Mitochondrion 11 (2011) 342–350

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

Blood cells from Friedreich ataxia patients harbor frataxin deficiency without a loss of mitochondrial function

Mary A. Selak [a,*], Elise Lyver [b], Elizabeth Micklow [b], Eric C. Deutsch [c], Özlem Önder [d], Nur Selamoglu [d], Claire Yager [a], Simon Knight [b], Martin Carroll [b], Fevzi Daldal [d], Andrew Dancis [b], David R. Lynch [c], Jean-Emmanuel Sarry [b,*]

[a] Children's Hospital of Philadelphia Research Institute, Children's Hospital of Philadelphia and University of Pennsylvania, Philadelphia, PA, USA
[b] Division of Hematology/Oncology, University of Pennsylvania School of Medicine, Philadelphia, PA, USA
[c] Departments of Neurology and Pediatrics, University of Pennsylvania School of Medicine, and Children's Hospital of Philadelphia, Philadelphia, PA, USA
[d] Department of Biology, University of Pennsylvania, Philadelphia, PA, USA

ARTICLE INFO

Article history:
Received 2 August 2010
received in revised form 15 November 2010
accepted 3 December 2010
Available online 13 December 2010

Keywords:
Friedreich ataxia
Frataxin
Blood cells
Mitochondria
Biomarkers

ABSTRACT

Friedreich ataxia (FRDA) is an autosomal recessive neurodegenerative disorder caused by GAA triplet expansions or point mutations in the *FXN* gene on chromosome 9q13. The gene product called frataxin, a mitochondrial protein that is severely reduced in FRDA patients, leads to mitochondrial iron accumulation, Fe-S cluster deficiency and oxidative damage. The tissue specificity of this mitochondrial disease is complex and poorly understood. While frataxin is ubiquitously expressed, the cellular phenotype is most severe in neurons and cardiomyocytes. Here, we conducted comprehensive proteomic, metabolic and functional studies to determine whether subclinical abnormalities exist in mitochondria of blood cells from FRDA patients. Frataxin protein levels were significantly decreased in platelets and peripheral blood mononuclear cells from FRDA patients. Furthermore, the most significant differences associated with frataxin deficiency in FRDA blood cell mitochondria were the decrease of two mitochondrial heat shock proteins. We did not observe profound changes in frataxin-targeted mitochondrial proteins or mitochondrial functions or an increase of apoptosis in peripheral blood cells, suggesting that functional defects in these mitochondria are not readily apparent under resting conditions in these cells.

1. Introduction

Friedreich ataxia (FRDA) is an autosomal recessive neurodegenerative disorder caused by a GAA triplet expansion or point mutations in the frataxin gene (*FXN*) on chromosome 9q13 (Campuzano et al., 1996). Its gene product (FXN) is a ubiquitously expressed, nuclear-encoded mitochondrial protein that is severely reduced in FRDA patients. Deficits of FXN are associated with mitochondrial iron accumulation, deficient Fe-S cluster biogenesis, heme protein deficiency, increased sensitivity to oxidative stress, deficits of respiratory chain complex activities and *in vivo* impairment of tissue energy metabolism (Babcock et al., 1997; Foury and Cazzalini, 1997; Wilson and Roof, 1997; Rotig et al., 1997; Chantrel-Groussard et al., 2001; Pandolfo, 2002). Clinically, FRDA mostly impacts neural and cardiac tissue, even though frataxin is ubiquitously expressed in all tissues. Muscle and islet cells are also affected in these patients to a lesser extent. Thus, the tissue specificity of this mitochondrial disease is complex and poorly understood. Platelets and mononuclear cells are a non-invasive, readily accessible material that can be purified easily and are the main source of mitochondria in blood. We hypothesized that sub-clinical abnormalities exist in the mitochondria of blood cells from FRDA patients, because (i) frataxin is also expressed in blood cell mitochondria, (ii) iron homeostasis and heme synthesis are essential for these cells, (iii) and several antioxidant enzymes as well as glutathione homeostasis in blood cells of FRDA patients are impaired while serum iron and ferritin concentrations are normal. If mitochondria from blood cells are abnormal, such cells would provide a powerful, readily accessible means to analyze the impact of frataxin deficiency on mitochondrial metabolism of FRDA patients by proteomic and functional analyses. They could also provide tissue for use in identifying clinically useful biomarkers for diagnoses and evaluating progression of therapies.

In the present study, we have developed proteomic, metabolic and functional approaches to analyze the changes in mitochondria from

Abbreviations: 2-DGE, two-dimensional gel electrophoresis; ETCI, electron transport chain complex I; FRDA, Friedreich ataxia; FXN, frataxin; *FXN*, frataxin gene; HSP, heat shock protein; NHV, normal healthy donor; PBMC, peripheral blood mononuclear cell.

* Corresponding authors. Selak is to be contacted at USUHS, Department of Neurology, Room B-3009, 4301 Jones Bridge Road, Bethesda, MD 20814, USA. Sarry, Division of Hematology/Oncology, University of Pennsylvania School of Medicine, BRBII/III, 7th floor, Rm 723, 421 Curie Bld, Philadelphia, PA 19104, USA. Tel.: +1 215 573 7014.

E-mail address: jesarry@sas.upenn.edu (J.-E. Sarry).

1567-7249/$ – see front matter © 2010 Elsevier B.V. and Mitochondria Research Society. All rights reserved.
doi:10.1016/j.mito.2010.12.003

FRDA peripheral blood cells from FRDA patients. We have demonstrated that, while the frataxin protein level is decreased in blood cells of FRDA patients, this deficiency is associated only with a decrease in two mitochondrial heat shock proteins. However, we did not observe any profound change in target mitochondrial proteins or mitochondrial functions or an increase of apoptosis in peripheral blood cells.

2. Materials and methods

2.1. Patient characterization and samples process

Blood samples of 25 patients with FRDA and 15 normal healthy volunteers (NHV) as controls were obtained from subjects seen in the Department of Neurology, University of Pennsylvania School of Medicine. All protocols were approved by the IRB at the University of Pennsylvania and written informed consent was obtained from each subject.

Peripheral blood mononuclear cells (PBMCs) and platelets were purified by differential centrifugation and Ficoll gradients. The purity of the cell preparations was assessed by Wright–Giemsa and FACS analysis. We obtained 1–5 10^9 platelets and 50–100 10^6 peripheral blood mononuclear cells isolated from 30 mL of citrated peripheral blood draw of normal healthy volunteers and FRDA patients. We routinely observed purity greater than 85 and 96% for mononuclear cells and platelets, respectively, using FACS analysis and Giemsa–Wright Hema3 staining. Apoptosis was assayed by flow cytometry using 7-AAD and Annexin-V-APC as recommended by the manufacturer.

2.2. Lateral flow immunoassay (dipstick assay)

MitoSciences dipstick (MSF31) assays were used to measure frataxin levels in PBMCs and platelets. Briefly, 10 μg of PBMC protein or 5 μg of platelet protein in 25 μL of extraction buffer was mixed with 25 μL blocking buffer and added to individual wells on a 96-well plate with gold-conjugated mAb at the bottom of each well. After samples were allowed to equilibrate with the antibodies, dipsticks were inserted into the well and sample was allowed to wick up the membrane, where frataxin was immunocaptured onto designated capture zones on the dipstick. Capture zones on developed dipsticks were quantified with a Hamamatsu immunochromato reader (MS1000 Dipstick reader). A standard curve using a range (1–50 μg) of total protein extracted from PBMCs and platelets was run to determine an appropriate concentration of sample to use within the working range of the assay. Raw milliabsorbance units (mABS) were corrected for protein concentration and normalized to the control goat anti-mouse IgG band (internal positive control), and the data were expressed as percentages of the average controls run on the same assay.

2.3. SDS-PAGE and western blot analysis

40 μg of PBMC and platelet proteins was solubilized in SDS-PAGE sample loading buffer containing 80 mM dithiothreitol. Samples were denatured by heating to 95 °C for 5 min prior to electrophoresis on a 13% Laemmli-type gel. Proteins were electrophoretically transferred to Protran BA83 nitrocellulose membranes (Schleicher & Schuell) and briefly stained with a solution of amido black to position the protein markers. Membranes were incubated in the blocking solution (5% dry milk in TBST buffer (0.1 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20)) followed by incubations with appropriate primary antibodies, washing steps, and horseradish peroxidase-conjugated donkey anti-rabbit IgGs as secondary antibodies (Amersham Biosciences). The signal was detected by exposing the membrane to an x-ray film after incubating with luminol and p-coumaric acid as ECL substrates. The primary antibodies used were as follows: rabbit polyclonal antibodies against the GAPDH, cytochrome *c*, ACO1 and lipoic acid were purchased from Calbiochem. Anti-VDAC1, SOD1, SOD2, SDHC and FXN antibodies were mouse monoclonal antibodies obtained from Molecular Probes/Invitrogen and MitoSciences.

2.4. 2-D gel electrophoresis

Mitochondrial lysates were processed for 2-DGE as described previously (Sarry et al., 2006). Proteins in the lysates were precipitated by the addition of 10% (w/v) trichloroacetic acid dissolved in ice-cold acetone and centrifuged at 16,000 g for 20 min. After two washes with ice-cold acetone containing 0.05% (w/v) dithiothreitol, protein pellets were resuspended in isoelectric focusing loading buffer containing 2 M thiourea, 6 M urea, 4% (w/v) Chaps and 50 mM dithiothreitol, incubated with stirring at room temperature for 1 h, and centrifuged at 16,000 g for 15 min. Aliquots (300 μL) of the isoelectric focusing buffer containing 200 μg protein and supplemented with 0.2% (v/v) pH 3–10 ampholines were loaded onto the IPG strips (pH 3–10 NL; 11 cm; Bio-Rad) by passive rehydration for 12 h and active rehydration at 50 V for 8 h. Isoelectric focusing was performed for 120 kVh in a Protean IEF cell (Bio-Rad) at 20 °C. Voltage was increased linearly from 50 to 200 V for the first 3 h, held at 200 V for another 3 h, before being increased linearly to 1000 V over the next 3 h and held at that voltage for another 3 h. In the final two periods of 6 h each, the voltage was increased linearly to 5000 V and held at that value. Upon completion of the isoelectric focusing separations, the isoelectric focusing strips were equilibrated with 50 mM Tris/HCl (pH 8.8) containing 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 2% (w/v) dithiothreitol for 20 min with gentle stirring before the addition of 2.5% (w/v) iodoacetamide, and alkylation of the samples for 20 min. Electrophoresis in the second dimension was performed on Criterion 8.7 × 13.3 cm precast 8–16% (w/v) polyacrylamide gradient gels (Bio-Rad) at 50 V for the first hour and at 200 V for the second hour in a Protean Cell Criterion apparatus (Bio-Rad).

For analyses of the protein profiles, the gels were stained with colloidal Coomassie Brilliant Blue G-250 (GE Healthcare), scanned with an Epson flatbed scanner, and the TIFF files generated using Adobe Photoshop 7.0 were subjected to spot detection, matching and quantification using the PDQuest software package (v. 7.2, Bio-Rad). Three-dimensional Gaussian images of the spots were generated and their volumes were estimated after background subtraction. The intensity of each spot was normalized to the sum of the total spots on the gel such that the integrated and normalized intensity for each spot was expressed as a percentage of the sum of the intensities of all the spots in the Gaussian image.

2.5. Protein identification by mass spectrometry

The Coomassie Brilliant Blue-stained protein spots on the gels were excised manually and transferred to 96-well microtiter plates for fully automated washing, destaining, tryptic digestion, elution, and preparation for LC-QIT-MS/MS in a Multiprobe II MassPREP Station (Waters/Micromass, Inc., Milford, MA). Routinely, the excised gel plugs were washed several times for 15 min, first with aqueous 50 mM ammonium bicarbonate (pH 7.8) and then with 50% (v/v) acetonitrile containing 50 mM ammonium bicarbonate before dehydration for 5 min in acetonitrile. The gel plugs were digested at 37 °C in 25 μL 50 mM ammonium bicarbonate (pH 7.8) containing 6 μg mL^{-1} trypsin (sequencing grade, modified, Promega) for 7 h. The resulting peptide digests were successively extracted in 20 μL 0.1% (v/v) formic acid in 2% (v/v) acetonitrile, and with 0.1% (v/v) formic acid in 50% (v/v) acetonitrile.

The LC-QIT-MS/MS analyses were performed on an LCQ Ion Trap MS (Deca XP Plus, Thermo-Finnigan, San Jose, CA). The LC-QIT-MS/MS was interfaced to a Famos autosampler (LC-Packings, Sunnyvale, CA) which was used to sample the tryptic digests after their resuspension