Antisense Inhibition of Decorin Expression in Myoblasts Decreases Cell Responsiveness to Transforming Growth Factor β and Accelerates Skeletal Muscle Differentiation*

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Decorin is a member of the family of the small leucine-rich proteoglycans. In addition to its function as an extracellular matrix organizer, it has the ability to activate the epidermal growth factor receptor, and it forms complexes with various isoforms of transforming growth factor β (TGF-β). Decorin is expressed during skeletal muscle differentiation and is up-regulated in dystrophic muscle. In this study we investigated the role of decorin in TGF-β-dependent inhibition of myogenesis. To probe the function of decorin during myogenesis, C2C12 myoblasts were stably transfected with a plasmid expressing antisense decorin mRNA. The resulting inhibition of decorin expression led to the expression of myogenin, a master transcription factor for muscle differentiation, under growth conditions and accelerated skeletal muscle differentiation as determined by the expression of creatine kinase. In contrast myogenin expression was inhibited by adenovirally induced decorin expression or by adding exogenous decorin. Reduced synthesis of decorin resulted in a 7-fold decreased sensitivity to TGF-β-mediated inhibition of myogenin expression. In contrast, adenovirally induced decorin expression in wild type cells resulted in a 5-fold increased sensitivity to TGF-β-mediated inhibition of myogenin expression. Transfection studies with the TGF-β-dependent promoter of the plasminogen activator inhibitor-1 coupled with luciferase revealed that the transducing receptors for TGF-β1 and TGF-β2 were involved in the different responses of wild type and antisense decorin myoblasts. These results demonstrate that a reduction of decorin expression or of decorin availability results in a decreased responsiveness to TGF-β. These findings strongly suggest a new role for decorin during skeletal muscle terminal differentiation by activating TGF-β-dependent signaling pathways.

The process of myogenic development involves an ordered sequence of molecular events, which includes commitment of muscle precursor cells, cessation of cell division, myoblast terminal differentiation, and formation of myotubes that express muscle-specific genes involved in the specialized functions of the myofiber (1). A network of muscle regulatory factors (2) governs these processes. One of the factors responsible for the induction of terminal differentiation is myogenin, a transcription factor of the basic helix-loop-helix family that activates skeletal muscle-specific products such as creatine kinase, myosin heavy chain, acetylcholine receptor, etc. (3, 4). Terminal differentiation is critical as well during skeletal muscle formation as during muscle regeneration after injury (5).

The onset and progression of this process are controlled by a complex set of interactions between myoblasts and their environment. The presence of the extracellular matrix (ECM)1 is essential for normal myogenesis (6–9). The ability of myoblasts to differentiate is controlled in a negative manner by the extracellular concentration of specific mitogens, such as FGF-2, HGF/SF, and TGF-β (10–13). In the presence of these inhibitory growth factors, myoblasts continue to proliferate and fail to fuse or to express muscle-specific gene products. Conversely, the reduction in the concentration of these growth factors below a critical level results in cell culture experiments in an irreversible arrest in the G0 phase and in terminal differentiation.

It has been demonstrated that the activities of FGF-2, HGF/SF, and TGF-β can be regulated by binding to proteoglycans (14, 15). Cell surface heparan sulfate proteoglycans, in particular, have been suggested to play a role in modulating the activities of heparin-binding growth factors (16, 17). They modulate terminal myogenesis probably by acting as low affinity receptors for some growth factors such as FGF-2 (18) and HGF/SF (19). We have shown that the constitutive expression of syndecan-1, a heparan sulfate proteoglycan whose expression is down-regulated during terminal skeletal muscle differ-

1 The abbreviations used are: ECM, extracellular matrix; CEE, chicken embryo extract; FCS, fetal calf serum; FGF-2, basic fibroblast growth factor; HGF/SF, hepatocyte growth factor/scatter factor; TGF-β, transforming growth factor β; TGF-β-R, transforming growth factor-β receptor; PAGE, polyacrylamide gel electrophoresis.
entiation, inhibits the differentiation of myoblasts in culture (20, 21). In contrast, abolishing the expression of syndecan-3, which is another heparan sulfate proteoglycan synthesized by myoblasts, results in an acceleration of skeletal muscle differentiation by a mechanism being dependent on FGF-2, too (22).

Decorin is a member of the small leucine-rich proteoglycans gene family, which consists of a core protein and a single covalently linked glycosaminoglycan chain (23). Decorin binds several types of collagen in vivo, among them being types I, II, and VI, and promotes fibril stability. By interacting with ECM molecules like fibronectin and thrombospondin it influences cell adhesion. Additionally, at least in some types of cells, decorin activates the epidermal growth factor receptor, thereby triggering a signaling cascade that leads to phosphorylation of mitogen-activated protein kinase, to the induction of p21, and to growth suppression (24, 25). Great attention has been given to the observation that its core protein interacts with TGF-β (26–29). There are independent binding sites of decorin core protein for TGF-β and type I collagen (30). Affinity measurements of the interaction between decorin and TGF-β indicate the presence of at least two sites with Kd values of 1–20 and 20–200 nM for the high and low affinity binding sites, respectively (31). The biological consequences of the interaction between decorin and TGF-β are still a matter of debate. In analogy to the curative effect of blocking antibodies against TGF-β on the course of the anti-Thy-1-induced glomerulonephritis (32), the beneficial effect of decorin in the same disease model has been interpreted as the consequence of the inactivation of the cytokine by the proteoglycan. The reversal of TGF-β-mediated effects in this and other disease models by decorin gene transfer (27–29) supported this conclusion. On the other hand, the addition of decorin to osteoblasts resulted in an increased biological activity of TGF-β (33). Therefore, the precise mechanism whereby decorin modulates TGF-β activity remains to be elucidated.

In skeletal muscle decorin is found mainly in the perimysium (34). The synthesis and expression of decorin is up-regulated during skeletal muscle differentiation (35). Interestingly, the synthesis of decorin is also augmented in dystrophic mdx mouse skeletal muscle (36). In the present study we investigated the function of decorin during muscle differentiation. C2C12 myoblasts were transfected with an expression plasmid containing antisense decorin cDNA. Suppression of decorin production was accompanied with accelerated terminal differentiation and with a significant decrease in the sensitivity to TGF-β-dependent inhibition of myogenesis. It will be shown that this inhibition is mediated directly by interfering with the signaling cascade triggered upon binding of TGF-β to its transducing receptors. These findings demonstrate that the responsiveness of myoblasts to TGF-β, an inhibitor of skeletal muscle differentiation, is directly modulated by decorin expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—The C2C12 cell line was purchased from ATCC, Manassas, VA. Trizol LS, LipofectAMINE, Dulbecco’s modification of Eagle’s minimal essential medium, CEE, horse serum, FCS, Opti-MEM I, Hanks’ balanced salt solution, G418, and human FGF-2 were obtained from Life Technologies, Inc. Wizard plus maxi prep and prime-a-gene isolation system dual-luciferase reporter assay system, pGL3 basic vector, and pRL were from Promega, Madison, WI. F-12 medium, creatine kinase assay kit, bovine cartilage decorin, and fluorescein isothiocyanate- and tetramethylrhodamine B isothiocyanate-conjugated goat antirabbit IgG were from Sigma. TGF-β1 and TGF-β2 were obtained from R & D Systems, Minneapolis, MN. H3[35S]SO4 carrier-free (1050–1600 Ci/mmol) and [α-32P]dCTP (3000 mCi/mmole) and /α-32P]dCTP (1 Ci/mmol) were obtained from PerkinElmer Life Sciences. S & S Nytran plus membranes were from Schleicher & Schuell GmbH, Dassel, Germany. pSTP-Lux is a TGFβ-inducible luciferase reporter construct kindly donated by Dr. Fernando Lopez-Casilla, National University of Mexico, Mexico City, Mexico. Briefly, a modified plasmid containing adenovirus E4 promoter sequences was used to construct the 3TP promoter; a TGF-β-responsive element from positions –636 to –740 in the human plasminogen activator inhibitor-1 promoter was synthesized and inserted upstream of the adenovirus E4 sequences; a fragment (XhoI-EcoRI) containing the 3TP promoter cloned into XhoI-HindIII digested luciferase expression vector generating pSTP-Lux (37).

The pMyoLuc reporter construct was synthesized as described for pMyoCAT (20) with some modifications. Briefly, a 670-base pair DNA fragment corresponding to the myogenin promoter region, from +5 to 600, was subcloned into the pGL3 basic vector, which contains the CDNA encoding the firefly luciferase enzyme.

The construction of a replication-deficient adenoviral vector containing the full-length human decorin CDNA under the control of the EF-1 (38), the purification of decorin from mouse skeletal muscle (34), and the production of species–specific decorin antisera (38) have been described previously.

**Cell Culture**—The mouse skeletal cell line C2C12 was grown and induced to differentiate as described previously (20, 39). Collagen gels were prepared using collagen type I from rat tail, and the lattice was performed as described previously (38), with some modifications: cell-free collagen lattice was obtained by mixing 450 μl of collagen type I (5 μg/ml), 675 μl of medium of minimum medium, and 75 μl of 0.1 M NaOH in hydrophobic dishes and incubated at 37° for 1 h. The final lattice was cultured on myoblasts monolayers for different period of time.

**Stable Transfection and Isolation of Clones—**C2C12 cells were plated at a density of 6000 cells/cm2 in 100-mm dishes in growth medium. 24 h later the cells were transfected with the mammalian expression plasmid pCMVneo containing a 1372-bp CDNA insert containing the full-length mouse decorin sequence, in the antisense orientation, kindly donated by Dr. Renato Iozzo, Thomas Jefferson University, Philadelphia. The complete insert and a pCMVneo plasmid were digested with EcoRI to generate the pCMVneo-decorin recombinant plasmid; positive clones were selected by ampicillin resistance, and the orientation of the insert was determined by polymerase chain reaction; the antisense orientation was confirmed by DNA sequencing. Control cells were transfected with pCMVneo containing no insert. For transfections, 40 μg of plasmid DNA and 30 μg of LipofectAMINE in a total volume of 600 μl of Opti-MEM I were used according to the instructions of the manufacturer. After 6 h at 37° C, FCS and CEE were added to final concentrations of 10 and 0.5%, respectively, and the cells were incubated overnight. The next day the cells were rinsed twice with Hanks’ balanced salt solution and cultured in normal growth medium. Medium was replaced and cells were harvested (460 μg/ml) after 3 days. After 2–5 weeks colonies were selected using cloning rings.

**RNA Isolation and Northern Blot Analysis**—Total RNA was isolated from cell cultures using Trizol. RNA samples were electrophoresed in 1.2% agarose/formaldehyde gels, transferred to Nytran membranes, and hybridized with probes for creatine kinase, myogenin, MyoD, and tubulin as described previously (20, 21, 40). Blots were hybridized with radiolabeled probes in a hybridization buffer at 65° C. Hybridized membranes were washed twice at 65° C and exposed to Kodak x-ray film. For quantitative determination of the mRNA levels the intensity of the hybridization signals were measured by densitometric scanning (epson scanning densitometer).

**Gel Filtration Chromatography and SDS-PAGE**—Conditioned media from wild type and antisense decorin transfected cells, obtained after metabolic labeling during 18 h with 100 μCi/ml [35S]sulfate, were fractionated on a DEAE-Sephacl column (0.5 ml of resin) pre-equilibrated in 10 mM Tris-HCl, pH 7.5, 0.2 mM NaCl, 0.1% Triton X-100 and eluted with a linear NaCl gradient (0.2–1.0 M) at a flow rate of 5 ml/h. Fraction of 1.0 ml were collected, and radioactivity and conductivity were determined. Pooled fractions containing radioactive proteoglycans were then chromatographed on an analytical Sepharose CL-4B column (100 x 1 cm) equilibrated and eluted with 1% SDS, 0.1 M NaCl, 50 mM Tris-HCl buffer, pH 8.0. Samples (0.5 ml) were applied to the column together with prefractinated dextran blue (2000) and phenol red to mark void and total volumes, respectively. Columns were eluted at a flow rate of 5.0 ml/h, and effluent fractions of 0.8 ml were collected and aliquots counted for radioactivity. Selected fractions obtained from the Sepharose CL-4B column were analyzed by SDS-PAGE as described previously (41).

**Decorin Immunoprecipitation and Enzymatic Degradation**—Immunoprecipitation of secreted labeled decorin followed by SDS-PAGE and fluorography were carried out as described previously (42). Treatment of purified decorin with chondroitinase ABC was performed as described previously (41).
RESULTS

Stable Transfection of C2C12 Myoblasts with Antisense Decorin cDNA—Skeletal muscle myoblasts up-regulate decorin during differentiation (35). Because decorin binds TGF-β, a strong inhibitor of skeletal muscle differentiation, we reasoned that the down-regulation of decorin expression would affect skeletal muscle differentiation by modulating TGF-β activity. To test this hypothesis C2C12 myoblasts were stably transfected with a plasmid containing full-length decorin cDNA in the antisense orientation or with the control vector. Of the nine clones showing reduced [35S]sulfate incorporation into proteoglycans, two were chosen for more detailed studies. Myoblasts were labeled with [35S]sulfate followed by chromatographic analysis of the conditioned media on a Sepharose CL-4B column. Fig. 1A shows that both antisense clones, A4 and A6, synthesized significantly lower levels of sulfated proteoglycan compared with wild type cells.

The specificity of the effect on decorin synthesis is shown in Fig. 1B. SDS-PAGE followed by fluorography of three selected fractions obtained after the Sepharose CL-4B chromatography showed that in contrast to wild type cells, the antisense clones A4 and A6 did not synthesize detectable amounts of decorin. Decorin migrates like an Mr 100,000–110,000 globular protein indicated by the bracket, C, synthesis of proteins was evaluated in wild type (WT) and stably transfected myoblasts (A6) and in transfected cells infected with an adenovirus containing the full-length sequence for human decorin (WTi and A6i). The cultures were metabolically labeled with [35S]methionine (50 μCi/ml for 18 h), and cell extract (left panel) and medium (right panel) were concentrated and subjected to 8% SDS-PAGE followed by fluorography.

Immunofluorescence Microscopy—For immunostaining cells were grown on coverslips for 72 h. For staining of decorin the cells were rinsed with phosphate-buffered saline and fixed with 3% paraformaldehyde for 30 min at room temperature. They were then permeabilized with 0.05% Triton X-100 and incubated with the primary antibodies for 1 h at room temperature. After blocking unspecific binding sites with Biotto they were incubated for 1 h at room temperature with affinity-purified tetramethylrhodamine B isothiocyanate-labeled secondary antibodies diluted in blotto. The coverslips were viewed with a Nikon Diaphot inverted microscope equipped for epifluorescence. Immunostaining of fibronectin, biglycan, fibromodulin, lumican, and perlecan was carried out as described previously (7, 39).

Analysis of Creatine Kinase Activity—Myoblasts and myoblasts induced to differentiate for the indicated days were washed twice with phosphate-buffered saline, lysed by incubation with phosphate-buffered saline containing 0.1% Triton X-100 for 10 min at 25 °C, and harvested by scraping. Creatine kinase activity was determined using the creatine phosphokinase assay kit. All data points represent the means of duplicate determinations from two independent experiments.

Transient Transfection—Transfections with decorin adenoviral constructs or insert-free adenoviral preparations were performed as described previously (38). C2C12 cells, which had been plated the day before (6000 cells/cm2 in 25 cm2 flasks), were treated with 7.5 × 104 plaque-forming units of adenovirus containing the decorin sequence or with adenovirus in 1.3 ml/25 cm2 flask of Dulbecco’s modification of Eagle’s minimal essential medium/F-12 containing 2% heat-inactivated FCS. After 90 min of incubation 4 ml of standard medium/25-cm2 flask was added and incubation continued for an additional period of 23 h. Subsequently, the medium was changed for fresh medium, and the cells were grown for RNA analysis for 2 days.

For transfection with pMyoLuc and pRL, cells were plated at a density of 8000 cells/cm2 in six-well plates for 48 h and transfected for 6 h using Opti-MEM I containing 5 μg of pMyoLuc and 0.01 μg of pRL DNA and 12 μg of LipofectAMINE. Following transfection, 10% FCS and 0.5% CEE were added to the medium, and the cells were cultured for a further 14 h. Cells were then trypsinized and plated in 24-well plates in normal growth medium. Under these conditions, decorin or TGF-β1 is added at the indicated concentrations. After 24 h, the cells were harvested and assayed for dual luciferase activity.

For transfection with TGF-β1-inducible luciferase reporter construct (3TP-Lux), the cells were plated in growth medium 1 day before transfection at a density of 8000 cells/cm2 in 60-mm plates. Subsequently, the cells were incubated for 6 h in Opti-MEM I containing 2 μg of reporter plasmid (3TP-Lux), 2 μg of β-galactosidase plasmid and 15 μg of LipofectAMINE. After transfection the cells were incubated for 14 h in Opti-MEM I containing 10% FCS and 0.5% CEE. The cells were then washed twice with Hanks’ balanced salt solution and incubated for 2 days in growth medium followed by 1 day in differentiation medium or 30 h in differentiation medium containing TGF-β1, TGF-β2 (0–1.0 ng/ml), or FGF-2 (0–30 ng/ml). The cells were harvested and assayed for luciferase (37) and β-galactosidase activities (39), as a control of the transfection among different plates. All transfections were performed at least three times.

DNA and Protein Determination—DNA (43) and protein (44) were determined in aliquots of cell extracts as described.
further confirm the decreased synthesis of decorin in the antisense-transfected cells, antibodies against decorin were used to stain myoblast cultures. Fig. 2 shows that decorin was readily detectable in control transfected cells (35), whereas significantly lower levels were seen in the antisense clones. As a control measure, the localization of biglycan, fibromodulin, lumican, fibronectin, and perlecan was also assessed in control and transfected myoblasts and was found to indicate no changes in localization. Taken together, these results show unequivocally that stable clones with strongly reduced decorin expression were obtained by transfection with antisense decorin cDNA.

**Skeletal Muscle Differentiation Is Accelerated in Antisense Decorin-Transfected Myoblasts**—To evaluate whether the diminished synthesis of decorin in the antisense clones had any effect on skeletal muscle differentiation, the expression of myogenin, a master regulatory gene of skeletal muscle differentiation, was determined. Total RNA was isolated from antisense clones and wild type cells and analyzed by Northern blotting, using myogenin, MyoD, and tubulin cDNA probes. The transcript sizes for myogenin (Myo), MyoD, and tubulin (Tub) are indicated in each panel.

To further determine the relationship between decorin repression and myogenin induction under growth conditions, the induction of decorin expression was achieved episomally by infection using adenovirus containing the full-length sequence of human decorin or by adding purified decorin to the myoblast cultures. Fig. 4A, top left panel, shows that after viral infection, antisense cells did not express myogenin when maintained under growth conditions (compare A6i and A6), and wild type cells did not express myogenin in growth conditions (WT) or after infection with the adenovirus containing the decorin sequence (WTi), as expected. Wild type and antisense cells expressed myogenin after triggering differentiation in the above conditions (data not shown). Use of species-specific antibodies demonstrated the virus-mediated expression of decorin in antisense and wild-type cells after infection with the adenovirus containing decorin sequence (Fig. 4A, right panel). The viral infection did not affect the overall synthesis of proteins as shown in Fig. 1C. Fig. 4B shows that the application of exogenous decorin reverted myogenin expression. The effect of bovine cartilage decorin is shown in the left panel, whereas the effect of skeletal muscle-purified decorin is shown in the right panel. Treatment of purified skeletal muscle decorin with chondroitinase ABC had no effect on the reversion of myogenin expression (Fig. 4C). For both types of decorin, the IC_{50} was around 50–100 nm. To evaluate whether this decorin concentration was relevant to the conditions encountered by the cells, the amount of decorin in myoblast-conditioned medium was measured by Western blot assay, using skeletal muscle decorin as the standard. Conditioned medium, obtained after 24 h of culture under growth conditions, contained decorin at a concentration of ~70 nm (data not shown). These results strongly suggest that inhibition of decorin synthesis triggers the expression of myogenin under growth conditions and that this effect can be reversed by the endogenous production of decorin or by its exogenous application.

It is well established that skeletal muscle differentiation, including the expression of creatine kinase, is dependent on the expression of myogenin. In accordance with this fact was the
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In contrast creatine kinase mRNA was not detected until 4 days of differentiation. Creatine kinase activity was also detected earlier in the antisense-transfected cells than in wild type myoblasts (Fig. 5B). These results demonstrate that the expression of creatine kinase in a skeletal muscle specific marker, controlled by the expression of myogenin, is accelerated in antisense decorin-transfected cells compared with wild type cells.

Inhibition of Decorin Expression Decreases the Sensitivity of Myoblasts to TGF-β-dependent Inhibition of Myogenin Expression—To determine whether the accelerated expression of myogenin and other skeletal muscle markers observed in the antisense decorin-transfected cells resulted from a change in TGF-β signaling activity, we measured the TGF-β-dependent inhibition of myogenin mRNA expression as a function of growth factor concentration. Wild type- and antisense-transfected myoblasts were triggered to differentiate for 30 h in the presence of different concentrations of TGF-β1. As shown in the Fig. 6A (top left panel), exposure of wild type cells to TGF-β1 resulted, as expected, in a significant inhibition of myogenin mRNA expression. However, the antisense-transfected cells were considerably less sensitive toward the cytokine (bottom left panel). Whereas in wild-type cells half-maximal inhibition required 0.1 ng/ml, 0.7 ng/ml was needed to achieve the same effect in the cells with suppressed decorin synthesis (Fig. 6A, right panel). This change in responsiveness to TGF-β1 was specific, since no significant difference in the inhibitory activity of FGF-2 on myogenin expression was observed in both cell types (Fig. 6B, left and right panels). To confirm this modulatory effect of decorin on myogenin expression, wild type and wild type cells infected with adenovirus containing the full-length sequence for human decorin were triggered to differentiate for 30 h in the presence of different concentrations of

Fig. 5. Creatine kinase expression is augmented in antisense decorin-transfected clones. A, creatine kinase expression in wild type (WT) and antisense decorin-transfected cells (A6) was evaluated by Northern blot analysis. 10 μg of total RNA, isolated from myoblasts incubated in differentiation medium for 0 h, 2, or 4 days, was separated by electrophoresis, blotted onto nylon membranes, and hybridized with a 32P-labeled creatine kinase cDNA probe (CK). The transcript size is indicated. The ethidium bromide (EtBr)-stained gel is shown in the lower part of the panel, and the ribosomal RNAs are indicated. B, creatine kinase activity was quantitated as a function of time of differentiation as described under “Experimental Procedures.”

Fig. 4. The expression of myogenin in antisense decorin clones is reverted by specific decorin re-expression and by the addition of exogenous decorin. A, left panel, myogenin expression was evaluated in wild type cells and antisense decorin-transfected cells (WT and A6) and in transfected cells infected with an adenovirus containing the full-length sequence for human decorin (WTi and A6i). Northern blot analysis for myogenin under growth conditions was performed. 10 μg of total RNA isolated from myoblasts was separated by electrophoresis, blotted onto nylon membranes, and hybridized with a 32P-labeled myogenin probe (Myo). The methylene blue-stained nylon membrane is shown in the lower part of the panel, and the ribosomal RNAs are indicated. The transcript size is indicated. Right panel, the extent of decorin synthesized by the adenovirus-infected cells described in A was evaluated by specific immunoprecipitation with antibodies against human decorin as described under “Experimental Procedures.” B, A6 cells were incubated for 48 h with decorin purified from bovine cartilage (left panel) or from mouse skeletal muscle (right panel). Northern blot analysis for the effect of bovine decorin is shown in the left panel as described above. The reversion effect of skeletal muscle decorin on myogenin expression was evaluated using A6 myoblasts transiently cotransfected with pMycLu and pRL plasmids. The cells were incubated for 24 h in growth medium, harvested, and dual luciferase activity was determined as described under “Experimental Procedures.” C, A6 cells were incubated with skeletal muscle decorin pretreated with chondroitinase ABC (Cabc) and assayed as explained in the legend of B. Decorin concentrations are as follows: D1 = 0.1 μg/ml and D2 = 0.5 μg/ml. The results shown are the means from two different experiments performed in duplicate.

finding that the enzyme became induced more rapidly in antisense-transfected clones than in wild type cells when the cells were transferred to differentiation medium (Fig. 5). The expression of muscle-specific creatine kinase was measured in antisense-transfected clones and compared with wild type cells. As shown in Fig. 5A, creatine kinase mRNA levels were detected after 2 days of induced differentiation in antisense cells. In contrast creatine kinase mRNA was not detected until 4
TGF-β1. As shown in Fig. 6C, decorin-transfected cells exposed to TGF-β1 were more susceptible to the cytokine-induced inhibition of myogenin mRNA expression than wild type cells. Whereas in decorin-infected cells half-maximal inhibition required 0.018 ng/ml, the same effect in noninfected wild type cells required 0.09 ng/ml.

**Modulation of TGF-β Signaling by Decorin Suppression**—To determine whether decorin suppression had an influence on TGF-β signaling, a reporter construct was used where the promoter of plasminogen activation inhibitor-1 was coupled with luciferase cDNA. This reporter, p3TPLux, is activated upon binding of TGF-β to its transducing receptors (37). The antisense clone responded less to TGF-β1 and TGF-β2 when compared with wild type cells. The promoter activity was also diminished in the absence of exogenously added TGF-β, suggesting that the autocrine response to the cytokine was reduced (data not shown). To further examine this point, we titrated the response of the antisense clone, control transfect, and wild type cells to TGF-β1 and TGF-β2. Fig. 7 shows that TGF-β1 and -β2 increased reporter activity in all three cell lines. The antisense cells, however, showed a significant decrease in response mediated by TGF-β transducing receptors compared with control transfected and wild type cells (57 and 68%, respectively). Interestingly, the half-maximal effect was measured at similar cytokine concentration.

The levels of TGF-βRI, determined by the immunoprecipitation of metabolic labeled extract of myoblasts and the levels of betaglycan or (TGF-β-RIII), determined by Western blot analyses, were the same in antisense-transfected and wild type cells (data not shown). These results demonstrate, therefore, that the lowered sensitivity toward TGF-β in the myoblasts with suppressed decorin expression was mediated by the transducing TGF-β receptors.

**DISCUSSION**

The expression of decorin is up-regulated during skeletal muscle differentiation (35). The physiological relevance of this process is not yet known. Mice with deleted decorin genes have not been reported to exhibit clinical signs of abnormal muscle development (45), although detailed studies on muscle differentiation in these mice are not available in the literature. The absence of a striking muscular phenotype in decorin-deficient mice, however, may simply be an indication of the redundancy of the small leucine-rich proteoglycans and of the possibility that other members of this family may fulfill the function of decorin in skeletal muscle during development. We have, therefore, studied muscle differentiation in a less complex model, i.e., in cultured C2C12 myoblasts. Decorin expression was inhibited by stable transfection with full-length decorin antisense cDNA.

![Fig. 6. Concentration dependence of TGF-β-mediated inhibition of myogenin expression in control and antisense decorin cells.](image1)

![Fig. 7. Concentration dependence of TGF-β1 and -β2 on the plasminogen activator inhibitor-1 promoter activity in myoblasts of different capacity for decorin production.](image2)
As a result of abolishing decorin expression, skeletal muscle differentiation was triggered in myoblasts under growth conditions. This was evidenced by the accelerated expression of the transcription factor myogenin, a master regulator of expression of muscle-specific genes, which is silent in wild type cells under growth conditions (46, 47). Expression of myogenin in the non-decorin-expressing cells was reverted by infecting the cells with an adenovirus containing the full-length sequence for endogenously produced decorin and by adding exogenous decorin from two different sources. The data obtained support the hypothesis that decorin is able to suppress myoblast differentiation. This effect could be either the consequence of a direct signaling function of the proteoglycan or an indirect one mediated by the ability of decorin to interact with ECM components and growth factors.

Recent studies indicated that, at least in some tumor cells, decorin is influencing gene expression by direct interaction with a cell membrane receptor. Clear evidence was obtained that decorin binds to the epidermal growth factor receptor with a relatively high apparent $K_d$ value of about 90 nM (25). Binding was followed by receptor phosphorylation and an induction of p21, an inhibitor of cyclin-dependent kinases (24). Another candidate molecule for signaling is the 51-kDa endocytosis receptor protein, although evidence has not yet been obtained that decorin endocytosis is accompanied by altered gene expression (48–50). Myoblasts have been shown to respond to epidermal growth factor (51) and C$_2$C$_12$ myoblasts endocytose decorin very efficiently. In the present study, however, we have been unable to show unequivocally that decorin itself is a signaling molecule for C$_2$C$_12$ myoblasts. When decorin, purified under nondenaturing conditions, was added to the culture medium of wild type myoblasts undergoing differentiation, no significant change in myogenin expression was detected. As mentioned above, however, decorin is able to interact with a variety of serum components like vitronectin, thrombospondin, and fibronectin, and it is not known whether these interactions have an influence on the signaling properties of decorin. The observation that decorin undergoes a secretion-recapture pathway (52) is also compatible with the idea that it is the endogenously produced and not the exogenously added decorin that directly influences cell behavior.

The data obtained in the present investigation can best be explained by the assumption that decorin forms complexes as well with endogenously produced as with exogenously added TGF-$\beta$ and that these complexes allow a more efficient presentation of the cytokine to its signaling receptors than the free effector molecule. Myoblasts, being unable to synthesize relevant amounts of decorin or being deprived of the proteoglycan due to its sequestering to collagen lattices, are, therefore, less prone to the inhibitory effect of TGF-$\beta$ on myoblast differentiation. What molecular mechanisms are involved in the activation of TGF-$\beta$ by decorin remains to be investigated. This effect is specific to TGF-$\beta$, because antisense-transfected cells treated with FGF-2, another strong inhibitor of myogenin (10, 21, 22), exhibited the same FGF-2-dependent inhibition of myogenin expression (21). Inhibition of syndecan-3 expression produces a 13-fold increase in the IC$_{50}$ for FGF-2 dependent inhibition of myogenin expression (21). Inhibition of syndecan-3 expression produces a 13-fold increase in the IC$_{50}$ for FGF-2 inhibitory activity (22). The presence in the plasma membrane of syndecan-1 and -3 is critical for presentation of FGF-2 to the transducing receptors. Although decorin has been normally considered as an ECM proteoglycan, it has been shown to be associated to the plasma membrane through a specific decorin endocytosis receptor in a variety of eukaryotic cells (48–50, 53). The ability of decorin to interact with the plasma membrane via its specific receptor raises the possibility that decorin might modulate the TGF-$\beta$ activity in a similar fashion to that described for FGF-2 by heparan sulfate proteoglycans (16, 17).

Decorin possesses multiple functions besides modulation of TGF-$\beta$ activity. Decorin has been implicated in the control of collagen fibrillogenesis (54), cell proliferation (55), and corneal transparency (56). Some of these functions reside in the core protein, whereas others are attributed to the glycosaminoglycan chain (26). Some of the binding sites for molecules, such as TGF-$\beta$ and collagen type I, have been mapped and reside in different parts of the decorin core protein (30). The versatility of the decorin binding activity suggests that decorin may function as a modulator of fundamental biological processes during skeletal muscle differentiation, where its expression is up-regulated (35). In addition, decorin is an important structural constituent of the skeletal muscle ECM in normal and dystrophic mice (34, 36). It remains a challenge for further work to study the relative contributions of these growth factors and ECM components for muscle development.

In summary our results support the idea that decorin plays an important role during skeletal muscle differentiation by promoting the ability of the differentiation inhibitory growth factor TGF-$\beta$ to interact with its signaling receptors.

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Antisense Inhibition of Decorin Expression in Myoblasts Decreases Cell Responsiveness to Transforming Growth Factor β and Accelerates Skeletal Muscle Differentiation
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Additions and Corrections

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Cecilia Riquelme, Juan Larrain, Elke Schönher, Juan Pablo Henriquez, Hans Kresse, and Enrique Brandan

Pages 3593 and 3594: Figs. 4 and 6 were reversed although the legends are correct. The correct figures and legends are shown below.

FIG. 4. The expression of myogenin in antisense decorin clones is reverted by specific decorin re-expression and by the addition of exogenous decorin. A: left panel, myogenin expression was evaluated in wild type cells and antisense decorin-transfected cells (WT and A6) and in transfected cells infected with an adenovirus containing the full-length sequence for human decorin (WTi and A6i). Northern blot analysis for myogenin under growth conditions was performed. 10 μg of total RNA isolated from myoblasts was separated by electrophoresis, blotted onto nylon membranes, and hybridized with a 32P-labeled myogenin probe (Myo). The methylene blue-stained nylon membrane is shown in the lower part of the panel, and the ribosomal RNAs are indicated. The transcript size is indicated. Right panel, the extent of decorin synthesized by the adenovirus-infected cells described in A was evaluated by specific immunoprecipitation with antibodies against human decorin as described under “Experimental Procedures.” B, A6 cells were incubated for 48 h with decorin purified from bovine cartilage (left panel) or from mouse skeletal muscle (right panel). Northern blot analysis for the effect of bovine decorin is shown in the left panel as described above. The reversion effect of skeletal muscle decorin on myogenin expression was evaluated using A6 myoblasts transiently cotransfected with pMyoLuc and pRL plasmids. The cells were incubated for 24 h in growth medium, harvested, and dual luciferase activity was determined as described under “Experimental Procedures.” C, A6 cells were incubated with skeletal muscle decorin pretreated with chondroitinase ABC (Cabc) and assayed as explained in the legend of B. Decorin concentrations are as follows: D1 = 0.1 μM and D2 = 0.5 μM. The results shown are the means from two different experiments performed in duplicate.

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FIG. 6 Concentration dependence of TGF-β-mediated inhibition of myogenin expression in control and antisense decorin cells. Wild type (WT) and antisense decorin-transfected cells (A6) were incubated for 30 h in differentiation medium containing the indicated concentrations of TGF-β1 (A, left panel) and FGF-2 (B, left panel). RNA was isolated from the cells, and 10 μg of total RNA was analyzed by Northern blot with a 32P-labeled myogenin (Myo) cDNA probe. Right panels show the graphical representations of TGF-β1 (A) and FGF-2 (B)-dependent inhibition of myogenin expression in antisense decorin cells (open circles) and wild type cells (closed circles). Values correspond to the means of three independent experiments. C shows TGF-β1-dependent inhibition of myogenin expression in wild type cells (closed circles) and wild type cells infected with adenovirus containing the full-length sequence for human decorin (open circles). Myogenin expression was evaluated as described in the legend of Fig. 4B.