Correction of Hyperbilirubinemia in Gunn Rats Using Clinically Relevant Low Doses of Helper-Dependent Adenoviral Vectors

David Dimmock,* Nicola Brunetti-Pierri,[†] Donna J. Palmer, Arthur L. Beaudet, and Philip Ng

Abstract

Crigler–Najjar syndrome type I is a severe inborn error of bilirubin metabolism caused by a complete deficiency of uridine diphospho-glucuronosyl transferase 1A1 (UGT1A1) and results in life-threatening unconjugated hyperbilirubinemia. Lifelong correction of hyperbilirubinemia by liver-directed gene therapy using a helper-dependent adenoviral (HDAd) vector has been previously reported in the Gunn rat, a model of Crigler–Najjar syndrome, but was only achieved using high doses ($\geq 3 \times 10^{12}$ viral particles [vp]/kg), which are likely to elicit a severe toxic response in humans. Therefore, in this study, we investigate strategies to achieve correction of hyperbilirubinemia in the Gunn rat using clinically relevant low HDAd doses. We have found that correction of hyperbilirubinemia in the Gunn rat can be achieved with a low dose of 5×10^{11} vp/kg by using an HDAd vector bearing a more potent UGT1A1 expression cassette. Furthermore, by using hydrodynamic injection of the improved HDAd vector, correction of hyperbilirubinemia in the Gunn rat can be achieved using an even lower dose of 5×10^{10} vp/kg. Although hydrodynamic injection as performed in rats is not acceptable in humans, clinically attractive, minimally invasive methods have been successfully developed to mimic hydrodynamic injection of HDAd vector in non-human primates. Therefore, using an improved expression cassette combined with a more efficient method of vector delivery permits correction of hyperbilirubinemia in the Gunn rat using clinically relevant low HDAd doses and may thus pave the way to clinical application of HDAd vectors for Crigler–Najjar syndrome gene therapy.

Introduction

CRIGLER–NAJJAR SYNDROME (OMIM 218800) is an autosomal recessive inborn error of bilirubin metabolism caused by the lack of hepatic uridine diphospho-glucuronosyl transferase 1A1 (UGT1A1) activity. Being a highly hydrophobic compound, bilirubin cannot be eliminated in the bile unless it is conjugated with glucuronic acid by UGT1A1. Deficiency of UGT1A1 results in the accumulation of unconjugated bilirubin in various organs, causing jaundice and brain damage, and without treatment, patients may suffer significant neurological impairment and death (Jansen, 1999). Patients with Crigler–Najjar syndrome type I have complete deficiency of UGT1A1 activity, are refractory to phenobarbital treatment, have life-threatening elevations of bilirubin, and are generally managed with phototherapy, which results in degradation of unconjugated bilirubin throughout childhood and adolescence. Although effective, phototherapy is cumbersome and inconvenient, as it requires up to 14 hr of exposure, and its efficacy may diminish with age because of increased skin thickness and decreased sur-

face/mass ratio. Moreover, despite this treatment, patients remain at risk of brain damage when intercurrent infections may increase production of bilirubin above levels that can be controlled by the phototherapy (van der Veere *et al.*, 1996). Liver transplantation is the only currently available cure for Crigler–Najjar syndrome type I, and it provides complete metabolic correction (Schauer *et al.*, 2003; Ozcay *et al.*, 2009). However, it is complicated by perioperative mortality, organ rejection, and the long-term morbidity associated with chronic immunosuppression (Meyburg and Hoffmann, 2005). Hepatocyte transplantation, which is less invasive than orthotopic liver transplantation, resulted in partial albeit transient correction of the hyperbilirubinemia. Nevertheless, this study showed that about 5% of hepatocytes with normal enzymatic activity can result in significant improvement of serum bilirubin levels (Fox *et al.*, 1998).

Lifelong correction of hyperbilirubinemia using helper-dependent adenoviral (HDAd) vectors expressing UGT1A1 has been reported in the Gunn rat, a model of Crigler–Najjar syndrome (Toietta *et al.*, 2005). In that study, complete and lifelong phenotypic correction was achieved using 1×10^{13}

Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030.

*Present address: Division of Genetics, Department of Pediatrics, Medical College of Wisconsin, Milwaukee, WI 53226.

[†]Present address: Telethon Institute of Genetics and Medicine; and Department of Pediatrics, Federico II University of Naples, 80131 Naples, Italy.

viral particles (vp)/kg and 3×10^{12} vp/kg, and lifelong partial correction was achieved with a dose of 6×10^{11} vp/kg. These encouraging results suggest that Crigler-Najjar patients may benefit from HDAd-mediated gene therapy. Unfortunately, these doses are too high for human patients because they will likely elicit an acute toxic and potentially lethal response considering that a partial ornithine transcarbamylase (OTC)-deficient patient died following administration of 6×10^{11} vp/kg of a recombinant adenovirus vector (Raper *et al.*, 2003). Because adenovirus-mediated acute toxicity is dose-dependent (Nunes *et al.*, 1999; Morral *et al.*, 2002; Brunetti-Pierri *et al.*, 2004), it is imperative that strategies be developed to permit efficient high hepatocyte transduction at much lower dose. In this regard, we have previously shown that HDAd vector delivered by hydrodynamic injection in mice significantly increases hepatic transduction efficiency and reduces vector systemic dissemination and activation of the acute inflammatory response (Brunetti-Pierri *et al.*, 2005). Unfortunately, hydrodynamic injections, as performed in rodents, cannot be performed in humans because of the rapid injection of a large volume. However, we have successfully developed minimally invasive and clinically attractive methods to mimic hydrodynamic injection of HDAd vector in non-human primates without the need for rapid, large volume injection. It is important that these methods result in high-efficiency hepatocyte transduction using clinically relevant low vector doses (Brunetti-Pierri *et al.*, 2007, 2009). In addition to higher-efficiency hepatocyte transduction, strategies to improve the potency of the expression cassette by inclusion of *cis*-acting elements to enhance transgene expression may also improve the therapeutic index of the vector to permit dose reduction. Therefore, in the current study, we investigate the efficacy of hydrodynamic injection of an HDAd vector bearing an improved UGT1A1 expression cassette in Gunn rats.

Materials and Methods

HDAd vectors

HDAd28E4LacZ contains a MCMV-LacZ expression cassette and has been described in detail previously (Palmer and Ng, 2003). The expression cassette of the HDAd-rat phosphoenolpyruvate carboxykinase promoter (PEPCK)-UGT1A1-HCR is identical to that of the HDAd-PEPCK-UGT1A1 (Toietta *et al.*, 2005) except for the insertion of a ~ 1.7 -kb *XhoI*-*SalI* fragment containing the human apoE Hepatic Control Region (HCR) (Simonet *et al.*, 1993) into the *XhoI* site immediately 5' of the polyadenylation site (Fig. 1). Details regarding vector construction will be provided by the authors upon request. HDAds were produced using the helper virus AdNG163 (Palmer and Ng, 2004) and the 116 producer cell line (Palmer and Ng, 2003) as described in detail elsewhere (Palmer and Ng, 2003, 2008). Helper virus contamination levels were determined as described elsewhere (Palmer and Ng, 2003) and were found to be $<0.05\%$ for all vector preparations. DNA analyses of HDAd genomic structure was confirmed for all vectors as described elsewhere (Palmer and Ng, 2003).

Animal studies

Female homozygous Gunn rats (*HsdBlu:Gunn J/J*) were purchased from Harlan (Indianapolis, IN) and were

FIG. 1. Expression cassettes in (A) HDAd-PEPCK-UGT1A1 and (B) HDAd-PEPCK-UGT1A1-HCR. HDAd-PEPCK-UGT1A1-HCR was constructed by inserting the HCR into the *XhoI* site of HDAd-PEPCK-UGT1A1-HCR. intron, human apolipoprotein A1 intron; WPRE, woodchuck hepatitis post-transcriptional regulatory element; poly A, polyadenylation site.

maintained on Teklad rodent feed (Harlan, Indianapolis, IN) under 12-hr light/dark cycles. Eight-week-old female rats (weighing 150–180 g) were used for all vector experiments. Wistar rats used as controls of the analysis of bilirubin conjugation were purchased from Harlan (Indianapolis, IN). Animal experiments were performed according to a protocol approved by the Baylor College of Medicine (Houston, TX) Institutional Animal Care and Utilization Committee. Conventional injections were performed injecting 0.5 ml of vector diluted in lactated Ringer's solution into the tail vein. The hydrodynamic injections were performed by injecting the vector diluted in lactated Ringer's solution (100 ml/kg) prewarmed to 37°C via the tail vein in less than 15 sec using a Thermo winged infusion set and a 25-ml syringe, as previously reported (Maruyama *et al.*, 2002). The animals injected with HDAd28E4LacZ vector were sacrificed 72 hr post-injection for bromochloroindolyl galactopyranoside (X-Gal) histochemistry and β -galactosidase activity, which were performed as previously described (Brunetti-Pierri *et al.*, 2005). Blood samples from rats injected with HDAd-PEPCK-UGT1A1-HCR were collected by lateral saphenous vein puncture at baseline and at the various times after vector injection. Total serum bilirubin was assayed using the diazo dye method on a Roche COBAS Integra 400 Plus (Roche Diagnostics, North America, Indianapolis, IN), according to the manufacturer's instructions. Bile was collected by catheterization of the bile duct at 60 weeks post-vector and analyzed using high-performance liquid chromatography, as previously described (Toietta *et al.*, 2005). Statistical analyses were performed with the *t* test.

Results and Discussion

We first sought to determine whether hydrodynamic injection of HDAd vector would result in an increase of hepatic transduction in rats. To accomplish this, HDAd28E4LacZ at 1×10^{12} vp/kg was conventionally or hydrodynamically injected into wild-type rats. At 72 hr post-injection, the livers were harvested for analyses. Consistent with our previous results in mice (Brunetti-Pierri *et al.*, 2005), conventional injection of 1×10^{12} vp/kg (Fig. 2A) yielded qualitatively fewer β -galactosidase-positive cells than hydrodynamic injection (Fig. 2B) in wild-type rats as determined by X-gal histochemistry. Splenic transduction was also investigated. We found little to no splenic transduction following either conventional (Fig. 2C) or hydrodynamic injection (Fig. 2D) of

FIG. 2. X-gal histochemistry of representative rat (A and B) liver and (C and D) spleen 72 hr after conventional (A and C) or hydrodynamic (B and D) injection of HDAd28E4LacZ at 1×10^{12} vp/kg. (E) β -Galactosidase activity in liver tissues is significantly higher after hydrodynamic injection compared with conventional injection of HDAdLacZ at a dose of 1×10^{12} vp/kg ($p = 0.035$) (A) ($n \geq 3$ per group). Color images available online at www.liebertonline.com/hum.

HDAd28E4LacZ at 1×10^{12} vp/kg as determined by X-gal staining. To quantitate the amount of hepatic transgene expression, total proteins were extracted from the livers, and the amount of β -galactosidase activity was determined. The results revealed that hydrodynamic injection resulted in fourfold higher activity than conventional injection ($p = 0.035$) (Fig. 2E).

Toietta *et al.* (2005) showed that conventional tail vein injection of HDAd-PEPCK-UGT1A1 into Gunn rats resulted in lifelong phenotypic correction of hyperbilirubinemia, albeit using high vector doses ($\geq 3 \times 10^{12}$ vp/kg). As a first step towards improving the therapeutic index of the vector, we modified HDAd-PEPCK-UGT1A1, the vector used by Toietta *et al.* (2005), to create HDAd-PEPCK-UGT1A1-HCR by inserting into the UGT1A1 expression cassette the human apolipoprotein E HCR (Fig. 1). The HCR is a powerful *cis*-acting liver-specific enhancer element shown to greatly increase liver-specific expression of associated genes (Simonet *et al.*, 1993). We then administered, by conventional injection, four doses (5×10^{11} vp/kg, 1×10^{11} vp/kg, 5×10^{10} vp/kg, and 1×10^{10} vp/kg) of HDAd-PEPCK-UGT1A1-HCR into Gunn rats (Fig. 3A). With HDAd-PEPCK-UGT1A1-HCR at 5×10^{11} vp/kg, total serum bilirubin was normalized for at least 60 weeks. At 1×10^{11} vp/kg, total serum bilirubin did not normalize but did result in an average 60% reduction compared with pretreatment levels ($p = 0.000296$). The dose of 5×10^{10}

FIG. 3. Serum bilirubin levels in Gunn rats injected at various doses by (A) conventional or (B) hydrodynamic injections ($n \geq 3$ per group). The normal range in wild-type rats is 1.25 ± 0.86 mg/dl.

vp/kg by conventional injection also resulted in a partial correction with an average reduction of 44% compared with pretreatment levels ($p = 0.005$). Finally, a dose of 1×10^{10} vp/kg by conventional injection resulted in a 16% reduction of serum bilirubin compared with pretreatment levels ($p = 0.0019$). These results demonstrate that inclusion of the HCR decreases the therapeutic dose of HDAd vector because we could achieve normalization of serum bilirubin at a dose of 5×10^{11} vp/kg with HDAd-PEPCK-UGT1A1-HCR, whereas Toietta *et al.* (2005) could only achieve normalization of serum bilirubin at doses of at least 3×10^{12} vp/kg with HDAd-PEPCK-UGT1A1. Furthermore, we achieved a 60% reduction in serum bilirubin with HDAd-PEPCK-UGT1A1-HCR at 1×10^{11} vp/kg, whereas an HDAd-PEPCK-UGT1A1 dose of 6×10^{11} vp/kg was required to achieve a comparable level of reduction by Toietta *et al.* (2005). Our conclusion that inclusion of the HCR can significantly improve the therapeutic index of HDAd is consistent with the results reported by Brunetti-Pierri *et al.* (2008). In that study, an HCR-bearing HDAd vector expressing OTC was able to achieve phenotypic correction in OTC-deficient mice with at least a fivefold lower dose than an HDAd vector lacking the HCR.

We next determined whether hydrodynamic injection of the vector could further reduce the therapeutic dose. To accomplish this, HDAd-PEPCK-UGT1A1-HCR at 5×10^{10} vp/kg and 1×10^{10} vp/kg was hydrodynamically injected into Gunn rats, and serum bilirubin levels were determined at

FIG. 4. Excretion of bilirubin glucuronides in the bile 60 weeks post-vector injection shown in high-performance liquid chromatograms of bile collected by cannulation of the bile duct in (A) untreated Gunn rats, (B) wild-type (WT) control rats, (C) Gunn rats receiving 5×10^{11} vp/kg by conventional intravenous injection, (D) Gunn rats receiving 5×10^{10} vp/kg by hydrodynamic injection, (E) Gunn rats receiving 1×10^{11} vp/kg by conventional injection, (F) Gunn rats receiving 1×10^{10} vp/kg by hydrodynamic injection, and (G) Gunn rats receiving 5×10^{10} vp/kg by conventional injection.

various times post-injection (Fig. 3B). The results revealed that complete normalization of the serum bilirubin levels was achieved with 5×10^{10} vp/kg, whereas a 25% reduction of serum bilirubin levels compared with baseline ($p = 0.0007$) was achieved with a dose of 1×10^{10} vp/kg (Fig. 3B). These results demonstrate that hydrodynamic injection can further improve the therapeutic index of HDAd-PEPCK-UGT1A1-HCR such that clinically relevant lower vector doses can be used to achieve a therapeutic effect, at least in the Gunn rat model.

To confirm that the decline in serum bilirubin in the vector-injected animals was due to glucuronidation of unconjugated bilirubin resulting in excretion of bilirubin monglucuronide (BMG) and diglucuronide (BDG) in the bile, bile was collected from one animal at each dose and injection condition at 60 weeks and analyzed by high-performance liquid chromatography (Fig. 4). As expected, BMG and BDG were absent in bile from untreated Gunn rats (Fig. 4A) but present in nonjaundiced wild-type control rats (Fig. 4B). Consistent with lowering of serum bilirubin, BDG and BMG were observed in the bile of Gunn rats treated by conventional injection of 5×10^{11} vp/kg (Fig. 4C) and 1×10^{11} vp/kg (Fig. 4E) as well as by hydrodynamic injection of 5×10^{10} vp/kg (Fig. 4D). Little to no BDG and BMG were observed in animals injected hydrodynamically with 1×10^{10} vp/kg (Fig. 4F) or conventionally with 5×10^{10} vp/kg (Fig. 4G). Taken together, these results demonstrate that conversion of unconjugated serum bilirubin to BMG and BDG for excretion is mediated by HDAd-PEPCK-UGT1A1-HCR in a dose-dependent manner and that lower vector doses can be effective if delivered by hydrodynamic injection.

Acute serum bilirubin elevations are concerning in patients with Crigler-Najjar syndrome. Therefore, we investigated whether administration of HDAd vector was associated with acute bilirubin elevations. We did not observe elevations in serum bilirubin at 6 hr after either hydrodynamic or conventional injection of HDAd vector (data not shown). The lack of acute elevation of serum bilirubin following the hydrodynamic injections suggests that such acute stress may not be associated with significant decompensation in the Gunn rat.

Although hydrodynamic injection as performed in the rat is not possible in humans because of the rapid injection of a large volume, we have previously developed clinically attractive methods that successfully mimic hydrodynamic injection of HDAd vector in nonhuman primates (Brunetti-Pierri *et al.*, 2007, 2009). In these minimally invasive methods, HDAd vector is injected into the animals following inflation of balloon occlusion catheters percutaneously positioned in the inferior vena cava to occlude hepatic venous outflow that results in increased intrahepatic pressure to mimic the hydrodynamic effect but without the need to rapidly inject large volumes (Brunetti-Pierri *et al.*, 2007, 2009). These balloon occlusion catheter-based methods yielded up to an 80-fold improvement in hepatic transduction in non-human primates compared with systemic peripheral intravenous injection, and these improvements are greater than the improvement afforded by hydrodynamic versus conventional injection of HDAd vector into mice or rats.

In summary, we have demonstrated that long-term normalization of serum bilirubin in the Gunn rat can be achieved using clinically relevant low vector doses ($< 6 \times 10^{11}$

vp/kg) of an HDAd vector bearing a higher-potency hUGT1A1 expression cassette and delivered by hydrodynamic injection. Given that clinically attractive methods to mimic hydrodynamic injection in non-human primates have been successfully developed, the strategy describing here may be beneficial to Crigler-Najjar syndrome type I patients.

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Author Disclosure Statement

The authors have nothing to disclose.

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Address correspondence to:

Dr. Philip Ng

Department of Molecular and Human Genetics

Baylor College of Medicine

One Baylor Plaza, T619

Houston, TX 77030

E-mail: png@bcm.edu

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