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Lipidic Nanoparticles Comprising Phosphatidylinositol Mitigate Immunogenicity and Improve Efficacy of Recombinant Human Acid Alpha-Glucosidase in a Murine Model of Pompe Disease

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ABSTRACT

Enzyme replacement therapy with recombinant human acid α-glucosidase (rhGAA) is complicated by the formation of anti-rhGAA antibodies, a short circulating half-life, instability in the plasma, and limited uptake into target tissue. Previously, we have demonstrated that phosphatidylinositol (PI) containing liposomes can reduce the immunogenicity and extend plasma survival of factor VIII (FVIII) in a mouse model of hemophilia A. In this article, we investigate the ability of PI liposomes to be used as a delivery vehicle to overcome the issues that complicate therapy with rhGAA. In a murine model of Pompe disease, administration of PI-rhGAA mitigated the immunogenicity of rhGAA, resulting in a significantly lower formation of anti-rhGAA antibodies. PI-rhGAA also showed minimal improvements to the pharmacokinetic parameters and efficacy measures compared to free rhGAA. Overall, these data suggest that PI-rhGAA may have the potential to be a useful therapeutic option for improving the treatment of Pompe disease.

Introduction

Pompe disease is an inherited autosomal recessive condition caused by dysfunction or deficiency of acid α-glucosidase (GAA). GAA is the sole lysosomal enzyme responsible for the breakdown of glycogen into glucose within the cells throughout the body.[1] When GAA is no longer properly functioning, glycogen progressively accumulates causing the symptoms of the disease.

The treatment for Pompe disease currently consists of enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA). Although therapy with rhGAA has been effective in patients, results have been variable, and complicating problems have emerged.[2] One of the most prominent issues of ERT with rhGAA is the formation of anti-rhGAA antibodies that have the potential to completely abrogate the efficacy of therapy.[3] While the exact impact of these antibodies has yet to be completely elucidated, it is clear that in patients with high sustained antibody titers greater than 51,200 titer units, therapy is no longer efficacious and prognosis is poor.[4] In clinical trials, high levels of antibodies have been shown to cause an average of a 50% increase in clearance (CL) of rhGAA but the response was quite variable, ranging between 5% and 90%.[5] In addition to the prominent issue of the formation of anti-rhGAA antibodies, therapy is also complicated by pharmacokinetic (PK) issues. The PK of rhGAA is characterized by a short circulating half-life, instability in plasma, inefficient targeting, and limited uptake in target tissues.[6-9] A solution to these immunogenic and PK concerns would immensely improve therapy for Pompe patients.

Phosphatidylinositol (PI) is a naturally occurring anionic phospholipid. Liposomes containing PI have been shown to avoid the uptake by the reticuloendothelial system, specifically by the Kupffer cells of the liver.[10] In the past, our laboratory has demonstrated the benefits of using PI containing liposomes to improve delivery of another therapeutic protein, full length factor VIII (FVIII), and B-domain–deleted FVIII.[11,12] PI liposomes have been shown to increase the stability of FVIII in plasma, significantly improve the circulating half-life of FVIII, and extend the duration above the minimum effective concentration.[11,13] In addition, PI-FVIII reduces the immunogenicity of FVIII by shielding immunodominant FVIII epitopes from recognition by the immune system.[11,14] All these properties make PI liposomes a highly attractive candidate for use as a delivery vehicle for rhGAA to overcome the issues that complicate currently available therapy for

Abbreviations used: rhGAA, recombinant human acid α-glucosidase; PI, phosphatidylinositol; FVIII, factor VIII; GAA, acid α-glucosidase; 4-MUG, 4-methylumbelliferyl-α-D-glucopyranoside; PI-rhGAA, PI liposomes mixed with free rhGAA; ERT, enzyme replacement therapy.

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Pompe disease. Here, we explore the potential for PI containing liposomes to improve the PK of rhGAA and demonstrate its ability to reduce the formation of anti-rhGAA antibodies.

Methods

Materials

rhGAA was provided by Genzyme Corporation (Cambridge, MA). Dimyristoylphosphatidylcholine, PI from soybean, and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-(rhodamine B sulfonyl) (rhodamine-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol, acarbose, 4-methylumbelliferyl-α-D-glucopyranoside (4-MUG), amyloglucosidase from *Aspergillus niger*, glycogen, glucose (HK) reagent, 3,3′,5,5′-Tetramethylbenzidine, and horseradish peroxidase–conjugated goat anti-mouse IgG were purchased from Sigma-Aldrich (St. Louis, MO).

Liposome Preparation

PI liposomes were prepared by mixing the required lipids dissolved in chloroform at a fixed ratio of 50:50:5 dimyristoylphosphatidylcholine:PI:cholesterol. The lipids were mixed in a Kimax tube, and chloroform was removed by rotary evaporation to form a thin lipid film on the walls of the tubes. The films were reconstituted with buffer (phosphate-buffered saline [PBS], pH 5.5), and liposomes were formed through 3 cycles of heating at 37°C for 4 min and vortexing for 1 min. Liposomes were then made to the correct size using high pressure extrusion 20 times through 2 stacked polycarbonate membranes with an 80 nm pore size. The mean diameter of the resulting liposomes was 110 nm as measured by dynamic light scattering (Nicomp 380 Particle Sizer; Particle Sizing Systems, Port Richey, FL) Concentration of lipid was confirmed by phosphorus assay.[15] PI was mixed with rhGAA (PI-rhGAA) at a 1:1000 ratio and allowed to incubate for 30 min at room temperature for association.

Zeta Potential

After liposomes were made, the zeta potential of the PI liposomes was analyzed for surface charge. 0.1 μmol/mL PI liposomes were formulated in PBS and measured using a Zeta Potential Analyzer (Brookhaven's NanoBrook Omni, Holtsville, NY). Samples were allowed to equilibrate for 60 s, and the zeta potential value was calculated using the Smoluchowski equation. Measurements were taken at 25°C, and the number of cycles was set at 20. Three separate samples were run in triplicate.

Characterization of PI-rhGAA

rhGAA Activity in Buffer or Plasma

GAA activity was measured by activity assay. The activity is determined by the ability of GAA to cleave a fluorescent substrate, 4-MUG, and fluorescent emission measured. Samples and rhGAA standards were added to substrate solution (0.1 M 4-MUG, 0.1 M citric acid, 0.2 M disodium hydrogen phosphate, pH 4.5) containing 10 μM acarbose to inhibit contribution from neutral alpha-glucosidases which naturally reside in the plasma. The samples were allowed to incubate for 2 h at 37°C, after which the reaction was stopped by the addition of 0.5 M sodium carbonate (pH 10.7), and the fluorescence was read at an excitation of 365 nm and an emission of 448 nm using a SpectraMax Gemini fluorescence plate reader (Molecular Devices, Sunnyvale, CA). Concentrations of samples were interpolated from the standard curve.

Association Efficiency

Estimation of the amount of protein associated with PI liposomes was assessed. For this study, one mole percent rhodamine-PE was added to the lipid mixture before lipid film formation, and then liposome preparation was continued as previously explained. Rhodamine-PE was added to fluorescently label liposomes to aid in detecting liposomes as they eluted off the column. The addition of rhodamine-PE did not alter the formation of PI liposomes. A size exclusion column was prepared using G-150 Sephadex beads to separate free rhGAA from PI-associated rhGAA by size exclusion. Column eluent was collected in fractions and analyzed for liposome content by quantifying rhodamine-PE fluorescence at an excitation of 560 nm and emission of 583 nm and for protein content by carrying out an activity assay for GAA activity. Association efficiency was determined by quantifying the amount of protein in fractions collected after the liposomes had completely eluted off the column in comparison to free protein control.

GAA Knockout Mice

GAA knockout (KO) mice were originally developed by Raben et al.[16] Breeding pairs heterozygous for the GAA KO were purchased from The Jackson Laboratories (Bar Harbor, ME). Mice were then bred in house to achieve a colony that is homozygous for the KO. The presence of the KO was confirmed by polymerase chain reaction as described by the Jackson Laboratories.[17] All animal work was conducted in accordance and under approval from the Institutional Animal Care and Use Committee at SUNY Buffalo. Before injection, all formulations were confirmed to contain endotoxin levels less than 0.05 EU by limulus amebocyte assay (Charles River Laboratories, Wilmington, MA.)

Immunogenicity Study

The relative immunogenicity and ability of PI to induce tolerance was assessed in GAA KO mice. GAA KO mice ($n = 6$/group) received 4 weekly intravenous injections of either 20 mg/kg free rhGAA or PI-rhGAA. Mice were prophylactically treated each week just before their treatment injection with a 30 mg/kg intraperitoneal injection of diphenhydramine to prevent injection hypersensitivity reactions. Just before each weekly injection, a blood sample was taken via the saphenous vein to follow the progression of titers throughout the duration of the study. Two weeks after the final injection, mice were sacrificed, and blood was collected. Plasma was separated by centrifugation at 5000 *g* for 5 min, and samples were stored at −80°C. All samples were analyzed by anti-rhGAA antibody titers by ELISA.

Anti-rhGAA Antibody Titers

Anti-rhGAA antibody titers were measured by ELISA as described previously with modification.[18] Briefly, 96-well plates were coated with 2.5 μg/mL rhGAA and allowed to incubate for 2 h at 37°C. Plates were then washed and blocked with PBS containing 1% bovine serum albumin. Plates were washed again and serially diluted samples and controls were added to the plates. After 1 h at 37°C, plates were washed again and goat anti-mouse IgG horseradish peroxidase detection antibody was added at a 1:10,000 dilution. After one more hour at room temperature, the plates were washed for a final time, and then 3,3′,5,5′-Tetramethylbenzidine substrate was added and allowed to develop for 10 min before the reaction was stopped with sulfuric acid. Absorbance was measured using a SpectraMax190 UV spectrophotometer (Molecular Devices) at 450 nm. Titer levels were determined by calculating a statistically significant cutoff value with plasma from sham-treated animals as previously described.[19]