Gene therapy with adeno-associated viral vectors for inherited hyperbilirubinemia: towards a clinical trial for Crigler-Najjar syndrome

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Careful vector design guaranties long-term liver transduction with a low dose of an adenoviral vector

Manuscript preparation

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Abstract

Adenoviral vectors featured prominently in early liver directed gene therapy trials. After the death of a patient upon systemic injection due to the acute toxicity elicited by the vector, the clinical application of corrective gene therapy gave rise to concern. The clinical use of these viral vectors for liver directed gene therapy is highly dependent on dose reduction. Furthermore, since several studies showed that low expression of adenoviral genes resulted in loss of corrected cells in animal models, focus has been on the development of gutless helper dependent adenoviral (HD-Ad) vectors, devoid of all viral genes. However, the production of these HD-Ad vectors in clinically relevant quantities without contamination with replication competent adenovirus (RCA) has not yet been accomplished. In this respect E1, E3 deleted second generation adenoviral vectors may still be of interest since these can be produced RCA-free in the Per.C6 cell line. Aim of this study is to design adenoviral vectors that provide efficient gene transfer at a low vector dose and establish prolonged expression of the therapeutic gene. In the hyperbilirubinemic Gunn rat, the model for Crigler-Najjar syndrome, a vector in which the expression of the human UDP-glucuronosyltransferase (UGT1A1) was driven by the liver specific LP-1 promoter, provided prolonged correction of serum bilirubin. Inverting the LP1-UGT1 expression cassette enhanced the efficacy resulting in complete correction with the same low vector dose. In addition, also a hybrid adenoviral vector, in which the LP1-UGT1A1 cassette was flanked by inverted terminal repeats (ITR’s) of adeno-associated virus type 2, improved the vector efficiency even further. With this vector a dose of $3 \times 10^{11}$ vp/kg was sufficient to provide complete correction. In conclusion, our data indicate that careful design of the expression cassette does result in a highly efficient E1, E3 deleted adenoviral vector that provides sustained correction of a therapeutic gene upon administration of a clinical relevant low vector dose.
Introduction

The major advantage of adenoviral vectors for liver directed gene therapy is their natural tropism for this organ\(^1\). Upon systemic administration > 90% of all viral particles ends up in the liver. In addition, adenoviral vectors are able to mediate high levels of gene expression in hepatocytes, do allow the insertion of large sizes of foreign DNA in their genome and can be produced in high titer stocks. Furthermore, the adenoviral genome persists in an extrachromosomal (episomal form) in the nucleus which precludes a risk for insertional mutagenesis. These characteristics rendered these vectors promising for liver directed gene therapy to treat inherited liver diseases\(^2\). Also in the hyperbilirubinemic Gunn rat adenovirus mediated correction appeared effective but transient\(^3\). The transient nature of the correction appeared to be due to *de novo* synthesis of adenoviral proteins encoded by these vectors causing an adaptive immune response resulting in rejection of the transduced hepatocytes.

An option that has been used to overcome this was the removal of all viral coding sequences from the vector. These so-called helper-dependent Ad (HD-Ad) or “gutless” vectors only contain the adenoviral long terminal repeats (LTRs) and packaging signal\(^4\). The complete absence of viral gene expression, minimizes the adaptive immune response towards the cells transduced by HD-Ad\(^5\). This results in a longer expression of the encoded therapeutic genes in hepatocytes in vivo. Using an HD-Ad encoding the human UGT1A1 gene, Toietta et al obtained life-long correction of serum bilirubin levels in the Gunn rat\(^6\). The amount of vector injected (3x10\(^{12}\) vector particles (vp) per kilogram (kg)) however, is likely to provoke a strong innate immune response resulting in severe toxicity. Dose reduction therefore is essential. Recently, a similar correction of serum bilirubin levels was obtained with a six-fold lower dose of an HD-Ad upon systemic administration\(^7\). All these data indicated that long term effective therapy of liver disorders, including CN syndrome, seems feasible using HD adenoviral vectors.

Production of HD-Ad is still a challenge. Although the yield has increased, the generation of replication competent helper virus (RCA) in the production systems cannot be prevented completely. With the most optimal production procedures the percentage of RCA is below 0.05%, but a therapeutic dose of 5\(x\)10\(^{11}\) vp still contains 2,5\(x\)10\(^7\) RCA. This may have caused the transient transaminitis and thrombocytopenia in one patient in a phase I trial for hemophilia A. Although these parameters normalized 19-days post-infusion the trial was put on hold\(^8\). Second generation adenoviral vector batches can be produced RCA-free in Per.C6 cells\(^9\). Therefore, these vectors may still be of
interest for clinical use if the adaptive immune response can be prevented. To accomplish these additional vector improvements, promoter selection and orientation of the expression cassette are essential\textsuperscript{10,11}.

In this study we sought to evaluate the possible use of these improved second generation adenoviral vectors constructs for sustained correction hyperbilirubinemia in the Gunn rat. We show that carefully designed E1, E3 deleted adenoviral vectors do provide long term correction in this animal model upon the systemic injection of a clinically relevant low dose of vector.

**Material and Methods**

**Construction and production of Ad/AAV and adenoviral vectors.** The cDNA encoding human UGT1A1 was released from pSVK3 plasmid\textsuperscript{12}. After intermediate subcloning in pBS plasmid, the UGT1A1 cDNA was cloned in pShuttle-CMV plasmid in both orientations. For the Ad/AAV-LP1-UGT1A1 vectors the entire expression cassette was extracted from a scAAV-LP1-UGT1A1 with a simian virus (SV) 40 polyadenylation (polyA) sequence and cloned in both orientations into pShuttle plasmid. The adenoviruses were produced using the AdEasy\textsuperscript{13} vector, with titers for Ad/AAV-LP1-UGT1A1 of $6,50 \times 10^{12}$ vector particles (vp)/mL and $1,50 \times 10^{11}$ infectious particles (ip)/mL; Ad-LP1-UGT1A1 of $3,00 \times 10^{12}$ vp/mL and $1,50 \times 10^{11}$ ip/mL; Ad-CMV-UGT1A1 of $2,90 \times 10^{12}$ vp/mL and $1,30 \times 10^{11}$ ip/mL; Ad-rev-LP1-UGT1A1 of $4,70 \times 10^{12}$ vp/mL and $1,00 \times 10^{11}$ ip/mL.

**Animal experiments.** All animal experiments were performed in accordance with the Animal Ethical Committee guidelines of the Academic Medical Center of Amsterdam and guidelines for animal welfare from the French Ministère de l’Agriculture. Male Gunn rats from our own colonies, 8-10 weeks of age, with a weight between 180 and 200g, were fed *ad libitum* and randomly assigned to different treatment groups. Different groups of Gunn rats were tail vein or intraportally injected with the different adenoviral vectors. Portal injections, blood sampling and bile collection were performed as described previously\textsuperscript{14}.

**DNA and RNA analysis.** 100 ng of genomic DNA, isolated according to\textsuperscript{15}, was used to determine the UGT1A1 gene copy number by qPCR in the Roche LightCycler\textsuperscript{480} with the fast-start SYBRgreen kit (Fw: 5’-GACGCCTCGTTGACATCAG-3’; Bw: 5’-
CACGCTGCAGGAAAGAATC-3'). Dilutions of UGT1A1 plasmid into rat genomic DNA and normalizing to the rat endogenous β-actin gene (Fw: 5’-AGCCATGTACGTAGCCATCCA-3’; Rv: 5’-TCTCCGAGTCCATCACAATG-3’) was used to generate standard curves. To determine the expression of the UGT1A1 gene, total liver RNA was isolated using Trizol and 1μg was used for cDNA synthesis using oligo-dT and a primer for 18S and Superscript3. qPCR was performed using the same primers for UGT1A1 as used to determine genomic copies and primers for 18S (Fw: 5’-CGAACCTCCGACTTTCGTTCT-3’ and a Rv: 5’-TTCGGAACTGAGGCCATGAT-3’). Vector genomic copies per diploid genome equivalent (vc/dGE) and UGT1A1 mRNA/18S were calculated using the LinRegPCR software\textsuperscript{16}.

**Bilirubin quantification.** Total bilirubin in serum was determined by the routine clinical chemistry department using a standard colorimetric assay. Unconjugated bilirubin and bilirubin conjugates in bile were analyzed and quantified by HPLC as described\textsuperscript{14,17} with the modification that an Omnisphere column (Varian, The Netherlands) was used\textsuperscript{14,17}.

**Results**

**CMV transgene driven expression results in short-term hyperbilirubinemia correction**

In this study we construct a second generation E1, E3 deleted adenoviral vector, where the adeno E1 region was used to insert the human UGT1A1 gene behind a cytomegalovirus (CMV) promoter (Ad-CMV-UGT1A1) promoter.

A single systemic injection of 3x10\textsuperscript{10} ip/kg or 6,7x10\textsuperscript{11} ip/kg of Ad-CMV-UGT1A1 resulted in a complete correction of the serum bilirubin levels in the Gunn rats (Fig.1). After three weeks the serum bilirubin levels in these animals rapidly rose to the pre-injection levels. This pattern of transient correction confirms the data previously reported for first and second generation adenoviral vectors in this animal model\textsuperscript{3}. In addition the pattern of loss of correction is very similar to their study suggesting an adaptive immune response.
Fig. 1 – Ad-CMV-UGT1A1 tail vein administration in Gunn rats. Serum bilirubin levels of male Gunn rats (n=4) upon tail vein injection of $3 \times 10^{10}$ ip/kg ($6.7 \times 10^{11}$ vp/kg) of Ad-CMV-UGT1A1. Black arrow indicates the time point of tail vein injection of the scAAV2/8-CMV-UGT1A1 vector.

Fig. 2 – Schematic representation of the adenoviral vectors. (a) Ad-LP1-UGT1A1, an adenoviral vector UGT1A1 driven by a liver specific promoter. (b) Ad-rev-LP1-UGT1A1 with the same transgene cassette as (a) in the reverse orientation. (c) In the hybrid vector (Ad/AAV), the expression cassette driven by the LP1 promoter is flanked by AAV-ITR. (d) Adenoviral vector with UGT1A1 driven by cytomegalovirus (CMV) promoter. LTR, long terminal repeat; ITR, inverted terminal repeat; LP1, liver specific promoter; rev, reverse orientation; CMV, cytomegalovirus promoter.
A liver specific promoter prolongs the correction of hyperbilirubinemia

The use of a constitutive promoter like CMV may provide expression of adenoviral proteins in all tissues including antigen presenting cells which will increase the adaptive immune response resulting in the loss of transduced hepatocytes. A tissue specific promoter that restricts the transgene expression and putative expression of adenoviral proteins to the hepatocytes may therefore prolong the expression. To investigate this option, the CMV promoter was replaced by the hepatocyte specific LP1 promoter (Fig.2B).

Tail vein administration of $3 \times 10^{10}$ ip/kg ($6 \times 10^{11}$ vp/kg) of Ad-LP1-UGT1A1 resulted in a 60-70% reduction of serum bilirubin that in contrast to Ad-CMV-UGT1A1 was persistent (Fig.3).

![Graph showing serum bilirubin levels over weeks](image)

**Fig.3** - LP1-UGT1A1 inverted cassette presents a better hyperbilirubinemia correction in Gunn rats. Serum bilirubin levels of Gunn rats upon a tail vein injection of $3 \times 10^{10}$ ip/kg ($6 \times 10^{11}$ vp/kg) (n=5) of Ad-LP1-UGT1A1, $3 \times 10^{10}$ ip/kg ($1,4 \times 10^{12}$ vp/kg) (n=4) or $1 \times 10^{10}$ ip/kg ($4,7 \times 10^{11}$ vp/kg) (n=3) of Ad-rev-LP1-UGT1A1.

**Inverted transgene cassette improves adenoviral efficiency**

In the Ad-LP1-UGT1A1 the presence of the transcriptional active Ad LTR upstream of the UGT1A1 gene may affect both selectivity and efficacy of the LP1 promoter. As a consequence, expression may not be entirely hepatocyte-specific. In addition, this
may result in low expression of adenoviral proteins for instance due to aberrant splicing\textsuperscript{10}. Inverting the expression cassette would prevent this. Therefore the efficacy of a vector in which the orientation of the LP1-UGT1A1 transgene cassette in the E1-region was inverted (Ad-rev-LP1, Fig.2C) was determined. Tail vein injection of an identical dose (3x10\(^{10}\) ip/kg) of this vector normalized serum bilirubin levels, showing inverting the expression cassette improved the efficiency of the vector. Even a three fold lower dose 1x10\(^{10}\) ip/kg (4,7x10\(^{11}\) vp/kg) of the inverted vector was still sufficient to provide a 90% reduction of the serum bilirubin levels that persisted until the time of sacrifice 48 weeks (Fig.3). The improved vector efficiency of the inverted construct suggested that the nearby presence of the Ad LTR and/or the expression of adenoviral proteins in the Ad-LP1-UGT1A1 reduced its efficacy.

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{bilirubin.png}
\caption{Fig.4 – Ad/AAV-LP1-UGT1A1 administration results in a dramatic reduction of serum bilirubin levels in hyperbilirubinemic rats. Gunn rats were injected with 1.5x10\(^{10}\) ip/kg (7x10\(^{11}\) vp/kg) (n=3) and two individual animals 7.5x10\(^{9}\) ip/kg (3x10\(^{11}\) vp/kg) of Ad/AAV-LP1-UGT1A1 serum bilirubin levels were monitored.}
\end{figure}

\textbf{Adeno/AAV hybrid vector presents an unprecedented higher efficiency}

The LP1 promoter was previously used in AAV vectors to provide hepatocyte specific and efficient expression of transgenes in rodents and non-human primates\textsuperscript{18}. This suggests that AAV ITRs do not interfere with LP1 activity and specificity. Therefore, we tested if flanking the LP1-UGT1A1 expression cassette by AAV2 ITR’s could improve the efficacy of Ad-LP1-UGT1A1. Upon tail vein injection of this vector with a dose as
low as 7.5x10^9 ip/kg (3x10^{11} vp/kg) resulted in more than 95% reduction of serum bilirubin levels (Fig.4). This correction was sustained until the end of the experiment and serum bilirubin levels remained very low after 48 weeks while in control animals serum bilirubin levels show a gradual increase in time. Whether this increase efficacy is due to shielding of the LP-1 promoter from the putative influence of the adeno LTR or whether it prevents expression of adenoviral proteins is unclear.

Fig.5 - Flanking the LP1-UGT1A1 expression cassette with the AAV2 ITRs enhances the expression in liver. Ratio between the UGT1A1 mRNA levels and the transgene genomic copies in the liver of animals treated with Ad-LP1-UGT1A1 (n=4), Ad-rev-LP1-UGT1A1 (n=13) and Ad/AAV-LP1-UGT1A1 (n=6). The UGT1A1 mRNA levels are normalized with 18S rRNA and UGT1A1 transgene copies/rat diploid genome in livers.

**A cassette flanked by AAV2 ITRs enhances the transgene expression.**

To investigate the mechanism causing the difference in efficacy, the gc level of the different cassettes in the liver was determined using quantitative real-time PCR. The copy number per rat genome (vc/dGE) was determined by taking the ratio between the copies of the UGT1A1 and that of the endogenous rat β-actin gene. This revealed that the transgene copy number of the inverted LP1 construct was 0.14 +/- 0.09 while for the forward orientation this was 0.06 +/- 5. Although due to the large variation in this the difference in gc content between these two constructs did not reach statistical significance, the somewhat higher levels seen with Ad-inv-LP1-UGT1A1 are in agreement with the higher level of correction of serum bilirubin seen with this vector. The gc content of the hybrid vector, 0.22 +/- 0.13 was not significantly
increased compared to the vector with the inverted expression cassette. The lack of a significant difference in the percentage of vector genomes in liver a year after transduction indicates that the difference in correction seen between these vectors is not immune mediated. The absence of a humoral immune response to human UGT1A1 in all these rats (not shown), as opposed to a strong response was seen in the rats injected with the CMV driven expression, provides additional supports for this observation. Therefore it was investigated whether interference of the adeno LTR on the LP1 promoter could play a role in the different efficacy of these constructs. The expression of UGT1A1mRNA per genomic copies was determined for all constructs. For Ad-LP1-UGT1A1 and Ad-inv-LP1-UGT1A1, these levels were comparable (Fig.5). However, for the hybrid vector a significant 8-fold increase of UGT1A1 mRNA per genomic vector copy was seen.

**Discussion**

The liver tropism of adenovirus type 5 renders it an interesting option for liver directed gene therapy to correct inherited liver disorders, including Crigler-Najjar syndrome. Early studies showed that in Gunn rats E1 deleted adenoviral vectors provided a significant but transient correction. The absence of long term correction imposed a major drawback to this vector. Additional studies showed that an adaptive immune response to adeno viral proteins expressed at a low level encoded by these first generation vectors as well as to the transgene product caused the rapid decline in efficacy. Several strategies have been explored to prolong transgene expression and to allow repeated treatment with the same vector. For instance, lifelong tolerance to viral antigens could be induced by injecting the adenoviral vector in neonatal Gunn rats. This resulted not only in long term gene expression, but also allowed efficient liver transduction upon re-administration. Similarly, oral tolerance was evaluated and resulted in protracted expression using this vector. However, tolerization to a widespread human pathogen like adenovirus is out of reach for clinical application. Another strategy that was followed was to express the E3 region of the viral genome, which is known to induce immune suppression together with the therapeutic transgene. Significant results were obtained in Gunn rats, however this strategy was not further developed. Another approach is the deletion of all viral genes in the recombinant genome. The resulting HD-Ad vectors are, indeed, superior to early generation Ad vectors in terms of safety and long term efficacy. However, up till
now the production of the high-titer batches of HD-Ad needed to correct a liver disorder cannot be performed without contamination with RCA that are used in the production process\textsuperscript{28}. Although under optimal productions the RCAs levels are very low (0.05%), in a clinical relevant dose of $1 \times 10^{10}$ infectious particles the contamination is still of $5 \times 10^5$ RCAs. Presence of RCA seemed to have caused the adverse effects causing the stopping of a phase I trial for hemophilia A\textsuperscript{8,27}. In this respect RCA-free second generation adenoviral vectors produced on the Per.C6 cell line seem preferable\textsuperscript{29}. A major prerequisite for this is that the adaptive response to these vectors can be prevented.

The aim of this study was to improve the transgene cassette of second-generation adenoviral vectors to increase the efficiency and to prevent the adaptive immune response in the Gunn rat using a production process that is easily scalable and does not result in significant contamination with RCA. In this setting, we observed an efficient but transient correction when expression of the human UGT1A1 cDNA was controlled by the constitutive CMV promoter. This is in agreement the previous studies with adenoviral vectors in this animal model\textsuperscript{20,21}, a liver specific promoter resulted in a prolonged correction. However, others have reported that the use of a liver specific promoter is not sufficient. In dogs for instance the use of the liver specific albumin promoter did not result in sustained expression\textsuperscript{30}. The discrepancy between that study and ours may be the dose used. In these dogs a dose of $3 \times 10^{12}$ vp/kg was needed for efficient correction. Injection of a lower dose resulted in a milder immune response but was too low to obtain effective correction. It suggests that for long term correction, dose reduction is required but may not be enough to overcome the adaptive immune response. One cause of this response may be that the liver specific promoter leakiness or aberrant splicing results in low expression of adenoviral protein such as pIX encoded by the adenoviral vector\textsuperscript{10}. Inverting the orientation of this construct may overcome this problem. The efficient correction obtained with the construct in the inverted orientation seen in this study demonstrated that the tissue specific LP1 promoter was suitable for this approach. However, the comparable gc levels in liver of both the forward and the inverted vector after a year do indicate that immune response towards both, if any, are comparable.

The long term highly effective correction of serum bilirubin levels obtained with the hybrid vector in which the expression cassette is flanked by the AAV2 ITRs is very interesting. The similar gc levels in liver after a year suggest that this increased efficacy
is not due to a reduced immune response towards the transduced cells. Instead we observed an increase in UGT1A1 mRNA level indicating that flanking of the expression cassette by the AAV ITRs enhanced the transcriptional activity. This increase may be due to the transcriptional activity of the A/D element present in these ITR’s\(^\text{31}\). Such a mechanism would be in agreement with the effective hepatocyte specific expression provided by the scAAV-LP1-vectors\(^\text{18}\). Another explanation would be a negative effect of the adenoviral LTRs on the transcriptional activity of the LP1 promoter. However, the comparable efficiency of the expression cassette in the forward orientation, in which the LP1 promoter is in the proximity of the Ad-LTR, and the inverted cassette renders this explanation unlikely. Another more trivial explanation would be that the gc levels of the hybrid vector are underestimated due to the presence of the AAV-ITRs\(^\text{32}\). However, the distance between the amplicon and the AAV-hairpin is about 800 bp, so such an effect is unlikely. Furthermore, cutting the total liver DNA extensively with EcoR1, the sites for which are located between both ITRs and the amplicon, did not result in higher gc levels.

In conclusion we generated effective second generation adenoviral vectors that allow sustained correction of hyperbilirubinemia in the Gunn rat. Since these vectors can be produced RCA free in high titer this overcomes one of the hurdles imposed on clinical use of adenoviral vectors for liver directed gene therapy. Furthermore, our data suggest that flanking the expression cassette with AAV2 ITR’s enhances the efficacy without compromising the specificity of expression, allowing further dose reduction and thus improving safety.
References


