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Friedreich's ataxia: Oxidative stress and cytoskeletal abnormalities

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ABSTRACT

Friedreich's ataxia (FRDA) is an autosomal recessive disorder caused by mutations in the gene encoding frataxin, a mitochondrial protein implicated in iron metabolism. Current evidence suggests that loss of frataxin causes iron overload in tissues, and increase in free-radical production leading to oxidation and inactivation of mitochondrial respiratory chain enzymes, particularly Complexes I, II, III and aconitase. Glutathione plays an important role in the detoxification of ROS in the Central Nervous System (CNS), where it also provides regulation of protein function by glutathionylation. The cytoskeletal proteins are particularly susceptible to oxidation and appear constitutively glutathionylated in the human CNS. Previously, we showed loss of cytoskeletal organization in fibroblasts of patients with FRDA found to be associated with increased levels of glutathione bound to cytoskeletal proteins. In this study, we analysed the glutathionylation of proteins in the spinal cord of patients with FRDA and the distribution of tubulin and neurofilaments in the same area. We found, for the first time, a significant rise of the dynamic pool of tubulin as well as abnormal distribution of the phosphorylated forms of human neurofilaments in FRDA motor neurons. In the same cells, the cytoskeletal abnormalities co-localized with an increase in protein glutathionylation and the mitochondrial proteins were normally expressed by immunocytochemistry. Our results suggest that in FRDA oxidative stress causes abnormally increased protein glutathionylation leading to prominent abnormalities of the neuronal cytoskeleton.

1. Introduction

Friedreich's ataxia (FRDA) is an autosomal recessive disorder, neuropathologically characterized by prominent degeneration of the spinal cord pathways, with predominant neuronal loss in the dorsal root ganglia and in the Clarke's columns, together with degeneration of their long tracts as well as involvement of the pyramidal tracts [1].

Most FRDA patients are homozygous for expanded GAA triplet-repeat sequences (E alleles) in intron 1 of the *FXN* gene on chromosome 9q13 [2]. This mutation causes the formation of a "sticky" triplex DNA structure that interferes with correct transcription and reduces the synthesis of frataxin, a mitochondrial protein of 210 aminoacids, which is normally expressed at high levels in the human spinal cord [3,4].

There is evidence that frataxin is closely involved in the early steps of the iron–sulphur cluster (ISC) biosynthesis and acts as an iron-storage protein, keeping iron in a non-toxic and bioavailable form [5–7]. Therefore, an excess of unbound iron in mitochondria, resulting from frataxin deficiency, might induce the oxidation of cellular components and the inactivation of mitochondrial enzymes, through its capacity to produce free radicals [8]. Accordingly, yeast lacking the frataxin homologue gene (YFH1) shows a severe defect of mitochondrial respiration, intramitochondrial iron accumulation and increase sensitivity to oxidative stress [9]. Moreover, selective knockdown of frataxin in murine heart muscle causes decreased activity particularly of mitochondrial Fe–S dependent enzymes, i.e. Complexes I, II, III and aconitase, and late-stage iron accumulation [10].

The role of oxidative stress in the pathophysiology of the disease is still controversial. No evidence of oxidative stress has been found in studies of conditional knockout mouse models [11], whereas

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"humanized" GAA repeat expansion mouse models of FRDA exhibited oxidative stress leading to progressive neuronal and cardiac pathology [12].

Moreover, other studies have found increased plasma and urinary levels of oxidative stress markers in FRDA patients [13–17] and have demonstrated that some antioxidants, such as idebenone and vitamin E, might have a protective role [18,19].

Several lines of evidence suggest that the disarray of microfilament and microtubule network represents one of the early events in the degenerative process of neurons exposed to oxidative stress [20,21]. In differentiated PC-12 cells, for instance, neurons exposed to 100 μM H_2O_2 present microtubules depolymerization and cell death [22]. Furthermore, it has been demonstrated that oxidative stress might negatively interfere with the phosphorylation of neurofilaments, leading to an impairment of the axonal transport [23].

We moved from the observation of abnormal cytoskeletal organization in fibroblasts of FRDA patients that were associated to abnormally increased levels of glutathione bound to cytoskeletal proteins [14].

Presently, we studied by immunohistochemistry the spinal cord from four autoptic cases of FRDA to better understand the pathogenesis of neuronal degeneration in FRDA. For this purpose, we first tried to confirm in the human spinal cord of FRDA patients with frataxin deficiency some evidence of mitochondrial dysfunction in the respiratory chain enzymes. Then, as abnormally increased glutathionylation of proteins represents a sensitive redox-marker of tissue oxidative stress, we studied the amount and the distribution of glutathionylated proteins in the same area of the CNS using a monoclonal antibody (ab) that specifically reacts with glutathione bound to proteins (GS-Pro). Finally, because cytoskeletal protein thiols are particularly susceptible to oxidation, we analysed the expression of some cytoskeletal proteins that are essential for neuronal integrity and function.

2. Materials and methods

2.1. Human samples

Tissues were obtained at autopsy, performed 2 to 10 h after death, from four patients with FRDA. They included two females (patient 2 and 4; 25 and 33 years old, respectively) and two males (patient 1 and 3; 47 and 24 years old, respectively). All patients met the diagnostic Harding criteria for typical FRDA [24] that include onset before the age of 25, progressive limb and gait ataxia, absent tendon reflexes in the legs, followed by (within 5 years of presentation) dysarthria, areflexia at all four limbs, signs of pyramidal tract dysfunction in the legs, and distal loss of position and vibration sense [3,24]. The diagnosis was genetically confirmed by detecting the abnormal GAA expansion in the frataxin gene in cases 1 and 2. Technical reasons (i.e. the amount of tissue available) did not allow to perform the molecular genetic analysis in the remaining two cases. As controls, we used comparable specimens from ten age-matched individuals with no history of neurological diseases and no neuropathological lesions at post-mortem examination. None of the patients used as controls had suffered major respiratory stress/distress or other conditions (i.e. prolonged hypoxia, infective diseases or toxic injury, etc.) that could lead to tissue oxidative stress. Consent was obtained from our Institutional Ethic Committee for using human material for research. Samples used for immunohistochemical studies were fixed in 10% neutral formalin at room temperature for several weeks, sliced, and embedded in paraffin. Immunohistochemical studies were performed on transverse sections through upper and lower cervical segments, and through lower lumbar segments of the spinal cord.

2.2. Molecular genetic analyses

Molecular analyses were performed on tissues extracted from paraffin-embedded material as reported in [25]. Detection of E alleles in tissues and sizing of the "constitutional or most common" allele were estimated by a conventional polymerase chain reaction (PCR)-based methodology as outlined in [26]. Briefly, adopting oligonucleotide primers GAA–104F: (5′–3′) GGCTTAAACTTCCCACACGTGTT and GAA–629R: (5′–3′) AGGACCATCATGGCCACACTT we PCR amplified genomic DNA using the Boehringer-Mannheim Long Template PCR system. PCR conditions were 94 °C for 2 min, 17 cycles at 94 °C for 10 s, 68 °C for 2 min 30 s followed by 20 cycles of 94 °C for 10 s, 68 °C for 2 min 30 s with the addition of 20 s to the extension time per cycle, and a final extension at 68° for 10 min. This generates a PCR product of $(500 + 3n)$ base pairs, where n is the number of GAA repeats. The size of the PCR product was estimated using appropriate size standards (Invitrogen) on agarose 0.8% gel stained with ethidium bromide. The GAA repeat expansions were 615/681 for case 1 and 650 for case 2.

2.3. Immunohistochemistry

Transverse 4 μm-thick formalin fixed and paraffin-embedded sections of the samples were studied immunohystochemically with the following antibodies (abs) diluted from 1:5 to 1:500 in phosphate-buffered saline (PBS):

- monoclonal ab against glutathione bound to proteins (*GS-Pro*) (Virogen, Watertown, MA) [14,27,28];
- monoclonal ab against human frataxin (*frataxin*) (Immunological Sciences, Rome, Italy);
- monoclonal ab against the 30 kDa ISC-containing subunit II of Complex II (*CII Ip*) (Molecular Probes, Eugene, OR, USA);
- monoclonal ab against subunit IV of cytochrome *c* oxidase (*COX IV*) (Molecular Probes, Eugene, OR, USA);
- monoclonal ab against the human C-terminal β-tubulin (*tubulin*) (Sigma, St. Louis, MI, USA);
- monoclonal ab against the N-terminal heavy chain of human neurofilaments (*NF-H*) (Lab Vision, Fremont, CA, USA);
- monoclonal ab against the nonphosphorylated epitope in neurofilament H (*SMI 32*) (Covance, Berkeley, CA, USA);
- monoclonal ab against the phosphorylated form in NF-H (*SMI 34*) (Covance, Berkeley, CA, USA);
- polyclonal ab against tyrosinated tubulin (*Tyr-Tub*) (Chemicon International, Temecula, CA, USA);
- polyclonal ab against detyrosinated tubulin (*Glu-Tub*) (Chemicon International, Temecula, CA, USA).

Sections were immunostained with the avidin–biotin–peroxidase complex (ABC) according to previous published techniques [28,29].

Nuclear counter-staining was performed with Mayer hematoxylin solution. The primary ab was omitted in immunocytochemical control sections. In order to compare staining intensity, the tissues of patients and controls were processed together in single runs, exactly in the same labelling conditions of antibody exposure and background blocking.

3. Results

3.1. Immunohistochemistry

Samples of the spinal cord, which is known to be predominantly affected by neuronal degenerative lesions in FRDA [1], were studied immunohistochemically.

To confirm whether the reduced expression of frataxin was associated with mitochondrial respiratory chain enzymes deficiency in the spinal cord, cervical and lumbar sections from control individuals and patients were incubated with abs against frataxin and against the subunit II of Complex II (CII Ip) and the subunit IV of cytochrome *c* oxidase (COX IV) of the mitochondrial respiratory chain. Immunoreactivity for frataxin was absent or markedly reduced in the gray matter neurons as well as in cells and axons of the white matter of the four FRDA patients (Fig. 1), while a normal immunostaining was