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Reversal of advanced disease in lysosomal acid lipase deficient mice: A model for lysosomal acid lipase deficiency disease

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ABSTRACT

Lysosomal acid lipase (LAL) is an essential enzyme that hydrolyzes triglycerides (TG) and cholesteryl esters (CE) in lysosomes. Mutations of the *LIPA* gene lead to Wolman disease (WD) and cholesterol ester storage disease (CESD). The disease hallmarks include hepatosplenomegaly and extensive storage of CE and/or TG. The effects of intravenous investigational enzyme therapy (ET) on survival and efficacy were evaluated in *Lipa* knock out, *lal*−/− mice with advanced disease using recombinant human LAL (rhLAL). Comparative ET was conducted with lower doses (weekly, 0.8 and 3.2 mg/kg) beginning at 16 weeks (study 1), and with higher dose (10 mg/kg) in early (8-weeks), middle (16-weeks) and late (24-weeks) disease stages (study 2). In study 1, rhLAL extended the life span of *lal*−/− mice in a dose dependent manner by 52 (0.8 mg/kg) or 94 (3.2 mg/kg) days. This was accompanied by partial correction of cholesterol and TG levels in spleen and liver. In study 2, the high dose resulted in a significant improvement in organ size (liver, spleen and small intestine) and tissue histology as well as significant decreases in cholesterol and TG in all three groups. In the treated livers and spleens the cholesterol and TG levels were reduced to below treatment initiation levels indicating a reversal of disease manifestations, even in advanced disease. ET diminished liver fibrosis and macrophage proliferation. These results show that LAL deficiency can be improved biochemically and histopathologically by various dosages of ET, even in advanced disease.

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1. Introduction

Lysosomal acid lipase (LAL) deficiencies are rare autosomal recessively inherited diseases, caused by the insufficient LAL activity, and the resultant accumulation of cholesteryl esters (CE) and triglycerides (TG) in the lysosomes of many tissues [1]. LAL occupies a central position in the control of plasma lipoprotein levels and to the prevention of cellular lipid overload in liver, spleen and macrophages [2,3]. This central role of LAL is clearly evident from its spectrum of deficiency diseases, including Wolman disease (WD) and cholesteryl ester storage disease (CESD) [2–4]. WD is an infantile-onset lethal disease with an incidence of 1/300,000 live births and leading to death with a median age of ~3.5 months [5]. Affected WD infants displayed large accumulations of CE and TG in the lysosomes of Kupffer cells and hepatocytes, as well as in macrophages throughout the viscera. This leads to progressive liver failure, severe hepatosplenomegaly, steatorrhea, pulmonary fibrosis [6,7], and adrenal calcification and insufficiency [2,8]. Engorged macrophages in intestinal villi lead to severe malabsorption and cachexia [2,6]. In comparison, CESD can be a more indolent progressive disease characterized by microvesicular steatosis leading to liver fibrosis and cirrhosis, accelerated atherosclerosis and premature demise [4,9,10]. The incidence of CESD has been estimated at about 1/130,000 in the Caucasian and Hispanic populations [11,12]. The CNS is not directly involved in WD or CESD [13]. The various approaches to treat WD have included bone marrow and stem cell transplantation [7,8], and for CESD various hypocholesterolemic agents [14–18]. For the former, successful transplantation has been rare. For the latter, no consistent phenotypic or outcome effects have been apparent on the major organs involved in the disease, e.g., liver and intestine.

LIPA or *Lipa* encodes LAL in humans or mice, respectively. To understand the disease pathophysiology, LAL's role in lipid metabolism, and to evaluate the therapeutic approaches, a *Lipa* knock-out mouse (*lal*−/−) was created [19]. The *lal*−/− phenotype resembled human CESD, and

Abbreviations: LAL, lysosomal acid lipase; TG, triglycerides; CE, cholesteryl esters; WD, Wolman disease; CESD, cholesterol ester storage disease; ET, enzyme therapy; rhLAL, recombinant human LAL; 4-MUO, 4-methylumbelliferyloleate; ORO, Oil Red-O.

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its biochemical and histopathologic phenotypes mimic human WD. The *lal*−/− mice are normal appearing at birth, but develop liver enlargement by 4 weeks. Hepatosplenomegaly, lymph node enlargement, intestinal villus infiltration by foamy macrophages and accumulation of CE and TG are prominent by 8 weeks. [19]. Enzyme therapy (ET) has been studied in this model using human recombinant LAL (rhLAL) produced in several different eukaryotic systems [20–22]. These studies clearly showed the potential for control of the manifestations if ET had begun early in the course of the disease [22,23]. Also, sebelipase alfa, investigational recombinant human LAL produced in chicken egg white, showed improvement of the more rapidly progressive phenotype in a naturally occurring LAL-deficient rat [24]. In humans, short-term sebelipase alfa (0.35 to 3 mg/kg) administration showed improved liver transaminases, serum cholesterol, and TG profiles [23,24], and similar to the studies in mice indicated short-term safety and therapeutic effect in human LAL deficiency.

Because of the rapidity of progression of early onset LAL deficiency, there is a critical need to determine the ability of rhLAL to reverse and/or rescue *lal*−/− at advanced stages of the disease. Here, survival, histopathological, and biochemical studies were conducted in *lal*−/− mice at various stages of disease involvement using rhLAL produced in human fibrosarcoma cells. Significant beneficial effects were evident on survival and the overall disease reversibility in various tissues in advanced disease.

2. Materials and methods

2.1. Materials

The following were from commercial sources: Anhydrous ammonium acetate, O-phthalipadehyde, sodium methoxide, sodium metaperiodate, florisil, Triton X-100, 4-methylumbelliferyl oleate (4-MUO), Nonane and Triolein (Glyceryl trioleate) (Sigma, St. Louis, MO); monoolein, diolein and oleic acid, and cholesterol (CH-800-S22-V) (Nuchek Prep, Elysian, MN); Triglyceride Liquid Reagent Set and Triglyceride standard (Pointe Scientific, Inc. Canton, MI); and mouse IgG and IgE OptEIA kits and BD Falcon™ Clear 96-well Microtest™ Plate (BD Biosciences, San Diego, CA). All other chemicals were reagent grade.

Purified recombinant human lysosomal acid lipase (rhLAL) was produced from a human fibrosarcoma cell line (Lot RDLAL046, Shire Human Genetic Therapies, Inc., Lexington, MA). The specific activity of rhLAL was 8177 U (μmol/min)/mg enzyme protein. The stock rhLAL was at the concentration of 3.3 mg/ml in 20 mM citrate, pH 5.3. The potential bacterial endotoxin was 4.8 EU/mg of rhLAL protein and bioburden was 0 CFU/ml. The rhLAL protein also contains mono-M6P and di-M6P according to the manufacturer's certificate.

2.1.1. Mice

The *lal*−/− mice were produced in these laboratories and genotyped as described [19]. These mice were backcrossed into the FVB/NJ for 10 generations for use in these studies and all mice were on this background. The mice were housed in the pathogen-free barrier facility and according to Institutional Animal Care and Use Committee (IACUC) standard procedures at Cincinnati Children's Hospital Research Foundation. The CCHMC IACUC reviewed and approved these studies under protocol 1B05049. Mice were monitored daily and weighed weekly.

2.1.2. Enzyme therapy studies

Both male and female *lal*−/− mice were included. WT mice and untreated/saline-injected *lal*−/− mice were used as controls. Mice were anesthetized by inhalation of isoflurane. rhLAL was injected intravenously by tail vein bolus. Two studies were conducted (Fig. 1). Study 1 determined the effect of rhLAL at doses of 0.8 and 3.2 mg/kg on lipid levels and survival rate. rhLAL at either dose was administered weekly beginning at 16 weeks to *lal*−/− mice and continued for up to 12 to 52 weeks. Fifteen *lal*−/− mice were used in each study cohort. Five WT littermates were treated with enzyme and fifteen *lal*−/− mice were injected with saline as controls. Also, age-matched saline-injected *lal*−/− and WT mice were included as controls. WT mice receiving rhLAL showed no adverse events and had no changes on lipid levels in organs.

Study 2 evaluated the feasibility to reverse the disease by 10 mg/kg rhLAL at early (8-wk), middle (16-wk) or advanced (24-wk) disease stages with 3 treatment terms of either 8, 16 or 20/22 weeks (Fig. 1). Three to ten *lal*−/− mice were used in each cohort. Age- and sex-matched *lal*−/− mice were untreated or saline-injected as controls.

The enzyme was reassessed periodically during the course of the experiments to ensure activity stability and protein integrity by Western blotting [25]. Aliquots of rhLAL for activities were determined using 4-methylumbelliferyloleate (4-MUO) as substrate [26]. One unit is 1 μmol of 4-MUO cleaved per min under standard assay conditions. The stability of rhLAL activity was verified and showed less than a 10% variation in specific activity over the entire study period.

Previously, IgG/IgE-mediated adverse events were observed in *lal*−/− mice receiving this rhLAL (unpublished observation). To avoid these adverse events, all *lal*−/− mice that received saline or rhLAL were treated with methylprednisolone sodium succinate (2 mg/kg), by intramuscular injection 24 h prior to rhLAL injection. Methylprednisolone sodium succinate (2 mg/kg) together with triprolidine (16 mg/kg) was given to all mice by intraperitoneal injection 1 h prior to enzyme dosing. No gross effects on treated mice were observed during the treatment, and no mice died of from enzyme injection related side effects [22].

Liver, small intestine, spleen, adrenal gland, and lymph nodes were collected one week after the last injection and the organ weights were recorded. A uniform section of the proximal jejunum was collected for histology. The remaining intestine, duodenum to ileum was weighed and frozen for lipid analyses. Tissue samples for histology and lipid analyses of liver, spleen and lymph node were consistently dissected from the central regions of the organs.

2.1.3. Histology and immunohistochemical studies

Livers, spleens, lymph nodes, adrenal glands, and small intestines were placed in Bouin's fixative for paraffin blocks and in PBS buffered 4% paraformaldehyde for frozen blocks. Paraffin sections were stained with H&E and Trichrome. Frozen sections were processed for Oil Red-O and anti-CD68 antibody staining [25,27]. The stained sections were scanned with an Aperio ScanScope XT and evaluated for signal levels using Aperio ImageScope software.

2.1.4. Lipid analyses

2.1.4.1. Lipid extraction and purification. Tissue lipids were extracted and purified via the method of Folch [28]. Liver, lymph node and spleen samples (~100 mg) were homogenized in 3.9 ml of chloroform/methanol (2:1, v/v) for ~20 s with PowerGen 125. The homogenates were shaken for 15 min and filtered through Whatman No. 4 filter paper into 60 ml-separatory funnels. Additional chloroform/methanol (2:1, v/v) was added to rinse the filter papers and to bring the total volume in the separatory funnels to 8 ml. Then 1.92 ml of 100 mM KCl was added. The funnels were slowly inverted 5 times and allowed to partition. The organic layer was collected and the solvents evaporated under a stream of N_2 and stored at −20 °C.

For extraction of lipids from the duodenum to ileum sections of small intestines, the intestine samples were rotated overnight at room temperature in 30 ml of chloroform/methanol (2:1, v/v). The extracts were filtered through Whatman No. 4 filter paper and rinsed with 10 ml of chloroform/methanol (2:1, v/v). The solvents were evaporated under a stream of N_2. The dried residues were dissolved in 4 ml of chloroform/methanol/water (2:1:0.1, v/v/v) and decanted into a 60 ml-separatory funnel. The remaining residues were solubilized in

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