POSTNATAL CENTRALIZATION OF MUSCLE FIBRE NUCLEI IN CENTRONUCLEAR MYOPATHY

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Abstract—Postnatal centralization of muscle fibre nuclei, which were previously located subsarcolemmally, is described in a case of centronuclear myopathy (CNM) in a male patient with generalized muscle weakness since birth. A muscle biopsy was taken at the age of 11 months; no particular abnormalities were observed at this stage apart from an unusual variation in fibre size. A distinctly below average muscle fibre diameter, increased endomyosial connective tissue, and features typical for CNM were found in a biopsy taken 9 yr later. Immunohistochemical studies using antibodies to desmin, vimentin, laminin and type IV collagen revealed altered staining patterns compared with normal fibres. The abnormalities in the patterns of cytoskeletal proteins point to a defective regulation of the composition and organization of the cytoskeletal network during development, paralleled by abnormalities in the extracellular matrix.

Key words: Centronuclear myopathy, pathogenesis, cytoskeleton, basement membrane, extracellular matrix, immunohistochemistry.

INTRODUCTION

The first case of a muscular disease characterized by numerous centrally located nuclei in the muscle fibres was documented by Spiro et al. [1]. Since the morphological findings in the muscle biopsy suggested the presence of myotubes, the disease was named "myotubular myopathy". The general term "centronuclear myopathy" (CNM) was suggested by Sher et al. [2]. Several authors described variations in clinical picture, age of onset, mode of inheritance and progression, which indicated that the term centronuclear myopathy may in fact cover more than one disease [3-7]. The pathogenesis of the disorder is not clear although some explanations have been put forward [8-11]: (1) an arrest of, or delay in, muscle fibre maturation; (2) an impairment of maturation preventing migration of nuclei to a peripheral location; and (3) degenerative processes causing secondary nuclear migration to the centre of the fibres.

Disorganized cytoskeletal filaments have been observed in several myopathies [12,13] but few studies have investigated whether the presence of central nuclei is related to a disorganization of the cytoskeletal network. Because of the putative role of the cytoskeleton in the positioning of the nucleus, in its interaction with the cell membrane [14,15], it is tempting to speculate that some disorder in the cytoskeletal protein system might lead to centralization of nuclei in CNM. Using antibodies to intermediate filaments of the desmin (skeletin) type, no specific abnormalities in muscle biopsies of two patients presenting a slowly progressive form of CNM were observed [13], whereas in four cases of a severe, neonatal form of CNM desmin patterns comparable with foetal muscle were described [16].

Studies on the distribution pattern of the basement membrane constituents laminin and type IV collagen in CNM have not revealed differences in the localization of these components when compared with normal muscle [17-19]. Both laminin and type IV collagen were always located in a sharply delineated ring representing the basement membrane surrounding muscle fibres, blood vessels and capillaries.
In this report we describe a patient who suffered from a generalized muscle weakness since birth and from whom a muscle biopsy was taken at the age of 11 months. The biopsy showed no obvious abnormalities apart from too large a mean fibre diameter, and too large a variation in fibre size. A second biopsy was taken at the age of 10 yr. The biopsy findings appeared to be typical for CNM. In addition to conventional ultrastructural and histochemical characterization of the muscle biopsies, we performed immunohistochemical studies with antibodies to the intermediate filament proteins desmin and vimentin, and the basement membrane components laminin and type IV collagen, to investigate the organization and composition of the cytoskeletal network and the basement membrane of the pathologic myofibres.

**PATIENT AND METHODS**

**Case history**

The patient, a boy, was born as the first child of healthy, non-consanguineous parents after an uneventful pregnancy and delivery. From birth onwards he experienced hypotonia, feeding difficulty and reached motor milestones with some delay. He showed facial weakness and ptosis, but no other signs of external ophthalmoplegia. He walked at 18 months but was never able to run. A biopsy of the quadriceps muscle was performed elsewhere (by Dr M. H. Brooke) at the age of 11 months (biopsy 1). The biopsy was definitely abnormal, but a clear diagnosis could not be made. He had a number of life threatening respiratory infections, during one of which he needed ventilatory support. He experienced increasing difficulty in walking and showed progressive lordosis, fixed hip flexion and equinovarus deformity of the feet. When we first saw him at the age of almost 10 yr, he was a short stunted boy, with a height of 126 cm (below third percentile), a head circumference of 51 cm (between 10th and 50th percentile) and weighed 18.5 kg (below third percentile). General examination revealed no abnormalities and no cardiomyopathy was diagnosed. There was facial diplegia and bilateral ptosis without limited extra-ocular muscle movement, and generalized weakness, more pronounced in the distal than the proximal muscles. He was seriously handicapped by pes equinus left and right, with a slight varus and adductus deformity. The serum creatine kinase was not increased and the results of electromyographic investigations were equivocal. A needle biopsy of the quadriceps muscle (vastus lateralis) was performed and the tissue was embedded in epoxy-resin for ultrastructural examination (biopsy 2). A Z-lengthening procedure was performed on the right Achilles tendon and after uneventful recovery the left Achilles tendon was also treated. During the operation, a biopsy of the soleus muscle was taken (biopsy 3). The wounds healed well. Subsequently an ankle orthosis was made on both sides, but the course was seriously complicated by delayed weaning from the respirator due to tracheomalacia. This was eventually treated by
surgical removal of the eroded part of the trachea. Further recovery was uncomplicated.

**Histochetistry and morphometry**

Sections of biopsy 1 stained with haematoxylin-eosin (H&E) and ATPase (high pH) were kindly put at our disposal by Dr. Brooke. Frozen cryostat sections of biopsy 3 were used for staining with H&E and for ATPase (high pH) and other histochemical stainings [20]. Muscle fibre diameters were estimated using a MOP-Videoplan measuring instrument (Kontron, Munich, F.R.G.). In cases of ellipsoid profiles, the lesser diameter was considered to be the profile diameter [20].

**Immunohistochemistry**

Immunohistochemistry could only be performed on material from biopsy 3, which was frozen immediately in liquid nitrogen and stored at −20°C. Air-dried, 6 µm cryostat sections were fixed in methanol (−20°C, 5–10 min) and acetone (−20°C, 10–30 s). After air drying they were incubated with an antibody or antisera for 30 min at room temperature. The sections were rinsed in PBS (30 min), and when double labelling was performed incubated with a second antibody or antisera. After rinsing in PBS (30 min) they were incubated with the appropriate anti-mouse and/or anti-rabbit immunoglobulins, labelled with fluorescein isothiocyanate (Nordic Immunology, Tilburg, The Netherlands), Texas Red (Southern Biotechnology Associates, Birmingham, AL, U.S.A.) or peroxidase (Dakopatts, Glostrup, Denmark). After washing in PBS for 30 min, the sections were mounted in Gelvatol (Monsanto, St. Louis, MO, U.S.A.) or processed for the detection of peroxidase as described elsewhere [21]. Slides were viewed with a Leitz Dialux 20 EB microscope, equipped with epifluorescent illumination. Pictures were taken using 400 ASA Tri-X film (Kodak, Rochester, NY, U.S.A.) with an automatic camera.

The following polyclonal and monoclonal antibodies were used in this study:

1. the mouse monoclonal antibody 4E10, specific for human laminin [22],
2. a mouse monoclonal antibody to type IV collagen, raised to human kidney glomeruli [23],
3. the mouse monoclonal RV 202, specific for vimentin, the intermediate filament protein found in most types of mesenchymal cells [24],
4. an affinity-purified polyclonal rabbit antiserum (pY1M), raised against vimentin isolated from calf lens by preparative gel electrophoresis [25],
5. the mouse monoclonal antibody RD 301, specific for desmin, the muscle specific intermediate filament protein [24],
6. a polyclonal rabbit antiserum (pDes), raised against chicken gizzard muscle desmin as described before [25], and
7. the mouse monoclonal antibodies 330-R5B4 and 330-R5D4, specific for embryonic myosin [26].

The monoclonal and polyclonal antibodies to vimentin and desmin are available from Euro-Diagnostics B.V., Apeldoorn, The Netherlands.

Soleus muscle from a 9-yr-old patient suffering from a clinical (possibly toxic) polymyopathy (Figs 2 and 3), and a 6-yr-old patient suffering from Duchenne muscular dystrophy, in whose biopsy material large groups of basophilic regenerating fibres were present (Fig. 4), were used as controls.

**Electron microscopy**

Material from biopsy 2 was prefixed in buffered 2% glutaraldehyde and postfixed in 2% OsO4. Following embedding in Epon 812, ultrathin sections were double contrasted (uranyl acetate, lead citrate), and examined in a Philips EM 300 or a Philips EM 301 electron microscope.
RESULTS

Histochemical observations

Histochemical and quantitative morphometrical findings are depicted in Fig. 1 and summarized in Table 1. The first biopsy was characterized by a normal ratio of type I to type II fibres. Only 0.7% of the fibres contained centrally located nuclei (Fig. 1a,c). However, there was a distinct variation in fibre diameter and the average diameter was too large for that age. Biopsy 2, taken 9 yr later from the same muscle, showed numerous fibres with central nuclei and a mean muscle fibre diameter far below normal. Fibres showed a somewhat rounded appearance (Fig. 6a), endomysial connective tissue was increased, and adipose tissue was spread throughout the whole biopsy, replacing about 25% of the muscle tissue. Some fibres (especially the fibres with the largest diameters) showed small or large central “holes”, a perinuclear halo or a radial, spoke-like appearance of the cytoplasm. In longitudinal sections, internal nuclei were located in chains with or without internuclear spaces. Nuclei were somewhat rounded but not swollen and did not contain prominent nucleoli. In biopsy 3, taken from another muscle, the same abnormalities were present (Fig. 1b, d.). In H&E stained sections no bluish, basophilic fibres were observed.
Immunochemical observations (Figs 2 and 3)

Monoclonal and polyclonal antibodies to desmin, vimentin, laminin and type IV collagen were used to characterize abnormalities of the cytoskeleton and the basement membrane in biopsy 3. In numerous fibres desmin organization was disturbed (Fig. 2b–o), resulting in either protein accumulations in the peripheral region of the fibres (Fig. 2i), or in irregularly organized aggregates surrounding the nuclei (Fig. 2b–g), irregular punctate reactivity in the myoplasm (Fig. 2h,k–m) or a circumscribed reactivity between the nuclei (Fig. 2j). Vimentin, normally not present in mature muscle fibres but only in the interstitial tissue (Fig. 3a), was detected as cytoplasmic aggregates in numerous diseased muscle fibres (Fig. 3b). Staining by antibodies to laminin (Fig. 3c,d) and type IV collagen (Fig. 3e,f) was more intense than in the control biopsy; each fibre was completely surrounded by a thickened layer of laminin (Fig. 3d) and type IV collagen (Fig. 3f). Blood vessel walls also showed increased reactivity with the laminin and type IV collagen antibodies while the proliferated endomysial connective tissue showed no reactivity. These patterns were comparable with those obtained with peroxidase conjugated secondary antibodies, and were neither observed when incubation with the primary antibodies was omitted, nor in control muscle. In order to identify possible regenerating fibres we used two antibodies to embryonic myosin. No staining was observed in any of the muscle fibres of our patient, including those with disturbed desmin distribution, with either antibody (Fig. 4a,b), while in a control patient a large group of regenerating fibres showed obvious staining with
both antibodies to embryonic myosin as well as the antibodies to desmin (Fig. 4c,d).

Ultrastructural observations (Figs 5 and 6)

Apart from the presence of central nuclei, most muscle fibres showed a regular arrangement of the contractile apparatus. However, spoke-like organization of the myofibrils, with widened intermyofilamentous spaces was observed as well as structural aberrations such as replicated triads, target fibres, Z-disk streaming, and other myofilamentous disorganizations (Fig. 5c,d). In several fibres membrane-bound depositions of electron-grey or -opaque material were observed between the filaments (not illustrated). Perinuclear spaces, present in most of the fibres, were occupied by mitochondria, lysosomal structures, small lipofuscin-like granules, glycogen, Golgi complexes, intermediate filaments (Fig. 5a,b) as well as filamentous bodies (Fig. 5c). A replicated basal lamina surrounding muscle fibres and blood vessels was also seen (Fig. 6b,c).

DISCUSSION

Centronuclear myopathy (CNM) consists of several subtypes with different characteristics [3,8,27,28], the most commonly observed of which are centrally located nuclei in a significant number of muscle fibres and a type I fibre predominance. Several authors [3,27-29] have suggested that CNM can be subdivided into the following categories: (1) a neonatal type with autosomal recessive inheritance, ("myotubular myopathy") [3]; (2) a severe neonatal type with X-chromosome linked inheritance ("X-linked myotubular myopathy", XLMTM) [3]; and (3) an autosomal dominant or sporadic type, with onset in childhood or adult life ("centronuclear myopathy") [3]. Considering the time of onset, the negative family history and the anamnestic data, the patient described in this report probably belongs to type 3.

There has been much speculation about the pathogenesis of the disease. The original assumption of Spiro et al. [1] that fibres with central nuclei were non-matured foetal myotubes was questioned by several authors [2,8,9]. In contrast to normal foetal myotubes, which have prominent nuclei and small amounts of contractile elements, most fibres in CNM muscle contain relatively large areas with fully differentiated sarcomeres. The presence of myofibrillar disruptions, atrophy-associated abnormalities and
Fig. 5 Alterations in the organization of the cytoskeleton as observed by electron microscopy. (a) Centrally located nuclei (n) with an internuclear space occupied by numerous organelles. At the right regular arranged myofilaments and the sarcolemma with a fibroblastic extension (b) Disarrangement of intermediate sized filaments (inset higher magnification), mitochondria (arrows) and a Golgi area (asterisk) between bundles of myofilaments. (c) Perinuclear area containing glycogen, filamentous bodies (arrowheads), and replication of triads (arrow); n indicates the nucleus. (d) Irregular electron-dense accumulations of cross-sectioned Z-band material. Bars indicate 1 μm (a,b,c) or 10 μm (inset d).

Subsarcolemmal nuclei are more suggestive of other pathological processes rather than an arrest in foetal muscle development. In XLMTM, however, evidence is accumulating that fibres with central nuclei may indeed be non-developed foetal muscle fibres [16,30,31].

Few follow-up studies have been performed with CNM patients. The cases that have been documented so far showed no obvious histologic changes in consecutive biopsies [4] or decreased numbers of central nuclei [32]. One case with pathological features of both CNM and multicore disease has been described that in some aspects resembles our case [33]. Unlike a muscle biopsy taken during the first year of life, biopsies taken several years later revealed the presence of
internal nuclei in a large number of fibres. It seems to indicate that in this patient muscular weakness preceded the translocation of nuclei. It has been proposed by Hülsmann et al. [9] that postnatal, secondary migration of nuclei to a more central position may be due to an autolytic or autodegenerative process.

We present a case in which a boy suffering from generalized muscle weakness was biopsied 9 yr after the first examination. During this period, a number of changes had occurred, both within and between the muscle fibres. Apart from an unusual variation in fibre size, muscle fibre maturation appeared normal. Subsequently, fibres did not grow to normal sizes and nuclei migrated to the centre of the fibres. These observations point to a secondary migration of nuclei to a central position in this case of CNM. Furthermore, the presence of hypertrophic fibres in biopsy 1 and the presence of atrophic fibres in biopsies 2 and 3 indicate a delay or even an arrest in muscle fibre growth. This abnormal development is associated with pathological changes, such as the appearance of fibres with large vacuoles, or radial or spoke-like structures, and a proliferation of endomysial tissue with
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excessive adipose tissue replacing muscle fibres.

Several authors have proposed that proliferation of connective tissue may play a role in the pathogenesis of various myopathies [18,19, 29]. Presumably, proliferated endomysial tissue encloses muscle fibres in such a way that growth in width, and possibly also in length, is hindered. In combination with the presence of a reduplicated sarcolemma, endomysial tissue proliferation may interfere with the contact of the fibres with anchoring structures to the supporting tissue [34], and consequently also with the co-ordinated contraction of muscle fibres and force-transmission. It has been suggested that in muscular dystrophic mice, the presence of increased connective tissue and an abnormal sarcolemma caused growth impairment and maturational defects in pathologic muscle fibres [35].

Our studies showed a distinct and even increase in laminin and type IV collagen immunoreactivity surrounding all muscle fibres (Fig. 3d,f). This is most likely to be related to the thickening and reduplication of the basal laminae around all muscle fibres and blood vessels (Fig. 6b,c). We are aware of one other immunohistochemical study of a CNM muscle biopsy [19], using anti-type IV collagen and laminin antibodies. In this case no significant differences were found when compared with control tissue. Irregular, focally increased immunoreactivity for laminin and type IV collagen is found in muscular dystrophies and around individual atrophic fibres in a variety of other neuromuscular disorders [17]. However, the regularity in the increase of endomysial type IV collagen and laminin throughout the muscular tissue, in the absence of extensive fibrosis of the perimysium, seems rather exceptional [17]. In this context it seems worthwhile to note that the desmin intermediate filament organization is severely disturbed within a high percentage of muscle fibres. We consider it highly unlikely that the aberrant desmin patterns are related to an immaturity of the fibres or are the result of regenerative processes. In a variety of myopathies, the desmin staining pattern is intense and diffusely distributed in immature and regenerating muscle fibres [36,37]. Also arguing against muscle fibre regeneration is the absence of basophilic fibres and large, vesicular nuclei with prominent nucleoli. To further exclude the possibility of fibre regeneration in this patient, we used antibodies specific for embryonic myosin. These antibodies reveal distinctly positive fibres in normal foetal or neonatal human skeletal muscle tissue [26], stain regenerating fibres (Fig. 4d), but showed no positive reaction at all in our patient (Fig. 4b).

Focal accumulation of intermediate-sized filaments in muscular disorders is often considered a rather non-specific abnormality [12,13,38]. In our case however, we observed a generalized disturbance of the desmin organization. It has been demonstrated in various cell types, including muscle cells, that desmin filaments contact both the nuclear surface and the plasma membrane [14,39,41]. It is tempting to speculate that in our CNM case, a disturbance of the regulatory mechanisms that govern the organization of the desmin intermediate filaments may result in an abnormal localization of the nuclei. In addition, the occurrence of intracellular cytoskeletal abnormalities and the extracellular thickening of endomysial structures may also be related. Under normal conditions vimentin is only present in immature or regenerating muscle fibres [16,41]. Therefore the presence of a punctate localization of vimentin immunoreactivity in underdeveloped, but clearly not regenerating, muscle fibres may be due to an abnormal regulation of intermediate filament gene-expression. In this respect it is interesting to refer to Sarnat's suggestion [16] that persistence of "foetal" desmin and vimentin filaments hinders nuclear migration to a subsarcolemmal position during maturation of muscle fibres in XLMTM.

From the above it will be clear that further studies on abnormalities in the development of the myofibre cytoskeletal system and of extracellular matrix components are needed for a better understanding of the pathogenesis of the various forms of CNM. The different staining patterns obtained with antibodies to desmin and vimentin might be of diagnostic significance in discerning between XLMTM and the childhood onset type of CNM.

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