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Higher apoptotic state in Fabry disease peripheral blood mononuclear cells. Effect of globotriaosylceramide

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ABSTRACT

Fabry disease is an X-linked lysosomal storage disorder (LSD) due to deficiency of the enzyme α-galactosidase A, resulting in intracellular deposition of globotriaosylceramide (Gb3). Accumulation of Gb3 is probably related to tissue and organ dysfunctions. Diverse pathological mechanisms are elicited in LSDs, giving together the phenotypic expression of each disease. The purpose of the present study is to investigate if apoptosis could play a role in Fabry disease pathogenesis and to understand the mechanisms involved in the proapoptotic state. We have demonstrated that Fabry disease peripheral blood mononuclear cells display a higher apoptotic state, which is reduced by enzyme replacement therapy (ERT), and is mediated, at least in part, by activation of the intrinsic pathway of caspases. We could rule out the implication of "unfolded protein response-ER stress" in this apoptotic process. To further confirm the suggestion that Gb3 is associated to apoptotic cell death, we treated normal cells with Gb3 at concentrations found in Fabry patients. Addition of Gb3 resulted in a dose-dependent induction of apoptosis involving the intrinsic pathway. In summary, PBMC from Fabry patients display a higher apoptotic state, which could be mainly related to elevated Gb3.

1. Introduction

Fabry disease is an X-linked lysosomal storage disorder (LSD) due to deficiency of the enzyme α-galactosidase A (GLA, EC 3.2.1.22) [1,2]. Absent or reduced enzyme activity leads to impaired catabolism of neutral glycosphingolipids, particularly globotriaosylceramide (Gb3), resulting in intracellular deposition of such lipids [3]. Intracellular accumulation of Gb3 is probably related to tissue and organ dysfunctions, but the exact molecular mechanism that translate the primary insult into cell damage remains to be determined. Other concurrent pathological mechanisms are elicited in LSDs, giving together the phenotypic expression of each disease [4].

Abnormalities in the number of immune cell subsets in Fabry patients have been recently described [5]. Moreover, there is evidence of an inflammatory activated state in endothelium and leukocytes in Fabry disease [6,7]. Inflammation and apoptosis have been extensively studied in mouse models of different LSDs, and seem to be common findings. Studies in animal models of LSDs have demonstrated increased apoptosis of chondrocytes in mucopolysaccharidosis (MPS) VI [8], a reduction in the expression of the repressor of apoptosis Bcl-2 in brains from Gaucher mice [9] and increased cell death in brain of Niemann–Pick type C mice [10]. Moreover, cells from human patients suffering from different LSDs are more susceptible to apoptosis [11].

Three major pathways of apoptotic cell death can be distinguished. The extrinsic pathway that signals through plasma membrane death receptor [12], the intrinsic pathway involving mitochondria [13], and the endoplasmic reticulum (ER) stress pathway [14] that is triggered by a continuous loading of mutated misfolding proteins. These pathways are characterized by the recruitment of different specific initiator caspases: caspase 8, caspase 9 and caspase 4/12, respectively. They converge to a final common route with the activation of the common effector caspase 3, which cleaves regulatory and structural proteins, culminating in the death of the cell [15].

We hypothesized the existence of a disturbance of the immune balance in Fabry disease leukocytes, possibly associated to a proinflammatory state, that leads to a higher apoptotic status. In this study we evaluated the apoptotic state of peripheral blood mononuclear cells (PBMC) from Fabry disease using different apoptosis markers, and analyzed the relevance of ER stress and elevated Gb3 levels to this

Abbreviations: Gb3, globotriaosylceramide; LSD, lysosomal storage disorder; ER, endoplasmic reticulum; MPS, mucopolysaccharidosis; PBMC, peripheral blood mononuclear cells; MFI, mean fluorescence intensity; ERT, enzyme replacement therapy; GLA, α-galactosidase A; DGJ, deoxygalactonojirimycin; ΔΨm, mitochondrial membrane potential; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; FL, fluorescence.

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condition. We found an increased apoptotic state in Fabry PBMC, that is not due to ER stress, and that could be related to elevated Gb3.

2. Materials and methods

2.1. Patient's samples

Blood samples from 32 Fabry patients, 16 males and 16 females (mean age: 26.25 ± 15.49 years old; range: 3–60 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. Seventeen 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.

Thirty healthy individuals (mean age: 27.93 ± 12.10; range: 10–55 years old) matched for age and sex served as controls.

Peripheral blood samples (10 ml) were collected by venipuncture in heparin as anticoagulant and immediately processed. Mononuclear cells from whole blood (PBMC) were isolated by Ficoll Hypaque (Sigma, St Louis, MO, USA) gradient separation.

2.2. Flow cytometric determination of apoptosis by Annexin V and TUNEL tests

2.2.1. Apoptosis by Annexin V

Fifty microlitres of whole blood were placed in a tube, and 300 µl of binding buffer (HEPES 10 mM, NaCl 140 mM, $CaCl_2$ 2.5 mM, pH = 7.40), 2 µl of Annexin V-FITC (Becton Dickinson, Mountain View, CA, USA) and 1 µl propidium iodide (Sigma, St Louis, MO, USA) were added. After an incubation of 30 minutes at 4 °C in darkness, samples were analyzed by flow cytometry in a FACScalibur (Becton Dickinson, Mountain View, CA, USA). Mononuclear cells were gated according to forward scatter/sideways scatter (FSC/SSC) characteristics, and percentage of Annexin V positive cells and mean fluorescence intensity (MFI) were determined.

2.2.2. Apoptosis by TUNEL

TUNEL (Promega, Madison, WI, USA) labeling of PBMC was carried out according to the manufacturer's protocol. Briefly, one million mononuclear cells were fixed with 1% paraformaldehyde at 4 °C during 20 min. After washing cells with PBS, 70% ethanol was added and incubated at −20 °C for at least 4 h. Following washing, equilibration buffer was added and incubated during 5 min at room temperature. After centrifugation at 300 *g*, the supernatant was discarded and a reaction mixture, composed of recombinant Terminal Deoxynucleotidyl Transferase and nucleotide mix containing fluorescein-12-dUTP was added. The reaction was stopped with 20 mM EDTA and permeabilization of cells was carried out by the addition of 0.1% Triton, 5 mg/ml BSA in PBS solution. After centrifugation, a solution of 5 µg/ml of propidium iodide containing 250 µg of DNase-free RNase A in PBS was added, and incubated in darkness during 30 minutes. Percentage of TUNEL positive cells and MFI were analyzed by flow cytometry in a FACScalibur (Becton Dickinson, Mountain View, CA, USA).

Mononuclear cells were identified and gated according to forward scatter/sideways scatter (FSC/SSC) characteristics. For each sample, 100,000 events were acquired and data analysis was done using Cell Quest software program (Becton Dickinson, Mountain View, CA, USA). MFI was obtained for each marker and the percentage of positive cells was determined using negative controls as a threshold reference. For each Fabry patient's sample, at least one normal control was processed in parallel on the same run. Intra-run variability was assessed by performing quadruplicate runs on control samples for both Annexin V and TUNEL assays.

2.3. Immunoblotting determination of caspases activation

Whole-cell proteins lisates were obtained by homogenizing PBMC from Fabry patients (F) or normal controls (N) in a solution containing 10 mM HEPES pH = 7.9, 1 mM EDTA, 60 mM KCl, 0.2% Nonidet P40, 1 µM DTT, 1 µM PMSF, 0.1 µg/ml aprotinin, 0.1 µg/ml leupeptin, 1 µM sodium vanadate and 5 µM NaF (Sigma, St Louis, MO, USA). Total protein content was determined using bicinchoninic acid assay (Pierce Laboratories, Rockford, IL), and 10 µg of total protein from each sample were resolved by SDS-PAGE, and then blotted onto nitrocellulose with an electrotransfer device (BioRad, Hercules, CA, USA). Samples were loaded in pairs comprising a normal and a Fabry sample, which were processed together. The membranes were probed with primary antibodies against caspase 3 (Cell Signaling, Danvers, MA, USA), caspase 8 (Cell Signaling, Danvers, MA, USA), caspase 9 (Cell Signaling, Danvers, MA, USA), caspase 12 (Affinity bioreagents, Rockford, IL, USA), caspase 4 (Sigma, St Louis, MO, USA) followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (BioRad, Hercules, CA, USA), and developed with a chemiluminiscent reagent containing 1.25 mM luminol (Fluka, St Louis, MO, USA), 0.2 mM p-coumaric acid (Fluka, St Louis, MO, USA), 2.9 mM hydrogen peroxide, 10 mM Tris, pH = 8.8. Detection of beta actin by a specific monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as loading control.

2.4. Expression analysis of genes related to ER stress by qPCR

Total RNA was isolated from PBMC of each individual by means of total RNA isolation system (Macherey Nagel, Duren, Germany) following the manufacturer's protocols. The isolated total RNA samples were then reverse transcribed using random hexamers and reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR (qPCR) for the genes HSPA5 (BiP), DDIT3 (CHOP), PPP1R15A (GADD34), EDEM1 and HSP90B1 was performed using SYBR Green PCR Master Mix (BioRad, Hercules, CA, USA). The sequence-specific primers were designed using PerlPrimer [16]. Human β-actin gene was used as internal control. Comparative threshold (CT) method was used for data analysis. Data were expressed as relative quantitation of gene expression ($2^{-\Delta Ct}$) in the cells of Fabry patients versus normal controls. Thapsigargin-treated (1 µM, 1 h) PBMC were used to verify ER stress related activation for each gene used.

2.5. In vitro treatment of PBMCs

One million PBMC from normal volunteers were plated onto 96-well culture plates in AIM V medium (Invitrogen, Carlsbad, CA, USA) containing varying amounts (up to 40 µM) of Gb3 (Matreya, Pleasant Gap, PA, USA) in DMSO or DMSO alone, and incubated at 37 °C in a 5% CO_2 atmosphere for different times. GLA-inhibited controls were produced by preincubating PBMC for 24 h with 20 mM deoxygalactonojirimycin (DGJ, Sigma, St Louis, MO, USA) prior to Gb3 addition. The specificity of the results obtained with Gb3 was assessed by replacing Gb3 with glucosylceramide (Matreya, Pleasant Gap, PA, USA). After in vitro treatment, apoptosis by Annexin V, TUNEL and JC-1 was determined.

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2.6. Measurement of mitochondrial membrane potential by JC-1

Mitochondrial membrane potential ($\Delta\Psi m$) changes were measured by the use of J-aggregate-forming lipophilic cationic fluorochrome 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) reagent (Molecular Probes, Eugene, OR, USA) and flow cytometry in PBMC from normal controls exposed *in vitro* to Gb3. Membrane depolarization was assessed by first calculating a ratio between green and red fluorescence for each event, and then measuring the percentage of cells above a threshold value and calculating the derived parameter's geometric mean.

2.7. Data analysis

Statistical analysis was performed on obtained data using Student's *t*-test, Mann–Whitney *U* test or non-parametric ANOVA as appropriate. A value of $p<0.05$ was regarded as statistically significant.

3. Results

3.1. Apoptosis determination in PBMCs from Fabry disease patients

The apoptotic state of PBMCs from untreated and ERT-treated Fabry patients was analyzed by Annexin V and TUNEL flow cytometric methods, in order to evaluate early and late apoptotic events, respectively. Histograms show higher Annexin V (Fig. 1A) and TUNEL (Fig. 1B) fluorescence in samples from Fabry untreated patients, as compared to ERT-treated patients and normal controls. Ratios of MFI obtained from Fabry patients (untreated and treated) to normal controls by Annexin V and TUNEL are shown in Figs. 1C and D. It can be seen that a substantial number of untreated Fabry patients display an MFI ratio greater than one, indicating a higher apoptotic state of the cells from these patients compared to normal controls. Moreover, ERT-treated patients display overall lower MFI ratios than those observed in untreated Fabry, specially in Annexin V assay, which shows a significant difference between both groups of patients ($p=0.034$).

3.2. Apoptotic pathway displayed in Fabry disease

In order to investigate if apoptosis is mediated, at least in part, by caspases, immunoblotting of caspase 3 (effector and common final caspase) in PBMCs from Fabry patients and controls individuals was carried out. Samples from Fabry patients showed higher levels of caspase 3 as compared to normal controls (Fig. 2). No signs of active caspase 3 could be detected in any of the samples, probably because of very low quantities of this polypeptide below the sensitivity level of this assay.

To investigate the apoptotic pathway triggered in Fabry disease, we analyzed the levels of initiator caspases specific for the 3 different pathways: caspase 8 (extrinsic), caspase 9 (intrinsic) and caspase 12 and 4 (ER stress). Levels of caspase 8 were undetectable both in Fabry and normal controls' samples (not shown). Levels of caspase 9 and 12 (Fig. 2), both the full length and cleaved active products were higher in samples from Fabry patients in comparison to normal controls, as shown by immunoblotting. Levels of caspase 4 were variable when comparing Fabry patients and controls. No differences were noted in levels of any of the caspases assayed between untreated and ERT Fabry patients.

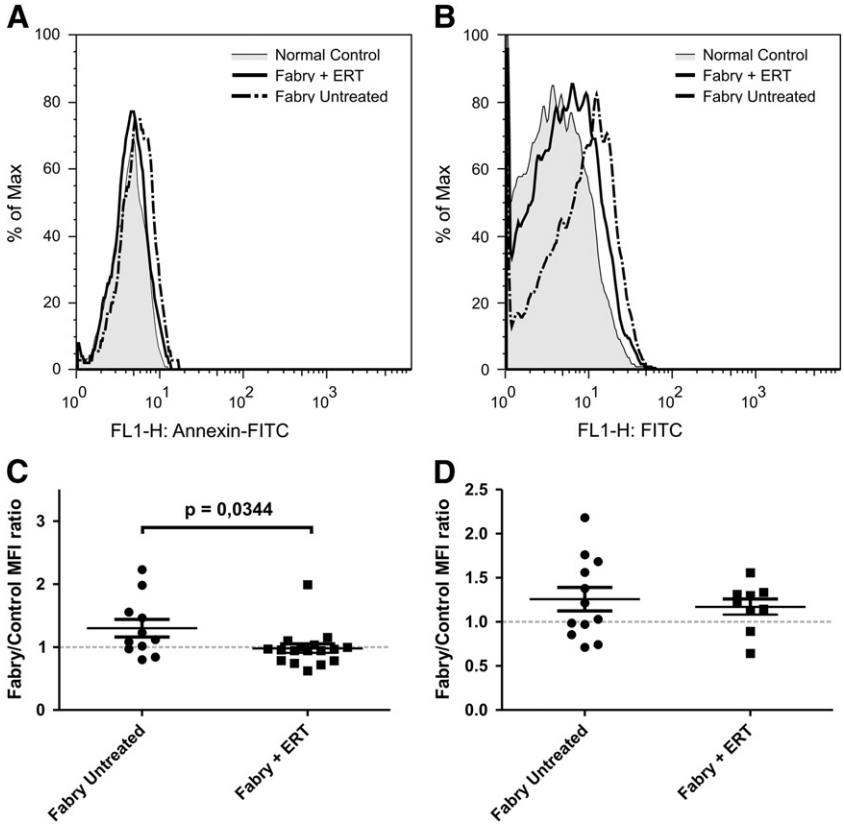

Fig. 1. Apoptosis determination by Annexin V and TUNEL flow cytometric methods in PBMC from untreated and ERT-treated Fabry patients in comparison to normal controls. The figure shows the histograms (top) and the ratio between MFI values for Fabry patients to normal controls (bottom) obtained by Annexin V (A and C) and TUNEL (B and D).

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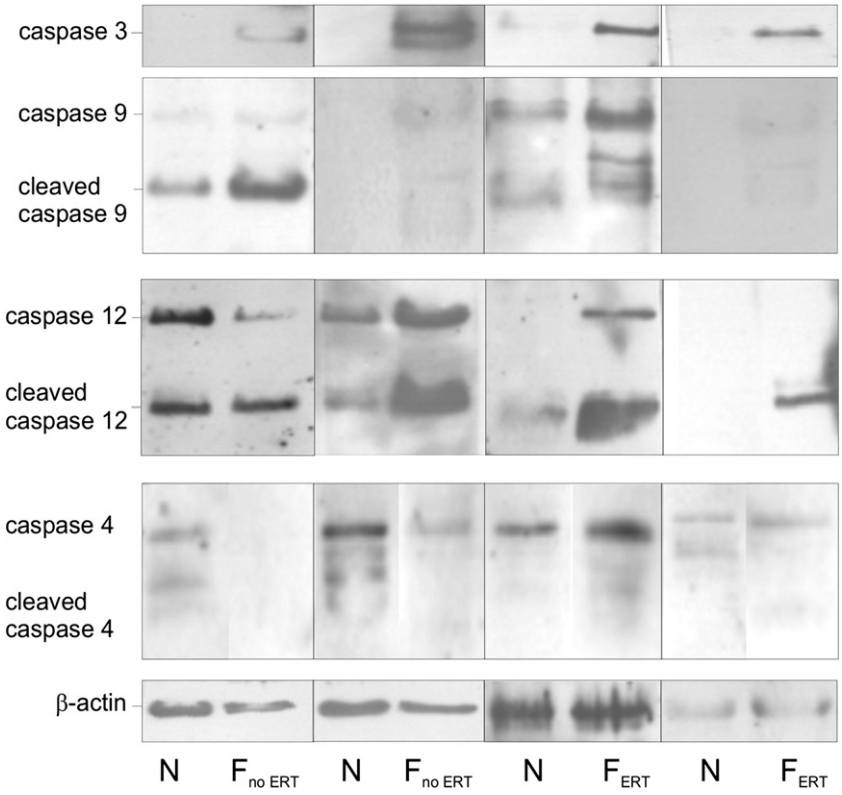

Fig. 2. Immunoblotting of caspases 3, 9, 12 and 4 in PBMC from Fabry untreated ($F_{no\ ERT}$) and treated (F_{ERT}) patients and normal controls (N). β-actin was used as loading control.

3.3. Involvement of ER stress in Fabry apoptosis

Levels of caspase 12 were shown to be higher in Fabry patients, however variable results were found for caspase 4 between Fabry patients and controls. To further analyze if ER stress is involved in cell death in Fabry disease, we decided to test the expression of 5 genes related to ER stress: HSPA5 (BiP), DDIT3 (CHOP), PPP1R15A (GADD34), EDEM1 and HSP90B1 (Fig. 3). The expression was highly variable in the different Fabry samples analyzed. For the genes tested, we observed some samples with high expression levels, especially among Fabry patients undergoing ERT, but not among untreated patients. No significant differences were detected in expression levels between Fabry patients and controls. These results would rule out the implication of ER stress in apoptotic cell death in Fabry PBMCs.

3.4. In vitro treatment of normal PBMCs with Gb3

In order to analyze if exposure to Gb3 is able to induce apoptosis, we treated normal PBMC with 20 μM Gb3, a concentration comparable to that found in plasma of Fabry male patients, for different times. Apoptosis analysis by Annexin V revealed higher apoptotic levels in cells treated with Gb3 as compared to control cells, from 1 to 6 h (Fig. 4A). TUNEL assay showed a maximum of apoptosis at around 3 h.

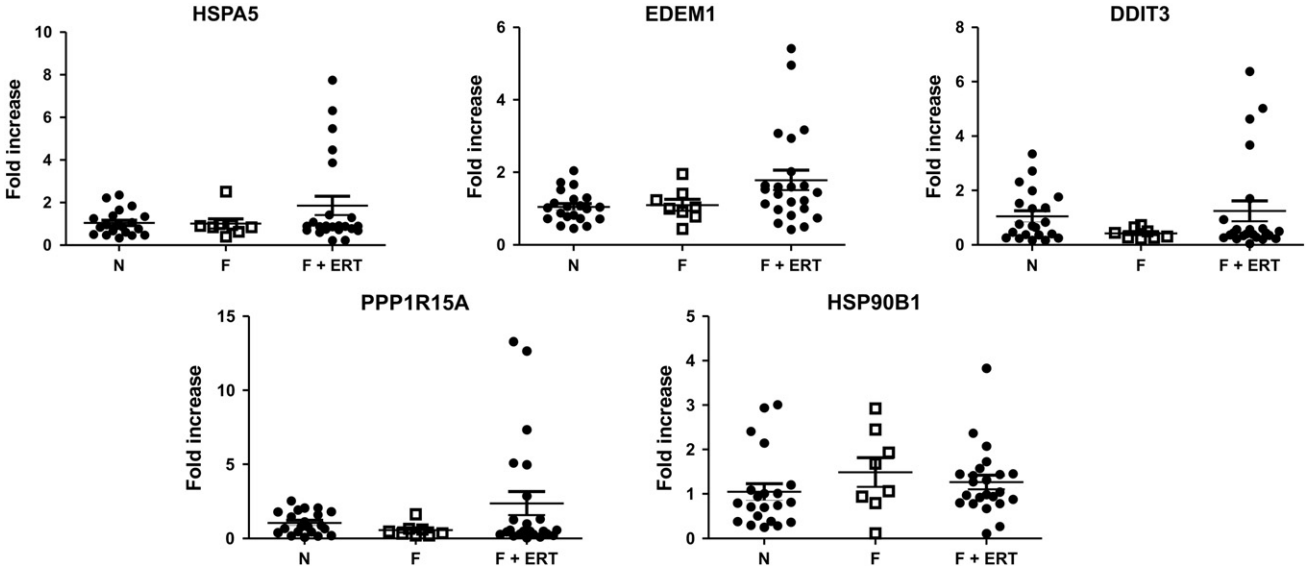

Fig. 3. Expression analysis of genes related to ER stress by qPCR in PBMC from Fabry untreated ($F_{no\ ERT}$) and treated (F_{ERT}) patients and normal controls (N).

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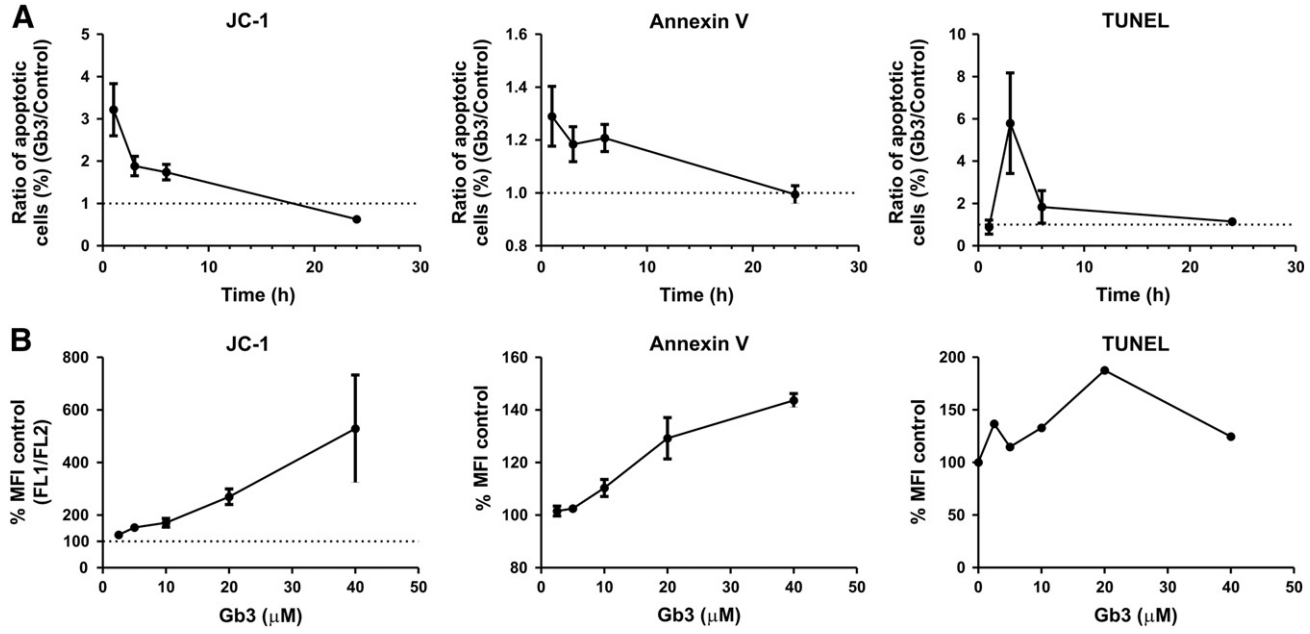

Fig. 4. Flow cytometric determination mithocondrial membrane depolarization by JC-1, and of apoptosis by Annexin V AND TUNEL in normal control's PBMC treated with Gb3. Panel A shows the timely evolution of these tree parameters during treatment with 20 μM Gb3. Panel B shows the Gb3 dose-dependent response measured by JC-1 at 1 h, Annexin V at 1 h or TUNEL assay at 3 h, respectively. Data were expressed as a ratio between treated cells and controls.

Moreover, vital staining with Trypan Blue also revealed higher number of dead cells in the Gb3 treated PBMC (not shown).

To test if Gb3-induced apoptosis is mediated by intrinsic pathway, we also assayed the changes in mitochondrial membrane potential ($\Delta\Psi m$). The percentage of positive JC1 cells and the rate of MFI green/red FL indicated an induction of depolarization of mitochondrial membrane by the addition of Gb3. These results are compatible with the activation of the intrinsic pathway of apoptosis.

To further validate these findings, we performed a dose-response analysis on normal PBMC by culturing them with increasing amounts of Gb3, ranging from 2.5 to 40 μM. Both apoptosis, as measured by Annexin V at 1 h or TUNEL assay at 3 h, and loss of $\Delta\Psi m$, as measured by JC-1 at 1 h, showed a strong positive correlation with Gb3 dose, confirming the effect of pathological Gb3 levels on normal PBMC (Fig. 4B).

When these same dose–response experiments were performed on normal PBMC that were pre-incubated with DGJ at inhibitory concentrations of GLA activity, responses remained unchanged, indicating that Gb3 elicits apoptosis in these conditions regardless of the level of GLA activity present in the cells. Furthermore, if Gb3 was replaced by glucosylceramide in these experiments, the apoptotic effects disappeared, showing that the observed phenomena are specific to Gb3 (data not shown).

4. Discussion

The purpose of the present study was to search if apoptosis could play a role in Fabry disease pathogenesis, and to investigate the possible underlying causes that trigger these apoptotic events. We have found that Fabry disease PBMC display elevated apoptosis markers, evidenced by evaluation of early and late events. Furthermore, mononuclear cells from untreated Fabry patients presented a higher apoptotic state than patients undergoing ERT.

Moreover, we could demonstrate that apoptosis is mediated, at least in part, by caspases activation, through the detection of higher levels of the effector caspase 3 in samples from Fabry disease patients. In accordance with this finding, a study by microarray hybridization in Fabry disease children detected two apoptosis related genes as the only differentially expressed from 54,000 genes tested: neuronal apoptosis inhibitory protein and apoptosis-inducing factor (AIF) [17]. Since we did not observe differences in procaspase 3 levels between untreated and treated patients, the mechanism by which ERT reduces apoptosis could involve events that are downstream caspase 3.

Apoptosis appears to be a common finding in other LSDs, such as infantile neuronal ceroid lipofuscinosis [18], MPS [19], Niemann Pick [20], Tay-Sachs and Sandhoff [21]. This phenomenon of apoptosis could reflect a problem related to lysosomal deposition itself but independent to the specific substrate accumulated. The mechanism or pathway that leads to apoptosis could be specific for each disorder. In Tay-Sachs and Sandhoff diseases the inflammatory reaction leads to a phase of rapid neuronal apoptosis [22]. Increased dermatan sulfate levels and/or inflammatory cytokines induced apoptosis of chondrocytes in MPS VI cells [8]. Apoptosis mediated by intrinsic pathway was reported in Gaucher murine model brain, where a downregulation of the repressor of apoptosis Bcl-2 was detected [9]. Extrinsic caspase pathway is involved in apoptosis in GM1/GM2 gangliosidosis [23] and Niemann–Pick type C [10]. In the case of Fabry disease PBMC, we have detected in this work the contribution of the intrinsic pathway in apoptotic cell death.

We observed higher levels of active caspase 12 in samples from Fabry patients, but this caspase is probably not functional in human beings [24]. For this reason, we analyzed levels of caspase 4, the probable human caspase counterpart related to ER stress, and variable results were observed. To further elucidate the possible contribution of ER stress in apoptotic cell death, we tested the expression of 5 genes related to ER stress. We have not found any significant differences in the expression of these genes between untreated patients and normal controls, ruling out the possibility of the involvement of ER stress in Fabry PBMC apoptotic cell death. A similar finding was reported in a neuronopathic model of Gaucher, where no activation of the unfolded protein response was observed [25]. The conclusion from the report of Wei et al., stating that ER stress was shown to be a common mediator of apoptosis both in neurodegenerative and non-neurodegenerative LSDs [11] should not be generalized to all LSDs. Intriguingly, a small

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group among patients undergoing ERT displayed an elevated profile of expression of ER stress genes, an unexpected observation that should be further investigated.

To investigate the hypothesis that Gb3 is associated to apoptotic cell death, we treated normal cells with Gb3 at concentrations found in Fabry patients. In fact, addition of Gb3 produced a dose-dependent induction of apoptosis in normal cells. Moreover, it produced an alteration of $\Delta\Psi m$, confirming the involvement of mitochondrial intrinsic pathway in Gb3-induced apoptosis. These results were specific to Gb3, as glucosylceramide treatment failed to reproduce them.

Interestingly, these results remained unaltered when GLA activity was inhibited by prior incubation with DGJ, indicating that the effects observed for Gb3 treatment on PBMC are not related to Gb3 degradation within the lysosomes, which in turn could explain the short times at which these effects are readily detectable.

Loss of $\Delta\Psi m$ preceding nuclear disintegration, a typical feature of apoptosis, was associated with an increase in reactive oxygen species and elevation in cytosolic calcium [26]. The decline of $\Delta\Psi m$ during apoptosis could be associated to the activity of permeability transition pore channel that participates in calcium efflux from mitochondria due to an elevation in mitochondrial calcium [27]. Moreover, the loss of $\Delta\Psi m$ has been shown to be involved in the release of the mitochondrial oxidoreductase AIF [28], the gene that was shown to be overexpressed in Fabry disease [17]. During apoptosis signaling, both AIF and cytochrome c are released from mitochondria to cytosol, leading to activation of caspases pathway. Future studies from our group are directed to elucidate the reactive oxygen species (ROS) and calcium involvement in the observed change of $\Delta\Psi m$ in Gb3 mediated and Fabry disease-apoptosis.

5. Conclusions

In summary, PBMC from Fabry patients display a higher apoptotic state, which can be partially reverted by ERT. Apoptosis in Fabry disease PBMCs could be mainly attributed to elevated Gb3 in these patients and is mediated by intrinsic pathway associated to depolarization in mitochondrial membrane.

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The funding source had no involvement neither in the study design; in the collection, analysis, interpretation of data; in the writing of the report; nor in the decision to submit the paper for publication.

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