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Recommended Citation

Mian, A., McCormack, W., Mane, V., Kleppe, S., Ng, P., Finegold, M., O'Brien, W., Rodgers, J., Beaudet, A., Lee, B. (2004). Long-term correction of ornithine transcarbamylase deficiency by WPRE-mediated overexpression using a helper-dependent adenovirus. *Molecular Therapy*, 10(3), 492-499.

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Long-Term Correction of Ornithine Transcarbamylase Deficiency by WPRE-Mediated Overexpression Using a Helper-Dependent Adenovirus

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Available online 17 July 2004

The urea cycle disorders (UCDs) are important models for developing gene replacement therapy for liver diseases. Long-term correction of the most common UCD, ornithine transcarbamylase (OTC) deficiency, has yet to be achieved in clinical or preclinical settings. The single human clinical trial using early-generation adenovirus (Ad) failed to show any biochemical correction. In adult OTC-deficient mice, an E1/E2-deleted Ad vector expressing the mouse OTC gene, but not the human, was only transiently therapeutic. By using post-transcriptional overexpression in the context of the less immunogenic helper-dependent adenoviral vector, we achieved metabolic correction of adult OTC-deficient mice for >6 months. Demonstrating this result were normalized orotic aciduria, normal hepatic enzyme activity, and elevated OTC RNA and protein levels in the absence of chronic hepatotoxicity. Overexpressing the human protein may have overcome two potential mechanisms accounting for poor cross-species complementation: a kinetic block at the level of mitochondrial import or a dominant negative effect by the mutant polypeptide. These data represent an important approach for treating human inborn errors of hepatocyte metabolism like the UCDs that require high-level transduction and gene expression for clinical correction.

Key Words: liver-directed gene therapy, phosphoenolpyruvate carboxykinase (PEPCK) promoter, woodchuck hepatitis virus post-transcriptional regulatory element (WPRE)

INTRODUCTION

Because the urea cycle disorders (UCDs) are characterized by the triad of hyperammonemia, encephalopathy, and respiratory alkalosis, they are models of classic inborn errors of hepatocyte metabolism [1,2]. Despite aggressive pharmacotherapy, patients are at high risk for repeated episodes of hyperammonemia and cumulative neurological morbidity and mortality [3–5]. Hence, risk-benefit considerations make the development of clinical gene replacement therapy for this group of disorders appropriate for long-term correction as well as for bridging therapy to alternative treatments such as liver transplantation [6–9].

One group of researchers achieved partial correction in a large-animal bovine model of a UCD, citrullinemia (argininosuccinic synthetase deficiency), using a first-generation adenovirus (FGV) despite transduction of 30% of hepatocytes [10]. Several studies over the past decade have found the therapeutic effect of several different Ad vectors to be transient in ornithine transcarbamylase (OTC)-deficient mouse models [11–13]. The longest period of biochemical correction (2 months) was achieved with an E1/E2-deleted Ad expressing the mouse OTC (mOTC) gene [11]. A relatively high dose (5×10^{11} total particles per mouse) of a FGV expressing human OTC (hOTC) could not correct the biochemical defect in adult OTC-deficient

mice [11]. A report by Ye *et al.* suggests that the differences between mouse and human mitochondrial leader peptide sequences of OTC may lead to inefficient import of hOTC into mouse mitochondria [14]. An isolated report of short-term correction in *spf-ash* mice with FGV expressing hOTC at a relatively low dose of 5×10^8 pfu/mouse has not been reproduced [13].

Morsy *et al.* showed that an Ad vector expressing hOTC was therapeutic in neonatal *spf-ash* mice; however, measured OTC activity in treated mice was significantly lower than that in wild-type littermates [12]. This was attributed to a dominant negative effect of the endogenous mutant protein on the activity of the wild-type protein delivered by the Ad. Morsy *et al.* also suggested that increased enzyme activity in the intestine at early time points may have contributed to the rapid reduction of urinary orotic acid [12], and this was consistent with earlier transgenic studies documenting the importance of small-bowel expression of OTC [15].

Another obstacle to clinical efficacy is acute toxicity associated with direct vector–host cell interaction and the host innate immune response [16–19]. In a phase 1/2 human clinical trial, researchers administered an E1/E4-deleted Ad vector expressing hOTC into the right hepatic artery of human patients with partial OTC deficiency (OTCD). One patient treated with 6×10^{11} viral particles per kilogram suffered fatal acute toxicity [20]. Of concern was the absence of any substantial metabolic correction as measured by stable isotope study. One general strategy to reduce a potential host immune response is to restrict the pattern of transgene expression by using tissue-restricted promoters. This may be important for avoiding an adaptive immune response to the therapeutic protein product [21–23]. Another development that has markedly increased the therapeutic index for Ad gene therapy has been the generation of the helper-dependent adenoviral vector (HDV). This vector should avoid the host cell-mediated immune response to viral proteins and direct hepatotoxicity associated with viral gene expression [24]. Additionally, with the exception of expression of the intracellular structural protein dystrophin [25], data obtained so far with HDVs have focused on secreted proteins. Hence, the UCDs represent an important model for developing HDV-mediated correction of intracellular enzymatic deficiencies of hepatocyte metabolism. Increasing gene expression offers another potential avenue for increasing the therapeutic index, especially for a non-cell-autonomous process that would allow for an associated reduction in vector dose. The ability of specific viruses to enhance gene expression post-transcriptionally could serve well in this arena [26,27]. For example, Loeb *et al.* have postulated that the inclusion of the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) in the sense orientation

downstream of the cDNAs will enhance stability of mRNA [26]. Hence, it may be an effective tool for increasing the level of protein expression in gene therapy [28].

We hypothesized that by combining HDV-mediated delivery, tissue-restricted expression of an intracellular protein product, and post-transcriptional enhancement of expression using the WPRE, we can achieve high-level and efficient expression of hOTC and correct the metabolic phenotype in *spf-ash* mice with little, if any, chronic toxicity. This would constitute an important model for correcting a host of inborn errors of hepatocyte metabolism including organic acidemias, fatty acid oxidation defects, and amino acidopathies.

RESULTS

Generation and Characterization of HDVs

The phosphoenolpyruvate carboxykinase (PEPCK) promoter is predominantly expressed in the liver and to some degree in the kidney [29]. These are also the sites where the urea cycle is active, making this an appropriate tissue-restricted promoter for expression of UCD proteins. The region of the rat PEPCK promoter from –2,088 to +69 bp was both necessary and sufficient to direct hepatocyte-specific, developmental, hormonal, and dietary regulation of the gene encoding PEPCK [30]. We generated two HDV plasmids derived from the C4HSU plasmid [31]. Both contained the PEPCK promoter upstream of the hOTC cDNA (Fig. 1A). One study had shown that the 600-bp WPRE enhanced gene expression in an orientation-specific but transgene-, promoter-, and vector-independent manner [32]. Hence, we inserted this downstream of the hOTC cDNA in one vector (Fig. 1A). Restriction digestion analysis of vector and parental plasmid DNA showed the expected bands and excluded gross vector rearrangements during rescue (Fig. 1B). Southern hybridization using a packaging signal-specific probe followed by quantification by phosphoimaging indicated 0.09% helper contamination for HDV-hOTC and 0.12% for HDV-hOTC-WPRE, respectively (Fig. 1B, bottom panel).

Long-Term HDV-Mediated Correction of Orotic Aciduria in OTCD Mice

In OTCD there is accumulation of carbamyl phosphate that can be diverted to cytoplasmic pyrimidine synthesis. Hence, OTCD patients and mice have increased excretion of urinary orotic acid [33,34]. In clinical practice, orotic aciduria is an important biochemical measure of urea cycle activity in OTCD. Hence, successful gene replacement therapy of OTCD should normalize urinary concentrations of this metabolite. To determine whether HDV-mediated gene therapy could correct orotic aciduria, we treated *spf-ash* mice with intravenous injection of saline, HDV-PEPCK-hOTC, or HDV-PEPCK-hOTC-WPRE.

FIG. 1. HDV structure and helper virus contamination. (A) Schematic showing the two vectors used in this study. The first contains hOTC cDNA downstream of the PEPCK promoter (P). In the second construct, WPRE (W) was inserted downstream of hOTC cDNA in an orientation- and position-specific manner. Ψ , Ad packaging signal; L-ITR, left inverted terminal repeat; R-ITR, right inverted terminal repeat. (B) Restriction digests of viral vector (lanes 1 and 3) and parental plasmid (lanes 2 and 4) DNAs. Arrow indicates the band containing the bacterial origin of replication and ampicillin resistance cassette expected in the case of the parental plasmid only (lanes 2 and 4), but visualized better with the *XmnI* digest. Identical bands in vector and plasmid lanes indicate absence of gross vector rearrangements. After a prolonged exposure a helper virus-specific band was apparent in the vector lane (bottom panel). M, 1 kb molecular weight standard; H, helper virus; 1, HDV-PEPCK-hOTC; 2, pC4HSU-PEPCK-hOTC; 3, HDV-PEPCK-hOTC-WPRE; 4, pC4HSU-PEPCK-hOTC-WPRE.

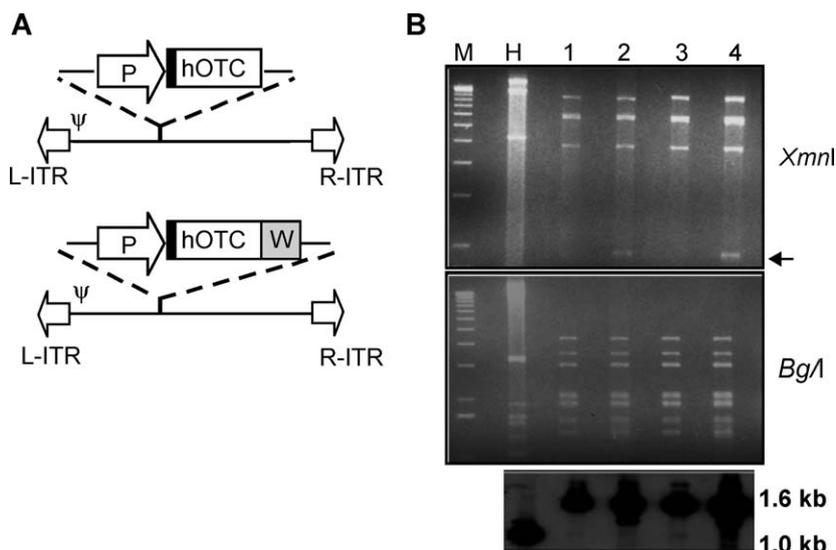


Fig. 2 shows the profile of urinary orotic acid excretion. *Spf-ash* mice treated with PBS demonstrated consistently elevated urinary orotic acid compared with wild-type mice, though with considerable variability that is reflective of the daily pattern of food and water intake. Mice treated with 1.0×10^{13} particles/kg of HDV-PEPCK-hOTC showed a significant decline in orotic acid concentrations 1 week after injection (115 vs. 29 mM/mol of creatinine (cr); $P = 0.04$) (Fig. 2). However, by 2 weeks after injection, there was no difference between this and the PBS-treated group. In contrast, urinary orotic acid of mice treated with 1.0×10^{13} particles/kg of the WPRE-containing vector decreased to within the normal range (18 ± 7 mM/mol cr; $n = 10$ age- and sex-matched littermates) 1

week after injection. Thereafter, these mice showed a complete and prolonged correction of orotic aciduria that lasted for the duration of the experiment (Fig. 2). A subgroup of these mice that were not sacrificed at the endpoint (25 weeks) have continued to show normal urinary orotic acid at 12 months after injection ($n = 2$; data not shown). These mice had no mortality.

Complete Normalization of Hepatic OTC Activity in HDV-Treated Mice

Histochemical assays on liver sections from mice injected with HDV-PEPCK-hOTC demonstrated little staining in a majority of hepatocytes (Figs. 3C and F). This result was supported by the biochemical assay on liver homogenates (Fig. 3H), which showed hepatic OTC activity comparable to livers from mice treated with PBS. In contrast, mice treated with the WPRE-containing vector demonstrated considerable liver OTC activity by histochemical staining in a majority of hepatocytes (Figs. 3D and G). Histochemical staining supported the presence of high-level hepatocyte transduction and transgene expression required for clinical correction of a defect of hepatocyte metabolism. To quantify this, we measured OTC activity in homogenized liver in these groups of mice. The HDV-PEPCK-hOTC-WPRE-treated group had comparable activity to that of wild-type mice. Moreover, the HDV-PEPCK-hOTC-WPRE group had significantly more activity than PBS-treated (19,000 vs. 852 $\mu\text{mol/h/g}$; $P < 0.0001$) or HDV-PEPCK-hOTC-treated groups (19,000 vs. 700 $\mu\text{mol/h/g}$; $P < 0.0001$).

Enhanced Steady-State Concentrations of OTC mRNA and Protein Mediated by WPRE

To determine whether differences in vector transduction efficiency might account for differences in phenotypic correction mediated by HDV-PEPCK-hOTC-

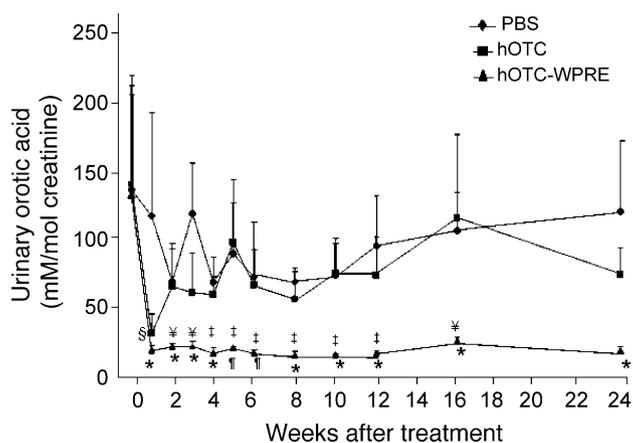


FIG. 2. Long-term correction of orotic aciduria in *spf-ash* mice by WPRE-containing HDV. Orotic aciduria was completely abolished in mice treated with the WPRE-containing HDV, and this effect lasted for >6 months. Values represent mean \pm SD of between 4 and 10 determinations. $^{\$}P < 0.05$ HDV-hOTC vs. PBS; $^*P < 0.005$, $^{\ddagger}P = 0.02$, HDV-hOTC-WPRE vs. PBS; $^{\vee}P < 0.02$, $^{\dagger}P < 0.005$, HDV-hOTC-WPRE vs. HDV-hOTC.

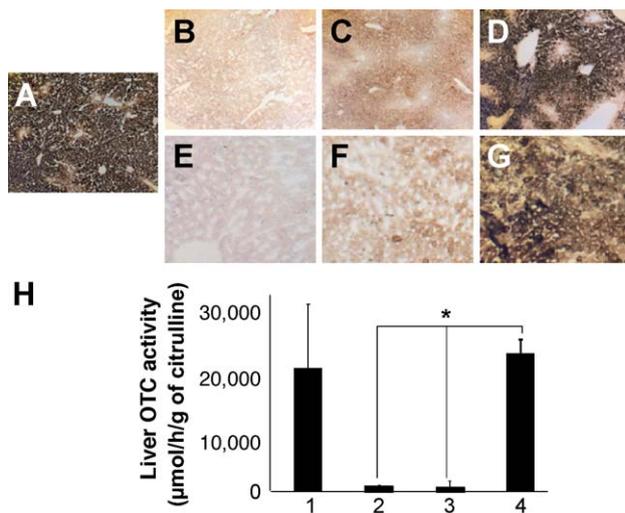


FIG. 3. Complete normalization of liver OTC activity in *spf-ash* mice treated with WPRE-containing vector. (A) For comparison, OTC histochemical activity in liver from a wild-type male littermate is shown. Liver tissue from PBS-injected *spf-ash* mice shows very low levels of histochemically active OTC, either at 5 weeks (B) or 25 weeks (E) after treatment. In contrast, mouse liver from the HDV-PEPCK-hOTC-treated group shows low histochemical staining above background, at both time points (C and F). In liver from mice treated with WPRE-containing vector, OTC histochemical activity is similar to wild-type levels (D and G). (H) Liver OTC activity measured by a quantitative biochemical assay shows normal levels in the HDV-PEPCK-hOTC-WPRE-treated group of *spf-ash* mice at 25 weeks after injection. 1, Wild-type male littermate; 2, *spf-ash*, PBS; 3, *spf-ash*, HDV-PEPCK-hOTC; 4, *spf-ash*, HDV-PEPCK-hOTC-WPRE. Values represent mean \pm SD of three independent determinations. * $P < 0.0001$.

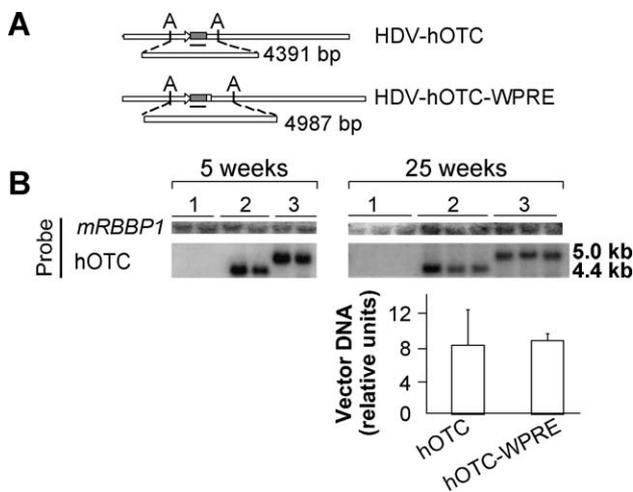


FIG. 4. Efficacy of WPRE-containing vector is not due to differences in hepatocyte transduction. (A) *AsclI* restriction map for the two vectors used in the study. Small black bar represents probe used in Southern blotting. (B) Southern blot. The vector/mouse genome ratio was similar between the two vectors (quantification shown for week 25). 1, PBS; 2, HDV-hOTC; 3, HDV-hOTC-WPRE.

WPRE vs. the HDV without the WPRE, Southern blot analyses were done on liver-derived DNAs from mice at weeks 5 and 25 after injection. Using a probe specific for vector (hOTC) and a probe specific for an endogenous mouse gene (*Rbbp1*) for normalization, quantitative Southern blot analysis showed comparable vector DNA for the two HDVs (Fig. 4B). This suggested that both vectors had similar transduction rates in liver and that potential differences in infectivity did not account for the different degree of phenotypic correction.

Northern hybridization analysis of liver RNA showed significantly higher steady-state levels of transgene mRNA in the mice treated with the WPRE-containing vector (Fig. 5A). After normalizing to mouse *Gapdh* mRNA, quantification showed that HDV-PEPCK-hOTC-WPRE-treated mouse livers had a 9-fold greater amount of hOTC mRNA when compared with HDV-PEPCK-hOTC-treated mouse livers. Western blot analysis was consistent with this finding, with significantly higher levels of total OTC protein in liver from mice treated

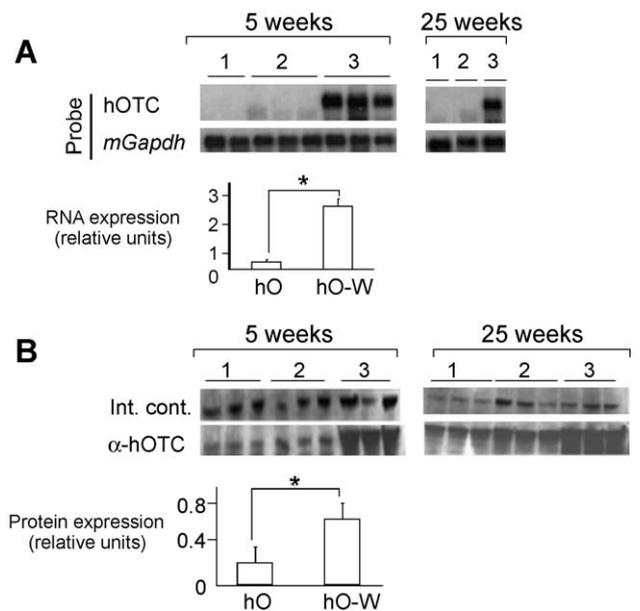


FIG. 5. Increased steady-state levels of OTC mRNA and protein in mice treated with HDV-hOTC-WPRE. (A) Northern blot. The relative intensities of hOTC mRNA and mouse *Gapdh* were quantified at week 5 only. At steady state, the amount of hOTC mRNA was significantly elevated in the livers of *spf-ash* mice treated with the WPRE-containing vector compared with the other group (* $P < 0.0005$ for week 5). 1, PBS-treated mice; 2 and hO, HDV-PEPCK-hOTC-treated mice; 3 and hO-W, HDV-PEPCK-hOTC-WPRE-treated mice. (B) Western blot. hOTC (lanes in groups 2 and 3) and mOTC (lanes in group 1) were detected by a polyclonal antibody generated to hOTC. The OTC protein was normalized to an internal control band (int. cont.). At steady state, the amount of hOTC protein was significantly elevated in the livers of *spf-ash* mice treated with HDV-PEPCK-hOTC-WPRE (* $P < 0.02$ for week 5). 1, PBS-treated mice; 2, and hO, HDV-PEPCK-hOTC-treated mice; 3 and hO-W, HDV-PEPCK-hOTC-WPRE-treated mice.

with the WPRE-containing vector (Fig. 5B). The polyclonal antibody reacts with both the mouse and human proteins and detected the mOTC in the PBS-treated *spf-ash* mice. The sizes of the detected bands were appropriate based upon respective translation of the open reading frames.

Tissue-Restricted Expression Alone Does Not Confer Long-Term Correction

To determine whether the prolonged correction of the hOTC-WPRE cassette was primarily due to absence of viral gene expression and/or tissue-restricted expression of the protein, we generated a FGV vector harboring the identical PEPCK-hOTC-WPRE transgene. The FGV vector expressing hOTC from the tissue-restricted PEPCK promoter (FGV-PEPCK-hOTC-WPRE) was unable to normalize urinary orotic acid in the affected mice for a prolonged period, even though the construct contained the WPRE (Fig. 6A). As shown in Fig. 6A, a transient decline in urinary orotic acid could not be sustained. This course of correction is highly reminiscent of that obtained by Ye *et al.* using a E1/E2-deleted Ad vector expressing the mouse OTC cDNA from the cytomegalovirus promoter [11]. In contrast to Southern blot data from HDV-treated mice (Fig. 4B), liver tissue from FGV-treated mice showed a marked decline in vector DNA from week 5 to 15 (Fig. 6B), consistent with the urinary orotic acid data for the latter group of mice.

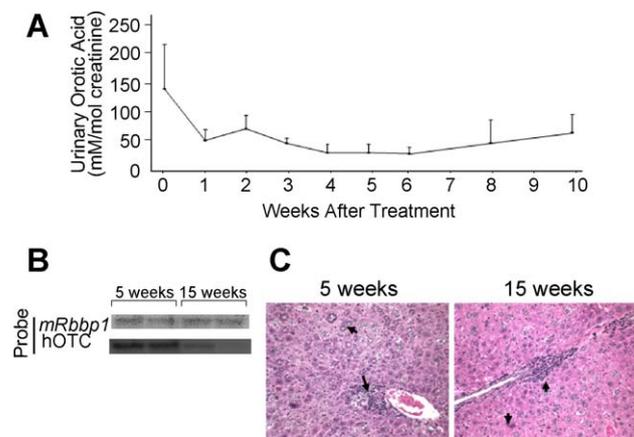


FIG. 6. Tissue-restricted expression mediated by FGV-PEPCK-hOTC-WPRE is unable to achieve long-term correction. (A) A transient drop in orotic acid production is followed by an upward trend in urinary orotic acid. Note that urinary orotic acid levels never entered the normal range, as was observed with HDV-mediated transfer. Values represent mean \pm SD of between three and seven determinations. (B) Southern blot shows that FGV-mediated transfer is accompanied by loss of vector genome between week 5 and 15 after injection. (C) Liver histology shows FGV-mediated hepatotoxicity. Arrows indicate foci of lymphocytic infiltration consistent with necro-inflammation and single-cell necrosis of hepatocytes, respectively. The dose was 1×10^{13} particles/kg in both the HDV and FGV cases.

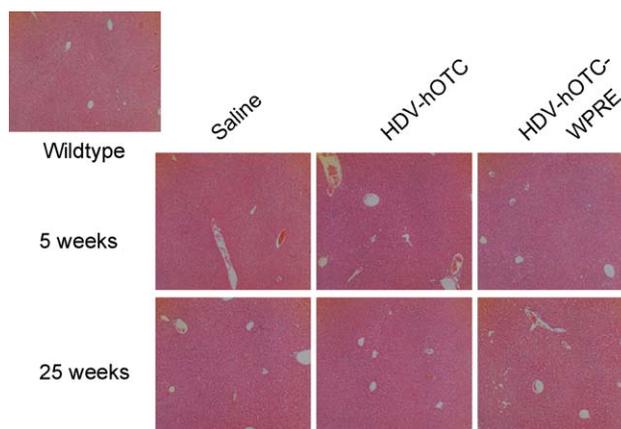


FIG. 7. Complete absence of liver histopathology in HDV-treated mice when compared with PBS-treated mice on H and E staining.

Absence of Chronic Toxicity with the HDV

First generation Ad vector treatment is associated with hepatotoxicity, both early and late after injection [6,35,36]. Compared with PBS-treated mice, however, those receiving HDV showed no significant elevation in liver function tests. Serum ALT at 1 month after injection was as follows: 52 ± 17 U/liter (PBS) vs. 74 ± 1 U/liter (HDV-PEPCK-hOTC) vs. 37 ± 12 U/L (HDV-PEPCK-hOTC-WPRE). Histological analysis confirmed the absence of hepatitis, lymphocytic infiltration, fibrosis, or steatosis in PBS- and in HDV-treated mice at both early and late time points (Fig. 7). In contrast, mice treated with the first-generation vector expressing hOTC (FGV-PEPCK-hOTC-WPRE) demonstrated significant hepatotoxicity. There was evidence of liver injury with variable extent of hepatocyte anisonucleosis (variations in size, amount of chromatin, vacuoles, multilobation, and multiple nuclei), as shown in Fig. 6C. Furthermore, at 1 month after injection the average serum ALT in the FGV-injected mice was 332 U/liter, which was five times greater than in untreated or HDV-treated mice.

DISCUSSION

A helper-dependent Ad vector carrying the WPRE and expressing hOTC in a tissue-restricted fashion was able to correct the biochemical phenotype in OTCD mice for >6 months. This is the first demonstration of long-term clinical correction in this model using the human transgene. In fact, our results also demonstrate long-term clinical correction of an intracellular enzymatic protein deficiency in hepatocytes by an Ad vector. Together, the data support a potential approach for correction of genetic deficiencies that require high-level transduction and gene expression in the liver. Until now, most studies have focused on correction of metabolic phenotypes of non-cell-autonomous processes like hemophilia and β -glucuronidase deficiency. In these conditions, transduc-

tion of a smaller percentage of target cells could theoretically correct the clinical phenotype by overexpressing a secreted protein.

Metabolic parameters including serum glutamine and ammonia have been assessed as endpoints, but because of the significant variability throughout the day, partially reflective of food intake, we and others have used urinary orotic acid as a primary metabolic endpoint of preclinical efficacy. As in severely affected OTC female patients who have partial activity of the urea cycle, urinary orotic acid correlates best with clinical status. Hence, we used this as our main biochemical and metabolic endpoint for clinical correction. Several groups have attempted to correct the *spf-ash* phenotype using Ad vectors in preclinical studies. In one experiment, Ye *et al.* showed that a FGV containing a ubiquitously expressed hOTC cDNA did not correct the biochemical phenotype, whereas an identical vector containing mOTC cDNA did so transiently at a relatively high dose (2×10^{11} – 5×10^{11} total particles/mouse) [11]. To determine whether overexpression of the human OTC could overcome the hypothesized kinetic block of mitochondrial entry, we injected 3×10^{11} particles/mouse of each vector, a dose similar to what Ye *et al.* had shown to work transiently in mice. At 2- and 10-fold lower doses of HDV, there was no correction of orotic aciduria, despite the WPRE (data not shown). The extremely short-lived partial correction that we observed with HDV-PEPCK-hOTC was similar to the pattern reported for early-generation Ad vectors expressing the human transgene. Because hOTC is poorly imported into mouse mitochondria, it may undergo accelerated degradation in the cytoplasm [14]. Furthermore, because steady-state protein concentration will depend on the rate of protein turnover, there might not be sufficient protein for sustained therapeutic effect, thereby explaining the transient correction rather than long-term low-level correction. In fact, both the western blot analyses and the histochemical staining of liver tissue show that the HDV-PEPCK-hOTC-treated mice did not have steady-state levels of OTC protein (mouse or human) greater than what is seen in the untreated mice, which retain ~5% residual activity. Our data show that the WPRE is an effective *cis*-acting sequence for considerably enhancing transgene expression, a variable that could increase the therapeutic index of Ad-mediated gene therapy. One could argue that in human hepatocytes, where impeded import of hOTC is not an issue, the steady-state level of protein may be even higher than that observed in the mouse system. This would make such an approach even more efficacious in the human setting.

The presence of a potentially nonfunctional, albeit lower steady-state concentration of mOTC protein may influence immune responses in gene therapy experiments. These mice are not immune deficient but do express the mOTC protein. Hence, an immune response to the very similar hOTC is less likely. In fact, in another

system in which we have compared the B6 mouse immune response to another urea cycle enzyme, argininosuccinic acid synthetase (ASS), which is similarly homologous between humans and mice, we could not detect mouse cytotoxic immune responses to human ASS (unpublished data). Ye *et al.* suggested that a cell-mediated immune response to hOTC or to residual viral protein expression could also contribute to the short duration of correction that they observed [11,36]. It is possible that residual viral protein expression in early Ad vectors may concurrently act as an adjuvant to the host immune response to the transgene protein. Our data suggest that we can overcome these potential mechanisms in the absence of viral gene expression with the HDV and by restricting transgene expression primarily to hepatocytes. At least in this model, the finding of transient correction with the FGV carrying an identical transgene, the immunogenic effect of viral proteins with associated direct toxicity probably constitute the predominant influences in limiting correction at these higher doses. We know that liver-specific expression of secreted proteins can avoid a humoral immune response in otherwise responsive mice [21,23]. Although the use of tissue-restricted promoters offers theoretical advantages even in our case, it alone in the FGV backbone could not confer clinical correction.

Kiwaki *et al.* did not observe any correction when the hOTC cDNA was driven by the ubiquitously expressing $Sr\alpha$ promoter, whereas the same promoter-transgene combination was therapeutic in *spf-ash* mice according to Morsy *et al.* [12]. In the latter case, it is possible that neonatal gene therapy induced immune tolerance to the transgene. From a risk-benefit perspective, severe neonatal OTCD would be an optimal candidate disorder for developing gene replacement therapy. The use of HDV would minimize concerns of chronic toxicity and hepatitis due to the host adaptive immune response.

These studies also demonstrate effective production of HDVs using a recently described suspension culture [37]. This is an important advance that facilitates the production of infectious vectors, with minimal helper virus contamination. This is crucial for permitting future clinical trials. Although the use of HDVs, tissue-restricted promoters, and gene-regulatory strategies for increasing gene expression will improve the therapeutic index of these therapies and minimize the adaptive immune response and chronic toxicity, a remaining obstacle for translating these findings to the clinic is an acute toxicity associated with systemic Ad delivery [38]. This acute toxicity is evident within hours after delivery and is characterized, in part, by direct interaction of the vector with host cells, an innate immune response to transduced cells, and a cytokine response. Ultimately, this acute toxicity can escalate into a systemic inflammatory response that is characterized by an amplification of the cytokine burst during an intermediate phase of toxicity

that may be further complicated by *de novo* viral gene expression [18,19]. Although HDVs and FGVs probably have similar acute toxicity profiles, the absence of viral gene expression in HDVs might attenuate the host response during the intermediate phase of toxicity.

In conclusion, we have successfully demonstrated long-term correction of OTCD in a mouse model by WPRE-mediated tissue-restricted overexpression of hOTC from a HDV. Importantly, complete clinical correction occurred without associated chronic toxicity. Incorporation of the *cis*-acting WPRE can increase protein expression, whereas the tissue-restricted expression might further decrease an adaptive immune response to the transgene product, although it alone could not confer long-term correction in the presence of viral gene expression. It seems that in this model, the absence of viral gene expression was the primary variable facilitating long-term correction. These data also demonstrate correction of an intracellular enzymatic defect requiring high-level transduction and gene expression. This might constitute a general approach that improves the therapeutic index of Ad-mediated gene replacement therapies of enzymatic deficiencies of hepatocyte metabolism.

MATERIALS AND METHODS

Generation and characterization of Ad vectors. hOTC cDNA with *NotI* linkers was cloned downstream of the rat PEPCK promoter to generate pPEPCK-hOTC. This plasmid was digested with *AscI*, and the resultant PEPCK-hOTC fragment inserted into the adenoviral backbone plasmid pC4HSU [31] to generate the parental HDV plasmid pC4HSU-PEPCK-hOTC. Plasmid pWPRE was digested with *Clal* to release the WPRE, which was then inserted into the *MluI* site of pPEPCK-hOTC, to generate pPEPCK-hOTC-WPRE. The remaining steps to generate the HDV plasmid pC4HSU-PEPCK-hOTC-WPRE were the same as just mentioned. All cloning sites were confirmed by DNA sequence analysis. The identity of recombinant HDV plasmids was confirmed by restriction enzyme digestion with *HindIII* and *BamHI*.

The HDV plasmids were linearized with *PmeI* before transfection into 293Cre4 cells. HDVs were rescued and amplified with 293Cre4 cells and helper virus AdLC8cluc as described in detail elsewhere [39–41]. Suspension 293N3Scre8 cells [30] were used in the final step of HDV production [37]. Purification, quantification by OD₂₆₀ and viral DNA extraction were done as described in detail elsewhere [37,41].

For structural characterization, DNA digested with *XmnI* or *BglII* was run on an ethidium bromide-stained 1% agarose gel. For comparison, the parental plasmid was digested with *PmeI* in addition to the enzymes just named. For determination of helper contamination, DNA digested with *BglII* was transferred to a nylon membrane for Southern hybridization analysis. The [α -³²P]dCTP random prime-labeled adenoviral packaging signal probe ψ [41] was used to distinguish between the 1.6-kb vector-specific and the 1.0-kb helper-specific bands. Autoradiography of the blot was conducted using a Storm phosphorimager and quantitated using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

A FGV was generated using the AdEasy system (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. Briefly, the PEPCK-hOTC-WPRE cassette was cloned into a shuttle plasmid, linearized, and co-transformed with an adenoviral backbone plasmid into *Escherichia coli*. Homologous recombination produced a FGV plasmid, which was linearized to expose the inverted terminal repeats and used to transfect 293 cells. The vector FGV-PEPCK-hOTC-WPRE was produced at high titer

through successive rounds of amplification. The vector was titered by infecting 293 cells in culture at several different dilutions of vector. The cells were then overlaid with agarose, and individual plaques were counted after 10 days and used to calculate the viral particle/pfu ratio.

Mouse injection and sample collection. Breeding pairs of *spf-ash* mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). A colony was maintained by mating heterozygote females with either wild-type littermates or hemizygous OTCD males in the Center for Comparative Medicine at Baylor College of Medicine. To account for possible inter-animal variation, wild-type littermates were used as controls to obtain baseline values for metabolic and other parameters. In this hypomorphic mouse model, there is production of mouse OTC at 5% of normal [11]. The mice received a normal protein load of regular chow. All mice used in the study were 12- to 16-week-old OTCD males and their wild-type male littermates. A standard vector dose of 1.0×10^{13} particles/kg (3.0×10^{11} per mouse) was injected into tail veins of affected mice. As a control, an equal volume of PBS was injected into tail veins of 10 affected mice. Blood was obtained by retro-orbital bleeding at specified times before and after vector administration (3 days before injection; 3 days, 4 weeks, and 25 weeks after injection). Urine was obtained by placing mice in diuresis cages (Tecniplast, Phoenixville, PA, USA) for 12 h. At weekly intervals, urine samples were obtained before and after injection. Mice were sacrificed at specified time points (5 and 25 weeks after injection for the HDV-injected mice and 5 and 15 weeks for the FGV-injected mice, respectively) and liver samples processed as mentioned later. Lower vector doses (5×10^{12} and 1×10^{12} particles/kg, respectively) were also injected into OTCD mice. No differences in appearance or life expectancy were noted among HDV- and PBS-treated mice.

Biochemical and histochemical assays. Urinary orotic acid was determined by electrospray tandem mass spectrometry in the Biochemical Genetics Laboratory at Baylor College of Medicine. Quantification was accomplished on a Waters 2695 HPLC coupled to a Micromass Quattro LC mass spectrometer using a variation of the method of Ito *et al.* [42]. Urinary creatinine was determined by the Jaffe colorimetric method [43]. For OTC enzymatic assay, liver tissue was homogenized in PBS using a glass homogenizer. The homogenate was centrifuged at maximum speed (14,000 RPM) for 5 min, supernatant was transferred to a new tube, and OTC enzyme activity measured as previously described [11]. OTC histochemical staining was done as previously described [11]. On light-microscopic observation, dark brown deposits of lead sulfide indicated the sites of OTC activity.

Southern, northern, and western analyses. Total DNA was extracted from mouse liver, and 25 μ g digested with *AscI* were fractionated on a 1.0% agarose gel and transferred to a nylon membrane. Total liver RNA was isolated with TRIzol (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol, and 20 μ g of RNA fractionated on a 1.2% glyoxal gel were transferred to a nylon membrane. DNA fragments used as probes in Southern and northern hybridizations were gel-purified and labeled with [α -³²P]dCTP by random priming. Total liver protein was isolated, fractionated on a 12% polyacrylamide gel (ISC BioExpress, Kaysville, UT, USA), transferred to Immobilon-P membrane (BioRad, Hercules, CA, USA), probed with a nonspecific polyclonal antibody against hOTC (provided by Mendel Tuchman, Children's National Medical Center, Washington, DC, USA) and immunoreactive OTC detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA). Autoradiography of the blots was done using a Storm phosphorimager and quantitated using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Serum chemistry and liver histology. Serum alanine aminotransferase (ALT) was analyzed at the Center for Comparative Medicine at Baylor College of Medicine. Histological analyses were done on Sections 4 μ m thick. The sections were fixed in 10% formalin, embedded in paraffin, and

then stained with H&E using a standard protocol at the Texas Children's Hospital Pathology Laboratory.

Statistical analysis. Statistical analysis was limited to Student's *t*-test for unpaired samples. Statistical significance was assigned at $P < 0.05$. All results are expressed as mean \pm SD of at least three independent observations.

ACKNOWLEDGMENTS

We appreciate technical assistance from Donna Palmer, Roxanne Walden, Dorene Rudman, Racel Garcia, Christian Clarke, and Shannon Bloesch. We thank Marina Jerebetsova and Xuhei Ye for sharing experimental protocols. We are grateful to J. Donello and Kazuhiro Oka for providing the WPRE and PEPCK plasmids, respectively. The polyclonal hOTC antibody was a gift from Hiroki Morizono and Mendel Tuchman. The pC4HSU plasmids were a gift from Merck. We acknowledge Olivia Hernandez for administrative assistance. This work was supported by NIH grant DK56787 (B. Lee) and the Baylor College of Medicine MRRC grant P30 HD24064 (H. Zoghbi).

RECEIVED FOR PUBLICATION JULY 31, 2003; ACCEPTED MAY 21, 2004.

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