Decorin Regulates Endothelial Cell Motility on Collagen I through Activation of Insulin-like Growth Factor I Receptor and Modulation of α2β1 Integrin Activity*

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The proteoglycan decorin is expressed by sprouting but not quiescent endothelial cells, and angiogenesis is dysregulated in its absence. Previously, we have shown that decorin core protein can bind to and activate insulin-like growth factor-I receptor (IGF-IR) in endothelial cells. In this study, we show that decorin promotes α2β1 integrin-dependent endothelial cell adhesion and migration on fibrillar collagen type I. We provide evidence that decorin modulates cell-matrix interaction in this context by stimulating cytoskeletal and focal adhesion reorganization through activation of the IGF-IR and the small GTPase Rac. Further, the glycosaminoglycan moiety of decorin interacts with α1β1 integrin, at a site distinct from the collagen I-binding A-domain, to allosterically modulate collagen I-binding activity of the integrin. We propose that induction of decorin expression in angiogenic, as opposed to quiescent, endothelial cells promotes a motile phenotype in an interstitial collagen I-rich environment by both signaling through IGF-IR and influencing α2β1 integrin activity.

Decorin is a component of the extracellular matrix, often found in association with collagen I-rich matrices (1, 2). As a member of the small leucine-rich repeat proteoglycan family, decorin is composed of a leucine-rich repeat core protein, and a single covalently attached glycosaminoglycan chain of varying length and composition (3, 4). Embryonic vasculogenesis and development is unaffected by the absence of decorin (5), which may be explained by compensation from other members of the small leucine-rich repeat proteoglycan family (6). However, the absence of decorin cannot be compensated for in the event of postnatal angiogenesis. Neangiogenesis in wounded cornea is reduced in the absence of decorin (7). Conversely, neangiogenesis is enhanced during dermal wound healing (8). These studies indicate a regulatory role for decorin in inflammation-associated angiogenesis. In accordance with these observations, decorin is not expressed by quiescent endothelium but is induced in nascent blood vessels formed under inflammatory conditions (7, 9, 10). Further, endothelial cells synthesize decorin only when undergoing angiogenic morphogenesis (11), and expression can be induced by the inflammatory mediators interleukin-6 and -10 (12).

However, investigation of whether decorin acts as a pro- or antiangiogenic factor has yielded conflicting results. In a collagen I environment, endogenous decorin enhances tube formation (10), but as a substrate, it inhibits this process (13). Exogenous decorin did not influence tube formation on matrigel (14) but inhibited vascular endothelial growth factor (VEGF)4-induced tube formation on this substrate (15). Suppression of VEGF activity through down-regulation of this growth factor has been previously described (14), although VEGF down-regulation could not be demonstrated in another study (7). These differences may in part depend on experimental design, including the manner in which decorin is presented, and may reflect a context-dependent response to decorin. Additionally, however, the use of denaturing agents and/or precipitation steps during isolation may compromise decorin activity, whereas differential post-translational modifications could also contribute. Of particular relevance, two studies have noted varying levels of activity of different decorin preparations in inhibiting tube formation (13, 15). Endothelial tube formation is itself a complex process requiring extensive rearrangement of cell-matrix interactions. Therefore, it is essential to investigate the role of decorin in directly regulating endothelial cell adhesion and motility. Decorin has been shown to inhibit endothelial cell adhesion and migration on collagen I (15, 16), which could

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1 Integrin Activity*

4 The abbreviations used are: VEGF, vascular endothelial growth factor; IGF-I, insulin-like growth factor-I; IGF-IR, insulin-like growth factor-I receptor; FCS, fetal calf serum; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxy-methyl)propane-1,3-diol; FAK, focal adhesion kinase; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; GTPγS, guanosine 5′-O-(thiotriphosphate).

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involve inhibition of VEGF activity (15) or promotion of peri-
cellular matrix organization (16). Alternatively, decorin could
inhibit cell-matrix interactions by masking integrin binding
sites (17). Although these mechanisms would certainly be
expected to contribute to modulation of tube formation by
decorin, direct interactions of decorin with cell surface recep-
tors could play an essential role in this process. For example, we
have shown that decorin core protein signals through the insu-
lin-like growth factor-I receptor (IGF-IR) in endothelial cells
(18). Moreover, IGF-IR activation modulates motility in endo-
thehial and other cells by influencing integrin affinity for their
matrix ligands (19–21). Decorin has also been reported to sup-
port platelet adhesion via interaction with the collagen I bind-
ing integrin α2β1 (22). This interaction could also be relevant
for control of endothelial cell behavior by decorin, since α2β1
integrin activity critically modulates endothelial cell motility
and capillary morphogenesis in a collagen I environment (23).

Coordinated signals from the extracellular matrix via inte-
grins and from growth factors to their receptors control endo-
thehial cell motility and angiogenic morphogenesis. We there-
fore hypothesized that the mechanism(s) by which decorin
regulates endothelial cell motility could involve direct activa-
tion of IGF-I receptor and/or α2β1 integrin on endothelial
cells. In this study, we investigated whether activation of these
receptors is involved in modulation of endothelial cell motility
by decorin.

EXPERIMENTAL PROCEDURES

Protein Production—Decorin was purified from conditioned
medium of human skin fibroblasts without denaturing and/or
precipitation steps using anion exchange chromatography
(DEAE-Sepharose) essentially as described (24). Decorin-con-
taining fractions were then applied to a Resource Q-15 column
(Amersham Biosciences), washed with 0.7 M NaCl in 20 mM
Tris-HCl, pH 7.4, and eluted with 1 M NaCl in 20 mM Tris-HCl,
pH 7.4. Decorin was dialyzed into PBS and concentrated to
>0.5 mg/ml by ultrafiltration (Centriprep YM-10; Amicon) to
preserve activity (25), aliquoted, and stored at −20 °C. Decorin
core protein was obtained by digestion with chondroitin ABC
lyase (Seikagaku Fine Biochemicals), at 0.2 milliunits/enzyme/µg of decorin in 0.1 M Tris-HCl, 0.03 M sodium acetate,
pH 8.0, at 37 °C for 2 h, followed by purification on DEAE-
Trisacryl M (Serva) as described (26). Decorin proteoglycan
and core protein purity was checked by SDS-PAGE followed by
silver staining. Immunoreactivity was confirmed using a poly-
clonal rabbit antiserum to human decorin, 1:500 (27). A 90-kDa
core protein complex immunoreactive with decorin antibodies
yielded exclusively sequences consistent with decorin as deter-
mined by matrix-assisted laser desorption/ionization-time-of-
flight mass spectrometry and tandem mass spectrometry
(Cardiff University Services Facility).5 Decorin activity was ver-
ified by induction of Akt phosphorylation in EA.hy926 cells as
previously described (28). Soluble human integrins α2β1 and
α1β1 and GST-α2A-domain fusion protein were recombini-
nantly produced and isolated as described (29, 30). Rhodocetin
was purified from the venom of the Malayan pit viper (29).

Dermatan sulfate (porcine skin, 90% l-iduronic acid, 10% chon-
droitin 4/6-sulfate) was obtained from Sigma and dissolved in
double-distilled water at 1 mg/ml. Integrin-modulating mono-
clonal antibodies JA221 and 9EG7 were produced as described
(30). The recombinant minicollagen FC3 contains the integrin-
binding motif GFPGER within a guest collagen triple helix of 10
GPP triplets.6

Cell Culture—The human endothelial cell line EA.hy926 (31)
was propagated in MCDB 131 medium (Invitrogen) supple-
mented with 10% fetal calf serum (FCS), 100 µM hypoxanthine,
0.4 µM aminopterin, 16 µM thymidine (Invitrogen), 100
units/ml penicillin, and 100 µg/ml streptomycin. For experi-
ments, cells were seeded on a fibrillar collagen I substrate and
cultured in Waymouth MAB 87/3 medium supplemented with
antibiotics and 0.5% heat-inactivated FCS for a minimum of
24 h. Collagen gels were prepared by coating hydrophilic cul-
ture dishes with acid-extracted rat tail tendon collagen imme-
diately following neutralization with serum-free medium/
NaOH as described (10). Where indicated, decorin (0.1–0.6
µM) was immediately mixed into the gels prior to setting.
Where required, cells were treated with 10 µM tyrphostin
AG1024 (10 mM stock in DMSO; Alexis) for 1 h prior to stim-
ulation.

Cell Migration Assay—A migration assay based on the work
of Korff and Augustin (32) was developed. Briefly, uniform
spheroids were generated by incubating 1,000 EA.hy926 cells/
well in hydrophobic 96-well plates (Greiner Bio-one) in Way-
mouth MAB 87/3 medium supplemented with 5% FCS, anti-
obitics, and 30% (v/v) methyl-cellulose (from filtered stock
solution of 1.3 g/ml methyl-cellulose (Sigma) in Waymouth
medium) for 48 h. Spheroids were removed using a stripette
and collected by centrifugation at 725 × g for 2 min, washed
with serum-free medium, and resuspended in medium supple-
mented with 0.5% heat-inactivated FCS and antibiotics to a
volume of 100 µl/spheroid. A 100-µl suspension was trans-
ferred per well of a 96-well plate containing 50 µl of fibrillar
collagen I or mixed decorin-collagen I gels. Wells were
inspected microscopically, and those containing a single spher-
oid located close to the center of the well were selected for
analysis. Plates were incubated at 37 °C and 5% CO2, and migra-
tion was recorded by capturing phase-contrast images at time
of plating and every 24 h thereafter for 5 days. Radial outgrowth
was determined from diameter measurements (n = 10).

Cell Attachment Assay—Cells were trypsinized and resus-
pended in serum-free medium, and 15,000 cells/well were
seeded into a 96-well tissue culture plate containing collagen I
or mixed decorin-collagen gels. Where required, cells were
mixed with soluble decorin immediately prior to seeding. Cells
were allowed to adhere for the indicated times at 37 °C and
washed in PBS. Adherent cells were quantified using crystal
violet staining as previously described (33).

Immunocytochemistry—Cells were seeded in serum-free
medium at 100,000/well of a 24-well plate onto glass coverslips
coated with fibrillar collagen I or mixed decorin-collagen gels.
After incubation at 37 °C and 5% CO2 for the indicated time

5 G. Martin, and D. Aeschlimann, and E. Schönherr, unpublished data.
6 S. Niland and J. A. Eble, manuscript in preparation.
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period, adherent cells were fixed in 4% paraformaldehyde/PBS for 10 min and permeabilized with 0.2% Triton X-100 in PBS. Nonspecific binding sites were blocked with 1% BSA/PBS. F-actin was visualized by staining with 1 μg/ml fluorescein isothiocyanate-conjugated phalloidin (Sigma) and focal adhesions with antibodies against vinculin (6 μg/ml mAb VIN-11-5; Sigma), detected with TRITC-conjugated anti-mouse IgG (1:100; ICN/Cappel).

FAK Signaling—Adherent cells (5 × 10^5 cells/35-mm dish) were cultured on collagen I gels under low serum conditions (0.5% FCS) for 48 h prior to stimulation. Alternatively, trypsinized cells (1 × 10^6 cells/well of a 24-well plate) were seeded in serum-free medium onto fibrillar collagen I gels in the absence or presence of decorin or onto mixed decorin-collagen I gels. At the indicated times, protein extracts were prepared as previously described (33). Equal amounts of protein, as determined by BCA assay (Pierce), were separated on 4–12% SDS-PAGE BisTris gels (Invitrogen) under reducing conditions and transferred to nitrocellulose membranes (Protran; Whatman). After blocking with TBS containing 5% nonfat dried milk and 0.1% Tween 20, membranes were probed with antibodies to Tyr(P)397 FAK (0.5 μg/ml; Calbiochem), followed by incubation with horseradish peroxidase-conjugated antibodies to rabbit IgG (1:2000; DakoCytomation) in 1% nonfat dried milk, 0.1% Tween 20 in TBS for 1 h each at room temperature. Membranes were washed between incubations and prior to development in TBS, 0.1% Tween 20 and developed with Supersignal ECL reagent. As a control for equal loading, membranes were probed for β-tubulin (TUB2.1, 1:500; Sigma), detected with horseradish peroxidase-conjugated antibodies to mouse IgG (1:2000, DakoCytomation), or stripped (60 mM Tris/HCl, pH 7.4, 0.7% 2-mercaptoethanol, 2% SDS for 30 min at 50 °C) and reprobed with antibodies to FAK (1:100, PC314; Oncogene).

GTase Assays—Adherent cells (3 × 10^6 cells/10-cm dish) cultured on fibrillar collagen I gels were serum-starved for 24 h prior to stimulation with 0.7 μM decorin. At the indicated times, cells were washed with PBS and lysed in 500 μl of lysis buffer supplied with the Rac activation assay kit (Cytoskeleton Inc.) per dish. The active form of the small GTases was captured from 1 ml of cell lysate at 700 μg/ml protein using GST-tagged PAK-PBD beads as specified by the manufacturer, and Rac-1 was detected by immunoblotting. Alternatively, active Rac-1 was quantified using the G-LISATM Rac activation assay kit (Cytoskeleton Inc.) as specified by the manufacturer. Briefly, 3.5 × 10^5 cells/35-mm dish were cultured on collagen I gels, serum-starved for 24 h, and pretreated with inhibitors for 1 h prior to stimulation with 0.7 μM decorin for 15 min.

Integrin Binding Studies—Microtiter plates were coated overnight at 4 °C with 30 μg/ml decorin, 12.5 μg/ml FC3 peptide in PBS, or 10 μg/ml collagen I in 1 ml acetic acid. After washing with TBS (50 mM Tris/HCl, pH 7.4, 150 mM NaCl) containing 2 mM MgCl_2 (TBS/Mg), nonspecific binding sites were blocked with 1% BSA in the same buffer (blocking buffer). Integrins were diluted in blocking buffer containing 1 mM MnCl_2, unless otherwise indicated, and mixed with additives or competitors at specified concentrations immediately prior to plating. For analysis of pH dependence, ampholines (pH 4–10; Amersham Biosciences) at 40 mg/ml were used as buffering agents. The integrin solution was incubated with immobilized substrates for 2 h at room temperature. After washing, bound integrin was chemically fixed and quantified by ELISA using a rabbit antiseraum against the β1 subunit (1:600) as described (30). Experiments with GST-integrin α2A-domain followed the same procedure, and binding was detected using polyclonal rabbit antibodies to the GST moiety (Molecular Probes) (30).

Statistics—Statistical significance within matched groups was assessed using repeated measures analysis of variance and Dunnett multiple comparison test with p < 0.05 considered statistically significant (*, p < 0.05; **, p < 0.005).

RESULTS

Decorin Enhances α2β1 Integrin-dependent Endothelial Cell Adhesion and Migration on Fibrillar Collagen I—To investigate decorin effects on endothelial cell motility during sprout formation from preexisting vessels, we adapted a three-dimensional spheroid model. This system has established cell-cell contacts and mimics the quiescent endothelial cell phenotype (32). Transferring spheroids onto the surface of fibrillar collagen I gels induced radial outward migration of endothelial cells (Fig. 1A). Decorin, as a soluble medium supplement (as a model for endothelial cell-secreted decorin), enhanced this process and promoted formation of elongated, sproutlike extensions (Fig. 1A), an effect that was dose-dependent (Fig. 1B). Enhanced proliferation did not appear to contribute significantly to this observation, since migration was conducted in low serum (0.5% FCS) and reached a plateau at extended time points (data not shown), indicating the presence of a finite cell population. Further, decorin does not appear to modulate endothelial cell proliferation (15, 16). To delineate the mechanism of endothelial cell motility in our system, we used rhodocetin, a specific inhibitor of the major collagen I-binding integrin, α2β1. Rhodocetin reduced outward migration of endothelial cells from spheroids in a dose-dependent manner, and cells adopted a rounded morphology (Fig. 1, A and C), indicating that motility was, at least in part, mediated by α2β1 integrin. Since decorin deposited by fibroblasts regulates collagen I fibrillogenesis, it could affect α2β1 integrin-mediated cell-collagen I interactions by altering the supramolecular structure of collagen aggregates. To test this hypothesis, collagen was polymerized in the presence of decorin. Under these conditions, endothelial cell migration was also enhanced (Fig. 1D). Alternatively, since decorin has been reported to support platelet adhesion by direct interactions with α2β1 integrin (22), decorin-containing substrates could similarly support endothelial cell attachment and migration. By itself, however, decorin failed to support endothelial cell attachment (Fig. 2A). Rhodocetin inhibited adhesion to collagen I (Fig. 2A), indicating that adhesion was mediated, at least in part, by α2β1 integrin. In contrast, decorin promoted endothelial cell attachment to collagen I when presented either as a medium supplement or in a collagen-bound form (Fig. 2A). Enhancement of adhesion by both soluble and collagen-bound decorin was dose-dependent and was apparent up to at least 3 h after seeding (Fig. 2B and data not shown). To investigate which moiety of decorin was involved in enhancing adhesion, endothelial cells were seeded onto collagen gels in the presence of either decorin core protein or glyco-
aminoglycan alone or onto collagen gels that had been prepared in the presence of these moieties. Neither moiety of decorin by itself had any similar effect, indicating that the intact proteoglycan is required to enhance adhesion to collagen I (supplemental Fig. 1). These data demonstrate that decorin, irrespective of its presentation as either a soluble factor or in association with collagen I fibrils, influences the cellular interactions with collagen and that α2β1 integrin plays a central role in this system.

Decorin Induces a Motile Phenotype by Activating IGF-IR and the Small GTPase Rac—To further delineate decorin effects on motility, the cellular phenotype induced by decorin during adhesion to fibrillar collagen I was investigated. In cells adhering to collagen I alone, mature focal adhesions associated with actin stress fibers were formed within 1 h (Fig. 3, A–C, Col I, arrows). In contrast, when cells were seeded onto collagen gels in the presence of soluble decorin or onto collagen-bound decorin, large vinculin-positive focal complexes (Fig. 3, A–C, Dcn and Dcn-Col I, arrows), ruffles, and microspike-like structures (Fig. 3, A–D, Dcn and Dcn-Col I, arrowheads) were frequently observed at the periphery of cells.

In a manner reminiscent of these morphological changes, activation of the small GTPase Rac-1 induces membrane ruf-
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FIGURE 3. Decorin modulates endothelial cell morphology and activates Rac via IGF-IR. Endothelial cells were seeded onto collagen gels in the absence (Col I) or presence of 0.5 μm decorin (Dcn) or onto mixed decorin-collagen gels (Dcn-Col I) in serum-free medium. After 1 h, actin stress fibers (A, top) and focal adhesions (A, bottom) were visualized with fluorescein isothiocyanate-phalloidin and anti-vinculin (TRITC), respectively. Decorin treatment induces ruffles and microspike-like structures (arrowheads) and formation of large peripheral adhesion complexes (arrows). Size bar, 20 μm. Higher magnifications of areas in A indicated in white are shown in B and in C, where the images shown in B were overlaid with the corresponding images of actin stress fibers. D, confocal images of cells seeded onto collagen gels in the absence of decorin or onto mixed decorin-collagen gels. Size bar, 10 μm. After 1 h, actin stress fibers were stained as described for A. To analyze Rac activity, serum-starved adherent endothelial cells were stimulated with 0.7 μm decorin in serum-free medium for the indicated times, and active Rac was precipitated from cell lysates with p21-activated kinase binding domain beads. To visualize Rac levels in unstimulated cells, cell lysate was incubated for 15 min with nonhydrolyzable GTPγS. Active Rac was detected by Western blotting with antibodies specific for Rac (D). For quantitative analysis, an ELISA-style assay was used, with binding domain of Rac effector protein (Rac G-LISA™) as the immobilized phase, and bound Rac was detected with specific antibodies. Cells cultured for 24 h in serum-free medium were treated with 10 μM AG1024 to block IGF-IR signaling or an equal volume of vehicle (DMSO) for 1 h prior to stimulation with 0.6 μM decorin for 15 min, and cell lysates were assessed for active Rac (F). Levels of active Rac in cell lysates were assessed by ELISA and represent the mean ± S.E. of five replicate analyses for each condition. In some experiments, basal Rac activity was lower in the presence of the IGF-IR inhibitor than in its absence. Independent of this basal activity, decorin induced Rac always to a similar degree and only in the absence of AG1024.

FIGURE 4. Decorin enhances endothelial cell motility by activating IGF-1 receptor. A and B, spheroids were treated with 10 μM AG1024 to block IGF-IR signaling or with an equal volume of vehicle (DMSO) for 1 h prior to stimulation with soluble (Dcn; A) or matrix-bound decorin (Col I-Dcn; B). Endothelial cell migration on collagen I was quantified as in Fig. 1. Data are shown as the mean of 10 spheroids ± S.E. In C, decorin as an additive is not degraded (left); nor do endothelial cells produce endogenous decorin under the assay conditions (absence of inflammatory cytokines) (right). Conditioned medium was analyzed at the conclusion of migration experiments by digestion with chondroitin ABC lyase (ABCase) and immunoblotting with antibodies to decorin core protein, detected with anti-rabbit horseradish peroxidase (left) or anti-rabbit biotin followed by streptavidin-horseradish peroxidase (right). The left panel indicates that soluble decorin was not degraded over the time course of the experiment (Std, purified decorin standard; proteoglycan and core protein; Dcn, solubilised decorin-conditioned medium). The right panel indicates that decorin was released from decorin-containing collagen gels (1, chondroitin ABC lyase; 2, conditioned medium from Col I-Dcn gel; 3, conditioned medium from Col I gel). Migration of molecular mass markers is indicated on the right. Dcn PG, intact proteoglycan showing up as a characteristic “smear”; arrows, monomeric decorin core protein of 43 and 45 kDa, corresponding to two or three N-linked oligosaccharides, respectively (24); arrowhead, decorin complex of ~90 kDa (resistant to boiling in SDS and reduction), consistent with decorin core dimer; asterisk, contaminant originating from chondroitin ABC lyase that reacted with secondary antibodies.

flinging and formation of peripheral focal complexes (34). Therefore, we investigated whether decorin induces Rac activation. Indeed, Rac activation occurred within 15 min of incubation of decorin with adherent cells (Fig. 3E). Since we have shown that decorin can activate IGF-IR signaling pathways in these cells (18), we investigated whether Rac activation by decorin involved signaling through this receptor. To this end, serum-starved endothelial cells were treated with AG1024, an inhibitor of IGF-IR, or vehicle (DMSO) for 1 h prior to stimulation with decorin. Decorin enhanced levels of active Rac 1.3-fold but could not stimulate Rac activity when IGF-IR activation was blocked (Fig. 3F). To ascertain whether activation of IGF-IR by decorin was therefore responsible for enhanced motility, endothelial cell spheroids migrating on collagen I were treated with AG1024 prior to the addition of decorin. Under these conditions, decorin did not promote endothelial cell migration from spheroids (Fig. 4A). Similarly, blocking IGF-IR activation prevented collagen I-bound decorin from promoting endothelial cell migration (Fig. 4B), indicating that decorin promotes endothelial cell motility by signaling through IGF-1R.

Matrix metalloprotease activity is essential for sprout formation and invasion through interstitial matrices, and endothelial cells in contact with fibrillar collagen I up-regulate MMP2 (matrix metalloprotease 2) (35). Since decorin is a substrate of MMP2 (36), we tested conditioned media at the conclusion of the migration assay for decorin or proteolytically processed products. Since the endothelial cells did not produce endogenous decorin under our noninflammatory culture conditions (Fig. 4C, right, lane 3), in line with previous results (12), the detected decorin must have originated from exogenously added decorin. Only the intact proteoglycan could be detected where decorin had been added to the
media (Fig. 4C, left), indicating that the intact proteoglycan rather than proteolytically processed products modulates endothelial cell motility in our system. However, since the collagen I-immobilized decorin was also found in the conditioned media (Fig. 4C, right, lane 2), the effects of collagen-bound decorin cannot be easily distinguished from that of soluble decorin.

Decorin Counteracts the Effects of Rhodocetin on \( \alpha_2\beta_1 \) Integrin Activity—Activation of \( \alpha_2\beta_1 \) integrin and/or IGF-1R can modulate activity of focal adhesion kinase (FAK), an intracellular integrin-associated protein involved in control of focal adhesion turnover and cell motility. To investigate whether decorin influences FAK activity, endothelial cells were cultured on fibrillar collagen I and incubated with either decorin or rhodocetin. Rhodocetin reduced FAK autophosphorylation at Tyr397 (Fig. 5A), in accordance with its role as an antagonist of \( \alpha_2\beta_1 \) integrin. In contrast, decorin did not influence FAK Tyr397 phosphorylation under the same conditions (Fig. 5B). To test whether decorin instead influenced FAK autophosphorylation during formation of new cell-extracellular matrix contacts, endothelial cells were seeded onto fibrillar collagen I in the presence of soluble decorin or onto mixed decorin-collagen I gels. Decorin did not induce any changes in FAK Tyr(P)397 compared with cells adhering to collagen I alone (Fig. 5C). Thus, in contrast to rhodocetin, classical integrin-mediated signaling by collagen I is not altered by decorin. Further, decorin-mediated enhancement of adhesion appears to be independent of alterations in classical integrin-mediated signaling.

Decorin Modulates \( \alpha_2\beta_1 \) Integrin Binding Activity to Collagen I—Since decorin promotes motility on collagen I, an \( \alpha_2\beta_1 \) integrin-dependent process, we investigated whether decorin could modulate \( \alpha_2\beta_1 \) integrin binding activity to collagen I. In accordance with its role in blocking \( \alpha_2\beta_1 \) integrin-collagen I interaction, rhodocetin induced a rounded morphology in endothelial cells cultured on collagen I (Fig. 6A). In contrast, decorin induced more subtle changes in cell morphology (similar to those shown in Fig. 3A), and cells remained spread (Fig. 6A), consistent with undisrupted \( \alpha_2\beta_1 \) integrin-collagen I interaction.
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interaction and persistent FAK activation. However, when adherent cells were preincubated with decorin for 1 h prior to treatment with rhodocetin for a further 1 h, decorin counteracted the morphological changes induced by rhodocetin (Fig. 6A) and, correspondingly, prevented the reduction in FAK Tyr<sup>972</sup> phosphorylation induced by rhodocetin (Fig. 6B). Simultaneous co-incubation of decorin and rhodocetin showed similar results (Fig. 6C). Therefore, although decorin by itself did not alter collagen I-induced signaling through α2β1 integrin, it interfered with inhibition of collagen I-α2β1 integrin interaction by rhodocetin. By ELISA and dot blot assays, we did not detect any direct interaction between rhodocetin and decorin (data not shown). These data therefore provide evidence that decorin and rhodocetin could target the same molecule on endothelial cells: α2β1 integrin.

Decorin Interacts with α2β1 Integrin via Its Glycosaminoglycan Chain at a Site Distinct from That of the Collagen I- and Rhodocetin-binding A-domain—To further study direct interaction between α2β1 integrin and decorin, we used an established solid-phase assay (29). The α2β1 integrin bound to immobilized decorin in a dose-dependent manner, approaching saturation (Fig. 7A). Similar to collagen I binding of α2β1 integrin, the interaction was divalent cation-dependent, since it was abolished in the presence of EDTA (Fig. 7A). For binding in the presence of Mn<sup>2+</sup> ions and the integrin-activating antibody 9EG7, the apparent K<sub>D</sub> value for α2β1 integrin-decorin interaction was estimated according to Heyn and Weischet (37) to be in the range of 30–35 nM. The α2β1 integrin recognizes collagen I and rhodocetin via the classical ligand-binding A-domain within the α2 subunit. To test whether decorin similarly interacts with α2β1 integrin, we employed a previously characterized GST-α2A fusion protein (30). No binding of α2A-domain to decorin could be detected up to 9 μM α2A-domain, conditions under which binding to collagen had reached saturation (Fig. 7B). Further, this interaction of decorin is specific for α2β1 integrin, since no interaction of decorin with the related collagen I-binding integrin, α1β1, could be detected (Fig. 7C). This could also indicate that the binding site for decorin is located at least partially within the α2 subunit.

The interaction of α2β1 integrin with decorin was then further analyzed under conditions that enhance or inhibit binding to collagen type I (30). Binding of α2β1 to either immobilized collagen or decorin was similarly enhanced in the presence of the activating mAb 9EG7 and Mn<sup>2+</sup> over Mn<sup>2+</sup> alone (Fig. 7D). However, another activating mAb, JA221, that enhanced integrin binding to collagen did not influence binding to decorin (Fig. 7D). Further, Ca<sup>2+</sup> ions, which decrease the affinity of α2β1 integrin for collagen I, could not inhibit interaction of the integrin with decorin (Fig. 7D). Interestingly, the inhibitor rhodocetin, which competitively blocks collagen I-A-domain interaction (30), abolished binding of α2β1 integrin to collagen type I but enhanced integrin binding to decorin (Fig. 7D). We also tested whether rhodocetin could similarly promote binding of the isolated α2 A-domain to decorin but did not observe any interaction under these conditions (data not shown). These data suggest that decorin interacts with a site on the α2 subunit distinct from the classical ligand-binding A-domain.

To delineate the decorin moiety responsible for interaction with α2β1 integrin, binding of soluble α2β1 integrin to immobilized decorin was assessed in the presence of increasing concentrations of either the intact proteoglycan or its moieties, glycosaminoglycan side chains or purified core protein. Both dermatan sulfate (Fig. 7E) and chondroitin 4/6-sulfate (data not shown) dose-dependently and completely blocked integrin binding to decorin. In contrast to the glycosaminoglycan-bearing entire decorin molecule, decorin core protein did not consistently or strongly interfere with this interaction (Fig. 7F). These data indicate that the glycosaminoglycan moiety of
decorin plays a key role in the interaction of $\alpha_2\beta_1$ integrin with decorin.

**Decorin Allosterically Modulates $\alpha_2\beta_1$ Integron-Collagen I Interaction**—Since rhodocetin may preferentially bind to and/or stabilize an inactive conformation of $\alpha_2\beta_1$ integrin (30) and enhance interaction of $\alpha_2\beta_1$ integrin with decorin, this could indicate that decorin preferentially interacts with an inactive integrin conformation. Under mildly acidic conditions, $\alpha_2\beta_1$ integrin also adopts a less active conformation, with reduced binding affinity for collagen I (30). Thus, interaction of $\alpha_2\beta_1$ integrin with collagen I was inhibited below pH 7 (Fig. 8A). In contrast, interaction with decorin was maximal under mildly acidic conditions (Fig. 8A). However, below pH 5, integrin interaction with decorin was abolished, presumably because of denaturation of $\alpha_2\beta_1$ integrin under strongly acidic conditions (Fig. 8A).

To test whether decorin binding to $\alpha_2\beta_1$ integrin influences the interaction with collagen I, a recombinant minicollagen, FC3, was employed. FC3 contains an $\alpha_2\beta_1$ integrin binding site but does not interact with decorin. $\alpha_2\beta_1$ integrin bound dose-dependently to FC3 in a cation-dependent manner (Fig. 8B). However, premixing of $\alpha_2\beta_1$ integrin with decorin reduced integrin interaction with FC3 (Fig. 8B). Since decorin does not interact with either FC3 or the collagen I-binding A-domain of the integrin $\alpha_2$ subunit, a competitive mode of inhibition does not appear likely. However, the consistently reduced binding signals indicate that decorin could compromise the collagen-binding capability of $\alpha_2\beta_1$ integrin by inducing/stabilizing a less active conformation.

**DISCUSSION**

The role played by decorin in angiogenesis is controversial and is further complicated by the large number of potential interactions of decorin with both matrix components and cell surface receptors. Angiogenesis is mediated by growth factors acting in concert with extracellular matrix-derived signals; hence, decorin could contribute to angiogenesis in a contextual manner, as a matrix organizer and/or through direct signaling. In this study, we sought to examine the role of decorin by using an easily manipulated *in vitro* model in order to minimize interference from the complexity of the physiological angiogenic environment.

Using this model, we present evidence that decorin regulates angiogenesis through the following two molecular mechanisms. 1) Decorin-IGF-IR interaction on endothelial cells triggers Rac activation, which is in line with actin cytoskeleton rearrangement and enhanced motility. 2) Decorin influences activity of $\alpha_2\beta_1$ integrin, one of the major collagen I receptors on endothelial cells.

At first glance, the concentrations of decorin used in this study (100–700 nM) may seem rather high, although they are consistent with those found in collagen I-rich connective tissues (38, 39). Albeit unknown, the local concentration of soluble decorin synthesized by sprouting endothelial cells could conceivably be high and might also locally accumulate by association with collagen I. Further, since decorin may be dimeric in solution (4), the concentration of a biologically relevant form of decorin may in fact be substantially less than that calculated based on a monomeric molecular mass. It is also to be expected that greater concentrations of decorin might be required to elicit effects similar to those of IGF-I. First, the affinity of decorin for the IGF-I receptor is 10-fold less than that of IGF-I (18), and second, the amount of decorin available to interact with the IGF-I receptor would be less than that added, due to competing interactions of decorin with both collagen I matrices and with alternative cell surface receptors (such as $\alpha_2\beta_1$ integrin). For comparison, maximal stimulation of endothelial cells is achieved with 50–200 ng/ml (7–28 nM) IGF-I, whereas 100 nM decorin already stimulated adhesion and motility (although higher concentrations are required for statistical significance). Given these considerations, the concentrations employed in this study do not seem unreasonable.

Decorin on its own did not support endothelial cell adhesion but induced a motile phenotype and promoted adhesion and migration on fibrillar collagen I, suggesting that it acts as a modulator of integrin-extracellular matrix interactions. In contrast, decorin previously inhibited endothelial cell adhesion and migration on collagen I (15, 16). Different sources of decorin or use of natively purified decorin, as in the current study, could have contributed to these contrasting results. Further, we studied motility of endothelial cells from three-dimensional spheroids rather than single cell suspensions or monolayers. Integration of endothelial cells into spheroids prevents apoptosis, induces quiescence, and results in established cell-cell contacts (32), which may result in differential cell responses, and more closely reflect the *in vivo* situation during endothelial cell sprouting from existing vessels.

Additional to direct activation by extracellular ligands, integrin activity and cell motility are controlled by inside-out signaling, whereby growth factor receptor activation disrupts intracellular integrin subunit interactions to influence ligand binding at the ectodomain (40). Therefore, inside-out signaling by decorin through IGF-IR could be responsible for modulation of $\alpha_2\beta_1$ integrin-mediated motility, in accordance with our observation that decorin-mediated enhanced motility was dependent on IGF-IR activity. It could be argued that the IGF-IR inhibitor AG1024 had effects other than blocking IGF-IR signaling, since treatment with inhibitor alone already
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In contrast to the antagonistic α2β1 integrin inhibitor rhodocetin, decorin alone could not alter collagen I-α2β1 integrin-triggered activation of FAK. This may seem surprising, since peptides derived from decorin core protein altered VEGF-induced FAK phosphorylation (15). However, it was not shown whether these peptides alone modulated FAK phosphorylation, and in agreement with our data, IGF-I-induced, FAK-independent motility has been previously observed (52, 53). Further, decorin alone could not alter FAK phosphorylation; nor as a substrate could it support integrin activation required to mediate endothelial cell adhesion. These observations are in agreement with a lack of interaction of decorin with the A-domain of the α2 subunit, since interactions with this domain would be expected to result in transduction of ligand occupancy to intracellularly associated FAK. Hence, we consider decorin neither as an antagonist nor as an agonist of α2β1 integrin but rather as a modulator of its collagen I binding activity.

In the cell-free system, the glycosaminoglycan moiety, but not the core protein of decorin, was principally responsible for interaction with α2β1 integrin. Nevertheless, the intact proteoglycan was required to modulate cell adhesion to collagen I. This could indicate that a simultaneous interaction of decorin with collagen I (via the core protein) and α2β1 integrin (via the glycosaminoglycan) could act as a bridge and thereby modulate collagen I-α2β1 integrin interaction, potentially increasing the number of α2β1 integrin-collagen contacts of a cell. However, it could also be speculated that decorin glycosaminoglycan-α2β1 integrin interaction in conjunction with decorin core protein-IGF-IR interaction (18) could influence stability and cross-talk within cell surface receptor complexes. Indeed, the large number of potential interactions of decorin with multiple matrix components and cell surface receptors makes it difficult to clearly ascertain the contribution of individual interactions to a complex process such as angiogenesis. Despite this, direct interaction of decorin with cell surface receptors of endothelial cells has been highlighted in this study as likely to play an important role in regulating endothelial cell motility. Further, the activities of both α2β1 integrin and small Rho GTPases, which have been shown in this study to be modulated by decorin in endothelial cells, are critically involved in vessel formation and maturation (23, 54, 55), providing potential mechanisms for decorin in modulating capillary morphogenesis.

In conclusion, we suggest that decorin modulates endothelial cell behavior independent of masking integrin binding sites and of its role in collagen fibrillogenesis. We also provide evidence that decorin influences α2β1 integrin-mediated endothelial cell responses by signaling through IGF-IR and/or by allosteric modulation of α2β1 integrin activity.

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