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≡ **Abstract** ≡

Recombinant Adeno-associated Virus-Mediated Gene Transfer in Homocystinuria Mice

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Homocystinuria is a metabolic disorder caused by a deficiency of cystathionine β-synthase (CBS). Patients with homocystinuria show clinical symptoms such as mental retardation, lens dislocation, vascular disease with life-threatening thromboembolisms and skeletal deformities. Generally, the major treatments for CBS deficiency include pharmacologic doses of pyridoxine or dietary restriction of methionine. However, there is no effective treatment for this disease up till today and gene therapy can be an attractive novel approach to treatment of the disease. We investigated whether a recombinant adeno-associated virus could be used as a CBS gene transfer vector to reduce the excessive homocysteine level in the homocystinuria mouse model. Recombinant adeno-associated virus vector encoding the human CBS gene (rAAV-hCBS), driven by EF1-a promoter, was infused into CBS-deficient mice ($CBS^{-/-}$) via intramuscular (IM) and intraperitoneal (IP) injection. IP injection was more efficient than IM injection for prolongation of lives and reduction of plasma homocysteine levels. After 2 weeks of gene transfer by IP injection, serum homocysteine level was significantly decreased in treated mice compared with the age-matched controls and the life span was extended about 1.5 times. Also, increased expression of CBS gene was observed by immunohistochemical staining in livers of treated $CBS^{-/-}$ mice and microvesicular lipid droplets was decreased in cytoplasm of liver. These results demonstrate the possibility and efficacy of gene therapy by AAV gene transfer in homocystinuria mice.

Introduction

Homocystinuria (MIN 236200) is an autosomal recessively inherited disorder caused by deficiency of cytathionine b-synthase (CBS). The major clinical symptoms include mental retardation, lens dislocation, vascular disease with life-threatening thromboembolisms, skeletal deformities[1, 2]. A large number of mutations in different regions of the human CBS have been found in patients with homocystinuria. Mutations in the CBS gene can alter mRNA stability or enzyme stability, activity, PLP binding, heme binding, or allosteric regulation[3].

A lack of CBS activity causes homocysteine accumulation as well as export of homocysteine from the cell, leading to hyperhomocysteinemia, which may be toxic to cells. Moreover, it perturbs the methylation cycle, such as intracellular accumulation of S-adenosyl homocysteine, which has consequences for cell metabolism[4]. The elevated homocysteine concentration has been shown to be a potential risk factor for cardiovascular diseases[5,6], neural tube defects[7] and Alzheimer's disease[8]. Several studies show that homocysteine induces endothelial dysfunction and injury[9,10].

Current treatment of CBS deficiency includes (i) the administration of pyridoxine to putatively stimulate the residual CBS activity; (ii) restriction of dietary methionine intake to decrease the load in the affected pathway; (iii) supplementation of cysteine to correct cysteine deficiency; and (iv) administration of betaine, folic acid and cobalamin to facilitate the remethylation of homocysteine back to methionine[11]. Homocysteine (Hcy) reducing therapy delays the development of the clinical symptoms, and markedly reduces the risk of vascular events[12,13], suggesting involvement of Hcy in the pathogenesis. However, approximately 50 % of CBS-deficient patients are biochemically responsive to pharmacological doses of pyridoxine and treatment must be continued for life[2]. Effective and long-term treatment to reduce homocysteine level in the severe homocystinuria is needed. Gene therapy is an attractive novel approach to treatment this disease because it is effective, sustained and stable other than treatments. However, gene therapy for homocystinuria]has not been tried.

In this study, we used the recombinant adeno-associated virus (AAV) vector as a gene delivery vehicle. rAAV is an attractive vector for use in gene therapy as wild-type AAV is not associated with human disease, but is naturally defective requiring helper adenovirus or herpes simplex virus (HSV) coinfection for replication. rAAV vectors deleted for all viral proteins, leaving only the two 145-bp inverted terminal repeats which are sufficient for packaging and

integration[14,15], thereby reducing the risk of toxicity and immune responses[16,17]. rAAV has been proven to transduce effectively both dividing and nondividing cells such as those of the eye[18], heart[19], brain[20], liver[21], lungs[22], and muscle[23], and lead to stable long-term gene expression[24,25]. It has been widely used for gene therapy studies in inherited diseases such as hemophilia B[26,27], cystic fibrosis (CF)[28] and Fabry disease[29] with promising results.

In this study, we tested the efficacy of delivery of human CBS cDNA in a murine model of homocystinuria, the $CBS^{-/-}$ and $CBS^{+/-}$ mouse, using recombinant adeno-associated virus vectors.

Results

1. Construction of transgene

The transgene, human CBS cDNA, was driven by a human elongation factor1 - a promoter endowed with more stability by woodchuck hepatitis virus posttranscriptional regulatory element, WPRE, and the polyadenylation site was provided by the BGH poly (A). Human CBS cDNA encodes for 551 amino acids and mutations in C-terminal region of gene contained as; deletion of C-terminal, Δ420 - 551 (419 a.a + stop codon) and point mutation (Q451G) (Fig. 1.).

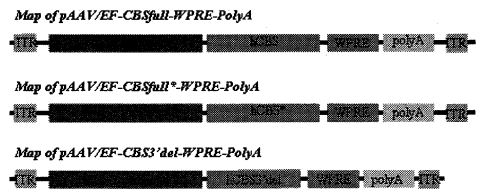


Fig. 1. Schematics of vector constructs. CBS*: Point mutation in the regulatory domain (Glu451Gly). CBS 3'del: Deletion in the regulatory domain (419a.a + Stop). WPRE : Woodchuck hepatitis virus posttranscriptional regulatory element

2. Expression and activity assay of rAAV-hCBS after in vitro transduction

We used these cells as A negative control cell line for the in vitro assay because CBS activity is not present in NIH3T3 cells. NIH3T3 cells were infected at MOI of 5,000 and 20,000 at 60% cell density. Two days after infection, protein was isolated from rAAV-hCBS transduced and untransduced cells, and used for detection of enzyme expression and activity (Fig. 2). The rAAV-hCBS transduction resulted in human CBS expression in the transduced cells, but there was no expression in the untransduced control (Fig. 2A). Protein bands (63 kDa) were observed in HepG2 positive control cells and transduced cells, using a monospecific anti-hCBS antibody. The enzyme activity assay measuring the conversion of [^{14}C] serine to [^{14}C] cystathionine also showed results

consistent with the Western blot analysis (Fig. 2). These in vitro studies confirmed that the rAAV-EF1a-hCBS vector was capable of delivering a functional gene to the cells. Wild-type of CBS gene showed the same enzyme activity as mutant forms. Wild-type CBS gene showed increased enzyme activity for 1.5 ~ 2 times in the presence of AdoMet. Deletion form of C-terminal of CBS gene resulted in the absence of stimulatory effect to AdoMet.

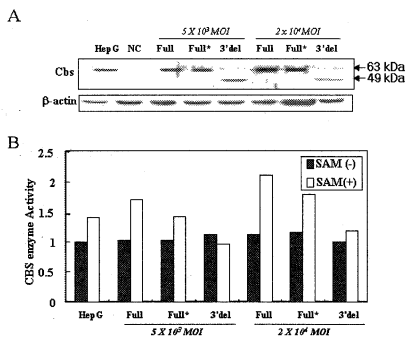


Fig. 2. Western blot analysis and enzyme activity assay in NIH 3T3 cells infected with rAAV-hCBS.

3. Elongation of life span

Homozygous mutants completely lacking cystathionine b- synthase were born at the expected frequency from matings of heterozygotes. but they suffered from severe growth retardation, such as delayed eye opening and facies typical of very young animals. In addition, a majority of them died within 5 weeks after birth with low body weight[30]. In this study, average life span of untreated $CBS^{-/-}$ mouse was about 15.6 ± 1.78 days. We measured extent of the elongation of life span in treated mice. After injection of rAAV-CBS, the life span was lengthened approximately 3-7 days in treated mouse. Intraperitoneal injection of rAAV-CBSfull was the most effective method of gene delivery that showed elongated life span as 21.4 ± 2.94 days. Intraperitoneal injection was superior to intramuscular in effectiveness (Fig. 3).

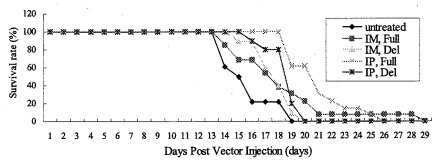


Fig. 3. Survival rate of $CBS^{-/-}$ mice after rAAV-CBS administration through various routes. IM; intramuscular. IP; intraperitoneal

4. Homocysteine level in Plasma

The plasma homocysteine levels in 20-day-old homozygotes were approximately 40 times higher than those of age-matched normal littermates. Heterozygotes have about twice the normal homocysteine levels (Fig.4). At two weeks after delivery of rAAV-hCBS, plasma homocysteine levels were measured. Basal plasma homocysteine level of untreated $CBS^{-/-}$ mice was 401.66 ± 38.67 mM. At 2 weeks after injection, plasma homocysteine levels in mice infused with viral vectors (2 x 10^{12}) viral particles) decreased to 241.83 ±

54.58 mM in rAAV-CBSfull and 301.6 ± 63.97 mM in rAAV-CBSdel, respectively (Fig. 4). Homocysteine concentration was decreased to less than half of the levels observed in untreated homocystinuria mice.

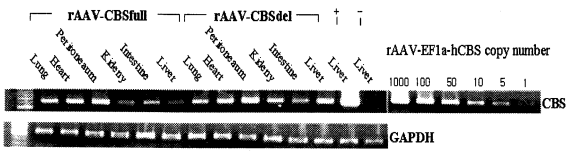


Fig 4. Plasma homocysteine concentration in $CBS^{-/-}$ mice administered rAAV-CBS via IP. $CBS^{-/-}$ mice were administered with 2 X 10^{12} viral particles via intraperitoneal route and sacrificed at 2 weeks after injection. Values are presented as the mean ±SD (N=3).

5. Distribution of recombinant virus in various tissues

RNA was extracted from various organs at 2 weeks after an intraperitoneal injection and analyzed for the tissue distribution of viral transgene vector. The human CBS specific band was detected in treated mouse. Recombinant viral vectors were distributed in major organs such as heart, lungs, liver, intestine, peritoneaum, and kidneys (Fig. 5).

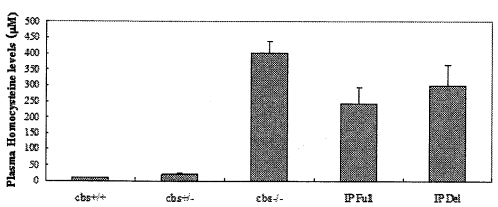


Fig. 5. Analysis of tissue distribution of rAAV-CBSfull & rAAV-CBSdel after intraperitoneal injection in $CBS^{-/-}$ mouse

6. Histological observation and immunohistochemical stain of the liver from homocystinuria mice injected with rAAV-hCBS

Most homozygotes at weaning were runted and their eyes were smaller than normal and not completely open. Gross examination of their organs showed no obvious differences except that the color of the livers was very light in contrast to the reddish-brown color of those from heterozygotes and wild-type mice. The 20-day-old homozygous mutants were sacrificed for histological examination. Hepatic morphology of homozygous CBS-deficient mice was observed by light microscope. Fat droplets were prominent in the liver homocystinuria mice. The cytoplasm was filled with microvesicular lipid droplets (Fig.6 A). In rAAV injected mouse, color of the livers was somewhat changed to reddish-brown and reduction of microvesicular fat droplets was observed by histological examination (Fig.6 B, C). However, many macrovesicular fat change were observed in treated mouse. Immunochemical staining with the anti-hCBS antibody showed that CBS protein was detected in cytoplasm of liver treated with rAAV-hCBS. Mice administered

rAAV-CBSfull was more detected than rAAV-CBSdel (Fig. 6 E, F). This result was consistent with serum homocysteine concentration.

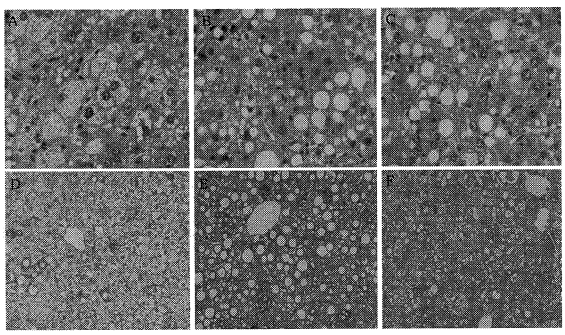
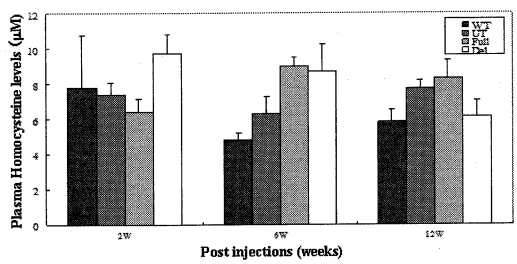


Fig. 6. Histological examination (A-C) and immunohistochemical staining (D-F) of mouse livers after administration of rAAV-hCBS.

7. Administration of rAAV-CBS into CBS$^{+/-}$ mouse

Homozygous CBS-deficient mice exhibit growth retardation, hepatic dysfunction, and shortened survival. Because of severity of the phenotype CBS$^{-/-}$ mice may have limited utility for model of gene therapy. The heterozygotes grew normally and were heathy. They have twice of normal plasma homocysteine levels. In previous studies, it had been shown that heterozygous mice were useful experimental model for hyperhomocysteinemia. We tested the efficacy of gene transfer of rAAV-CBS using CBS$^{+/-}$. Homocysteine concentration of plasma was measured at 2, 6 and 12 weeks after injection. CBS$^{+/-}$ mouse had twice higher plasma homocysteine concentrations compared with CBS$^{+/+}$ (11.3±0.95 mM vs. 22.6±1.8 mM) at 2 weeks after birth. But plasma homocysteine concentrations decreased with age in wild-type and heterozygous mouse and difference of concentration between two groups, 1:2 ratios, was not observed. Homocysteine level was ineffective in treated mice (Fig. 7). There was no significant in enzyme activity (data not shown) in injected mice, consistent of plasma homocysteine level.

Fig. 7. Homocysteine concentration in plasma



of CBS$^{+/-}$mouse administered rAAV-CBS via hepatic portal vein. CBS$^{+/-}$ mice were administered with 1 X 10^{12} viral particles and killed at 2, 6 and 12 weeks after injection. Values are presented as the mean ± SD (n=3).

Conclusion

In conclusion, our data showed intraperitoneal administration of a rAAV-CBS can result in elongation of life span, decrease of homocystein level in plasma and expression of CBS gene in the murine model of

homocystinuria. These findings suggest that an AAV-mediated gene transfer may be useful therapeutic candidate for the treatment of homocystinuria. In addition, new suitable mouse model should be needed for further investigation of therapeutic strategies for the patients with homocystinuria.

References

1) Mudd SH, Levy HL, Skovby F. Disordes in transsulfuration. In: Scriver CR, Beaudet A, Sly W, Valle D, editors. The Metabolic and Molecular bases of Inherited Disease. New York: McGraw-Hill; 2001. p.2007-2056.

2) Mudd SH, Skovby F, Levy HL, Pettigrew KD, Wilcken B, Pyeritz RE, et al. The history of homocystinuria due to cystathionine b-synthase deficiency. Am J Hum Genet 1985;37:1-31.

3) Miles EW, Kraus JP. Cystathionine beta-synthase: structure, function, regulation, and location of homocystinuria-causing mutations.J Biol Chem 2004;279:29871-4.

4) Boers GH, Smals AG, Trijbels F. Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. N Engl J Med 1985;313:709-15.

5) Guba SC, Fink LM, Fonseca VA. Hyperhomocysteinemia-an emerging and important risk factor for thromboembolic and cardiovascular disease. Am J Clin Pathol 1996;106: 709-22.

6) Robert K, Vialard F, Thiery E, Toyama K, Sinet PM, Janel N, et al. Expression of the cystathionine beta synthase (CBS) gene during mouse development and immunolocalization in adult brain. J Histochem Cytochem 2003;51:363-71.

7) Mills JL, McPartlin JM, Kirke PN, Lee YJ, Conley MR, Weir DG, et al. Homocysteine metabolism in pregnancies complicated by neural-tube defects. Lancet 1995;345: 149-51.

8) Clarke R, Smith AD, Jobst KA, Refsum H, Sutton L, Ueland PM. Folate, vitamin B12, and serum total homocysteine levels in confirmed Alzheimer disease. Arch Neurol 1998;55:1449-55.

9) Weiss N, Heydrick S, Zhang YY, Bierl C, Cap A, Loscalzo J. Cellular redox state and endothelial dysfunction in mildly hyperhomocysteinemic cystathionine beta-synthase-deficient mice. Arterioscler Thromb Vasc Biol 2002;22: 34-41.

10) Denis CV, Terraube V, Robert K, Janel N. Elevated plasma von Willebrand factor in a murine model of severe hyperhomocysteinemia. Thromb Haemost 2003;90:362-4.

11) Orendac M, Zeman J, Stabler SP, Allen RH, Kraus JP, Bodamer O, et al. Homocystinuria due to cystathionine beta-synthase deficiency: novel biochemical findings and treatment efficacy. J Inherit Metab Dis 2003;26:761-73.

12) Wilcken DE, Wilcken B. The natural history of vascular disease in

homocystinuria and the effects of treatment. J Inherit Metab Dis 1997; 20:295-300.

13) Schwahn BC, Wendel U, Lussier-Cacan S, Mar MH, Zeisel SH, Leclerc D, et al. Effects of betaine in a murine model of mild cystathionine-beta- synthase deficiency. Metabolism 2004; 53:594-9.

14) McLaughlin SK, Collis P, Hermonat PL, Muzyczka N. Adeno-associated virus general transduction vectors: analysis of proviral structures. J Virol. 1988;62:1963-73.

15) Samulski RJ, Chang LS, Shenk T. Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. J Virol. 1989;63:3822-28.

16) Halbert CL, Rutledge EA, Allen JM, Russell DW, Miller AD. Repeat transduction in the mouse lung by using adeno-associated virus vectors with different serotypes. J Virol 2000;74:1524-32.

17) Snyder RO, Miao CH, Patijn GA, Spratt SK, Danos O, Nagy D, et al. Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. Nat Genet 1997;16:270-6.

18) Bainbridge JW, Mistry A, De Alwis M, Paleolog E, Baker A, Thrasher AJ, et al. Inhibition of retinal neovascularisation by gene transfer of soluble VEGF receptors Flt-1. Gene Ther 2002;9:320-6.

19) Melo LG, Agrawal R, Zhang L, Rezvani M, Mangi AA, Ehsan A, et al. Gene therapy strategy for long-term myocardial protection using adeno-associated virus-mediated delivery of heme oxygena-se gene. Circulation 2002;105:602-7.

20) Davidson BL, Stein CS, Heth JA, Martins I, Kotin RM, Derksen TA, et al. Recombinant adeno-associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system. Proc Natl Acad Sci U S A. 2000;97:3428-32.

21) Snyder RO, Miao CH, Patijn GA, Spratt SK, Danos O, Nagy D, et al. Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors.Nat Genet. 1997;16:270-6.

22) Flotte TR. Recombinant adeno- associated virus gene therapy for cystic fibrosis and alpha(1)-antitrypsin deficiency. Chest 2002;121:98S-102S.

23) Herzog RW, High KA. Adeno-associated virus-mediated gene transfer of factor IX for treatment of hemophilia B by gene therapy. Thromb Haemost 1999;82:540-6.

24) Bueler H. Adeno-associated viral vectors for gene transfer and gene therapy. Biol Chem 1996;380:613-22.

25) Synder RO. Adeno-associated virus-mediated gene therapy. J Gene Med 1999;1:166-75.

26) Kay MA, Manno CS, Ragni MV, Larson PJ, Couto LB, McClelland A, et al. Evidence for gene transfer and expression

of factor IX in haemophilia B patients treated with an AAV vector. Nat Genet 2000;24:257-61.

27) Manno CS, Chew AJ, Hutchison S, Larson PJ, Herzog RW, Arruda VR, et al. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. Blood 2003;101:2963-72.

28) Flotte TR, Zeitlin PL, Reynolds TC, Heald AE, Pedersen P, Beck S, et al. Phase I trial of intranasal and endobronchial administration of a recombinant adeno-associated virus serotype 2 (rAAV2) -CFTR vector in adult cystic fibrosis patients: a two-part clinical study. Hum Gene Ther 2003;14:1079-88.

29) Takahashi H, Hirai Y, Migita M, Seino Y, Fukuda Y, Sakuraba H, et al. Long-term systemic therapy of Fabry disease in a knockout mouse by adeno-associated virus-mediated muscle-directed gene transfer. Proc Natl Acad Sci USA 2002;99:13777-82.

30) Watanabe M, Osada J, Aratani Y, Kluckman K, Reddick R, Malinow MR, et al. Mice deficient in cystathionine beta-synthase: animal models for mild and severe homocyst(e)inemia. Proc Natl Acad Sci USA 1995;92:1585-89.