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Short Communication

Light and Electron Microscopic Localization of β-amyloid Protein in Muscle Biopsies of Patients with Inclusion-body Myositis

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In 11 of 11 inclusion-body myositis (IBM) patients, including one hereditary case, vacuolated muscle fibers contained large and multiple small inclusions immunoreactive for β-amyloid protein (βAP). All IBM muscle biopsies had characteristic cytoplasmic tubulo-filaments (CTFs) by electron microscopy. None of 14 control muscle biopsies contained the βAP immunoreactive (IR) inclusions characteristic of IBM. On the light microscopy level, βAP-IR inclusions colocalized with ubiquitin immunoreactivity. By immunogold electronmicroscopy, βAP immunoreactivity was localized to a) amorphous, poorly defined structures, b) dense floccular material, c) clusters of loosely packed amyloidlike fibrils 6–8 nm in diameter, and d) poorly defined loose fibrillar structures 6–8 nm in diameter. βAP immunoreactive structures were often in proximity to CTFs, but CTFs themselves never contained βAP-IR. Our study provides the first demonstration of βAP accumulations in abnormal human muscle. This finding suggests that in addition to Alzheimer's disease, Down syndrome, and Dutch-type hereditary cerebrovascular amyloidosis, βAP may play an important role in the pathogenesis of other diseases, including ones outside the central nervous system, for example, IBM. (Am J Pathol 1992, 141:31–36)

Inclusion-body myositis (IBM) is diagnosed by a combination of features.[1–6] Clinically adult-onset, usually sporadic, progressive muscle weakness, thinning of the forearms, male predominance, and often a poor or no response to immunosuppression treatment are present. Light-microscopic (LM) pathologic features include: degrees of inflammation varying from abundant to none; muscle fibers with rimmed vacuoles, which usually contain red-staining material with the modified trichrome reaction[7]; and a few atrophic angular, panesterase-dark muscle fibers, suggestive of a denervation component. By routine histochemistry, IBM can be difficult to distinguish from polymyositis. Electronmicroscopy (EM) reveals that abnormal muscle fibers contain cytoplasmic tubulo-filaments (CTFs), 15–21 nm external and 3–6 nm internal diameter; these are the ultrastructural diagnostic criteria of IBM.[1–5]

Autosomal recessive "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.[8–10] The pathogenesis of these disorders and the origin of CTFs are unknown.

We demonstrated in both sporadic and hereditary IBM that vacuolated muscle fibers contain strong ubiquitin immunoreactivity, which by immunoelectronmicroscopy was localized to CTFs.[11,12] It has also been shown that the vacuolated muscle fibers contain Congo-red positivity indicating amyloid,[13] but the type of amyloid protein was not identified. Because β-amyloid protein (βAP) is localized in ubiquitinated senile plaques in the Alzheimer's disease (AD) brain,[14–17] we investigated whether in IBM muscle biopsies βAP is a constituent of the amyloid deposits that coexist with ubiquitin in vacuolated muscle fibers.

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Material and Methods

Patients

βAP immunolocalization was performed in diagnostic muscle biopsy sections from 25 patients, ages 5–73 years, with the following diagnoses: sporadic IBM, 10; autosomal-recessive hereditary IBM in an Iranian Jew, 1; polymyositis, 7; Duchenne muscular dystrophy, 1; amyotrophic lateral sclerosis, 4; normal muscle, 2. The median age of IBM patients was 64 years and the median age of the non-IBM controls was 60 years. Diagnosis of all patients was based on clinical, laboratory, muscle-biopsy 18-reaction histochemistry,[18] and ultrastructural studies. All IBM patients had CTFs by electronmicroscopy and ubiquitinated inclusions by immunocytochemistry. All patients with sporadic IBM, except the patient with hereditary IBM, had crystal-violet positive (metachromatic red) amyloid inclusions[19] in vacuolated muscle fibers. Crystal-violet positive amyloid inclusions were also positive with thioflavine S.

Immunocytochemistry

Light microscopic immunocytochemistry was done on 10-μm transverse sections of fresh-frozen muscle biopsies, using peroxidase-antiperoxidase (PAP) and fluorescence stainings, following the same general procedures as described.[11,20] Two well-characterized antibodies were used: 1) mouse monoclonal antibody G-OP-1, directed against sequence 8-17 of β-amyloid synthetic peptide,[21] diluted 1:200; and 2) rabbit polyclonal antibody R1280, directed against sequence 1-40 of the synthetic peptide,[22] diluted 1:2000. Ubiquitin (Ub) was localized with a monoclonal antibody, clone 042691GS (Chemicon, Temecula, CA), diluted 1:20. In our previous study, this antibody proved to be specific and produced the same results as several other well-characterized monoclonal and polyclonal Ub antibodies.[11,12,23] Double immunolocalization of βAP and Ub was performed using fluorescence staining, as we have described.[20,23]

Specificity of βAP immunoreactivity was determined by: a) omitting the primary antibody, b) replacing the primary antibody with non-immune serum, and c) absorbing the primary monoclonal antibody with synthetic βAP peptide sequence 8-17.

For electronmicroscopic (EM) immunocytochemistry, βAP was localized on 10-μm unfixed frozen sections adhered to the bottom of 35-mm Petri dishes, according to our method for ultrastructural localization of Ub immunoreactivity.[11] After 48 hours of incubation in the monoclonal antibody against βAP, sections were incubated 69–72 hours in diluted goat anti-mouse-IgG serum conjugated to 10-nm gold (Amersham, Arlington Heights, IL). Then the sections were fixed in paraformaldehyde-glutaraldehyde, postfixed in osmium, and embedded *in situ* in the Petri dish according to our method for cultured muscle.[24] After being embedded, the section in the dish was viewed under phase-contrast microscopy and compared with an adjacent cross-section that had been stained with crystal violet to visualize metachromatic-red amyloid inclusions. The same muscle fibers that contained amyloid-positive inclusions in vacuoles with the crystal violet stain were identified in the adjacent gold-labeled Epon-embedded section. From the latter, a small (1-mm diameter) area containing the identified fiber (or fibers) was marked with a modified 16-gauge needle attached to the microscope.[25] The Epon disk was removed from the dish, and the marked areas were cored-drilled out as described.[24,25] The drilled-out cores, each containing at least one vacuolated muscle fiber, were mounted in an Epon blank block,[25] trimmed, and thin-sectioned. The thin sections were counterstained with uranyl acetate lead citrate, and examined by EM.

Results

Light Microscopy

Vacuolated muscle fibers of sporadic and hereditary IBM biopsies contained dark βAP-IR inclusions within large and small vacuoles (Figure 1). In these positive patients, βAP-IR inclusions were present in almost 100% of their vacuolated fibers. The βAP-IR inclusions had an amorphous pattern and were located subsarcolemmally or more internally in the fibers. At a given cross-sectional level, in hereditary IBM the inclusions tended to be larger and single, whereas in sporadic IBM they were often small and multiple throughout the fiber (Figure 1). In some abnormal and highly vacuolated muscle fibers, βAP-IR accumulations appeared to be extending outside the boundary of the muscle fiber. In sporadic IBM, rarely were there nonvacuolated muscle fibers containing a thin subsarcolemmal rim of βAP-IR (Figure 1A). In the hereditary IBM biopsy, some muscle fibers contained a strong, wide subsarcolemmal βAP accumulation that extended to the interior of the fiber (Figure 1G). Comparison of the immunolocalization of UB and βAP after double immunostaining showed that Ub-IR was colocalized with βAP-IR in all βAP-positive fibers (Figure 1E–H).

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

One hundred percent of the crystal-violet positive muscle fibers had βAP-IR. However, 100% of the βAP-positive muscle fibers in hereditary IBM and 1.5% of the

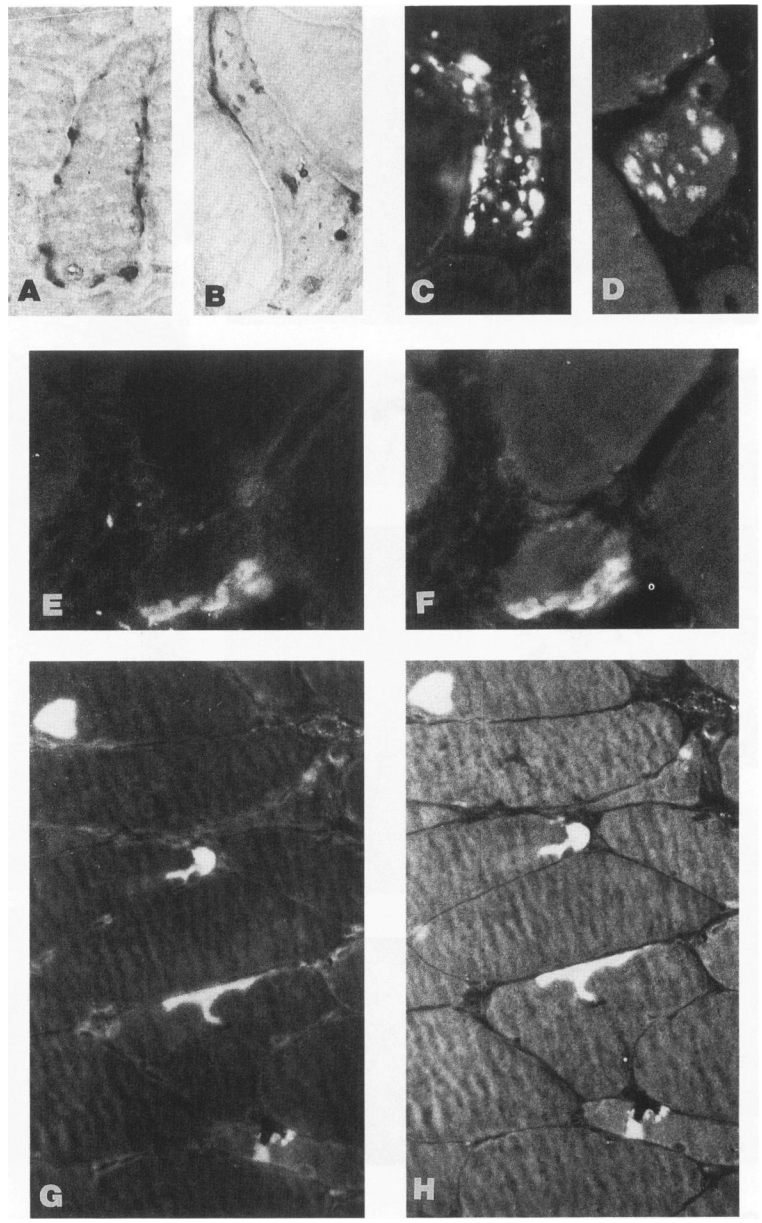

Figure 1. *Light microscopic immunocytochemistry of IBM muscle biopsies.* **A,B**: *PAP reaction.* **C,D**: *Fluorescent staining, all of βAP immunoreactivity in vacuolated muscle fibers of sporadic IBM, ×1250.* **E–H**: *Double immunostaining for βAP* **(E,G)** *and ubiquitin* **(F,H)**. *βAP is localized with Texas red and ubiquitin with green FITC;* **(E,F)** *sporadic IBM ×1250;* **(G,H)** *hereditary IBM ×813. There is close colocalization between βAP and ubiquitin in* **(E,F)** *and* **(G,H)**.

βAP-positive muscle fibers in sporadic IBM were crystal-violet negative. None of the biopsies of seven patients with polymyositis and none of the other control patients' biopsies had the βAP-IR inclusions that were characteristic of IBM.

EM Immunocytochemistry

Most of the structures immunodecorated by the βAP antibody consisted of irregular clusters of nearly amorphous material; those structures were either irregular or rounded (Figure 2A–C,F,I). In such structures, βAP was localized either throughout the entire area or on the looser peripheral parts that aggregated into short thin fibrils 6–8 nm in diameter. βAP-IR was also present on poorly defined dense, floccular material (Figure 2D,E). Amorphous structures intensively immunodecorated by βAP antibody were sometimes seen close to CTFs; however, βAP-IR was never seen on them (Figure 2F). Inside some of the muscle fibers, large clusters of loosely packed amyloid-like fibrils 6–8 nm in diameter had small patches of βAP-IR (Figure 2G). Also immunodecorated by βAP antibody were some loose fibrillar structures (Figure 2H,J). All of these βAP-IR structures seemed to be located inside the muscle fibers. In addition, outside of abnormal muscle fibers, small patches of βAP-IR material were present lying in the extracellular space and intermingled with collagen fibers.

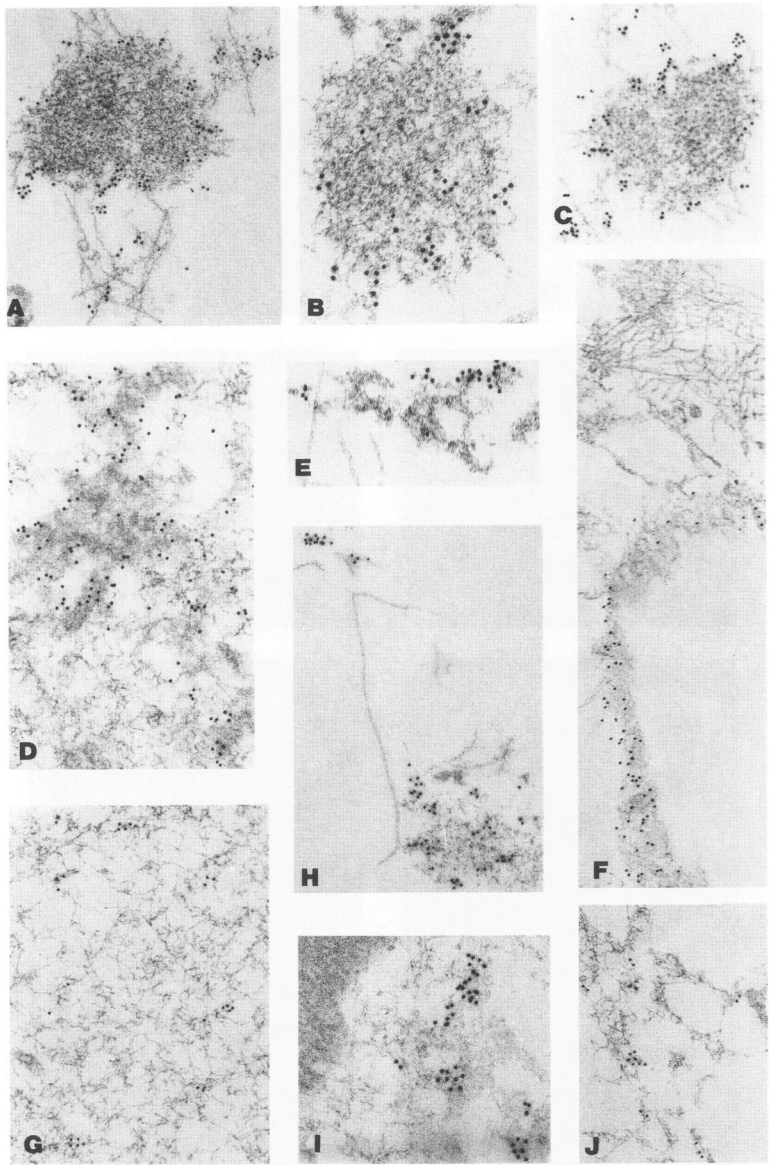

Figure 2. *Electron microscopic immunocytochemistry of βAP using gold particles.* A,B,C,F,I: *Immunodecoration by anti-βAP of various of amorphous or nearly amorphous structures.* A–C: *Gold particles are present on less-tightly packed peripheral material which appears fibrillar.* D,E: *Abundant gold particles are on floccular material.* G,H,J: *There are small patches of immunodecoration in a cluster of loose fibrillar material. Long, larger diameter (approximately 15 nm) filaments in* (A,E,H) *and a cluster of those filaments in* (F) *are not immunodecorated.* I: *Portion of a myofibril is at the upper left,* (A) ×*43,000;* (B) ×*81,000;* (C) ×*33,000;* (D) ×*67,000;* (E) ×*59,000;* (F) ×*43,000;* (G) ×*42,000;* (H) ×*33,000;* (I) ×*62,000;* (J) ×*33,000.*

Discussion

This study provides, to our knowledge, the first demonstration of βAP accumulation in diseased human skeletal muscle. It thereby indicates that βAP accumulations are not exclusively in AD, Down syndrome, Dutch-type hereditary cerebrovascular amyloidosis, and advanced age.

βAP was discovered in and first sequenced from the amyloid fibrils in blood vessels of AD patients.[26] Subsequently, it was also isolated from senile plaques of AD brain.[27,28] βAP has received considerable attention regarding the pathogenesis of AD.[16,17,29] βAP is composed of a 4 kda polypeptide, which is produced by proteolytic cleavage of the much larger amyloid precursor protein.[16,17,30]

In AD brain, βAP deposits occur in: a) typical Congo-red positive senile plaques composed mainly of 8-nm diameter amyloid fibrils and dystrophic neurites, and b) so-called diffuse "pre-amyloid" plaques that are Congo-red negative and do not contain typical amyloid fibrils.[31,32] (Congo-red positivity in the form of anisotropic dichroism and crystal-violet positivity in the form of a metachromatic red color are indicators of amyloid, and presumably are based on its β-pleated sheet configuration; they do not indicate the specific type of protein composing the amyloid.) In AD brain, the Congo-red–negative but βAP-positive plaques are considered to be an early pathologic change.[29] Another characteristic of the AD brain, intraneuronal neurofibrillary tangles (NFTs), which are composed of 10-nm paired helical filaments (PHF), lack βAP-IR[15–17,29,33] (although extracellular

"ghost" NTFs do contain βAP-IR.[15,33] Ubiquitin-IR is present not only in both kinds of plaques, Congo-red–positive and negative, but also in the intraneuronal NFTs.[14,15,32,34] Similar abnormalities to those in AD brain occur in brains of older patients with Down syndrome and to a slight extent in advanced aging.[16,17,29]

Because in both IBM muscle and AD brain the amyloid accumulations are immunoreactive with βAP and Ub, they may result from similar cellular events. Electron microscopically, the βAP-IR in IBM is present in structures similar to those described as βAP-positive in AD brain.[15]

Even though by light microscopy βAP-IR and Ub-IR are closely colocalized in IBM muscle fibers, the spatially intermixed abnormal subcellular organelles by electron microscopy show differences: 15–21-nm diameter CTFs are strongly positive for ubiquitin-IR but negative for βAP-IR, whereas the 6–8-nm amyloid-like filaments have prominent βAP-IR (their ubiquitin status has not yet been clarified).

Some muscle fibers had βAP-positive accumulations but, because they were crystal-violet and thioflavin-S negative, apparently did not contain amyloid in β-pleated sheets. Those muscle fibers, widely prevalent in our one case of hereditary IBM, may represent early changes of IBM and therefore be analogous to the finding in AD brains where βAP accumulations in the "diffuse" Congo-red–negative plaques seem to represent early changes.[29]

The presence of βAP accumulations deeply internal in some muscle fibers suggests their intracellular origin. In other vacuolated muscle fibers, the origin of the βAP seems less certain because the fiber appears fragile and the surface membrane might have been transiently broken, such that βAP could have moved in from outside the muscle fiber. By EM, some of the vacuolated fibers contained, seemingly within muscle fibers, a few βAP-IR filaments intermingled with collagen fibrils. Because collagen fibrils are an extracellular component, those βAP filaments may have been generated inside the muscle but become intermixed with exogenous collagen fibrils that had entered through a broken surface membrane, or both may have been generated outside of the fiber.

Future studies will be required to determine the precise origin and pathogenic steps of abnormal βAP accumulation in IBM muscle. Important questions are: a) is βAP in IBM-muscle produced intracellularly or is it transported from the extracellular region, or both; b) is amyloid precursor protein increased in abnormal muscle fibers; c) is the immunoreactive βAP protein of IBM derived from, or does it have homology with, another normal cellular protein, and if so, which one; d) because CTFs (like the intraneuronal NFTs in AD brain[14,15]) contain Ub-immunoreactivity[11] but not βAP-immunoreactivity, does βAP have any relationship to CTF protein; and e) do βAP accumulations occur in any other muscle diseases that we have not yet studied?

Because the accumulations of βAP in IBM muscle and AD brain have many similar features, their pathogenesis may be similar. Accordingly, detailed molecular studies of pathogenic mechanisms in the more readily accessible biopsied living IBM muscle (as compared with brain), including use of cultured IBM muscle biopsies,[35] could be advantageous for understanding both diseases.

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