Expression of Myogenic Proteins in Vitro in Myoblasts and Myotubes of Normal and Dystrophic Muscles of (ReJ 129) Mice.

Lenny Brocks, Peter van der Ven and Ad M. Stadhouders

Department of Cell Biology and Histology, Faculty of Medical Sciences, University of Nijmegen, The Netherlands.

Abstract

The expression of several muscle specific proteins was studied in cultured myoblasts and myotubes of hindlimb muscles of normal (Dy/Dy or Dy/dy) and dystrophic (dy/dy) mice of the ReJ 129 strain.

In myoblasts desmin and myosin stained in a diffuse way. Vimentin and TRITC-phalloidin staining reaction consisted of bundles of filaments, while nebulin and tropomyosin showed no immuno-reactivity in myoblasts.

In the myotubes desmin showed an irregular filamentous pattern, with only occasionally an indication of periodicity. No reaction was seen with vimentin, while myosin, nebulin, tropomyosin antibodies and TRITC-phalloidin antiserum each stained in a characteristic regular banding pattern.

No clear differences were seen in the reaction patterns of all the antibodies used in this study between myosatellite cells and myotubes in normal and in dystrophic mouse cultures.

Key Words: murine muscular dystrophy, mouse, muscle culture, cytoskeleton, contractile proteins, development.

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The term muscular dystrophy refers to a group of genetically determined diseases with progressive degeneration of skeletal muscle. Of the various human muscular dystrophies the basic defect is known only of a few, particularly of the severely progressive X-linked Duchenne's dystrophy (DMD) and Becker's muscular dystrophy (BMD), which is milder in severity. A large protein called dystrophin is either absent or defective [25, 19, 15] in these diseases. Although it is likely that dystrophin plays a role in the maintenance of architecture and function of the myofibre membrane, the exact function of this protein is still a puzzle. Dystrophin is also missing in the X-linked mdx mouse muscular dystrophy. However, despite the homology from a genetical point of view with human DMD, the two conditions are both clinically and pathologically very different. In many other types of muscular dystrophy, occurring either in humans or animals, the molecular basis and pathogenetic mechanisms of the disorder are unknown. Research on animal models other than the mdx mouse is relevant to obtain more knowledge about basic biological processes of progressive muscular dystrophy, in particular to provide more insight into the pathogenetic mechanisms that lead to the specific pathology and/or tissue restauration in the various muscular dystrophic conditions. The Bar Harbor ReJ 129 dy/dy strain is of particular interest, because this model, although genetically different from human DMD/BMD, shows various clinical and histopathological similarities with human DMD/BMD.

Muscular dystrophy in the mouse strain ReJ 129 dy/dy is a highly progressive, autosomal, recessive disease, caused by one gene. Muscle fibre necrosis, abundant connective tissue infiltration and pronounced structural disorganization, eventually leading to complete impairment of the muscle [26, 36, 31], strongly resembles the histopathology seen in DMD. Number of recent studies indicate that altered myofibre differentiation may play a role in the development of the disease. Wirtz et al [36], using enzyme histochemical methods to identify the various muscle fibre types, reported a delayed maturation of part of the myofibres in the dystrophic hindlimb muscle. Reggiani et al [29], using immunohistochemical techniques, showed that with regard to myosin heavy chain (MHC) expression, the myofibre maturation in dystrophic animals is retarded. The

disappearance of neonatal MHC was found to be delayed in the all studied hindleg muscles of dystrophic mice, except for the soleus.

In the process of myofibre maturation the differentiation of the cytoskeleton is important in constructing the ordered organization of myofibrils. It is known that both desmin and vimentin are distributed as longitudinal filaments in immature myotubes, whereas during maturation, vimentin disappears and desmin is redistributed in a cross-striated pattern [10, 13]. Brocks et al [4] studying mice older than 10 days of age found no differences in vimentin and desmin expression between normal and dystrophic mice. However, this does not rule out the possibility that altered myofibre cytoskeletal differentiation may have occurred during earlier stages of muscle genesis, for instance in the prenatal period.

To study whether differences between normal and dystrophic mice can be established in the earliest stages of myofibre formation and differentiation, a myosatellite cell culturing system was arranged and the expression of a number of cytoskeletal and contractile proteins during early myotube formation was studied.

Materials and Methods

Animals

Normal (Dy/Dy or Dy/dy) and dystrophic (dy/dy) mice of the strain ReJ 129, originally obtained from Jackson Laboratories Bar Harbor, Maine, USA, were used in this study. The mice were kept under standardized conditions at the Central Animal Laboratory of the University of Nijmegen and had free access to food and water.

Three week old mice with distinct clinical signs of dystrophy were used for isolation of myoblasts. Clinically normal animals of the same age were used as controls. Animals were killed with an overdosis of pentobarbital sodium.

Culture media and buffers

Phosphate-buffered saline (PBS) contained: 145 mM NaCl, 5.4 mM KCl, 5 mM Na_2HPO_4, 25 mM glucose, 25 mM sucrose, 50 IU/ml penicillin, 10 g/ml streptomycin; pH 7.2. Incubation medium contained 0.15 % trypsin (Difco, Detroit, MI, USA), 0.1 % collagenase (Sigma, St. Louis, MO, USA), 0.1 % bovine serum albumin (BDH, Poole, UK) in phosphate buffer. Growth medium (a high nutritional culture medium) was composed of Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Paisley, UK) with 20 % fetal calf serum (HyClone Laboratories, Inc., Logan, UT, USA), 2 % chicken embryo extract (Flow Laboratories, Irvine, UK), 2 mM Glutamine (Gibco BRL, Paisley, UK), 100 U/ml penicillin (Gibco BRL, Paisley, UK) and 1 g/ml streptomycin (Gibco BRL, Paisley, UK). Differentiation medium (a low nutritional culture medium) contained 10 % horse serum (Flow Laboratories, Irvine, UK) instead of fetal calf serum.

Isolation of myosatellite cells

Hindleg muscles were excised under sterile conditions. Two different methods for isolation of myosatellite cells were used.

In the first method mononucleate cells were obtained using essentially the protocol of Yasin et al [37]. Briefly, small fragments of muscle tissue were kept in incubation medium at 37°C for 15 min. The top layer containing mononuclear cells was collected in growth medium. This procedure was repeated three times. After the third and fourth time the muscle fragments were triturated through a 10 ml pipette 10 times to release more mononuclear cells. The collected fractions were centrifuged for 10 min, 50 χg at room temperature. The pellets were resuspended in fresh growth medium and plated into 35 mm culture dishes (Costar, Cambridge, UK). The suspension was filtered (30 µm nylon mesh) to remove possible intact myofibres and plated into 35 mm culture dishes or on glass coverslips.

An alternative second method to obtain mononucleate cells was used, which was essentially according to the prescription of Askanas [1]. Briefly, the hindleg muscles were cut into small pieces (1 mm^3 each) and put into 35 mm culture dishes (6 or 7 per dish). After an abundant outgrowth of cells had emerged the muscles pieces were placed in new culture dishes. This procedure was repeated several times. An homogeneous mononucleate cell layer was established by dissociating the cell cultures using a incubation of 2 mM EDTA in buffer for 1 min followed by incubation with 0.02 % trypsin in buffer for 10 min. After centrifugating 5 min, 50 χg at room temperature the cells were resuspended in fresh growth medium.

Culturing of the cells

Further treatment of the cell cultures obtained through either of the two different methods was identical. The cells were cultured in a humidified incubator (Heraeus, Hanau, FRG) at 37 C and 7 $^1/_2$ % CO_2 in air. Culture medium was changed every second day. Growth medium was replaced by differentiation medium just before the cultures reached confluency or if myotubes arose.

Immunohistochemistry

The cultured cells were fixed three days after the appearance of myotubes. The petri-dishes were rinsed with PBS (137 mM NaCl; 13 mM $Na_2HPO_4.H_2O$; 3 mM KH_2PO_4; all from Merck, pH 7.4) and subsequently the cells were fixed in cold (-20°C) methanol for 1 min. The cells were then air dried for at least 60 min. Thereafter they were incubated with one of the antibodies described below in a moist chamber at 37°C for 60 min. After washing twice (15 min each) in PBS at room temperature the cells were incubated for 60 min at room temperature either with fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyanate (TRITC)-conjugated Swine-anti-Rabbit IgG, or with FITC- or TRITC-conjugated Rabbit-anti-Mouse IgG (DAKO-immunoglobulins a/s, Denmark), depending on the primary antibody used. After

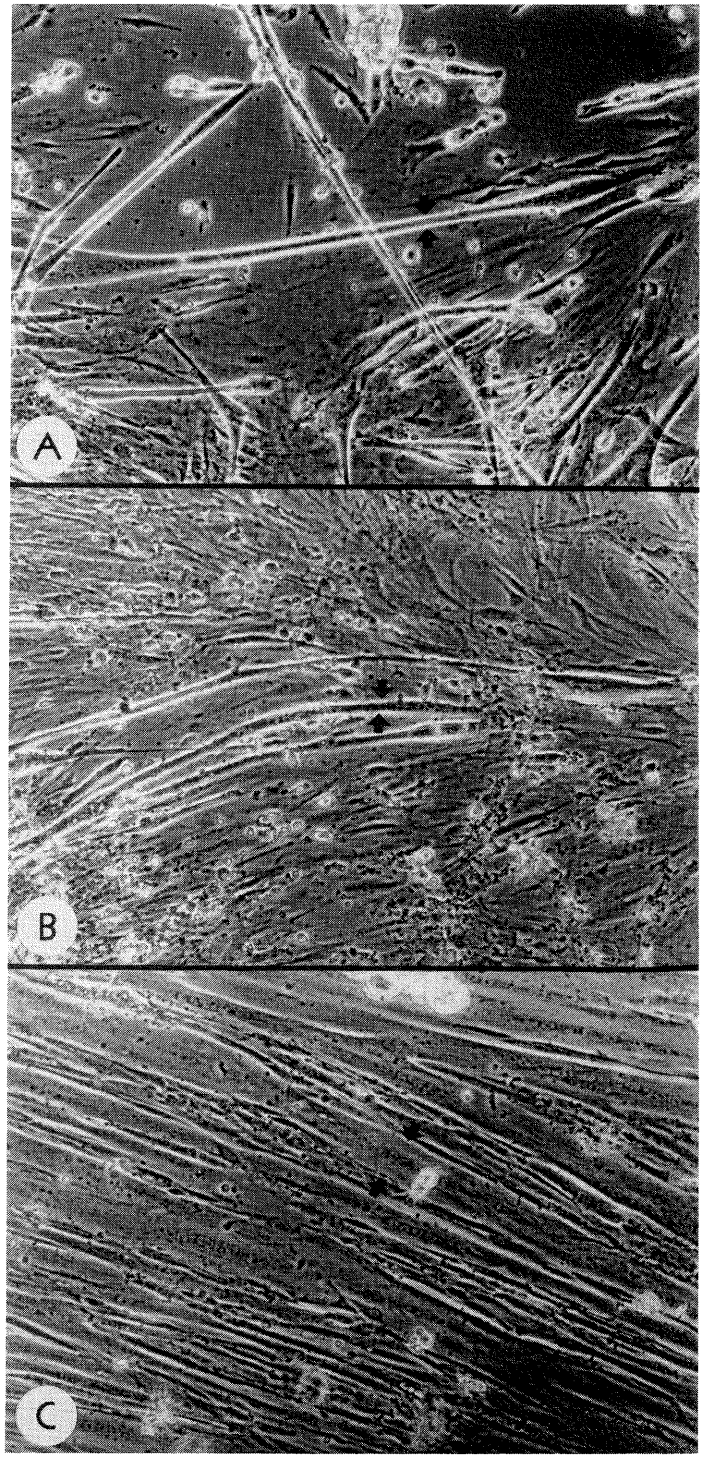

Figure 1. Phase contrast micrographs of cultured normal (a) and dystrophic (b) mouse and normal human (c) muscle cells. Arrows indicate the diameter of the myotubes. Magnification 120 x.

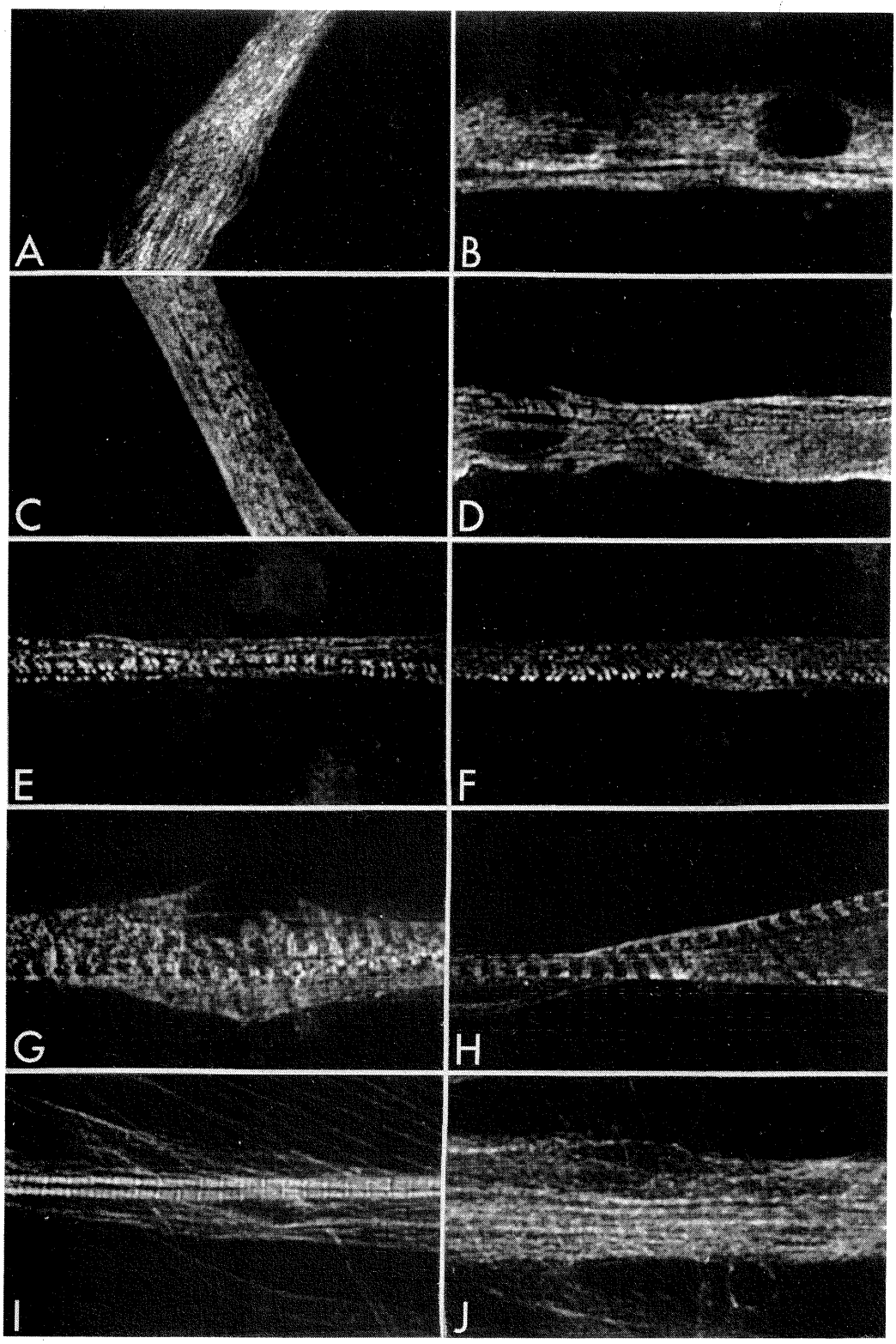

Figure 2. Immunofluorescence CSLM micrographs of cultured myotubes, arisen after fusion of myosatellite cells derived from normal (a,c,e,g,i) or dystrophic (b,d,f,h,j) hindleg mouse muscles of 20-22 day-old mice, and cultured for at least three days after the first appearance of myotubes, incubated with K5 desmin (a,b), MF20 myosin (c,d), NB2 nebulin (e,f), CH1 tropomyosin (g,h) or TRITC-phalloidin (i,j). Magnification 1400 x.

washing twice during 15 min in PBS at room temperature, the cells were mounted in Fluorostab (Euro-diagnostics, BV, Apeldoorn, The Netherlands).

Antibodies and reagents:

The following antibody preparations were used:

- A polyclonal rabbit antiserum directed against chicken gizzard muscle desmin (pDes). Preparation and characterization of this antiserum have been described by Ramaekers et al [27].
- An affinity purified rabbit polyclonal antiserum to bovine lens vimentin (pVim). Purification and characterization of this antiserum have been described by Ramaekers et al [28].
- A mouse monoclonal antibody to titin (9D10) [35, 11, 12, 13], obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Pharmacology and Molecular Sciences, John 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.
- A purified monoclonal antibody directed against chicken breast titin (T11) purchased from Sigma (St. Louis, MO, USA). Purification and characterization have been described by Fürst [8].
- A mouse monoclonal antiserum to striated muscle myosin (MF20) reacting with all sarcomeric mouse myosins (adult and embryonic) [2], which was a kind gift of Dr. D. Fischman, New York, USA.
- A mouse monoclonal antibody directed against chicken breast nebulin (NB2), purchased from Sigma (St. Louis, MO, USA). Purification and characterization have been described by Fürst [8].
- A mouse monoclonal antibody directed against chicken muscle tropomyosin (CH1) [22], 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.
- Tetramethylrhodamine isothiocyanate-labelled phalloidin (TRITC-phalloidin, Molecular Probes, Inc., Eugene, OR, USA) was used. This reagent stains filamentous actin (F-actin). A 3.3 M stock solution was diluted 1 : 25 in PBS.

Microscopic examination

The cells were examined and photographed with a Leitz MPV compact fluorescence microscope (Wetzlar, FRG) or with a Lasersharp MRC-600 confocal scanning laser microscope (Biorad, Oxfordshire, England).

Results

Microscopic characteristics of cultured muscle cells.

Cell isolation according to both procedures (see Materials & Methods) resulted in a high fraction of fibroblast-like cells in the cell cultures. Especially in dystrophic cell cultures the percentage of fibroblast-like cells was always extremely high (compare fig. 1a with 1b). These cells proliferate much faster than do the myosatellite cells and therefore quickly overgrow the cell culture system. This may interfere in the development and differentiation of the muscle cells. For this reason no cultures older than 7 days were used for analysis.

Myosatellite cells in dystrophic muscle cell cultures do not differ in morphology from those in normal cultures. They are spindle-shaped, mononucleate cells, with a high nucleus/cytoplasma ratio and are often somewhat ruffled at one or both ends. By simple phase-contrast microscopy the myosatellite cells are hardly distinguished from fibroblast-like cells, because the latter vary in shape from large, flat stellate cells to more spindle-shaped ones.

Fusion of myosatellite cells mostly occurred before cultures had reached confluency. Also the exchange of the high nutritional culture medium by the low nutritional medium was not necessary to induce myosatellite fusion. Fusion results in the formation of syncitia containing clusters of nuclei. These syncitia matured into myotubes with positioning of nuclei in a central core within four days of culturing (fig. 1a). Mouse myotubes are short and narrow-shaped. This contrasts with myotubes of human muscle cell cultures (compare fig. 1a,b with 1c; see arrows). Spontaneous rhytmic contraction of myotubes could be observed regularly. Dystrophic cultures differed from normal ones in that less myotubes were formed. On the other hand the myotubes formed in dystrophic cultures were morphologically not different from those in normal cultures (fig. 1b) with the exception that in dystrophic cell cultures myotubes were often shorter.

Antibody reactivity pattern

Although it is known that several of the antibodies used in this study cause profuse unspecific background staining in murine tissue, this was not obvious in the tissue cultures. The 9D10 monoclonal titin, although reactive in human muscle cultures [33], gave no reaction in mouse muscle cultures. Also with T11 monoclonal no positive reaction was observed in our muscle cultures. T11 was also negative in mouse muscular tissue sections and in muscular tissue of other species like man (unpublished results).

Mononuclear cells:

The K5 Polyclonal desmin antiserum appeared in the myoblasts derived from normal mouse muscle in a diffuse staining pattern with a random distribution of fluorescence. Non-muscle cells such as fibroblasts and fat cells did not stain with the desmin antiserum. With the K36 polyclonal vimentin antiserum myosatellite cells as well as fibroblast-like cells showed an intense reaction, consist-

ing of bundles of filaments in almost all mononuclear cells. The MF20 monoclonal myosin antibody stained muscle cells only. The MF20 staining reaction was mostly diffuse with a random distribution of fluorescence in the cell cytoplasm. The myoblasts showed no immuno-reactivity with the antibodies to nebulin and tropomyosin. Incubation with TRITC-phalloidin resulted in a staining reaction consisting of bundles of filaments in both mononuclear muscle and non-muscle cells.

Myotubes:

With K5 desmin antiserum an irregular filamentous reaction pattern was observed (fig. 2a) running along the whole length of the myotube. Only occasionally an indication of periodicity in these filaments could be detected. No reaction was seen with K36 vimentin antiserum. Myosin, nebuline and tropomyosin antibodies stained each in a characteristic regular double banding pattern (fig. 2c,e,g). Striation of myosin was mostly weak consisting of wide bands (fig. 2c). Nebuline showed a strong striation pattern consisting of distinct pairs of bands alternating with dark zones (fig. 2e). The antibody to tropomyosin reacted in a pattern of paired bands with a distinct non-reactive zone in between (fig. 2g). The paired bands were separated by broad unstained regions. TRITC-phalloidin reagent showed strong striations in almost all myotubes. The striation pattern consisted of strong lines appearing with a relatively constant distance (fig. 2i). Sometimes a couple of weaker band were seen in the dark zone.

Myotubes from normal and dystrophic mouse cultures reacted identical with all the antibodies used in this study (fig. 2b,d,f,h,j), which implicates that the antibodies used reacted to the same amount and in the same pattern of distribution in dystrophic myotubes as in normal myotubes. With the antibodies to tropomyosin and nebuline and with TRITC-phalloidin, striation was found in dystrophic cell cultures, although less frequently than in normal ones.

The results of the antibody staining reactions are summarized in table 1.

Discussion

Characteristics of antibody and reagent reactivity

Incubation with K5 desmin let many cells unstained. They presumably represent mainly fibroblastic cells and fat cells. The percentage of negative cells was even higher in the dystrophic cultures. Desmin cross-striation indicates a high degree of maturity of the myotubes [Van der Ven, personal communication; 33]. In the mouse myotubes sometimes an indication of periodicity was seen (fig. 2a,b). Although this periodicity does not coincide with the length of the sarcomeres, it might indicate one of the first steps leading to cross-striation, i.e. the mutual arrangement of myofibrils and their sarcomeres. The MF20 myosin was confined to the A-band staining sarcomeric myosin as seen in fig. 2c,d. The dark zone coincides with the Z-line. Kruger et al [20] showed that nebulin antibodies recognize epitopes within the I-band. Consequently the striation pattern seen with nebulin consists of a pair of distinct bands in the I-zone of the sarcomere (fig. 2e,f). The anti-tropomyosin staining is confined to the I-band. The narrow non-reactive line in between corresponds to the Z-line (fig. 2g,h). TRITC-phalloidin, staining strong lines alternated with weaker bands, reacts with filamentous actin (fig. 2i,j). Van der Ven et al [33] observed a similar reaction pattern in human myotubes. They suggest that the alternating weak and strong bands represent the thin filaments and the actin filaments beneath the sarcolemma at the level of the M-line respectivily.

Normal versus dystrophic muscle culture

Morphology

Normal cultures contained a high percentage of mononucleated non-muscle cells. In dystrophic cultures the number of non-muscle cells was extreme. This was most likely related to the age of the animals we used in our experiments, since a rapid progression of the disease can be observed during the second and third postnatal week. Especially during the third week infiltration of connective tissue and the appearance of macrophages and fat cells in

Table 1. Expression-patterns of muscle specific proteins during mouse myofibrillogenesis in vitro. Comparison between normal and dystrophic cells.

antibody/ reagent	antigen	normal myoblasts	dystrophic myoblasts	normal myotubes	dystrophic myotubes
K36	vimentin	filamentous	filamentous	negative	negative
K5	desmin	filamentous	filamentous	filamentous	filamentous
MF20	myosin	diffuse	diffuse	cross-striated	cross-striated
CH1	tropomyosin	negative	negative	cross-striated	cross-striated
NB2	nebuline	negative	negative	cross-striated	cross-striated
TRITC-phalloidin	F-actin	filamentous	filamentous	cross-striated	cross-striated

dystrophic hindleg muscle is high. Less myotubes and often shorter ones were observed in the dystrophic cultures. We suggest that this is due to the high amount of non-muscle cells, which interfers with the process of myosatellite cell-cell recognition and fusion. However, we can not exclude that other factors are of importance. Another possible explanation is that the readiness to fuse of the dystrophic myoblasts is less than that of the normal ones due to the genetic defect. Due to a slower than normal process of myoblast fusion in dystrophic cultures myotube growth could be less than in normal cultures. Totsuka et al [31] developed a model, in which it is proposed that growth of dystrophic myofibres is defective. In their "bone-muscle imbalance" hypothesis they assume that an age-related aggravation in this imbalance, due to a defective growth of the muscle in relation to a normal growth of the bone, is responsible for pathogenesis and progress of muscular dystrophy in the dy mouse. In the light of this hypothesis our findings are of interest, although better methods to exclude the interference of non-muscle cells during myotube formation are necessary to elucidate this point.

Antibody reactivity pattern

In the present investigation the expression of several cytoskeletal and contractile muscle specific proteins in cultured myofibres of dystrophic or normal origin are compared. Several studies suggest that cytoskeletal proteins are important in myofibre development and differentiation [7, 34]. Coexpression and sometimes even colocalization of different Intermediate Filament (IF) proteins is a common feature in developing embryonic tissues including skeletal muscle [3, 10, 21]. The expression of one protein can even trigger the appearance or differentiation of another [6, 9]. In the early stages of myofibrillogenesis the IF protein vimentin is expressed in the developing muscle cells, either without or in combination with desmin [3, 6, 9, 10]. Hill et al [14] observed that titin and myosin synthesis and their assembly into ordered striated filaments are linked during myofibrillogenesis in postmitotic mononucleated myoblasts. Colley [6] also showed that titin is already present in postmitotic myoblasts. They observed that titin appeared around the nucleus in a stable basket-like distribution and suggested that this protein could play an organizing role during subsequent early stages of myofibrillogenesis. Another indication for this organizing role of titin is given by Schaart et al [30], who showed that in mouse embryos cross striation of titin precedes differentiation of desmin. In our study no qualitative differences were found between normal and dystrophic cultured myotubes with the antibodies and antisera we used. Although less frequently striation was observed with the antibodies to tropomyosin and nebulin and with TRITC-phalloidin. The basic explanation for this phenomenon is the same one as for the earlier mentioned shorter myotube formation in dystrophic than in normal cultures. Due to a high amount of non-muscle cells interfering with myoblast fusion, or a lesser than normal readiness of dystrophic myoblasts to fuse, myotube formation could be a slower process in dystrophic cultures, although started at the same time as in normal cultures. This results in the presence of more different stages of differentiating myotubes and consequently less striation (striation is seen in later stages in the differentiating process). Further study is needed to elucidate if myofibre development and differentiation in normal and dystrophic muscle are different in these early stages.

Our earlier studies [29, 36] showed a difference in maturation of dystrophic myofibres compared with normal ones. An explanation that such a difference was not significantly present in these culture studies could be that differentiation of the myofibres in vitro is not far enough to elucidate a possible missing protein. Nerve supply, normally involved in maturation of myofibres and not present in culture, could also be an important factor.

In dystrophic dy animals muscular tissue damage becomes strongly progressive at the time the animals get more active, i.e. walking activity is more prominent. This makes it likely that stresses associated with contraction play a key role in the pathogenetic mechanisms. In this context it is of interest that in DMD/BMD the missing protein product dystrophin is a sarcolemma-associated cytoskeletal protein, suggesting that the function of dystrophin in normal muscle is to protect the fibre by providing mechanical strength for the sarcolemma. Recently it has been shown that dystrophin-like proteins (DMDL for Duchenne muscular dystrophy-like) are expressed in a wide range of tissues at varying levels [24]. The dystrophin-related locus is on the human chromosome 6 that maps close to the dy mutation on mouse chromosome 10 [23] and has been designated Dmdl. These findings strongly suggest that Dmdl are of importance in the dy muscular dystrophy. Support for this supposition comes from the findings that in the dy mouse both the peripheral nerves [16, 17] and neuromuscular junctions [32] are abnormal, while also defects were observed in the elasticity of dystrophic dy muscle [5]. Immunolocalization of Dmdl showed that these proteins were particularly present in the neuromuscular and myotendinous junctions, as well as peripheral nerves and vasculature of skeletal muscle [18]. These findings may be taken to support the suggestion that in the dy mouse defects in Dmdl proteins may play a key role in the pathogenesis, despite the fact that there is no apparent mutation or deletion in the Dmdl gene detected so far in dy mice [24].

We conclude that although muscle cell cultures can be a useful tool to study disorders in protein expression, in our case we couldn't observe any significant differences between normal and dystrophic muscle cultures, with regard to the muscle specific proteins we used. New methods should be explored to get a better separation between muscle cells and other types of mononuclear cells to exclude the possible interference of non-muscle cells on myotube formation and differentiation. Further study is needed to elucidate if myofibre growth and differentiation

in dystrophic muscles is slowed down or in another way defective.

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Address correspondence to:

Lenny Brocks, Department of Cell Biology and Histology, Faculty of Medical Sciences, University of Nijmegen, St. Adelbertusplein 1, 6500 HB Nijmegen, The Netherlands

References

[1] Askanas V, Gallez-Hawkins G: Synergistic influence of polypeptide growth factors on cultured human muscle. *Arch Neurol* 1985; 42: 749-752.

[2] Bader D, Tomoh M, Fischman DA: Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. *J Cell Biol* 1982; 95: 763-770.

[3] Bennett GS, Fellini SA, Toyama Y, Holtzer H: Redistribution of intermediate filament subunits during skeletal myogenesis and maturation in vitro. *J Cell Biol* 1979; 82: 577-584.

[4] Brocks L, Jap PHK, Ramaekers FCS, Stadhouders AM: Vimentin and desmin expression in degenerating and regenerating dystrophic murine muscles. *Virch Arch B Cell Pathol* 1991; 61: 89-96.

[5] Brocks L, Wirtz P, Loermans H, Binkhorst R: Effects of early immobilization on the functional capacity of dystrophic (ReJ 129 dy/dy) mouse leg muscles. *Int J Exp Path* 1992; 73: 223-229.

[6] Colley NJ, Tokuyasu KT, Singer SJ: The early expression of myofibrillar proteins in round postmitotic myoblasts of embryonic skeletal muscle. *J Cell Sci* 1990; 95: 11-22.

[7] Fischman DA: Myofibrillogenesis and the morphogenesis of skeletal muscle, in Engel AG and Banker BQ (eds): *Myology; Basic and Clinical.* McGraw-Hill, New York, 1986, pp 5-30.

[8] Fürst DO: The organization of titin filaments in the half-sarcomere revealed by monoclonal antibodies in immunoelectron microscopy: a map of ten nonrepetitive epitopes starting at the Z-line extends close to the M-line. *J Cell Biol* 1988; 106: 1563-1572.

[9] Fürst DO, Osborn M, Weber K: Myogenesis in the mouse embryo: Differential onset of expression of myogenic proteins and the involvement of titin in myofibril assembly. *J Cell Biol* 1989; 109: 517-527.

[10] Gard DL, Lazarides E: The synthesis and distribution of desmin and vimentin during myogenesis in vitro. *Cell* 1980; 19: 263-275.

[11] Gassner D: Myofibrillar interaction of blot immunoaffinity-purified antibodies against native titin as studied by indirect immunofluorescence and immunogold staining. *Eur J Cell Biol* 1986; 40: 176-184.

[12] Greaser ML, Handel, SE, Wang, S-M, Schultz E, Bulinski JC, Lessard JL: Assembly of titin, myosin, actin and tropomyosin into myofibrils in cultured chick cardiomyocytes, in Stockdale F, and Kedes L, (eds): *Cellular and Molecular Biology of Muscle Development, UCLA Symposium on Molecular and Cellular Biology, New Series*, Alan R Liss, New York 1989; 93: 246-257.

[13] Handel SE, Wang S-M, Greaser ML, Schultz E, Bulinski JC, Lessard JL: Skeletal muscle myofibrillogenesis as revealed with a monoclonal antibody to titin in combination with detection of the α- and γ-actin. *Develop Biol* 1989; 132: 35-44.

[14] Hill CS, Duran S, Lin Z, Weber K, Holtzer H: Titin and myosin, but not desmin, are linked during myofibrillogenesis in postmitotic mononucleated myoblasts. *J Cell Biol* 1986; 103: 2185-2196.

[15] Hoffman EP, Monaco AP, Feener CA, Kunkel LM: Conservation of the Duchenne muscular dystrophy gene in mice and humans. *Science* 1987; 238 347-350.

[16] Jaros E, Jenkison M: Defective differentiation of peripheral nerves in the dystrophic mouse. *Develop Brain Res* 1983; 6: 231-242.

[17] Jaros E, Jenkison M: Quantitative studies of the abnormal axon-schwann cell relationship in the peripheral motor and sensory nerves of the dystrophic mouse. *Brain Res* 1983; 258: 181-196.

[18] Khurana TS, Watkins SC, Chafey P, Chelly J, Tomé FMS, Fardeau M, Kaplan J-C, Kunkel LM: Immunolocalization and developmental expression of dystrophin related protein in skeletal muscle. *Neuromuscular Disorders*; 1: 185-194.

[19] Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener CA, Kunkel LM: Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell* 1987; 50: 509-517.

[20] Kruger M, Wright J, Wang K: Nebulin as a length regulator of thin filaments of vertebrate skeletal muscles: Correlation of thin filament length, nebulin size, and epitope profile. *J Cell Biol* 1991; 115: 97-107.

[21] Lazarides E: Intermediate filaments: A chemically heterogeneous, developmentally regulated class of proteins. *Ann Rev Biochem* 1982; 51: 219-250.

[22] Lin JJ, Helfman DM, Hughes SH, Chou CS: Tropomyosin isoforms in chicken embryo fibroblasts: purification, characterization, and changes in Rous sarcoma virus-transformed cells. *J Cell Biol* 1985; 100: 692-703.

[23] Love DR, Hill DF, Dickson G, Spurr NK, Byth BC, Marsden RF, Walsh FS, Edwards YH, Davies KE: An autosomal transcript in skeletal muscle with homology to dystrophin. *Nature* 1989; 339: 55-58.

[24] Love DR, Morris GE, Ellis JM, Fairbrother U, Marsden RF, Bloomfield JF, Edwards YH, Slater CP, Parry DJ, Davies KE: Tissue distribution of the dystrophin-related gene product and expression in the mdx and dy mouse. *Proc Natl Acad Sci USA* 1991; 88: 3243-3247.

[25] Monaco AP, Neve RL, Colletti-Feener CA, Bertelson CJ, Kurnit DM, Kunkel LM: Isolation of candidate cDNAs for portions of the Duchenne muscular dystrophy gene. *Nature* 1986; 323: 646-650.

[26] Platzer A: Embryology of two murine muscle diseases: muscular dystrophy and muscular dysgenesis, in Harris JB (ed): *Muscular Dystrophy and Other Inherited Diseases of Skeletal Muscle in Animals*, New York Academy of Sciences. 1979, pp. 94-114.

[27] Ramaekers FCS, Moesker O, Huijsmans A, Schaart G, Westerhof G, Wagenaar SjSc, Herman CJ, Vooijs GP: Intermediate filament proteins in the study of tumor heterogeneity: an in-depth study of tumors of the urinary and respiratory tracts. *Ann NY Acad Sci* 1985; 455: 614-634.

[28] Ramaekers FCS, Puts JJG, Moesker O, Kant A, Huijsmans A, Haag D, Jap PHK, Herman CJ, Vooijs GP: Antibodies to intermediate filament proteins in the immunohistochemical identification of human tumours: an overview. *Histochem J* 1983; 15: 691-713.

[29] Reggiani C, Brocks L, Wirtz P, Loermans H, te Kronnie G: Myosin isoform expression in hindlimb muscles of normal and dystrophic (ReJ129 dy/dy) mice. *Muscle Nerve* 1992; 14: 199-200.

[30] Schaart G, Viebahn C, Langmann W, Ramaekers F: Desmin and titin expression in early postimplantation mouse embryos. *Developm* 1989; 107: 585-596.

[31] Totsuka T, Watanabe K, Uramoto I: A bone-muscle imbalance hypothesis for the pathogenesis of murine muscular dystrophy, in Ebashi S and Ozawa E, (eds): *Muscular Dystrophy: Biomedical aspects* Japan Sci. Soc. Press, Tokyo, Springer-Verlag, Berlin, 1983, pp 29-38.

[32] Tremblay JP, Grégoire L, Sasseville R, Guay G, Belhumeur C: Reduction of postjunctional fold density and depth in dystrophic mice. *Synapse* 1988; 2: 148-156.

[33] Van der Ven P, Schaart G, Jap PHK, Sengers RCA, Stadhouders AM, Ramaekers FCS: Differentiation of human skeletal muscle cells in culture. Maturation as indicated by titin and desmin striation. *Cell Tissue Res* 1992; in press.

[34] Wang K: Cytoskeletal matrix in striated muscle: The role of titin, nebulin and intermediate filaments. *Adv Exp Med Biol* 1984; 170: 285-305.

[35] Wang S-M, Greaser ML: Immunocytochemical studies using a monoclonal antibody to bovine cardiac titin on intact and extracted myofibrils. *J Muscle Res Cell Motil* 1985; 6: 293-312.

[36] Wirtz P, Loermans HMTh, Peer PGM, Reintjes AGM: Postnatal growth and differentiation of muscle fibres in the mouse. II A histochemical and morphometrical investigation of dystrophic muscle. *J Anat* 1983; 137: 127-142.

[37] Yasin R, v Beers G, Nurse KSE, Al-Ani A, Landon DN, Thompson EJ: A quantitative technique for growing human adult skeletal muscle in culture starting from mononucleated cells. *J Neurol* 1977; 32: 347-360.