Heparan sulfate proteoglycans are increased during skeletal muscle regeneration: requirement of syndecan-3 for successful fiber formation

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Accepted 9 August 2003
Journal of Cell Science 117, 73-84 Published by The Company of Biologists 2004
doi:10.1242/jcs.00828

Summary

Skeletal muscle regeneration is a highly complex and regulated process that involves muscle precursor proliferation and differentiation and probably requires the participation of heparin binding growth factors such as FGFs, HGF and TGFβ. Heparan sulfate proteoglycans, key components of cell-surfaces and ECM, modulate growth factor activities and influence cell growth and differentiation. Their expression in forming muscle masses during development and in cell culture, suggest their participation in the regulation of myogenesis. In the present study, heparan sulfate proteoglycan expression in skeletal muscle regeneration induced by barium chloride injection was evaluated. Expression of muscle differentiation markers and neuromuscular junction (NMJ) components was characterized. Immunobots with anti-Δ-heparan sulfate antibody showed that four major species – perlecan, glypican, syndecan-3 and syndecan-4 – were transiently up-regulated. The first three were detected at the surface or basement membranes of newly formed myotubes by specific indirect immunofluorescence. Syndecan-3, a satellite cell marker, showed the earliest and most significant increase. Experiments involving myoblast grafting into regenerating muscle showed that C2C12 cell clones, with inhibited syndecan-3 expression resulting from antisense transfection, presented a normal proliferation rate but an impaired capacity to fuse and form skeletal muscle fibers. These data constitute the first in vivo evidence suggesting the requirement of a specific heparan sulfate proteoglycan for successful skeletal muscle regeneration.

Key words: Proteoglycans, Myogenesis, Heparan sulfate, Perlecan, Syndecan-3, Skeletal muscle regeneration

Introduction

Post-natal skeletal muscle formation occurs in mammals during the body growth period and also throughout the rest of the life as a damage-induced regenerative response. Satellite cells, mononuclear cells located at the periphery of mature myofibers and beneath its basal lamina, constitute the main source of muscle precursor cells after birth. Satellite cells normally rest in a quiescent state, but signals released after skeletal muscle injury induce their activation, proliferation and differentiation into mature myofibers in a process that resembles, in some aspects, embryonic myogenesis (Buckingham, 1992). Skeletal muscle differentiation is regulated by the expression of specific combinations of muscle regulatory transcription factors by precursor cells. Among them, a family of basic helix-loop-helix transcription factors called muscle regulatory factors (MRFs) is crucial for muscle differentiation (Seale and Rudnicki, 2000).

A complete understanding of the molecular mechanisms that regulate satellite cell activation and muscle differentiation has remained elusive. In vivo, the onset and progression of skeletal muscle regeneration are controlled by a complex set of interactions between muscle precursor cells and their environment. The presence of extracellular matrix (ECM), as has been demonstrated by in vitro studies (Melo et al., 1996; Osses and Brandan, 2001), is essential for normal myogenesis, both through direct interactions of ECM molecules with plasma membrane receptors and through the modulation of growth factor activities. Growth factors are crucial regulators of skeletal muscle differentiation. Among them, some heparin-binding growth factors such as members of the fibroblast growth factor (FGF) family, hepatocyte growth factor/scatter factor (HGF) and transforming growth factor β (TGFβ) have been implicated in satellite cell activation, migration, proliferation and as regulators of skeletal muscle differentiation (Hawke and Garry, 2001).

Heparan sulfate proteoglycans, which are present in almost all ECMs and on most eukaryotic cell surfaces, influence biological processes by interacting with a large number of physiologically important macromolecules. Cell-surface heparan sulfate proteoglycans can sequester soluble ligands thereby increasing their local concentration, and they can also modulate ligand-receptor encounters (Bernfield et al., 1999). HGF and FGF signaling is markedly enhanced by heparan sulfate in order to transduce an intracellular signal through its receptors (Rapraeger et al., 1991; Yayon et al., 1991). Heparan sulfate interactions with other growth factors such as TGFβ isoforms, do not affect their binding to their membrane receptors, but protect the growth factors from inactivation (Lyon et al., 1997).
The role of heparan sulfate proteoglycans in skeletal muscle physiology and during skeletal muscle differentiation has been previously evaluated. In mature skeletal muscle tissue, heparan sulfate proteoglycans also act as co-receptors for the asymmetric form of acetyl cholinesterase (AcChE) at the NMJ (Brandan et al., 1985; Peng et al., 1999). Several studies have been performed with C2C12 cells, a satellite cell line derived from regenerating adult mouse skeletal muscle that undergoes in vitro terminal myogenic differentiation after serum removal from the culture medium (Blau et al., 1985; Yaffe and Saxel, 1977). Inhibition of proteoglycan sulfation by chlorate treatment of C2C12 cultures (Meló et al., 1996; Osses and Brandan, 2001) or intact myofibers (Cornelison et al., 2001) affects the proper progression of the myogenic program in vitro. Moreover, in vivo administration of synthetic polymers that imitate heparan sulfate glycaminoglycans accelerates both the regeneration and re-innervation of skeletal muscle (Desgranges et al., 1999).

Heparan sulfate proteoglycans are diverse and can be transmembrane, as syndecans, bound by a glycosyl phosphatidylinositol (GPI) linkage to plasma membrane lipid, as glypicans, or secreted into the basement membrane, as perlecan and agrin (Perrimon and Bernfield, 2000). Glypicans and syndecans represent the two main cell-surface heparan sulfate proteoglycans, and, in mammals, four separate syndecan and six separate glypican genes have been identified. We have shown that during C2C12 myogenensis the expression of perlecans, syndecan-1 and syndecan-3 is down-regulated (Fuentealba et al., 1999; Larraín et al., 1997a; Larraín et al., 1997b), whereas the expression of glypican is up-regulated (Brandan et al., 1996). This differential expression may reflect differing functions or macromolecular specificity during the process. Syndecans have been reported to modulate FGF2 activity in vitro myogenensis (Fuentealba et al., 1999; Larraín et al., 1998; Rapaeger et al., 1991) and to participate in the modulation of growth factor activities, cell-cell adhesion and cell-matrix adhesion in developmental and adult wound repair processes (reviewed by Bernfield et al., 1999; Carey, 1997; Rapaeger, 2001). It has been reported recently that syndecan-3 and syndecan-4 are expressed by developing myocytes during embryonic limb skeletal muscle formation (Cornelison et al., 2001; Olguín and Brandan, 2001), and that the expression continues in adult muscle tissue but is restricted to satellite cells (Cornelison et al., 2001). Glypican-1 colocalizes with laminin in adult rat skeletal muscle (Brandan et al., 1996; Campos et al., 1993). Perlecan is an intrinsic constituent of skeletal muscle basal membrane that participates in the binding of α-dystroglycan (Peng et al., 1998) and ECM molecules such as laminin (Yamagata et al., 1993), and it is present at NMJs where it is necessary for AchE localization (Arikawa-Hirasawa et al., 2002). In this study, heparan sulfate proteoglycan expression was studied in a model of skeletal muscle regeneration induced by barium chloride injection, and the functional participation of syndecan-3 was evaluated by myoblast transfer experiments into regenerating muscle with antisense clones.

**Materials and Methods**

**Animals and experimental muscle injury**

C57BL/10 ScSn male mice 8- to 12-weeks old were studied. The animals were kept at room temperature with a 24 hour night-day cycle and fed with pellets and water ad libitum. Injury of normal muscles was performed by barium-chloride injection (Caldwell et al., 1990) in mice under ketamine/xylazine anesthesia [80/12 mg/kg body weight (b.w.), intraperitoneally (i.p.)]. Briefly, 69 μl of an aqueous 1.2% mass/volume (m/w) BaCl2 solution were 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), TAs were dissected and removed under anesthesia, and the animals were sacrificed. Tissues were rapidly frozen and stored at −80°C until processing. All protocols were conducted in strict accordance and with the formal approval of the Animal Ethics Committee of the P. Universidad Católica de Chile.

**Western blot analysis of proteoglycans**

Protein extracts were prepared with a protocol slightly modified from that previously described by Brandan and Inestrosa (Brandan and Inestrosa, 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 hours. 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-Sephadex anion-exchange chromatography, equilibrated and washed with the same urea buffer and eluted with 1.0 M NaCl. The extracts were finally equilibrated with heparitinase buffer (100 mM Tris-HCL, 50 mM NaCl, pH 7.5). Protein content was determined as described previously (Riquelme et al., 2001).

Appropriate samples, containing equivalent amounts of proteins, were digested with heparitinase or chondroitinase ABC (Seikagaku, Tokio, Japan) 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-A-heparan sulfate monoclonal antibody 3G10 (Seikagaku, Tokio, Japan) or anti-perlecan polyclonal antibody as described previously (Olguín and Brandán, 2001), and visualized by enhanced chemiluminescence (Pierce, IL, USA). In parallel, blots of chondroitinase ABC-treated samples were detected with anti-decorin polyclonal antibodies [LF-113; kindly donated by Dr L. Fisher, NIDR, NIH, Bethesda, MD (Fisher et al., 1995)]. Corresponding samples from the total homogenate in urea buffer were also analyzed by immunoblot as described above with monoclonal antibodies against myogenin (Olguín and Brandán, 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, USA].

**RNA isolation and northern blot analysis**

Total RNA was isolated from skeletal muscles as described previously (Brandan et al., 1992; Chomczynski and Sacchi, 1987). Twenty micrograms RNA samples were electrophoresed in 1.2% agarose/formaldehyde gels and transferred to Nytran membranes (Schleicher & Shuell, Dassel, Germany) and hybridized with random primed [32P]dCTP-labeled cDNA probes for syndecan-3, glypican-1 and perlecan in hybridization buffer at 65°C as described previously (Brandan et al., 1996; Fuentealba et al., 1999; Larraín et al., 1997a). 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 GAPDH or 18S 32P-labeled probes kindly donated by Dr J. Chianale (Department of Gastroenterology, Faculty of Medicine, P. Universidad Católica de Chile, Chile).

**Immunohistochemistry**

Cryostat sections (6 μm) of control and treated TAs at different...
times after BaCl2 injection were fixed for 20 minutes in 3% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, blocked with 8% BSA in PBS and incubated at 4°C overnight with primary antibodies as described previously for syndecan-3 at a 1:50 dilution (Fuentetabla et al., 1999), glycican (1:150) (Brandan et al., 1996) and perlecan (1:1500) (Larrain et al., 1997a). Anti-Δ-heparan sulfate antibody was used in a 1:500 dilution, and anti-α-dystroglycan (Upstate Biotechnology, NY, USA) and anti-embryonic myosin were used at 1:100 dilutions. Sections were then washed and incubated with either anti-rabbit-FITC, anti-mouse-TRITC or anti-mouse alkaline phosphatase-conjugated secondary antibodies (all diluted 1:100; Pierce, IL, USA) for 1 hour at room temperature. For nuclear staining, sections were incubated with 1 μg/ml Hoechst 33258 in PBS for 10 minutes. After rinsing, the sections were mounted with fluorescent mounting medium (Dako Corporation, CA, USA) under glass coverslips and viewed and photographed with a Nikon Eclipse microscope equipped for epifluorescence. Alkaline phosphatase activity was detected with NBT/BCIP (Sigma Chemical Co, MO, USA) and developed for 2-5 minutes at room temperature according to the manufacturer instructions.

For staining with anti-Δ-heparan sulfate antibody, the sections were pre-treated with 2.5 mU heparitinase for 2 hours at 37°C (Friedl et al., 1997). For detection of nicotinic acetyl choline receptors (AChR), sections were first fixed for 5 minutes in 1% paraformaldehyde, rinsed and incubated in rhodamine-conjugated α-bungarotoxin (Molecular Probes, OR, USA) for 1 hour (Bowe et al., 2000). Sections were then washed, fixed and processed as described above.

Constitutive β-galactosidase expressing myoblasts

The mouse skeletal muscle cell line C2C12 (ATCC, VA, USA) was grown in Dulbecco’s Modified Eagle’s medium (DMEM) containing 10% fetal calf serum and 0.5% chick embryo extract (Larrain et al., 1998; Larrain et al., 1997b). For the antisense stable transfectants, 400 μg/ml G418 was added to the growth medium. C2C12 myoblasts with an inhibited syndecan-3 expression [clones AN-10 and AN-8 (Fuentetabla et al., 1999)] and wild-type C2C12 myoblasts were infected with a replication-defective retroviral vector containing the lacZ sequence (kindly provided by Dr María Rosa Bono, MIFAB and Immunology Laboratory, Universidad de Chile, Chile). This vector can only replicate in the NB5 mouse fibroblast cell line. Briefly, myoblasts were grown on six-well culture plates until reaching semi-confluence. Cells were incubated for 2 hours (37°C, 8% CO2 and agitation) with 0.5 ml per well of NB5 conditioned medium from overnight culture, plus 2 μl Polibrene (4 mg/ml; Sigma Chemical Co, MO, USA). After incubation, 2 ml of myoblast growth medium were added and the whole culture medium was replaced by fresh growth medium the day after.

In all cases, β-galactosidase-positive clones were isolated after clonal dilution by testing both the β-galactosidase activity and the persistence of the parental phenotype by morphological and specific molecular skeletal muscle markers, and will be herein referred to as C2C12I, AN-10I and AN-8I.

Myoblast transfer experiments

Once stable clones of C2C12I, AN-10I and AN-8I cells were obtained, cells were multiplied to obtain large populations for injection. The cells were removed with 0.05% trypsin-0.53 mM EDTA (Life Technologies, MA) and collected by centrifugation at 1,300 g for 10 minutes. 10⁶ cells from each group were resuspended in 20 μl of HBSS and separately injected with a 702 Hamilton syringe into mice TAs 24 hours after barium chloride injection. Myoblasts were injected slowly when the needle was being withdrawn from the muscle, after inserting it longitudinally, so they were delivered along the whole length of the TA. Three to seven muscles were injected with each of the clones for every different experimental time point, in each experimental series. Muscles were dissected out 1, 3, 5 and 7 days later and frozen in isopentane chilled in liquid nitrogen. Cryostat sections (16 μm) were obtained at the proximal and middle thirds of each muscle.

Histochemical detection of β-galactosidase activity

lacZ reporter gene-derived β-gal activity was detected using the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). X-gal was used at a final concentration of 1 mg/ml in PBS containing 25 mM potassium ferrocyanide, 25 mM potassium ferricyanide, 2 mM MgCl2, 0.01% sodium deoxycholate and 0.02% Nonidet P-40. Sections were fixed in 3.7% formaldehyde in PBS, pH 7.4 containing 2 mM MgCl2 and 1.25 mM EGTA for 10 minutes and incubated in X-gal solution overnight at 37°C. Sections were post-fixed and skeletal muscle fibers detected by immunohistochemistry against α-dystroglycan or embryonic myosin as described above.

β-galactosidase-positive cells and myotubes positive for both β-galactosidase and α-dystroglycan or embryonic myosin were counted in 3-12 sections from the different zones of each muscle. Statistical difference among the fraction of β-galactosidase-positive fibers from the different clones was evaluated by unpaired Student’s t-tests. To assess the diameter of β-galactosidase-positive fibers, measurements were made from at least five photomicrographs, taken with a 40x objective lens, for each of the counted sections from day 7 after transplantation. The minimum diameter of all β-galactosidase-positive fibers was measured by an independent observer after importing the images into Adobe Photoshop 5.0 software.

Cell proliferation studies

Proliferation of the transplanted cells was evaluated by 5-bromo-2’-deoxyuridine (BrdU) labeling with the In Situ Cell Proliferation Kit, FLUOS (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, animals were injected 20 hours after cell transplantation with 100 μmol/kg b.w. BrdU i.p., and 4 hours later the mice were sacrificed and TA were harvested and processed as described above. After β-galactosidase detection, BrdU incorporation was determined according to the manufacturer instructions. The preparations were finally incubated with anti-BrdU FLUOS fluorescein-conjugated F(ab')2 antibody fragments for 45 minutes at 37°C, rinsed and mounted. A pre-incubation with 20 μg/ml sulphorhodamin 101 (Molecular Probes, OR, USA) was used to reduce non-specific fluorescence. Four muscles from each group were studied. Two to four sections from each muscle were completely scanned and images were acquired with bright-field and fluorescence microscopy. Cells positive for β-galactosidase and for BrdU and β-galactosidase were counted after merging the images with Adobe Photoshop 5.0 software. In vitro proliferation was estimated after plating the same number of cells (5,000 cells/cm²) of each clone and then removing them with 0.05% trypsin-0.53 mM EDTA at different time points. After collection of the cells by centrifugation at 1,300 g, they were resuspended in the same volume of growth medium and a sample was counted in a Neubauer chamber.

Results

Morphological and molecular characterization of the muscle necrotic-regeneration model

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). As can be observed in Fig. 1A, in the first 3 days after the injection abundant mononuclear cells, predominantly polymorphonuclear leukocytes and macrophages, are present in the enlarged intercellular space separating necrotic fibers. Spindle-shaped mononuclear cells

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that may represent activated satellite cells are apparent by the third day after the injection, and the first regenerated myotubes \( \text{\textendash} \) slim and with the nucleus in a central position \( \text{\textendash} \) appear by day 4 or 5. By the seventh day, most of the fibers have a central nucleus but their diameters are still highly heterogeneous. Myotubes continue maturing and by day 15 after the injection the tissue has recovered most of its normal morphology, with persistence of central nuclei and a slight increase in the interstitial spaces. The process of regeneration after barium chloride-induced damage, as evaluated by the proportion of fibers with centrally located nuclei, is homogeneous along the whole TA length and involves over 90\% of the fibers (data not shown).

Basement membranes are initially preserved after myofiber degeneration and are supposed to serve a scaffolding function for muscle precursor cells and to conserve positional information of the original NMJ that facilitates re-innervation (Sanes et al., 1978). Its components are replaced at specific times in the new basement membranes synthesized by the regenerating fibers (Gulati et al., 1983). \( \alpha \)-Dystroglycan is an extracellular glycoprotein that is part of the dystrophin-associated protein complex in the sarcolemma of skeletal muscle fibers and has been reported to participate, together with heparan sulfate proteoglycans, in asymmetric AChE clustering at the synaptic basal lamina. \( \alpha \)-Dystroglycan and AChR localization were followed during skeletal muscle regeneration by double-labeling with immunofluorescence and binding of \( \alpha \)-bungarotoxin conjugated with rhodamine, respectively (Fig. 1A). \( \alpha \)-Dystroglycan is present at the surface of the myofibers and concentrates at NMJ as is observed in control TA and in regenerated muscle 15 days after barium chloride injection. As has been previously described (Kaariainen et al., 2000; Vater et al., 1995), after damage, \( \alpha \)-dystroglycan staining can be observed firstly associated with residual basal lamina (day 3) and then at the surface of regenerating myotubes (days 5 and 7). \( \alpha \)-Bungarotoxin labeling is localized in NMJ in control sections. Three days after barium chloride injection, AChR clusters are still detected (although in a very reduced numbers, data not shown), probably associated with remnants of degenerating fibers. By the fifth day, \( \alpha \)-bungarotoxin labels the perimeter of some of the regenerating myotubes, but in others patches are observed. After 15 days, AChR clusters, albeit still reduced in number, are morphologically similar to those observed in control muscle.

During barium chloride-induced regeneration the myogenic process may be followed in vivo by the expression of markers such as MRF myogenin or the transient expression of the
embryonic isoform of skeletal muscle myosin. These markers are absent from normal adult muscle, but, after injury, transient up-regulation of myogenin was detected from days 3 to 5 after barium chloride injection, by western blot analysis, while embryonic myosin appeared as a later differentiation marker and was only detected between days 5 and 7 (Fig. 1B).

Thus, after barium chloride injection the process of skeletal muscle regeneration occurs during a precise time window and can be followed by its morphological features and the expression of molecular markers of differentiation.

Changes in heparan sulfate proteoglycans expression in skeletal muscle regeneration

Expression of heparan sulfate proteoglycans during skeletal muscle regeneration was first studied by immunofluorescence with the monoclonal antibody anti-Δ-heparan sulfate 3G10 (Fig. 2) that recognizes a neo-epitope generated in proteoglycan core proteins that are substituted with heparan sulfate after glycosaminoglycan digestion with the enzyme heparitinase from Flocobacterium heparinum (Steinfeld et al., 1996). In heparitinase-treated sections, anti-Δ-heparan sulfate immunodetection presented a similar staining pattern to that described above for α-dystroglycan: in normal TA, staining of skeletal muscle fibers, surface and basement membrane was observed (Fig. 2A), and during regeneration an increased signal from heparan sulfate proteoglycan core proteins was detected associated with recent regenerating myotubes (day 4 and 5 after the injection are shown in Fig. 2B,C). Anti-heparan sulfate proteoglycans staining returned to its basal pattern after the regeneration process was completed (day 15, Fig. 2D). Heparan sulfate proteoglycans were also detected during the whole regeneration process as small ring-like structures around myofibers that were probably blood vessels. However, no staining associated with infiltrating cells was observed.

Detection of anti-Δ-heparan sulfate 3G10 epitope by western blot analysis allows the putative identification of various species in heparitinase-treated skeletal muscle samples, that may correspond to different heparan sulfate proteoglycan core proteins. As shown in Fig. 2G, a transient increase of several heparan sulfate proteoglycans was observed during skeletal muscle regeneration, especially on days 5 and 7 after barium chloride injection. Up-regulated core proteins migrated as (i) a triplet of 64-66 kDa, (ii) a band of 120 kDa and (iii) several high molecular mass bands, with three main bands of 300, 210 and 180 kDa. According to the electrophoretical migration pattern these proteoglycans were probably glypican, syndecan-3 and perlecan, respectively, as has been previously characterized in C2C12 myoblasts extracts (Fig. 2H) based on their specific biochemical properties (Fuentealba et al., 1999; Larrain et al., 1997a; Larrain et al., 1997b). Interestingly, the 120 kDa band, corresponding to syndecan-3, was already increased by the third day after the injection. With longer exposure times, another 35 kDa band, which was probably corresponded to syndecan-4, appeared 5 and 7 days after barium chloride injection (Fig. 2G, lower inset). Up-regulation of these heparan sulfate proteoglycans shows a certain degree
Specific heparan sulfate proteoglycans expression in skeletal muscle regeneration: early up-regulation of syndecan-3

In order to advance the identification of the specific heparan sulfate proteoglycans that are up-regulated during skeletal muscle regeneration, the localization of the specific above mentioned proteoglycans in the tissue was studied by indirect immunofluorescence with polyclonal antibodies against the corresponding species (Fig. 3).

In control muscle, syndecan-3, glypican and perlecan were detected at the sarcolemma or endomisium in the periphery of myofibers and were absent from the perimisium (Fig. 3A,E and I, respectively). Syndecan-3 reactivity was weak and there was also some cytoplasmic staining for glypican (Fig. 3E). After injury, an increased staining for these three proteoglycans was apparent from day 4, preferentially surrounding the newly formed myotubes rather than with remnant myofibers in the same sections (Fig. 3B,F,J). This pattern of expression was maintained and more evident at day 5, where staining was absent from interstitial strips with abundant cellular infiltration (Fig. 3C,G,K). By the 15th day, the expression of syndecan-3, glypican and perlecan returned to basal levels (Fig. 3D,H,L).

Syndecan-3 expression in myoblast plasma membrane is important for successful fiber formation in myoblast transplant studies

Among the heparan sulfate proteoglycans that increase during skeletal muscle regeneration, syndecan-3 stands out as a possible regulator of the process for its low expression in normal muscle, its expression by quiescent and activated satellite cells (Cornelison et al., 2001), its early increase in regeneration (Figs 3 and 4) and for its localization in forming limb skeletal muscle masses during embryonic development (Olguin and Brandan, 2001). We have previously described two sub-clones of the skeletal muscle cell line C2C12 in which, by the stable transfection of an antisense-sequence encoding plasmid, syndecan-3 expression is inhibited (Fuentealba et al., 1999). In view of the lack of studies on the role of heparan sulfate proteoglycans in skeletal muscle regeneration in vivo, the specific role of syndecan-3 in this process was studied by
myoblast transplantation into regenerating muscle (Hagiwara et al., 1995; Lee et al., 2000; Morgan et al., 1992).

Subclones of C2C12, AN-10 and AN-8 expressing the lacZ gene, which fully differentiate like their parental clones, were selected. No differences were observed in myoblast proliferation among the subclones in normal growing conditions (Fig. 5F), or in their fusion index 5 days after the myoblasts were induced to differentiate in vitro (data not shown). For in vivo studies, 24 hours after the injury induced by the barium chloride injection, 10⁶ of AN-10i or C2C12i cells were injected intramuscularly along the whole length of the TAs of separate mice. After different time periods, the animals were killed and muscles were harvested for histological and immunohistochemical analysis. Proliferation of C2C12i and AN-10i myoblasts was assessed in vivo by BrdU labeling 24 hours after transplantation (Fig. 5A-D). No difference was observed in the fraction of β-galactosidase-positive cells that incorporated BrdU from either group at this time point (32.9±4.9% C2C12i vs. 29.0±3.05% AN-10i myoblasts, P=0.23, unpaired Student’s t-test), suggesting that inhibition of syndecan-3 expression does not impair the ability of myoblasts to proliferate in the damaged muscle tissue (Fig. 5E).

In vivo differentiation of the grafted cells was evaluated 3, 5 and 7 days after transplantation. At all these time points, multiple β-galactosidase-positive cells were detected in transverse sections from different zones of the muscles transplanted with either C2C12i or AN-10i myoblasts. Positive nuclei could be detected either centrally in regenerated myotubes or in mononuclear cells from the interstitial space (Fig. 6A). Differentiating cells or myotubes were detected by anti-embryonic myosin (preferred for day 3) or anti-α-dystroglycan immunostaining (on days 5 and 7). No qualitative differences were detected in the temporal expression of these markers, and quantification of embryonic myosin-positive or α-dystroglycan-positive grafted cells or fibers on serial sections from day 5 yielded comparable results (Fig. 7). No differences were found in the number of β-galactosidase-positive cells expressing these markers in C2C12i and AN-10i myoblasts by day 3, when only about 2% of the cells expressed embryonic myosin in both groups (Fig. 6B). However, from the fifth day after transplantation a significantly smaller fraction of AN-10i than C2C12i cells could be detected in α-dystroglycan-positive regenerating myotubes (18.6±0.9% AN-10i vs. 27.4±2.3% C2C12i, P<0.01, Student’s t-test; Fig. 6C). By day 7, 77±4.2% of C2C12i nuclei were forming new myofibers, while only 42±5.3% of AN-10i were detected within fibers (P<0.001, Student’s t-test; Fig. 6D). There was no difference in the diameter of the β-galactosidase-positive fibers formed after the injection of either C2C12i or AN-10i myoblasts on day 7 (21.7±6.4 μm for C2C12i fibers vs. 21.8±6.9 μm for AN-10i, mean diameter±s.d.), indicating that, broadly speaking, the in vivo differentiation process was similar in both groups and was probably commanded by the native muscle precursor cells.

These results, which suggest that myoblasts with inhibited syndecan-3 expression do not readily differentiate into myofibers in vivo, were confirmed in a second series of experiments comparing the differentiation of C2C12i with AN-10i and AN-8i myoblasts 5 days after transplantation (Fig. 7). A similar reduction in the ratio of β-galactosidase-positive cells expressing α-dystroglycan or embryonic myosin was observed for both antisense clones as compared with C2C12i (in the case of α-dystroglycan-positive myotubes, the ratio was decreased by 40.6±8.8% for AN-10i cells and 57.3±22.6% for AN-8i cells, mean ± s.d.; P<0.01 in each case, Student’s t-test).
In infiltrating inflammatory cells and remodeling ECM that can affect reparative myogenesis. In the presence of multiple signals, the sequence of satellite cell activation, proliferation of muscle precursor cells, and the transition through differentiation must be tightly regulated. Heparan sulfate proteoglycans, known modulators of growth factors activities, are potentially involved in the regulation of these events. In this study, heparan sulfate proteoglycan expression was evaluated during mouse skeletal muscle regeneration induced by barium chloride injection. A transient up-regulation of the following heparan sulfate proteoglycans was observed: syndecan-3, glypican, perlecan and probably also syndecan-4. Increased expression of these proteoglycans was temporally coincident with expression of early markers of the differentiation process and was localized in newly formed myotubes. Up-regulation was earlier and more dramatic for syndecan-3, a cell surface heparan sulfate proteoglycan that has been previously implicated in myogenesis in cell culture studies and because of its pattern of expression during development (Cornelison et al., 2001; Fuentealba et al., 1999; Olguin and Brandan, 2001). We show that C2C12 cells with inhibited syndecan-3 expression were unable to fully differentiate when transplanted into regenerating skeletal muscle, constituting the first in vivo evidence of the participation of a specific proteoglycan in skeletal muscle regeneration.

Barium chloride induces necrosis and regeneration of skeletal muscle fibers, with a similar sequence of events to other widely used toxic models of injury (Hawke and Garry, 2001). Transient expression of early skeletal muscle markers of differentiation temporally circumscribe the myogenic process (Fig. 1). Active remodeling of basement membranes provides signals for a rapid and successful regeneration (Caldwell et al., 1990). In the case of an ischemic or toxic injury, new myofibers develop within the original basement membrane tubes, which permit the formation of NMJ at original synaptic sites (Sanes et al., 1978). Part of the information stored in these basement membrane specializations may depend on heparan sulfate proteoglycans such as agrin or perlecan (Gautam et al., 1996; Peng et al., 1999). In the present study, AChR clusters were observed in regenerating myotubes (Fig. 1A). Heparan sulfate proteoglycans were expressed with a different temporal pattern from the observed for NMJ components such as AChE or its collagene tail colQ (data not showed).

Heparin binding growth factors such as FGFs, HGF and TGFβ are among the principal known regulators of skeletal muscle regeneration. They may be normally present in muscle tissue and released upon injury or secreted auto- or paracrinally by the cells during the regeneration process (Clarke et al., 1993; Jennische et al., 1993; Sakuma et al., 2000; Tatsumi et al., 1999). Quiescent and activated myoblasts express the c-met HGF receptor as well as FGF receptors [especially FGFR-1 and FGFR-4 (Cornelison and Wold, 1997; Kastner et al., 2000)]. However, adult satellite cells in vitro are initially responsive to HGF, but not to FGF2 and show a delayed entry into the cell cycle (Johnson and Allen, 1993; Johnson and Allen, 1995). Growth factor responsiveness, and consequently skeletal myogenesis, may be regulated by ligand and receptor availability (Scata et al., 1999), as well as by the presence of heparan sulfate proteoglycans at the cell surfaces or at basement membranes (that may be either presenting or
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The up-regulation of different heparan sulfate proteoglycans during muscle regeneration was detected by immunoblot with 3G10 anti-D-heparan sulfate antibody (Fig. 2). Perlecan, glypican, syndecan-3 and probably syndecan-4 were the main increased heparan sulfate proteoglycan species, detected temporally and topologically with newly formed differentiating myotubes (Figs 2 and 3). A detailed localization analysis showed that in the first days, elongated cells at the periphery of damaged myofibers expressed syndecan-3 at the cell surface (Fig. 4). These cells, which express embryonic myosin, may be to nascent myotubes beginning to fuse beneath the basal lamina of morphologically intact myofibers. However, they may also belong to a subpopulation of activated satellite cells ready to differentiate after a first division round (Rantanen et al., 1995). The expression of embryonic myosin in mononuclear satellite-like cells has been described previously during skeletal muscle regeneration (Gorza et al., 1983; Sartore et al., 1982).

Among other signals, growth factors such as FGF2 or TGFβ, and some inflammatory cytokines have been reported to regulate the expression of different heparan sulfate proteoglycans (Bernfield et al., 1999; Iozzo et al., 1997; Niu et al., 1998), and may participate in their up-regulation during skeletal muscle regeneration. Differences between protein expression and mRNA levels have been previously reported for heparan sulfate proteoglycans and attributed to post-transcriptional regulation (Bernfield et al., 1999). In this study, up-regulation of both transcripts and core protein expression could only be detected for syndecan-3 (Fig. 4). For glypican-1 mRNA only a weak increase that followed a slight fall in its expression, could be observed on the same days that core protein up-regulation was detected in extracts and tissue sections. Concomitant up-regulation of other members of glypican family with similar relative molecular mass cannot be discounted.

Although in vitro evidence suggested heparan sulfate proteoglycans were involved in skeletal myogenesis (Cornelson et al., 2001; Fuentealba et al., 1999; Larrain et al., 1998), and in vivo studies suggested potential therapeutic benefits of artificial heparan sulfate mimetics for muscle regeneration (Desgranges et al., 1999), no previous in vivo data on the participation of naturally occurring heparan sulfate proteoglycans has been published. Mutant mice null for the expression of syndecan-1 or syndecan-4 do not present a pathological phenotype and the main abnormality detected so far has been defective skin repair (Bernfield et al., 1999; Echtermeyer et al., 2001; Stepp et al., 2002). Syndecan-3-deficient mice are born in the expected Mendelian ratios, have a normal lifespan and the only phenotype described to date are changes in hippocampal long-term potentiation and in spatial memory and an abnormal feeding behavior (Kaksonen et al., 2002; Reizes et al., 2001). Mice lacking perlecan show lethal defects related to the rupture of stress-sensitive basement membranes, but the only alteration that has been described in skeletal muscle is the absence of AChE at NMJs (Arikawa-Hirasawa et al., 2002; Costell et al., 1999). Interestingly, the role of other molecules in the regeneration of adult muscle was not evident in mutant mice initially. MyoD+/− and Fgf6−/− mutant mice do not show an unusual phenotype and can reach the adult stage, but their muscles regenerate with decreased efficiency compared to wild-type mice (Floss et al., 1997; Megeney et al., 1996).

In the present work, syndecan-3 participation in skeletal muscle regeneration was evaluated by myoblast transplantation studies of C2C12i myoblasts and two clones of myoblasts with...
inhibited expression of syndecan-3 (AN-10i and AN-8i) in regenerating muscle. Analysis of syndecan-3 expression in C2C12 myoblasts showed that it is lost when the cells differentiate and that anti-sense blockade of syndecan-3 expression hastens their differentiation in vitro, a phenotype that can be reversed by heparin addition (Fuentealba et al., 1999). In vivo, AN-10i cells, when injected into regenerating muscle, show a normal proliferation rate during the first days after injury (Fig. 5). C2C12 and AN-10 myoblasts also proliferate at similar rates in vitro under normal growing conditions, but syndecan-3-defective myoblasts have a decreased proliferative response to FGF2 (L. Fuentealba and E.B., unpublished results). This suggests that, in vivo, the specific contribution of FGF2 to myoblast proliferation is probably redundant with the several other mitogens that are present in the tissue.

Both antisense clones, AN-10i and AN-8i, showed a decreased capacity to form or be incorporated into new myotubes in vivo (Figs 6 and 7), suggesting at least a partial syndecan-3 requirement for a successful regeneration. We have shown previously that myoblasts with an inhibited syndecan-3 expression differentiate earlier than wild-type C2C12 myoblasts in vitro after reducing the serum content of culture medium. We think this is not the case in the transplantation experiments, as the proportion of grafted cells expressing differentiation markers such as embryonic myosin or α-dystroglycan increased progressively after transplantation of both C2C12i- and syndecan-3-deficient myoblasts, but it was lower in the antisense cells at all the time points studied. The differences observed in the total number of the transplanted cells detected in the tissue seem to be idiosyncratic for the different clones, and probably more dependent on their survival capacities and not related to their differentiation potential. However, grafting experiments using fourfold fewer wild-type myoblasts, did not result in any change in the fraction of cells positive for both β-galactosidase and α-dystroglycan over total β-galactosidase-positive cells, suggesting that, at least in the range studied, no minimal critical mass of myoblasts is required for differentiation.

Absence of syndecan-3 from muscle cells surfaces may alter the effects of growth factors on differentiation through directly affecting their binding to their signaling receptors, but may also affect cell-cell and cell-matrix interactions as members of the syndecan family may also act in the fine-tuning of integrin-induced changes of cytoskeletal organization (Rapraeger, 2001; Woods, 2001; Woods and Couchman, 2000). Surface proteins from the transmembrane 4 superfamily, convergent in some properties with the syndecans (Woods and Couchman, 2000), act as promoters of myoblast fusion and protect myotubes from apoptosis (Tachibana and Hemler, 1999). The partial magnitude of differentiation impairment observed for the cells with inhibited syndecan-3 expression may reflect the activity of native syndecan-3 from the host muscle or the redundancy of regulatory mechanisms acting over skeletal muscle regeneration. The more complex microenvironment that surrounds cells in vivo, comprising multiple signals affecting the differentiation process at different levels, may also explain possible differences between in vivo satellite cell behavior and in vitro studies (Schultz, 1996).

In this study, the expression of a heparan sulfate proteoglycan during skeletal muscle regeneration was studied. An increased expression of syndecan-3, glypican and perlecan by differentiating muscle cells was observed, and a requirement for syndecan-3 for successful regeneration in
myoblast transplant experiments was found. Future studies should explore the specific mechanism of syndecan-3 participation in skeletal muscle regeneration, and address the function of the other up-regulated proteoglycans in this process.

The authors are indebted to Dr John R. Hassell for generously providing anti-perlecan antibody and to Dr David J. Carey for generously providing anti-glypican and anti-syndecan-3 antibodies. This work was supported in part by grants from FONDAP-Biomedicine No. 13980001, FONDECYT 1990151 (to E.B.), and 2000113 (to J.C.C.). The research of E.B. was supported in part by an International Research Scholars grant from the Howard Hughes Medical Institute. E.B. and N.I. were supported by a Presidential 2000113 (to J.C.C.). The research of E.B. was supported in part by Biomedicine No. 13980001, FONDECYT 1990151 (to E.B.), and generously providing anti-glypican and anti-syndecan-3 antibodies. providing anti-perlecan antibody and to Dr David J. Carey for process.

should explore the specific mechanism of syndecan-3 myoblast transplant experiments was found. Future studies

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