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Authors

Järveläinen, HT
Iruela-Arispe, ML
Kinsella, MG
et al.

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Expression of Decorin by Sprouting Bovine Aortic Endothelial Cells Exhibiting Angiogenesis *in Vitro*

HANNU T. JÄRVELÄINEN,*† M. LUISA IRUELA-ARISPE,† MICHAEL G. KINSELLA,*
LINDA J. SANDELL,‡§ E. HELENE SAGE,† AND THOMAS N. WIGHT*¹

*Department of Pathology, †Department of Biological Structure, and ‡Department of Orthopaedics and Biochemistry, University of Washington, Seattle, Washington 98195; §Veterans' Administration Medical Center, Seattle, Washington 98108; and ¶Department of Medical Biochemistry, University of Turku, Kiinamyllynkatu 10, SF-20520 Turku, Finland

In our recent studies, we have demonstrated that monolayer cultures of bovine aortic endothelial (BAE) cells that do not express type I collagen also fail to express and synthesize decorin, a small chondroitin/dermatan sulfate proteoglycan that interacts with type I collagen and regulates collagen fibrillogenesis *in vitro*. However, BAE cells exhibiting a spontaneous sprouting phenotype and a predisposition toward the formation of cords and tube-like structures (an *in vitro* model for angiogenesis) initiate the synthesis of type I collagen during their morphological transition from a polygonal monolayer to an angiogenic phenotype. In the present study, we examined whether BAE cells also initiate the synthesis of the proteoglycan decorin during this morphological transition. We show by Northern blot analysis and by immunochemical methods that BAE cell cultures containing sprouting cells and cords, but not monolayer cultures of these cells, express and synthesize decorin ($M_r \sim 100,000$). We also show that type I collagen expression by BAE cell cultures is initiated concomitantly. However, the localization of decorin and type I collagen in cord and tube-forming BAE cell cultures is not completely identical. Type I collagen is detected only in sprouting BAE cells and in endothelial cords, whereas decorin is also apparent in BAE cells surrounding the cords and tubes. Our results indicate that the synthesis of decorin as well as type I collagen is associated with endothelial cord and tube formation *in vitro*. © 1992 Academic Press, Inc.

INTRODUCTION

Angiogenesis, the formation of new capillaries from preexisting vessels, is a critical event in a variety of both normal and pathological processes that include embryogenesis, inflammation, wound healing, and tumor growth [1]. There is evidence that angiogenesis also plays a role in the development of coronary atherosclerosis [2]. Although the cellular events leading to the for-

mation of new capillaries are not entirely clear, it is known that angiogenesis involves protease production and migration and proliferation of endothelial cells and their differentiation into capillary tubes [3]. The assembly of endothelial cells into tubes resembling capillaries has been demonstrated in culture [4]; this model system has been used to study factors that regulate the angiogenic behavior of endothelial cells.

It is now well established that not only growth factors [1, 5–7], but also various components of the extracellular matrix [5, 8–16] contribute to the formation of endothelial tubes during angiogenesis. One of the extracellular matrix molecules that is involved in angiogenesis both *in vivo* and *in vitro* is type I collagen [12, 17] especially when present in fibrillar form [13, 17]. A molecule that is associated with all major type I collagen-rich tissues [18] and that is also known to interact with type I collagen fibrils [19, 20] and to affect collagen fibrillogenesis *in vitro* [21–23] is decorin, a small interstitial chondroitin/dermatan sulfate proteoglycan (M_r 90–180 kDa). In our recent studies, we have demonstrated that bovine aortic endothelial (BAE) cells that grow as non-sprouting monolayers synthesize neither decorin nor type I collagen [24]. However, sprouting BAE cells that form cords and tubes initiate the synthesis of type I collagen [12]. Thus, in this study we asked whether decorin synthesis is also initiated during the formation of endothelial cords and tubes *in vitro*. Our results demonstrate that decorin synthesis is induced in BAE cell cultures when they modulate from a cobblestone-like monolayer to a sprouting phenotype. We propose that decorin, together with type I collagen, is specifically related to the angiogenesis-like behavior of BAE cells in culture.

MATERIALS AND METHODS

Cell culture. Endothelial cells were isolated from adult bovine aorta, cloned, and subcultured as previously described [25]. Strains of BAE cells exhibiting a spontaneous sprouting phenotype in culture [26, 27] were used in these experiments. A BAE cell strain with a nonsprouting phenotype served as a negative control for decorin synthesis [24]. The cells were grown in Dulbecco's modified Eagle's me-

¹ To whom reprint requests should be addressed at Department of Pathology, SM-30, University of Washington, Seattle, WA 98195. Fax: (206) 543-3644.

dium (DMEM; GIBCO Laboratories, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT), containing 250 µg/ml amphotericin B (Sigma Chemical Co., St. Louis, MO) and 100 U/ml penicillin and streptomycin (Sigma).

Metabolic labeling of cultures. Cultures of BAE cells at different stages of cord and tube formation were preincubated for 30 min in serum- and sulfate-free DMEM, followed by a 24-h incubation with sulfate-free DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum and containing the same antibiotics as above as well as 100 µCi/ml carrier-free Na₂[³⁵S]O₄ (ICN Biomedicals, Inc., Irvine, CA). Total [³⁵S]sulfate incorporation in proteoglycans (PGs) was determined by precipitation with cetylpyridinium chloride [28, 29].

RNA extraction and Northern blot analysis. Total RNA was isolated from cell cultures as described [30]. The ratios of absorbance at 260 vs 280 nm of RNA samples used in Northern blot analyses were greater than 1.8. RNAs were resolved by electrophoresis for 15–16 h on 1% (w/v) agarose gels containing 16.7% (v/v) of 37% (w/w) formaldehyde (Fisher Laboratories, Inc., Pittsburgh, PA) [31]. Following electrophoresis, RNAs were transferred from agarose gels to nitrocellulose membranes (Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, MD) [32], and the membranes were baked at 80°C for 2 h. Blots were prehybridized for at least 2 h at 42°C in a solution containing 50% (v/v) formamide (Sigma), 6× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 25 mM Na₂HPO₄, 0.02% (w/v) bovine serum albumin (Sigma), 0.02% (w/v) Ficoll-400 (Sigma), 0.02% (w/v) polyvinylpyrrolidone-40 (Sigma), 0.5% SDS, 100 µg/ml salmon sperm DNA (Sigma), and 250 µg/ml Torula yeast RNA (Calbiochem Corp., San Diego, CA). Hybridizations of the blots with ³²P-labeled cDNA probes were carried out at 42°C in the same solution as above. The minimum hybridization time was 16 h after which the blots were washed twice with 2× SSPE/0.1% (w/v) SDS (1× SSPE = 0.15 M NaCl, 0.2 M NaH₂PO₄, and 0.02 M Na₂EDTA) and twice with 0.1× SSPE/0.1% (w/v) SDS for 15–20 min each at 42–65°C. The blots were subsequently air-dried and exposed to Kodak XAR-2 film (Eastman Kodak Co., Rochester, NY) at –70°C.

cDNA probes. The following cDNA probes were used: for decorin, a cDNA (lambda Pg28) for the full-length core protein of bovine bone decorin, kindly provided by Dr. M. Young (National Institute of Dental Research, Bone Research Branch, Bethesda, MD) [33]; for type I collagen, a cDNA (HF677) corresponding to the C-terminal region of human fibroblast proα1(I) collagen, kindly provided by Dr. F. Ramirez (Mount Sinai Hospital, New York, NY) [34]; for thrombospondin, a cDNA (lambda TS-33) corresponding to the amino-terminal region of human umbilical vein endothelial cell thrombospondin, kindly provided by Dr. P. Bornstein (University of Washington, Seattle, WA) [35]; for 28S ribosomal RNA, a cDNA fragment of 280 base pairs of bovine 28S RNA [12]. Nick translation (Nick Translation System, Bethesda Research Laboratories Life Technologies, Inc.) or random priming (Multiprime DNA Labeling System, Amersham Corp.) was used for labeling of the cDNAs with 5'-[α-³²P]dCTP (Amersham Corp., Arlington Heights, IL). The specific activity of the ³²P-labeled cDNA probes was approximately 1 × 10⁸ cpm/µg DNA.

Antisera. The following antisera were used: a polyclonal rabbit anti-bovine decorin antiserum, R10 [36], which is highly specific for bovine decorin and does not cross-react with bovine biglycan [24]; a polyclonal rabbit anti-bovine type I collagen antiserum, kindly provided by Dr. L. Fouser (University of Washington, Seattle, WA), which reacts with α1 and α2 chains of type I collagen [12].

Immunoprecipitation. All reagents unless specified were purchased from Sigma Chemical Co. (St. Louis, MO). Immunoprecipitation of [³⁵S]sulfate-labeled culture medium with decorin antiserum, R10, was carried out by a modification of the method of Kimura *et al.* [37], as described by Hering and Sandell [38]. To the medium was added ¼ vol of 5× final lysis buffer (5× final lysis buffer = 250 mM

Hepes, pH 8.0, containing 10% lithium dodecyl sulfate, 50 mM Na₂EDTA, 5 mM phenylmethylsulfonyl fluoride, and 1.8 mM pepstatin A). The samples were denatured by heating at 100°C for 5 min and reduced by adding 0.4 M dithiothreitol to a final concentration of 10 mM. The cooled samples were alkylated by incubation in the dark at room temperature for 2 h following addition of 0.8 M iodoacetamide to a final concentration of 20 mM. The samples were subsequently diluted by the addition of 9 vol of 50 mM Hepes, pH 7.4, containing 1% (w/v) Nonidet P-40, 1% (w/v) sodium deoxycholate, 0.15 M NaCl, 10 mM Na₂EDTA, 0.1 M 6-aminohexanoic acid, 10 mM N-ethylmaleimide, 5 mM benzamidinium-HCl, and 1 mM phenylmethylsulfonyl fluoride. The samples were centrifuged, and the supernatants were transferred to new tubes. Decorin antiserum or normal rabbit serum was added, and the samples were left at 4°C overnight. Normal rabbit serum served as a negative control to confirm the specificity of the immunoprecipitations. To each immunoreaction mixture was added protein A-Sepharose beads in washing buffer (the same composition as the dilution buffer above but with the addition of SDS to a final concentration of 1%). The samples were placed on a rotating mixer for 3 h at 4°C and were subsequently centrifuged at 800g for 5 min at 4°C. The initial supernatants were drawn off, and the protein A-Sepharose pellets were washed six times with washing buffer, and four times with 10 mM Tris-HCl, pH 6.8. After the final wash, the pellets were lyophilized and then suspended in 40 µl of sample buffer [39] containing 2-mercaptoethanol. Samples were heated at 100°C for 5 min, cooled to room temperature, and centrifuged through Bio-Rad Econo-Columns (Richmond, CA) in order to remove the Sepharose beads before further analysis of the immunoprecipitates by SDS-PAGE.

SDS–polyacrylamide gel electrophoresis/fluorography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of [³⁵S]sulfate-labeled culture medium products was carried out before and after immunoprecipitation essentially as described by Laemmli [39] on 4–12% gradient gel with a 3.5% stacking gel. The positions of the radioactive bands were visualized by fluorography of dried gels previously treated with Enlightening enhancer (DuPont/New England Nuclear, Boston, MA) and exposed at –70°C on Kodak XAR-2 film (Eastman Kodak Co.). ¹⁴C-labeled protein molecular weight standards purchased from Bethesda Research Laboratories Life Technologies, Inc., were used to estimate the sizes of PGs.

Immunocytochemistry. BAE cells were cultured on coverslips for various periods of time and immunostained with antisera described above using an avidin–biotin–peroxidase technique [12]. Briefly, the cultures were washed three times with phosphate-buffered saline (PBS), fixed with buffered 3% paraformaldehyde for 30 min at 4°C, and rinsed with 70% methanol containing 3% H₂O₂ for 30 min to inactivate endogenous peroxidases. Nonspecific binding sites were blocked by treatment with 1% normal goat serum in PBS for 2 h at 4°C. The cells were exposed to primary antibodies for 1–2 h at 4°C in a humidified chamber. Subsequently, the cells were rinsed for 30 min with three changes of PBS and incubated sequentially with biotinylated goat antibodies against rabbit IgG for 1 h at 4°C, PBS for 5 min, and avidin–biotin–peroxidase complex for 30 min. The complex was developed by exposing the cells to a solution of 3,3'-diaminobenzidine-4-HCl (1 mg/ml in 0.05 M Tris-HCl at pH 7.6, containing 0.02% H₂O₂) until a brown stain appeared (10–15 min). The cells were then rinsed with tap water for an additional 10 min. A solution of 1% toluidine blue was used as a counterstain, after which the cells were dehydrated, rinsed with xylene, and mounted in Permount. Negative controls included replacement of the primary antibody by preimmune rabbit serum.

RESULTS

Sprouting Strains of Bovine Aortic Endothelial Cells at Different Stages of Cord/Tube Formation

At confluence, cultures of sprouting strains of BAE cells, like their nonsprouting counterparts, form a cob-

FIG. 1. Bovine aortic endothelial cell cultures shown at different stages of endothelial cord and tube formation. (A) Nonsprouting strain of BAE cells at confluence (after 45 days in culture). The cells exhibit a typical cobblestone-like morphology. Bar = 100 μ m. (B) Sprouting strain of BAE cells at confluence (Day 3 of culture). The cells form a monolayer similar to that seen in the nonsprouting strain of BAE cells shown at this same stage in A. Bar = 100 μ m. (C) Sprouting strain of BAE cells at postconfluence (5 days after reaching visual confluence). A second population of elongated cells, termed sprouts (arrow), can be observed. Bar = 100 μ m. (D) Sprouting strain of BAE cells in the process of forming cords and tubes (arrow; 15 days in culture). Bar = 100 μ m.

blestone-like monolayer when the cells are grown on plastic in the presence of 10% fetal bovine serum (Figs. 1A and 1B). At this stage, endothelial cells are primarily polygonal in shape and they are contact-inhibited. After reaching confluence (generally 5 days), sprouting strains of BAE cells contain a second population of elongated cells (Fig. 1C, arrow) that have been termed sprouts [27]. These morphologically atypical BAE cells that usually grow under the endothelial monolayer do not represent a contaminating cell type but correspond to endothelial cells expressing specific endothelial markers [12]. After approximately 15 days in culture,

sprouting BAE cells associate with one another and form cords (Fig. 1D, arrow) or tube-like structures [12]. This morphological transition does not take place in nonsprouting strains of BAE cells cultured under similar conditions even after extended culture times (e.g., 45 days).

Synthesis of Decorin by Cultures of Bovine Aortic Endothelial Cells Exhibiting an Angiogenic Phenotype

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of [35 S]sulfate-labeled products from the culture media of nonsprouting and sprouting strains of BAE cells demonstrates that, at confluence, both types of BAE cell strains produce [35 S]sulfate-labeled macromolecules of similar molecular size (Fig. 2A, lanes 1 and 2, asterisks). In addition to [35 S]sulfate-labeled macromolecules that do not enter the separating gel or that remain at the top of the gel, two broad bands of radioactivity are present with M_r of $\sim 230,000$ and $\sim 140,000$, respectively. Our previous studies have shown that these two bands were sensitive to digestion with chondroitin ABC lyase, indicating that they contained chondroitin or dermatan sulfate chains [24]. Although the M_r $\sim 140,000$ -labeled band migrated to a position in SDS-PAGE typical for decorin, antibodies to decorin failed to immunoprecipitate this band. However, antisera to biglycan immunoprecipitated this band as well as the band at M_r $\sim 230,000$ [24]. Following morphological transition from a cobblestone-like monolayer to a sprouting and cord and tube phenotype, BAE cells synthesized macromolecules that differed significantly in M_r from those products secreted by the same cells at confluence or by nonsprouting BAE cells at confluence (Fig. 2A, lanes 3 and 4, arrows). Although these cultures produce the same radiolabeled bands that remained at the top of the gel, the two lower molecular weight bands had shifted to positions corresponding to M_r of $\sim 200,000$ and $\sim 100,000$, respectively. To determine whether either of these two radiolabeled bands contained decorin, the same [35 S]sulfate-labeled medium samples as shown in Fig. 2A were resolved by SDS-PAGE after immunoprecipitation of the samples with a polyclonal antiserum specific for bovine decorin (Fig. 2B). This antiserum immunoprecipitated [35 S]sulfate-labeled macromolecules of M_r $\sim 100,000$ from sprouting and cord and tube-forming BAE cell cultures (Fig. 2B, lanes 5 and 7) but did not immunoprecipitate any [35 S]sulfate-labeled products from the media of cobblestone-like monolayer cultures of sprouting or nonsprouting strains of BAE cells (Fig. 2B, lanes 1 and 3).

Expression of Decorin mRNA by Sprouting Strains of Bovine Aortic Endothelial Cells at Different Stages of Cord and Tube Formation

Northern blot analysis with a full-length cDNA probe corresponding to bovine decorin core protein was per-

FIG. 2. Synthesis of decorin by sprouting and tube-forming BAE cell cultures. A nonsprouting strain of BAE cells at confluence and a sprouting strain of BAE cells at different stages of endothelial cord and tube formation were labeled with 100 $\mu\text{Ci}/\text{ml}$ of [^{35}S]sulfate for 24 h. Aliquots from the culture media were analyzed by SDS-PAGE on a 4–12% gradient with a 3.5% stacking gel before (A) and after (B) immunoprecipitation with a polyclonal decorin antiserum, R10, or normal rabbit serum. The lanes are as follows. (A) Lane 1: nonsprouting strain of BAE cells at confluence. Lane 2: sprouting strain of BAE cells at confluence. Lane 3: sprouting strain of BAE cells at postconfluence (5 days after reaching visual confluence). Lane 4: sprouting strain of BAE cells that was forming cords (Day 15 of culture). The asterisks point out the major size classes of [^{35}S]sulfate-labeled macromolecules in the medium of monolayer cultures of nonsprouting and sprouting strains of BAE cells. The arrows point out the two bands of [^{35}S]sulfate-labeled macromolecules with shifted M_r s. (B) Lanes 1 and 2: nonsprouting strain of BAE cells at confluence. Lanes 3 and 4: sprouting strain of BAE cells at confluence. Lanes 5 and 6: sprouting strain of BAE cells at postconfluence. Lanes 7 and 8: sprouting strain of BAE cells that was forming cords. Lanes 1, 3, 5, and 7 represent medium samples after immunoprecipitation with decorin antiserum, and lanes 2, 4, 6, and 8 are the respective samples after immunoprecipitation with normal rabbit serum.

formed on RNAs isolated from cultures of the sprouting strain of BAE cells at different stages of cord and tube formation. The results demonstrate that BAE cell cultures which contain sprouting cells, cords and tubes, but not monolayer cultures of the same cells, expressed decorin mRNA (Fig. 3). In both sprouting and cord and tube-forming cultures, two sizes of decorin mRNA could be detected (Fig. 3) that appeared identical to those previously described for bovine arterial smooth muscle cells [24] and other cell types [40]. Hybridization of the blot shown in Fig. 3 with a cDNA probe for type I collagen demonstrated that transcripts for this protein were present only in the sprouting and cord and tube-forming cultures, as previously described [12]. This result indicates that expression of decorin and type I collagen is coincident during the process of angiogenesis *in vitro*. Hybridization of the blot with a cDNA probe for throm-

bospondin, another extracellular matrix glycoprotein, demonstrated that all strains of BAE cells expressed significant amounts of mRNA for this molecule (Fig. 3) [41]. Hybridization of the blot with a cDNA probe for 28S ribosomal RNA revealed that nearly equal amounts of RNA were loaded in the different lanes (Fig. 3).

Immunolocalization of Decorin and Type I Collagen in Bovine Aortic Endothelial Cell Cultures at Different Stages of Cord and Tube Formation

Since we have previously demonstrated that type I collagen is selectively expressed by sprouting BAE cells and by BAE cells comprising cords or tube-like structures [12], we compared the distribution of this protein with that of decorin. Type I collagen immunoreactivity was absent in nonsprouting BAE cell cultures but present in sprouting BAE cells and cells in cords and tubes (Figs. 4A–4C). Decorin immunoreactivity was apparent in sprouting and cord- and tube-forming cultures, but not in confluent monolayer cultures of the same cells (Figs. 4D–4F). In sprouting cultures, reactivity for decorin was particularly associated with elongated cells (Fig. 4E, arrows) as was observed for type I collagen immunoreactivity (Fig. 4B, arrows). In cord- and tube-forming cultures, decorin immunoreactivity was seen not only in endothelial cords, but also in the cells surrounding the cords (Fig. 4F).

FIG. 3. Expression of decorin and type I collagen by sprouting strain of BAE cells at different stages of cord and tube formation. Total RNAs (10 $\mu\text{g}/\text{lane}$) from BAE cell cultures at different stages of cord and tube formation (lane 1, subconfluent; lane 2, confluent; lane 3, sprouting; lane 4, cords and tubes) were resolved on a denaturing 1% agarose gel and transferred to a nitrocellulose filter as described under Materials and Methods. The blot was probed with ^{32}P -labeled cDNA probes for decorin, type I collagen, thrombospondin, and 28S ribosomal RNA as indicated. The sizes of different mRNA transcripts are shown.

FIG. 4. Immunostaining of BAE cells at different stages of cord and tube formation with antibodies specific for decorin and type I collagen. (A and D) BAE cells at confluence; (B and E) BAE cells at postconfluence (i.e., after sprouting, arrows); (C and F) BAE cells after forming cords and tube-like structures (arrow). Cultures in A–C were immunostained with type I collagen antibodies, and cultures in D–F were immunostained with decorin antibodies.

DISCUSSION

In the present study, we have demonstrated that angiogenic cultures of BAE cells initiate the synthesis of decorin during their morphological transition from a cobblestone-like monolayer to a sprouting phenotype. We have also shown that expression of type I collagen by these cultures is initiated concomitantly. Our immunohistochemical data indicate that cells exhibiting the sprouting phenotype and those that form cords and tubes stain for both decorin and type I collagen, suggesting that the same cells synthesize both of these molecules.

A number of extracellular matrix molecules have been implicated in the angiogenic response including laminin, fibronectin, SPARC, collagen, and proteoglycans (PGs) [9, 10, 12–14, 42, 43]. Although the precise

role of these individual components in the angiogenic response is unclear, the fact that these molecules influence such cell processes as adhesion, migration, and cell shape suggests that altered extracellular matrix protein synthesis may be important in modulating the phenotypic changes characteristic of BAE cells during angiogenesis. The role that decorin plays in the sprouting and cord- and tube-forming process is not understood. However, since decorin and type I collagen synthesis are specifically induced as BAE cells sprout and form cords and tubes, these two molecules may act in concert to promote angiogenesis. It is now well established that the fibrillar form of type I collagen is important for endothelial cells to form capillary tubes both *in vitro* and *in vivo* [13, 17]. Since decorin interacts with type I collagen [19, 20] and regulates fibril formation *in vitro* [21–23], we suggest that decorin promotes angiogenesis by influenc-

ing the formation of type I collagen fibrils. The mechanism by which fibrillar collagen facilitates or directs tube formation is not understood but these fibrils could contribute a scaffold around which endothelial cells associate [13].

Decorin may have a more direct effect on the angiogenic phenotype by influencing endothelial cell migration. For example, BAE cells induced to migrate following *in vitro* wounding increase the synthesis of PGs enriched in dermatan sulfate [44]. Small chondroitin/dermatan sulfate PGs facilitate cell migration by decreasing cellular adhesion by binding to collagen and fibronectin and interfering with the cell binding sites of these extracellular matrix molecules [45–47]. Decorin might also promote angiogenesis through its ability to bind growth factors such as transforming growth factor- β [48], an association that could modulate the response of cells to morphogens. This interaction may be analogous to that recently described for heparan sulfate PGs and basic fibroblast growth factor in the differentiation of myoblasts [49].

The mechanism(s) responsible for the induction of decorin expression in cultured BAE cells during sprouting is not known at the moment. However, a mechanism that has been shown to control gene activity is the degree of DNA methylation [50, 51]. At least in colon carcinoma hypomethylation of decorin gene has been shown to be associated with increased decorin expression [52].

The level of signal of decorin in sprouting and cord and tube-forming BAE cell cultures is high (Fig. 2), although the number of endothelial cells expressing this protein remains relatively small (Fig. 4). There are several possibilities not examined during the course of this study that could explain this observation. Two of these possibilities are: (1) the synthesis of decorin in a selective BAE cell population is extremely active, and/or (2) the breakdown of decorin in sprouting and cord- and tube-forming BAE cell cultures is low. However, since the number of BAE cells going through a modulation to an angiogenic phenotype may vary markedly between individual experiments, the level of signal for decorin in sprouting and cord- and tube-forming BAE cell cultures may also show a significant variation.

In summary, in the present study we have demonstrated that decorin, together with type I collagen, is associated with the morphological transition of cultured BAE cells from a polygonal monolayer to a sprouting phenotype. We propose that these two extracellular matrix molecules play a role in angiogenesis.

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