Hepatocyte-specific gene expression by a recombinant adeno-associated virus vector carrying the apolipoprotein E enhancer and $\alpha_1$-antitrypsin promoter

Research Article

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Summary

An adeno-associated virus vector was constructed to express exogenous genes to the liver. The original plasmid construct carried two expression units; a neomycin resistant gene and human $\alpha_1$-antitrypsin cDNA under the control of hepatocyte specific transcription elements. Cells were transfected with the constructed plasmid DNA with another packaging plasmid, and recombinant adeno-associated viruses (rAAV) were then recovered after adenovirus infection. Alternatively, rAAV were recovered by transduction of DNAs of the packaging plasmid and adenovirus into pre-selected cells carrying constructed proviral DNA. When the transducing abilities were evaluated based on G418 resistant colony formation on HeLa cells, the latter method was found to give almost 10-fold more rAAV. We then isolated G418 resistant colonies and established several independent clones for the HeLa and Hepa1A cells infected with the rAAV. All of the eight clones derived from Hepa1A cells produced significant amounts of the human $\alpha_1$-antitrypsin protein. In contrast, none of the five clones derived from HeLa cells produced a detectable level of $\alpha_1$-antitrypsin. Our results suggest that liver-specific promoter and enhancer maintain the tissue specificity in the rAAV construct, and that the rAAV vector system would be useful in hepatocyte directed gene therapy.

I. Introduction

Liver-directed gene therapy could revolutionize treatments for many genetic disorders such as phenylketonuria, familial hypercholesterolemia and hemophilia (Ledley 1993). Adenoviral vectors efficiently transduce a gene into hepatocytes, easily achieve its expression at a therapeutic level for many diseases, but do not allow a long-term expression and repetitive administration because of the high immunogenicity (Jaffe et al. 1992; Okuyama et al. 1998). Retroviral vectors are also able to transduce an exogenous gene into hepatocytes, and a long term expression of the transduced gene has been identified in several experiments using rat or dog liver (Rettinger et al. 1994; Kay et al. 1992; Kay et al. 1993; Hafenrichter et al. 1994). However, the expression level is
generally too low for therapeutic treatments of patients because the transducing efficiency is extremely low.

We have previously demonstrated that a retroviral vector expressing an exogenous gene under the control of a human apolipoprotein E enhancer and α1-antitrypsin promoter as well as an original retroviral LTR promoter dramatically increase the level of protein production after administration into the rat liver (Okuyama et al. 1996). The apolipoprotein E enhancer has been detected through studies on a gene cluster of apoE/C-I/C-II in human chromosome 19. Studies on transgenic mice disclosed that a 154 bp region located 15 kb downstream of the apolipoprotein E gene was responsible for the high level of expression in hepatocytes (Shachter et al. 1993; Simonet et al. 1993). A 420 bp segment of the 5' flanking region of the human α1-antitrypsin gene contains distinct HNF-1 and HNF-2 binding sites, and both sites are responsible for strong and tissue-specific expression of α1-antitrypsin (Li et al. 1988). Recently we found that rats administered the retroviral vector expressing human coagulation Factor X under the control of the 420 bp of α1-antitrypsin promoter produced a therapeutic level of functional Factor X (Le et al. 1997). These observations suggested that with the retroviral vectors designed to express an exogenous gene under the control of promoter-enhancer complex of apolipoprotein E and α1-antitrypsin, one is able to achieve significantly high levels of transgene expression. However, retroviral vectors can transduce foreign genes only into dividing cells, thus inducing the regeneration with partial hepatectomy which is essential for retroviral gene-transduction into hepatocytes. Although the mortality for a 70% hepatectomy is relatively low and the procedure could be justified for life threatening genetic deficiencies, alternative methods that circumvent partial hepatectomy are desirable.

Adeno-associated virus is a replication-defective parvovirus that is being developed as a vector for human gene therapy (Laughlin et al. 1986). One advantage of AAV as a vector is that it can transduce genes into postmitotic cells like cells of the Central Nervous System (Kaplitt et al. 1994), lung epithelial cells (Flotte et al. 1993), or muscle fiber cells (Fisher et al. 1996). Since most hepatocytes in vivo are also in the growth arrested state, AAV vectors is expected to be suitable for in vivo hepatocyte-directed gene therapy. However, little is known about the transduction and expression efficiency of rAAV in hepatocytes in vitro as well as in vivo (Flotte et al. 1995; Fisher et al. 1997; Snyder et al. 1997). In this study, we generated a recombinant adeno-associated virus containing hepatocyte-specific expression unit, and evaluated its transduction efficiency, tissue specificity, and level of expression in gene-transduced cells of hepatocyte origin.

II. Results

A. Generation of rAAV vector containing a liver-specific promoter and enhancer

The structure of the rAAV vector TRNAEAT containing liver-specific transcriptional elements is shown in Fig. 1. The vector contains the human α1-antitrypsin (hAAT) cDNA as a reporter gene downstream of the 420 bp 5' flanking sequence of its own gene. The enhancer region located in the apoE/C-I/C-II gene locus is necessary for high level expression of the apoE gene in livers of transgenic mice (Schacter et al. 1993). In an attempt to further enhance expression of the hAAT promoter from an rAAV vector, four copies of ApoE enhancer were placed upstream of the hAAT promoter (Simonet et al. 1993). In addition to these 2.8-kb hepatocyte-specific expression units of hAAT, an 1.5 kb expression cassette of neomycin resistant gene under the control of mouse phosphoglycerate kinase promoter (Soriano et al. 1991; Adra et al. 1987) was also introduced into the AAV vector plasmid pTR (+).

B. Comparison of the efficiency of rAAV generation

We produced rAAV vectors via two different methods. Method I was a conventional co-transfection method described before (Zolotukhin et al. 1996). Briefly, HeLa cells were co-transfected with the vector plasmid pTRNAEAT and packaging plasmid pM45 (Peel et al. 1997) at a molar ratio of 1:2 using the calcium-phosphate method, followed by the infection of human adenovirus 5 at MOI 10. For Method II, we established a stable HeLa cell line “HeLa-TRNAEAT” carrying proviral sequences of AAV-TRNAEAT by transfecting HeLa cells with pTRNAEAT following the selection in G418. HeLa-TRNAEAT cells were transfected with pM45 and infected with Ad5. Then, wild type HeLa cells were infected with the rAAV generated by these two methods and selected with G418 to determine the Neo-resistant titers. The rAAV titers of the viral stock from Method I was 0.8±0.4x10^3 cfu/ml, while those from Method II was 1.0±0.2x10^3 cfu/ml (Fig. 2). We were able to generate rAAV stocks with 10 fold higher titer using Method II. Moreover, since more consistent and reproducible results were obtained with the latter method, we used Method II for generating rAAV for further analysis.

C. Testing the rAAV vectors for infectivity to human and rodent hepatoma cell lines

We determined the Neo^R titers of HepG2 and Hepa1A cells to evaluate the infectivity of the rAAV to cell lines of hepatocyte origin. The Neo^R titers for HepG2 and Hepa-
Figure 1. Schematic presentation of recombinant adeno-associated virus ‘TRNAET’. Two expression cassettes were introduced in an opposite direction to each other between two terminal inverted repeat sequences of AAV. Expression of neomycin resistant gene is under the control of phosphoglycerate kinase promoter, and expression of human $\alpha_1$-antitrypsin cDNA is expected to be under the control of human $\alpha_1$-antitrypsin promoter and apolipoprotein E enhancer. Tr, inverted terminal repeat sequence of adeno-associated virus; Neo-R, expression cassette for neomycin resistant gene; ApoEEn, 4 copies of apolipoprotein E enhancer; hAATcDNA, cDNA for human $\alpha_1$-antitrypsin; A, polyA signal of SV40.

Figure 2. Neo$^R$ titers of rAAV ‘TRNAET’ in HeLa cells. The viral stocks of rAAV ‘TRNAET’ were prepared by two different methods, Method I and Method II (see details in text), and Neo$^R$ titers of each viral stock were determined in HeLa cells. Data is presented as the average ± SE.

Figure 3. Infectivity of rAAV in different cell lines. Neo$^R$ titers of the rAAV prepared with Method II were determined with 4 different cell lines, HeLa (Lane 1), NIH3T3 (Lane 2), HepG2 (Lane 3), and Hepa1A (Lane 4). Data is presented as the average ± SE.

1A cells were 0.8±0.2x10$^5$ cfu/ml and 0.6±0.1x10$^5$ cfu/ml, respectively. The same viral solution was used to calculate the titer in HeLa cells and NIH3T3 cells (Fig. 3). The relative infectivity of the rAAV in HepG2 and Hepa1A cells compared to those of HeLa cells were 0.75 and 0.67, respectively. These observations suggested that it is possible to transduce exogenous genes into cell lines of hepatocyte origin with similar efficiency as into HeLa or NIH3T3 cells using the rAAV gene transfer system.
**Figure 4.** Detection of proviral genome sequence in Hepa1A and HeLa cells infected with rAAV "TRNAEAT". A 400 bp DNA region of human \( \alpha_1 \)-antitrypsin was amplified using purified genomic DNA of rAAV-infected cells as templates. (A) PCR results for the eight Hepa 1A clones. Template DNA samples of PCR reactions were as below, pTRNAET (Lane P), wild type Hepa1A (Lane 0), rAAV-infected Hepa1A clones No.1-No.8 (Lane 1-Lane 8), and wild type HepG2 (Lane G). Lane M stands for DNA size markers, \( H_{ind} \) II digested Lambda DNA (left) and \( Sae3AI \) digested PUC 19 (right). The 400 bp amplified DNA fragments were identified in Lanes 1 to 8, indicating that proviral sequences of the rAAV were introduced into all of the eight clones. This signal was not identified in Lane 0 and Lane G instead. This corresponds to the DNA amplified from human genome for \( \alpha_1 \)-antitrypsin. (B) PCR results for five independent HeLa clones. Template DNA samples of PCR reactions were wild type HeLa cells (Lane 0), and rAAV-infected HeLa clones No.1-No.5 (Lane 1-Lane5). Lane P, Lane G, and Lane M were same as for Figure 4A. PCR of the five HeLa clones and wild type HeLa cells resulted 1.8 kb amplified DNA fragments, corresponding to the endogenous human hAAT gene. The hAAT protein secreted into the media of each clone was assayed by ELISA using human-specific antibody for \( \alpha_1 \) -antitrypsin. All eight clones of Hepa1A cells secreted hAAT into the media. The average amount of hAAT protein secreted from the rAAV-infected Hepa1A cells was 103.8 ng /10^6 cells /24 hours (Fig. 5A). This represented 31% of hAAT secreted from one million cells of wild type HepG2, and was similar to the level of hAAT secreted from Hepa1A cells infected with retroviral vector “apoE(-)haat-LTR” carrying an identical liver-specific expression cassette for hAAT (Table 1). On the other hand, none of the HeLa clones obtained by the infection of rAAV-TRNAEAT secreted detectable levels of hAAT protein in cultured media, although expression units for hAAT were administered into the host cell chromosome (Fig. 5B). These results suggested that rAAV-TRNAEAT was able to express exogenous genes exclusively in cells of hepatocyte origin.

**III. Discussion**

In this report we have shown that a recombinant adeno-associated virus vector is able to transfer an exogenous gene into human or rodent cells of hepatocyte origin with a similar efficiency as in HeLa cells or in NIH3T3 cells, and
that it is possible to obtain hepatocyte specific transgene expression using a liver-specific promoter and enhancer.

We previously generated a retroviral vector expressing $\alpha_1$-antitrypsin cDNA under the control of the enhancer-promoter complex of apolipoprotein E and $\alpha_1$-antitrypsin. This retroviral vector, apoE(-)haat-LTR, showed markedly increased expression of $\alpha_1$-antitrypsin in rat liver in vivo (Okuyama et al. 1996). The rAAV vector TRNAEAT contains the same liver-specific expression cassette, and the levels of transgene expression in Hepa1A cells infected with rAAV-TRNAEAT were similar to those in the same cells infected with the retroviral vector apoE(-)haat-LTR. These in vitro results suggest that a high level of expression could be expected in vivo using rAAV-TRNAEAT, if similar gene transduction efficiency with retroviral vectors is obtainable using the rAAV gene transfer system.

In order to evaluate the level of expression in rat liver in vivo, however, it is necessary to prepare high titer viral stocks. It is difficult to generate high titer AAV viral particles in large scale with the conventional co-transfection method (Peel et al. 1997). To circumvent this problem, we established a HeLa cell line encoding the proviral genome sequence of the rAAV-TRNAEAT. Using this cell line, it was possible to obtain more than 10 fold higher titer viral stocks easily, and this result was highly reproducible (Fig. 2). Flotte et al. (1995) tried a similar approach using 293 cells, and were successful in generating rAAV with a 5 fold higher titer compared with the conventional co-transfection method.

One of the potential advantages of rAAV for hepatic gene therapy is that it is possible to transduce genes into non-dividing cells (Podsakoff et al. 1994). Recently Snyder et. al. (1997) reported persistent transgene expression in mouse after a simple intraportal infusion of the rAAV expressing human Factor IX under control of MuLV LTR promoter/enhancer. This result suggests that the rAAV gene transfer system is promising for in vivo liver-directed gene therapy. However, one major disadvantage of in vivo hepatic gene transfer is that it is difficult to restrict gene transduction to hepatocytes, because there are many non-parenchymal cells, such as Kupffer cells and sinusoidal endothelial cells, in the liver in addition to hepatocytes. Here we demonstrated that cell-type specific transgene expression was achievable by rAAV carrying liver-specific promoter enhancer sequences. The vector system described here has the potential advantage of eliminating the risk of miss-targeting, a problem encountered when rAAV vectors are used as an in vivo gene delivering vehicle.

**Figure 5.** Quantification of human $\alpha_1$-antitrypsin secreted from rAAV-infected Hepa 1A and HeLa cells. The amount of human $\alpha_1$-antitrypsin protein secreted into the media was determined by ELISA. (A) The results of ELISA assay in wild type Hepa1A cells (Lane 0), in rAAV-infected Hepa1A clones No.1-No.8 (Lane1-Lane8), and in wild type HepG2 cells (Lane G). (B) The results of ELISA assay in wild type HeLa cells, rAAV-infected HeLa clones No.1-No.5 (Lane 1-Lane 5), and wild type HepG2 (Lane G). Data is presented as the average of three independent assays.

**IV. Materials and Methods**

**A. Plasmid construction**

The plasmid pIM45, encoding rep and cap genes of AAV, and pTR(+) for constructing vector plasmid of rAAV were generous gifts from Dr. Nick Muzyckzucka of the University of Florida. The structure of retroviral vector plasmid pAp(-) hAAT-LTR was described elsewhere (Okuyama et al. 1996). The plasmid pAp(-)hAAT-LTR was linealized at the unique BglII site, and then partially digested with BamHI. The 2.2 kb BglII-BamHI DNA fragment containing 4 copies of the 154 bp...
apolipoprotein E enhancer region, 400 bp of human $\alpha_1$-antitrypsin promoter sequence, and 1.2kb cDNA for human $\alpha_1$-antitrypsin was gel-isolated and ligated with BglII-digested pTR(+) generating the plasmid pTRNAET.

pTR(+) is a plasmid for constructing rAAV, using E.coli strain JC8111 (Deiss et al. 1990) as host cells for transformation. The plasmid pTRNAET was again digested and linealized with BglIII, blunt-ended with the Klenow fragment of E.coli DNA polymerase, and ligated with the 1.6 kb expression cassette of the neomycin resistant gene under the control of mouse phosphorylglycerol kinase promoter, isolated from another plasmid, pPGKNeo (Soriano et al. 1991). The plasmid pTRNAET was generated based on this cloning process.

**B. Production of rAAV vector “TRNAEAT”**

HeLa cells were maintained with DMEM (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Sanko Junyaku Co. Ltd., Tokyo Japan). The rAAV TRNAEAT was generated by two different methods. Method I involved conventional co-transfection. HeLa cells were transfected with pTRNAEAT and pIM45 at a molar ratio of 1:2 using calcium phosphate precipitation method described before (Chen and Okayama 1987). 24 hours after the transfection, the cells were infected with wild type adenovirus Ad5 with MOL2 for 2 hours. Three days after the transfection, the cells were harvested, lysed by freezing and thawing 5 times, and incubated at 60°C for one hour to inactivate co-existing adenoviruses. In Method II, we established the stable HeLa cell clone first. HeLa cells were transfected with the plasmid pTRNAEAT and selected with 600µg/ml G418 (GIBCO BRL) for 14 days, and established the HeLa cell clone HeLa-NAEAT carrying the proviral genome sequences in its chromosomes. To produce rAAV, HeLa-NAEAT was transfected with pIM45, and the transfected cells were treated in the same way as for Method I.

**C. Determination of neo R titers of the rAAV “TRNAEAT”**

HeLa and HepG2 cells were maintained with DMEM supplemented with 10% fetal bovine serum, NIH3T3 cells were maintained with DMEM with 10% calf serum (Sanko Junyaku Co. Ltd.), and HepA1A cells, cells from a mouse hepatoma cell line, (Darlington 1987) were maintained with 75% MEM (GIBCO BRL), 25% Waymouth (GIBCO BRL), 10% fetal bovine serum. Serial dilutions of the viral stocks were made with DMEM, and certain amounts were added into the media of the plates culturing HeLa cells for 4 hours. Then cells were washed with PBS twice, and fed with fresh media for two more days. Two days after the infection, 600µg/ml of G418 (GIBCO BRL) was added to the media, and the culture was continued until distinct colonies were identified. The titer of each viral stock solution was calculated by counting the numbers of the G418 resistant colonies on the plates. The neo R titers of the rAAV to NIH3T3 cells, HepA1A cells, and HepG2 cells were determined in the same way except for the concentrations of G418 used for the selection: 400 µg/ml in NIH3T3 cells, 600 µg/ml in HepA1A cells, and 800 µg/ml in HepG2 cells, respectively.

**D. Isolation of Hepa1A and HeLa cell clones infected with rAAV “TRNAEAT”**

Hep 1A cells and HeLa cells were infected with rAAV “TRNAEAT” for 4 hours at MOI. 0.1, and two days after the infection, 600 µg/ml of G418 was added to the media. About two weeks after the infection, several colonies were picked up, and further propagated. Finally we established eight Hepa1A clones, and five HeLa clones. Purified genomic DNA samples from these cells were used as templates of PCR reactions for detecting the 400bp DNA region of human $\alpha_1$-antitrypsin cDNA. Forward and reverse primer sequences were 5’-CACTCAGAGCCTTTGACCTGA-C’ and 5’-ACCCAGCTGGACACGTCTTT-3’. Thirty cycles of PCRs were performed at 1 minute of 95°C, 1 minute of 57°C, and 2 minutes of 72°C. Since the forward and reverse primers were synthesized based on the sequence of exon1 and 2 of human $\alpha_1$-antitrypsin gene, this PCR reaction was expected to generate a 1.8kb DNA fragment covering the whole of intron 1 and the part of exon 1 and 2 of the human genomic DNA (Long et al. 1984).

**E. Quantification of human $\alpha_1$-antitrypsin produced from rAAV-infected HeLa and HepA1 cells**

24 hour-cultured media were used for ELISA assay to quantify the amounts of human $\alpha_1$-antitrypsin secreted from the cells. The assay was performed in 96-well microtitration plates. Goat anti-human $\alpha_1$-antitrypsin antibody, and peroxidase-conjugated goat anti-human $\alpha_1$-antitrypsin antibody were purchased from Cappel (Durham, NC). After 2-hour incubation at room temperature with goat anti-human $\alpha_1$-antitrypsin antibody (2 mg/well), non-specific binding was blocked by overnight incubation with 200 ml of 3% BSA and 0.02% sodium azide in PBS at 4°C. After rinsing with washing buffer, 200ml of the cultured media or control samples (purified human $\alpha_1$-antitrypsin, Sigma, St. Louis MO) were added. The standard curve was made from 0 to 100 ng/ml. The microtitration plates were incubated for 3 hours at room temperature and washed four times with PBS. Then 200 ml of peroxidase-conjugated goat anti-human $\alpha_1$-antitrypsin antibody (15mg/ml) was added to each well. After incubation for 2 hours at room temperature, the wells were rinsed five times and 200ml of substrate solution containing 10 mg o-phenylenediamine hydrochloride (Sigma), 10ml 30% H2O2, and 25ml citrate-phosphate buffer pH5 was added. The reaction was stopped by the addition of 50ml of 3 M H2SO4.

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References


