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RESEARCH REPORT

A Short Synthetic Peptide Mimetic of Apolipoprotein A1 Mediates Cholesterol and Globotriaosylceramide Efflux from Fabry Fibroblasts

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Abstract Fabry disease is an X-linked sphingolipid storage disorder caused by a deficiency of the lysosomal enzyme α-galactosidase A (AGA, EC 3.2.1.22) resulting in the intracellular accumulation of globotriaosylceramide (Gb3). We found that Gb3 storage also correlates with accumulation of endosomal–lysosomal cholesterol in Fabry fibroblasts. This cholesterol accumulation may contribute to the phenotypic pathology of Fabry disease by slowing endosomal–lysosomal trafficking. We found that LDL receptor expression is not downregulated in Fabry fibroblasts resulting in accumulation of both cholesterol and Gb3. 5A-Palmitoyl oleoyl-phosphatidylcholine (5AP) is a phospholipid complex containing a short synthetic peptide that mimics apolipoprotein A1, the main protein component of high-density lipoprotein (HDL) that mediates the efflux of cholesterol from cells via the ATP-binding cassette transporter. We used 5AP and HDL to remove cholesterol from Fabry fibroblasts to examine the fate of accumulated cellular Gb3. Using immunostaining techniques, we found that 5AP is highly effective for depleting cholesterol and Gb3 in these cells. 5AP restores the ApoA-1-mediated cholesterol efflux leading to mobilization of cholesterol and reduction of Gb3 in Fabry fibroblasts.

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Introduction

Fabry disease is an X-linked glycosphingolipid storage disorder caused by a deficiency of the enzyme α-galactosidase A, which causes the accumulation of glycosphingolipids, particularly globotriaosylceramide (Gb3) in most cell types. Fabry disease is a progressive debilitating disorder affecting multiple organ systems with an incidence of ~1 in 40,000 males. Signs and symptoms include angiokeratoma, lymphedema, cornea verticillata, hypohidrosis, neuropathic pain, cardiac hypertrophy, proteinuria, progressive kidney failure, abdominal pain, diarrhea, fatigue, vertigo, and strokes. Fabry disease demonstrates significant morbidity even in childhood. Investigators analyzed dried blood spots collected from 34,736 newborns in an Austrian newborn screening program and found that 1 per 2,315 babies had a lysosomal storage disorder, much higher than the previous estimated incidence of 1 per 7,700 births. The most frequent mutations found were for Fabry's disease (Mechtler et al. 2012).

Enzyme replacement therapy (ERT) for Fabry disease is now the therapy of choice. It appears to show some beneficial effects, but the overall effects of ERT have been modest with regard to strokes, myocardial infarctions, and renal involvement.

A report exists about the postmortem findings of a male patient with Fabry disease who was on ERT for more than 2 years. He received α-galactosidase A infusions (agalsidase

beta; Genzyme Corporation, Cambridge, MA) at a dosage of 1 mg/kg every 2 weeks for the last 2.5 years of his life. The autopsy revealed that he had widespread atherosclerotic coronary artery disease that culminated in a massive acute myocardial infarction at age 47. Typical Fabry cardiomyopathy and glomerular nephropathy were found. With the exception of vascular endothelial cells, extensive glycolipid storage deposits were seen in all vascular and nonvascular cells and organ systems. In this patient, repeated infusions with α-galactosidase A over a prolonged period did not appreciably clear storage material in cells other than vascular endothelial cells. Also remarkable was the history of his blood cholesterol levels. His total blood cholesterol at age 36 was 201 mg/dL (5.21 nmol/L) and his HDL-cholesterol and triglyceride levels were 87 mg/dL (2.25 nmol/L) and 92 mg/dL (2.38 nmol/L), respectively. At age 43, total cholesterol was 241 mg/dL (6.24 nmol/L), LDL-cholesterol level was 161 mg/dL (4.16 nmol/L), and HDL-cholesterol level was 24 mg/dL (0.62 nmol/L). Normal ranges for total cholesterol should be below 200 mg/dL (5.21 nmol/L) for LDL cholesterol below 70 mg/dL (1.81 nmol/L) and for HDL cholesterol 40–60 mg/dL (1.04–1.55 nmol/L) (Schiffmann et al. 2006).

The presence of marked storage in cell types other than vascular endothelia cells such as smooth muscle cells and pericytes after more than 2 years of enzyme infusions suggests that the infused enzyme has limited access cells other than vascular endothelia cells. Thurberg analyzed pretreatment and posttreatment endomyocardial biopsies from 58 Fabry patients enrolled in a 5-month, phase 3, double-blind, randomized, placebo-controlled trial, followed by a 54-month open-label extension study of recombinant human α-galactosidase A. No clearance of GL-3 was observed in the cardiomyocytes during this trial (Thurberg et al. 2009).

Therefore, exploration of new therapies that may achieve more complete clearing of storage material is imperative.

It has been known for several decades that high-density lipoprotein (HDL) exerts a protective effect on atherogenesis. This effect is primarily mediated by the ATP-binding cassette (ABC) transporter ABCA1 that promotes the efflux of excess cholesterol from cells. ABCA1 resides on the plasma membrane, as well as in endocytic vesicles, that shuttle between late endocytic compartments and the cell surface. ABCA1 in late endocytic vesicles (late endosomes and lysosomes) can mobilize lipids for ApoA-1-mediated cellular efflux. A model for the pathway of ABCA1-mediated intracellular lipidation of ApoA-1 was postulated (Neufeld et al. 2004). ApoA-1 binds to the cell surface and is internalized along with ABCA1 into early endosomes. A portion of ABCA1 and ApoA-1 is delivered to late endocytic compartments. ABCA1 at the cell surface and in early endocytic compartments mediates the lipidation of ApoA-1. ApoA-1 traffics back to the cell surface where it is released as the nascent HDL particle.

The association of glycosphingolipid storage and cholesterol accumulation is well known (Puri et al. 2003). The physical interaction between GSLs and cholesterol is thought to cause intracellular cholesterol “trapping.” Cholesterol together with other lipids accumulates as primary or secondary storage in several lysosomal storage disorders and has been proposed to impede trafficking in the endolysosomal system (Walkley and Vanier 2009). This “traffic jam” can impair lysosomal function such as delivery of nutrients through the endolysosomal system leading to a state of cellular starvation (Schulze and Sandhoff 2011). ApoA-1-mediated cholesterol efflux occurs via ATP-binding cassette transporter A1 (ABCA1) and is a key regulator of cellular cholesterol balance. The accumulation of cellular cholesterol that accompanies glycosphingolipid storage is associated with an impaired capacity to efflux cholesterol to ApoA-1. Fabry disease fibroblasts express increased ABCA1 mRNA levels while still exhibiting a suppressed capacity to efflux cholesterol to ApoA-1 (Glaros et al. 2005). An agent that is able to increase ApoA-1-mediated cholesterol efflux and reduce lysosomal Gb3 would be of enormous benefit for the treatment of Fabry disease.

It has been found that the variant of ApoA-1 in which there is an arginine to cysteine substitution at amino acid 173 (ApoA-1Milano) appears to have superior anti-atherogenic properties compared with conventional ApoA-1. This finding led to the first positive clinical trial of HDL replacement therapy (Remaley et al. 2008). However, the production of therapeutic quantities of recombinant HDL and the Milano variant is costly. Fabry disease fibroblasts show impaired apolipoprotein A1-mediated cholesterol efflux compared with normal control fibroblasts (Glaros et al. 2005). We showed that Gb3 storage in Fabry fibroblasts correlates with the accumulation of endosomal–lysosomal cholesterol. LDL receptor expression is not downregulated when fibroblasts accumulate both cholesterol and Gb3.

It was recently found that short synthetic peptides that mimic ApoA-1, the main component of HDL, can mediate the efflux of cholesterol from cells (Sethi et al. 2008) and may be a cost-effective alternative to a full-length ApoA-1 as a therapeutic agent. The 5A peptide is a bihelical amphipathic peptide that can be complexed with the

phospholipid palmitoyl oleoyl-phosphatidylcholine (5AP). 5AP specifically effluxes cholesterol from cells by ATP-binding cassette transporter 1 (ABCA1) (Amar et al. 2010). In our studies, we used 5AP and HDL to remove cholesterol from Fabry fibroblasts to examine the fate of accumulated cellular Gb3. Using immunostaining techniques, we could show that 5AP is highly effective for depleting cholesterol and Gb3. 5AP restores the ApoA-1-mediated cholesterol efflux which leads to mobilization of cholesterol and reduction of Gb3 in Fabry fibroblasts.

Materials and Methods

Materials

Fabry fibroblasts GM 00107 with 0.0% of normal control alpha-galactosidase-A enzyme activity were from the Coriell Institute of Medical Research (Camden NJ, USA). Control Fibroblasts were from a normal healthy subject. Fetal bovine serum was obtained from HyClone Laboratories, Inc., Logan, UT. Lipoprotein-deficient bovine serum (LPDS), human low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were prepared by Advanced Bioscience Laboratories, Rockville, MD. Dr. Alan Remaley, National Heart, Lung, and Blood Institute (NHLBI) at NIH, provided the peptide 5AP. Filipin was purchased from Polysciences, Warrington, PA; mouse monoclonal anti-Gb3 antibody was from Seikagaku, Falmouth, MA; and Alexa-568 tagged IgG goat anti-mouse antibody and DiI-LDL were from Life Technologies, Grand Island, NY. Glass and plastic microscope culture chamber slides were from Nunc, Inc., Naperville, IL.

Methods

Fabry fibroblasts were cultured in McCoy's medium supplemented with 5% FBS (HyClone) and 100 units of penicillin–streptomycin/mL in a humidified incubator with 95% air and 5% CO_2 at 37°C. Fibroblasts were seeded at a density of 20,000 cells/chamber in human fibronectin-coated two-chamber glass slides. Cells were cultured in McCoy's medium supplemented with 5% LPDS (lipoprotein-depleted serum) and 100 units of penicillin–streptomycin/mL (McCoy's/5% LPDS) medium for 5 days for cholesterol depletion and cell synchronization. Cells were then incubated in fresh medium containing LDL (low-density lipoprotein) (50 µg/mL) (Sethi et al. 2008) for 24 h and then HDL (50 µg/mL) or peptide 5AP (50 µg/mL) was added to different groups. One experimental group was washed after LDL exposure, with phosphate-buffered saline (PBS), and subsequently grown another 24 h in McCoy's/5% LPDS as a cholesterol washout control.

Immunocytochemical Analyses

Filipin Staining: Filipin Staining Was Used to Visualize Unesterified Cholesterol

Cells in chamber slides were PBS washed, fixed in 3% paraformaldehyde for 30 min at room temperature, washed with PBS, and then incubated overnight with 0.05% filipin in PBS at 4°C. After incubation, cells were washed with PBS and mounted in *p*-phenylenediamine glycerol. Cells were viewed with a Zeiss LSM 410 confocal fluorescence microscope using excitation filter BP-350-364 for filipin. Then images were taken using a Zeiss Axiovert 100 TV fluorescent microscope. Eight images per slide were taken of random fields. The images were then analyzed using the I-vision program and fluorescent pixels per cell were calculated.

Gb3 Staining

Cells in chamber slides were washed in PBS and fixed in 3% paraformaldehyde for 30 min. Cells were immunolabeled using an indirect procedure in which all incubations were performed in blocker solution containing goat IgG (10 mg/mL) and phosphate-buffered saline with saponin (0.2%). Slides were incubated with primary antibody mouse monoclonal antibody against human Gb3 at a 1:500 dilution and followed detection with an Alexa-568-labeled anti-mouse antibody at a 1:100 dilution. Fluorescence was viewed with a Zeiss LSM 410 laser scanning confocal microscope, using a krypton–argon (Milles-Griot) laser with an excitation wavelength of 568 nm. Imaging and image analysis were performed as described for filipin.

LDL Uptake and Gb3 Accumulation

Monitoring of LDL uptake in Fabry and control fibroblasts with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI-LDL). DiI-LDL is a marker for receptor-mediated cellular endocytosis of LDL (Stephan and Yurachek 1993). Fabry and control fibroblasts were

seeded in 2-well chamber slides and 24-well plates at 20,000 cells per well and cholesterol depleted for 4 days. Cells were then incubated in fresh LPDS medium containing DiI-LDL (20 μg/mL). Twenty-four-well plates were analyzed using the Cytofluor 4000 plate reader at excitation of 540 nm and emission 620 nm at 0, 6, 12, and 24 h. After 24 h, wells were washed 2 times with PBS, the cells were dissolved with 0.05 N NaOH, and total protein was determined with the micro-BCA assay using bovine serum albumin as a standard. Images of the cells stained for Gb3 and cholesterol were taken at the same time using the Zeiss LSM 700.

Results

We monitored LDL uptake in Fabry and control fibroblasts with DiI-LDL as a marker for receptor-mediated cellular endocytosis of LDL. We found that DiI-LDL uptake was markedly increased in Fabry fibroblasts compared to controls, suggesting that LDL receptor expression is markedly upregulated in Fabry fibroblasts (Fig. 1).

The time course of DiI-LDL uptake and Gb3 increases in Fabry fibroblasts (Fig. 2).

A micrograph of Fabry Fibroblasts stained with filipin (blue) for cholesterol and immunostaining for Gb3 (red). The typical granular pattern of lysosomal staining is increased in fibroblast loaded with LDL and reduced in cells subsequently cultured with 5AP for 24 h (Fig. 3).

We treated cholesterol-depleted Fabry and control fibroblasts with LDL for 24 h to load them with cholesterol and then for 24 h with either LPDS, HDL, or 5AP for an additional 24 h. In Fabry fibroblasts Gb3 increases with LDL uptake and decreases with 5AP or HDL treatment. In control fibroblasts the Gb3 levels showed almost no fluctuation (Fig. 4).

T-Test Statistics

The difference between LPDS wash and 5AP is statistically significant, ($p < 0.05$).

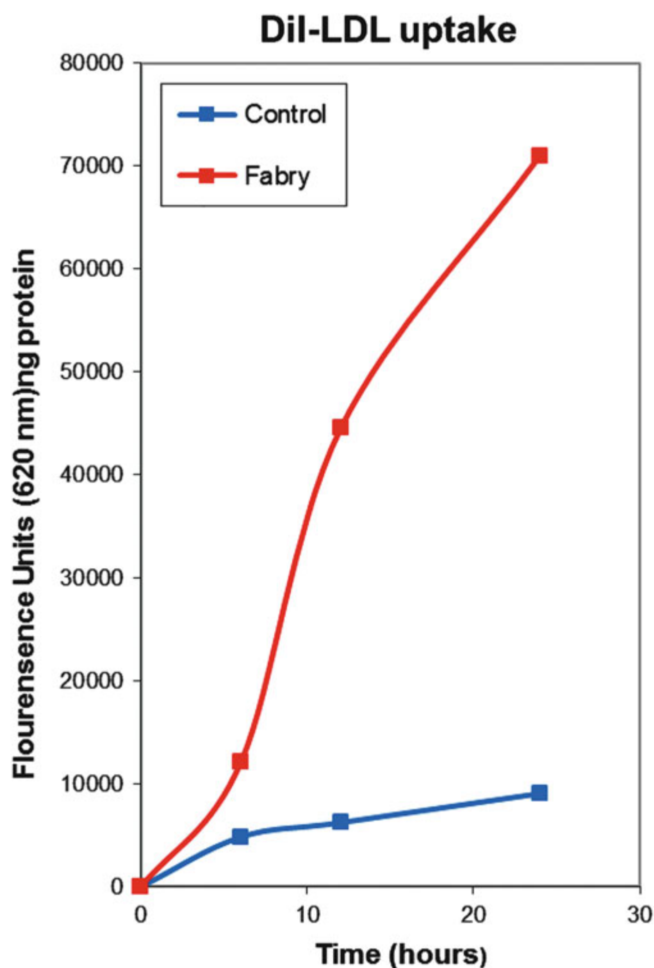

Fig. 1 DiI-LDL uptake in Fabry and control fibroblasts monitored over 6, 12, and 24 h measured with a fluorescence plate reader (Cytofluor 4000). Results are expressed as fluorescence units per mg of protein

The difference between LPDS wash and HDL is statistical very significant, ($p < 0.01$).

Our cell culture studies have shown that peptide 5AP is highly effective for depleting cholesterol and Gb3 from Fabry fibroblasts.

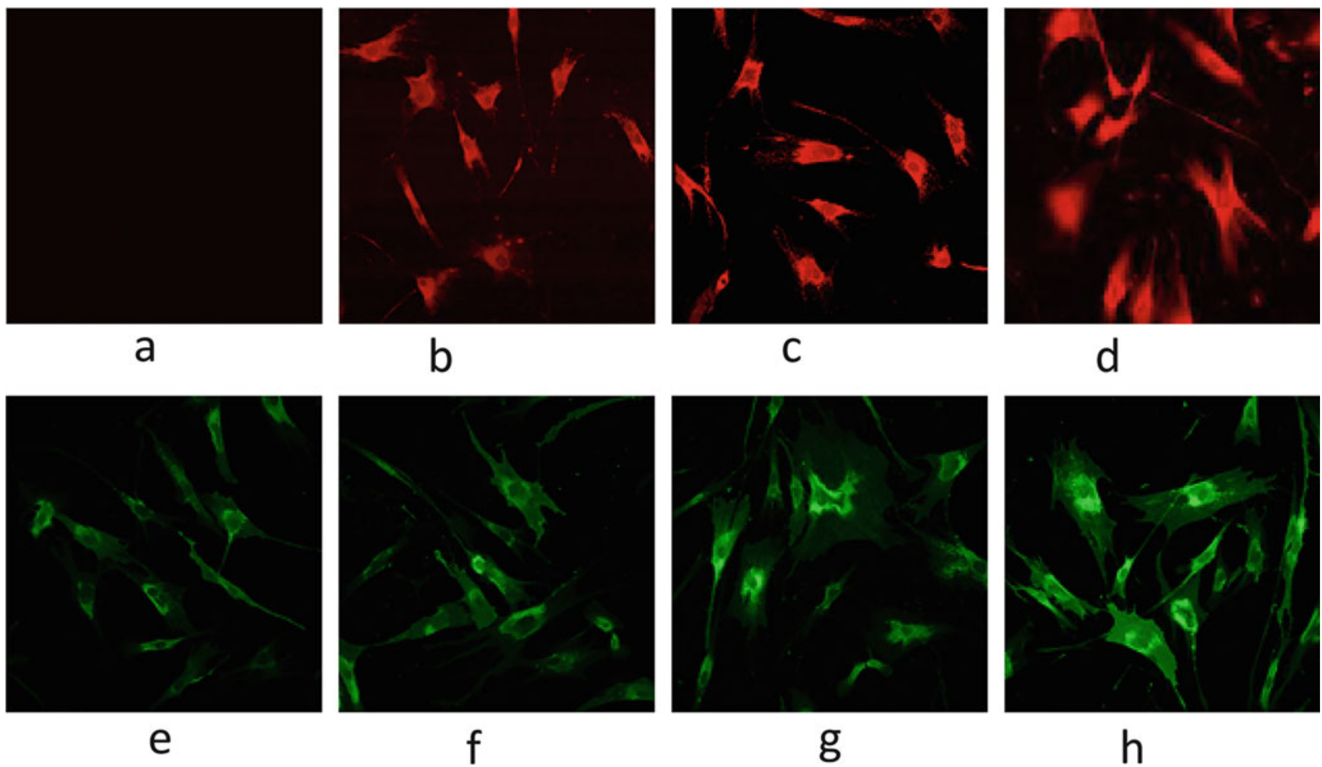

Fig. 2 Imaging of DiI-LDL uptake and staining for Gb3 in Fabry fibroblasts. *Upper row*: DiI-LDL visualized after 0 (**a**), 6 (**b**), 12 (**c**), and 24 (**d**) hours of incubation. *Lower row*: Gb3 increase visualized by Gb3 staining after 0 (**e**), 6 (**f**), 12 (**g**), and 24 (**h**) hours (original magnification 200×)

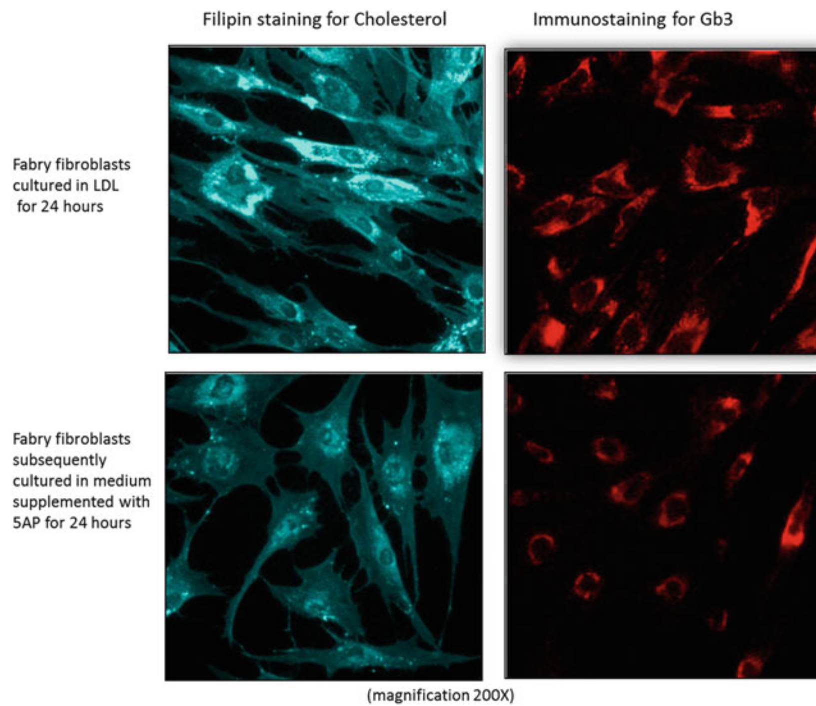

Fig. 3 Micrograph of Fabry fibroblasts stained with filipin for cholesterol, *left*, and Gb3, *right* shows a typical granular staining for lysosomes

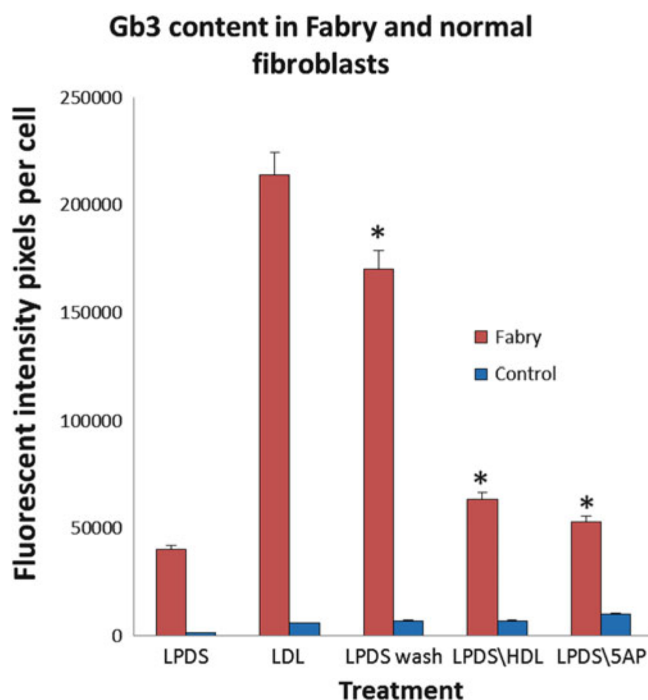

Fig. 4 Determination of Gb3 content in Fabry and control fibroblasts by image analysis. Cells were treated as described in methods, and the average intensity of pixels per cells was determined using I-vision software. The *error bars* indicate a 5% SE

Discussion

The postmortem findings of a male patient with Fabry disease, who was on ERT for more than 2 years, showed the presence of marked storage in cell types other than vascular endothelial cells, such as smooth muscle cells and pericytes. Accumulation of lipid after more than 2 years of enzyme infusions suggests that the infused enzyme has limited access to cells other than vascular endothelial cells (Schiffmann et al. 2006). Askari also found that substantial amounts of lysosomal and extralysosomal Gb3 immuno-reactivity remains in cells and tissues even after years of enzyme replacement (Askari et al. 2007).

Our finding that cholesterol efflux induced by 5AP treatment reduces the accumulation Gb3 in Fabry fibro-blasts offers promise for developing an improved treatment for patients with Fabry disease.

It is known that glycolipid storage promotes cholesterol accumulation and is often called cholesterol "trapping." Cholesterol can form a complex with glycolipids (GSL). For example, Fantani and coworkers (Fantini et al. 2013) found in the cholesterol–galactoceramide complex that the OH of cholesterol is a donor group that forms a hydrogen bond with the oxygen atom of the glycosidic linkage between galactose and ceramide. A similar hydrogen bond is formed between cholesterol and GM1. The other contacts between cholesterol and the GSL give rise to stabilizing van der Waals interactions between the apolar ceramide part of the GSL and cholesterol (Fantini et al. 2013). Cholesterol is thought to accumulate as a secondary storage product. This accumulation has been proposed to slow trafficking in the endosomal–lysosomal system, like a traffic jam in the lysosome. Blanchette-Mackie postulates that the late endosomal tubules that can be visualized by immunostaining or with a fluorescent tag become immobile and that depletion of cellular and lysosomal free cholesterol in Fabry fibroblasts correlates with reestab-lishment of the late endosomal tubular trafficking and results in mobilization of Gb3 from the lysosome (Joan Blanchette-Mackie, personal communication).

Glaros has shown that glycolipid accumulation inhibits the cholesterol efflux via the ABCA1/apolipoprotein A-1 pathway. The ApoA-1-mediated cholesterol efflux from fibroblasts derived from patients with genetic GSL storage diseases like Fabry disease was impaired compared with control cells (Glaros et al. 2005). We showed that the cholesterol uptake is also impaired in Fabry fibroblast. According to Brown and Goldstein, LDL receptor syntheses are shut down as a consequence of cellular cholesterol saturation (Brown and Goldstein 1975). Fabry fibroblasts do not slow down LDL-mediated cholesterol uptake and therefore cholesterol, as well as Gb3, accumulates in the lysosome. The effect of selective LDL apheresis in a Fabry patient was studied by Utsimi (Utsumi et al. 2006). They used this strategy to deplete the accumulated circulating Gb3.

It is known that circulating Gb3 is mostly transported by LDL particles in the plasma (Clarke et al. 1976; Chatterjee and Kwiterovich 1984). The patient that was treated by Utsimi suffered from severe Fabry-related pain and was able to remain without pain medication for 2–3 days after LDL apheresis. The markers for endothelial activation and inflammation were reduced as well.

We showed that the apolipoprotein A1 mimetic peptide 5AP mobilizes cholesterol and Gb3 from Fabry fibroblasts. We hypothesize that the apolipoprotein A1 mimetic peptide 5AP activates cholesterol efflux and effluxes Gb3 simultaneously. As a consequence of the lipid and cholesterol overload in Fabry fibroblasts, cholesterol and Gb3 form a complex in the lysosome as described by Fantani for other GSLs.

Whether the Gb3 can finally leave the body or is stored in a different compartment of the cell then the lysosome remains to be elucidated. We hope that Fabry mouse studies will help us to answer this question.

Synopsis

Cholesterol efflux facilitated by a mimetic apolipoprotein A1 peptide reduces globotriaosylceramide in lysosomes of Fabry fibroblasts.

Compliance with Ethics Guidelines

Conflict of Interests

Christine Kaneski, Alan Remaley, Stephen Demosky, Nancy Dwyer, Joan Blanchette-Mackie, John Hanover, and Roscoe Brady declare that they have no conflict of interest.

Ulrike Schueler has received a research grant from the Genzyme Corporation.

Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

Contribution

Ulrike Schueler designed, performed, and analyzed the experiments and wrote the manuscript.

Christine Kaneski grew the cells and provided technical support.

Alan Remaley provided the 5AP peptide and edited the manuscript.

Stephen Demosky provided technical support.

Nancy Dwyer provided imaging support.

Joan Blanchette-Mackie evaluated the experiments.

John Hanover evaluated the experiments and edited the paper.

Roscoe Brady evaluated the experiments, edited the paper, and mentored on the project.

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