Retroviral Overexpression of Decorin Differentially Affects the Response of Arterial Smooth Muscle Cells to Growth Factors

Jens W. Fischer, Michael G. Kinsella, Bodo Levkau, Alexander W. Clowes, Thomas N. Wight

Abstract—Decorin is a member of the family of small leucine-rich proteoglycans that are present in blood vessels and synthesized by arterial smooth muscle cells (ASMCs). This proteoglycan accumulates in topographically defined regions of atherosclerotic lesions and may play a role in the development of this disease. However, little is known about whether decorin has specific effects on the cellular events that contribute to atherosclerotic lesion formation. In the present study, rat ASMCs were transduced with a retroviral vector (LDSN) that carries the bovine decorin gene. Compared with vector control cells (LXSN), these cells constitutively overexpress decorin, as verified by Northern and Western analysis and by metabolic labeling. Experiments were performed to examine the responsiveness of decorin-overexpressing rat ASMCs to platelet-derived growth factor (PDGF) and transforming growth factor-β1 (TGF-β1), 2 growth factors that affect cell proliferation and extracellular matrix production in atherosclerosis. Decorin-overexpressing cells had decreased [3H]thymidine incorporation into DNA and increased the levels of the cyclin-dependent kinase inhibitors p21 and p27 in the first 24 hours of response to serum and PDGF-BB. However, these effects of decorin were not apparent at 48 or 72 hours after plating and did not result in reduced growth of decorin-overexpressing cells in response to serum and PDGF-BB. In contrast, the growth response of decorin-overexpressing ASMCs to TGF-β1, as well as the expression of TGF-β1-responsive genes, such as plasminogen activator inhibitor-1 and versican (an extracellular matrix proteoglycan), was diminished. These results indicate that decorin selectively inhibits the responsiveness of rat ASMCs to TGF-β1 and suggests that the induction of constitutive decorin overexpression by ASMCs in vivo may have therapeutic value in the inhibition of TGF-β1-mediated effects on the development of atherosclerotic lesions. (Arterioscler Thromb Vasc Biol. 2001;21:777-784.)

Key Words: transforming growth factor-β1 ■ cell proliferation ■ extracellular matrix decorin ■ decorin ■ arterial smooth muscle

Decorin is a member of the small leucine-rich chondroitin/dermatan sulfate proteoglycans and is present in the extracellular matrix (ECM) of a variety of tissues and cell types. In blood vessels, decorin is confined mainly to the adventitia but is also present in lesser amounts in the smooth muscle–rich media. However, in atherosclerosis, decorin accumulates in defined locations throughout the lesions in association with deposited lipoproteins, collagen fibrils, and the plaque neovasculature. Such findings suggest that decorin might play a role in lipid retention, as well as in fibrosis and neovessel growth during the development of the atherosclerotic lesion.

Decorin has been shown to influence the proliferative capacity of cells. For example, decorin inhibits the growth of Chinese hamster ovary (CHO) cells and various cancer cell lines. The growth-inhibitory effect of decorin in malignant cell lines involves an increase in the cyclin kinase inhibitor p21. Moreover, the upregulation of decorin expression in nongrowing confluent arterial smooth muscle cells (ASMCs) suggests a relationship between the expression of decorin and growth quiescence in ASMCs. Decorin also influences ECM production and organization. Decorin binds to several ECM proteins, such as collagen, fibronectin, and thrombospondin, and mediates aspects of matrix protein fibrillogenesis and fibril packing. In decorin-null mice, the regulation of collagen fibril formation is obviously disturbed, and fibrils with irregular size and shape are deposited in collagenous tissues. Overexpression of decorin in vivo disease models also alters ECM deposition. For example, an antifibrotic effect of decorin in vivo has been demonstrated in the bleomycin-induced hamster model of lung fibrosis and in an experimental animal model of glomerulonephritis. In addition, local overexpression of decorin in balloon-injured rat carotid arteries causes an increase in the density of collagen fibril packing within the neointima and decreases neointimal accumulation.
of versican and fibronectin, thereby reducing intimal volume.30
In addition to a direct effect on the assembly of the ECM proteins, decorin may mediate cellular and ECM changes by an influence on the activity of cytokines and growth factors that are involved in the regulation of cell proliferation and ECM production. For example, some studies have shown that decorin binds and inactivates transforming growth factor-β1 (TGF-β1)31–33 and reverses the effects of this cytokine on cells. The administration of purified decorin or gene therapeutic delivery of decorin reduces fibrosis in an experimental animal model of glomerulonephritis.28,29 in which fibrosis is dependent on TGF-β1.34,35 Recent studies have demonstrated that decorin overexpression can also block the TGF-β1-dependent suppression of immune surveillance of gliomas36,37 and the inhibition of lung epithelium morphogenesis by TGF-β1.38
We have recently found that the accumulation of ECM in the intima of balloon-injured carotids that were seeded with decorin-overexpressing cells is decreased.39 The decreased volume of the lesion involves a decreased matrix volume with no change in cell number. To explore the mechanism by which decorin modulates this response, we have examined whether ASMCs that overexpress decorin have altered growth and ECM production in response to TGF-β1, which is a cytokine that influences cell proliferation and ECM accumulation during atherosclerotic lesion development.39–41

Methods

Materials

HEPES, NaCl, EGTA, dithiothreitol, sodium fluoride, Na3VO4, β-glycerophosphate, leupeptin, aprotinin, urea, phenylmethylsulfonyl fluoride, Tween 20, chondroitin sulfate (type C), and TGF-β1 (No. T7039) were from Sigma Chemical Co; 6-aminohexanoic acid and benzamidine were from Eastman Kodak Co; chondroitin ABC lyase was from ICN Pharmaceuticals; and DEAE-Sephacel was from Pharmacia Biotech, Inc. Prestained and 14 C-labeled protein standards, glycine, SDS, N,N′,N,N′-tetramethylethylenediamine, ammonium persulfate, and all cell culture supplies were from Life Technologies, Inc. Triton X-100 was from Boehringer-Mannheim Corp. Na-[35S]sulfate (carrier free) and [3H]glucosamine were from ICN Radiochemicals, and 40% acrylamide solution was from Bio-Rad. Rabbit antibody (LF-94) against bovine decorin was generously provided by Dr L. Fisher, National Institute of Dental Research, National Institutes of Health, Bethesda, Md. Antibodies to TGF-β1 receptor type I (No. sc398) and type II (No. sc400) and to p27 (No. sc328) were from Santa Cruz Biotech, Inc. The polyclonal antibody to p21 (No. PC55) was from Oncogene Research Products. Recombinant human platelet-derived growth factor (PDGF)-BB was kindly supplied by Dr Charles Hart (Zymogenetics Inc, Seattle, Wash). Purified decorin, which was disassociatively extracted from bovine tendon and is active in a collagen fibrillogenesis assay, was kindly provided by Dr Kathryn Vogel, University of New Mexico, Albuquerque.

Construction of the Bovine Decorin Retrovirus (LDSN) and Stable Transduction of Fischer 344 Rat Smooth Muscle Cells

The cDNA of bovine decorin (PG28, courtesy of Dr Marid Young, National Institute of Dental Research, National Institutes of Health, Bethesda, Md) was inserted into the EcoRI site of the replication-defective retroviral vector LXSIN (courtesy of Dr A.D. Miller, Fred Hutchinson Cancer Research Center, Seattle, Wash)40 to prepare the retroviral vector for the expression of bovine decorin (LDSN). Subsequently, the packaging cell lines were established, and Fischer 344 rat ASMCs were transduced as described previously.26,30,42

Briefly, the retroviral vector containing the bovine decorin gene and the control vector (LXSN) were transfigted into the ecotropic packaging cell line PE501 by use of the calcium phosphate precipitation method.43 Transiently produced virus was harvested and used to infect the second amphotropic packaging cell line (PA317). After titering the virus production in NIH 3T3 TK- cells, the virus was harvested from clones producing between 5×105 and 5×106 virus/ml and used for stable transduction of Fischer 344 rat ASMCs in the fourth passage after primary culture. Selection media for the PA317 cells and the ASMCs contained the neomycin analogue G418 at 600 μg/ml and 800 μg/ml, respectively.

Cell Culture

ASMCs from male Fischer 344 rats were obtained as described previously.44 Transduced cells were used for experiments between 4 and 8 passages after the initial transduction. After selection by means of the neomycin analogue G418 (800 μg/ml), ASMCs were maintained on tissue culture plastic in DMEM supplemented with 10% calf serum.

Purified collagen (Vitrogen-100, Collagen Corp) was used according to the directions of the manufacturer to coat tissue culture plastic with monomeric collagen and polymeric collagen films. Briefly, for the monomeric coating, 100 μL of Vitrogen-100 solution was spread per well (24-well plates). The polymeric collagen coats were prepared by covering the tissue culture surface with a neutralized solution of Vitrogen-100 that was allowed to polymerize at 37°C for 1 hour. The polymeric and the monomeric collagen preparations were then air-dried overnight in the tissue culture hood. Before use, the wells were rinsed and rehydrated with serum-free tissue culture medium.

Growth Assays

To perform proliferation studies, cells were seeded at 2×104 cells per well into 24-well plates, grown for the indicated time periods, harvested by means of trypsinization, and fixed in 3.7% formaldehyde. Cell number was determined in a Coulter particle counter after dilution (1:10) with PBS. The growth curves were established either directly without preceding serum deprivation or after stimulation following serum withdrawal (48 hours). DNA synthesis was measured by incorporation of [3H]thymidine (10 μCi/ml), which was added 4 hours before the cells were harvested. Subsequently, cells were washed twice with ice-cold PBS and then incubated with ice-cold 10% trichloroacetic acid overnight at 4°C. After 2 additional washes with 10% trichloroacetic acid, the precipitated material was dissolved in 0.1N NaOH and analyzed in a liquid scintillation counter. After an aliquot had been stored for protein measurement (BCA, Pierce).

Metabolic Labeling and Proteoglycan Analysis

For metabolic labeling of proteoglycans, 100 μCi/ml carrier-free Na[35S]sulfate or 10 μCi/ml [3H]glucosamine was added to the culture medium. Incorporation of radiosulfate and [3H]glucosamine into total secreted glycosaminoglycans was determined from duplicated aliquots of culture medium by cetylpyridinium chloride precipitation.45

For separation of radiolabeled proteoglycans by SDS-PAGE, samples of proteoglycans in conditioned medium were partially purified and concentrated on 0.5 mL DEAE-Sephalac columns in 8 mol/L urea with 0.5% Triton X-100, 0.01 Tris-HCl, pH 7.5, and 0.25 mol/L NaCl (urea buffer), washed with ~10 vol urea buffer, and eluted by 3 mol/L NaCl in urea buffer. After the addition of 30 μg chondroitin sulfate carrier, the eluted material was precipitated at −20°C (2 hours) by the addition of 3.5 vol of 95% ethanol containing 1.3% potassium acetate. The pellet was dissolved in distilled water, and ethanol precipitation was repeated without the addition of carrier. After the final centrifugation, the supernatants were discarded, and the pellet was air-dried. Samples were resuspended in 8 mol/L urea, either with or without prior digestion by chondroitin ABC lyase (0.02 U) in enriched Tris buffer,46 pH 8, for 3 hours at 37°C. Subsequently, samples were boiled (3 minutes) in SDS-containing sample buffer with β-mercaptoethanol. Undigested radiolabeled samples were applied to a 4% to 12% gradient SDS-
polyacrylamide gel and detected by autoradiography of the dried gels. 14C-labeled protein standards were used to estimate the size of proteoglycan core proteins. In addition, digested and undigested samples were run by SDS-PAGE and were blotted for Western analysis (see below).

**Western Analysis**

Analysis of cyclin-dependent kinase inhibitors was performed as described previously. Briefly, cells grown on tissue culture plastic were rinsed with PBS and harvested in lysis buffer (50 mmol/L HEPES [pH 7.5], 150 mmol/L NaCl, 5 mmol/L EDTA, 2.5 mmol/L EGTA, 1 mmol/L dithiothreitol, 1 mmol/L NaF, 0.1 mmol/L Na3VO4, 10 mmol/L β-glycerol phosphate, 0.5 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin). Cell extracts were incubated 10 minutes on ice and centrifuged at 27 000g for 30 minutes. Subsequently, supernatants were analyzed for protein content (BCA, Pierce). Equal aliquots of protein (20 to 50 μg) were separated by 15% SDS-PAGE. After transfer to Immobion membranes (Millipore Corp), the proteins were immunooblotted with antibodies against p21 and p27. For detection of primary antibodies, a horseradish peroxidase–conjugated secondary antibody was applied and detected by use of the enhanced chemiluminescence method (Amersham-Pharmacia Biotech, Inc).

To detect decorin core protein before and after chondroitin ABC lyase digestion, Western analysis was performed as described previously. Briefly, samples were run on 10% SDS-polyacrylamide gels and were transferred to a nitrocellulose membrane (Millipore Corp), and the proteins were immunooblotted with antibodies against p21 and p27. For detection of primary antibodies, a horseradish peroxidase–conjugated secondary antibody was applied and developed by use of the enhanced chemiluminescence method (Amersham-Pharmacia Biotech, Inc).

**Northern Blot Analysis**

Total RNA was isolated from cultured ASMCs by the method of Chomczynski and Sacchi. Fifteen micrograms of total RNA was separated on 0.8% agarose gels containing formaldehyde. Subsequently, RNA was subjected to limited alkaline hydrolysis, transferred to Zetaprobe (Bio-Rad), and cross-linked by UV light. Membranes were prehybridized for 2 hours at 42°C in 50% (vol/vol) formamide (Life Technologies, Inc), 6× SSPE, 5× Denhardt’s solution, 0.5% SDS, 5% dextran sulfate, and 100 μg/mL salmon sperm DNA (Sigma). Probes were P-labeled by random priming, with the use of 2'-3'-dideoxyribonucleotides (Amersham-Pharmacia Biotech, Inc) as described previously. Hybridization with P-labeled cDNA probes (see below) was carried out at 42°C in the same solution for 16 hours, followed by 3 washes with 2× SSPE/0.1% SDS at 42°C and 2 washes with 0.3× SSPE/0.1% SDS at 65°C.

**cDNA Probes**

The same full-length bovine decorin cDNA (Pg28) used for construction of the LDSN vector was used to detect bovine decorin mRNA in the transduced ASMCs by Northern analysis. The rat versican cDNA probe against the V3 form of versican was used to determine versican mRNA levels by Northern blotting. The rat plasmaminogen activator inhibitor-1 (PAI-1) cDNA probe used in the present study for Northern analysis was generously provided by Dr T. Gelehrter (Albany Medical College, Albany, NY).

**Figure 1.** Expression of bovine decorin mRNA and core protein in aortic ASMCs from Fischer 344 rats. A, Northern blot probed with the cDNA of bovine decorin (Pg28). Control indicates mRNA extracted from the parental ASMCs; LXSN, mRNA from ASMCs transduced with the LXSN vector; and LDSN-1 to LDSN-4, 4 different cell lines transduced with bovine decorin cDNA. The endogenous rat or bovine decorin mRNA is ~1.8 kb. When expressed after transduction with the LXSN vector, the decorin mRNA transcript includes neomycin phosphotransferase sequence (794 bp), which increases the transcript size to ~2.6 kb. B, Western blot analysis of medium samples conditioned for 24 hours by the same cell lines as in panel A, with use of the species-specific antibody LF-94 against bovine decorin. Samples have been digested with chondroitin ABC lyase before the analysis. To allow for quantitative comparison, ASMCs were seeded at equal density to harvest mRNA and conditioned medium.

**Statistical Analysis**

Unpaired t tests were performed where appropriate, and a 2-tailed value of P<0.05 was considered significant.

**Results**

**Expression of Bovine Decorin Proteoglycan in Cultured Rat Vascular ASMCs**

After transfection, the ASMCs were analyzed for expression of bovine decorin. Northern blot analysis (Figure 1A) demonstrated bovine decorin mRNA expression in 4 ASMC lines transduced with the LDSN retrovirus compared with ASMCs transduced with the “empty” LXSN retrovirus and the parental cell line. The bovine decorin cDNA (Pg28) was used for hybridization. The expression of bovine decorin core protein was demonstrated by use of a species-specific antibody to bovine decorin (LF-94, Figure 1B). Figure 1 demonstrates that the expression levels in LDSN-1, LDSN-2, and LDSN-4 cells were similar. Thus, these cell lines were used for the subsequent experiments designed to study the function of decorin. The amount of bovine decorin secreted by the confluent transduced cells was ~30 μg per 10⁶ cells for 24 hours, as determined by comparison with a purified tendon decorin standard in a quantitative blotting assay (see Methods).

To determine whether the retrovirally expressed decorin contained a chondroitin sulfate/dermatan sulfate (CS/DS) glycosaminoglycan chain, Western blot analysis was performed before and after chondroitin ABC lyase digestion of samples derived from conditioned tissue culture medium. As shown in Figure 2A, a strong band at 90 kDa was detected in
ASMCs transduced with LDSN, compatible with $M_r$ estimates for decorin. Digestion of the sample with chondroitin ABC lyase shifted the band to 40 kDa, which indicates the presence of CS/DS glycosaminoglycan. To confirm the presence of newly synthesized CS/DS glycosaminoglycan chains, cells were metabolically labeled with $[3\,\text{H}]$glucosamine and $[35\,\text{S}]$sulfate. As shown in Figure 2B, incorporation of $[3\,\text{H}]$glucosamine and $[35\,\text{S}]$sulfate into total secreted proteoglycans is increased in the decorin-overexpressing cells. In addition, electrophoretic separation of secreted proteoglycans on SDS-PAGE showed markedly increased levels of $[35\,\text{S}]$-labeled proteoglycan at 90 kDa (Figure 2C) in ASMCs transduced with LDSN. No changes in cell morphology were observed on overexpression of decorin (not shown).

Effects of Decorin Overexpression on Levels of Cdk Inhibitors and Proliferation

DNA synthesis, measured by $[3\,\text{H}]$thymidine incorporation, was reduced in decorin-overexpressing cells 24 hours after plating in 10% serum (Figure 3A). This reduction of DNA synthesis was transient, inasmuch as no difference was observed at 48 hours and 72 hours. To determine whether changes occurred in the levels of cell cycle–regulatory proteins, such as the cdk inhibitors p21 and p27, Western analysis was performed. Twenty-four hours after plating, p21 and p27 levels were increased over the levels in LXSN cells (Figure 3B). If LXSN cells were plated (24 hours) in conditioned medium from LDSN cells, the levels of p21 and p27 were increased as well (Figure 3C), suggesting that secreted bovine decorin is responsible for the increase in cdk inhibitor levels. The effect of decorin overexpression on cdk inhibitors was also transient (72 hours, Figure 3B), in agreement with the transient decrease in DNA synthesis. The transient effect of decorin on $[3\,\text{H}]$thymidine incorporation and cdk inhibitors is not due to decreased decorin synthesis after 24 hours, because comparable levels of decorin are present in medium collected from 0 to 24 hours and from 24 to 48 hours after plating (Figure 3A, right).

To determine whether the initial inhibition of DNA synthesis in LDSN cells has an effect on cell proliferation, the growth of ASMCs was determined over the course of 6 days. For this purpose, ASMCs were grown for 2 days after plating, synchronized by serum withdrawal for 48 hours, and stimulated by serum or PDGF-BB. No difference in growth rate between LXSN and LDSN cells was found in response to 10% serum (Figure 4A) or 2% serum (not shown). In addition, proliferation in response to PDGF-BB (10 ng/mL) was identical in ASMCs transduced with either LXSN or LDSN (Figure 4B). Nor did retroviral overexpression of bovine decorin in bovine aortic ASMCs affect proliferation.

**Figure 2.** Secretion of bovine decorin proteoglycan (PG). A, Western analysis (LF-94) of equal volumes of conditioned medium derived from LXSN and LDSN cells with and without prior digestion by chondroitin ABC lyase. B, Quantification of total secreted glycosaminoglycans by metabolic labeling (100 $\mu\text{Ci/mL}^{[35\,\text{S}]}$sulfate and 100 $\mu\text{Ci/mL}^{[3\,\text{H}]}$glucosamine, 24 hours) followed by precipitation with cetylpyridinium chloride. Shown is a representative experiment in LDSN-1 cells. C, Autoradiography of SDS-polyacrylamide gradient gel (4% to 12%) loaded with equal counts (30 000 dpm) of $[35\,\text{S}]$sulfate-labeled secreted PGs.

**Figure 3.** Transient upregulation of cdk inhibitors p21 and p27 in decorin-overexpressing cells. A, Left, DNA synthesis of ASMCs quantified by $[3\,\text{H}]$thymidine incorporation. Cells were seeded sparsely into tissue culture wells (20 000/cm$^2$) containing cell culture medium supplemented with 5% serum and were harvested after the indicated times. Data were derived from 3 experiments; values are mean $\pm$ SEM. *P<0.05. A, Right, Western blot (anti-bovine decorin, LF-94) of bovine decorin in conditioned media 0 to 24 hours and 24 to 48 hours after plating. B, Western blot of cdk inhibitor levels in transduced ASMCs. Protein samples derived from ASMCs cultured under the same conditions as in panel A were run on 10% SDS-PAGE, blotted on Immobilon membranes, and probed with anti-p21 antibody or an antibody to p27. C, Left, p21 and p27 levels in LXSN cells that were incubated in medium that was conditioned either by confluent decorin-overexpressing cells (LDSN-1) or cells transduced with the empty LXSN vector. C, Right, Western blot (LF-94) showing the presence of bovine decorin in the conditioned medium used in this experiment.
(data not shown), indicating that the failure of decorin overexpression to inhibit proliferation is not species specific.

Because a fibrillar collagen substratum alters the proliferative response of ASMCs to PDGF, additional growth assays were performed on monomeric and polymeric collagen films, with or without the addition of 5 and 25 μg/mL purified decorin, to determine whether the effect of decorin on cell growth was dependent on the nature of the substrate. The addition of purified decorin had no effect on the growth of ASMCs transduced with LXSN or LDSN on either substrate (data not shown). The same decorin preparation used in these assays inhibited the migration of endothelial cells without affecting cell proliferation, indicating that purified decorin is biologically active. These data suggest that retroviral overexpression of decorin does not affect ASMC growth in response to serum and PDGF in vitro.

Response of ASMCs That Overexpress Bovine Decorin to Exogenous TGF-β1 Is Reduced

In confluent rat ASMCs, TGF-β1 stimulates DNA synthesis, whereas in subconfluent ASMC cultures, TGF-β1 inhibits ASMC proliferation. The stimulation of DNA synthesis in confluent ASMCs is mediated by the induction of the autocrine PDGF production. TGF-β1, in the presence of 5% serum, also stimulates DNA synthesis of confluent LXSN-transduced ASMCs and inhibits DNA synthesis in sparse ASMCs (Figure 5). Both of these density-dependent effects of TGF-β1 on [3H]thymidine incorporation by ASMCs were inhibited in decorin-overexpressing cells (Figure 5).

The induction of 2 TGF-β1-responsive genes, PAI-1 and the proteoglycan versican, was assayed in LXSN and LDSN ASMCs to confirm directly the neutralization of TGF-β1 activity by decorin. The addition of TGF-β1 to LXSN cells caused a pronounced increase in PAI-1 and versican mRNA levels, but a markedly lesser response was observed when LDSN cells were exposed to the growth factor (Figure 6). TGF-β1 induced the accumulation of PAI-1 mRNA in LXSN cells in a dose-dependent manner, with the strongest induction at 5 ng/mL, whereas the dose-response curve for versican mRNA is bell-shaped, with a peak of expression level at 0.1 ng TGF-β1 per milliliter (Figure 6B). In LDSN cells, the dose-response curves for the induction of PAI-1 and versican mRNA were shifted to higher TGF-β1 concentrations. Notably, the effects of low concentrations (0.01 and 0.1 ng/mL) of TGF-β1 were blocked completely in decorin-overexpressing cells, whereas the effect of 5 ng/mL was only partially inhibited.

Medium-switching experiments were performed to test whether decorin secreted by LDSN cells could inhibit the induction of PAI-1 and versican mRNA levels by TGF-β1 in LXSN cells (Figure 6B). Thus, LDSN-conditioned medium was applied to LXSN cells before stimulation with 2 ng/mL TGF-β1. In LDSN cells, the dose-response curves for the induction of PAI-1 and versican mRNA were shifted to higher TGF-β1 concentrations. Notably, the effects of low concentrations (0.01 and 0.1 ng/mL) of TGF-β1 were blocked completely in decorin-overexpressing cells, whereas the effect of 5 ng/mL was only partially inhibited.

Medium-switching experiments were performed to test whether decorin secreted by LDSN cells could inhibit the induction of PAI-1 and versican expression by TGF-β1 in LXSN cells (Figure 6B). Thus, LDSN-conditioned medium was applied to LXSN cells before stimulation with 2 ng/mL TGF-β1. In the presence of LDSN-conditioned medium, TGF-β1-mediated induction of PAI-1 and versican mRNA was reduced in LXSN cells compared with the induction observed in LXSN cells covered with LXSN-conditioned medium (Figure 6B). These findings suggest that secreted decorin in the medium of LDSN cells inhibits the activity of TGF-β1 in rat aortic ASMCs. No differences were observed between LXSN and LDSN cultures when PDGF-BB (10 ng/mL) was used to stimulate PAI-1 mRNA expression (data...
thus, no difference was seen between LDSN and LXSN cells at 48 hours and 72 hours after plating. Because the decorin content in the medium of LDSN cells at 48 hours is as high or higher than that in 24-hour conditioned medium, as shown in Figure 3A, decreased decorin concentration in the media at later times after plating cannot explain the transient effect on DNA synthesis. The transient nature of the decorin effect on cell cycle–regulatory proteins may be due instead to the neutralization of decorin by binding to other matrix components, such as fibronectin and collagen,25,62 because these ECM components are produced in large amounts in ASMCs.63–65 Alternatively, the deposition of ECM molecules, such as fibronectin and collagen, may influence the proliferative response of ASMCs to growth factors67,66 and override the effects of decorin on cell proliferation. For example, Koyama et al67 demonstrated that ASMCs grown on fibrillar collagen had increased levels of cyclin-dependent kinase inhibitors and a decreased growth response to PDGF compared with cells cultured on monomeric collagen. Thus, although decreased collagen synthesis or deposition by ASMCs that overexpress decorin might be expected to allow continued proliferation in response to PDGF and serum, the growth of rat ASMCs on fibrillar collagen did not result in significant differences in the response of LXSN or LDSN cells. Clearly, no difference in growth kinetics was detected between LXSN and LDSN cells at later times in culture, confirming the transient effect of decorin on DNA synthesis and p21/p27 levels in ASMCs.

Although the growth response of decorin-overexpressing cells to serum and/or PDGF is not affected, TGF-β1 responsiveness is dramatically impaired. TGF-β1 induces proliferation in dense cultures of ASMCs that is due to the induction of PDGF-AA19,55 and inhibits growth in sparse cultures. The stimulation of DNA synthesis in dense cultures and the inhibition in sparse cultures by exogenous TGF-β1 were blocked in decorin-overexpressing ASMCs (Figure 5). These results are similar to those of previous studies in which decorin inhibited the proliferation of CHO cells because of an inhibition of TGF-β1 utilization.14 It is thought that the interaction between TGF-β1 and decorin is mediated through a binding sequence in the decorin core protein and does not involve the glycosaminoglycan chain.4,31,33 Several reports have demonstrated an inhibition of TGF-β1 activity by decorin,14,28,29 although enhancement of TGF-β1 activity by decorin has also been described.67 Other work indicates that TGF-β1/decorin complexes may be selectively inactive,32 and thus, only some TGF-β1–dependent cellular responses are affected. The variability in these observations may be due to the use of different cell systems or differences in decorin preparations that were used, because the folding and glycosylation of the decorin core protein are dependent on the source and isolation procedure used.56

The observation that decorin decreases the effect of TGF-β1 on TGF-β1–responsive genes provides further evidence that decorin influences TGF-β1 activity. The effect of decorin on the responsiveness of ASMCs to TGF-β1 does not appear to be mediated by changes in TGF-β1 receptor expression, inasmuch as the TGF-β receptor I and II expression levels were slightly upregulated in the decorin-transduced cells. Therefore, these experiments indicate that decorin is a functional antagonist of TGF-β1 in rat ASMCs.

Figure 6. Transcriptional activity of TGF-β1 is reduced in decorin-overexpressing ASMCs. ASMCs were grown to near confluence, starved for 24 hours, and stimulated by addition of the active form of recombinant TGF-β1 to the conditioned medium. Total RNA was harvested after 6 hours, and PAI-1 mRNA levels were analyzed by Northern blotting. A, Induction of PAI-1 mRNA on addition of active TGF-β1 (4 ng/mL) is shown. B, Dose-response curve for the induction of PAI-1 and versican mRNA levels were analyzed by Northern blotting. A, B, and C, Western blots of cell extracts probed with antibodies to TGF-β1 receptor I and II (cond) medium reduced the response in LXSN cells (2 lanes at right). LX indicates control. C, Western blots of cell extracts probed with antibodies to TGF-β1 receptors I and II are shown.

Discussion

ECM accumulation and ASMC proliferation are hallmarks in the development of intimal thickenings associated with the development of atherosclerotic lesions.41 Our recent work has shown that insertion of decorin-overexpressing cells into injured arteries causes a decrease in intimal volume.30 This decrease is due primarily to a decrease in ECM volume rather than a change in cell number within the intima. The results of the present in vitro study support these in vivo observations and, furthermore, suggest that the effects of decorin overexpression may involve the modulation of TGF-β activity.

An initial decrease in DNA synthesis by decorin-overexpressing cells was observed during the first 24 hours after plating, but this relative decrease disappeared at later time points. The relative decrease in LDSN cells of [3H]thymidine incorporation and increase of cyclin-dependent kinase inhibitors p21 and p27 at 24 hours after plating are similar to the effects that were reported in studies of human cancer cell lines in which decorin inhibited growth by the upregulation of p21.15,17 However, in contrast to those studies, the inhibitory effect of decorin on DNA synthesis in ASMCs is transient; not shown). Western blot analysis indicated that ASMCs transduced with LXSN or LDSN expressed similar levels of the receptors I and II (Figure 6C). This observation indicates that differences in TGF-β1 receptor expression cannot explain the decreased response to TGF-β1 in decorin-overexpressing cells.
In these studies, the PAI-1 mRNA level was used as a well-established reporter for TGF-β1 activity. Earlier studies have also shown that TGF-β1 induces the expression of versican by ASMCs. Therefore, the current observation that the induction of versican mRNA by decorin-overexpressing ASMCs is altered in response to TGF-β1 is significant, inasmuch as versican is an important constituent of the ECM and an early response element in restenotic and atherosclerotic lesions. Moreover, decorin overexpression in vivo appears to significantly decrease the immunostaining for versican in rat carotid intimal lesions that develop in response to balloon catheterization, although we have not attempted to determine whether PAI-1 expression is altered in that model. Therefore, some of the effects of decorin overexpression during atherosclerotic lesion development in vivo may involve the antagonism of endogenous TGF-β activity.

In conclusion, the present study demonstrates that decorin overexpression clearly inhibits the response of ASMCs to TGF-β1. Moreover, despite transient effects on cell cycle–regulatory proteins and DNA synthesis, decorin overexpression by ASMCs had little effect on long-term growth in response to serum or PDGF stimulation. These data establish that decorin overexpression modifies cellular processes that are fundamental to the development of vascular fibrosis.

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References


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