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Modulation of Extracellular Matrix Proteins by Endothelial Cells Undergoing Angiogenesis In Vitro

M. Luisa Iruela-Arispe, Clement A. Diglio, and E. Helene Sage

Angiogenesis results in part from the response of endothelial cells to the integrated action of morphogenic factors and extracellular matrix proteins. In this study we identified specific components of the extracellular matrix that were modulated in endothelial cells derived from bovine aorta and rat cerebral microvessels, both of which spontaneously form cords and tubes under standard culture conditions. SPARC (secreted protein, acidic and rich in cysteine) was upregulated 4.2-fold in aortic and 10-fold in microvascular cultures that had organized into cords and/or tubes. This Ca^{2+} -binding glycoprotein was synthesized primarily by endothelial cells in the process of cord formation. Transcription of type I collagen was initiated in aortic endothelial cells undergoing angiogenesis in vitro and showed a 12-fold increase in similar cultures of microvascular cells. Type VIII collagen protein was upregulated to a lesser degree (4.3-fold in aortic and 1.8-fold in microvascular cells). Dense cytoplasmic staining for these two collagen types was seen in cells directly participating in the organization of cords. In contrast, the disparate levels of fibronectin observed in both types of endothelium indicated an indirect or secondary role for this glycoprotein in cord/tube formation in vitro. These results identify SPARC, type I collagen, and type VIII collagen as extracellular matrix components that are actively synthesized by endothelial cells undergoing angiogenesis in vitro. Moreover, expression of these proteins during the formation of tubes and cords appears to follow a biosynthetic program that is common to endothelial cells from both the macrovasculature and microvasculature. (*Arteriosclerosis and Thrombosis* 1991;11:805–815)

The inner lining of the cardiovascular system consists of a single layer of endothelial cells. Although metabolically quite active, these cells generally exhibit low rates of turnover. In addition to performing numerous functions related to hemostasis and thrombosis, endothelial cells give rise to new vessels in adult organisms. Under appropriate stimuli microvascular endothelium initiates a complex repertoire, including degradation of the basement membrane, migration, and proliferation, which contributes to angiogenesis.¹ The molecular mechanism(s) initiating the cascade of events that concludes with the formation of new capillaries are unknown. However, experimental evidence has im-

plicated 1) growth factors such as basic fibroblast growth factor^{2–4} and transforming growth factors- α and - β ,^{5–9} 2) extracellular matrix (ECM) components including collagens,^{10–12} laminin,^{13,14} and fibronectin,¹⁵ and 3) extracellular proteases.^{11,16,17} Identification of these factors as well as their specific roles and interactions with endothelial cells will contribute to our understanding of angiogenesis.

Several experimental models have been used to explore capillary formation in vitro via the modulation of cell behavior by ECM, growth factors, and other substances. Endothelial cells from diverse sources have been cultured in the presence of 1) tumor-conditioned medium,¹⁸ 2) fibrin clots,¹⁹ 3) phorbol esters,²⁰ 4) collagen gels or gels of modified basement membrane components,^{13,21–24} and 5) basic fibroblast growth factor.³ Although these systems have provided valuable insights into the mechanisms that direct cell behavior, studies of biosynthetic modulation can be interpreted more clearly when morphogenesis of capillary-like structures occurs in the absence of exogenous factors. Spontaneous angiogenesis under conventional culture conditions has been reported as an intrinsic characteristic of certain

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strains of endothelial cells. Capillary-like structures have been described in primary cultures of endothelial cells from umbilical vein,²⁵ calf aorta,²⁶ bovine adrenal cortex microvessels,²⁷ and adult bovine aorta.²⁸ To understand the specific mechanisms underlying the angiogenic process, we have identified ECM components that are consistently modulated when endothelial cells from both macrovessels and microvessels undergo spontaneous formation of capillary-like structures in vitro. In a previous study²⁸ we demonstrated that type I collagen was induced and that the secreted Ca^{2+} -binding glycoprotein, SPARC (secreted protein, acidic and rich in cysteine), was upregulated when bovine aortic endothelial cells (BAECs) spontaneously organized into tubular or cord-like structures in vitro. In the present study we have investigated whether levels of type I collagen and SPARC were modulated in a similar manner when cultured rat brain microvascular (resistance vessel) endothelial cells (RVECs) spontaneously formed tubes and/or cords. We also examined the expression of fibronectin and type VIII collagen as a function of angiogenesis in vitro. Our data show an upregulation of SPARC and collagen types I and VIII, but not fibronectin, in both macrovascular and microvascular endothelial cells that actively form vascular cords.

Methods

Cell Culture

BAECs were isolated as previously described²⁹ and were characterized by their ability to take up acetylated low density lipoproteins and to synthesize von Willebrand factor. In this study, we used three different BAEC strains that were selected and cloned based on their propensity to express the sprouting phenotype.³⁰ RVECs were isolated and characterized according to Diglio et al.³¹ Both cell types were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO/BRL, Gaithersburg, Md.) containing 10% heat-inactivated fetal calf serum (Flow, McLean, Va.), 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate, and 250 $\mu\text{g}/\text{ml}$ amphotericin B (Sigma Chemical Co., St. Louis, Mo).

Spontaneous organization of endothelial cords and/or tubes was monitored by phase-contrast microscopy. In general, this phenomenon occurred 10–20 days after the cultures reached confluence. Experiments were performed between passages 3–10 for BAECs and passages 25–30 for RVECs.

Metabolic Labeling and Analysis of Secreted Proteins

Metabolic labeling was performed on cultures of BAECs and RVECs that exhibited either 80–90% confluency (subconfluent) or that contained endothelial cords and/or tubes. Initially, the cells were incubated in serum-free DMEM supplemented with 50 $\mu\text{g}/\text{ml}$ sodium ascorbate and 64 $\mu\text{g}/\text{ml}$ β -aminopropionitrile fumarate for 1 hour. The medium was aspirated, and the cultures were incubated for 18–20 hours in fresh DMEM containing sodium ascorbate,

β -aminopropionitrile, and 50 $\mu\text{Ci}/\text{ml}$ L-[2,3,4,5-³H]proline (100 Ci/mol, Amersham, Arlington Heights, Ill.).

After this incubation, the medium was processed at 4°C. Clarification from cells and debris was done by brief centrifugation; the supernate was transferred directly into a mixture of proteinase inhibitors to produce a final concentration of 0.2 mM phenylmethylsulfonyl fluoride, 10 mM *N*-ethylmaleimide, 2.5 mM EDTA,²⁸ and 0.5 $\mu\text{g}/\text{ml}$ pepstatin A (Peninsula Laboratories, San Carlos, Calif.). The medium was dialyzed against 0.1N acetic acid and subsequently lyophilized. To correlate cell number with radiolabeled proteins, the cultures were counted by hemocytometer immediately after the medium was removed.

Lyophilized proteins were solubilized in sample buffer containing sodium dodecyl sulfate (SDS)³² and were counted by scintillation spectrophotometry. A volume equivalent to 250,000 cells was removed from each sample, reduced with 50 mM dithiothreitol, and heated for 1–3 minutes at 90–100°C. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on discontinuous polyacrylamide slab gels.³² Protein molecular-weight standards included myosin (H chain, 200 kd), phosphorylase b (97.4 kd), bovine serum albumin (68 kd), ovalbumin (43 kd), α -chymotrypsinogen (27.5 kd), and lysozyme (14.3 kd). Gels were stained with Coomassie brilliant blue R-250, destained, and incubated in Enhance (DuPont, Boston, Mass.) according to the manufacturer's instructions. Dried gels were exposed to RP X-Omat x-ray film (Kodak, Rochester, N.Y.) at –70°C.

Pepsin digestion. To identify type VIII collagen, lyophilized proteins were resuspended in 0.5 M acetic acid at 4°C, and pepsin (Worthington, Freehold, N.J.) was added to a final concentration of 50 $\mu\text{g}/\text{ml}$ for 4 hours at 4°C. The reaction was terminated by a twofold molar excess of pepstatin A. The digest was immediately frozen at –70°C and lyophilized. Digestion products were subsequently resolved by SDS-PAGE, and type VIII collagen was identified by Western immunoblotting.

Immunoblotting

SDS-PAGE gels were transferred to nitrocellulose (Schleicher & Schuell, Keene, N.H.) in a Bio-Rad transblotter (Richmond, Calif.) at 500 mA for 4 hours at 4°C. To verify efficiency of transfer, nitrocellulose sheets were stained with amido black (0.1% amido black in a solution of 10% acetic acid and 20% methanol). Subsequently, the blots were blocked in a solution of phosphate-buffered saline (pH 7.4) containing 1% nonfat dry milk and 0.05% Tween-20 at pH 7.8 (MT buffer) for 16 hours at 4°C. Primary antibodies were rabbit anti-mouse SPARC immunoglobulin G (IgG) (directed against a synthetic C-terminal peptide of SPARC)³³ at 9 $\mu\text{g}/\text{ml}$ and rabbit anti-bovine type VIII collagen IgG³⁴ at 10 $\mu\text{g}/\text{ml}$ (final concentrations in MT buffer). Incubations were performed in 10 ml antibody solution for 2–3 hours

at room temperature, followed by six washes in MT buffer (5 minutes each). Blots were then incubated in 10 ml MT buffer containing 5 μ Ci 125 I-Protein A (NEN, Boston, Mass.). After extensive washes with MT buffer (eight times, 15 minutes each), the blots were exposed to x-ray film. A scanning spectrophotometer (Beckman Instruments Inc., Fullerton, Calif.) was used to quantify band density within a range of linear absorbance.

Immunocytochemistry

Cells were cultured on Labtek slides (Nunc, Naperville, Ill.) until organization of endothelial cords was evident by phase-contrast microscopy. Cultures were then washed three times with serum-free DMEM and fixed in 3% buffered paraformaldehyde (pH 7.4) for 30 minutes at 4°C. After fixation, the cultures were washed in phosphate-buffered saline and incubated with 70% methanol containing 3% H₂O₂ for 30 minutes to inactivate endogenous peroxidases. Treatment with 1% normal goat serum in phosphate-buffered saline for 3 hours was performed to minimize nonspecific binding. Cells were then exposed to one of the following primary antibodies (final concentrations): rabbit anti-mouse SPARC IgG (directed against a synthetic N-terminal peptide of SPARC,³³ 25 μ g/ml); anti-type VIII collagen IgG,³⁴ 30 μ g/ml; rabbit anti-bovine fibronectin anti-serum, 14 μ g/ml, absorbed with bovine plasma proteins minus fibronectin (Telios, San Diego, Calif.); and sheep anti-bovine type I procollagen IgG, 35 μ g/ml (provided by Laurie Fouser, University of Washington). Anti-type I procollagen IgG was affinity absorbed on Sepharose–fibronectin followed by Sepharose–type I collagen. Specificity for type I procollagen was confirmed by Western blotting and enzyme-linked immunosorbent assay. After incubation with primary antibody for 2 hours at 4°C, the cells were rinsed in phosphate-buffered saline (three times, 30 minutes each), incubated with biotinylated goat anti-rabbit IgG for 1 hour at 4°C, rinsed in phosphate-buffered saline, and exposed to an avidin–biotin–peroxidase complex (Vector Labs, Burlingame, Calif.) (30 minutes). 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) was used to develop immune complexes. The reaction was stopped with tap water, and the cells were counterstained with an aqueous solution of 1% toluidine blue. Negative controls included replacement of the primary antibody by preimmune rabbit serum. Photography was performed with a Zeiss Photomicroscope II on Kodak Ektachrome film (160 ASA).

Northern Blot Analysis

Total RNA was extracted, as described by Chomczynski and Sacchi,³⁵ from cultures of 1) BAECs at 90% confluency, 2) BAECs containing endothelial cords, 3) RVECs at 90% confluency, and 4) RVECs containing endothelial cords. Concentrations of RNA were determined spectrophotometrically.

Seven micrograms of total RNA was denatured in a formamide/formaldehyde solution at 55°C for 15 minutes, and the samples were resolved on a denaturing 1.2% agarose gel.³⁶ After electrophoresis, the gel was stained with 0.5 μ g/ml ethidium bromide in diethylpyrocarbonate-treated water for 5 minutes and destained in diethylpyrocarbonate-treated water. RNA was then transferred to a nitrocellulose sheet by a Vacuum Blotting System (Pharmacia-LKB, Piscataway, N.J.) and cross-linked by ultraviolet irradiation in a Stratilinker (Stratagene, La Jolla, Calif.). Prehybridization was performed at 42°C for 16 hours in a solution containing 50% deionized formamide, 30% of 20 $\times$ standard saline citrate (1 $\times$ standard saline citrate is 0.15 M NaCl/0.015 M sodium citrate), 50 mM NaH₂PO₄, 10 μ g yeast total RNA, and 4% of a 50 $\times$ Denhardt's solution (1% Ficoll, 1% polyvinylpyrrolidone, and 1.1% bovine serum albumin). The cDNA probes used in this study were 1) a 557-bp *Bam*HI–*Eco*RI fragment of mouse SPARC cDNA³⁷; 2) a 1.1-kb *Eco*RI–*Eco*RI fragment of human α 1(I) collagen cDNA encoding a protein sequence involved in triple-helix formation³⁸; 3) a 2.2-kb *Pst*I–*Pst*I fragment of human fibronectin cDNA³⁹; and 4) a 280-bp *Eco*RI–*Eco*RI cDNA fragment from bovine 28S rRNA, which shows 99.5% sequence homology with rat 28S rRNA.

DNA fragments were labeled by a standard nick-translation protocol with deoxycytidine 5'-[α -³²P]triphosphate (Amersham, 10 mCi/ml) and chromatographed on Sephacryl S-400 (Promega, Madison, Wis.). Specific activity of probes was between 0.2 $\times$ 10⁹ and 1 $\times$ 10⁹ cpm/ μ g. Hybridization was performed with excess probe for 16 hours in the solution previously described for prehybridization containing 10⁶ cpm cDNA/ml. Posthybridization washes were performed at a final stringency of 0.1 $\times$ standard saline citrate in 0.1% SDS at 65°C. Nitrocellulose sheets were exposed to x-ray film, and the signal was scanned in a spectrophotometer. Normalization for loading of RNA was accomplished by scanning densitometry of the signal for 28S rRNA.

Results

Spontaneous formation of endothelial cords and tubes by adult BAECs²⁸ and RVECs⁴⁰ has been previously reported. In this study, we characterized this process and its incipient alterations in ECM biosynthesis in a strain of rat endothelium derived from the cerebral microvasculature. Comparison between aortic and resistance vessel endothelia was conducted under identical culture conditions for both types of cells and without exogenous factors other than those present in DMEM and fetal calf serum.

Spontaneous organization of endothelial cords by BAECs and RVECs was evident after 10–15 days in culture and occurred several days after the cells had formed a confluent monolayer (Figures 1A and 1D). BAECs required a second pattern of growth, termed sprouting, before cord formation (Figure 1B, arrow-

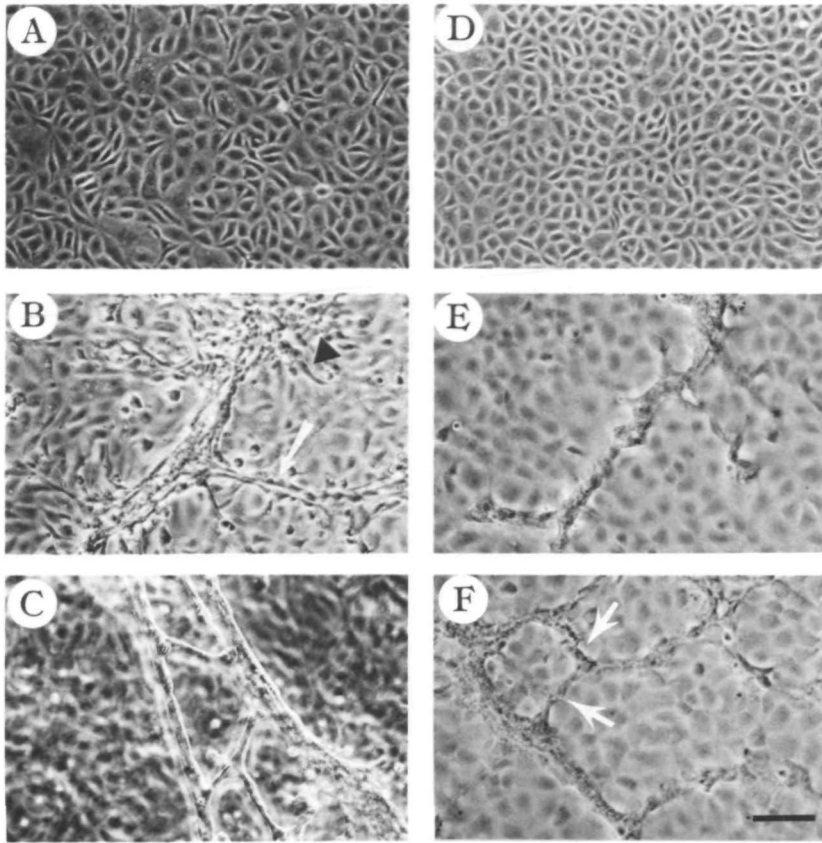


FIGURE 1. Phase-contrast photomicrographs showing formation of endothelial cords by bovine aortic endothelial cells (BAECs) and resistance vessel endothelial cells (RVECs). Panel A: Confluent monolayer of BAECs (3 days in culture). Panel B: Organization of endothelial cords by BAECs (arrow). Presence of sprouting cells is evident (arrowhead) (15 days in culture). Panel C: Arrays of endothelial cords displayed by BAECs (30 days in culture). Panel D: Monolayer of RVECs (2 days in culture). Panel E: Endothelial cord of RVECs (10 days in culture). Panel F: Network of endothelial cords organized by RVECs (arrows) (17 days in culture). Bar=100 μ m.

head). In contrast, RVEC cords appeared in culture without sprouting (Figure 1E). Cords from both cell types could be maintained in culture for as long as 2 months without overt cellular senescence or necrosis. Indeed, mitotic indexes from these cultures revealed a high rate of proliferation, particularly in areas where cells were contributing to the growth of cords. The number of cords tended to increase in long-term cultures, which formed organized arrays of intercommunicating networks resembling capillary beds *in vivo* (Figures 1C and 1F). Although we verified the presence of lumina in BAECs,²⁸ we have referred to these capillary-like structures as endothelial cords rather than endothelial tubes. It is our experience, however, that in a given culture of BAECs, cords and tubes coexist, and the formation of cords precedes that of tubes. RVEC cultures contained predominantly cords; patent tubes were found to be rare (C. Diglio et al, unpublished observations).

To compare the proteins secreted by endothelial cells before and after morphogenesis of tubes, we metabolically labeled subconfluent cultures and cord-containing cultures from both BAECs and RVECs. Figure 2 shows [³H]proline-labeled proteins secreted into the culture media by BAECs (lanes 1 and 2) and RVECs (lanes 3 and 4). Although the biosynthetic profiles differed in several respects, the ECM proteins fibronectin, thrombospondin, and type III collagen could be identified as prominent secretory products of both cell types. A similar analysis showed that the ECM proteins secreted by subcon-

fluent and confluent cultures were similar within the same cell type (data not shown). However, the secretory phenotype was altered after the cultures began to organize into cords. As shown in Figure 2, cord-forming RVECs secreted significantly higher levels of types I and III procollagen as compared with subconfluent cells (compare lane 3 with lane 4). The marked upregulation of type I procollagen (and its processed forms α 1[I] and α 2[I]) seen in cord-forming BAECs has been described²⁸ but is shown in lanes 1 and 2 for comparison with the microvascular cells. The identity of the collagen chains shown in Figure 2 was confirmed by Western blotting (data not presented).

Since the phenomenon of angiogenesis *in vitro* was at least partially dependent on the duration of subculture, we performed control experiments on post-confluent cultures. Thus, strains of BAECs that did not express the sprouting phenotype were plated for an equivalent period of time as that needed for strains undergoing angiogenesis *in vitro*. The spectrum of proteins secreted by the control cultures was similar to that seen in subconfluent cultures. However, the level of protein secretion was significantly lower, and certain proteins such as thrombospondin and SPARC were selectively diminished (data not shown). We have used sparsely plated cells rather than confluent cultures in these studies since the subconfluent cultures resembled cord-containing cultures more closely with respect to levels of protein synthesis and mitotic index.

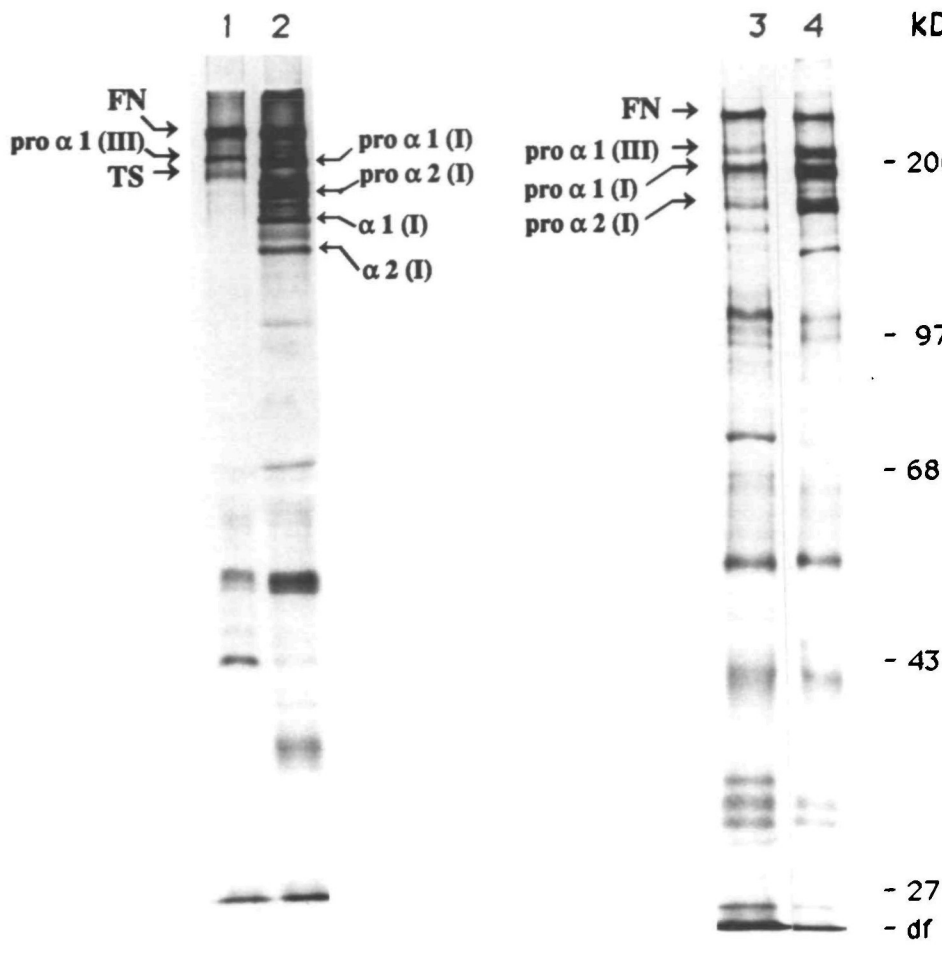


FIGURE 2. Photograph of SDS-PAGE gels showing secretory phenotypes of bovine aortic endothelial cells (BAECs) and resistance vessel endothelial cells (RVECs) before and after organization of endothelial cords in vitro. BAECs and RVECs were metabolically labeled with [2,3,4,5-³H]proline at 80% confluency (lanes 1 and 4) and after formation of endothelial cords (lanes 2 and 3). Radiolabeled proteins, secreted by 250,000 cells/sample, were resolved on a 4%/8% SDS-PAGE gel under reducing conditions and were visualized by autoradiography. Lane 1, subconfluent BAECs; lane 2, cord-containing BAEC cultures; lane 3, subconfluent RVEC cultures; and lane 4, cord-containing cultures. Globular protein molecular-weight standards are indicated on the right. Fibronectin (FN), thrombospondin (TS), type I and type III procollagen chains (pro α), and type I collagen chains (α) have been identified. Two separate gels are shown. SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; df, dye front.

In this comparative study we wished to address the changes in matrix production by endothelial cells undergoing cord formation, with specific emphasis on cells derived from a microvascular bed. Since we had observed, in an earlier study, an induction of type I collagen mRNA and an increase in SPARC mRNA by cord-forming BAECs,²⁸ we initially chose to examine these gene products in RVECs in the presence and absence of cords. The experiments were then extended to include fibronectin and type VIII collagen. Although both of these proteins have been characterized as products of endothelial cells, their participation as endogenous products in cord or tube formation has not been described in either aortic or resistance vessel endothelium.

Figure 3 shows representative immunoblots that illustrate the relative changes in type VIII collagen and SPARC protein in subconfluent and tube-forming BAECs and RVECs. In cultures that contained cords, levels of pepsin-treated type VIII collagen were 4.3-fold higher in BAECs and 1.8-fold higher in RVECs as compared with the respective subconfluent cultures (Figure 3A). These values were derived from equal numbers of cells.

We also detected a 4.2-fold increase in the secretion of SPARC by BAECs and a 10-fold increase by RVECs when these cells were forming cords (Figure 3B). mRNA levels for SPARC showed a similar trend (Figure 4A). Consistent results were obtained from replicate dishes within a single experiment, as well as from independent experiments. The increase in SPARC mRNA in subconfluent cultures versus cord-containing cultures was always greater than 3.5-fold for BAECs and eightfold for RVECs. These values were obtained from five independent preparations of RNA and from several different strains of endothelial cells. When confluent cultures rather than subconfluent cultures were used to calculate the ratio, the differences were even greater due to the reduced levels of SPARC mRNA and protein synthesis in contact-inhibited endothelial monolayers (data not shown).

We previously demonstrated the initiation of transcription of the $\alpha 1(I)$ collagen gene when BAECs undergo cord formation.²⁸ In support of this finding Figure 4B shows the presence of $\alpha 1(I)$ collagen mRNA in cord-containing cultures but not in subconfluent cultures. This figure also indicates a significant increase in $\alpha 1(I)$ collagen mRNA in cultures of

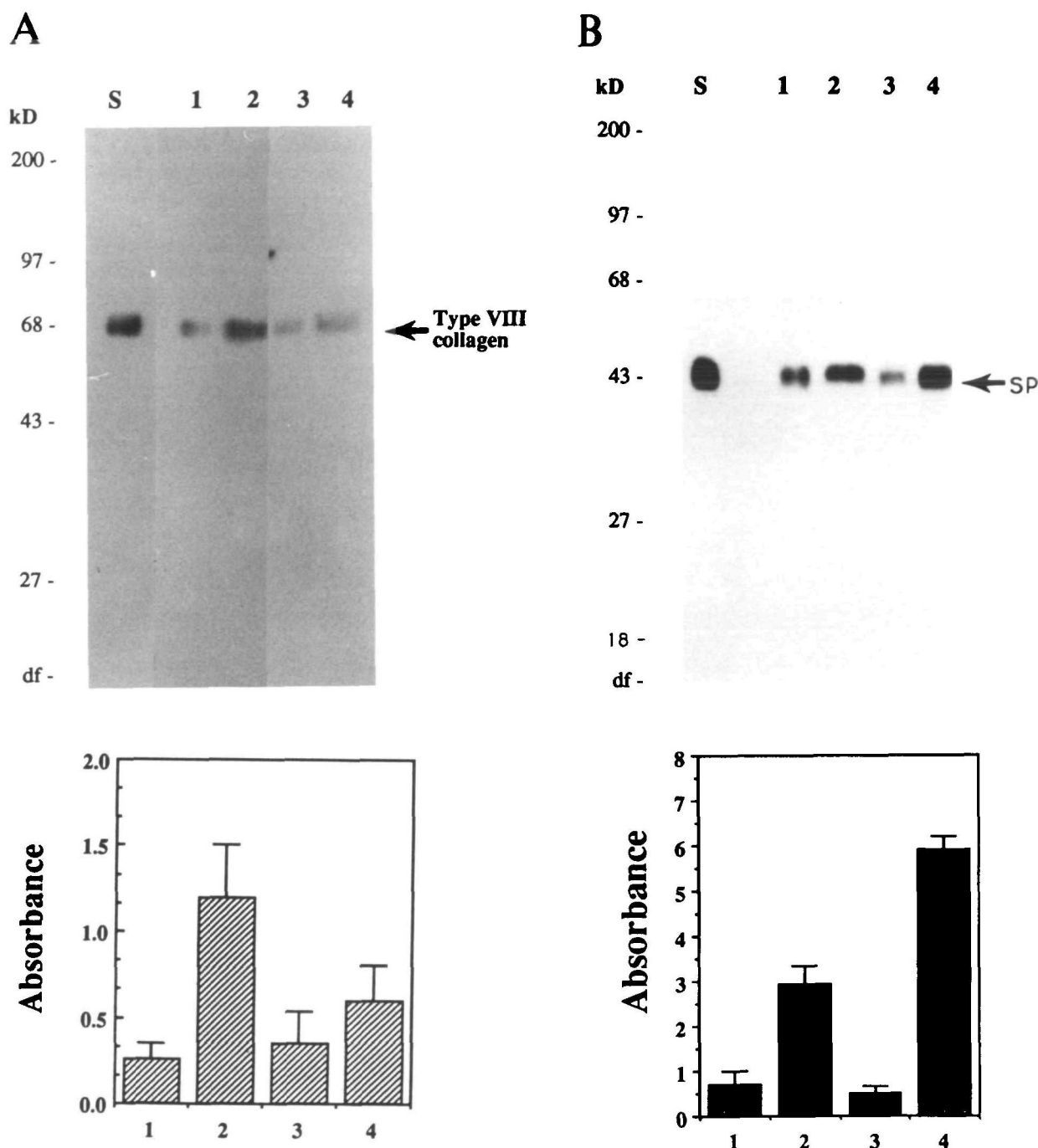


FIGURE 3. Increases in type VIII collagen and SPARC in bovine aortic endothelial cells (BAECs) and resistance vessel endothelial cells (RVECs) engaged in cord formation. Secreted proteins were resolved on a 5%/10% SDS-PAGE gel, transferred to nitrocellulose, and treated with anti-type VIII collagen immunoglobulin G (panel A) (in this case, proteins were previously digested with pepsin) or anti-SPARC peptide 4.2 immunoglobulin G (panel B), followed by 125 I-labeled protein A. Resulting autoradiograms are shown, and values determined by scanning densitometry are represented in histograms (lower portion of figure; mean $\pm$ SEM). Molecular weight markers are indicated at left. Lane 1, subconfluent BAECs; lane 2, cord-containing BAEC culture; lane 3, subconfluent RVECs; and lane 4, cord-containing RVEC culture. SPARC, secreted protein, acidic and rich in cysteine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; S, control protein (type VIII collagen chain in panel A and SPARC [SP] in panel B).

RVECs that undergo cord morphogenesis, although in this case, the expression of $\alpha 1(I)$ collagen mRNA was also observed in subconfluent cultures. Scanned

gels from four individual experiments revealed an average 12-fold increase in $\alpha 1(I)$ collagen mRNA in cord-forming cultures of RVECs.

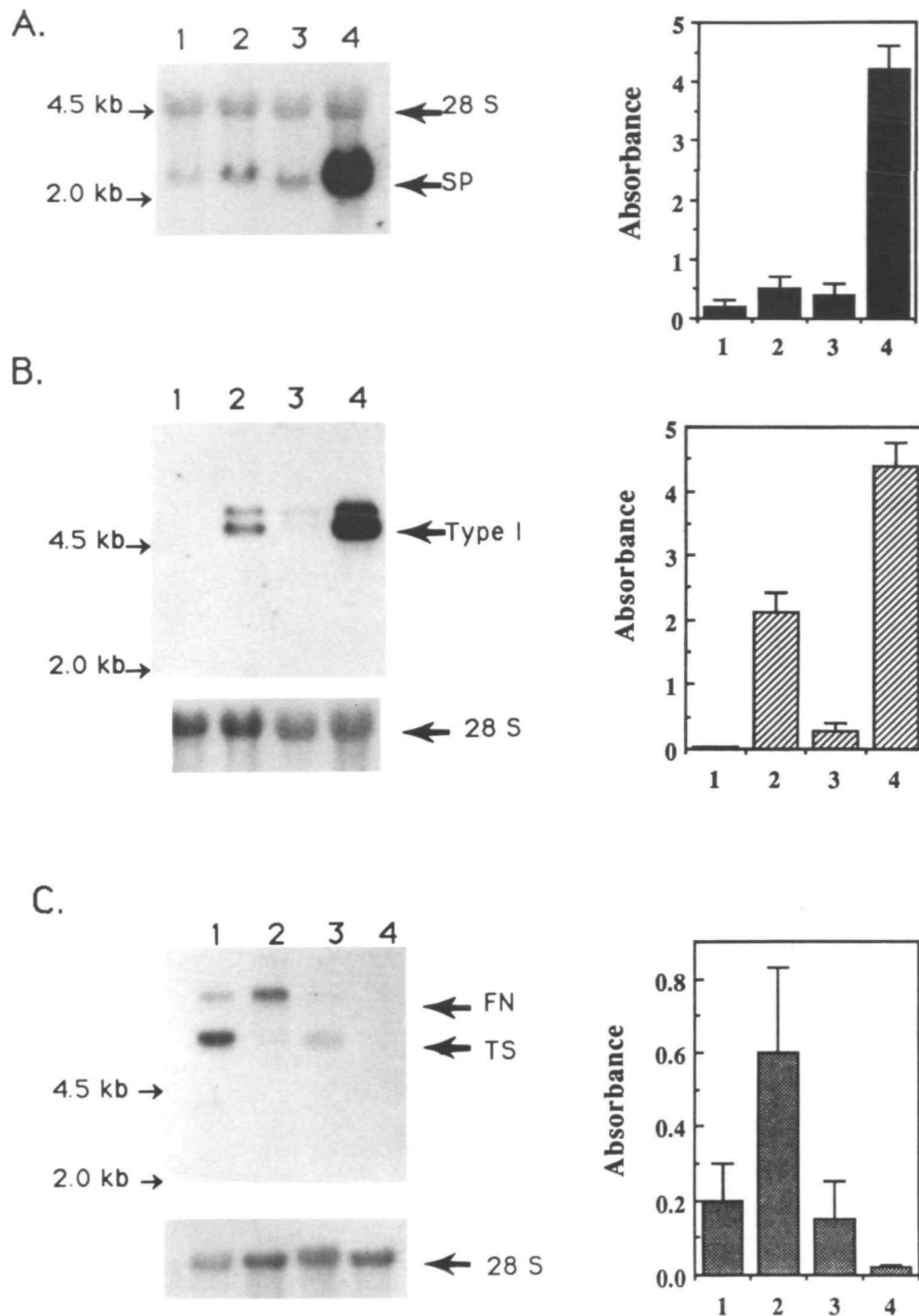


FIGURE 4. Abundance of SPARC, $\alpha 1(I)$ collagen, and fibronectin mRNAs during organization of endothelial cords in culture. Seven micrograms of total RNA from (lane 1) subconfluent bovine aortic endothelial cells (BAECs), (lane 2) cord-containing BAEC cultures, (lane 3) subconfluent resistance vessel endothelial cells (RVECs), and (lane 4) cord-containing RVEC cultures, after transfer from an agarose denaturing gel to nitrocellulose and hybridization with cDNA probes to (panel A) SPARC (SP), (panel B) $\alpha 1(I)$ collagen (type I), or (panel C) fibronectin (FN). A 28S cDNA probe was used to normalize hybridization signals for equal loadings of RNA. Values determined by scanning densitometry after normalization are shown in histograms at right (mean $\pm$ SEM). In panel C, hybridization signal corresponding to thrombospondin mRNA (TS) is also shown. SPARC, secreted protein, acidic and rich in cysteine.

From the data shown in Figure 2, fibronectin was secreted by both BAECs and RVECs in the presence and absence of cords. Levels of fibronectin mRNA did not show a consistent trend in the two cell types. We

detected a 3.2-fold increase in BAEC cultures containing endothelial cords and a 10-fold decrease in the levels of fibronectin mRNA in similar cultures of RVECs (Figure 4C). These changes reflect the average

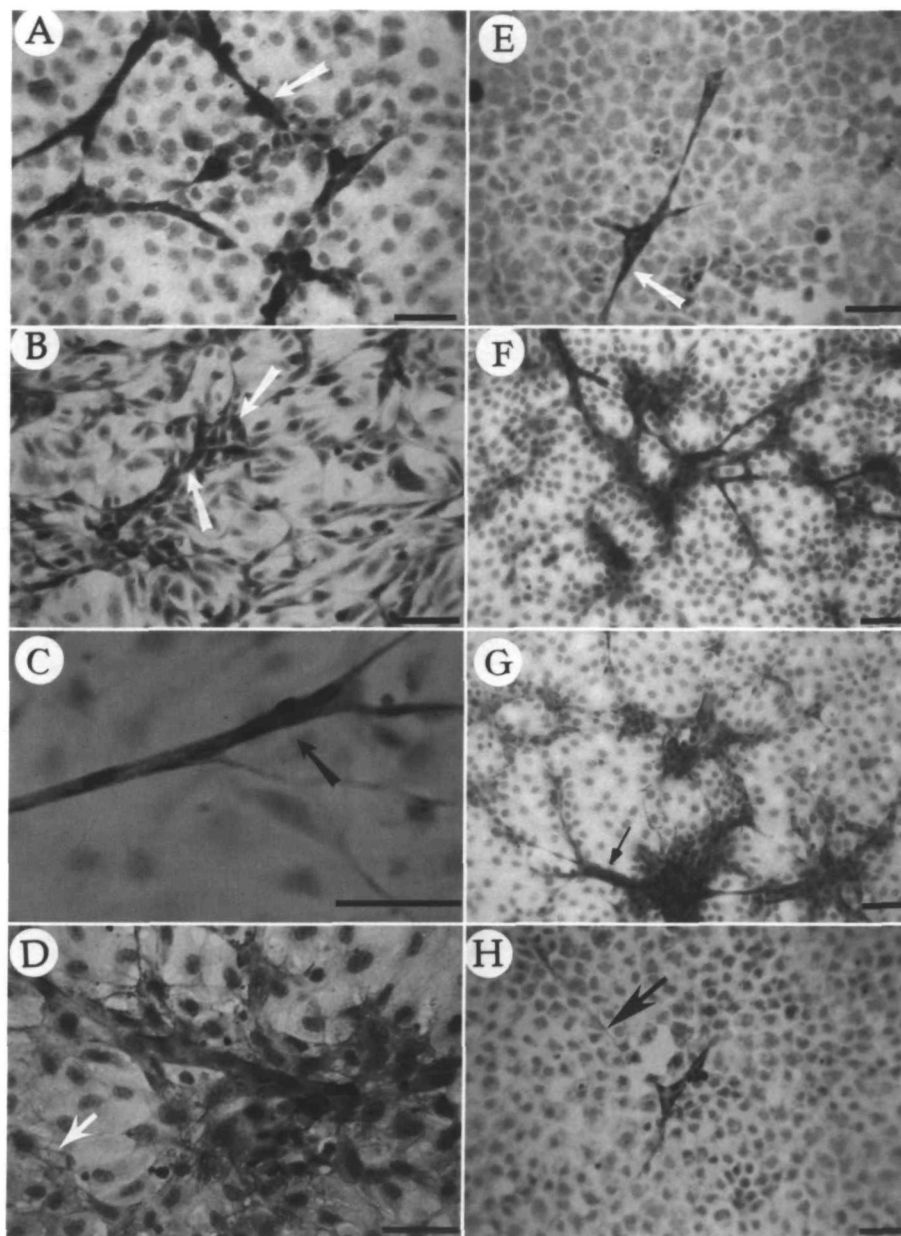


FIGURE 5. Photomicrographs showing immunolocalization of extracellular matrix (ECM) proteins in endothelial cord-containing cultures. Bovine aortic endothelial cells (panels A–D) and resistance vessel endothelial cells (panels E–H) were cultured on slides until formation of endothelial cords was evident by phase-contrast microscopy. Cultures were fixed as described in “Methods,” and immunolocalization of ECM proteins was performed by an avidin–biotin–peroxidase technique. Toluidine blue was used as a counterstain. Panels A and E, antibodies against type I procollagen demonstrating strong cytoplasmic staining in cords (arrows); panels B and F, SPARC preferentially associated with cords and with cells proximal to cords (arrows); panels C and G, anti-type VIII collagen antibodies identifying this collagen mainly in cords (arrows); panels D and H, anti-fibronectin immunoglobulin G revealing a fibrillar array between cells in the monolayer (arrow) and those in cords. However, there was no preferential association with cords. SPARC, secreted protein, acidic and rich in cysteine. Bars=100 μ m.

of four independent experiments. Variations of this sort are most likely due to differences in the regulation of the fibronectin gene that are cell-type specific.

We performed immunocytochemical studies to determine the distribution of these ECM proteins with respect to the endothelial cords. In Figures 5A and 5E, the network of BAEC cords was readily delineated by the reaction product, which is seen as a dark brown precipitate localizing the type I procollagen immune complex. In RVEC cultures, however, cytoplasmic reaction product could be detected in cells that were not involved in cord formation. These data are consistent with the fact that RVECs secrete type I collagen before cord formation. Type VIII collagen, which appeared to be predominantly intracellular (Figures 5C and 5G), was associated with the organized cords in both BAEC and RVEC cultures. This pattern was similar to that found for type I collagen.

Staining for type VIII collagen was also evident in those cells immediately adjacent to the cords, especially in BAEC cultures (Figure 5C).

SPARC was associated with both endothelial cords and notably with sprouting cells in BAEC cultures (Figure 5B, arrow). By time-lapse videomicroscopy, we have shown that sprouting BAECs participate in the initiation and remodeling of tubes and cords (data not presented). In contrast, a more generalized staining in RVEC cultures (Figure 5F) suggested that SPARC was associated with at least two cellular populations: those in cords and those in an apparent monolayer surrounding the cords. However, staining was seen preferentially in the cord-like arrays in RVEC cultures.

Panels D and H of Figure 5 depict staining for fibronectin in the cells and matrix of cord-containing cultures. Fibronectin appeared among these cells in a

delicate fibrillar pattern. After examining several cultures, we found that fibronectin was not specifically or preferentially associated with cords. This more generalized distribution was also found in confluent cultures of both types of endothelial cells.

Discussion

Many cellular responses are dictated by the composition and organization of the extracellular environment. From the early stages of development, a relation between cells and the ECM is established and modulated during the complex process of differentiation. Understanding the interactions between cells and their secreted ECMs will allow us to determine how specific molecules affect cellular phenotype, including those relevant to angiogenesis. In this article we have identified type I collagen, type VIII collagen, and SPARC as significant components synthesized by endothelial cells during the organization of endothelial cords *in vitro*.

We chose two different types of endothelial cells to perform these studies. Our results from macrovascular as well as microvascular cells were coincident with respect to the regulation of certain ECM proteins by endothelium. Biosynthetic heterogeneity in the ECM has previously been shown among arterial, venous, and capillary endothelial cells.^{41,42} For example, type I collagen was found to be a major secretory product of capillary endothelium but was not detected in the culture medium or in cell layers of venous or aortic endothelial cells.⁴¹ The predominant collagen secreted by BAECs is an interstitial (type III) collagen,²⁹ while umbilical vein endothelium synthesizes a basement membrane (type IV) collagen.⁴² Differences among arterial, venous, and capillary endothelial cells have also been shown with respect to the synthesis of coagulant factors.⁴³ Our present results clearly show, however, that aortic endothelial cells assume a secretory profile similar to that of capillary endothelium when both cell types undergo cord formation *in vitro*.

We have shown that the expression of type I collagen is initiated *de novo* in BAEC cultures that manifest a secondary morphological phenotype termed sprouting.^{28,44} The sprouting cells are responsible for the formation of endothelial tubes *in vitro*.²⁸ Why certain cultures spontaneously exhibit this sprouting growth pattern while others maintain a contact-inhibited monolayer is not understood. Our data would suggest that the aortic endothelial sheet is formed by a heterogeneous population of cells. The organization of cords by some clones and not by others would support this hypothesis.

Our results have also shown that levels of $\alpha 1(I)$ collagen mRNA are increased in RVECs engaged in cord formation. Furthermore, transcription of $\alpha 1(I)$ collagen mRNA was initiated in BAEC cultures undergoing angiogenesis *in vitro*. A significant amount of processing was observed for type I collagen, particularly by BAECs. This activity might also reflect the increase in collagenases and other proteases that

regulate capillary endothelial cell migration and invasion.⁴ The relevance of type I collagen to angiogenesis *in vivo* is presently unclear. Ingber and Folkman¹⁰ have claimed that metabolic reduction of collagen synthesis induced capillary regression in the chicken chorioallantoic membrane. Since type I collagen constitutes a major component of pericapillary connective tissue *in vivo*, sprouting endothelial cells are exposed to an ECM rich in type I collagen, which might provide a necessary substrate for the migration of endothelial cells during the formation of new capillaries. Alternatively, type I collagen, in concert with a surface receptor, might modulate a metabolic pathway in endothelial cells that would activate transcription of genes requisite to the process of angiogenesis.

The noncollagenous glycoprotein SPARC was also upregulated in cord-forming cultures of BAECs²⁸ and RVECs (this article). Described as a stress-induced culture shock protein secreted by endothelial cells *in vitro*,⁴⁵ SPARC was found associated with only certain kinds of endothelium *in vivo*.⁴⁶ Although the function of SPARC in blood vessels is presently unknown, its role as an inhibitor of cell spreading, as well as its ability to promote changes in cell shape and to bind to the ECM,⁴⁷ suggests that this protein might interfere with cell-matrix interactions. Immunolocalization of SPARC in cultures containing newly formed endothelial cords showed that cells in the cords as well as cells in the proximity of these structures expressed high levels of cytoplasmic SPARC. By time-lapse videomicroscopy, we have seen that endothelial tubes and cords are unstable and undergo continuous remodeling by disaggregation and incorporation of new cells. Since it is this active population of cells that expresses high levels of SPARC, we have proposed that SPARC promotes changes in focal adhesions and cell shape that release endothelial cells from their ECM and intercellular contacts. For example, these changes would allow the cellular migration necessary for otherwise contact-inhibited cells to organize into a cord or tube.

Despite the association of type VIII collagen with endothelial cords that was shown by immunocytochemistry, negligible increases in the levels of this protein were found in tube-forming cultures. Since type VIII collagen is significantly downregulated after cells reach confluence,⁴⁸ even the modest increases detected by Western blotting are likely to be relevant to the phenomenon of angiogenesis. Recent studies have shown that type VIII collagen is associated with the developing mammalian and avian cardiovascular system, particularly in the myocardium and endocardiac cushions, as well as in embryonic capillaries³⁴ and some large vessels.⁴⁹ *In vitro*, type VIII collagen appears to be preferentially expressed by proliferating and migrating cells.⁴⁸ Since we have shown that type VIII collagen was secreted by growing capillaries both *in vivo* and *in vitro*, we speculate that this protein might facilitate the assembly of endothelial cords and tubes.

McAuslan and colleagues^{50,51} have described modulation of fibronectin in cultures of sprouting BAECs. Although our data show increased mRNA levels for fibronectin in BAEC cord-containing compared with subconfluent cultures, fibronectin mRNA was clearly diminished in RVEC angiogenic cultures. These results reflect cell type-specific differences in the regulation of this gene. Jaye et al¹⁵ reported increased levels of fibronectin mRNA in cord-containing cultures of human umbilical vein endothelial cells over confluent cultures, and similar results were described for chick embryonic endothelium *in vivo*.⁵² Since it is primarily the microvascular endothelium that contributes to angiogenesis in the adult animal, it is likely that cells from these vessels respond to different stimuli and synthesize additional ECM proteins that subserve an auxiliary role to that of fibronectin. In this regard, Yang and Moses⁹ have recently shown that transforming growth factor β 1, which stimulates the synthesis of collagen and fibronectin,⁵³ induced larger vessels in the chick chorioallantoic membrane, but basic fibroblast growth factor stimulated preferentially the formation of small vessels.

In summary, we have identified specific ECM proteins that appear to be relevant to the phenomenon of cord formation by macrovascular and microvascular endothelial cells *in vitro*. Future studies will establish how these proteins interact with endothelial cells during the process of angiogenesis *in vivo*.

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