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AN EXPERIMENTAL STUDY ON THE INFLUENCE OF GENE THERAPY WITH ADENO ASSOCIATED VIRUS ON OXALATE METABOLISM OF PRIMARY HYPEROXALURIA TYPE 2 AND TYPE 3 MICE MODELS

REVIEW ARTICLE

Usha Adiga

Professor, Nitte (Deemed to be University), KS Hegde Medical Academy(KSHEMA), Department of Biochemistry,Mangalore, Karnataka, India575018

ushachidu@yahoo.com

Abstract

The primary hyperoxalurias (PHs)(PH1,2 and 3 types) are rare genetic disorders due to specific hepatic enzyme deficiencies resulting in overproduction of a waste product oxalate. This results in the excretion of oxalate in urine in large quantity leading to renal stones and gradually kidney damage. End stage renal failure frequently occurs and is followed by systemic oxalate deposition along with its devastating effects. Due to the lack of familiarity with PHs and their heterogeneous clinical expressions, the diagnosis is often delayed until there is advanced disease. It is important to look for new treatment modalities as the disease burden can be severe.Aim of the study is to find out whether gene therapy using recombinant adeno associated virus(AAV) as vector,can reduce oxalate levels and excretion in mice models.This may be helpful in reducing kidney damage. If this fact is proved by the study,the gene therapy can be used as a therapeutic option in primary hyperoxalurias (PHs). Therapeutic role of recombinant adeno associated virus as vector in PH1 has been experimentally proved.However there is no experimental data available on the role of this bacteria on PH2 and PH3 animal models. So the study intends to explore whether gene therapy using recombinant adeno associated virus as vector would be effective in reducing oxalate excretion.This experimental study may prove the beneficial role of PH2 and PH3 mice models and hence may throw light on using gene therapy as a therapeutic option in PH2 and PH3. If proved,new therapeutic approach may improve the quality of life of individuals with PH2 or PH3.

Keywords: *primary hyperoxaluria, gene therapy, mice models*

Introduction

Systemic levels of oxalate are derived from two sources, endogenously through metabolic production by the liver and by absorption across the gastrointestinal tract from dietary sources. Since mammals lack the enzymes necessary for its utilization, oxalate is a waste product that must be excreted, occurring primarily in the urine [1]. An imbalance in oxalate production/absorption and its excretion can lead to hyperoxaluria, a major risk factor for calcium oxalate kidney stone formation which affects approximately 1 in 11 of the population in the US [2,3].

Hyperoxaluria may be either inherited or acquired. The primary hyperoxalurias (PH) are inborn error of metabolism resulting in increased endogenous production of oxalate leading to excessive urinary oxalate excretion. To date, three distinct hereditary enzymatic deficiencies have been linked to PH, namely, PH type 1 (PH1), type 2 (PH2), and type 3 (PH3), due to inborn defeciencies of alanine : glyoxylate amino transferase (AGT), glyoxylate reductase / hydroxy pyruvate reductase (GRHPR) and 4-hydroxy-2-oxoglutarate aldolase (HOGA1) respectively. Due to marked hyperoxaluria, recurrent urolithiasis and progressive nephrocalcinosis are the principal manifestations of PH. As a result of kidney injury, glomerular filtration rate (GFR) declines leading to chronic kidney disease, and ultimately to end-stage renal disease (ESRD) and systemic involvement in PH1, the most severe type of PH.

Currently, the most effective treatment for primary hyperoxaluria is pre-emptive liver transplantation, or combined liver and kidney transplantation. However, this treatment has its own limitations including the scarce supply of suitable organs, significant morbidity and mortality, and the life-long requirement for immunosuppressive agents. Thus, new treatments for PH are required, and as such, somatic gene therapy may be an alternative option.

Somatic gene therapy is a promising approach, provided that sufficient hepatocytes can be efficiently transduced to limit oxalate production by the liver to amounts that can be excreted into the urine without kidney damage.

Recombinant adeno-associated virus (AAV) has emerged as one of the most promising gene transfer vectors for treatment of human diseases based on its ability to transduce both dividing and nondividing cells and to mediate long-term transgene expression without toxicity[4]. Preclinical studies using AAV in animal models for different diseases have demonstrated long-term, stable transgene expression in liver, muscle, and central nervous system [2-7]. In addition, several early phase clinical trials with AAV vectors have shown to be quite safe[8-11]. The host immune response has resulted in limited efficacy in some studies [12] , while sustained expression has also been reported even with a T lymphocyte response[16].

However there are no studies available to the best of our knowledge ,which focus to evaluate the effectiveness of gene therapy with AAV in PH2 and PH3 and thereby improving the conditions. Hence the present study is designed to assess the oxalate metabolizing capacity of the bacteria in PH2 and PH3 mice models with WT mice as controls.

Specific Objectives

Objectives of the study are to

- assess the effect of gene therapy with recombinant AAV in GRHPR knockout mice model(PH2 model) and HOGA1 knockout mice model(PH3 model)
- compare urinary oxalate excretion in PH2 and PH3 mice models before and gene therapy with recombinant AAV.

Preliminary data : Not available

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Methods

Study setting: The collaborative study will be carried out in animal house attached to the Department of Pharmacology and Molecular division of central research laboratory, KS Hegde Medical Academy, Mangalore

Study type: Experimental

Animal experimentation will be carried out after obtaining approval by the institutional animal ethics committee.

Animals will be divided in three groups.Six male/female mice (25–30 g) will be used in each group.

Group 1:GRPHR knockout mice

Group 2: HOGA1 knockout mice

Group 3: C57BL/6 [wild-type (WT)] mice as controls

GRPHR and HOGA1 knockout mice model will be obtained as described by Salido et al[17].The animals will be given free access to a standard mouse chow (diet 2018S, Harlan Teklad) and drinking water before the experimentation procedure.

The study will include the following steps;

1. Adult GRPHR KO mice and HOGA1 KO (12–16 weeks old) will be placed in metabolic cages, fed an oxalate-free diet and water *ad libitum*, and allowed to adjust for 3 days.

2. Twenty-four hour urine will be collected in acidified tubes and the basal rate of oxalate excretion will be determined.

3. AAV vectors will be constructed by inserting the human GRPHR cDNA/HOGA1 cDNA into an pro-AAV2 vector plasmid, under the control of a hybrid EalbAAT liver-specific promoter[18].

Recombinant AAV construction, production, and DNA analysis.

The AAV plasmids that contain the expression cassette flanked by two ITRs from the AAV2 and an appropriate stuffer sequence to adjust genome size to the optimal packaging capacity (4.1–4.9 kb) described for AAV will be used in this study . The expression cassette will have the following elements: the 5′ inverted terminal repeats from AAV2, a liver-specific EalbAAT promoter with regulatory sequences from the albumin enhancer[18].

Recombinant AAV8 vectors will be produced by calcium phosphate-mediated cotransfection in 293 cells of three different plasmids: pAdDeltaF6, p5E18-VD2/8 and the therapeutic (ssAAV-EalbAAT-AGXT-WPRE-polyA) or reporter gene (ssAAV -EalbAAT-GFP-WPRE-polyA) plasmid [19,20]. Similarly, AAV5 vectors will be produced using p5E18-VD2/5 . AAV will be harvested from transfected 293 cells by three cycles of freeze-thaw 48 hours later. The virus will be purified by ion exchange column chromatography and iodixanol gradient centrifugation followed by filtration and further concentration against PBS–5% sucrose. Virus titers (vg/ml) will be determined by quantitative-PCR performed in triplicate, using TaqMan (Applied Biosystems) protocols, and primers pr300fw (5′-CCCTGTTTGCTCCTCCGATAA-3′) and pr301rv (5′-GTCCGTATTTAAGCAGTGGATCCA-3′), which amplify a 95 bp fragment from the hAAT promoter region. AAV capsid composition and purity will be determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

4. AAV8 vectors will be administered at doses of 5×1012vector genomes per kg body weight (vg/kg), a dose considered sufficient to transduce the vast majority of hepatocytes, in male GRPHR KO mice and in HOGA1 KO (n = 10 animals per group), which will be followed for 50 days.

5. Purified, high titer (1.7–6.3×1011 vg/ml for AAV8 and 4.5–10×1011 vg/ml for AAV5) preparations

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of vectors in 0.2 ml phosphate buffered-saline (PBS)-sucrose will be administered intravenously via the tail vein.

6. The effect of gene transfer with AAV5 with respect to AAV8, and possible gender differences in vector-mediated expression will be compared by using different doses of AAV5 vectors at either 1.5×1013, 5×1012, or 5×1011vg/kg body weight, and AAV8 vectors at 5×1012 vg/kg, in both female and male GRPHR KO mice and HOGA1 KO mice (n = 6 animals per group)

7. 24-hour urine collections will be performed each week in narrow tubes containing 50 µl 6 N HCl. The oxalate oxidase assay was used to measure urine oxalate, while the Jaffe alkaline picrate test will be used to measure urine creatinine.

8. An additional group of five Grphr+/+ and HOGA1+/+ males, not injected with any vector, C57BL/6 will be used as wild-type controls.

9. Influence of gene therapy will be assessed by measuring urinary oxalate excretion in all the groups at 4 weeks and 8 weeks

10. The mice will be sacrificed, and blood will be collected for biochemical analysis. Serum samples will be used, diluted 1:100 in PBS, to check for the presence of anti-GRPHR and anti-HOGA1 mouse antibodies by immunoblotting of recombinant human GRPHR and HOGA1 proteins.

11. Liver samples will be harvested for histology, enzymes GRPHR and HOGA1 activity assays and western blot analyses. Kidney samples will be collected for histological analysis.

Histological analysis.

Tissues will be fixed in 4% buffered paraformaldehyde and either embedded in paraffin or cryoprotected in 20% sucrose and snap frozen in liquid nitrogen. Hematoxylin and eosin staining will be performed for in all tissues collected, and CaOx staining will be performed on kidney sections [21].

GRPHR and HOGA1 enzyme assay, western blot and immuno-histochemistry

GRPHR and HOGA1 enzyme activities and western blot analyses will be carried out as described [17]. Immunohistochemistry will be performed on frozen sections, incubating with 1:5,000 rabbit antihuman GRPHR and HOGA1 antibody for 2 hours, followed by 3, 5 minutes each, PBS washes and a 30-minute incubation in horseradish peroxidase–conjugated anti-rabbit serum . After another 3 PBS washes, a 3,3′-diaminobenzidine-H_2O_2 solution will be used as chromogen, and some sections will be counterstained with hematoxylin. Serial sections will be also stained with 1:5,000 anti-glutamine synthetase antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) to label perivenular zones.

Outcome measures:

- Evaluation of the effectiveness of gene therapy by measuring urinary excretion of oxalate after gene therapy
- Histological evaluation of liver and kidney after gene therapy as well as western blot and immunohistochemistry of the organs

Statistical analysis

The GraphPad Prism statistical software (version 5.03, GraphPad Software, Inc, La Jolla, CA) will be used for the analyses. Data will be presented as the mean ± standard error (SEM) or as percentages of difference between measured parameters. The data will be considered statistically significant at $p \leq 0.05$. Data normality will be verified by D'Agostino and Pearson omnibus normality test, and for data failing to meet the assumptions of approximate normality and equality of variance, the equivalent non-parametric tests will be performed.

Unpaired t test/Mann Whitney U test will be used to compare oxalate excretion in multiple experimental groups. Fischer's exact test will be used to find the association between the effect of gene therapy

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using recombinant AAV and altered oxalate excretion.The western blot and immunohistochemistry results will be analyzed.

Expected Outcome

The study may support the role of gene therapy of PH2 and PH3 mice models, with recombinant AAV as the vector. It may be effective in reducing oxalate excretion as well as the extent of kidney damage.It may be one of the most promising treatment option for primary hyperoxalurias. The new therapeutic approach may improve the quality of life of individuals with PH2 or PH3.

Mid term success indicators

- Lowered urinary oxalate levels at 4 was and 8 was of gene therapy
- Reduced serum creatinine levels
- Improved histological features of liver and kidney

Long term success indicators

- The experimental study has to be translated to the bedside research.The study findings need to be confirmed in humans by clinical trials.

Improved quality of life and life expectancy of patients with PH2 and PH3.

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