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Original Contribution

In inclusion-body myositis muscle fibers Parkinson-associated DJ-1 is increased and oxidized

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ABSTRACT

Sporadic inclusion-body myositis (s-IBM) is the most common muscle disease of older persons. The muscle-fiber molecular phenotype exhibits similarities to both Alzheimer-disease (AD) and Parkinson-disease (PD) brains, including accumulations of amyloid-β, phosphorylated tau, α-synuclein, and parkin, as well as evidence of oxidative stress and mitochondrial abnormalities. Early-onset autosomal-recessive PD can be caused by mutations in the DJ-1 gene, leading to its inactivation. DJ-1 has antioxidative and mitochondrial-protective properties. In AD and PD brains, DJ-1 is increased and oxidized. We studied DJ-1 in 17 s-IBM and 18 disease-control and normal muscle biopsies by: (1) immunoblots of muscle homogenates and mitochondrial fractions; (2) real-time PCR; (3) oxyblots evaluating DJ-1 oxidation; (4) light- and electron-microscopic immunocytochemistry. Compared to controls, in s-IBM muscle fibers DJ-1 was: (a) increased in the soluble fraction, monomer 2-fold ($P = 0.01$), and dimer 2.8-fold ($P = 0.004$); (b) increased in the mitochondrial fraction; (c) highly oxidized; and (d) aggregated in about 15% of the abnormal muscle fibers. DJ-1 mRNA was increased 3.5-fold ($P = 0.034$).

Accordingly, DJ-1 might play a role in human muscle disease, and thus not be limited to human CNS degenerations. In s-IBM muscle fibers, DJ-1 could be protecting these fibers against oxidative stress, including protection of mitochondria.

Introduction

Sporadic inclusion-body myositis (s-IBM), the most common, progressive muscle disease associated with aging, is manifested by pronounced muscle weakness and wasting, leading to severe disability (reviewed in [1]). There is no successful treatment. The pathology of s-IBM muscle biopsies is characterized by: (a) vacuolar degeneration and atrophy of muscle fibers, accompanied by intramuscle-fiber accumulations of misfolded, ubiquitinated, congophilic, multiprotein aggregates; and (b) lymphocytic inflammation (reviewed in [2–4]). An intriguing feature of the s-IBM muscle-fiber phenotype is its similarity to both the Alzheimer-disease (AD) brain and Parkinson-disease (PD) Lewy bodies, including accumulation of aggregated amyloid-β (Aβ), phosphorylated tau, and several other Alzheimer-characteristic proteins, as well as α-synuclein (α-syn) and parkin (reviewed in [2,4]). Also similarly to AD and PD, oxidative stress and mitochondrial abnormalities are evident in s-IBM [4,5].

DJ-1 is a ubiquitously expressed protein of the ThiJ/PfpI/DJ1 superfamily, which is highly conserved across species (reviewed in [6,7]). Mutations in the DJ-1 gene, preventing expression of DJ-1 protein, are a cause of early-onset autosomal-recessive PD [6–9]. In sporadic AD and PD brains, DJ-1 was reported to be increased and highly oxidized [10]. Although its precise functions are not yet known, DJ-1 has been proposed to act as an antioxidant [11–15] and be an important mitochondrial protective agent [14,15]. The role of DJ-1 in muscle diseases has not been studied, to our knowledge.

Because of the molecular and pathological similarities of s-IBM muscle fibers to AD and PD brains, we asked whether alterations of DJ-1 occur in s-IBM muscle fibers.

Material and methods

Muscle biopsies

Studies were performed on portions of diagnostic muscle biopsies obtained (with informed consent) from 17 s-IBM and 18 age-matched-control biopsies, including 2 dermatomyositis , 2 polymyositis , 1 morphologically nonspecific myopathy, 2 amyotrophic lateral sclerosis, 2 peripheral neuropathy, and 9 normal muscles (considered normal after all diagnostic tests were performed). Not all studies were performed on all biopsies (details below). s-IBM patients were ages

Abbreviations: Aβ, amyloid-β; AD, Alzheimer disease; DNP, 2,4-dinitrophenyl; DNPH, 2,4-dinitrophenylhydrazine; PD, Parkinson disease; qRT-PCR, quantitative real-time polymerase chain reaction; s-IBM, sporadic inclusion-body myositis; α-syn, α-synuclein.

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61–87 years, median age 73; normal control patients were ages 61–86, median age 72. Diagnoses were based on clinical and laboratory investigations, including our routinely performed 16-reaction diagnostic histochemistry of the biopsies. All s-IBM biopsies met s-IBM diagnostic criteria, as we described [16].

Immunoblots

These were performed to: (a) evaluate the specificity of DJ-1 antibodies rabbit polyclonal [Abcam, Boston, MA] and mouse monoclonal [Stressgene, Victoria, BC, Canada] in human muscle, both having been previously characterized in human and mouse brain and in various cultured cells [9,15,17]; and (b) determine the content of DJ-1 in s-IBM and normal-control muscle biopsies. Because previous studies reported a difference of DJ-1 content between the soluble and insoluble fractions of brain tissue and in cells overexpressing DJ-1 [17,18], in the present study we performed immunoblots of DJ-1 on soluble and insoluble fractions of total homogenates of 5 s-IBM and 5 age-matched normal-control muscle biopsies. The detergent-soluble/insoluble fractionation was modified from a method described previously [19]. In brief, twenty 10-μm-thick frozen-muscle sections were collected at -25°C, rapidly suspended in lysis buffer (20mM Hepes [pH 7.4] containing 120mM NaCl, 5mM EDTA, 10% glycerol, 1% Triton X-100 and supplemented with complete protease inhibitors [Roche Diagnostics, Mannheim, Germany]). Muscle fibers and their intracellular organelles were disrupted using an ultrasonic homogenizer. After incubation for 30min at 4°C, the suspensions were separated by centrifugation at 16,000*g* for 1h at 4°C. Supernatants were collected as the soluble fraction, and were considered to contain cytoplasmic proteins, as well as the content of the disrupted organelles, including mitochondria.

The insoluble pellet was washed four times with ice-cold lysis buffer, solubilized in SDS (final concentration 1%) for 1h at 60°C, and centrifuged at 16,000*g* for 30min at 4°C. The supernatants were collected as the insoluble fraction.

Twenty micrograms of protein (measured by the Bradford method) was loaded on 10% NuPAGE gels (Invitrogen, Carlsbad, CA), electrophoresed, transferred to nitrocellulose or PVDF membranes, and immunoprobed with an antibody against DJ-1 diluted 1:1500, as we described [20–22].

The blots were developed using the enhanced chemiluminescence system (Amersham Bioscience, Piscataway, NJ). Protein loading was evaluated by actin bands visualized with a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:2000. Quantification of immunoreactivity was performed by densitometric analysis using NIH Image 1.310 software. Intensity of the band of interest was calculated in relation to intensity of the actin band. Omission of a primary antibody was the control for reaction specificity.

RNA isolation and qRT-PCR

Total RNA from 5 s-IBM and 6 age-matched control muscle biopsies was isolated using an RNA isolation kit (BD Pharmingen, San Diego, CA), as recently described [22]. One microgram of RNA was subjected to genomic-DNA removal, and then cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Real-time PCR (qRT-PCR) was performed in duplicate at total volume of 25 μl, using 1 μl of cDNA, QuantiTect Primers (Qiagen) for PARK7/DJ1 or GAPDH, and QuantiFastSYBR Green PCR Master mix (Qiagen). PCR runs were performed on an Eppendorf Mastercycler *realplex*^{2}. Cycling conditions were 95°C for 5min, followed by 40 cycles of 95°C for 10s and 60°C for 30s. Relative gene expression was calculated by using the $2^{-\Delta\Delta CT}$ method, in which the amount of DJ-1 mRNA was normalized to an endogenous reference (GAPDH). The results are expressed as fold induction relative to controls. Correct PCR products were confirmed by agarose gel electropheresis and melting curve analysis.

Light-microscopic immunocytochemistry

To determine the localization of DJ-1 in human muscle fibers, we performed immunofluorescence on 10-μm-thick unfixed sections of fresh-frozen diagnostic muscle biopsies, as described [20–23], using either the rabbit polyclonal or the mouse monoclonal antibodies, diluted 1:100. In addition to the DJ-1 immunofluorescence, some sections were counterstained with the nuclear marker Hoechst 33342 (Invitrogen/Molecular Probes). Immunostainings were performed on 4 s-IBM, 4 age-matched normal controls, and all disease controls listed above. To block nonspecific binding of an antibody to Fc receptors, sections were preincubated with normal goat serum diluted 1:10 [20–23]. Controls for staining specificity were omission of the primary antibody, or its replacement with nonimmune sera or irrelevant antibody; these were always negative.

Gold immunoelectron microscopy

This was performed on 10-μm unfixed frozen sections adhered to the bottom of 35-mm petri dishes, as detailed previously [20–23]. For double immunostaining, sections were incubated concurrently in rabbit polyclonal antibody against DJ-1, combined with mouse monoclonal antibody against the mitochondrial marker porin/VDAC (Invitrogen/Molecular Probes), followed by incubation in two different specie-specific secondary antibodies, one conjugated to 12-nm gold particles and the other conjugated to 6-nm gold particles. Subsequently, the sections were fixed in a 2% paraformaldehyde–1.25% glutaraldehyde mixture, postfixed in 1% osmium tetroxide, and Epon-embedded in situ in the petri dish. The embedded sections in the dish were viewed under phase-contrast microscopy, and the muscle fibers of interest were marked, drilled-out, and processed for electron microscopy, as described [20–23].

Isolation of mitochondrial fractions

This was performed from four s-IBM and four age-matched control muscle biopsy samples using a four-step procedure, essentially as previously described by others [24]. Briefly, thirty 10-μm sections of fresh-frozen human muscle biopsies were placed in a prechilled microcentrifuge tube containing 200μl of isolation medium (320mM sucrose, 1mM EDTA, 10mM Trizma-base, pH 7.4). Step 1: after thorough but gentle manual homogenization on ice, the homogenate was centrifuged at 3000*g* for 5min at 4°C, and the pellet discarded. Step 2: the supernatant was centrifuged at 12,000*g* for 10min at 4°C. Step 3: the resulting pellet (crude mitochondrial fraction) was resuspended in 1ml isolation medium, and centrifuged at 3000*g* to remove nuclear contamination. Step 4: the resulting pellet was discarded and the supernatant was centrifuged at 12,000*g* for a further 10min. Steps 3 and 4 were repeated to further purify the mitochondrial fraction. The resulting pellet from the last 12,000*g* spin constituted the mitochondria-enriched fraction [24].

Immunoprecipitation, 2,4-dinitrophenyl (DNP) derivatization, and oxyblot

To evaluate the presence of carbonyl groups indicative of DJ-1 oxidation, we performed an immunoprecipitation/protein-derivatization/immunoblot procedure in four s-IBM and four age-matched control muscle biopsies, and in one dermatomyositis muscle biopsy. Because human muscle biopsies are rather sparse, we were not able to perform this procedure on more samples. The amount of 100μg of muscle homogenate prepared as for immunoblots (above) was immunoprecipitated for 1h at 4°C with the DJ-1 rabbit polyclonal antibody that had been cross-linked to protein-A Dynabeads (Invitrogen), according to the manufacturer's protocol. After extensive washing, the DJ-1- immunoprecipitated complex was eluted from the