Constitutively activated dystrophic muscle fibroblasts show a paradoxical response to TGF- β and CTGF/CCN2

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Abstract Transforming growth factor beta (TGF- β) and connective tissue growth factor (CTGF) have been described to induce the production of extracellular matrix (ECM) proteins and have been reported to be increased in different fibrotic disorders. Skeletal muscle fibrosis is a common feature of Duchenne muscular dystrophy (DMD). The *mdx* mouse diaphragm is a good model for DMD since it reproduces the muscle degenerative and fibrotic changes. Fibronectin (FN) and proteoglycans (PG) are some of the ECM proteins upregulated in dystrophic conditions. In view of understanding the fibrotic process involved in DMD we have isolated fibroblasts from dystrophic *mdx* diaphragms. Here we report that regardless of the absence of degenerative myofibers, adult *mdx* diaphragm fibroblasts show increased levels of FN and condroitin/dermatan sulfate PGs synthesis. Fibroblasts isolated from non fibrotic tissue, such as 1 week old mice diaphragms or skin, do not present elevated FN levels. Furthermore, *mdx* fibroblast conditioned media is able to stimulate FN synthesis in

control fibroblasts. Autocrine TGF- β signaling was unaltered in *mdx* cells. When control fibroblasts are exposed to TGF- β and CTGF, FN increases as expected. Paradoxically, in *mdx* cells it decreases in a concentration dependent manner and this decrease is not due to a downregulation of FN synthesis. According to this data we hypothesize that a pathological environment is able to reprogram fibroblasts into an activated phenotype which can be maintained through generations.

Keywords Muscular dystrophy · Fibronectin · Proteoglycans · Fibrosis · TGF- β · CTGF · Fibroblasts

Abbreviations

α -SMA	Alpha smooth muscle actin
C _{ABC}	Chondroitinase ABC
CTGF/CCN2	Connective tissue growth factor
DMD	Duchenne muscular dystrophy
ECM	Extracellular matrix
FN	Fibronectin
H _{ase}	Heparitinase
PGs	Proteoglycans
TGF- β	Transforming growth factor type β

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Introduction

Duchenne muscular dystrophy (DMD) is the most common skeletal muscle dystrophy in children, with a prevalence of 1:3,500 boys (Emery 2002). Phenotypic changes in muscle tissue include degeneration of muscle fibers, a certain amount of regenerative fibers, fatty tissue and inflammatory infiltration. Towards the end of the disease vast fibrotic tissue is observed which, together with the exhaustion of

muscle progenitor cells, further precludes muscle healing. The disease is due to the absence of the protein dystrophin in the muscle which leads to instability of the sarcolemma and disruption of the myofiber (Blake 2002). The animal model of the disease is the *mdx* mouse. The mouse lacks dystrophin due to a mutation in exon 23 which introduces a stop codon. The course of the disease in *mdx* mice produces slight degenerative changes in limb muscles and the mouse has an almost normal life span. However, the diaphragm of the *mdx* presents vast degenerative changes similar to DMD muscles (Stedman et al. 1991). One of these is the age dependent appearance of fibrotic tissue.

Tissue fibrosis is a common end stage feature of most chronic diseases. It has been widely studied in different systems. In skeletal muscle the increase of extracellular matrix (ECM) occurs in the endomysium and perimysium and can be evaluated through the detection of several ECM molecules, such as fibronectin (FN) and proteoglycans (PGs; Alvarez et al. 2002; Fadic et al. 2006). Under physiological conditions synthesis of these molecules increases during repair of damaged tissue and decreases with the rise of normal parenchyma (Leask and Abraham 2004). During this process there is also inflammatory cell infiltration and cytokine release. Key growth factors involved in the progression towards fibrosis are transforming growth factor beta (TGF- β), and connective tissue growth factor (CTGF; Denton and Abraham 2001; Kinbara et al. 2002; Leask and Abraham 2004; Yokoi et al. 2002).

Fibroblasts are key mediators of the fibrotic response and targets of the aforementioned growth factors. Upon injury, fibroblasts are activated to proliferate and form stress fibers which render them contractile, they express α -smooth muscle actin (α -SMA) and they also increase the synthesis of FN and collagen (Tomasek et al. 2002). Upon resolution of the injury, these activated fibroblasts (myofibroblasts) undergo apoptosis, however when dysregulated they continue to proliferate and remodel the ECM. Furthermore, the ability of ECM molecules to determine the fate of undifferentiated cells is well established. For example an organized ECM is necessary to induce the proper differentiation of C2C12 myoblasts (Osse and Brandan 2002). Thus, it is not surprising that an alteration in the balance of ECM components may have deleterious effects over the resident cells including both fibroblasts and myoblasts.

Much has been studied with regard to the characteristics of myoblasts in DMD but not much emphasis has been placed on fibroblasts since full-length dystrophin is not expressed in those cells. The aim of this work is to understand the pathophysiological role of fibroblasts in skeletal muscle fibrosis associated to muscular dystrophies. Here we report that regardless of the absence of degenerative myofibers, *mdx* skeletal muscle isolated fibroblasts

synthesize more FN and PGs even after 4 passages in cell culture. *Mdx* fibroblast conditioned medium was capable of increasing FN synthesis in control fibroblast cultures, however signaling molecules that have been shown to mediate profibrotic effects were unaltered in *mdx*. Furthermore, when the response to TGF- β or CTGF, growth factors known to have profibrotic properties (i.e. induction of ECM molecules) was studied in *mdx* fibroblasts, we found that both proteins decreased the levels of FN. These results suggest that fibroblasts from dystrophic muscle are constitutively activated maybe due to being exposed to a constantly injured tissue.

Materials and methods

Cell culture

Skeletal muscle fibroblasts were obtained from diaphragm muscles of C57/Bl6 and *mdx* mice aged 8–16 weeks unless otherwise stated. Fibroblasts were isolated from muscle explants as described previously (Fadic et al. 2006; Melone et al. 2000). Briefly, muscle biopsies were minced into pieces smaller than 1 mm², seeded onto 3.8 cm² well plates and covered with 500 μ L of growth medium (Dulbecco's modified eagle medium F-12, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μ g/ml anfotericin B, Invitrogen) supplemented with 50% (v/v) fetal calf serum (Hyclone). Explants were kept at 37°C, 5% CO₂ and 95% humidity. After 3–4 days fibroblasts migrated from the muscle explants. When the cells covered 70–90% of the well surface they were trypsinized and plated onto 21 cm² petri dishes, growth medium was switched to 20% FCS. Cells were used in passages 2 to 4.

Incubation with TGF- β 1 (R&D Systems, USA) or recombinant purified CTGF produced in our laboratory (Abreu et al. 2002; Vial et al. 2007) were carried out in serum free medium. Cells were serum starved for 18 h prior to the addition of growth factors where indicated.

All protocols were conducted in strict accordance and with the formal approval of the animal Ethics Committee of the P. Universidad Católica de Chile.

Gel filtration chromatography and SDS-PAGE

Cells grown in 21 cm² petri dishes were labeled with [³⁵S]-H₂SO₄ 200 μ C/ml, in serum free F-12 HAM medium for 6 h. Conditioned media from *mdx* and control fibroblast cultures, obtained after metabolic labeling, were fractionated through a DEAE-Sephacel column (0.5 ml resin) pre-equilibrated in 10 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 0.1% Triton X-100 and eluted with a linear NaCl gradient (0.2–1.0 M) at a flow rate of 5 ml/h. Fractions of 1.0 ml were

collected and radioactivity was determined. Pooled fractions containing radioactive PGs were then chromatographed on an analytical Sepharose CL-4B column (100 $\times$ 1 cm) equilibrated and eluted with 1% SDS, 0.1 M NaCl, 50 mM Tris-HCl buffer, pH 8.0. Samples (0.5 ml) were applied to the column together with prefractionated dextran blue and phenol to mark void and total volumes respectively. Columns were eluted at a flow rate of 5 ml/h, effluent fractions of 0.8 ml were collected and aliquots counted for radioactivity. Selected fractions from the Sepharose CL-4B column were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Brandan et al. 1992).

SDS-PAGE and western blot analysis of proteoglycans

Appropriate samples were digested with chondroitinase ABC (C_{ABC}) or heparitinase (H_{ase} ; Seikagaku, Tokyo, Japan) and then analyzed by SDS-PAGE followed by fluorography, using a 4–12% acrylamide gradient in the separation gel, as previously described (Caceres et al. 2000). Samples containing equivalent amounts of proteins were digested with H_{ase} or C_{ABC} and then analyzed by SDS-PAGE, the gel was dried and subjected to autoradiography. To identify heparan sulfate proteoglycans (HSPGs) or decorin, fractions equivalent in protein content were incubated with H_{ase} or C_{ABC} respectively and electrophoretically transferred onto immobilon membranes, detected with the anti- Δ -Heparan sulfate monoclonal antibody 3G10 (Seikagaku, Tokyo, Japan; Steinfeld et al. 1996) or anti-mouse decorin LF-113 polyclonal antibody (kindly donated by Dr. L. Fisher, NIDR, NIH, Bethesda, MD, USA; Fisher et al. 1995), and visualized by enhanced chemiluminescence (Pierce, IL, USA).

Immunoblot analysis

For immunoblot analyses, cell extracts obtained from fibroblasts were prepared in 1% SDS buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% sodium dodecyl sulfate and 1 mM PMSF). For analysis of phosphorylated proteins, cell extracts were prepared in radio immunoprecipitation assay buffer as previously described (Osses and Brandan 2002). Aliquots were subjected to SDS gel electrophoresis in 8% or 10% polyacrylamide gels, electrophoretically transferred onto polyvinylidene fluoride membranes (Immobilon-P) and probed with mouse anti- α SMA (1:10,000; Sigma-Aldrich, USA), rabbit anti-FN (1:10,000; Sigma-Aldrich, USA), mouse anti- α -tubulin (1:5,000; Sigma-Aldrich, USA); mouse anti-GAPDH (1:2,000; Chemicon, USA), rabbit anti-phospho-Smad2 (1:1,500; Calbiochem, Germany), mouse anti-phospho ERK1/2 (1:500; Santa Cruz, USA) and rabbit anti-phospho-p38 (1:1,000; Cell Signaling, USA). Anti-rabbit

and anti-mouse horseradish peroxidase (Pierce, USA). All immunoreactions were visualized by enhanced chemiluminescence (Pierce, USA).

Metabolic labeling and immunoprecipitation assays

Fibroblasts were grown on 35 mm² petri dishes. Metabolic labeling was performed incubating the cells with 150 μ Ci/ml of ³⁵S-methionine (Express protein label mix, Perkin Elmer, USA) for 30 min. Cells were then lysed with 1% SDS buffer. FN immunoprecipitation was performed incubating 30 μ g of protein with 5 μ l anti-FN antibody. Protein extracts were preincubated with 50 μ l of protein A-sepharose for 30 min at 4°C in 500 μ l of immunoprecipitation buffer, IPP (Tris-HCl 50 mM pH 7.4, 0.1% SDS, 0.5% NP40, 0.5% deoxicolate). Beads were discarded and supernatants were incubated overnight with 5 μ l of anti-FN polyclonal antibody. 50 μ l of protein A beads were added and incubated 1 h at room temperature. Sepharose was sedimented and washed three times with IPP buffer and three times with phosphate-buffered saline. Sepharose was resuspended in loading buffer and subjected to SDS-PAGE. After electrophoresis the gel was fixed with fixing buffer (25% Isopropanol, 10% acetic acid) for 30 min, then incubated in Amplify solution (Amersham Biosciences, UK), vacuum dried, and exposed to a phosphor imager screen (PerkinElmer, USA).

For the conditioned medium experiments, control and *mdx* fibroblasts were grown in 35 mm² petri dishes incubated with 1 ml serum free medium for 48 h. The medium was collected and centrifuged at 10,000 rpm for 5 min. A new set of control fibroblasts isolated from a different animal were grown on 35 mm² petri dishes and were incubated with 900 μ L of conditioned medium obtained previously. After 2 h of incubation, 300 μ Ci/ml of ³⁵S-methionine were added to each plate. Cells were labeled for 4 h and then lysed in 1% SDS buffer. Immunoprecipitation of FN was carried out as described above.

Crosslinking assays

Carrier-free TGF- β 1 (R & D systems, USA) was radio-labeled with Na[¹²⁵I] using chloramine T. Binding and crosslinking assays of [¹²⁵I]-TGF- β to cell surface using disuccinimidylsuberate (Pierce, USA) were performed as previously described (Lopez-Casillas et al. 2003).

Immunofluorescence microscopy

Cells to be immunostained were grown on coverslips. The medium was removed and the coverslips were rinsed with phosphate-buffered saline. Cells were fixed with 3% paraformaldehyde for 30 min on ice and subsequently

permeabilized for 2 min with 0.05% Triton X-100. Cells were incubated with blocking solution (Tris 50 mM pH 7.7, NaCl 100 mM, 2% bovine serum albumin) for 1 h at room temperature. Rabbit anti-SMAD3 (1:25, diluted in blocking solution; Zymed, USA) or goat anti-desmin (1:100; Sta. Cruz, USA) were incubated at room temperature during 1 h. Cells were rinsed with blocking buffer and incubated for 1 h at room temperature with rhodamine or fluorescein-conjugated secondary antibodies.

Results

Fibroblasts isolated from dystrophic diaphragm muscle synthesize increased amounts of fibronectin compared to control mouse muscle fibroblasts

To study the role of fibroblasts in fibrosis associated to skeletal muscle dystrophy we isolated these cells from diaphragm muscles of 3 to 4 month old *mdx* and control (C57Bl/6) mice. Diaphragm muscles were chosen because they present more pronounced degenerative and fibrotic changes than limb muscles (Stedman et al. 1991). Under contrast phase microscopy no major differences in morphology between control and *mdx* cells could be observed (Fig. 1a). In order to verify the purity of our fibroblast cultures we performed immunocytochemistry of desmin as a myogenic marker (Fig. 1b). The amount of contamination with myoblasts varied from 9–10% through different cultures, there was no difference between *mdx* and control fibroblast cultures regarding the amount of myoblasts. For the control mice 174 cells out of 1,655 were desmin positive (9.5%) and for *mdx* 227 out of 2,202 (9.7%). All the cells were vimentin positive (results not shown). Figure 1 also shows the pattern of coomassie stained proteins from control and *mdx* fibroblasts, SDS-PAGE resolved no major differences (Fig. 1c). We also observed little change in the total protein levels of α -SMA, a marker of myofibroblasts, in *mdx* as compared to control fibroblasts (Fig. 1d). FN was chosen as a marker of fibrosis. Surprisingly, FN was increased an average of fivefold in *mdx* compared to control fibroblasts in seven independent experiments (Fig. 2a). To determine if this was due to an increased synthesis we immunoprecipitated FN from cell cultures that had been metabolically labeled with 35 S-methionine as described in “Materials and methods” (Fig. 2b). *Mdx* fibroblasts synthesize more FN despite the absence of a degenerative surrounding. The increase in FN was tissue specific since fibroblasts isolated from skin of control and *mdx* mice present no differences (Fig. 2c). We hypothesized that fibroblasts might be activated in the presence of dystrophic muscle; therefore if the surrounding muscle did not present vast degenerative changes then the tissue fibroblasts might not be forced to increase the

Fig. 1 Control and *mdx* skeletal muscle fibroblasts do not present gross morphological differences. **a** Contrast phase microscopy of fibroblasts obtained from diaphragm muscles of control and *mdx* mice. **b** Evaluation of the purity of the fibroblast cultures. Indirect immunofluorescence for desmin in control and *mdx* fibroblasts. Bar 100 μ m. **c** Coomassie staining of an SDS-PAGE of protein extracts obtained from control and *mdx* fibroblasts. **d** Western blot analysis of α -SMA from protein extracts obtained from control and *mdx* fibroblasts. GAPDH was used as loading control

production of ECM molecules. Limb muscles of *mdx* mice do not show such pronounced degenerative changes, in view of this we isolated fibroblasts from mouse *tibialis anterior* muscles of adult mice and compared levels of FN. There was no difference in FN levels between *mdx* and control mouse *tibialis anterior* fibroblasts (Fig. 2d). Furthermore, when fibroblasts are isolated from diaphragm muscles of young *mdx* mice they do not have increased levels of FN either (Fig. 2e) as was observed in adult 8–12 week old mice (Fig. 2a).

Therefore, fibroblasts that had been isolated from dystrophic skeletal muscle tissue in which fibrosis is an important feature, as opposed to successfully regenerating

Fig. 2 *Mdx* skeletal muscle fibroblasts synthesize more FN than control mouse muscle fibroblasts. **a** Fibroblasts obtained from skeletal muscle explants of *mdx* and control mice. Representative immunoblot analysis (upper panel) of fibronectin (FN) from cell culture medium and tubulin (TUB) from protein extracts (lower panel). Densitometric quantification of seven independent experiments. * $p=0.008$. **b** Autoradiograph of fibronectin immunoprecipitation. Cells were metabolically labeled with ^{35}S -methionine for 30 min. Proteins were immunoprecipitated with anti-FN antibodies. **c** Western

blot analysis (upper panel) and densitometric quantification (lower panel) of FN, TUB and GAPDH obtained from skin-derived mouse fibroblasts. Cells were assayed as in (a) Fibroblasts were obtained from two control mice (C1 and C2) and two *mdx* mice (M1 and M2; **d** and **e**). Western blot analysis of FN and TUB from protein extracts of fibroblasts isolated from adult (12 week old) control and *mdx* mice *tibialis anterior* muscles (**d**) and from diaphragms of young mice (1 to 3 weeks of age; **e**). C control, M *mdx*

muscle (i.e. *tibialis anterior*), are reprogrammed to increase the synthesis of FN. We state here that they are “reprogrammed” because fibroblasts that are isolated from the same muscle but at an earlier stage in the disease (young *mdx* mice) do not present elevated levels of FN.

Chondroitin/dermatan sulfate proteoglycans are increased in dystrophic muscle isolated fibroblasts

Other members of the ECM that have been shown to be upregulated both in DMD and *mdx* muscle tissue are PGs (Alvarez et al. 2002; Caceres et al. 2000; Fadic et al. 2006). Both CDPG and HSPG species are increased in these settings. Fibroblasts were incubated with radioactive sulfate for 6 h. Incorporation of radioactive sulfate to macromolecules accumulated in the conditioned medium of cultured fibroblasts obtained from *mdx* and control skeletal

muscles was determined by anion exchange chromatography DEAE-Sephacel. Fig. 3a shows a chromatographic profile from control fibroblasts, with two peaks, the first peak eluted at a salt concentration of 0.5 M, and the second peak eluted at 0.7 M. The column profile was basically the same in fibroblasts from *mdx*, and controls suggesting that there are no significant changes in glycosaminoglycan chain sulfation density. However Fig. 3a also shows a 2–3 fold increase in radioactive material obtained from *mdx* muscle fibroblasts compared to controls. To determine if the size of PGs synthesized by *mdx* fibroblasts was different to controls, eluted fractions obtained from peak II (*), were fractionated through a Sepharose CL-4B column chromatography. Volumes were corrected so that the fractions contained the same amount of radioactive activity (c.p.m.). Similar profiles, with a broad peak of K_{av} 0.2–0.6, were observed from both *mdx* and controls (Fig. 3b). Digestion

Fig. 3 Increased synthesis of CDPGs in *mdx* skeletal muscle isolated fibroblasts. **a** The solid curves are DEAE-Sepharose profiles of conditioned medium corresponding to equal amounts of total cell extract proteins from *mdx* (solid circles) and control (open circles) isolated fibroblasts incubated with [35 S]-sulfate for 6 h. The line indicates the NaCl gradient profile. The recovery from both columns was higher than 90%. **b** Conditioned medium from each DEAE column, indicated by a bar and asterisk in (a), were concentrated and chromatographed on a Sepharose CL-4B column. Samples loaded onto the column had equal

amounts of c.p.m. Eight hundred-microliter fractions were collected and analyzed in a scintillation counter. **c** Samples equivalent to **b** were incubated with Chondroitinase ABC (C_{ABC}) previous to the chromatography on a Sepharose CL-4B column; almost all the radioactive material was displaced to the total volume of the column. The recovery from both columns ranged from 91% to 95%. **d** Samples obtained from conditioned media corresponding to equal amounts of total cell extract protein at 0, 2 and 6 h after the beginning of metabolic labeling with [35 S]-sulfate were concentrated and subjected to SDS-PAGE followed by autoradiography

of these samples with C_{ABC} displaced almost all the radioactive material near K_{av} 1.0, indicating that the peak II obtained from the DEAE-Sepharose corresponds mainly to CDPGs (Fig. 3c). Aliquots of conditioned medium from fibroblasts collected 1, 2 and 6 h. after beginning the metabolic labeling were separated by SDS-PAGE which shows that the increase in these molecules is due, at least in part, to an increase in PG synthesis (Fig. 3d). To visualize and further characterize the CDPGs that were increased the same experiment was performed with conditioned media from cells labeled for 6 h. Figure 4 shows an increase of two [35 S]-labeled species in *mdx* fibroblasts (brackets). According to their known molecular weights, the sensitivity to C_{ABC} , and resistance to H_{ase} , the upper band corresponds to biglycan and the lower to decorin (Fig. 4a; Brandan et al. 1992; Fadec et al. 2006), to corroborate if the increase in decorin corresponds to an increase in protein core, samples from conditioned medium digested with C_{ABC} were

detected using an anti-mouse decorin antibody LF-113, Fig. 4b shows an increase of the protein core of decorin. On the other hand (Fig. 4c,d), the synthesis of detergent-soluble PGs occurred mainly with HSPGs. We didn't find differences in the synthesis levels of these species determined by SDS-PAGE followed by autoradiography. To determine the amount of each HSPG in the above mentioned fraction, the monoclonal antibody anti- Δ -Heparan sulfate 3G10 was used to recognize the neo-epitope generated in PG core proteins that are substituted with heparan sulfate after glycosaminoglycan digestion with the enzyme H_{ase} from *Flavobacterium heparinum* (Steinfeld et al. 1996). Detection of the anti- Δ -Heparan sulfate 3G10 epitope by western blot analysis allowed the identification of various species corresponding to different HSPG core proteins, in H_{ase} treated samples after SDS-PAGE fractionation (Casar et al. 2004; Fuentealba et al. 1999; Olguin and Brandan 2001; Fig. 4d). There are no major differences in the amount of

Fig. 4 Decorin and biglycan, but not HSPGs synthesis is increased in *mdx* muscle fibroblasts. **a** Samples obtained from conditioned medium, corresponding to equal amounts of total cell extract proteins, of *mdx* and control fibroblasts were concentrated and subjected to SDS-PAGE followed by fluorography. On the left, upper and lower brackets indicate CSPG (biglycan and decorin, respectively). Sample treatments included: heparitinase (H_{ase}) and chondroitinase ABC (C_{ABC}). **b** Samples similar to **a** were concentrated and incubated with C_{ABC} , separated by SDS gel electrophoresis, transferred to nitrocellulose membranes, stained with specific antibodies against decorin to reveal decorin core protein using enhanced chemiluminescence. **c** Samples obtained from cell extract, corresponding to equal amounts of total proteins were concentrated and subjected to SDS-PAGE followed by fluorography, the bracket indicates HSPGs. **d** Samples similar to **c** were incubated with Hase, separated by SDS-PAGE, transferred to nitrocellulose membranes and stained with anti-stub specific monoclonal antibodies (3G10) to reveal the core proteins of HSPGs by enhanced chemiluminescence. Based on the molecular weights of the core proteins, it is possible to identify the following PGs: syndecan-3 (Syn-3); syndecan-1 (Syn-1); glypican (Gly); syndecan-2 (Syn-2) and syndecan-4 (Syn-4)

each HSPG. Therefore *mdx* fibroblasts synthesize specifically more soluble CDPGs than control fibroblasts.

Mdx fibroblast conditioned media stimulates fibronectin synthesis in control fibroblasts

The finding that, even after isolation, *mdx* fibroblasts continue to synthesize more ECM molecules drove us to find out if there could be an autocrine stimulation of these cells. Therefore we prepared conditioned medium from control and *mdx* fibroblasts as described in Materials and Methods and incubated only control mouse diaphragm fibroblasts with these. We sought to evaluate FN levels, consequently, to avoid measuring FN that was carried over in the conditioned media, we metabolically labeled the cells and immunoprecipitated FN. We observed an increase in FN associated radioactivity in the control fibroblasts that had been incubated with *mdx* conditioned medium (Fig. 5).

This result suggests that *mdx* fibroblasts are secreting some factor that can stimulate ECM synthesis into their culture medium, and this may be in part responsible for the maintenance of the fibrotic phenotype observed in these cells.

Fig. 5 Conditioned media from *mdx* dystrophic muscle isolated fibroblasts stimulates FN synthesis in control muscle fibroblasts. Autoradiography of immunoprecipitated FN from cell lysates of control fibroblasts that had been incubated for 6 h with conditioned media obtained from control (C) and *mdx* (M) fibroblasts. Control and *mdx* fibroblasts were serum starved for 48 h and the conditioned media was collected. A different set of control fibroblasts (from a different mouse) were incubated for 2 h with the conditioned media and 35S-labeled methionine was added to the culture. After 4 h post methionine addition cells were lysed and FN was immunoprecipitated as described in “Materials and methods”. Equivalent amounts of protein obtained from the cell lysates were subjected to SDS-PAGE and stained with coomassie blue as loading control

Mdx fibroblasts show increased binding of TGF- β 1 to betaglycan

Given the above results we hypothesized that *mdx* fibroblasts might be synthesizing and secreting increased amounts of TGF- β or other profibrotic growth factor. TGF- β has been vastly studied as a profibrotic growth factor in several systems (Leask and Abraham 2004). It is known to induce FN synthesis in fibroblasts and epithelial cells (Hocevar and Howe 2000; Niculescu-Duvaz et al. 2007). Also, in the golden retriever muscular dystrophy model of DMD, TGF- β mRNA is elevated compared to control dogs (Passerini et al. 2002).

We sought to determine if TGF- β 1 receptors or signaling were altered in *mdx* diaphragm fibroblasts which might explain the basal increase in FN in these cells. TGF- β 1 binding to its receptors was studied through crosslinking assays. Binding to TGF- β RI and II was unaltered. An increase in 125 I-TGF β 1 radioactive labeling was observed associated to betaglycan (TGF- β RIII) (Fig. 6a). Coomassie

staining of the same gel is shown as a loading control. Betaglycan is a coreceptor for TGF- β which can increase TGF- β signaling through the receptors I and II (Lopez-Casillas et al. 1994). TGF- β signaling through TGF- β RI and II can induce phosphorylation of SMAD2 and 3, and translocation of these proteins, bound to SMAD4, to the nucleus (Shi and Massague 2003). TGF- β can also signal through p38 and ERK proteins (Nakagawa et al. 2005) therefore we decided to evaluate the status of autocrine TGF- β signaling in *mdx* and control fibroblasts and found no differences between the phosphorylation status of SMAD2, p38 or ERK1/2 (Fig. 6b). However, TGF- β 's profibrotic effect has been ascribed to SMAD3 (Flanders 2004) so we evaluated SMAD3 cellular translocation as an indicator of the pathway's activation. Figure 6c shows intracellular staining of SMAD3 in two control (C1 and C2) and *mdx* (M1 and M2) fibroblast cultures. Cellular localization of SMAD3 does not vary between the different cell types.

Mdx diaphragm fibroblasts present more binding of TGF- β 1 to betaglycan but, regarding the signaling intermediates

Fig. 6 TGF- β 1 binding to betaglycan, but not TGF- β RI or TGF- β RII, is increased in *mdx* skeletal muscle derived fibroblasts but endogenous signaling through p38, SMAD2/3 and ERK1/2 is unaltered. **a** Fibroblasts were obtained from two controls (C1 and C2) and two *mdx* (M1 and M2) mouse diaphragms. Cells were cross-linked with 125 I-TGF- β 1 at 4°C. Cell extracts were subjected to SDS-PAGE, stained with coomassie brilliant blue and fluorographed. RI: TGF- β receptor 1, RII: TGF- β receptor 2. Coomassie staining of the same gel is shown as loading control. Western blot analysis of signaling intermediates in serum starved control and *mdx* fibroblasts (**b**) and indirect immunofluorescence of SMAD3 of two controls (C1 and C2) and two *mdx* (M1 and M2) fibroblast cultures (**c**). White bar indicates 100 μ m

that we evaluated, we did not find alterations in the basal signaling status of *mdx* vs. control fibroblasts that would account for the increase in FN and proteoglycan levels.

TGF- β 1 and CTGF decrease fibronectin levels in *mdx* fibroblasts

We did not observe differences in signaling in basal conditions between *mdx* and controls but we hypothesized that *mdx* fibroblasts might be more responsive to stimulation by growth factors which have been associated with an increase in ECM molecules. We, therefore, evaluated the levels and synthesis of FN in the presence of TGF- β or CTGF. As expected, incubation of control fibroblasts with TGF- β 1 increased FN levels (Fig. 7a). Paradoxically in *mdx* fibroblasts TGF- β 1 decreased FN levels in a concentration dependent manner (Fig. 7a). In order to rule out that there might be a biphasic component to TGF- β 's effect over previously activated fibroblasts, in other words, that higher concentrations of TGF- β in control fibroblasts might also have an inhibiting effect over FN levels, we incubated control fibroblasts with high concentrations of TGF- β 1 (Fig. 7b). Here we observed that FN levels increased in the presence of the growth factor but reached a plateau at 10 ng/ml of TGF- β 1 but did not decrease at very high

concentrations (i.e. 100 ng/ml). Since there is an increased basal synthesis of FN in *mdx* fibroblasts maybe TGF- β was decreasing FN synthesis in these cells. To evaluate this we incubated the cells with TGF- β for 24 h. and metabolically labeled them with 35 S-methionine for 30 min. We evaluated FN synthesis immunoprecipitating FN from the labeled media, and also total levels of the molecule by performing standard western blots. Figure 7c,d shows an increase in FN synthesis and protein levels in control fibroblasts whereas in *mdx* fibroblasts there is a basal increased synthesis of FN which remains unaltered under TGF- β treatment but the total protein levels are decreased when incubated with TGF- β . This result suggests that TGF- β may be increasing degradation of FN in *mdx* fibroblasts.

CTGF is a downstream target of TGF- β and a potent profibrotic growth factor (Abou-Shady et al. 2000; Leask and Abraham 2004; Ruperez et al. 2003). When control fibroblasts were incubated with CTGF FN levels increased (Fig. 7e), in *mdx* fibroblasts however CTGF also decreased FN levels in a concentration dependent manner both in the conditioned media and protein extracts (Fig. 7e). Fibroblasts were also incubated with insulin like growth factor 1 (IGF-1) and observed an increase in FN levels in control fibroblasts but a decrease in *mdx* (results not shown).

Fig. 7 Paradoxically, in *mdx* mice muscle fibroblasts TGF- β and CTGF, decrease FN levels. (a, b, c and d) Western blot analysis of fibronectin (FN) obtained from control and *mdx* fibroblasts. Fibroblasts were serum starved for 18 h. They were then incubated for 24 h with TGF- β 1 or 48 h with recombinant CTGF at the indicated concentrations in serum free media. FN from protein extracts and cell media (c and d as indicated) were analyzed by immunoblotting. Tubulin (TUB) from cell extracts was used as loading control (e). Autoradiograph of FN immunoprecipitation (a and c) and western blot analysis of total FN levels (b and d). Cells were incubated with 5 ng/ml TGF- β 1 in serum free media for 24 h, they were then metabolically labeled with 35 S-methionine for 30 min in the presence or absence of TGF- β 1. Proteins extracts were either immunoprecipitated with anti-FN antibodies, electrophoresed and autoradiographed or subjected to western blot analysis

Discussion

Most recent studies on DMD have focused on treatments that aim at restoring the missing gene/protein through gene therapy (van Deutekom and van Ommen 2003) or favor muscle regeneration through cell transplantation (Sampaioles et al. 2006) or drugs (Minetti et al. 2006; Tidball and Wehling-Henricks 2004). We have studied *mdx* diaphragm as a model of muscle degeneration and fibrosis in DMD and found that when fibroblasts are isolated from dystrophic muscle they behave differently than those from control muscles. Here we report that *mdx* diaphragm fibroblasts synthesize increased amounts of FN and CDPGs as compared to control mice fibroblasts. This feature is surprising given that the disease is originated because of the lack of dystrophin in the muscle fibers. In *mdx* mice the mutation occurs in the 23rd exon of the protein gene introducing a stop codon thus inhibiting the expression of the protein in the muscle. The gene however, has intra-gene promoters which account for extramuscular dystrophin isoforms. These include central nervous system specific isoforms and a small 71 kDa isoform (Dp71) present in most tissues. Dp71 is unaffected in *mdx* because its promoter is distal to the mutation that originates the pathology (Lederfein et al. 1993; Rapaport et al. 1993). Therefore, it is surprising that, otherwise genetically “normal” fibroblasts would show an altered behavior when they have been removed from the pathological environment. This is further corroborated by the finding that young *mdx* diaphragm fibroblasts do not have increased FN levels.

The process of fibroblast activation is secondary to a wide variety of stimuli. *In vivo* during tissue injury a resident fibroblast acquires the characteristics of a myofibroblast, namely the formation of actin stress fibers, the expression of α -SMA, and an increase in the synthesis of ECM molecules. The acquisition of this phenotype is dependent on the presence of cytokines released by inflammatory cell infiltration in the injured tissue and a change in the mechanical properties of the microenvironment due to a loss of the stress shielding properties of the remodeling ECM (Hinz et al. 2007). Hinz et al. further describe three conditions that must be present to induce the differentiation of a mature myofibroblast: (1) an increase in extracellular stress, (2) the presence of specialized ECM molecules (such as the EDA splice variant of FN) and (3) the presence of active TGF- β (Hinz et al. 2007). It has been observed that α -SMA expression can be induced *in vitro* in fibroblasts that are cultured on rigid plastic surfaces (Tomasek et al. 2002) as we have done in this work, which may explain why we observed no differences between the levels of this protein between control and *mdx* fibroblasts. Thus, at least with respect to α -SMA, they do not differ, however *mdx* fibroblasts also have increased ECM molecules synthesis and this may determine the activation status of these cells.

With regard to the presence of specialized ECM molecules, we did not evaluate if there were differences in the expression of EDA FN between controls and *mdx* but preliminary results from our lab show that *mdx* fibroblasts at least express this isoform (RT-PCR data, not shown). There are, nonetheless, several other ECM molecules upregulated too. Here we show that decorin and biglycan are also elevated in *mdx* fibroblasts. Decorin has been shown to modulate the effects of TGF- β both increasing and decreasing its signaling/effects (Cabello-Verrugio and Brandan 2007; Kolb et al. 2001; Riquelme et al. 2001; Sato et al. 2003b). A further role of decorin as an inducer of fibroblast activation has not been reported, although a role as an antifibrotic agent has been attributed to it.

The conditioned media experiments suggested that there might be an autocrine factor synthesized by the dystrophic fibroblasts capable of maintaining their activation. Given the evidence cited above we guided our attention to TGF- β and CTGF. TGF- β and CTGF are both known profibrotic growth factors involved in tissue remodeling following injury. In fibroblasts obtained from scleroderma patients CTGF is constitutively elevated with regard to control patient fibroblasts (Holmes et al. 2003). CTGF mediates TGF- β induced collagen deposition (Duncan et al. 1999). On the other hand TGF- β is increased in DMD patients and in a model of DMD in dogs (Bernasconi et al. 1999; Passerini et al. 2002) and CTGF is upregulated in microarrays performed on *mdx* muscle tissue (Porter et al. 2003). TGF- β signals through its receptors I and II and the coreceptor betaglycan (Shi and Massague 2003). The binding of TGF- β 1 to the receptors I and II was unaltered in *mdx* fibroblasts, however there was an increased binding to betaglycan. This increased binding observed in the fluorography may be due to an increased amount of betaglycan at the cell surface or at an increase in the affinity of TGF- β for that receptor. Since we did not observe more binding of TGF- β to receptors I and II it is less likely that the growth factor's bioavailability be increased. In this work we did not evaluate betaglycan's levels at the cell surface, however, it has been previously reported that adenovirally delivered betaglycan to C2C12 myoblasts induces ligand independent signaling through p38 (Santander and Brandan 2006). With this in mind we analyzed phosphorylated p38 levels in unstimulated fibroblasts and observed no differences between *mdx* and controls. The other signaling intermediates for TGF- β evaluated were not consistently altered, SMAD2 and 3 levels and localization, respectively, did not differ. On the other hand, we also looked at ERK1/2 phosphorylation status, this kinase may be activated through both TGF- β and CTGF stimulation, and again we observed no differences. Maybe, steady state levels of these mediators (which are dependent on the autocrine release of factors) do not

differ, nevertheless, the effect of exogenous growth factors over the cells might still be different between control and *mdx* muscle fibroblasts.

Accordingly, another surprising finding was the paradoxical response of *mdx* fibroblasts to well known profibrotic cytokines. When control mice fibroblasts are isolated and incubated with either TGF- β or CTGF there is an increase in FN levels, when *mdx* fibroblasts undergo the same treatment FN decreases to levels similar to control fibroblasts which are stimulated with either growth factor. This finding is extremely interesting since many potential treatments for DMD done in *mdx* mice are trying to modulate the activity of different growth factors, for example increasing IGF or decreasing myostatin (which is a member of the TGF- β family of growth factors; Bogdanovich et al. 2004; Wagner et al. 2002). There are also reports in which decorin has been used in *mdx* mice to promote muscle healing with promising results and this effect has been attributed to the inhibition of TGF- β and myostatin (Sato et al. 2003a; Zhu et al. 2007). In our *mdx* in vitro model blocking TGF- β or CTGF would have deleterious effects over ECM production, since in this setting they behave as antifibrotic factors. However, it has been reported that IGF-1 stimulates collagen synthesis in vitro in renal and intestinal fibroblasts (Lam et al. 2003; Simmons et al. 2002) but it decreases muscle fibrosis in vivo in *mdx* mice (Barton et al. 2002). In agreement with this last finding we also observed a decrease in FN levels in *mdx* fibroblasts incubated with IGF-1. Another question raised by these results is that TGF- β decreases FN levels with no effect on FN synthesis. Suggesting that it might be regulating catabolic pathways for FN such as endocytosis and intracellular degradation (Salicioni et al. 2002; Sottile and Chandler 2005). Furthermore their response to the growth factors may be altered due to intrinsic changes that might translate different epigenetic states with respect to control cells. In fact, it has been reported that collagen synthesis induced by TGF- β in fibroblasts is dependent on the histone acetylase p300 levels (Bhattacharyya et al. 2005).

In summary, we propose that fibroblasts are activated in vivo in the presence of dystrophic, fibrotic muscle tissue; when isolated, they maintain their activation status maybe due to autocrine stimulation (e.g. EDA FN, or another growth factor), the presence of an altered ECM, or an intrinsic epigenetic reprogramming. Furthermore we present a novel function for TGF- β and CTGF in decreasing the levels of FN in the dystrophic cells. These results might translate an alternative mechanism for the regulation of FN and PG synthesis in *mdx* fibroblasts that may be independent of extracellular stimuli, especially since they have been isolated from a pro-ECM synthesizing environment and still retain the ability to up regulate ECM molecules.

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