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Modulation of VE-Cadherin and PECAM-1 Mediated Cell–Cell Adhesions by Mitogen-Activated Protein Kinases

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Abstract Endothelial cell transition from a differentiated, quiescent phenotype to a migratory, proliferative phenotype is essential during angiogenesis. This transition is dependent on alterations in the balanced production of stimulatory and inhibitory factors, which normally keep angiogenesis in check. Activation of MAPK/ERKs is essential for endothelial cell migration and proliferation. However, its role in regulation of endothelial cell adhesive mechanisms requires further delineation. Here, we show that sustained activation of MAPK/ERKs results in disruption of cadherin-mediated cell–cell adhesion, down-regulation of PECAM-1 expression, and enhanced cell migration in microvascular endothelial cells. Expression of a constitutively active MEK-1 in mouse brain endothelial (bEND) cells resulted in down-regulation of VE-cadherin and catenins expression concomitant with down-regulation of PECAM-1 expression. In contrast, inhibition of MEK-1 restored parental morphology, cadherin/catenins expression and localization. These data are further supported by our observation that sustained activation of MAPK/ERKs in phorbol myristate acetate incubated HUVEC lead to disruption of cadherin-mediate cell–cell interactions and enhanced capillary formation on Matrigel. Thus, sustained activation of MAPK/ERKs plays an important role in disruption of cell–cell adhesion and migration of endothelial cells. J. Cell. Biochem. 90: 121–137, 2003.

Key words: adherens junction; angiogenesis; CD31; cell–cell interactions; endothelial cells

Angiogenesis is a multi-step process involving the dissolution of basement membrane, loosening of cell–cell and cell–matrix interactions, migration, proliferation, and differentiation resulting in formation of vessels. VE-cadherin is an endothelium specific cadherin required for normal development of the vasculature in the embryo and for angiogenesis in the adult [Vittet et al., 1997; Carmeliet et al., 1999]. Disruption of cadherin-mediated cell–cell interactions is observed prior to endothelial cell migration [Lampugnani et al., 1995]. Alteration in components of adherens junctions including VE-cadherin, γ-catenin, and β-catenin compromises formation of endothelial cell–cell interactions [Schnittler et al., 1997; Venkiteswaran et al., 2002]. In addition, interaction of VE-cadherin with intracellular signal transducing molecules can modulate growth factor mediated signal transduction pathways influencing angiogenesis [Zanetti et al., 2002].

PECAM-1/CD31 is a member of the immunoglobulin gene superfamily that is highly expressed on the surface of endothelial cells and to a lesser extent in hematopoietic cells. PECAM-1-mediated cell–cell interactions are essential during angiogenesis [DeLisser, 1997; Sheibani et al., 1997; Sheibani and Frazier, 1999; Cao et al., 2002]. Mice deficient in PECAM-1 develop

Abbreviations used: VE-cadherin, vascular cadherin; PECAM-1, platelet endothelial cell adhesion molecule-1; MAPK, mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK kinase; JNK, c-*jun* N-terminal protein kinase; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; TSP, thrombospondin.

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normally with subtle inflammatory response defects, both in diapedesis of leukocytes and re-establishment of the endothelium barrier [Duncan, 1999; Graesser et al., 2002]. Therefore, PECAM-1 mediated cell–cell interactions play an important role in vascular functions and integrity.

We have recently shown that expression of the Δ15 PECAM-1 isoform in an epithelial cell line (MDCK) prevents cadherin mediated cell–cell interactions while expression of Δ14&15 PECAM-1 isoform does not influence these interactions [Sheibani et al., 2000]. This was mediated through sustained activation of MAPK/ERKs in Δ15 PECAM-1 cells. Thus, PECAM-1 modulates cadherin-mediated cell–cell interactions through its ability to interact with various intracellular proteins activating MAPK/ERKs. The differential ability of PECAM-1 isoforms to activate MAPK/ERKs and modulate cadherin-mediated cell–cell interactions suggests that different PECAM-1 isoforms may have different roles during angiogenesis.

The MAPK cascade, triggered by a variety of external signals including VEGF, FGF-1, FGF-2, and HGF, plays a central role in the transduction of extracellular stimuli into diverse biological responses. Sustained activation of MAPK/ERKs is essential for HGF-induced epithelial cell motility [Tanimura et al., 2002]. VEGF, an angiogenic factor, stimulates proliferation, migration, and barrier dysfunction of endothelial cells through activation of multiple signal transduction pathways that vary depending on EC origin [Xia et al., 1996; Kroll and Waltenberger, 1997; Esser et al., 1998; Cohen et al., 1999; Yashima et al., 2001]. The VEGF-mediated proliferation of EC is dependent on activation of MAPK/ERKs through ras dependent and independent pathways [Kroll and Waltenberger, 1997; Yashima et al., 2001]. The activation of MAPK/ERKs is also a key step in FGF-1 and FGF-2 mediated signal transduction in endothelial cells and angiogenesis [LaVallee et al., 1998; Giuliani et al., 1999]. However, the role activated MAPK/ERKs play in endothelial cell–cell interactions requires further delineation. Here, we show that expression of a constitutively active mutant of the upstream ERK activator, MEK-1, in endothelial cells results in pronounced phenotypic changes in these cells. We observed changes in morphology, disruption of cadherin-mediated cell–cell adhesion, down-regulation of PECAM-1, and increased cell motility. Therefore, sustained activation of MAPK/ERKs influences the cell–cell interactions mediated through VE-cadherin and PECAM-1 and EC migration, which are essential during angiogenesis.

MATERIALS AND METHODS

Construction of the Expression Vector

The activating mutant of MEK-1 cDNA was obtained by digesting the expression vector pUSEamp(+)-S218D/S222D (Upstate, Lake Placid, NY) with *Hind*III/*Xho*I. This released a 1.3 kb insert which includes the HA-tag and MEK-1 cDNA. The insert was ligated with pMEP4 expression vector (Invitrogen, San Diego, CA) digested with same enzymes. This plasmid contains a hygromycin resistance sequence and allows expression of activated MEK-1 from the metallothionine promoter. The integrity of the recombinant plasmid was confirmed by restriction enzyme digestion and DNA sequencing. The metallothionine promoter is leaky in bEND cells [Sheibani and Frazier, 1998] and thus does not require induction.

Cells and DNA Transfection

The mouse brain endothelial cells, bEND, were maintained as described previously [Sheibani and Frazier, 1995]. The bEND cells were transfected with the pMEP4/MEK-1 or empty vector utilizing Lipofectin (Invitrogen) as described previously [Sheibani and Frazier, 1995]. Cells were selected in growth medium containing hygromycin B (50 μg/ml; Sigma, St. Louis, MO) for 2–3 weeks and approximately 70 stable clones were isolated. Individual clones were expanded and screened by Western blot for levels of MEK-1 expression using HA-tag antibody. Several stable clones, which express high levels of exogenous MEK-1 were obtained, screened for their levels of ERKs, and used for further characterization.

Human umbilical veins endothelial cells (HUVEC) (obtained from Dr. Cornelus, Washington University, St. Louis, MO) were maintained on gelatin-coated dishes in M199 medium supplemented with 20% heat-inactivated fetal bovine serum, 100 μg/ml endothelial cell mitogen, 100 μg/ml heparin, 25 mM HEPES, 2 mM L-glutamine. Cells from passage 3–7 were used for the experiment. The mouse endothelial cell line Py4-1 were obtained from Dr. Bautch

(University of North Carolina, Chapel Hills, NC) and maintained as described previously [Sheibani et al., 1999].

Western Blot Analysis

Western blot analysis was performed as described previously [Sheibani et al., 2000]. For screening of the bENDmek clones, cells were plated in 60 mm dishes, allowed to reach confluence, and removed by trypsin–EDTA in 4 ml of medium. Three milliliters of cell suspension was centrifuged and frozen down to keep as stock. One milliliter of cell suspension was centrifuged, washed with PBS, lysed in 0.1 ml of lysis buffer (20 mM Tris-HCl, pH 7.6, 2 mM EDTA) and used for Western blot analysis. For other experiments, 1×10^5cells were plated in 60 mm dishes and allowed to reach confluence (3–4 days). Cells were then rinsed twice with cold serum-free medium containing 0.5 mM Na_3OV_4, lysed on ice in 0.1 ml lysis buffer (50 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, 1 mM each $CaCl_2$ and $MgCl_2$, 1% NP-40, 0.5% deoxycholate, 100 mM NaF, 3 mM Na_3OV_4, and a cocktail of protease inhibitors (Roche Biochemicals, Indianapolis, IN), and protein concentrations were determined using the BCA protein assay (Bio-Rad, Hercules, CA). Equal amounts of protein lysate (25 μg) were analyzed by SDS–PAGE (4–20% Tris-Glysine gels, Invitrogen, San Diego, CA), and transferred to Nitrocllulose. Membranes were blocked, incubated with appropriate primary and secondary antibodies, and developed using ECL (Amersham, Piscataway, NJ). The antibodies to α-catenin, β-catenin, and γ-catenin were obtained from Transduction Laboratories (Lexington, KY). The antibodies to phospho-ERKs and ERK1/2 were from Promega (Madison, WI). The antibody to MEK1/2 was from Cell Signaling Technology (Beverly, MA). The antibodies to VE-cadherin (C-19), PECAM-1 (M-20), phospho-JNK (G-7), Phospho-P38 (D-8) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibody to TSP1 (A6.1) was from NeoMarkers, Inc. (Fremont, CA).

Indirect Immunofluorescence Assay

Cells (2×10^4) were plated on glass coverslips until they reached confluence. The coverslips were rinsed three times with PBS, fixed with 3% paraformaldehyde for 15 min for PECAM-1 staining, or fixed and permeabilized with ice-cold 3% paraformaldehyde containing 0.1% Triton X-100 for 15 min on ice for β-catenin, VE-cadherin and actin staining. Cells were then washed three times with TBS (20 mM Tris-pH 7.6; 150 mM NaCl), incubated with primary antibodies to β-catenin, VE-cadherin, and PECAM-1 in TBS with 1% ovalbumin for 30 min at 37°C. The rabbit anti-mouse β-catenin antibody (Sigma) was used at 1:300 dilution, the rabbit anti-human VE-cadherin polyclonal antibody (Bender Medsystems, Burlingame, CA) was used at 1:200 dilution and the rat anti-mouse PECAM-1 monoclonal antibody MEC13.3 (BD Pharmingen, San Diego, CA) was used at 1:500 dilution. Coverslips were then rinsed with TBS and incubated with fluorescein isothiocyanate-conjugated secondary antibody (1:100) (Pierce, Rockford, IL), prepared in TBS with 1% ovalbumin, for 30 min at 37°C. Following incubation, coverslips were washed three times with TBS and mounted in TBS with 50% glycerol. Cells were viewed on a Zeiss Axiophot fluorescence microscope equipped with a AxioCam digital camera (Carl Ziess, Chester, VA).

Three-Dimensional Culture of Endothelial Cells

Matrigel (Collaborative Research, Bedford, MA) was diluted to 10 mg/ml with serum-free medium, 0.5 ml was added per 35 mm tissue culture dish, and allowed to harden at 37°C for at least 30 min. Cells were removed by trypsin–EDTA, washed with growth medium, resuspended in growth medium (with and without inhibitor) at about 1.5×10^5 cells/ml, and 2 ml was gently added to each of the duplicate plates. The plates were monitored for 6 to 24 h and photographed with a Nikon microscope equipped with a digital camera. Each experiment was repeated at least twice with duplicate plates. PD98059 was used at 50 μM and bisindolymaleimide was used at 2 μM.

Scratch Wound Assays

Cells (2×10^5) were plates on 60 mm tissue culture dishes and allowed to reach confluence (2–3 days). After aspirating the medium, cell layers were wounded using a 1 ml micropipette tip. Plates were then rinsed with PBS, fed with growth medium, and wounds were observed and photographed at 0, 24, and 48 h. These experiment were repeated at least three times with duplicate plates. For inhibitor studies, cells were incubated with appropriate concentration

of inhibitors (PD98059- MEK inhibitor, 50 μM; PP1- Src inhibitor 10 μM; bisindolymaleimide- PKC inhibitor 2 μM, H89- PKA inhibitor, 1 μM; JNKL1- JNK inhibitor, 5 μM; SB28059- P38 inhibitor 10 μM; Forskolin- stimulate cAMP production 10 μM; 5-flurouracil- DNA synthesis inhibitor 10 μg/ml) overnight prior to wounding and after wounding for desired times.

Incubation With Pharmacological Agents

All the pharmacological agents including PKC stimulator PMA (phorbol 12-myristate 13-acetate) were obtained from Alexis Biochemicals Corporation (San Diego, CA), and stock solutions were prepared (1,000×) as recommended by the supplier. Na_3OV_4 was from Sigma. For Western blot analysis, cells (3×10^5) were plated in 60 mm dishes and the next day they were incubated with growth medium containing 50 nM PMA or 1 mM Na_3OV_4 for indicated times. In other assays, cells were pre-treated with 50 μM PD98059 for 30 min prior to the addition of 1 mM Na_3OV_4. Following treatment (1–24 h), cells were washed with cold serum free medium containing 0.5 mM Na_3OV_4, 0.5 ml of lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA) added to each plate, cells were scrapped and transferred to a microfuge tube. Equal amount of protein samples were used for SDS–PAGE and Western blot analysis as described above. For indirect immunofluorescence staining, cells (10^4) were plated on coverslips, the next day PD98059 was added at final concentration of 50 μM. Cells were fed 2 days later with growth medium containing PD98059 and allowed to reach confluence. Coverslips then washed, fixed, and stained with appropriate antibody as described above.

Northern Blot Analysis

The poly(A^+) RNA was isolated form logarithmically growing cells as described [Sheibani and Frazier, 1995]. RNA (5 μg) was electrophoresed in a 1.2% agarose gel containing formaldehyde, transferred to Zeta-probe membrane (Bio-Rad), prehybridized, and then hybridized in the presence of random primer ^{32}P-labeled cDNA probes. The cDNA probes used were the full-length human fibronectin cDNA, the 1.4 kbp of mouse TSP1 cDNA (both from Dr. D. Mosher, University of Wisconsin, Madison, WI), the full-length mouse PECAM-1 cDNA (from Dr. S.M. Albelda, University of Pennsylvania, Philadelphia, PA), the cDNA for rat αv (from Dr. M. Hammerman, Washington University, St. Louis, MO), the cDNAs for mouse α5 and β1 integrins (from Dr. D. Dean, Washington University, St. Louis, MO), the cDNA probe for mouse β3 and β5 integrins (from Dr. P. Ross, Washington University, St. Louis, MO), the cDNA probe for mouse β-catenin (from Dr. J. Nelson, Stanford, CA), the cDNA probe for mouse VE-cadherin was generated by RT-PCR using the 5′-CCATCGCCAAAAGAGAGA-3′ (forward) and 5′-TCTGGCAGCTTGAAGTGG-TAGA-3′ (reverse) primers and mRNA from bEND cells, and a 1.3-kbp Pst I fragment of rat glyceraldehydes-3-phosphate dehydrogenase cDNA to control for loading [Sheibani and Frazier, 1995].

RESULTS

Expression of the Constitutively Active MEK-1 in bEND Cells

The bEND cells were stably transfected with the pMEP4/MEK-1 (bENDmek) expression vector, which expresses a dominantly active MEK-1. Total cell lysates were prepared from a population of bENDmek or vector control cells and Western blotted with a HA-tag antibody (data not shown). The bENDmek cells express high levels of HA-tagged MEK-1, which is absent in parental or vector control cells. We isolated approximately 70 clones of bENDmek cells and screened them by Western blot analysis using HA-tag and/or MEK-1 antibodies. We identified a number of clones that expressed high levels of active HA-MEK-1. We next screened these clones for levels of activated MAPK/ERKs using a phospo-ERK antibody. In Figure 1A, we identified several clones, which have high levels of MEK-1 and phospho-ERKs. Clones that had high MEK-1 levels but did not exhibit high levels of phospho-ERKs were used as additional controls. Figure 1B shows expression of adherens junction components by Western blot analysis of cell lysates prepared from parental, vector control, and MEK-transfected bEND cells. The bENDmek clones with higher expression of phospho-ERKs exhibited a significant decrease in VE-cadherin, α- and γ-catenins expression. The expression of β-catenin was moderately affected. In addition, the PECAM-1 level was also decreased in cells with increased expression of phospho-ERKs.

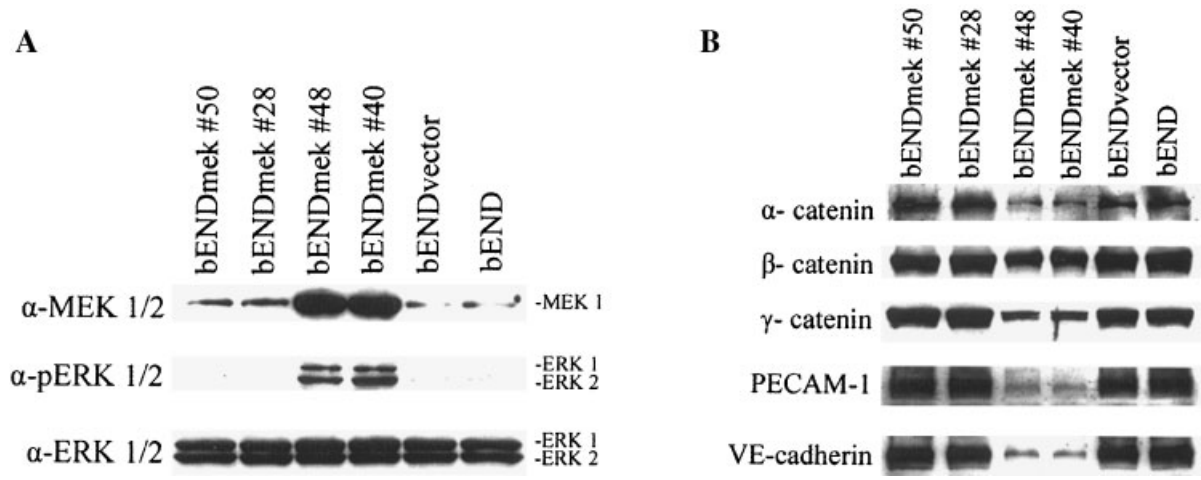

Fig. 1. Expression of MEK-1 results in activation of ERKs and altered expression of adherens junction component and PECAM-1. Total cell lysates were prepared from parental bEND, bEND vector, and several bENDmek clones and analyzed by Western blot analysis. The membrane was incubated with antibodies to MEK-1, phospho-ERKs, or ERKs (**A**) or antibodies to components of adherens junction or PECAM-1 (**B**). Please note that the bENDmek clones expressing high levels of MEK-1 also express increased amounts of phospho-ERKs and significantly lower levels of α-, β-, γ-catenins, VE-cadherin, and PECAM-1.

Sustained Activation of ERKs Results in Altered Cell Morphology

The bEND cells rapidly proliferate in culture and form a tightly packed monolayer with spindle cell morphology (Fig. 2A). The bEND cell morphology was not affected by expression of an empty pMEP4 vector (Fig. 2B). However, bENDmek cells with increased expression of phospho-ERKs formed a monolayer of cells that fail to form close cell–cell contacts with gaps between the cells (Fig. 2C,D). These results are consistent with our data from the Δ15 PECAM-1 transfected MDCK cells, which have high levels of phospho-ERKs with altered morphology [Sheibani et al., 2000]. The reduced expression of VE-cadherin and catenins (Fig. 1B) may contribute the inability of these cells to form cell–cell contacts exhibiting an altered morphology.

Enhanced Ability of bENDmek Cells to Form Tube on Matrigel

The tube forming ability of EC on Matrigel recapitulates the later stages of angiogenesis. EC plated on Matrigel rapidly migrate and organize into a capillary like network within 12–24 h without significant cell proliferation. We have previously shown that parental bEND cells organize very poorly on Matrigel and fail to form a capillary-like network. Instead, they from spheres, which with time grow in size and resemble hemangiomas seen in vivo [Sheibani et al., 1997; Sheibani and Frazier, 1998]. This was attributed, at least in part, to expression of high levels of PECAM-1 since bEND cells with significantly reduced levels of PECAM-1 organize better on Matrigel [Sheibani and Frazier, 1998]. Figure 3 shows the ability of the bENDmek and vector control cells to form capillary like networks on Matrigel. Vector transfected cells, similar to parental bEND cells, failed to organize on Matrigel (Fig. 3A). In contrast, bENDmek cells expressing high levels of phospho-ERKs form a capillary-like network. Thus, expression of activated MEK-1 in bEND cells (bENDmek) is sufficient to enhance migration and organization on Matrigel compared to control cells.

To further investigate bENDmek cells migratory phenotype we utilized monolayer-wounding assays. Monolayers of vector control or bENDmek cells were wounded and wound closure was monitored over the next 48 h. Minimal migration of vector control cells was observed (Fig. 4A–C). The majority of the wounded area remained uncovered with minimal sheet migration toward the wound. In contrast, bENDmek cells had significant number of cells migrating into the wounded area by 24 h. At 48 h, a significant portion of the wound is covered (Fig. 4D–F). Figure 4G shows the quantitative assessment of the data. Incubation of the wounded monolayers with 5-flurouracil

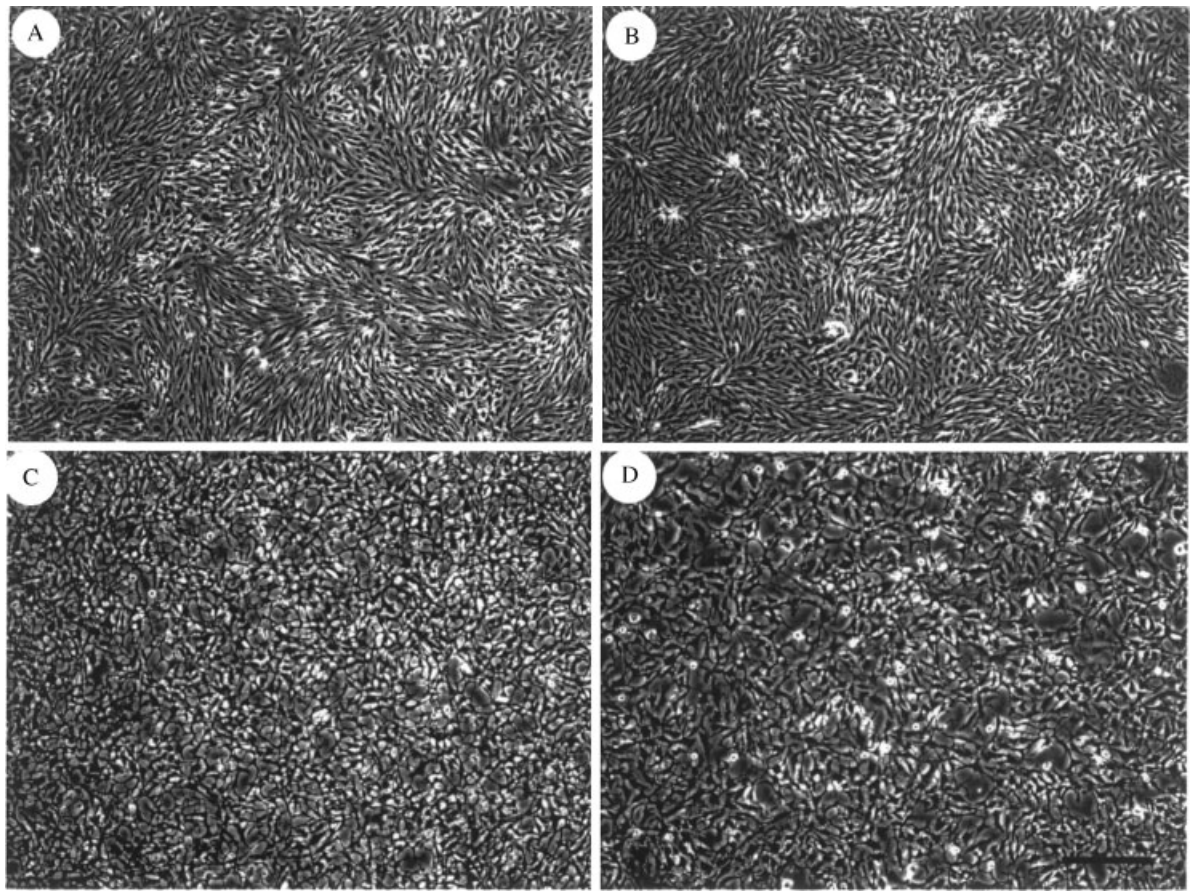

Fig. 2. Activation of MAPK/ERKs alters the morphology of bEND cells. Phase micrograph (4× objective) of the parental bEND cells (**A**), bENDvector (**B**), and bENDmek clones (**C**, #40 and **D**, #48) cultured in growth medium. Please note the lack of close cell–cell contact and gaps in bENDmek clones (compare A and B to C and D). Bar = 400 μm.

(inhibits cell proliferation) did not affect our results, indicating that the differences observed in cell migration ability were not the result of different rates of cell proliferation (not shown). Therefore, EC with sustained activation of ERKs exhibit enhanced migration.

Sustained Activation of MAPK/ERKs Is Essential for Altered Morphology

To demonstrate that sustained activation of ERKs is necessary for the altered morphology observed in bENDmek cells, we examined the

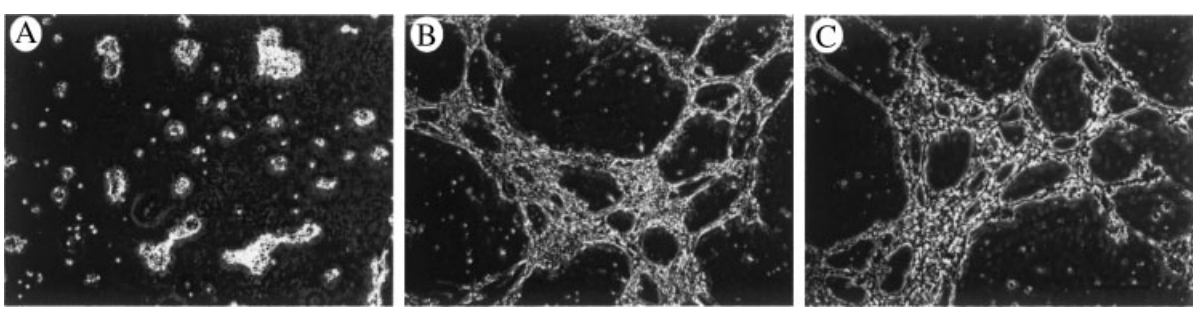

Fig. 3. Enhanced ability of bENDmek cells to organize and form cords on Matrigel. bENDvector (**A**) and bENDmek (**B**, clone 40 and **C**, clone 48) cells were plated on Matrigel (as described in ''Materials and Methods'') and photographed 24 h later. Please note the enhanced ability of bENDmek cells to migrate and organize to cords. Bar = 400 μm.

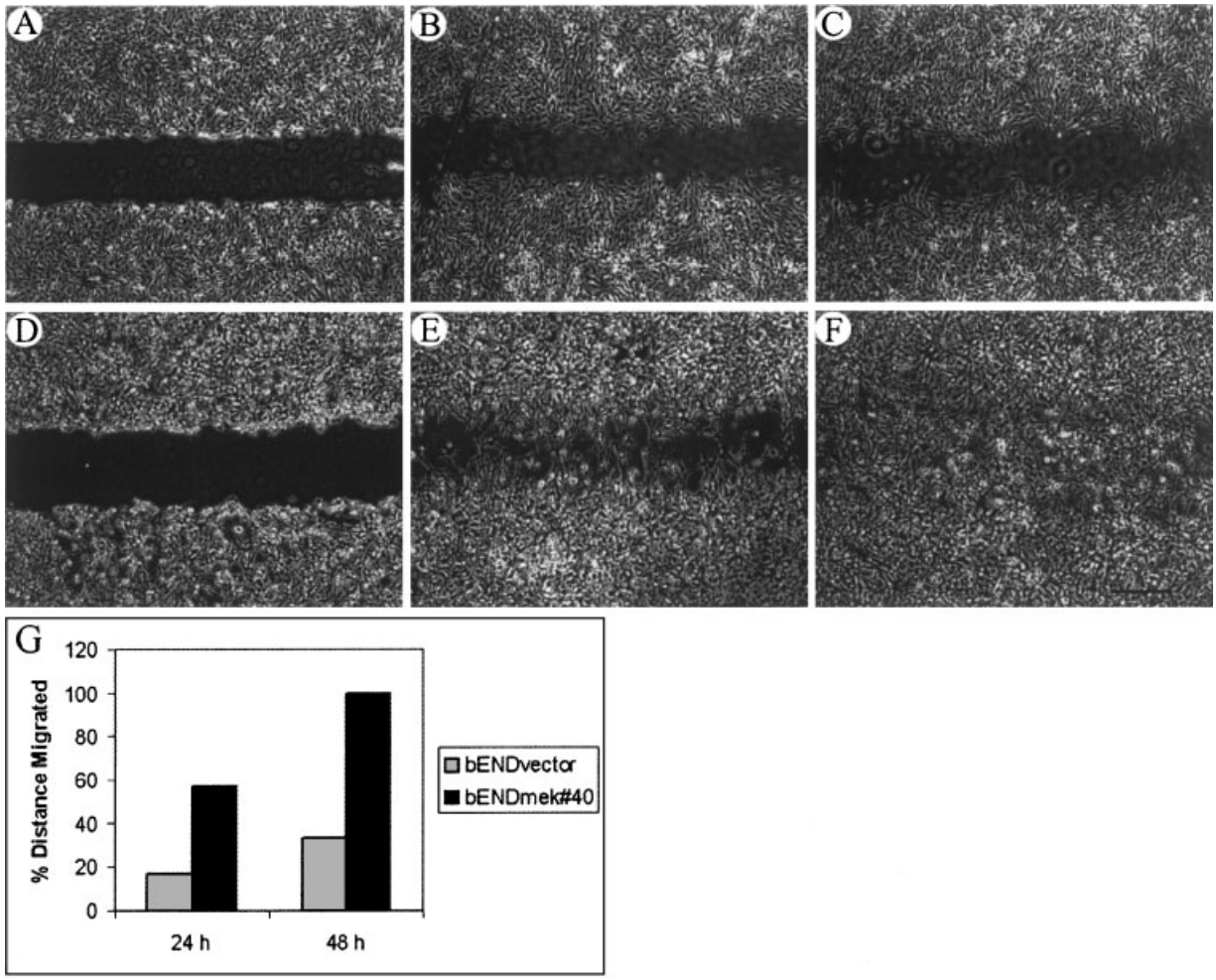

Fig. 4. Enhanced ability of bENDmek cells to migrate. bENDvector (**A–C**) and bENDmek (**D–F**) cells were plated and allowed to reach confluence. Monolayers were wounded (as described in ''Materials and Methods'') and wound closure was monitored after 24 h (B and E) or 48 h (C and F) by phase photography. A and D show the wounded monolayer at time zero. **G** shows the quantitative assessment of the data. Please note that bENDmek cells have migrated and covered the wound by 48 h. These experiments were repeated three times with similar results. A representative experiment is shown. Bar = 400 μm.

effects of PD98059 on the morphology of bENDmek cells (Fig. 5). Figure 5A–C show the phase morphology, D–F show actin staining, G–I show β-catenin staining, and J–L show VE-cadherin staining in the presence of DMSO (A, D, G, and J), PD98509 (B, E, H, and K), or following removal of PD98059 (C, F, I, and L). The bENDmek cells incubated with PD98509 for 4 days demonstrated a change in morphology such that they resemble parental cells and form close cell–cell contacts (compare Fig. 5A to 5B). These changes were reversible upon removal of PD98059 with cells regaining their altered morphology (Fig. 5C). This is consistent with changes in actin, VE-cadherin and β-catenin localization. The bENDmek cells exhibit more actin stress-fibers, and lack junctional staining for VE-cadherin and β-catenin. However, bENDmek cell incubated with PD98059 exhibit less actin stress fibers and more peripheral actin localization along with junctional VE-cadherin and β-catenin staining was observed. This was reversible upon removal of PD98059. Therefore, sustained activation of MAPK/ERKs is required for the altered morphology and lack of cadherin/catenins junctional localization observed in bENDmek cells.

Src Kinase Activity Is Essential for Enhanced Migration of bENDmek Cells

The Src kinase plays an important role in many aspects of angiogenesis. The Src kinase activity is essential for EC migration and VEGF mediated EC proliferation and altered cell

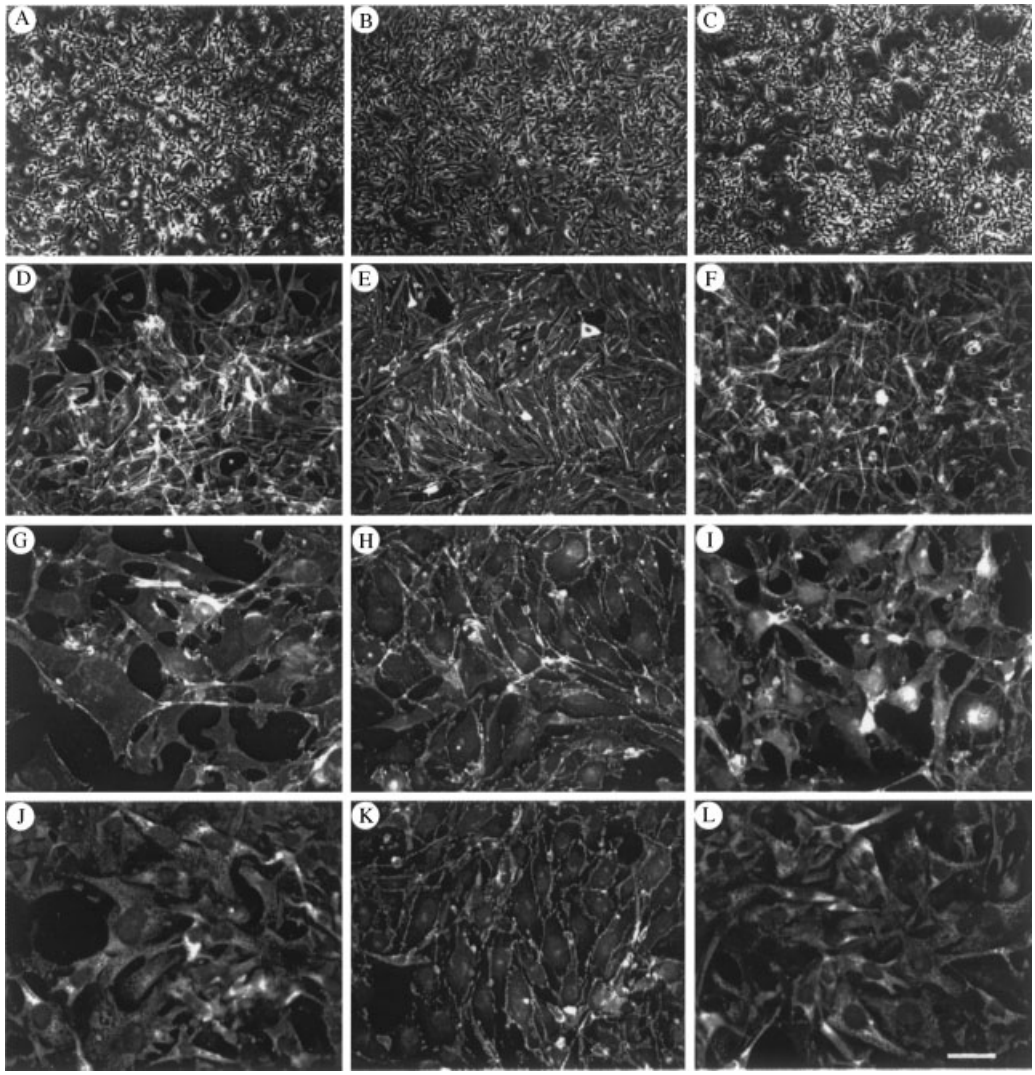

Fig. 5. Sustained activation of MAPK/ERKs is necessary for altered morphology and disruption of adherens junction. The bENDmek cells were plated on coverslips, incubated with DMSO (**A**, **D**, **G**, **J**) or PD98509 (**B**, **E**, **H**, **K**) for 4 days, and photographed before (A–C, phase 4×) or after immunostaining for actin (D–F), β-catenin (G–I), and VE-cadherin (J–L) (40×). In one set of samples, cells were incubated with PD98059 for 4 days, rinsed with medium to remove PD98059, incubated for two additional days in the absence of PD98059 and photographed (**C**, **F**, **I**, **L**). Please note that inhibition of MAPK/ERKs restores parental morphology and cadherin mediated adherens junction in a reversible manner. Bar = 50 μm.

permeability [Shi et al., 2000; Eliceiri et al., 2002; Cottrell et al., 2003; Mucha et al., 2003]. However, its role in modulation of PECAM-1 and VE-cadherin mediated cell–cell interactions requires further delineation. We next determined whether Src activity is necessary for enhanced migration of bENDmek cells. Figure 6 shows that incubation of bENDmek cells with PP1 (a specific inhibitor of Src kinase) inhibits migration. To determine whether this is mediated by inhibition of the ERKs, we examined the levels of ERKs in bENDmek cell incubated with PP1. Figure 6D shows that incubation of vector or MEK-1-transfected cells with normal phospho- ERKs level (clone #28) with PP1 inhibits basal levels of ERKs, although to much lower extent than PD98059. A similar result was observed in bENDmek cells incubated with PP1, in which the level of phospho-ERKs was partially down regulated.

The cross activation of JNK, but not P38 MAP kinase, by ERKs and subsequent JNK activity is essential for VEGF- [Pedram et al., 1998] or hypoxia-mediated vascular cell proliferation [Das et al., 2001]. We observed increased levels of phospho-JNK, but not P38, in bENDmek cells

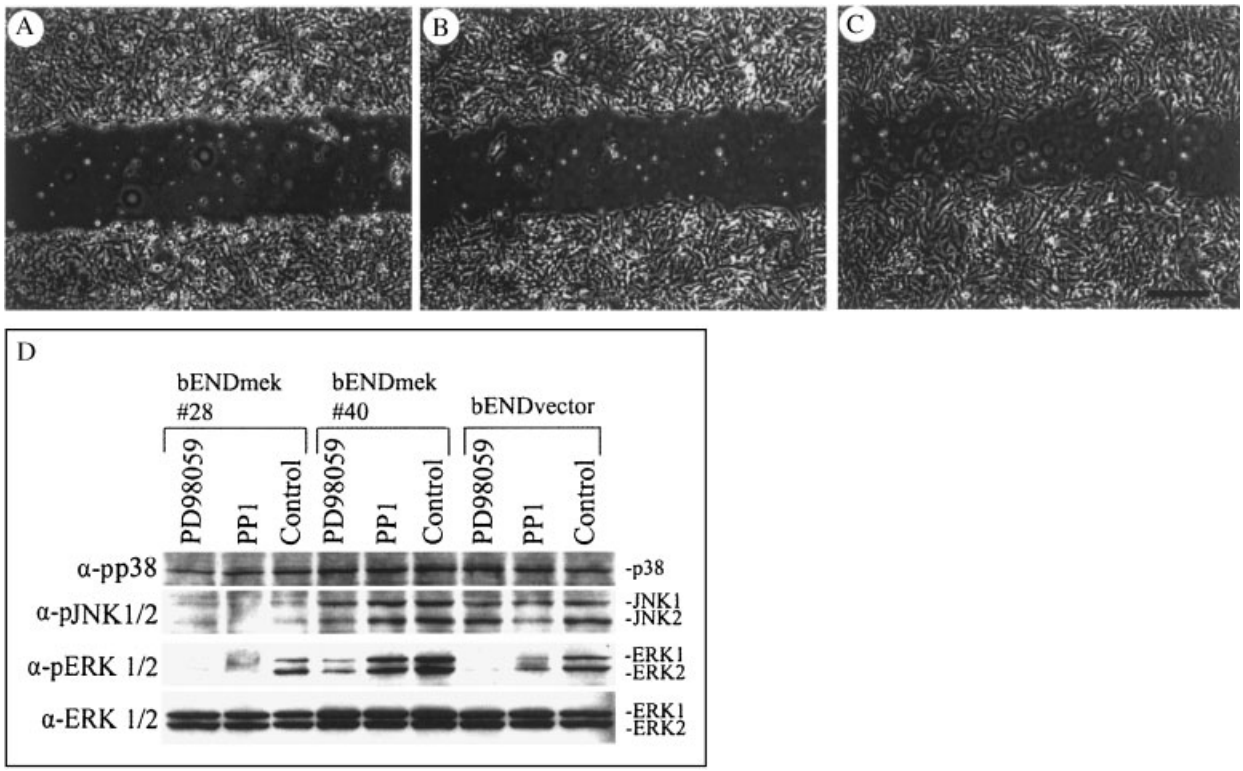

Fig. 6. Src activity is essential for migration of bENDmek cells. The ability of bENDmek cell (clone #40) to migrate was assessed in the presence of PP1 (a specific inhibitor of Src activity). Confluent monolayer of bENDmek cells incubated with PP1 (10 μM) overnight, wounded the next day, and wound closure was monitored after 0 h (**A**), 24 h (**B**), or 48 h (**C**) by phase photography. Please note minimal migration of cells after 48 h. The effects of PP1 and PD98059 on the levels of active ERKs, JNK, and P38 were assessed in the vector, and MEK-transfected clones with high ERKs (#40) or normal ERKs (#28) by Western blot analysis (**D**). Please note that PP1 partially inhibits the active ERK, with little effects on active JNK and P38, in all the cells. The bENDmek cells incubated with PP1 exhibit significantly higher levels of ERKs compared to vector or MEK-transfected (#28). These experiments repeated twice with very similar results. Bar = 400 μm.

(Fig. 6D) suggesting that activation of JNK kinase was regulated by ERKs [Pedram et al., 1998]. Expression of phospho-JNK and phospho-P38 was not significantly affected by incubation with PP1.

Sustained Activation of MAPK/ERKs Affects Expression of a Number of Genes With Potential Role in Angiogenesis

Activation of MAPK/ERKs pathway by growth factors, as well as other stimuli, results in activation of a number of transcription factors that lead to alterations in gene expression. Our Western blot analysis of lysates prepared from bENDmek cells expressing high levels of ERKs demonstrated altered expression of VE-cadherin, catenins, and PECAM-1. To determine whether the steady state mRNA levels of these genes were affected we examined their expression by Northern blot analysis. Consistent with our Western blot data, we observed a dramatic decrease in the steady state levels of VE-cadherin and PECAM-1 mRNA (Fig. 7). The β-catenin expression was moderately affected. We also observed an increase in expression of fibronectin and intgerins β1, αv, and β3. The changes in expression of these genes are consistent with the migratory, more angiogenic, and less differentiated phenotype demonstrated for these cells.

Sustained Activation of ERKs Result in Down Regulation of PECAM-1 and Enhanced Expression of TSP1

We have previously shown a reciprocal relationship between PECAM-1 and TSP1 expression in bEND cells [Sheibani and Frazier, 1995; Sheibani and Frazier, 1998]. The Northern data (Fig. 7) showed an increase in TSP1 mRNA in bENDmek cells compared to control bEND cells. This is consistent with our previous data where down-regulation of PECAM-1 by anti-sense

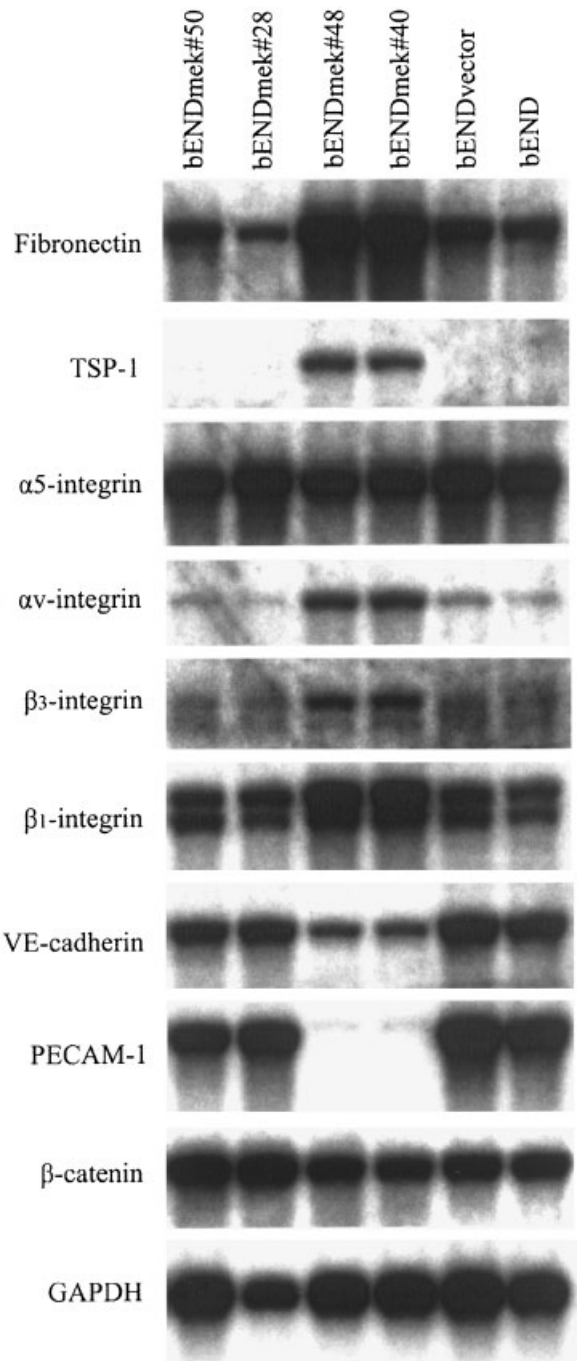

Fig. 7. Northern blot analysis of mRNA prepared from parental and MEK-transfected bEND cells. Poly(A^+) RNA (5 μg) isolated from various cell lines ran on 1.2% agarose formaldehyde gel, transferred, prehybridized, and hybridized to specific cDNA probes as described in ''Materials and Methods.'' Please note a significant decrease in expression of VE-cadherin and PECAM-1 mRNA. The β-catenin mRNA level was not significantly affected. The mRNA levels for TSP1, αv, β3, β1, and fibronectin were significantly upregulated in bENDmek clones with high levels of ERKs.

transfection of bEND cells turns on TSP1 expression [Sheibani and Frazier, 1998]. Figure 8 shows the localization of PECAM-1 in vector control and bENDmek cells. Vector control cells demonstrated PECAM-1 localization at sites of cell–cell contacts (Fig. 8A). However, PECAM-1 staining was significantly reduced in bENDmek cells without any junctional localization (Fig. 8B), consistent with our Western blot data (Fig. 1B). To determine whether TSP1 protein expression is increased in bENDmek cells with decreased PECAM-1 expression, we prepared conditioned medium from parental, vector control, or bENDmek cells. The level of TSP1 in serum-free medium was assessed by Western blot analysis (Fig. 8C). We detected very little or no TSP1 in medium prepared from parental bEND or vector control cells, as previously reported [Sheibani and Frazier, 1995]. However, a significant increase in production of TSP1 was observed in medium prepared from bENDmek cells with high levels of phospho-ERKs. Furthermore, sustained activation of ERKs was required for the increased expression of TSP1 since incubation of bENDmek cells with PD98509 reduced TSP1 expression to levels comparable to the parental or vector control cells. Therefore, the down-regulation of PECAM-1 in bENDmek cells can be attributed, at least in part, to increased expression of TSP1 as previously demonstrated [Sheibani et al., 1997].

Incubation of EC With Na_3VO_4 Results in Activation of ERKs

Incubation of EC and epithelial cells with the phosphatase inhibitor pervanadate results in dissolution of adherens junctions [Ozawa and Kemler, 1998], mediated in part, through altered phosphorylation of the adherens junction proteins. Incubation of EC with Na_3OV_4 results in rapid activation of ERKs, which can be partially inhibited by PD98059 (Fig. 9). Therefore, the changes observed in permeability of EC monolayer upon incubation with phosphatase inhibitors may in part be attributed to activation of ERKs by these phosphatase inhibitors.

Sustained Activation of MAPK/ERKs in HUVEC Disrupts Cell–Cell Adhesion and Promotes Tube Formation on Matrigel

To demonstrate that the disruption of cell–cell interactions upon sustained activation of

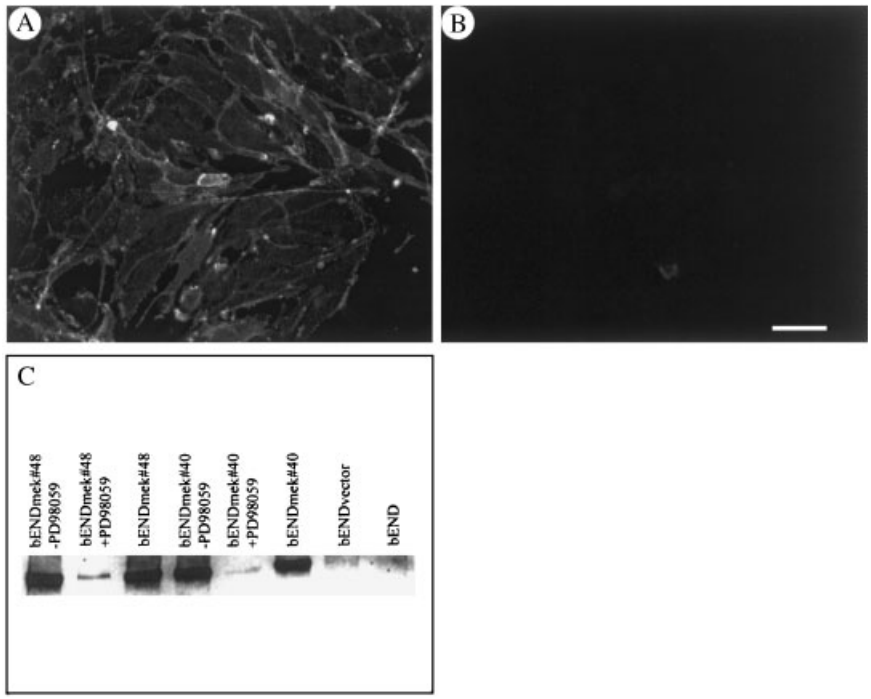

Fig. 8. Down-regulation of PECAM-1 expression in bENDmek is concomitant with up-regulation of TSP1 expression. bEND-vector (**A**) or bENDmek (**B**, clone 40) were plated, and stained for PECAM-1 as described in ''Materials and Methods.'' Please note expression and junctional localization of PECAM-1 in bEND-vector cells, which is severely compromised in bENDmek cells. In **panel C**, cells were plated and allowed to reach confluence. Cells were then fed with serum free medium containing PD98059 or DMSO for 2 days. Conditioned medium were collected, centrifuged to remove debris, and analyzed by Western as described in ''Materials and Methods.'' Please note that TSP1 expression is upregulated in bENDmek cells and is dependent on sustained activation of MAPK/ERKs. Bar = 50 μm.

ERKs occurs in other types of EC, we examined the consequence of sustained activation of ERKs on morphology and organization of cadherin/catenins in HUVEC. Incubation of HUVEC with PMA results in rapid activation of ERKs (within 30–60 min) persisting for at least 24 h, which significantly affects the morphology of these cells (Fig. 10). Figure 11 shows the morphology (A and B) and localization of β-catenin (C and D), VE-cadherin (E and F), and PECAM-1 (G and H) in HUVECs incubated with DMSO (control, left panel) or PMA (right panel). PMA treatment resulted in destabilization of EC junction, with altered localization of VE-cadherin, β-catenin, and PECAM-1. Therefore, sustained activation of ERKs in HUVEC also results in changes in

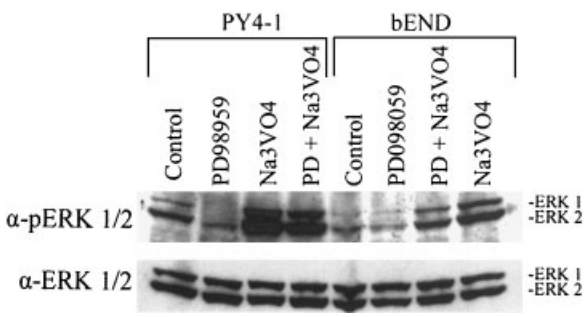

Fig. 9. Inhibition of phosphatases in endothelial cells activates MAPK/ERKs and disrupts cell–cell adhesion. Endothelial cells were incubated with DMSO (control), PD98059, Na_3OV_4, or PD98059 with Na_3OV_4 for 30 min, cells were lysed, and analyzed by Western blot analysis. Please note increased levels of phosphorylated ERKs in cells incubated with phosphatase inhibitor.

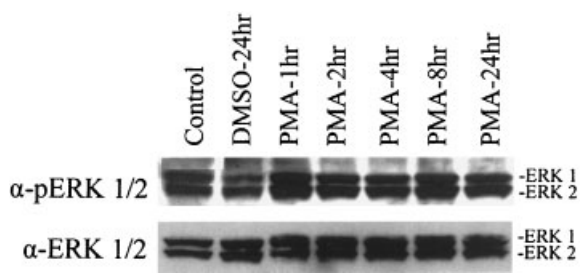

Fig. 10. Incubation of HUVEC with PMA results in sustained activation of MAPK/ERKs. Total cell lysates were prepared from HUVEC incubated with PMA for designated times, lysed, and analyzed by Western blot analysis. Please note the rapid activation of MAPK/ERKs by PMA, which remains activated for at least 24 h.

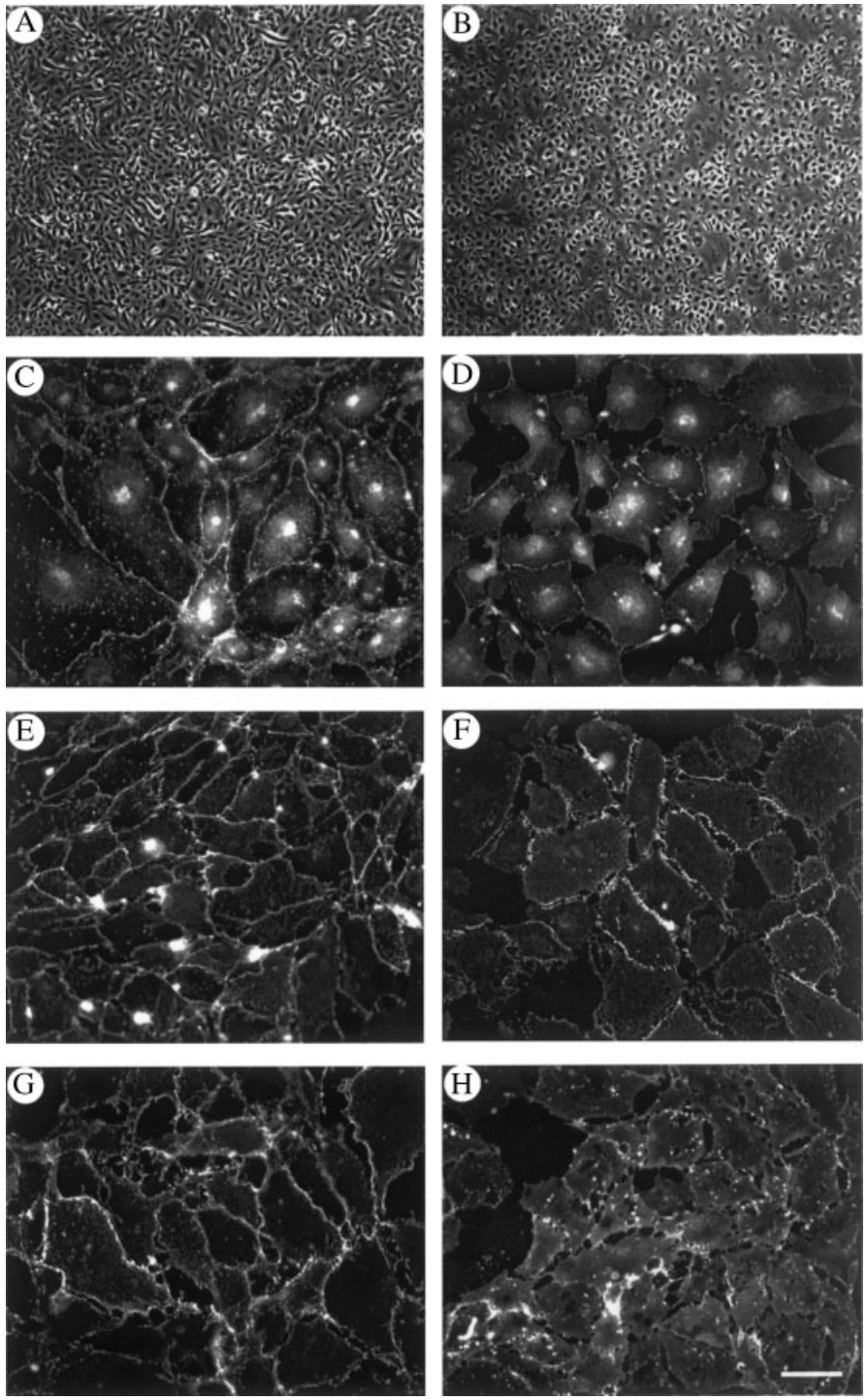

Fig. 11. PMA-mediated activation of MAPK/ERKs in HUVEC disrupts cadherin mediated adherens junction. HUVEC were plated on coverslips, incubated with DMSO (**A**, **C**, **E**, **G**) or PMA (**B**, **D**, **F**, **H**) for 24 h, and analyzed by indirect immunofluorescence staining. The panels A and B are phase micrographs showing the cell morphology. Panels C and D show β-catenin, E and F show VE-cadherin, and G and H show PECAM-1 staining. Please note that the cells have pulled apart upon incubation with PMA and junctions are disrupted with big gaps between cells (compare β-catenin staining). Bar = 50 μm.

morphology and localization of adherens junction components.

Incubation of EC with PMA can enhance their ability to form capillary networks on Matrigel [Davis et al., 1993; Wang et al., 2002]. This is mediated, at least in part, through activation of PKC. We next examined the ability of PMA treated HUVEC to form tubes on Matrigel. The PMA-treated HUVEC organizes significantly faster and formed more extensive network of tubes on Matrigel compared to control cells. This was significantly reduced in the presence of PD98509 (MEK inhibitor) and to a lesser extent by bisindolymaleimide (a PKC inhibitor) (data not shown). These changes were most obvious during early stages of tube formation. Therefore, sustained activation of ERKs in HUVEC contributing to destabilization of strong cell–cell adhesion enhancing the ability of cells to migrate and organize into capillary-like network.

DISCUSSION

VE-cadherin and PECAM-1 mediated cell–cell and integrin-mediated cell–matrix interactions play important roles during angiogenesis [Dejana et al., 1995; Dejana, 1996; Matsumura et al., 1997; Sheibani et al., 1997; Vittet et al., 1997; Bach et al., 1998; Eliceiri et al., 1998; Yang et al., 1999; Cao et al., 2002]. Sustained activation of MAPK/ERKs is essential for angiogenic activity of VEGF and FGFs [D'Angelo et al., 1995; Eliceiri et al., 1998]. Although interaction of EC with integrin $\alpha v\beta 3$ is essential for sustained activation of MAPK/ERKs during angiogenesis [Eliceiri et al., 1998], the role of VE-cadherin and PECAM-1 mediated endothelial cell–cell interactions requires further delineation. Here, we show that sustained activation of MAPK/ERKs: (1) disrupts cadherin-mediated cell–cell interactions through down regulation of VE-cadherin and catenins expression; (2) down-regulates PECAM-1 expression; (3) enhances cell migration in a Src-dependent manner; and (4) enhances the ability of EC to form capillary-like networks on Matrigel. Therefore, sustained activation of MAPK/ERKs is not only essential for migration of EC, but is also required for disruption of the cadherin-mediated cell–cell interactions.

PECAM-1 plays an important role during angiogenesis in vitro and in vivo [DeLisser, 1997; Sheibani et al., 1997]. This is mediated through the homotypic interaction of PECAM-1 molecules on EC. PECAM-1 undergoes alternative splicing and generates a number of isoforms, which differ in the length of their cytoplasmic domain, their ability to activate MAPK/ERKs, and their adhesive properties [Sheibani and Frazier, 1999; Sheibani et al., 2000]. In addition, the expression of PECAM-1 isoforms is regulated during vascular development. For example isoforms with exon 14 are expressed early while those that lack exon 14 are expressed later in developing kidney vasculature [Sheibani et al., 1999]. Together these data suggest that differential activation of MAPK/ERKs by PECAM-1 isoforms may facilitate cell migration and organization of capillary networks during early stages of angiogenesis without interference from strong cadherin-mediated cell–cell adhesion. However, cadherin-mediated cell–cell interactions are essential for normal physiological function of formed vessels [Rabiet et al., 1996; Hordijk et al., 1999]. Thus, down-regulation of MAPK/ERKs activity through cadherin-mediated cell–cell interactions promotes stabilization of the vascular phenotype in mature vessels [Viñals and Pouysségur, 1999].

To determine whether sustained activation of MAPK/ERKs affects cadherin-mediated cell–cell adhesions in EC, we expressed a dominant active form of MEK-1 in microvascular endothelial cells. We observed a dramatic effect on the morphology of cells with sustained activation of MAPK/ERKs. These cells also failed to form close cell–cell interactions suggesting alterations in the components of adherens junction. Sustained activation of MAPK/ERKs resulted in down-regulation of VE-cadherin and catenin expression (Figs. 1B and 7) and failure to form close cell–cell contacts (Figs. 2 and 5). Inhibition of MAPK/ERKs by PD98509 (a MEK inhibitor) restored the ability of cells to form cell–cell contacts and appropriately localize cadherin/catenins (Fig. 5), indicating that sustained activation of MAPK/ERKs is necessary for disruption of cadherin-mediated cell–cell interactions. The important role of MAPK/ERKs pathway in angiogenesis is further emphasized by embryonic lethality of MEK-1 null mutation resulting from embryonic vascular abnormalities [Giroux et al., 1998]. Therefore, MAPK/ERKs pathway plays an important role in modulating cadherin-mediated cell–cell adhesion not only in epithelial cells but also in endothelial cells.

Sustained-activation of ERKs also resulted in decreased expression of PECAM-1 (Fig. 1B and 7). Down-regulation of PECAM-1 may contribute to enhanced migration of EC [Schimment et al., 1992; Sheibani and Frazier, 1998]. This is consistent with the enhanced ability of these cells to form extensive cord-like structures on Matrigel and cover wounds in monolayer cultures (Figs. 3 and 4). We have previously shown that down-regulation of PECAM-1 in bEND cells by anti-sense transfection enhanced their ability to organize cord-like structures on Matrigel. In addition, we established that a threshold level may exist for PECAM-1 such that too much PECAM-1 may interfere with the ability of cells to organize and form capillary-like networks, while modest PECAM-1 expression was stimulatory [Sheibani et al., 1997; Sheibani and Frazier, 1998]. Therefore, decreased expression of PECAM-1 in cells with activated ERKs is consistent with their enhanced migratory phenotype. Furthermore, bENDmek cells expressed increased levels of fibronectin, β1, αv, and β3 integrins, all of which shown to be markers of angiogenic endothelium [Brooks et al., 1994; Kim et al., 2000a,b]. In fact, crosstalks between these integrins play an important role during vascular development and angiogenesis [Kim et al., 2000b; Hynes, 2002]. Therefore, sustained activation of ERKs results in modulation of expression of a number of genes with important roles in angiogenesis.

Thrombospondin-1 is a naturally occurring inhibitor of angiogenesis whose expression favors a differentiated, quiescent state of endothelium [Sheibani and Frazier, 1999]. We have previously shown a reciprocal relationship between PECAM-1 and TSP1 expression in bEND cells [Sheibani and Frazier, 1998]. Here we demonstrate decreased PECAM-1 expression is also concomitant with induction of TSP1 expression in a MAPK/ERKs dependent manner. Inhibition of MEK-1 with PD98059 suppressed TSP1 expression and re-established normal cell–cell adhesions. These cells normally express little or no TSP1 but express very high levels of PECAM-1 [Sheibani and Frazier, 1995]. The data presented here confirms our previous observation indicating that a reciprocal relationship exists between PECAM-1 and TSP1 [Sheibani and Frazier, 1998]. Thus, when PECAM-1 is high TSP1 expression is low and when PECAM-1 expression is low TSP1 expression is high. These findings demonstrate for the first time that MAPK/ERKs pathway may be an important modulator and/or coordinator of TSP1 and PECAM-1 expression in the endothelium during angiogenesis. Therefore, sustained activation of MAPK/ERKs contributes to the events that are necessary for angiogenesis such as loosening of strong cell–cell adhesion and promotion of EC migration and proliferation. We believe the induction of TSP1 expression in bENDmek cells is in response to a feed back mechanism to dampened these angiogenic responses. However, the ability of the MAPK/ERKs to regulate TSP1 expression in EC is a novel observation and requires further studies.

The MAPK/ERKs activity is essential for migration on extracellular matrix proteins [Klemke et al., 1997; McCawley et al., 1999]. The bENDmek cells exhibited an enhanced ability to migrate in a scratch wound assay (Fig. 4). This was dependent on sustained activation of ERKs and was inhibited in the presence of PD98059 (not shown). The enhanced migratory phenotype of bENDmek cells was also Src dependent. The Src specific inhibitor, PP1, blocked enhanced migration in bENDmek cells (Fig. 6) while PKC inhibitor (bisindolymaleimide), as well as other inhibitors (see "Materials and Methods") had no effect (data not shown). The incubation of bENDmek cells with PP1 did not significantly affect the levels of active ERKs compared to control cells (Fig. 6D). Therefore, the effect of Src on cell migration, for the most part, is independent of ERKs activity.

The activity of the Src is shown to be required for disruption of cadherin-mediated cell adhesion and migration in epithelial and endothelial cells [LaVallee et al., 1998; Owens et al., 2000] and for vascular permeability and angiogenesis [Eliceiri et al., 1999]. This is mainly believed to be mediated through increased phosphorylation of junctional proteins. We did not observe a significant difference in the levels of β-catenin tyrosine phosphorylation in bENDmek cells compared to control cells (data not shown). Thus, changes in the expression levels of adherens junction components in bENDmek cells, rather than their phosphorylation, mediate the disruption of cell–cell adhesion. It is not presently known what contributes to Src activation. Our data suggest Src activity, downstream of MEK-1, is essential for EC migration. However, the identity of the mechanisms that

lead to Src activation, downstream of MEK-1, remains unknown. The phosphorylation of Src on serine 17 by PKA (cAMP dependent kinase) activates Src [Schmitt and Stork, 2002]. It is not presently know whether MEK-1 (a threonine/tyrosine kinase) can phosphorylate Src affecting its activity. Src contains two consensus MEK-1 phosphorylation sites (TXY), ^{132}TGY (identical to ERKs) and ^{341}TEY. Therefore, threonine and/or tyrosine phosphorylation of Src on these residues by MEK-1 may activate Src. We have consistently observed a band around 66–68 kDa in our Western blots of total cell lysates incubated with phospho-specific ERK antibody, which recognizes the phosphorylated TGY residues in ERKs. This band is only present in the bENDmek cells. However, whether the MEK-1 consensus phosphorylation sites in Src can be recognized by MEK-1 and become phosphorylated is currently under investigation.

The direct phosphorylation of myosin light chain through promotion of myosin light chain kinase by Src and/or ERKs promotes EC contraction and monolayer permeability [Klemke et al., 1997; Mucha et al., 2003]. In addition, the activated ERKs and Src can directly phosphorylate connexin 43 affecting gap junctional communications [Cottrell et al., 2003]. The sustained activation of ERKs may also influence integrin mediated cell adhesion and Src activation [Hughes et al., 1997]. The Δ15 PECAM-1 expressing epithelial cells with sustained activation of ERKs exhibit a significant amount of active Src and altered matrix adhesive properties (personal communication). Thus, identification of pathway(s) that lead to activation of Src will aid in dissecting the mechanisms by which MAPK/ERKs and/or Src modulate cell adhesive properties.

In summary, we demonstrate that sustained activation of MAPK/ERKs is essential for disruption of cadherin-mediated cell–cell adhesion, as well as endothelial cell migration. However, inhibition of MAPK/ERKs activity by cell–cell contact is an essential step in promoting cell cycle withdrawal in vascular EC and formation of stable vessels [Viþals and PouyssÕgur, 1999]. Therefore, appropriate regulation of MAPK/ERKs and/or Src activity by PECAM-1 isoforms during angiogenesis may play a critical role in vascular development, and provide a suitable target for development of agents to modulate angiogenesis.

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