Vimentin and desmin expression in degenerating and regenerating dystrophic murine muscles

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Summary. The distribution of the intermediate filament proteins (IFP) desmin and vimentin was studied in gastrocnemius, plantaris and soleus muscles of the dystrophic mouse strain ReJ 129 during postnatal development. Special attention was paid to the overall morphological changes in the distribution of these cytoskeletal constituents in degenerating and regenerating muscle fibres. In contrast to their normal counterparts, the dystrophic mice (ReJ 129 dy/dy) appeared to develop four types of distinct muscle fibres with immunohistochemically detectable aberrant IFP patterns. The distribution of desmin IFP differed in the dystrophic muscle fibres as compared to the normal fibres in that juxtanuclear aggregates of IFP were frequently seen. In contrast to the recent literature we conclude that these cells are regenerated myofibres exhibiting defective nuclear migration.

Key words: Vimentin – Desmin – Cytoskeleton – Dystrophy – Mouse – Muscle

Introduction

Muscular dystrophy in the mouse strain ReJ 129 (dy/dy) is a monofactorial, autosomal recessively inherited defect. For many years this strain has served as a model for human muscular dystrophy. In the human system, the defective gene responsible for Duchenne muscular dystrophy (DMD) has been identified (Monaco et al. 1986; Koenig et al. 1987; Hoffman et al. 1987), while it has also been shown that the normal gene product, dystrophin, is deficient in muscles of individuals with DMD. However, the expression of dystrophin is normal in mouse strain ReJ 129 (own unpublished observation). Therefore this strain seems not to be a suitable model for DMD, but clearly suffers from a different kind of dystrophy, with clinical similarities to DMD. For example, the disease is progressive and interferes with postnatal development (Wirtz et al. 1983). The course of the disease in this mouse strain is unknown, but abnormalities in muscle (Banker 1968; Meier 1967; Platzer and Chase 1964) and nerve (Biscoe et al. 1975; Bradley and Jenkinson 1973; Jaros and Bradley 1978; Bradley and Jaros 1979) were observed at early stages. Especially, during the second and third postnatal weeks the dystrophic disease progresses rapidly (Wirtz et al. 1983), coinciding with the period that the animals become mobile.

Several studies have indicated that altered muscle development may play a role in the pathogenesis of dystrophy in the mouse (Dangl et al. 1987; Skau 1983; Tozuka et al. 1981). Recently, Reggiani et al. (1991) showed that isomyosin expression is altered in dystrophic mice. At the age of 10 days, even before the appearance of any marked histopathological features, more myofibres with immature (neonatal) myosin were detected as compared to normal controls.

Intermediate filaments (IF) have been shown to alter their composition and structural organization during maturation of myofibres. These cytoskeletal structures are suggested to be functional in the maintenance of the ordered organization of myofibres (Gard and Lazarides 1980) but in addition also for establishing the lateral registry of the sarcomeres. The main component of neonatal and adult muscle IF is desmin (Lazarides 1978, 1980; Thornell et al. 1983). Muscle fibre differentiation is accompanied by a profound redistribution of cytoskeletal components, paralleled by a switch in gene expression. In myoblasts a major IF protein is vimentin. Gene expression of this protein is suppressed upon differentiation while desmin gene expression is up regulated (Wahlen et al. 1976; Gard and Lazarides 1980; Fischman and Danto 1985).

Only a few workers have investigated the possible involvement of these IF proteins in the pathogenesis of muscular dystrophies (Thornell et al. 1980, 1983). In the present investigation, the expression of desmin and vi-
Mentin was studied during normal and dystrophic postnatal development of mice up to 2 months. The aim of this study was to examine whether or not altered IF protein expression or an altered IF protein distribution could be correlated with defective maturation of diseased muscles.

Materials and methods

Animals. Mice of the Rey 129 strain, raised from breeding pairs originally obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and maintained at the Central Animal Laboratory of the University of Nijmegen, were used. The mice were kept under standardized conditions and received food and water ad libitum.

Dystrophic (dy/dy) animals were obtained by mating heterozygous (Dy/dy) mice. Homozygous (Dy/Dy) mice served as controls.

Muscle tissue preparation. Animals were anaesthetized with sodium pentobarbital. Three hindleg muscles (gastrocnemius, plantaris and soleus) were excised and quickly frozen in isopentane chilled with liquid nitrogen. Muscle tissue from mice aged 3, 8, 10–11, 14–15, 17–18 and 20–21 days, and 1 and 2 months were examined.

Serial transverse 8 μm thick sections were cut in a Walter Dites cryostat (−25°C). The sections were stored on Multispot slides (Flow Laboratories) at −15°C until use.

Histology. To assess the overall morphological changes in the muscle the frozen sections were stained with Sirius red (SR) according to Sutter et al. (1964).

Methyl green pyronin (MGP) staining according to Trevan and Sharrock (1951) was used to detect RNA in regenerating fibres.

The sections were air dried for 60 min and pretreated for 5 min with phosphate buffered saline (PBS; pH 7.4) containing 0.5% Triton X-100. They were incubated with the appropriate antibody in a moist chamber at 37°C for 60 min. After washing twice in PBS for 15 min each at room temperature the sections were incubated with FITC-conjugated Swine-anti-Rabbit IgG (DAKO-immunoglobulins a/s, Denmark) for 60 min at room temperature. After two washes of 15 min each with PBS at room temperature the sections were stained with 0.01 M Ethidium Bromide (EtBr) for 30 sec to detect the DNA and RNA. After washing with PBS the sections were mounted in Fluorobat (Euro-diagnostics BV, Apeldoorn, The Netherlands).

Fig. 1. Immunofluorographs of muscle from 3 days a, b and 2 months old c, d normal mice in serial transverse sections viewed by confocal scanning laser microscope (CSLM), stained for vimentin (blue-green) and DNA (orange) a, c, or desmin (blue-green) and DNA (orange) b, d. White arrow indicates a myofibre in optimal cross section showing a fluorescent intermyofibrillar network. Black arrow indicates a myofibre, with a fluorescent pattern of concentric circles. Magnification 300×.


**Antisera.** The following polyclonal antisera were used:

- An affinity purified rabbit antiserum raised against vimentin, isolated from calf lens by preparative gel electrophoresis as described by Ramaekers et al. (1981).
- A rabbit antiserum directed against chicken gizzard muscle desmin prepared as described by Ramaekers et al. (1983).

The specificity of both antisera in mouse tissues has been extensively tested and proven (Pieper et al. 1989; Schaart et al. 1989).

**Microscopic examination.** The tissue sections were examined and photographed with a Leitz MPV compact fluorescence microscope (Wetzlar, FRO) and with a Lasersharp MRC-500 confocal scanning laser microscope (Biorad, Oxfordshire, England).

**Results**

**Vimentin and desmin in normal muscle**

Vimentin staining (Fig. 1a, c) is negative in the myofibres of muscles of all animals studied (from 3 days up to 2 months). Vimentin is distinctly present in connective tissue cells, tendons and nerves as well as in endothelial cells of capillaries and larger vessels, and some smooth muscle cells.

The distribution of desmin in myofibres of 3 day-old animals is coarsely granular in all muscles studied (Fig. 1b), although some staining is concentrated around the Z-discs.

From the age of 14 days onwards, the mature staining pattern appears (Fig. 1d) i.e. uniformly concentrated close to the sarcolemma with a high concentration at the Z-discs appearing in a fluorescent intermyofibrillar network in the optimal cross sections (Fig. 1d: white arrow) or in a fluorescent pattern of concentric circles and parallel lines (Fig. 1d: black arrow).

**Vimentin and desmin in dystrophic muscle**

The overall histological appearance shows no differences between muscles of healthy and dystrophic mice up to 10–11 days. Immunohistochemical staining reactions for desmin and vimentin show similar patterns in both healthy and diseased mice up to this age. After 10–11 days vimentin remains absent from the "first generation" myofibres in all animals studied. However, in areas with connective tissue proliferation (Fig. 2a, c and Fig. 3b, c), small, strongly vimentin-positive cells become apparent. Such cells are particularly obvious in muscles of 14 day-old animals. Staining of these cells with EtBr reveals a weak RNA reaction in the cytoplasm in addition to the strong reactivity in the nucleus, whereas incubation with methyl green pyronin (MGP) results in a red staining reaction in the cytoplasm in addition to a green nuclear staining (results not shown).

Vimentin expression in nerves, connective tissue, capillaries and large blood vessels as well as tendons is similar to that observed in normal muscle tissue.

In 10–11 day-old dystrophic muscles desmin shows a uniform staining at the periphery and a more or less granular central staining in the "first generation" myo-fibres (results not shown). Particularly at this age, but to a lesser extent also at all later stages, rounded desmin-negative myofibres are observed. Figures 2f and 3h show this cell type in 24 and 14 day-old animals, respectively. These cells are also negative for vimentin (Fig. 2e), and show a pale sarcoplasmic staining in Sirius red stained sections (Fig. 3i). The latter staining reaction reveals nicely the contours of these desmin-negative muscle fibres (compare Fig. 3i, j), showing that no significant difference in the diameter of these cells as compared to normal muscle fibres exists.

In dystrophic animals of 14 days and older, desmin shows the normal, adult distribution pattern (Figs. 2b, 3a). In these morphologically normal "first generation" myofibres desmin is uniformly distributed at the periphery with a reticular sarcoplasmic staining (or with a staining pattern of concentric circles or parallel lines). However, next to these fibres, the areas of proliferated connective tissue show small, strongly desmin-positive cells (Fig. 2b, d, 3b). When comparing these desmin staining patterns with the vimentin reaction in serial sections it appears that many of these cells co-express both types of IF protein (compare Fig. 2a with 2b, 2c with 2d and Fig. 3b with 3c).

In 17–18 day-old dystrophic animals, many small myofibres with centrally located nuclei are seen (Fig. 2d). These cells show a desmin distribution pattern that appears to be more mature than the desmin pattern observed in the small fibre cells, which co-express desmin and vimentin (compare Fig. 3b with 3d, 3e). In addition to a staining pattern associated with the Z-discs, these cells also contain centrally located desmin accumulations (Fig. 3d) or a desmin reaction surrounding the nucleus (Fig. 3e). They are negative for vimentin, while MGP gives a faint cytoplasmic staining reaction. Incubation with EtBr shows centrally located nuclei in the fibres and clearly demonstrates that the desmin accumulations are associated with the periphery of the nuclei (compare Figs. 2d and 3d). No RNA-related EtBr-fluorescence is seen in the fibre cytoplasm.

At the age of 21 days and older, small myofibres are still evident, but also many myofibres with centrally located nuclei are seen (Fig. 3f, g). These myofibres have diameters comparable to those of healthy mice of the same age, and contain perinuclear desmin-accumulations or a desmin reaction surrounding the nucleus.

At the age of 1 month and 2 months the total number of myofibres per muscle appears to be decreased, while the relative amount of connective tissue has increased (data not shown). Compared to muscles of 21 day-old dystrophic mice, 1 month old muscles contain more large myofibres with centrally located nuclei.

**Correlation between age and the occurrence of the four different types of muscle cells in the dystrophic mouse**

Table 1 correlates the occurrence of the pathologically altered types of muscle fibres with the age of dystrophic mice. The peak occurrence of the various aberrant types of muscle fibres is seen at different ages of the
Fig. 2. Immunofluorographs of dystrophic mouse muscle in serial transverse sections viewed by CSLM, stained for vimentin (blue-green) and DNA (orange) a, c, e, or desmin (blue-green) and DNA (orange) b, d, f. Magnification 300 x. a, b Two and a half weeks old mouse. Black arrows indicate cells co-expressing vimentin and desmin. c, d Three and a half weeks old mouse. Black arrows indicate cells co-expressing vimentin and desmin. White arrows indicate small cells with central nuclei expressing desmin. e, f Three and a half weeks old mouse. Stars indicate desmin negative cells.
Fig. 3. Different types of myofibres observed in dystrophic mouse muscle after staining for desmin a, b, d, e, f, g, h or vimentin c, as referred to in Table 1. I Sirius red staining of the fibres shown in j. j A desmin stained section (using peroxidase), in order to reveal the contours of the desmin negative cells. Magnification 600 × (a i/m h), 150 × i, j.
Table 1. Correlation of immunohistochemically defined muscle cell type and age of dystrophic mice

<table>
<thead>
<tr>
<th>Muscle cell type</th>
<th>Age in days</th>
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<tbody>
<tr>
<td>Large, desmin negative cells</td>
<td>8, 11, 14, 17, 21, 30, 60</td>
</tr>
<tr>
<td>Small, vimentin and desmin positive cells</td>
<td></td>
</tr>
<tr>
<td>Small, desmin positive cells with</td>
<td></td>
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<tr>
<td>perinuclear aggregates</td>
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<td>Large, desmin positive cells with</td>
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<td>perinuclear aggregates</td>
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+++, major fraction; ++, minor fraction; ±, sporadically present; −, not present

dystrophic process, suggesting a specific sequential appearance of these morphologically distinct types of fibres.

Discussion

In normal muscle development of various animal species it has been shown that putative myoblasts synthesize vimentin as a major constituent of their intermediate filaments (Gard and Lazarides 1980). After fusion of myoblasts, the rate of desmin synthesis increases considerably, while vimentin expression decreases (Fischman and Danto 1985). Initially, the organization of desmin and vimentin is diffuse in the myotubes but desmin gradually concentrates around the Z-discs (Bennett et al. 1979; Gard and Lazarides 1980).

Our results show that in the myofibres of healthy mice vimentin is absent already 3 days after birth and that desmin is fully organized in the myofibres around 14 days after birth. This coincides with the period of markedly increased animal mobility (Wirtz et al. 1983). The expression of myosin heavy chain (MHC) also changes at this stage, with the neonatal MHC being down regulated totally in the period between 14 and 21 days after birth. During the developmental stages, the amount of myofibres containing adult MHC types (slow or fast MHC) increases (Reggiani et al. 1991).

As is the case in healthy muscles, it appears that during development of dystrophic muscle vimentin is already absent in all myofibres shortly after birth. Up to 10–11 days after birth, desmin displays the same differentiated organization as in normal myofibres from homozygous healthy animals of the same age. Our results also demonstrate that the level of expression and mode of organization of desmin in histologically normal myofibres, which are seen at all stages of the dystrophic process in addition to affected fibres, is comparable to that observed in normal mice. So with regard to desmin and vimentin the cytoskeleton seems to be normally developed in non-affected myofibres of dystrophic animals. This is in contrast to what is seen in myosin HC expression during postnatal development. With regard to the myosin HC expression, Reggiani et al. (1991) reported a retarded maturation of all myofibres of dystrophic animals. At the age of 10 days, a distinctly higher percentage of myofibres containing neonatal myosin HC was detected in dystrophic mice as compared to normal mice. Using enzyme histochemical methods for myosin ATPase and oxidative activity of the myofibres Wirtz et al. (1983) already suggested a delayed maturation of dystrophic myofibres.

After the first 14 days of life, the disease becomes clinically evident resulting in an impaired mobility behaviour (Wirtz et al. 1983). From 14 days on a sequence of (immuno)histochemical changes can be observed in the dystrophic muscles. Firstly, the occurrence of large, rounded desmin negative myofibres is observed with their sarcoplasm staining weakly with Sirius red. This type of myofibre is observed starting from 10–11 days after birth. Since this type of fibre stains pale in Sirius red we conclude that these myofibres are in an initial phase of degeneration. Also Thornell et al. (1983) and Helliwell (1988) showed that necrotic myofibres can be identified by their complete lack of desmin reactivity.

Thereafter, many small cells, which are both desmin and vimentin positive, are observed within the zones of connective tissue proliferation. Both MGP and EtBr staining reactions indicate a high concentration of RNA in these cells. From the fact that these fibres are observed only subsequently to the appearance of degenerating fibres, we assume that these small fibres represent a stage of regeneration in the diseased muscle.

At a slightly later stage of regeneration these small fibres cease vimentin synthesis, but retain a desmin reactivity partially located at the Z-discs. In addition, they show either centrally located desmin-positive aggregates or circumscribed desmin-negative areas in the centre of the fibre. Judging from the positive DNA staining of the central spots it is concluded that these cells are young regenerating myofibres with central nuclei.

In adult animals (1–2 months old) the total number of myofibres per muscle has decreased. This is in agreement with Wirtz et al. (1983) and Reggiani et al. (1991). Also many fibres with centrally located nuclei are observed. Their average diameter is similar to that of normal muscle fibres. A mature distribution of desmin is observed in these cells with the exception that a perinuclear aggregate of desmin or a desmin reaction surrounding the nucleus is present. In our opinion these cells represent regenerating myofibres originating from the small regenerating fibre type, but failing to mature normally as concluded from the fact that migration of the nucleus towards the sarcolemma does not occur (at least not until the age of 2 months). Even at the age of 7 months many large myofibres with central nuclei are seen, as shown by Wirtz et al. (1983). Wirtz et al. (1982) reported that such myofibres with central nuclei were
not present in dystrophic muscle if regeneration was inhibited by irradiation at the pre-dystrophic phase.

Our study shows that the maturation of muscle in dystrophic ReJ 129 mice, at least as concluded from the expression of desmin, appears not to be delayed. This is again not in line with observations on myosin HC expression by Reggiani et al. (1991), who showed that even at the age of 10 days, before any marked histopathological features can be seen, more myofibres with immature (neonatal) myosin are observed in comparison to normal controls. These contrasting results may be explained by the fact that myosin is a late marker in myofibre differentiation (Schaart et al. 1989). Our results indicate that at least up to desmin organization differentiation of dystrophic myofibres is normal. Desmin organization is also normal in apparently normal myofibres. Furthermore, our results suggest that the myofibres with centrally located nuclei do not represent degenerating fibres, but are signs of myofibre regeneration, contrary to what has been suggested by Totsuka (1986, 1987), who concluded that degeneration occurs after the nuclei have moved to the centre of the myofibre. In our view, no migration of the nuclei from the centre to the sarcolemma occurs (at least not until the age of 2 months) in maturing, regenerating myofibres in dystrophic mice. This is in agreement with Wirtz et al. (1983), who showed that also in dystrophic mice of 5 months many large myofibres with centrally located nuclei are observed, and Reggiani et al. (1991). In summary, we conclude that degeneration of mature fibres takes place and that immature fibres are actually regenerating fibres and not developmentally arrested ones. Furthermore we showed that examination of desmin and vimentin expression in combination with histochemical staining on serial sections is an easy and useful method to identify distinctive early stages of degeneration and regeneration in diseased muscle.

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