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Immunophenotyping of congenital myopathies: disorganization of sarcomeric, cytoskeletal and extracellular matrix proteins

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Abstract

We have studied the expression and distribution patterns of the intermediate filament proteins desmin and vimentin, the sarcomere components titin, nebulin and myosin, the basement membrane constituents collagen type IV and laminin, and the reticular layer component collagen type VI in skeletal muscle of patients with “classic” congenital myopathies (CM), using indirect immunofluorescence assays. In all biopsy specimens obtained from patients with central core disease (CCD), nemaline myopathy (NM), X-linked myotubular myopathy (XLMTM) and centronuclear myopathy (CNM), disease-specific desmin disturbances were observed. Vimentin was present in immature fibres in severe neonatal NM, and as sarcoplasmic aggregates in one case of CNM, while the amounts of vimentin and embryonic myosin, observed in XLMTM, decreased with age of the patients. Abnormal expression of myosin isoforms was found in several CM biopsies, although the organization of myosin and other sarcomere components was rarely disturbed. Basement membrane and reticular layer proteins were often prominently increased in severe cases of CM. We conclude that (i) desmin is a marker for individual types of CM and might be used for diagnostic purposes; (ii) the expression patterns of the differentiation markers desmin, vimentin and embryonic myosin in XLMTM, point either to a postnatal muscle fibre maturation or to a variable time-point of maturational arrest in individual patients; (iii) the correlation between the distribution patterns of extracellular matrix proteins and clinical presentation points to a role of these proteins in pathophysiology of CM.

Keywords: Congenital myopathies; Central core disease; Nemaline (rod) myopathy; Centronuclear/myotubular myopathy; Muscle fibre maturation; Immunocytochemistry

1. Introduction

Defined as inherited non-progressive childhood neuromuscular diseases, characterized by specific clinical features and structural abnormalities within the skeletal muscle, the group of congenital myopathies (CM) now includes some forty distinct disorders. These rare diseases can be divided into three categories as recently reviewed by Goebel (1991): (i) “classic” CM, such as central core disease (CCD), nemaline myopathy (NM), centronuclear myopathy (CNM), X-linked myotubular myopathy (XLMTM) and congenital fibre type disproportion (CFTD); (ii) “accepted” CM, often named after the morphology of structural changes observed in the muscles (e.g. fingerprint body myopathy and zebra body myopathy); and (iii) “questionable” CM, a large group of less well defined muscular diseases.

CCD is the first CM reported in the literature. Shy and Magee (1956) described a family suffering from
non-progressive, mild muscle weakness, which after histologic examination revealed muscle fibres with abnormal central areas, that were later shown to be devoid of mitochondria and sarcoplasmic reticulum and deficient in oxidative enzymes and phosphorylase activity (Bodensteiner, 1994). The myofibrils in those areas or cores are either disrupted (unstructured cores) or show a normal cross-striation (structured cores). In the latter case myofibrils are often contracted or otherwise out of register with the fibrils outside the core (Bodensteiner, 1994).

NM was described for the first time by Shy et al. (1963) and Conen et al. (1963) as a mild, non-progressive muscle weakness characterized by rod-like structures within the myofibres, which was confirmed by Engel et al. (1964) and Kuitunen et al. (1972). Subsequent reports described patients with myofibrillar as well as nuclear rods (Paulus et al., 1988; Rifai et al., 1993). Apart from relatively benign cases, severely affected and progressive cases have also been described (Nonaka et al., 1989, 1990; Shimomura and Nonaka, 1989; Wallgren-Pettersson, 1989). Bodensteiner (1988) suggested to classify the neonatal and congenital forms as a mild, non-progressive, although progressive cases have also been described (Arts et al., 1978; Cartwright et al., 1990). A myopathy characterized by the presence of central nuclei in the muscle fibres was described for the first time by Spiro et al. (1966). This author introduced the term "myotubular myopathy". Together with "centronuclear myopathy" (Sher et al., 1967) this term has since then often been used for myopathies distinguished by non-peripherally located nuclei in muscle fibres. Nowadays most authors discern two or three different types of myopathies with centrally located nuclei (Dubowitz, 1985; Swash and Schwartz, 1988; Bodensteiner, 1994). A convenient classification proposed by Figarella-Branger et al. (1992), categorizes all non-X-linked types in one group, and the X-linked form in a second group. In this paper we will refer to the non-X-linked cases as centronuclear myopathies (CNM) and to the X-linked cases as X-linked myotubular myopathy (XLMTM). XLMTM, first described by Van Wijngaarden et al. (1969) and Barth et al. (1975), is undoubtedly the most severe CM. Symptoms are always present at birth and patients usually die within a few months. Muscle biopsies are characterized by small fibres with large central nuclei that show features of immaturity or a delayed development (Sarnat, 1990). The affected gene in XLMTM has been assigned to Xq28 (Darnfors et al., 1990; Lehjesjoki et al., 1990; Starr et al., 1990; Thomas et al., 1990).

CNM has its onset in childhood or in adult life (Goebel et al., 1984; Lovaste et al., 1987; Van der Ven et al., 1991) and usually results in somewhat milder symptoms. An autosomal recessive (Sher et al., 1967; Heckmatt et al., 1985) as well as an autosomal dominant (McLeod et al., 1972; Torres et al., 1985) inheritance has been suggested. Mostly, the often sporadic childhood or adult life onset cases are even more benign, although progressive cases have also been described (Baradello et al., 1989).

The molecular organization and distribution of structural muscle fibre components in CM have only scarcely been studied. Abnormal desmin distribution has been described in CCD (Thornell et al., 1983; Gallanti et al., 1992), NM (Jockusch et al., 1980; Thornell et al., 1980; Sarnat, 1992), XLMTM (Sarnat, 1990, 1992) and CNM (Van der Ven et al., 1991; Misra et al., 1992; Figarella-Branger et al., 1992), while aberrant expression of vimentin, other than in regenerating fibres, has been reported in XLMTM (Sarnat, 1990, 1992) and CNM (Van der Ven et al., 1991; Misra et al., 1992).

The sarcoplasmic rods characteristic for NM were shown to contain the Z-band protein \( \alpha \)-actinin (Jockusch et al., 1980; Jennekens et al., 1983; Paulus et al., 1988) and actin (Yamaguchi et al., 1982; Rifai et al., 1993). Whether desmin is a component of rods (Paulus et al., 1988; Rifai et al., 1993) or just associated with them (Jockusch et al., 1980) is still not clear. The presence of vimentin in rods has to date been described in a single report (Paulus et al., 1988). Intranuclear rods differ in composition from their sarcoplasmic counterparts in that only the presence of \( \alpha \)-actinin could be demonstrated (Paulus et al., 1988; Rifai et al., 1993).

Embryonic myosin was found to be expressed in XLMTM (Sawchak et al., 1991) and an overrepresentation of either fast or slow twitch fibres was demonstrated in cases of CCD (Dubowitz, 1985; Swash and Schwartz, 1988) and NM (Biral et al., 1985; Shimomura et al., 1989). Antibodies specific for basement membrane and reticular layer proteins have been used in very few studies concerning CM (Dunn et al., 1984; Hantai et al., 1985). In some cases of CNM only a thickening of the basement membrane of a few atrophic fibres was observed (Bertolotto et al., 1983), while in one case of CNM the increased expression of laminin and collagen type IV surrounding all muscle fibres and blood vessels was ultrastructurally shown to be the result of a reduplication of the basement membrane (Van der Ven et al., 1991).

We previously described abnormalities in the distribution of intermediate filament proteins and components of the basement membrane and the reticular layer in skeletal muscle of a case of CNM (Van der Ven et al., 1991). This report describes a systematic and extensive study in a large group of CM patients.
Table 1
Summary of clinical data of patients suffering from congenital myopathy

<table>
<thead>
<tr>
<th>Type of CM/PNo</th>
<th>Sex</th>
<th>Age</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCD1</td>
<td>F</td>
<td>8y</td>
<td>mild delay in milestones</td>
</tr>
<tr>
<td>CCD2</td>
<td>F</td>
<td>10y</td>
<td>generalized moderate muscle weakness</td>
</tr>
<tr>
<td>CCD3</td>
<td>M</td>
<td>10y</td>
<td>normal developmental milestones</td>
</tr>
<tr>
<td>CCD4</td>
<td>F</td>
<td>13y</td>
<td>progressive muscle weakness; daughter of CCD5</td>
</tr>
<tr>
<td>CCD5</td>
<td>M</td>
<td>34y</td>
<td>mild symptoms; father of CCD4</td>
</tr>
<tr>
<td>NM1</td>
<td>M</td>
<td>9m</td>
<td>severe neonatal form</td>
</tr>
<tr>
<td>NM2</td>
<td>F</td>
<td>14m</td>
<td>moderate, congenital form; marked delay in milestones</td>
</tr>
<tr>
<td>NM3</td>
<td>F</td>
<td>19m</td>
<td>severe neonatal form</td>
</tr>
<tr>
<td>NM4</td>
<td>M</td>
<td>23y</td>
<td>adult onset form; mild generalized muscle weakness</td>
</tr>
<tr>
<td>NM5</td>
<td>F</td>
<td>34y</td>
<td>adult onset form; progressive; severely affected; autosomal dominant trait</td>
</tr>
<tr>
<td>XM1</td>
<td>M</td>
<td>1d</td>
<td>severe neonatal form</td>
</tr>
<tr>
<td>XM2</td>
<td>M</td>
<td>1m</td>
<td>severe neonatal form</td>
</tr>
<tr>
<td>XM3</td>
<td>M</td>
<td>2m</td>
<td>severe neonatal form</td>
</tr>
<tr>
<td>XM4</td>
<td>M</td>
<td>4m</td>
<td>severe neonatal form</td>
</tr>
<tr>
<td>CNM1</td>
<td>F</td>
<td>8y</td>
<td>mild congenital form</td>
</tr>
<tr>
<td>CNM2</td>
<td>M</td>
<td>10y</td>
<td>childhood onset; progressive, generalized muscle weakness</td>
</tr>
</tbody>
</table>

CM: congenital myopathy; PNo: patient number; CCD: central core disease; NM: nemaline (rod) myopathy; XLMTM: X-linked myotubular myopathy; CNM: centronuclear myopathy; Age: age at time of biopsy.

with several antibodies specific for cytoskeletal, sarcomeric, basement membrane and reticular layer proteins. Serial sections were studied and double labelling experiments were performed to allow the comparison of different components within the same muscle fibre.

2. Materials and methods

Muscle biopsies

Skeletal muscle biopsies from 16 patients suffering from a congenital myopathy were examined in this study (Table 1). In all cases the diagnosis was made after clinical investigation and a standard series of histochemical investigations (Dubowitz, 1985). Electron microscopy was applied in a number of cases in order to confirm the diagnosis.

Immunohistochemistry

Immunohistochemical studies, using the indirect immunofluorescence technique, were performed as described before (Van der Ven et al., 1991), except that in most experiments non-fixed cryosections or sections treated for 5 min with 0.5% Triton X-100 in phosphate buffered saline were used. The characteristics of the

Table 2
Antibodies and antisera used in this study

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Designation</th>
<th>M/R</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desmin</td>
<td>RD301</td>
<td>M</td>
<td>ED</td>
<td>Ramackers et al. 1987</td>
</tr>
<tr>
<td></td>
<td>D3</td>
<td>M</td>
<td>F</td>
<td>Danto and Fischman 1984</td>
</tr>
<tr>
<td></td>
<td>pDes</td>
<td>R</td>
<td>ED</td>
<td>Ramackers et al. 1985</td>
</tr>
<tr>
<td>Vimentin</td>
<td>RV202</td>
<td>M</td>
<td>ED</td>
<td>Ramackers et al. 1985</td>
</tr>
<tr>
<td></td>
<td>pVim</td>
<td>R</td>
<td>ED</td>
<td>Ramackers et al. 1983</td>
</tr>
<tr>
<td>Myosin, sarcomeric (MHC)</td>
<td>MF20</td>
<td>M</td>
<td>F</td>
<td>Bader et al. 1982</td>
</tr>
<tr>
<td>Myosin, fast (MLC)</td>
<td>MF5</td>
<td>M</td>
<td>DSHB</td>
<td>Shimizu et al. 1985</td>
</tr>
<tr>
<td>Myosin, slow (MHC)</td>
<td>219-1D1</td>
<td>M</td>
<td>W</td>
<td>Wessels et al. 1990</td>
</tr>
<tr>
<td>Myosin, embryonic (MHC)</td>
<td>330-RSB4</td>
<td>M</td>
<td>W</td>
<td>Wessels et al. 1990</td>
</tr>
<tr>
<td></td>
<td>330-R5D4</td>
<td>M</td>
<td>W</td>
<td>Wessels et al. 1990</td>
</tr>
<tr>
<td>Nebulin</td>
<td>NB2</td>
<td>M</td>
<td>S</td>
<td>Fürst et al. 1988</td>
</tr>
<tr>
<td>Titin</td>
<td>9D10</td>
<td>M</td>
<td>DSHB</td>
<td>Wang and Greaser 1985</td>
</tr>
<tr>
<td>Laminin</td>
<td>2E8</td>
<td>M</td>
<td>DSHB</td>
<td>Engvall et al. 1986</td>
</tr>
<tr>
<td>Collagen type IV</td>
<td>M3F7</td>
<td>M</td>
<td>DSHB</td>
<td>Poellmer et al. 1983</td>
</tr>
<tr>
<td>Collagen type VI</td>
<td>5C6</td>
<td>M</td>
<td>DSHB</td>
<td>Hessle and Engvall 1984</td>
</tr>
</tbody>
</table>

M: mouse monoclonal antibody; R: rabbit antiserum; ED: available from EuroDiagnostica BV, Apeldoorn, The Netherlands; F: kind gifts of Dr D. Fischman, New York, NY, USA; DSHB: obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University, School of Medicine, Baltimore, MD, USA and the Department of Biology, University of Iowa, Iowa City, IA, USA, under contract N01-HD-6-2915 from the NICHD; W: kind gifts of Dr A. Wessels, Amsterdam, The Netherlands; S: purchased from Sigma Chemical Company, St. Louis, MO, USA; MHC: myosin heavy chain; MLC: myosin light chain.
antibodies used in this study are summarized in Table 2. None of the reactivity patterns was observed after omission of the primary antibodies. In double-labelling experiments, the observed reactivity patterns did not differ from those of the individual antibodies in parallel experiments.

3. Results

The results of the immunohistochemical studies on skeletal muscle sections of patients suffering from CCD, NM, XLMTM and CNM are summarized in Tables 3 and 4 and illustrated in Figs. 1–6.

Table 3

<table>
<thead>
<tr>
<th>Patient</th>
<th>Desmin</th>
<th>Vimentin</th>
<th>Slow/fast myosin</th>
<th>Embryonic myosin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCD1</td>
<td>strong staining of aggregates in cores</td>
<td>no staining of muscle fibres</td>
<td>almost exclusively (&gt;99%) slow-twitch fibres</td>
<td>only 1 positive fibre</td>
</tr>
<tr>
<td>CCD2</td>
<td>most cores negative with a fluorescent demarcation</td>
<td>strong staining of endomysium and remnants degenerated fibres</td>
<td>all fibres slow-twitch</td>
<td>only few (&lt;1%) positive fibres</td>
</tr>
<tr>
<td>CCD3</td>
<td>cores negative to strongly stained; often staining of aggregates</td>
<td>no staining of muscle fibres</td>
<td>all fibres slow-twitch</td>
<td>no positive fibres</td>
</tr>
<tr>
<td>CCD4</td>
<td>cores negative or with aggregates; fluorescent demarcation of cores</td>
<td>no staining of muscle fibres</td>
<td>predominance (&gt;99%) of slow-twitch fibres</td>
<td>no positive fibres</td>
</tr>
<tr>
<td>CCD5</td>
<td>in most cores staining of aggregates; fluorescent demarcation of cores</td>
<td>no staining of muscle fibres</td>
<td>predominance (80%) of slow-twitch fibres, cores exclusively in slow-twitch fibres</td>
<td>no positive fibres</td>
</tr>
<tr>
<td>NM1</td>
<td>patchy to diffuse overall staining of most fibres</td>
<td>diffuse staining of especially small fibres</td>
<td>small fibres predominantly fast-twitch, large fibres predominantly slow-twitch sometimes stained</td>
<td>small fibres often, large fibres</td>
</tr>
<tr>
<td>NM2</td>
<td>disturbed in some small fibres</td>
<td>no staining of muscle fibres</td>
<td>small fibres predominantly slow-twitch, large fibres predominantly fast-twitch</td>
<td>no positive fibres</td>
</tr>
<tr>
<td>NM3</td>
<td>increased staining of most fibres</td>
<td>no staining of muscle fibres</td>
<td>predominance of slow-twitch fibres (&gt;95%), few small fast-twitch fibres</td>
<td>very few positive fibres</td>
</tr>
<tr>
<td>NM4</td>
<td>organization exclusively disturbed in small fibres</td>
<td>no staining of muscle fibres</td>
<td>large fibres exclusively fast-twitch, small fibres exclusively slow-twitch</td>
<td>no positive fibres</td>
</tr>
<tr>
<td>NM5</td>
<td>organization disturbed in most fibres; presence of core-like structures</td>
<td>no staining of muscle fibres</td>
<td>ATPase: predominance (&gt;99%) of slow-twitch fibres</td>
<td>nd</td>
</tr>
<tr>
<td>XLMTM1</td>
<td>strong central staining in &gt;90% of the fibres; cross striations</td>
<td>strong central staining in most small fibres; cross striations</td>
<td>normal differentiation in fast-twitch and slow-twitch fibres</td>
<td>negative to strongly positive fibres throughout biopsy</td>
</tr>
<tr>
<td>XLMTM2</td>
<td>&gt;90% of the fibres; cross striations</td>
<td>strong central staining in small fibres</td>
<td>normal differentiation in fast-twitch and slow-twitch fibres; fast-twitch fibres smaller</td>
<td>negative to strongly positive fibres throughout biopsy</td>
</tr>
<tr>
<td>XLMTM3</td>
<td>strong central staining in most small and some large fibres</td>
<td>central staining in &lt;10% of the fibres</td>
<td>normal differentiation in fast-twitch and slow-twitch fibres</td>
<td>negative to strongly positive fibres throughout biopsy</td>
</tr>
<tr>
<td>XLMTM4</td>
<td>strong central staining in &gt;90% of the fibres; cross-striations</td>
<td>central staining in very few small fibres</td>
<td>normal differentiation in fast-twitch and slow-twitch fibres</td>
<td>few positive fibres</td>
</tr>
<tr>
<td>CNM1</td>
<td>organization disturbed in most small fibres</td>
<td>no staining of muscle fibres</td>
<td>small fibres &gt; 90% slow-twitch; predominance of slow-twitch fibres</td>
<td>few positive fibres</td>
</tr>
<tr>
<td>CNM2</td>
<td>organization disturbed in numerous fibres</td>
<td>punctate staining in several fibres</td>
<td>normal differentiation in fast-twitch and slow-twitch fibres</td>
<td>no positive fibres</td>
</tr>
</tbody>
</table>

nd: not done.
Central core disease (CCD; Fig. 1)

In all five CCD patients studied, desmin staining was normal apart from the central cores, which were often negative, and separated from the rest of the fibre by a fluorescent demarcation (Fig. 1A). A strong fluorescence of the cores (Fig. 1A) or a staining of desmin aggregates within the cores (Fig. 1B) was also observed. These different desmin patterns occurred next to each other within biopsies of individual patients. Vimentin reactivity was never found in CCD muscle fibres. In one patient (CCD2) the increased connective tissue compartment was strongly stained by anti-vimentin (Fig. 1C). All biopsies showed a conspicuous predominance of slow fibres. Embryonic myosin was never detected in more than a few small, regenerating fibres. Basement membrane protein expression was slightly or strongly

Fig. 1. Central core disease. Immunofluorescence micrographs of muscle tissue of patients CCD1 (D,K), CCD2 (A,C,E,G,H), CCD3 (B,F,I) and CCD4 (K) incubated with polyclonal anti-desmin (A,B), polyclonal anti-vimentin (C), anti-sarcomeric myosin heavy chain (D), anti-laminin (E,F), anti-collagen type IV (G), anti-collagen type VI (H,I), or anti-titin (J,K). Note the presence of desmin negative cores (arrow in A) and strongly stained cores (arrowhead in A) in one and the same tissue section (A). J and K depict the absence of titin in some cores (K, arrow) while other cores show a disturbance of the cross-striated pattern (arrow in J). Bar = 50 μm (A,B,D,J,K) or 100 μm (C,E,I).
increased in CCD4 and CCD2 (Fig. 1E) respectively, while normal reactivity was seen in the other cases (Fig. 1F). In one patient expression of the basement membrane protein collagen type IV was observed in the perimysial tissue (Fig. 1G). Endomysial and/or perimysial staining of collagen type VI was increased in four patients (Fig. 1H) and normal in one (Fig. 1I). A disturbance or absence of sarcomeric proteins like myosin (Fig. 1D) and titin (Figs. 1J, K) was observed in the cores of some patients but not in the apparently non-affected parts of the muscle fibres.

**Nemaline myopathy (NM; Fig. 2)**

Muscle fibre areas in biopsies of five NM patients containing nemaline rods were identified by the modified Gomori trichrome stain (Figs. 2A, B). In biopsies of the severe cases (NM1, NM3, NM5) almost all fibres contained rods (Fig. 2A) while in the other biopsies...
predominantly small, slow fibres were affected (Fig. 2B). An increased, diffuse desmin reactivity was observed in most fibres of patients with the severe neonatal form (Fig. 2F). In the moderately affected patients NM2 and NM4 only small fibres showed a disturbance in desmin distribution (Fig. 2E). This disturbance was not restricted to subsarcolemmal parts of the muscle fibres where rods usually are localized. The biopsy material of patient NM5 showed besides rods also core-like structures. Despite the abnormal desmin distribution (Fig. 2C), most fibres still showed a cross-striated staining pattern with anti-desmin (Fig. 2D). A diffuse vimentin staining reaction and the presence of embryonic myosin in several muscle fibres was only seen in the youngest NM patient (Figs. 2G, H). In severe neonatal cases small fibres were often of the fast-twitch type, while in the other cases the small, rod-containing fibres were predominantly of the slow-twitch type. Expression of basement membrane proteins was increased around fibres and blood vessels in severe neonatal cases (Fig. 2I). This phenomenon was accompanied by a punctate collagen type IV deposition in the perimysium (Fig. 2J). Anti-collagen type VI staining showed a slight to strong increase in endomysial and perimysial tissues in severe cases (Fig. 2J), but a normal connective tissue compartment in other cases (Fig. 2K).

**X-linked myotubular myopathy (XLMTM; Figs. 3–5)**

Biopsy specimens of our four XLMTM patients were characterized by a strong desmin staining of the central area of a large majority of the muscle fibres in all patients (Figs. 3A–C, 5A). Despite this abnormal desmin distribution, longitudinally sectioned muscle fibres showed a relatively normal cross-striated pattern when stained with anti-desmin (Fig. 3I) and anti-titin (Fig. 3J). The vimentin staining pattern (Figs. 4A–D, 5B) was comparable (though often weaker) to that of desmin (Figs. 3A–C, 5A) and included cross-stiations (Fig. 4B). However, many large fibres did not contain vimentin (Figs. 4A, 5B), while the number of vimentin containing fibres showed considerable variation among patients and seemed to decrease with the age (Figs. 4A–D). Upon staining with the anti-embryonic myosin antibodies a similar, age-dependent decrease in expression was observed (Figs. 4E–G). In serial sections of the muscle biopsy of patient XLMTM1 all fibres that expressed embryonic myosin were also stained by the anti-desmin and anti-vimentin antibodies (Figs. 5A–C). Some embryonic myosin negative fibres expressed desmin and vimentin, while most large fibres negative for embryonic myosin, expressed desmin but not vimentin (Figs. 5A–C). The large fibres in this biopsy that were negative for embryonic myosin were, how-

### Table 4

<table>
<thead>
<tr>
<th>Patient</th>
<th>Laminin/collagen type IV</th>
<th>Collagen type VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCD1</td>
<td>normal</td>
<td>endomysial staining slightly increased</td>
</tr>
<tr>
<td>CCD2</td>
<td>increased expression around muscle fibres and blood vessels; expression of collagen type IV in perimysium</td>
<td>increased staining of endomysial and perimysial connective tissue</td>
</tr>
<tr>
<td>CCD3</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>CCD4</td>
<td>slightly increased reactivity</td>
<td>slightly increased reactivity</td>
</tr>
<tr>
<td>CCD5</td>
<td>normal</td>
<td>normal to slightly increased reactivity</td>
</tr>
<tr>
<td>NM1</td>
<td>increased expression around muscle fibres and blood vessels; expression of collagen type IV in perimysium</td>
<td>all fibres embedded in increased amounts of strongly stained connective tissue</td>
</tr>
<tr>
<td>NM2</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>NM3</td>
<td>increased expression around muscle fibres and blood vessels; expression of collagen type IV in perimysium</td>
<td>slightly increased reactivity</td>
</tr>
<tr>
<td>NM4</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>NM5</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>XLMTM1</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>XLMTM2</td>
<td>slightly increased staining around muscle fibres and blood vessels</td>
<td>slightly increased staining of endomysium; perimysium normal</td>
</tr>
<tr>
<td>XLMTM3</td>
<td>slightly increased expression; expression of collagen type IV in perimysium</td>
<td>slightly increased staining of endomysial and perimysial tissue</td>
</tr>
<tr>
<td>XLMTM4</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>CNM1</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>CNM2</td>
<td>increased expression around all muscle fibres and blood vessels</td>
<td>increased staining of endomysium; perimysium normal</td>
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nd: not done.
ever, found to express the slow myosin heavy chain (Figs. 5D,E). Several other, smaller fibres co-expressed embryonic myosin and an adult form of myosin (Figs. 5D,E).

The two XLMTM patients that were studied with antibodies to laminin, collagen type IV and collagen type VI, showed an increased basement membrane labelling with anti-laminin and anti-collagen type IV around muscle fibres and blood vessels (Figs. 3D–F). Expression of collagen type IV in perimysial tissue was observed in one of these patients (Fig. 3F). Collagen type VI reactivity was increased in the endomysial tissue of both patients (Fig. 3G). Only one of the patients showed increased collagen type VI expression in the perimysial connective tissue. Like in all other cases of normal and pathological skeletal muscle, anti-nebulin diffusely stained the fibres in XLMTM. In most XLMTM fibres the reactivity in their central region was absent or less intense when compared to other parts of the fibres (Fig. 3H). Ultrastructurally, we

Fig. 3. X-linked myotubular myopathy. Immunofluorescence micrographs of muscle tissue of patients XLMTM1 (A), XLMTM 2 (B,D,E,G,I,J), XLMTM3 (F) and XLMTM4 (C,H) incubated with the monoclonal anti-desmin RD 301 (A,C) or polyclonal anti-desmin (B,I), anti-laminin (D), anti-collagen type IV (E,F), anti-collagen type VI (G), anti-nebulin (H) or anti-titin (J). Bar = 50 μm (A–E, G–J) or 100 μm (F).
observed accumulations of intermediate filaments in the perinuclear areas that showed increased intermyofibrillar space (not shown).

Centronuclear myopathy (CNM; Fig. 6)

Most of the results concerning patient CNM2 were described and illustrated before (Van der Ven et al., 1991). Upon NADH-TR staining the fibres in the CNM biopsies often showed a strong central reactivity and a peripheral halo (Fig. 6A) or a radial spoke-like appearance. Increased anti-desmin staining was observed in muscle biopsies of both patients studied. In patient CNM1 specifically small, slow twitch fibres (Fig. 6E) showed a disturbed desmin distribution pattern (Fig. 6B), while in patient CNM2 both fibre types were affected. In longitudinal sections a normal cross-striated desmin reactivity was observed surrounding the strongly stained centre of the fibres (Fig. 6C). As described before, patient CNM2 showed cytoplasmic aggregates of vimentin. No vimentin reactivity was observed in the muscle fibres of the other patient (Fig. 6D). Embryonic myosin was only detected in a few scattered fibres in one of the patients. Expression of basement membrane and reticular layer proteins was normal in patient CNM1 (Figs. 6F,G). Patient CNM2, however, showed an overtly increased expression of laminin and collagen type IV around muscle fibres and blood vessels and an increased endomysial collagen type VI expression (Fig. 6H). Expression and distribution of titin was only studied in patient CNM1. Compared to small fibres, large fibres seemed to be stained a little stronger and more regular (Fig. 6I).

4. Discussion

The normal function of skeletal muscle depends primarily on a correct assembly of myofibrils, in particular the organization of the sarcomeric units. Next to the motor proteins actin and myosin, several other muscle cell constituents play an important role in this process. The high molecular weight proteins titin and nebulin assumedly help organize the myosin and actin filaments into a regular geometric pattern (Fulton and
Isaacs, 1991; Trinick, 1992). The muscle specific inter­
mediate filament protein desmin (Fischman, 1986) and
probably also skelemin (Price, 1987) link adjacent my­
ofibrils to each other at the level of the Z-line and the
M-line, respectively, in order to keep myofibrils in
register during contraction. The same filament proteins
anchor myofibrils to a subsarcolemmal network con­
taining dystrophin that co-localizes with α-actinin, vin­
culin and other proteins known to occur in adhesion
plaques (Ahn and Kunkel, 1993). Dystrophin associates
with a laminin-binding transmembrane complex of
glycoproteins, while integrins are thought to provide a
further link to the interstitial connective tissue, the
reticular layer (Ahn and Kunkel, 1993; Ervasti and
Campbell, 1993), that contains the microfibril-forming
collagen type VI (Hessle and Engvall, 1984). The force
generated by myofibril contraction is not only transmit­
ted to the tendon via the myotendinous junction, but
also through the endomysial and perimysial tissue. It is
evident that any disturbance of the described protein­
protein interactions might cause muscle weakness. It is
therefore that we have undertaken this study on the
expression and localization of sarcomeric, cytoskeletal
and extracellular matrix components of diseased mus­
cle.

Desmin disorganization as a marker for congenital myo­
opathy

One of the most striking observations from our
study is the wide-spread disturbance of desmin local­
ization in CM. Altered expression patterns of the inter­
mediate filament proteins (IFPs) desmin and vimentin
have been described to be associated with regeneration
by several authors (Thornell et al., 1980, 1983; Helliwell
et al., 1989; Gallanti et al., 1992; Bornemann and
Schmalbruch, 1992). The presence of regenerating fi­
bres in a muscle biopsy is associated with several
myopathies (e.g. Duchenne muscular dystrophy, myosi­
tis, CM). Therefore, the increased IFP-expression ob­
served in these fibres, can not be considered specific
for CM. In all studied cases of CCD, NM, XLMTM
and CNM an abnormal desmin distribution was ob­
served, which could not be correlated to the presence
of regenerating fibres. The different desmin staining
patterns in CCD were described before (Thornell et
al., 1983; Gallanti et al., 1992) and are probably related

Fig. 5. X-linked myotubular myopathy. Immunofluorescence micrographs of serial sections (A–C, D–E) of muscle tissue of patient XLMTM1
stained with monoclonal anti-desmin (RD301, A), monoclonal anti-vimentin (B), anti-embryonic myosin (330-R5D4, C,D) or anti-slow myosin
heavy chain (E). Corresponding fibres are indicated by arrows. Bar = 50 μm.
to the presence of structured and unstructured cores (Bodensteiner, 1994). In NM, abnormal desmin staining patterns were exclusively found in all rod-containing fibres. Our results provided however, further evidence against desmin as a component of nemaline rods. Our results in XLMTM differ from those of Sarnat (1990), in that desmin staining in the centre of the fibres was much stronger when compared to the periphery, while Sarnat reported an overall staining of muscle fibres. The desmin staining patterns obtained in this study correspond, however, well to the ultrastructurally observed accumulations of intermediate filaments. The differences in staining patterns might result from the sensitivity of the immunofluorescence technique, or the specificity of the desmin antibodies. In our hands, anti-desmin monoclonal antibody D3 stained XLMTM myofibres less abundantly than RD301 and pDes. Differential staining patterns of serial skeletal

Fig. 6. Centronuclear myopathy. NADH-TR (A) or immunofluorescent (B–I) staining of muscle tissue of patients CNM1 (A–G, I) or CNM2 (H). Sections are incubated with polyclonal anti-desmin (B,C), polyclonal anti-vimentin (D), anti-fast myosin light chain (E), anti-laminin (F), anti-collagen type VI (G,H) or anti-titin (I). Corresponding fibres in A and B are indicated by arrows. Note the disturbed desmin pattern (arrow in C) in the centre of a longitudinally sectioned fibre. Bar = 50 μm (A,B,C,G,H) or 100 μm (D,E,F,I).
muscle sections with different antibodies to desmin were also noted by Hellilwell et al. (1989). Increased desmin staining in the centre of the fibre was observed in a number of CNM cases (Van der Ven et al., 1991, Misra et al., 1992). This reactivity pattern is not found in muscle biopsies of patients with e.g. myotonic dystrophy that are also characterized by numerous internal nuclei (Van der Ven et al., unpublished results; Sarnat, 1992) and can thus be considered as specific for CNM.

The abnormal desmin localization in CNM might be a result of developmental regression (Misra et al., 1992). The observation that nuclei centralize postnatally in some patients (Van der Ven et al., 1991) supports this idea. Desmin is found in a cross-striated pattern only in a progressed stage of myofibrillogenesis (Van der Ven et al., 1992, Van der Ven et al., 1993). It is therefore conceivable that desmin is one of the first proteins to be redistributed during dedifferentiation of muscle cells.

**Age-related changes in expression of differentiation markers in XLMTM**

In XLMTM patients skeletal muscle fibres show features of immaturity and it was therefore suggested that the disease is associated with a partial arrest of morphogenesis (Sarnat, 1990). On the one hand, the presence of vimentin and embryonic myosin, and the distribution pattern of desmin as described in the underlying study, point to a deranged process of maturation. On the other hand, our results confirm the observation that XLMTM myofibres are mature in other aspects of development, e.g. differentiation of fibre types (Sarnat, 1990; Sawchak et al., 1991; Soussi-Yanicos et al., 1991). The normal alignment of adjacent myofibrils (Sarnat, 1990), results in a cross-striated pattern upon staining with anti-desmin and anti-titin antibodies and provides further proof for a normal progression of certain maturation processes in this disease. Due to the lack of serial biopsies of XLMTM patients, progression of the disease and possible postnatal fibre-maturation have scarcely ever been studied. In two brothers, one born at 28 weeks of gestation and the other at term, markedly increased pathologic findings were found in the latter (Braga et al., 1990), suggesting prenatal progression. In one case of XLMTM described by Sarnat et al. (1981), no postnatal morphologic maturation was observed between the age of 5 days and 9 months. Examination of muscle biopsies from two other brothers suffering from XLMTM, revealed that the fibre diameter increases with developmental age (Sawchak et al., 1991), implicating that XLMTM muscle fibres mature postnatally. Our studies concerning 4 XLMTM patients that vary in age from 1 week to 4 months, and whose biopsies show an age-dependent down-regulation of vimentin and embryonic myosin, support the suggestion that XLMTM muscle matures postnatally. However, it cannot be excluded that the time point of maturational arrest varies in individual patients.

**Relation of clinical data with immunohistologic results:**

**A) central core disease**

Variations in clinical expression have been described for several muscle diseases. Some of these variations are the result of mutations in different genes. For example, mutations in the ryanodine receptor gene (Zhang et al., 1993) and the β-myosin heavy chain gene (Fananapazir et al., 1993), both resulting in a CCD phenotype may explain the considerable variation of clinical features in CCD patients. The clinical differences in CCD patients within one family illustrate that the same mutation can also result in different clinical phenotypes. Included in our study are a father and his daughter both with CCD. Although in both cases the same genetic mutation most probably underlies the CCD phenotype, clinical expressions varied considerably. Immunohistochemical experiments revealed that in the muscle biopsy of the severely affected daughter expression of basement membrane and reticular layer proteins were increased. Moreover, the daughter's muscle biopsy contained only slow-twitch fibres that were all affected, while the muscle biopsy of the father only showed a predominance of affected slow-twitch fibres. The presence of 20% non-affected fast-twitch fibres might explain the relatively mild symptoms in the father. In the biopsies of other investigated CCD patients, that were all clinically affected in early childhood, very few, if any, fast-twitch fibres were present. Moreover, the muscle biopsy of the most severely affected patient (CCD2), showed apart from necrotic fibres and replacement of muscle fibres by adipose tissue, also increased expression of basement membrane and reticular layer proteins.

**B) Nemaline myopathy**

When immuncytochemical data are compared with the phenotype in NM, three subgroups should be discerned: (i) the neonatal; (ii) the moderate congenital; and (iii) the adult onset form (Shimomura and Nonaka, 1989; Bodensteiner, 1994). Obvious signs of immaturity were observed in the biopsies of patients with the severe neonatal form, like the presence of several embryonic myosin and vimentin-positive fibres and the diffuse desmin distribution in all fibres. The desmin and vimentin staining patterns clearly differed from those in XLMTM. Furthermore, the severe neonatal NM cases showed an increase of laminin, collagen type IV and especially collagen type VI expression, associated with an unusual presence of collagen type IV in the perimysium. This was particularly obvious in case
changes in the expression and organization of structural proteins can however vary considerably between different types of CM, but also within one type of CM. Correlations between these changes at the molecular level on the one hand, and the clinical phenotype on the other hand were observed. In case of fibre type-specific defects, the percentage of non-affected fibres could be correlated with clinical presentation. These fibres may compensate for the weakness caused by the affected fibres. Severe clinical phenotypes are accompanied by an increased deposition of basement membrane and reticular layer proteins, implicating that these compartments may play a role in the development of muscular weakness. We can, however, not exclude that this increase represents the result of the course of the disease. The age-related differences in expression of embryonic myosin and vimentin in biopsies of XLMTM patients are either the result of postnatal maturation, or a variable time-point of maturational arrest in individual patients. The disturbed desmin staining patterns seen in our patients are unique for each type of CM and appear useful for diagnostic purposes.

References


5. Conclusions

The molecular phenotype of pathologic skeletal muscle fibres in CM can be severely disturbed when compared to normal myofibres. Intra- and extracellular


