Lifelong elimination of hyperbilirubinemia in the Gunn rat with a single injection of helper-dependent adenoviral vector

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Crigler–Najjar syndrome is a recessively inherited disorder characterized by severe unconjugated hyperbilirubinemia caused by a deficiency of uridine diphospho-glucuronosyl transferase 1A1. Current therapy relies on phototherapy to prevent kernicterus, but liver transplantation presently is the only permanent cure. Gene therapy is a potential alternative, and recent work has shown that helper-dependent adenoviral (HD-Ad) vectors, devoid of all viral coding sequences, induce prolonged transgene expression and exhibit significantly less chronic toxicity than early-generation Ad vectors. We used a HD-Ad vector to achieve liver-restricted expression of human uridine diphospho-glucuronosyl transferase 1A1 in the Gunn rat, a model of the human disorder. Total plasma bilirubin levels were reduced from >5.0 mg/dl to <1.4 mg/dl for >2 yr after a single i.v. administration of vector expressing the therapeutic transgene at a dose of 3 × 10¹² viral particles per kg. HPLC analysis of bile from treated rats showed the absence of bilirubin glucuronides at normal WT levels >2 yr after one injection of vector, and i.v. injection of bilirubin IIIa and XIIa in the same animals revealed excess bilirubin-conjugating capacity. There was no significant elevation of liver enzymes (alanine aminotransferase) and only transient, moderate thrombocytopenia after injection of the vector. A clinically significant reduction in serum bilirubin was observed with a dose as low as 6 × 10¹¹ viral particles per kg. We conclude that complete, long-term correction of hyperbilirubinemia in the Gunn rat model of Crigler–Najjar syndrome can be achieved with one injection of HD-Ad vector and negligible chronic toxicity.

Materials and Methods

Generation of HD-Ad Vector. A helper-dependent adenovirus is a vector with all viral coding sequences deleted and replaced by noncoding human genomic DNA. We generated a HD-Ad vector containing human UGT1A1 cDNA; the rat phosphoenol...
pyruvate carboxykinase (PEPCK) promoter (25); a fragment of the human apoA-I intron 1 containing the flanking region of exons 1 and 2 (base pairs 474–698, GenBank accession no. J00098); the woodchuck hepatitis virus posttranscriptional regulatory element (26); and the bovine β-globin polyadenylation site. The expression cassette was cloned into pBGSshuttle, and the corresponding Ad backbone was generated by homologous recombination in Escherichia coli as described (27). HD-Ad vector preparation followed a recently described protocol with minor modifications (28). The genomic structure of the vector was determined by HindIII digestion of viral DNA, and the parental plasmid was followed by agarose gel electrophoresis. Helper virus contamination was determined by Southern hybridization analysis of PsI-digested vector DNA with an Ad-inverted terminal repeat-specific probe and estimated to be less than 1.5% by PhosphorImager analysis.

Animals. Animals were Gunn rats, HsdBlu:Gunn (Harlan Bioproducts for Science, Indianapolis), maintained on standard chow under 12-h light/dark cycles. Homozygote (j/j), 8-week-old female rats (135–155 g) were used for vector administration. WT rats with the same genetic background [HsdBlu:Gunn (+/+)] were used as controls for HPLC studies. Animal experiments were performed according to a protocol approved by the Baylor College of Medicine in conformity with National Institutes of Health guidelines.

Administration of HD-Ad Vector. The Ad vector preparation, diluted into 1 ml of PBS, was injected via the tail vein. Six animals were injected with 6 × 10¹¹ viral particles (vp)/kg HD-Ad PEPCK-UGT1A1 (low-dose group), six received 3 × 10¹² vp/kg (intermediate-dose group), and two animals received a 1 × 10¹³ vp/kg dose (high-dose group). Another six rats received an equal volume of PBS (control group).

Effect of Phenobarbital. Four rats were injected i.v. (tail vein) with 6 × 10¹¹ vp/kg HD-Ad PEPCK-UGT1A1 and then treated i.p. (60 mg/kg per day) with phenobarbital (Sigma) for 8 consecutive days. Four control rats received vector followed by vehicle instead of phenobarbital. Total serum bilirubin levels in these two groups were then compared. For comparison, four saline-treated rats that had received no vector were administered phenobarbital as above, and their serum bilirubin levels were compared with those from a group of four saline-treated rats that had received only vehicle solution.

Blood Sample Analysis. Blood samples were collected from the lateral saphenous vein or tail vein. Alanine aminotransferase (ALT) levels were measured with a GP transaminase kit (Sigma), and total and direct-reacting serum bilirubin levels were determined by a diazo reagent-based method with a clinical chemistry kit (Sigma), according to the manufacturer’s instructions. Serum-unconjugated bilirubin was also measured by HPLC (29). Glucuronides in bile were identified by their absorbance spectra and comparing their retention times with authentic standards. Complete blood counts were performed at the Clinical Pathology Laboratory in the Center for Comparative Medicine at Baylor College of Medicine.

Analysis of Bilirubin Glucuronides in Bile. Animals were fitted with a short (7.5 cm, PE 50) biliary cannula under ketamine anesthesia and placed under an IR heating lamp in a darkened room. After ~30 min, a solution containing ~0.25 mg bilirubin-IIIα and ~0.25 mg bilirubin-XIIα dissolved in 1 ml of WT rat serum with the aid of 0.1 ml of 0.1 M argon-degassed NaOH or DMSO was injected as a bolus into the tail vein. Bile was collected in 20-μl aliquots just before isomer injection and at frequent intervals thereafter for the next 60 min. Bile samples were flash-frozen immediately in dry ice and kept under −80°C until analysis by HPLC.

RNA Analysis. Total RNA was extracted from liver homogenates by using the RNeasy Protect kit from Qiagen (Valencia, CA). RT-PCR used specific primers to amplify a 321-bp segment of the unique 5’ domain of the human UGT1A1 mRNA as described (11). Primers and conditions for normalizing RT-PCR by rat β-actin amplification have been described (30).

Protein Analysis. Liver homogenates were prepared in a solution of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4. Microsomes were obtained by centrifugation of liver homogenates (8,000 × g for 20 min and 105,000 × g for 90 min at 4°C). Protein content was determined by using the Micro BCA protein assay reagent kit (Pierce) with BSA as standard. Aliquots of 100 μg of total homogenates or 20 μg of microsomal proteins were separated on a 7.5% SDS/PAGE gel. Western blot analysis was performed as described (11) with specific human UGT1A1 mAbs (31).

Immunohistochemical Analysis. Liver biopsies were fixed in Formalde-Fresh solution (Fisher Scientific) and embedded in paraffin. Sections were deparaffinized with xylene and rehydrated by incubating successively with 100%, 95%, 70%, and 50% ethanol. Endogenous peroxidase was blocked by incubation for 15 min in peroxidase quenching solution (30% H₂O₂/ methanol, 1:9). A blocking kit (Vector Laboratories) was used to eliminate nonspecific binding of biotin and avidin. A specific anti-human UGT1A1 (dilution 1:900) was used as primary antibody. After overnight incubation with primary antibody, slides were washed in PBS and treated with a biotinylated secondary antibody (Zymed). Sections were washed with PBS, overlaid with a streptavidin-peroxidase complex for 5 min, and then treated with diaminobenzidine tetrahydrochloride substrate. The slides were washed, counterstained with hematoxylin, and mounted for light microscopy (32).

Statistical Analysis. Analysis of data and comparisons between control and treated groups were done with INSTAT (GraphPad, San Diego). The significance of differences was assessed with a two-tailed Student t test for unpaired data; P < 0.05 was considered statistically significant. Results are expressed as means ± SEM.

Results

HD-Ad Administration to Gunn Rats: Effect on Serum Bilirubin. Complete normalization of total serum bilirubin concentration was achieved within ~1 wk of a single i.v. administration of 3 × 10¹² vp/kg HD-Ad PEPCK-UGT1A1. Phenotypic correction, manifested as reduction in jaundice in the treated animals, was confirmed by analysis of serum (Fig. 1a) and direct, indirect (data not shown) and total bilirubin assays (Fig. 1b). Whereas pretreatment and saline-treated values of total bilirubin ranged from 5.8 to 10.5 mg/dl, animals treated with the intermediate and high doses of HD-Ad had values ranging from 0.4 to 1.4 mg/dl. The difference between total bilirubin values in the treated group was statistically significant compared with the control group throughout the experiment at all analyzed time points. Taking the 1-wk postinjection value as reference, no statistically significant difference was subsequently observed in total bilirubin level for the HD-Ad intermediate-dose and high-dose animals at any time point analyzed, indicating that the correction was stable for 2 yr. In addition, a 70% drop in total bilirubin concentration was observed after injecting a lower dose (6 × 10¹¹ vp/kg) of vector, and the bilirubin concentration remained <3.5 mg/dl for 110 wk. These prolonged effects on serum bilirubin were confirmed by HPLC analysis of serum from
high-, intermediate-, and low-dose animals 115, 102, and 107 wk, respectively, after vector injection (Fig. 1).

The viral preparation was administered to 8-week-old rats whose body weight (143 g) increased during the course of the study to 240 g after 2 yr. Interestingly, despite this increase, total bilirubin levels after HD-Ad injection remained stable for up to 2 yr after the first week.

Phenobarbital has been reported to induce bilirubin glucuronidation and lower plasma bilirubin levels in Gunn rats (33). To investigate whether it is possible to combine low-dose HD-Ad gene therapy with drug treatment to achieve complete correction of hyperbilirubinemia, a separate low-dose-treated group of rats was given phenobarbital (60 mg/kg per day for 8 consecutive days) as described (33) (Supporting Materials and Methods and Fig. 6, which are published as supporting information on the PNAS web site). Total bilirubin levels were then compared with a group receiving the same dose of vector but not treated with phenobarbital. No statistically significant difference was detected between the two groups, indicating that bilirubin clearance mediated by subphysiological UGT1A1 activity derived from HD-Ad-mediated expression under the rat PEPCK promoter cannot be increased by phenobarbital treatment. Nor, in contrast to ref. 33, did we observe a significant effect of phenobarbital in the Gunn rat controls.

**HD-Ad Administration to Gunn Rats: In Vivo Conjugation of Bilirubin.** To confirm that the persistent decline in plasma bilirubin in the treated animals was caused by elimination of bilirubin as glucuronides, we analyzed bile from one animal from each dosage group by HPLC 2 yr after HD-Ad injection and compared these chromatograms with those from a saline-treated homozygous Gunn rat control and normal nonjaundiced WT controls (Fig. 2). As expected, bilirubin glucuronides were not present in bile from the saline-treated homozygous control, but copious quantities of bilirubin monoglucuronides and diglucuronides were present in bile from the high- and intermediate-dose animals in quantities commensurate with those in WT controls. Bilirubin glucuronides were clearly present even in the low-dose rat some 107 wk after virus injection. However, the amounts were small, relative to those in WT animals and animals treated with higher doses of vector, and they contained a higher proportion of monoglucuronides.

Rats and humans normally have an excess capacity for bilirubin conjugation and only a small fraction of the normal complement of UGT1A1 is required to completely conjugate all bilirubin produced by heme catabolism (4, 34). To test the excess conjugating capacity of the treated Gunn rats, one animal from each treatment group was injected i.v. >2 yr after the original viral injection with an equimolar mixture of two synthetic...
bilirubin isomers, bilirubin-IIIα and bilirubin-XIIIα (35), and bile was collected in small (20-μl) aliquots immediately before the injection and at frequent intervals for the next hour for HPLC analysis. Bilirubins IIIα and XIIIα differ from natural bilirubin (Iαα) only in the positions of the methyl and vinyl groups on their lactam rings, and they are metabolized identically to the natural isomer, requiring UGT1A1 for conjugation and biliary excretion. However, being symmetrically substituted, they each form only one monoglucuronide in addition to a diglucuronide, and these metabolites can be readily distinguished from the glucuronides of endogenous bilirubin by HPLC. The total amount of isomers injected (0.5 mg; ~2 mg/kg) is ~35% of the daily production of bilirubin in the rat (36). Normal WT rats and a saline-treated Gunn rat control were also treated in the same way for comparison. In the saline-treated Gunn rat lacking endogenous UGT1A1 no biliary excretion of bilirubin glucuronides was observed, as expected. In the low-dose-treated rat there was no detectable excretion of bilirubin-IIIα or XIIIα glucuronides. However, in the WT controls and the intermediate-dose and high-dose rats, rapid excretion of the IIIα and XIIIα monoglucuronides and diglucuronides was observed without any notable diminution of the endogenous IXα glucuronide excretion (Fig. 3). Thus, the high-dose and intermediate-dose animals still had excess bilirubin-conjugating capacity, like the normal rat, 2 yr after injection of the vector, whereas the low-dose animal did not. Comparison of the complete bilirubin isomer glucuronide excretion profiles for these rats (data not shown) indicated that the excess conjugating capacity decreased in the order WT > high dose > intermediate dose, as might be expected.

mRNA and Protein Analysis. Restricted hepatic expression of human UGT1A1 mRNA 20 and 52 wk after treatment in intermediate-dose HD-Ad-treated animals was confirmed by RT-PCR using primers specific for a segment of the human UGT1A1 exon 1 (Fig. 4a). No human UGT1A1 mRNA was detected by RT-PCR in stomach, intestine, spleen, kidney, heart, ovary, or lungs, both in saline-treated and HD-Ad-treated animals (data not shown).

Immunoblot analyses of protein from total homogenates and microsomal preparations of liver biopsies from rats killed 20 wk after injection were performed by using a mAb specific for human UGT1A1 (31). Expression of a protein with the molecular mass of human UGT1A1 (52 kDa) was detected in HD-Ad-treated but not in saline-treated Gunn rats (Fig. 4b). This result was further confirmed by immunohistochemical analysis performed on liver sections from the same animals (Fig. 4 c and d).

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if they survive infancy unscathed; and (iv) an animal model, the Gunn rat, with a similar, although not identical, genetic defect is available (9); experimental therapy can be easily monitored by measuring short transgene expression, were defects of early-generation acute toxicity and immunogenicity of viral proteins, which cut hepatocytes without viral genome integration. Unfortunately, its usefulness has been questioned (39). Prolonged correction of an hepatic endoplasmic reticulum protein needed for correction of an inherited cell-autonomous metabolic disorder. Toxicity may not be linear and the window between therapeutic response to capsid proteins with rapid release of cytokines. In the current study, toxicity associated with treatment was mild as reflected by serum transaminase levels and complete blood counts. However, the relationship between vector dose and acute toxicity may not be linear and the window between therapeutic and toxic dose might, therefore, be narrow.

Several gene-transfer methods have been extensively evaluated by using the Gunn rat (38). Among nonviral approaches, gene correction via chimeraplasty has been attempted (17), but its usefulness has been questioned (39). Prolonged correction in Gunn rats has been achieved by using retroviral vectors (16, 40), but this approach requires invasive partial hepatectomy to deliver of HD-Ad vectors lacking viral genes, and its origin remains to be determined. Possible explanations are direct damage or activation of intravascular endothelial cells with subsequent platelet sequestration by the reticuloendothelial system (45); interaction between viros and platelets leading to platelet activation; and induction of an early, innate immune response to capsid proteins with rapid release of cytokines. In the present study, toxicity associated with treatment was mild as reflected by serum transaminase levels and complete blood counts. However, the relationship between vector dose and acute toxicity may not be linear and the window between therapeutic and toxic dose might, therefore, be narrow.

The current study uses a HD-Ad vector for long-term expression of an hepatic endoplasmic reticulum protein needed for correction of an inherited cell-autonomous metabolic disorder. Most cell-autonomous diseases, such as urea cycle disorders, require correction of the phenotype in a high percentage of hepatocytes, and hence, a higher dose of vector with increasing risk of disseminated intravascular coagulation. CN syndrome, as indicated by hepatocyte transplantation studies in humans and by gene therapy research in rats, requires transfection of fewer hepatocytes to normalize or significantly reduce the plasma bilirubin concentration. In the current study, complete long-term correction was achieved in Gunn rats with a HD-Ad dose.
causing only mild, transient thrombocytopenia and no apparent chronic toxicity. Even a lower dose, which did not induce any measurable alteration in acute toxicity indicators, significantly reduced hyperbilirubinemia by 70% for >2 yr. A similar reduction in human patients would be adequate to greatly diminish the risk of icterus toxicity and eliminate the burden of daily phototherapy. However, further reduction of the acute toxicity and confirmation of the long-term safety of HD-Ad vectors may be needed before they can be used for gene therapy in humans.

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