Neonatal helper-dependent adenoviral vector gene therapy mediates correction of hemophilia A and tolerance to human factor VIII

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Edited* by Yuet Wai Kan, University of California, San Francisco School of Medicine, San Francisco, CA, and approved December 15, 2010 (received for review October 27, 2010)

Neonatal gene therapy is a promising strategy for treating a number of congenital diseases diagnosed shortly after birth as expression of therapeutic proteins during postnatal life may limit the pathologic consequences and result in a potential "cure." Hemophilia A is often complicated by the development of antibodies to recombinant protein resulting in treatment failure. Neonatal administration of vectors may avoid inhibitory antibody formation to factor VIII (FVIII) by taking advantage of immune immaturity. A helper-dependent adenoviral vector expressing human factor VIII was administered i.v. to neonatal hemophilia A knockout mice. Three days later, mice produced high levels of FVIII. Levels declined rapidly with animal growth to 5 wk of age with stable factor VIII expression thereafter to >1 y of age. Decline in factor VIII expression was not related to cell-mediated or humoral responses with lack of development of antibodies to capsid or human factor VIII proteins. Subsequent readministration and augmentation of expression was possible as operational tolerance was established to factor VIII without development of inhibitors; however, protective immunity to adenovirus remained.

immune response | coagulation

Hemophilia A is an X-linked recessive disease caused by a deficiency of clotting factor VIII (FVIII). FVIII protein, in a complex with factor IX, regulates the conversion of factor X to factor Xa. The deficiency of FVIII results in a marked reduction in this conversion and is characterized by frequent bleeding episodes. The current treatment is the administration of plasma-derived or recombinant human FVIII protein. Complicating therapy in some patients is the development of inhibitory antibodies ("inhibitors") that prevent the function of the activated protein and thus result in a failure of hemostasis. The development of inhibitors occurs in ~20–25% of patients (1, 2) and is one of the most challenging complications of treatment in persons with hemophilia A, resulting in increased morbidity and cost of care. Although viral vector gene therapy for this disorder was initially met with enthusiasm, the failure of therapy in adult animals due to the development of inhibitory antibodies or neutralizing immune responses has blunted this initial excitement.

A number of reports suggest that there are advantages to initiating gene therapy in the neonatal period (3–8). These advantages include the following: (i) early gene expression to avoid the development of irreversible pathology; (ii) administering vector earlier results in greater vector to cell ratio, allowing for less vector administration; (iii) stem and progenitor cells, likely less accessible later in life, may be more easily transduced; and (iv) immune responses may be reduced or absent (7). A number of animal models have been treated in the neonatal period with encouraging results, including Crigler–Najjar syndrome (9), mucopolysaccharidosis type VII (10–12), Pompe disease (13), and spinal muscular atrophy (14).

Whereas using neonatal gene therapy for the treatment of secretory proteins in humans may allow for correction of disease during early postnatal development, the challenges of correcting

an otherwise newborn mammal and expecting significant correction to remain when adult size is attained are not without significant concern. Although the use of nonintegrating viral vectors may lead to enhanced safety and avoidance of potential oncogenesis that has been detected with other viral vector systems (15–17), it invokes further challenges in that transgene expression may not be stable due to vector loss with cytokinesis and animal growth along with an expansion in blood volume and dilution of secreted transgene-encoded proteins. Whereas sustained therapeutic levels may not be achieved with a single administration of vector, if operational immunologic tolerance is established, further administration of vector at a later time may successfully supplement expression. This aspect, gene therapy to induce tolerance for subsequent augmentation, has not been previously explored. Tolerance to vector-associated antigens (i.e., virus) would be unfavorable due to the risk of subsequent exposure to wild-type virus and potential for development of pathology with possible overwhelming viremia and death.

Here we show that with a helper-dependent adenoviral vector (Hd-AV) expressing factor VIII, protein levels of the coagulant result in improvement in the coagulation deficiency and a long-term substantial reduction in bleeding for at least 16 mo of life in FVIII-deficient mice. Operational tolerance to human factor VIII was established and augmentation of expression was possible with a second dose of vector. Importantly, immune tolerance to virus-associated antigens was not established as immunological ignorance was present during the period of viral uncoating.

Results

Tolerability of Virus Administration. Matings of FVB/n hemophilic males ($X_H Y$) and hemophilic females ($X_H X_H$) were set up to produce offspring that were all affected. Previously published data demonstrate that these mice develop antibodies to human factor VIII in adult animals when injected with Hd-AV expressing human FVIII (18). On the third day of life, mice were i.v. administered 5×10^{12} viral particles (vp)/kg of Hd-AV expressing B domain-deleted human factor VIII under control of the phosphoenol pyruvate carboxykinase (PEPCK) promoter and ApoE1 enhancer (Hd-AV-FVIII). The woodchuck hepatitis virus postregulatory enhancer (WPRE) was included as was the human growth hormone polyadenylation signal (Fig. 1). A total of 51 mice were injected with helper-dependent adenovirus on the third day of life. All newborn mice survived the injection as did the vehicle-injected (pharmaceutical grade saline) controls. Survival throughout the studies was high with only a small number of mice dying in the first week of life: Four of 51 injected

Author contributions: G.S.L. designed research; C.H. and G.S.L. performed research; R.G.C., M.S., and B.L. contributed new reagents/analytic tools; C.H. and G.S.L. analyzed data; and G.S.L. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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Fig. 1. Schematic of Hd-AV-FVIII virus carrying the B domain-deleted form of factor VIII. A total of 5×10^{12} vector particles/kg were administered on the third day of life. Subsequent adult injections were with 5×10^{12} vector particles/kg. (PEPCK, phosphoenol pyruvate carboxykinase promoter; ApoA1 intron, BDD-hFVIII, B domain-deleted human FVIII; WPRE, woodchuck hepatitis virus postregulatory enhancer; hGH pA, human growth hormone polyadenylation signal; ITR, inverted terminal repeat; ψ , packaging signal.)

with Hd-AV-FVIII died (92.2% survival) and 1 of 13 injected with saline died in the first week of life (92.3% survival) [$P =$ not significant (NS)].

Injected mice were weighed frequently to assess weight gain and they demonstrated typical growth (Fig. 2A). Alanine aminotransferase levels were examined on the 35th day of life and compared with those of saline-injected controls to assess toxicity. Hd-AV-injected mice demonstrated alanine aminotransferase (ALT) values of 33.0 ± 5.6 IU/mL ($n = 6$) whereas saline-injected animals demonstrated ALT values of 34.2 ± 6.2 IU/mL ($n = 5$) ($P = 0.74$). There was no difference between female and male mice.

Factor VIII Expression Is Long-Lived After Neonatal Administration. Human FVIII expression peaked 3 d after the injection [day of life (DOL) 6] at $648.8 \pm 124.2\%$ of normal (Fig. 2B). By day 9, expression was at $309.4 \pm 85.0\%$ as animal weight increased from 1.6 ± 0.2 g on DOL 3 to 3.4 ± 0.5 g on DOL 9 (Fig. 2A). Further decline was present on DOL 18 with $136.7 \pm 13.2\%$ and weight increased to 7.7 ± 0.6 g. By day 21, FVIII expression declined to $46.9 \pm 15.2\%$ and at 4 wk, FVIII expression was $5.0 \pm 0.6\%$ with a weight of 15.5 ± 1.7 g. Over the subsequent 16 mo, FVIII expression remained stable from $2.3 \pm 0.5\%$ to $6.8 \pm 1.8\%$ (Fig. 2C).

Factor VIII Is Functional Resulting in Shortening of Bleeding Time. To assess whether FVIII activity correlated with phenotypic correction in virus-treated mice, bleeding time was analyzed by tail clip assay at 5 wk and at 7 mo after neonatal FVIII knockout mice received Hd-AV-FVIII. Saline-injected wild-type mice (5 wk) were examined for comparison (range 44–56 s) (Fig. 3). The bleeding time in all Hd-AV-FVIII-injected mice (at 5 wk, range 57–310 s and at 7 mo, range 78–230 s) declined from that of saline-administered knockout mice (>600 s), demonstrating that functional factor VIII is produced and that partial correction of the phenotype is present.

Vector Copy Number Decreases in the First Weeks After Virus Administration. Mice were randomly selected at predetermined time points after injection of Hd-AV-FVIII. 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. At least three mice were analyzed at each time point. Vector copy number declined substantially between day 5 and day 30, becoming stable at approximately one copy per hepatocyte from 3 mo to ≥ 1 y of age (Fig. 4).

Antibodies to Helper-Dependent Adenovirus and Human FVIII Do Not Develop After Neonatal Injection. Antibodies to vector-associated proteins and FVIII were examined after neonatal injection of Hd-AV-FVIII. As adults (5 wk of age), mice were bled and plasma was examined for antibodies against human factor VIII by ELISA. Antibodies to FVIII were not present (Fig. 5A) in any of the mice tested ($n = 5$) as they continued to demonstrate expression of FVIII. Plasma was also examined for antibodies against helper-dependent adenoviral capsid proteins. Similarly, all mice demonstrated no antibodies against vector-associated antigens (Fig. 5B) ($n = 5$).

Augmentation of FVIII Expression Is Possible with a Subsequent Vector Injection. As corrected mice demonstrate long-term expression of factor VIII at ~ 3 – 7% , we sought to examine if we could augment expression by administering a second postnatal or adult dose of vector to adult animals; control mice, that had been ad-

Fig. 2. Factor VIII levels were assessed by ELISA. Hemophilia A mice were injected with saline or 5×10^{12} vp/kg of Hd-AV-FVIII on the third day of life. Blood was collected at regular intervals. (A) Animal growth over the first 6 wk of life until lean body mass is reached. Afterward weights tend to stabilize with increases primarily in fat. (B) Expression over the first 4 wk during rapid animal growth. (C) Expression from 1 to 16 mo of life. ($n = 3$ – 7 animals at each time point.)

Fig. 3. Neonatal helper-dependent adenoviral vector administration demonstrates improvement in bleeding phenotype. Bleeding times were assessed in adult mice after neonatal i.v. administration of 5×10^{12} vp/kg of Hd-AV-FVIII. [WT, wild type; FVIII^{-/-}, factor VIII deficient (hemophilic); Hd-AV, neonatal helper-dependent adenovirus injected; wks, weeks; mos, months.] ($n \geq 4$ animals per group.)

ministered saline on the second day of life, were included. Mice were administered 5×10^{12} vp/kg of Hd-AV-FVIII i.v. (Fig. 6) as both factor VIII levels and antibody development to vector-associated antigens were examined. Control adult animals (injected as neonates with saline) had development of FVIII expression to $34.5 \pm 18.0\%$ of normal 6 d after administration; concomitant with this was the development of an antibody response to Hd-AV and FVIII (Fig. 7A and B) and loss of factor VIII expression. However, the neonatally injected Hd-AV-FVIII animals demonstrated different findings. Factor VIII expression after supplemental adult vector administration resulted in augmentation from basal expression of 5%, to as high as 70% early, with persistence to 48% subsequently (Fig. 6) to at least 3 mo postadministration. Similar to that in control animals, an antibody-mediated response developed to the helper-dependent adenovirus in this group (Fig. 7D), suggesting that the lack of response at the time of the neonatal administration was due to immunologic ignorance and not tolerance.

Inhibitors Do Not Develop to Subsequent Administration of Recombinant FVIII or Vector. Control mice that were administered Hd-AV-FVIII only as adults developed antibodies to FVIII shortly after vector administration (Fig. 7A). However, mice that received Hd-AV-FVIII as neonates and were subsequently readministered vector as adults did not (Fig. 7C). Taken together, these data demonstrate that tolerance to FVIII in these hemophilic animals has been established.

Fig. 4. Viral copy number in liver was measured at selected time points by quantitative PCR. Viral copy number was measured shortly after injection on DOL3 until animals were ≥ 1 y of age (DOL, day of life; wks, weeks; mos, months; yr, year.) ($n \geq 3$ animals at each time point.)

Discussion

The treatment of patients with severe hemophilia A has improved owing to the availability of FVIII concentrates that are manufactured with improved viral safety, advancement of techniques for performing venipuncture during home treatment, and the widespread adoption of prophylaxis to prevent bleeding episodes (19–21). With these advances, however, the development of inhibitors (alloantibodies to FVIII) remains the most challenging complication of treating hemophilia A patients and results in increased morbidity and is a significant economic burden (22). The risk of inhibitor formation is influenced by the type of FVIII mutation (23–26). Inversions, large deletions, and nonsense mutations are associated with the highest risk, likely because the immune system recognizes normal factor VIII protein as a neoantigen (27). Other factors known to influence the risk of inhibitor development include the severity of hemophilia A, patient ethnicity, intensive factor exposure at the time of surgery, age at first infusion, prophylactic or on-demand treatment regimens, the source of FVIII, and the cumulative number of days of replacement therapy (28–31). In addition, numerous studies to develop gene replacement strategies for hemophilia A in both small and large animal models have been complicated by the development of inhibitors (32–35).

The ability to correct hemophilia A or alter its phenotype would lessen the burden of disease in these patients. Of equal importance, development of tolerance to factor VIII protein would allow for administration of factor VIII without fear of inhibitor development if gene therapy alone was not sufficient in correcting the bleeding diathesis. In the studies conducted herein we evaluated in mice the efficacy of neonatal administration of a single dose of helper-dependent adenoviral vector expressing human FVIII to correct hemophilia A and examined the immune response to both FVIII and vector-associated antigens with primary injection and with subsequent administration of vector.

Helper-dependent adenoviral vectors are devoid of all viral coding sequences, unlike first-generation vectors. Numerous studies have demonstrated their superiority to first-generation vectors in mediating long-term transgene expression with minimal chronic hepatotoxicity (36–39). However, like that of first-generation adenoviral vectors, administration results in acute toxicity consistent with activation of an acute inflammatory response characterized by elevation in liver enzymes and proinflammatory cytokines and chemokines related to viral capsid proteins (40). The results from the studies conducted herein demonstrate that after a single neonatal i.v. dose of helper-dependent adenovirus expressing FVIII, long-term low-level expression with alteration of the phenotype is possible; furthermore, this expression is not associated with evidence of acute toxicity or an innate inflammatory response related to the vector capsid that occurs when adenoviral vectors have been administered to both large (40, 41) and small animals (42, 43).

Although expression is high within 3 d of injection, a rapid decline occurs over the first 4 wk with stable expression of 3–7% thereafter to at least 16 mo. Subsequent augmentation of expression was possible with readministration of vector as the development of an immune response to FVIII protein (i.e., tolerance) and vector capsid (i.e., ignorance) was lacking. The early and rapid decline is not immune mediated; rather, episomal vector genome loss appears to be related to hepatocellular division resulting in (i) dilution of vector genomes in an expanding population of hepatocytes and (ii) likely by degradation of genomes by an as yet unknown mechanism. Furthermore, FVIII levels themselves decrease in part as the volume of blood greatly expands as animals increase in size from neonates to adults. Although it was not observed, there may be a risk associated with the very high levels of FVIII achieved in the early neonatal period. As demonstrated in adult patients, high FVIII levels are a risk factor for venous thrombosis (44–46). Whereas the FVIII levels attained in this study were very high 3 d after vector administration (~650% of normal), they rapidly declined to ~135% of normal by day 18. As elevated plasma FVIII levels are a dose-dependent risk

Fig. 7. Readministration of Hd-AV-FVIII is possible postnatally with different immune responses based on persistence of protein. Mice received i.v. either saline or 5×10^{12} vp/kg of Hd-AV-FVIII on the third day of life. At 2 mo of age, mice were bled and plasma was analyzed for antibodies to factor VIII and Hd-AV capsid proteins ("baseline") followed by i.v. injection of Hd-AV-FVIII with subsequent analysis to 4 wk. (A) Neonatal saline-injected mice demonstrating absence of anti-FVIII antibody (baseline) and response at 5, 18, and 28 d after adult injection of Hd-AV-FVIII. (B) Neonatal saline-injected mice demonstrating absence of anti-Hd-AV capsid antibody (baseline) and response at 5, 18, and 28 d after adult injection. (C) Neonatal Hd-AV-FVIII-injected mice demonstrating absence of anti-FVIII antibody (baseline) and response at 5, 18, and 28 d after subsequent adult injection of Hd-AV-FVIII. (D) Neonatal Hd-AV-FVIII-injected mice demonstrating absence of anti-Hd-AV capsid antibody (baseline) and response at 5, 18, and 28 d after adult injection. All groups include an appropriate positive and negative control for comparison. ($n = 5$ animals per group.)

Mice and Animal Procedures. FVIII exon 16 knockout hemophilia A mice, purchased from The Jackson Laboratory, were used in all of the experiments described in this study. The characteristics of the exon-16 strains of hemophilic mice have been previously reported (55, 56) and were backcrossed with FVB/n mice (Charles River) through 10 cycles before these studies. The immune characteristics of these mice to human FVIII have been described (32). All mice were housed under specific pathogen-free conditions; food and water were provided ad libitum. All experimental procedures were conducted in accordance with institutional guidelines for animal care and use. Newborn pups on the third day of life and adult mice at 2 mo of age from X_HX_H and X_HY matings were injected with Hd-AV-FVIII diluted in pharmaceutical grade saline (5×10^{12} viral particles/kg) in the superficial temporal vein or by tail vein injection, respectively. The injections were performed with a total volume of 40 μ L in pups and 200 μ L in adults. Males and females were equally represented throughout the study. When mice were killed, the livers were harvested and kept at -80°C until analysis. After the vector injection, the mice were bled by retro-orbital or venous bleeding. Plasma was frozen immediately and stored at -80°C until analysis.

Assays for Human Factor VIII Expression and Phenotypic Correction. Plasma used for assay of human FVIII was collected at regular intervals from bleeding using heparinized tubes. FVIII expression was quantitated by an ELISA specific for the human protein. FVIII standards were generated using pooled normal human plasma (Sigma-Aldrich). Detection was performed according to the manufacturer's instructions (Enzyme Research Laboratories). Phenotypic correction was assessed by tail transection bleeding time as described (57). The mouse tail was transected at 5 mm from the tip and was immediately immersed into 37°C saline. The bleeding time was determined as the time from the tail transection to the moment the blood flow stopped for >60 s. Bleeding time beyond 600 s was considered cutoff time for the purpose of analysis.

Examination of Immune Responses to Adenovirus and Human Factor VIII After Viral or Recombinant FVIII Injection. Immune response to capsid protein was measured by anti-adenovirus ELISA. Ninety-six-well immunoplates (NUNC) were coated with 1×10^8 viral particles/well of Hd-AV-LacZ that were prepared in 75 μ L 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 PBS, 200 μ L of blocking reagent (PBS with 5% FCS) was added to each well and incubated at 37°C for 2 h. Meanwhile, twofold dilutions of plasma samples from experimental and control groups, beginning from 1:40, were prepared in 1% BSA/PBST. After the plates were washed, 100 μ L of diluted plasma was added in duplicate and subsequently incubated for 2 h at 37°C . Fifty microliters of 1:1,000 dilution of HRP-conjugated goat-anti-mouse secondary antibody (BD Pharmingen) was added and incubated for 1 h at 37°C . The plates were washed as above and incubated with 50 μ L of o-phenylenediamine dihydrochloride substrate for up to 6 min. Color development was stopped by adding 50 μ L of 2.5 M sulfuric acid and was measured for absorbance at 492 nm; background was defined as the absorbance measured in the absence of primary and secondary antibody. The anti-human FVIII ELISA was similarly performed except the sample volume was 50 μ L/well. The immunoplates were initially coated with 0.122 IU/well of B domain-deleted human recombinant FVIII (Xyntha; Pfizer). A positive control anti-adenovirus antibody was generated by intraperitoneally administering a naive 8-wk-old adult mouse with 5×10^{10} vp of Hd-AV-LacZ mixed with Alum (Pierce). Blood was collected 4 wk later and serum was removed and stored at -80°C until needed. A mouse anti-human FVIII antibody was generated by injecting an adult mouse i.v. with Hd-AV-FVIII.

Determination of Serum ALT. Blood was collected from mice at 35 d of life and analyzed as described (IDEXX Veterinary Services) with results pooled and mean and SD calculated.

Vector Copy Number Analysis. At regular intervals, mice were killed and liver tissue was removed. Genomic DNA was extracted (DNAeasy Kit; Qiagen) and

quantitated by nanodrop (ND-1000 spectrophotometer; NanoDrop Technologies). Using real-time quantitative PCR (MyIQ2; Bio-Rad Laboratories) of the hGH poly(A) signal (forward primer, 5'AATCTTGGCTCACTGCAATCTCCG3'; reverse primer, 5'CATGCATGCTGGAATCCCAACAA3') with SsoFast EvaGreen detection (Bio-Rad Laboratories), quantification of vector genomes was determined in duplicate. The PCR product size was 89 bp, amplified by the following conditions: 98 °C for 5 s and 60 °C for 10 s for 33 cycles in 96-well plates (plates and TempPlate RT Optical film were both from USA Scientific) as described above using 1 μ L of DNA per reaction as template (reaction volume, 15 μ L; final primer concentration, 333 nM). The efficiency of this PCR is 107.3%, $R^2 = 0.998$. A value of 150 vector particles/1 ng was used for vector copy number determination. Liver tissue from saline-injected mice ($n = 3$) at each time point was used for nontemplate controls and demonstrated no

amplification. Determination of copy numbers was based on known dilutions of plasmid pAAV-CBA-OVA-hGHpa.

Statistical Analysis. Statistical calculations were performed using the SPSS version 17.0 statistical software package. Statistical significance was determined by paired t test with P value set at ≤ 0.05 . Data are presented as mean $\pm$ SD unless stated otherwise.

ACKNOWLEDGMENTS. We thank Tamara Horwich, MD, MS, for her assistance with the statistical analysis. We thank Joseph Springer, Robin Rosenblatt, and Fides Lay for their technical assistance. This work was supported by grants from the National Institutes of Health (5K08HD057555-03, 4, 3K08HD057555-03S1, and 1R01NS071076-01A1 to G.S.L.), the Stein–Oppenheimer Foundation (to G.S.L.), and K99HL098692 (to M.S.).

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