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Transient up-regulation of biglycan during skeletal muscle regeneration: delayed fiber growth along with decorin increase in biglycan-deficient mice

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Abstract

The onset and progression of skeletal muscle regeneration are controlled by a complex set of interactions between muscle precursor cells and their environment. Decorin is the main proteoglycan present in the extracellular matrix (ECM) of adult muscle while biglycan expression is lower, but both are increased in *mdx* mice dystrophic muscle. Both of these small leucine-rich proteoglycans (SLRPs) can bind other matrix proteins and to the three TGF-β isoforms, acting as modulators of their biological activity. We evaluated biglycan and decorin expression in skeletal muscle during barium chloride-induced skeletal muscle regeneration in mice. A transient and dramatic up-regulation of biglycan was associated with newly formed myotubes, whereas decorin presented only minor variations. Studies both in vitro and in intact developing newborn mice showed that biglycan expression is initially high and then decreases during skeletal muscle differentiation and maturation. To further evaluate the role of biglycan during the regenerative process, skeletal muscle regeneration was studied in biglycan-null mice. Skeletal muscle maintains its regenerative capacity in the absence of biglycan, but a delay in regenerated fiber growth and a decreased expression of embryonic myosin were observed despite to normal expression of MyoD and myogenin. Transient up-regulation of decorin during muscle regeneration in these mice may possibly obscure further roles of SLRPs in this process.

Keywords: Biglycan; Decorin; Embryonic myosin; Proteoglycans; Skeletal muscle regeneration

Introduction

In mammals, skeletal muscle formation continues postnatally during growth of muscle masses and as a damage-induced regenerative response. Muscle regeneration maintains muscle function in aging and delays functional impairment caused by progressive neuromuscular diseases such as Duchenne muscular dystrophy. Satellite cells, mononucleated cells located at the periphery of mature myofibers and beneath its basal lamina, constitute the main source of muscle precursor cells for growth and repair. After skeletal muscle injury, released signals induce their reentry into the cell cycle and their migration into the damaged zone where they proliferate and differentiate into mature myofibers.

Differentiation of muscle precursor cells is regulated by the expression of specific combinations of muscle regulatory transcriptions factors, especially from a family of basic helix-loop-helix transcription factors denominated muscle regulatory factors (MRFs). Among the MRFs, myf-5 and MyoD are expressed first by activated satellite cells, associated with proliferation of determined muscle precursors, and are then followed by the expression of myogenin, which triggers terminal differentiation, and by MRF-4, which is expressed in differentiated muscle (Cornelison and Wold, 1997; Seale and Rudnicki, 2000). Myogenin expression, in cooperation with other transcription factors such as myocyte enhancer factor 2 (MEF-2), leads to muscle precursor cell fusion and

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to the expression of muscle specific proteins, such as creatine kinase or myosin heavy chains, which follow a transition from developmental to adult isoforms.

Growth factors such as transforming growth factor-β (TGF-β), insulin-like growth factors (IGF), hepatocyte growth factor (HGF), and members of the fibroblast growth factor (FGF) family are among the known extracellular signals that control skeletal muscle differentiation. A satellite cell line derived from regenerating adult mouse skeletal muscle, C2C12, undergoes in vitro terminal myogenic differentiation after serum removal from the culture medium (Blau et al., 1985; Yaffe and Saxel, 1977). The surrounding extracellular matrix (ECM) is also likely to play an important role in growth control and differentiation. It acts not only as a scaffold for the cells but as a reservoir of growth factors and cytokines, regulating their activation status and turnover. Also, several ECM molecules exhibit direct signaling functions (Boudreau and Bissell, 1998; Kresse and Schonherr, 2001; Lukashev and Werb, 1998). In the C2C12 cells, disorganization of the ECM, caused by the inhibition of proteoglycan sulfation, affects the proper progression of the myogenic program independently of myogenin expression (Melo et al., 1996; Osses and Brandan, 2001). In vivo, muscle injuries that destroy the muscle basal lamina generally present a poorer functional recovery than injuries that minimally disrupt its integrity and orientation (Sanes, 2003).

Decorin and biglycan are two chondroitin sulfate or dermatan sulfate (CS/DS) ECM proteoglycans of the SLRP family, which bind to TGF-βs, collagens, and other matrix proteins (for a review, see Ameye and Young, 2002; Iozzo, 1998). In adult skeletal muscle tissue, decorin is the most abundant proteoglycan and is found mainly in the perimysium (Brandan et al., 1992). The synthesis and expression of decorin are up-regulated during skeletal muscle differentiation in vitro (Brandan et al., 1991). However, decorin expression in myoblasts is required for preventing terminal differentiation: antisense inhibition of its expression in C2C12 myoblasts accelerates skeletal muscle differentiation by decreasing sensitivity to TGF-β signaling (Riquelme et al., 2001). Biglycan, on the other hand, is expressed by secondary myotubes during fetal muscle formation (Bianco et al., 1990) and, in the adult, is localized preferentially in the endomysium and in certain NMJs, probably complexed to the dystrophin-associated protein complex through its binding to α-dystroglycan (Bowe et al., 2000). Both decorin and biglycan are up-regulated in the ECM of the dystrophic muscle of the *mdx* mouse (Bowe et al., 2000; Caceres et al., 2000; Porter et al., 2002). This mouse model of Duchenne muscular dystrophy presents a much milder phenotype than human patients because of the functional compensation of dystrophin by utrophin (Grady et al., 1997) and enhanced muscle regeneration (Pagel and Partridge, 1999). Decorin and biglycan are also increased in the skeletal muscle of Duchenne muscular dystrophy patients, as has been described with microarray analyses (Haslett et al., 2002), but this increase appears to be mostly associated with fibrotic tissue (Alvarez et al., unpublished results).

In this study, decorin and biglycan expressions were evaluated in a mouse model of skeletal muscle regeneration. Interestingly, their expression during muscle regeneration was differentially regulated. A dramatic and transient increase in biglycan expression associated with regenerating myotubes was observed, whereas decorin remained relatively unchanged. Biglycan expression was studied during animal muscle growth and in vitro myogenesis, and its role in skeletal muscle regeneration was assessed by studies in biglycan-null mice.

Materials and methods

Animals

C57BL/6 wild-type male mice of 2–12 weeks of age and biglycan-deficient mice of 8–12 weeks of age were studied. These mice were generated by gene targeting by homologous recombination as described previously (Chen et al., 2002; Xu et al., 1998). Biglycan gene, *Bgn*, is located on chromosome X so wild-type male mice have a genotype of *Bgn*+/0 and the mutant male mice, which do not express either biglycan mRNA or its protein, are *Bgn*−/0. The animals were kept at room temperature with a 24-h night–day cycle and fed with pellets and water ad libitum. All protocols were conducted under strict accordance and with the formal approval of Brown University's Institutional Animal Care and Use Committee and the Animal Ethics Committee of the P. Universidad Católica de Chile.

Experimental muscle injury

Injury of normal muscles in animals of 8–12 weeks of age was performed by barium chloride injection (Caldwell et al., 1990) in mice under ketamine/xylazine anesthesia (80:12 mg/kg bw ip). Briefly, 60 μl of 1.2% m/v $BaCl_2$ aqueous solution was injected along the whole length of the left Tibialis anterior muscle (TA). Contralateral non-injected muscles were used as controls. After different recovery times (range 2–15 days), TA were dissected and removed under anesthesia and the animals were sacrificed. Tissues were rapidly frozen and stored at −80°C until processing.

Cell culture

The mouse skeletal muscle cell line C2C12 (ATCC, VA), derived from adult mouse leg muscle, was grown and induced to differentiate as described previously (Larrain et al., 1997, 1998). Differentiating cells were synchronized by the addition of AraC, an inhibitor of cell proliferation, to the differentiation medium since the second day.

RNA isolation and Northern blot analysis

Total RNA was isolated from skeletal muscles as described (Brandan et al., 1992; Chomczynski and Sacchi, 1987). Eight to 10 μg RNA samples were electrophoresed in 1.2% agarose/formaldehyde gels, transferred to Nytran membranes (Schleicher and Shuell, Dassel, Germany), and hybridized with random primed [^{32}P]-dCTP-labeled cDNA probes for biglycan, decorin, MyoD, and myogenin in hybridization buffer at 65°C as described previously (Brandan et al., 1992; Brandan et al., 1996; Riquelme et al., 2001). The biglycan probe, 625 nt long, was amplified by PCR from mouse liver cDNA as described below. Hybridized membranes were washed twice at 65°C and exposed to Kodak X-ray film. To normalize signal intensity, blots were later stripped and rehybridized with a GAPDH or an 18S ^{32}P-labeled probes kindly donated by Dr. J. Chianale (Department of Gastroenterology, Faculty of Medicine, P. Universidad Católica de Chile, Chile).

RT-PCR analyses

Two micrograms of total RNA from each sample were used for cDNA generation with a recombinant M-MLV reverse transcriptase (Invitrogen, CA) and random hexamers as primers. The cDNAs were amplified using the following primers: biglycan, 5′-TCCCCAGGAACATTGACCAT-3′ and 5′-GTTCAAAGCCACTGTTCTCCA-3′; embryonic myosin heavy chain, 5′-GGAGACACGGATCAGAGAGC-3′ and 5′-CAGCCTGCCTCTTGTAGGAC-3′; and 18S RNA, 5′-TAGAGCTAATACATGCCGACG-3′ and 5′-TTAATCATGGCCTCAGTTCCG-3′. Samples were denatured at 94°C for 5 min, followed by amplification rounds consisting of denaturing at 94°C for 30 s, annealing at 60°C or 57°C (biglycan) for 90 s and extension at 72°C for 90 s through 32 cycles in the case of biglycan and embryonic myosin, and 24 for 18S RNA; thus, all reactions were performed within the linear range of amplification for each pair of primers. Final extension at 72°C for 10 min was allowed. The products were size-fractionated by electrophoresis through 1.5% agarose gels and stained with ethidium bromide. Images of the gels were acquired with Gel Doc system and analyzed with the software Quantity One® (Bio-Rad Laboratories, CA).

Labeling of skeletal muscle proteoglycans

Four or 6 days after the induction of muscle injury, a group of mice was injected intraperitoneally with 3 mCi sodium [^{35}S]-sulfate (New England Nuclear, MA) in 0.15 M NaCl each, divided into four individual injections separated by 3 h (Brandan and Inestrosa, 1987). Twenty four hours later, the animals were sacrificed and TA muscles were excised and subjected to protein extraction protocols as described below.

Protein extraction

Protein extracts were prepared with a protocol slightly modified from the one previously described by Brandan and Inestrosa in 1987. Briefly, skeletal muscle was homogenized in 4 M 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, previous to enzymatic treatments. For alkaline extraction of biglycan (modified from Bowe et al., 2000), the tissue was homogenized in 50 mM Tris, pH 12.0, containing protease inhibitors (10 μg/ml leupeptin, 100 μg/ml benzamidine, 50 μg/ml aprotinin, 100 μg/ml trypsin inhibitor, and 1 mM PMSF) at 4°C, maintained under agitation for 1 h at room temperature, and centrifuged 30 min at 20,000 × *g* and 4°C. The supernatants were neutralized with 10 mM Tris–HCl, pH 6.0 to a pH between 7 and 8, and then concentrated by DEAE-Sephacel anion-exchange chromatography, as described above. Protein content was determined as described previously (Riquelme et al., 2001).

Enzymatic digestions

Chondroitinase ABC (Sigma, MO) and heparitinase (Seikagaku Corporation, Tokio, Japan) digestion of proteoglycans were performed as previously described (Brandan and Inestrosa, 1987; Brandan et al., 1992).

Determination of proteoglycan-associated radioactivity

Aliquots of the digested material from the extracts of metabolically labeled muscle were spotted on dry Whatman 3 MM filter discs impregnated by prior soaking in 2.5% cetylpyridinium chloride (CPC; Caceres et al., 2000). The filter discs were washed sequentially in 25 mM sodium sulfate, distilled water, and 95% ethanol and then dried for scintillation counting. A 2-h incubation with 20% trichloroacetic acid was performed in between washes with distilled water. GAG-containing material binds quantitatively to these discs, but disaccharides generated by the digestion or cleavages do not (Rapraeger and Bernfield, 1985).

SDS-PAGE analysis of proteoglycans

Appropriate samples were digested with chondroitinase ABC or heparitinase and then analyzed by SDS-PAGE followed by fluorography using a 4–15% acrylamide gradient in the separation gel as previously described (Caceres et al., 2000).

Western blot analysis

For proteoglycan detection, appropriate samples containing equivalent amounts of proteins were incubated with chondroitinase ABC and then analyzed by SDS-PAGE using a 4–15% acrylamide gradient in the separation gel. Proteins were electrophoretically transferred to nitrocellulose membranes, detected with anti-Δ-mouse biglycan LF-106 or anti-mouse decorin LF-113 polyclonal antibodies (both were kindly donated by Dr. L. Fisher, NIDR, NIH, Bethesda, MD; Fisher et al., 1995) and visualized by enhanced chemiluminescence (Pierce, IL). Corresponding samples from the total homogenate in urea buffer were also analyzed by immunoblot as described above, with monoclonal antibodies against myogenin (Olguin and Brandan, 2001) and embryonic myosin F1.652 [developed by Dr H. Blau (Silberstein et al., 1986) and obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA].

Histology and immunohistochemistry

Cryostat sections (6 μm) of control and treated TA at different times after $BaCl_2$ injection were fixed for 20 min in 3% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, and stained with hematoxilin–eosin (H.E.) or blocked with 8% BSA in PBS and treated with 2.5 mU chondroitinase ABC for 2 h at 37°C (Bianco et al., 1990) previous to an overnight incubation at 4°C with primary antibodies against biglycan LF-51 (in 1:300 dilution), decorin LF-113 (in 1:700 dilution), and embryonic myosin F1.652 (1:100). Sections were then washed and incubated with either anti-rabbit FITC or anti-mouse TRITC (all diluted 1:100, Pierce) for 1 h at room temperature. For nuclear staining, sections were incubated with 1 μg/ml Hoechst 33258 in PBS for 10 min. After rinsing, the sections were mounted with fluorescent mounting medium (Dako Corporation, CA) under glass cover slips and viewed and photographed with a Nikon Eclipse microscope equipped for epifluorescence. Specificity of anti-biglycan and anti-decorin antibodies was assayed by the observation of absence of staining after preincubation with an over tenfold excess of the purified proteoglycans subjected to chondroitinase ABC digestion.

For the assessment of fiber diameters, images from H.E.-stained cross sections from *Bgn*+/0 and *Bgn*−/0 muscles were obtained with the Nikon ACT-1 version 2.12 software and a Nikon DXM1200 CCD digital camera. For each muscle (n = 2–3 for each different time point), two ×10 field images were selected from the zones that presented the greatest variability in the diameters of the fiber. The lesser diameter of each fiber in the field (over 1000 for each muscle) was measured with the Adobe PhotoShop 5.0 software.

Results

Increased synthesis of CS/DS proteoglycans during skeletal muscle regeneration

Injection of a 1.2% barium chloride solution induces necrosis of skeletal muscle fibers followed by the regeneration of the tissue (Caldwell et al., 1990; Casar et al., 2004). Briefly, as we have previously described (Casar et al., 2004), an enlargement of the intercellular space separating necrotic fibers, together with the appearance of abundant mononucleated cells, is observed the first 3 days after the injection. These cells are mainly inflammatory cells—predominantly

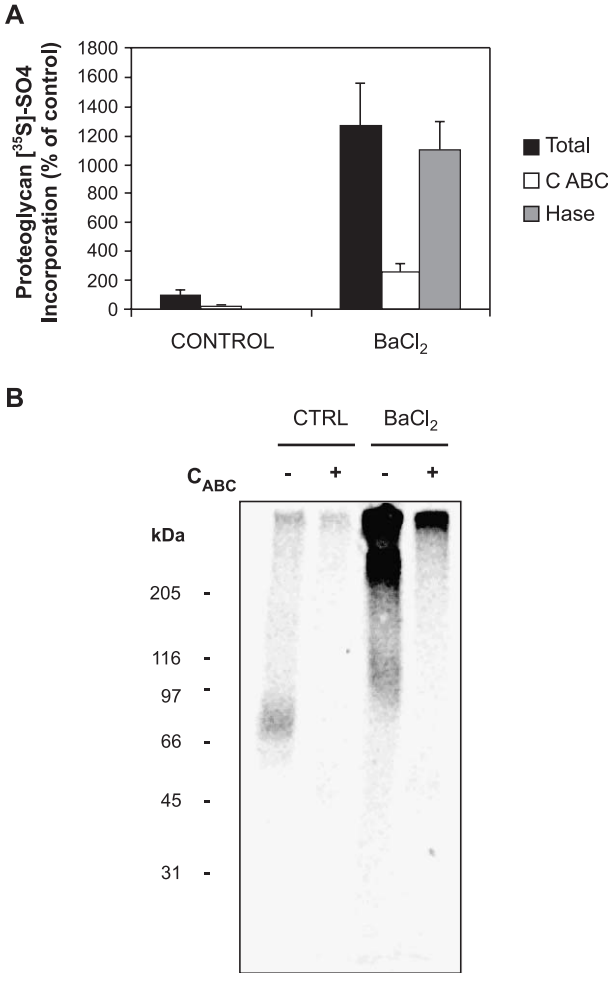

Fig. 1. Proteoglycan synthesis is increased in skeletal muscle regeneration. (A) After metabolic labeling with [^{35}S]-sulfate, radioactivity associated with proteoglycans in samples from control muscle and 5 days after barium chloride injection was determined by binding to CPC-impregnated filters as described in Materials and methods. In regenerating muscle, a greater than ten-fold increase in sulfate incorporation into proteoglycans was observed, maintaining a proportion of about 80% CS/DS proteoglycans. (B) SDS-PAGE fractionation in 4–15% gradient gels of the labeled muscle samples and fluorography shows that the observed increase in proteoglycan content is mainly associated to CS/DS species of 100–130 and 200–300 kDa and a band of CS/DS and HS proteoglycans of higher apparent molecular mass. Migration of molecular mass standards is shown on the left (C ABC, chondroitinase ABC; Hase, heparitinase).

polymorphonuclear leukocytes and macrophages—and spindle-shaped activated and proliferating satellite cells. Regenerated myotubes, with the nuclei in central position, appear by day 4 or 5. By the seventh day, most fibers in a cross section of different zones of an injected TA present central nuclei, but their diameters are still highly heterogeneous. By the 15th day after the injection, the tissue has recovered most of its normal morphology, but regenerated fibers present central nuclei and a slight increase in the interstitial space is still observed.

As a global approach to proteoglycan synthesis during skeletal muscle regeneration, intraperitoneal injection of [^{35}S]-sulfate was performed the fourth day after barium chloride injection, that is, a time when massive muscle differentiation has started, but some degree of muscle precursors proliferation can still be found. After 24 h of metabolic labeling, both the injured and the contralateral spared muscle were harvested and protein extracts were prepared and enriched in proteoglycans by anion-exchange chromatography. Proteoglycan content in these extracts was evaluated by binding to CPC impregnated filters. In control muscle, as has been previously described (Andrade and Brandan, 1991; Brandan et al., 1991), most of the label was associated to CS/DS proteoglycans and the remaining was sensitive to heparitinase digestion. In regenerating muscle, a remarkable—about tenfold—increase in proteoglycan-associated sulfate labeling was observed, being still around 80% of the radioactivity sensitive to chondroitinase ABC digestion. Aliquots from these extracts, equivalent in protein content, were fractionated by SDS-PAGE and subjected to fluorography for a further approach to the identity of the different proteoglycan species involved (Fig. 1). The main species in normal muscle is decorin, a CS/DS proteoglycan that migrates around 70–90 kDa (Brandan et al., 1992), which appears together with a less intense band of high molecular weight heparan sulfate proteoglycans (i.e., sensitive to heparitinase digestion, data not shown). In regenerating skeletal muscle, in contrast, two CS/DS species appear, which migrate as broad heterogeneous bands around 120 and 250 kDa, respectively, and likely correspond to the forms of biglycan substituted with one or two glycosaminoglycan chains (Fisher et al., 1989). An increase in the label of higher relative molecular mass species is also observed in regenerating muscle, mostly associated with an increase in

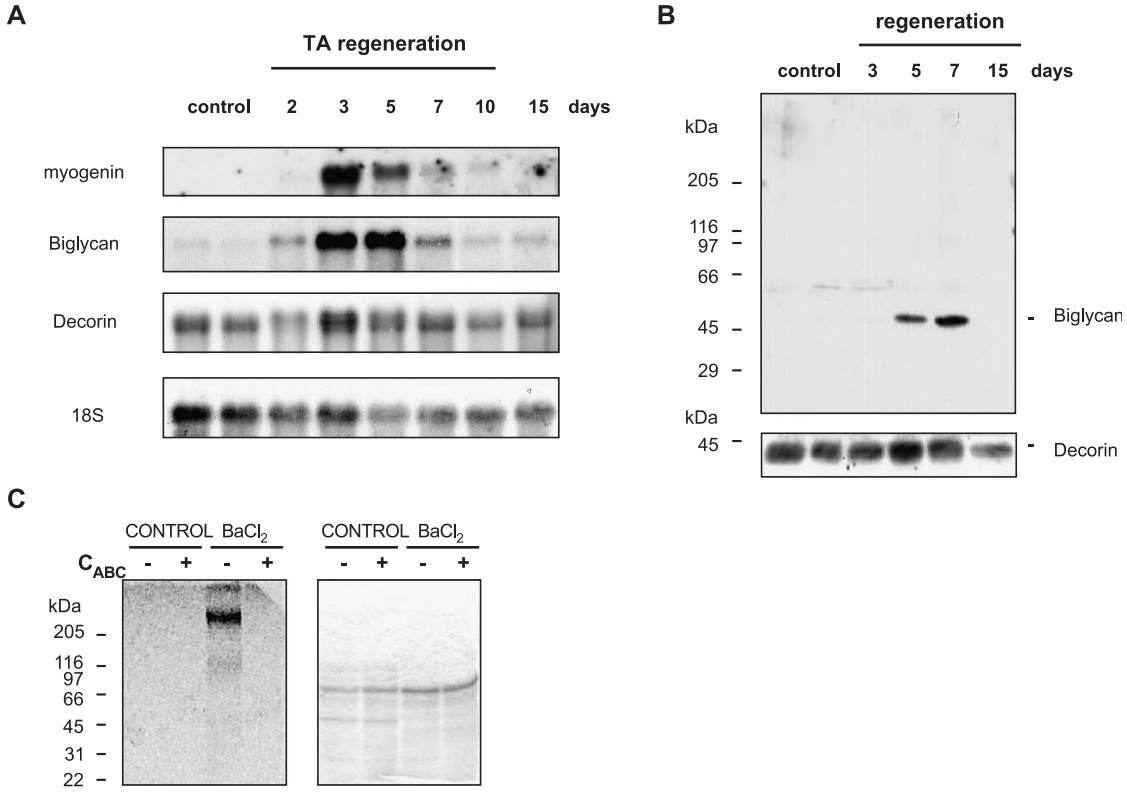

Fig. 2. Biglycan expression is transiently increased during skeletal muscle regeneration. (A) In Northern blot studies, biglycan steady state mRNA levels increase from the second day after the barium chloride injection until around the seventh day, parallel to the early stages of myogenesis as indicated by the induction of myogenin expression. Decorin transcript levels, in contrast, do not show major variations and only a slight increase is observed during regeneration as compared to basal levels. Hybridization with a probe for 18S ribosomal RNA is presented as a gel-loading control. (B) Biglycan core protein increases transiently during skeletal muscle regeneration as is detected on the fifth and seventh day after the injection in Western blot studies of chondroitinase ABC-treated extracts. Decorin core protein levels only show minor variations and is high at all time points. (C) SDS-PAGE of samples of TA subjected to metabolic labeling of proteoglycans with [^{35}S]-sulfate 6 days after barium chloride injection and alkaline solubilization shows an increase of two CS/DS species with an electrophoretic migration pattern previously described for biglycan (Fisher et al., 1989). Coomassie blue staining of the proteins in the gel is showed on the right (C_{ABC}, chondroitinase ABC).

heparan sulfate proteoglycans, as we have recently described (Casar et al., 2004). However, this high molecular weight smear appears to be partially sensitive to chondroitinase ABC digestion, probably reflecting an increase in other CS/DS proteoglycans of high molecular weight such as versican or complex formation by smaller proteoglycans such as decorin and biglycan. Together, these results show that quantitative and qualitative changes in small CS/DS proteoglycans occur in skeletal muscle during regeneration.

Transient increase of biglycan expression during skeletal muscle regeneration

Characterization of CS/DS SLRP expression during barium-chloride-induced skeletal muscle regeneration in TA muscle was performed. The expression of biglycan and decorin transcripts was studied in mouse TA skeletal muscle during regeneration by Northern blot (Fig. 2A). In adult skeletal muscle, biglycan mRNA expression is comparatively low, being almost undetectable in the conditions studied. During regeneration, a transient and dramatic increase in biglycan mRNA expression is observed; it starts around the second day after barium chloride injection, peaks between the third and fourth day after the injection, and then decreases to reach the basal level towards the seventh day. This time course is coincident with the early differentiation of regenerated myotubes, as reflected by up-regulation of the transcripts of the MRF myogenin. Decorin mRNA, in contrast, appears to show small variations during the process, presenting only a slight increase that is observed on the first days after the injection.

Western blots were performed to study if differences in mRNA levels were reflected in changes of the core protein levels of these proteoglycans (Fig. 2B). After fractionation and blotting of chondroitinase ABC-treated samples, anti-biglycan polyclonal antiserum LF-106 recognizes a protein that migrates as a globular protein of 48 kDa and whose content is transiently increased on days 5 and 7 after $BaCl_2$ injection. Anti-decorin antibodies recognize a protein that migrates with a slightly smaller relative molecular mass, detectable in control muscle and whose content persists without major variations during regeneration. As LF-106 antibodies

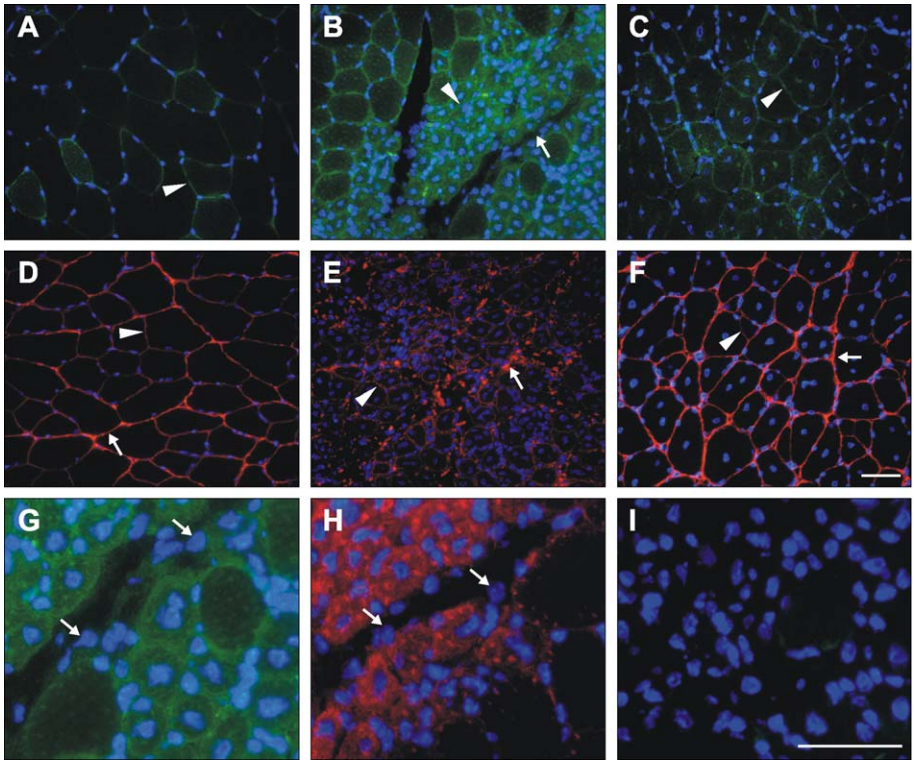

Fig. 3. Biglycan increase is localized in nascent new myotubes in regenerating skeletal muscle. Indirect immunofluorescence studies in cross sections of control (A and D) and regenerating TA at five (B, E, G, H, and I) and 15 days (C and F) after barium chloride injection. An increased biglycan immunodetection is observed in areas of newly formed myotubes on the fifth day after barium chloride injection (as detected with an FITC-conjugated second antibody, A–C, G). In control muscle, biglycan is localized preferentially in the endomysium, whereas decorin (detected with TRITC-conjugated antibodies, D–F) is localized also at the endomysium, but mostly at the perimysium. During regeneration, decorin is detected at the periphery of myotubes and myofibers and in the interstitial connective tissue. By the 15th day after barium chloride injection, distribution of both proteoglycans resembles that of control muscle. The endomysium and regenerating myotubes basement membranes are indicated by an arrowhead; the perimisium and interstitial tissue during regeneration are indicated by an arrow. In G–I, higher magnification microphotographs show that during regeneration, biglycan increase is associated to newly formed myotubes (G), which show a positive staining for embryonic myosin in adjacent tissue sections (detected with TRITC-conjugated antibodies, H), and is absent from interstitial cells (arrows). Specificity of the staining was confirmed by absorbing anti-biglycan antibodies with an excess of chondroitinase ABC-digested purified mouse biglycan (I). The scale bar indicates 50 μm.

do not recognize the glycanated forms of biglycan, mice were metabolically labeled with [^{35}S]-sulfate on the sixth day after barium chloride injection and both TA muscles were harvested 24 h later and subjected to alkaline solubilization to partially isolate biglycan (modified from Bowe et al., 2000). SDS-PAGE and fluorography of these extracts show a significant increase during regeneration of two CS/DS species that migrate as broad bands around 250 and 120 kDa, respectively (Fig. 2C). Taken together, these results show that during skeletal muscle regeneration in the TA, biglycan is the main CS/DS proteoglycan presenting an important transient increase in its expression, at both mRNA and proteoglycan levels.

The tissue localization of up-regulated biglycan was studied by indirect immunofluorescence (Fig. 3). Biglycan immunoreactivity in normal adult skeletal muscle is localized in the endomysium, with a relatively high background from the muscle fiber's cytoplasm (Fig. 3A). During regeneration, its immunoreactivity persists selectively localized around skeletal muscle fibers, with greater intensity around newly formed myotubes at the fourth (not shown) and fifth day after the injection (Figs. 3B and G). These new myotubes are characterized by their small diameter, presence of central nuclei, and expression of embryonic isoform of myosin in their cytoplasm (Fig. 3H). Interestingly, biglycan staining is absent from interstitial mononuclear cells (arrows, Fig. 3G). By the 15th day after $BaCl_2$ injection, biglycan staining is similar to that observed in control muscle (Fig. 3C). Decorin immunoreactivity, on the other hand, is localized preferentially at the perimysium and also at the endomysium of normal muscle tissue (Fig. 3D). This localization is not changed after the injury when decorin is detected both surrounding the nascent myotubes and persistent fibers and in the enlarged strips of connective

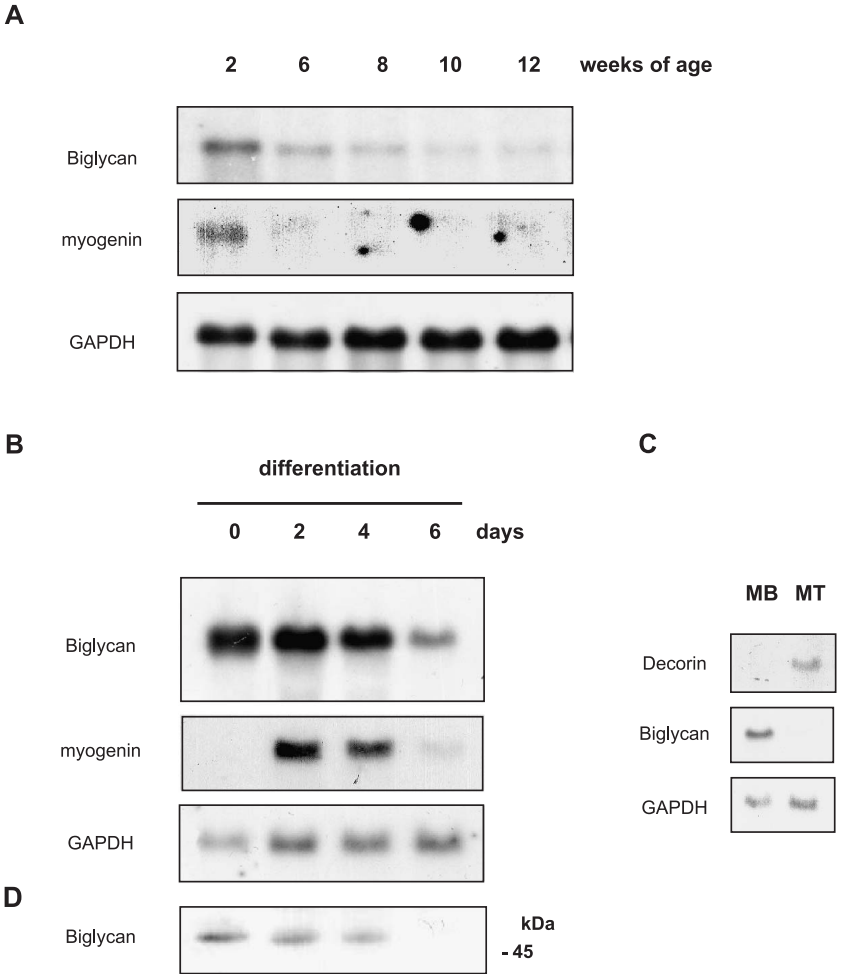

Fig. 4. Biglycan expression decreases with skeletal muscle differentiation and maturation, both in vitro and in vivo. (A) Biglycan mRNA expression in hindlimb skeletal muscle is high in 2-week-old mice, when muscle formation is still underway as is apparent by the maintained expression of myogenin transcripts, and then decreases progressively with age until adulthood. (B) In the skeletal muscle cell line C2C12, biglycan mRNA expression is high in myoblasts and is down regulated with muscle differentiation. (C) Decorin expression, on the contrary, is low in myoblasts and high in myotubes. Northern blots of total RNA samples (15 μg) were analyzed with [^{32}P]-labeled probes. Hybridization with a GAPDH probe was used as a gel-loading control. (D) Biglycan core protein decreases with skeletal muscle differentiation in the conditioned media of C2C12 cells. Western blot of chondroitinase ABC treated samples of conditioned media from C2C12 cells at different days after the induction of differentiation, concentrated by DEAE chromatography. Molecular mass standards are shown on the right.

tissue where most mononuclear cells are localized (Fig. 3E). As is the case for biglycan, by day 15, decorin distribution is similar to that observed in normal muscle, although with a small increase of the signal around muscle fibers, as a slight enlargement of the endomysial space is still observed (Fig. 3F). These histological results show that the observed up-regulation of biglycan is associated with regenerating myotubes and suggest that its transient increase may relate to the initial phases of muscle formation.

Biglycan expression decreases with maturation and differentiation of skeletal muscle

In view of the low levels of biglycan in adult skeletal muscle and its transient increase during regeneration, we next determined the time course of biglycan mRNA during normal postnatal development and maturation of mouse hind limb skeletal muscle by Northern blot (Fig. 4A). During the first weeks after birth, muscle formation continues as required by the animal growth (Buckingham, 1992). Interestingly, biglycan mRNA expression is higher at the youngest age studied (2 weeks), coincident with ongoing muscle formation as reflected by detectable myogenin mRNA expression. Biglycan mRNA expression then gradually decreases to the low levels characteristic of the adult.

Changes in the expression of biglycan during myogenic differentiation in vitro were studied in C2C12 cells. Biglycan transcripts are highly expressed by proliferating C2C12 cells. After the induction of differentiation, it progressively

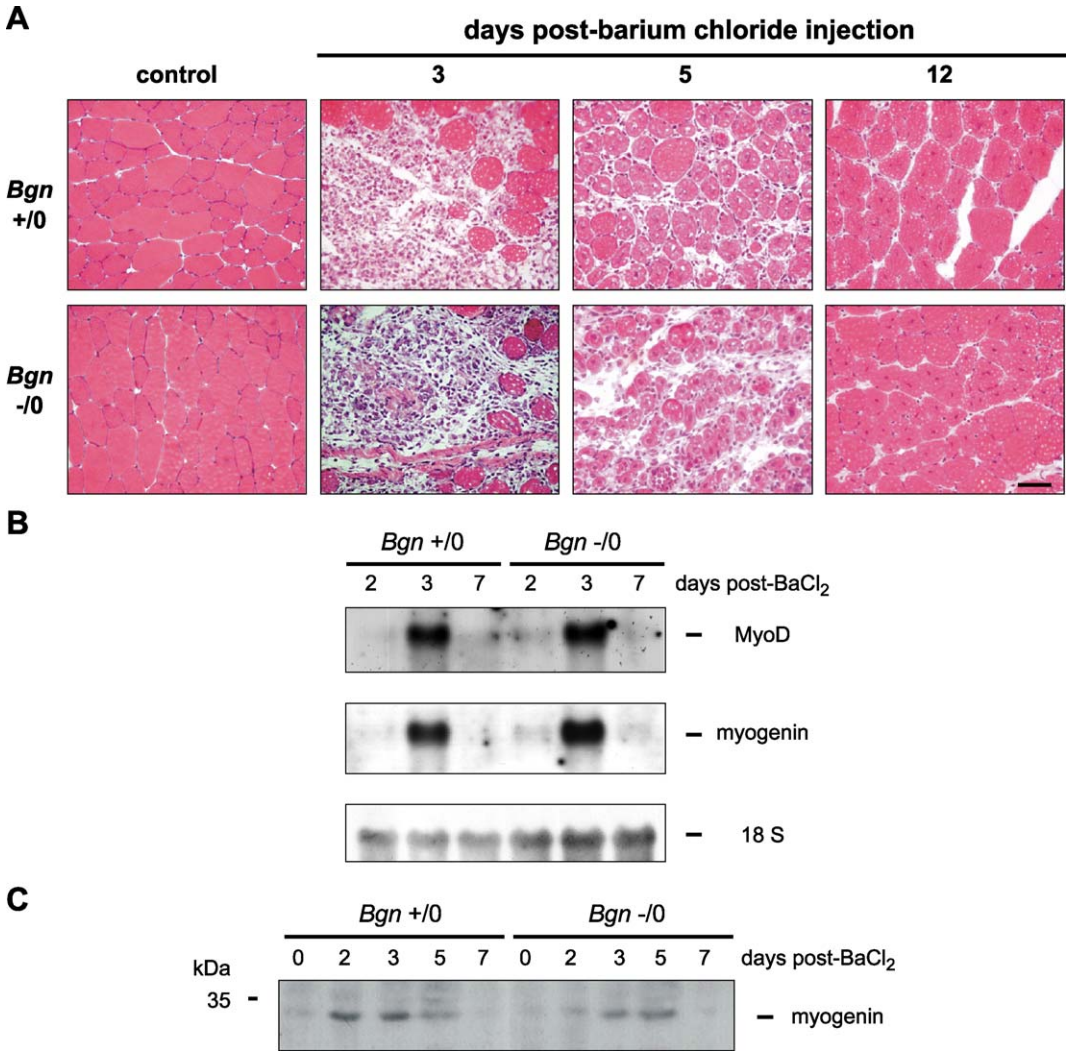

Fig. 5. Morphological progression and MRF expression during skeletal muscle regeneration of biglycan-null mice. (A) The morphological progress of skeletal muscle regeneration after barium chloride injection is similar in wild-type (*Bgn*+/0) and biglycan-null mice (*Bgn*−/0). H.E. staining of cross sections. The scale bar indicates 50 μm. (B) The temporal pattern of expression of the transcripts for the MRFS MyoD and myogenin in regenerating muscle from *Bgn*−/0 as compared to wild-type mice is shown. Northern blots of samples of total RNA (8 μg) from TA muscle at different days after barium chloride injection, analyzed with [^{32}P]-labeled probes. Hybridization with a probe for 18S ribosomal RNA was used as a gel-loading control. (C) Myogenin expression during skeletal muscle regeneration in wild-type and biglycan-null mice was also studied by Western blot. No difference is observed in the overall time course of its expression in *Bgn*−/0 muscle, although a slight delay in the onset of myogenin expression is apparent. A standard of molecular mass is shown on the right.

decays until the sixth day, when abundant myotubes are present in the culture (Fig. 4B). Myogenin mRNA is detectable only after differentiation is induced, and at the fourth day it starts to decrease. As in the in vivo studies, biglycan's core protein presented a similar pattern to its mRNA expression and was down-regulated with myogenesis of C2C12 cells (Fig. 4D). The expression pattern observed for biglycan in these cells is opposite to the pattern observed for decorin (Brandan et al., 1991), which has a low expression by myoblasts and increases with differentiation (Fig. 4C).

Taken together, the in vivo and in vitro results suggest that biglycan is expressed by activated or proliferating muscle precursor cells and during the first steps of myogenesis and is then down-regulated with low levels persisting around the periphery of mature fibers in vivo.

Delayed fiber growth and diminished expression of myosin isoforms during skeletal muscle regeneration in biglycan-null mice

The transient up-regulation of biglycan during skeletal muscle regeneration suggests a function for this SLRP in adult myogenesis. To test this hypothesis, we studied regeneration in mice with targeted inactivation of biglycan by homologous recombination. Biglycan-null mice show abnormalities in collagen fibril formation and a late onset (>6 months) osteoporotic phenotype characterized by impaired bone formation, osteoarthritis, and ectopic tendon ossification (Ameye et al., 2002; Corsi et al., 2002; Xu et al., 1998). These mice also present a mild myopathy, with instability of the sarcolemma and scattered foci of necrotic and regenerating myofibers (Rafii et al., 2000). Skeletal muscle regeneration was induced in 8-week-old mice by

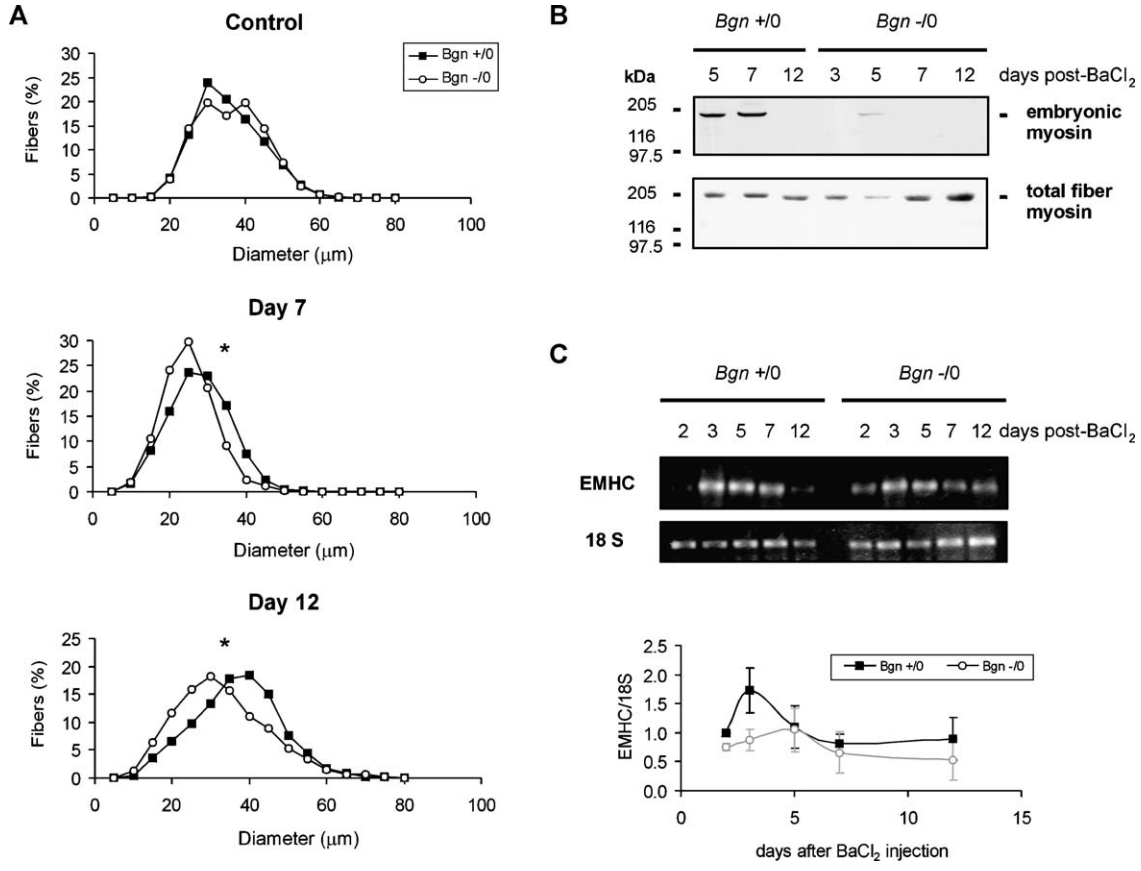

Fig. 6. Skeletal muscle regeneration in *Bgn*−/0 mice shows slower growth of the regenerated fibers and decreased expression of embryonic myosin. (A) The distribution of muscle fiber diameters is presented for the wild type (*Bgn*+/0), and biglycan-null (*Bgn*−/0) mice are presented for midbelly sections from control muscle and muscles after 7 and 12 days from barium chloride injection (n = 2–3 muscles in each group for each of the different time points studied). No difference in the fiber diameter distribution was observed in basal conditions in *Bgn*+/0 and *Bgn*−/0 muscle, but the distribution was significantly different 7 days after the injection of barium chloride into muscle and the difference was even more notorious by day 12 (*P < 0.001, Mann–Whitney rank sum test). (B) Embryonic myosin expression during skeletal muscle regeneration was studied by Western blot in extracts from *Bgn*+/0 and *Bgn*−/0 muscle from different days after barium chloride injection. A decreased content of this isoform was detected in biglycan-null muscle during regeneration, although levels of total muscle myosin were comparable to the observed in wild-type mice. Molecular mass standards are shown on the left. (C) Semiquantitative RT-PCR was performed to study embryonic myosin heavy chain (EMHC) transcripts expression during skeletal muscle regeneration. Amplification of 18S cDNA was used as a loading control. Densitometric quantification of the products graphically shows that in biglycan-null muscle, the increase in EMHC expression is lower and is delayed as compared to wild-type animals (lower panel, n = 3; average ± SD, n = 3).

barium chloride injection in TA muscle. No qualitative difference in the morphological progression of skeletal muscle regeneration was observed in *Bgn*−/0 as compared to wild-type mice. Three days after the injection, extensive degeneration of myofibers and abundant mononuclear cells were observed; by the fifth day, numerous newly formed myotubes were apparent; and from days 7 to 12, tissue morphology was grossly restored by centrally nucleated myofibers (Fig. 5A). The expression of mRNA of the MRFs MyoD and myogenin, early markers and regulators of the myogenic process, was studied by Northern blot (Fig. 5B). No differences in their temporal pattern of expression were observed between wild-type and *Bgn*−/0 mice. The temporal pattern of myogenin protein expression was also similar in both groups in Western blot studies, although a slight delay in the onset of its expression was apparent in *Bgn*−/0 mice (Fig. 5C).

When muscle fiber diameters were assessed, a difference was observed in skeletal muscle fiber growth during regeneration (Fig. 6A). No difference was found in the basal fiber diameter distribution in the TA of any of the groups of mice, indicating that skeletal muscle formation and myofiber growth in this skeletal muscle are not affected by long-term in the absence of biglycan. However, at the seventh day after barium chloride injection, the frequency distribution of the diameters of the regenerated fibers was significantly different in *Bgn*−/0 compared to wild-type muscle ($P < 0.001$, Mann–Whitney rank sum U test), where the myotubes in null-mice muscle were slightly thinner, even though their modal diameter was similar. This difference became more evident at the 12th day after the injection where *Bgn*−/0 muscle showed a smaller modal fiber diameter, suggesting that a delay in myotube enlargement is observed in regenerating *Bgn*−/0 muscle.

Interestingly, and also coincident with possible abnormalities occurring after muscle precursor cell fusion, a second difference in the regenerative process in biglycan-null muscle was observed when the expression of a later differentiation marker was studied: *Bgn*−/0 muscle presented a decreased embryonic myosin expression, even though the total content of skeletal muscle myosin was similar in both groups of mice (Fig. 6B). This decrease in embryonic myosin content reflects a delayed and lesser peak of expression of the transcripts for the embryonic isoform of myosin

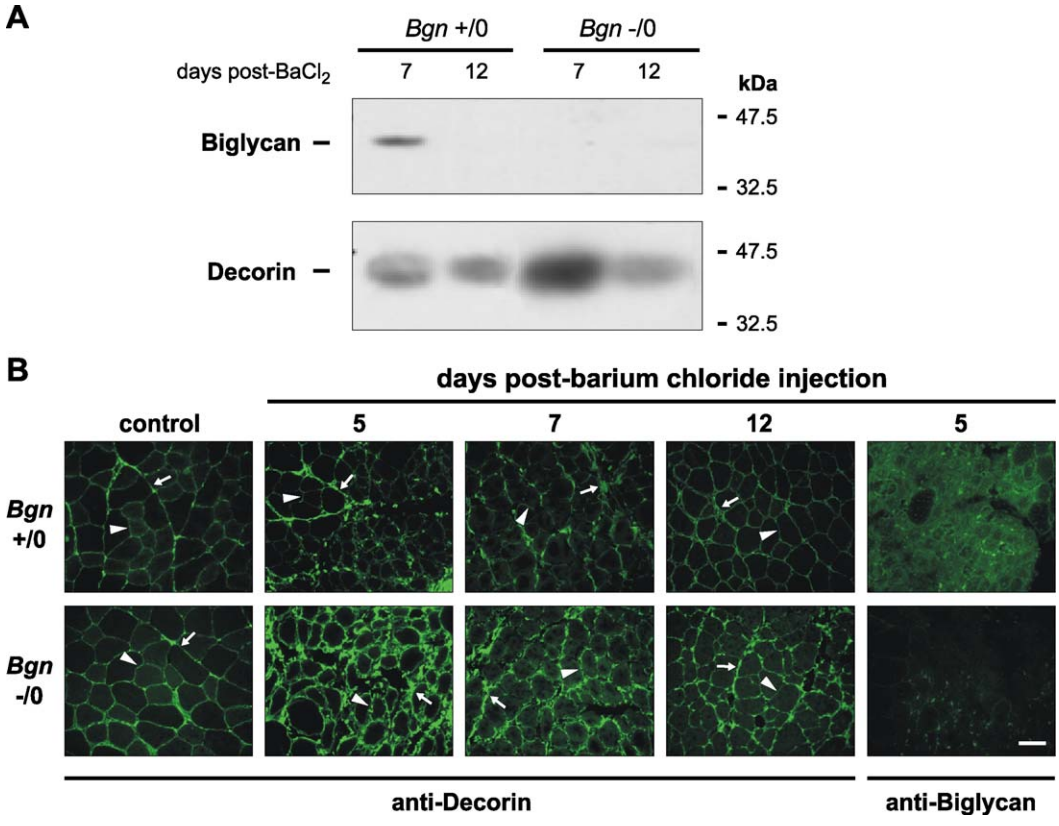

Fig. 7. Decorin is increased in *Bgn*−/0 mice during skeletal muscle regeneration. (A) Western blots of chondroitinase-treated samples from DEAE-enriched extracts from regenerating muscle of wild-type (*Bgn*+/0) and biglycan-null (*Bgn*−/0) mice. In *Bgn*+/0 muscle, an up-regulation of biglycan but not of decorin is observed on the seventh day after barium chloride injection. In the absence of biglycan, decorin up-regulation in regenerating muscle is observed on the same day. Molecular mass standards are shown on the right. (B) By indirect immunofluorescence studies, an increase of decorin in the endomysium of regenerating skeletal muscle is observed on the fifth and seventh days after barium chloride injection. As a control, biglycan immunodetection is shown in the last column. Some degree of faint unspecific binding is detected in *Bgn*−/0 sections. The endomysium and regenerating myotubes basement membranes are indicated by an arrowhead; the perimisium and interstitial tissue during regeneration are indicated by an arrow. The scale bar indicates 50 μm.

heavy chain in biglycan-null muscle, as detected by semi-quantitative RT-PCR (Fig. 6C).

Decorin is up-regulated in regenerating Bgn−/0 skeletal muscle

In mice with ablated individual SLRP genes, the possibility of a partial compensation by related SLRP members has been described (Ameye and Young, 2002). We therefore studied decorin core protein content in chondroitinase ABC-treated DEAE-enriched extracts from control and regenerating muscle. In Fig. 7A, Western blot studies on samples from the seventh day after barium chloride injection and from the 12th day, that is, towards the end of the regeneration process (and when proteoglycan content is not different from basal conditions), are presented. As expected, in wild-type muscle, a transient increase of biglycan core protein is observed and decorin core protein does not present a significant variation. In contrast, an increase in decorin's core protein content was observed in biglycan-null muscle. This increase in decorin expression was confirmed by indirect immunofluorescence studies in cross sections from regenerating TA (Fig. 7B). Increased immunodetection of decorin core protein was observed in *Bgn*−/0 muscle from the fifth and seventh day after barium chloride injection. Interestingly, this augmented detection was localized in the interstitial tissue but was more marked in the periphery of newly formed myotubes, the usual biglycan localization (Fig. 3). These results show that in biglycan-deficient skeletal muscle, there is a transient increase in the amount of decorin during regeneration.

Taken together, the results from the studies in biglycan-null mice show that biglycan expression influences fiber maturation and embryonic myosin expression during skeletal muscle regeneration but is dispensable for the expression of critical regulators as MyoD or myogenin and for the morphological completion of the process. Moreover, skeletal muscle regeneration in the absence of biglycan goes through with an unexpected decorin up-regulation, which makes it interesting to speculate a possible redundant function of these SLRPs in skeletal muscle regeneration.

Discussion

Decorin and biglycan are the only proteoglycan members of the class I of SLRP family, as the third member of this family, the related protein asporin, lacks the dipeptide sequence required for glycosaminoglycan attachment (Henry et al., 2001; Lorenzo et al., 2001). They contain 10 leucine-rich repeats and present a high degree of homology, having probably originated by gene duplication events during evolution. They carry one and two CS or DS chains, respectively, and the attachment of CS versus DS chains is variable and tissue specific (Ameye and Young, 2002; Hocking et al., 1998). Decorin, biglycan, and some other SLRPs are multifunctional proteins that bind to different types of collagen, modulating collagen fibrillogenesis (reviewed in Iozzo, 1998). They also bind the three isoforms of TGF-β through at least two sites, of high and low affinity, with K_d values of 1–20 and 20–200 nM, respectively (Hildebrand et al., 1994). Some degree of controversy exists on the biological activity of the proteoglycan/TGF-β complex, mostly based in the case of decorin, which has been studied more extensively. In models of fibrotic diseases, decorin application improved the course of the disease (Border et al., 1992; Isaka et al., 1996; Kolb et al., 2001), and no effect was observed after biglycan administration (Kolb et al., 2001). Decorin overexpression also inhibited TGF-β growth response in CHO cells and arterial smooth muscle cells (Fischer et al., 2001; Yamaguchi et al., 1990) but an opposite effect has been observed in other systems (Riquelme et al., 2001; Takeuchi et al., 1994). In the particular case of skeletal muscle, inhibition of decorin expression by the stable transfection of an antisense sequence encoding vector in C2C12 myoblasts produced an accelerated differentiation as consequence of a reduced sensitivity to TGF-β signaling (Riquelme et al., 2001). Similarly, a decreased growth response to TGF-β was recently described in bone marrow stromal cells from *Bgn*−/0 mice (Chen et al., 2002). These different observations may be explained in part as an effect of localization, with the proteoglycan sequestering TGF-β when bound to the ECM and favoring its interaction with its transducing receptors when associated to the cell surface. In general terms, decorin and biglycan have unique and divergent distribution patterns, with decorin being preferentially associated with collagen-rich connective tissues and biglycan localized at the cell surface of certain specialized cell types. However, as shown in Fig. 3, in skeletal muscle, decorin is also localized in the proximity of the cell surface, as is evident from its immunostaining signal at the basement membrane of myofibers in basal adult muscle and during regeneration. Specific binding of biglycan and decorin to separate cell surface molecules has been described. Biglycan interacts through its CS chains with α-dystroglycan (Bowe et al., 2000), and decorin interacts through its core protein with the epidermal growth factor receptor (Santra et al., 2002). Decorin can exert direct signaling effects on growth and differentiation processes, up-regulating $p21^{WAF1/CIP1}$ (De Luca et al., 1996; Santra et al., 1997), and an effect for biglycan on different signaling pathways is also suspected (Kresse and Schonherr, 2001).

In this context, the observation of an increase in CS/DS proteoglycan expression during skeletal muscle regeneration that is mainly dependent on biglycan up-regulation, normally of low expression in adult skeletal muscle, is of particular interest (Figs. 1–3). Increased biglycan expression is also detected in growing skeletal muscle after birth and in proliferating C2C12 myoblasts, being slowly down-regulated with skeletal muscle differentiation (Fig. 4). Considering the role that has been recently recognized for proteoglycans in the control of cell growth and differenti-

ation (as described above), this expression pattern is suggestive of the possible participation of biglycan in the myogenic process. Studies of skeletal muscle regeneration in biglycan-null mice show differences in the expression of differentiation markers and in the enlargement of regenerated myofibers during their maturation (Fig. 6) but do not support a more definite role, as these mice maintain a relatively normal capacity to form and regenerate skeletal muscle at the beginning of adult life (Fig. 5). However, these differences in the regenerative process and the concomitant transient up-regulation of the related proteoglycan decorin (Fig. 7) suggest the participation of biglycan in the regulation of skeletal muscle regeneration and the possible appearance of alternate regulatory mechanisms for the maintenance of the tissue response.

Skeletal muscle regeneration is a complex process that is not yet completely understood. Following skeletal muscle injury, several signals are released from degenerating myofibers, infiltrating inflammatory cells and remodeling ECM that can affect reparative myogenesis. Between days 3 and 5 after toxic muscle injury, a morphological shift from a proliferative phase of muscle precursor cells to a phase of muscle differentiation occurs (Yan et al., 2003). Biglycan up-regulation is observed during the early phase of skeletal muscle differentiation and is detected, accordingly, in the periphery of newly formed myotubes. In vitro studies of the C2C12 cell line show that its expression is high in myoblasts and then decreases with differentiation. C2C12 myoblasts in growth conditions proliferate and express MyoD and myf-5, properties that make them resemble activated satellite cells. Thus, the in vivo and in vitro data on biglycan expression in skeletal muscle, taken together, suggest it is expressed at low levels by mature myofibers, but after injury is probably up-regulated, both in activated muscle precursor cells and growing myotubes, to progressively decrease again with maturation of the fibers. Considering the possible role of biglycan as a modulator of TGF-β signaling, it is interesting to note that this pattern of expression bears some similarities to the described expression of TGF-β2 during muscle regeneration, which was found increased in fusing satellite cells and newly formed myotubes (McLennan and Koishi, 1997). Biglycan, or decorin in its absence, may have a role in the fine-tuning of TGF-β2 signaling in nascent myotubes.

Biglycan expression, however, is not necessary for skeletal muscle regeneration, as is shown by the studies in *Bgn*−/0 mice. The concomitant up-regulation of decorin in regenerating biglycan-deficient muscle, however, precludes us from a full appreciation of the respective roles of the SLRPs in the regulation of this process. Despite the fact that decorin and biglycan bear distinct functions, as is evident by the different phenotypes of each knockout mice (Danielson et al., 1997; Xu et al., 1998), an important degree of redundancy has been revealed by the study of double-deficient mice (Corsi et al., 2002). The phenotype of the double knockout is severe: they cannot breed and the number of double-deficient animals obtained for heterozygous breeders is much lower than the expected Mendelian frequencies. When analyzed, these mice show a bone phenotype that is much more severe than biglycan single-deficient mice and skin fragility that is reminiscent of the decorin single deficient phenotype. The difficulty in the breeding of these double mutant mice makes it extremely arduous to obtain sufficient number of animals to perform studies to follow the regeneration process.

A possible redundancy in the functions of biglycan and decorin during skeletal muscle regeneration, however, would not be sufficient to maintain completely the features of the process observed in wild-type mice. The observed delay in regenerated fiber growth probably reflects differences in events later than myogenin expression and cell fusion. Interestingly, this delay in fiber enlargement resembles the phenotype described for skeletal muscle regeneration in tensin-null mice (Ishii and Lo, 2001), an intracellular sarcolemma protein that is recruited to focal adhesions, where it binds to actin filaments and interacts with phosphotyrosine-containing proteins, and that is also localized in some NMJs. Biglycan, and not decorin, participates in the dystrophin-associated protein complex, and *Bgn*−/0 mice present a mild myopathy with signs of membrane disruption and necrosis in a subgroup of fibers (Bowe et al., 2000; Rafii et al., 2000). A role for dystroglycan and the correct assembly of the dystrophin-associated protein complex for successful skeletal muscle regeneration have been recently proposed (Cohn et al., 2002). Although the temporal expression patterns of biglycan and α-dystroglycan differ during muscle regeneration (data not shown), both molecules are present during this time, and thus it is possible that a mechanism involving their interaction may come into play during regeneration.

Forming muscle masses express a succession of myosin heavy chain isoforms during embryogenesis, from embryonic to adult, through neonatal and perinatal isoforms (reviewed in Weiss and Leinwand, 1996). The functional importance of these transitions and of the developmental expression of myosin heavy chain isoforms is not well understood, as neither are the complete mechanisms regulating their expression. However, the observed differences in the transient expression of embryonic myosin may reflect some mechanism of control by ECM signals on the myogenic programs elicited during regeneration independently of MRF expression.

In summary, class I SLRP expression during skeletal muscle regeneration in mouse TA was studied. A dramatic, transient up-regulation of biglycan was observed while decorin, the most abundant proteoglycan in normal adult skeletal muscle, only exhibits minor variations during the process. These proteoglycans are regulated in opposite directions during in vitro myogenesis, with decorin expression highest in myotubes and biglycan's most prominent in proliferating myoblasts. Studies of skeletal muscle regeneration in biglycan-deficient mice suggest that although biglycan expression is dispensable for the overall occurrence of the regenerative process, its absence affects regenerated fiber

growth and the transient expression of nonadult myosin isoforms. Moreover, transient decorin up-regulation in the absence of biglycan appears as a possible compensatory mechanism and allows us to speculate that SLRPs play a role in skeletal muscle regeneration.

Acknowledgments

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