RESEARCH ARTICLE

Involvement of Clusterin and the Aggresome in Abnormal Protein Deposits in Myofibrillar Myopathies and Inclusion Body Myositis

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Myofibrillar myopathies (MM) are characterized morphologically by the presence of non-hyaline structures corresponding to foci of dissolution of myofibrils, and hyaline lesions composed of aggregates of compacted and degraded myofibrillar elements. Inclusion body myositis (IBM) is characterized by the presence of rimmed vacuoles, eosinophilic inclusions in the cytoplasm, rare intranuclear inclusions, and by the accumulation of several abnormal proteins. Recent studies have demonstrated impaired proteasomal expression and activity in MM and IBM, thus accounting, in part, for the abnormal protein accumulation in these diseases. The present study examines other factors involved in protein aggregation in MM and IBM. Clusterin is a multiple-function protein which participates in Aβ-amyloid, PrP^{res} and α-synuclein aggregation in Alzheimer disease, prionopathies and α-synucleinopathies, respectively. γ-Tubulin is present in the centrosome and is an intracellular marker of the aggresome. Moderate or strong clusterin immunoreactivity has been found in association with abnormal protein deposits, as revealed by immunohistochemistry, single and double-labeling immunofluorescence and confocal microscopy, in MM and IBM, and in target structures in denervation atrophy. γ-Tubulin has also been observed in association with abnormal protein deposits in MM, IBM, and in target fibers in denervation atrophy. These morphological findings are accompanied by increased expression of clusterin and γ-tubulin in muscle homogenates of MM and IBM cases, as revealed by gel electrophoresis and Western blots. Together, these observations demonstrate involvement of clusterin in protein aggregates, and increased expression of aggresome markers in association with abnormal protein inclusions in MM and IBM and in targets, as crucial events related with the pathogenesis of abnormal protein accumulation and degradation in these muscular diseases.

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INTRODUCTION

The term myofibrillar myopathies (MM) refers to a clinically and genetically heterogeneous group of inherited or sporadic muscle diseases characterized morphologically by the presence of non-hyaline structures corresponding to foci of dissolution of myofibrils, and hyaline lesions composed of aggregates of compacted and degraded myofibrillar elements. Immunohistochemical studies have demonstrated intracytoplasmic accumulation of several proteins, including desmin, αB-crystallin and ubiquitin (9, 18, 20, 38, 39, 43, 55). Mutations in 3 genes—desmin, αB-crystallin and myotilin—have been identified as causing MM (19, 20, 37, 53, 54, 62). However, the causative gene remains to be discovered in most cases suffering from MM (8, 55).

Inclusion body myositis (IBM) is a sporadic inflammatory myopathy characterized by the presence of rimmed vacuoles, eosinophilic inclusions in the cytoplasm, rare intranuclear inclusions, and accumulation of several abnormal proteins including Aβ-amyloid, PrP and ubiquitin. Electron microscopic studies have shown filamentous cytoplasmic inclusions, paired-helical filaments, flocculomembranous and amorphous material, and myelin-like bodies (2, 23, 40, 45, 64, 66).

The ubiquitin-proteasomal pathway plays a crucial role in non-lysosomal protein degradation (17). Misfolded proteins or unassembled subunits of larger protein complexes and retro-translocated proteins from the endoplasmic reticulum are also subjected to proteasomal degradation (30). Proteasomes are present in the cytoplasm and in the nuclei. In the cytoplasm, proteasomes associate with the centrosomes, cytoskeletal networks and the outer surface of the endoplasmic reticulum. Under conditions of impaired proteolysis proteasomes and ubiquitinated proteins accumulate at these locations. In case of the pericentrosomal area those aggregates have been termed aggresomes (65). However, under appropriate circumstances aggresomes are cleared by autophagy (12, 60).

The reasons for the accumulation of distinct proteins in MM and IBM are not known, but impaired protein degradation is associated with MM and IBM as suggested by abnormal expression levels and aberrant localization of several subunits of the proteasome 19S and 26S, and by the up-regulation of immunoproteasomal subunits in muscle fibers containing abnormal protein deposits (11, 13). Moreover, endoplasmic reticulum stress and unfolded protein response has been reported in IBM (61). Abnormal proteasomal expression and activity does not explain, however, why several proteins cluster and aggregate into abnormal protein deposits in MM and IBM, although mutant ubiquitin and aggresomes may play a role in protein accumulation (13, 14).

Clusterin (Apolipoprotein J, SP40,40) is a sialoglycoprotein with a nearly ubiquitous tissue distribution. Clusterin is assembled as a heterodimeric molecule, formed by the anti-parallel alignment of α- and β-chains, in which the cysteine-rich centers, linked by 5 disulfide bridges, are flanked by 2

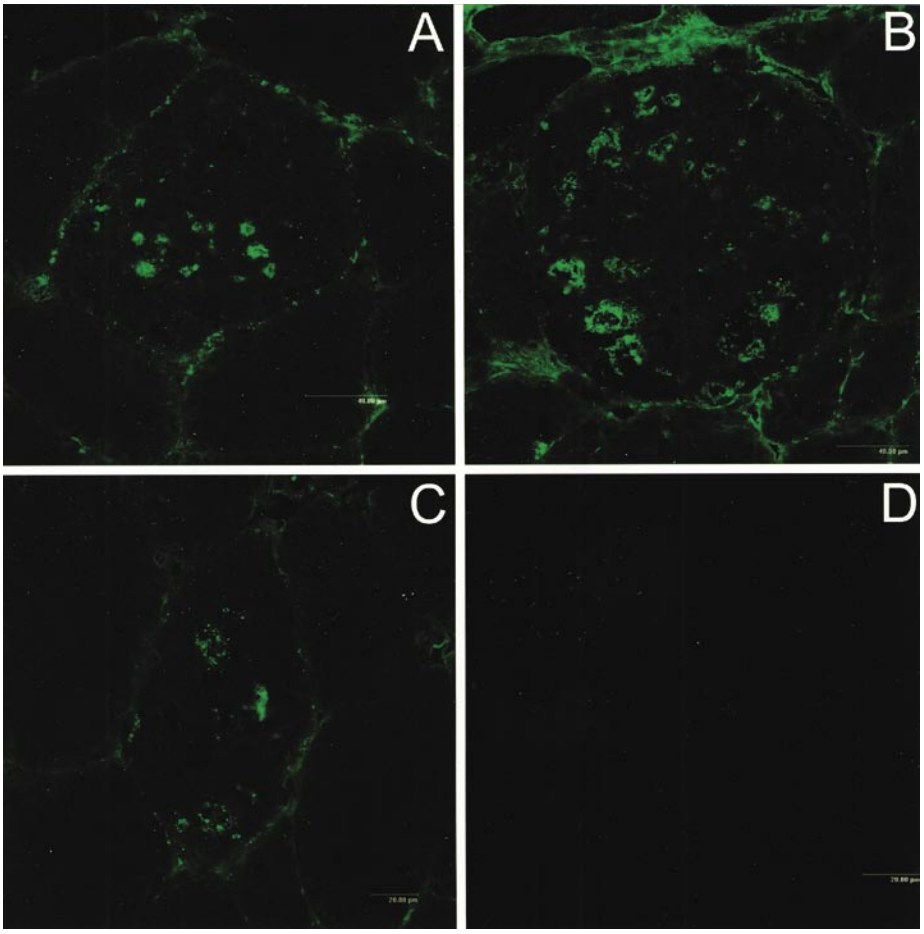

Figure 1. Multiple clusterin immunofluorescent deposits (green) in selected muscle fibers in MM. Note variable immunofluorescence on the periphery of muscle fibers (**A-C**). One section stained only with the secondary antibody is used as a negative control (**D**).

predicted coiled α-helices and 3 predicted amphipathic α-helices (25). Its major form, the 75 to 80 kDa heterodimer, is secreted and present in physiological fluids including plasma, cerebrospinal fluid and semen. Clusterin has the capacity to interact with a wide range of molecules, including itself, lipids, amyloid, components of the complement membrane attack complex, and immunoglobulins (25, 36, 47, 52). Clusterin is associated with abnormal protein accumulations in several neurodegenerative diseases, including amyloidopathies (ie, βA-amyloid plaques in Alzheimer disease), prionopathies and α-synucleinopathies (3, 5, 15, 25, 36, 47, 52). Little is known about clusterin in disorders of the skeletal muscle, although clusterin immunoreactivity has been reported in target structures of denervated fibers, in which there is an aberrant accumulation of several components of the myofibrils (44).

Aggregation of proteins is facilitated by several factors including covalent modifications, phosphorylation, proteolytic cleavage, ubiquitination, and mutated proteins (6, 48). γ-Tubulin mediates the link between microtubules and the centrosome and functions as a regulator of the microtubule organizing center (41, 67). In addition, recent studies have suggested that centrosomes are involved in the package of abnormal proteins (24); aggregated proteins translocate to the aggresome by active transport (29). Interestingly, the expression of aggresome markers (ie, γ-tubulin) is increased and co-localizes with aberrant protein accumulations in several conformational diseases characterized by the production of specific inclusions. For example, γ-tubulin co-localizes with α-synuclein deposits of Lewy bodies in Parkinson disease (35, 42).

The present study examines the characteristics of clusterin deposits and γ-tubulin expression in association with abnormal protein accumulations in MM and IBM. Muscle samples with no morphological abnormalities, and samples from patients with denervation atrophy with targets were processed in parallel for comparative purposes. Immunohistochemistry, and single and double-labeling immunofluorescence and confocal microscopy have been used to demonstrate clusterin and γ-tubulin co-localization with ubiquitin, αB-crystallin and other proteins including myotilin in abnormal protein aggregates. Myotilin, a recently identified Z-disk-related protein, is present in abnormal protein aggregates in MM (54). Morphological studies have been complemented with Western blots to further demonstrate increased clusterin and γ-tubulin expression in affected muscles in MM and IBM.

MATERIAL AND METHODS

General aspects. Eight patients with MM from 6 unrelated families were included in the present study. They were 4 men and 4 women aged from 28 to 79 years of age (mean age: 63 years). The age at onset was between 15 and 70 years. Three cases were sporadic, and the disease was autosomal dominant in the remaining cases. One sporadic patient had a de novo R406W mutation, and one patient with autosomal dominant MM had a single amino acid deletion at position 366 in the desmin gene (7, 27).

In all cases, the diagnosis was established after examination by light and electron microscopy of the muscle biopsies which were carried out after informed consent following the guidelines approved by the ethics committee. The neuropathological criteria were the presence of non-hyaline and hyaline cytoplasmic inclusions, desmin- immunoreactive on cryostat sections, and the observation of electron-dense granular aggregates by electron microscopy examination. The clinical, electrophysiological, neuropathological, and genetic studies of four of the cases have been detailed elsewhere (43).

Ten patients with IBM were included in this study. The patients were 6 men and 4 women aged 61 to 83 years (mean age 70.9). In all cases, the diagnosis was performed following well-established combined clinical and neuropathological criteria (21). All of them suffered from slowly progressing proximal and distal muscle weakness. In each patient, the muscle biopsy showed the characteristic morphological abnormalities

with the presence of endomysial inflammatory infiltrates, rimmed vacuoles in muscle fibers and intracytoplasmic inclusions consisting of 15 to 18 nm filaments identified by electron microscope.

In addition, 2 cases with denervation atrophy and the presence of targets, and 2 controls were used for comparative purposes.

Clusterin and γ-tubulin immunohistochemistry. Cryostat sections were incubated with 2% hydrogen peroxide and 10% methanol for 30 minutes at room temperature, followed by 5% normal serum for 2 hours, and then incubated overnight with one of the primary antibodies. The primary goat anti-clusterin (anti-apolipoprotein J) purified polyclonal antibody (Chemicon) was used at a dilution of 1:200. The mouse monoclonal γ-tubulin antibody (Abcam; GTU88) was used at a dilution of 1:5000. After washing, the sections were processed with the EnVision System Peroxidase (DAB) procedure (Dako) following the instructions of the supplier, and the immunoreaction was visualized with 0.005% diaminobenzidine (DAB) and 0.001% H_2O_2 in 200 ml PBS. Control of the immunoreaction was tested by incubating the sections without the primary antibodies.

Some sections were pre-incubated with proteinase K (Dako) for 15 minutes diluted in 1 ml buffer, and then processed for clusterin immunohistochemistry. Both sections without and with proteinase digestion were processed in parallel.

Serial consecutive sections were processed for desmin, ubiquitin and αB-crystallin immunohistochemistry as detailed elsewhere (11).

Double-labeling immunofluorescence and confocal microscopy. Cryostat sections were stained with a saturated solution of Sudan black B (Merck) for 30 minutes to block the autofluorescence of lipofuscin granules, rinsed in 70% ethanol and washed in distilled water. The sections were incubated at 4°C overnight with the goat anti-clusterin antibody at a dilution of 1:200 and rabbit polyclonal anti-ubiquitin (Dako), mouse monoclonal anti-αB-crystallin (Novocastra) or mouse monoclonal myotilin (Novocastra) at dilutions of 1:100, 1:500 and 1:150, respectively, in a vehicle solution composed of Tris buffer, pH 7.2, containing 15 mmol/L NaN_3, and protein (Dako). Secondary antibodies were Alexa488 anti-goat and Alexa546 anti-mouse or anti-rabbit (both from Molecular Probes), and these were used at a dilution of 1:400. Clusterin was green and ubiquitin, αB-crystallin and myotilin were red. Other sections were incubated with rabbit polyclonal anti-γ-tubulin (Sigma) at a dilution of 1:100, and goat polyclonal anti-clusterin (Chemicon) at a dilution of 1:500, or mouse monoclonal anti-αB-crystallin (Novocastra) at a dilution of 1:500. Secondary antibodies were Alexa546 anti-rabbit (green) and Alexa488 anti-goat or anti-mouse (red). After washing in PBS, the sections were incubated in the dark with the cocktail of secondary antibodies and diluted in the same vehicle solution as the primary antibodies for 45 minutes at room temperature. After washing in PBS, the sections were mounted in Immuno-Fluore Mounting medium (ICN Biomedicals), and sealed and dried overnight. Sections were examined in a Leica TCS-SL confocal microscope. Sections incubated with only one primary antibody and the corresponding secondary antibody, and sections incubated only with the secondary antibodies, were used for the control of the immunoreactions.

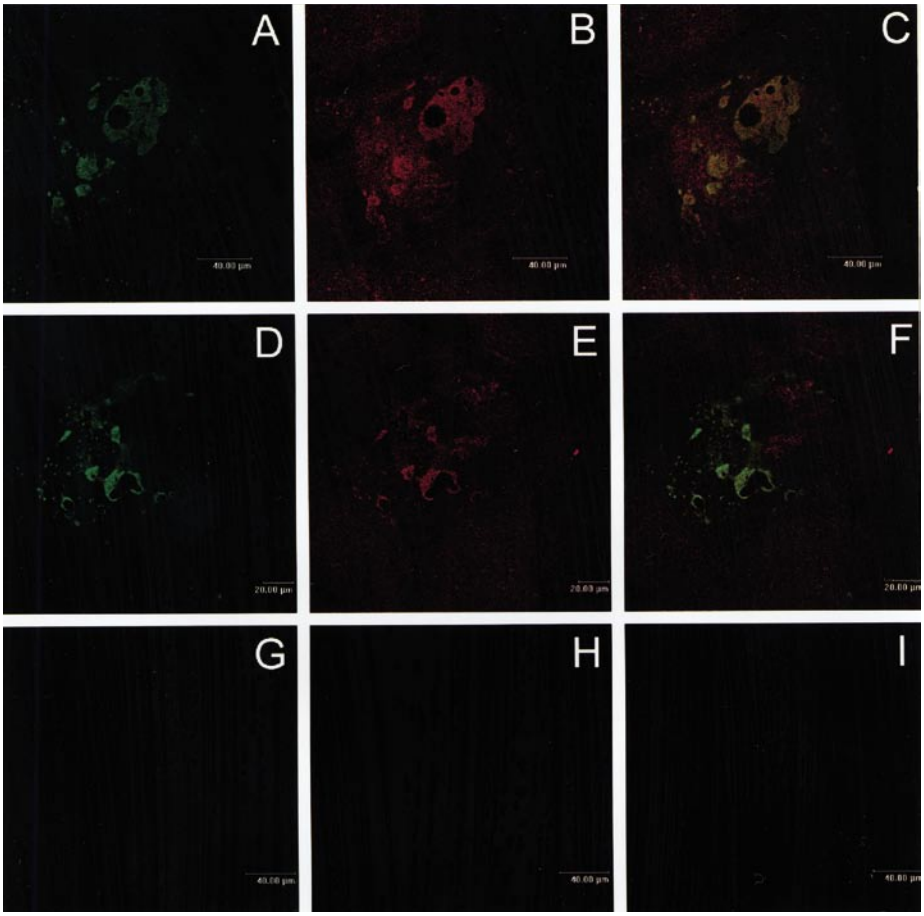

Figure 2. Double-labeling immunofluorescence to clusterin (green, panels **A, D**) and myotilin (red, panels **B, E**) in abnormal muscle fibers in MM. Note co-localization of clusterin and myotilin (merge yellow, panels **C, F**). One section of the same case stained only with the secondary antibodies is used as a negative control (**G-I**).

Electrophoresis and Western blotting. For Western blot studies, about 0.1 g of frozen muscle was homogenized in a glass tissue grinder in 20 volumes (w/v) of cold buffer containing 1 mM phenylmethylsufonyl fluoride, 5 μg/ml aprotinine, 10 μg/ml leupeptine and 5 μg/ml pepstatine (all of them from Sigma) to inhibit endogenous phosphatases. After centrifugation at 9500g for 5 minutes, the supernatants were mixed with loading buffer containing 75 mM Tris HCl (pH 6.8), 20% glycerol (Merck), 10% μ-mercaptoethanol (Sigma), 15% SDS (Bio-Rad) and 0.001% bromophenol blue (Bio-rad), and heated at 95°C for 5 minutes. Sodium dodecylsulphate-polyacryamide gel electrophoresis (10% SDS-PAGE)

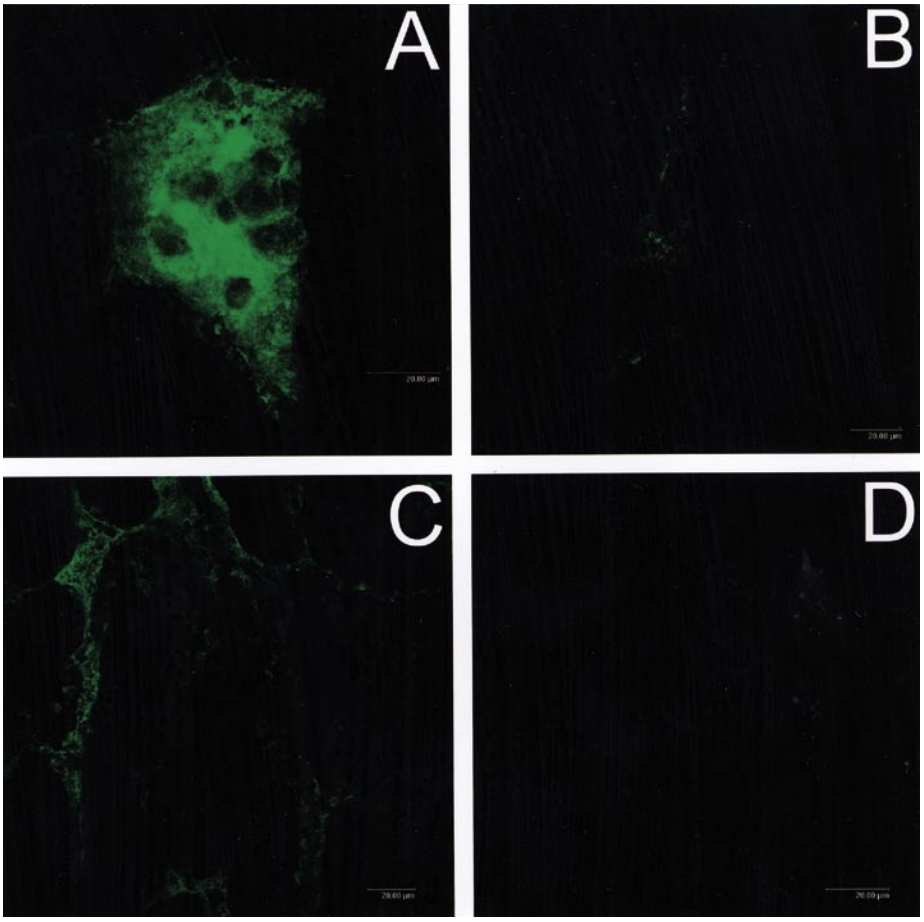

Figure 3. Clusterin immunoreactivity of variable intensity in abnormal fibers in IBM (**A-C**). Panel **D** shows the negative staining of an abnormal fiber from a section processed with only the secondary antibody.

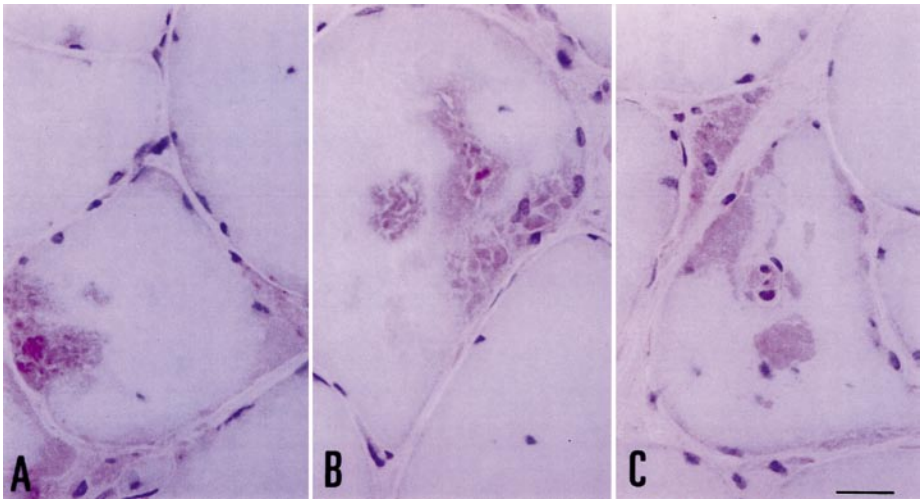

Figure 4. γ-Tubulin immunohistochemistry in MM (A-C) showing focal irregular immunostaining localized at the periphery or in the center of muscle fibers. Cryostat sections slightly counterstained with hematoxylin. Bar = 50 μm.

was carried out using a mini-protean system (Bio-Rad) with low-range molecular weight standards (Sigma). Proteins were transferred to nitrocellulose membranes using an electrophoretic transfer system (Bio-Rad). Unspecific blocking was performed by incubating the membranes in TTBS containing 5% skimmed milk for 20 minutes. Membranes were incubated with the goat anti-clusterin antibody (Chemicon) used at a dilution of 1:500 or with the mouse monoclonal γ-tubulin antibody (Abcam) used at a dilution of 1:10 000. After rinsing, the membranes were incubated with anti-goat IgG/HRP for one hour at room temperature. The membranes were washed, and then developed with the chemiluminescence ECL system (Amersham) followed by apposition of the membranes to autoradiographic films at 4°C overnight. Control of protein loading was tested by the staining of membranes with 0.19% Ponceau (Sigma). The densitometry of the bands was analyzed with STATGRAPHICS Plus 5.0. The monoclonal anti-β-actin antibody (Sigma) at a dilution of 1:5000 was used as a control of protein loading.

RESULTS

Clusterin immunohistochemistry. Clusterin immunoreactivity in normal muscles was restricted to slight positivity in the periphery of muscle fibers. Normal muscle fibers were barely immunostained for clusterin at the concentrations of the antibody used in the present study. In contrast, moderate or strong clusterin immunoreactivity was found in fibers with abnormal protein accumulations, as revealed in consecutive serial sections immunostained for ubiquitin, αB-crystallin, desmin or myotilin, in MM and IBM. In all cases, clusterin was not resistant to proteinase incubation.

Single and double-labeling clusterin immunofluorescence and confocal microscopy. Clusterin deposition in MM was characterized by multiple positive irregular aggregates in muscle fibers or by confluent immunofluorescent aggregates covering a part of the muscle fiber (Figure 1). The intensity of the immunoreaction was variable from one affected fiber to another, indicating variable amounts of the protein in particular lesions. Clusterin co-localized with abnormal protein deposition in selected fibers, as revealed in double-labeled sections stained for clusterin and myotilin (Figure 2). Similar findings were seen in sections double stained for clusterin and αB-crystallin and ubiquitin (data not shown).

Clusterin was also present in abnormal fibers in IBM, and again the intensity of the immunostaining was variable from one affected fiber to another (Figure 3). This was associated with abnormal deposits of other proteins including ubiquitin (data not shown).

γ-Tubulin immunohistochemistry. No γ-tubulin immunoreactivity was seen in normal muscle fibers. Yet focal increase in γ-tubulin immunoreactivity was present in the periphery or in central aggregates in MM and IBM cases (Figure 4). Serial sections immunostained for γ-tubulin and desmin, or clusterin or ubiquitin revealed co-localization of these proteins in individual muscle fibres.

Single and double-labeling γ-tubulin immunofluorescence and confocal microscopy. γ-Tubulin-immunoreactive aggregates were found in individual fibers in MM and IBM. Moreover, γ-tubulin aggregates co-localize with clusterin (Figure 5) and with αB-crystallin in individual muscle fibers in MM.

Clusterin and γ-tubulin in targets. Clusterin and desmin were present in targets in denervation atrophy (Figure 6A, B). Double-labeling immunofluorescence and confocal microscopy disclosed co-localization of clusterin and ubiquitin (among other proteins) in these abnormal structures. γ-tubulin immunoreactivity was also found in targets in denervation atrophy (Figure 6C).

Gel electrophoresis and Western blots. Western blots showed several clusterin-immunoreactive bands between 34 and 80 kDa, probably corresponding to heterodimers and aggregates with other proteins, in control and MM cases. The expression levels were significantly higher in MM cases than in controls ($p<0.05$) (Figure 7).

Similarly, γ-tubulin expression levels were significantly higher in MM cases when compared with controls ($p<0.05$) (Figure 8).

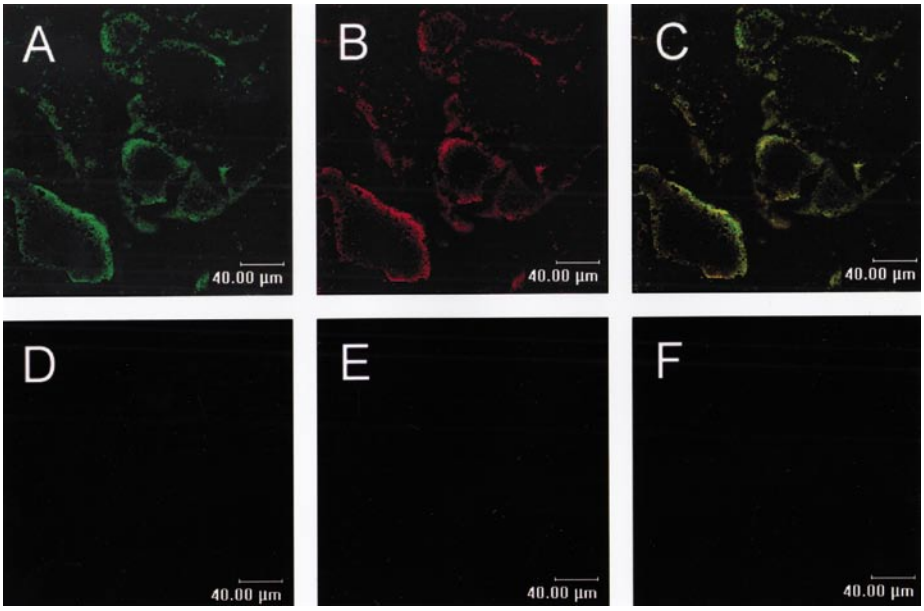

Figure 5. γ-Tubulin aggregates (green) co-localize with clusterin (red) (**A-C**, C merge) in individual muscle fibers in MM. Lower panel (**D-F**), control section incubated only with the secondary antibodies.

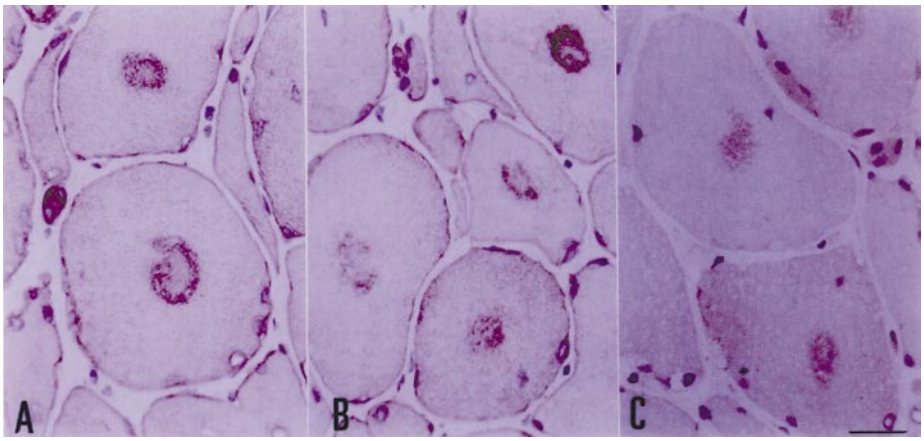

Figure 6. Clusterin (**A**) and desmin (**B**) are present in targets in denervation atrophy. γ-Tubulin immunostaining is also observed in targets (**C**). Cryostat sections slightly counterstained with haematoxylin. Bar = 50 µm.

DISCUSSION

The present results have shown clusterin immunoreactivity in association with abnormal protein deposits in MM and IBM. Clusterin deposition also occurs in targets in denervation atrophy. These findings indicate clusterin association with abnormal protein aggregates.

Previous studies have shown increased clusterin expression in Alzheimer disease (AD) brain tissue, which is largely due to increased clusterin expression in astrocytes and in a percentage of senile plaques (31, 33). Clusterin interacts with soluble βA-amyloid (32), thus suggesting a possible role for clusterin in preventing amyloidogenesis in AD (68). However, a recent study has shown that clusterin promotes amyloid plaque formation in a mouse model of AD (10). Increased clusterin has been found in the central nervous system of human and animal prion diseases (3, 15, 34, 47, 50). Recent studies have shown that clusterin aggregates with PrP in plaque-like deposits in CJD, and, therefore, that clusterin is likely involved in PrP^{res} plaque formation in CJD (15). Taking together the results in AD and CJD, it can be inferred that, although clusterin might have some effect on soluble protofibrils, it associates, rather, with βA-amyloid and PrP plaques in both diseases. In line with the functional role of clusterin in protein aggregation, recent studies have shown clusterin association with Lewy bodies in cases with Dementia with Lewy bodies, and with α-synuclein deposits in glial cells in multiple system atrophy (51).

Similarly, clusterin would facilitate aggregation of abnormal proteins in selected fibers with aberrant protein deposits in MM and IBM, and in targets in denervation atrophy.

The present study has also demonstrated increased γ-tubulin expression in association with abnormal protein deposits in MM and IBM. Aggresomes have been previously reported in relation to the biogenesis of several intracellular inclusions composed of abnormal protein aggregates, including Mallory bodies, expanded polyglutamine inclusions, and Lewy bodies (35, 42, 46, 57). The present results add protein

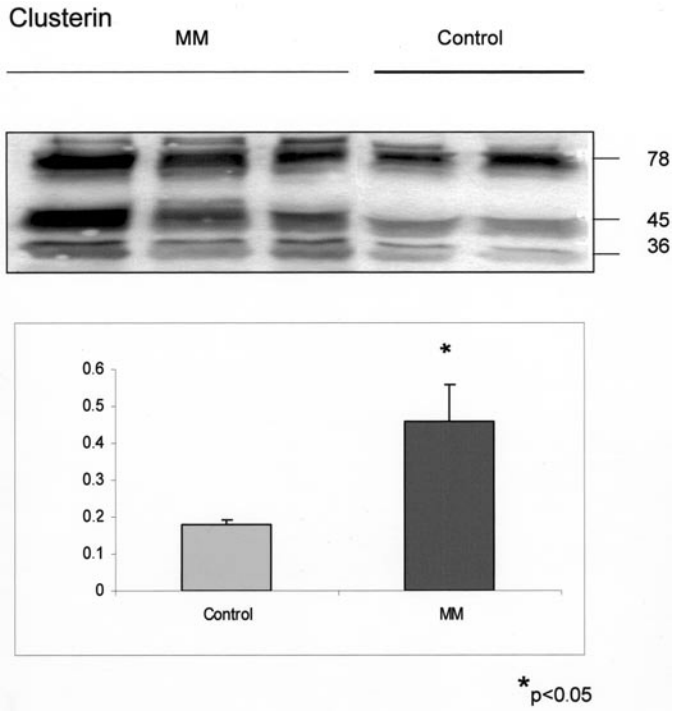

Figure 7. Gel electrophoresis and Western blotting of total muscle homogenates. Clusterin expression levels are higher in MM cases when compared with controls (p<0.05). Ordinate scale indicates arbitrary units.

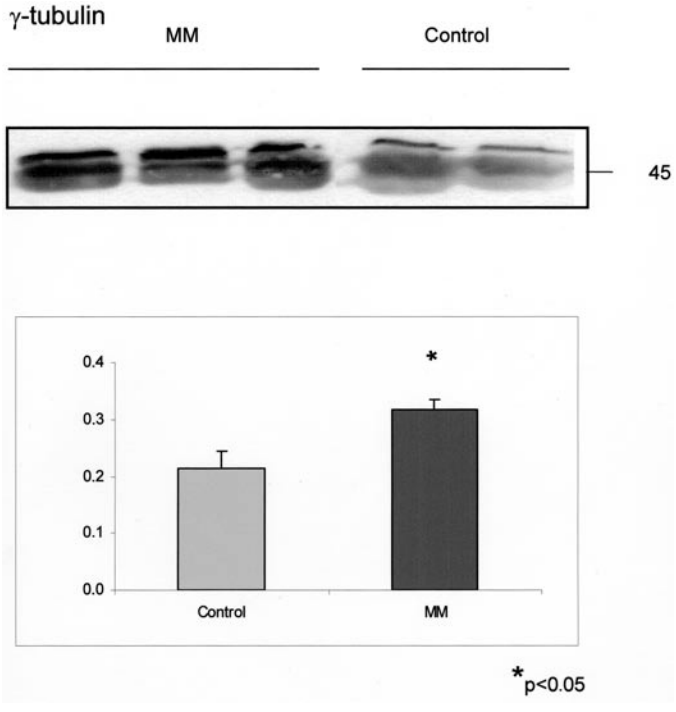

Figure 8. Gel electrophoresis and Western blotting of total muscle homogenates. γ-Tubulin expression levels are significantly higher in MM cases when compared with controls (p<0.05). Ordinate scale indicates artificial units.

inclusions in MM and IBM to abnormal aggresome structures related with intracellular protein inclusions in other disease-specific cell types. Similar conclusions can be applied to targets in denervation atrophy. In favor of this hypothesis is the fact that R120G mutation in the αB-crystallin gene, which causes desmin-related cardiomyopathy, forms aggresomes in vitro (4) and is accompanied by aggresome-related dense bodies containing desmin and αB-crystallin in transgenic mice (49).

Aggresomes have been described in the spinal cord during development (56), but also in relation with abnormal protein aggregates in liver and in Parkinson disease and polyglutamine diseases in the central nervous system (1, 16, 26, 42, 63). This is the first description of aggresomes in relation with abnormal protein aggregates in MM. Whether these accumulations are cytotoxic or not is a matter of controversy (22, 42). However, recent studies in limited paradigms have shown that aggresomes composed of α-synuclein and synphilin are cytoprotective (58). Moreover, inhibition of aggresome formation prevents intracellular inclusions and increases cell toxicity (24, 28, 59).

In summary, the present study has shown that abnormal protein accumulations in MM and IBM, and also in targets in denervation atrophy, are enriched in clusterin and γ-tubulin. These findings suggest that clusterin participates in protein aggregation and that aggresomes play a pivotal role in protein accumulations in MM and muscular disorders with abnormal protein aggregates.

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