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

Peter F.M. van der Ven [a,*], Paul H.K. Jap [a], Henk J. ter Laak [b], Ikuya Nonaka [c], Peter G. Barth [d,e], Rob C.A. Sengers [f], Ad M. Stadhouders [a], Frans C.S. Ramaekers [g]

[a] *Department of Cell Biology and Histology, University of Nijmegen, Trigon Building, PO Box 9101, 6500 HB Nijmegen, The Netherlands*
[b] *Department of Neurology, University Hospital, Nijmegen, The Netherlands*
[c] *National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan*
[d] *Department of Neurology, University of Amsterdam, Academic Medical Center, Amsterdam, The Netherlands*
[e] *Department of Pediatrics, University of Amsterdam, Academic Medical Center, Amsterdam, The Netherlands*
[f] *Department of Pediatrics, University Hospital, Nijmegen, The Netherlands*
[g] *Department of Molecular Cell Biology and Genetics, University of Limburg, Maastricht, The Netherlands*

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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

* Corresponding author. Tel.: (+31-80) 614673/614329; Fax: (+31-80) 540525.

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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 of NM and the adult onset form as separate disease entities. Evidence was presented that NM is inherited as an autosomal dominant trait (Arts et al., 1978; Kondo and Yuasa, 1980), although in some families autosomal recessive inheritance was suggested (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; Lehesjoki 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 α-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 α-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; Hantaï 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

Type of CM/PNo	Sex	Age	Remarks
CCD1	F	8y	mild delay in milestones
CCD2	F	10y	generalized moderate muscle weakness
CCD3	M	10y	normal developmental milestones
CCD4	F	13y	progressive muscle weakness; daughter of CCD5
CCD5	M	34y	mild symptoms; father of CCD4
NM1	M	9m	severe neonatal form
NM2	F	14m	moderate, congenital form; marked delay in milestones
NM3	F	19m	severe neonatal form
NM4	M	23y	adult onset form; mild generalized muscle weakness
NM5	F	34y	adult onset form; progressive; severely affected; autosomal dominant trait
XLMTM1	M	1d	severe neonatal form
XLMTM2	M	1m	severe neonatal form
XLMTM3	M	2m	severe neonatal form
XLMTM4	M	4m	severe neonatal form
CNM1	F	8y	mild congenital form
CNM2	M	10y	childhood onset; progressive, generalized muscle weakness

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

Antigen	Designation	M/R	Source	Reference
Desmin	RD301	M	ED	Ramaekers et al. 1987
	D3	M	F	Danto and Fischman 1984
	pDes	R	ED	Ramaekers et al. 1985
Vimentin	RV202	M	ED	Ramaekers et al. 1985
	pVim	R	ED	Ramaekers et al. 1983
Myosin, sarcomeric (MHC)	MF20	M	F	Bader et al. 1982
Myosin, fast (MLC)	MF5	M	DSHB	Shimizu et al. 1985
Myosin, slow (MHC)	219-1D1	M	W	
Myosin, embryonic (MHC)	330-R5B4	M	W	Wessels et al. 1990
	330-R5D4	M	W	Wessels et al. 1990
Nebulin	NB2	M	S	Fürst et al. 1988
Titin	9D10	M	DSHB	Wang and Greaser 1985
Laminin	2E8	M	DSHB	Engvall et al. 1986
Collagen type IV	M3F7	M	DSHB	Foellmer et al. 1983
Collagen type VI	5C6	M	DSHB	Hessle and Engvall 1984

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
Distribution patterns of the intermediate filament proteins desmin and vimentin, and of the different myosin isoforms in the cases of congenital myopathy

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

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

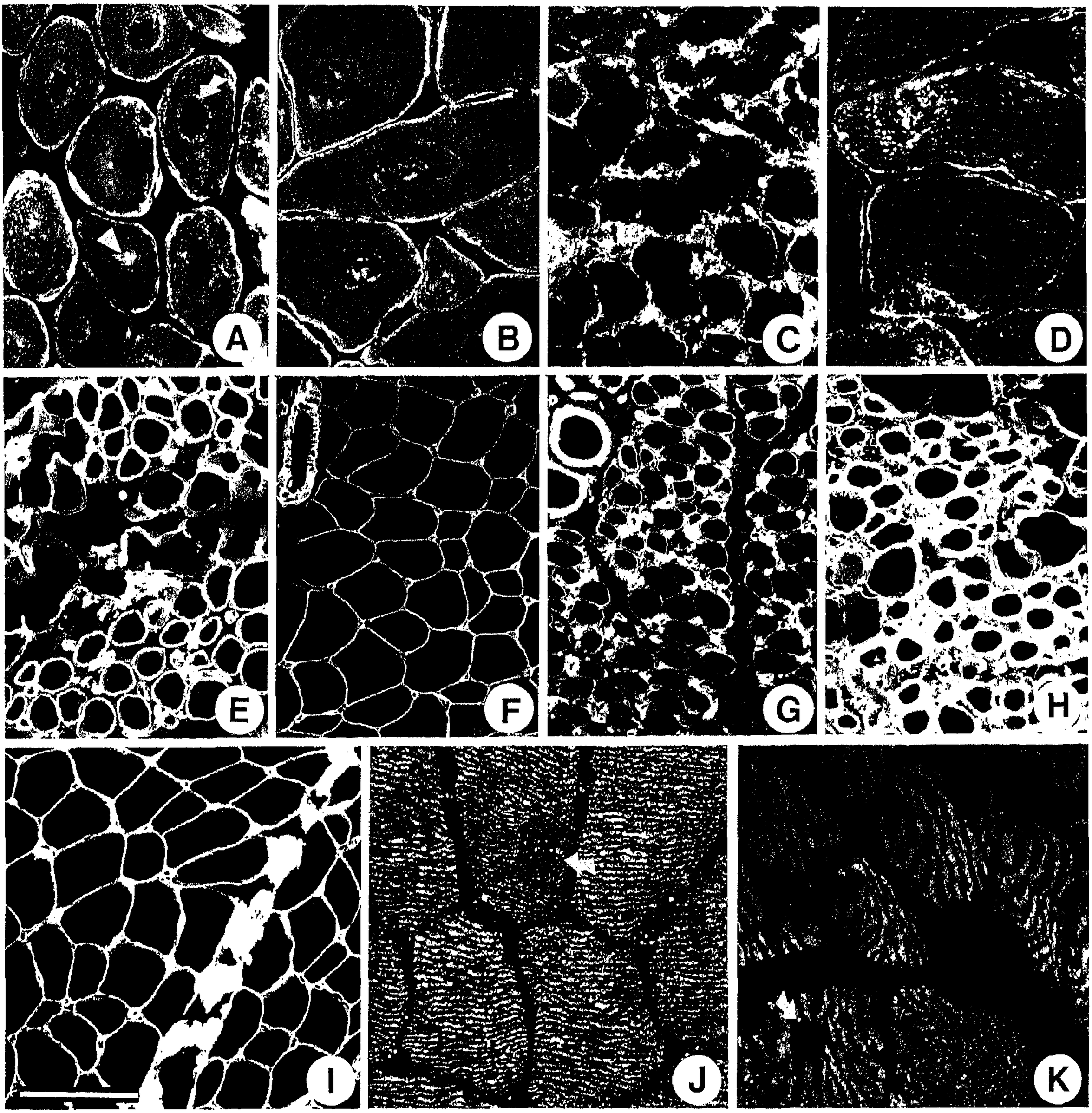

Fig. 1. Central core disease. Immunofluorescence micrographs of muscle tissue of patients CCD1 (D,K), CCD2 (A,C,E,G,H), CCD3 (B,F,I) and CCD4 (K) incubated with polyclonal anti-desmin (A,B), polyclonal anti-vimentin (C), anti-sarcomeric myosin heavy chain (D), anti-laminin (E,F), anti-collagen type IV (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).

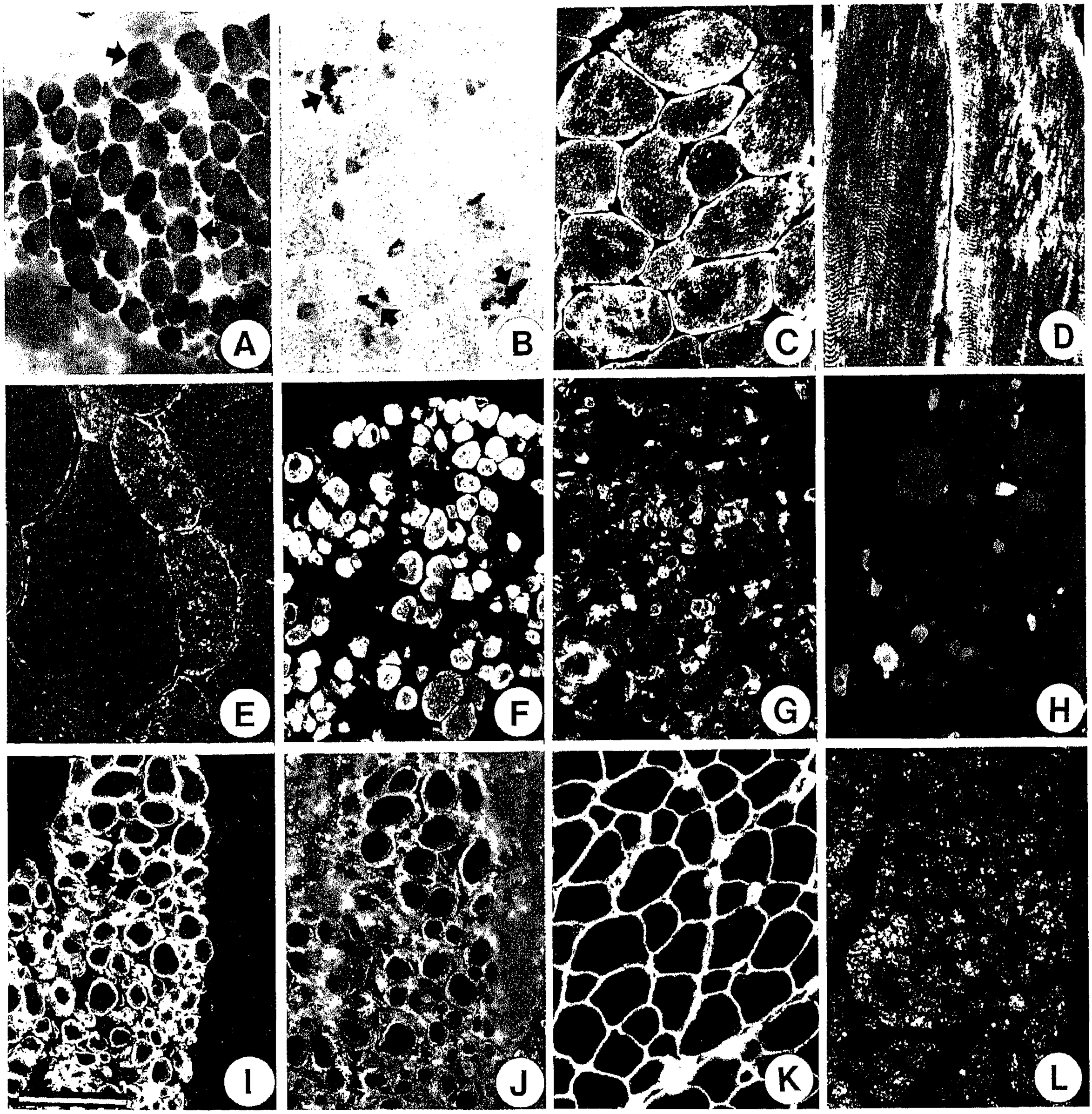

Fig. 2. Nemaline myopathy. Gomori trichrome (A,B) or immunofluorescent (C-L) staining of muscle tissue of patients NM1 (F–J), NM2 (B,K), NM3 (A,L), NM4 (E) or NM5 (C,D). Sections are incubated with monoclonal anti-desmin RD301 (C,D), polyclonal anti-desmin (E,F), polyclonal anti-vimentin (G), anti-embryonic myosin (330-R5B4, H), anti-collagen type IV (I), anti-collagen type VI (J,K) and anti-titin (L). Some Gomori trichrome stained areas in A,B are indicated by arrows. Bar = 50 μm (A,B,D–L) or 100 μm (C).

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. 2I). 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). Reaction patterns with antibodies to sarcomeric proteins such as titin (Fig. 2L) varied considerably according to the accuracy of cross-sectioning of the fibres. The patterns were not considered to be abnormal.

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-striations (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
Distribution patterns of the basement membrane components laminin and collagen IV and the reticular layer constituent collagen VI

Patient	Laminin/collagen type IV	Collagen type VI
CCD1	normal	endomysial staining slightly increased
CCD2	increased expression around muscle fibres and blood vessels; expression of collagen type IV in perimysium	increased staining of endomysial and perimysial connective tissue
CCD3	normal	normal
CCD4	slightly increased reactivity	slightly increased reactivity
CCD5	normal	normal to slightly increased reactivity
NM1	increased expression around muscle fibres and blood vessels; expression of collagen type IV in perimysium	all fibres embedded in increased amounts of strongly stained connective tissue
NM2	normal	normal
NM3	increased expression around muscle fibres and blood vessels; expression of collagen type IV in perimysium	slightly increased reactivity
NM4	nd	nd
NM5	nd	nd
XLMTM1	nd	nd
XLMTM2	slightly increased staining around muscle fibres and blood vessels	slightly increased staining of endomysium; perimysium normal
XLMTM3	slightly increased expression; expression of collagen type IV in perimysium	slightly increased staining of endomysial and perimysial tissue
XLMTM4	nd	nd
CNM1	normal	normal
CNM2	increased expression around all muscle fibres and blood vessels	increased staining of endomysium; perimysium normal

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

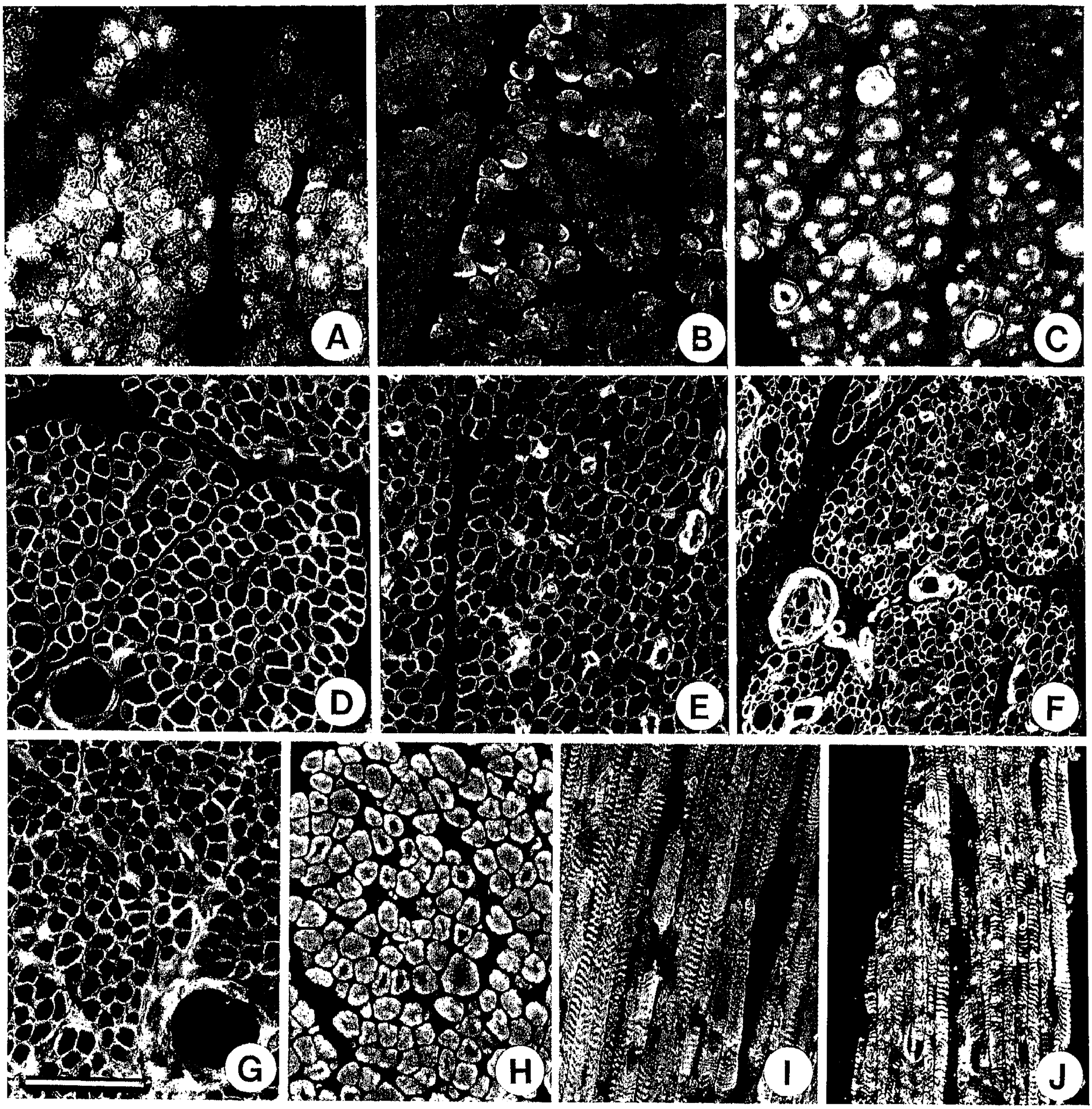

Fig. 3. X-linked myotubular myopathy. Immunofluorescence micrographs of muscle tissue of patients XLMTM1 (A), XLMTM 2 (B,D,E,G,I,J), XLMTM3 (F) and XLMTM4 (C,H) incubated with the monoclonal anti-desmin RD 301 (A,C) or polyclonal anti-desmin (B,I), anti-laminin (D), anti-collagen type IV (E,F), anti-collagen type VI (G), anti-nebulin (H) or anti-titin (J). Bar = 50 μm (A–E, G–J) or 100 μm (F).

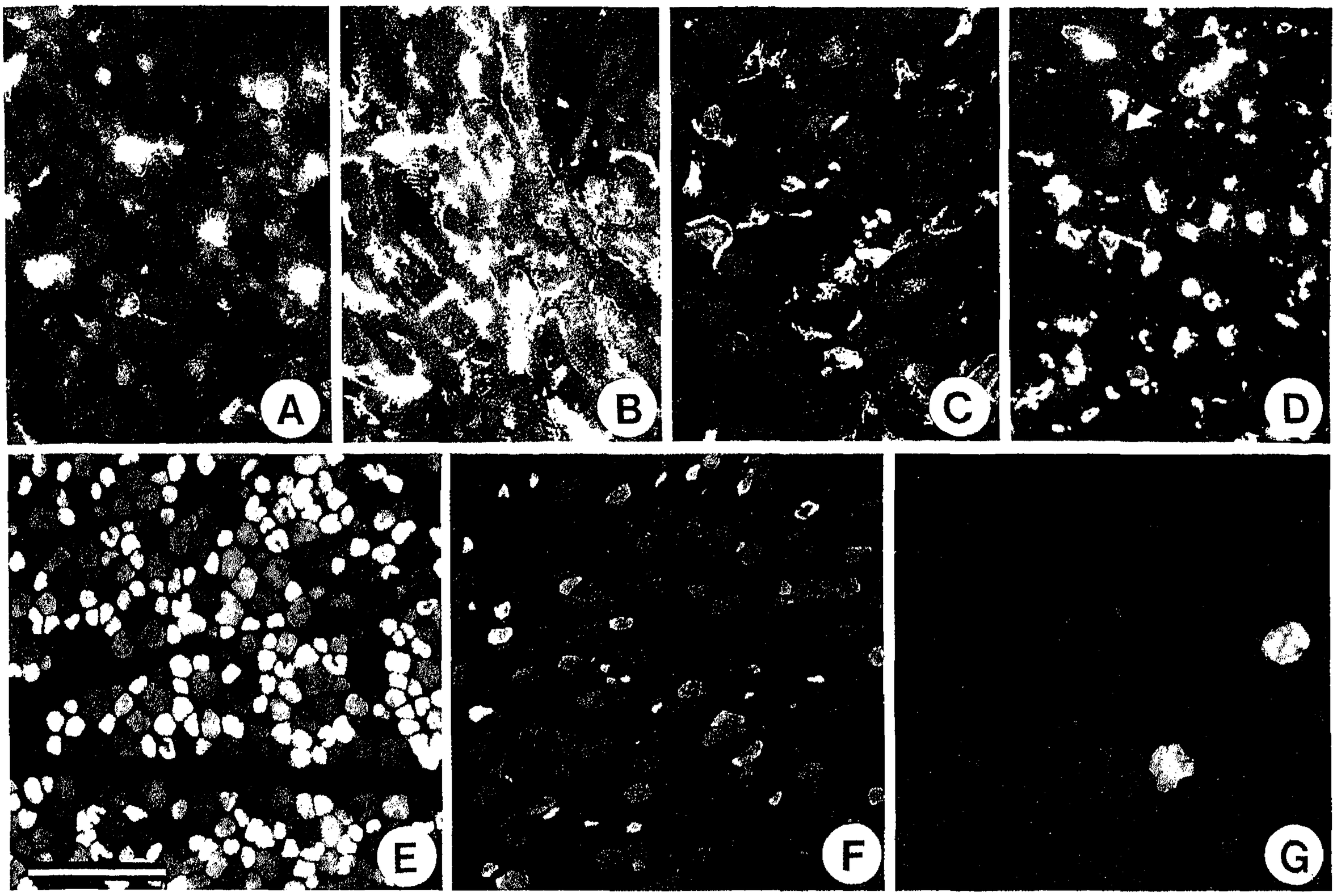

Fig. 4. X-linked myotubular myopathy. Immunofluorescence micrographs of muscle tissue of patients XLMTM1 (A,B,E), XLMTM 2 (C), XLMTM3 (F) and XLMTM4 (D,G) incubated with the monoclonal vimentin antibody RV202 (A,B,D) or polyclonal vimentin antiserum (C), or the monoclonal antibodies to embryonic myosin 330-R5D4 (E) or 330-R5B4 (F,G). Note the age-dependent decrease in the number of vimentin positive fibres from A through D, and the decrease in the number of embryonic myosin positive fibres from E through G. The section shown in D contains a single vimentin-positive fibre (arrow). Bar = 50 μm.

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 intermediate filament protein desmin (Fischman, 1986) and probably also skelemin (Price, 1987) link adjacent myofibrils 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 containing dystrophin that co-localizes with α-actinin, vinculin 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 transmitted 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 muscle.

Desmin disorganization as a marker for congenital myopathy

One of the most striking observations from our study is the wide-spread disturbance of desmin localization in CM. Altered expression patterns of the intermediate 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 fibres in a muscle biopsy is associated with several myopathies (e.g. Duchenne muscular dystrophy, myositis, CM). Therefore, the increased IFP-expression observed 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 observed, 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

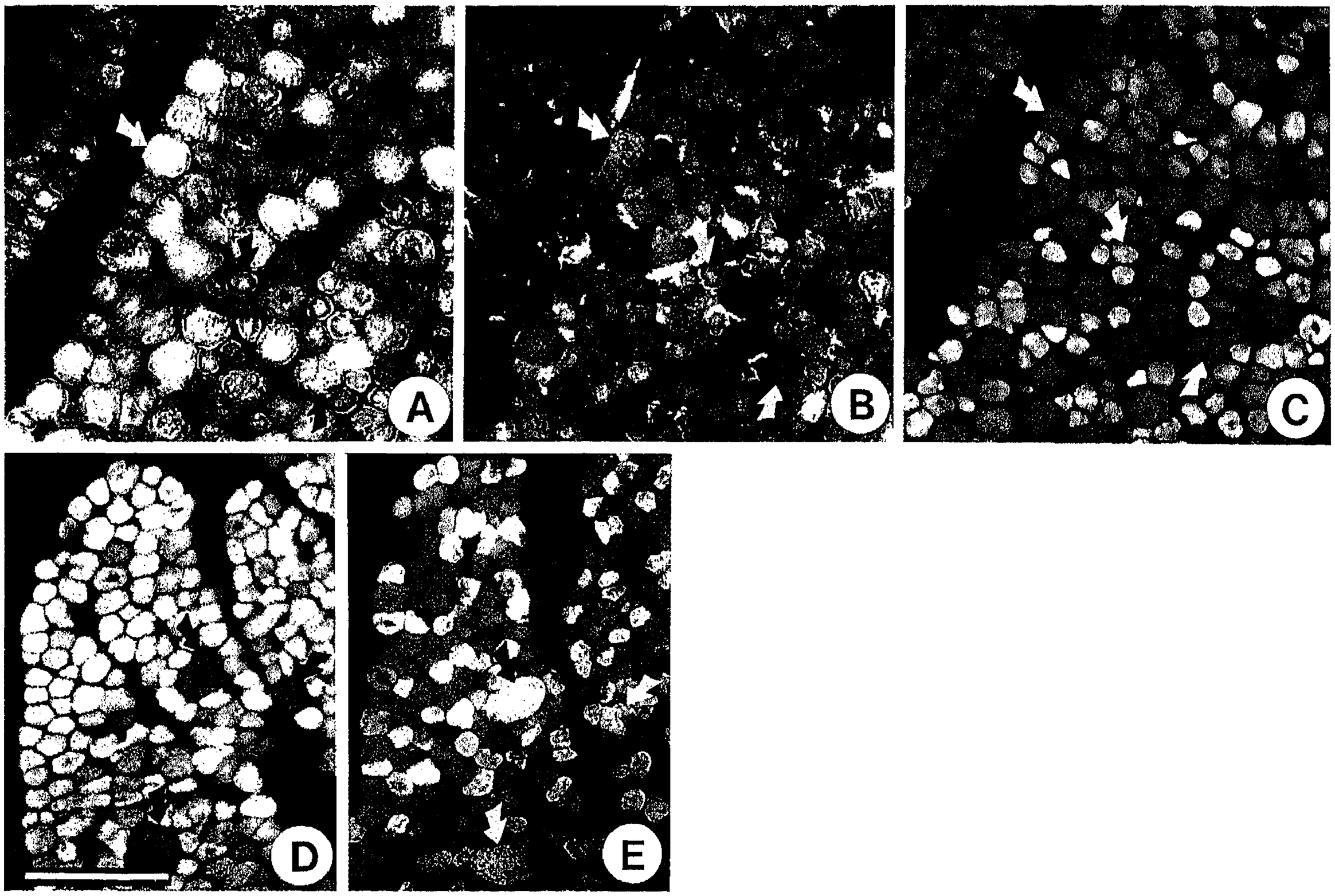

Fig. 5. X-linked myotubular myopathy. Immunofluorescence micrographs of serial sections (A–C, D–E) of muscle tissue of patient XLMTM1 stained with monoclonal anti-desmin (RD301, A), monoclonal anti-vimentin (B), anti-embryonic myosin (330-R5D4, C,D) or anti-slow myosin heavy chain (E). Corresponding 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

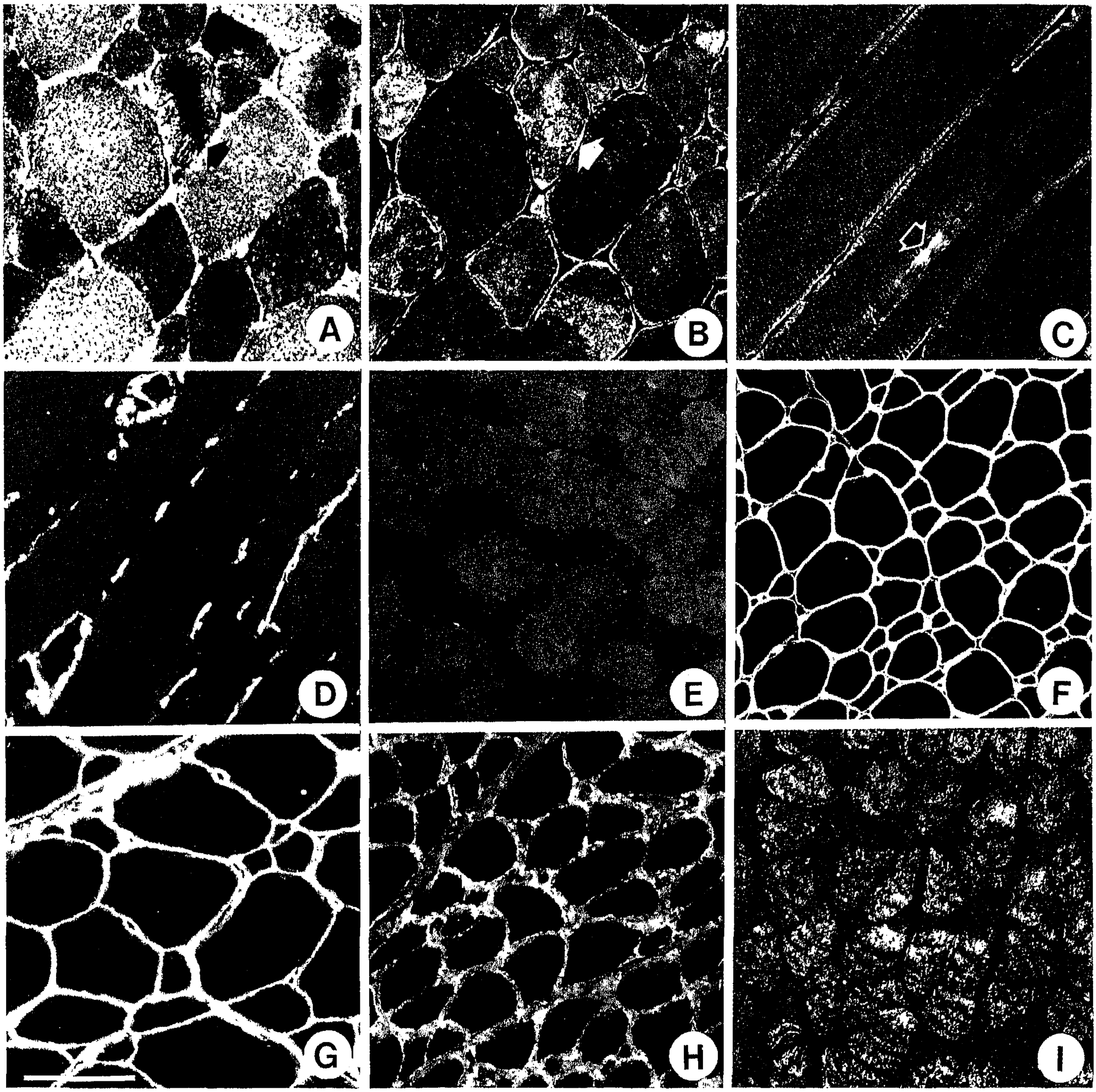

Fig. 6. Centronuclear myopathy. NADH-TR (A) or immunofluorescent (B–I) staining of muscle tissue of patients CNM1 (A–G, I) or CNM2 (H). Sections are incubated with polyclonal anti-desmin (B,C), polyclonal anti-vimentin (D), anti-fast myosin light chain (E), anti-laminin (F), anti-collagen type VI (G,H) 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 Helliwell 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-Yanicostas 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.

Relation of clinical data with immunohistologic results: B. Nemaline myopathy

When immunocytochemical 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

NM3. These patterns were not observed in an age-matched, much less affected patient with the moderate congenital form of NM. Muscle weakness in NM patients was reported not to be associated to the number of rods, but to the degree of predominance and/or atrophy of slow-twitch fibres (Bodensteiner, 1994). In our cases the severely affected adult-onset NM patient shows a high predominance of slow-twitch fibres, while in the adult patient with mild symptoms distinctly less slow-twitch fibres were observed. This observation supports the suggestion of Shimomura and Nonaka (1989) that progression in NM is associated with an ongoing change of fibre types from fast-twitch to slow-twitch. In this respect, it is interesting to note that both, Volpe et al. (1982) and Miike et al. (1986) provided indications for a change of myosin expression from fast to slow in NM.

Linkage studies have recently assigned the autosomal dominant form of NM, to chromosome 1 between 1p13 and 1q25.1 (Laing et al., 1992), while linkage of the autosomal recessive form to this region was excluded (Tahvanainen et al., 1994). This indicates that these forms of NM are caused by different genetic mutations, and might explain the different observed staining patterns.

Relation of clinical data with immunohistologic results: C. Myotubular / centronuclear myopathy

When compared to other types of CM, XLMTM patients are a relatively homogeneous group with a high mortality rate (Bodensteiner, 1994). Our immunohistochemical findings are relatively uniform and, apart from the differences already discussed, in accordance with the literature as far as IFPs and myosin isoforms are concerned (Sarnat, 1990; Sawchak et al., 1991; Soussi-Yanicostas et al., 1991). Notable is the increased expression of collagens type IV and VI as well as laminin in this severe type of myopathy. A correlation between an increased expression of extracellular matrix proteins and the severity of CM, is also supported by our finding that in one of the CNM cases, in contrast with a second, less-affected case, high amounts of these constituents were found. Furthermore, the muscle biopsy of the former patient showed abnormalities in both fibre types as well as the presence of vimentin aggregates in numerous fibres, while in the biopsy of the latter, all fast-twitch fibres were unaffected, and no vimentin was detected in any of the fibres.

5. Conclusions

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

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