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Phenotypic Correction of Ornithine Transcarbamylase Deficiency Using Low Dose Helper Dependent Adenoviral Vectors

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Abstract

Background—Helper-dependent adenoviral vectors (HDAd) can mediate long-term phenotypic correction in the ornithine transcarbamylase (OTC)-deficient mice model with negligible chronic toxicity. However, the high doses required for metabolic correction will result in systemic inflammatory response syndrome in humans. This acute toxicity represents the major obstacle for clinical applications of HDAd vectors for the treatment of OTC deficiency. Strategies for reducing the dose necessary for disease correction are highly desirable since HDAd acute toxicity is clearly dose-dependent.

Methods—We analyzed a potent expression cassette and the hydrodynamic injection for the ability to reduce the HDAd dose necessary for phenotypic correction in OTC-deficient *spf-ash* mice.

Results—We have developed a vector containing a potent expression cassette expressing the OTC transgene which allowed phenotypic correction at lower doses. Our results suggest that vector expressing greater OTC levels allows correction of orotic acid overproduction at lower doses that make clinical translation more relevant. We were able to further reduce the minimal effective dose by delivering the vector through the hydrodynamic injection technique.

Conclusions—Vectors containing the expression cassette used in this study combined with other strategies for improving HDAd therapeutic index will likely permit application of these vectors for the treatment of OTC deficiency as well as other urea cycle disorders.

Introduction

Ornithine transcarbamylase (EC 2.1.3.3) deficiency (OTCD; MIM #311250) is the most common inherited disorder of urea synthesis and it results in frequent episodes of hyperammonemia invariably leading to poor neurological outcomes and high mortality [1]. Pharmacological treatment with ammonia-conjugating agents has improved survival but not the neurodevelopmental outcome [2,3]. At this time orthotopic liver transplantation (OLT) represents the best available therapeutic approach to treat these patients and it results in nearly complete metabolic correction. However, it does not result in reversal of neurologic deficits [4] although improvements are observed with early OLT and more aggressive metabolic management [3,5]. Clearly the available treatments strategies for OTCD are insufficient and the risk-benefit assessment makes this disease an excellent candidate for new therapeutic approaches such as gene replacement therapy [6]. Gene therapy for OTCD has in fact been attempted in a human clinical trial but the results have been unsatisfactory. In this pilot trial

using a third-generation adenoviral vector expressing the human OTC (hOTC) there was lethal toxicity at the highest vector dose and no evidence of gene transfer [7,8]. The systemic administration of first generation adenoviral vector (FGAd) as well as of second- and third-generation Ad vectors results in a strong host immune response involving both innate and adaptive immunity. The innate immune response is rapidly developed after virus administration, is dose-dependent and does not require viral gene expression. The adaptive cellular and humoral response is developed within days of viral delivery, and is caused by viral gene expression from the transduced cells. Helper-dependant adenoviral vector (HDAd), in contrast to FGAd, do not cause an adaptive cellular immune response against the transduced cells, however, they can still elicit an acute toxic and potentially lethal reaction due to the activation of the innate immunity [9,10]. This acute toxicity is the major factor limiting clinical application of HDAd vector. Several factors have been implicated in the activation of this toxic response [11], but the precise pathogenesis remains still unclear. However, regardless of the precise mechanism, it is clear that this acute toxicity is dose-dependent and that a threshold of innate immune activation must first be attained before severe and lethal acute toxicity manifests. Therefore, strategies aimed at the reduction of the minimal effective dose can improve the therapeutic index and facilitate clinical translation of the treatment.

Our group has previously reported long-term correction of the biochemical phenotype in OTCD mouse model using HDAd vector carrying the WPRE and expressing hOTC under the control of a tissue-restricted promoter [12]. However, high vector doses (1×10^{13} vp/kg) were necessary to achieve correction. For the problems related to the acute toxicity of the HDAd, these results do not translate directly in the clinical arena because such high doses of Ad vector are potentially lethal in humans. Nevertheless, this study as well as several others showing long-term transgene expression with no chronic toxicity underscore the importance of continuing to investigate strategies to improve the therapeutic window of these vectors [13]. The easiest strategy to reduce the inflammatory response caused by systemic HDAd administration is simply to reduce the dose while maintaining the same levels of transgene expression. This strategy is likely to be successful given that the acute toxicity is clearly dose-dependent. Toward this end, we have evaluated the efficiency of a vector containing a liver-specific enhancer, the hepatic locus control region from the apolipoprotein E gene (LCR) in increasing transgene expression [14]. Aiming at a further reduction in dose, we have subsequently investigated whether delivering the HDAd through the hydrodynamic injection technique would allow a supplemental reduction in the dose necessary for phenotypic correction.

Materials and methods

Helper-dependent adenoviral vectors

Both HDAds (HDAd-hOTC-W and HDAd-hOTC-WL) used in this study were produced in 116 cells [15] with the helper virus AdNG163 [16] as described in detail elsewhere [15]. Helper virus contamination levels were determined as described elsewhere [15] and were found to be <0.05% for both vector preparations. DNA analyses of HDAd genomic structure was confirmed for both vectors as described elsewhere [15].

Infectivity assay

The physical titer of the HDAd, expressed as vp/ml, was determined by absorbance at 260 nm with correction for vector genome size as described in detail elsewhere [17]. The HDAd vectors were diluted in phosphate buffered saline (PBS) and equal volumes of each were mixed together. Virion DNA was extracted directly from an aliquot of this mixture as described elsewhere [17] and another aliquot of this same mixture was used to co-infect 60-mm dishes of 90% confluent 293 cells at an m.o.i. of 100 total vp/cell (50 vp of each virus/cell). Infection conditions were performed as described in detail elsewhere [15] and co-infected cells were

washed extensively with PBS following the 1-h absorption to remove unabsorbed virions. Four hours post-co-infection, the cells were detached from the plates and washed with PBS and intracellular DNA was extracted as described in detail elsewhere [17]. Virion and intracellular DNAs were digested with *AscI* and analyzed by Southern blot hybridization with 2200-bp probe fragment hybridizing with the PEPCK promoter which is common to both vectors. Phosphorimager analyses were performed to quantitate the intensities of the HDAd-hOTC-W and HDAd-hOTC-WL -specific bands.

Animal experiments and analyses

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. The mice received a normal protein load of regular chow. 9 to 12 week old *spf-ash* male mice were used for all the experiments. For hydrodynamic injections, HDAd was diluted in a total volume of 100 ml/kg of sterile Ringer's solution (147 mM NaCl, 4 mM KCl, 1.13 mM CaCl₂) pre-warmed at 37°C and injected into the tail vein in using a 27 gauge needle at a rate of 0.4 ml/sec. Conventional injections were performed with a volume of 200 µl. All treatment groups consisted of at least five mice. Urine samples were collected before the injection and at weekly or monthly intervals after the injection. Mice from the group injected with 1×10^{12} vp/kg were sacrificed at a specified time point (6 months after injection) and liver samples processed as mentioned later. 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. [18]. Urinary creatinine was determined by the Jaffe colorimetric method [19].

OTC histochemistry on liver tissues was done as previously described [20]. Total DNA extracted from liver using the QIAamp DNA extraction kit (Qiagen) was quantitated by absorbance at 260 nm. Quantitative real time PCR was performed using the LightCycler FastStart DNA Master SYBER Green I (Roche) in a total volume of 20 µl with 2 µl of template DNA, 4mM MgCl₂, 5 µM of each HDAd-specific primers (5'-TCTGAATAATTTTGTGTTACTCATAGCGCG-3' and 5'-CCCATAAGCTCCTTTTAACTTGTTAAAGTC-3'. The cycling conditions consisted of 95°C for 10 minutes followed by 45 cycles at 95°C for 10 seconds, 60°C for 7 seconds, and 72°C for 20 seconds. Serial dilutions of a plasmid bearing the PCR target sequence were used as a control to determine the amounts of HDAd and results were analyzed with LightCycler software version 3.5 (Roche).

Statistical analyses were performed with the t-Test.

Results

HDAd vector comparison

The two vectors used in the present study were: 1) HDAd-hOTC-W containing (5' to 3') the liver-restricted phosphoenolpyruvate carboxykinase (PEPCK) promoter (21), the human ApoAI intron, the hOTC cDNA and the human growth hormone poly A; 2) HDAd-hOTC-WL containing also a liver-specific enhancer LCR inserted between the hOTC cDNA and the poly A (Figure 1). Both vectors were generated using the pΔ21.7 backbone (14). The HDAd-hOTC-W contained the same expression cassette of the vector previously used by Mian et al. [12] but in a different backbone.

To compare the infectivity of the two vector preparations a co-infection assay of the two preparations was performed [16]. In this assay, the physical titer of the vectors is first

determined by absorbance at 260 nm. Subsequently equal amount in vp of the two vectors are mixed together and virion DNA (V. DNA) is then extracted from an aliquot of this mixture and subjected to Southern analysis (Figure 2). An aliquot of this same mixture is also used to co-infect 293 cells, and intracellular DNA (I. DNA) is extracted from the cells 4 h post-co-infection and subjected to Southern analysis (Figure 2). During the first 4 hours of infection the virus is known to be entered in the nucleus but viral DNA replication has not occurred yet [17]. In this assay the infectivity of the two vector preparations is assessed by comparing the ratio between the intensities of the vector-specific bands in the I. DNA with the intensities of the vector specific bands in the V.DNA. The ratio between the two vector bands is identical in the I. DNA (1.3) and in the V. DNA (1.3), proving that the infectivity of the two vectors is the same.

Phenotypic correction in *spf-ash* mice

Once we had established that the two vectors had identical infectivity, we injected *spf-ash* mice with the HDAd vectors at various doses ranging from 5×10^{11} vp/kg to 1×10^{13} vp/kg. As expected from our previous study, a reduction of urinary orotic acid was seen with both vectors at 1×10^{13} vp/kg (data not shown). At 5×10^{12} vp/kg we observed correction with both vectors (Figure 3). However, this correction was sustained only for the group treated with the HDAd-hOTC-WL. The animals injected with HDAd-hOTC-W exhibited urinary orotate reduction only for the first 8 weeks and at 12 weeks differences in orotate were statistically different ($p < 0.05$) (Figure 3). Animals injected with the HDAd-hOTC-WL showed phenotypic correction also at the dose of 1×10^{12} vp/kg (Figure 3) but no correction was seen with 5×10^{11} vp/kg of HDAd-hOTC-WL (data not shown).

Histochemical analysis and vector genome copy determination

OTC histochemical analyses were performed on liver sections collected 6 months in the mice injected with 1×10^{12} vp/kg of HDAd-hOTC-W and HDAd-hOTC-WL. We have selected this dose for these analyses because clear differences in the phenotypic correction between the two vectors were evidenced at this dose. The histochemical analyses showed comparable staining with both vectors (Figures 4A and B) which appears to be less than wild-type (Figure 4C) but significantly higher than uninjected *spf-ash* controls (Figure 4D). The histochemical analysis of HDAd-hOTC-WL injected mice showed intense localized staining in a few cells. This intense staining may be due to hOTC overexpression in transduced hepatocytes.

On the same liver samples we also determined the number of vector genome copies in the two treatment groups by Real time PCR using HDAd-specific primers. We found that the genome copy number were not statistically different between the two groups (Figure 5). Therefore, at a given number of vector genome copies in the liver, the HDAd-hOTC-WL vector is superior to the vector without LCR in achieving phenotypic correction. These results suggest that phenotypic correction can be more efficiently achieved by over-expression of hOTC in the transduced hepatocytes.

Hydrodynamic injection of HDAd-hOTC-WL

We have previously shown that the hydrodynamic injection of HDAd results in higher efficiency hepatic vector uptake and transgene expression compared to conventional injection [14]. Although this method cannot be applied in humans due to the large volume injected, a recent method to mimic the hydrodynamic injection has been developed and can potentially translate in large animals the results of hydrodynamic injection [22]. Therefore, we have investigated whether the hydrodynamic injection delivery could allow further reduction in the dose necessary for phenotypic correction. Toward this end, we have tested by this delivery method the more potent vector (HDAd-hOTC-WL) at the dose of 5×10^{11} vp/kg which did not result in correction by the conventional systemic injection (Figure 6). We indeed observed that

5×10^{11} vp/kg of HDAd-hOTC-WL administered hydrodynamically resulted in phenotypic correction (Figure 6) thus confirming the previous observation that the hydrodynamic delivery can increase percentage of hepatocyte transduction and levels of transgene expression [14].

Discussion

Adenoviral vectors and more recently novel AAV serotypes 8 and 9 [23] have been shown to correct the phenotype of the *spf-ash* mice. In this study we expand on the findings presented we have previously generated using the HDAd [12] and we have explored two main strategies to improve the therapeutic index of the vector. As previously reported we have used urinary orotic acid as a primary metabolic endpoint of preclinical efficacy because this parameter best correlates with clinical status and because other metabolic markers such as serum glutamine and ammonia have great diurnal variability in mice [12].

In our first approach to reduce the effective dose of vector, we tried to increase the transgene RNA of our expression cassette by the inclusion of the LCR, a liver specific *cis* acting element. In these experiments, two vectors (HDAd-hOTC-W and HDAd-hOTC-WL) of equivalent physical and infectious titers were directly compared *in vivo*. Traditional infectious titer assays, based on cytopathic effect following infection of 293 cells, cannot be employed for HDAd lacking of all viral coding sequences. Therefore, for our study we used the method recently developed by Palmer and Ng for determining HDAd infectivity [16]. This method allows for a robust comparison of infectivity among vectors *in vitro* and in the present study we have validated this assay also *in vivo*. The vector copy numbers in the livers of the two treatment groups were in fact not different as predicted from the *in vitro* determination of the infectivity. This approach could serve as a model for comparisons of two or more HDAd vectors *in vivo*.

The vector containing the LCR resulted in phenotypic correction at both to 5×10^{12} and 1×10^{12} vp/kg while the vector without LCR did not result in any correction at 1×10^{12} vp/kg and in transient correction at 5×10^{12} vp/kg. The loss of expression was not seen with the same vector (without LCR) injected at the higher dose of 1×10^{13} vp/kg. We do not understand why at 5×10^{12} vp/kg the phenotypic correction was not sustained with the HDAd-hOTC-W. However, we have previously experienced a similar result in hemophilia B dogs [24] using a similar expression cassette but without the WPRE element and was also reported by Brown et al. in hemophilia A dogs [25]. It is unlikely that the loss of vector genome has occurred in our case because this loss was not observed with the HDAd-hOTC-WL vector at the same dose and because at the lower dose of 1×10^{12} there were no differences in vector genome copies between the two vectors (Figure 5).

It is important to note that the hOTC has been used in this study as a therapeutic gene, and differences between mouse and human mitochondrial leader peptide sequences of OTC may lead to inefficient import of hOTC into mouse mitochondria [20]. This would suggest that even doses lower than 1×10^{12} vp/kg of HDAd expressing mOTC would correct the *spf-ash* mouse phenotype by conventional injection.

The second strategy we have investigated to improve the therapeutic window was delivering the HDAd vector through the hydrodynamic injection. This injection technique consists of a rapid injection of high volume in the tail vein of a mouse and it was initially developed for naked plasmid DNA delivery to the hepatocytes. As performed in rodents is not feasible in larger animals due to the large volume required. However, methods of localized hydrodynamic injection directly into the isolated liver may be possible [26,27]. Moreover, a novel delivery method using balloon catheters has been recently developed in nonhuman primates for HDAd delivery and it is very attractive for clinical applications given its limited invasiveness [22]. Delivering our improved vector through this delivery method has the potential to greatly

improve the therapeutic window of the HDAd for OTCD. Based on the data on the improved expression cassette and hydrodynamic injection, we believe we have achieved at least one order of magnitude improvement in the therapeutic index for this approach of OTCD gene therapy. Assuming that clinical use of the hOTC in humans will be further more efficient than the present studies of hOTC in *spf-ash* mice, we may have effective safety margin greater than two orders of magnitude.

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Figure 1.

Diagram of the two vectors used in the study; the HDAd-hOTC-W, contains in the pΔ21.7 backbone (14) (5' to 3') the liver-restricted phosphoenolpyruvate carboxykinase (PEPCK) promoter, the human ApoAI intron, the hOTC cDNA, the WPRE element, and the human growth hormone poly A. The second vector, HDAd-hOTC-WL contains also a liver-specific enhancer LCR inserted between the hOTC cDNA and the poly A.

Figure 2.

Determination of the infectivity of the two vectors. The physical titer of the vectors is first determined by absorbance at 260 nm. Subsequently equal amount in vp of the two vectors are mixed together and virion DNA (V. DNA) is then extracted from an aliquot of this mixture and subjected to Southern analysis. An aliquot of this same mixture is also used to co-infect 293 cells, and intracellular DNA (I. DNA) is extracted from the cells 4 h post-co-infection and subjected to Southern analysis (1 and 2 are duplicates). The infectivity of the two vector preparations is determined by comparing the ratio between the intensities of the vector-specific bands in the I. DNA with the intensities of the vector specific bands in the V.DNA. The ratio between the two vectors is identical in the I. DNA (1.3) and in the V. DNA (1.3), proving that the infectivity of the two vectors is the same.

Figure 3. Urinary orotate excretion in *spf-ash* mice injected with 5×10^{12} and 1×10^{12} vp/kg of HDAd-hOTC-WL and HDAd-hOTC-W. Urinary orotate/mg of creatinine is presented as percentage of pretreatment levels and are the mean $\pm$ standard deviation of at least 5 mice.

Figure 4.

Histochemical analyses on liver sections collected 6 months after the injection of 1×10^{12} vp/kg of HDAd-hOTC-WL (A) and HDAd-hOTC-W (B), in wild-type (C) and in uninjected *spf-ash* controls (D). On light microscopic observation dark brown deposits of lead sulfate indicate the sites of OTC activity.

Figure 5.

Real time PCR to determine vector genome copy number in livers samples collected 6 months after the injection of 1×10^{12} vp/kg of HDAd-hOTC-WL (A) and HDAd-hOTC-W. No difference in vector genome copies were found among the two groups.

Figure 6.

Urinary orotate excretion in *spf-ash* mice injected with 5×10^{11} vp/kg of HDAd-hOTC-WL administered through conventional or hydrodynamic injection. Urinary orotate/mg of creatinine are presented as percentage of pretreatment levels and are the mean $\pm$ standard deviation of at least 5 mice.