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Normal innervation and differentiation of X-linked myotubular myopathy muscle cells in a nerve-muscle coculture system

Olivier M. Dorchies[a], Jocelyn Laporte[b], Stéphanie Wagner[a], Colette Hindelang[b], Jean-Marie Warter[a], Jean-Louis Mandel[b], Philippe Poindron[a,*]

[a]*Laboratoire de Pathologie des Communications entre Cellules Nerveuses et Musculaires (UPRES 2308), UFR des Sciences Pharmaceutiques, BP 24, 74 route du Rhin, 67401 Illkirch Cedex, France.*

[b]*Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, BP 163, 1 rue Laurent Fries, 67404 Illkirch Cedex, France.*

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Abstract

To study the pathogenesis of X-linked recessive myotubular myopathy (XLMTM), we used a nerve-muscle coculture system which allows the reconstitution of functional motor units in vitro after coupling of human skeletal muscle cells with embryonic rat spinal cord explants. We used three skeletal muscle cell lines derived from subjects with known mutations in the *MTM1* gene (two from embryonic tissues, associated with mutations predicted to give a severe phenotype, and one from a neonate still alive at 3 years 6 months and exhibiting a mild phenotype). We compared these three XLMTM muscle cell cultures with control cultures giving special attention to behaviour of living cocultures (formation of the myofibres, contractile activity, survival), expression of muscular markers (desmin, dystrophin, α-actinin, troponin-T, myosin heavy chain isoforms), and nerve-muscle interactions (expression and aggregation of the nicotinic acetylcholine receptors). We were unable to reproduce any 'myotubular' phenotype since XLMTM muscle cells behaved like normal cells with regard to all the investigated parameters. Our results suggest that XLMTM muscle might be intrinsically normal and emphasize the possible involvement of the myotubularin-deficient motor neurons in the development of the disease.

Keywords: X-linked myotubular myopathy; Nerve-muscle coculture; Myotubularin; In vitro differentiation

1. Introduction

X-linked myotubular myopathy (XLMTM) is a congenital muscle disorder characterized by a generalized hypotonia and muscle weakness in affected newborn males [1]. The respiratory insufficiency at birth is responsible for a high neonatal mortality. Patients who survive past 1 year of age suffer from various often lethal medical complications [2]. Some patients, however, display milder forms with spontaneous improvement of the respiratory function allowing prolonged life into adulthood [3]. *MTM1*, the gene implicated in XLMTM has been identified in 1996 [4], improving the efficiency of pre- and postnatal diagnosis and genetic counselling. Myotubularin, the product of the *MTM1* gene is ubiquitously expressed. It was expected on the basis of sequence analysis to be a tyrosine phosphatase [5]. Although myotubularin has been demonstrated to have a dual specificity phosphatase activity on serine/threonine and tyrosine residues in vitro [6], it would rather act in vivo as a phosphatase for phosphatidylinositol 3-phosphate [7,8]. Despite these recently acquired molecular data, our knowledge on the pathogenesis of XLMTM is mainly restricted to clinical and histopathological reports.

Characteristic features of XLMTM muscle consist in centrally-located nuclei in a variable proportion of fibres. These abnormally-positioned nuclei are surrounded by a halo devoid of contractile elements and ATPase activity [1] but containing aggregated mitochondria [9]. Disorganized myofibrills are seen at the periphery of the fibres. Immunocytochemical studies have reported many variations in the expression of N-CAM, utrophin, laminin α5 chain and HLA1 antigen [10]. Others investigators have found abnormalities in the expression of desmin, vimentin and fetal myosin heavy chain (MHC) [11,12]. However, these histological defects do not seem to be consistent and specific features of XLMTM samples [13]. In addition to these conflicting data, abnormal expression of desmin could not be reproduced in vitro in cell cultures of XLMTM myotubes [14].

Because XLMTM fibres share some histological characteristics with fetal myotubes, it has been suggested that the

* Corresponding author. Tel.: +33-388-676927; fax: +33-388-660190.
E-mail address: philippe.poindron@pharma.u-strasbg.fr (P. Poindron).

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Table 1
Characteristic of muscle cell cultures used in this study.

XLMTM muscle cultures				Control muscle cultures		
Designation	Age	Muscle	Mutation	Designation	Age	Muscle
Severe phenotype[a]						
DY46	12 w	Leg	R474X	Q1014TE	9 w	Leg
EG84	11 w	Arm	fs238	Q1016TE	9 w	Leg
Mild phenotype [b]						
NL15	10 d	Quadriceps	R241C	904TE	3.5 y	Leg
				1037TE	3.5 y	Paravertebral

[a] Age of embryonic development.
[b] Age of postnatal life.

disease results from an arrest of the normal development of muscle fibres during late myogenesis, or from a defect in the structural organization of the fibres. However, other possibilities such as a dysfunction in the maintenance of normally-differentiated fibres could not be ruled out, nor could be a defect in motor innervation [15,16].

To explore these hypotheses, we used a nerve-muscle coculture system [17] in which human muscle cells grown de novo can be efficiently innervated by motor neurons from rat embryonic spinal cord explants. This system allows the in vitro maturation of human muscle cells into contracting fibres that display numerous criteria of morphological, biochemical, and pharmacological differentiation [17–20]. It has proven to be a powerful tool to study the pathogenic mechanisms of various muscular [21,22] and neuromuscular disorders [23–25]. The present study is the first devoted to exploration of the behaviour of molecularly-confirmed XLMTM muscle cells in culture.

2. Materials and methods

2.1. Muscle samples and muscle cell cultures

Muscle samples were obtained from male individuals by biopsy or following voluntary termination of pregnancy. XLMTM samples were obtained via the ENMC consortium for centronuclear/myotubular myopathy. They were screened for mutations in the *MTM1* gene [26,27]. Cell cultures DY46 and EG84 were established in our laboratory from leg muscles of a 12-week old embryo and arm muscles of a 11-week old embryo, respectively. These embryos had mutations predicted to give a severe phenotype with complete loss of functional protein [28]. Cell culture NL15 is from a patient with a missense mutation, associated with a milder phenotype. The biopsy was performed in the quadriceps 10 days after birth. Control muscle tissues were provided by the Tissue Bank of the Association Française contre les Myopathies from subjects diagnosed as free of intrinsic muscular or neuronal disease. Control samples were chosen to match, when possible, XLMTM samples. Fetal XLMTM tissues were compared with closely age-matching normal fetal tissue. However, we cannot be provided with a quadriceps sample from a healthy newborn male, so that the cell culture NL15 was compared with a cell culture established from paravertebral muscle of a young boy (3.5-year old). Table 1 summarizes the features of the muscle cell cultures used in this study.

2.2. Nerve-muscle cocultures

Human muscle cultures were established according to a technique described previously [29,30] with slight modifications. Briefly, muscle cells were cultured from a dissociated cell suspension, plated onto 0.1% gelatin-coated 35-mm Petri dishes (Falcon) (2×10^5 cells per dish), and grown in a proliferation medium consisting of F14 medium (Life Technologies) supplemented with 2 mM glutamine (Life Technologies), 10 μg/ml insulin (Sigma), 10 ng/ml epidermal growth factor (EGF) and fibroblast growth factor (FGF) (both from Tébu), 10% fetal bovine serum (Flobio) and an antibiotic-antimycotic mixture (Life Technologies).

Cocultures were performed as described by Askanas et al. [17]. Three to four days after muscle satellite cell fusion began, explants consisting of whole transverse slices in spinal cord of 12–13 day-old rat embryos, with the dorsal root ganglia (DRG) attached, were placed onto the muscle monolayer, 4–7 explants per dish. DRG are necessary to achieve a sufficient ratio of innervation [31]. Days of 'innervation' are arbitrarily defined as the number of days after beginning the coculture. Innervated cultures were maintained in a coculture medium composed of Eagle's minimum essential medium (Life Technologies), supplemented with 25% medium 199 (Life Technologies), 5% fetal bovine serum, 10 μg/ml insulin, and antibiotic-antimycotic mixture. Media were changed twice a week. All the cultures were kept at 37°C in a 95% air–5% CO_2 humidified atmosphere, and were frequently monitored by phase contrast microscopy.

In the following, cocultures are referred as *control* or *XLMTM* cocultures.

2.3. Immunocytochemistry

Immunocytochemical labelings were performed at room

Table 2
Primary antibodies used in this study

Antigen	Designation	Dilution
Desmin	Polyclonal	1:30
Vimentin	Clone V9	1:200
Actin	Clone AC40	1:400
α-actinin[a]	Clone EA-53	1:1600
Dystrophin[b]	Clone MANDYS8	1:800
Tropomyosin	Clone TM311	1:800
Troponin-T	Clone JLT-12	1:1000
Phosphotyrosine	Clone 4G10	1:50

[a] Sarcomeric α-actinin.
[b] The antibody recognizes an epitope located in the rod domain of the protein.

temperature on 3-week old cocultures using a panel of antibodies against phosphotyrosine residues, proteins of the contractile apparatus, cytoskeleton and membrane-bound proteins (Table 2). All the primary antibodies are monoclonal, produced in mouse, except anti-desmin which is polyclonal from rabbit. Primary antibodies were purchased from Sigma, except 4G10, from Euromedex. Other reagents were from Interbiotech.

Cells were fixed and permeabilized with a cold mixture of 95% methanol/5% acetic acid, for 20 min. Alternatively, cocultures were fixed in a 4% paraformaldehyde solution in phosphate buffered saline (PBS), for 30 min, and then permeabilized with a 0.1% Triton X-100 solution in PBS, for 30 min. Non-specific binding sites were blocked by an additional 30 min incubation in a 3% bovine serum albumin (BSA) solution in PBS. All the subsequent steps are performed in PBS containing 0.5% BSA. Samples were incubated with the primary antibody for 1 h, washed, incubated with the secondary antibody (see Table 2 for details) for 1 h, and washed again. Monoclonal primary antibodies were revealed with a Cy3® (indocarbocyamine)-conjugated Goat anti-Mouse (GAM) antibody. Antibodies against desmin and vimentin were chosen to allow double-labelling experiments. Desmin was revealed with a biotinylated Donkey anti-Rabbit (DAR) antibody (diluted 1:200) followed by (4,6-dichlorotriazinyl)aminofluorescein (DTAF)-conjugated streptavidin (diluted 1:500). Samples were mounted in a mixture composed of 75 vol. glycerol, 20 vol. PBS, and 5 vol. propyl gallate as an anti-bleaching compound. Fluorescence was visualized under a Nikon epifluorescence microscope Optiphot-2.

2.4. Myosin heavy chains

Areas of innervated fibres were delineated and carefully dissected out from the Petri dishes. Actomyosin was extracted according to Butler-Browne et al. [32] and myosin heavy chain (MHC) were separated on 6% SDS-polyacrylamide gels containing 25% glycerol, for 36 h at 70 V. MHC were revealed by silver staining (Silver Stain Plus, Biorad). Identification of isoforms was made possible by the use of actomyosin extracts from reference muscle tissues, separated along with the samples to be analyzed.

2.5. Autoradiographic visualization of nicotinic acetylcholine receptor (nAChR) aggregation

It has been previously demonstrated by electron microscopy that in this coculture system, nAChR aggregates correspond to genuine nerve-muscle contacts [17]. nAChR were labeled by autoradiography with ^{125}I-alpha bungarotoxin [^{125}I-αBTX] (Amersham Pharmacia Biotech) [33]. Briefly, after a 1 h incubation at 37°C with the snake venom (4 nM, 100–250 Ci/mmol) diluted in the medium, cocultures were rinsed five times, fixed in a 2.5% glutaraldehyde solution in PBS, dehydrated, air-dried, and covered with Kodak NTB-2 emulsion. After a 7–20 days exposure, autoradiographs were developed using Kodak D-19 developer.

2.6. Transmission electron microscopy

Three- and 5-week-old cocultures were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) for at least 20 min at 4°C, post-fixed in 1% osmium tetroxide in the same buffer for 1 h at room temperature, dehydrated in graduated alcohols, and embedded in epoxy resin. After embedding, the areas of nerve-muscle cocultures designated for ultrastructural studies were identified in phase contrast microscopy, removed from the Petri dish as small resin cubes ($<$1 mm side). These small blocks were re-embedded in epoxy resin in an appropriate orientation in order to perform sections longitudinally to the muscle fibres. Semithin sections were stained with toluidine blue and observed with a light microscope. Ultrathin sections were contrasted with uranyle acetate and lead citrate and subsequently examined with a Philips electron microscope.

3. Results

3.1. Behaviour of the living cultures

3.1.1. Aneural muscle cell cultures

The relative amount of myogenic versus non-myogenic cells (mainly fibroblasts) originating from muscle samples was greatly variable even for samples sharing the same features (e.g. nature of the muscle biopsy, age of the donor). For instance, cultures from DY46 were over 95% fibroblastic-like, while EG84 gave cultures nearly pure in myoblasts. However, both control and XLMTM myoblasts in aneural cultures of muscle cells usually fused into myotubes within 3 days after confluence has been reached. Myotubes remained non-contractile and unstriated even when maintained for long period of time in proliferation medium or in coculture medium. This study was not aimed to describe the characteristics of aneurally-cultured myotubes. However, no obvious differences in myotube size

and shape, nor in the distribution of nuclei have been observed.

3.1.2. Nerve-muscle cocultures

Whatever the origin of the muscle cells, neurites are seen outgrowing from the spinal cord explants 24–48 h after coupling with cultured myotubes. They make contacts with them and progressively induce a morphological transformation into long (up to 1 cm) contracting muscle fibres. The first contractions are usually observed after 5–6 days in coculture. Quickly thereafter, innervated muscle fibres located in the vicinity of spinal cord explants are virtually continuously contracting. By the end of the second week of innervation, they generally exhibit regularly-spaced cross-striations throughout their length (Fig. 1).

For each muscle cell sample, the mean yield of innervation (percentage of muscle cell-spinal cord explant coupling that lead to efficient myotube innervation and differentiation into contracting muscle fibres) was around 35–40%. Innervated muscle fibres from all cell cultures, including XLMTM, survived and contracted for up to 1 year. Some slight behavioural variations from one normal cell culture to another, and between independent experiments performed with one given cell culture, are commonly observed in this nerve-muscle coculture system (unpublished observation). They are not specific for and cannot be related with any disease state, and might be due to intrinsic individual variations in the original muscle tissues in the group which is considered.

In vitro innervated muscle fibres usually have a several-fold decreased diameter as compared with parent fibres in the muscle sample from which the cells originated [34]. Nuclei have about the same size as the fibre diameter, and often seem to be 'centrally-located' (for illustration, see troponin-T labeling in Fig. 3B,E). As a consequence, the position of the nuclei in nerve-muscle cocultures should not be reasonably considered as a phenotypic feature of immaturity of the innervated muscle fibres. Yet, some in vitro differentiated control and XLMTM fibres showed unequivocally peripheral, subsarcolemmal-located nuclei, the inner part of the fibre being occupied by well-organized myofibrills.

3.2. Expression of differentiation markers

3.2.1. Double labeling for desmin and vimentin

Desmin and vimentin were labeled simultaneously on the same cocultures. Differentiated muscle fibres (DMF) were found to strongly express desmin (Fig. 2A,C). Myotubes and some mononucleated cells (presumably myoblasts) were faintly stained at the antibody dilution we used (1:30). Preliminary experiments have shown that higher dilution (1:200) of the anti-desmin gave no signal, neither for normal nor in XLMTM myofibres formed in our nerve-muscle cocultures. Although infrequent, a cross-striated appearance of the muscle fibres after desmin labeling has been observed for cocultures established from all the cell cultures studied. The commonly found diffuse expression of desmin in muscle fibres might be due to a dysregulation of desmin expression in vitro, which may account for hiding the sarcomeric distribution of desmin. Indeed, when observed in phase contrast microscopy, these fibres appeared to be well cross-striated. Vimentin was found to be expressed to the same extent in almost all the cells in the cocultures. It is still present in DMF of all 3-week-old cocultures (Fig. 2B,D).

3.2.2. Expression of dystrophin and sarcomeric proteins

Sarcomeric α-actinin, dystrophin, troponin-T, and tropomyosin were found specifically in the DMF (Fig. 3, tropomyosin not shown). Neither the underlying muscle cell layer containing fibroblasts, myoblasts and myotubes, nor the cells outgrowing from the spinal cord explants were stained. Conversely, all the fibres found to be differentiated on morphological criteria expressed these four markers.

Labeling for sarcomeric α-actinin (Fig. 3A,D) underlined the normal sarcomeric organization of the myofibrills in the DMF. Location of nuclei appeared as poorly-stained black zones. Actin was detected in all cells (not shown). The highest intensity of labeling was found in DMF, where the sarco-

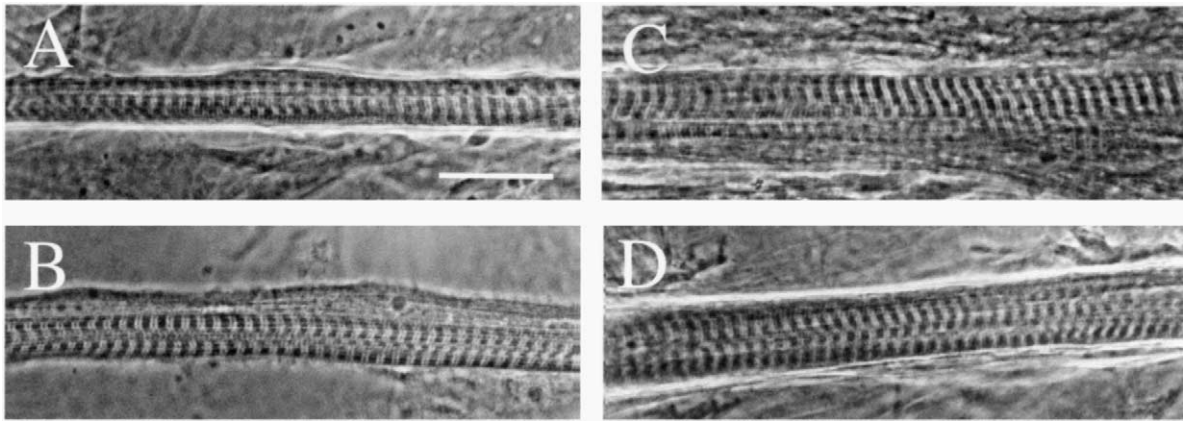

Fig. 1. High magnification microphotographs of isolated cross-striated fibres in 3-week-old living cocultures. (A): embryonic control muscle; (B): postnatal control muscle; (C): embryonic XLMTM muscle (severe phenotype); (D): postnatal XLMTM muscle (mild phenotype). Bar, 20 μm.

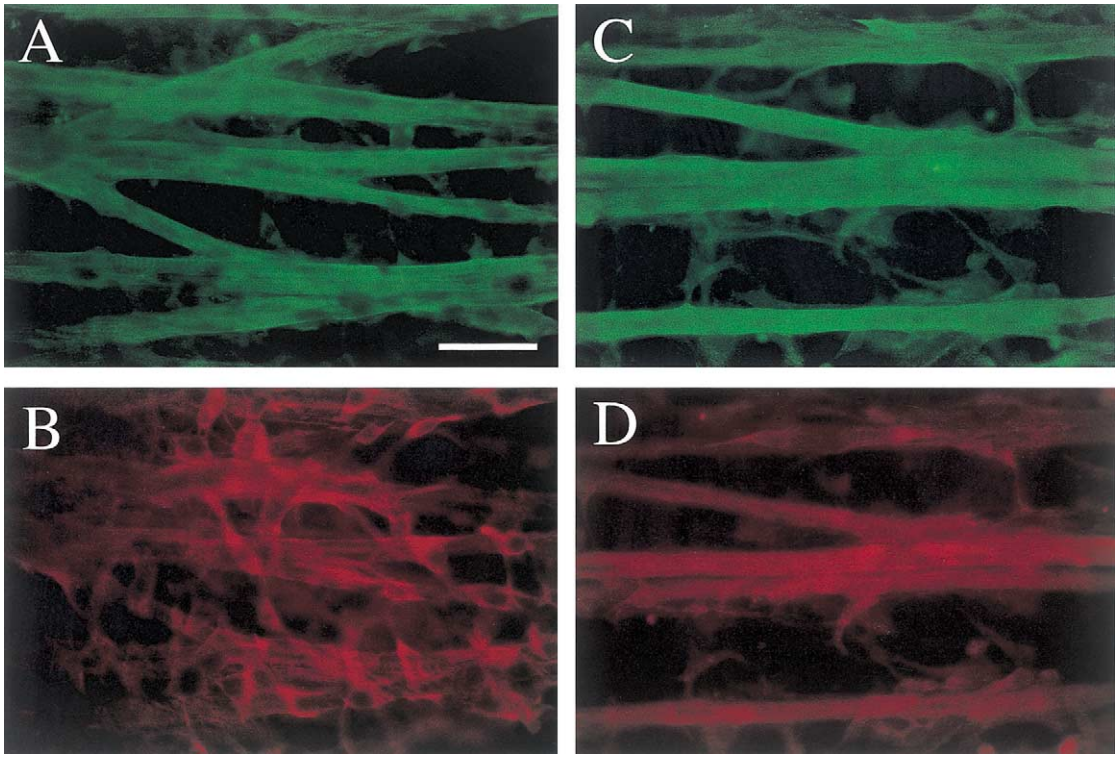

Fig. 2. Double labeling for desmin and vimentin. Immunocytochemistry was performed on 3-week-old cocultures. Same field of control DMF (A,B) and XLMTM DMF (C,D). (A,C): desmin labeling; (B,D): vimentin labeling. Bar: 50 μm. Innervated fibres have contracted for approximately 2 weeks and often have organized as a cell layer above the non-contractile components, left at the bottom of the dish. For this reason, in pictures (A,C,D), DMF are almost the only cells to be in the focal planes, whereas in picture (B), DMF are covered with desmin-negative mononucleated cells (presumably fibroblasts).

meric location of actin was partially masked by a faint cytoplasmic expression.

Troponin-T (Fig. 3B,E) and tropomyosin (not shown) were strongly expressed in DMF. In most cases, labeling was evenly distributed. Some fibres or portions of fibres showed sparse dot or fibrill-like appearance. Position of nuclei was easily identified as dark and elongated shapes in the cytoplasm. Some rather peripheral nuclei are seen in XLMTM DMF (Fig. 3D, lower fibre; Fig. 3E, central fibre).

In the DMF, the dystrophin labeling (Fig. 3C,F) was associated with the sarcolemma. Absence of a significant expression of dystrophin at ectopic sites (e.g. cytosol) was assessed by varying the focus during observation of the fluorescence.

We performed electrophoretic separation of MHC isoforms from DMF innervated for 3 and 5 weeks in vitro (Fig. 4). Actomyosin extracts for all types of cocultures predominantly contain the fetal MHC isoform accompanied by a small amount in slow MHC isoform. This pattern is in agreement with results from others investigators [35] obtained with normal muscle in an organotypic nerve-muscle coculture system.

3.3. Ultrastructure of differentiated muscle fibres

Ultrastructure of DMF was observed on ultrathin longitudinal sections of the fibres. Both normal and XLMTM fibres exhibited well-organized myofibrills spanning the entire width of the fibres (Fig. 5A,C). The typical bands and lines could be readily identified (Fig. 5B,D). In the most differentiated fibres, Z-bands are most often 'in register'. All the samples observed displayed some fibres at less well organized stages. However, they do not present obvious signs of degeneration and are believed to belong to the 'second-wave' of innervated fibres, recruited by nerve terminal sprouting. Some nuclei were in subsarcolemmal position in both types of cocultures (Fig. 5A,C). None of the more centrally-located nuclei were found to be surrounded by accumulated mitochondria or other organites. Other structures (e.g. sarcoplasmic reticulum, ribosomes, glycogen content) were similar between control and XLMTM cocultures and did not reveal any feature which could be representative for a disease state.

3.4. Expression and clustering of nAChR

After 3 weeks in coculture, DMF could be macroscopically visualized after autoradiographic labeling with ^{125}I-αBTX, due to their richness in nAChR. This outlined that DMF developed to the same extent for both types of cocultures (Fig. 6). At higher magnification, nAChR are aggregated in clusters, corresponding to genuine nerve-muscle contacts [17,33]. Clusters shared the same qualitative pattern of distribution on DMF from both control and

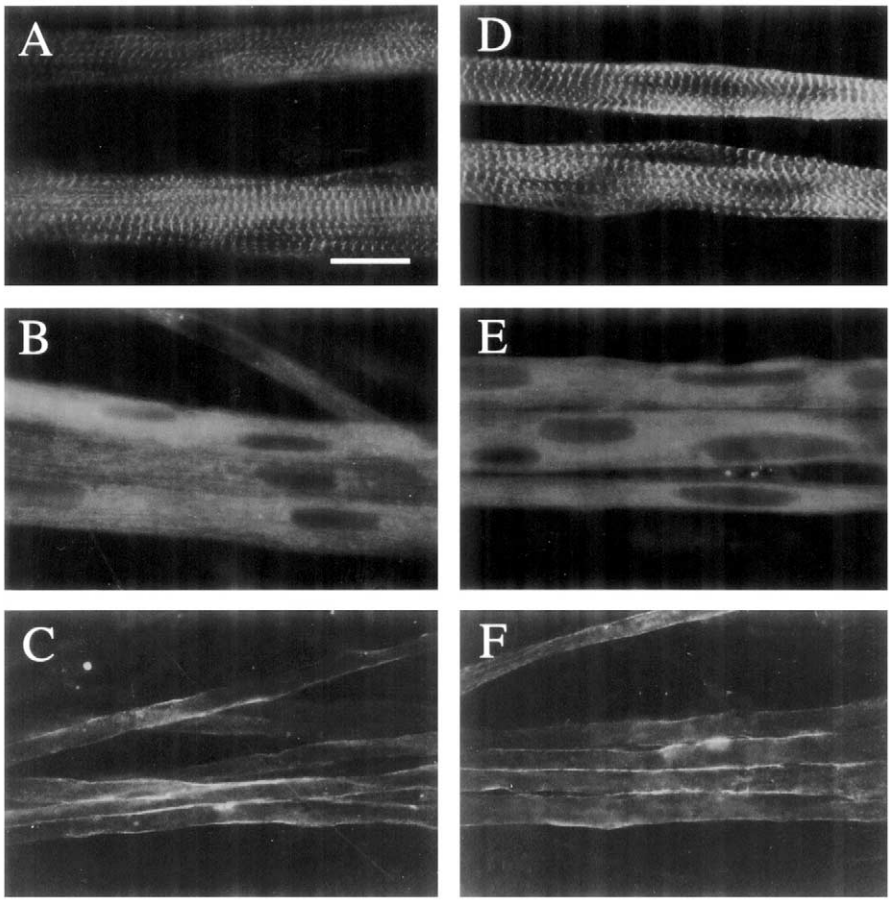

Fig. 3. Expression of sarcomeric α-actinin (A,D), troponin-T (B,E) and dystrophin (C,F) in DMF from 3-week-old cocultures. (A,B,C): control DMF; (D,E,F): XLMTM DMF. Bar: 20 μm (A,B,D,E); 50 μm (C,F).

XLMTM origin. However, quantitative analysis was not performed because of known variations among muscle samples, regardless of the disease.

4. Discussion

We investigated various aspects of muscular differentiation and innervation of XLMTM muscle cells in vitro, in a nerve-muscle coculture system. In this model, morphological changes of innervated myotubes and induced contractile activity can be readily followed by light microscopy observation. Moreover, biochemical changes in the reconstituted motor units can be conveniently investigated by immunocytochemistry and other cytological approaches.

4.1. Impairement of myotubularin function does not affect differentiation of muscle fibres in vitro

We could not find any difference in the behaviour of

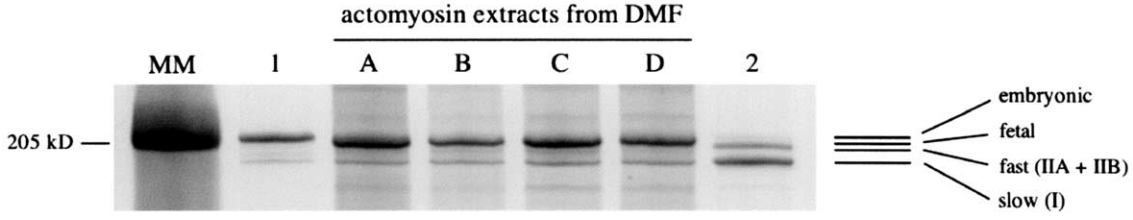

Fig. 4. MHC isoform expression in DMF. Actomyosin was extracted from DMF in 5-week old cocultures involving embryonic control muscle cells (lane A); postnatal control muscle cells (lane B); embryonic XLMTM muscle cells (severe phenotype) (lane C); postnatal XLMTM muscle cells (mild phenotype) (lane D). Lanes A–D correspond to 10 mm^2 of DMF. Lanes 1 and 2 correspond to reference actomyosin muscle extracts, from respectively 9-week old embryonic leg muscle (which contains embryonic and slow isoforms) and 15-year old paravertebral muscle (which contains fast and slow isoforms). The relative order of migration depends on the glycerol content in the gel. For the experiment shown here, the glycerol content was decreased from the regularly-used 30 to 25%. This is responsible for an unusual order of migration, depicted by the diagram on the right side of the figure. The major band in lanes A–D was identified as fetal isoform because of its non-embryonic, non-fast and non-slow gel mobility.

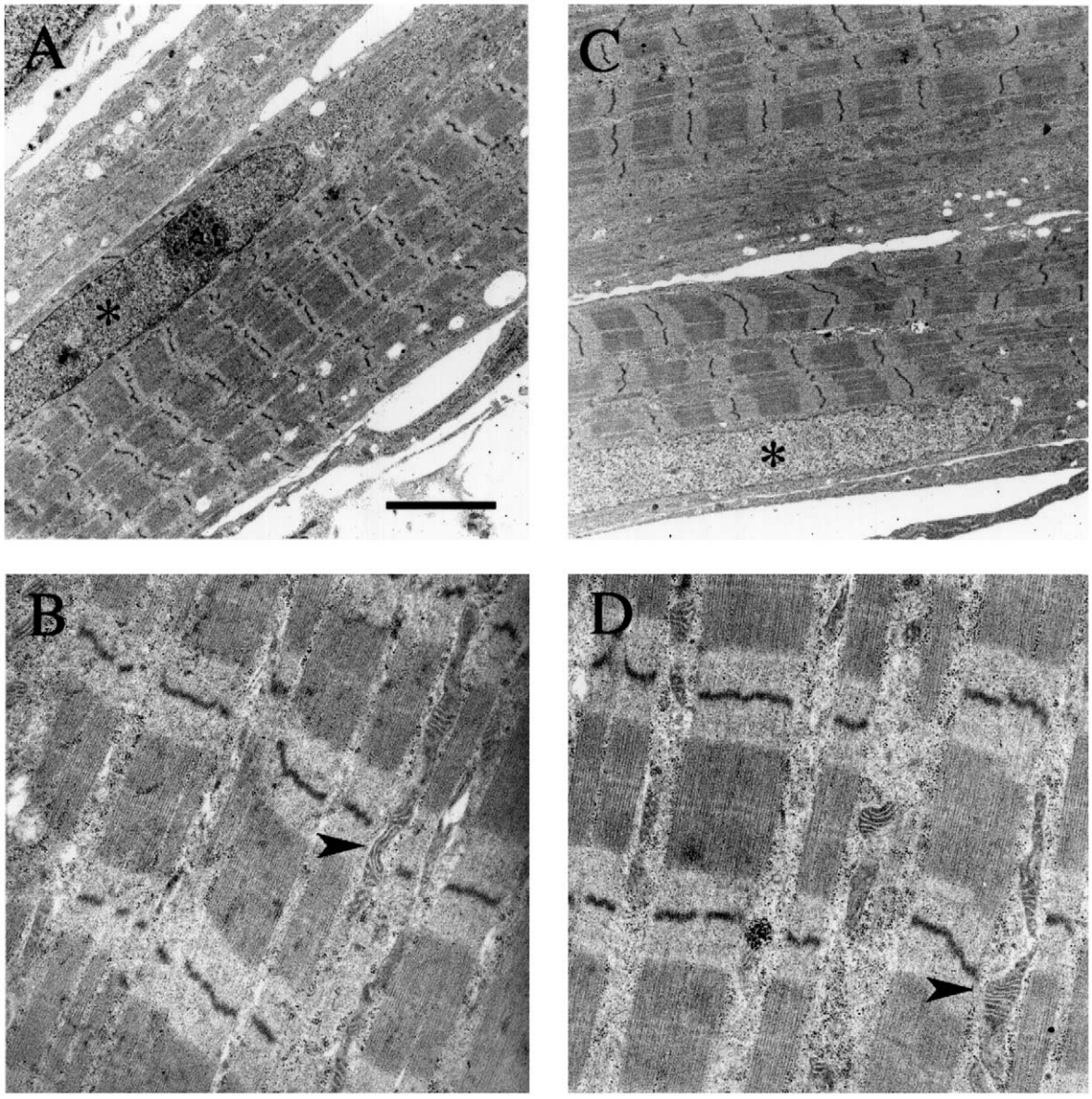

Fig. 5. Ultrastructure of 3-week-old in vitro-differentiated muscle fibres. (A,B): control DMF; (C,D): XLMTM DMF. (A,C): low magnification pictures of DMF with cross-striations on their full width, and subsarcolemal nuclei (asterisks) that lie immediately contiguous to well-organized myofibrills. (B,D): higher magnification views of relaxed myofibrills, showing the typical band pattern. Arrowheads indicate mitochondria infiltrated in the cytoplasm surrounding myofibrills. Bar: 3 μm (A,C); 1 μm (B,D).

living cultures during dynamic steps of muscle differentiation. We particularly focused on morphological and contractile aspects. Milestones of morphological transformation of myotubes into DMF were temporally and qualitatively conserved for both control and XLMTM muscle cells, whatever the clinical phenotype. Autoradiographic labeling of nAChR with ^{125}I-αBTX (Fig. 6) outlines that DMF developed to the same extent for both types of cocultures, leading to equivalent amounts of muscle mass, packed in large areas close to the spinal cord explants. Muscle fibres are morphologically fully differentiated after 3 weeks in coculture (Figs. 1 and 5). Longer time in vitro allows subtle maturation events to occur, mainly involving T-tubules and neuromuscular junctions (NMJ) but does not improve sarcomeric arrangement [17]. Immunocytochemical labeling for α-actinin revealed the regularly-spaced cross-striations in DMF from both groups. All the DMF specifically express high amounts of actin, α-actinin, troponin-T, tropomyosin and dystrophin (Fig. 3). No difference was observed in the global content of phosphotyrosine residues between control and XLMTM DMF (not shown). DMF from both control and XLMTM cocultures contain mainly fetal isoform with little slow isoform (Fig. 4). Such a content should be compared with a fetal-like MHC profile [36,37]. We did

not find, however, a difference between control and XLMTM DMF. This results is in accordance with a study carried out by Soussi-Yanicostas et al. [38] who found that the MHC isoform patterns of XLMTM and normal muscle tissues are roughly identical.

Our results demonstrate that myotubularin is not essential in vitro for the normal progress of myogenesis encompassing myoblast proliferation, myotube formation and innervation, and differentiation into muscle fibres. One could suggest that the overall muscular differentiation reached in our nerve-muscle coculture system is still insufficient to make the myotubular phenotype to appear. This interpretation is rather unlikely since clinical signs indicative for a possible XLMTM can be detected in utero by reduced fetal movements. Moreover, the MHC content could not by itself account for the global differentiation of the system. When nerve-muscle cocultures are established according to Askanas' protocol with postnatal muscle tissue from patients with type I or II spinal muscular atrophy [23,25] or inclusion body myositis [22], respectively childhood-onset neuromuscular disorder and adult-onset muscular disorder, obvious phenotypes appear in vitro despite the fetal-like MHC contents of the muscle fibres.

4.2. Pathological features of XLMTM muscle are not reproduced in vitro

Askanas et al. [39] observed in aneural muscle cell cultures established from biopsy specimen of two children with molecularly-uncharacterized XLMTM phenotype, an unusual ability to proliferate through numerous passages. Myotubes were described as very immature compared to control even after several weeks in culture. However, we could not observe any of these abnormalities in our aneural cultures established with four molecularly-confirmed XLMTM samples, nor could Van der Ven et al. [14] on their samples, including a severe case with a posteriori identified E17X stop mutation, associated with absence of functional myotubularin.

The abnormal persistence of desmin and vimentin described by Sarnat [11] is not believed to be relevant for XLMTM since it is a rather common feature of congenital muscle disorders [13], and it has been also described in rare cases of adult-onset myotubular myopathy [40]. Moreover, Van der Ven et al. [14] gave evidence that persistence of intermediate filaments they observed on clinically-diagnosed (and a posteriori molecularly confirmed) XLMTM tissues could not be reproduced in vitro in myotube cultures subsequently obtained from those examined tissues. They explained their observations by an insufficient differentiation of the myotubes and by a possible neural involvement in the XLMTM muscular defects. Our results on double labeling experiments for desmin and vimentin (Fig. 2) demonstrate that there is no phenotypic difference in expression of these proteins during the late nerve-dependent steps of myogenesis, although the human muscle components were allowed to reach much further differentiation as compared with aneurally-cultured myotubes. Our results

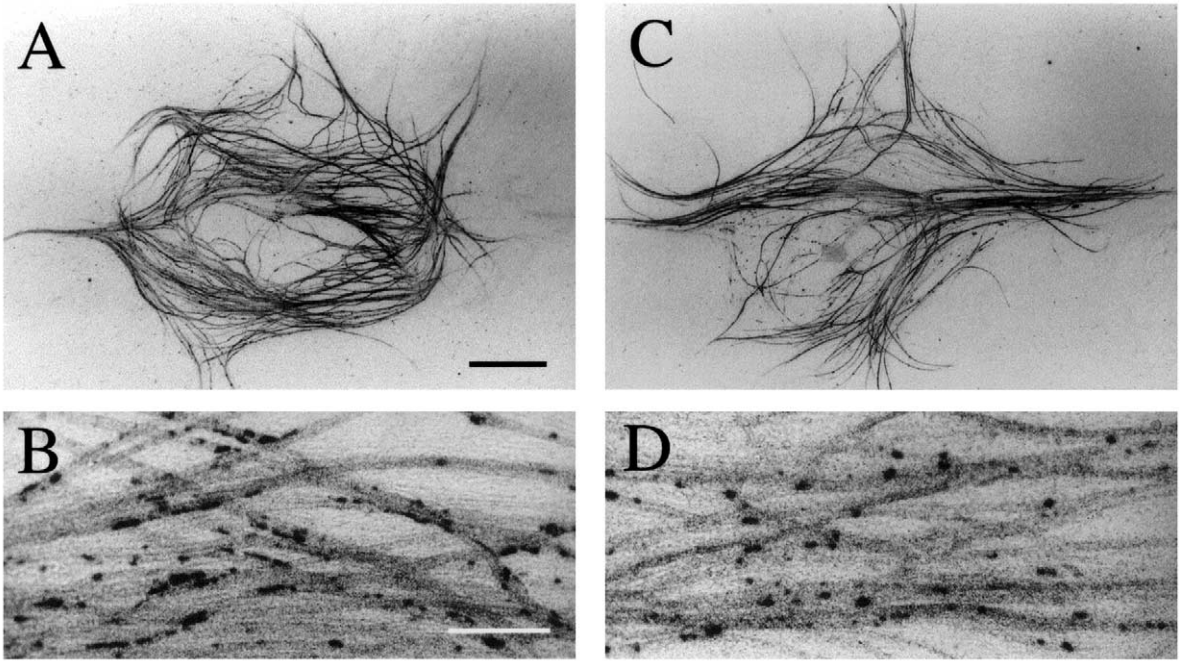

Fig. 6. Expression and aggregation of nAChR. The receptors were visualized after autoradiographic labeling using ^{125}I-αBTX. After exposure and development, innervated fibres appear in black in the dishes because of their richness in nAChR. Control muscle (A,B); XLMTM muscle (C,D). (A,C) Macroscopic views of representative areas occupied by innervated fibres. Note that single fibres spread over near 1 cm. Bar: 2 mm. (B,D) Higher magnification views of DMF showing clustered nAChR indicative of nerve-muscle contacts. Bar: 50 μm.

show that, in contrast to congenital myopathy muscle samples [41], DMF derived from XLMTM muscle cells seem not to overexpress desmin, and behave like normal cells with regard to their intermediate filament expression. This supports the hypothesis from Van der Ven et al. [14] that the abnormal expression of desmin and vimentin is not an intrinsic characteristic of XLMTM muscle. At the ultrastructural level (Fig. 5), it was impossible to detect any defect in the sarcomeric organization of XLMTM DMF in comparison with control ones. Nuclei, whatever their central or subsarcolemmal location, are not surrounded by aggregated organelles as those seen in XLMTM muscle tissue [9]. Taken together, our results indicate that despite the impairment of myotubularin function in the muscular components of the nerve-muscle cocultures, the numerous histopathological abnormalities of XLMTM muscle reported by others [9–11,41] are not reproducible in vitro. While associated with a pathological stage, they are likely to be indirect or non-specific consequences of myotubularin deficiency.

4.3. Responsiveness of XLMTM muscle cells to innervation

Control and XLMTM muscle cells share the same capability to be efficiently innervated as shown by appearance of myofibre contraction. Absence of myotubularin in human muscle cells do not disturb or delay the neuritogenesis of normal rat spinal neurons. It affects neither the capability to make nerve-muscle contacts, nor the motor neuronal coverage reflected by the area occupied by DMF in the vicinity of cord explants (Fig. 6) (measured as defined in [42]).

Structural abnormalities in NMJ [15,16] or in peripheral nervous system [43] have rarely been reported. Although this aspect is poorly-documented, XLMTM patients seem to have normal NMJ [9]. This study was not aimed to investigate the ultrastructure of NMJ in nerve-muscle coculture as was done by others [17,44,45]. However, they are expected to be normal for the following reasons: XLMTM myotubes do not affect neuritogenesis, are normally responsive to innervation (Figs. 1 and 3), and exhibit a normal nerve-induced muscle differentiation; XLMTM muscle fibres show a normal expression and clustering of nAChR (Fig. 6); and finally motor neurons are from normal rat spinal cord explants and are expected to express myotubularin.

4.4. XLMTM, a myopathy with intrinsically normal muscle cells?

It is surprising to observe that despite the numerous parameters we investigated, XLMTM muscle cells innervate and differentiate as well as control muscle cells do. Myotubularin can be detected after immunoprecipitation on cultured human and mouse myoblasts and myotubes, and on normal mouse adult tissue (J.L., in preparation). For these reasons, it is expected to be expressed in normal DMF in nerve-muscle cocultures. Myotubularin as been shown to decrease the level of phosphatidylinositol 3-phosphate in yeast [7,8]. This phospholipid is known to be involved in intracellular membrane trafficking. This suggests that possible function(s) of myotubularin on normal muscle might take place in late stages of myogenesis, by modulating muscle fibres responsiveness to growth and differentiation promoting stimuli, or by impairing vesicular transport to the plasma membrane or structural integrity of the fibres.

It is not known whether related members from the *MTM1* gene family [5] may compensate for the myotubularin deficiency in human. *MTM1*, *MTMR1*, and *MTMR2* form a subgroup of genes with high sequence homology in human and all three have an ubiquitous expression. However, it seems unlikely they would share exactly the same functions as they have diverged for a long time during evolution [5]. Moreover, mutations in the *MTM1* gene can be detected in about 85% of clinically diagnosed XLMTM cases [28]. *MTMR1* has not been found mutated in any case so far investigated. Finally, *MTMR2* has been very recently identified as one of the genes causing Charcot–Marie–Tooth type 4B [46], an autosomal recessive demyelinating neuropathy. Taken together with the various medical complications affecting most of the long-term survivors with XLMTM [2], the role of *MTMR2* clearly illustrate that myotubularin and related phosphatases must have pleiotropic roles which are not restricted to muscle.

To conclude, our findings strongly support the hypothesis that XLMTM muscle might be intrinsically normal. They are fully consistent with a possible involvement of the peripheral nervous system in the pathogenesis of the disease. As XLMTM is a developmental disorder, the fact that not only muscles but also motor neurons are myotubularin deficient in XLMTM patients should be taken into account to explain the muscular defects observed in the disease. Because of the continuous vital and trophic dialogue motor nerves and muscle cells exert on each other, one cannot exclude that a motor neuronal substrate should be modified by myotubularin to trigger and/or maintain muscle differentiation via an anterograde mechanism, the absence of such a mechanism leading to the myotubular phenotype.

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