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

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

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The Doctoral Dissertation
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

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December 2004

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December 2004

ACKNOWLEDGEMENTS

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항상 가까운 곳에서 힘이 되어주시고 믿음으로 지켜봐 주신 부모님들, 소중한 가족들 모두에게 진심으로 감사드립니다. 한결같은 마음으로 힘든 고비마다 항상 옆에서 격려해주고 사랑과 믿음으로 지켜준 남편, 그리고 건강하고 밝게 자라준 너무나 사랑하는 윤정이, 윤서에게 감사와 사랑의 마음을 전합니다.

2004년 12월

저자 박은숙 올림

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ABSTRACT

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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. However, there is no effective treatment for this disease up to now 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-α 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-mediated gene transfer in homocystinuria patients.

Key Words: homocystinuria, cysthathionine β - synthase (CBS), homocysteine, adeno-associated virus (AAV), gene therapy, mouse

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I. INTRODUCTION

Homocystinuria (MIN 236200) is an autosomal recessively inherited disorder caused by deficiency of cytathionine β–synthase (CBS). The major clinical symptoms include mental retardation, lens dislocation, vascular disease with life-threatening thromboembolisms and 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].

The human CBS enzyme, a pyridoxal 5-phosphate-dependent enzyme, converts homocysteine to cystathionine in the trans-sulfurration pathway of the methionine cycles. The enzyme is 551 amino acids in length, forms a homotetramer of 63kDa subunits and requires pyridoxal

phosphate and heme for activity[4-6]. Each subunit binds two substrates (homocysteine and serine) and is further regulated by S-adenosyl-L-methionine (AdoMet), allosteric regulator which activates the enzyme ~ 2-fold[7]. Structure of enzyme consists of the catalytic domain located in the N-terminal 409 amino acids and a regulatory domain located in the C-terminal 142 amino acids[8,9]. Mutations found in patients with homocystinuria are distributed widely in the catalytic and regulatory domains of human CBS (for a continuously updated list of more than 130 mutations, see http://www.uchsc.edu/sm/cbs/cbs)[10]. Interestingly, deletion of the C-terminal regulatory region or specific point mutations within this region can functionally suppress the phenotype of several CBS mutant alleles found in homocystinuria when expressed in yeast[8,11].

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[12]. The elevated homocysteine concentration has been shown to be a potential risk factor for cardiovascular diseases[13,14], neural tube defects[15] and Alzheimer's disease[16]. Several studies have shown that homocysteine induces endothelial dysfunction and injury[17,18].

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[19]. Homocysteine (Hcy) reducing therapy delays the development of the clinical symptoms, and markedly reduces the risk of vascular events[20,21], 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 (rAAV) 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[22,23], thereby reducing the risk of toxicity and immune responses[24,25]. rAAV has been proven to transduce effectively both dividing and nondividing cells such as those of the eye[26], heart[27], brain[28], liver[29], lungs[30], and muscle[31], and lead to stable long-term gene expression[32,33]. It has been widely used for gene therapy in inherited diseases such as hemophilia B[34,35], cystic fibrosis (CF)[36] and Fabry disease[37] with promising results.

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

II. MATERIALS AND METHODS

1. Animals and Procedures

All animal experiment was carried out in accordance with our institutional guidelines. C57BL/6J-Cbs^{tm1Unc} mice were obtained from the Jackson Laboratory. The genotype of the mice was identified by PCR using genomic DNA obtained from tail biopsies as described[38]. Both homozygous CBS-deficient mice ($CBS^{-/-}$) and heterozygous CBS-deficient mice ($CBS^{+/-}$) were used as models of hyperhomocysteinemia.

2. Cell Lines

Human embryonic kidney cell line, 293T, hepatoma cell line, HepG2, and NIH3T3 cell lines were propagated in Dulbecco modified Eagle medium supplemented with heat-inactivated 10 % fetal bovine serum and antibiotics.

3. Cloning of human CBS cDNA

To synthesize human CBS cDNA, liver total RNA was isolated from a HepG2 cell line, human hepatoma cell, and then RT-PCR was carried out as described. Full length and mutated CBS cDNA, point mutation and deletion of regulatory domain of CBS gene were constructed. Full-length normal and mutated human CBS cDNA were cloned into

pGEM-T Easy Vector (Promega). Verification of the identity of the hCBS cDNA was performed by DNA sequencing. The nucleotide sequences were referred to NCBI GenBank (Accession No.NM_000071).

4. Production of rAAV-hCBS Viral Vector

The vector plasmid, pAAV-EF-hCBS-WPRE-BGHpoly(A), based on AAV type 2, was constructed as previously described[39] with minor modification. The human CBS cDNA was cloned into the unique EcoRI site of the vector backbone. Large-scale production of rAAV-EF-hCBS vector was carried out in an adenovirus-free system by triple transfection with the vector plasmid and two additional plasmids, the rep/cap plasmid, pAd/AAV, and the adenovirus helper plasmid, pAd12. All three plasmids, pAAV-EF-hCBS-WPRE-BGHpoly(A), pAd/AAV, and pAd12, were transfected into 293T cells using calcium phosphate. After 48 h, cells were collected by centrifugation and resuspended in 5 mL of tissue dissociation buffer (140 mM NaCl, 5 mM KCl, 0.75 mM K_2HPO_4, 25 mM Tris-HCl, pH 7.4). The resuspended cells were lysed by freezing and thawing. The cell pellet was thawed at 37 °C, and Benzonase (Sigma, St. Louis, MO) was added to a final concentration of 20 U/mL. Sodium deoxycholate (Sigma) was added to a final concentration of 0.5 % and the suspension was incubated for 1 h. The homogenate was purified and concentrated by cesium chloride (Sigma) density gradient ultracentrifugation. Fractions with a refractory index of 1.371~1.373 were pooled and dialyzed (PIERCE Biotechnology, Rockford, IL) against PBS. Refractory indices were

determined using a refractometer (Leica, Buffalo, NY). The rAAV-hCBS genomic titer was determined by real-time quantitative PCR using the ABI7700 (Perkin-Elmer/Applied Biosystems, Foster City, CA), in which the signal from aliquots of test material was compared with a standard signal generated using the linearized pAAV-EF-hCBS-WPRE-BGHpoly(A) plasmid.

5. Administration of rAAV viral vectors

HCU mice ($Cbs^{-/-}$) used for in vivo gene transfer were 5 - 6 days of ages. A 50-100 μl of containing 2 x 10^{12} of rAAV-hCBSfull and rAAV-hCBSdel was slowly delivered using an insulin syringe with a 29-gauge needle by IP and IM injections, respectively. Ten-week-old $CBS^{+/-}$ mice were anesthetized with ketamin/xylazine and injected with 1 x 10^{12} viral particles of rAAV-hCBSfull and rAAV-hCBSdel into hepatic portal vein. Blood samples were collected at various time points. The liver, kidneys, heart, intestine, peritoneum and lungs were isolated from each rAAV-injected $CBS^{-/-}$ and $CBS^{+/-}$ mouse along with age-matched wild-type control and untreated $CBS^{-/-}$ and $CBS^{+/-}$ mice for analysis.

6. Plasma Homocysteine assay

Blood samples obtained were collected into tubes containing 0.5M EDTA and immediately refrigerated at 4 ℃. Blood samples were centrifuged at 1,000g for 10 min at 4 ℃. The plasma obtained was

separated and stored at -80 ℃. Plasma homocysteine level was determined by HPLC (HPLC/fluorescence detector, Bio-Rad 2800series, USA) with fluorometric detection, using a commercially available protocol. The method involves deproteinization of a small sample volume of plasma (50 µl) followed by HPLC and homocysteine level was determined by HPLC reagent kit (Bio-Rad).

7. CBS enzyme assay

CBS enzyme assay was performed as previously described methods[40]. Briefly, the cells and the frozen tissue were homogenized in 0.1 M KH_2PO_4 buffer (pH 7.0) containing 3 mM dithiothreitol, and sonicated. The homogenate was centrifuged at 13,000 × g for 20 min at 4 °C, and the supernatant was used for the CBS enzyme assay. The CBS enzyme assay was performed in 100 µL containing 0.1 M Tris-HCl (pH 8.6), 250µM pyridoxal 5-phosphate, 0.5 mg/ml bovine serum albumin, 0.1 µCi [^{14}C]serine (Amersham, Buckinghamshire, UK), 1 mM cystathionine and 100 µg of crude extract, and the mixture was preincubated for 5 min at 37 °C in the presence or absence of 1.0 mM SAM (S-adenosyl-L-methionine). The reaction was initiated by addition of L-homocysteine (final concentration, 10.0 mM) and incubation for 2 hr at 37 °C. The assay was terminated by cooling to 0 °C in an ice bath. Proteins were precipitated by centrifugation at 12,000 × g for 10 min. The 50 µL of supernatant was then concentrated to 10 µL and analysed by a thin layer chromatography (TLC Silica Gel 60 plates, Merck, Darmstadt,

Germany) and developed in butanol/ acetic acid/ distilled water (60:15:25, v/v/v) at room temperature. The [^{14}C]cystathionine was separated from [^{14}C]serine by ascending TLC. The enzyme activity was assayed by measuring the production of [^{14}C]cystathionine from [^{14}C]serine. Quantification for radioactivity of [^{14}C]serine and converted [^{14}C]cystathionine was done using the ImageQuant, after visualization with a PhosphoImager (Molecular Dynamics).

8. Western blot analysis

Protein extracts were isolated from transduced NIH3T3 cells and HepG2 cells. Proteins (30 μg per lane) were separated by SDS-PAGE using 10 % polyacrylamide gels and transferred to nitrocellulose membrane (Life Technologies, Gaithersburg, MD). The membrane was hybridized with a 1:7,500 dilution of rabbit antibody against human CBS and subsequently with a horseradish peroxidase-conjugated anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The signals were then visualized using ECL-Plus enhanced chemiluminescence (Santa Cruz Biotechnology).

9. Tissue distribution

To assay distribution of recombinant viral vector in liver and other organs, RT-PCR was performed. Total RNA was isolated using a TRI REAGENT (MRC Inc. USA). RT reaction was conducted using 100

pmol/μL random primer (TAKARA SHUZO, Japan). For detection of specific human cystathionine β– synthase sequence, the following primers were used with 25 pmol: forward primer, 5´CAAGTGTGAGTTCTTCAACGCG3´ reverse primer, 5´GGGATGAAGTCGTAGCCGATC3´. These primers amplified a 615 bp product. Primers to mouse GAPDH were used as an internal control (530 bp) (forward primer, 5´CCCACACTGTGCCCATCTAC3´ reverse primer, 5´AGTACTTGCGCTCAGGAGGA3'). After PCR amplification, samples were electrophoresed on 1% agarose gels and visualized with ethidium bromide.

10. Histologic examination

The liver tissues were fixed in 4% formaldehyde, embedded in paraffin, and sectioned into 2μm. Paraffin sections were stained with hematoxylin and eosin, and examined by a specialized pathologist.

11. Immunohistochemical staining

Livers were removed from the mice administered rAAV-hCBS and age-matched control. The sections were incubated with anti-CBS antibody (1:2,000 dilution) at 4 °C overnight. Anti-rabbit IgG used as secondary antibody was incubated for 1h at RT. The immunoreaction was visualized by treating the sections with DAB+substrate chromogen reagent for 20 sec at RT. Tissues were counterstained with hematoxyline, dehydrated through

graded concentration of alcohol, cleared in xylene, and coverslipped.

12. Statistical analysis

The statistical significance levels of differences between groups were determined using *t* test. The data are presented as mean ± SD. The p value less than 0.05 was used as a significance range.

III. RESULTS

1. Construction of transgene

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

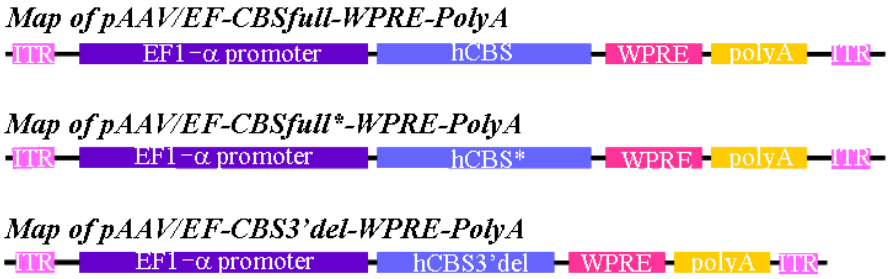

Figure 1. Schematics of vector constructs. CBS*: Point mutation in the regulatory domain (Glu451Gly). CBS 3'del: Deletion in the regulatory domain (419a.a + Stop). WPRE : Woodchuck hepatitis virus posttranscriptional regulatory element.

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

We used NIH3T3 cells as a negative control cell line for the *in vitro* assay because CBS activity is not present in theses 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 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. 2B). These *in vitro* results confirmed that the rAAV-EF1α-hCBS vector was capable of delivering a functional transgene to the cells. We compared enzyme activity with full length and c-terminal mutant forms in CBS gene. rAAV contained full-length of CBS gene showed higher activity compared to mutant form. CBS enzyme activity is stimulated 2-3-fold by the addition of S-adenosylmethionine (AdoMet)[7]. In this study, rAAV contained wild-type CBS increased enzyme activity 1.5 ~ 2 times in presence of Adomet (S-adenosyl-L-methionine, SAM). Mutant form was somewhat less active than wild-type CBS and the deletion form exhibited no AdoMet stimulation.

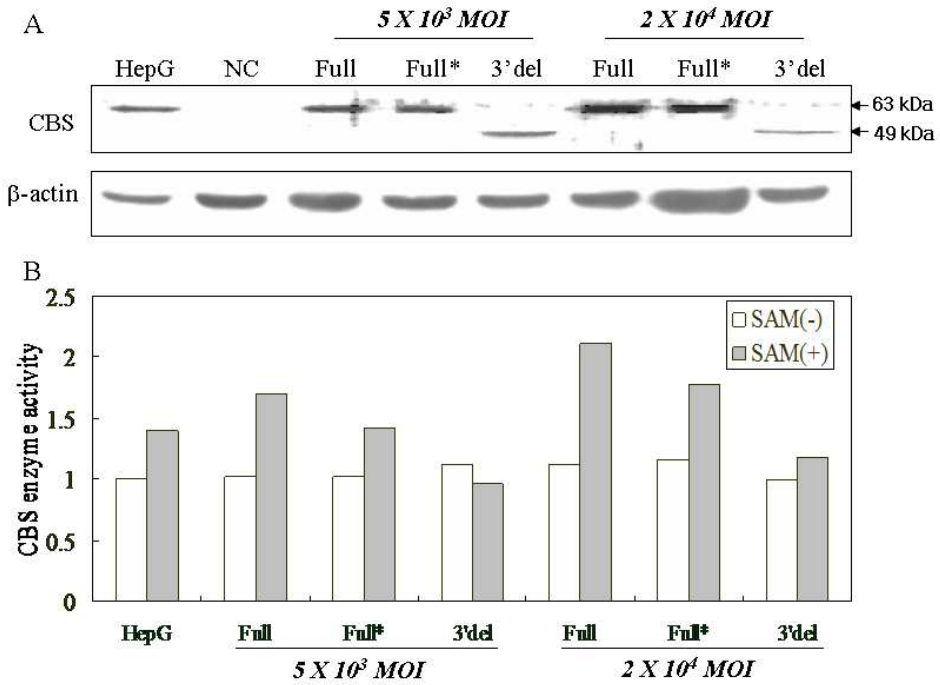

Figure 2. Western blot analysis and enzyme activity assay in NIH 3T3 cells infected with rAAV-hCBS. Analysis was performed at 2 days after transduction with rAAV-hCBS at MOI of 5,000 and 20,000. (A) Cell extracts were separated on 10% SDS-PAGE and blotted onto membrane and probed with human CBS antibody. Goat anti-rabbit IgG was used as the secondary antibody. 63 kDa and 49kDa bands were detected on immunoblots of cells transduced with rAAV-hCBS (lane 3-8) and not found in untransduced (lane 2). The positive control band in HepG2 cells was sited at 63 kDa (lane 1). (B) Each cell extract was assayed for CBS activity with and without 100μM S-adenosylmethionine (SAM) as described. CBS enzyme activity was detected by thin layer chromatography. Full; wild-type CBS, Full*; point mutation in c-terminal region, Q451G, 3'del; deletion of c-terminal region, Δ420 - 551.

3. Elongation of life span

Homozygous mutants completely lacking cystathionine β- 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[38]. 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, Table1).

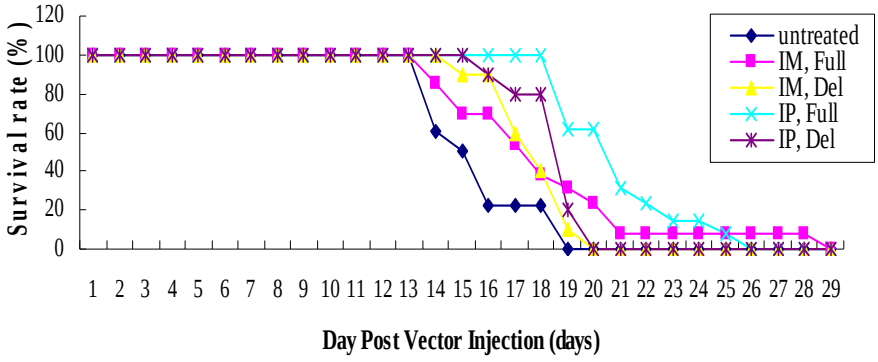

Figure 3. Survival rate of $CBS^{-/-}$ mice after rAAV-CBS administration through various routes. IM; intramuscular. IP; intraperitoneal

Table 1. Changes in Life span of homocystinuria mouse by administration of rAAV-CBS

	Untreated (n=18)	IM; Full (n=15)	IM; Del (n=13)	IP; Full (n=15)	IP; del (n=13)
life span (days)	15.6(±1.78)	18.3(±4.02)	17.5(±1.31)	21.4(±2.94)	18.4(±1.34)

*4. Homocysteine level in Plasm*a

The plasma homocysteine levels in 20-day-old homozygotes were approximately 40 times higher than those of age-matched control 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 μM. 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 μM in rAAV-CBSfull and 301.6 ± 63.97 μM in rAAV-CBSdel, respectively (Fig. 4). Homocysteine concentration was decreased to less than half of the levels observed in untreated homocystinuria mice.

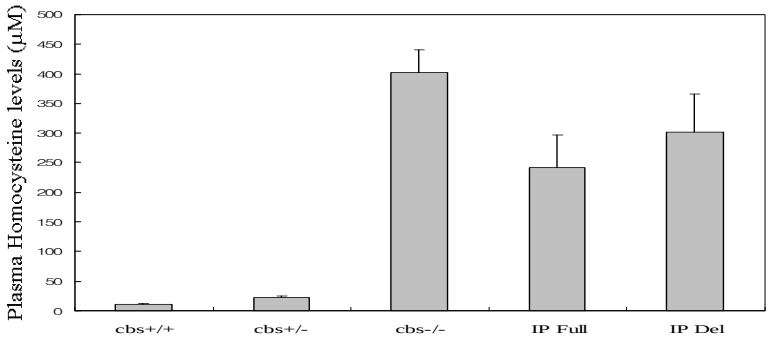

Figure 4. Plasma homocysteine concentration in $CBS^{-/-}$ mice administered rAAV-CBS via IP. $CBS^{-/-}$ mice were administered with 2 X 10^{12} viral particles via intraperitoneal route and sacrificed at 2 weeks after injection. Values are presented as the mean ± SD (N=3).

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).

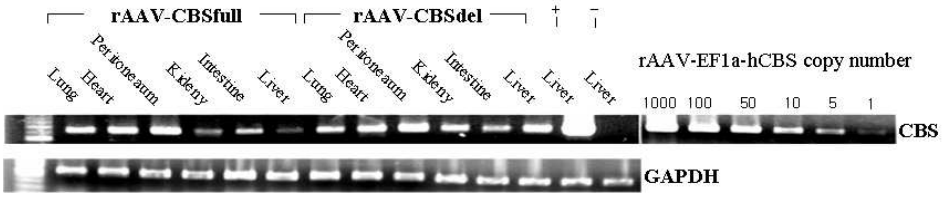

Figure 5. Analysis of tissue distribution of rAAV-CBSfull & rAAV-CBSdel after intraperitoneal injection in $CBS^{-/-}$ mouse. PCR was performed to analyze distribution of rAAV vector in various tissues. RNA was extracted from injected and noninjected mouse and vector-specific region was amplified. All tissues of injected mouse showed vector-specific fragment and no signal was detected in untreated mouse. Internal control, mouse GAPDH amplification was performed for equal quantification. +; wild-type mouse, -; untreated $CBS^{-/-}$ 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 of 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. Immunohistochemical 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.

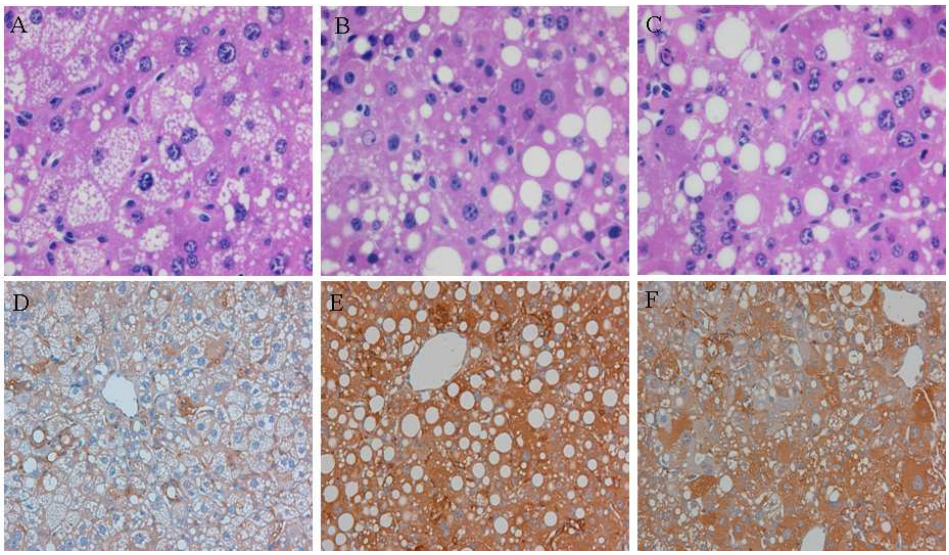

Figure 6. Histological examination (A-C) and immunohistochemical staining (D-F) of mouse livers after administration of rAAV-hCBS. Tissue specimens from untreated homocystinuria mouse (A, D), and injected homocystinuria mouse with rAAV-CBSFull (B, E) and rAAV-CBSDel (C, F) respectively were analyzed at 2 weeks postinjection. CBS protein is detected with immunohistochemical staining in liver of mice injected with rAAV-CBS.

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 μM vs. 22.6±1.8 μM) 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 change of enzyme activity (data not shown) in injected mice, consistent of plasma homocysteine level.

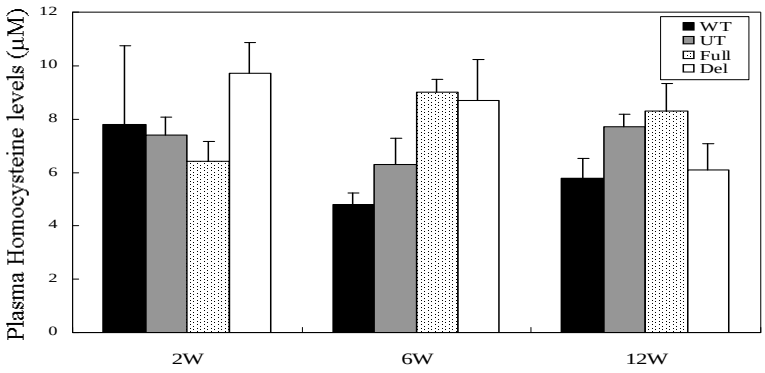

Figure 7. Homocysteine concentration in plasma of $CBS^{+/-}$ mice administered rAAV-CBS via hepatic portal vein. $CBS^{+/-}$ mice were administered with 1 X 10^{12} viral particles and killed at 2, 6 and 12 weeks after injection. Values are presented as the mean ± SD (n=3).

IV. DISCUSSION

To date, treatment strategies of homocystinuria include pyridoxine and folic acid administration in pyridoxine responsive patient, and low methionine diet, with or without the addition of betaine, in pyridoxine non-responsive patients[20]. The clinical manifestations of the disease can be avoided with appropriate therapy but only in those children treated from soon after birth. There is no effective therapy for homocystinuria. However, gene therapy has several potential advantages over classical pharmacological treatment. In order to develope safe and promising therapeutic strategy for homocystinuria, we studied 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 AAV vectors have shown to be promising tool for a number of gene therapy applications such diseases as haemophilia[34], cystic fibrosis[30], α–1 antitrypsin deficiency[30], malignant brain tumors[41], and for gene transfer to the eye[42]. In this study, we demonstrated that AAV-mediated transduction into the homocystinuria mouse brought about improved histologic changes of liver, expression of CBS gene in treated mouse and elongation of life span. In contrast, expected result could not be obtained concerning the long-term effect. Reasons for that were as follows; first, recombinant AAV vectors cannot deliver the normal CBS gene to the liver at sufficient levels to overcome homocystinuria by IP injection although injected mice expressed transgene and their life span

were prolonged. Alternative administration route, liver directed injection may be attempted for potent effect. However, it was impossible to inject via hepatic portal vein, because $CBS^{-/-}$ mice were too small to perform the technique. Second, mouse model wasn't suitable for gene therapy because homozygous deletion of CBS gene was mortal and was not able to continue experiments. Untreated $CBS^{-/-}$ mice used in this study suffered from severe growth retardation and a majority of them died within 2 weeks after birth. In addition, plasma homocysteine levels in $CBS^{-/-}$mice were higher than other study reported previously, 401.66 ± 38.67 μM vs. 205 ± 86 μM. Probably, mice fed standard dietary diet instead of special diet, standard A04 rodent chow[43]. Therefore, new mouse model for homocystinuria will be needed for gene therapy study. In human, the most of pathogenic mutation were missense mutation within the CBS gene. In the future, homocystinuria mouse model with partial defect gene will be developed to treat using gene therapy.

The C-terminal of human CBS gene was known as negative regulatory domain, consisting approximately 140 amino acid residues. This region is required for tetramerization of the human enzyme and AdoMet activation[44]. Deletion of the C-terminal regulatory domain of CBS could at least partially suppress the functional effects of missense mutations located in the catalytic domain[8]. The mutant Δ414-551, lacking both of the proposed CBS1 and CBS2 domains, was about 5-fold more active than the wild type enzyme control in the absence of AdoMet[45]. Enzyme activity was compared with full length and C-terminal mutant forms in CBS gene. rAAV contained full-length of CBS gene was higher than mutant form in

enzyme activity. rAAV contained wild-type CBS increased enzyme activity 1.5 ~ 2 times in presence of AdoMet. Mutant form was somewhat less active than wild-type CBS and deletion form exhibited no AdoMet stimulation. rAAV containing C-terminal deletion is expected to show higher level of enzyme activity than that of wild type. However, wild-type form of CBS gene showed higher enzyme activity than mutant form of the gene. This would probably be explained with difference of CBS gene expression system. In previous report, truncation mutants were expressed in yeast and *Escherichia coli* whereas we expressed it in human cell line. Further experiments remain to be performed, including comparison of enzyme activity in variety of mutant forms and expression systems of CBS gene.

Efficacy of gene transfer also was tested in $CBS^{+/-}$ mouse. CBS-deficient mice ($CBS^{+/-}$) have approximately 50% reduction in cystathionine β- synthase mRNA and enzyme activity in the liver and have twice normal plasma homocysteine levels[38]. Thus, heterozygous mutants will be useful as gene therapy models. Plasma homocysteine levels in $CBS^{+/-}$ mouse had about twice the normal homocysteine levels at 2 weeks after birth. But $CBS^{+/-}$ mouse injected rAAV weren't changed in plasma homocysteine level compared with untreated mouse. There are several possible explanations for this finding. $CBS^{+/-}$ mouse had 2 times higher plasma homocysteine concentrations compared with $CBS^{+/+}$ (11.3 ± 0.95 μM vs. 22.6 ± 1.8 μM) 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. At 12, 16 and 22 weeks, homocysteine concentration in wild-type and heterozygous mouse had 7.8, 4.8, 5.8 μM vs. 7.4, 6.3, 7.7 μM, respectively. Consequently, it is impossible to reduce homocysteine concentration by gene transfer. These results show that $CBS^{+/-}$ mouse is not suitable to study for gene therapy as homocystinuria mouse model.

The results were showed possibility of rAAV-mediated gene therapy of homocystinuria as follows; elongation of life span, decrease of homocystein level in plasma, expression of CBS gene and decrease of microvesicular lipid droplets in injected mice. These data demonstrate possibility and efficacy of gene therapy by AAV gene transfer in homocystinuria mice.

V. CONCLUSION

In conclusion, these data showed intraperitoneal administration of rAAV-CBS can result in elongation of life span, decrease of homocysteine 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 for the treatment of homocystinuria.

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ABSTRACT(IN KOREAN)

재조합 adeno-associated virus를 이용한 Homocystinuria mice로의 유전자 전달

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Homocystinuria는 선천성 대사질환의 하나로 상염색체 열성으로 유전되는 유전질환으로써 CBS (Cysthathionine β-synthase) 유전자가 그 원인유전자로 알려져 있다. 유전자의 결핍으로 urine내의 homocysteine의 농도가 크게 증가하여 지능저하, 골격계의 이상, 혈관의 혈전증의 임상증상을 초래하는 것으로 보고되고 있다.

Homocystinuria 질병의 치료방법은 약물치료를 기본으로 하고 있으나 아직까지 효과적인 치료방법이 알려져 있지 않다. 따라서 본 연구에서는 질병을 보다 안전하고 효과적으로 치료하기 위한 방법으로 유전자치료가 가능한지를 알아보고자 하였다. 최근 유전자치료에 효과적이라고 알려져 있는 virus vector system을 이용하여 CBS유전자의 재조합바이러스를 만들어 in vitro system에서 유전자의 발현과 활성도를 측정하여 환자의 유전자치료에 이용할 수 있는지를 알아보고자 하였다. Viral vector system 중에서 homocystinuria와 같은 상염색체열성의 유전질환에 효과적으로 이용할 수 있다고 알려진 adeno-associated viral vector를 이용하여 본 연구를 수행하였다.

Adeno-associated virus type 2를 기본바이러스로 하여 transgene (CBS cDNA)을 삽입하여 construct를 제작하였다. CBS유전자는 효소의 활성을 억제하는 기능을 가지고 있는 regulatory domain을 가지고 있다고 알려져 있어 CBS유전자의 regulatory domain을 돌연변이 시킨 것과 제거시킨 것도 cloning하여 정상 CBS 유전자와 그 효율을 비교하였다. *In vitro* system에서 해당 유전자가 세포내로 효율적으로 전달되고 있음을 확인하였고 construct간의 효소활성을 비교한 결과 정상의 유전자에서 효율이 가장 효과적이었다.

Homocystinuria의 동물질환모델을 이용하여 치료효과를 알아본 결과

치료군에서 질환모델의 수명의 연장과 plasma 내의 homocysteine의 농도저하 그리고 간의 cytoplasm내의 microvesicular lipid droplets이 감소되고 전달된 유전자가 발현되고 있음을 확인하였다. 그러나 치료효과가 장기간 지속되지 못하는 단점을 나타내었다. 이러한 단점을 극복하기 위해서는 유용한 동물질환모델의 개발과 질병의 원인 등에 대한 다양한 연구가 먼저 선행되어야 할 것이다.

이상의 본 연구의 결과를 통해 AAV를 매개로한 유전자치료가 homocystinuria 질병치료에 효과적인 치료방법의 하나로써 그 가능성을 제시하고 있으며 앞으로 인간의 질환 환자의 치료에 이용될 수 있을 것으로 기대된다.

핵심되는 말 : Homocystinuria, CBS유전자, viral vector, recombinant adeno-associated virus, 유전자 치료