Overexpression of Decorin by Rat Arterial Smooth Muscle Cells Enhances Contraction of Type I Collagen In Vitro

Hannu Järveläinen, Robert B. Vernon, Michel D. Gooden, Aleksandar Francki, Stephanie Lara, Pamela Y. Johnson, Michael G. Kinsella, E. Helene Sage, Thomas N. Wight

Objective—Overexpression of decorin reduces neointimal thickening in balloon-injured carotid arteries of rats by decreasing the volume of neointimal extracellular matrix (ECM). We examined the hypothesis that decorin regulates ECM volume by stimulating cell-mediated contraction of collagen-rich ECMs.

Methods and Results—Rat arterial smooth muscle cells (ASMCs) transduced with bovine decorin cDNA by retroviral transfection (LDSN) exhibited enhanced contraction of collagen gels in vitro when compared with vector-only transduced (LXSN) cells. Addition of recombinant decorin to LXSN or LDSN cells did not stimulate contraction of collagen gels. Enhanced contraction of collagen by LDSN cells was unaffected by the metalloproteinase inhibitor GM6001. LDSN cells exhibited increased expression of type I collagen mRNA when compared with that of LXSN cells. Correspondingly, collagen gel contraction by LDSN cells was reduced by inhibition of collagen synthesis by 3,4-dihydroxyproline (DHP). Antibodies to α1β1-integrin, but not to α2β1-integrin, blocked collagen contraction by both LXSN and LDSN cells. However, LXSN and LDSN cells expressed similar levels of α1- and β1-integrin mRNAs.

Conclusions—Decorin synthesized de novo by ASMCs increases type I collagen synthesis and enhances contraction of collagen gels. Regulated synthesis of decorin may be a useful therapeutic approach to reduce ECM volume in vascular disease. (Arterioscler Thromb Vasc Biol. 2004;24:67-72.)

Key Words: extracellular matrix ■ decorin ■ collagen, type I ■ muscle, smooth ■ integrin

Although inhibition of arterial smooth muscle cell (ASMC) proliferation is one approach to control intimal expansion in the early phases of vascular disease, an alternative strategy would be to limit the accumulation of extracellular matrix (ECM), which constitutes ≈80% of the volume of intimal lesions 4 weeks after injury. It is known that the ECM within atherosclerotic and restenotic lesions is a complex mixture of various components such as collagens and proteoglycans that have distinct temporal and spatial patterns associated with the genesis of vascular lesions.1 However, it is not clear why specific ECM components accumulate with time after vascular injury; nor is it clear whether this ECM accumulation can be controlled.

Some progress has been made in the regulation of tissue volume in response to injury. For example, decorin, a small, leucine-rich proteoglycan, inhibits the accumulation of ECM in animal models of glomerulonephritis and lung fibrosis.2,3 Furthermore, local overexpression of decorin by ASMC-mediated gene transfer at the site of arterial injury significantly reduces neointimal formation by reducing the volume of ECM in a rat vascular injury model in the absence of altered cell proliferation.4

The mechanism(s) by which decorin expression reduces ECM volume is unclear. A number of studies have shown that decorin interacts with fibrillar collagen to partly regulate collagen fiber diameter (for review, see Hocking et al5), as well as collagen packing.4,6 Thus, it is conceivable that decorin might regulate the volume of ECM solely via decorin–collagen binding interactions that influence the size and spacing of collagen fibrils.7 Such interactions could in turn have a profound effect on the capacity of cells to alter the packing of the collagen fibrils.

A variety of cell types, including ASMCs, pull strongly on their surrounding ECM by a process referred to as traction.8 Traction forces, which originate in the cytoskeleton and are transmitted to pericellular ECM by coupling molecules that include the transmembrane integrins, mediate the reorganization of ECM in a variety of situations that include wound repair, pathological fibrosis, and developmental morphogenesis. In vitro, cells dispersed within lattices (gels) of fibrillar type I collagen exert traction forces that can significantly reduce the volume of the surrounding collagen matrix—a phenomenon that is a well-established model for physical interactions between cells and ECM.
In the present study, we use a model of collagen gel contraction (CGC) to explore the hypothesis that decorin influences the volume of ECM by regulation of contractile forces generated by resident ASMCs.

Methods

Assays of CGC

The bovine decorin retrovirus (LDSN) was constructed as described previously. Assays of cell-mediated CGC were performed according to the method of Vernon and Gooden, using disc-shaped gels of 12.7 mm diameter comprised of 0.5 mg/mL type I collagen. In selected experiments, gels were supplemented with recombinant human decorin (purchased from Dr. F. Welser, EMP Genetech, Denzlingen, Germany) before or immediately after polymerization of the collagen. The concentration of exogenously added recombinant human decorin in the collagen gels was 835 nM, equivalent to a 1:2 molar ratio of decorin to collagen.

Collagen gel cultures were maintained from 16 to 48 hours and subsequently were fixed with 1% neutral-buffered formalin. Digital images of fixed gels were recorded under darkfield illumination with a Leitz-Wild stereomicroscope. Areas of gels were measured with the public domain National Institutes of Health Image program (http://rsb.info.nih.gov/nih-image/) according to the method of Vernon and Gooden. In selected experiments, actin cytoskeletons of vector-only transduced (LXSN) and LDSN cells in contracted collagen gels were labeled with phalloidin conjugated to Alexa Fluor 488 (Molecular Probes) and viewed by epifluorescence illumination with the Leitz-Wild stereomicroscope.

For studies of the effects of inhibition of matrix metalloproteinases (MMPs) on cell-mediated contraction of collagen, LDSN cells in polymerized collagen gels were supplemented with 20 μmol/L of the proteinase inhibitor GM6001 (Ilomastat; Chemicon International). For studies involving inhibition of collagen secretion, LDSN cells in polymerized collagen gels were supplemented with 1 to 2 mmol/L of proteinase inhibitor GM6001 (Ilomastat; Chemicon International).

For studies of integrin function, suspended ASMCs were incubated for 30 minutes in DMEM/10% calf serum supplemented with purified Armenian hamster monoclonal antibodies (mAbs) against rat integrin subunits-α1, -α2, or -β1 (clones Ha 31/8, Ha 1/29, and Ha 2/5, respectively, from PharMingen). The concentration of anti-α1, -α2, or -β1 mAbs present during the incubation was 5 μg/mL, 10 μg/mL, and 10 μg/mL, respectively. Subsequently, the incubation media, with suspended cells, were combined with collagen solutions that contained mAbs at the same concentration. The mixtures of collagen and cells were polymerized, cultured for 48 hours, and measured to assess contraction as described above.

Transmission Electron Microscopic Analyses

LXSN and LDSN cells cultured in collagen gels were fixed in half-strength Karnovsky solution, postfixed with 1% OsO₄ in 0.1 mol/L Na cacodylate buffer (pH 7.4), dehydrated, and embedded in Epon. Thin sections were cut and stained with uranyl acetate/lead citrate and subsequently were fixed with 1% neutral-buffered formalin. Digital images of fixed gels were recorded under darkfield illumination with a Leitz-Wild stereomicroscope. Areas of gels were measured with the public domain National Institutes of Health Image program (http://rsb.info.nih.gov/nih-image/) according to the method of Vernon and Gooden.

Results

Our previous studies demonstrated that rat ASMCs stably transfected with the retroviral vector containing the bovine decorin gene (LDSN) expressed intact bovine decorin in amounts of ~30 μg per 10⁶ cells over a 24-hour period. Western blot analysis of LDSN cells used in the present study confirmed similar levels of bovine decorin expression (data not shown).

In CGC assays, LDSN cells exhibited significantly greater contraction of collagen than did LXSN cells over a 4-fold range of cell number per gel (Figure 1A). The enhanced ability of LDSN cells to contract collagen corresponded to a robust generation of traction by these cells (Figure 1B) as indicated by the formation of cellular aggregates (a consequence of compression of ECM between adjacent cells) and by the appearance of networks of aligned collagen fibrils between the cellular aggregates. In contrast, LXSN cells did not aggregate or form fibrillar networks (Figure 1C). Active

The reverse transcription–polymerase chain reaction (RT-PCR) with oligonucleotide primers complementary to the rat cDNAs for these proteins.
engagement of collagen gels by resident LDSN cells was confirmed by transmission electron microscopy. LDSN cells were irregular in shape and exhibited an abundance of cellular projections and clefts that were intimately associated with bundles of extracellular fibrillar collagen (Figure 1D). In contrast, LXSN cells lacked cellular projections and were not surrounded by collagenous bundles (Figure 1E).

Addition of recombinant human decorin to collagen either before or after gelation did not affect contraction of the gels by either LXSN or LDSN cells (Figure 2). These results indicate that newly synthesized decorin is required for enhanced contraction of collagen gels by ASMCs.

The remodeling of connective tissues after injury is mediated in part by synthesis of ECM and by concomitant degradation of matrix by collagenolytic MMPs. For example, an increase in MMP activity occurs after balloon dilation of rat\(^1^6\) and pig\(^1^7\) carotid arteries. Moreover, inhibition of metalloproteinases by batimastat, a broad-spectrum inhibitor of MMPs, reduced constrictive narrowing of rat and porcine arteries after balloon dilation.\(^1^8,1^9\) In the present study, we assayed the contribution of MMP activity to the contraction of collagen gels by ASMCs by performing CGC assays in the presence of GM6001, which, like batimastat, is a broad-spectrum inhibitor of MMPs. We found that GM6001 had little or no effect on the robust contraction of collagen by LDSN cells (Figure 3), confirming a lack of an MMP effect on cell-mediated CGC.\(^2^0\)

Studies in vitro have shown that the effectiveness with which type I collagen gels are contracted by certain cell types is directly correlated with the capacity of the cells to secrete new type I collagen.\(^2^1\) Thus, it has been proposed that the newly synthesized pericellular collagen acts as a binding agent that transfers forces of cellular traction to the fibrils of the surrounding collagen gel. We compared the synthesis of type I collagen by LXSN and LDSN cells by RT-PCR analysis of mRNA coding for the \(\alpha_1\)-subunit of type I collagen. We found that LDSN cells cultured either on a substrate of tissue culture plastic or within gelled collagen expressed significantly greater levels of collagen mRNA than did LXSN cells cultured under similar conditions (Figure 4A). Subsequently, we examined the effect of collagen synthesis on contraction of collagen gels by LDSN cells. CGC assays were performed in the presence of L-DHP, a proline analog that impairs the secretion of collagen as a consequence of its inability to be hydroxylated by prolyl-4-hydroxylase.\(^2^2\) Compared with controls lacking L-DHP, the contraction of collagen was inhibited significantly by L-DHP at concentrations of 1 to 2 mmol/L (Figure 4B). Furthermore, cells in which collagen synthesis was inhibited lacked cellular projections and were not surrounded by collagenous bundles, resembling LXSN control cells (data not shown).
Traction forces generated by the cytoskeleton are transmitted to the surrounding ECM by specific molecules that span the plasma membrane. Foremost among these force-transducing receptors are the integrins, a family of \( \alpha \)- and \( \beta \)-subunits that associate noncovalently as \( \alpha \beta \)-heterodimers in 20 different combinations on the cell surface. The \( \alpha_1 \beta_1 \)- and \( \alpha_2 \beta_1 \)-integrins are important receptors for type I collagen, and both integrins mediate the contraction of collagen gels by a variety of cell types. Accordingly, we explored the possibility that differences in the function of the \( \alpha_1 \beta_1 \)- or \( \alpha_2 \beta_1 \)-integrins might contribute to differences in contraction of collagen gels by LXSN and LDSN cells. Contraction of collagen by LXSN or LDSN cells was completely blocked by a mAb that interfered with the binding of the rat \( \beta_1 \)-integrin subunit to collagen (Figure 5A). Moreover, contraction of collagen by LXSN and LDSN cells was inhibited by a mAb against the rat \( \alpha_1 \)-integrin subunit (Figure 5B), but not by a mAb against the rat \( \alpha_2 \)-integrin subunit (Figure 5C). RT-PCR analyses indicated that LDSN and LXSN cells cultured in type I collagen gels expressed similar levels of mRNA corresponding to the \( \alpha_1 \)- and \( \beta_1 \)-subunits of integrins.

**Discussion**

The involvement of ECM in the thickening of the vascular intima after balloon injury is well established. In fact, the majority of experimental studies show that cell proliferation only accounts for initial thickening in the first few weeks after injury, with the majority of subsequent thickening owing to accumulation of ECM. Furthermore, recent studies of advanced human atherosclerotic plaques and human in-stent restenosis show enhanced ECM accumulation as being a major determinant of lesion severity. Such results suggest that modulation of ECM composition or structure is likely to be of potential therapeutic value for treatment of cardiovascular disease.
Recent studies demonstrate that introduction of decorin-overexpressing ASMCs into injured rat carotid arteries is associated with a significant reduction in neointimal ECM volume with no change in cell density. Furthermore, lumen diameter was preserved in the decorin-overexpressing neointimas, whereas constrictive remodeling is usually associated with reduction of lumen diameter. Consistent with preservation of lumen diameter is the observation of enhanced packing of the collagen fibrils in the decorin overexpressing neointimas. Although the capacity of decorin to influence the organization of fibrillar ECM is not understood mechanistically, decorin might act directly on ECM to facilitate the packing of ECM fibrils, or act indirectly to influence the compression of ECM by resident cells. Consistent with the latter hypothesis is our finding in the present study that overexpression of decorin by LDSN ASMCs is accompanied by an increased capability of the cells to contract fibrillar collagen in vitro.

There is growing evidence that the capacity of cells to synthesize collagen influences their ability to contract surrounding collagenous matrices in vitro. For example, contraction of collagen gels by different clonal lines of bovine aortic endothelial cells is directly correlated with type I collagen synthesis. Moreover, contraction of collagen gels by murine embryonic fibroblasts is inhibited by arrest of type I collagen synthesis via inactivation of the collagen gene. Correspondingly, we observed in the present study that contraction of collagen gels by rat ASMCs is inhibited by L-DHP at concentrations previously shown to inhibit collagen synthesis by porcine carotid SMCs. From these observations, it may be inferred that newly synthesized pericellular collagen fibers might serve as bridges to couple the force-generating elements of the cytoskeleton to the bulk of the supportive ECM. Accordingly, an increase in the synthesis of pericellular collagen would enhance the physical coupling between the cell and the surrounding ECM and, consequently, would increase the effectiveness of traction-mediated ECM remodeling. However, whether changes observed in the pericellular organization of collagen in the LDSN cultures totally arose by new collagen synthesis awaits further study.

Decorin is reported to alter CGC by MG-63 osteosarcoma cells, although a lack of a decorin effect has been shown for CHO cells, indicating possible differences in the response between different cell types. It is conceivable that in addition to regulating the quantity of pericellular collagen, decorin might alter the thickness and/or spacing of the newly synthesized collagen fibrils in a manner that would maximize the transmission of traction forces to the surrounding ECM. There is precedent for the hypothesis that control of the multimeric structure of ECM affects the remodeling of collagen by the cells. For example, the degree to which cells polymerize a surrounding, insoluble fibronectin matrix is directly correlated with the effectiveness of cell-mediated contraction of collagen gels in vitro.

Decorin-mediated modulation of CGC must, in some capacity, involve collagen receptors on the surface of the ASMCs. The present study demonstrates that of the 2 integrins that are the major receptors for type I collagen (α1β1 and α2β1), it is α1β1-integrin that is the primary mediator of CGC by both LXSN and LDSN ASMCs. Other studies have shown that α1β1-integrin is the principal collagen receptor involved in the reorganization of collagenous matrix after injury. We observed that the expression of α1β1-integrin mRNA did not differ between LXSN and LDSN cells in vitro; therefore, it is unclear at present whether the functions of this integrin are regulated directly by decorin. It is conceivable that decorin could influence the disposition of α1β1-integrin at the cell surface indirectly via regulation of the availability of pericellular ECM ligands. In this context, it is noteworthy that inhibition of collagen synthesis by L-DHP did not affect the total expression of β1-integrins by porcine carotid SMCs but did disrupt normal clustering of β1-integrins on the SMC surface and inhibit engagement of collagen-coated substrates by the cells, as measured by spreading and migration assays in vitro.

The increase in collagen content and organization in decorin-overexpressing neointimas in vivo may confer substantial biomechanical advantages to the tissue. For example, decorin bound to fibrillar collagen increases the tensile strength of collagen fibers, an effect that could provide stability to the newly-remodeled tissue. Considerable recent interest has focused on the protective role of collagen in stabilizing the fibrous cap in vascular lesions and preventing plaque rupture. It is very possible that decorin could play an important therapeutic role in stabilizing plaque structure through collagen interactions.

In summary, manipulating the expression of decorin in ASMCs genetically causes dramatic changes in cell-mediated CGC. Such an effect could have a major impact on the ECM volume and biomechanical properties of vascular lesions. The utility of decorin-based gene therapy to treat cardiovascular disease awaits further study.

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References

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