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Reduced glucosylceramide in the mouse model of Fabry disease: Correction by successful enzyme replacement therapy

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ABSTRACT

Fabry disease is an X-linked lysosomal storage disease (LSD) caused by deficient activity of α-Galactosidase A (α-Gal A). As a result, glycosphingolipids, mainly globotriaosylceramide (Gb3), progressively accumulate in body fluids and tissues. Studies aiming at the identification of secondary lipid alterations in Fabry disease may be potentially useful for the monitorization of the response to enzyme replacement therapy (ERT) and development of future therapies. The focus of this study was to evaluate if α-Gal A deficiency has an effect on two key groups of molecules of sphingolipids metabolism: glucosylceramides (GlucCers) and ceramides (Cers). Studies performed in a mouse model of Fabry disease showed reduced level of GlucCer and normal level of Cer in plasma, liver, spleen, kidney and heart. Moreover, analysis of GlucCer isoforms in Fabry knockout mice showed that GlucCer isoforms are unequally reduced in different tissues of these animals. ERT had a specific effect on the liver's GlucCer levels of Fabry knockout mice, increasing hepatic GlucCer to the levels observed in wild type mice. In contrast to Fabry knockout mice, plasma of Fabry patients had normal GlucCer and Cer but an increased GlucCer/Cer ratio. This alteration showed a positive correlation with plasma globotriaosylsphingosine (lyso-Gb3) concentration. In conclusion, this work reveals novel secondary lipid imbalances caused by α-Gal A deficiency.

1. Introduction

Fabry disease is an X-linked lysosomal storage disease (LSD) caused by mutations in the *GLA* gene encoding the lysosomal hydrolase α-Galactosidase A (α-Gal A). Reported incidences, ranging from 1:476,000 (Poorthuis et al., 1999) to 1:117,000 (Meikle et al., 1999) in the general population, may largely underestimate the true prevalence of the disease. Indeed, recent estimated prevalences in Fabry newborn screenings range from 1:3100 (Spada et al., 2006) to 1:13,341 (Wittmann et al., 2012), suggesting that Fabry disease is more frequent than previously anticipated. Loss-of-function of α-Gal A results in a progressive accumulation of substrates with terminal α-galactosyl moieties, particularly globotriaosylceramide (Gb3), in bodily fluids and in the lysosomes. The accumulation of lipid substrates causes a multisystemic disease with a wide array of clinical signs and symptoms that predominantly affect the heart, central nervous system and kidney. Despite being an X-linked disorder, heterozygous female patients also often display clinical symptoms (Wang et al., 2007). With age, progressive damage to major organ systems occurs, culminating in severe cardiac and cerebrovascular complications, end-stage renal failure and premature mortality (Germain, 2010).

Enzyme replacement therapy (ERT) with recombinant α-Gal A is the primary treatment for Fabry disease. It has been shown that ERT attenuates renal and cardiac clinical manifestations of Fabry patients (Banikazemi et al., 2007). Moreover, ERT reduces the primary accumulation of Gb3 (Schiffmann et al., 2006; Young et al., 2005) that was, for a long time, considered as a surrogate marker of Fabry disease. Indeed, elevated levels of Gb3 are frequently present in urine of patients with Fabry disease (Fuller et al., 2005) and plasma of classic Fabry hemizygotes (Togawa et al., 2010; Young et al., 2005). However, plasma Gb3 is variably elevated in males with milder variant forms of Fabry disease and heterozygotes (Togawa et al., 2010; Young et al., 2005). Furthermore, several studies also indicate that Gb3 levels poorly reflect Fabry disease manifestations and therapeutic outcome (Bekri et al.,

Abbreviations: GlucCer, glucosylceramide; Cer, ceramide; ERT, Enzyme replacement therapy; Gb3, globotriaosylceramide; GSL, glycosphingolipid; LSD, lysosomal storage disease; Lyso-Gb3, globotriaosylsphingosine.

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2006; Schiffmann et al., 2013; Whitfield et al., 2005; Young et al., 2005). Recently, highly elevated levels of globotriaosylsphingosine (lyso-Gb3) were discovered in plasma (Aerts et al., 2008) and urine (Auray-Blais et al., 2010) of patients with Fabry disease. Plasma lyso-Gb3 levels correlate with the risk of cerebrovascular disease in hemizygotes and overall disease severity of heterozygotes (Rombach et al., 2010), suggesting that it may play a direct role in the pathogenesis of Fabry disease.

Apart from primary lipid accumulation, considerable evidence suggests that secondary alterations in lipid compounds, without a direct link to the primary protein defect, can themselves be actively involved in the pathogenesis of several LSDs (Walkley and Vanier, 2009). Moreover, the identification of secondary lipid alterations in LSDs may potentially contribute to the discovery of novel ways of discerning patients from healthy subjects and monitoring therapeutic efficacy. Indeed, previous studies evaluating the efficacy of therapy on animal models of Mucopolysaccharidoses (MPS) (Ciron et al., 2006; Cressant et al., 2004; Desmaris et al., 2004; Ellinwood et al., 2007; Frisella et al., 2001) and Niemann–Pick type C (NPC) (Griffin et al., 2004) demonstrated correlations between the normalization of secondary lipid alterations and the amelioration of primary biochemical markers or clinical features. To our knowledge, similar studies have not been performed in the mouse model of Fabry disease (α-Gal A knockout mice), which is a valuable tool to elucidate underlying mechanisms of Fabry disease pathophysiology. Despite having a normal lifespan without organ failure (Ohshima et al., 1999), biochemically, Fabry knockout mice have been shown to reproduce abnormalities found in Fabry patients, including a significant Gb3 (Rodrigues et al., 2009) and lyso-Gb3 accumulation (Aerts et al., 2008). Therefore, it is of considerable interest to identify secondary lipid alterations associated with the mouse model of Fabry disease.

In patients with Fabry disease, secondary urinary alterations in Ceramide (Cer) and Glucosylceramide (GlucCer) isoforms, with specific acyl-chain lengths, have been reported (Fuller et al., 2005). More specifically, secondary increases in Cer 16:0 and 24:1 in Fabry hemizygotes and decreases in GlucCer 22:0 and 24:0 in heterozygotes were found. Apart from its role as a central molecule in glycosphingolipid (GSL) metabolism (Fig. 1), Cer is a bioactive molecule and a mediator in cellular senescence, differentiation, apoptosis (Hannun and Obeid, 2008) and inflammation (Wu et al., 2007). In contrast to the growth-inhibitory effects of Cer, GlucCer (Fig. 1) exerts pro-mitogenic effects in a variety of tissues and cell types (Datta and Radin, 1988; Marsh et al., 1995; Shayman et al., 1991). Therefore, these bioactive molecules appear to be critical for the maintenance of cell and tissue homeostasis. The present study was designed to evaluate if α-Gal A deficiency causes secondary alterations in GlucCer and Cer, including their respective isoforms, in the Fabry disease mouse model and in the plasma of patients with Fabry disease.

2. Materials and methods

2.1. Animal studies

α-Gal A knockout mice were provided by the National Institutes of Health (Bethesda, MD, USA) and a colony was maintained at the *Instituto de Biologia Molecular e Celular* (IBMC, Porto, Portugal). Given the genetic background of this mouse colony, C57BL/6J mice were used as controls. Food and water were provided ad libitum. The IBMC animal facility is licensed for breeding and experiments with laboratory rodents. Animal experiments were carried out in accordance with the EU Directive 2010/63/EU. All animals used in this study were 12-week-old males.

For the ERT experiments, α-Gal A (agalsidase alfa, Replagal©, Shire Human Genetics) was administered weekly to Fabry knockout mice by intravenous injection, via the tail vein, of 100 μL at 1.5 or 0.2 mg/kg body weight, from the fourth to the eleventh week of life. 8 weekly administrations of 1.5 mg/kg α-Gal A were selected because it has previously been demonstrated that this protocol successfully reduces Gb3 accumulation in Fabry knockout mice (Macedo et al., 2012). The dosage of 0.2 mg/kg was chosen because it is the approved clinical dosage of agalsidase alfa. One group of Fabry knockout mice received weekly infusions of saline solution during the same period. At week twelve, animals were sacrificed, organs were harvested and kept at −80 °C until use. Sample sizes were determined based on a power analysis. Power analysis was performed taking into consideration previous information regarding the standard deviations (our own unpublished data), for a significance level of 0.05 and power of 0.8. The total numbers of animals used in this study were: 11 wild type mice; 7 Fabry knockout mice; 5 Fabry knockout mice treated with 0.2 mg/kg α-Gal A; and 3 Fabry knockout mice treated with 1.5 mg/kg α-Gal A.

2.2. Patient samples

Plasma samples from unaffected individuals and Fabry patients were kept at −20 °C until analysis. The control groups contained 20 males

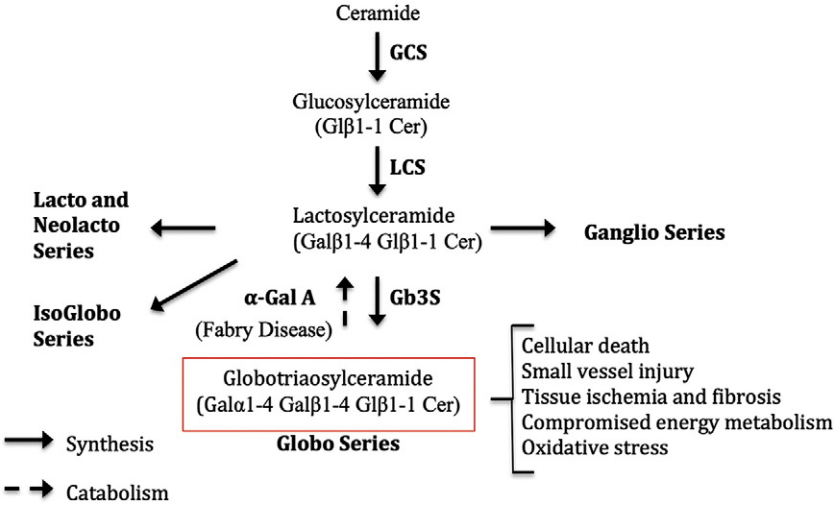

Fig. 1. Biosynthesis flow chart of GSLs from Cer. Impaired α-Galactosidase A (α-Gal A) activity leads to the primary accumulation of Globotriaosylceramide (Gb3, red box) in Fabry disease and subsequent cellular dysfunction. Also depicted are the enzymes responsible for the synthesis of GlucCer from Cer, LacCer from GlucCer, and Gb3 from LacCer: GCS — Glucosylceramide synthase; LCS—Lactosylceramide synthase; Gb3S—Globotriaosylceramide synthase.