http://www.kidney-international.org

OPEN

see commentary on page 5

Establishing 3-nitrotyrosine as a biomarker for the vasculopathy of Fabry disease

Liming Shu[1], Anuradha Vivekanandan-Giri[1], Subramaniam Pennathur[1], Bouwien E. Smid[2], Johannes M.F.G Aerts[3], Carla E.M. Hollak[2] and James A. Shayman[1]

[1]*Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan, USA;* [2]*Department of Endocrinology and Metabolism, Academic Medical Centre, Amsterdam, The Netherlands and* [3]*Department of Medical Biochemistry, Academic Medical Centre, Amsterdam, The Netherlands*

The endothelial dysfunction of Fabry disease results from α-galactosidase A deficiency leading to the accumulation of globotriaosylceramide. Vasculopathy in the α-galactosidase A null mouse is manifested as oxidant-induced thrombosis, accelerated atherogenesis, and impaired arterial reactivity. To better understand the pathogenesis of Fabry disease in humans, we generated a human cell model by using RNA interference. Hybrid endothelial cells were transiently transfected with small interfering RNA (siRNA) specifically directed against α-galactosidase A. Knockdown of α-galactosidase A was confirmed using immunoblotting and globotriaosylceramide accumulation. Endothelial nitric oxide synthase (eNOS) activity was correspondingly decreased by >60%. Levels of 3-nitrotyrosine (3NT), a specific marker for reactive nitrogen species and quantified using mass spectrometry, increased by 40- to 120-fold without corresponding changes in other oxidized amino acids, consistent with eNOS-derived reactive nitrogen species as the source of the reactive oxygen species. eNOS uncoupling was confirmed by the observed increase in free plasma and protein-bound aortic 3NT levels in the α-galactosidase A knockout mice. Finally, 3NT levels, assayed in biobanked plasma samples from patients with classical Fabry disease, were over sixfold elevated compared with age- and gender-matched controls. Thus, 3NT may serve as a biomarker for the vascular involvement in Fabry disease.

Kidney International (2014) **86,** 58–66; doi:10.1038/ki.2013.520; published online 8 January 2014

KEYWORDS: endothelial cell; endothelial nitric oxide synthase; Fabry disease; globotriaosylceramide

Correspondence: *James A. Shayman, Department of Internal Medicine, 1150 West Medical Center Drive, Ann Arbor, Michigan 48109-0676, USA. E-mail: jshayman@umich.edu*

Received 18 March 2013; revised 4 November 2013; accepted 14 November 2013; published online 8 January 2014

Fabry disease (FD) is an X-linked lysosomal storage disease arising from a deficiency in α-galactosidase A (GLA). A loss of GLA activity results in the accumulation of glycosphingolipids with galα-1,4 gal linkages including globotriaosylceramide (Gb3), galabiosylceramide, and globotriaosylsphingosine (lyso-Gb3). FD has a pleiotropic phenotype that includes renal disease, cardiomyopathy, and vasculopathy. The vasculopathy is the basis for the life-threatening complications of FD, including stroke, hypertrophic cardiomyopathy, and renal failure. Whereas enzyme replacement therapy with recombinant GLA has emerged as a therapeutic option for FD, its effectiveness in preventing the long-term cardiovascular morbidities has been questioned.[1] The ability to evaluate and deliver effective therapy is limited by a poor understanding of the pathogenesis of the vasculopathy and by the absence of a biomarker that corresponds directly with the presence and degree of vascular dysfunction.

The Gla null mouse has been an useful model from exploring the vascular pathophysiology of FD. Although this mouse does not exhibit a spontaneous vascular phenotype, several inducible models of vascular disease have been reported. These include oxidant-induced thrombosis,[2] accelerated atherogenesis,[3] and impaired vasorelaxation.[4] A common mechanism that could potentially link these experimentally observed abnormalities is endothelial nitric oxide synthase (eNOS) dysfunction.[5] eNOS dysfunction may result in either decreased nitric oxide (NO) bioavailability or enzyme uncoupling, which generates a potent oxidant, peroxynitrite, a reactive nitrogen species.[6,7] The relationship between GLA and eNOS was explored by determining whether these changes could be recapitulated in a human endothelial cell line. We report that when the Gb3 content of EA.hy926 cells is increased with GLA knockdown, there is an associated uncoupling of eNOS with the formation of 3-nitrotyrosine (3NT), a specific marker for reactive nitrogen species. The eNOS dysfunction was specifically associated with the loss of GLA activity in that comparable changes were not observed with β-glucocerebrosidase (GBA) knockdown. High circulating levels of 3NT were measured in the plasma and aortic extracts of Gla knockout mice.

Finally, the concentrations of protein-bound oxidized amino acids were measured in plasma samples from classic

FD patients and compared with age- and gender-matched controls. A more than fivefold elevation in 3NT was observed in the FD samples compared with controls, raising the possibility that 3NT represents an useful biomarker for vasculopathy in FD.

RESULTS

EA.hy926 cell studies

Previous work in GLA-deficient mice demonstrated endothelial dysfunction associated with decreased NO bioavailability and eNOS uncoupling.[5] To determine whether similar abnormalities could be documented in a human vascular endothelial cell line, immortalized EA.hy926 cells, derived from fused HUVEC and A549 cells, were employed. EA.hy926 cells retain endothelial characteristics, including Factor VIII-related antigen expression, eNOS gene expression, and Weibel–Palade bodies.[8,9] Importantly, EA.hy926 cells also are characterized by measureable Gb3 and GLA (Figure 1a and b).

The silencing efficacies of Dicer-substrate RNAs (dsiRNA) (27-mer) and traditional small interfering RNA (siRNA) (21-mer) were assessed to determine whether it would be possible to fully suppress the expression and activity of GLA in cultured EA.hy926 cells. The conventional 21-mer siRNA against human GLA demonstrated low efficiency and short blocking times (data not shown). By contrast, anti-human GLA-dsiRNA completely knocked down GLA in cultured EA.hy926 cells for up to 3 days at a relatively low siRNA concentration (10 nM) as confirmed using immunoblotting (Figure 1a). As both siRNA and dsiRNA interference are transient, a second transfection was performed 3 days later. Under these conditions, GLA silencing was observed up to day 6. The lower expression of GLA was associated with the accumulation of Gb3 (Figure 1b). A correlation between increased Gb3 and the degree of GLA knockdown was observed. Compared with control-dsiRNA–transfected cells, the Gb3 levels in GLA-dsiRNA–transfected cells increased with the duration of the incubation with GLA-dsiRNA, ranging from 210% of control on day 2 after a single transfection up to 275% on day 6 with a double transfection (Figure 1c).

The specificity of the Gb3 changes was evaluated by use of GBA-dsiRNA. *GBA* encodes β-glucocerebrosidase, the lysosomal glycosidase that degrades glucosylceramide (GlcCer) to ceramide. GBA expression in cultured EA.hy926 cells was suppressed to undetectable levels. The silencing effect lasted until day 6 as measured using immunoblotting (Figure 2a). This silencing effect was observed following both single transfection and double transfection with the 27-mer anti-human GBA-dsiRNA. The corresponding loss of GBA activity resulted in the accumulation of GlcCer (Figure 2b). The specificity of this effect was demonstrated by the absence of any corresponding change in galactosylceramide, a cerebroside that is not a substrate for GlcCerase.

GlcCer mass increased fivefold in GBA-dsiRNA–transfected cells relative to the levels in control-dsiRNA cells

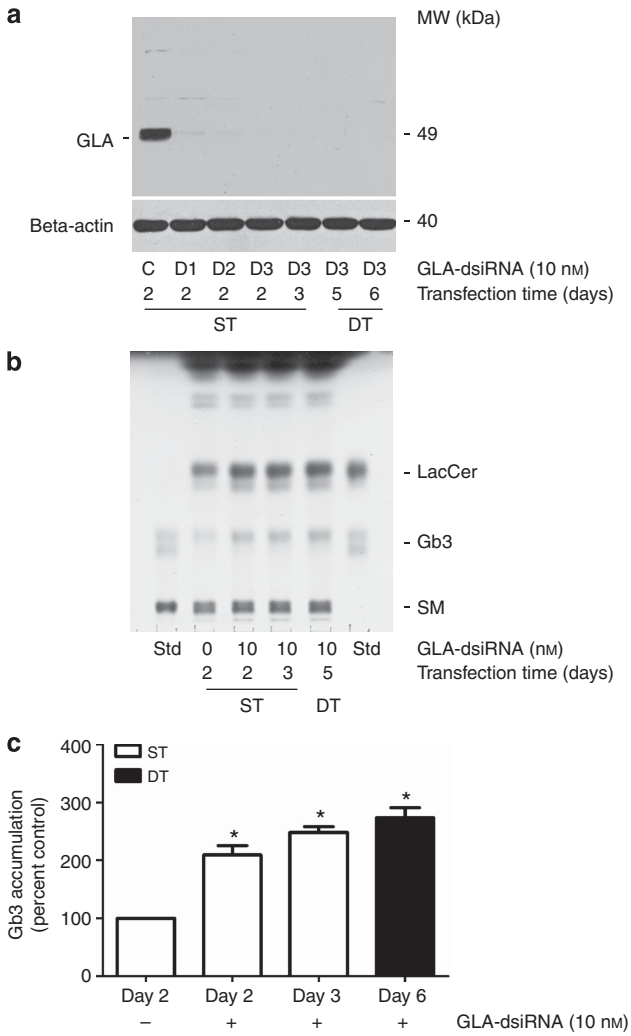

Figure 1 | Alpha-galactosidase A (GLA) knockdown raises globotriaosylceramide (Gb3) in a time-dependent manner. (**a**) A representative immunoblot for complete knockdown of the GLA from cultured EA.hy926 cells by three anti-human GLA-dsiRNA (Dicer-substrate RNA) duplexes (D1, D2, D3) at the indicated exposure times. (**b**) A representative thin layer chromatogram of neutral sphingolipids demonstrating Gb3 accumulation in GLA-dsiRNA–transfected EA.hy926 cells harvested on days 2 and 3 after a single transfection (ST) and on day 5 and 6 after double transfections (DT), respectively. LacCer, lactosylceramide; SM, sphingomyelin; Std., standards. (**c**) Gb3 levels in EA.hy926 cells as determined by scanning densitometry with the ImageJ software (NIH, Bethesda, MD). The values were normalized to Gb3 levels in cells transfected with a non-targeting control-dsiRNA. The data represent the mean ± s.e. from six independent experiments. *$P < 0.01$.

(Figure 2c). A small but reproducible reduction in GlcCer from its peak level occurred on day 4 after first transfection likely due to transient effect of siRNA. When a double GBA-dsiRNA transfection was performed, the cellular GlcCer levels on day 6 were as high as those observed on day 3 with a single transfection (Figure 2c). Although GlcCer is a precursor in Gb3 synthesis, no increase in Gb3 levels were observed in cells subjected to GBA-dsiRNA transfection (Figure 2d and e).