Molecular Biology of the Cell
Vol. 8, 1329–1341, July 1997

Thrombospondin-1, a Natural Inhibitor of Angiogenesis, Regulates Platelet-Endothelial Cell Adhesion Molecule-1 Expression and Endothelial Cell Morphogenesis

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Submitted March 28, 1997; Accepted April 30, 1997
Monitoring Editor: Gregor Eichele

Expression of thrombospondin-1 (TS1) in polyoma middle-sized T (tumor)-transformed mouse brain endothelial cells (bEND.3) restores a normal phenotype and suppresses their ability to form hemangiomas in mice. We show that TS1 expression results in complete suppression of platelet-endothelial cell adhesion molecule-1 (PECAM-1) expression and altered cell–cell interactions in bEND.3 cells. To further investigate the role of PECAM-1 in regulation of endothelial cell–cell interactions and morphogenesis, we expressed human (full length) or murine (Δ15) PECAM-1 isoforms in TS1-transfected bEND.3 (bEND/TS) cells. Expression of either human or murine PECAM-1 resulted in an enhanced ability to organize and form networks of cords on Matrigel, an effect that was specifically blocked by antibodies to PECAM-1. Anti-PECAM-1 antibodies also inhibited tube formation in Matrigel by normal human umbilical vein endothelial cells. However, PECAM-1-transfected bEND/TS cells did not regain the ability to form hemangiomas in mice and the expressed PECAM-1, unlike the endogenous PECAM-1 expressed in bEND.3 cells, failed to localize to sites of cell–cell contact. This may be, in part, attributed to the different isoforms of PECAM-1 expressed in bEND.3 cells. Using reverse transcription-polymerase chain reaction, we determined that bEND.3 cells express mRNA encoding six different PECAM-1 isoforms, the isoform lacking both exons 14 and 15 (Δ14&15) being most abundant. Expression of the murine Δ14&15 PECAM-1 isoform in bEND/TS cells resulted in a similar phenotype to that described for the full-length human or murine Δ15 PECAM-1 isoform. The Δ14&15 isoform, despite the lack of exon 14, failed to localize to sites of cell–cell contact even in clones that expressed it at very high levels. Thus, contrary to recent reports, lack of exon 14 is not sufficient to result in junctional localization of PECAM-1 isoform in bEND/TS cells.

INTRODUCTION

Platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31)[1] is a member of the immunoglobulin (Ig) super family that is expressed on endothelial cells (ECs) of large and small vessels as well as on platelets, leukocytes, and hematopoietic precursors. It contains six Ig-like domains, a short hydrophobic transmem-

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[1] Abbreviations used: EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; MBEC, mouse brain endothelial cell; PECAM-1, platelet–endothelial cell adhesion molecule-1; TS1, thrombospondin-1.

brane domain, and a cytoplasmic tail of variable length due to alternative splicing of exons 10 through 16 (Newman *et al.*, 1990, 1992; Albelda *et al.*, 1991; Xie and Muller, 1993; DeLisser *et al.*, 1994b; Kirschbaum *et al.*, 1994; Newman, 1994). PECAM-1 has been implicated in leukocyte–EC interactions, transendothelial migration, EC–EC adhesion, angiogenesis, and development of the early cardiovascular system (Bogen *et al.*, 1992; Horak *et al.*, 1992; Baldwin *et al.*, 1994; DeLisser *et al.*, 1994b; Berman and Muller, 1995; Bischoff, 1995; Muller, 1995). In some cultured ECs, such as human umbilical vein endothelial cells (HUVECs), PECAM-1 is mainly localized to the sites of cell–cell contact in regions distinct from adherence junctions or tight junctions (Ayalon *et al.*, 1994; DeLisser *et al.*, 1994b; Dejana *et al.*, 1995, Romer *et al.*, 1995). In addition, differences in the detergent extractability of PECAM-1 compared with cadherins also suggest differences in the mode of association with known cytoskeletal proteins (Ayalon *et al.*, 1994; Romer *et al.*, 1995). PECAM-1 mediates cell–cell adhesion via both homophilic and heterophilic mechanisms. The binding sites for homophilic adhesion have been shown to require Ig domains 1 and 2 (Sun *et al.*, 1996). Heterophilic cell–cell adhesion is dependent on divalent cations and is inhibited by antibodies that bind either Ig domain 2 or 6 through a mechanism that is not yet understood (Muller *et al.*, 1992; DeLisser *et al.*, 1993). In addition, the $\alpha v\beta 3$ integrin has been implicated as a heterotypic ligand for PECAM-1 that may mediate leukocyte–EC interactions facilitating transmigration of the endothelium (Piali *et al.*, 1995; Buckley *et al.*, 1996). Integrin-associated protein, which has been recently shown to be a receptor for thrombospondin-1 (TS1; Gao *et al.*, 1996) and associates with $\alpha v\beta 3$, is also essential for EC transmigration (Cooper *et al.*, 1995). However, the role of these molecules in EC interactions remains elusive. Finally, PECAM-1-mediated cellular interactions may be affected by deletions or alternative splicing of the PECAM-1's cytoplasmic domain (Baldwin *et al.*, 1994; DeLisser *et al.*, 1994a; Kirschbaum *et al.*, 1994; Yan *et al.*, 1995), suggesting an important role for the intracellular domain in regulating PECAM-1-dependent cellular interactions.

Brain ECs stem from the perineural vascular plexus by angiogenesis and exhibit blood–brain barrier function (Risau, 1995). This differentiated characteristic, which is apparently induced by the brain environment in an attempt to maintain homeostasis, is rapidly lost in culture. Brain ECs have been shown to express PECAM-1 when cultured under conditions that attempt to maintain blood–brain barrier function (Rubin *et al.*, 1991); otherwise, it is rapidly lost after passage in culture (studies presented herein). Mouse brain endothelial cells (MBECs) are very susceptible to transformation by polyoma middle T (tumor) oncogene (Williams *et al.*, 1989; Montesano *et al.*, 1990). Such transformed cells (bEND.3 cells) grow rapidly in culture, express little or no TS1 (a natural inhibitor of angiogenesis), and form hemangiomas in mice (RayChaudhury *et al.*, 1994; Sheibani and Frazier, 1995). We have recently demonstrated that constitutive reexpression of TS1 in bEND.3 cells restores many aspects of a normal phenotype. The bEND/TS cells exhibit an altered morphology, a slower growth rate, a normal proteolytic balance, an ability to organize better on Matrigel, and lose most dramatically the ability to form tumors in mice (Sheibani and Frazier, 1995). These data indicate that TS1 has a major role in the regulation of EC phenotype.

To better understand the mechanisms of TS1 action on ECs, we have examined expression of several genes in TS1-transfected bEND.3 (bEND/TS) cells whose products have been implicated in regulation of EC phenotype. One such gene is PECAM-1. bEND.3 cells express high levels of PECAM-1 localized to the sites of cell–cell contact. The high expression of PECAM-1 in bEND.3 cells may contribute to hemangioma formation by participating in the recruitment of, and anastomosis with, host ECs. The mechanisms by which transformation of normal MBEC results in high expression of PECAM-1 in bEND.3 cells, and its suppression upon transfection with TS1 are not known. The purpose of the present study, therefore, was to clarify the relationship between TS1 and PECAM-1 expression and examine the function of PECAM-1 in EC morphogenesis.

MATERIALS AND METHODS

Construction of Expression Vectors

The expression plasmid pREP8/hPECAM-1 was generated by ligating a 2.6-kbp *Hin*dIII-*Not*I fragment of human PECAM-1 (hPECAM-1) cDNA containing the entire coding sequence with pREP8 vector (Invitrogen, San Diego, CA) digested with *Hin*dIII and *Not*I. The pREP8/mPECAM-1 was generated by ligating a 2.3-kbp blunted *Hin*dIII-*Xba*I fragment of murine PECAM-1 (mPECAM-1) cDNA containing the entire coding sequence with pREP8 vector digested with *Not*I, blunted, and dephosphorylated (Figure 1). The size and orientation of all inserts were confirmed by restriction digestion. The pREP8 vector provides constitutive expression and L-histidinol (hisD) selection. Cells were initially selected in the presence of 2.5 mM L-histidinol and then increased to 10 mM.

Cell Lines and DNA Transfection

The parental bEND.3 cells and its TS1-transfected clones were maintained as recently described (Sheibani and Frazier, 1995). The normal MBECs, two different strains, early passage (p4) and late passage (p22), were plated on gelatinized plates and fed with DMEM containing 10% fetal calf serum. The HUVECs were obtained from Dr. L. Cornelias (Washington University, St. Louis, MO), used between passages 3 and 5, maintained on gelatinized plates in M-199 with Earle's salts buffered with 25 mM HEPES and supplemented with 100 μg/ml EC mitogen, 100 μg/ml heparin, and 20% fetal calf serum. Cells were transfected using Lipofectin as described (Sheibani and Frazier, 1995). The TS1-expressing bEND.3 cells express *neo* and *hyg* genes conferring G418 and hygromycin B resistance, respectively. The vectors used to express PECAM-1 contain the *his*

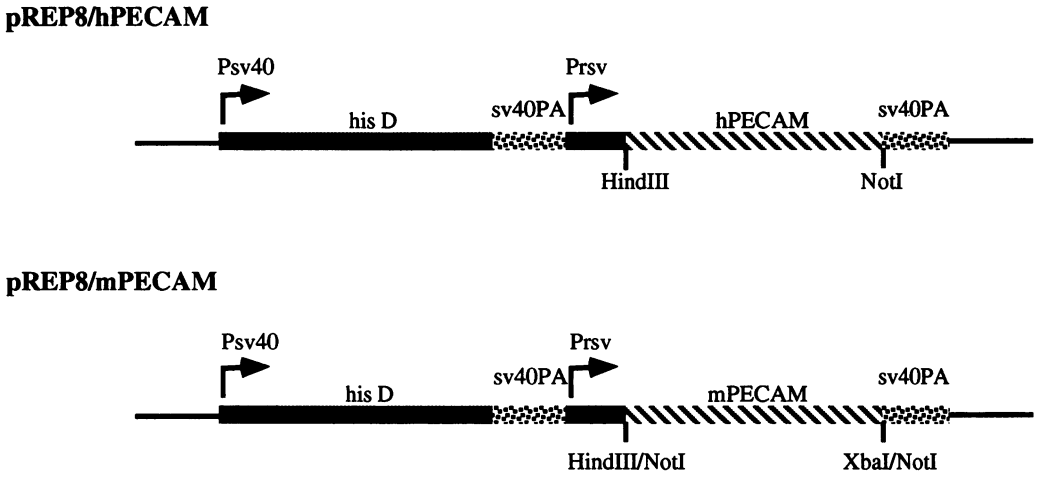

Figure 1. PECAM-1 expression vectors. The vectors that express h- or mPECAM-1 were constructed as described in MATERIALS AND METHODS.

gene, which allows growth in medium containing L-histidinol. The cells were grown in the presence of 2.5–10 mM L-histidinol in regular growth medium. After 2–3 wk, resistant colonies were expanded and enriched by cell sorting, and individual clones were isolated. Clones were expanded and screened by Western blotting.

Immunofluorescent Staining

Cells were plated on glass coverslips and allowed to reach confluence. Cells were washed in phosphate-buffered saline (PBS), fixed with 3% paraformaldhyde for 15 min, and then permeablized with ice-cold 0.5% Nonidet P-40 for 1 min. Coverslips were washed with PBS and incubated with anti-PECAM-1 antibody prepared in TBS (20 mM Tris-HCl, pH 7.6, 150 mM NaCl) with 1% ovalbumin at 37°C for 30 min. After washing with TBS, the coverslip was incubated with a 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Pierce, Rockford, IL) in TBS with 1% ovalbumin at 37°C for 30 min. Cells were viewed on a Nikon phase–epifluorescence microscope using a 40× fluorescence lens and photographed with Ecktochrom 400 ASA film.

Fluorescence-activated Cell Sorting Analysis

Cells grown on 100-mm tissue culture plates were removed by 0.04% EDTA, 0.05% bovine serum albumin (BSA) in PBS, washed with TBS, resuspended in TBS with 1% goat serum, and kept on ice for 20 min. Cells were pelleted, resuspended in TBS with 1% BSA containing anti-PECAM-1 antibody (10 μg/ml), and kept on ice for 30 min. Cells were washed twice with TBS containing 1% BSA, resuspended in TBS containing 1% BSA and a 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Pierce), and kept on ice for 30 min. Cells were washed twice with TBS containing 1% BSA and resuspended in TBS with 1% BSA, and flow cytometry was performed by using a FACScan (Becton Dickinson, San Jose, CA). Sorting was performed by using a FACS Vantage (Becton Dickinson).

Northern Blot Analysis

Poly(A)$^+$ RNA was isolated from logarithmically growing cells as described (Sheibani *et al.*, 1991). RNA (5 μg) was electrophoresed in a 1.2% agarose gel containing formaldehyde, transferred to Zeta-Probe membranes (Bio-Rad, Richmond, CA), prehybridized, and then hybridized in the presence of random primer ^{32}P-labeled cDNA probes. The cDNA probes used were the mPECAM-1 and hPECAM-1 entire coding regions, the 1.4-kbp *Bam*HI 5′ fragment of human TS1 cDNA, and a 1.3-kbp *Pst*I fragment of rat glyceraldehyde-3-phosphate dehydrogenase cDNA (Sheibani and Frazier, 1995).

Western Blot Analysis

Cells were removed from the plate by trypsin-EDTA and washed with TBS. Approximately 5 × 10^5 cells were resuspended in 0.1 ml of lysis buffer (20 mM Tris-HCl, pH 7.6, 2 mM EDTA) and stored at −70°C until ready for analysis. Cell lysates were thawed, mixed with 6× SDS sample buffer, boiled, and analyzed by SDS-PAGE and blotting as described previously (Sheibani and Frazier, 1995). The antibodies used were PECAM 1.3 (a monoclonal antibody (Mab against hPECAM-1 at 0.5 μg/ml) and a polyclonal anti-mouse PECAM-1 at 1 μg/ml (a gift from Dr. B.A. Imhof, Basel Institute for Immunology, Basel, Switzerland).

Three-dimensional Culture

Matrigel (Collaborative Research, Bedford, MA) was diluted to 10 mg/ml with serum-free medium and 0.5 ml was added per well of 24-well tissue culture plates and allowed to gel at 37°C for at least 30 min. Cells were removed by trypsin-EDTA, washed with growth medium, resuspended at 1 × 10^5 cells/ml, and 0.5 ml was gently added to each of duplicate wells. The plates were monitored for 6 to 24 h and photographed with a Nikon microscope. Each experiment was repeated at least twice. For antibody inhibition experiments, 8-well chamber slides (LabTeck) were incubated with 150 μl of Matrigel at 37°C for 30 min to allow the Matrigel to harden. Approximately 10^4 cells were mixed with the antibody, control IgG, or TBS alone in 150 μl and gently overlaid on top of the Matrigel. The cultures were photographed after 18 h.

Tumorigenesis Assay

The tumorigenesis assays were performed as described recently (Sheibani and Frazier, 1995). Briefly, cells were removed by trypsin-EDTA, washed with growth medium, resuspended at 2 × 10^7 cell/ml in growth medium, and 0.25 ml of cell suspension was injected subcutaneously on each side into the rear flanks of 6-wk-old male nude mice (Harlan Sprague Dawley, Indianapolis, IN; two sites per mouse and three mice per cell line). The mice were maintained in a sterile environment and examined daily. The care of the mice was provided by the Washington University veterinary staff and was conducted according to institutional guidelines.

Cloning and Isolation of mPECAM-1 Isoforms from bEND.3 Cells

The mRNA isolated from bEND.3 cells was used as a template for reverse transcription (RT) in a reaction mixture containing a gene-specific primer (primer 2253, see below) and SuperScript II RNase H^- reverse transcriptase as recommended by the manufacturer (Life Technologies, Gaithersburg, MD). To amplify the cytoplasmic domain of mPECAM-1 isoform from the reverse transcribed cDNA the following primers were used: a sense primer (5′-^{1395}TATGAAAGCAAAGAGTGA^{1412}-3′) flanking the *Bst*EII restriction site within the extracellular domain and an antisense primer (5′-CGAATGC^{2253}ATCCAGGAATCGGCTGCTCTTC^{2235}-3′) complementary to a region 70 bp downstream from the stop codon of mPECAM-1 and carrying a new 5′ *Nsi*I recognition sequence to facilitate subsequent cloning. The polymerase chain reaction (PCR) product was digested with *Bst*EII and *Nsi*I and ligated into a pcDNAI/neo vector (Invitrogen) containing mPECAM-1 (lacking exon 15) that was digested with the same enzymes, thus replacing the existing cytoplasmic domain. The resulting ligation mixture was used to transform competent DH5α cells. The antibiotic-resistant colonies were screened by digestion of miniprep plasmid DNA. Clones that carried the plasmid with the expected size insert were sequenced (PRISM ready reaction Dyedeoxy Terminator cycle sequencing kit, Perkin Elmer-Cetus, Norwalk, CT) to confirm their identity by using the above sense and antisense primers. Abundance of mRNA for particular PECAM-1 isoforms were determined by direct sequencing of 40 clones.

RESULTS

PECAM-1 Expression in bEND.3 and TS1-transfected bEND.3 Cells

bEND.3 cells proliferate rapidly in culture and are capable of forming hemangiomas in mice within 1–2 d. The rapidity of this process is attributed to the ability of bEND.3 cells to recruit host ECs (Williams *et al.*, 1989). PECAM-1, an EC adhesion molecule, could be involved in this process by mediating interactions between the bEND.3 cells and the host ECs. This is consistent with our observation that normal MBECs, after several passages (p4 or p22) in culture, rapidly lose expression of PECAM-1, but when transformed with polyoma mT (bEND.3 cells), they maintain a high expression level of PECAM-1 (Figure 2). Others have shown that early-passage normal bovine brain ECs in culture express PECAM-1, localizing to sites of cell–cell contact, under conditions that attempt to maintain the blood–brain barrier function of ECs (Rubin *et al.*, 1991). Therefore, alterations in blood–brain barrier function of normal MBECs in culture may be associated with rapid loss of PECAM-1 expression. The bEND.3 cells express very high levels of PECAM-1 that localizes to intercellular borders once cell–cell contact is achieved in culture (Figure 3). The production of PECAM-1 mRNA and protein in TS1-transfected bEND.3 (bEND/TS) cells is suppressed to undetectable levels (Figures 2 and 3). This is consistent with the differences observed in the morphology of bEND/TS compared with parental bEND.3 cells (Figure 3, compare b with h). The bEND.3 cells form closely packed cell layers

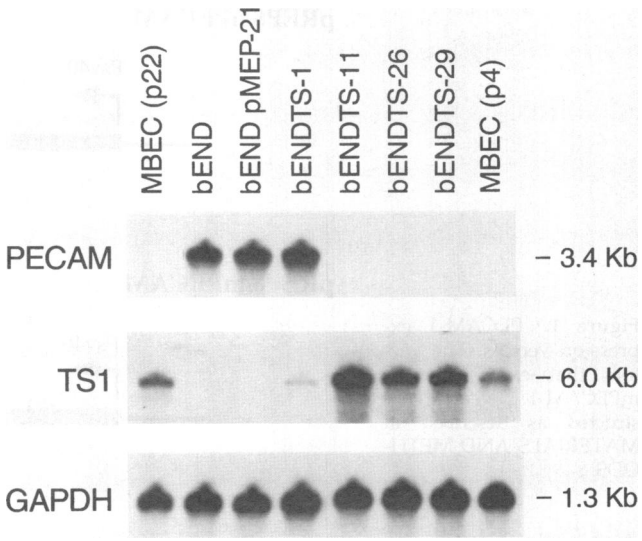

Figure 2. Analysis of the steady-state PECAM-1 mRNA levels in normal cultured MBECs, bEND.3, and vector (pMEP-21) or bEND/TS clones. Poly(A)$^+$ RNA was prepared from logarithmically growing cells, size fractionated on 1.2% agarose-formaldehyde gels, and transferred to Zeta-Probe membranes. The mRNA for PECAM-1 was detected with a mouse cDNA probe for PECAM-1. This blot was also probed with the cDNA for GAPDH to control for loading. Note that MBECs express TS1 but not PECAM-1, whereas polyoma middle-sized T-transformed bEND.3 cells have regained PECAM-1 expression but lost TS1. Transfection of bEND.3 cells with TS1 cDNA (bEND TS-11, TS-26, and TS-29 cells) results in suppression of PECAM-1 transcription concomitant with loss of their rapid growth and ability to form tumors (see text). The bEND TS-1 is a revertant clone with bEND.3 cell phenotype; it had bEND.3 cell morphology, expressed PECAM-1, and lost TS1 expression.

with spindle cell-like morphology similar to the cells of Kaposi's sarcoma, whereas the bEND/TS cells fail to establish close cell–cell contacts and appear to be disorganized (Figure 3h). It is possible that the lack of PECAM-1 protein in bEND/TS cells could, in part, contribute to the inability of these cells to adopt a closely packed spindle cell-like morphology in culture and recruit host ECs to form hemangiomas in mice. Southern blot analysis of DNA isolated from bEND/TS cells confirmed that there are no gross rearrangements in the *PECAM-1* gene when compared with the parental bEND.3 cells (our unpublished results). The functionality of the *PECAM-1* gene was further confirmed by the ability of revertant cells, which appeared in some of the bEND/TS clones and ultimately took over the culture, to reexpress PECAM-1 (Figure 2, clone TS-

Figure 3 (facing page). Indirect immunofluorescence staining of bEND.3 cells with a hamster Mab to mPECAM-1 (2H8) followed by fluorescein-conjugated goat anti-rat IgG. (a and b) Parental bEND.3 cells. (c–f) Two representative vector-transfected bEND.3 clones

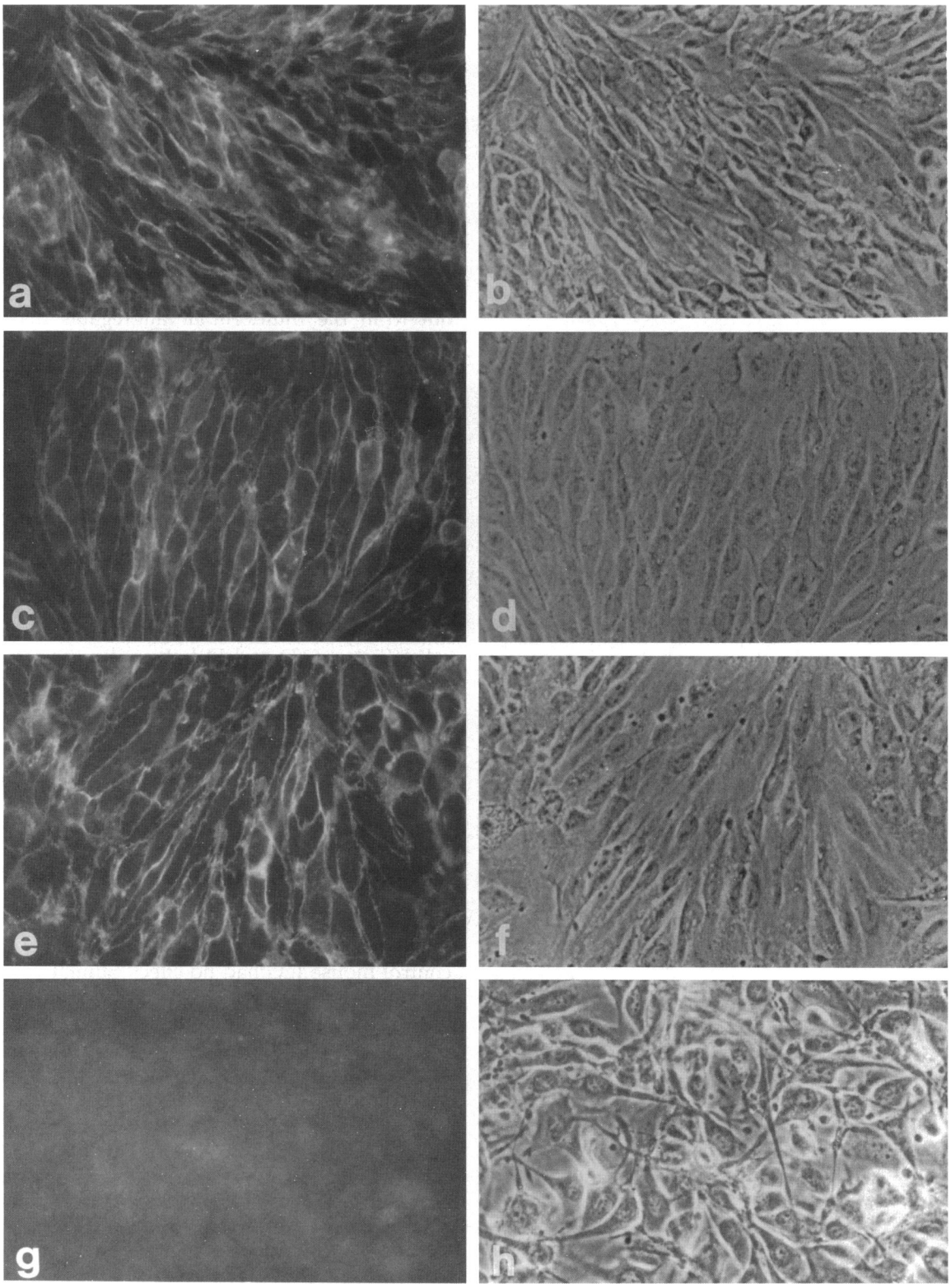

Figure 3 (cont). (one in c and d, the other in e and f). (g and h) A representative bEND/TS clone. Note that transfection of bEND.3 cells results in loss of PECAM-1 expression, consistent with the Northern blot results shown in Figure 2. g was overexposed to show complete lack of junctional staining and exposure time similar to other panels showed no staining.

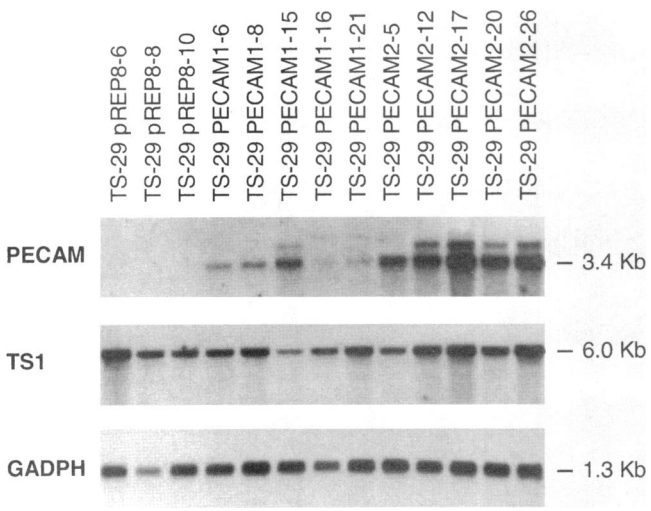

Figure 4. Analysis of the steady-state PECAM-1 mRNA levels. Poly A+(A)+ RNA was prepared from bEND/TS cells transfected with pREP8 (vector) or pREB8/hPECAM-1 (clones). The mRNA for PECAM-1 was detected with a cDNA probe specific for hPECAM-1. This blot was also probed with the cDNA for human TS1 and the cDNA for GAPDH to control for loading.

1). This occurred concomitant with the reestablishment of a bEND.3-cell–like morphology, reexpression of PECAM-1 and its localization to sites of cell–cell contact, and loss of TS1 production in the revertants. The bEND/TS cells thus provided us with a PECAM-1-null EC line to investigate the function of this molecule in EC–EC interactions and morphogenesis; an important issue because the majority of the PECAM-1 functional studies have been performed in non-EC lines.

Expression of hPECAM-1 and mPECAM-1 in bEND/TS Cells

To examine the role of PECAM-1 in EC–EC interactions and morphogenesis, four different clones of bEND/TS cells were supertransfected with vectors that expressed hPECAM-1 or mPECAM-1 cDNA (Figure 1). After selection with histidinol, cell populations were examined for expression of PECAM-1 by fluorescence-activated cell sorter (FACS) analysis and Northern blot analysis. One of the bEND/TS clones (TS-29) transfected with PECAM-1 was used for further characterization. These cells were stained with anti-PECAM-1 antibody and sorted twice to enrich for a population of cells expressing PECAM-1. After the second sort, approximately 40% of the cells stained positive for PECAM-1 and clones derived from the PECAM-1-positive cells were selected for further analysis. Northern blot analysis confirmed that these cells expressed PECAM-1 mRNA and mRNA encoding TS1 (Figure 4). Similar results were obtained with mPECAM-1-transfected bEND/TS cells.

Enhanced EC Morphogenesis after PECAM-1 Expression in bEND/TS Cells

We characterized the PECAM-1-transfected bEND/TS clones in two different ways. First, we examined the ability of these cells to form organized structures on Matrigel. We observed that high PECAM-1-expressing clones had an enhanced ability to migrate and form extensively branched cords of cells on Matrigel compared with vector-transfected or parental bEND/TS cells (Figure 5). These cells form cords by migrating out from the multicellular aggregates that initially form after plating ECs on Matrigel. These multicellular aggregates are much smaller in cell number, and many more cells are in compact cords when compared with those formed by vector-transfected cells. The vector-transfected cells (Figure 5B), like the bEND/TS parental cells (Figure 5A) and other bEND/TS clones that we had examined previously, form large multicellular aggregates and broad streams of cells on Matrigel with proportionally fewer cells forming compact well-defined cords. The role of PECAM-1 in enhanced morphogenesis was confirmed by the ability of specific anti-PECAM-1 Mabs to block or break up the more compact cords formed by PECAM-1-expressing cells (Figure 6, D and F) but not by the control cells (Figure 6B). Figure 6, A, C, and E, shows cellular differentiation in the absence of anti-PECAM-1 Mabs. These experiments were carried out with at least a dozen clones of transfected cells and repeated twice with identical results. We also tested the effect of anti-PECAM-1 antibodies on HUVECs, which normally express high levels of PECAM-1 at cell–cell junctions (DeLisser *et al.*, 1994b) and form extensive networks of tubes on Matrigel. As shown in Figure 7, anti-hPECAM-1 Mab (PECAM 1.3: 0.5 mg/ml) blocked the ability of these cells to organize and form tubes on Matrigel but lack of antibody or presence of control mouse IgG had no effect. Similar results were also observed with a polyclonal anti-hPECAM-1 antibody, SEW 16 (our unpublished data).

Lack of Hemangioma Formation by PECAM-1-expressing bEND/TS Cells

We next tested the ability of the PECAM-1 expressing bEND/TS cells to form hemangiomas in mice, a function lost upon TS1 expression (Sheibani and Frazier, 1995). The parental bEND.3 cells form hemangiomas in 1–2 d. The PECAM-1-transfected bEND/TS cells did not regain the ability to form hemangiomas when followed for periods of up to 6 wk after injection, suggesting that the PECAM-1 isoforms expressed in the presence of continued expression of TS1 did not result in a transformed phenotype. The expression of PECAM-1 in bEND/TS cells did not affect cell mor-

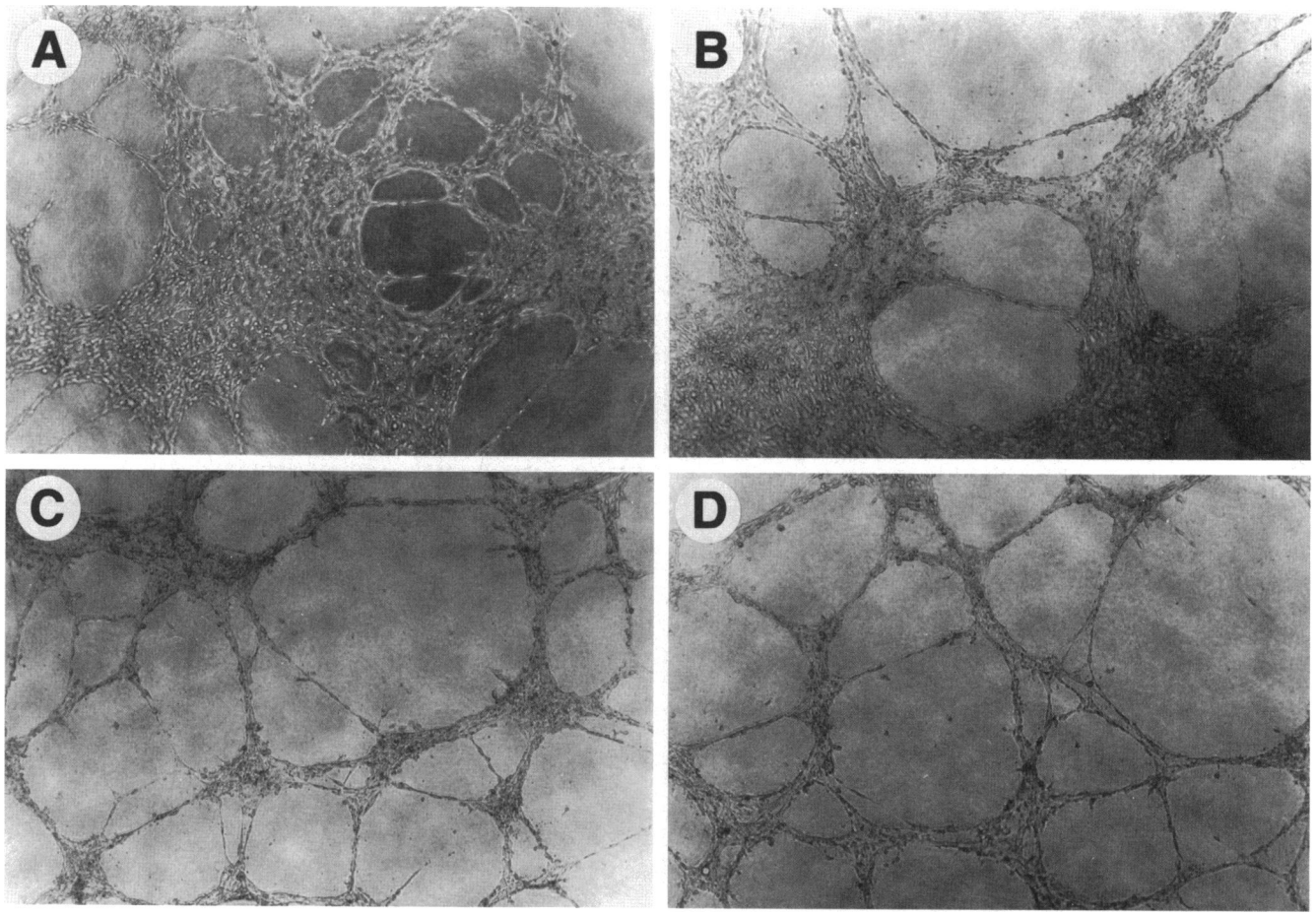

Figure 5. Differentiation of ECs in three-dimensional Matrigel cultures. Phase micrographs are shown. (A) bEND/TS parental cells. (B) Cells transfected with control vector. (C and D) Two different clones of bEND/TS cells transfected with hPECAM-1 (PECAM-8 and PECAM-12). These results are representative of more than a dozen similar experiments performed with vector-, hPECAM-1-, or mPECAM-1-transfected clones of bEND/TS cells. Note that the PECAM-1-transfected bEND/TS cells exhibit an enhanced ability to organize and form networks of cords on Matrigel.

phology (Figure 8, compare B and D). Furthermore, the PECAM-1 expressed on the surface of these cells did not localize to the sites of cell–cell contact (Figure 8, compare A and C), whereas the PECAM-1 endogenously expressed in the bEND.3 parental cells (Figure 3a) localized to sites of cell–cell contact. These results suggested that different isoform(s) of PECAM-1 might be expressed in bEND.3 cells accounting for the differences in localization found for the transfected versus the endogenous PECAM-1.

Identification of the mPECAM-1 Isoform(s) Expressed in bEND.3 Cells

Although expression of hPECAM-1 or mPECAM-1 (above) seems to result in an enhanced ability of bEND/TS cells to organize on Matrigel, these are not as well organized as tubes formed by normal MBECs. This may be due to a defect in homotypic cell–cell adhesion. It has been recently shown that in mouse embryo at least seven different isoforms of PECAM-1 are expressed that are generated by alternative splicing of the PECAM-1 cytoplasmic domain. These include the full-length, Δ12, Δ14, Δ15, Δ12&15, Δ14&15, and Δ12,14&15 isoforms. The mPECAM-1 isoforms lacking exon 14 mediated homophilic, but not heterophilic, adhesion when expressed in L cells (a non-EC line), whereas those that contained exon 14 mediated only heterophilic adhesion (Yan *et al.*, 1995). To identify the naturally occurring isoform(s) of PECAM-1 expressed in parental bEND.3 cells, mRNA encoding the transmembrane region and cytoplasmic tail of bEND.3 PECAM-1 was isolated and sequenced by reverse transcription-polymerase chain reaction (RT-PCR). The primers were positioned such that they spanned exons 9 through 16 of PECAM-1 (Yan *et al.*,

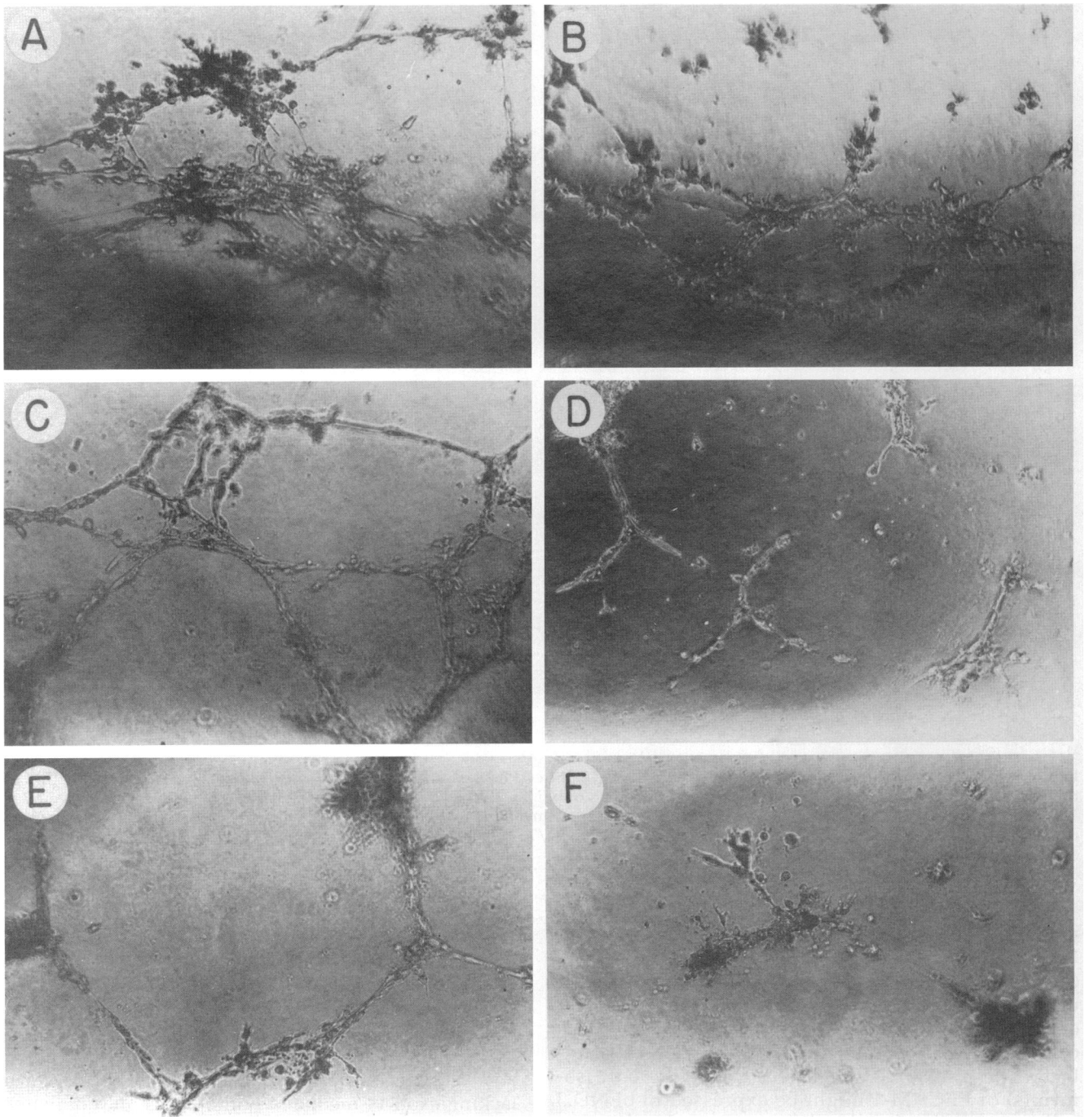

Figure 6. Differentiation of ECs in three-dimensional Matrigel cultures in the presence of anti-PECAM-1-specific antibody. Phase micrographs are shown. (A and B) bEND/TS parental cells. (C–F) Two different PECAM-1-transfected bEND/TS clones (one in C and D, the other in E and F). (A, C, and E) Without the antibody. (B, D, and F) With anti-hPECAM-1 Mab (PECAM-1.3; at 0.5 mg/ml). Experiments carried out in the presence of a polyclonal antibody against hPECAM-1 (SEW16) showed similar results.

1995). Analysis of the RT-PCR products by electrophoresis revealed multiple bands, suggesting the expression of several PECAM-1 isoforms in bEND.3 cells. These cDNAs were cloned and sequenced as described in MATERIALS AND METHODS. The cDNA sequences obtained for the cytoplasmic domains were identical to the sequences reported for full-length, Δ12, Δ14, Δ15, Δ12&15, and Δ14&15

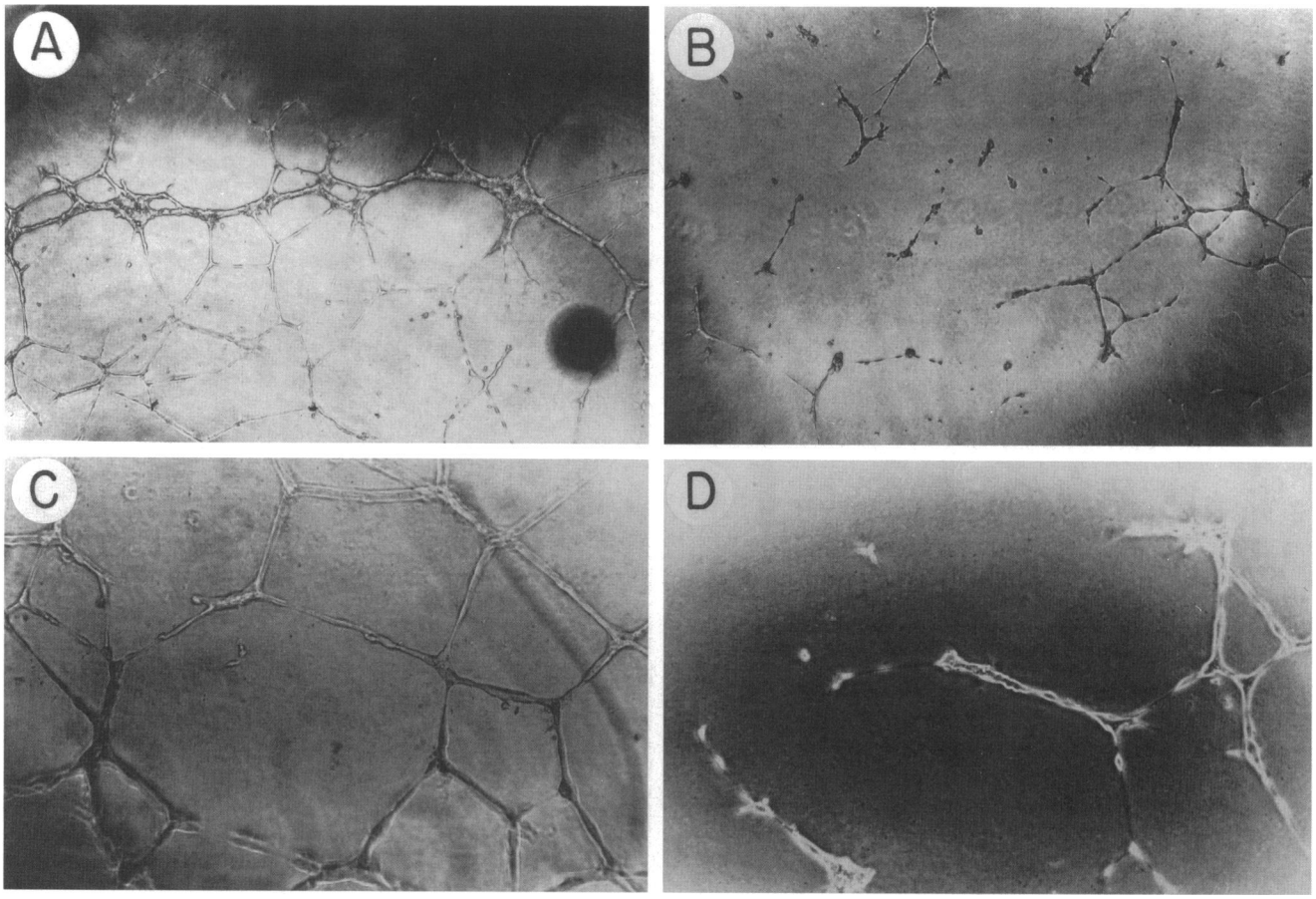

Figure 7. Anti-PECAM-1 antibody inhibits tube formation in cultured HUVECs. Phase micrographs are shown. (A) Control (no antibody). (B) Anti-hPECAM-1 Mab (PECAM-1.3; at 0.5 mg/ml). (C and D) Higher magnification of the same cultures, respectively. These experiments were repeated at least twice with two different isolates of HUVECs. Similar results were observed with the polyclonal antibody (SEW16) against hPECAM-1.

mPECAM-1 isoforms (Yan *et al.*, 1995). The isoform that lacked both exons 14 and 15 (Δ14&15) was the most abundant isoform detected. However, it is not known which of these isoforms are translated. The mPECAM-1 isoform expressed by cDNA transfection in bEND/TS cells (above) lacked only exon 15 (Δ15), but the hPECAM-1 isoform did not lack any exons (full length). The ability of the bEND.3 PECAM-1 isoform to localize to sites of cell–cell contact, where it can participate in homophilic interactions, is consistent with expression of a PECAM-1 isoform lacking exon 14 (Yan *et al.*, 1995). Thus, we expressed the mPECAM-1 Δ14&15 isoform in bEND/TS cells. The phenotype generated by this reexpression of the most abundant isoform in bEND.3 cells was identical in all respects to that seen after expression of either full-length hPECAM-1 or Δ15 mPECAM-1 described above. In particular, the Δ14&15 mPECAM-1 did not become localized to sites of cell–cell contact and the cells expressing it did not revert to the parental bEND.3 growth habit and morphology. Thus, TS1 expression in bEND.3 cells may be involved not only in regulation of PECAM-1 expression but also in modulating its localization and adhesion characteristics.

DISCUSSION

The bEND.3 cells are polyoma middle-sized T-transformed MBECs that express little or no TS1 and exhibit intense PECAM-1 staining at sites of cell–cell contact (Figures 2 and 3a). In contrast, normal MBECs express high levels of TS1 and rapidly lose PECAM-1 expression in culture. Thus, it appears that transformation of early-passage MBECs results in maintenance of high PECAM-1 expression and repression of TS1. This endogenous PECAM-1 isoform localizes to sites of cell–cell contact and may take part in interactions with host ECs during hemangioma formation in mice. However, the expression of TS1, a natural inhibitor of angiogenesis, is down-regulated in these cells, whereas it is highly expressed in normal MBECs in culture (Sheibani

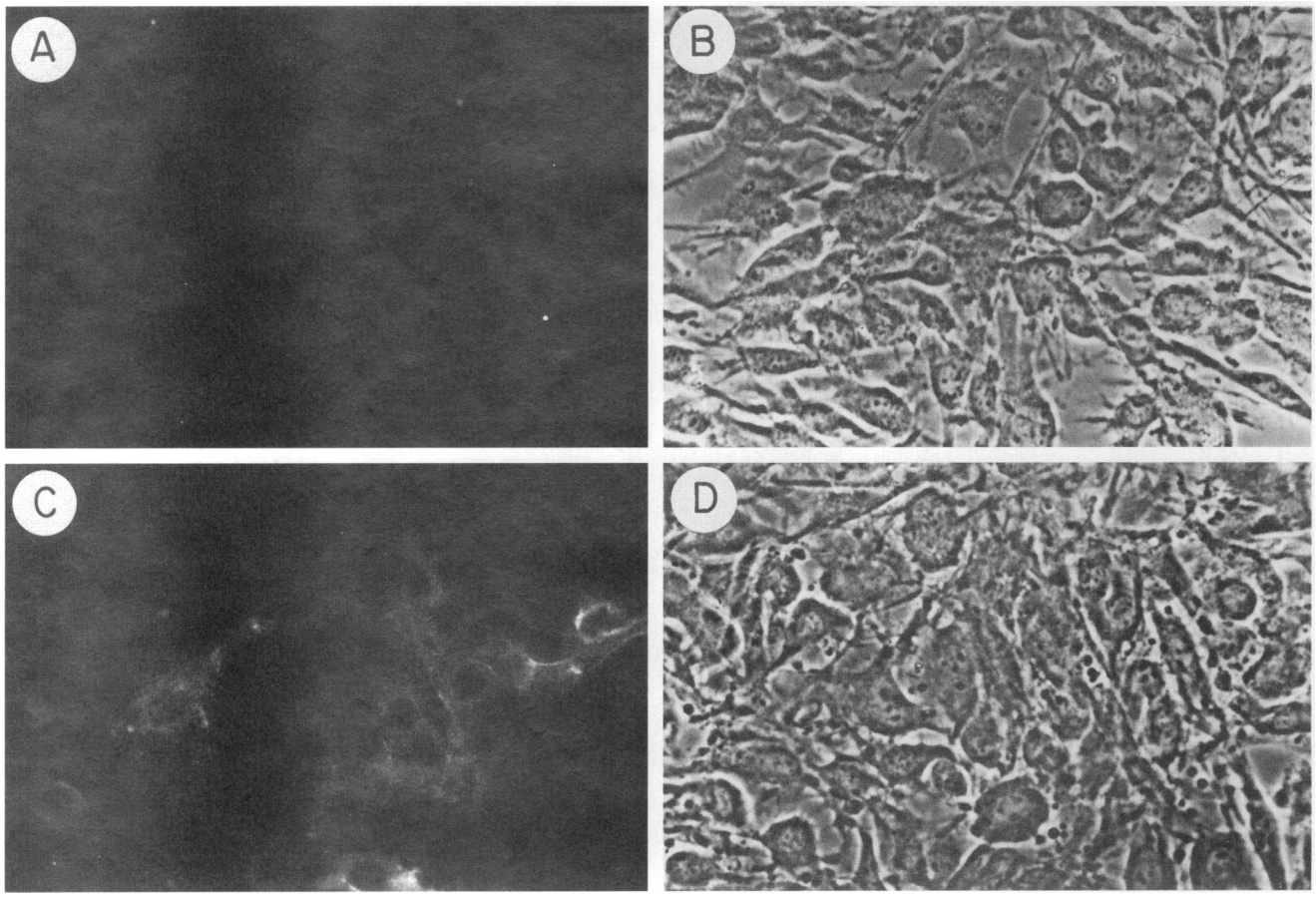

Figure 8. Indirect immunofluorescence staining of bEND/TS cells with a hamster Mab to mPECAM-1 (2H8) followed by fluorescein-conjugated goat anti-rat IgG. (A and B) Representative vector-transfected bEND/TS cells. (C and D) Representative mPECAM-1-transfected bEND/TS cells. Identical results were observed with hPECAM-1 (pREP8/hPECAM).

and Frazier, 1995). Reestablishment of TS1 expression in bEND.3 cells by cDNA transfection had a dramatic effect on their phenotype. The bEND/TS cells exhibited many characteristics of normal MBECs in culture, including suppression of PECAM-1 expression (Figures 2 and 3g). We did not detect any gross rearrangements in the *PECAM-1* gene of bEND/TS cells. Furthermore, revertant clones arising in some of the bEND/TS cells, which lose expression of TS1, and reexpress PECAM-1 (Figure 2, clone TS-1), suggest that the *PECAM-1* gene is intact and functional.

PECAM-1 mediates adhesion via interactions with PECAM-1 in some cells, leading to homophilic adhesion, but in other cells it appears to activate signal transduction pathways necessary to stabilize other adhesive events (Sun *et al.*, 1996). Cell–cell and cell–matrix interactions along with growth factors provide important cues that regulate the organization of ECs into vessels (Drake *et al.*, 1992; Damsky *et al.*, 1993; Gamble *et al.*, 1993; Brooks *et al.*, 1994). These effects are mediated through cell surface receptors interacting with different extracellular matrix molecules or neighboring cells, both functions of PECAM-1. It is not known how TS1 expression negatively affects PECAM-1 production in bEND.3 cells. It is possible that interaction of TS1 with a receptor on bEND.3 cells may lead to down-regulation of PECAM-1 expression. This is consistent with the important role of PECAM-1 in mediating initial cell–cell interactions during differentiation of ECs (DeLisser *et al.*, 1994b), but not being required later after cell–cell contacts are established. In contrast, TS1 is expressed at later stages of EC differentiation, a role consistent with its angiostatic and prodifferentiative activity (Reed *et al.*, 1995; Sheibani and Frazier, 1995). Thus, constitutive expression of TS1 in bEND.3 cells may provide an inappropriate signal suppressing PECAM-1 expression and interfering with its early functions during EC morphogenesis. Studies are currently underway to examine such a possibility and to identify the signaling pathways involved that result in suppression of PECAM-1 expression.

Most studies implicating PECAM-1 in homophilic and heterophilic cell–cell interactions have utilized expression in non-EC lines (Muller *et al.*, 1992; DeLisser *et al.*, 1993, 1994b; Yan *et al.*, 1995). The regulation of the adhesive state of PECAM-1 and its role in EC morphogenesis remains poorly understood. The bEND/TS cells, which express no PECAM-1, provide a system in which to examine the role of this cell adhesion molecule during EC morphogenesis. By themselves, the bEND/TS cells cannot prove the causal role of PECAM-1 in any particular process because other changes brought about by TS1 expression may be responsible for the observed effects. To address this issue, we reexpressed mPECAM-1 and hPECAM-1 in bEND/TS cells. Expression of either hPECAM-1 (full length) or mPECAM-1 (Δ15) in bEND/TS cells improved their ability to form a more highly branched extensive network of compact cords, despite the fact that the PECAM-1 isoforms expressed in these cells did not obviously localize to sites of cell–cell contact in culture as did the endogenous mPECAM-1 expressed in bEND.3 cells. This difference in localization may explain the inability of the transfected hPECAM-1 or mPECAM-1 isoforms to reestablish the parental bEND.3 cell morphology in bEND/TS monolayer cultures. Furthermore, bEND/TS cells expressing these isoforms of m- or hPECAM-1 did not regain the ability to form tumors in mice even 6 wk after injection, suggesting an important role for homophilic interactions in recruitment of host ECs and hemangioma formation in vivo. The bEND.3 cells that express high levels of PECAM-1 rapidly form hemangiomas (1–2 d) in mice and fail to differentiate on Matrigel. They form cyst-like structures on Matrigel instead of organized compact cords seen in bEND/TS cells or bEND/TS cells expressing hPECAM-1 or mPECAM-1. This suggests that a threshold level for PECAM-1 may exist so that too much PECAM-1 may result in strong cell–cell and/or cell–matrix interactions, preventing EC morphogenesis on Matrigel. This idea is supported by the fact that when PECAM-1 levels are down-regulated by antisense expression in bEND.3 cells to levels comparable to those of PECAM-1-transfected bEND/TS cells, we see an enhanced ability of these cells to form organized cords on Matrigel compared with cells expressing either high PECAM-1 (bEND.3) or no PECAM-1 (bEND/TS) (Sheibani and Frazier, unpublished data). The studies of Gamble *et al.* (1993) further support the idea that an optimal level of cell–cell or cell–matrix interaction is required for EC morphogenesis on three-dimensional matrices. These authors found that anti-integrin antibodies could actually enhance the ability of ECs to differentiate and form tubes on very dense three-dimensional matrices.

The differences in phenotype of the cells expressing endogenous versus transfected PECAM-1 could also be attributed to the different functional properties of the isoforms of PECAM-1 expressed in these cells. The h- and mPECAM-1 cDNAs encoded full-length and Δ15 PECAM-1, respectively, but the endogenous PECAM-1 expressed in the bEND.3 may be different. It is also not known what isoform(s) of PECAM-1 is expressed in normal MBECs. The cytoplasmic domain of PECAM-1 may affect both cytoskeletal interactions or downstream signaling events. Full-length hPECAM-1 containing all cytoplasmic exons mediates heterotypic calcium-dependent cell–cell interactions. However, mutant hPECAM-1, which lacked one-third or two-thirds of the cytoplasmic domain, supported only calcium-independent homotypic cell–cell interactions (DeLisser *et al.*, 1994a). Alterations in the cytoplasmic domain of mPECAM-1 have recently been shown to occur by alternative splicing of the cytoplasmic tail (exons 10–16), thus generating a number of PECAM-1 isoforms that are expressed in the developing mouse embryo (Yan *et al.*, 1995). Expression of these isoforms in L cells (a non-EC line) indicated that full-length mPECAM-1 and three isoforms that contained exon 14 mediated calcium-dependent and heparin-blockable heterophilic cell–cell interactions. However, three mPECAM-1 variants that lacked exon 14 mediated calcium- and heparin-independent homotypic cell–cell interactions. Thus, exon 14 appeared to modulate the adhesive properties of the extracellular domain of mPECAM-1 or downstream signaling affecting other cell adhesion systems. These observations may also explain the differences observed in adhesive properties of hPECAM-1 versus mPECAM-1 when expressed in L cells reported earlier (Xie and Muller, 1993) and differences in the adhesive properties of the mPECAM-1 and hPECAM-1 expressed in bEND/TS cells compared with the mPECAM-1 endogenously expressed in parental bEND.3 cells. The PECAM-1 cDNAs that we expressed in the bEND/TS cells both contained exon 14, which might have explained the inability of these PECAM-1 isoforms to localize to sites of cell–cell contact and mediate homotypic EC adhesion, and their inability to participate in homotypic interactions with host ECs to form hemangiomas in vivo. Conversely, the intense PECAM-1 staining of bEND.3 cells at sites of cell–cell contact, and the ability of these cells to recruit host ECs in hemangioma formation, might indicate that the PECAM-1 isoform(s) expressed by bEND.3 cells lacked exon 14. To investigate this possibility, we identified the PECAM-1 isoforms expressed in bEND.3 cell by sequencing the cytoplasmic tail of PECAM-1 isoforms generated by RT-PCR. The bEND.3 cells expressed six different isoforms of PECAM-1 previously detected in the whole mouse embryo (Yan *et al.*, 1995). The isoform that lacked both exons 14 and 15 was the most abundant. Expression of this isoform in bEND/TS cells produced a phenotype

similar to that observed with full-length hPECAM-1 or Δ15 mPECAM-1 isoforms. Furthermore, the lack of exon 14 was not sufficient to result in junctional localization of PECAM-1 in bEND/TS cells, even in clones that expressed very high levels of PECAM-1 (at least three times the levels reported by Yan *et al.*, 1995). The identification of PECAM-1 isoforms that are translated awaits development of specific antibodies that will discriminate among these isoforms. These studies clearly demonstrate that PECAM-1 plays an important role in EC adhesion, morphogenesis, and perhaps transformation, where its expression and adhesive functions may be regulated by TS1 along with alternative splicing of its cytoplasmic domain. To our knowledge, this is the first report of PECAM-1 expression in an EC line to investigate its role in cell–cell interactions and morphogenesis. The cell lines generated herein provide a valuable tool for dissecting the downstream signaling events that emanate from cell surface engagement of the various PECAM-1 isoforms. This should allow us to gain a better understanding of the functions of PECAM-1 in inflammation, angiogenesis, and vascular development.

ACKNOWLEDGMENTS

We thank Drs. B.A. Imhof (Basel Institute for Immunology, Basel, Switzerland), S.A. Bogen (Boston University, Boston, MA), and S.M. Albelda (University of Pennsylvania, Philadelphia, PA) for kind gifts of mPECAM-1 antibodies and mPECAM-1 cDNA (lacking exon 15). We thank Dr. C.M. Sorenson for critical reading of the manuscript, M. Ehsanie for excellent technical support, the protein and nucleic acid chemistry laboratory of Washington University (Dr. G. Grant, Director) for synthesis of primers, the core nucleotide sequencing laboratory of Washington University for sequencing the recombinant plasmids on an Applied Biosystems (model 373A) sequencer, and P. Chand at the FACS facility for FACS analysis and cell sorting. This work was supported by grants CA-65872 (W.A.F.) and HL-40926 (P.J.N.).

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