AAV-based neonatal gene therapy for hemophilia A: long-term correction and avoidance of immune responses in mice

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INTRODUCTION

Neonatal gene therapy is a promising strategy for treating multiple congenital diseases that can be diagnosed shortly after birth. A number of reports suggest that there may be advantages, including the following: (1) therapeutic gene expression may allow avoidance of the development of irreversible pathology; (2) with earlier vector administration, there is greater vector-to-cell ratio allowing less vector; and (3) stem and progenitor cells, likely less accessible later in life, may be more easily transduced. In addition, transduction and expression of genes prior to maturation of immunity may enable the induction of operational immune tolerance and prolonged transgene expression. A number of animal models have been treated in the neonatal period with encouraging results, including Crigler-Najjar syndrome, mucopolysaccharidosis type VII, Pompe disease, methylmalonic acidemia, hemophilia B and spinal muscular atrophy. However, in some models in immunocompetent adult animals, delivery and expression of genes have resulted in immune responses to the gene product and/or to vector-associated antigens that have limited or abrogated expression.

Hemophilia A is an inherited disease of coagulation owing to a deficiency of functional factor VIII characterized by spontaneous and prolonged bleeding in joints, muscles and internal organs. Patients with this disease require invasive, lifelong management and can become untreated owing to immune responses to factor VIII protein (FVIII); neutralizing antibodies or ‘inhibitors’ can complicate therapy in up to 25% of patients. Gene therapy is a compelling goal for treatment of hemophilia as the therapeutic window is wide and amounts as little as 1–2% of normal physiologic levels are therapeutic and result in substantial improvement in the clinical phenotype. However, hemophilia A requires expression of FVIII to be persistent for gene transfer to be considered an effective therapy.

The success in canine studies was not successfully translated in the human clinical trial with adeno-associated virus-2 (AAV2) for gene therapy of hemophilia B; multiple pieces of evidence suggest that failure of this trial was due to the development of an anamnestic immune response to the AAV serotype-2 capsid proteins, and not factor IX with specific immune-mediated destruction of vector-transduced cells. A recent and ongoing clinical trial enrolled severe hemophilia B patients without histories of inhibitor development to receive AAV8 with a liver-specific promoter for expression of factor IX. Two patients, one in a lower-dose cohort and one in a medium-dose cohort achieved levels of factor IX of 2–4%, whereas two patients in the highest dose cohort did have brief periods of elevated hepatic transaminases weeks after administration suggestive of an immune response, they have both maintained factor IX expression. This most recent clinical trial suggests that in selected non-inhibitor-prone hemophilia B patients the barrier to achieving sustained expression of therapeutic amounts of protein may have been reached. While our approach with neonatal transgene expression in hemophilia A may avert complications in the perinatal period, such as intracranial hemorrhage in hemophilic neonates, it may also allow avoidance of a humoral immune response to the factor VIII protein. Earlier studies of neonatal murine gene transfer for hemophilia A using AAV serotype-2 and retroviral vectors did demonstrate prolonged FVIII protein expression in some...
animals (22 weeks with AAV (n = 8) and up to 14 months with retroviral vectors (n = 2)); however, these were complicated by the development of inhibitory antibodies in many of the animals.

Recombinant adeno-associated viral vectors (rAAVs) have specific advantages as gene transfer vehicles. These include (1) long-term gene expression with minimal toxicity following administration in adult mammals;18 (2) a broad host cell range, with the ability to infect dividing and growth-arrested cells; and (3) the availability of procedures for generating high-titer, helper virus-free preparations.19 Furthermore, identification of additional AAV serotypes that demonstrate higher levels of transduction in adult mammalian tissues, including the liver, has been described.20 Although not completely understood, the mechanisms underlying these differences in biological performance have been attributed to differences in cell entry, trafficking, viral uncoating and genome processing.22-24 One of the more recently described serotypes is rh10, a clade E primate AAV25 with neonatal growth.20 With no known pathogenicity of wild-type vector copy-number persistence during the period of rapid gene transfer, including higher levels of transgene expression and vector copy-number persistence during the period of rapid neonatal growth.20 With no known pathogenicity of wild-type AAVs, the features of rAAVs in general, and with the properties of the most recently described serotypes, suggest that they may be well-suited for neonatal gene transfer and thus require thorough evaluation in appropriate animal models.

In these studies we demonstrate that initiating therapy for hemophilia A shortly after birth using recombinant AAV serotype rh10 expressing factor VIII results in in vivo production of biologically active protein initially at supraphysiological levels, which declines to relatively stable therapeutic levels; this results in an improvement of the bleeding phenotype by tail clip and a functional FVIII assay (Coatest). This persistent expression is lifelong in the murine model of hemophilia A after co-injection of rAAVs, one expressing the heavy chain (HC) of FVIII and the other expressing the FVIII light chain (LC). Importantly, no antibodies develop against factor VIII protein subsequent to vector administration or with protein challenge in the presence of adjuvant.

RESULTS

Tolerability of virus administration

Matings of FVB/n hemophilic males (X,Y) and hemophilic females (XhXh) were set up to produce offspring that were all affected. Previously published data demonstrate that these mice develop antibodies against human factor VIII (hFVIII) in adult animals when injected with hFVIII.26 C57Bl/6 mice were purchased for reporter gene (that is, luciferase) studies. On the second day of life, mice were intravenously administered either pharmaceutical saline (negative controls, n = 12) or AAVrh10 (n = 54). Among the AAVrh10-injected groups, mice received either AAVrh10-chicken β-actin promoter/CMV enhancer (CBA)-luciferase (n = 20), or AAVrh10 serotypes expressing both the FVIII-LC and FVIII-HC (n = 34), each under the control of the CBA promoter (Figure 1).

Wild-type C57Bl/6 mice were administered pharmaceutical saline (negative controls) (n = 3) or 2.0 × 10^{12} gc kg^{-1} (genome copies/kg) AAVrh10 expressing firefly luciferase (n = 20). Affected hemophilia A neonatal mice received either 2.0 × 10^{12} gc kg^{-1} AAVrh10 carrying each of FVIII-HC and FVIII-LC (referred to as moderate dose) (n = 26), or 7 × 10^{12} gc kg^{-1} AAVrh10 carrying each of FVIII-HC and FVIII-LC, or saline (referred to as high dose) (n = 8). Hemophilia A mice were followed longitudinally except for a subset killed at 6 months of life after receiving 2 × 10^{12} gc kg^{-1} AAVrh10 FVIII-HC and FVIII-LC on day 2 of life (n = 4).

All of the animals having received AAVrh10 expressing factor VIII and AAVrh10 expressing luciferase appeared well during the neonatal and juvenile periods, and did not show any evidence of growth retardation compared with pharmaceutical saline-injected controls. The alanine aminotransferase levels of mice having received 2.0 × 10^{12} gc kg^{-1} each of FVIII-HC and FVIII-LC at 30 days of age (n = 5 per group) were similar to those of the controls (49.7 ± 4.0 vs 49.2 ± 19.6 IU l^{-1}, respectively (P = NS)).

Luciferase gene expression is long-lived after neonatal administration

Bioluminescent imaging (BLI) was performed on mice having received the neonatal injection of 2.0 × 10^{12} gc kg^{-1} AAVrh10-
CBA-luciferase to examine the distribution and longevity of the expression of the reporter gene (Figures 2a–c). Mice were imaged from 2 days after injection to 96 weeks of life, the length of the study \((n = 6 - 8\) mice at each time point), to generate a time course plot allowing analysis of the level of expression. Mice were imaged from the lateral aspect beginning 48 h after vector administration.
(fourth day of life) and from the ventral surface beginning on day 9; photon diffusion patterns were acquired. Subsequent images were obtained on weeks 2 through 6, 8, 12, 26, 52, 78 and 96. Expression was detected at the earliest time point and this was the peak as detected by BLI in the course of these studies.

The time course of expression (Figure 2c) demonstrates that the greatest period of decline occurs over the first 2 weeks during this period of rapid growth. This decline is substantial (graphical data demonstrate a negative slope and images show a substantive loss of red and yellow (higher intensity) areas, and more predominant appearance of blue and purple (lower intensity), or areas of no expression); however, by 4 weeks whole-animal BLI time course demonstrates stability throughout the length of these studies.

**Figure 2.** Persistence of luciferase expression after vector administration in neonatal mice. In vivo imaging of firefly luciferase after intravenous injection of (a) $2.0 \times 10^{12}$ gc kg$^{-1}$ AAV or (b) saline on the second day of life demonstrates photon diffusion patterns. The images are shown at 2 days (48 h after injection), 9 days, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, 12 weeks, 26 weeks, 52 weeks, 78 weeks and 96 weeks after vector administration, and at 48 h, 2 weeks, 4 weeks, 12 weeks, 52 weeks and 96 weeks after saline administration. Total flux in photons/second was acquired. For all time points except at 48 h, images were acquired with the mice in the ventral position. Images at 48 h and 9 days, and from 2 to 96 weeks, were set with the same references such that side-by-side comparison can be made. Note changes in the legend of the pseudocolor scale per group between these two groups. (c) BLI was followed longitudinally in animals as was luminometry in selected tissues at (d) 21 days and (e) 25 months after vector administration. (f) Representative histological liver sections examined at indicated time points after intravenous injection of 2-day-old mice with $2 \times 10^{12}$ gc kg$^{-1}$ per mouse of AAVrh10-CBA-luciferase ($\times$ 640 magnification) (n = 6 - 8 animals per group at each time point for a - c, and n = 5 animals per group for d and e). The error bars represent mean ± s.d. AAV, adeno-associated virus.
Furthermore, we examined the expression of luciferase by tissue luminometry up to 25 months of life (Figures 2d and e). Comparing expression in multiple tissues at 21 days of life (Figure 2d) demonstrates that the highest expression was in the liver in moderate-dose AAVrh10-CBA-luciferase-injected animals; all other tissues demonstrated lower levels of expression. At 25 months of life (length of study), similar levels of expression with the same general organ distribution was demonstrated (Figure 2e). Although some bioluminescent images do demonstrate an increased intensity in the craniofacial region of some mice, this is due to the intravascular injection, with local expression at the site of injection, as this intensity of expression was not found in the brain; during the injection some injectate does enter the local tissues owing to the elevated intravascular pressure. Luminometry of individual tissues demonstrates that the highest expression of the transgene-encoded protein was in the liver; animals receiving saline only on the second day of life demonstrated no luciferase expression or bioluminescence as expected.

Immunohistochemistry for luciferase expression in hepatocytes was performed to further examine the decline in expression after neonatal administration of vector (Figure 2f). Using a fluorescein isothiocyanate-conjugated secondary antibody to detect the anti-luciferase antibody, widespread hepatic expression of luciferase is demonstrated by fluorescein-positive cells in the liver by 1 week after administration. Subsequent images demonstrate a decline in the number of luciferase-expressing hepatocytes. Examination of hepatocytes at 6 and 25 months demonstrates low but stable expression in groups of clustered cells.

Factor VIII expression in the hemophilia murine model
A similar time course of expression was detected with factor VIII antigen. Mice receiving $2.0 \times 10^{12}$ gc kg$^{-1}$ each of AAVrh10 FVIII-HC + LC demonstrated supranormal levels of hFVIII antigen of $230.2 \pm 79.1$ ng ml$^{-1}$ at 1 week of life (Figure 3a). The level declined to $20.2 \pm 10.2$ ng ml$^{-1}$ by 1 month of life. From 2 until 22
months of age (length of study) FVIII antigen levels ranged from 4.2 ± 1.3 to 10.9 ± 5.2 ng ml⁻¹. At 22 months, the average factor VIII antigen level was 8.6 ± 5.6 ng ml⁻¹. Mice receiving 7.0 × 10¹² gc kg⁻¹ each of AAVrh10 FVIII-HC + LC also demonstrated supranormal levels of hFVIII antigen of 470.6 ± 87.1 ng ml⁻¹ of normal at 1 week of life (Figure 3b). The level declined to 122.3 ± 56.6 ng ml⁻¹ by 1 month, and from 2 until 20 months of age FVIII antigen levels ranged from 42.9 ± 13.0 to 10.5 ± 2.4 ng ml⁻¹ (length of study). At 20 months, the average FVIII antigen level was 19.9 ± 10.9 ng ml⁻¹.

Mice receiving 2.0 × 10¹² gc kg⁻¹ each of the hFVIII chain were examined for functional factor VIII activity. In order to assess FVIII function (1) a chromogenic assay based on FVIII activity was performed and (2) bleeding time was analyzed by tail clip assay 8 months after neonatal FVIII-knockout mice received AAVrh10 expressing FVIII-HC and FVIII-LC. Mice demonstrated supraphysiological levels of FVIII activity 3 days after vector administration (205.6 ± 24.6%), which stabilized at 3.6 ± 0.1-8.9 ± 5.6% from 2 to 22 months of life (n = 5-7 mice per time point) (Figure 3c). With the tail clip bleeding time assay, saline-injected wild-type mice (n = 5) were examined for comparison (range 44-56 s). The bleeding time in all mice injected with AAVrh10 expressing FVIII-HC + LC (n = 8) (range 85-168 s) shortened from that of saline-administered knockout mice (n = 3) (>600 s) (Figure 3d), also demonstrating that functional factor VIII is produced and that partial correction of the phenotype is present.

In a subset of animals that received 2.0 × 10¹² gc kg⁻¹ AAVrh10 FVIII-HC + LC as neonates, examination of the quantity of HC and LC along with the chromogenic assay was performed in the same animals to assess if there was an imbalance between the secretion of the two chains. Using samples from 6-month-old animals collected at the same time (n = 4), the LC was expressed 6.9 times more than the HC (compared with standardized plasma, HC was 1.8 ± 0.2% and LC was 12.4 ± 0.7%). FVIII activity by Coatest at the same time point with the same animals was 2.6 ± 0.2%.

Genome copy number declines with animal growth

Genome copy number per hepatocyte was examined after injection with the viral vector. Livers were removed and DNA was prepared for nucleic acid analysis. Viral DNA was quantitated by real-time PCR to determine the total copy number per genomic DNA. Three days after injection there were 69.6 ± 34.7 copies per hepatocyte (1.04 × 10⁵ ± 5.21 × 10⁴ gc ng⁻¹ liver DNA). Subsequently, mice were randomly selected at predetermined time points after injection of AAVrh10 expressing FVIII-HC + LC to examine vector copy-number stability. Vector copy number declined from day 5 to 153.9 ± 57.6 gc ng⁻¹ liver DNA at 1 month and 84.9 ± 37.1 gc ng⁻¹ liver DNA at 2 months, with 30.3 ± 19.4 gc ng⁻¹ liver DNA at 1 ½ years of life (n = 3-6 mice per time point) (Figure 4).

Antibodies against AAV and FVIII do not develop after neonatal injection

Antibodies against vector-associated proteins and hFVIII were examined after neonatal injection of the AAV. As adults, mice injected with 2.0 × 10¹² gc kg⁻¹ AAVrh10-FVIII-HC + LC as neonates were bled at 1 month, 3 months, 6 months, 9 months and 1 year, with plasma examined for antibodies against hFVIII by ELISA. Antibodies against FVIII were not present (Figure 5a, n = 8) in any of the mice tested as they continued to demonstrate expression of FVIII. Plasma was also examined for antibodies against rh10 AAV capsid proteins. All mice demonstrated no antibodies against vector-associated antigens (Figure 5b, n = 6). Mice injected with 7.0 × 10¹² gc kg⁻¹ AAVrh10-FVIII-HC + LC as neonates similarly did not demonstrate antibodies against FVIII or vector-associated antigens when tested (data not shown).

Antibodies do not develop against recombinant FVIII after challenge with adjuvant

As corrected mice (having received 2.0 × 10¹² gc kg⁻¹ AAVrh10-FVIII-HC + LC as neonates) demonstrate long-term expression of factor VIII at ≥5%, we sought to examine if these animals were immunologically unresponsive to exogenously administered hFVIII; control mice, that had been administered saline on the second day of life, were included. Mice were administered intraperitoneal route 1 unit of B-domain-deleted hFVIII as an immunogen mixed with alum (an adjuvant) at 8 weeks of life (n = 3); antibody development against hFVIII was examined. Control adult animals (injected as neonates with saline) had development of anti-hFVIII antibodies when plasma was analyzed.
have examined the administration and persistence of episomal viral gene therapy vectors in the context of \textit{in vivo} cellular proliferation associated with growth and maturation of tissues;\textsuperscript{20,38,39} however, like neonatal tissues it has been demonstrated in adult mice that loss of episomal genomes, the primary source of rAAV-mediated gene expression,\textsuperscript{36} does occur with cell division\textsuperscript{40,42} and proliferation after hepatic resection.\textsuperscript{36,42,43} Daily postnatal weight gain in mice peaks at the time of weaning, up to 0.9 g per day by day 20 postpartum, with subsequent decline afterwards.\textsuperscript{44} The thoracic (heart, lung) and abdominal organs (liver, pancreas, gastrointestinal tract), and the brain reach their adult size by day 35 of life.\textsuperscript{25} During this time of rapid animal growth and organ size maturation, loss of AAV-mediated vector genome occurs.\textsuperscript{25,34} Tissues undergoing active cell cycling during neonatal life such as hepatocytes (unlike the adult liver) result in a substantial loss of vector after administration, with stability only achieved when adult size is attained.\textsuperscript{20,38}

However, postmitotic tissues (for example, brain, skeletal muscle and heart) have lower rates of vector genome loss during this time.\textsuperscript{20} This decline in replication-incompetent vector number is explained only partly by organ growth with vector dilution, as we\textsuperscript{20} and others\textsuperscript{38} have previously demonstrated that this substantial early vector loss after administration is suggestive of active degradation rather than being solely dilutinal. While the mechanism is not known, we hypothesize that the un-integrated genomes in the nucleus are not linked to chromatin, and thus with cellular division and breakdown of the nuclear envelope during prophase, epimeres may move to the cytoplasm and then are degraded. We believe that this is similar to what occurs with cytotoxic plasmid DNA: Naked plasmid DNA entering the cytoplasm is unstable, is subjected to cytosolic nucleases and is rapidly turned over.\textsuperscript{31,45} In addition, the factor VIII level, as a secreted protein, is further affected by its dilution in the expanding blood volume with animal growth. Thus, the rapid cellular proliferation, active genome degradation and expanding volume of distribution in the early neonatal period make the potential for efficacious therapeutic gene replacement strategies with episomal vectors more challenging. While integrating vectors may be able to overcome limitations of vector loss that are likely to occur in humans as detected in mice, concerns of oncogenesis and insertional mutagenesis remain.\textsuperscript{46,47}

In the studies conducted here we were able to obtain long-term persistent expression of the reporter gene luciferase, and expression and function of factor VIII with AAVrh10-mediated gene delivery. Supraphysiological levels of factor VIII were obtained shortly after vector administration, declining to stable levels of $\geq 5\%$, which persisted up to 22 months (length of study) when $2.0 \times 10^{12}$ gc kg$^{-1}$ AAVrh10 FVIII-HC + LC, or $\geq 19\%$ at 20 months when $7.0 \times 10^{12}$ gc kg$^{-1}$, was administered neonatally (length of study). Chain-specific ELISA confirmed that more LC is produced compared with HC as demonstrated by others.\textsuperscript{16,48,49} An improvement in bleeding phenotype was observed with tail clipping in this clinically representative model of hemophilia A. Other studies by our group have demonstrated a similar longevity of expression, altered bleeding phenotype and lack of immune response in the murine model of hemophilia A using a helper-dependent adenoviral vector expressing FVIII.\textsuperscript{50}

Luciferase expression was widespread and high-level shortly after administration, with a similar decline detected by BLI, when compared with imaging shortly after administration; tissue studies with luciferase demonstrate that expression is maintained in multiple organs long-term. While some bioluminescent images demonstrate higher levels of expressions in the craniofacial region, organ luminometry does not demonstrate these levels of expression as this appears to be from expression in the overlying soft tissues at the site of injection secondary to unavoidable viral extravasation owing to the elevated venous pressure of injection during the time of vector administration.
Vector persistence in these studies has demonstrated that genome copies endure for greater than 1 year with relative stability, making continual expression of FVIII possible; in addition, persistence of gene expression has been demonstrated in multiple studies to be important in both the induction and maintenance of immune tolerance.51-53 Expression in hepatocytes appears to be maintained long-term in small clusters of cells, also previously demonstrated by others using AAVs.8,38,39 Re-administration of serotype rh10 AAV appears to be possible to subsequently augment FVIII expression as the animals mature, as a humoral immune response to this adeno-associated viral serotype did not develop after early postnatal administration; these studies, although preliminary and limited, demonstrate with subsequent vector administrations (2 × 10^{12} gc kg^{-1} at 3 weeks of age) that human FVIII antigen in plasma increased on average to 70 ng ml^{-1} when examined at 3 months of age (data not shown). In humans, however, it is unclear if this will be possible owing to (1) the stage of development of immune ontogeny at birth and (2) to the seroprevalence of anti-AAV antibodies in the human population. Whereas the prevalence of neutralizing antibodies to AAVrh10 is not known, seroprevalence to AAV2 has been reported to be as high as 30%54 to 72%55 of the population. Neutralizing antibodies against more novel serotypes are lower (for example, AAV7 ~15% in United States of America53; AAV8 16%54; AAV9 19%56 to 72%55 in United States of America and France, respectively; and AAV9 33.5%55 in France). In a study limited to AAV2, AAV5 and AAV6, children (age <18 years) had a lower prevalence of neutralizing antibodies than adults, and serotypes 5 and 6 were lower than AAV2. Taken together, these results support the development of more novel serotypes of AAV directed to future clinical applications in this area.

Unlike hemophilia B, where it is estimated that development of inhibitory or neutralizing antibodies occurs in 3% of those afflicted,12 hemophilia A is a disease where 20-25% develop inhibitors56 and may be a more appropriate model for examining the ability of the neonatal immune system to avoid this pathological consequence. Antibodies against FVIII can complicate treatment of acute bleeding episodes in human patients as they neutralize the activity of the infused clotting factor, making it difficult to establish hemostasis effectively, and despite years of investigation, it is not possible to predict which patients will develop inhibitory antibodies. The ability to induce anergy or tolerance to FVIII would be advantageous in preventing inhibitor formation, thus surmounting the major complication for this disease, and would allow subsequent administration of gene therapy vectors or factor infusions if necessary without the worry of immune consequences.

Avoidance of humoral immune responses to FVIII in these murine studies was possible by both initiating the expression of the transgene before immune ontogeny was complete and by maintaining expression continuously. In contrast to human development, mice are born at an immunologically immature stage with few B cells and almost no T cells in the peripheral lymphoid tissues, and they do not develop alloreactivity until birth.57,58 As a result, autologous antibodies that are spontaneously generated that the neonatal murine response to some antigens may not be fully developed until they are 4-5 days old58 marmalids with gestational periods of 30 days or longer generally have immunocompetence at birth.54 While B-cell tolerance to selected antigens has been established in humans up to 2 years of life,59 humans are born with a considerably more mature immune system capable of generating effective T- and B-cell responses with populated peripheral lymphoid tissues; human fetal thymocytes first respond to phytohemagglutinin at 14 weeks of gestation.60 While the studies described here demonstrate lack of an immune response after neonatal administration of AAV in mice, further investigation is necessary in appropriate large animal models with an immune ontogeny similar to that of humans.
Bioluminescent imaging

At predetermined times after AAV administration, mice were placed on a warming table and administered luciferase substrate (D-luciferin; Caliper, Hopkinton, MA, USA; at 150 mg kg$^{-1}$, intraperitoneally) and imaged using the bioluminescence optical imager (IVIS 200; Xenogen, Alameda, CA, USA) as described previously. At each time point a region of interest was generated surrounding each animal (excluding the tail) in order to quantify the total photons emitted by luciferase activity.

Immunohistochemistry

For detection of luciferase, liver sections (5 µm) were deparaffinized and rehydrated, followed by a heat-mediated antigen retrieval step performed using sodium citrate buffer (10 mM, pH 6.0). Tissue was permeabilized using 0.2% Triton X-100. Samples were then blocked for 20 min with protein buffer (Dako, Carpinteria, CA, USA (cat no. X0909)) at room temperature followed by incubation with the firefly luciferase antibody (Abcam; Cambridge, MA, USA (cat no. ab81823)) at a 1/100 dilution overnight at 4 °C. Bound primary antibody was detected with a fluorescein isothiocyanate-conjugated donkey anti-goat IgG secondary antibody (Abcam; cat no. ab6881) at a 1/100 dilution for 1 h at room temperature. Following washing with 1 x TBS, tissue was counterstained with DAPI (4',6-diamidin-2-phenylindole) (Vector Laboratories, Burlingame, CA, USA (cat no. H-1500)). Images were captured using an Olympus IX71 fluorescent microscope using the cellSens software (Olympus, Center Valley, PA, USA).

Assays for luciferase activity

Mice were killed by isoflurane overdose. Tissues were removed and placed in flat-bottom, 2.0-ml small capped tubes (USA Scientific, Ocala, FL, USA) with cell culture lysis reagent (Promega (E153A)) and placed on ice. Tissues were homogenized with a hand-held homogenizer (Omni International, Marietta, GA, USA). Specimens were centrifuged at 13 200 r.p.m. for 5 min and then placed at 4 °C. A luminometer (Berthold Detection Systems, Oak Ridge, TN, USA) was used to measure total light emission according to the manufacturer’s instructions. All samples were measured for 10 s. Luciferase levels were determined in duplicate and recorded as relative light units (RLUs).

A Lowry-based protein assay was performed (Bio-Rad, Hercules, CA, USA) and specimens were analyzed on a microplate reader (BioTek, Winooski, VT, USA) at 750 nm. Samples were performed in duplicate and compared with protein standards. Mean and s.e.m. was calculated. Luciferase levels by luminometry were normalized after protein concentrations were determined.

Assays for measuring hFVIII antigen and activity level

hFVIII antigen levels were examined by ELISA using a polyclonal antibody against human FVIII-LC and FVIII-HC that are species-specific and do not cross-react with murine FVIII. Plasma used for this assay of human FVIII was collected at regular intervals from retroorbital bleeding using heparinized tubes. Factor VIII antigen was quantitated by following the manufacturer’s instructions (Enzyme Research Laboratories, South Bend, IN, USA) using 20 µl plasma, performed in duplicate. FVIII standards were generated using normal human pooled plasma (Sigma-Aldrich, St Louis, MO, USA). The background antigen of hFVIII in control animals was negligible. An ELISA to quantitate LC and HC antigen levels, based on the technique of Sabatino et al. and (personal communication with DE Sabatino), was established using antibodies specific for human FVIII. A monoclonal antibody (1:500 diluted in coating buffer) against the LC (ESH-2; American Diagnostica, Stamford, CT, USA) or HC (6345; Abcam) (both provided by Denise Sabatino, University of Pennsylvania) was used to capture the protein. After blocking with 5% milk, a rabbit anti-hFVIII polyclonal antibody conjugated to horseradish peroxidase as a secondary antibody (Enzyme Research Laboratory) was added. After washing the OPD substrate was added, and after 10 min the reaction was stopped and the plate was read at 450 nm. A standard curve was generated using serial dilution of normal human pooled plasma as above.

FVIII activity was quantitated by chromogenic assay (COATEST SP FVIII, Chromogenix; Instrumental Laboratory Company, Lexington, MA, USA) using citrated plasma collected at selected time points following the manufacturer’s instructions for microplate method. FVIII levels were calculated from a standard curve generated using a series of dilutions of normal human pooled plasma (Hemosil Calibration Plasma; Instrumental Laboratory Company).

Phenotypic correction was assessed by tail transection bleeding time as described. Mouse tail was transected at 5 mm from the tip and was immediately immersed into 37 °C saline. Bleeding time was determined as the time from tail transection to the moment blood flow stopped for more than 60 s. Bleeding time beyond 600 s was considered as the cut-off time for the purpose of analysis.

Examination of immune responses to AAV and human factor VIII after viral injection

Immune response to viral capsid protein was measured by anti-AAVrh10 ELISA. A positive control for anti-AAVrh10 antibody was generated by intraperitoneally administering a naïve 8-week-old adult mouse with 5 x 10$^{10}$ gc of AAVrh10-luciferase mixed with Alum (Pierce Biotechnologies). Blood (> 200 µl) was collected 4 weeks later, and serum was removed and stored at –80 °C until needed. Ninety-six-well immunoplates (NUNC, Rochester, NY, USA) were coated with 1 x 10$^3$ viral particles per well of AAVrh10-luciferase that were prepared in 75 µl of 50 mM carbonate buffer (pH 9.6). To inactivate the virus, the plates were exposed to UV light for 30 min. Following an overnight incubation at 4 °C and washing with 1 x phosphate-buffered saline, 200 µl of blocking reagent (1 x phosphate-buffered saline with 5% fetal calf serum) were added to each well and incubated at 37 °C for 2 h. Meanwhile, two fold dilutions of plasma samples from experimental (neonatally injected with AAVrh10 FVIII-LC and FVIII-HC) and control groups, beginning 1:40, were prepared in blocking buffer. After the plates were washed, 100 µl of diluted plasma were added in duplicate and subsequently incubated for 2 h at 37 °C. Ninety-six-well microtiter plates were coated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody at 1:1000 dilution (BD Pharmingen, Franklin, NJ, USA) was added and incubated for 1 h at 37 °C. The plates were washed as above and incubated with 50 µl of OPD substrate for up to 6 min. Color development was stopped by adding 25 µl of 2.5 M sulfuric acid and was measured for absorbance at 492 nm; background was defined as absorbance measured in the absence of primary and secondary antibody.

Total antibodies against hFVIII were detected by an antigen-specific ELISA technique using purified commercial factor VIII protein concentrate. The anti-hFVIII ELISA was similarly performed to the ELISA described above except the sample volume was 50 µl per well. The immunoplates were initially coated with 0.122 IU well$^{-1}$ purified human recombinant FVIII (1 U = 100 ng hFVIII protein) (Xyntha; Wyeth). The background levels of anti-hFVIII antibodies measured in control hemophilia A mice were negligible. A mouse anti-human FVIII antibody was generated by injecting an adult mouse intravenously with recombinant human FVIII and was used as positive control for these studies. Results were presented as average ± s.d.

Determination of serum alanine aminotransferase

Blood was collected from mice at 30 days of life and analyzed as described (IDEXX Veterinary Services Inc., West Sacramento, CA, USA); 65 µl per animal were subjected to analysis. The results were pooled, and mean and s.d. were calculated.

Statistical analysis

Statistical calculations were performed using the SPSS version 17.0 statistical software package (Chicago, IL, USA). Data are presented as mean ± s.d. unless stated otherwise. Groups were compared by t-test and a P-value of less than 0.05 was considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.
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