β-Amyloid Precursor Epitopes in Muscle Fibers of Inclusion Body Myositis

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Sporadic inclusion body myositis (IBM) and hereditary inclusion body myopathy (hIBM) are severe and progressive muscle diseases, characterized pathologically by vacuolated muscle fibers that contain 15- to 21-nm cytoplasmic tubulofilaments (CTFs). Those vacuolated muscle fibers also contain abnormally accumulated ubiquitin and β-amyloid protein (Aβ), and they contain amyloid in β-pleated sheets as indicated by Congo red and crystal violet positivity. Using several well-characterized antibodies, we have now demonstrated that, in addition to Aβ, two other epitopes, N-terminal and C-terminal, of the β-amyloid precursor protein (βPP) are abnormally accumulated in IBM vacuolated muscle fibers and similarly in hIBM. At the light microscopy level, immunoreactivities of N- and C-epitopes of βPP closely colocalized with Aβ and ubiquitin immunoreactivities. However, by immunogold electronmicroscopy, even though N-, C-, and Aβ epitopes of βPP and ubiquitin colocalized at the amorphous and dense floccular structures, only Aβ was localized to the 6- to 10-nm amyloid-like fibrils and only ubiquitin was localized to CTFs. βPP immunoreactive structures were often in proximity to CTFs, but CTFs themselves never contained βPP immunoreactivities. The fact that Aβ but not C- or N-terminal epitopes of βPP localized to the 6- to 10-nm amyloid-like fibrils suggests that free Aβ might be generated during βPP processing and, after aggregation, may be responsible for the amyloid present within IBM muscle fibers. Our study demonstrates that three epitopes of βPP accumulate abnormally in diseased human muscle, and therefore this phenomenon is not unique to Alzheimer's disease, Down's syndrome brain, and Dutch-type cerebrovascular amyloidosis.

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Sporadic inclusion body myositis (IBM) is the most common muscle disease in patients age 55 years and older. There is progressive, often pronounced, proximal and distal muscle weakness, thinning of the forearms, male predominance, and commonly a poor or no response to immunosuppression treatment [1–6]. Light microscopic features include degrees of inflammation varying from abundant to none; muscle fibers with vacuoles that are usually rimmed by, and contain some, red-staining material with the modified trichrome reaction [7]; and a few atrophic angular, panesterase-dark muscle fibers, suggesting a denervation component [5, 6, 8]. By routine histochemistry, IBM can be difficult to distinguish from polymyositis. Electronmicroscopy (EM) reveals that abnormal muscle fibers contain cytoplasmic tubulofilaments (CTFs), 15- to 21-nm external and 3- to 6-nm internal diameter; these have been considered the characteristic ultrastructural feature of IBM [1–5]. The pathogenesis of sporadic IBM is not known. Autosomal recessive [9–11] and autosomal dominant [12] "hereditary IBM" designates rare patients with progressive muscle weakness, CTFs in vacuoles of abnormal muscle fibers, and atrophic muscle fibers, but no inflammation in the biopsy, i.e., "hereditary inclusion body myopathy." The pathogenesis of these hereditary disorders is unknown.

Vacuolated muscle fibers of both sporadic and hereditary IBM have recently been shown to contain (1) abnormal accumulations of ubiquitin (Ub) and β-amyloid protein (Aβ) [13–16], and (2) Congo red positivity indicative of amyloid in β-pleated sheets [17]. By light microscopy, Ub and Aβ colocalized with each other; however, by immuno-EM Ub was localized to CTFs [13, 14], while Aβ was localized to amorphous, poorly defined structures and to 6- to 10-nm amyloid-like fibrils [16]. Abnormally accumulated α_1-antichymotrypsin immunoreactivity was also demonstrated in the vacuolated muscle fibers of sporadic and hereditary IBM by light microscopy [18]. There is a pathological similarity between abnormal muscle fiber intracellular inclusions of IBM and the extracellular senile plaques of Alzheimer's disease (AD) brain because both contain accumulations of amyloid fibrils, Ub, Aβ, and α_1-antichymotrypsin ([13–21], reviewed in [22, 23]).

Aβ is a 39–43 amino acid polypeptide that is a part

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of the much larger β-amyloid precursor protein (βPP) [24–26]. Abnormal accumulation of Aβ in AD brain is thought to result from abnormal proteolytic cleavage of βPP, which produces the amyloidogenic fragment Aβ (reviewed in [27, 28]). In the dystrophic neurites of senile plaques of AD brain, in addition to Aβ there are abnormally accumulated C-terminal and N-terminal sequences of βPP [29–38]. In respect to sporadic and hereditary IBM, we now have asked whether other epitopes of βPP, in addition to Aβ, are abnormally accumulated in IBM and, if so, how they relate ultrastructurally to Aβ and Ub immunoreactivities. Using both light and EM immunocytochemistry, we localized three sequences of βPP, viz, the N-terminal 45–62, C-terminal 676–695, and Aβ sequence, and colocalized them with each other and with Ub.

Material and Methods

Patients

Immunolocalizations were performed on sections of diagnostic muscle biopsies (obtained with informal consent), from 33 patients, ages 5 to 76 years, with the following diagnoses: sporadic IBM, 14; autosomal–recessive hereditary IBM in an Iranian Jew, 1; polymyositis, 9; Duchenne muscular dystrophy, 1; amyotrophic lateral sclerosis, 4; normal muscle, 5. Median age of IBM patients, 65 years; of the non-IBM controls, 62 years. Diagnosis of all patients was based on clinical and laboratory studies, including 18-reaction histochemistry of the muscle biopsy [39]. Biopsies of all IBM patients had vacuolated muscle fibers with modified trichrome staining and CTFs by electronmicroscopy (Fig 1). The majority of CTFs appeared as twisted helical filaments, having twist repeats of 41 to 55 nm. Some patients had clusters of densely packed small fibrils, 6 to 10 nm in diameter, which frequently were adjacent to CTFs (see Fig 1). All patients with sporadic IBM, but not the one with the hereditary IBM, had crystal violet–positive (metachromatic red) and Congo red–positive amyloid inclusions in vacuolated muscle fibers (see Fig 1).

Light Microscopic Immunocytochemistry

Immunocytochemical stainings were performed on 10-μm transverse sections of fresh-frozen muscle biopsies, using peroxidase–antiperoxidase (PAP) and fluorescence methods, following the same general procedures as described [13, 14, 16]. Six well-characterized antibodies against βPP sequences were used (details in Table 1). These antibodies were shown to be very specific in our previous studies [15, 16, 43]. In addition, the following two commercially available antibodies against βPP sequences (Boehringer) were used: (1) rabbit polyclonal against sequence 1–40 of Aβ, diluted 1:20 to 1:40, and (2) mouse monoclonal, clone 22C11, that recognized sequence 60–100 of N-βPP diluted 1:20 to 1:40. Ub was localized with a monoclonal antibody, clone 042691GS (Chemicon, Temecula, CA) diluted 1:50 to 1:200 depending on the lot. In our previous study, this antibody proved to be specific and produced the same results as several other well-characterized monoclonal and polyclonal anti-Ub antibodies [14–16, 43, 44].

For PAP reactions, sections were preincubated for 60 minutes in either normal goat or rabbit serum diluted 1:10, followed by 48 hours of incubation at 4°C in the appropriately diluted primary antibody, and then by 1 hour of incubation in a secondary antiserum. This was followed by a PAP complex, visualized by the DAB reaction.

For fluorescence staining, the procedure was essentially the same as for PAP except that the secondary antibodies were labeled either with fluorescein isothiocyanate (FITC) or rhodamine. In some instances, the secondary antiserum was biotinylated and the reaction product was visualized by streptavidin–Texas red. Double immunolocalization of βPP epitopes with each other and with Ub was performed with double fluorescence labeling using FITC, Texas red, or rhodamine as described [16, 43, 44].

Specificity of βPP immunoreactivities was determined by (1) omitting the primary antibody, (2) replacing the primary antibody with nonimmune serum, (3) absorbing the primary monoclonal antibody G-OP-1 with synthetic Aβ peptide sequence 8–17, and (4) absorbing primary polyclonal antiserum C8 with synthetic peptide 676–695.

Gold Immuno-Electronmicroscopy

βPP sequences and Ub were localized on 10-μm unfixed frozen sections adhered to the bottom of 35-mm Petri dishes [13, 16]. For single immunolocalization, sections were incubated in a primary antibody for 48 hours at 4°C, and then incubated in diluted secondary antibody conjugated to 10-nm gold particles (Amersham-Janssen Auro Probe EM) for 69 to 72 hours at 4°C. The sections were then fixed in a 2% paraformaldehyde 1.2% glutaraldehyde mixture, postfixed in osmium, and embedded in situ in the Petri dish, according to our method for cultured muscle [45]. 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 EM but stained with the PAP reaction. The exact same muscle fibers that contained PAP-positive inclusions were identified in the adjacent gold-labeled Epon-embedded section, marked, and 1-mm-diameter cores drilled out [45, 46]. From the cores, each containing at least one vacuolated muscle fiber, thin sections were cut, counterstained with uranyl acetate and lead citrate, and examined by EM. This method has proved reliable for ultrastructural identification of CTFs in IBM vacuolated muscle fibers [47] and for ultrastructural immunogold labeling [13, 16, 48].

For ultrastructural double immunolocalization, the method was essentially the same, except that sections were incubated simultaneously in two antibodies directed against different antigens and raised in different species. This was followed by incubation in two correspondingly appropriate secondary antibodies, one labeled with 5 nm of gold and the other with 15 nm of gold. To avoid misinterpretation caused by possibly different penetration of two sizes of gold particles, in each experiment studies were performed in duplicate, alternating the size of gold particles conjugated with each secondary antibody.

Results

Light Microscopic Immunocytochemistry

Vacuolated muscle fibers of sporadic and hereditary IBM biopsies contained very strongly immunoreactive

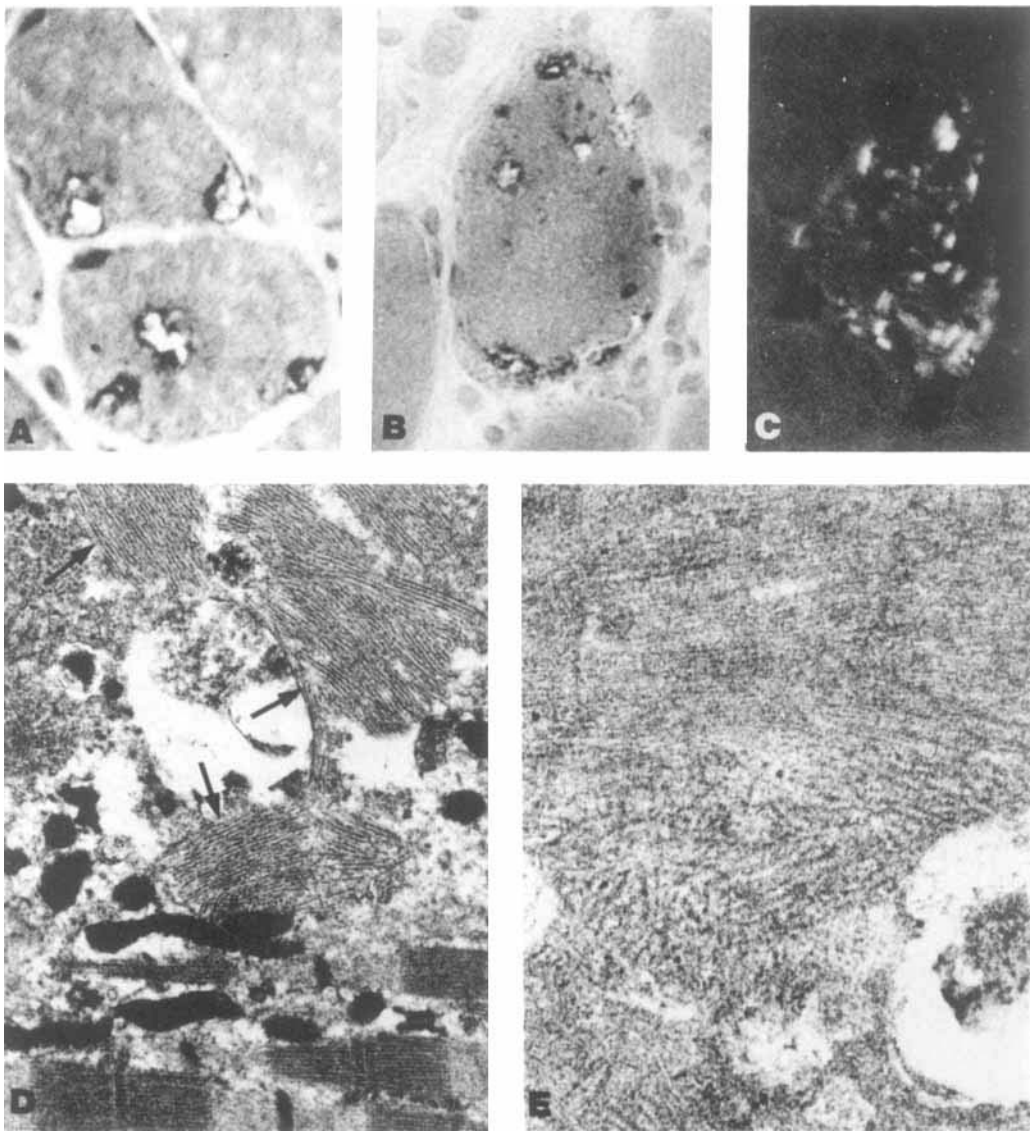


Fig 1. Overview of inclusion body myositis muscle biopsy. (A–C) Cross sections, light microscopy. (D, E) Electronmicroscopy. (A, B) Muscle fibers containing interior and subsarcolemmal vacuoles (modified trichrome staining, ×1,250). There is a slight mononuclear cell infiltration adjacent to the abnormal fiber in B. (C) Amyloid deposits in a vacuolated muscle fiber (Congo red staining viewed in polarized light, ×1,600). (D) Characteristic groups of cytoplasmic tubulofilaments (CTFs) (arrows). *(E) A large area of densely packed 6- to 10-nm amyloid-like fibrils (upper half of field) adjacent to CTFs (lower half of field). (D: ×11,000, E: ×49,000.)*

Table 1. Antibodies Used to Localize β-Amyloid Precursor Protein Epitopes in Inclusion Body Myositis Muscle

Designation	Antigen	Dilution	Reference
C8	C-terminal, sequence 676–695 (C-βPP)	1:1,000	40
R37	C-terminal, sequence 681–695 (C-βPP)	1:200	30
SP18	N-terminal, sequence 45–62 (N-βPP)	1:500	32
T97	N-terminal, sequence 18–38 (N-βPP)	1:200	35
R1280	Aβ, sequence 1–40	1:1,000	41
G-OP-1	Aβ, sequence 8–17	1:200	42

All antibodies are rabbit polyclonal, except G-OP-1, which is mouse monoclonal.
Aβ = β-amyloid protein.

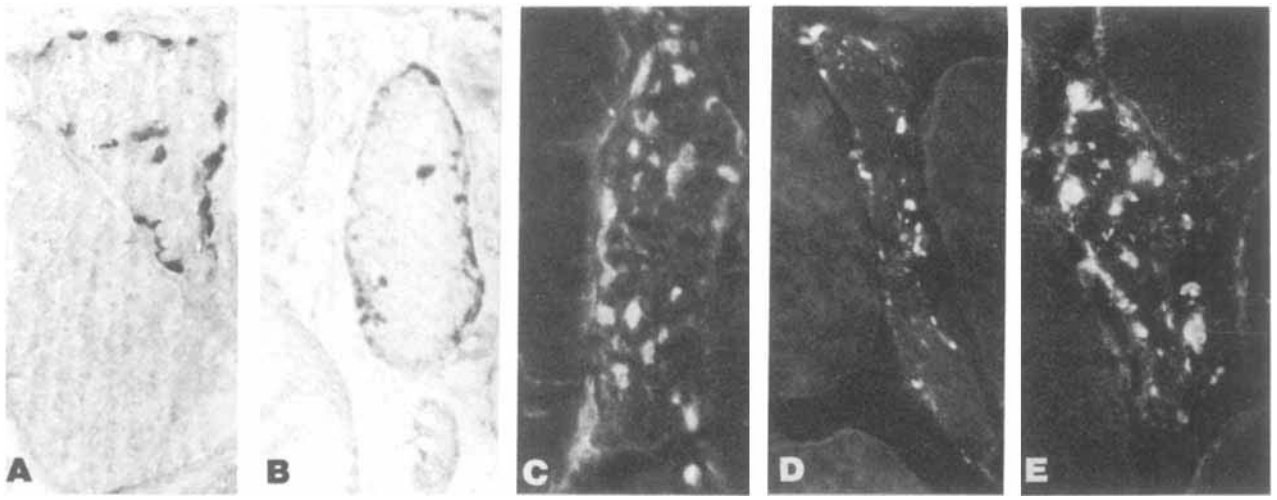
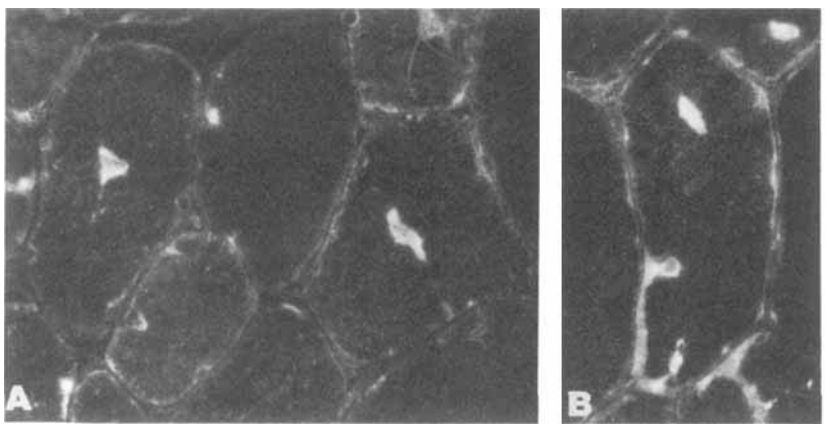


Fig 2. Light microscopic immunocytochemistry of three β-amyloid precursor protein (βPP) sequences in abnormal muscle fibers of sporadic inclusion body myositis. (A, B) Peroxidase–antiperoxidase reaction: N-terminal sequence of β-amyloid precursor protein (N-βPP) (A), C-terminal sequence of βPP (C-βPP) (B). (C–E) Fluorescence staining (C: N-βPP; D: Aβ; and E: C-βPP). Abnormal muscle fibers contain clusters of βPP immunoreactivity distributed within the cytoplasm. In addition, muscle fibers in A and B also have a patchy staining in the peripheral region of muscle fiber. (A, B: ×1,400 before 3% reduction; C, E: ×1,500 before 3% reduction; D: ×1,000 before 3% reduction.)

Fig 3. Fluorescence staining of C-terminal sequence of β-amyloid precursor protein epitope in muscle biopsy of hereditary inclusion body myopathy. (A, B) Muscle fibers have large interior cytoplasmic inclusions; a muscle fiber also has a strong subsarcolemmal immunoreactivity, part of which extends toward the interior of the fiber (B) (×1,050).

(IR) inclusions of all βPP sequences within large and small vacuoles (Fig 2). (For simplicity, the designation IR will be omitted after each substance localized in this study, but that term must be understood.) The βPP-inclusions were located in the subsarcolemma or internally in the fibers. Occasional abnormal muscle fibers, especially ones containing only one or two small vacuoles, had a thin rim of sarcolemmal-like staining (see Fig 2). In some very abnormal and highly vacuolated muscle fibers, βPP accumulations appeared to extend slightly outside the boundary of the muscle fiber (see Fig 2). In an occasional vacuolated muscle fiber, in addition to strong immunoreactivity within vacuoles and/or their close proximity, there was also a slight, somewhat diffuse immunoreactivity in what appeared to be vacuole-free cytoplasm with antisera C8 and R37 (both against the C-terminal of βPP) and T97 (against the N-terminal of βPP). In the hereditary IBM biopsy, some muscle fibers contained a strong, wide subsarcolemmal accumulation of βPP sequences that extended to the interior of the fiber (Fig 3). Use of Boehringer



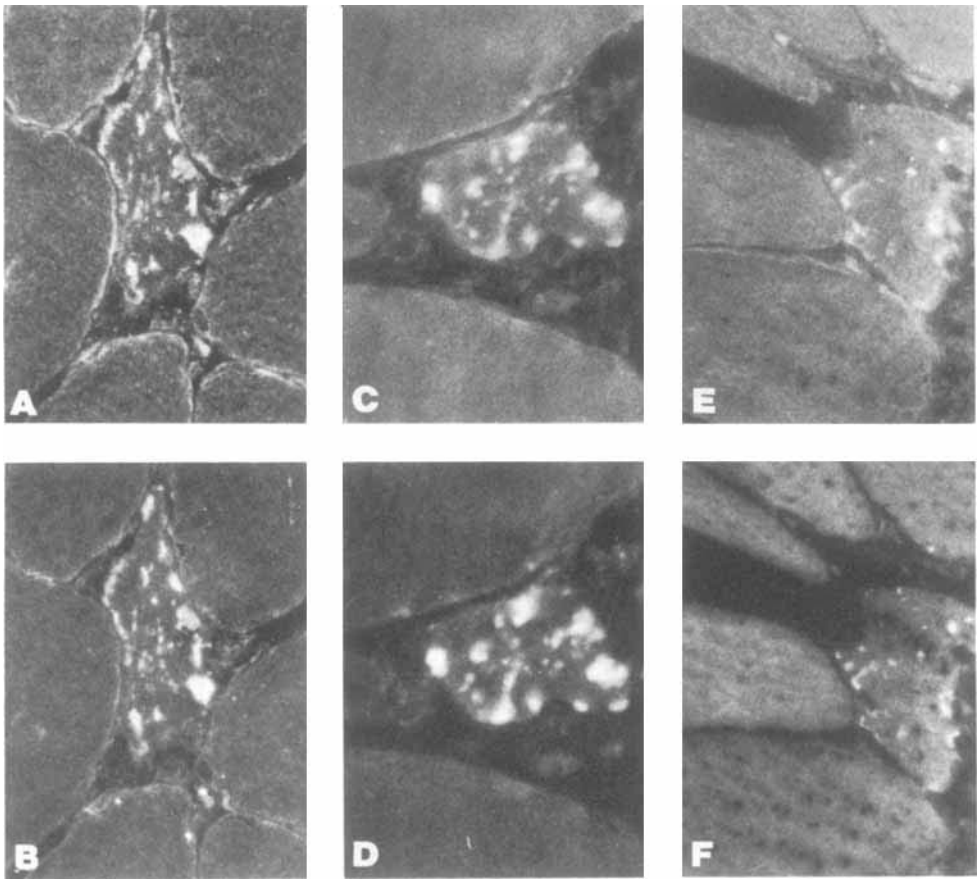


Fig 4. Light microscopic double immunostaining on the same section of tissue of abnormal muscle fibers in inclusion body myositis. C-terminal sequence of β-amyloid precursor protein (C-βPP) (A) plus β-amyloid protein (Aβ) (B); N-terminal sequence βPP (N-βPP) (C) plus Aβ (D); C-βPP (E) plus ubiquitin (F). There is close colocalization of N and C epitopes of βPP with Aβ and with ubiquitin. (All ×1,400.)

antisera against βPP sequences provided the same abnormal pattern of βPP sequence accumulation but with considerably weaker reactivity.

Light microscopic double immunostainings showed that (1) C- and N-terminals of βPP closely colocalized with Aβ (Fig 4A–D), and (2) that βPP was colocalized with Ub in all βPP-positive fibers (Fig 4E, F).

When the primary antibody was omitted, absorbed, or replaced by a nonimmune serum, the immunoreaction did not take place.

None of the biopsies of 9 patients with polymyositis and none of the other control patients' biopsies had the βPP inclusions that were characteristic of IBM vacuolated muscle fibers. Small regenerating muscle fibers in IBM, polymyositis, and Duchenne muscular dystrophy had slight, evenly distributed immunoreactivity of C- and N-terminal βPP (with C8, T97, and R37 antisera).

As previously described in normal human muscle [45, 46], in all biopsy categories there was strong immunoreactivity of all βPP sequences and Ub at the postsynaptic domain of the neuromuscular junctions. Intramuscular nerve twigs, in all biopsies that contained them, were immunoreactive with all βPP sequences except Aβ 8–17 (G-OP-1) and Aβ 1–40 (R1280).

Gold Immuno-Electronmicroscopy (Results Summarized in Table 2)

Most of the structures identified by all the βPP antibodies consisted of irregular clusters of nearly amorphous dense material, which were either irregular or rounded (Figs 5, 6). In those structures there was colocalization of all three βPP sequences (see Fig 5). The three βPP sequences were also colocalized as loose, floccular material (see Fig 5), which was often in close proximity to, or adjacent to, the 15- to 21-nm CTFs; however, the CTFs themselves never contained immunoreactivity of any of the βPP epitopes (see Fig 6). In proximity to the loose, floccular material, there were also loosely packed amyloid-like fibrils 6 to 10 nm in diameter; these had Aβ but not C- or N-terminal βPP immunoreactivity (see Fig 5).

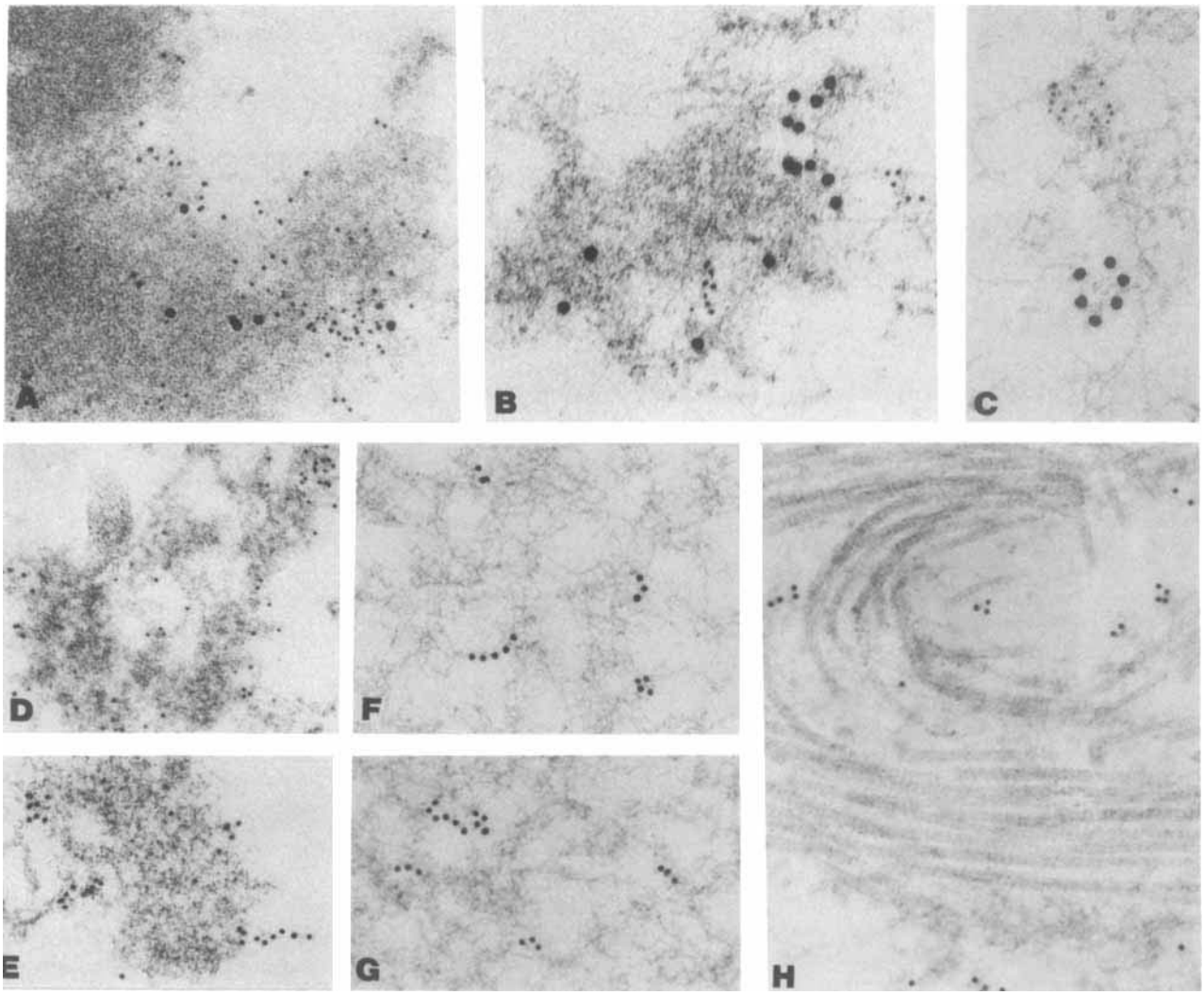


Fig 5. Double (A–C) and single (D–H) immunogold electronmicroscopy of inclusion body myositis muscle biopsies. (A) C-terminal sequence of β-amyloid precursor protein (C-βPP) is marked with 15-nm gold particles, β-amyloid protein (Aβ) is marked with 5-nm gold particles. (B) N-terminal sequence of βPP (N-βPP) is marked with 15-nm gold particles, Aβ with 5-nm gold particles. (C) N-βPP is marked with 5-nm gold particles, Aβ with 15-nm gold particles. While there is close colocalization of N- and C-βPP with Aβ on the denser amorphous and looser floccular structures in A and B, only Aβ is localized to the 6- to 10-nm loose fibrillar structures (B, C). (A: ×107,000 before 3% reduction; B: ×134,000 before 3% reduction; C: ×128,000 before 3% reduction.) (D) C-βPP is localized to amorphous structures (5-nm gold particles) (×108,000 before 3% reduction). (E–G) Aβ is localized to amorphous structures and fibrillar material extending from the amorphous material (E) and organized in loose clusters (F, G) (10-nm gold particles) (E: ×55,000 before 3% reduction; F, G: ×65,000 before 3% reduction). (H) Intermingled with collagen fibrils lying slightly outside an abnormal muscle fiber are anti-C-βPP antibody immunodecorated structures (10-nm gold particles) (×67,000 before 3% reduction).

Table 2. Subcellular Localization of β-Amyloid Precursor Protein Epitopes and Ubiquitin in Inclusion Body Myositis Muscle

Structure	Aβ	N-βPP	C-βPP	Ubiquitin
Cytoplasmic tubulofilaments (CTFs)	–	–	–	+
6–10-nm amyloid-like fibrils	+	–	–	+ (but less abundantly than Aβ)
Amorphous structures	+	+	+	+
Floccular material	+	+	+	+

Aβ = β-amyloid protein; N-βPP and C-βPP = N-terminal and C-terminal sequences of β-amyloid precursor protein.

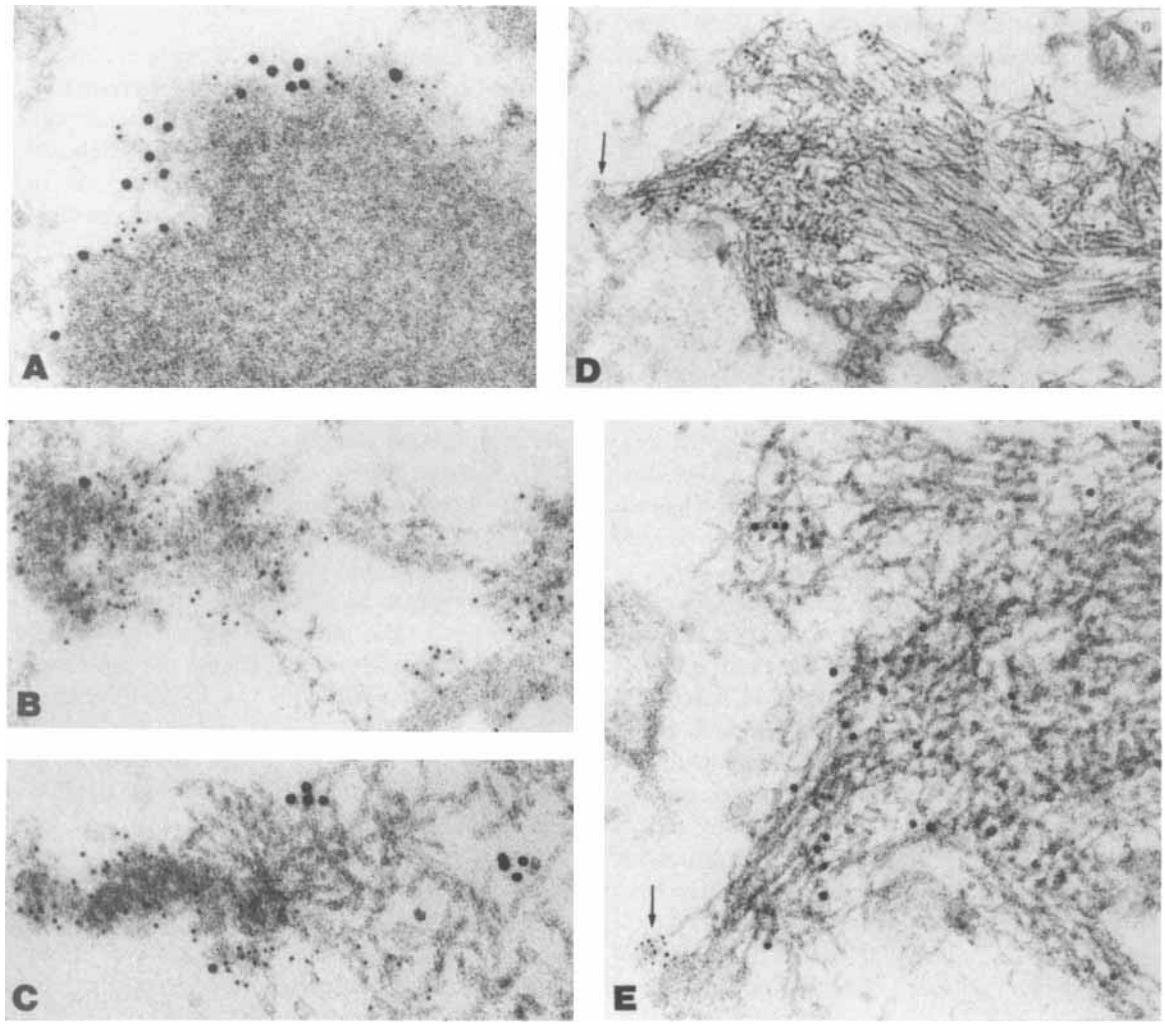


Fig 6. Double immunogold electronmicroscopy demonstrating localization of ubiquitin and three epitopes of β-amyloid precursor protein (βPP) in abnormal muscle fibers of inclusion body myositis. Ubiquitin is marked with 15-nm gold particles, βPP epitopes with 5-nm gold particles. (A) Both ubiquitin and β-amyloid protein (Aβ) are present on less tightly packed material at the periphery of an amorphous structure. (B) Ubiquitin and C-terminal sequence βPP (C-βPP) are present on floccular material (×108,000). (C) Ubiquitin and N-terminal sequence of βPP (N-βPP) are colocalized at the dense amorphous material (left) adjacent to 15- to 21-nm cytoplasmic tubulofilaments (CTFs) (center and right), but only ubiquitin is localized to CTFs (×108,000). (D, E) Lower and higher power of CTFs immunodecorated by anti-ubiquitin antibody. At the periphery of the collection of CTFs, there is a small patch of an amorphous material immunodecorated by anti-Aβ antibody (arrows) *(D: ×33,000; E: ×85,000).*

Even though most of the βPP loci were inside the muscle fibers, occasionally immediately outside very abnormal muscle fibers there were also patches of βPP material lying in the extracellular space and intermingled with collagen fibers (see Fig 5).

The most abundant Ub was present on CTFs (see Fig 6), which contained no βPP. On the amorphous and floccular structures, Ub immunoreactivity colocalized with all three βPP epitopes (see Fig 6) but in some areas appeared to be less abundant there (see Fig 6B, C).

Discussion

This study demonstrated that N-, C-, and Aβ epitopes of βPP were abnormally accumulated in the vacuolated muscle fibers of sporadic and hereditary IBM. Most of those accumulations were Congo red and crystal violet positive, indicating amyloid in β-pleated sheets. However, some vacuolated fibers in sporadic IBM and all the vacuolated fibers in our 1 patient with hereditary IBM had three-epitope βPP positive accumulations

that were Congo red negative, suggesting that their Aβ sequences were not in an amyloid β–pleated sheet configuration, but perhaps still in relatively intact βPP molecules. Accordingly, such accumulations may represent early changes of this IBM feature. The Congo red negativity of these Aβ-containing loci is like that of diffuse plaques in AD brain, which are considered to be an early abnormality [49, 50]. The presence of βPP accumulations deeply internal within some muscle fibers suggests their origin was intracellular.

Even though immunoreactivities of βPP epitopes colocalized with each other and with Ub at the light microscopic level, their ultrastructural localization differed. While all three βPP epitopes were colocalized to several types of abnormal subcellular structures, only Aβ was localized to the 6- to 10-nm-diameter amyloid-like fibrils. None of the βPP epitopes was localized on the 15- to 21-nm-diameter CTFs; these contained only strong Ub immunoreactivity.

In some very abnormal muscle fibers, the accumulations of βPP appeared to extend outside the muscle fiber boundary. This may have been due to a fragility of the fiber's surface membrane, which could have been transiently broken. Sometimes associated with those fibers were a few βPP structures intermingled with collagen fibrils, either inside the muscle fiber or outside but very close to it. Because collagen fibrils are an extracellular component, the occasional βPP structures intermixed with them just outside the fiber may have been generated (1) inside the muscle fiber but emerged through a broken membrane or by exocytosis, or (2) outside the fiber. The first possibility appears more likely because (1) it has been reported that βPP is synthesized in the normal adult muscle fiber [51], (2) βPP is present within normal human muscle fibers at the postsynaptic domain of the neuromuscular junction [43], and (3) in normal human muscle fibers regenerating in culture, βPP mRNA is diffusely present as identified by in situ hybridization (Sarkozi E, Askanas V, McFerrin J, Engel WK, unpublished observation).

βPP, the product of a chromosome-21 gene [52], exists in virtually all tissues (reviewed in [22]). βPP is considered to be a cell-surface glycoprotein containing a large extracellular N-terminal domain, a transmembrane Aβ domain, and a short cytoplasmic C-terminal domain [24–26]. The βPP gene produces at least three alternatively spliced transcripts, encoding βPP containing 695, 751, or 770 amino acids. The last two contain in their extracellular region an insert of a Kunitz-type protease inhibitor (reviewed in [22, 28]). The biological functions of βPP are unknown, but it may play a role in (1) cell-to-cell and cell-to-matrix interaction [52–56], (2) neurite growth [53], and (3) maintaining cell integrity and shape [57]. The recent demonstration that βPP epitopes are strongly accumulated postsynaptically at normal human neuromuscular junctions [43] suggests that βPP may have an important function there.

Prior to our studies of IBM muscle, abnormal accumulation of βPP epitopes was reported only in (1) brain senile plaques of Alzheimer's disease and Down's syndrome, and (2) cerebral blood vessels of Alzheimer's disease and Dutch-type hereditary cerebral hemorrhage (reviewed in [22, 27, 28]). Our results demonstrate that all three epitopes of βPP (N-terminal, C-terminal, and Aβ) are abnormally accumulated in vacuolated muscle fibers of sporadic and hereditary IBM. Further studies will be required to answer several questions. Does this accumulation result from increased generation of βPP, abnormal processing of it, or both? Is βPP abnormality the initial step in a cascade of pathogenic events leading to sporadic and hereditary IBM, including formation of CTFs, or is it secondary to another pathogenic mechanism? Is the Ub increase a primary pathogenic process or is it secondary to a need for Ub to facilitate degradation of abnormal proteins? Do similar abnormal βPP accumulations occur in any other muscle disease that we have not yet studied?

The fact that Aβ, but not C- and N-terminal epitopes of βPP, is localized to the amyloid-like fibrils suggests that free Aβ is generated during βPP processing and may be responsible for the amyloid present in IBM muscle fibers.

Conclusion

Our study indicates that abnormal accumulation of βPP occurs in diseased human muscle and thus is not unique to the brain and cerebral vessels. Normal and pathological human muscle, including use of cultured normal and genetically abnormal (such as hereditary IBM) muscle [58], should provide living human tissue more readily accessible than brain for a wide range of molecular studies of βPP synthesis and processing. Such analyses could lead to a better understanding of sporadic and hereditary IBM, Alzheimer's disease, and perhaps other diseases. Whether there is a mutation of the βPP gene in hereditary IBM remains to be determined.

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