Lysosomal storage of subunit c of mitochondrial ATP synthase in Batten's disease (ceroid-lipofuscinosis)

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Immunochemical studies demonstrate that subunit c of mitochondrial ATP synthase is stored in the late-infantile, juvenile and adult forms of Batten's disease. It does not accumulate in the infantile form, or in other conditions involving lysosomal hypertrophy. These results suggest that the defective metabolism of subunit c is central to the pathogenesis of these three forms of Batten's disease.

INTRODUCTION

The terms Batten's disease (BD) and ceroid-lipofuscinosis are both used to designate a group of inherited neurodegenerative disorders characterized by the intra-lysosomal accumulation, within neurons, of a storage material with the histochemical properties of lipofuscin/ceroid. A histochemically similar pigment is present in aging brain. Four major forms of the disease have been delineated: infantile (IB), late-infantile (LIB), juvenile (JB) and an adult form, also known as Kufs disease. They can be distinguished on the basis of clinical course, neurophysiological findings and the ultrastructural appearance of the storage material [1]. BD is found in all populations, though it is particularly prevalent in Finland (1 in 10000 births) [2]. Transmission is by an autosomal recessive mode of inheritance and the genes for JB and IB have been mapped by linkage analysis to DNA polymorphisms on chromosome 16 [3] and chromosome 1 [4] respectively; the genetic locations of the other forms of the disease have not yet been found. Several genetic animal models of the disease have also been identified and an ovine form (OB) has been studied extensively [5,6].

Storage material from sheep has as its major component a single polypeptide with the terminal sequence of subunit c of the mitochondrial ATP synthase (subunit c) [6]. The entire mature protein is present, and there is no evidence for any abnormally modified constituent amino acids [7]. Subunit c is a very hydrophobic membrane protein, containing 75 amino acids, with a molecular mass of 7602 Da. Evolutionarily it is highly conserved; indeed, the amino acid sequences are identical in cattle [8], sheep and humans [9]. There is even detectable sequence similarity with the equivalent protein in fungi and bacteria [8]. Subunit c is defined as a proteolipid because it can be extracted by organic solvents. It is reported to migrate on PAGE under denaturing conditions at an anomalously low molecular mass and may aggregate to form multimers [7]. Analysis by gas-phase sequencing of isolated storage material has demonstrated the presence of subunit c as a dominant component in LIB and JB [9]. In this study we have investigated whether storage of subunit c is a specific marker for BD and have extended the previous studies to include Kufs disease.

MATERIALS AND METHODS

Materials

Storage material derived from OB was a gift from R. D. Jolly (Palmerston North, New Zealand). BSA, ovalbumin, Freund's adjuvant, CNBr-Sepharose 6B and Protein A-agarose were from Sigma (Poole, Dorset, U.K.). Immunochemical reagents for Western blotting were from Bio-Rad (Hemel Hempstead, Herts., U.K.) and those for immunocytochemistry were from Dako (High Wycombe, Bucks., U.K.). Bicinchoninic acid was from Pierce (Chester, Cheshire, U.K.). The synthetic peptide was from Alta Bioscience (Birmingham, U.K.). Nitrocellulose (0.2 µm pore size) was obtained from Schleicher and Schuell (Dassel, Germany). CNBR-Sepharose 6B was from Pharmacia (Uppsala, Sweden). All other reagents were from BDH (Poole, Dorset, U.K.).

Clinical material

The diagnoses were established on clinical evidence, by electrophysiological tests, and by histochemical examination of diagnostic biopsies. Confirmation of diagnosis and definitive classification into a particular disease subtype was established by electron microscopy of biopsies or of post-mortem tissue. Cases studied included five cases of IB, nine cases of LIB, six cases of JB and three cases of Kufs disease; case reports of two of the Kufs disease patients have been published as case 2 in [10] and case 1 in [11]. Controls for biochemical analyses included three cases of G_{M1}-gangliosidosis, two cases each of G_{M2}-gangliosidosis and Niemann-Pick disease type C, single cases of Hunter disease, Krabbe leucodystrophy, maple syrup urine disease, congenital heart disease, and four old-age controls (ages 63–79 years). Controls for immunocytochemistry included brains from cases of sialic acid storage disease, G_{M1}-gangliosidosis, G_{M2}-gangliosidosis, metachromatic leucodystrophy, orthochromatic leucodystrophy, others with non-specific changes and normal old-age brain.

Isolation of storage material and lipofuscin

Storage material from brain in each form of BD and lipofuscin from old-age brain were prepared using a method adapted from

Abbreviations used: BD, Batten's disease; IB, infantile form of Batten's disease; LIB, late-infantile form of Batten's disease; JB, juvenile form of Batten's disease; OB, form of Batten's disease affecting sheep; PBS, phosphate-buffered saline; LDS, lithium dodecyl sulphate.

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[5]. All operations were performed at 4 °C. Washing steps were carried out by resuspension in 0.4 mM-Tris/HCl, pH 7.4, followed by centrifugation for 30 min at 5400 *g* (or 20000 *g* after CsCl steps). Final products were resuspended in water.

Tissue (cortex, dissected grey matter) was homogenized (10 ml/g of tissue) in 250 mM-sucrose/25 mM-KCl/50 mM-Tris/HCl/5 mM-MgCl₂/10 mM-2-mercaptoethanol, pH 7.4, with 10 passes of a Teflon/glass homogenizer (20–30 passes for IB brain). After filtration through washed 113 filter paper (Whatman, Maidstone, Kent, U.K.), the homogenate was centrifuged at 1000 *g* for 30 min. The pellet was resuspended (2.5 ml/g of tissue) in 0.4 mM-Tris/HCl, pH 7.4, and aliquots (1 ml) were sonicated for 20 s at an amplitude of 6 μ m (peak to peak) with a 150 W ultrasonic disintegrator fitted with a micro probe (MSE, Loughborough, Leics., U.K.). They were then pooled and centrifuged at 5400 *g* for 30 min. The resulting pellet had two layers; the upper light-coloured layer was resuspended by gently swirling with small volumes of buffer and discarded. The darker-coloured lower layer was resuspended and centrifuged at 5400 *g*, and the upper part of the pellet was again discarded. The pellet was finally resuspended (0.5 ml/g of tissue), sonicated and centrifuged at 5400 *g* for 30 min to produce the final product.

Storage material prepared from LIB and JB brains was further purified by density centrifugation. The above product was resuspended, adjusted with 30% (w/w) CsCl to a density of 1.15 g/ml and a concentration of 1 ml/g of tissue. It was then centrifuged at 30000 *g* for 30 min in a PrepSpin 65 ultracentrifuge, with a 10 $\times$ 10 ml angle rotor (MSE). The pellet was resuspended at 2 ml/g of tissue in 15% (w/w) CsCl, and 1 ml aliquots were layered over 6 ml gradients [20–30% (w/w) CsCl] and centrifuged at 150000 *g* for 16 h. A fluorescent band at a density of 1.20–1.24 g/ml was aspirated, diluted to 4 ml/g of tissue and washed three times.

The purification was monitored by fluorescence microscopy. Finally, a quantitative assessment of purity was made by electron microscopy as described [12].

Preparation and purification of antisera

Polyclonal antibodies to subunit c were raised by immunizing rabbits (four sites; intramuscular) with isolated storage material (dissolved in 0.1% LDS). The first injection was given in complete Freund's adjuvant and subsequent injections were given in incomplete adjuvant. The antibody was raised against storage material isolated from either LIB brain (SCLI) or OB pancreas (SCOV). SC36 was raised by immunizing rabbits with a synthetic peptide corresponding to residues 36–48 of the subunit c sequence, coupled via bis-diazobenzidine to ovalbumin. An affinity column was prepared by coupling, to CNBr-Sepharose 6B, subunit c which had been purified by proteolipid extraction from storage material derived from OB liver and demonstrated to be homogeneous by electrophoresis with silver staining. Antibodies were bound to this affinity material in phosphate-buffered saline (PBS) [13] and eluted with 100 mM-glycine, pH 2.4. IgG was isolated from the eluted fractions by affinity chromatography using Protein A-agarose [13]. SC36 was further purified by treatment with ovalbumin coupled to CNBr-Sepharose 6B.

Immunochemical methods

After electrophoretic transfer of proteins to nitrocellulose at 2.4 mA/cm² using a semi-dry apparatus, following the manufacturer's instructions (Pharmacia), membranes were blocked with 1% BSA in washing buffer (PBS containing 0.05% Tween 20). They were then incubated with primary antibody dissolved in blocking solution. After washing, membranes were incubated with a goat anti-rabbit IgG/alkaline phosphatase conjugate,

followed by washing and reaction with *p*-Nitrotetrazolium Blue/5-bromo-4-chloro-3-indolyl phosphate, by following the manufacturer's instructions (Bio-Rad).

For immunocytochemistry, either cryostat sections of frozen brain or paraffin sections of formalin-fixed brain were cut into 5 μ m sections, blocked in 1% BSA in PBS and incubated with antibody for 16 h. After washing with PBS, they were incubated with biotinylated pig anti-rabbit IgG, then with an avidin-biotin complex coupled to horseradish peroxidase. Colour was developed with 0.05% diaminobenzidine/0.03% H₂O₂ in 0.1 M-phosphate buffer, pH 7.4.

Other methods

Sub-mitochondrial particles were prepared from rat liver mitochondria as described [14], and proteolipids were extracted from mitochondria as described [15]. Proteins were separated by PAGE [16] [17% (w/v) acrylamide] at 10 V/cm for 40 min followed by 17 V/cm for 2–4 h at 20–25 °C. Samples were denatured under non-reducing conditions by heating at 80 °C for 5 min. Silver staining was carried out as described [7]. Protein was determined using a bicinchoninic acid method (Pierce), and dry weight was determined gravimetrically.

RESULTS AND DISCUSSION

Storage material was isolated and analysed by PAGE (Fig. 1). Material derived from OB (lane 1) and from LIB (lanes 2 and 3) contained predominantly a single band with an apparent molecular mass of 4.4 kDa (subunit c). The minor higher-molecular-mass proteins detected in lane 3 are probably derived from the 35% contamination in this preparation (mainly mitochondria and nuclear fragments). Storage material of very high purity isolated from two cases of JB revealed, after electrophoretic separation, a low-molecular-mass band which co-migrated with subunit c, as well as a number of higher-molecular-mass protein bands (Fig. 1, lanes 4 and 5). The intensity of the 4.4 kDa band was much less than was found in LIB. Neither high-purity storage material isolated from IB (lanes 6 and 7) nor lipofuscin isolated from old-age brain (lanes 8 and 9) showed staining in this region after electrophoresis, although each preparation

Fig. 1. LDS/PAGE of isolated storage material and mitochondrial proteins, with detection of proteins by silver staining

Isolated storage material derived from OB pancreas (lane 1), LIB brain (lanes 2 and 3), JB brain (lanes 4 and 5) and IB brain (lanes 6 and 7) was analysed. Lanes 8 and 9 contain lipofuscin isolated from old-age brain; lane 10 contains sub-mitochondrial particles and lane 11 contains a proteolipid extract of mitochondria. The minimum purity of the preparations of storage material was in excess of 80%, with the exception of the material analysed in lanes 3 (65%) and 9 (42%). A total of 0.9 μ g dry weight was separated in lanes 2 and 3, and 1.8 μ g dry weight in lanes 1 and 4–9; 1.25 μ g of protein was separated in lane 10 and 0.1 μ g of protein was separated in lane 11.

Fig. 2. Western blot of isolated storage material and mitochondrial proteins

Samples, as in Fig. 1, were electrophoretically transferred to nitrocellulose, blocked and probed with SCL1. Similar results were obtained with SCOV and SC36. The top arrow indicates the start of the separating gel.

contained a number of higher-molecular-mass bands. The subunit c band in storage material co-migrated with the lowest-molecular-mass bands detected in preparations of sub-mitochondrial particles (lane 10) and in a proteolipid extract of mitochondria (lane 11).

Affinity-purified polyclonal antibodies to subunit c were used to study subunit c in Western blots of the above samples (Fig. 2). Very strong binding was found with the 4.4 kDa band in storage material derived from OB and LIB (lanes 1–3). Binding to the same band was also found in storage material derived from JB (lanes 4 and 5). No immunoreactive components were detected in preparations of storage material from IB or in lipofuscin from old-age brain (lanes 6–9). Sub-mitochondrial particles were also shown to contain a prominent component which co-migrated with subunit c (lane 10), confirming that the antibody detected a mitochondrial inner membrane protein. Analysis of a mitochondrial proteolipid extract revealed enrichment of subunit c, confirming its proteolipid character (lane 11). Minor bands were detected at 15 kDa in some samples and probably result from aggregation of subunit c [7]. No staining was obtained after incubation with IgG from pre-immune serum.

Brain homogenates were separated by electrophoresis and analysed by Western blotting (Fig. 3). Subunit c was detected at very high concentrations in LIB brain (lanes 1 and 2) (a total of six cases analysed). Lower, but still appreciable, concentrations were detected in JB brain (lanes 3 and 4) (four cases). Subunit c was barely detectable in IB brain (lanes 5 and 6) (three cases); indeed, it was present at even lower concentrations than in age-matched controls (lanes 9 and 10). Old-age controls (lanes 7 and 8) (four cases studied) had slightly higher concentrations than the young controls, as did cases of G_{M1} -gangliosidosis (lane 11) (three cases) and G_{M2} -gangliosidosis (lane 12) (two cases). Similar concentrations were also found in the other controls studied. Though there was some variation from case to case, densitometric scanning confirmed that concentrations in JB were significantly higher than in any of the control brains studied. The absence of immunoreactive bands, other than that already noted at 15 kDa, confirms the specificity of the antibody. No staining was obtained after incubation with IgG from pre-immune serum.

Immunolocalization studies demonstrated that antisera reacted very strongly with storage material in neurons in cryostat and paraffin sections of LIB brain (Fig. 4a). The strongest

Fig. 3. Western blot of brain homogenates

Brain cerebral cortex (dissected grey matter) was homogenized in water, containing 1 μ g of leupeptin/ml, 0.1 μ g of pepstatin/ml, 1 μ g of antipain/ml and 2 mM-phenylmethanesulphonyl fluoride. Protein (10 μ g) was separated by electrophoresis, blotted and probed with SCL1, as described for Fig. 2. Samples were LIB brain (lanes 1 and 2), JB brain (lanes 3 and 4), IB brain (lanes 5 and 6), non-lysosomal storage disease controls aged 63 years (lane 7), 71 years (lane 8), 13 years (lane 9) and 12 years (lane 10), G_{M1} -gangliosidosis (lane 11) and G_{M2} -gangliosidosis (lane 12). Similar results were obtained with SCOV and SC36.

Fig. 4. Immunolocalization studies

Paraffin sections of brain were probed with SC36 and a nuclear counterstain (haematoxylin) was added. Tissues were from LIB (a), JB (b), Kufs disease (c), IB (d), G_{M2} -gangliosidosis (e) or normal brain with abundant lipofuscin from a 91-year-old patient (f). Magnification $\times 500$. Similar results were obtained with SCL1 and SCOV.

staining was consistently observed in LIB brain (nine cases analysed). Significant, but less intense, staining was seen in JB (Fig. 4b) (four cases). All three cases of Kufs disease showed significant antibody binding, comparable in intensity with that seen in JB (Fig. 4c); not all neurons showed antibody binding, but the pattern reflected the distribution of storage material demonstrated by histochemical methods. No antibody staining was observed in any of these cases when pre-immune serum was substituted as the primary antibody. No subunit c staining was detected in IB (Fig. 4d) (four cases), or in tertiary lysosomes

within lysosomal storage disease controls (six cases) or other controls (six cases) (Figs. 4e and 4f). The pre-extraction of cryostat sections of LIB brain with chloroform/methanol/water (40:20:3, by vol.) removed all immunoreactivity detectable with SCOV and SC36.

This study demonstrates that subunit c storage is specific for LIB, JB and Kufs disease. Since subunit c concentrations are normal in other lysosomal storage diseases, and subunit c is absent from lipofuscin of old-age brains, it is clear that subunit c is not a normal component of tertiary lysosomes. Though no frozen tissue was available from Kufs disease for Western blot analysis or isolation of storage material, the intensity of antibody staining in tissue sections of Kufs disease suggests that subunit c was stored to a similar extent to that found in JB. The failure to detect subunit c storage in IB suggests that this is a biochemically distinct disease, a suggestion supported by other biochemical evidence [17,18] and by genetic evidence indicating that IB is not allelic to JB [3,4].

Immunostaining failed to detect antibody binding in controls, suggesting that these methods do not detect subunit c present within mitochondria. This result is unsurprising, however, since in its native conformation subunit c is believed to be buried within the membrane and surrounded by other subunits of ATP synthase [8]. Though the generation of antibodies to subunit c in higher eukaryotes has not previously been reported, a similar inability to locate subunit c in holo-ATP synthase has been found in yeast [19]. Extraction with organic solvents removed subunit c, as detected by immunostaining; it is unlikely that solvent extraction denatures the antigen, since antibody binds strongly to dried chloroform/methanol extracts of subunit c (results not shown). This result, therefore, confirms that the antibodies to subunit c recognize a proteolipid in tissue sections.

Detection of subunit c could prove useful in the diagnosis of BD. This will be of particularly value in the diagnosis of Kufs disease, which is often problematic because histochemical methods do not clearly distinguish storage material from depositions of lipofuscin (which can be appreciable in older patients). The method can help to distinguish JB and LIB from other conditions in which lysosomes proliferate. Furthermore, immunolocalization studies have already been of value in establishing a diagnosis in a paediatric patient with a somewhat atypical clinical presentation in whom very marked subunit c storage was demonstrated (B. D. Lake, N. A. Hall & E. M. Brett, unpublished work).

Our results suggest that LIB, JB and Kufs disease are analogous to the ovine model. The very high concentrations of subunit c found, particularly in LIB storage material, suggest that the primary cause of this disease is a defect in the metabolism of subunit c. Though this could result from overproduction, this appears unlikely, since in OB the two structural genes for

subunit c have normal sequences and mRNA levels are normal [9]. The disease could result from an abnormality in intracellular targeting of subunit c, although there is no evidence for a defect in the mitochondrial respiratory chain, or for the presence of morphologically abnormal mitochondria. It seems most likely, therefore, that the disease involves a defect in the catabolism of this protein.

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