Original Article

Helper-dependent adenoviral vectors for liver-directed gene therapy of primary hyperoxaluria type 1

R Castello1, R Borzone1, S D’Aria1, P Annunziata1, P Piccolo1 and N Brunetti-Pierri1,2

Primary hyperoxaluria type 1 (PH1), caused by deficiency of the liver peroxisomal enzyme alanine:glyoxylate aminotransferase (AGT), is considered a good disease candidate for gene therapy. PH1 is estimated to account for about 1% of pediatric cases of end-stage renal failure and to occur in 1:120,000 live births in Europe. The lack of AGT activity results in a severe disease with overproduction of oxalate, which forms insoluble calcium salts that accumulate in the kidney resulting in nephrocalcinosis and urolithiasis. Calcium oxalate stone deposition occurs also in several other organs causing myocarditis, arrhythmias, stroke and peripheral vascular occlusions. In most patients, the first symptoms (for example renal colic, asymptomatic gross hematuria) occur before the age of 5 years and ~50% of patients present with end-stage renal disease at the time of diagnosis. Combined liver–kidney transplantation that replaces the biochemically defective organ is currently the only available treatment for most patients with PH1. Following liver transplantation, the rate of endogenous oxalate synthesis drops to normal levels as expected based on pattern of AGT expression. Before the use of liver–kidney transplantation, 80% of the patients died by the age of 20 years. Alternative, less invasive approaches, such as small molecule chaperones and oral administration of bacteria to degrade oxalate have been investigated. However, it is unknown whether these approaches will be effective enough to avoid liver and kidney transplantation that remains the only long-term treatment for patients with PH1. Organ transplantation is far from being an ideal treatment. Short-term peri-transplant and long-term morbidities associated with lifelong immunosuppression continue to be significant problems. Hepatocyte transplantation, which is a less invasive transplantation strategy, is unlikely to be successful because the number of engrafted hepatocytes required for correction of PH1 is highly likely to be beyond the capacity of this procedure. In addition, long-term persistence of transplanted hepatocytes has not been demonstrated so far and hepatocyte transplantation would still require lifelong immunosuppression.

Given the limitations of current therapy and the severity of the disease, PH1 is an attractive target for gene therapy that has been previously investigated in two preclinical studies. The first study showed transient correction of hyperoxaluria in the PH1 mouse model using a first-generation adenoviral (FGAd) vector. However, FGAd vectors are not suitable for long-term correction of inherited diseases because they express low levels of viral genes that elicit an immune response against the transduced cells resulting in loss of transgene expression. A second study showed sustained correction of hyperoxaluria and expression of AGT in transduced livers of PH1 mice transduced with serotypes 5 or 8 of adeno-associated viral (AAV) vectors. In contrast to FGAd, helper-dependent adenoviral (HDAd) vectors are attractive for gene therapy of inherited diseases because they can provide long-term transgene expression without chronic toxicity, as shown by several studies in small and large animal models. However, the dose-dependent acute inflammatory response elicited against the viral capsid remains a major obstacle for clinical applications of these vectors for systemic delivery. To achieve efficient hepatocyte transduction by intravenous injections, high doses of adenoviral vector are required in both rodents and nonhuman primates. Several studies have shown a nonlinear dose response, with low doses yielding very low to undetectable levels of transgene expression, but higher doses resulting in disproportionately high levels of transgene expression in both mice and nonhuman primates. In mice, this steep threshold effect can be transiently saturated and cells of the reticulo-endothelial system, including Kupffer cells...
in the liver, have a significant role in the nonlinear dose response. Therefore, efficient hepatic transduction is achieved only after intravenous injection of high doses of adenoviral vectors. Such systemic high vector dose results in activation of an acute inflammatory response with potentially severe and lethal consequences. The mechanism(s) responsible for this Ad-mediated activation of the acute inflammatory response is not completely understood, however, it is clearly dose dependent. To overcome the obstacle of the acute toxicity, a balloon occlusion catheter method for preferential hepatic delivery of HDAd has been previously developed. This method resulted in higher levels and long-term transgene expression from a single injection, and minimal toxicity in nonhuman primates (baboon and rhesus) with clinically relevant doses.

In PH1, combined liver and kidney transplantation has sufficient risk to make the attempt of hepatocyte gene therapy justifiable from the perspective of a risk:benefit ratio. This is particularly the case with HDAd vectors because they result in long-term transgene expression after a single injection. The main goal of this study was to evaluate the efficacy of HDAd vectors for liver-directed gene therapy in a mouse model of PH1.

RESULTS

The available mouse model of PH1 is homozygous for the deletion of exons 4–8 of the Agxt gene (Agxt−/−) and has been previously shown to have hyperoxaluria independent of the dietary oxalate content but normal growth and lifespan. Agxt−/− mice were not found to develop nephrolithiasis spontaneously but only after enhancement of oxalate production by oral administration of 0.5–0.7% ethylene glycol (EG), a precursor of glycolate and glyoxalate. In contrast to these studies, we did not observe development of calcium oxalate stones in Agxt−/− mice that received 0.6% of EG for 4 weeks in their drinking water (n = 5; Supplementary Table 1). Therefore, we investigated the higher dose of 1.25% EG in the drinking water for 4 weeks in Agxt−/− (n = 21) and wild-type mice (n = 10) as controls. Survival was 95% in Agxt−/− and 100% in wild-type mice and mild-to-moderate accumulation calcium stones were observed in kidneys of 6/21 (28.5%) Agxt−/− mice, whereas none of the wild-type controls showed nephrolithiasis (Supplementary Table 1 and Supplementary Figure 1). Therefore, calcium oxalate stone formation under 1.25% EG challenge does not appear to be a sensitive marker of the disease, at least in our colony of Agxt−/− mice. Nevertheless, Agxt−/− mice show oxalate excretions of 2.48 μmol per 24 h that were significantly different from oxalate excretion in age- and gender-matched wild-type SV129 controls (n = 7; 0.5–0.8 μmol per 24 h, 95% confidence interval; Figure 1).

We constructed an HDAd vector expressing the human AGT under the control of a liver-specific expression cassette (HDAd-AGT) that was injected intravenously into Agxt−/− mice at the doses of 1x10^{13}, 5x10^{12} or 1x10^{12} viral particles (VP) per kg body weight (n = 5 mice per group). As control, an additional group of Agxt−/− mice (n = 5) were injected with saline. Following vector administration, 24-h urines were collected at multiple time points for 24 consecutive weeks (Figure 1). Significant reduction of hyperoxaluria was observed in Agxt−/− mice injected with all three vector doses compared with saline-injected controls. Agxt−/− mice injected with 1x10^{13} or 5x10^{12} VP per kg of HDAd-AGT showed a statistically significant 3.6- and 2.7-fold reduction of urinary oxalate levels compared with saline-injected mice, respectively (Gaussian Processes, Bayes factor 35.98 and 26.08, respectively; P < 0.05 at each time point by Wilcoxon–Mann–Whitney test; Figure 1). Urinary oxalate excretions in mice injected with the two higher doses were within the normal range detected in wild-type SV129 mice. Partial reduction of urinary oxalate levels that were above the normal range were detected with the lowest dose of 1x10^{12} VP per kg (Gaussian Processes, Bayes factor 9.98; Figure 1). In all mice the reduction of urinary oxalate was sustained for the entire period of observation of 24 weeks (Figure 1).

Real-time PCR showed a dose-dependent increase of HDAd vector genome copies in the liver at the three tested doses (Supplementary Figure 2). By immunofluorescence performed on livers harvested at 28 weeks post-vector injections, a larger number of hepatocytes positive for AGT expression at the doses of 1x10^{13} and 5x10^{12} VP per kg was observed while a smaller percentage of AGT-expressing liver cells was detected at the dose 1x10^{12} VP per kg (Figure 3). By western blotting, livers of mice injected with 5x10^{13} and 1x10^{12} VP per kg of HDAd-AGT revealed...
robust AGT expression, whereas no protein was detected in saline-injected Agxt−/− mice (Figure 4a). AGT enzyme activity on liver homogenates from Agxt−/− mice injected with vector was higher than saline-injected controls at all three tested doses (Figure 4b; P < 0.05). The dose of 1x10^13 VP per kg resulted in supraphysiologic levels of enzyme activity (P < 0.05; 1x10^13 VP per kg vs wild-type controls) and 5x10^12 VP per kg resulted in enzyme activity that was similar to wild-type controls (Figure 4b). Agxt−/− mice that received 1x10^12 VP per kg showed 67% of normal enzyme activity (Figure 4b). Vector-encoded human AGT protein was also found to be enriched in the peroxisomal fraction of liver lysates thus showing that vector-encoded AGT was predominantly localized in peroxisomes (Figure 4c).

DISCUSSION

PH1 is an attractive candidate for gene therapy: its pathophysiology is well characterized, it has a favorable risk–benefit ratio, direct measures of clinical benefit are available and a sufficient number of patients would be available for enrollment in a clinical trial. Moreover, in humans AGT is only expressed in the liver and thus, liver-directed gene therapy offers the possibility of replacing the total body’s requirement for AGT.

In this study, we showed that a single injection of an HDAd vector expressing the AGT results in long-term correction of hyperoxaluria in a mouse model of PH1. Phenotypic correction was achieved with the two highest doses of 1x10^13 and 5x10^12 VP per kg of vector whereas partial reduction was detected with the lowest dose of 1x10^12 VP per kg. AAV vectors also resulted in similar levels of correction of hyperoxaluria at relatively high doses. The requirement of high vector doses for phenotypic correction is not surprising and was predicted by disease pathophysiology. In PH1 a large portion of the liver, although structurally normal, produces an excess of toxic oxalate and has to be replaced/corrected. For gene therapy to be effective, a large proportion of hepatocytes has to be transduced to minimize the deleterious effect of uncorrected hepatocytes that will continue to produce oxalate. Although hepatocytes suffer no damage from the enzymatic defect, PH1 behaves like cell-autonomous defects in which one corrected hepatocyte cannot compensate for overproduction of toxic metabolites in its neighboring cells. This model explains the partial correction observed with the lower dose of 1x10^12 VP per kg despite the increased levels of total AGT activity measured in livers. Higher percentages of hepatocyte transduction achieved with the higher doses of 5x10^12 and 1x10^13 VP per kg, as shown by immunofluorescence, are needed for correction of hyperoxaluria in Agxt−/− mice.

The AGT is an example of a dual localized protein with targeting sequences directing the protein to two distinct cellular compartments. In mice and rats, AGT is present both in the mitochondria and peroxisomes, whereas in humans it is exclusively located in the peroxisomes. Consistent with previous studies, we also
showed that the vector-encoded human AGT protein is correctly targeted to peroxisomes in mouse hepatocytes.

In recent years, there has been clear success in the clinic using AAV vectors for hemophilia B that resulted in long-term expression of factor IX. Although no immune reaction was observed at lower vector doses, participants who received the higher dose of vector developed a transient, asymptomatic elevation of serum alanine aminotransferase levels associated with detection of AAV-capsid-specific T cells in peripheral blood. A short course of glucocorticoid therapy was associated with rapid normalization of alanine aminotransferase levels and sustained factor IX in the therapeutic range. This and the previous trial with AAV2 in hemophilia B patients have highlighted the limitations of immunologic responses against transduced hepatocytes, and while short-course corticosteroid therapy appeared to be sufficient to blunt the immune response, it still needs to be determined whether long-term expression can be achieved in diseases such as PH1 that require an higher percentage of hepatocyte transduction compared with hemophilia.

AAV vectors have shown in general excellent safety profile in human trials. However, the safety of AAV has been challenged by two studies that documented hepatocellular carcinoma and vector genomic integration after AAV gene delivery in mice. Moreover, a recent study reports that natural infections in humans with AAV serotype 2 result in chromosomal insertions activating proto-oncogenes in the liver and it suggests that the AAV integrations cause the tumors, similarly to the hepatitis B. Although the risk of hepatocellular carcinoma development remains to be fully understood, it is important that other vector systems continue to be investigated, particularly for disorders such as PH1 that require higher percentage of liver transduction and therefore, higher vector doses. HDAd genomes appear to exist in the nucleus of transduced cells as replication-deficient linear monomers both in cell culture and in mouse livers. Intracellular HDAd genome is assembled into chromatin through association with cellular histones that promotes efficient transgene expression. Several studies in cell culture have investigated the frequency of HDAd genome integration and found random integration frequencies to be $10^{-3}$ to $10^{-5}$ per cell whereas an even lower integration frequency in hepatocytes has been detected in vivo. Therefore, HDAd vector appears to be predominantly episomal with very low frequency of genomic integration.

The acute toxicity elicited by high doses of HDAd vector is an obstacle preventing their clinical applications. This acute toxic response is dose dependent: it has been consistently shown in nonhuman primates that low vector doses result in little, if any, acute toxicity and hepatic transduction, whereas high vector doses, required for efficient hepatocyte transduction, lead to severe acute toxicity that can be lethal. If this acute response can be avoided, then HDAd should be able to provide long-term transgene expression without further chronic toxicity. Strategies allowing the use of lower vector doses are attractive for clinical applications because they can overcome the issue of acute toxicity. The method of balloon catheter-assisted delivery of HDAd in nonhuman primates resulted in high level of hepatic transduction and multi-year transgene expression with relatively low and clinically relevant doses, minimal evidence of acute toxicity and no chronic toxicity. The use of such delivery method has potential for severe disorders such as PH1 that is currently treated with invasive, high-risk surgical transplant procedure and require high percentage of hepatocyte transduction to obtain clinical benefit.

**MATERIALS AND METHODS**

**HDAd vector**

The HDAd-AGT vector bears the PEPCK-WL-hAGT expression cassette including the liver-specific promoter of phosphoenolpyruvatecarboxykinase (PEPCK) and other regulatory elements including the woodchuck hepatitis virus post-regulatory element, the locus control region from the apoE locus and the bovine growth hormone poly-adenylation signal as described elsewhere. HDAd was produced in 116 cells with the helper virus AdNG163 as described previously. The cells were regularly tested and found to be negative for Mycoplasma by real-time PCR. DNA analyses of HDAd genomic structure was confirmed as described elsewhere.

**Figures**

- Figure 4. AGT expression, activity and subcellular localization. (a) Western blot for AGT in liver lysates of Agxt−/− mice injected with three doses of HDAd-AGT expressed as VP per kg. The amount of protein extract loaded for the lystate of mice injected with $1 \times 10^{13}$ VP per kg was $1 \mu g$ (1/10) whereas $10 \mu g$ (1) of proteins were loaded for all remaining lysts. Livers of saline-injected mice were used as negative control. Calnexin was used as loading control. (b) Hepatic AGT enzyme activity showed a dose-dependent increase following injection of HDAd-AGT vector ($n=3$ per group; *$p<0.05$ vs saline). (c) Western blot analysis of subcellular organelle-enriched fractions of livers of mice injected with $1 \times 10^{13}$ VP per kg of HDAd-AGT: the AGT protein band is more abundant in the whole cell lyses (W) and in the peroxisome-enriched fraction (P) whereas nuclear (N) and mitochondrial (M)-enriched fractions show minimal amount of AGT. Peroxisomal protein PMP70 was used as a peroxisomal marker and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a cytosolic marker. The vector doses are expressed as VP per kg.
AGT western blot, enzyme activity and stainings

Liver specimens were homogenized in radio-immunoprecipitation assay buffer and complete protease inhibitor cocktail (Sigma-Aldrich). Samples were incubated for 4 h at 4°C and centrifuged at 13,200 g for 10 min. Pellets were discarded and cell lysates were used for western blots. Subcellular fractionation was performed as described elsewhere. Whole cell lysates were quantified and equivalent cellular fractions were loaded for each one of the three enriched fractions. Proteins were loaded on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane; blots were blocked with Tris-buffered saline-Tween-20 containing 5% non-fat milk for 1 h at room temperature followed by incubation with primary antibody overnight at 4°C. The primary antibodies used were: rabbit anti-human AGT (#HPA035370, Sigma-Aldrich), rabbit anti-AGT antibody19 was used at 1:1000 dilution in blocking solution for an overnight incubation. The AlexaFluor-488 anti-rabbit antibody made in goat serum, bovine serum albumin and phosphate-buffered saline; donkey serum, bovine serum albumin and phosphate-buffered saline; and 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). The percentage of AGT-positive cells was determined manually analyzing more nuclei were counted as one. Oxalate staining on kidneys were performed as described elsewhere.59

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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