LIPID IMBALANCE IN THE PROGRESSIVE NEUROLOGICAL METABOLIC DISORDER, FARBER DISEASE

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ABSTRACT
Farber disease is a neurodegenerative metabolic inherited disease caused by the deficient activity of acid ceramidase which leads to ceramide accumulation within lysosomes. Besides the structural role in biomembranes ceramide also acts as signalling molecule. The present study investigated whether intracellular trafficking of lipid molecules is blocked in diseased fibroblasts. The observation of secondary lysosomal glycosphingolipids and cholesterol storage in Farber cells reinforces the importance of a normal level and/or intracellular distribution of ceramide for proper cell function.

KEYWORDS
Biological Sciences, Science and Health, Disease, Molecular pathophysiology.

RESUMO
A Doença de Farber é uma doença hereditária metabólica neurodegenerativa causada pela deficiente actividade da ceramidase ácida que conduz à acumulação de ceramida nos lisossomas. Para além da função estrutural nas biomembranas, a ceramida também actua como molécula sinalizadora. O presente estudo investigou se o tráfego intracelular de moléculas lipídicas está comprometido em fibroblastos destes doentes. A acumulação lisossomal secundária de glicoesfingolípidos e colesterol observada em células de Farber sublinha a importância de um nível e/ou distribuição intracelular de ceramida adequados para o correcto desempenho da função celular.

PALAVRAS-CHAVE
Gestão de Ciência, Tecnologia e Inovação em Saúde, Actividades Científicas e Tecnológicas, Ciências da Saúde, Medicina, Pesquisa Científica e Desenvolvimento Tecnológico, Pesquisa Aplicada.

ABBREVIATIONS
AC, Acid ceramidase; DMEM, Dulbecco's Modified Eagle Medium; BODIPY, Boron dipyrromethene difluoride; BSA, Bovine serum albumin; Cer, Ceramide; FD, Farber disease; GSLs, Glycosphingolipids; HPTLC, High performance thin-layer chromatography; HSF, Human skin fibroblasts; LacCer, Lactosylceramide; LDL, Low density lipoprotein; LPDS, Lipoprotein deficient serum; PBS, Phosphate buffered saline; RT, Room temperature; SL, Sphingolipids; SMase, sphingomyelinase.

1. **INTRODUCTION**

Lipids are a heterogeneous group of compounds that include sphingolipids (SLs) and steroids. SLs are characterized by the presence of long-chain sphingoid backbone and display a great structural diversity and complexity. SLs are ubiquitous components of membranes of eukaryotic cells, many of which function also as bioactive signalling molecules, such as ceramide (Cer). Cer consists of sphingosine or a related-long chain base which is N-acylated with a fatty acid. It is the common precursor of glycosphingolipids (GSLs) and sphingomyelin. Multiple metabolic pathways converge upon Cer, namely the *de novo* biosynthetic pathway, the sphingomyelinase (SMase) pathway and GSLs catabolism. Differential activation of these distinct pathways of Cer formation is possible due to spatial separation of enzymes that contribute to each pathway, for example, endoplasmic reticulum, plasma membrane and lysosomes, respectively. Due to its role in cell signalling, in last decades Cer and other SLs have been implicated in diverse biological processes, including the regulation of cell growth, differentiation, migration, senescence, apoptosis, inflammation and angiogenesis (Bartke and Hannun; Wennekes et al.).

SL turnover is clearly important as exemplified by the severity of inherited pathologies associated with the SL catabolism, the sphingolipidoses (Yu et al., "Role"). Most of these diseases are due to the deficiency of a specific hydrolytic lysosomal enzyme or its activator "cofactor", both directly responsible for degradation of a specific SL, or of a lysosomal membrane transport protein involved in efflux from lysosomes of the final catabolic product. Defects in any of these proteins typically lead to lysosomal storage. Neuronal cells are likely to be especially vulnerable to lipid accumulation as most of these diseases are characterized by severe mental and motor decline, and premature death is often observed in the first decade of life. For most of these diseases the primary defects, from the gene to protein and storage materials, have been identified and characterized more than 20 years ago (Kolter and Sandhoff). However, the exact cascade of events from protein dysfunction to neuronal impairment have not been fully cleared yet. More recent studies of membrane lipid transport using fluorescent GSL analogues in fibroblasts suggested that cholesterol may play a major role in modulating the intracellular targeting of SLs, specifically lactosylceramide (LacCer), in normal and in a variety of sphingolipid storage diseases irrespectively to the nature of the primary storage compound (Pagano et al., "Membrane"; Puri et al.). Several other studies have documented the accumulation of glycosphingolipids, phospholipids and cholesterol as secondary storage materials in sphingolipidosis (Walkley and Vanier).

Farber disease (FD, MIM 228000) is a fatal neurodegenerative pathology caused by the deficient activity of lysosomal acid ceramidase (AC, N-acylsphingosine deacylase, E.C. 3.5.1.23). AC is the last hydrolase in the catabolism of SLs, leading to the realease of sphingosine and fatty acid. Clinically the disease is characterized by the appearance of painful and swollen joints, subcutaneous nodules, hoarseness, and various degrees of visceral and neurological impairment. Although death typically occurs within the first decade, a milder course (until adulthood) of the disease can be observed. The defective AC activity caused by mutations in the *ASAH1* gene leads to Cer storage within lysosomes (Li et al.; Moser). Although Cer is unlikely to escape from the lysosomes due to its hydrophobicity, the possibility of altered downstream cell events cannot be presently excluded.

Aiming to understand if lysosomal ceramide accumulation would also implicate cholesterol redistribution and alterations in the intracellular targeting of GSLs, several lipid analyses were performed in the lysosomal acid ceramidase-deficient cell model, Farber disease's fibroblasts.

2. **MATERIALS AND METHODS**

2.1. **PATIENTS AND CELL LINES**

Normal human skin fibroblasts (HSF) used in this study were obtained from healthy volunteers. Cultured fibroblasts from Farber disease patients were purchased from NIGMS Human Genetic Cell Repository (Coriell Institute for Medical Research, New Jersey, USA). Fibroblast cell cultures were maintained in DMEM medium, supplemented with 10% of fetal calf serum and antibiotics, at 37 ºC in an atmosphere containing 5% CO_2. After trypsinization the cells were washed with cold 0.9% NaCl and frozen at -20 ºC or immediately processed.

2.2. **MATERIALS**

Nonhydroxy fatty acid ceramide standard from bovine brain was obtained from Sigma and hydroxy fatty acid ceramide standard from Avanti Polar Lipids, Inc (Alabaster, USA). Lipoprotein deficient serum (LPDS), Filipin reagent and IgM m-chain specific secondary antibody were purchased from Sigma Aldrich (USA). Culture medium and antibiotics were obtained from Gibco BRL (UK). BODIPY-C5-Cer was purchased from Molecular Probes (Invitrogen, Barcelona). All other reagents were of the highest quality available.

2.3. **QUANTITATION OF CERAMIDE**

Lipids were extracted from about 80% confluent cultured skin fibroblasts basically according to the procedure previously described (Tohyama et al.). Lipids were separated by high performance thin-layer chromatography (HPTLC, Perkin-Elmer model 1220) and visualized by incubating the plate at 120 ºC after spraying with 1% solution of anisaldehyde in 80% of sulphuric acid. For lipid quantification, the plate was digitalized and bands quantified by UnScan-It Software (Silk Scientific, Inc, Utah, USA).

2.4. **BODIPY-C5-CER AND BODIPY-LACCER CELL INCUBATION ASSAY**

For BODIPY-C5-Cer, the procedure was performed as described (Pagano et al., "A Novel"). For BODIPY-LacCer, HSF were plated in glass cover slips and incubated in LPDS media at 37 ºC for 4 days, followed by a 24h incubation in normal medium containing 100µg/mL of low density lipoprotein (LDL) to enrich cellular membrane with sterol. Procedure was carried out as previously described (Puri et al.). For each cell line the procedure was repeated at least twice.

2.5. **FILIPIN STAINING**

HSF were plated in glass cover slips and incubated in LPDS media at 37 ºC for 4 days, followed by a 24h incubation in normal medium containing 100µg/mL of LDL to enrich cellular membrane with sterol. After fixation in methanol-free formaldehyde cells were stained for free-cholesterol with filipin (Teixeira et al.). For each cell line the procedure was repeated at least twice.

2.6. **GM2 GANGLIOSIDE IMMUNOFLUORESCENT STAINING**

Cells were fixed with formaldehyde in PBS pH 7.4 for 10 min at RT, rinsed and permeabilized with Triton X-100 10 min at RT. After washing cells were blocked with phosphate buffered

saline/bovine serum albumin (PBS/BSA) for 30 min. Cells were then incubated with monoclonal primary antibody anti-G_{M2} ganglioside (IgM), a kind gift from Dr. T. Tai from Metropolitan Institute of Medical Science in Japan, as described (Teixeira et al.). For each cell line the procedure was repeated at least twice.

2.7. **FLUORESCENCE MICROSCOPY**

Mounted cover slips were observed under a Nikon fluorescence microscope, using the adequate filters. Cell images were captured using the 100x objective with a Nikon DS-2MBWc camera. Images were processed with the ImageJ software (version 1.38).

2.8. **STATISTICAL ANALYSIS**

Results were expressed as mean ±SD. Data was analyzed using 2-tailed Student's ***t*** test and considered significant if P value was <0.05.

3. **RESULTS**

The level of intracellular Cer accumulation was firstly assessed in AC-deficient human fibroblasts after total lipid extraction and separation by HPTLC (Fig. 1A and 1B). Total Cer level was clearly increased in FD cells and found to be significantly elevated comparing with normal cells. To examine the distribution of endocytosed Cer normal control and FD cells were treated with the analogue BODIPY-C5-Cer and analysed by fluorescence microscopy (Fig. 1C). When cells were observed in the green+red channel (λexc 450-490nm; λem ≥ 520nm), a bright perinuclear and reticular pattern of fluorescence corresponding to the Golgi apparatus was observed in control and FD cells but the fluorescence intensity was considerably higher in FD cells. This fluorophore exhibits a shift in its emission maximum from green (~515 nm) to red (~620 nm) wavelengths with increasing concentration of Cer. Thus, when BODIPY-C5-Cer is used to label living cells as it was performed in the present study, this property allows to differentiate membranes containing high concentration of the fluorescent lipid and its metabolites (the corresponding analogues of sphingomyelin and glucosylceramide) from other regions of the cell where smaller amounts of the probe is present (Pagano et al., "A novel"). This shift was particularly noticed in the region of Golgi complex in FD cells (data not shown) indicating a higher probe concentration in Golgi membranes of FD than control cells.

Secondary GSL accumulation has been documented in human fibroblasts (Pagano et al., "Membrane") and mouse models of several Lysosomal Storage Diseases (McGlynn et al.). Moreover this storage is accompanied by sequestration of free cholesterol in a manner similar to that observed in primary gangliosidoses (Puri et al.). To address this hypothesis filipin staining and G_{M2} ganglioside immunostaining were performed in AC-deficient human fibroblasts to detect free cholesterol and G_{M2} ganglioside, respectively (Fig. 2). AC-deficient cells cultured in the presence of LDL showed filipin staining in perinuclear granules reflective of lysosomal cholesterol accumulation. In contrast, in normal control fibroblasts relatively little cytoplasmatic fluorescence was observed. Intralysosomal accumulation of G_{M2} ganglioside was assessed by immunofluorescence using a specific monoclonal antibody. As can be observed in the Fig. 2, discrete punctate fluorescent structures outlining the region of the nucleus were noticed in FD cells, which were not detected in control cells. Intracellular sorting of endocytosed GSLs was next studied by pulse-labeling of AC-deficient fibroblasts with the

fluorescent sphingolipid analogue BODIPY-LacCer (Fig. 2). In normal control cells BODIPY--LacCer was transported to the Golgi apparatus after endocytosis whereas in AC-deficient cells BODIPY-LacCer accumulates in punctate structures more likely endosomes/ lysosomes.

4. **DISCUSSION**

Over the past decade it has become apparent that the maintenance of a balanced level among specific SL and cholesterol is essential also for proper neuronal function. This becomes particularly noticeable when considering the SL storage diseases, such as Gaucher, Tay-Sachs, and Niemann-Pick A/B diseases, in which glucosylceramide, G_{M2} ganglioside and sphingomyelin accumulate, respectively, due to defective activity of the lysosomal enzymes responsible for their degradation. In these diseases cholesterol is trapped within lysosomes as a result of physical association of cholesterol with SL. A similar phenomena is observed in Niemann Pick type C disease which is characterised by lysosomal storage of unesterified cholesterol as the primary consequence of the disease (Puri et al.; Walkley and Vanier). Thus a possible pathogenic event common to several sphingolipidoses may reside in blocking of intracellular transport to or from lysosomes with subsequent endosomal/lysosomal jam (Liscum).

In Farber cells the prominent storage of Cer in lysosomes is considered as a primary consequence of the gene defect. Here we show that Cer levels are increased and its distribution may be perturbed in FD fibroblasts. It is possible that Cer turnover (and/or its metabolites, sphingomyelin and glucosylceramide) is delayed at the Golgi apparatus, implicating alterations in the membrane composition and function including the distribution of GSLs at the trans-Golgi. Supporting this hypothesis an increased level of sphingomyelin most likely due to delayed degradation was reported in FD fibroblasts (Van Echten-Deckert et al.). This issue must be further investigated as the effect of lysosomal storage may not affect only the function of lysosomes but also the structure and function of other cell compartments, particularly membrane microdomains enriched in these molecules.

Similarly to other sphingolipidosis (Pagano et al., "Membrane"), in FD fibroblasts LacCer is likely to be targeted to the endosomes/lysosomes rather than to the Golgi complex. As expected, these cells also showed accumulation of free cholesterol and complex GSLs in vesicular structures resembling lysosomes. Thus, secondary accumulation of GSLs related to cholesterol disturbed homeostasis might explain their intralysosomal trapping in AC--deficient human fibroblasts. Further work is currently being undertaken to clarify if the AC--deficiency is directly implicated in lipid imbalances here reported.

In conclusion, this study reinforces the notion that a correct balance of specific SL is required for normal cell functioning and, in this regard, Cer may display a central role. Membrane lipid rafts are assemblies of sphingolipids and cholesterol that act as signalling platforms to which different classes of proteins are associated (Lajoie et al.). Of notice ceramide-rich membrane domains (ceramide-rich lipid rafts) on the plasma membrane have also been reported (Xu et al.). Moreover displacement of sterol by Cer seems to have marked effects on lipid raft composition and physical properties (Alanko et al.; London; Yu et al., "Ceramide"). Interestingly, lipid and sphingolipid abnormalities including an increased level of Cer have also been reported in Alzheimer's disease (He et al.) which presents mechanisms of neurodegeneration related with lysosomal dysfunction (Nixon et al.). Furthermore lipid rafts have been implicated in the amyloidogenic processing of the amyloid precursor protein

(Vetrivel and Thinakaran). Thus, a deep knowledge of the nature, the subcellular locations, and the molecular mechanisms for sequestration of secondary storage compounds is crucial to understand if trafficking abnormalities caused by primary or secondary lysosomal dysfunction can be a common theme in neurodegenerative conditions regardless of the disease ethiology, and may also provide important information for biomarker identification and therapeutic options.

5. **ACKNOWLEDGMENTS**

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FIGURE 1A

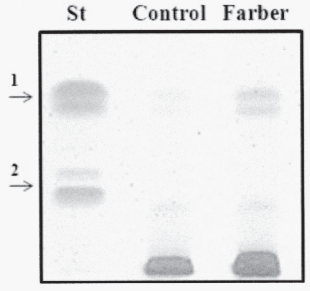

FIGURE 1B

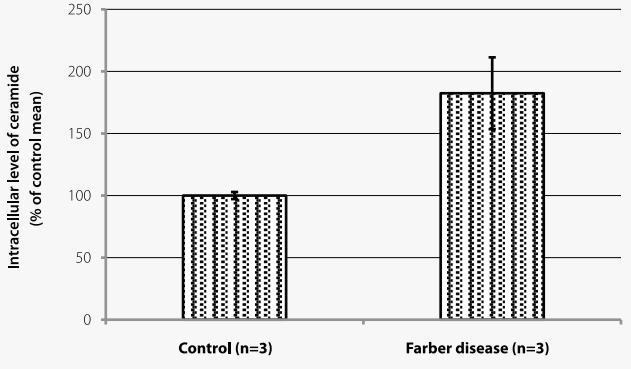

FIGURE 1C

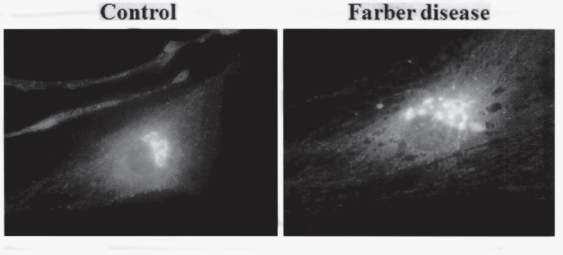

FIGURE 1 - Study of ceramide in cultured human skin fibroblasts. Normal control cells and FD cells were subjected to lipid extraction and analysis by TLC (A). The level of ceramide was quantified by densitometric scanning of the TLC plate (B). Data was expressed as percentage of the control mean value. St, standard ceramide; 1, ceramide with non--hydroxylated fatty acids; 2, ceramide with hydroxylated fatty acids. The band doublet represents fatty acids of distinct length (C16-C18 and C20-C24). The intracellular distribution of endocytosed BODIPY-C5-Cer was studied by fluorescence microscopy (C). Cells were observed with the following spectral window: excitation wavelength 450-490nm, and emission wavelength ≥ 520 nm.

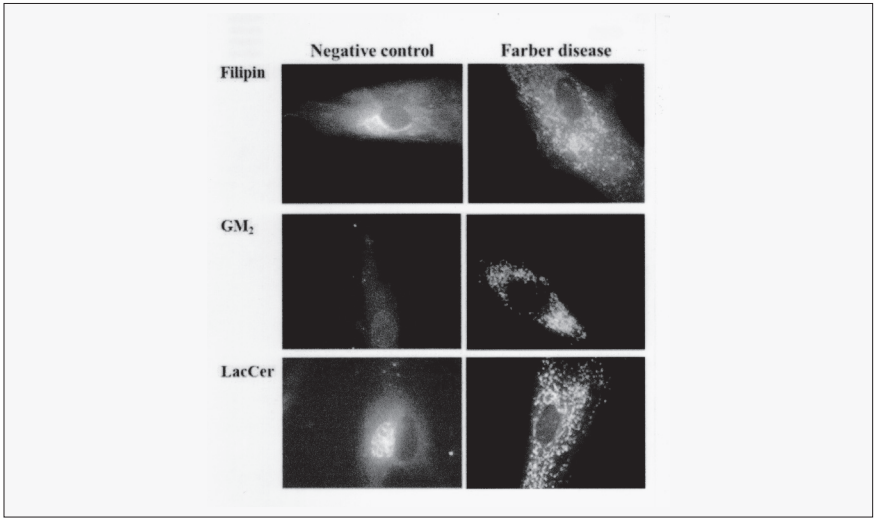

FIGURE 2 - Secondary lipid accumulation in cultured fibroblasts. Cells from normal controls and FD patients were analysed for free cholesterol (filipin staining) and glycosphingolipids as described in Materials and Methods. The representative pattern of normal and diseased fibroblasts is presented.