SERCA1 and calsequestrin storage myopathy: a new surplus protein myopathy

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We describe four patients, from four different families, affected by a mild myopathy or asymptomatic elevated serum creatine kinase levels, in whom toluidine blue-stained semithin sections of muscle specimens revealed inclusions of different size and shape. The inclusions did not stain by routine histochemical studies. The sarcoplasmic or endoplasmic reticulum calcium 1 (SERCA1) ATPase and/or calsequestrin reactivity of inclusions, by immunohistochemistry, and the SERCA1- and calsequestrin-increased expression, by immunoblot, suggested that inclusions were constituted by an excess of proteins normally present in the terminal cisternae of sarcoplasmic reticulum. Our cases, both sporadic and familial, represent a new type of surplus protein myopathy.

Keywords: surplus protein myopathy; sarcoplasmic or endoplasmic reticulum calcium 1 (SERCA1) ATPase; calsequestrin; inclusions; vacuolar myopathy

Abbreviations: ATPase = adenosine triphosphatase; CK = creatine kinase; H&E = haematoxylin and eosin; PAS = periodic acid – Schiff; SERCA1 = sarcoplasmic or endoplasmic reticulum calcium 1; SR = sarcoplasmic reticulum


Introduction

A certain number of myopathies are characterized by inclusions that can be related or not to pre-existing structures (Goebel and Anderson, 1999). Since particular proteins may accumulate in these inclusions, the term ‘surplus protein myopathy’ was introduced to define this subgroup of myopathies (Goebel and Warlo, 2001). The surplus protein, either in granular or filamentous form, may be localized inside the sarcoplasm or in the nucleus. Myopathies with surplus proteins in the sarcoplasm include desmin-related myopathies (Goldfarb et al., 1998; Vicart et al., 1998), actinopathies (Nowak et al., 1999), hereditary inclusion body myopathies (Askanas and Engel, 1998) and myosin storage myopathy (Bohlega et al., 2003; Tajsharghi et al., 2003; Laing et al., 2005). Accumulation of nuclear protein poly(A) binding protein 2 (PABP2) within muscle fibre nuclei was observed in oculopharyngeal muscular dystrophy (Brais et al., 1998).

We report a myopathy, observed in sporadic and familial cases, characterized by the presence of clear vacuoles in sections stained with haematoxylin and eosin (H& E) and Gomori-modified trichrome, and by multiple deep or pale blue inclusions in semithin resin sections stained with toluidine blue. By immunohistochemistry, these inclusions were shown to react for sarcoplasmic or endoplasmic reticulum calcium 1 (SERCA1) ATPase and/or calsequestrin, and by immunoblot the SERCA1- and calsequestrin-increased expression was demonstrated, thus indicating that the inclusions were constituted by an excess of proteins present in the sarcoplasmic reticulum (SR). Therefore, our cases represent a novel form of surplus protein myopathy.

Patients and methods

Case 1: A 58-year-old woman came to our attention for mild increase of serum creatine kinase (CK) levels. Two years before, she had suffered painful contractures in the posterior left thigh muscles that spontaneously disappeared within 1 year, whereas CK remained 2- to 3-fold the upper normal limit. Neurological examination, as well as thyroid function and EKG, were normal. EMG showed low-amplitude, short-duration polyphasic potentials. Nine years after the first examination, the patient was symptomless, the clinical examination was normal and serum CK was still mildly elevated. Two siblings of the patient, a 35-year-old man and a 25-year-old woman, had normal serum CK values; another son died at a young age from drug overdose. The patient’s 89-year-old...
mother; a 60-year-old brother; two sisters, respectively, 56 and 50 years old; and the youngest sister’s daughter, aged 18 years, had similar mild elevated CK. A 61-year-old brother had normal serum CK while in a 47-year-old sister CK was not determined.

The 56-year-old sister had a diagnosis of vacuolar myopathy made elsewhere; ultrastructural examination of the muscle biopsy was not done. Nine years after the diagnosis she did not complain of any neuromuscular symptom (phone interview).

Case 2: A 73-year-old man had been experiencing lower limb myalgia, mostly during the evening hours, and muscle weakness for 3 years. Neurological examination revealed difficulty in getting up from a squatting position. Serum CK level ranged from 5- to 4-fold above normal. EMG showed many polyphasic potentials; spontaneous activity was not recorded. Thyroid function, lactate and cancer markers were normal. His past medical history disclosed a transitory left hemiparesis occurring ~20 years before.

Case 3: A 39-year-old black man, who was born in Ghana and who had moved to Italy 7 years ago, had been complaining of limb myalgias and fatigue for 3 months. Clinical examination was normal. Serum CK level was 2- to 3-fold of normal; thyroid function, aspartate aminotransferase (AST), alanine aminotransferase (ALT), ionized plasma calcium and potassium were within the normal range; serology for HIV and hepatitis was negative. EMG was normal.

Case 4: A 28-year-old man came to our observation because of elevated serum CK (from 10- to 25-fold of normal value), which was also present in his father and in his 2-year-old son. The patient did not complain of any muscular symptoms, and the neurological examination was normal. Routine laboratory assays and EKG were normal. EMG showed some short-duration, low-amplitude polyphasic potentials. The patient has been followed for 10 years. He is now aged 38 and still symptomless. Serum CK level remains high. His 12-year-old son is well with unremarkable neurological examination, but serum CK is always over 5 times the normal value.

None of the patients had a significant past medical history and took any drug.

**Morphological studies**

**Histology and histochemistry**

All patients underwent an open biopsy of vastus lateralis muscle. Serial 8-μm-thick cryosections were stained with H&E, modified Gomori trichrome, adenosine triphosphatase (ATPase, pre-incubation at pH 4.3, 4.6 and 10.4), succinate dehydrogenase (SDH), cytochrome c oxidase (COX), reduced nicotinamide adenine dinucleotide (NADH), periodic acid-Schiff (PAS) with diastase digestion, Sudan black, acid phosphatase, Congo red, Alizarin red, Alcian blue at different pH and phosphorylase.

**Electron microscopy**

A small fragment of muscle tissue was fixed in 4% glutaraldehyde in phosphate buffer, post-fixed in 2% osmium tetroxide, dehydrated and embedded in Spurr resin. Semithin sections were stained with toluidine blue and PAS. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Zeiss EM 109 electron microscope.

**Immunohistochemistry and confocal microscopy**

Immunohistochemistry was performed on serial 6.5-μm-thick sections with antibodies to fast myosin, dystrophin, desmin, vimentin, ubiquitin, caveolin-3, SERCA1 ATPase, SERCA2 ATPase, Golgi 58K, tubulin β II, calsequestrin, calreticulin and calnexin (Table 1). The reactions were revealed by peroxidase and immunofluorescence methods (Vattemi et al., 2004).

<table>
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<tr>
<th>Antigen</th>
<th>Source</th>
<th>Dilution</th>
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<td>Fast myosin</td>
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<td>Dystrophin</td>
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<td>Vimentin</td>
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<tr>
<td>Ubiquitin</td>
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<td>SERCA2</td>
<td>Novocastra</td>
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<td>Tubulin β II</td>
<td>Novocastra</td>
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<td>Affinity Bioreagent</td>
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<td>Calnexin</td>
<td>BD Transduction Laboratories</td>
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Double immunofluorescence was performed with the rabbit polyclonal antibody to calsequestrin, diluted 1 : 50 in combination with a mouse monoclonal antibody to (i) SERCA1, diluted 1 : 300 and (ii) desmin, diluted 1 : 100. Sections were blocked with normal goat serum for 1 h and incubated overnight with the primary antibody to calsequestrin and SERCA1 or desmin. The day after, slides were incubated first with the biotinylated anti-mouse, then with streptavidin-Texas Red and finally with a FITC-conjugated goat anti-rabbit. The reaction was examined by confocal microscopy (LSM 510, Carl Zeiss, Oberkochen, Germany).

Controls for staining specificity were omission of the primary antibody or its replacement with non-immune sera at the same concentration.

**SDS–PAGE electrophoresis and immunoblot analysis**

In two patients (Cases 1 and 2) and in normal controls, SERCA1 and calsequestrin expression was evaluated by immunoblot (Vattemi et al., 2004). Briefly, ten 20-μm-thick frozen muscle sections were homogenized with a lysis buffer, sonicated and centrifuged at 1500 g for 10 min. Protein concentration was determined in the supernatants with the Bradford method. Aliquots corresponding to 20 μg of proteins were loaded on a 10% T polyacrylamide gel and separated by electrophoresis. Samples were transferred to a nitrocellulose membrane that was blocked with non-fat dried milk for 60 min at room temperature and incubated overnight at 4°C with the specific antibody. After intervening washes, the membrane was incubated with peroxidase-conjugated goat anti-mouse IgG for SERCA1 or anti-rabbit IgG for calsequestrin. Bands were visualized with the ECL Advance Western Blotting Detection Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Protein loading was normalized for sarcomeric actin. Bands were quantified densitometrically using the Quantity One software (Bio-Rad).

**Biochemistry**

Biochemical analysis was performed on muscle homogenates. In Patient 4, branching enzyme activity was measured with a
radioactive assay (Servidei et al., 1987), and debrancher and phosphofructokinase activities were determined by a spectrophotometric method (Layzer et al., 1967; DiMauro et al., 1979). Acid and neutral maltase were measured by a fluorimetric assay with the artificial substrate 4-methylumbelliferyl alpha-D-glucoside (Mehler et al., 1977) in Patients 1, 2 and 4. Respiratory chain enzyme activities were assayed in the four patients (DiMauro et al., 1987).

Results

Light microscopy on frozen sections

The striking feature, observed by H&E and Gomori trichrome stains, was represented by multiple vacuolar spaces of different size within the muscle fibres of all the patients (Fig. 1A). The percentage of fibres containing vacuoles ranged from 3 (Case 3) to 15% (Case 1). The larger vacuoles...
(≈20 × 10 μm) contained fine amorphous material forming subtle septa and had a lobulated border. The smaller vacuoles (2–3 μm in diameter) had a round or oval shape. Most of the fibres containing vacuoles reacted as type 2B. Vacuoles did not stain with SDH, phosphorylase, ATPase, Congo red, Alcian blue, Alizarin red and Sudan black; a faint NADH reaction was observed within some vacuoles or it partially outlined their contour. A thin rim of PAS positive material, which disappeared after diastase digestion, was observed around the vacuoles. Acid phosphatase-counterstained sections revealed the presence of some greenish material that was removed by trypptic digestion inside the vacuoles (Fig. 1B).

Slight fibre size variation was observed in Cases 2 and 4; in Case 3, type 1 and 2B fibres were smaller than type 2A. In Case 1, type 1 fibre diameter ranged from 60 to 120 μm while type 2 fibre diameter ranged from 20 to 50 μm. Type 1 fibre predominance was observed only in Patient 2 while the other patients had a checkerboard distribution.

On a given section, one or two necrotic fibres were present in all the four cases (<0.5%); one COX negative fibre (<0.5%) was observed in Cases 1, 3 and 4 while Case 2, the oldest patient, had eight COX negative fibres (2%).

**Light microscopy on semithin resin sections**

The main findings were single or multiple inclusions that could be quadrangular or round in shape within the muscle fibres. We conventionally denominated type 1 the quadrangular deep blue ones and type 2 the round, pale blue ones (Fig. 1C and D).

Type 1 inclusions were more numerous than type 2; they could reach the number of 20 per fibre. The short side of the quadrangle measured nearly 4 μm, the long side ≈6 μm; occasionally, they appeared placed inside a clear space or abutting on it. Type 2 inclusions were less numerous, from 5 to 8 per fibre, and their diameter was nearly 6 μm.

Both types of inclusions were sometimes gathered, to form a conglomerate mass of ≈20 μm in diameter, in the centre of the fibres or, less frequently, in the subsarcolemmal space. In longitudinal sections the inclusions could be arranged in rows. A subtle rim of PAS positive material encircled the single inclusions, thus creating an alveolar-like structure (Fig. 1E). In longitudinal sections the sarcomeric structure of the fibre was preserved. The inclusions were present within normal fibres.

**Electron microscopy**

The type 1 inclusions (Fig. 2A and B) had mostly rectangular or quadrangular shape, but could also be polygonal or have an irregular contour. They were constituted by compact homogeneous amorphous material, with a density similar to Z-lines, and were delimited by other components of the muscle fibres. Some inclusions were partially or entirely bounded by a single membrane (Fig. 2C); others had no membrane. Type 2 inclusions were constituted by less compact homogeneous and amorphous material of intermediate density (Fig. 2A and D). They exhibited rounded or irregular sinuous borders and were bounded by a single membrane (Fig. 2F); small round profiles were dispersed within the material. Both types of inclusions were surrounded by glycogen particles and were within normal myofibres. They could be single or gathered in one type group (Fig. 2D) or in a mixed group (Fig. 2E). The content of the inclusions did not have any discernible periodicity at high resolution.

Proliferation of SR tubules was frequently found around the inclusions. Honeycomb formation of T-tubules was rarely observed near the inclusions. In longitudinal sections the myofibrils appeared displaced and compressed by the inclusions but had normal sarcomeric structure. Myofilaments were preserved and Z-lines had normal thickness. Only few mitochondria were degenerating. Occasional myelin figures were observed. In Case 1, a collection of IBM-type tubulo-filaments was found. Nuclei were normal. Some morphological findings suggested different steps in the evolution of these inclusions. Probably, the earliest trace of type 1 inclusion was an irregular area of amorphous material with Z-line density (Fig. 2G), localized among the myofibrils and surrounded by glycogen particles. In a second phase, this material became a compact homogeneous structure with more defined and linear borders, partially limited by a membrane. The earliest step in type 2 inclusion formation seemed to be the accumulation of homogeneous material of intermediate density in dilated SR cisternae (Fig. 2H). In a second step, the vesicles became larger structures containing the same material.

**Immunohistochemistry and confocal microscopy**

SERCA1 antibody stained the sarcoplasmic reticulum ATPase in type 2 fibres and displayed single or multiple patches of very strong reactivity mostly inside type 2 fibres (Fig. 3A). A focal strong reaction was also observed inside the fibres with calcequestrin antibodies. Confocal microscopy showed a frequent co-localization of both antibodies (Fig. 3B).

Developmentally regulated cytoskeletal protein, desmin, vimentin and ubiquitin were absent in the vacuoles. The boundaries of the vacuoles were reinforced by fast myosin and desmin (Fig. 3C), sometimes by vimentin and occasionally by dystrophin. Calreticulin, calnexin, Golgi 58 K, tubulin, caveolin-3 and SERCA2 were not abnormally expressed. Rare fibres were positive for both SERCA1 and SERCA2.

**Immunoblot analysis**

By immunoblotting, SERCA1 and calcequestrin were strongly increased in the patients’ muscle compared with that of controls (Fig. 3D).
Fig. 2 Electron micrographs. (A) A type 1 inclusion (arrowhead) with a rectangular shape and containing high-density amorphous material and a type 2 inclusion (arrow) with more round shape and containing amorphous material of intermediate density. ×13 000 original magnification. (B–C) A long row of type 1 inclusions of different size and shape (B) ×13 000 original magnification; the border of some inclusions is partially limited by a single membrane (arrow) (C) (insert in B). (D) A large group of type 2 inclusions separated from each other by a rim of glycogen particles; the structure of surrounding sarcomeres appears normal. ×13 000 original magnification. (E) A conglomerate mass of type 1 (arrowhead) and type 2 (arrow) inclusions. ×15 000 original magnification. (F) The single membrane limiting the type 2 inclusion derives from a T-tubule (arrow). (G) A small lake of high-density amorphous material among the myofibrils probably represents the earliest trace of type 1 inclusions. ×83 000 original magnification. (H) Accumulation of amorphous material of intermediate density in dilated cisternae of the SR probably represents the earliest step in the formation of type 2 inclusions. ×20 000 original magnification.

SERCA1 and calsequestrin storage myopathy

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Fig. 3 Immunohistochemistry, confocal microscopy and immunoblot analysis. (A) SERCA1 immunostaining showing single or multiple dots localized more frequently in atrophic muscle fibres exhibiting higher immunoreactivity (type 2 fibres). (B) Confocal images for SERCA1 (green fluorescence) and calsequestrin (red fluorescence) showing deposits containing both SERCA1 and calsequestrin material (yellow) and some deposits containing only calsequestrin or SERCA1. (C) Confocal images for calsequestrin (red fluorescence) and desmin (green fluorescence) demonstrating calsequestrin deposits inside the vacuoles, which are lined by deposits containing both calsequestrin and desmin (yellow). (D) SERCA1 and calsequestrin immunoblots demonstrating an increased expression of both proteins in the patients’ muscle (P) compared with control muscles (C) and densitometric analysis of the stained bands.
Biochemistry

Acid maltase, branching, debranching, phosphofructokinase and respiratory chain enzyme activities were normal in the assayed patients.

Discussion

We first observed Patients 1 and 4 in 1996. Since then, other cases with the same histopathological features came to our attention. None of them had a severe clinical picture: Patient 3 had a mild lower limb girdle myopathy; Patient 2 complained of myalgia and fatigability, while Patients 1 and 4 had familial asymptomatic elevated serum CK. In neither of the two families the disease progressed over a 10-year follow-up, nor was a further increase of CK values observed. The two familial cases suggested an autosomal dominant inheritance.

The histological striking features of our cases were the presence of many inclusions within the muscle fibres of which we distinguished two types according to their morphology in resin-embedded samples stained with toluidine blue: type 1 inclusions were quadrangular and stained deep blue while type 2 inclusions were round and pale blue. They were not detectable on H&E and modified Gomori trichrome-stained sections where single/multiple clear vacuoles of different size and shape were evident within the muscle fibres. The observation of a faint greenish material inside the vacuoles in acid phosphatase-counterstained sections that was removed by trypic digestion suggested a protein content of vacuoles. Both types of inclusions could be single, multiple or gathered preferentially in the centre of the fibre; in longitudinal sections they could be arranged in rows. The material of type 2 inclusions resembled that occasionally found in muscle fibres in other different conditions, including a vacuolar myopathy of undetermined aetiology (Engel and Banker, 1994), a case of chronic progressive and relapsing neuromyopathy (Lach et al., 1990), and a case of myopathy with tubular aggregates (Lewis et al., 1971). To our knowledge, type 1 inclusions, as we conventionally called them, have never been described.

The quadrangular shape of the space limiting the accumulated material recalled the sarcoplasmic crystals described in an adult patient with diffuse pain and exercise-unrelated muscle fatigue; however, these crystals, having a compact granular structure, were not membrane-bound on electron microscopy and stained in cryostat sections as eosinophilic, bright red material with modified Gomori trichrome stain; recently, they were found to be tubulin positive (Vu et al., 2001). The ultrastructural morphology of type 2 inclusions, suggesting storage of material in dilated SR cisternae, prompted us to study proteins localized in SR. As a marker of the SR proteins we chose SERCA, the Ca$$^{++}$$-ATPase isoform localized in the wall of the SR tubes, as well as in the wall of the cisternae, where it does not interface with T-tubules (MacLennan et al., 1997); fast calsequestrin, a Ca$$^{++}$$ storing protein, associated with the cysternal aspect of the ryanodine receptor (Anderson et al., 1995); and calreticulin and calnexin, both chaperone proteins, the first resident in the SR lumen, the second an integral membrane protein of the endoplasmic reticulum (Wada et al., 1991). We also used a monoclonal antibody to Golgi 58 K as a selective marker for the cytoplasmic face of the Golgi apparatus (Gao et al., 1998). Many inclusions strongly reacted with SERCA1 antibody while others reacted with calsequestrin; in addition, in many of them we observed, by confocal microscopy, a co-localization of both proteins. SERCA1 and calsequestrin were also upregulated at protein level. SERCA2, calreticulin, calnexin and Golgi 58 K were not abnormally expressed. These data demonstrated that the inclusions contained, at least partially, an excess of proteins normally present in SR, mainly in terminal cisternae.

In addition, the tubular aggregates, observed in some human myopathies, and in fast twitch skeletal muscles from aged male inbred mouse, are shown to be positive for SERCA1 and calsequestrin by immunohistochemistry; it is agreed that they arise from dilated terminal cisternae of SR (Salvati et al., 1985). Differing from human tubular aggregates, our inclusions did not stain with modified Gomori trirome no show strong NADH reactivity. Their staining behaviour, instead, resembled that observed in tubular aggregates of aged male inbred mic (Agbulut et al., 2000).

The vacuolar spaces found in cryosections appeared peripherally reinforced by increased desmin immunoreactivity. This finding has to be regarded as non-specific since it may be observed at the periphery of central cores, encircling hyaline bodies, around nuclei in adult central nuclear myopathy and in rimmed vacuoles in type 2 glycogenosis (Vita et al., 1994).

In conclusion, our patients are affected by a new type of surplus myopathy, with benign evolution, characterized by inclusions composed of an excess of SERCA1 and calsequestrin, resident proteins in the SR cisternae.

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References


