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Differential Expression of Extracellular Proteins is Correlated with Angiogenesis *In vitro*

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Strains of bovine aortic endothelial cells, grown on plastic under conventional culture conditions and in the absence of growth factor supplementation, exhibited a sprouting phenotype and a predisposition toward the formation of cords and tubular structures. We examined endothelial cells at different stages of tube formation. Analysis of metabolically labeled proteins showed that the synthesis of type I collagen was initiated in sprouting cells and during the formation of tubular structures. SPARC (secreted protein, acidic and rich in cysteine) a Ca^{2+}-binding protein associated with cellular shape change and morphogenetic processes (Sage H, Vernon RB, Funk SE, Everitt EA, Angello J: J Cell Biol 109:341, 1989), was upregulated during spontaneous tube formation. Levels of messenger RNA for type I collagen and SPARC corroborated the stage-specific increases observed for these proteins. Differential levels of transcription were apparent in multilayered cells directly involved in tube formation, in comparison with cells comprising either the tubes or the confluent monolayers at a distance from the tubes. Analysis of DNA synthesis indicated that multilayered, sprouting cells in the proximity of the endothelial tubes were actively proliferating, whereas cells that had been incorporated into tubes showed low levels of DNA synthesis.

Immunolabeling studies revealed a dense accumulation of SPARC and type I collagen in the cytoplasm of cells that were situated near the growing tubes. Two other secreted proteins, type III collagen and thrombospondin, were expressed constitutively by subconfluent cultures and were increased in those cells contributing to tube formation. We propose that type I collagen and SPARC are specifically related to the angiogenesis-like phenomenon displayed by bovine aortic endothelial cells *in vitro*. Type I collagen might facilitate the active migration of endothelial cells, or the stabilization of the resulting tubes, with SPARC directing the re-organization and dynamic assembly of the tubular network.

Additional key words: Endothelial cells, SPARC, Type I collagen, extracellular matrix.

The formation of new capillaries (angiogenesis) from pre-existing vessels is critical to embryonic development, growth of tumors, tissue repair, and in a limited number of normal physiologic processes including endometrial cycling. The assembly of cultured endothelial cells into capillary-like tubes (angiogenesis *in vitro*) has been used extensively as a model system to study mechanisms that might direct this process *in vivo*. Questions addressed in the context of this angiogenesis model have centered on the identification of signals responsible for stimulating endothelial cells to migrate, proliferate, and organize into new capillaries (11). Within the last two decades, both the influence of growth factors and the role of the extracellular matrix (ECM) have emerged as potential regulatory effectors of endothelial cell behavior during angiogenesis *in vitro*. For example, basic fibroblast growth factor has been shown to stimulate tube formation *in vitro* (39) and neovascularization *in vivo* (11), while transforming growth factor-α and transforming growth factor-β induced angiogenesis *in vivo* (12, 46).

A number of investigators have suggested, however, that neovascularization and angiogenesis are not only mediated by growth factors, but are also dependent on the precise regulation of the synthesis and degradation of the ECM (2, 19, 32). For example, alterations in the secretion of native collagen (18), or in the abundance and assembly of other ECM proteins (8, 17) promote regression of growing capillaries in the chorioallantoic membrane. Investigation of the role of ECM *per se* in the organization of capillaries from endothelial cells has elicited different experimental approaches, *e. g.*, the effects of selected ECM components on the migration, proliferation, cytoskeletal organization, and shape of endothelial cells *in vitro* (1, 30, 32) and *in vivo* (18). Several groups have also used substrates to enhance tube formation *in vitro* (56). Kubota *et al.* (24) demonstrated that endothelial cells from umbilical vein cultured on type I collagen and laminin form tubes within 18 hours in culture. Similarly, specific short amino acid sequences in laminin seem to promote the organization of umbilical

vein endothelial cells into networks (14). Data describing changes in gene expression and secretory phenotype during the morphogenesis of endothelial cells into capillaries are limited. Intrinsic changes in fibronectin mRNA levels were observed when umbilical vein endothelial cells organized into tubular structures in the presence of endothelial cell growth factor (21). These and other studies have addressed the effects of exogenous agents on endothelial cell behavior. The appearance of newly synthesized proteins, transcriptional and/or translational modulation of previously active genes, or cessation of a specific transcriptional activity during the spontaneous organization of endothelial tubes would contribute to our understanding of this morphogenetic process.

In this study, we initially characterize the spontaneous organization of tubular structures by sprouting strains of bovine aortic endothelial (BAE) cells. We then show progressive changes in secretory phenotype during the assembly of these cells into tubular networks. Our data demonstrate initiation of expression of type I collagen, an upregulation of secreted protein, acidic and rich in cysteine (SPARC), and a specific association of type III collagen and thrombospondin with capillary-like structures. We propose that type I collagen in angiogenic cultures is a necessary substrate for the attachment and spreading of endothelial cells during the formation of endothelial tubes. SPARC might play an important role in inducing changes in the cytoskeleton that promote cell rounding and migration, and ultimately contribute to active reorganization of endothelial tubes.

EXPERIMENTAL DESIGN

Characterization of the system *in vitro* was performed by microscopy and cell proliferation assays. Subsequently, a combination of biochemical analyses of secreted proteins, Northern blots, and immunocytochemistry were used to identify changes in the levels and distribution of ECM components during the morphogenesis of endothelial tubes.

Cell Culture

Endothelial cells were isolated from adult bovine aorta, cloned, and passaged as previously described (51). Clones expressing a sprouting phenotype (7) were selected for further study. The cells were grown in Dulbecco's modified Eagle's medium (GIBCO Laboratories, Grand Island, New York) containing 10% heat-inactivated fetal calf serum (lot 1111593, Hyclone Laboratories, Logan, Utah), 250 μg/ml of amphotericin B (Sigma Chemical Company, St. Louis, Missouri), and 100 units/ml each of penicillin and streptomycin (Sigma). Experiments were performed with sprouting BAE cells between passages 4 and 11. Strains of BAE cells that did not manifest a sprouting phenotype were used as controls in biosynthetic experiments.

Spontaneous formation of capillary tube-like structures was observed after 15 days in culture. In some cases these structures were isolated from the total culture by several rinses with EDTA, since the tubes detached readily from the dishes. Isolated tubes were placed in a solution of 0.02% trypsin and EDTA to dissociate the component cells. After 5 minutes at room temperature, the trypsin was inactivated by Dulbecco's modified Eagle's medium containing 10% fetal calf serum, and the cells were collected by centrifugation. In a typical experiment, more than 70% of the tubes were removed from the culture dish. This recovery was quantitated as follows: sprouting BAE cells were grown in 24-well Costar plates until profuse tubular formation was observed. Initially, we counted the number of tubes/mm^2. Subsequently, the cultures were washed with EDTA until the detachment of capillary-like structures occurred. This procedure was monitored by phase contrast microscopy. Finally, we counted the number of remaining tubes/mm^2 after the EDTA washes. By subtraction, the number of tubes extracted by EDTA was 73.5 ± 8.4%. This number represents the average of 24 independent experiments (1 culture dish/experiment) (Table 1).

Cells comprising tubes were examined for their capacity to endocytose acetylated low density lipoproteins (ac-LDL) and to synthesize von Willebrand factor (vWF). Endocytosis of ac-LDL labeled with 1,1'dioctadecyl-1-3,3,3',3',-tetraethylindocarbocyanine (Dil-ac-LDL) (Biochemical Technologies, Inc., Stoughton, Massachusetts) was analyzed by incubation of the cultures with 5 μg/ml of Dil-ac-LDL in Dulbecco's modified Eagle's medium (42). vWF was identified by a polyclonal antibody preparation against human plasma vWF (rabbit IgG fraction, lot No. 015; Dako Corporation, Santa Barbara, California) in conjunction with an avidin-biotin-peroxidase technique (ABC Reagent, Vector Laboratories, Burlingame, California) (16).

Cell Proliferation Assays

Cells from a non-sprouting BAE strain and a sprouting BAE strain were plated in 35-mm tissue culture dishes and cultured under standard conditions. After 2, 4, 7, 15, 30, and 45 days in culture, the cells were trypsinized and counted by a hemocytometer. DNA synthesis was measured in subconfluent, confluent, and 45-day (tube-forming) BAE cultures by incorporation of 5-bromo-2'-deoxyuridine (BrdU) (Cell Proliferation Kit, Amersham, Arlington Heights, Illinois). Differential incorporation of BrdU was also quantified in 45-day tube-forming cultures with respect to three areas: (I) cells in the walls of tubes, (II) cells exhibiting a polygonal morphology in areas devoid of tubes, and (III) multilayered cells in the proximity of tubes (sprouting cells). Eight cultures plated in Labtek slides (Nunc, Inc., Naperville, Illinois) were

TABLE 1. RECOVERY OF INTACT ENDOTHELIAL TUBES FROM BAE CELL CULTURES[a]

Measured parameter	Numerical values[b]
Number of tubes/mm^2	17 (±4)
Percentage of tubes extracted by EDTA	73.5 (±8.4%)
Number of cells/tube	84 (±15)
Percentage of total cell population recovered as tubes	60 (±7.5%)

[a] Long-term cultures of tube-forming BAE cells were treated with EDTA to remove tubes selectively, as described in the "Experimental Design" section.

[b] Values (mean ± SD) represent the average of 24 different cultures plated on 16 mm^2 dishes.

preincubated for 2 hours with the labeling reagent (BrdU containing 5-fluoro-2'-deoxyuridine, which inhibits thymidylate synthetase and thereby maximizes incorporation of BrdU). After several washes with phosphate-buffered saline (PBS), the incorporated BrdU was detected immunocytochemically with an anti-BrdU monoclonal antibody, followed by anti-mouse IgG-peroxidase in conjunction with diaminobenzidine. A solution of 1% toluidine blue was used as a counterstain. The slides were mounted and cells were photographed and counted with a ×25 objective. For the counting procedure, we selected regions of tube formation, which simultaneously contained areas I, II, and III. All cells present in the field were counted and classified within the three areas. For each area at least 1,000 cells were counted. Finally, we counted labeled nuclei and obtained the mitotic index ± SE as shown in Table 2.

In one set of experiments, incorporation of BrdU was analyzed in (a) 8 cultures of sprouting BAE cells containing tube-like structures, plated for 45 days on Labtek slides, (b) 4 cultures of subconfluent BAE cells exhibiting a nonsprouting phenotype, and (c) 4 cultures of confluent, nonsprouting BAE cells. Differences between the values in each slide were not greater than 15% in the subconfluent population, 10% in the confluent population, and 8.5% in the cultures containing endothelial tubes. The average of the values obtained was determined for each culture, and total cell number was obtained by trypsinizing and counting duplicate cultures with a hemocytometer.

Table 2. Regional Distribution of BrdU Incorporation in Tube-Containing Cultures of BAE Cells

Areas	Mitotic index (in %) (±SD)
I	1.00 (±0.05)
II	3.27 (±20.6)
III	14.25 (±0.08)

[a] The values indicated are the averages of three different cultures plated on Labtek slides. Cultures were incubated with BrdU and incorporation was visualized by an immunoperoxidase reaction, as described in the "Experimental Design" section.

Biochemical Analyses

The spectrum of ECM proteins secreted by BAE cultures was analyzed by metabolic label with [^{3}H]proline and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Since we were interested in observing changes in the biosynthetic profile of BAE cultures during the formation of endothelial tubes, the labeling procedure was done at specific time points of tube formation (see the "Methods" section).

Molecular Biology

Levels of type I collagen and SPARC were measured by Northern blots. In addition, linearity of signal was verified by slot blots.

Total RNA was extracted from cultures at the following stages: Subconfluency, confluency, tube-containing cultures, and isolated tubes. RNAs were hybridized with an α1(I) collagen (6) probe and a SPARC probe (34) (see the "Methods" section for details). Autoradiograms were scanned with an Integrator-Zeineh soft laser scanning densitometer, model SLR-504-XL (Biomed Instruments, Fullerton, California). Values were corrected for equal loadings and plotted as a histogram.

RESULTS AND DISCUSSION

Formation of Endothelial Tubes *In vitro*

BAE cells in culture most frequently exhibit an epithelioid polygonal morphology. At confluence, the cells usually form a contact-inhibited monolayer resembling the corresponding endothelium *in vivo* (Fig. 1*a*). However, in some BAE cultures, a second pattern of growth, termed sprouting, has been observed (Fig. 1*b*). The "sprouting" phenomenon exhibited by some strains of BAE cells has been extensively described as a second morphologic pattern of endothelial cell growth (36, 57). In our studies these cells appeared 5 days after confluence and proliferated rapidly. Within the next 7 days these sprouting cells organized into cord-like structures that communicated via branching networks (Fig. 1*c* and *d*). Time-lapse videomicroscopy revealed that these structures were highly dynamic and showed active dissolution and re-organization (data not shown). In cultures plated for longer periods of time, the number and length of these tubes were increased.

Cross-sections of sprouting cultures revealed that the network of cords originally consisted of tubes with lumina formed by 1 to 4 endothelial cells that were situated below the monolayer (Fig. 1*f*). That these tubes could be isolated from the total culture by several washes with EDTA (Fig. 1*e*), suggests that the contacts between the tubes and surrounding cells are more sensitive to the removal of divalent cations than the intercellular contacts that occur in other areas of the culture. Of the total number of tubes present in the culture, 73.5% were extracted by this procedure. Also, we were able to estimate that an average of 84 cells comprised a tube. These results are summarized in Table 1. Based on differences in morphology and growth characteristics, these endothelial cultures appeared to contain at least 3 populations: (a) endothelial cells that organized into tubes, (b) polygonal cells constituting a typical cobblestone monolayer, and (c) endothelial cells surrounding tubes, frequently with a sprouting morphology. This apparent distinction was not due to the presence of mixed populations, as each BAE strain was derived by dilute-plate cloning and retained on the basis of the morphology as shown in Figure 1*a*. Certain strains were, however, chosen according to their propensity for tube formation. Moreover, trypsinization and replating of an isolated tube, shown in Figure 1*e*, resulted in a monolayer of polygonal cells identical in appearance to that shown in Figure 1*a*. The presence of vWF, as well as the capacity to engage in active endocytosis of Dil-ac-LDL, demonstrated that cells involved in tube formation and the monolayer from which these cells arose retained specific endothelial markers (data not shown).

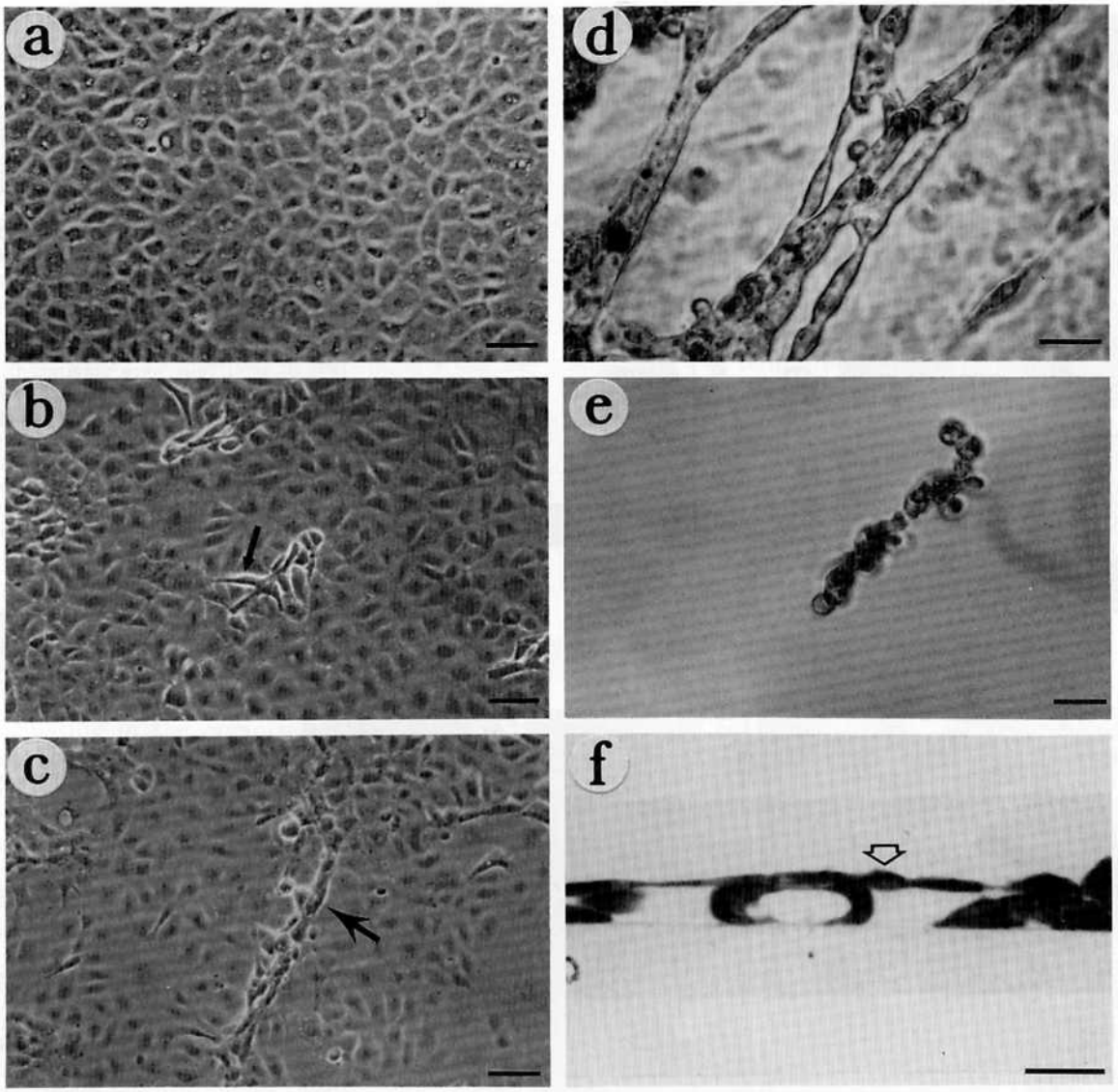

FIG. 1. Formation of endothelial tubes by BAE cells *in vitro*. *a*, A confluent monolayer of BAE cells is shown with a characteristic polygonal morphology (2 days in culture). *b*, Five days postconfluence, the culture shows a second population of elongated cells (*arrow*) termed sprouts. *c*, After 15 days in culture, sprouting cells organize into cords (*arrow*). *d*, At higher magnification, the intercommunicating endothelial cords are easily distinguished from the monolayer (out of focus) (45 days in culture). *e*, Tube-like structures can be preferentially dissociated from the culture by treatment with EDTA. *f*, Cross-section of a 15-day BAE culture demonstrates the presence of tubes. In this particular section, the lumen is enclosed by two cells. The *open arrow* indicates the monolayer. Bar, 20 μm.

DIFFERENCES IN RATES OF PROLIFERATION IN TUBE-FORMING CULTURES

Nonsprouting BAE cells, which do not form tubes, and sprouting BAE cultures, the latter selected according to different stages of tube formation, were trypsinized and counted at specific time points. The growth curve corresponding to the nonsprouting BAE cells reached a plateau at confluence (4 days postsubculture) (Fig. 2*B*). In contrast, the sprouting BAE cells continued to grow until mature tubes were formed (30 to 45 days post subculture) (Fig. 2*A*). Analysis of the incorporation of BrdU in a 45-day culture corroborated the results shown in Figure 2. In both confluent monolayers (C) and in 45-day tube-forming cultures (*T*), 0.5% of the total cell population incorporated BrdU, whereas 5% of the cells

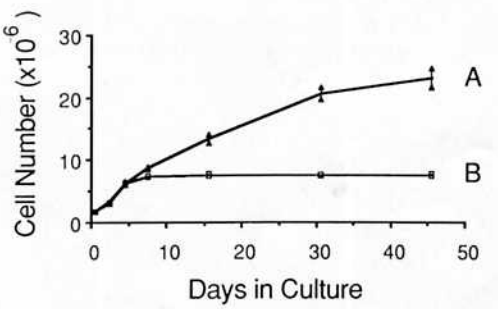

FIG. 2. Kinetics of BAE cell growth differ between tube-forming and nonsprouting cultures. Growth curves of a sprouting tube-forming strain (*A*) and a nonsprouting strain (*B*) are shown. Cells were plated in duplicate at the same density (1×10^6 cells/ml) and were trypsinized and counted after 2, 4, 7, 15, 30, and 45 days in culture.

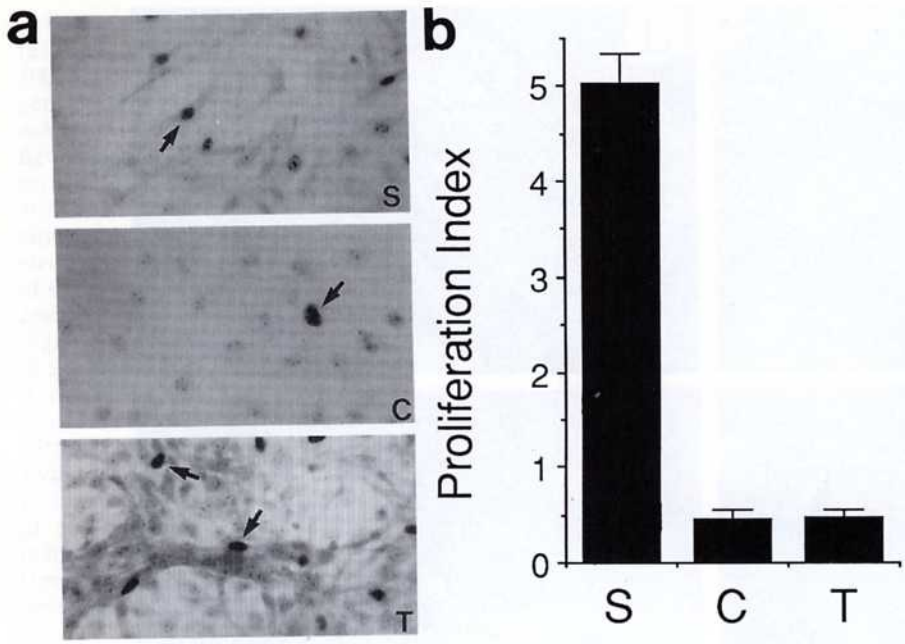

FIG. 3. Incorporation of BrdU in sprouting strains of BAE cells at progressive stages of tubular organization. *a*, Subconfluent (*S*) and confluent (*C*) BAE cultures, as well as those displaying extensive tubular organization after 45 days (*T*), were incubated with BrdU for 2 hours, and the incorporated analog was identified by an immunoperoxidase staining reaction. Labeled cells (*e.g., arrows*) were counted to generate the data shown in the histogram (*b*). The numbers of total cells were obtained from duplicate dishes. Bar, 20 μm. *b*, BAE cultures as shown in (*a*) were labeled with BrdU for 2 hours, and cells undergoing DNA synthesis were counted. Proliferation index is defined as the percentage of the total cell population that incorporated BrdU (±SD).

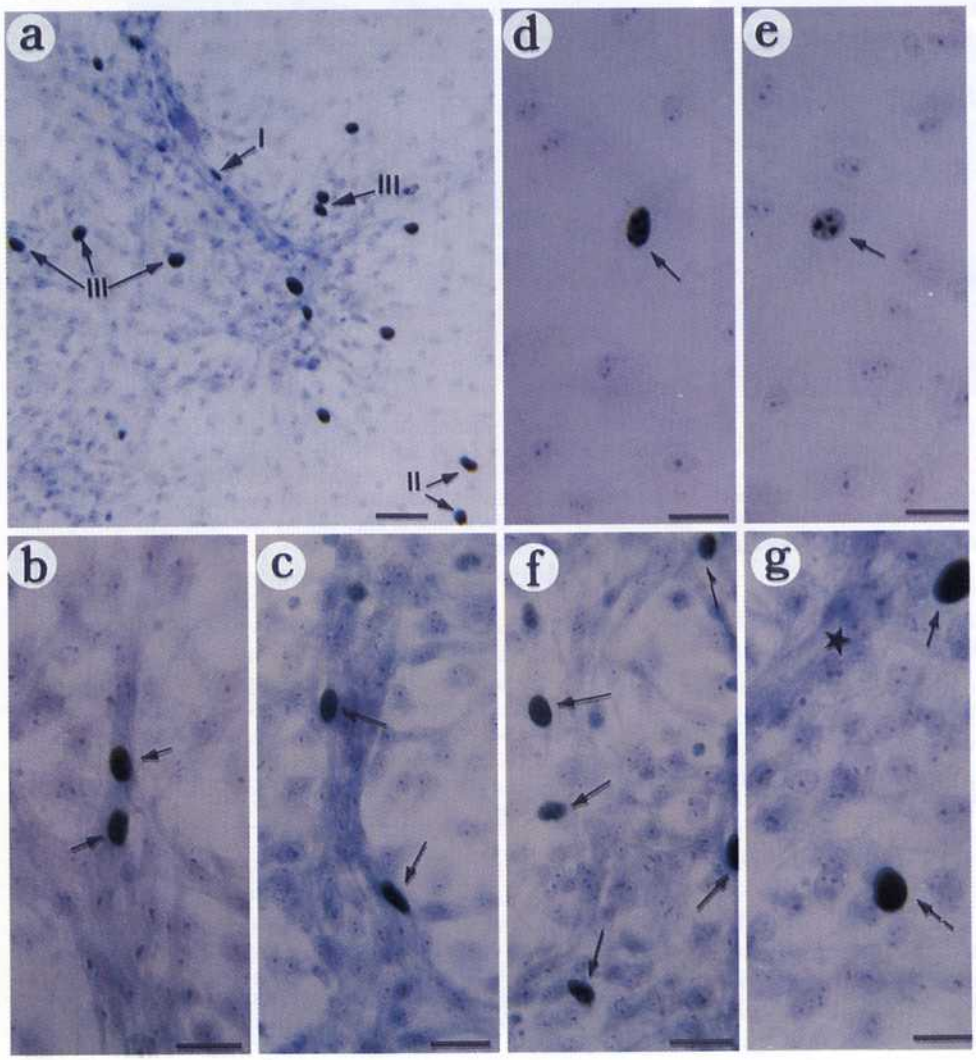

FIG. 4. Tube-forming cultures of BAE cells exhibit differential incorporation of BrdU. Cultures (45 days) displaying extensive tubular organization were incubated with BrdU as described in the legend to Figure 3. Three areas were counted: area I corresponds to cells in the tubes; area II corresponds to cells in the monolayer; area III represents the population that does not belong to either monolayer or tubes. This population is associated with multiple cell layers and is most often found in the proximity of capillary-like tubes (star). *a*, Low magnification showing the three areas; *b* to *g*, Higher magnifications of area I (*b*, *c*), area II (*d*, *e*), and area III (*f*, *g*). Bar, 20 μm.

in the subconfluent cultures (*S*) were engaged in DNA synthesis (Fig. 3).

In vivo, two of the earliest events in angiogenesis are the proliferation and active migration of endothelial cells. We questioned whether cells undergoing tube formation *in vitro* exhibited preferential synthesis of DNA. Mature, tube-forming BAE cultures (45 days) were labeled with BrdU and counted. Three different areas were characterized: (I) cells in the walls of the tubular structures, (II) cells in the monolayer and not involved in the organization of tubes, and (III) the remaining cell population, which included cells in the proximity of tubes and those associated with multilayers. Several examples of areas I, II and III are shown in Figure 4. There were significant differences in the incorporation of BrdU in the three populations. Cells in the proximity of the tube-like structures had a mitotic index of 14.25%. These cells exhibited a spindle-shaped (sprouting) morphology (Fig. 4*f* and *g*), and were often seen in multilayers. By video-microscopy, they participated in the formation of cords (data not shown). BAE cells that were not actively participating in tube formation displayed a polygonal morphology and exhibited a mitotic index of 3.27% (area II) (Fig. 4*d* and *e*). The cells present in the tubes themselves were considerably less active in DNA synthesis (mitotic index = 1%) (area I) (Fig. 4*b* and *c*). These results are summarized in Table 2. Since the proliferation index of the total culture was 0.5%, there appeared to be incidences of mitosis in areas related to tube formation.

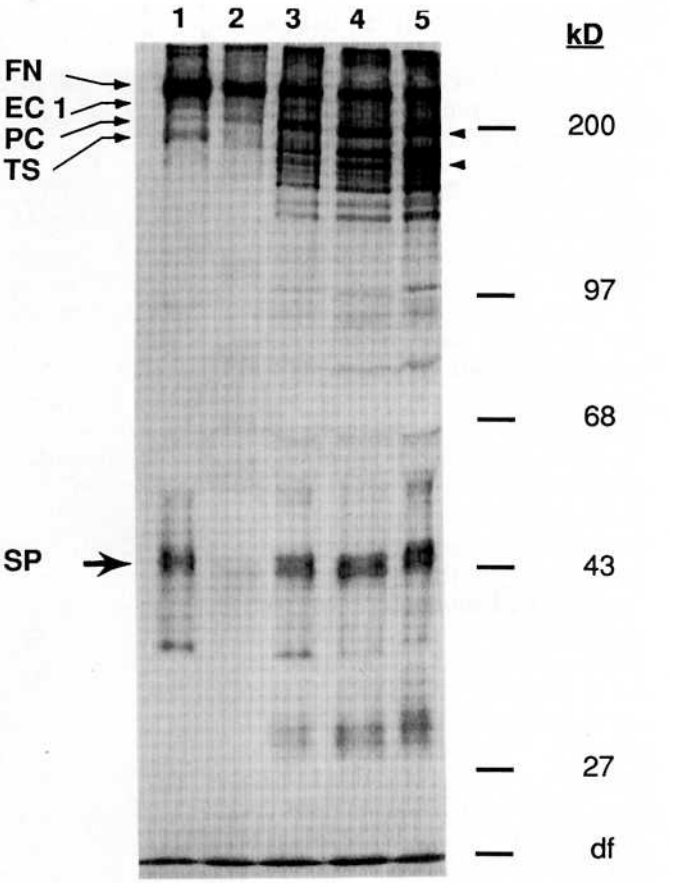

FIG. 5. Secretory phenotypes of BAE cells at different stages of organization into endothelial tubes. Cells at five different stages of tubular organization were metabolically labeled with [^{3}H]proline for 18 hours, and proteins in the culture medium were precipitated in 10% TCA. Pellets were analyzed by SDS-PAGE on a 4/8% gel under reducing conditions, and proteins were visualized by fluorescent autoradiography. *Lane 1*, subconfluent cultures (2 days); *lane 2*, confluent cultures (4 days); *lane 3*, appearance of tubular structures (15 days); *lane 4*, mature tubes (30 days); *lane 5*, mature tubes and more extensive tubular organization (45 days). Globular protein molecular weight standards are shown on the *right*. Unlabeled *arrowheads* indicate the migration of proα 1(I) and proα 2(I) chains, and *arrows* denote the α_1 and α_2 collagen chains. Fibronectin (*FN*), thrombospondin (*TS*), type VIII collagen (*EC 1*), pro α1 (III) collagen chains (*PC*), and SPARC (*SP*) are identified. *df*, dye front.

ORGANIZATION OF TUBE-LIKE STRUCTURES IS ASSOCIATED WITH CHANGES IN SECRETED PROTEINS

Strains of BAE cells forming tubes *in vitro* were metabolically labeled, and proteins secreted into the culture medium were analyzed by SDS-PAGE. The spectrum of secreted proteins changed significantly during the organization of endothelial tubes. The results were identical in two different strains of tube-forming BAE cells. Proteins secreted at progressive times during tube formation are shown in Figure 5 and were identified by a combination of Western blotting (not shown) and mobility on SDS-PAGE in the absence (not shown) and presence of DTT. Subconfluent BAE cells synthesized high levels of thrombospondin, fibronectin, and SPARC, as well as lesser amounts of procollagen types III (PC) and VIII (EC1) (Fig. 5, *lane 1*). A similar pattern was seen in confluent cultures, although the levels of thrombospondin and SPARC were considerably diminished (Fig. 5, *lane 2*). In early tube-forming cultures, however, synthesis of type I collagen (unlabeled *arrowheads* identify proα1(I) and proα2(I) collagen chains, and *arrows* indicate the migration of α1(I) and α2(I) collagen) was initiated, and SPARC was secreted at a higher rate (*lane 3*). Later stages of tubular organization (30 and 45 days, *lanes 4* and *5*, respectively) exhibited the biosynthetic pattern described for early tube-forming cultures (*lane 3*). In tube-containing cultures, the synthesis of type I collagen is also associated with various intermediate forms that are products of the proteolytic processing at the N and C terminal domains, resulting in new chains: p_C α1(I), p_N α1(I), p_C α2(I), and p_N α2(I). These bands migrate between pro α1 (I) and α2 (I) (49), as identified in Figure 5. Isolated tubes also synthesized type I collagen and SPARC (data not shown). Fibronectin, type III procollagen, and type VIII (pro)collagen were present in all cultures.

Figure 6 shows digests of culture medium proteins after incubation with bacterial collagenase. This procedure confirmed the identification of collagenous proteins and intermediate forms as stated in Figure 5. Moreover, it can be seen that nonsprouting BAE cells cultured for 50 days (*lanes 7* and *8*) had a secretory profile highly similar to that observed for subconfluent sprouting cells (*lanes 1* and *2*). The changes in secreted protein coincident with tube formation therefore are not a consequence of

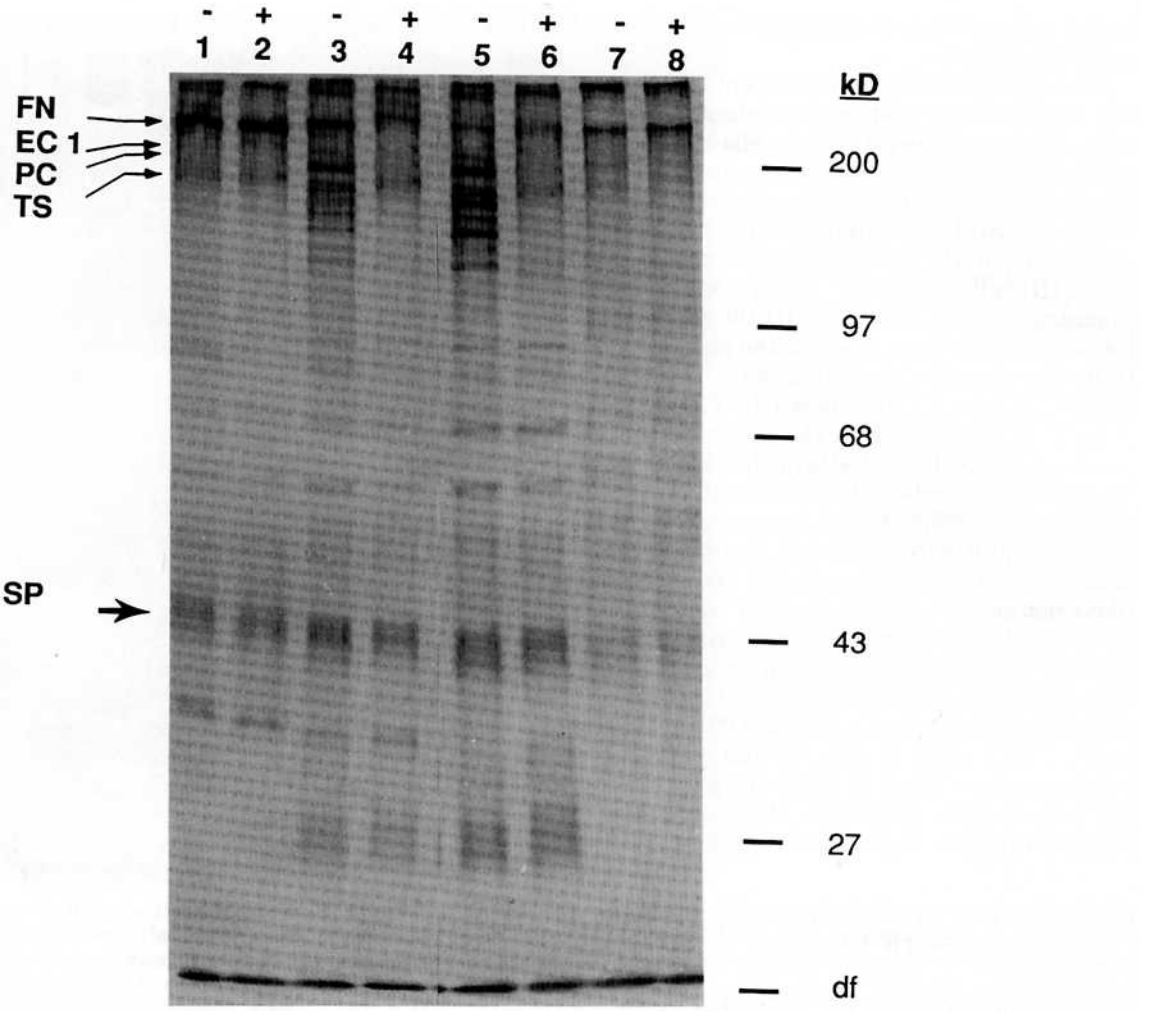

FIG. 6. Identification of collagenous proteins secreted by BAE cells in the process of tube formation. Culture medium proteins were initially precipitated in 10% TCA. Each pellet was resuspended in Tris-saline containing 10 mM $CaCl_2$ and incubated with bacterial collagenase. Undigested proteins were recovered by precipitation in TCA, and the digest was analyzed by SDS-PAGE on a 4%/8% gel in the presence of 50 mM DTT. Fluorescent autoradiograph shows secreted proteins from subconfluent cultures (*lanes 1* and *2*), tube-forming cultures after 15 days (*lanes 3* and *4*), and cultures exhibiting extensive tubular organization after 45 days (*lanes 5* and *6*). In addition, a nonsprouting strain of BAE cells, that had been in culture for 50 days, was analyzed (*lanes 7* and *8*). + and − indicate the presence and absence of collagenase, respectively. Proteins are identified as stated in the legend to Figure 5.

longevity *in vitro*, but are most likely associated with overt morphologic changes in the cells themselves.

mRNA Levels of Type I Collagen and SPARC are Correlated with Formation of Endothelial Tubes *In vitro*

The induction of type I collagen protein in tube-forming BAE cell cultures was confirmed by analysis of the corresponding mRNA. Total RNA was extracted and purified from cultures at subconfluent density, from cultures at confluent density, from cultures containing confluent cells as well as organized tubes, and from isolated tubes. Five different concentrations of total RNA from each stage of tube formation, ranging from 0.2 to 5 μg, were blotted onto nitrocellulose and probed with a nick-translated α1(I) collagen cDNA (Fig. 7*c*). To verify the size of mRNA that hybridized to the probe, Northern blots were also performed (Fig. 7*a*). Type I collagen mRNA was absent in subconfluent and confluent BAE cultures (Fig. 7*a*, *lanes 1* and *2*), but it was clearly present in tube-containing cultures as two bands of 4.7 and 5.7 kb (Fig. 7*a*, *lane 3*). These data demonstrate that the organization of tube-like structures is correlated with an initiation of transcriptional activity of the α1(I) collagen gene.

In the same context, we also analyzed these blots for the presence of SPARC mRNA. SPARC protein, initially identified as a culture shock product, is associated with some types of endothelial cells and in areas of active proliferation and morphogenesis *in vivo* (54). As shown in Figure 5, this protein was present during early to late stages of tube formation. However, we were interested in comparing the levels of SPARC mRNA in cells from subconfluent, confluent, and tube-containing cultures, as well as in isolated endothelial tubes. Data from slot blots indicated that the cultures which contained the tubes (*T*) expressed the highest levels of SPARC mRNA, followed by the subconfluent (*S*) cultures and the confluent cultures (*C*) (Fig. 7*b* and *d*). Confluent cultures containing tubes (*T* in Fig. 7) also contained the highest propor-

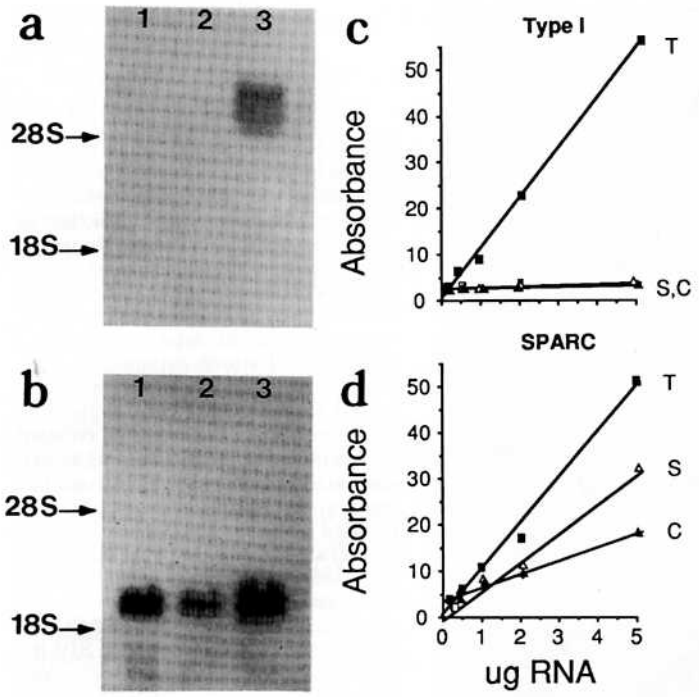

FIG. 7. Levels of type I collagen and SPARC mRNA during the formation of endothelial tubes *in vitro*. *a* and *b*, Northern blots of total RNA from subconfluent cultures (*lane 1*), confluent cultures (*lane 2*), and confluent cultures containing tubular networks (*lane 3*) were hybridized with (a) α1(I) collagen and (b) SPARC cDNAs. *c* and *d*, Increasing amounts of total RNA from the same subconfluent cultures (*S*), confluent cultures (*C*), and confluent cultures containing tubes (*T*) were slot-blotted and hybridized with (*c*) α1(I) collagen and (*d*) SPARC cDNAs. Hybridization levels were quantitated by scanning densitometry.

tion of proliferating BAE cells (corresponding to area III in Fig. 4). By Northern blot analysis (Fig. 7*b*), a SPARC mRNA of 2.2 kb (34) was identified that confirmed the relative abundance shown in Fig. 7*d*. In five separate experiments, the average increase in SPARC mRNA between subconfluent (Fig. 7*b*, *lane 1*) and tube-containing cultures (*lane 3*) was 4.5-fold. There was an increase of approximately 10-fold in SPARC mRNA between confluent (Fig. 7*b*, *lane 2*) and tube-containing cultures (*lane 3*). We found levels of SPARC and type I collagen mRNA in isolated tubes that were intermediate between the respective levels in confluent and tube-containing cultures (data not shown).

Extracellular Proteins are Differentially Distributed in Tube-Forming Cultures

We have identified specific secreted proteins associated with the formation of endothelial tubes *in vitro*. It was therefore of interest to determine the distribution of these proteins among the organized tubes and surrounding cells. To address this question, collagen types I, III, and IV, as well as thrombospondin and SPARC, were localized by immunocytochemistry in 45-day cultures of BAE cells with profuse tube formation.

Immunoreactivity corresponding to the presence of type I procollagen and collagen was specifically associated with the cells involved in tubular organization; there was no reactivity in cells that formed the monolayer (Fig. 8*a*). Subconfluent and confluent BAE cultures were also negative for this collagen (not shown).

The distribution of type I collagen was distinct from the other collagen types that we examined. Type III collagen, the major collagen species produced by growing BAE cells *in vitro*, was identified extracellularly as fine fibrils around the tubes and between the cells (Fig. 8*b*). No staining was seen when these cultures were treated with antibody to type IV collagen, a known component of basement membranes (data not shown). Since isolated tubes were found to contain α1(I) collagen mRNA, we suggest that the source of at least some of the tubular staining seen in Figure 8*a* was the intratubular endothelium.

Noncollagenous proteins were also distinctive in their distribution relative to endothelial tubes. Thrombospondin appeared as fine fibrils around the tubular structures, and intracellular reactivity, frequently detected in BAE cultures for this protein, was also observed (Fig. 8*c*). Since the metabolic labeling experiments demonstrated low levels of secretion of thrombospondin by tube-containing cultures, we speculate that this protein accumulates extracellularly and is probably associated with a fibril-forming matrix protein, *e.g.*, type V collagen (41).

SPARC was frequently observed in intracellular granules, especially in those cells which appeared to be involved in the organization of tubes (Fig. 8*d*). In mature tube-containing cultures, filamentous arrays were associated with some of the endothelial tubes. This pattern could result from the binding of SPARC to specific collagen types (55).

Discussion

Fundamental questions concerning mechanisms that regulate the morphogenesis of capillaries include the identification of exogenous and endogenous factors that activate angiogenic pathways in endothelial cells. Experimental models illustrating one result of this activation, endothelial tube formation *in vitro*, have been described. Several of them have included the use of exogenous components, such as growth factors (31, 39) and defined substrates (14, 24, 38, 43), to elicit 3-dimensional networks of endothelial cords. Spontaneous formation of tubes has also been described. Endothelial cells from human umbilical vein (29), calf aorta (9), and the microvasculature of bovine adrenal cortex (37) formed a meshwork of intercommunicating tubes in the absence of exogenous soluble or insoluble factors, except those present in the media of these cultures.

Our studies show that adult BAE cells are also able to organize into multicellular tubes that, in several respects, resemble capillaries *in vivo*. This characteristic seems to be specific to BAE cultures that exhibit a secondary morphology called sprouting (36, 57). Sprouting cells were elongated, grew beneath the endothelial monolayer, and initiated the formation of cords in BAE cultures. Eventually, the cords acquired a central lumen completely surrounded by cells. However, the monolayer of polygonal endothelial cells from which the sprouting cells arose was always present during the process. In this study we have examined some of the factors associated

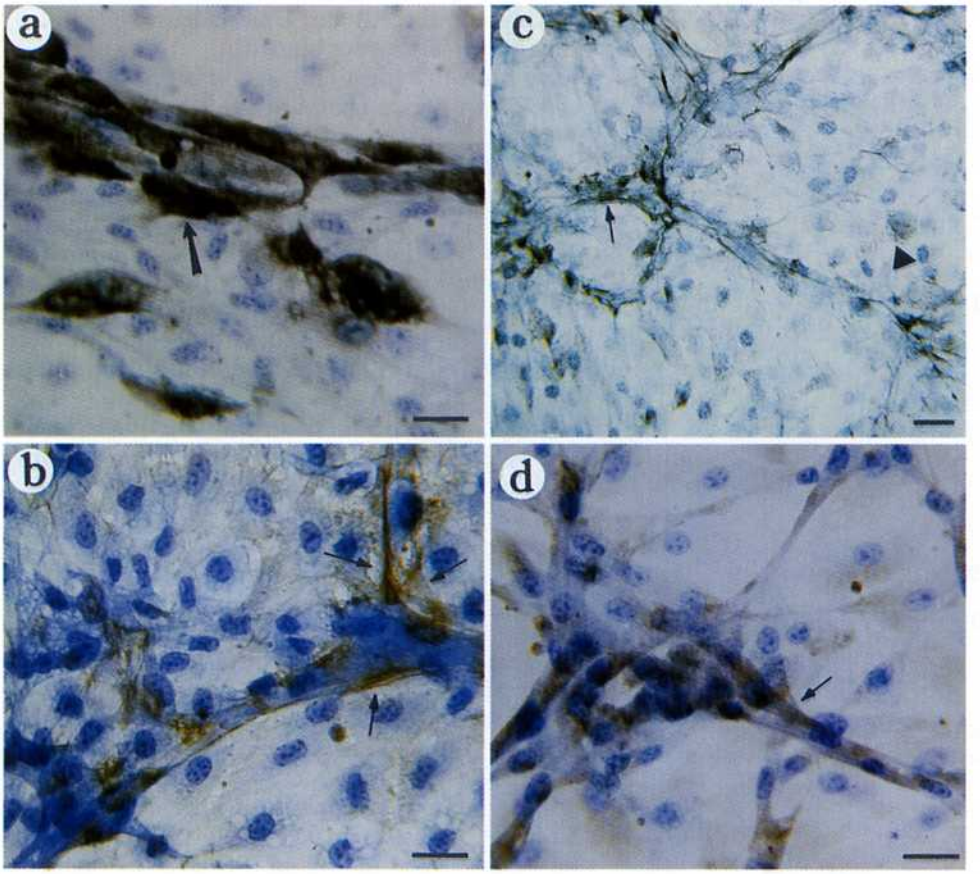

FIG. 8. Immunolocalization of extracellular proteins in cultures of BAE cells engaged in tube formation. Cells were grown on glass cover slips until tubular structures became apparent. Immunocomplexes were visualized by an avidin-biotin-peroxidase technique, and toluidine blue was used as a counterstain. *a*, Antibodies against type I procollagen demonstrated strong cytoplasmic reactivity (*arrow*) that was preferentially localized to endothelial cells engaged in tube formation. *b*, Anti-type III collagen IgG identified a fibrillar staining associated mainly with tube-like structures (*arrows*). *c*, Anti-thrombospondin IgG revealed a fibrillar array (*black stain*) of protein around cells that were organizing into cord-like networks (*arrow*). Some cells also showed reactivity in the cytoplasm (*arrowhead*). *d*, Anti-SPARC antibodies identified intracellular SPARC in several areas, but SPARC was preferentially localized in cells that comprised tubes (*arrow*). Bar, 20 μm.

with the formation of endothelial sprouts and tubes, and have presented evidence that both type I collagen and SPARC are actively transcribed during the intercellular association of endothelial cells into tube-like structures.

In vivo, angiogenesis takes place as a series of sequential steps and is initiated in part by local degradation of the basement membrane in the parent vessel (13). Cells then protrude through the digested basement membrane and exhibit active migration. At this stage of the process, the cells assume a bipolar configuration and are termed sprouts. Both proliferation and formation of vascular lumina are the major events associated with the last stage. After the new vessel is formed, endothelial cells exhibit a very low mitotic index, although they do retain mitogenic activity (11).

The formation of tubes *in vitro* therefore resembles, in some respects, the process of angiogenesis *in vivo*. It is probable, however, that there are many variations in the angiogenic response that are dependent on, *e.g.*, the initiating stimulus or the location of the progenitor vascular bed, and that systems *in vitro* may be biased toward a particular subset of endothelial cells. In culture, the appearance of sprouting is concomitant with proliferation and cellular organization. We were interested in identifying the population of cells (*e.g.*, polygonal or sprouts) that was responsible for tube formation, and in determining the synthesis and accumulation of ECM proteins that might be preferentially associated with these cells. Our results demonstrated that areas where endothelial cords were forming contained sprouting cells that exhibited high levels of proliferation. After the cells were incorporated into tubes, mitosis was considerably reduced. This new population of proliferative, sprouting cells produced elevated levels of SPARC and initiated synthesis of the interstitial procollagen, type I.

Previous studies have shown that BAE cells in culture synthesize fibronectin, types III and VIII collagen, thrombospondin, and SPARC (4, 28, 48, 49, 51). Culture medium from sprouting BAE cells contains, in addition, type I collagen (7, 26). Our data show that this collagen type was also secreted in significant amounts after the organization of endothelial tubes. These results were confirmed by both immunocytochemistry and analysis of cellular mRNA. It was interesting to note that bovine endothelial cells cultured from aorta, veins, and retinal capillaries did not synthesize type I collagen (52, 53), whereas endothelial cells from bovine adrenal capillaries secreted considerable amounts of this protein (53). An explanation for this apparent discrepancy was the conditions under which the capillary cells were grown: the adrenal cells, cultured on plastic in the presence of tumor cell-conditioned media, overgrew and sprouted (10), whereas the retinal cells, grown on fibronectin-coated dishes, remained as monolayers at confluence (48).

In a recent paper, Ingber and Folkman (20) remarked on the important of mechanical-tension interactions between cells and their substrate to promote cord formation. It is possible that fibrillar collagens, and particularly type I collagen, may be responsible for providing a permissive substrate for spreading and migration of endothelial cells during tubular organization (56). *In vivo*, type I collagen constitutes the major component of

the pericapillary connective tissue. Thus, sprouting endothelial cells are exposed to an ECM composed mainly of type I collagen. From our data, it seems evident that this protein is related to the phenomenon of angiogenesis. However, the interaction of type I collagen with endothelial cells might be mediated specifically through cell-surface receptors, which can also modulate cellular gene expression and metabolism (58). The relevance of type I collagen to angiogenesis *in vivo* has not been directly addressed. An interesting model to answer this question is a strain of mice termed *mov*-13. These mice have an insertional mutation in the first intron of the α1(I) collagen gene, and homozygous animals die between day 12 and 14 of gestation by rupture of blood vessels (27). Apparently, vasculogenesis can occur in these animals in the absence of type I collagen. Kratochowil *et al.* (23) have recently shown that branching morphogenesis of several organs also occurred normally in homozygous *mov*-13 mice (*i.e.*, in the absence of type I collagen).

Another protein of interest to us was the vascular-related, culture shock protein, SPARC. Our data confirmed that SPARC mRNA was upregulated during the organization of endothelial cords. By immunocytochemistry, the protein was also quite prominent in cells that were participating in the organization of cords. Since SPARC has been shown to bind to selected components of the ECM (55), it is possible that SPARC remains in these tubes as a complex with collagen types I or III. A clue to the role of SPARC in this morphogenic process is the ability of the protein to promote changes in cell shape (55). Addition of SPARC to BAE monolayers resulted in a rounded morphology which was reversed when the rounded cells were replated in the absence of SPARC. Moreover, BAE cells cultured on dishes coated with type III collagen/SPARC grew in the apparent absence of spreading. As an anti-spreading factor, SPARC could modulate Ca^{2+} at the cell surface and participate in a signaling pathway that either initiates or facilitates cellular proliferation or migration (54, 55). We believe that during tube formation *in vitro*, SPARC is permissive for the migration, disaggregation, and reorganization of tube-like structures, perhaps through the activation of Ca^{2+}-sensitive metalloproteinases, which are known to be expressed by angiogenic endothelium (2, 15, T. F. Lane, M. L. Iruela-Arispe, and H. Sage, unpublished data).

Although endothelial cells in culture share many features with endothelium *in vivo* (28, 40, 47), endothelial cells cultured from different vessels exhibit differential regulation of genes encoding certain ECM proteins (53). For example, endothelial cells from adrenal capillaries produced type I collagen, endothelia from pulmonary artery, aorta, and vena cava produced predominantly type III collagen, while human endothelial cells from large and small vessels secreted basement membrane (type IV) collagen (48, 53). Our data show, however, that BAE cells are capable of manifesting several properties of capillary endothelium, including its secretory profile and formation of tubular networks. It therefore appears that regulation of endothelial cell behavior with respect to tube formation can be affected by the appropriate stimuli in cells derived from macrovessels. We have also noted a lack of type IV collagen and laminin surrounding the newly formed endothelial tubes. Although Western blots showed low levels of type IV collagen associated with cell layers in confluent BAE cultures, we were unable to detect this protein immunocytochemically in tube-containing cultures (data not shown). Staining for laminin revealed positive cytoplasmic reaction in both confluent cultures and tube-containing cultures; however, this localization was neither restricted to, nor increased in, endothelial cords (data not shown). Indeed, Risau and Lemmon (45) have demonstrated that the appearance of laminin *in vivo*, as well as the formation of a basement membrane, are relatively late events in angiogenesis.

We have defined different groups of cells involved in the process of tube formation. These populations exhibited differences in rates of proliferation, levels of DNA synthesis, and in the relative amounts of secreted proteins and their corresponding mRNAs. We do not believe that these populations represent different clones of cells with genetically predetermined programs, since replated sprouts and tubes always form, initially, a confluent monolayer that does not transcribe α1(I) collagen mRNA. It is more likely that reciprocal and dynamic interactions between the cells and their microenvironment result in the modulation of gene expression that we observed.

The ability of endothelial cells to initiate an angiogenic response is dependent on many factors. We have identified two extracellular molecules that are significant to the angiogenic process. Elucidation of their roles in the establishment of new vessels should provide new insight into the mechanisms that direct this phenomenon.

METHODS

Light Microscopy Studies

To analyze the structure of the endothelial cords, BAE cultures at specific times of tube formation (2, 7, and 15 days) were fixed in Karnovsky's solution (pH 7.6) at 4° C for 30 minutes, rinsed with 0.1 M cacodylate buffer (pH 7.6) containing 7% sucrose, and subsequently dehydrated in increasing percentages of ethanol (50, 70, 80, 95, and 100%) (10 minutes each). Cultures were then embedded in Epon 812 by initial treatment with a solution of absolute ethanol:Epon (1:1 by volume) at room temperature for 30 minutes, followed by Epon alone under the same conditions, and finally Epon for 24 hours at 70° C. The resulting solid discs were fractured into small blocks and sectioned in an ultramicrotome with glass knives at a thickness of 1 μm. Sections were stained with a solution of 0.5% toluidine blue and 1.1% borax at 60° C for 2 minutes, washed with distilled water, and air-dried.

Metabolic Labeling of Cell Cultures

Sprouting BAE cells at specific time points (2, 4, 15, 30, and 45 days after subculture) were preincubated for 30 minutes to 1 hour in serum-free medium supplemented with 50 μg/ml of sodium ascorbate and 64 μg/ml of β-aminopropionitrile (GIBCO) (51). The cultures were then incubated for 18 to 20 hours in fresh preincubation media containing 50 μCi/ml L-[2, 3, 4, 5, ^{3}H]proline (100 Ci/mole, Amersham). Radiolabeled proteins were purified and concentrated by precipitation in 10% trichloroacetic acid (TCA) (51).

Precipitated proteins were solubilized in SDS-PAGE buffer (25), reduced with 50 mM DTT, heated 1 to 3 minutes at 90 to 100° C, and counted in a scintillation counter. SDS-PAGE was performed on discontinuous polyacrylamide slab gels (25) at 30 mA. Acrylamide concentrations for each gel are specified in the figure legends. Protein molecular weight standards included: myosin (H chain) (200 kilodaltons (kd)); phosphorylase b (97.4 kd); bovine serum albumin (68 kd); ovalbumin (43 kd); α-chymotrypsinogen (27.5 kd), and lysozyme (14.3 kd). After electrophoresis was completed, gels were stained with a solution of 0.2% Coomassie brilliant blue R-250 in 45% methanol/9% acetic acid, and destained in 50% methanol/10% acetic acid. Signal from radiolabeled proteins was amplified with a solution of 2,5-diphenyloxazole (3). Dried gels were exposed to X-Omat X-ray film (Kodak, Rochester, New York) at −70° C. Some samples that had been subjected to TCA were later incubated with bacterial collagenase (Advance Biofactures Corporation, Lynbrook, New York). The collagenase used in this procedure had no tryptic activity. Protein pellets were resuspended in 500 μl of Tris-saline (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5) containing 10 mM $CaCl_2$ and 5 units of collagenase. The digestion was performed at 37° C for 1 hour, and the remaining proteins were recovered by precipitation in 10% TCA.

Immunocytochemistry

Cells cultured on cover slips were washed three times with PBS, fixed in 3% buffered paraformaldehyde for 30 minutes at 4° C, and rinsed with 70% methanol containing 3% H_2O_2 for 30 minutes to inactivate endogenous peroxidases. Cells were also permeabilized with cold acetone for 10 seconds. Nonspecific binding sites were reduced by treatment with 1% normal goat serum in PBS for 2 hours at 4° C. Fixed cells were exposed to a rabbit primary antibody at the following dilutions: anti-bovine type III collagen, 1:100 (51); anti-human type IV collagen, 1:100 (22); anti-mouse SPARC, 1:250 (54), and anti-human thrombospondin, 1:200 (44) in 1% normal goat serum in PBS. Antibodies to type I procollagen (provided by Dr. Laurie Fouser) were affinity absorbed with fibronectin and lathyritic rat skin collagen, and their specificity was verified on Western blots. A positive reaction was obtained by enzyme-linked immunosorbent assay for the anti-type I procollagen IgG, at a dilution of 1:250. These same antibodies were used at a dilution of 1:100 on fixed cells. Incubation with the primary antibody was performed in a humidified chamber at 4° C for 1 to 2 hours. Subsequently, the cells were rinsed for 30 minutes with three changes of PBS and incubated sequentially with biotinylated goat antibodies against rabbit IgG for 1 hour at 4° C, PBS (5 minutes), and avidin-biotin-peroxidase complex (30 minutes). 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% H_2O_2) until a brown stain appeared (10 to 15 minutes). The cells were subsequently rinsed with tap water for an additional 10 minutes. A solution of 1% toluidine blue or hematoxylin-eosin 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 either PBS or preimmune rabbit serum.

RNA Extraction and Analysis

Total RNA was purified from sprouting strains of BAE cells at 4 different states of proliferation and organization: (a) subconfluent cultures, (b) confluent cultures, (c) cultures containing tube-like structures, and (d) isolated, purified tubes. We used the single-step method of RNA isolation as described by Chomczynski and Sacchi (5). The resulting RNA pellet was washed in 70% ethanol, air-dried, and resuspended in 0.1% diethylpyrocarbonate (DEPC)-treated water; concentrations were measured spectrophotometrically. To relate RNA yield to cell number, two additional plates at equivalent stages of proliferation were trypsinized and the cell number was determined by hemocytometer.

RNA samples were denatured in a formamide/formaldehyde solution (33) for 15 minutes at 55° C. Subsequently, they were resolved by electrophoresis in a denaturing 1.2% agarose minigel (33) at 50 mA. The gel was stained with 0.5 μg/ml of ethidium bromide in DEPC-treated water for 5 minutes at room temperature and destained with several changes of DEPC-treated water. RNA was transfered to a nitrocellulose membrane with a Vacuum Blotting System (Pharmacia-LKB, Piscataway, New Jersey). After transfer, the membrane was baked at 80° C for 2 hours. Both prehybridization and hybridization were performed in a solution containing 50% deionized formamide, 30% (20 × SSC) (SSC is 3M NaCl; 0.3 mM sodium citrate), 50 mM sodium phosphate, 1 μg/ml yeast total RNA, 4% 50 × Denhardt's solution (1% Ficoll, 1% polyvinylpyrrolidone, and 1% bovine serum albumin) at 42° C for 16 to 24 hours.

The levels of SPARC and α1(I) collagen mRNA were examined by hybridization to their corresponding cDNAs. The SPARC probe was a 557 bp *Bam*H1-*Eco*R1 fragment from mouse cDNA (34) and the α1(I) collagen probe was a 1.1 kb *Eco*R1 *Eco*R1 fragment from human cDNA corresponding to a sequence which participates in triple helix formation (6). DNA fragments were isolated from gel slices with the adsorbent Gene Clean (Bio 101, La Jolla, California) and were labeled with [^{32}P]CTP (Amersham, 10 mCi/ml) by random priming (Multiprime DNA Labeling System, Amersham), followed by Gene Clean. The specific activities ranged from 2.5 to 8.0 × 10^8 cpm/μg. The post-hybridization washes were carried to a final stringency of 0.1 × SSC containing 0.1% sodium dodecyl sulfate at 65° C.

For slot blots, the RNA was denatured in a solution of 8% 20 × SSC and 2.2% formaldehyde in DEPC-treated water and heated for 15 minutes at 55° C. RNA extracted from BAE cells was applied directly to nitrocellulose filters at five different concentrations: 0.2, 0.5, 1, 2, and 5 μg. Control samples included total RNA extracted from fetal calf ligament fibroblasts (positive for SPARC and type I collagen) and F-9 teratocarcinoma cells (negative for these mRNAs). The nitrocellulose was then washed twice with 10 × SSC and baked for 2 hours at 80° C. Hybridization and washing were performed as described for Northern blots. After exposure to X-ray film, the autoradiograms were scanned. Normalization for loading of RNA samples onto gels was accomplished by hybridizing the blots with a [^{32}P]-28S rRNA cDNA probe.

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