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Enhanced endothelial delivery and biochemical effects of α-galactosidase by ICAM-1-targeted nanocarriers for Fabry disease

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ABSTRACT

Fabry disease, due to the deficiency of α-galactosidase A (α-Gal), causes lysosomal accumulation of globotriaosylceramide (Gb3) in multiple tissues and prominently in the vascular endothelium. Although enzyme replacement therapy (ERT) by injection of recombinant α-Gal improves the disease outcome, the effects on the vasculopathy associated with life-threatening cerebrovascular, cardiac and renal complications are still limited. We designed a strategy to enhance the delivery of α-Gal to organs and endothelial cells (ECs). We targeted α-Gal to intercellular adhesion molecule 1 (ICAM-1), a protein expressed on ECs throughout the vasculature, by loading this enzyme on nanocarriers coated with anti-ICAM (anti-ICAM/α-Gal NCs). *In vitro* radioisotope tracing showed efficient loading of α-Gal on anti-ICAM NCs, stability of this formulation under storage and in model physiological fluids, and enzyme release in response to lysosome environmental conditions. In mice, the delivery of ^{125}I-α-Gal was markedly enhanced by anti-ICAM/^{125}I-α-Gal NCs in brain, kidney, heart, liver, lung, and spleen, and transmission electron microscopy showed anti-ICAM/α-Gal NCs attached to and internalized into the vascular endothelium. Fluorescence microscopy proved targeting, endocytosis and lysosomal transport of anti-ICAM/α-Gal NCs in macro- and micro-vascular ECs and a marked enhancement of Gb3 degradation. Therefore, this ICAM-1-targeting strategy may help improve the efficacy of therapeutic enzymes for Fabry disease.

1. Introduction

Fabry disease is a lysosomal storage disorder (LSD) caused by a genetic deficiency of α-galactosidase A (α-Gal) [1]. This enzyme hydrolyzes terminal α-D-galactosyl residues from neutral glycosphingolipids transported into lysosomes from the blood, plasma membrane and intracellular compartments [1]. As a result of the enzyme deficiency, blood group B substances, galabiosylceramide, and mainly globotriaosylceramide (Gb3) accumulate in body fluids and tissue lysosomes [1]. Gb3 deposits are found in endothelial, perithelial, and smooth-muscle cells in the vasculature, cells of the reticuloendothelial and myocardial tissues, renal epithelial cells, and perineural cells of the autonomic nervous system, causing multi-organ dysfunction and premature death [1].

The clinical manifestations of Fabry disease are variable, yet life-threatening complications commonly arise from progressive cerebrovascular, cardiac and renal impairments caused by the prominent vasculopathy [1]. Vascular lesions lead to myocardial ischemia, hypertension, atherogenesis, stroke, aneurysm, thrombosis, and renal failure [1]. The lung function can also be compromised by airflow obstruction, edema, and pulmonary embolism [1]. These effects of the vasculopathy typical of Fabry disease are strongly associated with endothelial dysfunction, and hence, endothelial cells (ECs) are a main target for the therapeutic intervention of this malady [1–3].

An available treatment for Fabry disease is enzyme replacement therapy (ERT) using recombinant galactosidases that contain mannose-6-phosphate (M6P) [4]. They can bind to M6P receptors on the surface of cells and be transported to lysosomes via clathrin-coated pits [5,6]. When injected in the circulation, these enzymes accumulate in tissues and attenuate Gb3 levels [7–10]. However, despite the clear utility of ERT, patients show varying effects and modest response to vasculopathy in the cardiovascular and neurological systems [11,12]. The altered expression or function of M6P receptors in cells affected by

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LSDs and the formation of immune-complexes that impair enzyme binding to cell receptors may contribute to limiting ERT outcome [13–16].

The efficacy of ERT for Fabry disease may benefit from strategies enhancing enzyme delivery to organs and vascular ECs via M6P-independent pathways. Targeting of α-Gal to intercellular adhesion molecule 1 (ICAM-1) may help accomplish this goal. ICAM-1 is a transmembrane glycoprotein and an adhesion molecule for leukocytes in inflammation [17]. It is expressed on vascular ECs and other cells in the body, and its expression is upregulated under pathologies, including Fabry disease [18,19]. ICAM-1 can be targeted by antibodies and affinity peptides for delivery of protein conjugates, contrast and therapeutic agents, and drug delivery vehicles such as liposomes and polymer nanocarriers in cells and animals [20–31].

We have shown that ICAM-1-targeted nanocarriers efficiently enhanced delivery of acid sphingomyelinase (ASM, a lysosomal enzyme deficient in types A-B Niemann-Pick disease) to mouse organs and macrovascular ECs [23,26,27]. ASM trafficked to lysosomes by cell adhesion molecule (CAM)-mediated endocytosis, bypassing clathrin-mediated uptake utilized by current ERTs [27]. Yet, the efficacy of this strategy to enhance the delivery of α-Gal or other enzymes and the degree of such enhancement are unpredictable. Different efficacy patterns can arise from potential variations in the enzyme loading capacity of nanocarriers and/or different enzyme pharmacokinetics, which are in part imposed by biochemical properties of the enzyme itself. In addition, the delivery of lysosomal enzymes to microvascular ECs (the major endothelial surface in the body, phenotypically and functionally different from macrovascular ECs) [32,33], has not been tested. Whether ECs with Gb3 storage typical of Fabry disease can efficiently internalize and transport nanocarriers to lysosomes also remains to be determined.

In this work, we have loaded α-Gal on model ICAM-1-targeted nanocarriers (anti-ICAM/α-Gal NCs) and used radioisotope tracing, fluorescence and electron microscopy to study this formulation *in vitro*, cell cultures and animals in comparison to non-targeted α-Gal. Our results demonstrate: (a) stability of anti-ICAM/α-Gal NCs under storage and enzyme release in response to lysosome environmental conditions, (b) enhanced enzyme delivery to organs and targeting to ECs in mice, and (c) efficient endocytosis, lysosomal transport, and Gb3 degradation in a Fabry disease model of micro- and macro-vascular ECs.

2. Materials and methods

2.1. Antibodies and reagents

Monoclonal antibodies to human or mouse ICAM-1 (anti-ICAM) were R6.5 and YN1, respectively [26]. Secondary antibodies were from Jackson Immunoresearch (West Grove, PA). Neutral α-Gal from *Escherichia coli* (Calbiochem; San Diego, CA) or coffee bean (Sigma Aldrich; St. Louis, MO) were chosen to distinguish this activity from the endogenous acidic lysosomal counterpart. α-Gal from *E. coli* was used in experiments in cell culture. α-Gal from coffee bean was used in experiments requiring ^{125}I labeling and in functional activity assays. Fluorescein isothiocyanate (FITC)-labeled and non-fluorescent 100-nm-diameter polystyrene particles were from Polysciences (Warrington, PA). Cell media and supplements were from Cellgro (Manassas, VA) or Gibco BRL (Grand Island, NY). Na^{125}I and Pierce Iodination Beads were from Perkin Elmer–Analytical Sciences (Wellesley, MA) and Thermo Scientific (Rockford, IL). All other reagents were from Sigma Aldrich (St. Louis, MO).

2.2. Preparation of anti-ICAM/α-Gal nanocarriers and enzyme release

Prototype anti-ICAM/α-Gal NCs were prepared by adsorbing anti-ICAM or a mix of anti-ICAM and α-Gal (95:5 or 50:50 antibody-to-enzyme mass ratio) onto the surface of 100-nm-diameter polystyrene particles, as described [27]. Where indicated, a mix of anti-ICAM and ^{125}I-α-Gal was used to trace the enzyme cargo (95:5 unlabeled-to-labeled enzyme molar ratio) [23]. Non-bound counterparts were separated by centrifugation [23]. The final diameter of the particles was kindly measured by NanoSight Limited using Nanoparticle Tracking Analysis (NanoSight LM20 System, Salisbury, Wilshire, UK).

The release of ^{125}I-α-Gal from anti-ICAM/^{125}I-α-Gal NCs was determined at 30 min, 1, 5, 8, 24, 48, and 72 h after particle preparation by centrifugation to separate free enzyme from particle-bound fraction. Release was assessed after 2 rounds of centrifugation at 13.8 g, resuspension by pipetting, and sonication. Enzyme release was also tested during incubation in storage buffer (phosphate buffer saline, PBS, supplemented with 1% bovine serum albumin, BSA), complete cell medium (described below), or fetal bovine serum (FBS), at 4 °C or 37 °C, pH 7.4 or pH 4.5, and in the absence or presence of enzyme substrate analog (2-μg/ml N-Dodecanoyl-NBD-ceramide trihexoside, NBD-Gb3; Matreya, LLC, Pleasant Gap, PA).

2.3. Pharmacokinetics and visualization of anti-ICAM/α-Gal nanocarriers in mice

Anesthetized C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were injected intravenously with ^{125}I-α-Gal or anti-ICAM/^{125}I-α-Gal NCs to track the biodistribution of the enzyme, or FITC-labeled anti-ICAM/α-Gal NCs to track carrier particles (30 μg/kg α-Gal, 1.5X10^{13} particles/kg). Blood was collected from the retro-orbital sinus 1, 15, and 30 min after injection. Brain, heart, kidneys, liver, lungs, and spleen were collected 30 min or 24 h after injection. Alternatively, a set of animals was perfused with PBS prior to organ collection to eliminate blood and the circulating nanocarrier fraction. The radioactivity and weight of the samples were determined to calculate the following parameters: the percentage of injected dose (%ID), the percentage of injected dose per gram of tissue to compare among organs of different size (%ID/g), the localization ratio to compare tissue-to-blood distribution (LR; %ID/g organ : %ID/g in blood), and the specificity index to compare targeted-to-non-targeted counterparts (SI; LR of anti-ICAM/α-Gal NCs : LR of α-Gal). For fluorescence measurements, organ sections were imaged by confocal microscopy (Leica TCS SP5 X) using Leica Lite 2.0.2 Software (Leica Microsystems, Wetzlar, Germany). For transmission electron microscopy (TEM) studies, organs were fixed in 2.5% glutaraldehyde and 0.1 M sodium cacodilate buffer and processed from 80–90-nm-thin resin-embedded sections [26]. These studies were performed according to IACUC and University regulations.

2.4. In vivo ICAM-1 expression

To complete previous data on ICAM-1 expression in mice [23], brain was collected from C57Bl/6 mice and homogenized at 4 °C in lysis solution (1× pheylmethylsulfonyl fluoride, 1× protease inhibitor cocktail, 0.5% sodium dodecyl sulfate, and 0.5% Triton X-100 in PBS). Protein electrophoresis, membrane transfer, immunoblot with rat anti-mouse ICAM, chemiluminiscence detection with horseradish peroxidase-conjugated goat anti-rat immunoglobulin G (IgG), and protein band densitometry were performed as described [23]. Data were normalized to actin levels, used as a control [23].

2.5. Cell culture models

Human umbilical vein endothelial cells (HUVECs; Clonetics, San Diego, CA) were cultured in M-199 medium supplemented as described [34]. Human brain microvascular endothelial cells (HBMECs; Applied Cell Biology Research Institute, Kirkland, WA) were cultured at 37 °C, 5% CO_2 and 95% humidity in RPMI-1640 supplemented with 20% fetal bovine serum (FBS), 2-mM glutamine, 30-μg/mL endothelial cell growth supplement, 100-μg/mL heparin,