ABNORMAL EXPRESSION OF INTERMEDIATE FILAMENT PROTEINS IN X-LINKED MYOTUBULAR MYOPATHY IS NOT REPRODUCED IN VITRO

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Abstract—Expression patterns of the intermediate filament proteins (IFPs) desmin and vimentin, in biopsy material taken from a 1 day old boy with fatal neonatal X-linked myotubular myopathy (XLMTM) were compared with the expression of these proteins in cultured myotubes, from the same patient. Immunohistochemical studies revealed the persistence of high levels of desmin in virtually all, and vimentin in most, of the myofibres within the patient's biopsy. Analysis of intermediate filament expression in differentiating, cultured muscle cells did not reveal overt differences between XLMTM cultures and cultures of control muscle. Titin distribution patterns indicated a normal process of myofibrillogenesis in XLMTM myotubes. We conclude that the failure to properly regulate IFP-expression is not intrinsic to XLMTM muscle fibres. The possibility that this failure is due to a defective external, possibly neural factor, is discussed.

Key words: X-linked myotubular myopathy, skeletal muscle cell cultures, muscle fibre immaturity, intermediate filament proteins, titin, immunohistochemistry.

INTRODUCTION

Under the denomination 'centronuclear myopathy' (CNM) a heterogeneous group of diseases is classified, with diverging clinical pictures and patterns of inheritance. [1] The first case of CNM was reported by Spiro et al. [2], who described an adolescent, male patient with muscle fibres resembling myotubes of fetal muscle. The term myotubular myopathy was proposed and an arrest of muscle development at the cellular level was suggested to be the primary cause of the disease. Other authors [3] provided arguments against the suggested pathogenesis of the condition, and the more descriptive term 'centronuclear myopathy' was put forward as an alternative [4]. Nowadays, most authors [5–8] prefer to use the term 'myotubular myopathy' for the severe neonatal form of CNM, that is associated with an X-linked, recessive pattern of inheritance [9]. The muscle fibres of the patients with this X-linked myotubular myopathy (XLMTM) have certain features characteristic for fetal myotubes and fail to reach a terminal level of maturity [6, 10].

In this report we describe the expression patterns of the skeletal muscle fibre differentiation markers desmin and vimentin in biopsy material and in cultured muscle cells obtained from a new case of a large, well-documented XLMTM family with six confirmed and 18 suspected cases over six generations [11, 12]. The purpose of this study was to investigate whether or not the characteristic abnormalities in the expression patterns of the intermediate filament proteins (IFPs) desmin and vimentin in XLMTM muscle [10, 13] are reproducible in vitro, which might indicate an intrinsic defect of XLMTM muscle fibres. Since changes in expression and distribution patterns of titin are
indicative for the successive stages of myotube differentiation and sarcomere formation [14], antibodies to this protein were used to study the process of myofibrillogenesis and to identify the maturational stages of the myotubes.

PATIENT

The patient, a boy at risk for XLMTM with fatal neonatal asphyxia [11], was born as the second child of healthy non-consanguineous parents after a pregnancy which was complicated by polyhydramnios in the last trimester. Birth was at a postmenstrual age of 35 weeks. He presented with severe respiratory failure, pronounced muscular hypotonia and no spontaneous movements. The tests were undescended. He died within 24 h of birth. The clinical diagnosis of XLMTM was confirmed by histopathologic examination of biopsied muscle tissue. Skeletal muscle tissue of a patient who died from cardiac failure on second day of life, obtained after informed consent within 1 h of death, served as control muscle.

MATERIALS AND METHODS

Muscle cell cultures

Satellite cells were isolated enzymatically from muscle biopsies of the XLMTM and the control patient, mainly as described before [15, 16]. Cells were cultured, trypsinized and frozen in liquid nitrogen in culture medium containing 10% dimethylsulphoxide, and stored until use. Upon thawing, cells were seeded, further cultured and induced to differentiate as described earlier [17]. An inverted microscope (Leitz Diavert, Wetzlar, FRG) equipped with phase-contrast optics and an automatic camera, was used to examine and photograph the cultures before and after methanol/acetone (1:1) fixation. The circumference of XLMTM (n = 78) and control (n = 74) myotube-nuclei was measured using PC-Image (Foster Findlay Associates Ltd, Newcastle upon Tyne, U.K.).

Indirect immunofluorescence assays

Indirect immunofluorescence assays were performed on methanol/acetone (1:1) fixed cells as described before [17], except that in double-labelling experiments Texas Red-labelled goat anti-mouse IgG was used to detect the antibodies to desmin or vimentin, and fluoresceine isothiocyanate-labelled goat anti-mouse IgM to detect the antibody specific for titin. Both conjugates were obtained from Southern Biotechnology Associates, Birmingham, AL, U.S.A.

Antibodies

Mouse monoclonal antibodies used in this study are: (1) RD301 (available from Euro-Diagnostica BV, Apeldoorn, The Netherlands) and DE-R-11 (purchased from DAKO A/S, Glostrup, Denmark), both specific for desmin [18, 19]. These antibodies revealed the same staining patterns in our studies. (2) RV202, specific for vimentin [18], available from Euro-Diagnostica BV, Apeldoorn, The Netherlands. (3) 330-R5B4 and 330-R5D4, specific for embryonic myosin heavy chain (MHC) [20]. (4) 219-1D1, specific for slow MHC. (5) 9D10, an antibody of the IgM subtype, specific for titin [21], obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University, School of Medicine, Baltimore, MD, U.S.A. and the Department of Biology, University of Iowa, Iowa City, IA, U.S.A., under contract N01-HD-6-2915 from the NICHD.

RESULTS

Intermediate filament proteins and myosin isoforms in XLMTM skeletal muscle tissue

Sections of XLMTM skeletal muscle revealed high levels of desmin (Fig. 1A) and vimentin (Fig. 1B) expression. Both proteins were often found concentrated in the centre of the muscle fibres and showed a cross-striated distribution pattern in longitudinal sections (Figs. 1C,D). Most of the larger, normal size fibres were not stained by the anti-vimentin antibody (Fig. 1B), while hardly any fibre was negative when stained with the anti-desmin antibodies (Fig. 1A). Embryonic MHC was detected in numerous fibres with both antibodies used (Figs. 1E,F). Slow MHC was expressed in a mosaic checkerboard pattern (Fig. 1G). Especially the vimentin and embryonic myosin staining patterns varied between different fascicles; e.g., while some fascicles contained only very few vimentin-negative or embryonic MHC-negative fibres (Figs. 1B,F), in other parts of the biopsy
numerous fibres negative or only weakly positive for these components were found (Fig. 1E).

Intermediate filament proteins in differentiating cultured skeletal muscle cells (Figs. 2, 3, 4)

When differentiation of muscle cells was induced by withdrawal of nutrients from the culture medium, within a few days many multinucleated cells were observed in cultures of XLMTM (Fig. 2A), similar to what could be observed in the control cells (Fig. 2B). In both cases, differentiation was studied until long myotubes were formed. Phase-contrast microscopy revealed no notable, structural differences between XLMTM (Figs. 2C,E) and normal cultures (Figs. 2D,F), except that nucleoli of XLMTM myotubes were more prominent (Figs. 2C,E). The circumference of nuclei in XLMTM myotubes (161.2 ± 13.9 pixels) did not differ significantly when compared to nuclei in control myotubes (157.4 ± 11.7 pixels).

To study the development of myofibrils in muscle cell cultures, the antibody specific for titin was used. The titin organization was used as a marker for the developmental degree of, often asynchronously differentiating, individual myotubes [14]. In both XLMTM and control cultures titin staining patterns, representing all maturational stages between a punctate stain-
Fig. 2. Phase-contrast micrographs of cultured, methanol/acetone (1:1) fixed skeletal muscle cells of the patient with XLMTM (A,C,E) and an age-matched patient without known muscle disorder (B,D,F), 5 days after the induction of differentiation by withdrawal of nutrients from the culture medium. Note the presence of many myotubes containing large groups of nuclei in XLMTM (A) as well as in control cultures (B). Prominent nucleoli are only present in the nuclei of XLMTM myotubes (C,E) and not in the nuclei of control myotubes (D,F). Bar indicates 70 μm (E,F), 110 μm (C,D) or 225 μm (A,B).

ing pattern (Figs. 3A,C; 4E,G) and the characteristic mature doublet banding pattern (Figs. 3A,C; 4I,K), were observed. These patterns correspond to a differentiational age of about 2 and 6 days, respectively [14, 17]. In double labelling experiments with the anti-desmin and anti-vimentin antibodies, no differences between the XLMTM and the control cultures were observed. The strongest desmin expression was found in mononuclear cells and young myotubes showing immature titin distribution patterns (Fig. 3). In myotubes that contain myofibrils with a mature cross-striated appearance when stained with anti-titin (Figs. 3A,C), desmin levels were reduced but never negative (Figs. 3B,D). Cells or myotubes with a punctate titin distribution pattern showed a comparable intensity and pattern of vimentin staining as titin negative cells (Figs. 4A–H). Upon further maturation, when filamentous titin staining patterns were observed, a slight but significant reduction of vimentin levels was observed (Figs. 4A–H). This reduction in vimentin expression was progressive during further differentiation, and in myotubes containing cross-striated myofibrils, vimentin expression was reduced beyond detection levels (Figs. 4I–L). In some myotubes myofibrils showed a variable degree of maturation (Figs. 4A,C,K). In the regions with the most mature appearing myofibrils, vimentin expression was the lowest (Figs. 4B,D,L).

DISCUSSION

X-linked myotubular myopathy is a rare, though well documented and severe muscle disease with an onset before or at birth. Consequently, most families show a history of spontaneous abortions and stillbirths, while
most male newborn patients do not survive their first year of life [1], due to severe hypotonia and respiratory failure as a consequence of generalized muscle weakness [11]. Other tissues including the heart, the brain, the spinal cord and peripheral nerves have been reported not to be affected [3, 10]. However, some authors described signs of degeneration and regeneration in the sciatic nerve [5], and immature neuromuscular junctions [6].

Although most XLMTM skeletal muscle fibres have a small diameter, they show a differentiation in fibre types, alignment of adjacent myofibrils, the development of a sarcotubular system and other signs of maturation [10, 12, 13]. The oblique or longitudinal orientation of triads, the localization of nuclei, glycogen and mitochondria in the centre of the fibres, and the occurrence of high amounts of vimentin and desmin, are as in normal fetal myotubes and point to an arrest in morphogenesis of differentiating myofibres [10]. We have compared the desmin and vimentin expression patterns in skeletal muscle fibres with those in cultured muscle cells in a case of a well documented XLMTM family [11, 12], in order to investigate whether the failure to properly regulate IFP expression is intrinsic to the muscle fibres or induced by an external signal.

Expression of differentiation markers is characteristic for XLMTM muscle fibres

The in situ expression patterns of the skeletal muscle differentiation markers desmin, vimentin, embryonic MHC and slow MHC in our case are comparable to those described for other XLMTM patients [10, 13, 22, 23]. On the one hand, the high amounts of embryonic MHC, desmin and especially vimentin definitely confirm the immaturity of the fibres. On the other hand, the aspect of maturity is
Fig. 4. Double-label immunofluorescence micrographs of different stages of double-stained, differentiating cultured muscle cells from the patient with XLMTM (A,B,E,F,I,J) and a control patient (C,D,G,H,K,L). Vimentin staining patterns (B,D,F,H,J,L) are compared to the staining patterns of anti-titin (A,C,E,G,I,K). Note that the maturation of the myotubes, as indicated by titin staining patterns (see also legends to Fig. 3), is accompanied by a decrease in vimentin levels in XLMTM as well as in control cultures. Bar indicates 50 μm (E–L) or 100 μm (A–D).
Desmin and Vimentin in XLMTM

stressed by the cross-striated titin (not shown here, but illustrated in Ref. 13) and desmin staining patterns, indicating proper myofibrillar alignment. Furthermore, the myosin staining patterns indicate a normal differentiation into slow-twitch and fast-twitch fibre types. Silver et al. [24] described a variation in fibre size between individual fascicles within a single muscle. Our immunohistochemical findings show that this phenomenon is associated with variable expression levels of embryonic MHC, and therefore, variable degrees of maturation of individual fascicles. Normal size fibres, typically present in low numbers in XLMTM muscle [5, 10], were found to be stained by anti-slow MHC, and not, or only weakly, by anti-embryonic MHC and anti-vimentin, illustrating their advanced stage of differentiation (see also Ref. 13). Nevertheless, the expression of desmin also remains high in these otherwise mature appearing fibres. This finding, taken together with the finding that in some XLMTM patients virtually no muscle fibres express vimentin or embryonic myosin but large amounts of desmin [13], indicates that the persistence of desmin filaments is characteristic for XLMTM, which has been suggested to play an important role in the pathogenesis of this disease [10]. Linkage studies have recently mapped the XLMTM locus to Xq28 [25-28]. Considering the chromosomal localization of the genes encoding desmin and vimentin (chromosomes 2 and 10, respectively [29]), these genes can be excluded as candidate genes for XLMTM. Sarnat suggested that mutations in vimentin or desmin suppressor genes might cause XLMTM [30].

Normal myofibrillogenesis and IFP expression in cultured XLMTM myotubes

The expression levels and localization patterns of desmin and vimentin dramatically change during myogenesis [31, 32]. The process of human muscle cell differentiation in vivo, can be mimicked in vitro. We previously showed that vimentin disappears from maturing, aneurally cultured myotubes, and that desmin amounts increase in early stages of differentiation, but decrease concomitant with reorientation of desmin filaments [14]. Furthermore, virtually all stages of myofibrillogenesis could be identified by staining with anti-titin antibodies [14, 17]. This culture system has been used by us to compare IFP expression in XLMTM and control myotubes during several stages of myofibrillogenesis.

The reactivity patterns obtained with anti-titin antibodies reveal that all stages of myofibrillogenesis observed in normal skeletal muscle cultures [14], were also observable in differentiating XLMTM myotubes. Since mature banding patterns of titin are always accompanied by a mature localization of other sarcomere constituents [17], we conclude that the myofibrils in XLMTM muscle cultures are normally matured. In XLMTM cultures, the expression patterns of desmin and vimentin were not altered when compared to the age-matched control muscle cells and muscle cultures studied earlier [14, 17]. In other words, the persistence of IFPs as seen in the biopsies of our patient and other XLMTM patients [10, 13], is not reproduced in vitro. This observation indicates that the abnormal regulation of IFP expression is not an intrinsic, primary defect of XLMTM muscle fibres.

Muscle cultures derived from XLMTM patients have been studied before by Askanas et al. [12]. At the light microscopical level these authors observed myotubes with unusually great numbers of large, clustered nuclei, when compared to control cultures. We observed large groups of nuclei in both XLMTM and control myotubes after five days of differentiation, and thus considered this phenomenon normal for in vitro muscle cell differentiation. Other reported signs of immaturity, such as the lack of striated myofibrils in cultured XLMTM myotubes, could not be reproduced in our XLMTM cultures. This discrepancy may be the result of the improved culture conditions we have used [14, 33]. Our results confirm, however, the presence of prominent nucleoli in XLMTM myotubes, indicating a more active protein synthesis in XLMTM myotubes.

Immaturity of XLMTM myofibres: a result of a neural defect?

Skeletal muscle cell determination and differentiation during early embryogenesis are regulated by the different members of the MyoD gene family [34]. These genes are not X-chromosomal and therefore not candidate genes for XLMTM. One of the transcription factors encoded by these genes, myogenin, is responsible for muscle fibre maturation [34]. In mice lacking functional myogenin genes, not only are few myotubes formed, but they also do not
develop mature myofibres. Cultured myoblasts
derived from these mice with null-mutations
in the myogenin genes, differentiate normally [35],
indicating that the in vitro conditions lack an
inhibitory factor, that negatively regulates
myogenesis [34].

At advanced developmental stages, muscle
cell maturation is regulated by innervation [36].
In most XLMTM patients a normal morphol-

ogy of nervous tissue as well as neuromuscular
junctions (NMJ) was reported [10, 24]. A
normal morphology is, however, no guarantee
for normal functioning. Furthermore,
XLMTM-linked abnormalities of the sciatic
nerve [5] as well as immature NMJ [6], were
reported in other patients. Defects in a certain,
possibly neural, muscle fibre maturation-regu-
lating factor, might therefore result in delayed
or arrested skeletal muscle development in
XLMTM patients [3, 6, 8, 37]. The fact that
absence of neural influence in our aneural
muscle cultures is associated with normal
muscle fibre differentiation, at least with
respect to the regulation of IFP-expression,
supports this assumption. Unfortunately, IFP-
expression was not studied in the experiments
by Askanas et al., who observed that the addition
of fetal rat neural extracts to XLMTM
muscle cultures, did not change the characteris-
tics of the muscle cells [12]. The main observa-
tion of these authors was a low level of
adenylate cyclase in their XLMTM muscle
cultures. They concluded that the abnormalities
were intrinsic to the muscle cells, and suggested
that the inability of XLMTM muscle fibres to
respond to neural, maturing influences caused their maturational arrest [12]. Our results
concerning IFP-expression do not confirm their
hypothesis.

It should be considered that, like in McArdle
disease [38] and some other muscle diseases
[39], in XLMTM the missing or defective
protein is not expressed during the early stages
of skeletal muscle fibre maturation that can be
studied in vitro. The abnormalities in XLMTM
muscle cultures noted earlier in a patient from
the same family [12], provide evidence against
this possibility.

CONCLUSION

Although cultured XLMTM muscle cells
were reported to show abnormalities in the
maturation of the myotubes [12], the character-

istic persistence of high levels of IFPs of the
desmin and vimentin type in the muscle fibres
is not found in vitro. This observation indicates
that the failure to regulate in situ IFP expres-
sion is not intrinsic to the muscle fibres. This
failure might be the result of a defective, possi-

bly neural factor, that is withheld from the
muscle cells in tissue culture conditions. Studies
to test this hypothesis, using coculture experi-
ments with XLMTM myotubes and rodent
embryonic motor-neurons are in progress. Our
results, taken together with those of Askanas
et al. [12], might implicate that XLMTM is
caused by abnormalities in both, skeletal
muscle and neural tissues.

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