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Fabry disease peripheral blood immune cells release inflammatory cytokines: Role of globotriaosylceramide

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ABSTRACT

Fabry disease is an X-linked lysosomal disorder (LD) due to deficiency of the enzyme α-galactosidase A (αGal), which leads to the accumulation of neutral glycosphingolipids, mainly globotriaosylceramide (Gb3). Several mechanisms contribute to the diverse physiopathological alterations observed in this disease, and it has been suggested that an underlying proinflammatory state could play a significant role. The aim of this study is to investigate the presence of a proinflammatory state in the different subsets of peripheral blood mononuclear cells (PBMC) and to understand the mechanisms that contribute to its onset and perpetuation. We have shown that cultured PBMC from Fabry patients present a higher proinflammatory cytokine expression and production. Moreover, we determined that among PBMC, dendritic cells and monocytes present a basal proinflammatory cytokine production profile, which is further exacerbated with an inflammatory stimulus. Finally we established that normal, monocyte-derived dendritic cells and macrophages display the same proinflammatory profile when cultured in the presence of Gb3 and an inhibitor of αGal. Furthermore, this effect can be abolished using a TLR4 blocking antibody, indicating that TLR4 is necessary in the process. In summary, our results demonstrate the presence of a proinflammatory state involving two key subsets of innate immunity, and provide direct evidence of Gb3 having a proinflammatory role, likely mediated by TLR4, a finding that could help in the understanding of the underlying causes of the inflammatory pathogenesis of Fabry disease.

1. Introduction

Fabry disease is an X-linked lysosomal disorder (LD) due to deficiency of the enzyme α-galactosidase A (αGal, EC 3.2.1.22, codified by the gene GLA) [1,2]. Absent or reduced enzyme activity results in impaired catabolism of neutral glycosphingolipids, particularly globotriaosylceramide (Gb3), which in turn leads to intracellular deposition of such lipids [3]. Although tissue and organ dysfunctions can be partly attributed to direct buildup of Gb3, the exact molecular mechanisms that link this accumulation to the eventual cell and tissue damage have not yet been clearly established. In a number of different LDs, other concurrent pathological mechanisms are elicited, which together contribute to the phenotypic expression of each disease [4].

Growing evidence shows immune system irregularities associated to lysosomal disorders [5]. In several different LDs, immune cells from affected patients display a constitutive proinflammatory pattern of cytokine expression [6–8]. For Fabry disease in particular, a number of immune alterations have been described. Leucocytes and endothelial cells have been shown to exhibit an activated profile. Deposition of globotriaosylceramide occurs in numerous cell types including vascular endothelial and smooth muscle cells. Fabry disease is associated to vascular injury and a prothrombotic state, that could be related to the finding of higher surface expression of integrins involved in cell adhesion [9]. The evidence for endothelium activation was confirmed lately by Shen et al. [10]. These authors showed that accumulated Gb3 induces oxidative stress and up-regulates adhesion molecules. The mechanisms of endothelial compromise may be associated to the decreased nitric oxide bioavailability detected in a Gla null mouse model, which could account for aberrant vascular phenotypes [11]. A pro-oxidative and proinflammatory state that correlates with urinary Gb3 levels has been recently described [12]. Our group has previously reported alterations in the number of leukocyte subpopulations, as well as in their surface marker levels [13]. Later on, we have described a higher apoptotic rate in peripheral blood mononuclear cells (PBMC), which could be directly attributed to the effects of elevated Gb3 levels [14].

In this work, we investigate the existence of an underlying proinflammatory state in the PBMC of Fabry patients, in particular

Abbreviations: LD, lysosomal disorder; PBMC, peripheral blood mononuclear cells; DC, dendritic cells; MΦ, macrophage; NK, natural killer; Gb3, globotriaosylceramide; lysoGb3, globotriaosylsphingosine; αGal, α-galactosidase A; GLA (Gla), human (murine) α-galactosidase A gene; ERT, enzyme replacement therapy; DGJ, 1-deoxygalactonojirimycin; TLR4, Toll-like receptor 4; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide.

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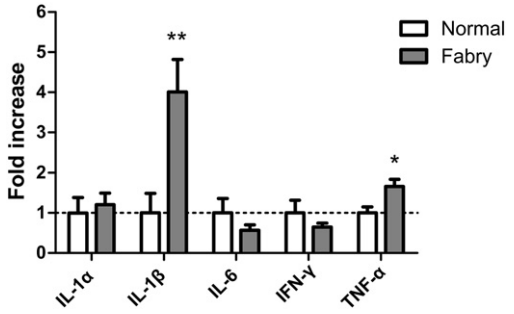

Fig. 1. Expression of cytokines in PBMC from Fabry patients and normal controls. Values shown correspond to average expression normalized to the mean of normal controls (fold increase), accompanied by its SEM. Statistically significant differences between both groups are indicated (*$p<0.05$, **$p<0.01$; Mann–Whitney test).

in the subsets related to the innate immune system, and the possible effect of excessive Gb3 levels on the initiation and maintenance of this state.

2. Materials and methods

2.1. Patient's and normal control's samples

Blood samples from 29 Fabry patients, 15 males and 14 females (mean age: 30.2 ± 18.4 years old; range: 3–74 years old) were taken for this study. Diagnosis of Fabry disease was established by clinical examination reduced enzymatic activity and genetic test. Clinical manifestations of the patients included angiokeratoma, acroparesthesia, cornea verticillata, abdominal pain, proteinuria and hypohidrosis; however none of the patients suffered from renal insufficiency, heart failure or cerebrovascular accidents at the time of enrolment in this study. Twenty-two patients were under enzyme replacement therapy (ERT) with agalsidase alfa (Replagal™, Shire Human Genetic Therapies, Cambridge, MA, USA) at the moment of this study. The protocol was approved by the scientific committee of AADELFA (Medical Association for Study of Lysosomal Disorders and Fabry disease in Argentina) according to provisions of the Declaration of Helsinki in 1995. The nature and purpose of the study were explained to all volunteers and all patients gave their informed consent prior to participation in this study.

Fifteen healthy individuals (mean age: 26.7 ± 11.1; range: 6–46 years old) served as controls.

Peripheral blood samples (10 ml) were collected by venipuncture using heparin as anticoagulant and immediately processed.

Buffy coats were obtained from serologically negative blood bank donors, and processed within 18 h from extraction.

PBMC were isolated from blood or previously diluted buffy coat by Ficoll Hypaque (Sigma, St Louis, MO, USA) gradient separation.

2.2. Analysis of cytokine expression and production

Total RNA of PBMC from Fabry patients and healthy donors was isolated using a commercially available kit (Macherey Nagel, Duren, Germany) and then reverse transcribed using random hexamers and reverse transcriptase (Invitrogen, Carlsbad, CA, USA). A quantitative PCR (qPCR) was performed using SYBR Green PCR Master Mix (BioRad, Hercules, CA, USA) and the relative expression level of the genes IL-1α, IL-1β, IL-6, IFN-γ and TNF-α was determined using β-actin expression as a normalizer. The sequence-specific primers were designed using PerlPrimer [15]. The values obtained were expressed as fold increase of Fabry samples compared to normal samples.

Normal and Fabry PBMC were seeded on 96-well culture plates at 5×10^5 cells/well in 300 μl AIM-V medium (Invitrogen, Carlsbad, CA, USA), and cultivated at 37 °C, 5% CO_2 for 24 h. The concentration of IL-1β, IL-6, IFN-γ, TNF-α and IL-13 was determined in the supernatants of the above cultures, using capture ELISA (BD OptEIA, BD Biosciences, San Diego, CA, USA).

2.3. Flow cytometric analysis of intracellular cytokine content in innate immunity cell subsets

Isolated PBMC from five Fabry patients not undergoing ERT and three healthy donors (mean ages 38.6 ± 18.1 and 31.7 ± 2.5 years-old, respectively) were seeded on 96-well plates at 1×10^6 cells/well in 300 μl AIM-V medium with the addition of 1 μg/ml Brefeldin A (GolgiPlug, BD Biosciences, San Diego, CA, USA), in the presence or absence of 1 μg/ml LPS (Sigma), and were incubated at 37 °C, 5% CO_2 for 14 h. After washing the cells with PBS, they were blocked with 10% inactivated normal human serum for 15 min at 4 °C, and then stained for different combinations of surface markers: CD56 (eBioscience, San Diego, CA, USA) and CD3 (BD Biosciences, San Diego, CA, USA), LIN-1 (BD Biosciences, San Diego, CA, USA) and HLA-DR (eBioscience, San Diego, CA, USA), and CD14 (BD Biosciences, San Diego, CA, USA). Afterwards, the cells were washed twice, and fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences, San Diego, CA, USA) following the manufacturer's protocol. Finally, cells were stained for IL-1β, IL-6 and TNF-α, washed, and analyzed by flow cytometry.

2.4. In vitro culture of monocyte-derived dendritic cells and macrophages

Monocytes were purified from PBMC isolated from normal controls' buffy coats as described elsewhere [16]. Briefly, cells were washed with sterile pyrogen-free saline, centrifuged at 100 *g* for 10 min, and resuspended in AIM-V medium. Fifty $\times 10^6$ cells were seeded in 75 cm^2 culture flasks and cultured for 1 h at 37 °C and 5% CO_2. Non-adherent cells were removed by washing twice with warm saline, and remaining cells were cultured in AIM-V for 6 days, with the addition of 36 ng/ml recombinant human GM-CSF and 20 ng/ml rhIL-4, or 30 ng/ml recombinant human M-CSF (R&D Systems, Minneapolis, MN, USA), to stimulate their differentiation to dendritic cells (DC) or macrophages (MΦ), respectively. Obtained cells were gently resuspended in AIM-V, seeded in 12-well culture plates (5×10^5 cells/well) and cultured for 24 h in the presence of 20 μM Gb3 (Matreya, Pleasant Gap, PA, USA) and/or 200 μM 1-deoxygalactonojirimycin (DGJ, Sigma). Stock Gb3 solution was prepared by dissolving the drug in DMSO, and performing subsequent dilutions in sterile water or culture medium. As Gb3 vehicle contained DMSO, the same final amount of this solvent (0.1%) was ensured in all conditions. Finally, the concentration of IL-1β, IL-6, and TNF-α was determined in the supernatants of the above cultures, using capture ELISA as mentioned before.

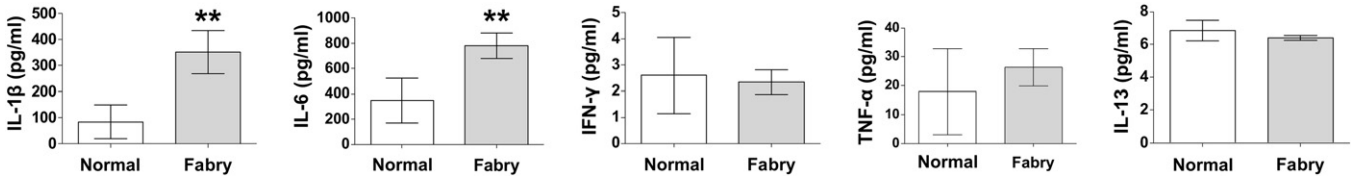

Fig. 2. Production of cytokines in 24 h PBMC cultures from Fabry patients (gray bars) and normal controls (white bars), measured in supernatants by capture ELISA. Each value is represented as a mean together with its SEM. Statistically significant differences ($p<0.01$, unpaired t-test, with Welch's correction if appropriate) are marked with two asterisks.