Novel Immunolocalization of α-Synuclein in Human Muscle of Inclusion-Body Myositis, Regenerating and Necrotic Muscle Fibers, and at Neuromuscular Junctions

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Abstract. α-synuclein (α-syn) is an important component of neuronal and glial inclusions in brains of patients with several neurodegenerative disorders. Sporadic inclusion-body myositis (s-IBM) is the most common progressive muscle disease of older patients. Its muscle phenotype shows several similarities with Alzheimer disease brain. A distinct feature of s-IBM pathology is specific vacuolar degeneration of muscle fibers characterized by intracellular amyloid inclusions formed by both amyloid-β (Aβ) and paired-helical filaments composed of phosphorylated tau. We immunostained α-syn in muscle biopsies of s-IBM, disease-control, and normal patients. Approximately 60% of Aβ-positive vacuolated muscle fibers (VMF) contained well-defined inclusions immunoreactive with antibodies against α-syn. In those fibers, α-syn co-localized with Aβ, both by light microscopy, and ultrastructurally. Paired-helical filaments did not contain α-syn immunoreactivity. In all muscle biopsies, α-syn was strongly immunoreactive at the postsynaptic region of the neuromuscular junctions. α-syn immunoreactivity also occurred diffusely in regenerating and necrotic muscle fibers. In cultured human muscle fibers, α-syn and its mRNA were expressed by immunocytochemistry, immunoblots, and Northern blots. Our study provides the first demonstration that α-syn participates in normal and pathologic processes of human muscle. Therefore, its function is not exclusive to the brain and neurodegenerative diseases.

Key Words: α-synuclein; Cultured human muscle; Inclusion-body myositis; Necrotic muscle fibers; Neuromuscular junctions; Regenerating muscle fibers.

INTRODUCTION

Sporadic inclusion-body myositis (s-IBM), the most common progressive muscle disease in older persons, is of unknown etiology and pathogenesis (reviewed in 1). Light-microscopic features of the s-IBM muscle biopsy include various degrees of mononuclear-cell inflammation, vacuolated muscle fibers (VMFs), congo-red positivity (indicating amyloid) within the VMFs, and 15–21-nm cytoplasmic paired-helical filaments (PHF) (1). An intriguing aspect of s-IBM-VMFs is abnormal accumulation of a group of proteins that are accumulated in Alzheimer disease (AD) brain. For example, on a given section, 70%–80% of the VMFs and some of the non-VMFs contain accumulations of amyloid-β protein (Aβ), C- and N-terminal epitopes of amyloid-β-precursor protein (AβPP), apolipoprotein E, presenilin-1, ubiquitin, and other “Alzheimer-characteristic” proteins (1). AβPP-mRNA is also increased in inclusion-body myositis (IBM)-VMF (2). Like AD brain, IBM muscle PHFs are composed of phosphorylated tau (3), while Aβ is localized to 6–10-nm filaments and floccular and amorphous material (4).

α-synuclein is a 140 amino-acid protein whose normal functions are virtually unknown (reviewed in 5–8). Missense mutations in the α-synuclein (α-syn) gene are linked to some forms of familial Parkinson disease (PD) (9). α-syn is an important component of Lewy bodies in several neurodegenerative diseases (5–13) and of glial and neuronal cytoplasmic inclusions in multiple system atrophy (14–16). Recently, α-syn immunoreactivity has been shown in axonal swellings in brains of various conditions, including primary and secondary neuroaxonal dystrophy (19). The exact role of α-syn in AD brain remains uncertain (13, 17, 18, 20).

In this study we examined whether α-syn might play a role in the pathogenesis of IBM and in other pathologic and normal processes of human muscle.

Fig. 1. PAP immunostaining of α-syn in IBM. Two abnormal muscle fibers (arrows in A, B) have mainly dotty α-syn immunoreactive inclusions. Magnification, ×900.
Fig 2. Double immunofluorescence labeling of α-syn (A, D, G) with Aβ (B) and desmin (E, H). C, F, I: Double exposure. A, B: in IBM, α-syn immunoreactive inclusions co-localize with Aβ inclusions, in the form of small plaquettes and a patchy subplasmalemmal staining. D, E: Small regenerating muscle fibers identified by strong desmin immunoreactivity (E) are strongly immunoreactive for α-syn (D). G, H: Necrotic, desmin-negative fiber (H) is strongly α-syn immunoreactive (G). Small circular line of immunopositivity (arrows) in both G and H, (yellow in I), possibly indicates an attempt of this fiber to regenerate. Magnification, ×900.

MATERIALS AND METHODS

Patients

We studied diagnostic muscle biopsy specimens (obtained with informed consent) from 30 patients with the following diagnoses: 9 s-IBM, 4 polymyositis, 3 morphologically non-specific myopathy, 2 myotonic dystrophy, 6 amyotrophic lateral sclerosis, and 6 normal muscle. All diagnoses were based on clinical and laboratory studies, including 18-reaction diagnostic histochemistry of the muscle biopsy specimen (21).

Biopsies of all IBM patients had muscle fibers with vacuoles on Engel-Gomori staining (22), 15- to 21-nm PHFs by electronmicroscopy and by SMI-31 immunoreactivity (23), and congo-red positivity using fluorescence enhancement (24).

Light-Microscopic Immunocytochemistry

Immunocytochemical stainings were performed on 10-μm transverse sections of fresh-frozen muscle biopsies, using peroxidase-antiperoxidase (PAP) and fluorescence methods as described (3, 4, 23, 25–28).

Two antibodies specifically recognizing α-syn were used: mouse monoclonal antibody MAb LB509 (12) (Zymed, San Francisco, CA), diluted 1:50 or 1:100; and rabbit polyclonal antibody directed against amino terminal carboxyl epitope of...
α-syn (16) (Chemicon, Temecula, CA), diluted 1:100. To block nonspecific binding of antibody to Fc receptors, sections were preincubated with 1:10 diluted normal goat serum, as described (3, 4, 23, 25–28). Controls for staining specificity were omission of the primary antibody, or its replacement with nonimmune sera or irrelevant antibodies as described (3, 4, 23, 25–28).

Double immunofluorescence was performed using polyclonal antisera against α-syn combined with either a) mouse monoclonal antibody SMI-31 (Stemberger Monoclonals, Inc., Lutherville, MD), diluted 1:1000, recognizing phosphorylated tau of IBM-VMFs (3, 23), or b) well-characterized monoclonal antibody 6E10 against Aβ (Senetek, Maryland Heights, MO), diluted 1:200, followed by species-specific secondary antibodies labeled either with fluorescein isothiocyanate (FITC) or Texas red, as described (3, 4, 23, 25–28). Regenerating and necrotic muscle fibers were identified with either polyclonal or monoclonal antibodies against desmin (Zymed), diluted 1:100, as described (27), double-labeled with either monoclonal or polyclonal antibody against α-syn.

Neuromuscular junctions were identified by α-bungarotoxin binding as described (27, 28) and double-labeled with either monoclonal or polyclonal antibody against α-syn.

Immunoelectronmicroscopy

This was performed using 10-μm unfixed frozen sections adhered to the bottom of 35-mm Petri dishes, as described (3, 4, 23, 25–27). In brief, after incubation for 36 h at 4°C in a primary antibody, the sections were incubated in either gold- or HRP-conjugated secondary antisera. Subsequently, the sections were fixed in a 2% paraformaldehyde and 1.2% glutaraldehyde mixture, postfixed in osmium, and embedded in Epon in situ in the Petri dish (29). The embedded section in the dish was viewed under phase-contrast microscopy and compared with an adjacent cross-section that had been incubated with the same antibody as for immunoelectronmicroscopy but stained with the PAP reaction. The identical VMFs that contained PAP-positive inclusions were identified in the adjacent gold-labeled Epon-embedded section, were marked, and 1-mm diameter cores were drilled out (29). From the cores, each containing at least 1 vacuolated muscle fiber, thin sections were cut, counterstained with uranyl acetate and lead citrate, and examined by electronmicroscopy.

For ultrastructural double immunolocalization, the method was essentially the same, except that sections were incubated simultaneously in 2 antibodies directed against different antigens and raised in different species. This was followed by incubation in 2 correspondingly appropriate secondary antibodies, 1 labeled with 5-nm gold and the other with 15- or 10-nm gold as described (4).

Cultured Human Muscle Fibers

Tissue cultures of normal human muscle were established as previously described, from satellite cells of portions of diagnostic muscle biopsies from patients who, after all tests were performed, were considered free of muscle disease (reviewed in 30). Cultures were maintained in F14 medium (Gibco), supplemented with 10% fetal bovine serum (HyClone), insulin (10 ug/ml, Sigma), and an antibiotic/antimycotic mixture (Sigma)

### RESULTS

#### Light Microscopic Immunocytochemistry

**IBM:** Approximately 50% of IBM-VMFs in all IBM muscle biopsies contained small strongly immunoreactive α-syn inclusions located internally in the fibers (Figs. 1, 2). Some VMFs had also patchy subplasmalemmal staining and a slight diffuse cytoplasmic immunoreactivity. On sections double-immunostained for both Aβ and α-syn, α-syn co-localized with Aβ (Fig. 2B). However, only 60%–70% of Aβ-positive muscle fibers had α-syn immunoreactive inclusions.

#### Gold Immunoelectronmicroscopy

Results summarized in the Table. Within IBM-abnormal muscle fibers α-syn immunoreactivity was localized to 6- to 10-nm-diameter amyloid-like fibrils, and amorphous and floccular material (Fig. 3). On those structures, α-syn was close to Aβ (Fig. 3), α-syn was not localized on the 15–21-nm PHFs (Fig. 4). While PHFs were strongly labeled with antibodies recognizing phosphorylated tau, α-syn was located on the 6–10-nm amyloid-like fibrils and/or amorphous material close to the PHFs, but was not on the PHFs themselves (Fig. 4). There was no α-syn immunoreactivity associated with normal myofibrils, nor on the myelin-like whorls, various lysosomal inclusions, and lipofuscin granules typically present in IBM vacuolated muscle fibers.

#### Other Diseased and Normal Muscle

None of the normal muscle biopsies had α-syn immunoreactive inclusions that were characteristic of IBM abnormal muscle fibers. Regenerating muscle fibers,
Fig. 3. Gold immunoelectronmicroscopy of IBM abnormal muscle fibers. A, B: Single labeling of α-syn. Gold particles (10-nm) label 6–10-nm filaments and amorphous material. Adjacent portions of the myofiber (asterisk) (B) are not immunolabeled. C–F: Double immunolabeling of α-syn (5-nm gold particles) and Aβ (C, F; 10-nm gold particles; D–E, 15-nm gold particles). α-syn and Aβ immunodecorate 6–10-nm filaments and amorphous inclusions. On those structures, Aβ is close to α-syn. Magnifications: A, ×53,000; B, ×42,600; C, ×32,000; D, ×30,000; E, ×42,660; F, ×85,000.

identified by positive desmin immunoreactivity, in all biopsies that contained them, had diffusely increased cytoplasmic α-syn immunoreactivity (Fig. 2D–F). Very strong α-syn immunopositivity was also present in necrotic muscle fibers, identified by lack of desmin immunoreactivity (27) (Fig. 2G–I). In all biopsies, there was strong α-syn immunoreactivity at the postsynaptic domain of the neuromuscular junctions, which closely colocalized with α-bungarotoxin bound to the nicotinic acetylcholine receptors (Fig. 5). When the primary antibody was omitted or replaced by either a nonimmune serum or an irrelevant antibody, the immunoreaction did not take place.

Cultured Human Muscle Fibers

Three-week-old cultured muscle fibers had a) strong α-syn immunoreactivity; b) an 18-kDa α-syn band on immunoblots; and c) a 1.5-Kb α-syn band on Northern blots (Fig. 6).

DISCUSSION

In 1993, a 140 amino acid protein was isolated from AD amyloid plaques and named “NAC” (non-Aβ component of AD amyloid) (17). The cloned DNA encoding NAC precursor was named “NACP” (17). NAC localized to the 6–10-nm amyloid fibrils in AD brain and has been shown to have strong tendency to form β-structures (17). NACP was detected as 19-kD protein in the cytosolic component of brain homogenates (17). Its mRNA was strongly expressed in brain, and in low concentrations in other tissues, but not in liver (17). In 1994, Jake et al (33) cloned from human brain 2 proteins, 1 identical to human NACP and the other to rat synuclein; they named them α- and β-synuclein, respectively. Synelphin, cloned from zebra finch brain by George et al, in 1995 (34), is now known to be a homologue of α-synuclein. β-synuclein and γ-synuclein are 2 other proteins of the synuclein family (reviewed in 5–8). Normal biological functions of the synucleins are relatively unknown.

α-synuclein, but not β-synuclein, plays an important role in neurodegenerative diseases of the brain. Missense mutations in the α-syn gene cause some forms of hereditary PD (9), and α-syn is a major component of abnormal brain structures in various neurodegenerative disorders (reviewed in 5–9).

Our data suggest that α-syn plays a role in pathologic and normal processes of human muscle. In s-IBM, immunolocalization of α-syn in VMFs was similar to that described in AD brain; α-syn was localized to amyloid-like fibrils, where it co-localized with Aβ, and it was not present on the phosphorylated-tau containing paired-helical filaments (13).
Fig. 4. Gold immunoelectronmicroscopy of IBM abnormal muscle fibers. A–C: Double immunolabeling of α-syn (10-nm gold) and SMI-31 (5-nm gold). SMI-31 antibody, which recognizes phosphorylated tau on IBM-PHFs (3, 23), labels the PHFs. The α-syn label is on structures close to PHFs but is not on the PHFs. Magnification, ×100,000.

Fig. 5. Two normal neuromuscular junctions. There is a very strong α-syn immunopositivity (A), co-localizing with postsynaptically bound α-bungarotoxin (α-BT) (B). Yellow color in (C) (double exposure) indicates co-localization of α-syn with α-BT. Magnification, × 900.

As in the brain of various neurodegenerative disorders, the mechanism and the initiating event and pathogenic steps leading to accumulation of α-syn in IBM muscle fibers are not yet known. We have previously demonstrated that accumulation of AβPP epitopes precedes other abnormalities in IBM muscle fibers (1, 31, 32). In the present study, only 60%–70% of the Aβ-containing muscle fibers had α-syn immunoreactivity, suggesting that accumulation of Aβ may precede α-syn deposition. It was previously demonstrated that α-syn associates with Aβ containing amyloid fibrils in AD brain (17), and that in vitro α-syn a) binds to pre-existing Aβ (35, 36) and b) stimulates Aβ aggregation (37). In vitro, α-syn is prone to self-aggregation and can form amyloid fibrils (38–41). Accordingly, one can speculate that in IBM muscle fibers a) α-syn binds to previously accumulated Aβ; b) some
of the congophilic inclusions are composed of both Aβ and α-syn, and c) binding of α-syn to Aβ and vice-versa, might accelerate and increase their fibrillogenesis there.

In vitro, oxidative stress has been shown to induce aggregation of α-syn into amyloid-like fibrils (42). Since oxidative stress seems to play a role in IBM pathogenesis (43), this mechanism may contribute to post-translational modification of α-syn and its aggregation in IBM.

Even though α-syn is traditionally considered a presynaptic protein, this designation appears too narrow. The previous identification of α-syn in glial cytoplasmic inclusions in multiple system atrophy (44), and our demonstration of α-syn in IBM abnormal muscle fibers indicates its presence and probable role in various cell types. Our finding of increased α-syn immunoreactivity in human regenerating and necrotic muscle fibers and the presence of α-syn and its mRNA in cultured human muscle fibers points to its possible role both in human muscle development and necrotic muscle cell death. Moreover, α-syn immunoreactivity at the postsynaptic domain of human nerve-muscle junctions, both by light microscopy (this paper), and by immunoelectronmicroscopy (Askanas and Alvarez, unpublished observation) suggest that α-syn may play a role in postsynaptic nerve-muscle communication, such as in signaling and/or maintaining the nerve-muscle junction. It is also possible that at the nerve-muscle junction α-syn is at least partially bound to Aβ, since all AβPP epitopes, including Aβ (28), and AβPP-mRNA (2) are normally accumulated there.

CONCLUSION

Our studies demonstrate that abnormal accumulation of α-syn occurs in diseased human muscle and thus is not unique to the brain disorders. Since α-syn is synthesized by human muscle in culture, normal and pathologic cultured human muscle models, such as, a) normal cultured muscle after AβPP gene transfer (31, 32), and b) cultured genetically-abnormal muscle from hereditary inclusion-body myopathy (45) should provide living human model of the tissue affected by α-syn accumulation in vivo and more readily available than brain, for a wide range of molecular studies of α-syn transcription, translation, and processing. Such studies might contribute to a better understanding of functions of α-syn in normal and abnormal human muscle, and perhaps be relevant to neurodegenerative diseases.

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