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Transcriptional Activity of the α1(I)-Collagen Promoter Is Correlated with the Formation of Capillary-like Structures by Endothelial Cells *in Vitro**

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Bovine aortic endothelial (BAE) cells spontaneously form structures *in vitro* that resemble capillary-like cords or tubes. This process is associated with changes in the expression of certain extracellular matrix proteins that include type I collagen. BAE cells exhibiting angiogenesis *in vitro* were transfected with plasmids containing either chloramphenicol acetyltransferase or human growth hormone genes directed by promoter sequences from the human α1(I)-collagen gene. Immunostaining for chloramphenicol acetyltransferase demonstrated that collagen promoter activity was restricted to cells involved in the formation of endothelial cords. In comparison to transfected monolayers of BAE cells, the transcriptional activity of the α1(I)-collagen promoter increased by 7-fold in cultures undergoing angiogenesis *in vitro*. The selective ability of angiogenic endothelium to utilize the α1(I)-collagen promoter is consistent with previous studies showing high levels of α1(I)-collagen mRNA in BAE cells actively engaged in the formation of tubes (Iruela-Arispe, L., Hasselaar, P., and Sage, H. (1991) *Lab. Invest.* 64, 174–186). We conclude that transcriptional activation of the α1(I)-collagen gene is closely linked to the morphologic alterations in cellular phenotype that accompany the transition of quiescent endothelial monolayers to the angiogenic state.

Molecular mechanisms directing the assembly and growth of new blood vessels (angiogenesis) are poorly understood, although this process plays critical roles in development and disease, *e.g.* the formation of filtering glomerular capillary tufts in the developing kidney (Ekblom, 1984) and vascular sprouts in the embryonic brain (Risau and Lemmon, 1988), as well as capillary proliferation in tumor metastasis and wound repair (Folkman, 1985). Several peptide growth factors have been identified that stimulate proliferation and migration of endothelial cells *in vivo* and *in vitro* (Folkman and Klagsbrun, 1987), but relatively little is known of the molecular interactions that promote organization of these cells into microvessels.

Postconfluent endothelial cells in culture occasionally exhibit foci of growth that eventually give rise to branching cords with lumina, a phenomenon that has been called angiogenesis *in vitro* (Folkman and Haudenschild, 1980). Early studies indicated that organization of tubular structures by human umbilical vein endothelial cells was favored by culture conditions that did not support proliferation (Maciag *et al.*, 1982). In most cases, endothelial cord or tube formation has been elicited *in vitro* by the growth of aortic and capillary cells on substrates of gelatin, specific types of collagen, matrigel (a basement membrane matrix), or laminin, and in media supplemented with various growth factors or phorbol esters (Madri and Williams, 1983; Schor *et al.*, 1983; Feder *et al.*, 1983; Montesano *et al.*, 1983, 1986; Montesano and Orci, 1985, 1987; Madri and Pratt, 1986; Kubota *et al.*, 1988; Grant *et al.*, 1989; Ingber and Folkman, 1989a). We have described clones of bovine aortic endothelial (BAE)[1] cells that spontaneously sprout (Cotta-Pereira *et al.*, 1980) and form cords and patent tubes when plated on plastic surfaces in the absence of exogenous growth factors (Iruela-Arispe *et al.*, 1991a). The sprouting cells that migrate beneath the endothelial monolayer assemble into structures that are composed of several layers of cells (Iruela-Arispe *et al.*, 1991a).

From studies on the biosynthesis of secreted proteins during spontaneous angiogenesis *in vitro*, we found that *de novo* expression of type I collagen mRNA and protein accompanied the phenotypic change in BAE cells from a monolayer of polygonal cells to a sprouting morphology (Cotta-Pereira *et al.*, 1980; Iruela-Arispe *et al.*, 1991a). A significant increase in type I collagen was also seen in cultures of rat capillary endothelial cells that formed cords, but in the apparent absence of sprouting (Iruela-Arispe *et al.*, 1991b). Immunocytochemical studies showed that staining for type I collagen was concident with the formation of endothelial cords and tubes in both macrovascular and microvascular cells (Iruela-Arispe *et al.*, 1991a, 1991b).

Our interest in the regulation of collagen synthesis led us to consider angiogenesis *in vitro* as a possible system in which to study transcriptional control of collagen gene expression. In previous studies on collagen *cis*-regulatory sequences, the levels of expression of reporter genes directed by different regulatory domains of the α1(I)-collagen promoter and first intron were compared by transient transfection of cell lines that either synthesized minimal levels of collagen, or expressed type I collagen constitutively (Bornstein and Sage, 1989; Ramirez and DiLiberto, 1990; Vuorio and de-Crombrugghe, 1990). Transfection of BAE cells undergoing spontaneous angiogenesis *in vitro*, however, would allow us to

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[1] The abbreviations used are: BAE, bovine aortic endothelial; CAT, chloramphenicol acetyltransferase; hGH, human growth hormone; bp, base pair; ECM, extracellular matrix; SPARC, secreted protein acidic and rich in cysteine.

determine specific collagen regulatory sequences that activate transcription in cells that do not initially synthesize type I collagen (Sage, 1984).

In this report we show that the $\alpha 1$(I)-collagen promoter is actively transcribed in BAE cells engaged in cord formation, whereas minimal or no transcription occurs in quiescent cells that form a contact-inhibited monolayer. These results provide further evidence for an active role of type I collagen in the morphogenesis of endothelial cords and tubes and suggest a novel approach to the study of transcriptional mechanisms that regulate collagen gene expression during cellular differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture—Transfections were performed on three cloned strains of BAE cells, one of which was nonsprouting and two of which exhibited a sprouting phenotype. Synthesis of von Willebrand factor and endocytosis of acetylated low density lipoprotein were confirmed for each endothelial strain (Iruela-Arispe *et al.*, 1991a). In this study, cords were not distinguished from patent tubes by microscopy; therefore, both of these endothelial structures have been designated as cords. All cultures were maintained in Dulbecco's modified Eagle's medium (GIBCO) and 10% fetal bovine serum (Hyclone, Logan, UT) containing antibiotics, as previously described (Iruela-Arispe *et al.*, 1991a). Cells were plated on 100-mm plastic tissue culture dishes for transfections or on Labtek® (Nunc, Naperville, IL) culture slides for immunocytochemistry.

Plasmids—Transcriptional activity of the type I collagen promoter was studied with two plasmids. pCOL-CAT contains the human $\alpha 1$(I)-collagen gene promoter, extending from −332 to +98, fused to a chloramphenicol acetyltransferase (CAT) reporter gene (Bornstein *et al.*, 1987). The plasmid pCOL-hGH consists of 804 bp of 5′-flanking sequence, the first exon, and 1230 bp of the first intron of the $\alpha 1$(I)-collagen gene, ligated to an hGH minigene (Bornstein and McKay, 1988).

Efficiency of DNA uptake was monitored in all experiments by cotransfection with pRSV-L (deWet *et al.*, 1987), in which a promoter from the Rous sarcoma virus long terminal repeat was fused to the coding sequence of the firefly luciferase gene. In selected experiments, transfectional efficiency was also examined with pSV2CAT, a plasmid in which the CAT gene has been placed under the control of the simian virus-40 early promoter (Gorman *et al.*, 1982).

Transient Transfection—Plasmid DNA was introduced into BAE cells by addition to cell cultures with the compound Lipofectin® (Bethesda Research Laboratories) (Felgner *et al.*, 1987). Cell layers were given fresh medium 18 h after transfection, removed after 48 h with a buffer containing 40 mM Tris-HCl, 1 mM EDTA, and 150 mM NaCl, and subsequently divided for extraction of protein (Gorman *et al.*, 1982) and RNA (Chomczynski and Sacchi, 1987).

Activities of CAT were determined on 100 μg of total cell lysate by measurement of acetylated forms of [^{14}C]chloramphenicol that were resolved by thin layer chromatography (Gorman *et al.*, 1982). Transcription of the hGH reporter gene was assayed by an RNase protection assay. 15 μg of total RNA was digested briefly with DNase I to avoid contamination from transfected plasmids; the RNA was subsequently hybridized with ^{32}P-labeled anti-sense hGH RNA (Bornstein and McKay, 1988). The protected RNA fragments were resolved by electrophoresis on a 6% acrylamide gel in 50% urea. Dried gels were exposed to X-Omat-RP film (Kodak, Rochester, NY) for 48 h, and radioactivity in protected bands was quantitated by computerized scanning densitometry of the autoradiogram. In all experiments, cell lysates were assayed for luciferase activity by quantitation of light emission when combined with D-luciferin and ATP (DeLuca and McElroy, 1978).

Northern Analysis of RNA—Samples of total RNA (7 μg) were resolved by electrophoresis on denaturing 1.2% agarose gels and transferred to nitrocellulose. The blots were hybridized at 42 °C with a ^{32}P-nick-translated human $\alpha 1$(I)-collagen cDNA probe (Chu *et al.*, 1982), washed to a final stringency of 0.1 × SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) containing 0.1% sodium dodecyl sulfate at 65 °C, and exposed to x-ray film. The collagen probe was subsequently removed with 0.1 × SSC containing 0.1% sodium dodecyl sulfate at 95 °C, and normalization for loading and transfer of RNA was performed by rehybridization of the blot with a ^{32}P-labeled 28 S rRNA cDNA probe (Iruela-Arispe *et al.*, 1991b).

Immunocytochemistry—Goat antibodies were raised against bovine type I procollagen purified from the culture media of fetal calf ligament fibroblasts. Antibodies were affinity-adsorbed with fibronectin and lathyritic rat skin (native type I) collagen. A positive reaction for bovine type I procollagen was demonstrated by enzyme-linked immunosorbent assay at an antibody concentration of 3.3 μg/ml. No reactivity was observed by enzyme-linked immunosorbent assay against human fibronectin and bovine types III, IV, V, and VIII collagen. The specificity of these antibodies was also verified by immunoblot. A concentration of 35 μg/ml was used for immunocytochemistry. Antiserum reactive toward CAT was developed in rabbits that were immunized with CAT (Sigma). Unfractionated antiserum reacted with CAT by enzyme-linked immunosorbent assay at a dilution of 1:2000 and was diluted to 1:75 for incubation with fixed cells.

Cultures grown on Labtek® slides were fixed in 3% buffered paraformaldehyde. Fixed cells were rinsed with 70% methanol containing 3% H_2O_2 for 30 min to inactivate endogenous peroxidases. Cells were blocked with 1% rabbit or goat serum, exposed to primary antibody for 1 h at 4 °C, and incubated sequentially with biotinylated secondary antibody, avidin-biotin-peroxidase complex, and 3,3′-diaminobenzidine-4-HCl. A solution of 1% toluidine blue was used as a counterstain.

RESULTS

The three strains of BAE cells used in this study were confirmed as endothelia by the accepted criteria of endocytosis of low density lipoprotein and production of von Willebrand protein (Iruela-Arispe *et al.*, 1991a). However, they exhibited different behaviors *in vitro*. One strain was nonsprouting and remained as a monolayer 50 days postconfluence. The other two strains exhibited a sprouting morphology at confluence but differed in the rates at which cord formation occurred. The first sprouting strain required culture for 14 days as a confluent monolayer before sprouts and cords were initiated, whereas the second sprouting strain formed a network of sprouts and cords within 72 h postconfluence.

To study the applicability of transient transfection to the phenomenon of angiogenesis *in vitro*, we used the plasmid pCOL-CAT, in which the transcription of the CAT gene is regulated by a short human $\alpha 1$(I)-collagen gene promoter. Precipitates of calcium phosphate were generally toxic to our cultures of BAE cells; however, use of the compound lipofectin did not affect the viability of BAE cells and resulted in a modest transfectional efficiency for pRSV-L that averaged 10^3 light units of luciferase activity/20 μg of cell lysate. Since transfection procedures are generally performed on cells cultured in monolayers or in suspension, we initially anticipated that plasmid DNA might not be accessible to cells that lie beneath a monolayer. In a phenotypically heterogeneous culture system containing several layers of cells, there were also potential problems with both the efficiency and selectivity of transfection. Therefore, we used antibodies to CAT to localize expression of CAT protein in BAE cells transfected with pCOL-CAT.

Fig. 1 is a summary of the immunolocalization studies performed on transfected BAE cells. When BAE cell monolayers were transfected with pCOL-CAT and subsequently incubated with antibodies to CAT, a few scattered cells with weak immunoreactivity were apparent (Fig. 1*B*, *open arrow*). However, transfected sprouting cultures exhibited strong reactivity for CAT that was selective for cells that exhibited sprouting and cord formation (Fig. 1*F*, *solid arrow*). In contrast, surrounding areas comprised of cells in a monolayer remained negative for CAT. This pattern of expression coincided closely with the immunolocalization of type I procollagen that we previously described for cells undergoing angiogenesis *in vitro* (Iruela-Arispe *et al.*, 1991a). BAE cells in confluent monolayers did not react with antibodies to type I procollagen (Fig. 1*A*); however, staining was apparent during

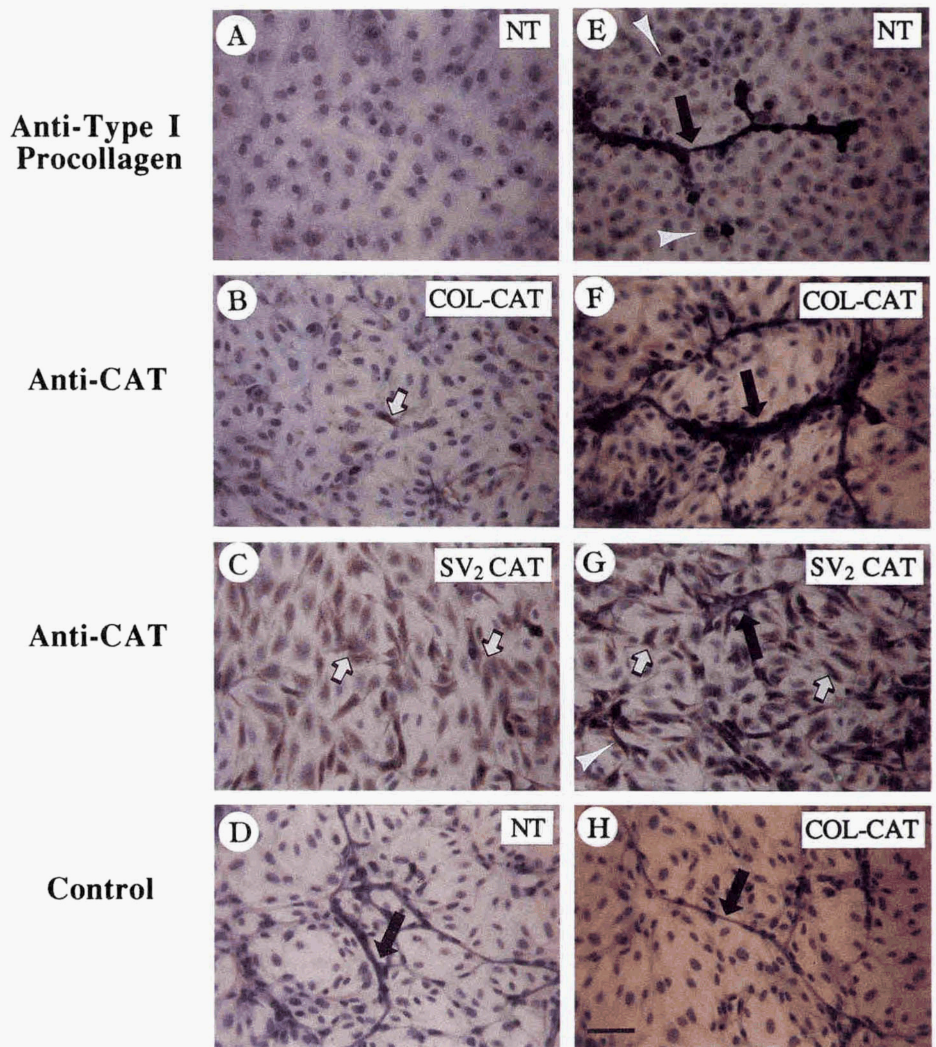

FIG. 1. **Immunolocalization of type I collagen and CAT proteins in BAE cells exhibiting angiogenesis *in vitro*.** Non-transfected (*NT*) monolayer (*A*) and cord-forming cultures (*E*) were incubated with anti-type I procollagen IgG. Endothelial cells in cords (*solid arrow*), as well as sprouting cells in the vicinity of cords (*white arrowhead*), stained positively for type I procollagen, whereas nonsprouting cells in the monolayer (*A*) were negative. Other cultures, transfected with pCOL-CAT (*B* and *F*) or a control plasmid, pSV2CAT (*C* and *G*), were subsequently exposed to rabbit anti-CAT antibodies. The *open arrow* (*B*) denotes a cell in the monolayer that expressed CAT as a result of transfection with the collagen promoter; in contrast, extensive staining was apparent in endothelial cords (*F*, *solid arrow*). Control transfections with the SV2 promoter showed staining for CAT in monolayers (*C*, *open arrows*), sprouts (*G*, *white arrowhead*), and cord-forming endothelial cells (*G*, *solid arrow*). *D* is a nontransfected sprouting culture incubated with anti-CAT antibodies, and *H* shows transfected cells incubated with secondary antibody alone. Although the toluidine blue counterstain enhanced the appearance of cord structures, no peroxidase-substrate reaction was observed in these controls. *Closed arrows* denote endothelial cords, *white arrowheads* identify sprouting endothelial cells, and *open arrows* point to non-sprouting endothelial cells in the monolayer. *Bar*, 100 μm.

sprouting and alignment of endothelial cells into cords (Fig. 1*E*).

The apparently selective pattern of CAT expression observed in cells transfected with the collagen promoter could potentially represent an enhanced transfectional efficiency manifested by migrating and sprouting cells. We therefore transfected BAE cells with pSV2CAT, a plasmid containing a viral promoter that is active in all phenotypes of BAE cells. A different pattern of immunostaining for CAT was observed. Reactivity with anti-CAT antibodies was present in most cells in the monolayer as well as in the cord-containing cultures. In the nonsprouting BAE cell monolayer shown in Fig. 1*C*, *open arrows* indicate only a few of many positively stained cells in which the diffuse peroxidase reaction is evident throughout the cytoplasm. Sprouting cultures also displayed this diffuse immunoreactivity (Fig. 1*G*). In contrast with the cultures transfected with pCOL-CAT, staining did not in all cases coincide with the position of endothelial cords. Indeed, cells positive for CAT in the surrounding monolayer (Fig. 1*G*, *open arrows*) obscured the cord structures (*solid arrow*) that had been demarcated in cells transfected with pCOL-CAT (Fig. 1*F*). Therefore, the pattern of expression for pCOL-CAT in BAE cell cultures appeared to be due to a selective activation of the $\alpha1$(I)-collagen promoter in cells specifically undergoing angiogenesis *in vitro*, since contiguous nonsprouting and sprouting cells exhibited equivalent efficiencies of transfection with pSV2CAT.

Quantitation of CAT levels in transfected BAE cells confirmed that expression of CAT was preferentially increased in cultures containing endothelial cords (Fig. 2*A*). Although CAT activity was nearly undetectable in a sprouting strain at subconfluent and confluent but presprouting densities (*lanes 1–4*), these cells exhibited on average an increase of 3.5-fold in the transcriptional activity of pCOL-CAT when they were transfected during sprouting and cord formation (*lanes 5* and *6*). Analysis of total RNA from these transfected cultures indicated that steady-state levels of $\alpha1$(I)-mRNA correlated with the activity of pCOL-CAT. Type I collagen mRNA was not evident in subconfluent and confluent BAE cell cultures (Fig. 2*B*, *lanes 1–4*) but was clearly present as two bands of 4.7 and 5.7 kilobases in total RNA from cord-containing cultures (*lanes 5* and *6*), despite the decreased levels of total RNA present in *lanes 5* and *6* (Fig. 2*C*).

Six separate experiments demonstrated a consistent and significant increase in the activity of the type I collagen promoter in sprouting cultures. In the transfections summarized in Table I, we compared the expression of two collagen promoter constructs in a nonsprouting and sprouting strain of BAE cells. The transcriptional activity of both $\alpha1$(I)-collagen promoters increased by an average of 10-fold in confluent cultures undergoing sprouting and cord formation, compared to confluent, nonsprouting cultures. This value is a conservative indication of the activity of the collagen pro-

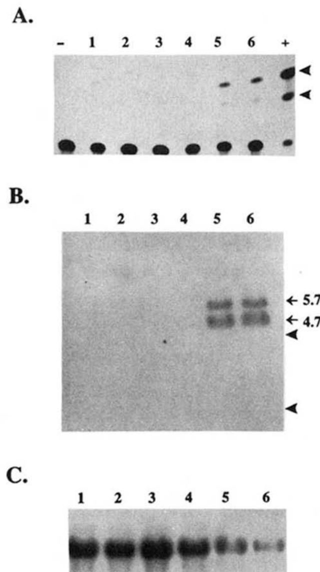

FIG. 2. **Correlation of α1(I)-collagen promoter activity and endogenous α1(I)-mRNA levels in BAE cells transfected with pCOL-CAT.** BAE cells were plated to permit stages of subconfluence (*lanes 1* and *2*), confluence (*lanes 3* and *4*), or sprouting and tube formation (*lanes 5* and *6*) at the time of transfection. Each culture was transfected with pCOL-CAT and pRSV-L. After 48 h, cells were collected and divided for extraction of protein and RNA. The thin layer chromatogram shown in *panel A* depicts forms of [^{14}C]chloramphenicol (*arrowheads*) that were acetylated by cell lysates from the different stages of culture. + and − denote assay controls performed with and without the CAT standard. *Panel B*, a Northern blot of total RNA from stages of culture corresponding to those shown in *panel A* was hybridized with a [^{32}P]α1(I)-collagen cDNA probe. Transcript sizes in kilobases are designated in relation to positions of 28 S and 18 S rRNA (*arrowheads*). *Panel C*, the blot shown in *panel B* was reprobed with ^{32}P-labeled 28 S rRNA cDNA to permit normalization for loading of total RNA.

TABLE I

Transcriptional activity of the α1(I)-collagen promoter in contact-inhibited (nonsprouting) and sprouting strains of BAE cells

BAE cells were plated and maintained for 14 days. One strain (nonsprouting) exhibited a stable, contact-inhibited monolayer at confluence; the second strain manifested a sprouting morphology at confluence and initiated the formation of cords. Duplicate cultures were transfected with pCOL-CAT or pCOL-hGH, and cell layers were divided for protein and RNA extraction 48 h later. In experiments I and II, collagen promoter activity is expressed as the average percent conversion ± S.E. of [^{14}C]chloramphenicol to its acetylated forms. Promoter activity for experiment III is expressed as the average ± S.E. of arbitrary units corresponding to the areas obtained by scanning densitometry of bands protected by the hGH riboprobe. CAT or hGH levels were normalized to luciferase activity to correct for the efficiency of transfection in each experiment.

Experiment	Morphology of confluent culture	Plasmid	Promoter activity	Fold increase
I	Nonsprouting	pCOL-CAT	1.0 ± 0.6	5.4
	Sprouts/cords		5.4 ± 0.8	
II	Nonsprouting	pCOL-CAT	1.3[a]	7.0
	Sprouts/cords		9.1 ± 2.1	
III	Nonsprouting	pCOL-hGH	0.26 ± 0.1	18.7
	Sprouts/cords		4.85 ± 1.74	

[a] Value from one transfection.

moter, since endothelial cords and sprouts represent only a fraction of the total cell population in sprouting cultures (*e.g.* see Fig. 1, *D–H*). This increase in transcriptional activity was not generally observed for other gene products in transfected, sprouting cells; for example, expression of the plasmids pRSV-L and pSV2CAT was equivalent or greater in confluent, nonsprouting cultures (data not shown).

The second collagen gene construct, pCOL-hGH, was used to confirm the specificity of the collagen promoter observed in the sprouting strain of BAE cells. The transcriptional activity of this plasmid increased by 18.7-fold when confluent sprouting cultures were compared with confluent nonsprouting cultures (Table I), in contrast to average increases of 5–7-fold in cells transfected with pCOL-CAT. The enhanced effect seen with pCOL-hGH was confirmed by RNase protection experiments as shown in Fig. 3. A protected band of 168 bp was apparent in RNA isolated from confluent cultures containing sprouts and cords (*lanes 5* and *6*), but a fragment of similar size was absent in RNA from confluent, nonsprouting cultures (*lanes 3* and *4*). Compared to pCOL-CAT, the plasmid pCOL-hGH contains an additional 472 bp of 5′-flanking region, as well as part of the first intron, of the α1(I)-collagen gene. The presence of these regulatory sequences might therefore account for the increase in activity of the α1(I)-collagen promoter seen in cord-forming cultures transfected with pCOL-hGH.

An additional control experiment was performed to assess the potential contribution of BAE cell proliferation and motility to transfectional efficiency and to the ability of sprouting cells to transcribe pCOL-CAT. This experiment was made possible by the use of a rapidly sprouting strain of BAE cells that formed sprouts and cords within 2 days after plating. In this manner we could measure cells transiently transfected at different degrees of confluence but with the same duration in culture. As shown in Table II, the rapidly sprouting strain of BAE cells consistently produced CAT at reduced levels (approximately 3-fold) when transfected at subconfluent densities, compared to levels observed when the cells were transfected during sprouting and early cord formation (experiments I and II). Moreover, there was a decrease by a factor of 5 in confluent cultures that had not yet begun to sprout, compared to cultures that had initiated sprouting and cord formation (experiment III). Thus, the predisposition of sprouting endothelial cells to transcribe α1(I)-mRNA (Fig. 2*B*) correlates with their ability to utilize the COL promoter.

DISCUSSION

In this study we have provided evidence in support of our previous observation that transcriptional activation of the α1(I)-collagen gene is closely linked to changes in the morphology of BAE cells during spontaneous angiogenesis *in vitro*

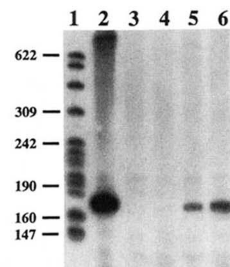

FIG. 3. **Selective expression of pCOL-hGH in BAE cells that form sprouts and cords.** Nonsprouting (*lanes 3* and *4*) and sprouting strains of BAE cells (*lanes 5* and *6*) were transfected with 6 μg each of pCOL-hGH and pRSV-L. Cell layers were divided for protein and RNA extraction after 48 h. 15 μg of total RNA was digested with DNase I and hybridized with ^{32}P-labeled antisense hGH RNA. After RNase digestion, the protected RNA fragments were resolved by electrophoresis on a 6% acrylamide sequencing gel. *Lane 1* shows molecular weight standards, and *lane 2* contains protected RNA from a human bone marrow stromal cell line transfected with pCOL-hGH.

TABLE II

Transcription of pCOL-CAT as a function of culture density and morphology in a rapidly sprouting strain of BAE cells

BAE cells from a rapidly sprouting strain were plated at different densities on day 1, such that stages of subconfluence, confluence, or sprouting/cord formation were attained after 48 h in culture. On day 3, each culture was transfected with 6 μg of pCOL-CAT, 6 μg of pRSV-L, and 50 μl of lipofectin, and cells were collected for assay on day 5. Promoter activity is expressed as the average percent conversion ± S.E. of [^{14}C]chloramphenicol by cell lysates from duplicate transfections. CAT levels were normalized to luciferase activity in each experiment.

Experiment	Density and morphology of culture[a]	Promoter activity	Fold increase
I	Subconfluent	7.8 ± 1.0	2.7
	Sprouts	21.0 ± 3.3	
II	Subconfluent	2.1 ± 0.4	2.8
	Sprouts/cords	5.8 ± 2.3	
III	Confluent	1.1 ± 0.1	5.3
	Sprouts/cords	5.8 ± 2.3	

[a] Appearance of sprouts and subsequent formation of cords are initiated only after cells reach confluence.

(Iruela-Arispe *et al.*, 1991a, 1991b). Although contact-inhibited macrovascular endothelial cells do not secrete interstitial type I collagen *in vitro*, capillary endothelial cells produce this component of the ECM constitutively (Sage, 1984; Iruela-Arispe *et al.*, 1991b). This difference in secretory phenotype might reflect one function of microvascular endothelium *in vivo*, *i.e.* the induction of an angiogenic response during embryogenesis or, in certain adult mammals, during ovulation, endometrial cycling, tumor growth, and tissue repair (Folkman, 1985). However, it appears that the initiation and the progression of angiogenesis *in vitro* by both macrovascular and microvascular endothelia are coincident with *de novo* induction or increased levels of transcription, respectively, of α1(I)-collagen mRNA (Iruela-Arispe *et al.*, 1991a, 1991b). Recent studies have also shown a transcriptional initiation of decorin mRNA in tube-forming BAE cells.[2] This proteoglycan, which binds to type I collagen, has been proposed to regulate the size and assembly of type I collagen fibrils (Scott, 1988). The appearance of banded collagen fibrils in the lumina of endothelial tubes *in vitro* is suggestive of a process whereby endothelial cells might utilize these macromolecular assemblies to organize into cords and tubes (Folkman and Haudenschild, 1980).[3] Degradation of these collagen-decorin cores could result in the formation of a lumen or define a branch point for another cordlike structure. Support for this hypothesis is found in a recent study by Jackson and Jenkins (1991), in which soluble type I collagen added to cultures of endothelial cells promoted the formation of tubular networks that contained fibrillar collagen within their lumina.

Our data also indicate that transient transfection with lipofectin can be used to study regulation of gene expression in a cell culture system that mimics capillary differentiation. The presence of multiple cell layers did not diminish transfectional efficiency, and the heterogeneity in cell shape did not favor the selective transfection of one cell type over another. We have previously shown by videomicroscopy that sprouting BAE cells engage in the formation, dissolution, and reorganization of endothelial cords (Iruela-Arispe *et al.*, 1988). The mechanisms that permit uptake of exogenous DNA by mammalian cells are largely unknown, but we speculate that transfectional efficiency was equal in sprouting BAE cells, endothelial cords, and surrounding monolayers because these cell populations are continuously engaged in active remodeling.

The significance of type I collagen to the process of angiogenesis *in vitro* is at the present time unclear. Various modifications in endothelial cell behavior that are necessary for the transition from a quiescent monolayer to a patent tube include the acquisition of a proliferative and migratory phenotype, alteration of cell-cell and cell-ECM contacts, induction of matrix-degrading enzymes followed by the synthesis of new gene products, and eventual withdrawal from the cell cycle following lumen formation (Folkman and Haudenschild, 1980; Furcht, 1986; Ingber and Folkman, 1989b). Specific glycoproteins of the vascular ECM that function in this pathway include laminin, which enhances the assembly of endothelial cells into cordlike structures (Kubota *et al.*, 1988; Grant *et al.*, 1989), and thrombospondin, which inhibits the growth and progression of cords and tubes (Iruela-Arispe *et al.*, 1991c). The anti-adhesive protein SPARC, which in BAE cells modulates cell shape, dissolves focal contacts, and retards the cell cycle, is also increased in sprouting cultures (Sage and Bornstein, 1991; Iruela-Arispe *et al.*, 1991a). The effect of SPARC on tube formation might be indirect, however, as it is associated with a decrease in the adhesive protein fibronectin[4] and a concomitant increase in plasminogen activator inhibitor-1 (Hasselaar *et al.*, 1991). We envision the role of type I collagen in promoting angiogenesis *in vitro* as different from those proposed for laminin, thrombospondin, and SPARC. Based on the requirement of endothelium for continual secretion of collagens during cell movement (Madri and Stenn, 1982), this fibrillar collagen could direct endothelial cell migration and promote intercellular adhesion.

A direct role for the ECM in the morphogenesis of capillary-like structures has been suggested by several investigators (Montesano and Orci, 1985; Furcht, 1986; Nicosia and Madri, 1987; Kubota *et al.*, 1988; Ingber and Folkman, 1989a, 1989b; Madri *et al.*, 1991), as many endothelial cells require exogenous ECM for the formation of cords and tubes *in vitro*. Moreover, regression of growing capillaries *in vivo* has been observed when chick chorioallantoic membranes were exposed to chemical agents that interfere with collagen cross-linking and triple-helix formation (Ingber and Folkman, 1988). Ingber and Folkman (1987, 1989a) have proposed that the mechanical properties of the ECM determine in part the response of endothelial cells to angiogenic factors. Whereas thin layers of gelatin, fibronectin, or type IV collagen provided a rigid support for endothelial cells that permitted attachment, spreading, and growth, more dense substrates of these proteins facilitated retraction and the formation of a capillary-like network (Ingber and Folkman, 1989a). The ability of purified components of ECM to modulate endothelial cell migration, proliferation, and/or tube formation must be considered in the context of specific morphoregulatory factors that either interact directly with cell-surface receptors or indirectly via extracellular macromolecules such as fibronectin, SPARC, thrombospondin, and various types of collagens (McAuslan *et al.*, 1985; Ingber and Folkman, 1989a, 1989b; Sage and Bornstein, 1991; Madri *et al.*, 1991). For example, gels composed of type I collagen specifically modulated the ability of transforming growth factor-β to induce angiogenesis *in vitro* (Madri *et al.*, 1988; Merwin *et al.*, 1990). The affinity

[2] H. T. Järveläinen, M. L. Iruela-Arispe, L. J. Sandell, E. H. Sage, and T. N. Wight, manuscript in preparation.

[3] M-L. Iruela-Arispe, T. Wight, and H. Sage, unpublished observations.

[4] T. F. Lane, M. L. Iruela-Arispe, and E. H. Sage, manuscript in preparation.

of acidic fibroblast growth factor for type I collagen has in fact been used to design an implant to direct angiogenesis *in vivo* at specific sites (Thompson *et al.*, 1988). It is therefore conceivable that one role for type I collagen in sprouting endothelial cells might be to sequester growth factors necessary for angiogenesis. However, our clones of BAE cells initiated synthesis of type I collagen concomitantly with the acquisition of a sprouting phenotype. Therefore, differentiation of these cells *in vitro* appears to be at least partially dependent on the transcriptional activation of specific genes, the expression of which provides an appropriate microenvironment of ECM.

We have shown that a relatively short segment of 5′ regulatory sequence in the α1(I)-collagen gene (*e.g.* from −332 to +98 of the collagen promoter in the case of pCOL-CAT) was able to direct specific expression of the reporter gene in sprouting and/or cord-forming endothelial cells, but was inactive in monolayers of endothelial cells that did not display the sprouting phenotype. Moreover, sprouting and cord formation were necessary for maximal levels of expression, since strains predisposed to sprouting at confluence expressed very low levels of CAT when they were transfected at a subconfluent stage. The apparent specificity of a relatively short promoter sequence is confirmatory of results from other investigators who have studied the regulation of the α1(I)-collagen gene by transient transfection of cells *in vitro*. For example, Brenner *et al.* (1989) have identified a segment of 181 bp 5′ of the transcriptional start site of the murine α1(I)-gene that directs efficient expression in NIH 3T3 cells. Several regulatory factors isolated from these cells have been shown to bind in the vicinity of that region (Karsenty and deCrombrugghe, 1990); in addition, a fibroblast-specific transcriptional repressor interacts with a sequence between −339 and −361 of the mouse gene (Ravazzolo *et al.*, 1991). Studies with the human α1(I)-collagen basal promoter, which is highly similar to its murine counterpart (Bornstein and Sage, 1989), have also indicated that the collagen sequence represented in pCOL-CAT is sufficient to direct cell-specific transcription with respect to the collagen gene (Bornstein and McKay, 1988).

The second construct used in our experiments, pCOL-hGH, contained 804 bp of 5′-flanking sequence, exon 1, and 1230 bp of intron 1. Sprouting and nonsprouting cultures transfected with pCOL-hGH exhibited differential levels of expression that were approximately 3-fold greater than those observed in similar cells transfected with pCOL-CAT. It is possible that the more efficient processing of the hGH transcript, relative to the CAT transcript, contributed to this difference (Bornstein *et al.*, 1990). However, nucleotide sequences extending from approximately −2500 in the promoter to +1400 within the first intron have been shown to affect the transcriptional rate of the α1(I)-collagen gene (Bornstein *et al.*, 1987, 1988; Rossouw *et al.*, 1987; Rippe *et al.*, 1989; Liska *et al.*, 1990; Boast *et al.*, 1990). Results from these studies have been difficult to reconcile, in part because of the presence of both positive and negative regulatory transcriptional elements within the first intron. Recent studies on transgenic mice, however, indicate that intronic sequences of the α1(I)-gene between +292 and +1440 are important for increased expression in the context of a short collagen promoter (Slack *et al.*, 1991). Our data showing elevated levels of CAT directed by pCOL-hGH in sprouting endothelial cells *in vitro* would support the data derived from transgenic animals.

Recognition and utilization of the α1(I)-collagen promoter by endothelial cells undergoing angiogenesis *in vitro* are consistent with the expression of type I collagen mRNA and protein by tube forming as compared to nonangiogenic cultures. Endothelial cells that can be modulated between states of contact-inhibited quiescence and proliferation leading to cord or tube formation should have considerable utility in defining the regulatory mechanisms that activate the expression of type I collagen and other genes during vascular maturation and endothelial cell differentiation.

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