene therapy. Blood 113: 538-545.

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