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Recombinant adeno-associated virus mediated gene transfer in a mouse model for homocystinuria

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Abbreviations: AAV, adeno-associated virus; CBS, cysthathionine β-synthase; rAAV-hCBS, recombinant adeno-associated virus vector carrying human cysthathionine β-synthase cDNA

Abstract

Homocystinuria is a metabolic disorder caused by a deficiency of cystathionine β-synthase (CBS). The major clinical symptoms of this disease are mental retardation, lens dislocation, vascular disease with life-threatening thromboembolisms, and skeletal deformities. The major treatments for CBS deficiency include pharmacologic doses of pyridoxine or dietary restriction of methionine. There is currently no effective long-term treatment to lower the elevated plasma levels of homocysteine. However, gene therapy could be an effective novel approach for the treatment of homocystinuria. A recombinant adeno-associated virus vector carrying human CBS cDNA (rAAV-hCBS) was constructed and administered to CBS$^{-/-}$ mice by intramuscular (IM) and intraperitoneal (IP) injections. Serum homocysteine concentrations significantly decreased in treated mice compared with age-matched controls two weeks after treatment. The treated CBS$^{-/-}$ mice had life spans 3-7 days longer compared with untreated CBS$^{-/-}$ mice. In CBS$^{-/-}$ mice treated with rAAV-hCBS *via* IP injection, the vector was detected in all organs examined including liver, spleen, and kidney, and CBS gene expression was observed by immunohistochemical staining in the liver. These results indicate the efficacy of gene delivery and demonstrate the possibility of gene therapy mediated by AAV gene transfer in this mouse model of homocystinuria.

Keywords: cystathionine β-synthase; dependovirus; gene therapy; homocysteine; homocystinuria; mouse

Introduction

Homocystinuria (MIN 236200) is a metabolic disorder inherited by an autosomal recessive pattern caused by a deficiency of cystathionine β-synthase (CBS; EC 4.2.1.22). Major clinical symptoms include mental retardation, lens dislocation, vascular disease with life-threatening thromboembolisms, and skeletal deformities (Mudd *et al*., 1985; 2001). CBS is a pyridoxal-5-phosphate-dependent enzyme, which converts homocysteine to cystathionine in the transsulfuration pathway. The enzyme is comprised of 551 amino acids and forms a homotetramer of 63 kDa subunits. The structure of the enzyme consists of a catalytic domain of 409 amino acids located in the N-terminal, and a regulatory domain of 142 amino acids located in the C-terminal (Kery *et al*., 1998; Shan and Kruger, 1998). 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 identified in homocystinuria when expressed in yeast (Shan and Kruger, 1998; Shan *et al*., 2001).

A lack of CBS activity causes the accumulation of homocysteine, the export of excess homocysteine from the cell, and results in hyperhomocysteinemia, which may be toxic to cells. Moreover, it perturbs the methylation cycle, such as the intracellular accumulation of S-adenosyl homocysteine, which has consequences for cell metabolism (Boers *et al*., 1985). Elevated homocysteine levels are a potential risk factor for cardiovascular disease (Guba *et al*., 1996; Robert *et al*., 2003b), and several studies have

shown that homocysteine induces endothelial dysfunction and injury (Weiss *et al*., 2002; Denis *et al*., 2003).

The major treatments for CBS deficiency include administration of pyridoxine, a diet low in methionine, a diet high in cysteine, and dietary supplementation with betaine. Homocysteine-reducing therapies delay the development of clinical symptoms and markedly reduce the risk of vascular events (Wilcken *et al*., 1997; Schwahn *et al*., 2004), suggesting that homocysteine is involved in the pathogenesis of thromboembolism. However, approximately 50% of patients are biochemically responsive to pharmacological doses of pyridoxine and the treatment must be continued throughout the individual's life (Mudd *et al*., 1985). A multi-center study that conducted long-term treatment to lower the markedly elevated plasma levels of homocysteine in patients showed unsatisfactory results (Yap *et al*., 2001). Most treated patients (even pyridoxine-responsive cases) have total homocysteine levels well above normal. These levels were several times higher in patients with CBS deficiency compared with the means for the respective normal population of each center (Yap *et al*., 2001). Therefore, effective and long-term treatments to reduce homocysteine levels in severe types of homocystinuria are needed, and gene therapy, which has not yet been studied, could provide a novel approach to treating the disease.

In this study, a recombinant adeno-associated virus (rAAV) vector was used as the gene delivery vehicle. rAAV is a promising vector for gene therapy because of its stability in the long-term expression of transgene products that have relatively weak immune responses (Snyder *et al*., 1997; Halbert *et al*., 2000). Numerous *in vivo* studies have demonstrated that recombinant AAV-2 vectors can efficiently transduce many tissues and lead to stable gene expression (Bueler *et al*., 1999; Snyder, 1999). AAV vectors have been widely used in gene therapy studies in inherited diseases such as hemophilia B (Kay *et al*., 2000; Manno *et al*., 2003), cystic fibrosis (CF) (Flotte *et al*., 2003), and Fabry disease (Takahashi *et al*., 2002) with promising results.

In this study, the efficacy of gene therapy using rAAV vectors in a murine model for homocystinuria, the homozygotes ($CBS^{-/-}$) and heterozygotes ($CBS^{+/-}$), has been studied.

Materials and Methods

Cloning of human CBS cDNA

To synthesize human CBS (hCBS) cDNA, liver RNA was isolated from a human hepatoma cell line (HepG2) and RT–PCR was carried out as per a general protocol. Full length (hCBSfull) cDNA of the CBS gene and mutated cDNA of the CBS gene, which included a point mutation and deletion of the regulatory domain of the CBS gene (hCBSdel), were amplified and cloned into a pGEM-T Easy Vector (Promega, Madison, WI). Verification of the identity of the hCBS cDNA was performed by DNA sequencing. The nucleotide sequences were referred to the NCBI GenBank (accession no. NM_000071).

Production of rAAV-hCBS vectors

The parent plasmid pAAV-EF-pL-WPRE-BGHpoly(A), based on AAV type 2, was created as previously described (Davidson *et al*., 2000) with minor modifications. Large-scale production of the rAAV-EF-CBS vector was carried out in an adenovirus-free system by triple transfection with the vector plasmid and helper plasmids (Davidson *et al*., 2000). The rAAV-hCBS vector was prepared and isolated by CsCl density gradient ultracentrifugation. The titer of rAAV-hCBS genomic DNA was determined by real-time quantitative PCR using the ABI7700 (Perkin-Elmer/Applied Biosystems, Foster City, CA), in which the signal from aliquots of a test material was compared with a standard signal generated using the linearized pAAV-EF-hCBS-WPRE-BGHpoly(A) plasmid.

Cell lines

A human embryonic kidney cell line, 293T, a human hepatoma cell line, HepG2, and the NIH3T3 cell line, were propagated in Dulbecco's Modified Eagle's Medium supplemented with heat-inactivated 10% fetal bovine serum and antibiotics.

Animals

All animal experiments were carried out in accordance with our institutional guidelines. C57BL/6J-Cbs^{tm1Unc} mice were obtained from the Jackson Laboratory (Maine). The heterozygous CBS-deficient mice ($CBS^{+/-}$) were bred to obtain homozygous CBS-deficient mice ($CBS^{-/-}$). Most homozygous $CBS^{-/-}$ mice die within two weeks after birth when fed a standard laboratory diet. In order to select homozygous mice, genotypes for the targeted CBS allele were identified four days after birth with genomic DNA obtained from tail biopsies as described (Watanabe *et al*., 1995). Five- or six-day-old $CBS^{-/-}$ mice were used for *in vivo* gene transfer. Culture medium (50-100 μl) containing 2×10^{12} rAAV-hCBSfull or rAAV-hCBSdel was slowly delivered to the mice using an insulin syringe with a 29-gauge needle by intramuscular (IM) or intraperitoneal (IP) injection. Animals were killed and examined two weeks after rAAV-CBS administration.

Ten-week-old $CBS^{+/-}$ mice were anesthetized with ketamin/xylazine and injected with 1×10^{12} viral particles of rAAV-hCBSfull or rAAV-hCBSdel into the hepatic portal vein. Animals were killed and analyzed two, six, and 12 weeks after rAAV-CBS administration. The liver, kidney, heart, intestine, peritoneum and lungs were isolated from each rAAV-injected $CBS^{-/-}$ and $CBS^{+/-}$ mouse, along with age-matched wild-type controls and untreated $CBS^{-/-}$ and $CBS^{+/-}$ mice, for further analysis.

Plasma homocysteine assay

Blood samples were collected in tubes containing 0.5 M EDTA and plasma homocysteine levels were determined by HPLC (HPLC/fluorescence detector, Bio-Rad 2800 series) with fluorometric detection according to a previously described protocol (Araki and Sako, 1987). The method involved deproteinization of a small volume of plasma (50 μl) followed by HPLC analysis and homocysteine levels were determined using a HPLC reagent kit (Bio-Rad, CA).

CBS enzyme assay

The CBS enzyme assay was performed according to previously described methods (Kraus, 1987). The enzyme activity was assayed by measuring the production of [^{14}C]-cystathionine from [^{14}C]serine. The reaction was initiated by addition of L-homocysteine (final concentration, 10 mM) and incubated for 1 h at 37°C in the presence or absence of 1 mM S-adenosylmethionine (SAM). The assay was terminated by cooling the sample to 0°C in an ice bath. Samples were applied to a thin layer chromatography (TLC) system (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. Quantification of radioactivity of [^{14}C]-serine and converted [^{14}C]-cystathionine was done using the ImageQuant, after visualization with a PhosphoImager (Molecular Dynamics, Sunnyvale).

Western blot analysis of hCBS

Protein extracts were isolated from transduced NIH3T3 cells and HepG2 cells. Protein extracts (30 μg per lane) were separated by SDS/PAGE using 10% polyacrylamide gels and transferred onto nitrocellulose membrane (Life Technologies, Gaithersburg, MD). The nitrocellulose 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 an ECL-Plus enhanced chemiluminescence detection system (Santa Cruz Biotechnology, Santa Cruz, CA).

Tissue distribution of recombinant viral vectors

In order to assay the distribution of recombinant viral vectors in different organs, RT–PCR was performed. Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH). The RT-PCR reaction was carried out with 100 pmol/μl of random primer (Takara, Japan). For detection of specific human cystathionine β-synthase sequences, the following primers were used at a concentration of 25 pmol: forward primer, 5'-CAAGTGTGAGTTCTTCAACGCG-3'; reverse primer, 5'-GGGATGAAGTCGTAGCCGATC-3'. These primers amplified a 615 bp product. Primers to mouse GAPDH were used as an internal control and amplified a 530 bp product (forward primer, 5'-CCCACACTGTGCCCATCTAC-3'; reverse primer, 5'-AGTACTTGCGCTCAGGAGGA-3'). After PCR amplification, products were analyzed on 1% agarose gels and visualized with ethidium bromide.

Histological examination

Livers were removed from mice administered rAAV-hCBS and age-matched control mice. The liver tissues were fixed in 4% formaldehyde, embedded in paraffin, and sectioned. Paraffin sections were stained with hematoxylin and eosin, and examined by a specialized pathologist.

Immunohistochemistry

Sections of the liver were incubated with anti-CBS antibody (1:2,000 dilution) at 4°C for overnight. Anti-rabbit IgG was used as secondary antibody in which sections were incubated for 1 h at room temperature. The immunoreactivity was visualized by treating the slides with DAB plus substrate chromogen reagent for 20 sec at room temperature. Tissues were counterstained with hematoxylin, dehydrated through a graded series of alcohol, cleared in xylene, and coverslipped.

Statistical analysis

The statistical significance of differences between groups was determined using Student's *t* test. The data are presented as mean ± SD. A *P* value of less than 0.05 was considered significant.

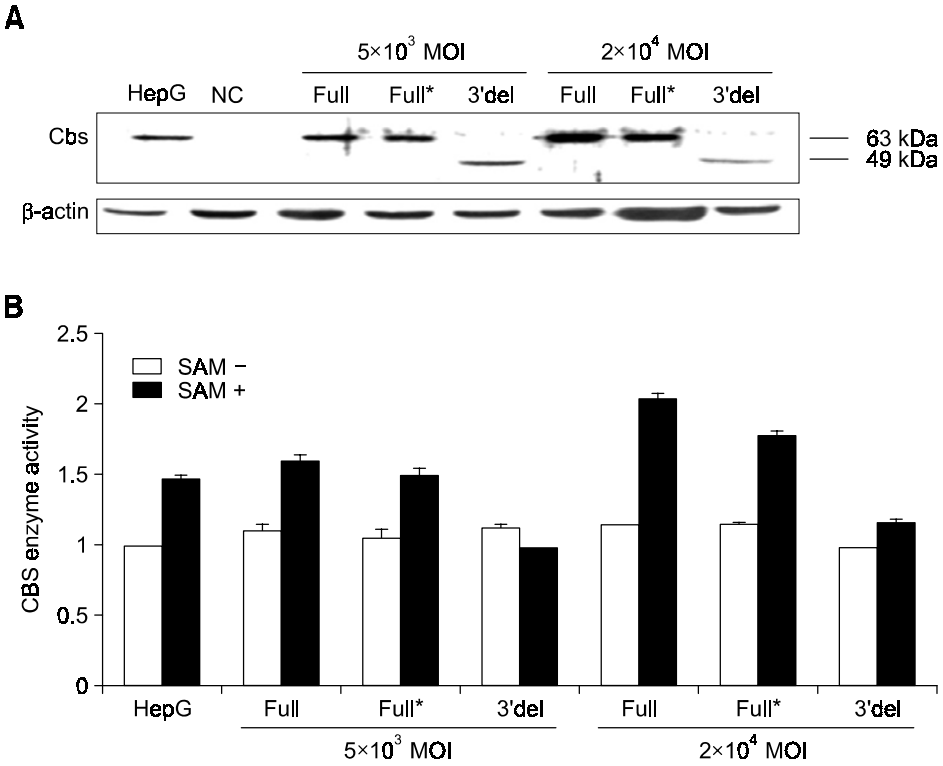

Figure 1. Western blot and enzyme assay of NIH3T3 cells transduced with rAAV-hCBS. Analysis was performed two days after transduction with rAAV-hCBS at MOI of 5,000 and 20,000. (A) Cell extracts were separated by 10% SDS-PAGE, blotted onto a nitrocellulose membrane and probed with human CBS antibody. Goat anti-rabbit IgG was used as the secondary antibody. Bands of 63 kDa and 49 kDa were detected in immunoblots of cells transduced with rAAV-hCBS (lanes 3-8). These bands were not visible in untransduced cells (lane 2). The 63-kDa-band is present in the positive control HepG2 cells (lane 1). (B) Each cell extract was assayed for CBS activity with and without 100 μM S-adenosylmethionine (SAM) as described. CBS enzyme activity was examined by thin layer chromatography. Full: wild-type CBS; Full*: Q451G mutation in C-terminal region; 3'del: deletion of C-terminal region (420-551). Values are presented as the mean ± SD.

Results

Construction of transgene

The transgene, hCBS cDNA, was driven by a human elongation factor 1-α promoter endowed with further stability by the woodchuck hepatitis virus posttranscriptional regulatory element, WPRE, and the polyadenylation site was provided by the BGH polyA . The full-length hCBS cDNA encodes for 551 amino acids. The mutant hCBS cDNAs constructed in the C-terminal region of the gene included a deletion in the C-terminal of 420-551 (419 amino acids plus the stop codon) and a point mutation (Q451G).

Expression of rAAV-hCBS after *in vitro* transduction

Since the CBS gene is not expressed in NIH3T3 cells, these cells were used as a negative control cell line for the *in vitro* assay. NIH3T3 cells were infected at an MOI of 5,000 and 20,000 at a cell density of 60%. Two days after infection, protein was isolated from rAAV-hCBS transduced and untransduced cells for analysis of enzyme expression and activity (Figure 1). CBS expression was observed in rAAV-hCBS transduced cells, and no CBS expression was observed in untransduced controls (Figure 1A). A band of 63 kDa was observed in HepG2 positive control cells and transduced cells using a monospecific anti-CBS antibody. The enzyme activity assay measuring the conversion rate of [^{14}C]-serine to [^{14}C]-cystathionine also showed results consistent with the western blot analysis (Figure 1). These *in vitro* studies confirmed that the rAAV-EF1α-hCBS vector was capable of delivering a functional gene to the cells. Enzyme activity was compared between full-length and C-terminal mutant forms of the CBS gene in the presence and absence of S-adenosyl-L-methionine (AdoMet). The wild-type CBS gene showed the same enzyme activity as the mutant forms of Q451G in the absence of AdoMet.

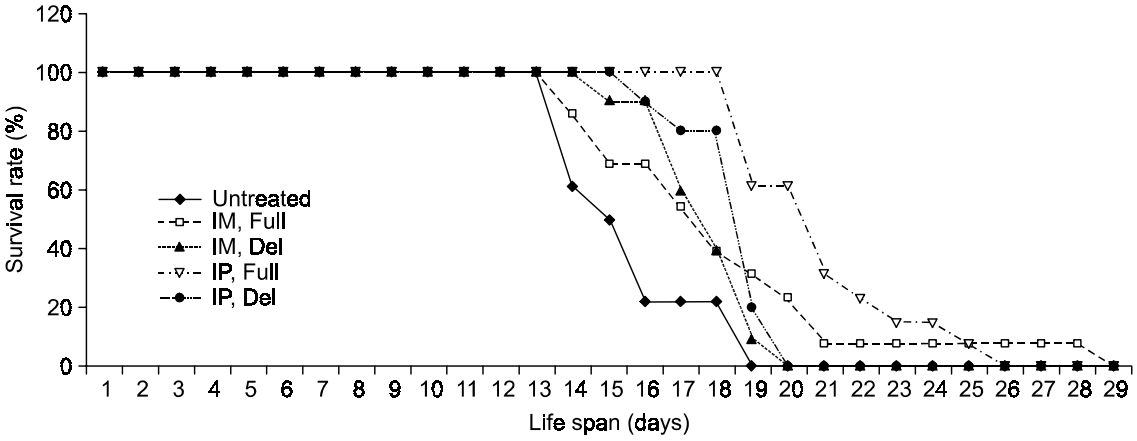

Figure 2. Survival rates of CBS$^{-/-}$ mice administered rAAV-hCBSfull *via* different routes. IM, intra muscular injection; IP, intraperitoneal injection.

In contrast, the 3'-deleted protein showed the same or slightly lowered enzyme activity than the wild-type CBS protein. In HepG2 cell line, the enzyme activity was increased for 1.5 times in the presence of Ado-Met. It was similar in full-length and C-terminal mutant forms of the CBS gene. The wild-type CBS gene showed increased enzyme activity of 1.5 to 2 times in the presence of AdoMet. The C-terminal deletion form of the CBS gene resulted in the absence of stimulatory effect of AdoMet (Figure 1B).

Elongation of life spans

Homozygous mutants suffered from severe growth retardation, such as delayed eye opening and the majority of them died within five weeks after birth with low body weight (Watanabe *et al*., 1995). The average life span of untreated CBS$^{-/-}$ mice was about 15.6 $\pm$ 1.78 days. After CBS$^{-/-}$ mice were injected with rAAV-hCBSfull, the life span lengthened by approximately 3-7 days. Intraperitoneal injection of rAAV-hCBSfull was a more effective method of gene delivery compared with IM injection, and mice given IP injections showed an elongated life span of 21.4 $\pm$ 2.94 days (Figure 2).

Homocysteine levels in plasma

Plasma homocysteine levels of homozygotes were approximately 40 times higher than those of age-matched control littermates at 2 weeks after birth. The homocysteine levels of heterozygotes were about two times higher compared with wild-type mice (Figure 3). Plasma homocysteine levels were measured two weeks after delivery of rAAV-hCBS. The basal plasma homocysteine level of untreated CBS$^{-/-}$ mice was 401.66 $\pm$ 38.67 μM. At two weeks after injection, plasma homocysteine levels in mice

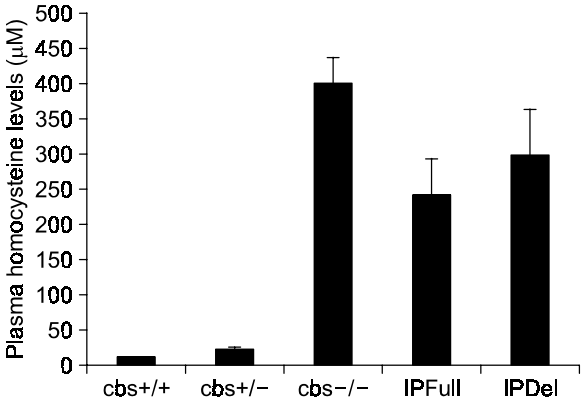

Figure 3. Plasma homocysteine concentrations in CBS$^{-/-}$ mice after the administration of rAAV-hCBS (2×10^{12} viral particles) *via* IP injection. Animals were killed after two weeks. Values are presented as means $\pm$ SD (n = 3).

infused with viral vectors rAAV-hCBSfull and rAAV-hCBSdel (2×10^{12} viral particles) decreased to 241.83 $\pm$ 54.58 μM and 301.6 $\pm$ 63.97 μM, respectively (Figure 3). The homocysteine concentration in the treated CBS$^{-/-}$ mice decreased to 40% of the levels observed in untreated CBS$^{-/-}$ mice.

Distribution of recombinant virus in various tissues

RNA was extracted from various organs two weeks after an IP injection with viral vectors rAAV-hCBSfull and rAAV-hCBSdel and analyzed for tissue distribution of the viral transgene vector. The hCBS-specific band was identified in treated mice. Recombinant viral vectors were distributed in major organs such as heart, lungs, liver, intestine, peritoneum, and kidneys (Figure 4).

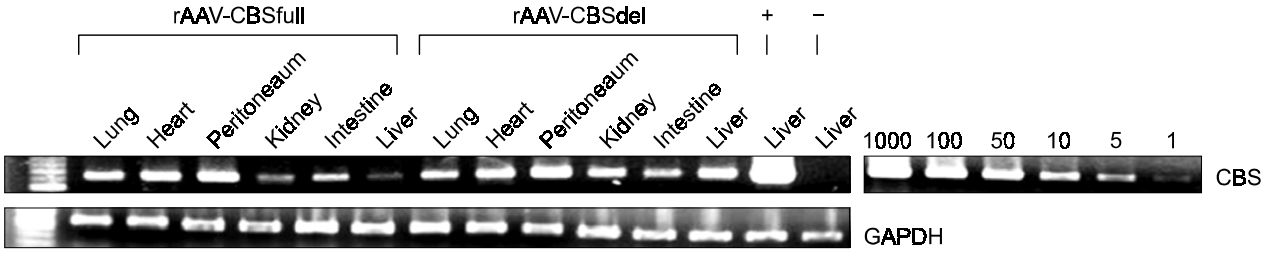

Figure 4. Analysis of tissue distribution of rAAV-hCBSfull and rAAV- hCBSdel after IP injection in $CBS^{-/-}$ mice. RT–PCR was performed to analyze the distribution of the rAAV vector in different tissues. The vector-specific fragment was amplified from all tissues examined in mice injected with rAAV-hCBS, whereas no signal was obtained in untreated mice. The mouse GAPDH gene was used as an internal control. +, wild-type mouse; -, untreated $CBS^{-/-}$ mouse.

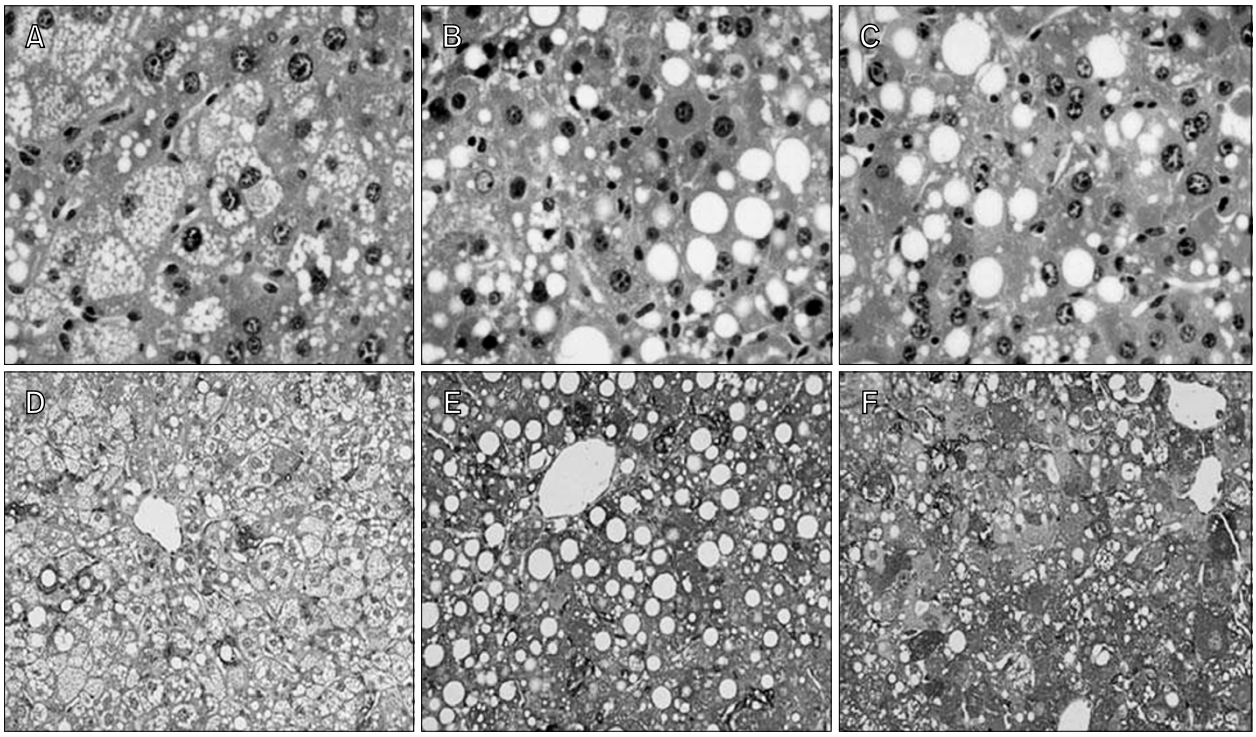

Figure 5. Histological examination (A-C) and immunohistochemical staining (D-F) of the liver after administration of rAAV-hCBS from untreated $CBS^{-/-}$ mice (A, D), and $CBS^{-/-}$ mice injected with rAAV-hCBSfull (B, E) and rAAV-hCBSdel (C, F). An increased amount of CBS protein was observed by immunohistochemical staining in mice injected with rAAV-hCBS (n = 3).

Histological and immunohistochemical study

Most homozygotes showed severe growth retardation and their eyes were not opened entirely. The livers from mutant homozygotes had the paler surface color in contrast to the reddish-brown color observed in those of heterozygotes and wild-type mice. Fat droplets were prominent in the liver of $CBS^{-/-}$ mice and the cytoplasm was filled with microvesicular lipid droplets (Figure 5A). In rAAV-injected mice, the gross color of the liver was somewhat changed to reddish-brown and microvesicular fat droplets reduced on histological examination (Figure 5B and C). However, many macrovesicular fat changes were observed in the treated mice. Immunohistochemical staining with anti-hCBS antibody showed that CBS protein was present in the cytoplasm of hepatocytes in mice treated with rAAV-hCBS. Mice administered rAAV-hCBSfull showed more enzyme protein compared with mice administered rAAV-hCBSdel (Figure 5). This result was consistent with serum homocysteine concentration.

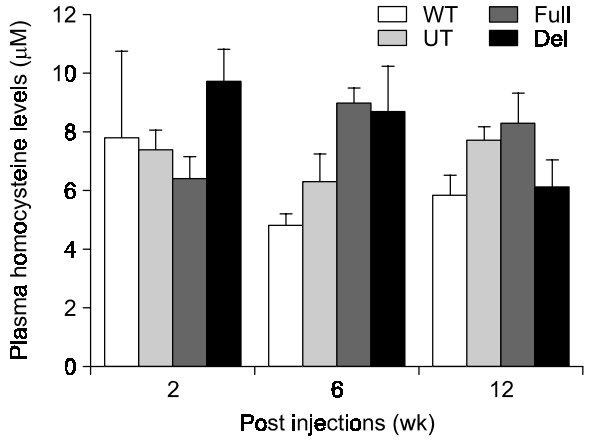

Figure 6. Plasma homocysteine levels in $CBS^{+/-}$ mice administered rAAV-hCBS *via* the portal vein. $CBS^{+/-}$ mice were administered with 1×10^{12} viral particles and killed at two, six and 12 weeks after injection. WT, wild-type mice ($CBS^{+/+}$); UT, untreated heterozygous mice ($CBS^{+/-}$); Full, heterozygous mice injected with rAAV-hCBSfull; Del, heterozygous mice injected with rAAV-hCBSdel. Values are presented as the mean ± SD (n = 3).

Administration of rAAV-CBS into $CBS^{+/-}$ mice

Since homozygous CBS-deficient mice exhibit growth retardation, hepatic dysfunction, and shortened life spans, they may have limitations as a model for studying gene therapy. The heterozygous mice grew normally and were relatively healthy, even though they had an approximate 50% reduction in CBS mRNA and CBS enzyme activity in the liver and twice the normal levels of plasma homocysteine (Watanabe *et al*., 1995). In this study, the efficacy of gene transfer with rAAV-CBS was tested in $CBS^{+/-}$ mice. Homocysteine concentrations in plasma were measured at two, six and 12 weeks after injection. The $CBS^{+/-}$ mice showed plasma homocysteine levels two times higher compared with $CBS^{+/+}$ mice (11.3 ± 0.95 μM vs. 22.6 ± 1.8 μM) two weeks after birth. Plasma homocysteine levels decreased with age in both wild-type and heterozygous mice, and homocysteine concentrations between wild-type and heterozygous mice were not significantly different at 12, 16 and 22 weeks (7.8 μM, 4.8 μM, 5.8 μM vs. 7.4 μM, 6.3 μM, 7.7 μM, respectively). Plasma homocysteine levels in heterozygous mice injected with rAAV-hCBSfull and rAAV-hCBSdel at 12, 16 and 22 weeks were 6.4 μM, 9 μM, 8.3 μM vs. 9.7 μM, 8.7 μM, 6.1 μM, respectively. Plasma homocysteine levels in heterozygous mice did not decrease after gene transfer (Figure 6). There was also no significant increase in enzyme activity in mice injected with rAAV-hCBSfull and rAAV-hCBSdel (data not shown).

Discussion

There are currently three recognized modalities for the treatment of homocystinuria caused by CBS deficiency. In the pyridoxine responsive group, only pyridoxine administration in combination with folic acid and/or vitamin B12 (Wilcken *et al*., 1997) is an effective treatment. The administration of pyridoxine putatively stimulates residual CBS activity. For the pyridoxine non-responsive group, a methionine-restricted, cysteine-supplemented dietary therapy (Robert *et al*., 2003a) should be employed even though its effectiveness is restricted. Pyridoxine, folic acid and vitamin B12 have continued to be administered in pyridoxine non-responders because they are cofactors of methionine metabolism. Additionally, betaine, a methyl donor that remethylates homocysteine to methionine, has been used as an adjunct to treatment (Wilcken *et al*., 1985). Early diagnosis and proper treatment is very important, especially during the newborn period or even later in infancy, to prevent or greatly reduce the severity of complications. However, lifelong dietary control is difficult in practice and novel approaches that are effective at reducing homocysteine levels in the long-term are needed. In this study, rAAV has been studied as a CBS gene-transfer vector to reduce excess homocysteine in a mouse model of homocystinuria.

Human CBS is predominantly expressed in the liver and the liver is the target organ for the treatment of other metabolic diseases. To deliver therapeutic genes, viral vectors are considered the most effective tools for efficient gene transfer to the liver *in vivo* and rAAV vectors represent a very promising tool for this process (Buning *et al*., 2003). Many studies have shown that AAV vectors can transduce foreign genes for long-term expression in animal models (Jung *et al*., 2001; Xu *et al*., 2001; Oh *et al*., 2004).

In the present study, improvements in CBS enzyme expression and prolongation of life span after AAV-mediated transduction into $CBS^{-/-}$ mice has been demonstrated. However, the long-term effect of gene transfer could not be observed because of the following reasons. Firstly, an IP injection of rAAV vectors cannot deliver the normal CBS gene into the liver at sufficient levels to overcome the clinical features of homocystinuria. The viral vector had to be injected intraperitoneally because the homozygous mice were too small to inject the vector *via* the hepatic portal vein. Secondly, the mouse model used in this study was not suitable for gene therapy experiments because homozygous null alleles of the CBS gene in mice are lethal in contrast to human. $CBS^{-/-}$ mice showed severe growth retardation and

most of them died within two weeks after birth. Plasma homocysteine levels in $CBS^{-/-}$ mice were higher in this study compared with previous studies, 401.66 $\pm$ 38.67 μM vs. 205 $\pm$ 86 μM, respectively (Robert *et al*., 2003a). This difference in homocysteine levels may be caused by a standard laboratory diet given in this study, instead of a special diet, in order to avoid the effect of diet therapy. $CBS^{-/-}$ mice are usually fed a standard A04 rodent chow, which is rich in choline chloride that is necessary for their survival (Robert *et al*., 2003a). Betaine-homocysteine methyltransferase in liver and kidney cells can transfer a methyl group to methionine from betaine, the oxidized form of choline, providing a secondary pathway for homocysteine remethylation (Finkelstein, 2000).

For a long-term therapeutic effect, new mouse models for homocystinuria induced by various types of mutation in the CBS gene will be preferentially needed to conduct gene therapy experiments on this disease. In humans, most pathogenic mutations are missense mutations within the CBS gene (Kraus *et al*., 1999). Further experiments remain to be evaluated; including potentials of alternative AAV serotypes, not AAV2 and the efficacy of rAAV delivered prenatally. Finally, the development of new therapeutics strategies will be necessary to correct homocystinuria patients.

The C-terminal of the human CBS gene is known to be a regulatory domain, consisting of approximately 140 amino acid residues. The allosteric activator, AdoMet, increases CBS activity by about three times and probably binds to the C-terminal regulatory domain (Shan *et al*., 2001). The C-terminal regulatory domain also encompasses the previously defined CBS domain, which is conserved in a wide range of otherwise unrelated proteins (Bateman, 1997). Mutations in this domain can constitutively activate the enzyme, indicating that it plays a role in the autoinhibitory function of the C-terminal region (Janosik *et al*., 2001; Shan *et al*., 2001). Additionally, the mutant 414-551, lacking proposed CBS domains, is about five-fold more active than the wild-type enzyme in the absence of AdoMet (Oliveriusova *et al*., 2002). In this study, the enzyme activity showed no significant difference between the wild-type and 3' deleted forms of the CBS gene *in vitro*. In contrast, the rAAV-hCBSfull injection was more efficient at generating biochemical and histological improvements in $CBS^{-/-}$ mice *in vivo* compared with the rAAV-hCBSdel injection.

In a previous study, CBS-deficient mice ($CBS^{+/-}$) showed approximately 50% reduction of CBS mRNA and enzyme activity in the liver, and plasma homocysteine levels were about two times higher, compared with wild-type mice (Watanabe *et al*., 1995). Plasma concentrations of homocysteine decreased by 22 weeks of age in $CBS^{+/-}$ mice and wild-type mice; however, the difference in plasma homocysteine levels between wild-type mice and $CBS^{+/-}$ mice was observed by 44 weeks of age (Watanabe *et al*., 1995). Compared with this previous study, in the present study, $CBS^{+/-}$ mice had plasma homocysteine concentrations two times higher than wild-type mice two weeks after birth and no difference in homocysteine concentration between wild-type and heterozygous mice was observed at 12, 16 or 22 weeks (7.8 μM, 4.8 μM, 5.8 μM vs. 7.4 μM, 6.3 μM, 7.7 μM, respectively). The plasma homocysteine level in heterozygotes did not change after gene transfer. These results suggest that the $CBS^{+/-}$ mouse is not suitable for studying the efficiency of gene therapy for homocystinuria.

In conclusion, our data show that rAAV-mediated gene therapy is a potential treatment for homocystinuria because CBS gene transfer in $CBS^{-/-}$ mice prolonged their life span, decreased homocysteine levels in plasma, and the transferred CBS gene was expressed in the liver. These findings suggest that AAV-mediated gene transfer may be a useful therapeutic candidate for the treatment of homocystinuria. Additionally, a more suitable mouse model is needed for further investigation of therapeutic strategies for patients with homocystinuria.

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