

## Increase in decorin and biglycan in Duchenne Muscular Dystrophy: role of fibroblasts as cell source of these proteoglycans in the disease

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### Abstract

Fibrosis is a common pathological feature observed in muscles of patients with Duchenne muscular dystrophy (DMD). Biglycan and decorin are small chondroitin/dermatan sulfate proteoglycans in the muscle extracellular matrix (ECM) that belong to the family of structurally related proteoglycans called small leucine-rich repeat proteins. Decorin is considered an anti-fibrotic agent, preventing the process by blocking TGF- $\beta$  activity. There is no information about their expression in DMD patients. We found an increased amount of both proteoglycans in the ECM of skeletal muscle biopsies obtained from DMD patients. Both biglycan and decorin were augmented in the perimysium of muscle tissue, but only decorin increased in the endomysium as seen by immunohistochemical analyses. Fibroblasts were isolated from explants obtained from muscle of DMD patients and the incorporation of radioactive sulfate showed an increased synthesis of both decorin and biglycan in cultured fibroblasts compared to controls. The size of decorin and biglycan synthesized by DMD and control fibroblasts seems to be similar in size and anion charge. These findings show that decorin and biglycan are increased in DMD skeletal muscle and suggest that fibroblasts would be, at least, one source for these proteoglycans likely playing a role in the muscle response to dystrophic cell damage.

**Keywords:** Duchenne muscular dystrophy • proteoglycans • biglycan • decorin • interstitial fibrosis

### Introduction

Duchenne Muscular Dystrophy (DMD) is a muscle degenerative disease, secondary to mutations in the

dystrophin gene, that remains invariably fatal in humans [1]. Since the discovery of the DMD gene about 20 years ago [2] the center of attention for therapeutic strategy has been focused towards the delivery of the missing gene/protein [3]. This has not yet been successful [4]. Increasing the understanding of the muscle cell response to the mutation may allow the design of alternative pharmacological therapies. In DMD the lack of dystrophin is followed by continuous

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cycles of degeneration and regeneration that result in extensive fibrosis and a progressive decrease of muscle mass. The muscle cell degeneration is thought to be secondary to a muscle cytoskeleton-extracellular matrix disruption [5]. This is presumed to lead to sarcolemmal instability which could render muscle fibers susceptible to necrosis [6]. The cause of fibrosis in DMD is not well understood. Dystrophin deficiency in the limbs of *mdx* mice, animal models of DMD [7], does not necessarily result in muscle fibrosis. It is known that dystrophy associated fibrosis is characterized by the accumulation of extracellular matrix (ECM) material [8]. The interaction of growth factors and cytokines with the ECM affects their bioavailability and biological activity. On the other hand, proteins which form part of the ECM have signaling functions *per se*. The ECM, therefore, would most likely have an important function in muscle fibrosis. [9–11]. Proteoglycans are one of the main constituents of the ECM, and we have shown that their synthesis, including both heparan sulfate and chondroitin/dermatan (CS/DS) sulfate groups, is increased in *mdx* mice [12]. Biglycan and decorin are two CS/DS proteoglycans of the small leucine-rich family of proteoglycans that belong to an expanding gene class whose distinctive feature is a structural motif, called the leucine-rich repeat. These proteoglycans are tissue organizers, orienting and ordering collagen fibrils during ontogeny and in pathological processes such as wound healing, tissue repair, and tumor stroma formation. Three-dimensional modeling of their prototype protein core proposes a binding surface appropriate for interactions with ligand proteins such as soluble growth factors.

Besides its function as an ECM organizer, decorin, has the ability to bind cell surface receptors, it activates the epidermal growth factor receptor and is capable of forming complexes with various isoforms of TGF- $\beta$ , thus modulating their activity [13]. The mRNA and protein levels of TGF- $\beta$ , a known profibrotic cytokine, are significantly elevated in *mdx* diaphragm [14]. Decorin is expressed during skeletal muscle differentiation [15] and its synthesis and expression are up-regulated during skeletal muscle differentiation *in vitro* [25]. It has a role during skeletal muscle terminal differentiation by activating TGF- $\beta$  dependent signaling pathways [16]. Biglycan is expressed in secondary myotubes during fetal muscle formation [15]. In adult muscle it is located in the endomysium and neuromuscular junctions, where it connects to the dystrophin-asso-

ciated proteins through binding to  $\alpha$ -dystroglycan [17]. In developing newborn mice biglycan expression is initially high and then decreases during skeletal muscle differentiation and maturation. Remarkably, a transient and important up-regulation of biglycan was associated with newly formed myotubes during injury induced muscle regeneration in mice [18]. There is little information about the putative role of these proteoglycans in dystrophic muscle fibrosis. In other organs decorin and biglycan seem to have dissimilar functions regarding fibrosis. The expression of decorin mRNA was increased, but biglycan mRNA expression was not elevated in kidney tubular epithelial cells and peritubular interstitium in a model of tubulointerstitial fibrosis [19]. *In vivo*, overexpression of TGF- $\beta$  resulted in marked lung fibrosis, which was significantly reduced by concomitant overexpression of decorin. Biglycan, however, had no significant effect on lung fibrosis induced by TGF- $\beta$  [20]. In a muscle regeneration model, myostatin (which belongs to the TGF- $\beta$  family of growth factors) null mice present smaller areas of fibrotic tissue parallel to an increased expression of decorin mRNA [21]. Neutralization of TGF- $\beta$ 1 by decorin administration resulted in a 40% reduction in the level of type I collagen mRNA in the diaphragm of dystrophic mice [14]. Biglycan may also have a role in the fibrotic process since it is overexpressed in atherosclerotic lesions and has been shown to cause an increase in thymidine incorporation and migration of vascular smooth muscle cells [22].

The only information available about decorin and biglycan levels in DMD comes from gene expression profiles of skeletal muscle biopsies that demonstrated increased amount of both proteoglycans [23]. Information regarding their cell source and as well as tissue localization is missing. We therefore decided to study both proteoglycans in DMD patients' muscle biopsies and cultured fibroblasts obtained from explants of DMD skeletal muscle tissue.

## Material and methods

### Muscle samples

Biopsies from individuals were obtained from *quadriceps* muscles during diagnostic or surgical procedures,

in accordance with university ethical rules. DMD Patient 1 (D1): 8 year-old boy first evaluated for delay in walking at 19 months of age, and subsequently developing progressive muscle weakness. He had no relatives with myopathies. CPK was 20,000 IU/ml. Dystrophin was absent in his muscle biopsy. Patient 2 (D2): 4 year-old boy with muscle pains since the age of 2 and a half, developing progressive muscle weakness shortly after that. Family history was negative for muscle disease. CPK was >18,000 IU/ml. EMG showed myopathic motor units with increased recruitment and muscle biopsy revealed muscle dystrophy. Immune histochemistry for dystrophin showed absence of the three-dystrophin domains. Samples obtained from 2 patients (C1 and C2) without muscle diseases, undergoing a surgical orthopedic procedure in the hip or femur, were considered as controls. After sampling the biopsies were snap frozen in isopentane and stored at -80°C until processing. Muscle biopsies used for cell cultures were kept in phosphate buffered saline until processed. Informed consent was obtained from all patients. The Ethics Committee of the Hospital Clínico de la P. Universidad Católica de Chile approved the study.

### **Isolation of skeletal muscle proteoglycans**

Protein extracts were prepared as previously described [24]. Briefly, the muscle sample was homogenized in 4M guanidine-HCL, 0.05 M sodium acetate (pH 5.8), and 1 mM PMSF at 4°C and maintained under agitation for 18 h. The supernatant was equilibrated by dialysis with 8 M urea, 0.2 M NaCl, 0.05 M sodium acetate, and 0.5% Triton X-100 to remove guanidine. Samples were concentrated by DEAE-Sephacel anion-exchange chromatography, equilibrated and washed with the same urea buffer, and eluted with 1.0 M NaCl. The extracts were finally equilibrated by dialysis with a buffer containing 100 mM Tris-HCl, 50 mM NaCl, pH 7.5, before enzymatic treatment.

### **SDS-PAGE analysis of skeletal muscle proteoglycans**

Appropriate samples were digested with chondroitinase ABC and then analyzed by SDS-PAGE followed by fluorography using a 3–15% acrylamide gradient gel as described [25].

### **Western blot analysis**

For proteoglycan detection, samples containing equivalent amounts of proteins were incubated with chondroitinase ABC and then analyzed by SDS-PAGE using a 3–15% acrylamide gradient. Proteins were transferred by electrophoresis to nitrocellulose membranes, detected with anti-mouse biglycan LF-106 or anti-mouse decorin LF-136 polyclonal antibodies (kindly donated by Dr. L. Fisher, NIDR, NIH, Bethesda, MD) [26], secondary horseradish peroxidase conjugated antibodies and visualized by enhanced chemiluminescence.

### **Immunohistochemistry**

Cryostat sections (8 µm) from DMD patients and controls were fixed with cold ethanol/acetic acid solution (1:1), rinsed with phosphate-buffered saline (PBS; pH 7.4), and blocked with an 8% BSA in PBS solution. They were then treated with 2.5 mU chondroitinase ABC for 2 hrs at 37°C, and incubated overnight at 4°C with primary antibodies against biglycan LF-51 (in 1:300 dilution) and decorin LF-136 (in 1:700 dilution). Sections were then washed and incubated with either anti-rabbit FITC or anti-mouse TRICT (1:100) for 1 hr at room temperature. For nuclear staining, sections were incubated with 1 µg/ml Hoechst 33258 in PBS for 10 minutes. After rinsing, sections were mounted with fluorescent mounting media (Dako Corporation, CA) under glass cover slips and photographed with a Nikon Eclipse microscope equipped for epifluorescence. Specificity of anti-decorin and anti-biglycan was verified by the absence of staining after preincubation with an over tenfold excess of the purified proteoglycans subjected to chondroitinase ABC digestion.

### **Cell culture**

Skeletal muscle samples were obtained from muscle biopsies of one DMD and one control patient and processed immediately after extraction. Fibroblasts were isolated from muscle explants as described previously [27]. Briefly, muscle biopsies were minced into pieces smaller than 1 mm<sup>2</sup>, seeded onto 3.8 cm<sup>2</sup> well plates and covered with 500 µL of growth medium (Dulbecco's modified eagle medium F-12, 100 U/ml

penicillin, 0.1 mg/ml streptomycin and 0.25 µg/ml anfotericin B, Invitrogen) supplemented with 50% (v/v) fetal calf serum (Hyclone). Explants were kept at 37°C, 5% CO<sub>2</sub> 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 cm<sup>2</sup> petri dishes, growth medium was switched to 20% FCS. Cells were used in passage 4 for all experiments.

### **Gel filtration chromatography and SDS-PAGE**

Cells grown in 21 cm<sup>2</sup> petri dishes were labeled with [<sup>35</sup>S]-H<sub>2</sub>SO<sub>4</sub> 200 µC/ml, in serum free F-12 HAM medium for 6 h. Conditioned media from DMD 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 proteoglycans were then chromatographed on an analytical Sepharose CL-4B column (100 x 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 SDS-PAGE [25].

## **Results**

### **Decorin is increased in Duchenne muscular dystrophy and localizes in the muscle perimysium and endomysium**

To evaluate decorin content in muscle samples from DMD patients and controls, western blot analysis with a specific anti-human decorin antibody (LF-136) was performed in chondroitinase ABC digested samples. As seen in Fig. 1A decorin core protein (filled arrow) and its glycanated form (open arrow)

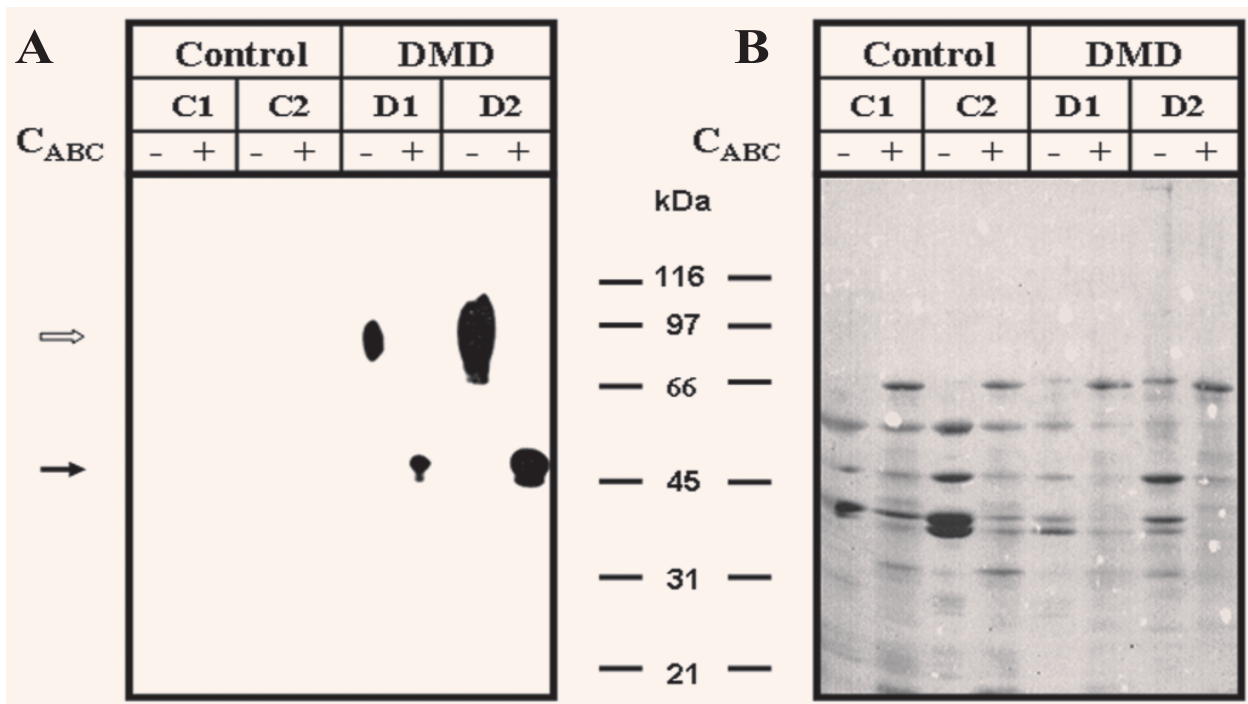
are increased in dystrophic muscle. No signal was detected in control muscles (Fig. 1A) when films were exposed for the same period of time. Protein profiles of DMD patients and control samples after Ponceau S staining of the same immunoblotted muscle homogenates is also shown (Fig. 1B). Decorin is secreted into the ECM and is located in the muscle perimysium [28]. To evaluate histological localization of the increased decorin muscle biopsy sections were stained with specific anti-decorin antibody. As seen in Fig. 2 decorin is increased both in the perimysium and endomysium as compared to control muscles. Nuclei are shown in blue. An increase in nuclei localized particularly in the perimysium can be observed and may correspond to inflammatory infiltration and fibroblasts. According to these results we conclude that decorin is increased in DMD muscle and localizes at the perimysium and endomysium.

### **Biglycan content is increased in Duchenne muscular dystrophy and is located mainly in the perimysium**

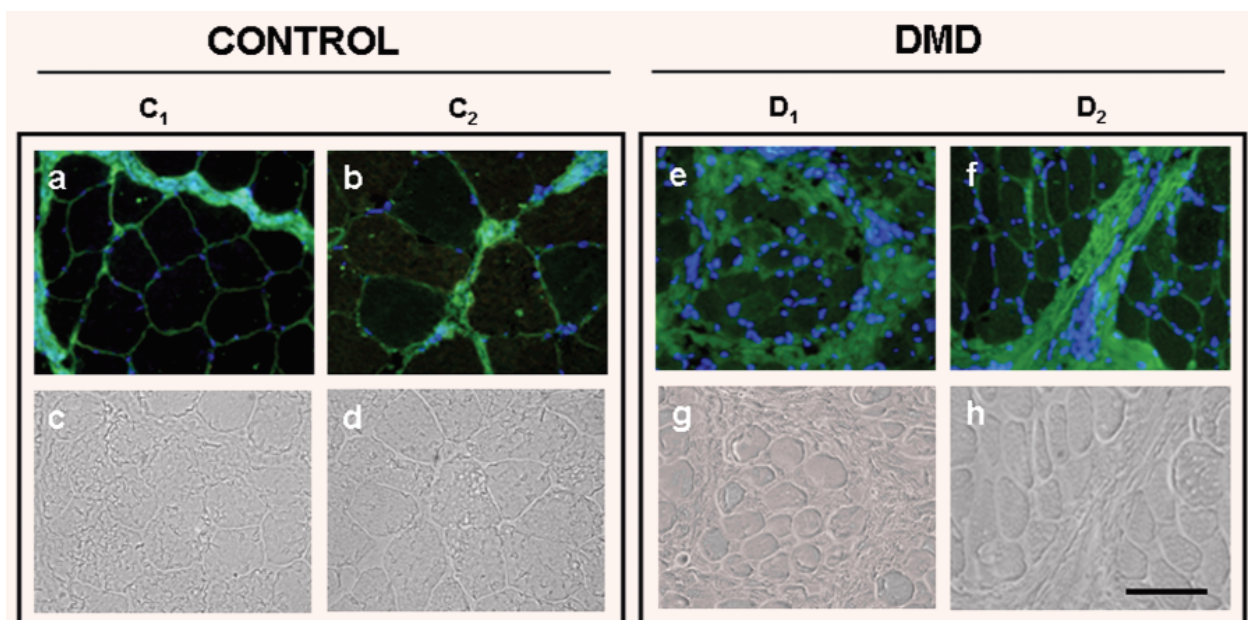
A similar procedure was performed to evaluate biglycan content. Western blot detection of biglycan in Fig. 3A shows an increase of the protein in the DMD patients as compared to controls. This antibody does not recognize the glycanated form of biglycan. Fig 3B shows Ponceau S staining of the same samples. As shown in Fig. 4 biglycan differs from decorin regarding tissue localization. The increase of the former in DMD muscle sections is mainly at the perimysium (Fig 4B).

### **Fibroblasts obtained from DMD skeletal muscle show an increase in the synthesis of chondroitin/dermatan sulfate proteoglycans**

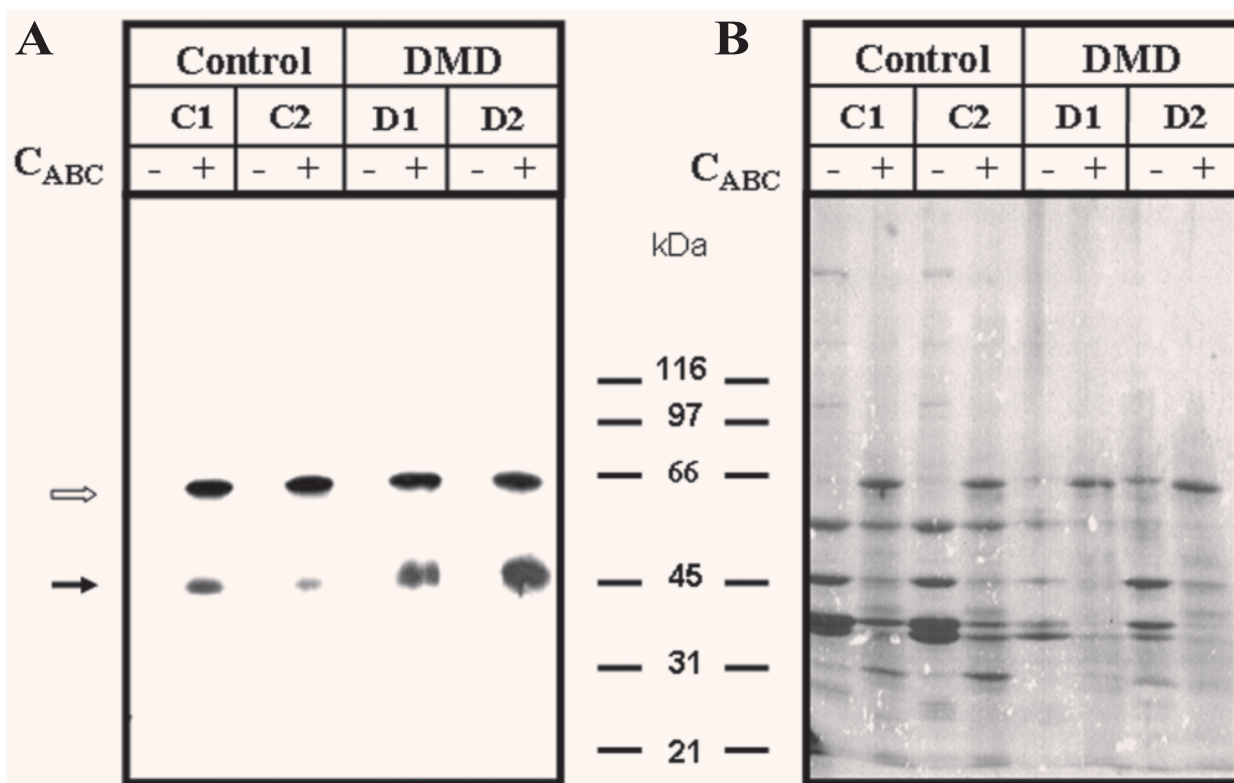
The above results show that both decorin and biglycan content is increased in Duchenne skeletal muscle. Muscle is composed mainly of skeletal muscle fibers and mononucleated cells such as fibroblasts and macrophages. We measured proteoglycan synthesis in cultured fibroblasts obtained from muscle biopsies to determine if this cell type is, at least in part, responsible for the observed increase in decorin and biglycan. Fibroblasts were isolated



**Fig. 1** Increases in the amount of decorin in DMD muscles. Western blot analyses were carried out using samples obtained from DMD and control biopsy extracts. C1 and C2 correspond to control biopsies and D1 and D2 to DMD patients. Fractions containing equivalent amounts of proteins were concentrated and incubated with and without chondroitinase ABC ( $C_{ABC}$ ), separated by SDS gel electrophoresis, transferred to nitrocellulose membranes, stained with specific antibodies against decorin to reveal decorin core protein using ECL. In (A), the open arrow shows decorin whereas the filled arrow corresponds to its core protein. In (B) the blot stained with Ponceau S is shown. Molecular weight standards are shown in the middle (kDa).



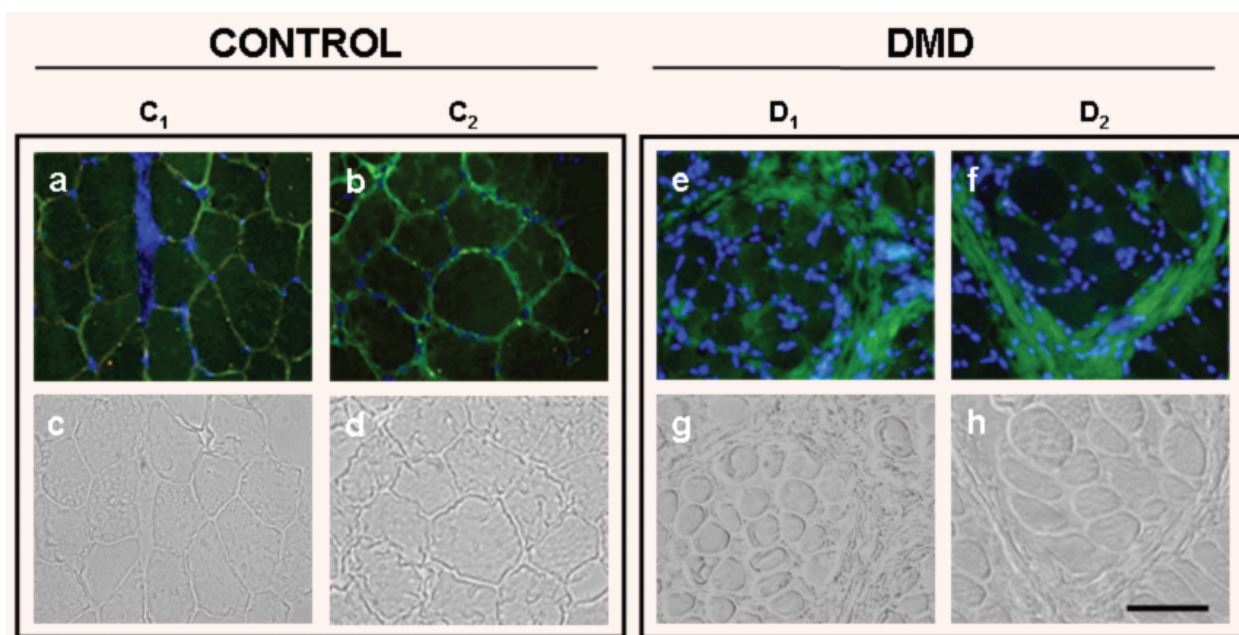
**Fig. 2** Decorin increases in both endomysium and perimysium of DMD muscle. Indirect immunofluorescence performed in cross sections of DMD ( $D_1$ ,  $D_2$ ) and control ( $C_1$ ,  $C_2$ ) muscles shows decorin increase in the interstitium around each individual muscle fiber and in the space surrounding muscle fascicles. Nuclei are stained with Hoechst. Corresponding phase contrast photographs are shown underneath each section. Scale bar = 100  $\mu$ m.



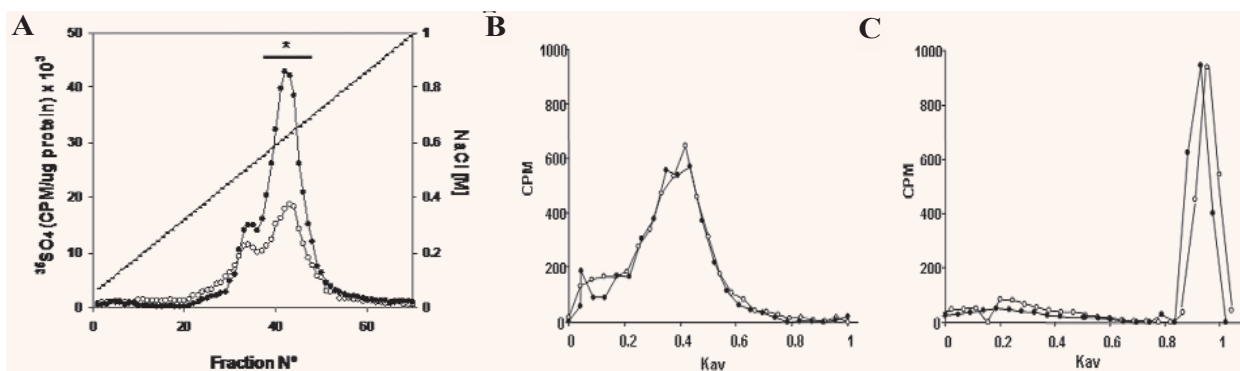
**Fig. 3** Increases in the amount of biglycan in DMD muscles. Western blot analyses were carried out using soluble samples obtained from DMD biopsy extracts. C1 and C2 correspond to control biopsies and D1 and D2 to DMD patients. Fractions containing equivalent amounts of proteins were concentrated and incubated with and without chondroitinase ABC (C<sub>ABC</sub>), separated by SDS gel electrophoresis, transferred to nitrocellulose membranes, stained with specific antibodies against biglycan to reveal biglycan core protein using ECL. In (A), the open arrow shows immunoreactivity to albumin present in the C<sub>ABC</sub> as preserving. The filled arrow corresponds to biglycan core protein. In (B) the blot stained with Ponceau S is shown. Molecular weight standards are shown in the middle (kDa).

from explants of DMD and control muscles as described in Material and Methods and incubated with radioactive sulfate for 6 h. Incorporation of radioactive sulfate to macromolecules accumulated in the conditioned medium of cultured fibroblasts obtained from DMD and control skeletal muscles was determined by anion exchange chromatography. Fig. 5A shows a twofold increase in radioactive material obtained from DMD muscle fibroblasts (closed circles) compared to controls (open circle). In both situations radioactive molecules eluted from the columns at a salt concentration in the 0.6 M range as described for chondroitin/dermatan sulfate proteoglycans [24, 28]. The column profile was basically the same in fibroblasts from DMD and controls suggesting that there are no significant changes in glycosaminoglycan chain sulfation density. To determine if the size of proteogly-

cans synthesized by DMD fibroblasts was different to controls, eluted fractions obtained from the DEAE-Sephacel columns containing the same amount of radioactive activity (c.p.m.), were fractionated through a Sepharose CL-4B column chromatography. Similar profiles, with a broad peak of Kav 0.2-0.6, were obtained from both DMD patients and controls (Fig. 5B). Digestion of these samples with chondroitinase ABC displaced almost all the radioactive material near Kav 1.0, indicating that the peak II obtained from the DEAE-Sephacel corresponds mainly to chondroitin/dermatan sulfate proteoglycans (Fig. 5C). To visualize the different radioactive soluble proteoglycans conditioned medium from fibroblast cell cultures were separated by SDS-PAGE and exposed to a radioactivity sensitive screen (Cyclone). Fig. 6 shows an increase of two [<sup>35</sup>S]-labeled species in DMD fibroblasts



**Fig. 4** Biglycan increases only in the perimysium of DMD muscle. Representative microphotograph of DMD (D1, D2) and control (C1, C2) muscle cross sections stained with antibodies against biglycan followed by a second FITC-conjugated antibody. The biglycan signal increases in the interstitial space around muscle fascicles. Nuclei stained with Hoechst. Corresponding phase contrast photographs are shown below each section. The scale bar indicates 100  $\mu\text{m}$ .

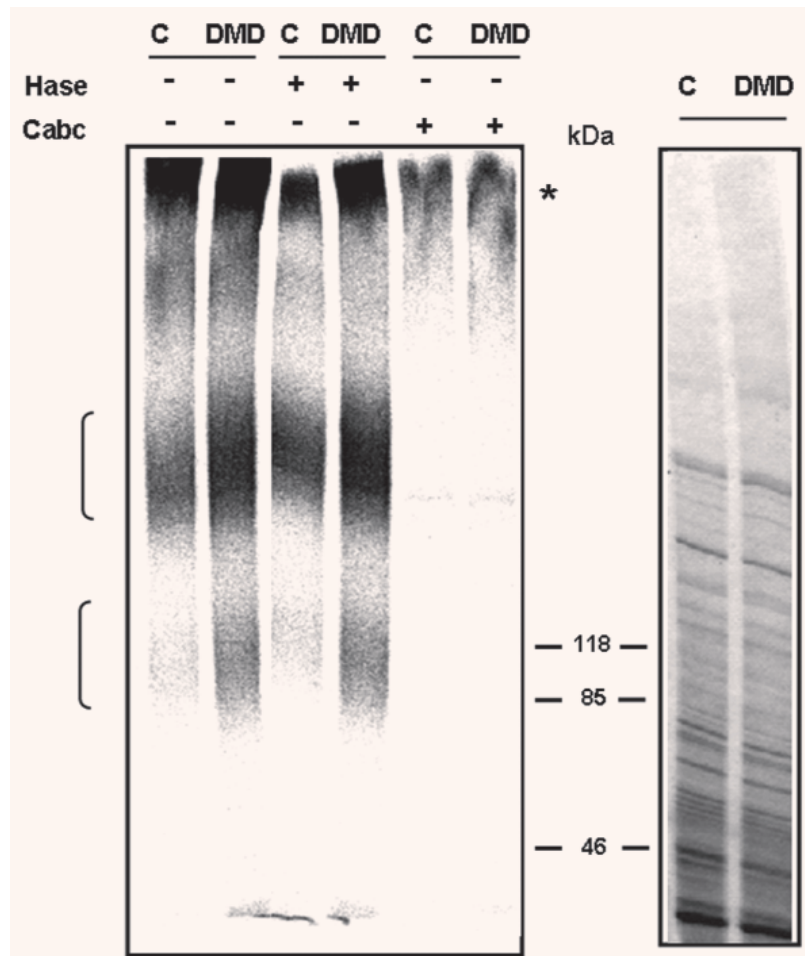


**Fig. 5** Fibroblasts isolated from muscle DMD synthesize higher amounts of chondroitin/dermatan sulfate proteoglycans. **A.** The solid curves are DEAE-Sephacel profiles of conditioned medium corresponding to equal amounts of total cell extract proteins from DMD (solid circles) and control (open circles) isolated fibroblasts incubated with  $^{35}\text{S}$ -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 in A, were concentrated and chromatographed on a Sepharose CL-4B column. Samples loaded onto the column had equal amounts of cpm. 800  $\mu\text{l}$  fractions were collected and analyzed in a scintillation counter. **C.** Samples equivalent to B, were incubated with chondroitinase ABC previously 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 87 to 96%.

(brackets). According to their known molecular weights and the sensitivity to chondroitinase ABC the upper band corresponds to biglycan and the lower to decorin (Fig. 6). Interestingly, radioactive

material associated to the higher molecular weight heparan sulfate proteoglycans levels does not differ among fibroblasts from DMD skeletal muscle patients and controls (Fig. 6, asterisk). As a loading

**Fig. 6** Synthesis of decorin and biglycan is increased in DMD muscle isolated fibroblasts compared to control. Samples obtained from conditioned medium, corresponding to equal amounts of total cell extract proteins, of DMD and control fibroblasts were concentrated and subjected to SDS-PAGE followed by fluorography. On the left, upper and lower brackets indicate chondroitin/dermatan sulfate proteoglycans (biglycan and decorin, respectively). The asterisk corresponds to heparan sulfate proteoglycans. Sample treatments included: Heparitinase (Hase) and Chondroitinase ABC ( $C_{ABC}$ ). Molecular weight standards are shown (kDa) in the middle. On the right, a coomassie staining of cell protein extracts shows equivalent amount of protein present in each sample.



control coomassie staining of cell protein extracts equivalent to the percentage of analyzed conditioned media is shown on the right of Fig. 6. Therefore, fibroblasts isolated from DMD muscle synthesize higher amounts of decorin and biglycan than fibroblasts isolated from control muscles.

## Discussion

The histological phenotype of dystrophic muscle differs substantially from normal muscle. DMD skeletal muscle is characterized by necrosis and regeneration, fiber size variation, proliferation of connective and adipose tissue and infiltration of inflammatory cells. Even though the histology of DMD skeletal muscle is well described the causative molecular pathways are poorly understood.

Connective tissue infiltration is considered a secondary response that may further compromise

muscle function in DMD. Biglycan and decorin are small extracellular proteoglycans that interact with cytokines and ECM proteins [13, 29]. They regulate the assembly of connective tissue and alter cell behavior during developmental and pathological processes. Decorin and biglycan have distinctive and differing distribution arrangements, with decorin being mainly related to collagen-rich connective tissues and biglycan restricted to the cell surface of certain cell types. Both decorin and biglycan are up-regulated in the ECM of *mdx* mouse dystrophic muscle [12, 17] and have been shown to be increased in dystrophic muscle through microarray analysis [23]. Little is known about their expression in other muscular dystrophies. In  $\alpha$ -sarcoglycan deficiency (adhelin/ Limb girdle dystrophy 2D) a reduction of heparan sulfate proteoglycans in the basal lamina of the muscle fiber has been described, compared with other muscle diseases, however, no changes were seen in decorin in this condition [30]. A reduction of decorin expres-



sion was reported in facioscapulohumeral muscular dystrophy myoblasts [31].

In this article we report an increase of both decorin and biglycan in skeletal muscle of DMD patients, and describe differences in their extracellular localization. We also show that cultured fibroblasts grown from DMD samples present an increased synthesis of decorin and biglycan but not heparan sulfate proteoglycans in the conditioned medium. This is the first report on the biochemical characteristics, localization and cell source of these species in DMD.

The role of both these molecules in DMD is not well understood. In other systems, decorin is considered to be a regulator of the scarring process. It has been postulated to have beneficial effects in atherosclerosis reducing inflammation and fibrosis in atherosclerotic plaques of ApoE(-/-) mice [32]. It also regulates the proper healing response to myocardial infarctions as its absence leads to abnormal scar tissue formation in a heart infarction model [33]. Subconjunctival decorin applications significantly affect eye conjunctival scarring and surgical outcome of glaucoma filtration treatments in rabbits [34]. Decorin exerts beneficial effects on renal tubulointerstitial fibrosis, primarily by influencing the expression of a key cyclin-dependent kinase inhibitor and by limiting the degree of apoptosis, mononuclear cell infiltration, tubular atrophy, and expression of TGF- $\beta$ 1 [35].

TGF- $\beta$ 1 is a known profibrotic cytokine which is secreted by inflammatory cells among others. In this case we noted an increase in the amount of nuclei which may correspond to inflammatory cell infiltration.

In muscular dystrophies, TGF- $\beta$ 1 plasma levels are significantly elevated in patients with DMD and congenital muscular dystrophy (CMD) [36]. TGF- $\beta$ 1 mRNA levels in skeletal muscle from laminin alpha2-negative and laminin alpha2-positive CMD patients are significantly greater than in controls. The TGF- $\beta$ 1 values are lower than those found in DMD, although the extent of fibrosis is greater in CMD [37].

Decorin is able to sequester TGF- $\beta$  and thus decrease its bioavailability [38]. But it has also been reported that decorin antisense ODN-treated tendinocytes showed suppression of TGF- $\beta$ 1 production [39]. Therefore, decorin increment in DMD muscle tissue might correspond to a cell effort to regulate fibrosis. Supporting this it has been shown that direct injection of decorin prevents TGF- $\beta$ 1-induced scar tissue *in vivo* [40], and efficiently pre-

vents fibrosis and enhances muscle regeneration in lacerated murine muscles [41, 42].

TGF- $\beta$  is able to inhibit myoblast differentiation *in vitro* [16], effect which can be attenuated by the addition of decorin. Interestingly, it has been reported that DMD myoblasts have a reduced proliferation rate together with a TGF- $\beta$ 1 increase in conditioned media. This may be related to the DMD muscle diminished regeneration capacity [43]. Blocking TGF- $\beta$ 1 could decrease fibrosis related to dystrophy since neutralization of TGF- $\beta$ 1 by decorin administration resulted in a 40% reduction in the level of diaphragm muscle type I collagen mRNA [44].

Biglycan expression is also related to fibrotic processes, but not necessarily in parallel to decorin changes. Biglycan is overexpressed in atherosclerotic lesions, and it has been suggested to be involved in the regulation of vascular smooth muscle growth and migration through cdk2- and p27-dependent pathways. Furthermore, changes in biglycan expression could be a factor influencing the susceptibility of arteries to vascular injury [22]. In a bleomycin-induced pulmonary fibrosis model, while decorin expression decreased, biglycan expression increased up to fourfold together with TGF- $\beta$ 1. When active TGF- $\beta$  was overexpressed *in vitro*. Kolb found that both decorin and biglycan were able to interfere with TGF- $\beta$  bioactivity in a dose-dependant manner [20]. *In vivo*, adenoviral overexpression of TGF- $\beta$  resulted in marked lung fibrosis. This effect was significantly reduced by adenoviral mediated overexpression of decorin. When biglycan was overexpressed in the same manner, it had no significant effect on lung fibrosis. This data suggest that differences in tissue distribution, and/or cell source may be responsible for the different effects of biglycan on TGF- $\beta$  bioactivity *in vivo* [20].

We describe decorin and biglycan increments in different tissue compartments in DMD muscle at a stage in which fibrosis does not yet seem to be the main histological feature. We do not know if, as the disease progresses, there would be changes in the distribution of these molecules. Differential expression patterns, either spatial or temporal, of decorin and biglycan have been reported in other forms of fibrotic diseases. Biglycan has been mainly associated to the fibrous areas of the diseased tissue. Decorin has been observed to have a spatial distribution which varies with the stage of the disease, be it inflammatory or chronic fibrotic.[45–47]. In these

biopsies biglycan was detected in the areas where connective tissue predominates and a fibrotic phenotype is being developed. Decorin on the other hand is seen surrounding the muscle fiber. This is an interesting observation if a protective role of decorin over the diseased fiber is argued. Furthermore, we have previously described an increase of decorin in the endomysium of *mdx* mice leg muscles where muscular dystrophy is less severe and the tissue has a greater regenerative capacity.

The decorin and biglycan increase in DMD may also play a role in a remodeling attempt in response to injury. Biglycan and decorin induce morphological and cytoskeletal changes in fibroblasts, resulting in an increase in migration. [48]. This increase may have other functions, such as trophic ones. It has been described that biglycan synthesized by meningeal fibroblast cell cultures markedly enhances survival of rat neocortical neurons [49].

We found that fibroblasts isolated from DMD muscle biopsy samples have increased synthesis of decorin and biglycan. In the heart, after myocardial infarction, angiotensin II (AngII) promotes ventricular remodeling and deposition of ECM. When stimulated with AngII, neonatal cardiac fibroblasts produce biglycan as the predominant proteoglycan. Furthermore, neutralizing antibodies to TGF- $\beta$ 1 inhibited biglycan induction in response to AngII [50]. In normal human skin and gingival fibroblast cultures TGF- $\beta$ 1 markedly increased the expression of biglycan mRNAs, and the enhancement of biglycan expression was coordinated with elevated type I procollagen gene expression in the same cultures. In contrast, the expression of decorin mRNA was markedly inhibited by TGF- $\beta$ 1 [51]. Our results also contrast with the increased amount of heparan sulfate proteoglycans found in whole muscle tissue in both DMD patients [52] and *mdx* mice [12]. This suggests that the skeletal muscle fiber itself may be the source of increased heparan sulfate proteoglycans. It is known that perlecan and glypican, heparan sulfate proteoglycans, are synthesized by differentiated myotubes [53, 54]. However it must be noted that the proteoglycans analyzed here come from the conditioned medium of the cell cultures, it is also possible that heparan sulfate species are been associated to the cell and ECM.

In summary, both decorin and biglycan are increased in DMD muscle samples. They have distinct extracellular localization and, at least in part,

this elevated amount is explained by an increased synthesis by fibroblasts. This phenomenon is similar to what we have described in the *mdx* mouse. Further experiments are required to determine if this is a compensatory response to muscle or, on the contrary, indicate the beginning of fibrosis.

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