

ABSTRACT: Sarcoplasmic accumulation of phosphorylated-tau has been widely stated to occur in and contribute to the pathogenesis of muscle disease in inclusion body myositis. Twenty inflammatory myopathy and 10 normal muscle samples along with a range of other tissues were stained with anti-“tau” antibodies (tau-5, pS422, and SMI-31). Myonuclear and sarcoplasmic fractions were prepared using differential solubilization and laser-capture microdissection, and immunoblots were performed using pS422 and SMI-31 antibodies. All three antibodies demonstrated anti-tau immunoreactivity in myonuclei from normal and diseased muscle, but not in nuclei from other tissues. Western blots showed pS422 and SMI-31 immunoreactivity against nuclear proteins outside the region expected for phosphorylated-tau. Antibodies previously reported to indicate abnormal accumulation of phosphorylated-tau in IBM myofibers react to normal myonuclei and recognize proteins other than tau. Normal myonuclei contain neurofilament H or other unidentified 200 kDa proteins with similar phosphorylated motifs accounting for SMI-31 immunoreactivity.

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NATURE OF “TAU” IMMUNOREACTIVITY IN NORMAL MYONUCLEI AND INCLUSION BODY MYOSITIS

MOHAMMAD SALAJEGHEH, MD,^{1,2} JACK L. PINKUS, PhD,^{1,2}
REMEDIOS NAZARENO, BS,^{1,2} ANTHONY A. AMATO, MD,¹
KENNETH C. PARKER, PhD,^{1,2} and STEVEN A. GREENBERG, MD^{1,2}

¹Department of Neurology, Division of Neuromuscular Disease, Brigham and Women’s Hospital, 75 Francis Street, and Harvard Medical School, Boston, Massachusetts 02115, USA

²Children’s Hospital Informatics Program, Boston, Massachusetts

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Inclusion body myositis (IBM) is a progressive inflammatory skeletal muscle disease with poorly understood pathogenesis. It has been stated that accumulation of phosphorylated forms of the microtubule-associated protein tau (MAPT) occurs in and contributes to IBM pathogenesis^{1,2} (see Suppl. Table 1 for exact text from 41 publications that echo this view). IBM has been called a “tauopathy,”² and

therapies recommended for clinical trials have been based in part on findings of reductions in “tau pathology” with lithium in a mouse model.³

The view that tau is present in IBM myofibers has been based entirely on its immunohistochemical detection by antibodies. The most frequently reported data have used SMI-31,⁴ an antibody whose immunoreactivity has been interpreted to indicate the presence of phosphorylated tau in IBM muscle,⁴ even though this antibody has published reactivity against other proteins, including the primary target it was developed against, phosphorylated neurofilament H⁵ (manufacturer’s datasheet, Covance, Denver, Pennsylvania) as well as neurofilament M, microtubule associated protein 1b (MAP1B),⁶ microtubule associated protein 2 (MAP2),⁷ a lamin intermediate filament,⁸ and possibly sequestosome-1.⁹ The specific proteins that constitute immunoreactive SMI-31 material in IBM muscle sections remains unknown. Furthermore, almost all studies that report tau immunoreactivity

Abbreviations: IBM, inclusion body myositis; DAB, diaminobenzidine tetrahydrochloride; DM, dermatomyositis; IF, immunofluorescent; IHC, immunohistochemistry; MAP1B, microtubule associated protein 1b; MAP2, microtubule associated protein 2; MAPT, microtubule associated protein tau; NF-H, neurofilament H; PM, polymyositis; TBS-T, Tris-saline-Triton X-100 buffer; TDP-43, Tar DNA binding protein 43

Key words: inclusion body myositis; myonuclei; tau; SMI-31; pS422; neurofilament H

Additional Supporting Information may be found in the online version of this article.

Correspondence to: M. Salajegheh; e-mail: msalajegheh@partners.org or S.A. Greenberg; e-mail: sagreenberg@partners.org

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in IBM have lacked quantitative data. In the only published quantitative studies of SMI-31, the percentage of IBM myofibers containing abnormal SMI-31 immunoreactivity was only 0.69%¹⁰ and 1.95%.¹¹ SMI-31-immunoreactive aggregates similar to those seen in IBM have also been reported in 5 of 10 patients with dermatomyositis.¹² One article reported that immunoreactivity with the antiphosphorylated tau antibody pS422 was present in all IBM myonuclei, but it did not report results of pS422 immunoreactivity in myonuclei of normal control samples.² Another publication commented on unpublished findings of tau immunoreactivity of IBM myonuclei, also without comment on control normal samples.¹³

To further clarify the nature of the reported immunoreactivity against "tau" we performed immunohistochemistry and immunofluorescent studies as well as immunoblots using the anti-tau antibodies tau-5, pS422, and SMI-31 on muscle sections and prepared sarcoplasmic and myonuclear fractions, respectively. We found that while IBM myonuclei were immunoreactive with anti-tau antibodies, such tau-immunoreactivity was present in all myonuclei from normal subjects and from all other patient muscle biopsy samples we examined. We furthermore found that the tau antibodies SMI-31 and pS422 most likely recognize myonuclear proteins other than tau.

MATERIALS AND METHODS

Patients and Samples. Muscle biopsy specimens from 20 patients with inflammatory myopathies (IBM $n = 10$; polymyositis $n = 8$; dermatomyositis $n = 2$) and 10 normals underwent immunohistochemical studies with anti-tau antibodies. Patients with IBM fulfilled criteria for definite or possible IBM¹⁴; patients with polymyositis (PM) or dermatomyositis (DM) fulfilled criteria for definite or probable PM or DM.¹⁵ No patient with IBM received corticosteroids for treatment of their myopathy at any time. Normal subjects had no symptoms, signs, laboratory findings, or pathological abnormalities of a neuromuscular disease. Muscle biopsies were performed for diagnostic purposes. Patients provided informed consent for research studies and were approved by our Institutional Review Boards.

Immunohistochemistry. Ten-micron cryostat sections were fixed in either cold (5°C) 4% paraformaldehyde (PFA) for 5 min and then soaked consecutively in cold (5°C) 0.05 M Tris buffer, pH 7.5, room temperature Tris buffer, or were fixed in

cold acetone ($-10^{\circ} \pm 5^{\circ}\text{C}$) for 5 min and soaked in Tris buffer at room temperature. Tissue sections were transferred to 0.05 M Tris-saline-Triton X-100 buffer (TBS-T), pH 7.5, supplemented with 4% porcine serum for immunohistochemistry or to TBS-T for immunofluorescence. The latter tissue sections were incubated for 30 min with Image-iTFX signal enhancer reagent (cat. no. I36933, Molecular Probes/Invitrogen, Eugene, Oregon), although omitting this step did not appear to diminish the fluorescence signal-to-noise ratio. These slides were rinsed and soaked in TBS-T, then soaked in 0.05 M Tris-Brij-35 buffer, pH 7.5, supplemented with 2% bovine serum albumin. Following all incubations, slides were rinsed and soaked in TBS-T and soaked in the same Tris-porcine serum buffer or Tris-bovine serum albumin buffer, respectively, prior to a subsequent step.

Primary antibodies used were rabbit polyclonal anti-Tau [pS422] phosphospecific antibody (cat. no. 44-764G, lot no. 0202, 226 μg IgG/ml, BioSource International/Invitrogen, Camarillo, California), mouse monoclonal anti-Tau (pan) proteins (cat. no. AHB0042, clone Tau-5, isotype IgG1, BioSource International/Invitrogen), and mouse monoclonal antibody (SMI-31, ascites fluid) to neurofilaments, phosphorylated epitope (cat. no. SMI-31R, clone SMI-31, isotype IgG1, Covance Research Products, Berkeley, California), mouse monoclonal anti-emerin antibody (cat. no. VP-E602, clone 4G5, isotype IgG1, Novacastra Laboratories, Newcastle upon Tyne, UK; obtained from Vector Laboratories, Burlingame, California).

For immunohistochemistry (IHC), tissue sections were incubated with polyclonal pS422 antibody (1:200, 1.13 μg /ml, 2 h) and subsequently incubated with horseradish peroxidase-conjugated polymer bound to goat antirabbit immunoglobulins (cat. no. K4011, 30 min, antirabbit EnVision+ System, Dako, Carpinteria, California). Antibody localization was effected by using a peroxidase reaction with 3,3'-diaminobenzidine tetrahydrochloride (DAB) as the chromogen. Tau-5 (1:50, 10 μg /ml) was added (100 μl) in titration mode to buffer (200 μl ; a minimal measured volume in test slides) covered tissue sections for antibody (1:150, 3.3 μg /ml, at 37°C for 40 min after acetone fixation or 32 min after PFA fixation) staining performed on an automated slide stainer with a horseradish peroxidase-labeled goat antimouse secondary antibody (BenchMark XT with ultraView Universal DAB Detection Kit, Ventana Medical Systems, Tucson, Arizona). Sections were counterstained with methyl green. Control studies were

performed with irrelevant IgG1 immunoglobulins matched for Tau-5, normal rabbit serum for pS422, and Tris buffer for endogenous peroxidase.

IHC and immunofluorescent (IF) staining with SMI-31 (PFA or no fixation, 1:10,000, overnight) utilized secondary antibody horseradish peroxidase (HRP)-conjugated polymer bound to goat anti-mouse immunoglobulins (cat. no. DPVM-110 HRP, 30 min, antimouse PowerVision, ImmunoVision Technologies) and Alexa Fluor 488-labeled goat antimouse immunoglobulins (1:400 dilution, 5 μ g/ml, 65 min, Molecular Probes/Invitrogen), respectively. Dual staining (IF) of PFA-fixed tissue sections with Tar DNA binding protein 43 (TDP-43) antibody was carried out overnight. An admixture of SMI-31 and TDP-43 antibodies contained each antibody at a final dilution as previously used.¹⁶ Secondary antibodies were an admixture of Alexa Fluor 555-labeled goat antirabbit immunoglobulins and Alexa Fluor 488-labeled goat antimouse immunoglobulins (each at 1:400 dilution and 5 μ g/ml, 65 min incubation; Molecular Probes/Invitrogen).

Preparation of Nuclear and Sarcoplasmic Fractions Using the Nuclear Extract Kit. Nuclear and sarcoplasmic fractions were prepared from 50 mg of freshly biopsied human muscle using the Nuclear Extract Kit (cat. no. 40010, Active Motif, Carlsbad, California) per the manufacturer's protocol. Protein concentration was measured using the Micro BCA Assay Kit (cat. no. 23235, Pierce, Rockford, Illinois), and the samples were stored at -80°C .

Laser-Capture Microdissection for the Purification of Nuclear and Sarcoplasmic Muscle Fractions. Muscle was cut into 7- μ m sections using a Leica microtome and placed on a non-plus glass slide. HistoGene kit (Arcturus no. KIT0401, Mountain View, CA) was used for staining as per the manufacturer's protocol. A Veritas microdissection system (Arcturus) and CapSure HS LCM caps (Arcturus no. LCM0214) were used for separate laser capture microdissection of nuclei and cytoplasmic regions of myofibers. The Veritas microdissection system allowed for rapid and automated capture of nuclei. After manual software-aided markup of hundreds of nuclei in a given section, automated laser capture of all marked regions by the device was performed. Forty μ l of extraction buffer (100 mM NH_4HCO_3 ; 1% SDS, 8 M urea, 10 mM DTT) was added to each cap, and the resulting solution was incubated for 2 h at 37°C . The extracted proteins were removed by centrifugation at 5,000g for

5 min. BCA assay (Pierce no. 23225) was used to determine protein concentration, and the samples were stored at -80°C .

Western Blots. Proteins in each sample were separated using SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting was carried out by incubating the membranes with rabbit anti HDAC1 (cat. no. 89910 Pierce; 1:250 dilution overnight at 4°C), pS422 (1:500 dilution overnight at 4°C), and SMI-31 (1:500 dilution overnight at 4°C), and after washing with goat antirabbit HRP (for HDAC1 and pS422; cat. no. ab6721 Abcam, Cambridge, Massachusetts; 1:5,000 dilution for 1 h at room temperature) and goat antimouse HRP (for SMI-31; cat. no. 62-6520 Zymed, South San Francisco, California; 1:2000 dilution for 1 h at room temperature). After stripping the blots using Restore Western Blot Stripping Buffer (Pierce no. 21062), they were incubated with rabbit anti-actin (Santa Cruz Biologicals, no. sc-1616, Santa Cruz, California) 1:10,000 dilution for 1 h at room temperature, and after washing with goat antirabbit HRP (Abcam no. ab6721) 1:10,000 dilution for 1 h at room temperature. SuperSignal West Pico Chemiluminescent Substrate (Pierce) and Kodak films were used for visualization of the bands.

RESULTS

Normal Myonuclear Localization of Anti-tau Antibody Immunoreactivities. In all normal muscle specimens, visible light microscopy showed the presence of pS422, tau-5, and SMI-31 immunoreactivities in myonuclei indicated by their colocalization with the DNA stain methyl green (Fig. 1). The localization of SMI-31 to myonuclei was further confirmed in immunofluorescent studies through colocalization with the DNA-binding fluorescent DAPI (Fig. 1) and another myonuclear protein TDP-43 (Fig. 2). SMI-31 immunoreactivity was present in 98% of 1,000 DAPI fluorescent nuclei counted (250 in each of four normal sections). pS422 and tau-5 immunoreactivities in immunoperoxidase-based reactions viewed with visible light microscopy were not quantified, but we estimated that about 75% of myonuclei were pS422 immunoreactive and 95% of myonuclei were tau-5-immunoreactive based on colocalization with methyl green. No staining of myonuclei was present with control primary IgG1 antibodies (for SMI-31 and tau-5), normal rabbit serum (for pS422), or Tris with secondary fluorescent labeled antibodies. All diseased muscle specimens also showed myonuclear

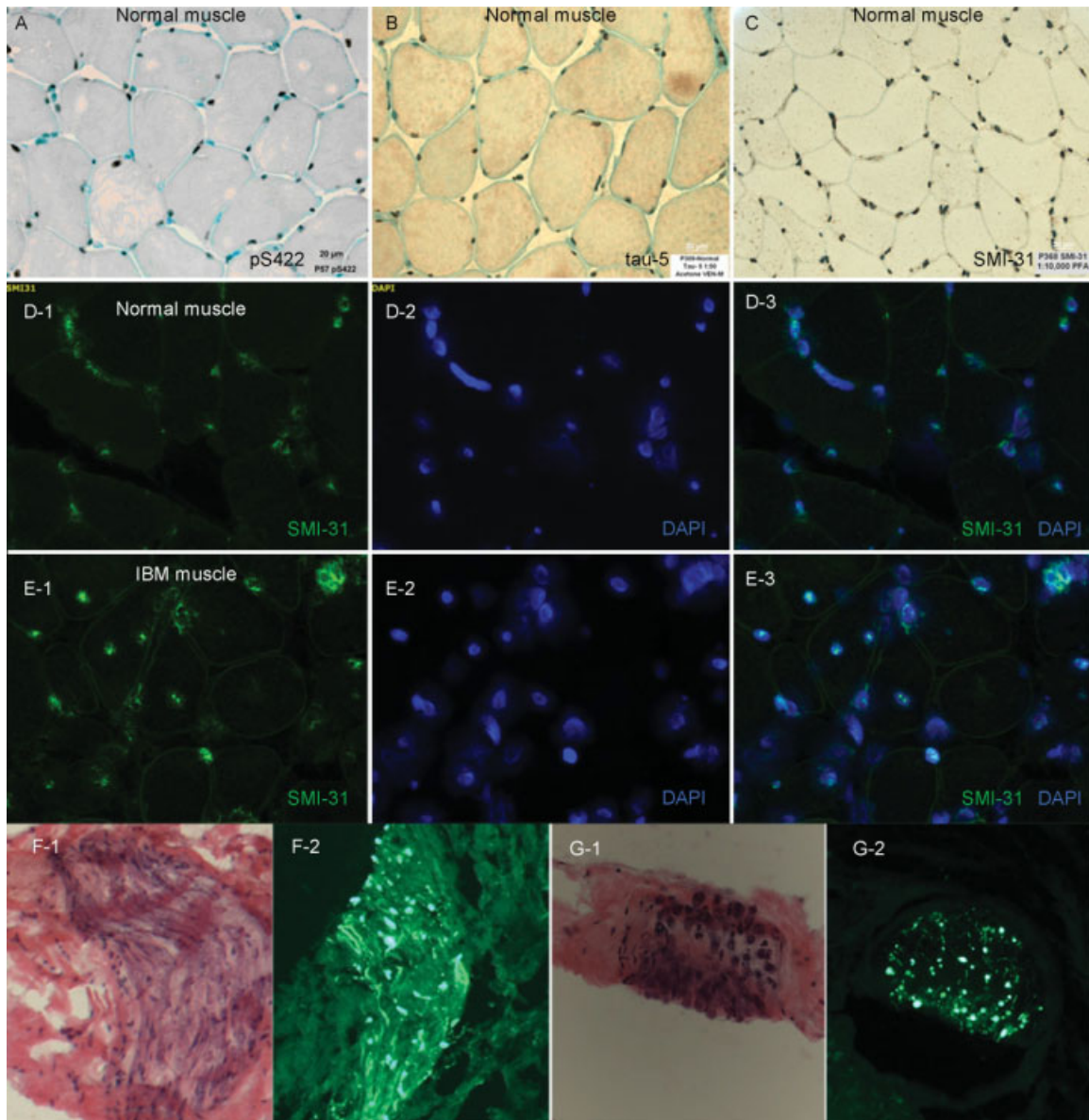


FIGURE 1. Normal myonuclear localization of anti-“tau” (pS422, tau-5, SMI-31) immunoreactivities in normal and IBM muscle. **(A)** pS422, **(B)** tau-5, and **(C)** SMI-31 light microscopic images, normal muscle. The pattern of staining of all of these suggests nuclear localization, and the visible colocalization with methyl green counterstain for pS422 confirms nuclear localization. **(D,E)** Confirmed nuclear localization of SMI-31 through its colocalization with the DNA stain DAPI in fluorescent studies, in **(D1–3)** normal muscle and **(E1–3)** IBM muscle. **(F,G)** The SMI-31 antibody stained intramuscular nerve appropriately as evidenced in adjacent hematoxylin and eosin (H&E) and SMI-31-stained sections, likely reflecting the presence of NF-H.

immunoreactivity to pS422, tau-5, and SMI-31, although these were not quantitated.

These findings in normal and diseased frozen muscle sections were not artifactual, as they were readily apparent in sections stained in different batches over a period of 18 months and were seen with both immunoperoxidase-based IHC reactions and immunofluorescent studies (for SMI31). In

≈8 years of IHC studies using 49 different antibodies, we have only seen myonuclear staining with antibodies with known nuclear targets (lamin A/C, emerin, Werner syndrome protein, fibrillarin, TDP-43, and valosin-containing protein). The SMI-31 antibody furthermore correctly stained neurons in intramuscular nerve in one of the biopsy specimens (Fig. 1F,G); peripheral nerve axons contain

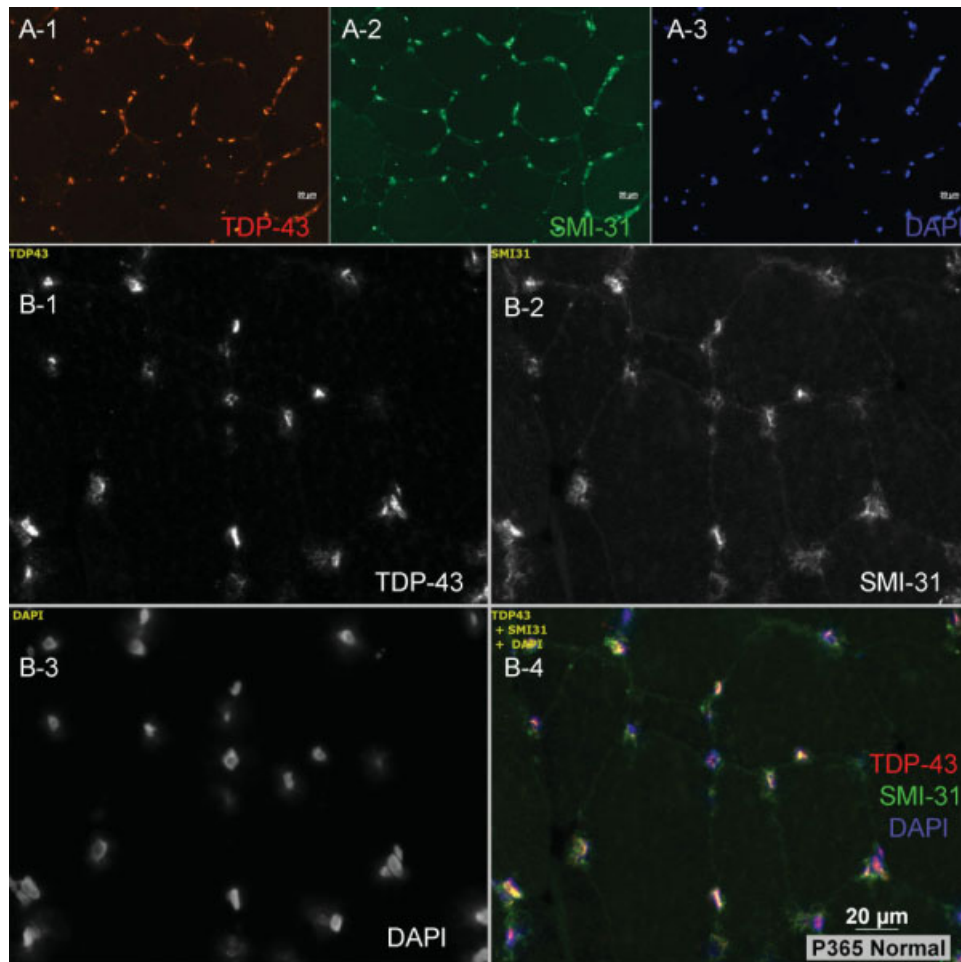


FIGURE 2. SMI-31 colocalization with myonuclear TDP-43 and DAPI in normal muscle. **(A1–3)** Triple fluorescent stained section of normal muscle for TDP-43, SMI-31, and DAPI viewed in true-color images show nuclear localization of SMI-31. **(B1–B4)** Higher magnification of section from a different normal sample showing black and white images of individual TDP-43, SMI-31, and DAPI fluorescent signal and colored merged image.

neurofilaments, most likely neurofilament H (NF-H), to which SMI-31 reacts.¹⁷ Furthermore, we studied pS422 and tau-5 immunoreactivity in a range of other biopsied tissues (lymph node, thymus, parathyroid gland, neck cancer, and placenta) using identical methods. We found nuclear pS422 and tau-5 immunoreactivity only in myonuclei (Suppl. Fig. 1).

Tau Immunoreactivity in Myonuclei May Reflect Cross-reactivity of These Antibodies to Proteins Other Than Tau. To understand whether the myonuclear immunoreactivities seen with antibodies SMI-31 and pS422 indicated the presence of tau, we purified myonuclei from normal muscle. Two approaches were used. First, we used a commer-

cially available nuclear preparation kit starting from whole muscle lysates (Active Motif protocol). This approach showed that pS422 and SMI-31 antibodies react to a number of normal muscle nuclear proteins in areas outside of the 48–67 kDa range where tau is known to be present (reviewed in Ref. 18), (Fig. 3A). In particular, SMI-31, an antibody that was developed specifically against the 200-kDa protein NF-H, showed strong staining to a 200-kDa protein and also to a 40-kDa protein.

The limitation of the first approach is that the preparation may contain nuclei from non-myofiber cell types, such as fibroblasts, endothelial cells, and circulating blood cells. To address this possibility, we employed a strategy of direct visualization and removal of myonuclei through laser capture microdissection to prepare purified myonuclear fractions

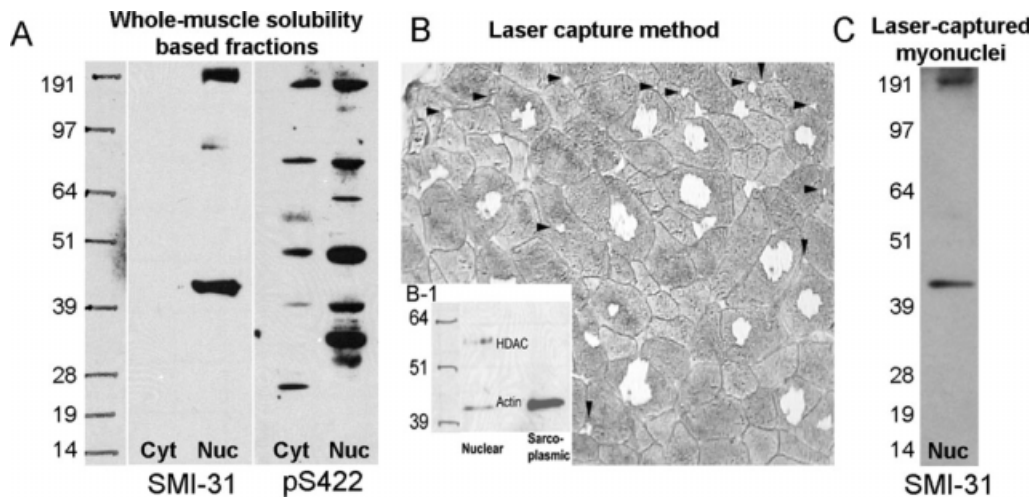


FIGURE 3. Anti-“tau” antibodies recognize proteins other than tau in muscle sarcoplasm and myonuclei. **(A)** Immunoblot from muscle “nuclear” and “cytoplasmic” extracts obtained using a commercial protocol from whole muscle specimens shows immunoreactivity of these antibodies to a number of proteins. While the 48–67-kDa bands for pS422 could represent tau (MAPT), other bands outside this range as well as those seen with SMI-31 (40 kDa and 200 kDa) most likely represent other non-tau proteins. **(B)** Preparation of myonuclear and sarcoplasmic fractions through laser capture microdissection (LCMD). Myonuclei were directly visualized and removed, leaving small gaps (arrowheads) for myonuclear fractions; larger gaps in myofibers are from removal of nonnuclei containing regions of sarcoplasm used for nuclei-free sarcoplasmic preparations. **(B-1)** Immunoblot of LCMD prepared fractions shows histone-deacetylase confined to the nuclear portion, and actin more abundant in the sarcoplasmic component, confirming enrichment of nuclear proteins in the nuclear fraction. **(C)** Immunoblot from these LCMD myonuclear fractions demonstrating SMI-31 immunoreactivity to an ≈40-kDa protein and a 200-kDa protein, the mass of NF-H, but not to any within the 48–67-kDa range expected for tau.

and separately myonuclei-free sarcoplasmic fractions (Fig. 3B). We confirmed that this approach enriched nuclear proteins and sarcoplasmic proteins separately through immunoblots detecting nuclear histone deacetylases (HDAC) and actin (Fig. 3B-1). Using these fractions, we again found that SMI-31 and pS422 antibodies immunoreact in immunoblots to a number of myonuclear proteins outside of the size range expected for tau. SMI-31 again reacted to exactly the same two proteins, a 200-kDa protein and a 40-kDa protein (Fig. 3C).

pS422 and SMI-31 Immunoreactivity in IBM Muscle. In all 10 IBM muscle samples, myofiber vacuoles were present and were sometimes lined with pS422 and SMI-31-immunoreactive material (Fig. 4). These were infrequent and were not quantitated or compared with the number of rimmed vacuoles seen on routine stains with hematoxylin and eosin or trichrome. Some of these colocalized with DAPI, likely indicating the remains of degenerated myonuclei (Fig. 4B).

We found very few IBM myofibers that contained nonnuclear SMI-31 immunoreactivity (0.83% of myofibers in 2,500 myofibers counted, 500 in each of five IBM samples) using a highly sensitive fluorescent method that also allowed for

distinction of nuclear and nonnuclear SMI-31 immunoreactivity based on colocalization with DAPI (Suppl. Fig. 2).

DISCUSSION

An important role for phosphorylated tau in inclusion body myositis has been claimed based on the

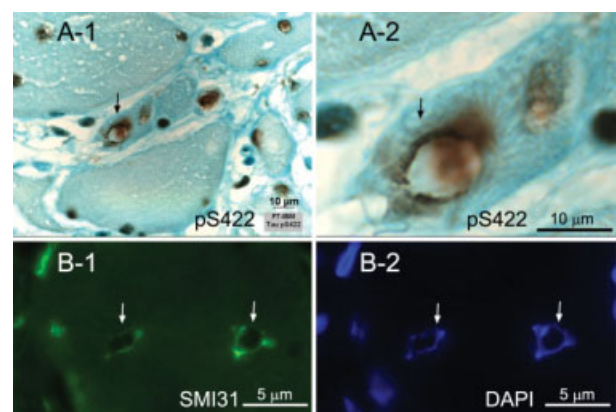


FIGURE 4. IBM vacuoles lined with pS422 and SMI-31 immunoreactive material. **(A-1)** pS422 immunohistochemistry with nuclear staining and a mass of pS422 immunoreactivity that includes a vacuole. **(A-2)** Higher magnification of A-1. **(B-1,B-2)** Double fluorescent stained IBM section showing two vacuoles lined with both SMI-31 and DAPI.

belief that this protein is in some way abnormally present in IBM myofibers, a view that appears to be widely accepted (Suppl. Table 1). This belief has provided support for mechanistic claims regarding IBM myofiber injury and for claims that “tau pathology” in a mouse model is a therapeutic biomarker applicable to IBM drug development.³ We were therefore surprised to find that three well known anti-“tau” antibodies showed immunoreactivity to most myonuclei in all normal and diseased specimens we examined. Indeed, a previous study characterizing IBM as a “tauopathy” reported immunoreactivity of all IBM myonuclei with the anti-“tau” antibody pS422, but it did not comment on whether such immunoreactivity was also present in myonuclei in normal muscle biopsy samples.²

Although tau accumulation has been stated to be a feature of IBM in at least 41 publications (as of 11/16/2008; see Suppl. Table 1), none of these review and data articles presented any quantitative data regarding the frequency of myofibers containing “tau” immunoreactive abnormalities. We found nonnuclear SMI-31 reactivity in 0.83% of myofiber cross-sections, data we have also reported in a separate article pertaining to TDP-43.¹⁶ Our findings are in agreement with the only other published quantitative data regarding SMI-31 abnormal immunoreactivity, reported in 0.69%¹⁰ and 1.95%¹¹ of myofibers. Studies with the anti-phosphorylated neurofilament antibody PHF1 reported a mean of 6.4 affected myofibers per IBM biopsy sample, but the total number of myofibers was not noted.¹⁹ Since typical muscle biopsy samples contain at least 1,000 myofiber cross-sections, these data most likely imply a frequency of less than 0.64% of myofibers. We are unaware of any other quantitative data regarding anti-“tau” immunoreactivity in IBM. In addition, the small percentage of IBM myofibers containing visible nonnuclear sarcoplasmic SMI-31 immunoreactivity may represent remnants of prior myonuclear degeneration,^{20–23} as suggested by the colocalization of SMI-31 with DAPI-lined vacuoles (Fig. 4).

To further clarify the nature of proteins against which pS422 and SMI-31 demonstrate immunoreactivity, we performed immunoblots using the two antibodies. We noticed that, while some of the immunoreactivity seen with the pS422 antibody was to bands within the 48–67 kDa range expected for phosphorylated tau (reviewed in Ref. 18), many of the identified bands fell outside this range. The highest molecular mass reported for tau has been in the 110–125 kDa range, for what has been labeled “big” or high molecular weight tau, in the

peripheral nervous system ganglia and some cell lines with peripheral neuron-like characteristics.^{24,25} Furthermore, when initiating our studies with the anti-“tau” antibody SMI-31, we were surprised to read in the manufacturer’s datasheet that this antibody’s primary target is NF-H.⁵ NF-H is a 200-kDa intermediate filament protein that can be extensively phosphorylated by many kinases including GS3K-beta²⁶ and CDK5²⁷ that can also phosphorylate tau. The crossreactivity reported for the anti-phosphorylated NF-H antibody SMI-31, with phosphorylated tau²⁸ and other proteins,^{6–9} has been related to the presence of shared phosphorylated serine-proline motifs between these proteins and NF-H. SMI31’s immunoreactivity in muscle sections therefore cannot be concluded to indicate the presence of tau. Indeed, we found, through Western blotting of myonuclear proteins obtained by laser capture microdissection, that normal myonuclei contain prominent 40-kDa and 200-kDa SMI-31 immunoreactive proteins. Given that NF-H is the 200-kDa protein against which SMI-31 was raised, and multiple publications have confirmed SMI-31 immunoreactivity to NF-H at 200 kDa,^{8,29} it is likely that this reactivity belongs to NF-H and that NF-H may be a normal myonuclear constituent. This is further supported by our immunofluorescent and immunohistochemistry studies that demonstrate the presence of SMI-31 reactivity in 98% of normal myonuclei examined. Several other studies have also reported the presence of SMI-31 immunoreactivity in nuclei in various cultured cell lines from neural lineage^{8,30} and adult neural rat brain cells, but the nature of the proteins has not been further clarified.³¹

Our studies have several implications with regard to IBM. First, we have concern regarding the validity of the interpretation of anti-“tau” immunoreactivity in IBM muscle sections as indicating the presence of tau.^{1–3} Second, since such immunoreactivity is already present in IBM myonuclei, interpreting it as present in the sarcoplasm of a myofiber requires a method that allows for concurrent visualization of nuclei, such as methyl green counterstaining or DAPI in fluorescent studies, as we have done here. No previous studies reporting anti-“tau” aggregates in IBM myofibers have excluded such “aggregates” from myonuclei. Even when confirmed as sarcoplasmic, the most natural interpretation of the significance of such anti-“tau” immunoreactivity is simply that it reflects prior nuclear degeneration.^{20–23} Third, we have concern regarding the rationale for therapeutic development of compounds that target tau

metabolism in patients with IBM,³ given the above considerations, and the paucity of visible myofiber cross-sections containing anti-“tau” immunoreactivity in all published studies that provide quantitative data. Fourth, although electron microscopy has shown accumulation of tubulofilaments in a small number of myonuclei and extranuclear regions of IBM myofibers, the identities of the molecules constituting these filaments are unknown.

Here we also report a method for purifying myonuclei from whole human muscle biopsy specimens. Normal muscle contains a variety of cell types, and their nuclei, such as endothelial cells, fibroblasts, and circulating blood cells, and diseased muscle, particularly inflammatory myopathy muscle, may contain large numbers of immune cells. Standard methods for preparing nuclear fractions from whole muscle lysates using differential centrifugation or solubilization may not yield pure myonuclear fractions. The approach we used here, through the collection of directly visualized myonuclei captured by laser capture microdissection, allows for accurate study of proteins present within normal human myonuclei. Furthermore, the collection of directly visualized nuclei-free regions of muscle sarcoplasm similarly eliminates contaminating proteins from connective tissue, nerve terminals, blood vessels, circulating and infiltrating blood cells, and soluble serum proteins present in “cytoplasmic” fractions prepared from whole muscle lysates.³²

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REFERENCES

1. Askanas V, Engel WK. Inclusion-body myositis: a myodegenerative conformational disorder associated with Abeta, protein misfolding, and proteasome inhibition. *Neurology* 2006;66:S39–48.
2. Maurage CA, Bussiere T, Sergeant N, Ghestem A, Figarella-Branger D, Ruchoux MM, et al. Tau aggregates are abnormally phosphorylated in inclusion body myositis and have an immunoelectrophoretic profile distinct from other tauopathies. *Neuropathol Appl Neurobiol* 2004;30:624–634.
3. Kitazawa M, Trinh DN, LaFerla FM. Inflammation induces tau pathology in inclusion body myositis model via glycogen synthase kinase-3beta. *Ann Neurol* 2008;64:15–24.
4. Mirabella M, Alvarez RB, Bilak M, Engel WK, Askanas V. Difference in expression of phosphorylated tau epitopes between sporadic inclusion-body myositis and hereditary inclusion-body myopathies. *J Neuropathol Exp Neurol* 1996; 55:774–786.
5. Sternberger LA, Sternberger NH. Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments in situ. *Proc Natl Acad Sci U S A* 1983;80: 6126–6130.
6. Gonzalez-Billault C, Del Rio JA, Urena JM, Jimenez-Mateos EM, Barallobre MJ, Pascual M, et al. A role of MAP1B in Reelin-dependent neuronal migration. *Cereb Cortex* 2005; 15:1134–1145.
7. Nukina N, Kosik KS, Selkoe DJ. Recognition of Alzheimer paired helical filaments by monoclonal neurofilament antibodies is due to crossreaction with tau protein. *Proc Natl Acad Sci U S A* 1987;84:3415–3419.
8. Weigum SE, Garcia DM, Raabe TD, Christodoulides N, Koke JR. Discrete nuclear structures in actively growing neuroblastoma cells are revealed by antibodies raised against phosphorylated neurofilament proteins. *BMC Neurosci* 2003;4:6.
9. Zatloukal K, Stumptner C, Fuchsbichler A, Heid H, Schnoelzer M, Kenner L, et al. p62 is a common component of cytoplasmic inclusions in protein aggregation diseases. *Am J Pathol* 2002;160:255–263.
10. van der Meulen MF, Hoogendijk JE, Moons KG, Veldman H, Badrising UA, Wokke JH. Rimmed vacuoles and the added value of SMI-31 staining in diagnosing sporadic inclusion body myositis. *Neuromuscul Disord* 2001;11: 447–451.
11. Banwell BL, Engel AG. AlphaB-crystallin immunolocalization yields new insights into inclusion body myositis. *Neurology* 2000;54:1033–1041.
12. Spuler S, Engel AG. SMI-31 immunoreactivity in inclusion body myositis. *Ann Neurol* 1997;42:815.
13. Engel WK, Askanas V. Inclusion-body myositis: clinical, diagnostic, and pathologic aspects. *Neurology* 2006;66:S20–29.
14. Griggs RC, Askanas V, DiMauro S, Engel A, Karpatis G, Mendell JR, Rowland LP. Inclusion body myositis and myopathies. *Ann Neurol* 1995;38:705–713.
15. Hoogendijk JE, Amato AA, Lecky BR, Choy EH, Lundberg IE, Rose MR, et al. 119th ENMC international workshop: trial design in adult idiopathic inflammatory myopathies, with the exception of inclusion body myositis, 10–12 October 2003, Naarden, The Netherlands. *Neuromuscul Disord* 2004;14:337–345.
16. Salajegheh M, Pinkus JL, Amato AA, Nazareno R, Baloh RH, Greenberg SA. Sarcoplasmic redistribution of nuclear TDP-43 in inclusion body myositis. *Muscle Nerve* 2009;40: 19–31.
17. Pestronk A, Watson DF, Yuan CM. Neurofilament phosphorylation in peripheral nerve: changes with axonal length and growth state. *J Neurochem* 1990;54:977–982.
18. Goedert M, Crowther RA, Garner CC. Molecular characterization of microtubule-associated proteins tau and MAP2. *Trends Neurosci* 1991;14:193–199.
19. Kannanayakal TJ, Mendell JR, Kuret J. Casein kinase 1 alpha associates with the tau-bearing lesions of inclusion body myositis. *Neurosci Lett* 2008;431:141–145.
20. Chou SM. Myxovirus-like structures and accompanying nuclear changes in chronic polymyositis. *Arch Pathol* 1968;86: 649–658.
21. Carpenter S, Karpatis G, Heller I, Eisen A. Inclusion body myositis: a distinct variety of idiopathic inflammatory myopathy. *Neurology* 1978;28:8–17.
22. Greenberg SA, Pinkus JL, Amato AA. Nuclear membrane proteins are present within rimmed vacuoles in inclusion-body myositis. *Muscle Nerve* 2006;34:406–416.
23. Greenberg SA, Watts GD, Kimonis VE, Amato AA, Pinkus JL. Nuclear localization of valosin-containing protein in normal muscle and muscle affected by inclusion-body myositis. *Muscle Nerve* 2007;36:447–454.
24. Drubin DG, Feinstein SC, Shooter EM, Kirschner MW. Nerve growth factor-induced neurite outgrowth in PC12 cells involves the coordinate induction of microtubule assembly and assembly-promoting factors. *J Cell Biol* 1985; 101:1799–1807.
25. Oblinger MM, Argasinski A, Wong J, Kosik KS. Tau gene expression in rat sensory neurons during development and regeneration. *J Neurosci* 1991;11:2453–2459.

26. Sasaki T, Taoka M, Ishiguro K, Uchida A, Saito T, Isobe T, Hisanaga S. In vivo and in vitro phosphorylation at Ser-493 in the glutamate (E)-segment of neurofilament-H subunit by glycogen synthase kinase 3beta. *J Biol Chem* 2002;277:36032–36039.
27. Sharma P, Barchi JJ Jr, Huang X, Amin ND, Jaffe H, Pant BC. Site-specific phosphorylation of Lys-Ser-Pro repeat peptides from neurofilament H by cyclin-dependent kinase 5: structural basis for substrate recognition. *Biochemistry* 1998;37:4759–4766.
28. Lichtenberg-Kraag B, Mandelkow EM, Biernat J, Steiner B, Schroter C, Gustke N, et al. Phosphorylation-dependent epitopes of neurofilament antibodies on tau protein and relationship with Alzheimer tau. *Proc Natl Acad Sci U S A* 1992;89:5384–5388.
29. Lewis SB, Wolper RA, Miralial L, Yang C, Shaw G. Detection of phosphorylated NF-H in the cerebrospinal fluid and blood of aneurysmal subarachnoid hemorrhage patients. *J Cereb Blood Flow Metab* 2008;28:1261–1271.
30. Glass TL, Raabe TD, Garcia DM, Koke JR. Phosphorylated neurofilaments and SNAP-25 in cultured SH-SY5Y neuroblastoma cells. *Brain Res* 2002;934:43–48.
31. Schilling K, Duvernoy C, Keck S, Pilgrim C. Detection and partial characterization of a developmentally regulated nuclear antigen in neural cells in vitro and in vivo. *J Histochem Cytochem* 1989;37:241–247.
32. Parker KC, Kong SW, Walsh RJ, Salajegheh M, Moghadaszadeh B, Amato AA, et al. Fast-twitch sarcomeric and glycolytic enzyme protein loss in inclusion body myositis. *Muscle Nerve* 2009;39:739–753.