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Dystrophic phenotype induced in vitro by antibody blockade of muscle α-dystroglycan-laminin interaction

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SUMMARY

Alpha-dystroglycan is a glycoprotein expressed on the surface of skeletal muscle fibres and other cell types. In muscle, α-dystroglycan provides a link between the myofibre cytoskeleton through its indirect binding to dystrophin, and the basal lamina through its binding to laminin-2, a protein of the extracellular matrix. The disruption of this linkage between the myofibre cytoskeleton and the extracellular matrix is a common feature of Duchenne and other muscular dystrophies, though the pathogenic mechanisms leading to muscle wasting remain unknown. By treating primary mouse muscle cultures with a monoclonal antibody which blocks α-dystroglycan binding to laminin, we show here the induction of a dystrophic phenotype in vitro. The phenotype is inducible in differentiated cultures only, is characterised by reduced myotube size, myofibril disorganisation, loss of contractile activity, reduced spontaneous clustering of acetylcholine receptors and is reversed by addition of excess exogenous laminin-2. Thus, α-dystroglycan may be part of a signalling pathway for the maturation and maintenance of skeletal myofibres. Detailed knowledge of this signalling pathway may provide insights into the molecular pathology of the various inherited muscular dystrophies, and identify valuable pharmacological targets and new therapeutic strategies.

Key words: Dystrophin, Dystroglycan, Merosin, Laminin, Myotube

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a progressive, X-linked disease with an incidence of around 1/3500, one third of which are sporadic with no previous family history (Emery, 1993). Both DMD and the more benign Becker muscular dystrophy (BMD) are caused by mutations in the gene that codes for dystrophin, a large cytoskeletal protein found beneath the plasma membrane of all muscle types and some neurons (Koenig et al., 1988; Hoffman et al., 1988; Byers et al., 1991). The N-terminal regions of dystrophin bind to sub-sarcolemmal F-actin whilst the cysteine rich and C-terminal domains bind to a tightly associated group of proteins and glycoproteins collectively known as the dystrophin glycoprotein complex (DGC) (Ervasti and Campbell, 1991).

The DGC consists of the dystroglycan, sarcoglycan and syntrophin sub-complexes (Tinsley et al., 1994). Dystroglycan exists as a noncovalently linked complex of a 156 kDa extracellular laminin and agrin-binding subunit (α-dystroglycan) and a 43 kDa transmembrane subunit (β-dystroglycan; Bowe et al., 1994; Yoshida et al., 1994). The α- and β-dystroglycans are post-translational products of a single gene, which take their name as a consequence of their interaction with dystrophin and the heavily glycosylated nature of α-dystroglycan (IbraghimovBeskrovnaya et al., 1992, 1993). As a consequence of its interaction with laminin and dystrophin the dystroglycan complex is generally attributed with a role in mediating a connection between the actin cytoskeleton of the muscle fibre and the extracellular matrix (Henry and Campbell, 1996; Brown and Dickson, 1996).

The functional importance of this group of proteins is emphasised by the finding that mutations in various components of the DGC, other than dystrophin, also result in muscular dystrophy. Furthermore a reduction in laminin α2, β1 and β2 chains has been observed in other muscular dystrophies, such as the Fukuyama congenital muscular dystrophy, the Finnish muscle-eye-brain disease, Walker-Warburg syndrome (Wewer and Engvall, 1996) and dystrophia muscularis in mice (Sunada et al., 1994). Overall these observations indicate that the integrity of the DGC and its association with laminin is essential for muscle fibre viability.

However the precise role of the DGC remains unclear. One

proposal is that it maintains the structural integrity of the sarcolemma during repeated cycles of contraction and relaxation. This hypothesis is supported by observations of an apparent increase in the fragility of dystrophin deficient membrane relative to controls (Menke and Jockusch, 1991; Menke et al., 1993; Menke and Jockusch, 1995; Pasternak et al., 1995). However, dystrophin and its associated protein complex may also be involved in mediating signal transduction events. Grb2, an SH2/SH3 adapter protein, binds to the cytoplasmic domain of β-dystroglycan (Yang et al., 1995).

The physiological function in skeletal muscle of the interaction between α-dystroglycan and laminin-2 does nonetheless, remain unclear. The present study was therefore undertaken to specifically examine the effect of blocking this interaction in cultured myotubes using the monoclonal antibody IIH6. This antibody has previously been shown to block α-dystroglycan binding to laminin both in biochemical and cellular assays (Durbeej et al., 1995; Ervasti and Campbell, 1993; Matsumura et al., 1997). We show that addition of the antibody fails to alter early events such as myoblast fusion and myotube development. However, its sustained presence in cultures leads to a reduction in myotube diameter, disorganisation of the myofibrillar apparatus, and a reduction in both spontaneous contractile activity and the clustering of acetylcholine receptors. Many of these features could be effectively out-competed by the addition of exogenous laminin-2. These observations implicate α-dystroglycan-laminin interactions with a role in the maturation and maintenance of skeletal myotubes and myofibres.

MATERIALS AND METHODS

Isolation and primary culture of mouse myoblasts

Glass coverslips (13 mm) were prepared by sequential incubation for 24 hours, 1 hour and 24 hours, respectively, in 1 M Mg acetate, 25 μg/ml poly-L-lysine and 5 μg/ml laminin (Sigma, Dorset, UK) Hind-leg muscle tissues from 4- to 5-week-old C57BL/10 mice were minced and dissociated by repetitive treatment with trypsin (2.5% w/v), collagenase (1% w/v) and bovine serum albumin (BSA) (1% w/v) in PBS for 15 minutes at 37°C. Digests were allowed to settle (1 minute), supernatants neutralised by addition of 10% (w/v) foetal calf serum (FCS) and the remaining muscle retreated twice with enzyme. Supernatants containing released cells were pooled, passed through a 50 μm and 20 μm nylon mesh and cells collected by centrifugation at 194 ***g*** for 10 minutes. Cells were resuspended in DMEM containing 10% (w/v) FCS (growth medium) and cultured on laminin-coated glass coverslips in 24-well plates. When myoblast cultures reached confluence, growth medium was changed to DMEM, 1% (w/v) FCS and 2% (w/v) heat-inactivated horse serum (differentiation media) to promote myoblast fusion and myotube formation.

Antibody mediated blocking of the interaction between α-dystroglycan and laminin-2

The hybridoma tissue culture supernatant of the antibody IIH6 which is known to block dystroglycan-laminin binding, was added to myotube cultures 1 day or 3 days after the initiation of myoblast fusion at final dilutions of 1:5 or 1:10, with subsequent medium changes every 48 hours. Cultures were then subjected to a range of quantitative analyses (see below). In initial studies the cell-surface association of the IIH6 antibody was examined by immunolabelling unfixed cultures at 4°C with biotinylated anti-mouse IgM and streptavidin conjugated with Texas Red (see below). To examine the specificity of IIH6, control cultures were grown either in the absence of added antibody or in the presence of 1:5 dilutions of hybridoma tissue culture supernatants of the monoclonal antibodies V1A41 (anti-α-dystroglycan IgG known not to block laminin binding) (Ervasti and Campbell, 1991, 1993) or X1XC2 (anti-dystrophin IgM) (Ervasti and Campbell, 1991, 1993). In addition the ability of added laminin-2 (merosin) to inhibit the effects of IIH6 was evaluated by the addition of 10-50 μg/ml human laminin-2 (Life Technologies, Paisley, UK) to culture media. For these studies myotube cultures, at 3 days post induction of fusion, were transferred to serum-free DMEM containing IIH6 antibody with or without laminin, and medium changed every 24 hours.

Immunocytochemistry

For immuno-labelling of myotubes, cultures were stained with a range of antibodies either live or following fixation in −20°C methanol for 5 minutes. Fixed cultures were incubated with antibodies at room temperature, whereas live cultures were incubated at 4°C to prevent internalisation of surface-bound reagents. The following antibodies were used. Dystrophin: monoclonals Dy8/6C5 (Nicholson et al., 1989) α-dystroglycan: sheep polyclonal FP-D (IbraghimovBeskrovnaya et al., 1992) and the monoclonal IIH6; β-dystroglycan: monoclonal 8GII-D10 (Novacastra, Newcastle, UK); desmin: monoclonal antibody (Dako Ltd). Primary antibody incubations (1 hour), were followed by a 1 hour incubation with biotinylated secondary antibodies (anti-mouse IgG; Serotec, Oxford, UK) or IgM (Amersham, Bucks, UK), anti-sheep Ig (Amersham), anti-rat IgG (Sigma), and finally with Texas Red-conjugated streptavidin (Amersham) for 20 minutes. Stained cultures were fixed in methanol for 5 minutes at −20°C. To determine if addition of laminin-2 to the culture medium was able to prevent IIH6 labelling of α-dystroglycan, cultures were incubated with varying concentrations of laminin-2 (10, 20, 50 μg/ml), and then stained, as described above. Staining of immuno-labelled cultures was examined and recorded with a Leica TCS 4D confocal laser scanning microscope.

Quantitative measurement of myotube number, diameter and contractile activity

For determination of myotube number and diameter, cultures were stained with desmin antibody to allow clear distinction of myotubes within the dense monolayer of surrounding fibroblasts and residual myoblasts. Distinct myotube numbers were counted in 4 randomly selected fields of 500×500 μm in each of 3 independent cultures. For myotube diameter estimates, random photomicrograph images were taken from at least 3 independent culture wells, and mean myotube diameter measured using the Lucia G image analysis system (Nikon, UK). For measurement of spontaneous myotube contractility, coverslips were equilibrated in 2 ml medium at 37°C for 30 minutes and then 4 randomly selected fields of 500×500 μm in each of 3 independent cultures examined for 1 minute. Observed events of myotube contraction were scored and summed for each culture. Under these conditions no significant change in temperature or pH of the medium occurred.

Detection of apoptotic nuclei in myotube cultures

Cultures were fixed in PBS containing 10% formalin for 10 minutes at room temperature, followed by ethanol/acetic acid (2:1 v/v) for 5 minutes at −20°C. Apoptotic nuclei were then detected by labelling with the Apoptag kit (Oncor, Gaithersburg, MD, USA) or with 0.5 μg/ml DAPI (20 minutes at room temperature: Sigma). NIH3T3 mouse fibroblast cultures treated with 1 μM staurosporine for 2 hours were used as positive controls for apoptotic nuclei detection.

Quantification of acetylcholine receptor (AChR) clusters in myotube cultures

To determine whether addition of IIH6 inhibited the spontaneous

clustering of AChRs on myotubes, cultures were grown in differentiation medium for 3 days, then switched to serum-free medium with or without addition of IIH6 or control antibodies. At 7 and 10 days post induction of fusion, cultures were rinsed in DMEM containing 1 mg/ml BSA (5 minutes at 37°C) and stained with rhodamine-conjugated α-bungarotoxin (Molecular Probes) for 1 hour at 37°C. Cultures were then rinsed in DMEM and fixed in 95% ethanol (5 minutes at −20°C). AChR clusters were analysed using a Leitz DMRXE microscope at ×400 magnification and their numbers summed from 100 random fields in 3 independent culture samples.

Electron microscopy

Cultures were fixed in 3% glutaraldehyde and 4% formaldehyde in 0.1 M Pipes buffer at pH 7.2 for 1 hour, post-fixed in 1% OsO_4 in 0.1 M Pipes for 1 hour and embedded in TAAB resin (TAAB Laboratories, Aldermaston, UK). Gold/Silver sections were cut using a Cambridge Huxley MKII ultramicrotome, stained with alcoholic uranyl acetate and Reynolds lead stain and viewed using a Zeiss EM 109.

RESULTS

The presence and membrane localisation of dystrophin, α-dystroglycan and β-dystroglycan in myotube cultures 5 days after the induction of fusion was confirmed by immuno-labelling with Dy8/6C5, IIH6 and 8G11-D10 antibodies (Fig. 1A,B,C). In the case of α-dystroglycan, the same pattern of staining was observed in both live and methanol-fixed cultures. Differentiated myotubes express laminin-2 (merosin) on their surface but very limited amounts of laminin-1 (Schuler and Sorokin, 1995; Vachon et al., 1996). The deposition of large amounts of laminin-2 on the surface of 4-5 days post fusion muscle cultures in the present study was also observed (not shown).

To examine whether the interaction between α-dystroglycan and laminin played any active physiological role in skeletal myotube function use was made of the monclonal antibody IIH6 which binds to α-dystroglycan and is known to block binding to laminin-1 both in biochemical and cellular assays (Durbeej et al., 1995; Ervasti and Campbell, 1993; Matsumura et al., 1997). IIH6 hybidoma supernatant was added to muscle cultures 1 day or 3 days after the switch to differentiation medium i.e. post-fusion, with media replenishment every 48 hours. For confirmation that IIH6 antibody was indeed binding to the surface of myotubes, treated cultures were stained live with secondary anti-mouse IgM and streptavidin-Texas Red. Intense labelling was observed on cultures exposed to 1:5 and 1:10 dilutions of IIH6 hybridoma supernatant, whereas 11H6 added at 1:20 dilution gave rise to a weaker staining pattern. Under these conditions with live intact cells, no staining was observed in control cultures incubated with the anti-dystrophin IgM XIXC2, or labelled with anti-mouse IgM secondary antibody alone.

In order to clearly identify and delineate myotubes and to evaluate numbers and size of these cells, IIH6-treated and control cultures were fixed at various time points, and immuno-stained for desmin (see examples in Fig. 3). The number of myotubes increased with time in both IIH6 treated and control cultures and no difference with respect to this parameter was found at either 1, 2 or 7 days post-fusion (Fig. 2A). At no stage up to 7 days post-fusion was evidence of significant apoptosis or necrosis apparent in either IIH6 treated or control myotubes as assayed by the Apoptag kit, DAPI labelling of nuclei or electron microscopy (not shown). However, 7 days post-fusion, a reproducible and highly significant reduction (P<0.001) in

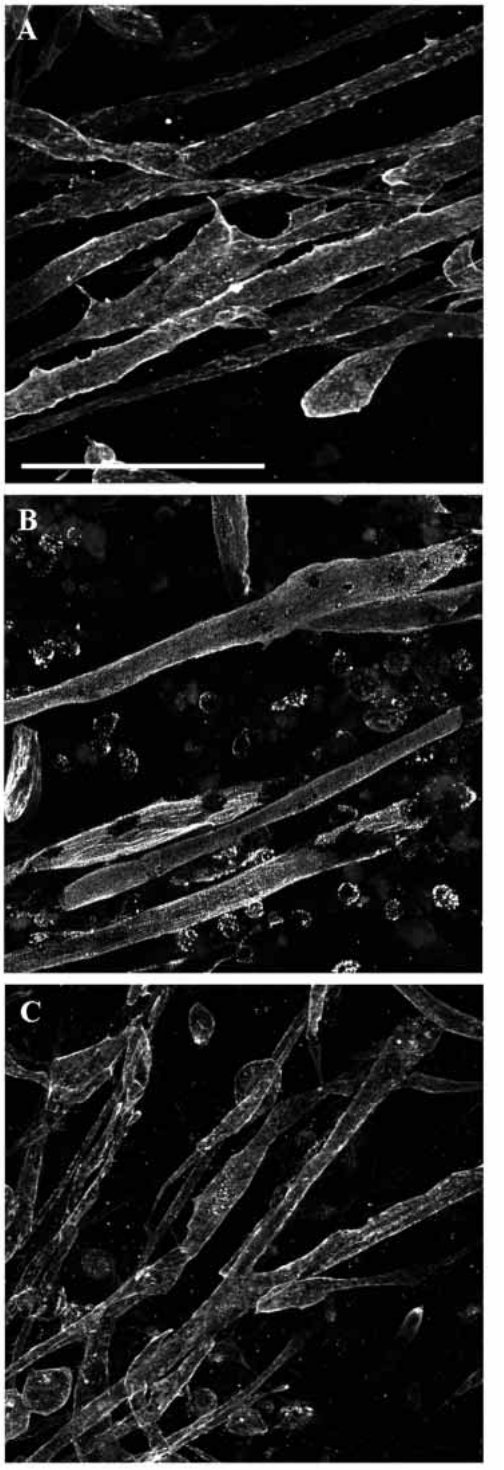

Fig. 1. Expression of dystrophin, α-dystroglycan and β-dystroglycan in mouse myotubes in vitro. Mouse myotube cultures at 5 days after induction of myoblast fusion were fixed and immunolabelled with (A) Dy8/6C5 antibody against dystrophin, (B) IIH6 antibody against α-dystroglycan, and (C) 8GII-D10 antibody against β-dystroglycan. All are maximum projection confocal images. Bar, 100 μm.

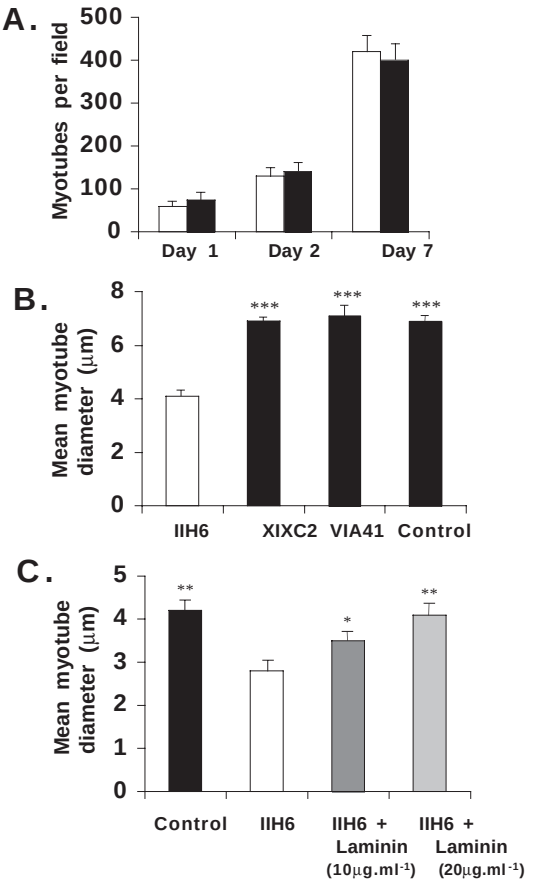

Fig. 2. Myotube morphometry in mouse muscle cultures treated with IIH6 antibody in the presence and absence of laminin-2. Mouse skeletal myoblast cultures were prepared, induced to undergo fusion and myotube formation, and then fixed and stained with desmin antibody under a variety of conditions and time intervals. (A) Effect of IIH6 on myotube formation. Cultures were induced to fuse, incubated in the absence (open bars) or presence (filled bars) of a 1:10 dilution of IIH6 hybridoma supernatant, and myotubes numbers counted in random fields (500×500 μm) from three independent cultures: values are means ± s.d. (n=3). Myotube number was not significantly affected by the presence of 11H6 antibody at either 1, 2 or 7 days post-fusion. (B) Effect of IIH6 on myotube size. Cultures were induced to fuse, and incubated in the absence of added antibody, in the presence of IIH6 antibody or with control anti-dystrophin IgM, XIXC2, and non-blocking anti-dystroglycan IgG, VIA41 (all at 1:10 dilutions of hybridoma supernatant). Myotube size (mean diameter ± s.d., n=3) was determined in independent culture wells at d3 (not shown) and d7. No difference was observed at d3, but at d7 a highly significant myotube atophy was found in specific response to IIH6 (t-test versus controls: ***P<0.001). (C) Reversal of IIH6 effects by laminin-2. Cultures at 3 days of fusion were further incubated in serum-free medium containing IIH6 antibody alone (1:10 dilution of hybridoma supernatant: open bar) and in the presence and absence of laminin-2 at 10 or 20 μg/ml (shaded bars). Myotube size (mean diameter ± s.d., n=3) was determined in independent culture wells 4 days later. Myotube atrophy induced by IIH6 was incrementally and significantly reversed by addition of 10 or 20 μg/ml laminin-2 (t-test versus IIH6-treated samples: *P<0.05; **P<0.01).

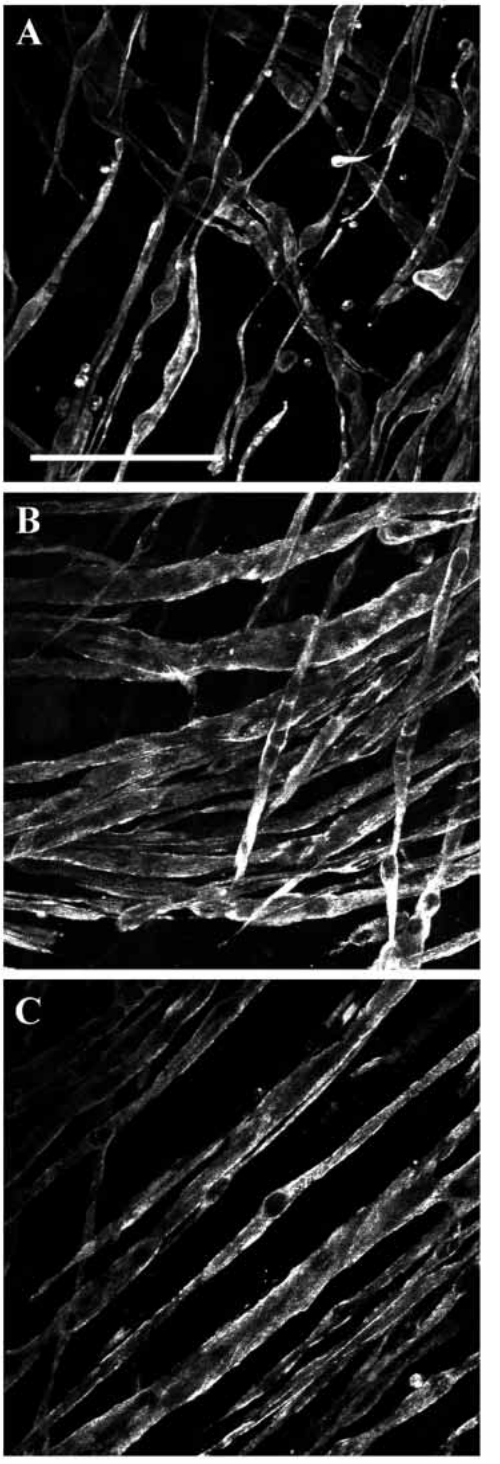

Fig. 3. Specific atrophy of myotubes in IIH6-treated cultures. Mouse myoblast cultures were induced to fuse, and incubated (A) in the presence of the blocking anti-dystroglycan IgM IIH6, (B) in the presence of non-blocking anti-dystroglycan IgG, VIA41 (both at 1:10 dilutions of hybridoma supernatant), or (C) in the absence of added antibody. At d7 of fusion cultures were fixed and immunostained with desmin to visualise myotubes. All are maximum projection confocal images. Bar, 50 μm.

the mean myotube diameter was evident in cultures exposed to IIH6 hybridoma supernatant at 1:5 and 1:10 dilution relative to untreated cultures or control cultures in the presence of either an alternative anti-α-dystroglycan monoclonal, V1A41

(serotype IgG), or an alternative IgM monoclonal, anti-dystrophin antibody X1XC2 (Figs 2B and 3). To further confirm the specificity of this phenotypic response to IIH6 exposure, 3 day myotube cultures were transferred to serum-free medium containing IIH6 alone or in combination with laminin-2, and examined at 7 days. The observed reduction in myotube diameter in response to IIH6 was found to be incrementally inhibited by the simultaneous addition of 10 or 20 μg/ml laminin-2 ($P<0.05$ and $P<0.01$, respectively: Fig. 2C). Myotubes grown on laminin-2 substrata exhibited the same morphological phenotype and responses as those grown on laminin-1 both in the absence and presence of 11H6 antibody. To evaluate whether the addition of laminin-2 to IIH6-treated cultures was able to displace IIH6 from sites on cell-associated α-dystroglycan, live myotubes incubated with IIH6 in the presence and absence of laminin-2 were stained for IIH6 binding by immunolabelling with biotinylated anti mouse IgM and streptavidin conjugated with Texas Red. However, no gross perturbation of the density and distribution of IIH6 immunolabelling or of the morphology of myotubes was observed in the presence of either 10, 20 or 50 μg/ml of laminin-2 (not shown).

To look for ultrastructural correlates of the myotube atrophy observed following treatment with IIH6, cultures were examined by transmission electron microscopy. At 7 days post-fusion, myotubes showed evidence of myofibril alignment and nascent Z-bands in both control and 11H6-treated cultures (Fig. 4A and B, respectively). However, by 10 days post-fusion, whilst organised sarcomeres were present in untreated and control antibody treated cultures (Fig. 4C,E,F), most 11H6-

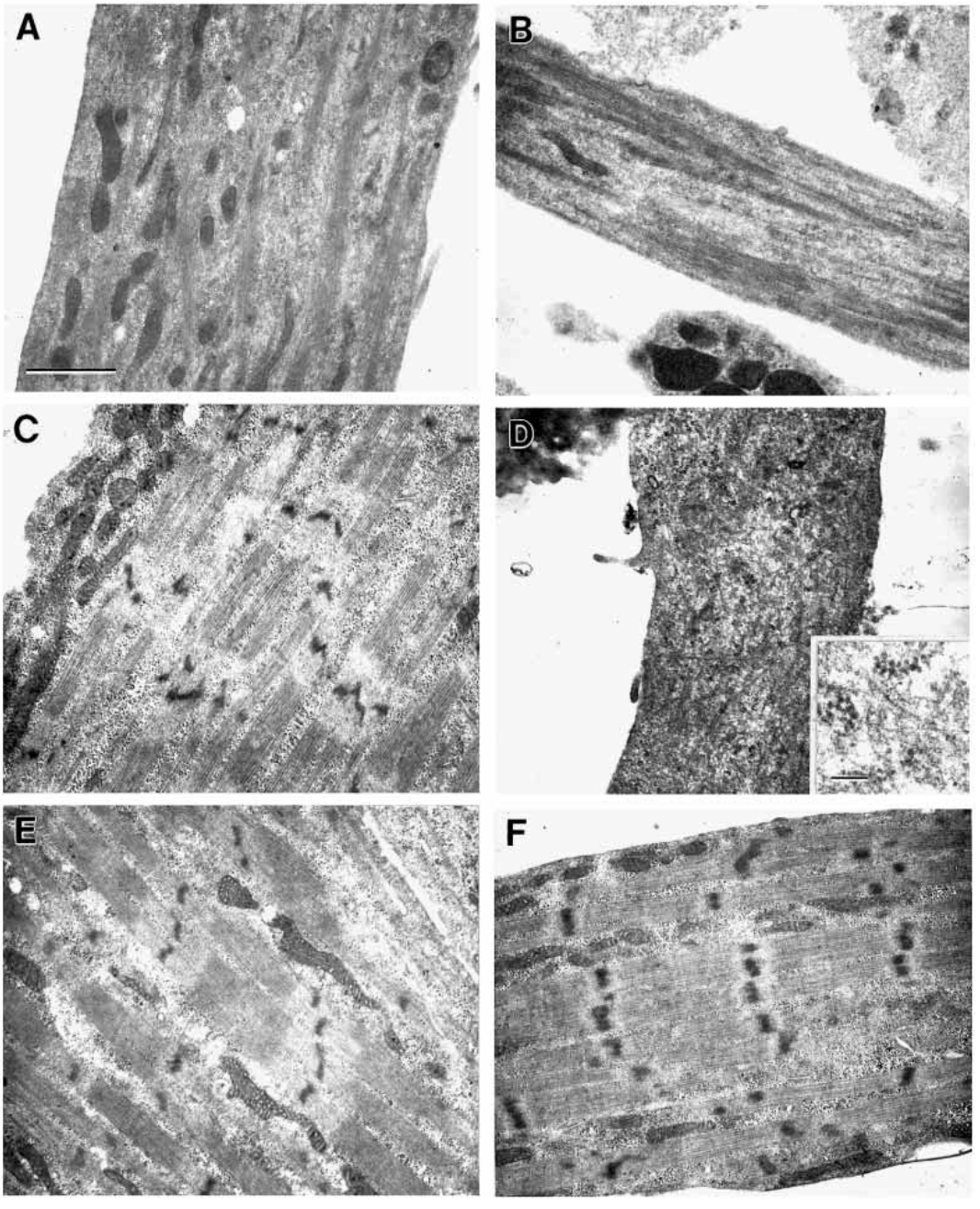

Fig. 4. Ultrastructural analysis of the effects of IIH6 on myofibril organisation. Mouse myoblast cultures were induced to fuse, and incubated (A and C) in the absence of added antibody, (B and D) in the presence of the blocking anti-dystroglycan IIH6 antibody, or (E and F) in the presence of control antibodies VIA41 or XIX (all at 1:5 dilutions of hybridoma supernatant). At 7 days (A,B) or 10 days (C-F) cultures were processed for transmission electron microscopic examination. While myotube size was clearly reduced in response to IIH6 at 7 days, no obvious differences were noted in myofibril organisation between (A) untreated, and (B) IIH6-treated cultures. At 10 days post-fusion, sarcomeric structure was well developed in (C) untreated cultures, and (E and F) control antibody treated cultures. In contrast, myofibril disorganisation was evident in IIH6-treated cultures (D and inset d). Bars: 1 μm (A-F); 0.1 μm (inset d).

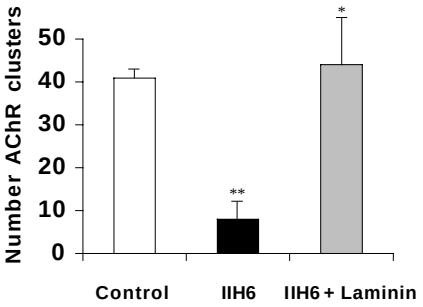

Fig. 5. Analysis of AChR clusters in control, IIH6 and laminin-2 treated myotubes. Mouse myoblast cultures were induced to fuse, and after 3 days incubated in serum-free media containing IIH6 antibody alone (1:10 dilution of hybridoma supernatant), or in the presence of laminin-2 (20 μg/ml). Three days later cultures were stained with rhodamine-conjugated α-bungarotoxin, and fluorescent patches of AChR clusters quantified in a unit area of groups of three independent replica cultures. Histobars represent means ± s.d. (n=3). T-test values versus control: **P<0.001; *non significant.

treated myotubes exhibited gross disorganisation of the myofibrillar structure, in addition to a marked reduction in size (Fig. 4D and inset d). Again the IIH6 phenotype could be prevented by addition of laminin-2 (not shown).

In addition to its widespread distribution on extra-junctional sarcolemma, α-dystroglycan is also present at the neuromuscular junction (NMJ). Alpha-dystroglycan has in fact been proposed to play a role in the induction of acetylcholine receptor (AChR) clustering on the surface of cultured myotubes, possibly by binding agrin and other components of the extracellular matrix (Campanelli et al., 1994; Gee et al., 1994; Noakes et al., 1995; Sugiyama et al., 1994). To examine the effects of IIH6 antibody on this phenomenon, rhodamine-conjugated α-bungarotoxin was used to label AChR clusters in treated and control myotube cultures at 10 days post-fusion. The density of AChR clusters was significantly reduced in IIH6 treated cultures compared to untreated controls (P<0.001) and again the effect could be reversed by the simultaneous application of laminin-2 (Fig. 5). Furthermore, in correlation with the observed myotube atrophy, ultrastructural disorganisation and a reduction in AChR clustering, the mean number of spontaneous contractile events in myotubes from 7 days IIH6-treated cultures (3.9±2.4: mean ± s.d., n=4) exhibited a very marked reduction (P<0.05) compared with control untreated cultures (0.4±0.25: mean ± s.d., n=4).

DISCUSSION

The laminins are a large family of basement membrane proteins consisting of one heavy (α) and two light chains (β and γ) which all exist in multiple isoforms. In skeletal muscle the principal laminin variants are laminin-2 (α2-β1-γ1) and laminin-4 (α2-β2-γ1) which are generally referred to collectively as laminin-2 (Wewer and Engvall, 1996). Muscle laminins are thought to interact with a range of cell surface components including the proteoglycan α-dystroglycan, a component of the dystrophin glycoprotein complex (DGC) whose malfunction underlies a number of inherited muscular dystrophies. The aim of the present study was to determine the effects of antibody-mediated inhibition of the interaction between α-dystroglycan and laminin in cultured myotubes using the blocking monoclonal antibody IIH6 which binds specifically to α-dystroglycan. IIH6-mediated blockade of the interaction between α-dystroglycan and laminin did not affect initial formation of myotubes but resulted subsequently in marked phenotypic changes characterised by reduced myotube size, myofibril disorganisation, loss of contractile activity and reduced spontaneous clustering of acetylcholine receptors. All of these effects were specific to the IIH6 IgM, were not produced by control IgM or alternative non-blocking antibodies to α-dystroglycan, and could be prevented by competition with added exogenous laminin-2. These observations raise the possibility that a disruption in the interaction between α-dystroglycan and laminin-2 may underlie many aspects of the muscle pathology of DMD, and other forms of muscular dystrophy where expression of the dystroglycan complex is severely reduced or laminin-2 in the extracellular matrix is absent (IbraghimovBeskrovnaya et al., 1992; Campbell, 1995; Roberds et al., 1993; Cullen et al., 1994; Ervasti et al., 1990; Ohlendieck et al., 1993).

Vachon et al. (1996, 1997) have recently shown that the interaction between laminin-2 and α7β1D integrin is essential for myotube and myofibre survival. Their data indicate the presence of a signalling/adhesion pathway active in the early phases of myotube differentiation. It is noteworthy that IIH6 treatment in the present study did not cause detachment or loss of myotubes from the substrate at any time, and that myotubes formed normally during the first three days after the initiation of fusion. Chronic exposure to the IIH6 for periods of 7-10 days after the onset of myotube formation was required to see the specific induction of atrophic changes by IIH6, making it unlikely that IIH6 exposure resulted in a direct reduction of myotube adhesion to the culture substrata. In our experiments we have disrupted the interaction between laminin-2 and α-dystroglycan and showed that most likely there is another signalling pathway which is active at later stages of myotube differentiation. The disruption of this pathway causes atrophy rather than apoptosis. However, in relation to the rescue of 11H6-treated myotubes by exogenous laminin-2, it remains possible that compensatory mechanisms may be activated via alternative receptor systems such as α7β1D integrin, and that combined interactive effects on the α7β1D and DGC signalling mechanisms may be responsible. This might in part explain the apparent discrepancy between the ability of added laminin-2 to inhibit IIH6 effects without affecting IIH6 immuno-localisation. Thus while the addition of exogenous laminin-2 to IIH6-treated cultures was able to effectively reverse many of the features induced by IIH6, it failed to cause gross displacement of the antibody from α-dystroglycan on the myotube surface. This was assessed by immunolabelling and may therefore have been due to the high affinity multi-valent binding of the IgM. This result was unexpected and is in contrast to the data of Cohen et al. (1997) in *Xenopus* myocytes. The failure of added laminin-2 to alter the distribution of IIH6 (and by implication of α-dystroglycan), in our studies may be due to species effects, differential culture conditions, or result from IIH6 inhibition of macrocluster formation.

The expression patterns and distribution of α7β1D integrin and the DGC suggest that they represent parallel extracellular

matrix attachment systems in skeletal muscle fibres (Hodges et al., 1997; Vachon et al., 1997). This is supported by the observation that α7β1D integrin occurs at activated levels in DMD and *mdx* muscle (Hodges et al., 1997). The co-operation of integrins with other surface molecules to modulate and integrate information on the local extracellular matrix and reinforce attachment is well established (Mercurio, 1995; Wewer and Engvall, 1996). In further support of this, bi-directional signalling between the sarcoglycan components of the DGC and the α5β1 integrin system has recently been shown in L6 myocytes (Yoshida et al., 1998). Current thinking attributes integrins with a role as mechanotransducers (Shyy and Chien, 1998). It might therefore be envisaged that due to aberrant signalling, dystrophic muscle fibres undergo pathological extracellular matrix remodelling with over-expression of components such as collagen types I, III and IV, and fibronectin and fibroblast growth factor (Hantai et al., 1985; Dimario et al., 1989). This could then result in an inappropriate adaption to mechanical stress and result in myofibre damage. While the precise conditions within diseased muscle cannot be fully replicated in vitro, the strength of cell culture studies lies in providing opportunities to identify contributory factors. For example, cultured myoblasts deficient in laminin-2 or with disruption of the laminin α1 chain gene exhibit normal differentiation in vitro but derived myotubes are unstable and undergo degeneration when they become contractile (Vachon et al., 1996; Kuang et al., 1998). Contraction-induced myofibre damage and lack of survival cues have thus been suggested to contribute to merosin-deficient congenital muscular dystrophy. Our work identifies a pathway by which myotube hypertrophy is disrupted without causing the pattern of myotube degeneration seen in the studies of Vachon et al. (1996, 1997) and Kuang et al. (1998), and shows for the first time that the interaction between the basement membrane and the DGC is essential for the maintenance of the differentiated myotube phenotype.

Finally it should be noted in the present study that the identity of the muscle laminin isoform whose interaction with α-dystroglycan is disrupted by IIH6 remains as yet to be strictly defined. Laminin α2, α5, β1 and γ1 chains are all components of the numerous patches of basal lamina on the surface of newly formed myotubes in vitro (Patton et al., 1998). The α1 chain is also present but largely confined to the ends of primary and secondary myotubes due to the synthetic activity of fusing myoblasts (Patton et al., 1998). In vivo, these regions of the myotube are where the myotendinous junction develops, and homologous areas in cultured myotubes are the talin/integrin-positive focal adhesions that attach myotubes to the culture substrata. Dystroglycan can also bind to laminin α-1, and it is possible that IIH6 could also be having an effect on this interaction. However, as discussed above, it is unlikely that IIH6 has any direct effect on adhesion of myotubes to culture substrata. Furthermore the data of Pall et al. (1996) indicate that that laminin-1 and laminin-2 have similar binding sites on α-dystroglycan. Taking these data together with the wide distribution of laminin-2 on mouse myotubes the most parsimonious explanation of the observed effects of the IIH6 antibody is that they result from blocking the interaction between α-dystroglycan and laminin-2. However, combinatorial interactions with other adhesion/signalling pathways cannot be ruled out.

In addition to its widespread extra-synaptic expression in muscle, α-dystroglycan is an abundant agrin receptor at the NMJ, although it is now not thought to mediate agrin signalling (Henry and Campbell, 1996). Instead it may have a predominantly structural role by binding to both agrin and laminin which are stable components of the NMJ basal lamina, thus ensuring adhesion of the post-synaptic membrane to the adjacent basal lamina (Sealock and Froehner, 1994). The mechanism, direct or indirect, of the reduction in the spontaneous clustering of AChRs in the presence of IIH6 seen in the present study remains to be defined, and the physiological relevance is unclear. Conflicting results on the effects of IIH6 on AChR clustering have been previously reported (Campanelli et al., 1994; Gee et al., 1994). One suggestion is that the binding of the large, polyvalent IgM to α-dystroglycan might inhibit cluster formation or perturb cluster morphology possibly by steric hindrance or cross-linking (Sealock and Froehner, 1994). Furthermore, an alternative pathway for AChR clustering via laminin-1 has recently been reported (Sugiyama et al., 1997). In the absence of any further information it is not possible to say whether a reduction in the number of AChR clusters on the myotube surface in response to IIH6 treatment was a direct or indirect effect, but only that the change could be reversed by the addition of laminin-2.

In summary the treatment of cultured myotubes with a monoclonal antibody that blocks α-dystroglycan binding to laminin leads to the induction of a dystrophic phenotype in vitro. Primary events in the fusion of myoblasts and in myotube formation appear to be unaffected, and myotube atrophy is inducible in differentiated cultures only. The phenotype is characterised by reduced myotube size, myofibril disorganisation, loss of contractile activity, reduced spontaneous clustering of acetylcholine receptors and may be reversed by addition of exogenous laminin-2. Thus, α-dystroglycan may be part of a signalling pathway for the maturation and maintenance of skeletal myofibres. Detailed knowledge of this signalling pathway may provide insights into the molecular pathology of the various inherited muscular dystrophies, and identify valuable pharmacological targets and new therapeutic strategies.

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