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Ubiquitinated inclusions in inclusion-body myositis patients are immunoreactive for cathepsin D but not β-amyloid

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Abstract

The nature of the inclusions in the human muscle disease inclusion-body myositis (IBM) has been the subject of debate. Parallels with Alzheimer's disease have been drawn after these inclusions were found to be ubiquitinated, and immunoreactive with antibodies to β-amyloid (Aβ) and certain amyloid-associated proteins. We have used a battery of antibodies against Aβ and associated proteins to immunostain muscle biopsies from patients with IBM. Although the inclusions are ubiquitinated, we could not show immunoreactivity for Aβ or the associated proteins investigated. We did, however, find that the ubiquitinated inclusions colocalised with the lysosomal marker, cathepsin D.

Keywords: Alzheimer's disease; Amyloid beta-protein; Muscle; Ubiquitin; Lysosomes; Human

Inclusion-body myositis (IBM) is a disease which most commonly affects middle-aged men, and causes progressive muscular atrophy. Histologically, rimmed vacuoles are characteristically seen in muscle fibres. Vacuolated fibres also contain filamentous inclusions, which some investigators have suggested are composed of β-pleated amyloid [4,22]. IBM inclusions have also been reported to contain ubiquitin [2,3,20], β-amyloid precursor protein (βAPP) [5,23,27,32] and β-amyloid (Aβ) [4,23,32], the same protein that is deposited in the brain in aging and Alzheimer's disease (AD). Some workers have found immunoreactivity for prion protein (PrP) [6], α_1-antichymotrypsin (ACT) [11], apolipoprotein E (Apo E) [9], cathepsins [32] and hyperphosphorylated tau [8] in these inclusions, which suggest a possible link between IBM and neurodegenerative disease, particularly AD, in terms of the protein content of the abnormal inclusions. We set out to confirm some of this previous work and to extend it using a battery of antisera to Aβ and proteins which are known to be associated with Aβ in AD brain.

Tissue from 16 patients with sporadic IBM was used. Ten micrometre sections of frozen (14 cases) and formalin-fixed, paraffin-embedded (six cases) tissue were cut and mounted on 3-aminopropyltriethoxysilane-coated slides. Sections from seven patients with non-IBM muscle disease were used as controls, and neocortical sections of AD (three cases) and Gerstmann–Sträussler disease (one case) brains served as positive immunostaining controls. Frozen sections were air-dried for 10–20 min, then fixed in acetone for 5 min. Paraffin sections were de-waxed and incubated in 3% H_2O_2 in methanol for 30 min.

Where Aβ, Apo E, PrP and cathepsin D antibodies were to be used on paraffin-embedded tissue, the sections were immersed in 95% formic acid for 5 min. For visualisation of βAPP epitopes, they were instead subjected to microwave antigen retrieval [29]. Immunocytochemistry was carried out, by incubating sections in primary antiserum (Table 1) after blocking with 5% fetal calf serum for 15 min. Incubations of up to 2 days were used in preliminary experiments but optimum staining was achieved at 1–2 h. Initially, eight Aβ antibodies were tested on frozen AD sections at different dilutions; five were chosen for the main part of the study. The sections were washed in Tris buffered saline, incubated for 30 min in the appropriate biotinylated secondary antibody (1/200, Vector), washed again, and incubated for 30 min with avidin-biotin complex (1/200, Vector). Peroxidase activity was revealed with 0.025% diaminobenzidine and 0.25% H_2O_2

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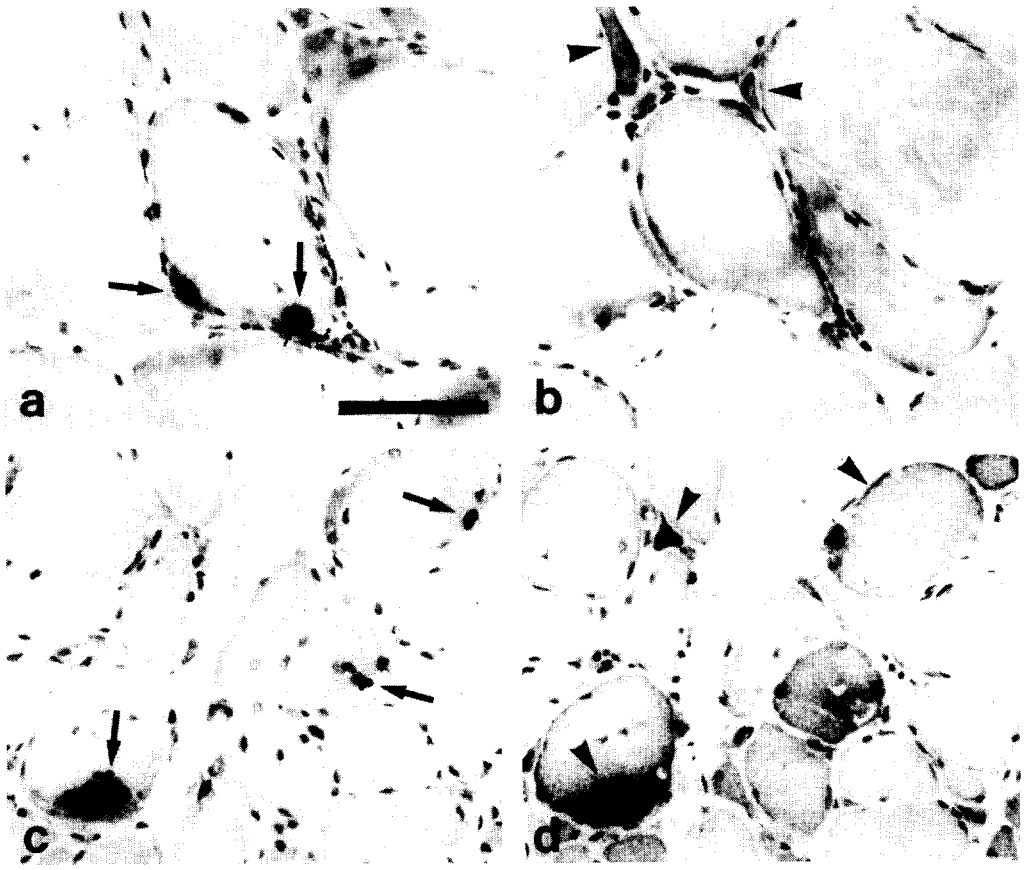

Fig. 1. (a) Ubiquitin immunostaining on a frozen section of muscle from a patient with inclusion-body myositis (IBM). Two labelled inclusions can be seen in one fibre (arrows). Haematoxylin counterstain. Bar = 100 μm (same magnification for all figures). (b) Serial section to (a) immunostained for Aβ with R1280. Note the light diffuse staining of atrophic fibres (arrowheads). This type of staining was not absorbed out by preincubation of the antibody with synthetic $A\beta_{1-40}$ peptide. (c) Ubiquitin staining on a frozen section of IBM muscle. Inclusions can be seen in three separate fibres (arrows). Obvious vacuolation can be seen in the fibre in the centre. (d) Serial section to (c) immunostained with R1280. In this case the antibody is non-specifically staining some atrophied fibres, the periphery of some diseased fibres and areas of muscle fibres around ubiquitinated inclusions (arrowheads). These patterns of R1280 immunostaining was not absorbed out by preincubation of the antibody with synthetic $A\beta_{1-40}$ peptide.

for 10 min. The sections were counterstained with haematoxylin, dehydrated and coverslipped with DPX. In cases which appeared to show some immunoreactivity with the antibody R1280, the reaction specificity was checked by preabsorption with the synthetic peptide HA4 ($A\beta_{1-40}$; gift from Dr. D. Selkoe). Frozen and paraffin sections

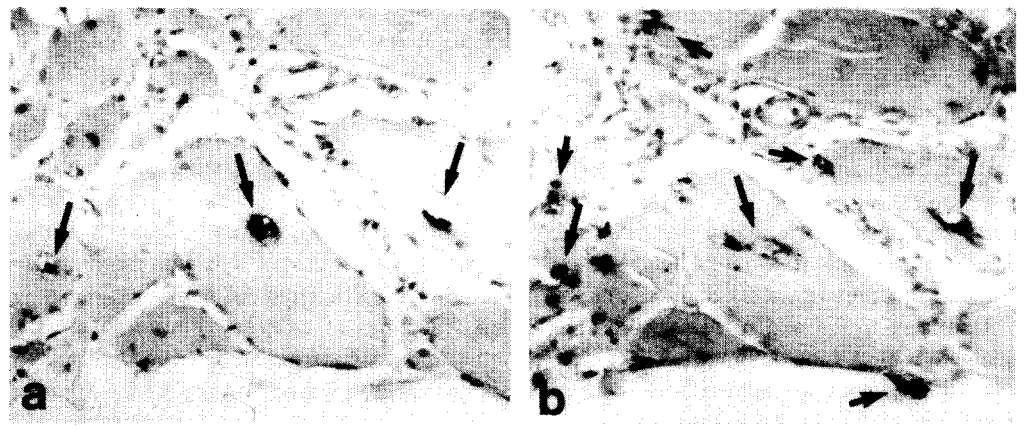

Fig. 2. (a) Ubiquitin immunostaining of inclusions on a paraffin section of IBM muscle (arrows). (b) Serial section to (a) with cathepsin D staining. Staining corresponding to ubiquitin-positive inclusions are shown by the larger arrows, but normal lysosomes are also stained (smaller arrows). Cathepsin D localises with all ubiquitin deposits in these sections.

Table 1

Antibody	Antigen	Concentration (species/type)	Source
MAB1510	Ubiquitin	1/200 (mm)	Chemicon
10D5 [15]	Synthetic $A\beta_{1-38}$	1/200–400 (mm)	Athena
R1280 [31]	Synthetic $A\beta_{1-40}$	1/1000 (rp)	D. Selkoe
R1282[a]	Synthetic $A\beta_{1-40}$	1/1000 (rp)	D. Selkoe
Y [16]	Synthetic $A\beta_{1-38}$	1/1000 (rp)	D. Selkoe
C [28]	Native $A\beta$ from AD brain	1/300 (rp)	D. Selkoe
R1736[b]	Synthetic peptide 595–611 of βAPP	1/1000 (rp)	D. Selkoe
22C11	βAPP (epitope within 60–100)	1/100 (mm)	Boehringer
LN27	βAPP (epitope within 1–200)	1/200 (mm)	Zymed
B207 [30]	βAPP	1/500 (gp)	B. Greenberg
Alz-50 [33]	Abnormally phosphorylated tau	1/5 (mm)	P. Davies
T-6402	Fetal tau	1/200 (rp)	Sigma
Am-3[c]	AD plaque cores	1/200 (rp)	K. Hirokawa/ H. Takahashi
NSE[d]	$PKC_{\beta II}$	1/50 (rp)	Saitoh
82943	Apo E	1/1000 (gp)	Atlantic
3D12	Apo E	1/100 (mm)	Sanbio
Cathepsin D [26]	Cathepsin D	1/1000 (rp)	W. Reid
1A8[e]	PrP	1/500 (rp)	C. Farquhar

$A\beta$, β-amyloid; mm, mouse monoclonal; rp, rabbit polyclonal, βAPP, β-amyloid precursor protein; PKCβII, protein kinase C-βII; gp, goat polyclonal; Apo E, apolipoprotein E; PrP, prion protein.

[a] R1282 prepared as R1280[14].

[b] Preparation similar to αC7[25] (R1736 also recognises $A\beta$ under certain conditions[29]).

[c] Am-3 recognises $A\beta$ and plaque-associated proteins [17].

[d] $PKC_{\beta II}$ is a phospholipid-dependant kinase, associated with $A\beta$ deposits in AD brain [21].

[e] Preparation similar to 1B3[13].

from nine cases were stained with Congo red, and viewed under polarised light and with fluorescence [7].

Ubiquitin immunoreactivity was found in association with rimmed vacuoles in frozen sections from seven cases of IBM (50%) and paraffin-embedded sections from three cases (50%). This labelling was focal and often within vacuoles. Some intranuclear inclusions were also labelled in both tissue preparations. None of the non-IBM controls showed ubiquitin staining, except weakly and diffusely within some atrophied fibres. None of the $A\beta$ antibodies labelled the ubiquitin-positive inclusions in IBM (Fig. 1A,B), despite showing strong immunoreactivity in AD brain. In both IBM and disease controls, antibody R1280 faintly labelled the periphery or sometimes the whole of some atrophied fibres. Similar but weaker labelling was also occasionally seen with R1282, antibody C and antibody Y. In two cases, antibody R1280 focally labelled the muscle fibre around the inclusion, but not the inclusion itself (Fig. 1C,D). The R1280 labelling was not eliminated by preabsorption of R1280 with synthetic $A\beta_{1-40}$ peptide. In one case of unidentified familial vacuolar myopathy, R1280 and antibody Y labelled calcium deposits within vacuoles. In both tissue preparations, the cathepsin D antibody labelled lysosomes in brain and in muscle sections from all cases (Fig. 2A,B). In normal muscle, lysosomal labelling was usually confined to the periphery of the fibres but in IBM, immunoreactivity was also associated with the majority of inclusions. Despite staining in positive control tissue, none was found in either frozen or paraffin sections of IBM or non-IBM muscle with antibodies to βAPP, tau, PrP, Apo E, $PKC_{\beta II}$ or Am-3, even after antigen retrieval techniques. No Congo red staining was found in any of our cases of IBM.

The discrepancies in $A\beta$ immunostaining are difficult to explain, particularly as one of the antisera used in this study (R1280) were also used in previous reports [4,11], however, we have shown that R1280 and other $A\beta$ antibodies can immunostain non-specifically in diseased muscle. It is possible that the $A\beta$ antigen present in IBM muscle is more fragile than that in AD brain, and thus destroyed by our tissue processing. We found that the inclusions were often labelled with cathepsin D and hence are associated with lysosomes. It has been suggested that IBM inclusions may originate from lysosomes although it is equally possible that this association may be secondary to their formation and ubiquitination.

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