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Novel demonstration of conformationally modified tau in sporadic inclusion-body myositis muscle fibers

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ABSTRACT

s-IBM is the most common muscle disease of older persons. Its muscle fiber molecular phenotype has close similarities to Alzheimer disease (AD) brain, including intra-muscle-fiber accumulations of (a) Aβ42 and its oligomers, and (b) large, squiggly or linear, clusters of paired-helical filaments (PHFs) that are immunoreactive with various antibodies directed against several epitopes of phosphorylated tau (p-tau), and thereby strongly resembling neurofibrillary tangles of AD brain. In AD brain, conformational changes of tau, including its modifications detectable with specific antibodies TG3 (recognizing phosphorylated-Thr231), and Alz50 and MC1 (both recognizing amino acids 5–15 and 312–322) are considered early and important modifications leading to tau's abnormal folding and assembly into PHFs. We have now identified conformationally modified tau in 14 s-IBM muscle biopsies by (a) light-and electron-microscopic immunohistochemistry, (b) immunoblots, and (c) dot-immunoblots, using TG3, Alz50 and MC1 antibodies. Our double-immunolabeling on the light- and electron-microscopic levels, which combined an antibody against p62 that recognizes s-IBM clusters of PHFs, revealed that TG3 immunodecorated, abundantly and exclusively, all p62 immunopositive clusters, while Alz50 labeling was less abundant, and MC1 was mainly diffusely immunoreactive. Interestingly, in the very atrophic degenerating fibers, TG3 co-localized with PHF-1 antibody that recognizes tau phosphorylated at Ser396/404, which is considered a later change in the formation of PHFs; however, most of TG3-positive inclusions in non-atrophic fibers were immunonegative with PHF-1. None of the 12 normal- and disease-control muscle biopsies contained conformational or PHF-1 immunoreactive tau. This first demonstration of conformational tau in s-IBM, because of its abundance in non-atrophic muscle fibers, suggests that it might play an early role in s-IBM PHFs formation and thus be pathogenically important.

Sporadic inclusion-body myositis (s-IBM), the most common, but untreatable muscle disease of older persons (after age 50), appears pathogenically multifactorial. Its muscle biopsy pathologic phenotype is comprised of uniquely multifaceted degeneration of muscle fibers, plus extracellular T-cell inflammation (recently reviewed in [1,2,4,7]). The triggering pathogenic event is not known. Also unknown is which aspect, degenerative or inflammatory, is the primary component of the s-IBM pathogenesis. However, it is becoming more apparent that an s-IBM-characteristic muscle-fiber degeneration leads to the muscle-fiber atrophy and death, and is responsible for severe muscle weakness.

Pathologically, the unique type of degeneration of s-IBM muscle fibers is characterized by (a) their vacuolization and atrophy, and (b) accumulation, mainly within their non-vacuolated cytoplasm, of ubiquitinated, congophilic multiprotein aggregates [1,2,4]. The exact mechanisms leading to the formation of these intra-muscle-fiber proteinacious aggregates is not yet known, but the co-existing impairment of the 26S proteasome and defective lysosomal degradation likely contribute [2,4,9,29]. Endoplasmic reticulum stress has been also demonstrated to importantly contribute to s-IBM pathogenesis [2,4].

An intriguing feature of the s-IBM muscle-fiber molecular phenotype is its close similarity to the Alzheimer disease (AD) brain which, in addition to other aspects, also includes ER stress and impaired protein degradation [20,25,27]. Other important similarities include intra-muscle-fiber accumulations of several Alzheimer-characteristic proteins, for example: (a) Aβ42 and its oligomers [1,2,22,24,28,34] and (b) large clusters of paired-helical filaments (PHFs) immunoreactive with various antibodies directed against several epitopes of phosphorylated tau (p-tau), which strongly resemble clusters of PHFs comprising intra-neuronal neurofibrillary tangles of AD brain [1–4,10,11,13,26,30]. Both the AD

Abbreviations: AD, Alzheimer disease; PHFs, paired-helical filaments; s-IBM, sporadic inclusion-body myositis.

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intraneuronal NFTs [23] and clusters of PHFs in s-IBM muscle fibers are congophilic [26].

Moreover, similarly to AD brain [14,31], several kinases involved in tau phosphorylation, including CDK5, active GSK3β, ERK1/2, and casein kinase 1α, are increased and accumulated in association with PHFs in s-IBM muscle fibers [19,33,36,37], suggesting that similar mechanisms leading to tau phosphorylation participate in both diseases.

In addition to phosphorylation, conformational changes of tau, involving its intramolecular folding into a pathologically significant orientation, were reported to importantly contribute in PHFs formation in AD brain [6,17,35]. Those conformational changes of tau—including tau amino acids 5–15 and 312–322 (recognized by Alz50 and MC1 antibodies), and tau phosphorylated on threonine 231, recognized by TG3 antibody—are considered early and important factors of posttranslational modification of tau leading to its abnormal folding and assembly into PHFs [15–17,35].

To determine whether conformationally modified tau (a) is present in s-IBM muscle, and (b) possibly precedes other PHFs abnormalities, we performed immunocytochemistry, immunoblots, and dot-immunoblots using TG3, Alz50, and MC1 antibodies [16,17,35] and compared their immunoreactivities to the immunoreactivity achieved with the well-characterized antibody PHF-1 (which recognizes phosphorylated Ser396/404 of the tau molecule, and immunoreactivity of which was previously demonstrated in s-IBM muscle fibers [26,37]).

Herein we have demonstrated for the first time conformationally modified tau in s-IBM muscle fibers, and we also show that, because of its abundance in non-atrophic, normally appearing muscle fibers, the conformational tau might play an early role in formation of the s-IBM PHFs, and thus be an important aspect of the s-IBM pathogenesis.

Studies were performed on portions of fresh-frozen diagnostic muscle biopsies obtained, with informed consent, from 14 s-IBM, 2 polymyositis, 2 amyotrophic lateral sclerosis, and 2 peripheral neuropathy patients, and 6 age-matched normal controls, who, after all tests were performed, were considered free of muscle disease. The s-IBM patients were ages 52–78 years, median 62; control patients were ages 50–56 years, median 52. Patient diagnoses were based on clinical and laboratory investigations, including our routinely performed 16-reaction diagnostic histochemistry of the muscle biopsies. All s-IBM biopsies met s-IBM diagnostic criteria by having muscle fibers with: vacuoles on Engel trichrome staining; paired-helical filaments by p62-immunoreactivity; and Congo-red-positivity using fluorescence enhancement [1,2].

a) Well-characterized monoclonal antibodies, MC1, Alz50 and TG3 against conformational tau referenced in [15–17,35] were generously provided by Dr. Peter Davies. Immunohistochemically, in AD brains, but not in normal brains, both Alz50 and MC1 recognize a similar pathological conformation of tau that is dependent on identical portion of epitope of non-phosphorylated but uniquely conformationally modified tau in a folded structure [15,16,35]. In AD brain, MC1 stains "pretangle neurons", and its immunopositivity is indicative of very early neuronal pathological changes [35]. Alz50 stains both pretangle-neurons, as well as early-formed paired-helical filaments [16]. We have previously demonstrated that some of the PHFs in s-IBM muscle fibers are immunopositive with Alz50 antibody [2,3].

   TG3, stains neuritic plaques and neurofibrillary tangles (NFTs) in AD brain [17,35], but it is not immunoreactive in normal brain. TG3 has a very high specificity for early AD pathology [35].

   In AD brain and in the 3xTg-AD mice, PHF-1 antibody stains well-formed NFTs, and its immunopositivity appears later in AD pathology [8,24,35]. Its specificity, and its exclusive staining of some of the s-IBM PHFs have previously been reported [26,37].

b) The specificity of the p62 antibody (H-290, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in s-IBM muscle has been previously described by us [30]. In s-IBM muscle fibers, p62 exclusively stained clusters of PHFs, where it co-localized with the staining achieved with AT100 antibody that recognizes phosphorylated tau [30].

To confirm the specificity of Alz50, MC1 and TG3 in s-IBM muscle biopsies, ten 10-μm thick fresh-frozen sections of s-IBM and age-matched control muscle biopsies were sonicated in RIPA buffer containing protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany) and Halt Phosphatase Inhibitor Cocktail (Pierce, Rockford, IL, USA) [9,29,30,33]. 100 μg of protein were heat-denatured for 10 min at 100 °C in Laemmli buffer. Subsequently, the proteins were loaded into 10% Bis–Tris gel and electrophoretically separated in NuPAGE MES SDS running-buffer. All reagents were obtained from Invitrogen (Carlsbad, CA, USA). After electrophoresis, samples were transferred to a nitrocellulose membrane (Amersham, Biosciences, Piscataway, NJ, USA). To prevent non-specific binding of antibodies, the nitrocellulose membranes were blocked in 5% blocking agent (Amersham)—they were then incubated overnight at 4 °C with either Alz50, MC1 or TG3 antibodies. Blots were developed using the Western Breeze chemiluminescent kit (Invitrogen). Protein loading was evaluated by the actin band (C-2, 1:500; Santa Cruz Biotechnology).

Dot-immunoblots were performed in 6 s-IBM and 4 control muscle biopsies using MC1, Alz50 or TG3 antibodies, as previously described for Aβ oligomers [28]. To preserve the original/intrinsic tau conformation, the samples were not heated and no detergent was added. They were sonicated in Tris-buffered saline (pH 7.4) supplemented with Halt Phosphatase Inhibitor Cocktail (Pierce) [16]. For both control and s-IBM samples, equal amounts of protein (8 μg for Alz50, and 10 μg for TG3 and MC1) was applied to a nitrocellulose membrane and air dried for 45 min. Subsequently, the membrane was blocked in 5% membrane-blocking agent (Amersham) in Tris-buffered saline and 0.02% Tween 20 for 1 h at room temperature. Subsequently, after 1 h incubation with one of the specific antibodies, dot-immunoblots were developed using the Western Breeze chemiluminescent kit (Invitrogen). All dot-immunoblots were performed in duplicate, and elimination of a primary antibody from s-IBM samples served as specificity control.

Immunofluorescence was performed, as described [3,9,26,30,36], on 10-μm transverse sections of 10 s-IBM, 1 normal-control, and 6 other disease-control muscle biopsies, as specified above. Double-immunofluorescence utilizing TG3 antibody with a rabbit polyclonal anti-p62 antibody was performed as described [3,9,26,30,36]. In addition, some sections immunostained with TG3 antibody were counterstained with the nuclear marker Hoechst 33342 (Invitrogen). To block non-specific binding of an antibody to Fc receptors, sections were pre-incubated with normal goat serum diluted 1:10 [3,9,26,30,36]. Controls for staining specificity were (i) omission of the primary antibody, or (ii) its replacement with non-immune sera or irrelevant antibody. These were always negative. In order to compare the frequency of muscle fibers immunoreactive with TG3 antibody to that of PHF-1 antibody, two closely adjacent serial sections from 4 s-IBM muscle biopsies were each stained with either TG3 or PHF-1 antibody—that was followed by an appropriate secondary antibody conjugated with a biotin and Streptavidin/horseradish peroxidase complex (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), diluted 1:50. The reaction product was visualized with a DAB/H_2O_2 solution [30]. On adjacent sections, the total number of muscle fibers and the number of muscle fibers containing