acta histochem. **103**, 379–396 (2001)
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acta
histochemica

The absence of MyoD in regenerating skeletal muscle affects the expression pattern of basement membrane, interstitial matrix and integrin molecules that is consistent with delayed myotube formation

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Received 3 January 2001 and in revised form 17 April 2001; accepted 21 April 2001

Abstract

MyoD is a member of a skeletal muscle specific family of transcription factors which directs the events of myogenesis during development and regeneration. Muscle cells that lack MyoD show delayed fusion *in vivo* and *in vitro* and defects have been observed *in vitro* in the attachment of MyoD(–/–) myoblasts to complex substrates such as Matrigel. Since interactions with the extracellular matrix (ECM) are important during myoblast fusion (i. e. myotube formation), it was hypothesised that expression of ECM molecules or their receptors may be altered in MyoD(–/–) muscle. The production of basement membrane molecules such as collagen type IV and several laminins, the interstitial molecules fibronectin and tenascin-C, and the cell surface molecules integrin $\alpha5$ and $\alpha6$ were quantitated *in vitro* using ELISA on cultured cells from MyoD(–/–) and wild type mice. Differences were observed in the production of fibronectin, tenascin-C, collagen type IV, laminin-1 and integrin $\alpha5$ between control and MyoD(–/–) myotubes *in vitro*. This corresponded with delayed fusion of myoblasts in MyoD(–/–) cultures. On the basis of these findings with respect to matrix expression *in vitro,* fluorescent immunohisto-

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http://www.urbanfischer.de/journals/actahist 0065–1281/01/103/4–379 $ 15.00/0

chemistry was carried out on adult whole muscle autografts to examine whether the expression of these molecules, as well as integrin $\alpha 7$, was altered in the complex *in vivo* environment. Some minor differences in expression patterns were observed in MyoD(–/–) as compared to normal BALB/c autografts. The overall expression of matrix components was consistent with the delayed onset of myotube formation. These results suggest that the delay in myotube formation in MyoD(–/–) muscle is not a direct result of altered expression of the matrix molecules collagen type IV, laminins, fibronectin, tenascin-C, and integrins $\alpha 5$, $\alpha 6$ or $\alpha 7$.

Key words: MyoD – regeneration – extracellular matrix – basement membrane – integrin – skeletal muscle

Introduction

Skeletal muscle has an excellent ability to regenerate. Muscle precursor cells, called satellite cells, that normally lie quiescent beneath the basement membrane, are activated in response to injury. Activated muscle satellite cells (usually called myoblasts) proliferate, differentiate, and eventually fuse into myotubes that mature into myofibres (Grounds, 1991).

MyoD is a skeletal muscle-specific transcription factor that plays a central role in muscle development and regeneration (Rudnicki and Jaenisch, 1994). However, mice that lack a functional MyoD gene seem to develop normally. They remain viable and fertile and show no morphological or physiological skeletal muscle abnormalities (Rudnicki et al., 1992), although myotube formation is slightly delayed in the developing limbs (Kablar et al., 1997). In regenerating whole muscle grafts in adult MyoD(–/–) mice, there is a two-day delay in myoblast fusion that is associated with a sustained level of myoblast proliferation (White et al., 2000) and this is also observed *in vitro* (Sabourin et al., 1999; Yablonka-Reuveni et al., 1999). In addition, MyoD(–/–) myoblasts *in vitro* do not adhere well to Matrigel (a basement membrane like substrate mainly composed of laminin-1, collagen IV, proteoglycans, nidogen and various growth factors) and these myoblasts clump together to form multi-layered foci (White et al., 2000). Similar clumping of replicating myoblasts is found on the basement membrane of isolated MyoD(–/–) myofibres in culture (Yablonka-Reuveni et al., 1999; Cornelison et al., 2000). The "clumping" of MyoD–/– myoblasts is striking and unexpected as basement components such as laminin-2 induce attachment, proliferation and migration of myoblasts as well as their fusion into myotubes (Kuhl et al., 1986; Ocalan et al., 1988; Schuler and Sorokin, 1995) in contrast to the interstitial matrix proteins fibronectin and collagen type I. This unusual behaviour of MyoD(–/–) myoblasts may be due to alterations in cell-matrix and/or cell-cell interactions and this may also be involved in delayed myoblast fusion.

Interactions with the extracellular matrix (ECM) are crucial for many aspects of myogenesis (Gullberg et al., 1998). The importance of the ECM in myoblast fusion has been demonstrated by strain-specific differences in the extent of myotube formation on Matrigel as compared with interstitial ECM molecules like collagen type I (Maley et al., 1995). Furthermore, disintegrins belonging to the ADAMS family that affect ECM interactions have been implicated in myogenic fusion (Yagami-Hiromasa et al., 1995). The present study tests whether disturbances in the expression of ECM molecules or their integrin receptors result from a lack of MyoD and account for the clumping of MyoD(–/–) myoblasts and their delayed fusion. Detailed immunohistochemical studies were carried out on MyoD(–/–) and control BALB/c whole muscle autografts *in vivo*, and on primary muscle cultures *in vitro* to identify differences in the expression of basement membrane molecules known to be expressed in muscle (laminin $\alpha 2$, $\alpha 4$, $\alpha 5$ chains and collagen IV), ECM proteins known to be up-regulated during inflammation (fibronectin, tenascin-C) and integrin receptors for laminin isoforms and fibronectin ($\alpha 5\beta 1$, $\alpha 6\beta 1$ and $\alpha 7\beta 1$). Antibodies against desmin, a muscle-specific cytoskeletal protein, were used to identify myogenic cells (Lawson-Smith and McGeachie, 1998; White et al., 2000).

Material and methods

Animals. A colony of MyoD (–/–) mice (originally a generous gift from Michael Rudnicki, Ottawa Hospital Research Institute, Ottawa ON, Canada; Rudnicki et al., 1992) was established in the Animal Resource Centre, Murdoch, Australia. Experiments were conducted in strict accordance with guidelines of the University of Western Australia Animal Ethics Committee and the National Health and Medical Research Council, Canberra, Australia. All animals were housed in individual cages under a 12 hour day/night cycle and allowed *ad libitum* access to food and water. The null mutation (exon 1 deletion) in MyoD(–/–) mice was confirmed using PCR with primers kindly provided by Dr Marcia Ontell (Pittsburgh PA, USA). The BALB/c strain was used as the wild type control in these studies for two reasons: (i) MyoD(–/–) mice are on a BALB/c enriched genetic background, and (ii) BALB/c muscle exhibits the least efficient regenerative capacity among the different strains investigated to date (Mitchell et al., 1992; Maley et al., 1995).

Primary cultures of skeletal muscle for in vitro studies. Primary cultures of skeletal muscle were established essentially as described previously (Maley et al., 1994, 1995). The hind limb and lower back muscles from 4 week old BALB/c and MyoD(–/–) mice were removed and minced using scissors. Sequential enzymatic digestion with collagenase, dispase and trypsin (Worthington, Freehold NJ, USA) was used to disrupt tissue structure. The digestion mixture was washed and coarsely filtered to remove tissue debris. The resulting cellular material was then seeded into flasks coated with 1% (v/v) gelatin (Sigma, St Louis MO, USA) in HAMS F10 (Sigma) supplemented with 20% (v/v) FCS (Life Technologies, Rockville MD, USA) and 25 ng/ml bFGF (Sigma).

Integrin expression on myogenic cells in vitro. Immunohistochemistry was used to examine expression of integrins $\alpha 5$ (binds fibronectin) and $\alpha 6$ (binds laminin) by fusing myoblasts *in vitro*. BALB/c and MyoD(–/–) myoblasts were plated at 2×10^4 cells per well on a gelatin-coated 8-well tissue culture slide in growth medium. After 24 h, the medium

Table 1. Details of primary and secondary antibodies used in immunohistochemistry

Primary antibodies

Specificity	Species	Dilution	Source/Reference
Fibronectin	Rabbit	1 : 400	Sigma
Tenascin C	Rat	1 : 200	Sigma
Collagen IV	Rabbit	1 : 200	Biodesign
Pan Laminin-1	Rabbit	1 : 400	Sigma
Laminin $\alpha 2$	Rat	Neat	(Schuler and Sorokin, 1995)
Laminin $\alpha 4$	Rat	1 : 200	(Ringlemann et al., 1999)
Laminin $\alpha 5$	Rat	Neat	(Sorokin et al., 1997)
Integrin $\alpha 5$	Rat	1 : 200	Pharmingen
Integrin $\alpha 6$	Rat	1 : 500	Pharmingen
Integrin $\alpha 7$	Rabbit	1 : 500	(Echtermeyer et al., 1996)
Desmin	Goat	1 : 75	Santa Cruz

Secondary antibodies used to detect binding of the primary antibodies listed above

Primary Source	Conjugate	Streptavidin	Dilution	Source/Reference
Rabbit	Cy3	N/A	1 : 500	Amersham
Rat	Biotin (1 : 200)	ALEXA546	1 : 10,000	Molecular Probes
Goat	ALEXA 488	N/A	1 : 1000	Molecular Probes

was removed and replaced with fusion medium (as described below). This was called time zero. The fusion medium was replenished daily. After 2, 4 and 6 days in fusion medium, cultures were fixed in 2% paraformaldehyde and stained for integrin $\alpha 5$ and $\alpha 6$ (for specifications of antibodies, see Table 1). Nuclei were visualised by Hoechst 33342 staining (Molecular Probes, Portland OR, USA).

Quantitation of matrix and integrin expression by cultured muscle cells in vitro. ELISA was used to quantify the production of ECM-associated proteins by muscle cells. Aliquots (100 µl) of 1×10^5 cells/ml in 'growth medium' (Hams F10 medium supplemented with 20% (v/v) fetal calf serum (FCS)) were added to each well of gelatin-coated 96-well ELISA plates and allowed to adhere. After 24 h, the medium was removed and replaced with fusion medium consisting of DMEM (Sigma) supplemented with 2% (v/v) horse serum (Sigma). This was designated time zero. The fusion medium was replenished daily. After 6 days in fusion medium, the presence of myotubes was confirmed by phase-contrast microscopy. This time point was selected on the basis of previous studies in our laboratory (unpublished observations) showing that myotubes were found frequently in cultures of cells from both strains. The levels of collagen type IV, laminins (as detected using a pan-laminin antibody), tenascin-C, fibronectin and the integrin subunits $\alpha 5$ and $\alpha 6$ were assessed by ELISA of the cell layers (for specification of antibodies, see Table 2). It should be noted that this method detects only protein that is attached to the exposed surface of cells: it does not detect protein at the lower side of the attached cells nor protein secreted into media. As such, this ELISA method allowed assessment of the possible contribution of ECM molecules and integrin receptors to myotube formation and correlations with our *in vivo* observations. Excess medium was washed from the wells with PBS and non-specific interactions with plastic were blocked with PBS containing 10% FCS and 1% BSA.

Table 2. Details of primary and secondary antibodies used in ELISA

Specificity	Species	Dilution	Source
Fibronectin	Rabbit	1 : 500	Sigma
Tenascin C	Rat	1 : 500	Sigma
Collagen IV	Rabbit	1 : 500	Biodesign
Pan Laminin-1	Rabbit	1 : 1000	Sigma
Integrin α5	Rat	1 : 200	Pharmingen
Integrin α6	Rat	1 : 500	Pharmingen
Secondary Antibodies			
Primary Source	Conjugate	Dilution	Source
Rabbit	Peroxidase	1 : 2000	Dako
Rat	Peroxidase	1 : 1000	Dako

After washing with PBS, primary antibodies were diluted in PBS containing 0.1% BSA and cells were incubated with the antibodies for 60 min at 37 °C. HRP-conjugated secondary antibodies (Table 2) were diluted in PBS containing 0.1% BSA and cells incubated with the antibodies for 60 min at 37 °C. To detect the presence of bound antibody, 100 µl of fresh ophenylenediamine (Sigma) dissolved in methanol and tri-sodium citrate buffer (pH 4) was added and cells were incubated for 15 min at room temp. The reaction was stopped using 50 μl of 12.5% sulphuric acid. The optical density (OD) of each well was read at a wavelength of 490 nm using a microplate reader (Bio-Rad, Hercules CA, USA). Statistical differences in the production of each individual proteins between MyoD(–/–) and BALB/c cultures were calculated using a Student's t-test.

Surgical procedure for whole muscle grafts. A total of 7 muscle grafts were examined (2 BALB/c and 5 MyoD(–/–) grafts). The procedure for autografting whole muscles has been described in detail elsewhere (Roberts et al., 1989). Briefly, the mice were anaesthetised with a gaseous mixture of 1.5% halothane and 0.2% nitrous oxide in oxygen, the extensor digitorum longus (EDL) muscle of each leg was relocated over the tibialis anterior (TA) muscle of the same leg, sutured proximally to the distal tendon of the quadriceps femoris muscle and distally to the distal tendon of the TA, and the skin was closed (with 6–0 braided silk suture). Following surgery, all mice were transferred to standard cages with food and water *ad libitum* and allowed to recover in a temperature and light controlled environment.

Tissue collection and processing. Mice were killed by cervical dislocation at 5 (2 MyoD null and 1 BALB/c) and 7 days (3 MyoD null and 1 BALB/c) after surgery. These periods of time were selected as they allowed examination of the various stages of myogenic regeneration (i. e. necrosis, myoblast proliferation, presence of small and large myotubes) within an individual graft sample. At the time of sampling, both grafted EDL and underlying TA were removed. Grafts were oriented for transverse cryostat sectioning on a cork block using Tragacanth Gum (Sigma), and snap frozen in isopentane (BDH, Poole, UK) cooled in liquid nitrogen. Samples were stored at –80 °C prior to sectioning. Frozen sections (8 µm thick) were collected onto silanated slides and air-dried and stored at –20 °C until staining.

Antibodies for immunohistochemistry. Specification and source of the primary antibodies are listed in Table 1. The secondary conjugates were biotinylated anti-rat IgG (Vector,

Burlington CA, USA), anti-rabbit antibody conjugated with Cy3 (Amersham Pharmacia, Amersham, UK) and anti-goat antibody conjugated with ALEXA488 (Molecular Probes) (Table 1). The biotinylated secondary antibodies were visualized using ALEXA546-streptavidin (Molecular Probes).

Staining protocols. All sections were double stained with antibodies against the cytoskeletal protein desmin to identify myoblasts and myotubes. Sections were re-hydrated with phosphate buffered saline (PBS) for 20 min and non-specific protein binding was inhibited by incubating the sections with 5% fetal calf serum in wash buffer (PBS containing 0.5% bovine serum albumin (BSA) and 0.1% glycine) for 60 min. Primary antibodies were diluted (as indicated in Table 1) in wash buffer and sections were incubated for 60 min at room temp. Anti-desmin and test antibodies were used simultaneously. After each incubation, excess antibody was washed from the sections with 3 changes of wash buffer. To detect binding of primary antibodies, secondary antibodies either directly conjugated to Cy 3 (against rabbit primary antibodies), ALEXA488 (against goat anti-desmin) or biotin (see Table 1 for details) were diluted in wash buffer and sections were incubated for 60 min. For rat-derived primary antibodies, a further incubation step with ALEXA546-streptavidin diluted in wash buffer was applied. Nuclei were stained with Hoescht 33342 for 20 sec and sections were mounted using a permanent aqueous mountant (polyvinyl alcohol). Slides were stored at –4 °C until analysis. Control staining in the absence of primary antibodies of a second section on each slide confirmed specificity of immunostaining.

Analysis of immunohistochemical staining. Sections were examined with a DM RBE fluorescence microscope (Leica, Wetzlar, Germany). In grafts, immunolocalisation of ECM components was analysed in relation to specific events in the regenerative process. Expression was carefully examined in myoblasts (desmin-positive mononucleated cells), cuffing myoblasts (desmin-positive cells within the basement membrane surrounding necrotic fibres), myotubes (larger desmin-positive cells with centralised nuclei, usually recently formed and close to the regenerative front) and more mature myotubes (larger desmin-positive cells with a central nucleus usually located toward the periphery of the grafts). Photomicrographs were made and images were scanned using a Sprint Scan 35 plus slide scanner (Polaroid, Cambridge MA, USA) and digitised using PhotoShop software (Adobe, San Jose CA, USA).

Results

Immunohistochemistry in vitro. To examine integrin expression on myoblasts and myotubes, primary muscle cultures were stained with antibodies against integrin $\alpha 5$ and $\alpha 6$. Integrin $\alpha 5$ staining was observed on myoblasts (desmin-positive mononuclear cells) and newly formed myotubes. Staining was localised in discrete zones at the periphery of cells. Integrin $\alpha 6$ was not detectable on desmin-positive mononucleated myoblasts, but was expressed heterogeneously on the surface of fibroblasts (data not shown).

Production of extracellular matrix molecules and integrin in vitro. ELISA was used to examine the expression of collagen IV, laminins, tenascin-C, fibronectin, and integrin $\alpha 5$ and $\alpha 6$ by fusing MyoD(–/–) and BALB/c myoblasts in vitro (Fig. 1). After 6 days in fusion medium, myotubes had developed in all cultures. However, MyoD(–/–) myotubes were significantly smaller (bi-nucleated myotubes) than in BALB/c cultures (up to 6 nuclei per myotube). Of all proteins examined, only expression of the integrin $\alpha 6$ subu-

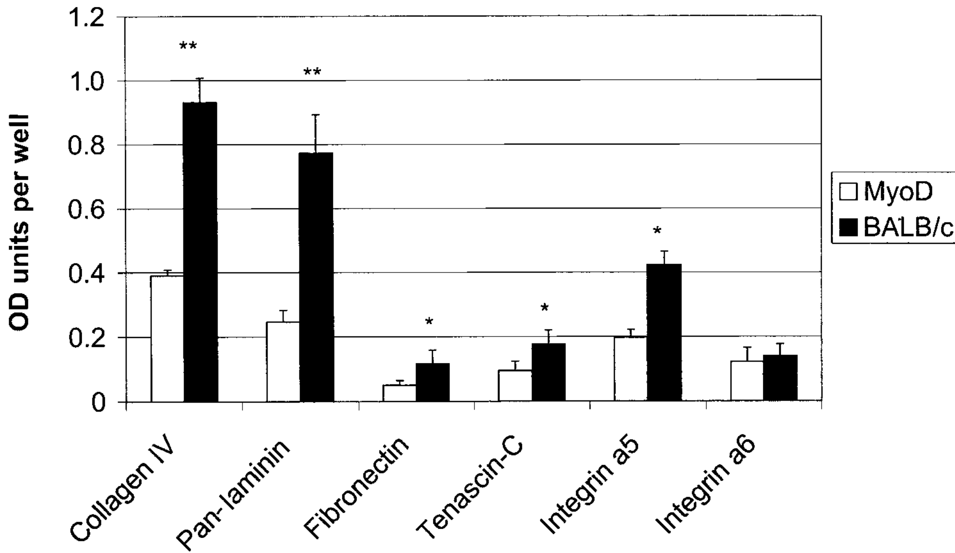


Fig. 1. ELISA quantification of extracellular matrix components and integrins expressed by fusing primary MyoD(–/–) and BALB/c myoblast cultures. ELISA was performed directly on cell layers at 6 days after addition of fusion medium and the amount of each protein is expressed as optical density (OD) per microgram of DNA. Note that only few myotubes were present in MyoD(–/–) cultures at this time point in contrast with BALB/c cultures where many myotubes are present. Error bars indicate standard deviation (n = 4). *, $P < 0.05$; **, $P < 0.01$.

nit showed no difference between fusing MyoD(–/–) and BALB/c myoblasts. Largest differences were observed in levels of the basement membrane components collagen IV ($P < 0.001$) and laminins ($P < 0.001$), as well as integrin $\alpha 5$ ($P < 0.001$). Smaller but significant differences were found in the production of interstitial matrix components tenascin-C ($P < 0.05$) and fibronectin ($P < 0.05$).

Expression of ECM molecules and integrins in adult skeletal muscle. Expression of all proteins was examined in underlying TA as baseline level of expression in uninjured adult skeletal muscle (Fig. 2).

Collagen type IV staining was present in basement membranes of muscle fibres, blood vessels and nerves in the undamaged TA (Fig. 2 a) and in grafts. In normal undamaged TA muscle of both MyoD(–/–) and BALB/c mice, laminin $\alpha 2$ was strongly expressed in the basement membrane (Fig. 2 b). In undamaged TA muscle, staining for laminin $\alpha 4$ was very weak and mainly restricted to blood vessels. In undamaged TA muscle, laminin $\alpha 5$ was mainly present in the basement membrane of blood vessels (Fig. 2 c).

Staining of interstitial components in undamaged skeletal muscle was weak. In TA muscle underlying the graft, fibronectin staining was found mainly in interstitial connective tissue with a mesh-like appearance surround-

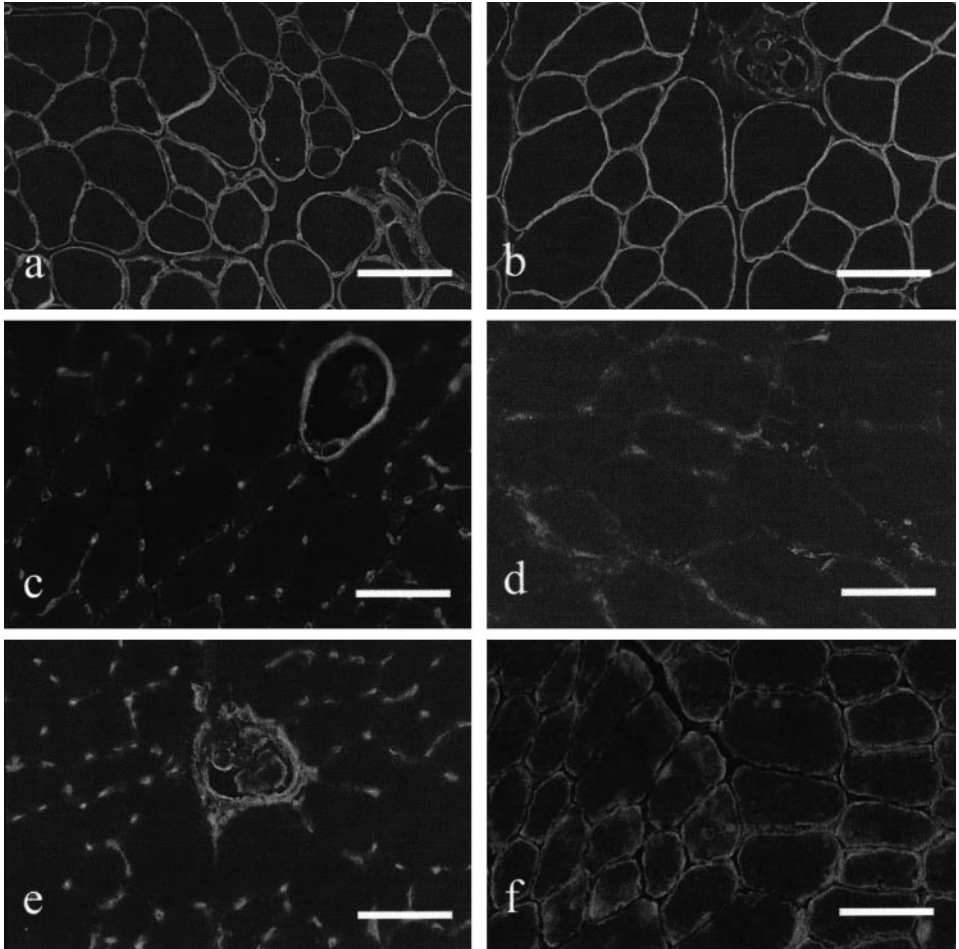


Fig. 2. Immunohistochemical staining of (undamaged) TA skeletal muscle underlying the grafts. The pattern of staining was identical for both MyoD(–/–) and BALB/c undamaged skeletal muscles. Staining for (**a**) collagen type IV, (**b**) laminin $\alpha2$, (**c**) laminin $\alpha5$, (**d**) integrin $\alpha5$, (**e**) integrin $\alpha6$, and (**f**) integrin $\alpha7$. Bars, 50 µm.

ing myofibres (data not shown because they only confirm previously published results). Weak intermittent interstitial staining of tenascin-C was observed around undamaged myofibres in TA (data not shown).

Focal patchy staining of integrin $\alpha5$ was present in undamaged adult skeletal muscle around myofibres (Fig. 2 d). Integrin $\alpha6$ staining was associated with blood vessels and peripheral nerves (Fig. 2 e), and integrin $\alpha7$ staining was present around myofibres (Fig. 2 f) of undamaged muscle.

Histology of whole muscle autografts. The pattern of regeneration in whole muscle grafts has been extensively described elsewhere (Roberts et al., 1997). In brief, when the tissue becomes revascularised (initially at the periphery of

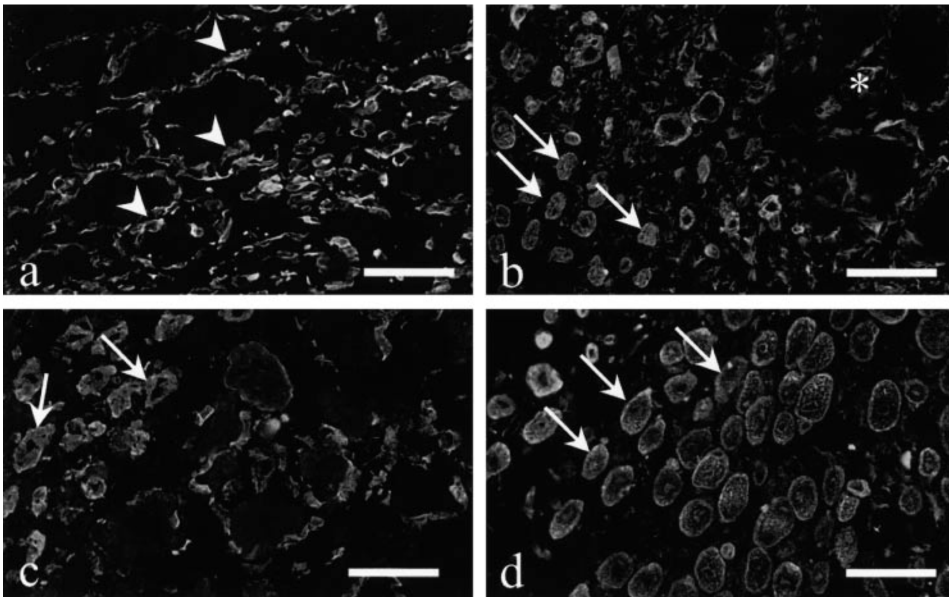


Fig. 3. Myotube formation in MyoD(–/–) and BALB/c autografts. Desmin immunohistochemistry was used to identify myoblasts and myotubes (Note: images a–d are similar fields as those in the double stained images in Fig. 4 a, g, h, d, respectively). A number of distinct differences between MyoD(–/–) and BALB/c autografts were observed. At day 5, myotubes were not found in MyoD(–/–) grafts (**a**), although many myoblasts were present and were 'cuffing' necrotic fibres (arrowheads). In contrast, many myotubes (arrows) had formed at this time point adjacent to necrotic muscle (*) in BALB/c grafts (**b**). At day 7, MyoD(–/–) grafts showed a level of myotube formation (arrows) that was comparable with that at day 5 in BALB/c grafts (**c**). At day 7, extensive myotube formation was found in BALB/c grafts and many large myotubes were present (arrows) (**d**). Bars, 50 μm.

the graft), removal of necrotic tissue and new muscle formation starts at the outer edges of the graft and progresses over time towards the necrotic centre. The 'regenerating zone' initially contains mononuclear cells that include inflammatory cells, fibroblasts and myoblasts. Many myoblasts are derived from satellite cells located beneath the basement membrane of myofibres and when the myoblasts encircle necrotic sarcoplasm they are often referred to as "cuffing cells" (see Fig. 3 a, c). Myotubes initially form at the periphery of the grafts and then progressively towards the centre, and increase in size as they mature (White et al., 2000). Thus, in a single transverse section of a graft, all stages of this zonal regenerative activity can be observed at different locations.

Desmin immunohistochemistry was used to identify myogenic cells and myotubes in autografts (Fig. 3). In BALB/c grafts, well-developed myotubes were present at day 5 after grafting (Fig. 3 b) and were extensively found throughout the graft by day 7 (Fig. 3 d) as has been described elsewhere (White et al., 2000). In contrast, the MyoD(–/–) grafts did not contain myo-

tubes at day 5 (Fig. 3 a). However, at 7 days many small myotubes had formed, mainly in the periphery, in MyoD(–/–) grafts (Fig. 3 c) and these grafts resembled BALB/c grafts at 5 days (Fig. 3 b).

Immunohistochemical staining of whole muscle grafts. The pattern of the immunohistochemical staining of grafts (Fig. 4) was carefully examined in (i) early "cuffing" and larger myoblasts, and (ii) small and large myotubes.

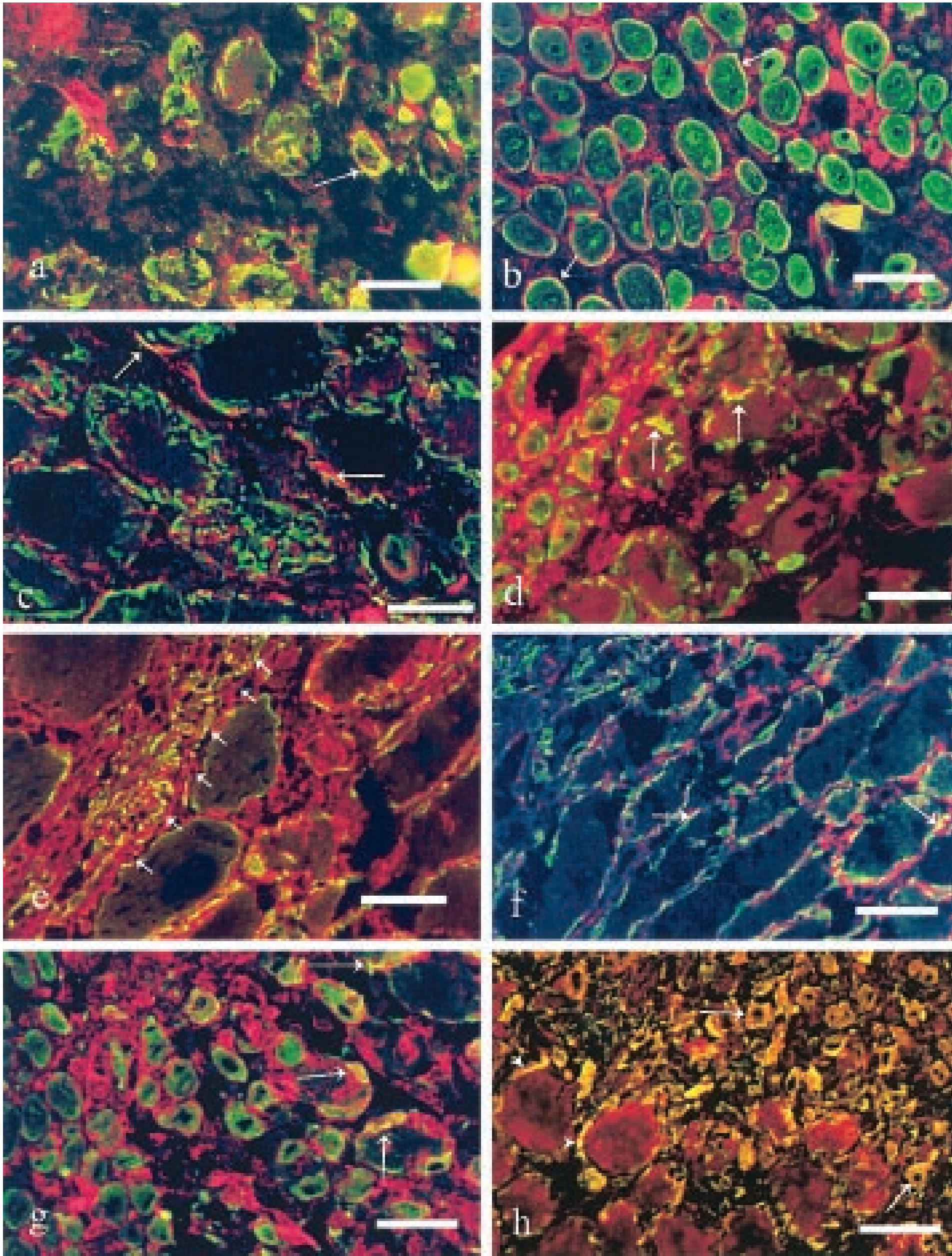
Collagen IV. In the necrotic central region of the graft, staining around myofibres was reduced and patchy, but immunoreactivity was retained throughout the experimental period. Staining for collagen type IV was found around newly formed myotubes, and the intensity of this staining increased as the myotubes matured. The pattern of collagen type IV staining was similar in both MyoD(–/–) and BALB/c autografts (data not shown).

Laminin $\alpha 2$. In grafts, the necrotic central region showed a discontinuous pattern of laminin $\alpha 2$ immunostaining in both strains. In all grafts, laminin $\alpha 2$ was present in the membranes of small myotubes and increased in intensity as the myotubes matured (data not shown).

Laminin $\alpha 4$. Staining was found only around blood vessels and some mature myotubes at the edges of day 7 MyoD(–/–) and BALB/c grafts; there was no staining of smaller less mature myotubes (data not shown).

Laminin $\alpha 5$. Weak discontinuous laminin $\alpha 5$ staining was observed around some cuffing cells, some myoblasts and some myotubes (Fig. 4 a, b). The myotube-associated staining appeared to be stronger in BALB/c than in MyoD(–/–) grafts. In small (newly developed) myotubes, staining was cytoplasmic (Fig. 4 a) whereas larger (older) myotubes were surrounded by intense staining for laminin $\alpha 5$ (Fig. 4 b).

Fig. 4. Immunohistochemical staining of regenerating whole muscle autografts of adult mouse skeletal muscle. All sections were double stained with desmin antibodies detected with green fluorescent ALEXA488 label, and the various matrix, laminin and integrin antibodies, were labelled scarlet with ALEXA546, thus colocalisation of the 2 fluorescent markers appears as yellow in the composite images (**a, b, d, f, g, h**). Laminin $\alpha 5$ expression (arrows) (**a, b**) in a day 7 BALB/c autograft is weakly stained in the cytoplasm of young myotubes (**a**) but is strongly stained at the periphery of larger mature myotubes (**b**). Tenascin-C staining (**c, d**) is present in close association with desmin-positive cuffing cells (arrows) in a day 5 MyoD(–/–) (**c**) and BALB/c autograft (**d**). Integrin $\alpha 5$ (**e, f**) staining (arrows) in a 5 day MyoD(–/–) autograft is present in areas of grafts occupied by large numbers of mononuclear cells (**e**) some of which are desmin positive (not shown) and is also strongly associated with some cuffing myoblasts (**f**). Integrin $\alpha 6$ staining (**g**) colocalises with desmin (arrows) in mature cuffing cells in a day 7 BALB/c autograft. Integrin $\alpha 7$ and desmin (**h**) show highly frequent colocalisation in a day 7 MyoD(–/–) autograft in an area where many myoblasts are present (arrowheads) and myotubes (arrows) are starting to form. Bar, 50 μm.


a
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Fibronectin. Staining of fibronectin was observed in the interstitial space between necrotic fibres of grafts, and strong staining was present around newly formed myotubes (data not shown). No difference in the staining pattern was observed in MyoD(–/–) grafts as compared with BALB/c grafts.

Tenascin-C. In all grafts there was strong interstitial staining of tenascin-C throughout the grafts, and tenascin-C colocalised with desmin in cuffing cells in both MyoD(–/–) and BALB/c autografts (Fig. 4 c, d). Strong staining was present around newly formed myotubes (data not shown), and this staining became more diffuse as the myotubes matured.

Integrin $\alpha 5$. In both strains, integrin $\alpha 5$ staining was observed in areas of grafts that contained many mononucleated cells (Fig. 4 e), some of which were desmin positive. Variable intensity of integrin $\alpha 5$ staining was observed in blood vessel walls and cuffing myoblasts (Fig. 4 f), and faint staining was present in some myotubes.

Integrin $\alpha 6$. Integrin $\alpha 6$ staining was variable around myogenic cells and staining was found in a few cuffing cells of both MyoD(–/–) and BALB/c grafts (Fig. 4 g). Staining was strong around the periphery of mature myotubes in one MyoD(–/–) graft, but not in other MyoD(–/–) and BALB/c grafts.

Integrin $\alpha 7$. Integrin $\alpha 7$ colocalised with desmin on all desmin-positive cells (myoblasts, myotubes and myofibres). Weak staining was also found in some blood vessel walls in grafts. A similar intensity of staining in all cell types was observed in both MyoD(–/–) (Fig. 4 h) and BALB/c autografts.

Discussion

The present study investigated whether disturbances in expression of ECM components or integrins are involved in sustained proliferation and delayed fusion of MyoD(–/–) myoblasts (in regenerating whole muscle autografts). Immunohistochemical assessment of expression of ECM components and integrins was compared after transplantation of whole muscle autografts and in primary muscle cultures of MyoD(–/–) and BALB/c mice. As previously reported (White et al., 2000), MyoD(–/–) autografts showed significant histological differences as compared to BALB/c autografts in the first week after transplantation. Whereas well-developed myotubes are present after 5 days in BALB/c grafts and were extensive throughout the graft at 7 days after transplantation, MyoD(–/–) grafts contained no myotubes at day 5 and small myotubes were present at day 7, mainly around the periphery of MyoD(–/–) grafts. At this period of time after transplantation, the appearance of BALB/c and MyoD(–/–) autografts is similar (White et al., 2000). The present study also confirmed that myotube formation is delayed *in vitro* which is in agreement with previous studies (Yablonka-Reuveni et al., 1999). After 6 days in fusion medium, MyoD(–/–) myotubes were smaller and only bi-nucleated as compared to BALB/c myotubes that contained up to six nuclei.

ELISA and immunofluorescent studies showed that the expression pattern of ECM components and integrins correlated with cellular events such as myotube formation during the regenerative process. Patterns found in MyoD(–/–) muscle closely mimicked the situation in BALB/c muscle (Grounds et al., 1998).

Regeneration can occur in the absence of a basement membrane (Caldwell et al., 1990), but the presence of a basement membrane scaffold facilitates effective regeneration. Major components of basement membranes are collagen IV and laminins. Our observations were consistent with previous findings: in undamaged muscle, collagen IV was present in basement membranes of myofibres, blood vessels and nerves (Ringlemann et al., 1999) and in regenerating muscle, collagen IV staining was found in basement membranes of developing myotubes as well but was not found in myoblasts (Sorokin et al., 2000). The strikingly smaller amounts of collagen type IV and laminins (as measured by ELISA) produced by cultured MyoD(–/–) muscle cells probably reflects the lack of proper formation of myotubes and associated basement membrane proteins at day 6 due to delayed MyoD(–/–) myoblast fusion as compared with control BALB/c cultures.

In skeletal muscle basement membranes, laminin-2, often referred to as merosin and characterised by the laminin $\alpha2$ chain, is the predominant form of laminin both during embryogenesis and in adult animals (Schuler and Sorokin, 1995). In the present study, there was strong expression of the laminin $\alpha2$ chain in the basement membrane of uninjured mature myofibres and myotubes, and staining was not observed in myoblasts or cuffing cells as has been reported previously (Grounds et al., 1998; Sorokin et al., 2000). The expression pattern in relationship with cellular events was similar in MyoD(–/–) and BALB/c muscles. Laminin $\alpha4$, a component of laminin 8 and 9, is transiently expressed in the basement membrane of young myotubes, and in endothelium and peripheral nerve basement membranes (Ringlemann et al., 1999; Sorokin et al., 2000): the same expression pattern was observed in the present study in both strains of mice. Laminin $\alpha5$, which is a component of laminin 10 and 11, is normally expressed in basements membranes of blood vessels, peripheral nerves and at neuromuscular junctions in mature myofibres (Sorokin et al., 1997). In previous studies on regenerating muscle, laminin $\alpha5$ was also found to be transiently expressed in the basement membrane of newly formed myotubes (Sorokin et al., 1997; Ringlemann et al., 1999). In the present study, weak laminin $\alpha5$ staining was indeed observed in (some) myotubes. In young myotubes, some laminin $\alpha5$ staining was localised in the cytoplasm, whereas in older myotubes laminin $\alpha5$ staining occurred extracellularly, and was mainly associated with blood vessels. The myotube-associated staining appeared to be stronger in BALB/c grafts than in MyoD(–/–) grafts, suggesting that laminin $\alpha5$ may play a role in early fusion processes. Laminin $\alpha5$ staining has also been found in cultured muscle cells (L. Sorokin, personal communication).

Apart from a 2–3 delay in myoblast fusion associated with the sustained proliferation of MyoD(–/–) myoblasts, the overall kinetics of regeneration in

MyoD(–/–) and BALB/c muscle in autografts are similar and infiltration of inflammatory cells into the graft occurs at the same time, within the first 48 h (White et al., 2000). During the inflammatory process, the interstitial ECM components fibronectin and tenascin-C are up-regulated and often co-localise in tissues (Chiquet-Ehrismann et al., 1988; Chiquet-Ehrismann, 1990). Fibronectin is strongly expressed in the interstitium between myofibers in undamaged muscle and is highly upregulated in the interstitium of both crush-injured muscle and EDL grafts (Grounds et al., 1998) as was also observed in the present study in both strains. Fibronectin is thought to play a role in cell adhesion, mediating the phagocytic removal of debris and the localisation of incoming fibroblasts, endothelial and myogenic cells. The exact role that fibronectin plays in cell fusion is not clear. Fibronectin is bound to the cell surface of proliferating myoblasts, but is lost during and after fusion (Kleinman et al., 1981; Schützle et al., 1984), and the level of fibronectin mRNA is also reduced upon fusion of myoblasts (Leibovitch et al., 1986). In addition, high levels of fibronectin promote replication and reduce fusion in mammalian cell lines (Podleski et al., 1979; Kleinman et al., 1981) further supporting a role for the high levels of fibronectin during proliferation and for low levels during fusion. However, an increase in fibronectin expression during myotube formation has been reported (Walsh and Phillips, 1981). Our tissue culture studies show an increased amount of fibronectin in fusing BALB/c cultures as compared with MyoD(–/–) at 6 days. This suggests, that fibronectin in primary muscle cultures is increased during fusion, since the extent of myotube formation is greater in BALB/c as compared with MyoD(–/–) cultures at this timepoint.

Tenascin-C occurs at the myotendinous junction in normal uninjured muscle, and also in the vicinity of new myotubes (Mackie et al., 1988; Daniloff et al., 1989; Irintchev et al., 1993; Grounds et al., 1998), which is consistent with its expression in tissues under mechanical stress and transient expression following muscle damage, respectively (Mackie et al., 1988; Daniloff et al., 1989; Irintchev et al., 1993; Grounds et al., 1998). Expression of tenascin-C correlates with muscle damage/regeneration and the presence of macrophages in several muscular dystrophies (Settles et al., 1996). Our observations suggest production of tenascin-C by myogenic cells, yet *in situ* hybridisation does not reveal any production of tenascin-C by skeletal muscle cells indicating that non-muscle cells synthesise most if not all tenascin (Gullberg et al., 1998). Tenascin-C levels were relatively low in MyoD(–/–) grafts at day 5 as compared with BALB/c and this correlated closely with the absence of myotubes. Where myotubes were present in day 7 MyoD(–/–) grafts, strong tenascin-C staining was observed and it was considered that tenascin-C increased in response to formation of myotubes. The lower amounts of tenascin-C produced by primary cultures of MyoD(–/–) muscle at 6 days was also considered to reflect the relatively poor myotube formation at this time point. The presence of fibroblasts (as well as myoblasts) could be responsible for the production of tenascin-C in such primary cultures. An alternative explanation

is that the lack of tenascin-C expression was responsible, at least in part, for the delayed myotube formation in MyoD(–/–) grafts.

The cell surface molecule integrin $\alpha5\beta1$ mediates cellular attachment to fibronectin in the interstitium (Gullberg et al., 1998). Integrin $\alpha5\beta1$ is associated with cell proliferation, oncogenic transformation, cell survival, cell migration, assembly of fibronectin matricies, wound healing, T-cell activation and gene expression (Taverna et al., 1998). The integrin $\alpha5$ antibody stained cuffing cells, myoblasts and myotubes *in vivo* and there was patchy staining around mature adult myofibres. A similar pattern observed in myoblasts and myotubes *in vitro*, and this may correspond to the presence of integrin $\alpha5\beta1$ in adhesion plaque-like structures at the surface of cultured myotubes (Enomoto et al., 1993). The integrin $\alpha5\beta1$ null mutation is lethal, but chimeric mice at birth show evidence of an ongoing myopathy including the presence of giant fibres, central nuclei, vacuoles, fibrosis and fibre degeneration (Taverna et al., 1998). Since integrin $\alpha5\beta1$ null myoblasts in culture do not demonstrate any myogenic defects (Taverna et al., 1998), this suggests that while integrin $\alpha5\beta1$ is not essential for proliferation, migration and differentiation of myoblasts, it may be necessary for long-term integrity of myotubes. Furthermore, integrin $\alpha5\beta1$ as well as integrin $\alpha7\beta1$ are localized in adhesion plaques on myofibres (McDonald et al., 1995) and at the myotendinous junction (Taverna et al., 1998), sites where considerable mechanical stress is exerted again supporting the idea of a stabilising role for this integrin. There were no apparent differences in staining patterns in MyoD(–/–) and control muscles in the present study.

Laminin binds to both integrin $\alpha7$ (the main isoform in mature skeletal muscle) and integrin $\alpha6$ (Burkin and Kaufman, 1999). As previously described (Sorokin et al., 2000), we found that integrin $\alpha6$ staining was only associated with blood vessels and peripheral nerves in uninjured mature muscle. After crush injury of skeletal muscle (in both dystrophic *dy/dy* and control ReJ mice) integrin $\alpha6$ staining was found on leukocytes and fibroblasts, but rarely on myogenic (desmin-positive) cells (Sorokin et al., 2000) although the intensity of integrin $\alpha6$ staining was higher in *dy/dy* muscle regenerating after crush injury. These observations are in contrast with the present study where intense staining of integrin $\alpha6$ was associated with some myotubes in both BALB/c and MyoD(–/–) autografts. The difference may be accounted for by the experimental models that are used to study regeneration. The present study was based on regeneration in a whole muscle graft where the basement membrane and matrix scaffold remain relatively intact (Roberts et al., 1997). However, more traumatic injuries such as crush injury used in the study of Sorokin et al. (2000) cause massive disruption of the muscle architecture and extensive interstitial connective tissue formation (Mitchell et al., 1992) and potentially a loss of integrin-mediated attachment.

Integrin $\alpha7\beta1$ is the predominant laminin-binding integrin in skeletal muscle and is present on the surface of myoblasts and myofibres (von der Mark et al., 1991; Burkin et al., 2001). Different splice variants of integrin $\alpha7$ exist and are concentrated at different locations along the myofibre (Burkin and

Kaufman, 1999). Variable α7 integrin staining was associated with myoblasts in mature muscle regenerating after crush injury (Sorokin et al., 2000). In the present study, antibodies against integrin α7 stained all myogenic cells intensely and colocalised strongly with desmin in both MyoD(–/–) and BALB/c autografts.

The most distinct differences that were observed between the 2 strains in the present study were the lower staining for tenascin-C *in vivo* in areas devoid of myotubes in MyoD(–/–) grafts, and less production of basement membrane components collagen type IV and laminins in 6 day cultures where MyoD(–/–) myotubes were fewer and smaller. Many other molecules, especially cell-surface cell adhesion molecules, remain to be investigated to determine their possible role in myoblast adhesion, proliferation and myotube formation. In conclusion, while some differences were noted in the expression of ECM proteins and integrins between regenerating MyoD(–/–) and BALB/c whole muscle autografts and myotubes in culture, these were considered to be a result of the delayed formation of myotubes rather than a direct causal mechanism.

Acknowledgments. This work was supported by a grant (MDG) from the National Health & Medical Research Council, Canberra, Australia and funding (JH) from the Dutch Duchenne Parent Project (**http://duchenne.nl**). The excellent technical assistance of Marilyn Davies is particularly and gratefully acknowledged. We thank H. von der Mark, University of Erlangen–Nuremberg, Germany, for kindly supplying the integrin α7 antibody and L. M. Sorokin, University of Erlangen–Nuremberg, Germany, for supplying antibodies against ECM molecules and for critical reading of the manuscript.

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