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Short Communication

Involvement of Endothelial PECAM-1/CD31 in Angiogenesis

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The adhesive interactions of endothelial cells with each other and the adhesion receptors that mediate these interactions are probably of fundamental importance to the process of angiogenesis. We therefore studied the effect of inhibiting the function of the endothelial cell-cell adhesion molecule, PECAM-1/CD31, in rat and murine models of angiogenesis. A polyclonal antibody to human PECAM-1, which cross-reacts with rat PECAM-1, was found to block *in vitro* tube formation by rat capillary endothelial cells and cytokine-induced rat corneal neovascularization. In mice, two monoclonal antibodies against murine PECAM-1 prevented vessel growth into subcutaneously implanted gels supplemented with basic fibroblast growth factor (bFGF). Taken together these findings provide evidence that PECAM-1 is involved in angiogenesis and suggest that the interactions of endothelial cell-cell adhesion molecules are important in the formation of new vessels. *(Am J Pathol 1997, 151:671–677)*

Angiogenesis is an essential feature of a number of important physiological processes (eg, wound healing, tissue repair, and development)[1] and pathological conditions (eg, tumor dissemination and diabetic retinopathy).[2] Regardless of the context, angiogenesis represents a complex multiple step process in which capillary endothelial cells sever their normal cell-cell attachments, migrate, and proliferate into the perivascular extracellular matrix in which they eventually reform cell-cell associations to create new capillaries.[3] The adhesive interactions of endothelial cells with each other and with the extracellular matrix along with the adhesion receptors that mediate these interactions are probably of fundamental importance to this process.[4–6]

The best characterized mediators of cellular adhesion with the extracellular matrix are the integrin family of receptors.[7,8] Endothelial cells express a number of these integrins, although the pattern of expression varies with the vessel type (large *versus* small), the source of the cells (cultured *versus in situ* cells), and the inflammatory stimulus.[9,10] Recently, the involvement $\alpha v\beta 3$ and $\alpha v\beta 5$ in *in vivo* angiogenesis has been demonstrated in avian, rabbit, and murine models of angiogenesis.[11–14]

Two vascular cell-specific cell-cell adhesion molecules have been identified that localize to endothelial cell intercellular junctions. The first is vascular endothelial cadherin, a 135-kd protein that, like other cadherins, mediates a calcium-dependent homophilic adhesion.[15–17] The other is platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31),[18–20] a 130-kd membrane glycoprotein of the immunoglobulin superfamily that is able to mediate both homophilic and heterophilic adhesion.[21] The distribution of these two molecules in endothelial junctions is close but clearly distinct. Cadherin molecules are found mostly in subapical locations associated with adherens junctions, whereas PECAM-1 is located at more basal positions along the lateral plasma membrane.[16] Also

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both associate with the actin cytoskeleton, although the cadherin interaction seems to be stronger.[16,22]

Although our understanding of the structure-function relationships of these endothelial intercellular adhesion molecules has advanced quickly, their role in angiogenesis is only now emerging.[23] Early studies[19] however did suggest a potential role for PECAM-1 in the formation of new vessels. Endothelial cells plated in the presence of anti-PECAM-1 antibodies adhered normally to the culture plate but failed to establish tight cell-cell contacts and the normal "cobblestone" appearance.[19] Intact confluent monolayers were not affected by the antibody. These findings suggested that PECAM-1 in intercellular junctions was inaccessible to the antibody or that PECAM-1 might be involved early on in the formation of endothelial cell-cell associations but was not required for maintaining those associations once they were established.

The purpose of this study was to investigate the possible role of PECAM-1 in angiogenesis. A polyclonal antibody to human PECAM-1, which cross-reacts with rat PECAM-1, was found to block *in vitro* tube formation by rat capillary endothelial cells and cytokine-induced rat corneal neovascularization. These findings were not limited to the rat endothelium as two monoclonal antibodies against murine PECAM-1 in mice prevented vessel growth into subcutaneously implanted gels supplemented with bFGF. Together these findings not only provide additional evidence that PECAM-1 is involved in angiogenesis but suggest that the interactions of endothelial cell-cell adhesion molecules are important in the formation of new vessels.

Materials and Methods

Antibodies

The following antibodies were used: A rabbit polyclonal antibody against angiotensin-converting enzyme (ACE) (a generous gift from Dr. F. Joseph Roll, University of California at San Francisco); a rabbit polyclonal antibody against human PECAM-1;[24] rat anti-mouse monoclonal antibodies (mAbs) Mec 13.3[25] and 390,[26] against murine PECAM-1; rat anti-murine ICAM-1, YN1;[27] and rabbit polyclonal antisera to rat β1 integrin (anti-R140) (generously provided by Dr. Clayton Buck, Wistar Institute, Philadelphia, PA).[28]

Cells

Rat microvascular endothelial cells (RFC) were isolated from the epididymal fat pads of Sprague-Dawley rats and cultured as previously described.[29] RFC were passaged and grown on 1.5% gelatin-coated tissue culture plates in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum mixed 4:1 with sterile-filtered conditioned bovine aortic endothelial cell media. They were characterized as endothelial cells by their ability to form tubes in culture, expression of von Willebrand factor and PECAM-1, and E-selectin inducibility in response to tumor necrosis factor-α treatment.[29–31]

Three-Dimensional Cultures

Three-dimensional cultures of RFC were made as previously described.[30,32] Briefly, purified type I collagen was solubilized in 10 mmol/L acetic acid at a concentration of 5.0 mg/ml and stored at 4°C. A measured amount of the collagen with 1/10 vol of 10× Dulbecco's modified Eagle's medium was neutralized with sterile 1 mol/L NaOH and the solution was kept on ice. Cultured RFC were added to the collagen preparation to achieve a concentration of 10^6 cells/ml; 700 μl of the cell/collagen suspension was added to 12 mm of Millicell-HA (Millipore Products Division, Bedford, MA) filter chambers set into the wells of a Costar 24-well tissue culture dish (Costar Corp., Cambridge, MA) and placed in a 37°C humidified 5% CO_2 incubator for 10 minutes to permit polymerization of the collagen. After gel formation, 1.5 ml of medium was added per well allowing the cells to be fed from both the top and bottom of the collagen gel. To elicit an angiogenic response TGF-β1 (0.5 ng/ml, R & D Systems, Minneapolis, MN) was added to culture.

Inhibition of in Vitro *Tube Formation*

To assess the role of PECAM-1 molecules in the process of *in vitro* tube formation, antibodies or synthetic peptides were added to three-dimensional cultures of RFC. Final working concentrations/dilutions of blocking reagents were as follows: anti-PECAM-1 (1:40); anti-β1 integrin (1:50); anti-angiotensin-converting enzyme (1:40); bovine serum albumin (BSA)-GRGDSP (5 mmol/L); and BSA-GRGESP (5 mmol/L).[28] After 4 days of growth, the cultures were rinsed three times with phosphate-buffered saline, removed from the filter chambers, and snap frozen in OCT embedding compound. Eight-micron cryostat sections were placed on albumin-coated glass slides, fixed for 1 minute at −20°C, and air dried. Greater than 12 individual sections were viewed under an inverted Olympus IM microscope (Olympus Corporation, New Hyde Park, NY) and photographed at a magnification of ×100 using XP-1 400 film. The photographs are representative of three experiments. Tube formation was quantitated by determining the total length of tube structures (vessel length/mm^2) in 12 or more micrographs from each experiment and performed in quadruplicate. The NIH Image program was used to determine lengths of tube formation.

Corneal Micropocket Model of Angiogenesis

In vivo anti-angiogenic activity was assayed in the avascular cornea of Long Evans rat eye as previously described.[33,34] Briefly, cytokine (ENA-78 or bFGF) without or with antibody (500 ng) was combined with sterile Hydron (Interferon Sciences Inc.) casting solution. Five microliters of aliquots (50 ng cytokines/pellet) were pipetted onto the flat surface of an inverted sterile polypropylene specimen container and polymerized overnight in a laminar flow hood under ultraviolet light. Before implantation, pellets were rehydrated with normal saline. Animals were

given intraperitoneally ketamine (150 mg/kg) and atropine (250 μg/kg) for anesthesia. Rat corneas were anesthetized with 0.5% proparacaine hydrochloride ophthalmic solution followed by implantation of the Hydron pellet into an intracorneal pocket (1 to 2 mm from the limbus). Six days after implantation animals received heparin (1000 U) and ketamine (150 mg/kg) intraperitoneally followed by 10-ml perfusion of colloidal carbon via the left ventricle. Corneas were harvested and photographed. Positive neovascularization responses were defined as sustained directional in-growth of capillary sprouts and hairpin loops towards the implant. Negative responses were defined as either no growth or only an occasional sprout or hairpin loop displaying no evidence of sustained growth.

Matrigel Model of Cytokine-Induced Murine Angiogenesis

This model has been extensively characterized by Passaniti et al.[35] Briefly, C57BL/6 mice were injected subcutaneously with 0.5 ml of Matrigel supplemented with bFGF (500 ng/ml) to induce the growth of vessels into the gel. Animals were then injected with BSA, rat IgG, mAb 390, mAb Mec 13.3, or mAb YN1 daily via the intraperitoneal route (50 μg/day) for 7 days. After the 7 days, the animals were killed and the gels were either snap frozen in OCT for immunohistochemical staining or were processed for hemoglobin analysis.[35]

Statistical Analysis

Differences among groups were analyzed using one-way analysis of variance. When statistically significant differences were found ($P < 0.05$), individual comparisons were made using the Bonferroni/Dunn test.

Results

Inhibition of PECAM-1 Function Blocks in Vitro *Endothelial Cell Tube Formation*

To determine the role of PECAM-1 during *in vitro* endothelial cell tube formation,[36] TGF-β1 treated rat microvascular endothelial cells were incubated with antagonists of PECAM-1. Unstimulated negative control cultures grown in medium without TGF-β1 revealed quiescent cells showing only rudimentary tubular formations (data not shown), whereas after 4 days of growth, TGF-β1-treated cultures displayed extensive tubular networks resembling *in vivo* capillary beds (Figure 1A). Incubation with normal rabbit serum (data not shown) and antibody against angiotensin-converting enzyme (a surface-expressed endothelial cell molecule) did not interfere with this tube formation (Figure 1B). In contrast, addition of an antibody to human PECAM-1 that cross-reacts with rat PECAM-1[37,38] throughout the duration of the 4-day culture significantly inhibited tube formation by RFC (Figure 1C).

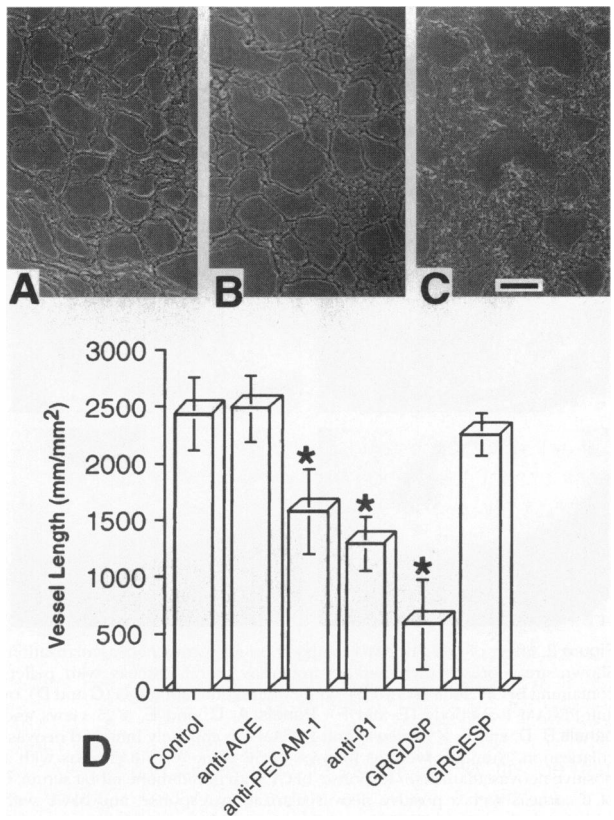

Figure 1. Inhibition of *in vitro* microvascular tube formation by anti-PECAM-1 antibody. Three-dimensional cultures were embedded *en bloc* with OCT compound, frozen, and 8-μm cryosections were generated, acetone-fixed, and viewed. The following conditions were examined: **A**, cultures treated with TGF-β1 at 0.5 ng/ml demonstrating complex branching tube formation; **B**, TGF-β1-treated cultures incubated with antibody to angiotensin-converting enzyme (ACE), an endothelial cell surface molecule not involved in cell-cell interactions; and **C**, TGF-β1-treated cultures incubated with antibody directed against PECAM-1 showing significantly impaired the formation of multicellular tubes. Magnification, ×100. Scale bar, 100 μm. (**D**) Tube formation was quantitated by determining the total length of tube structures (vessel length/mm²; see Methods). Vessel length/mm² was determined for control (BSA), anti-ACE, anti-PECAM-1, anti-β1, GRGDSP (RGD), and GRGESP (RGE) peptides present throughout the entire 4-day culture. Antagonists of PECAM-1 and β1 integrin function significantly inhibited tube formation. (n = 5 to 15). Data are presented as means ± SEM. Statistical differences were calculated by analysis of variance. *, $P < 0.05$ when compared with BSA.

Quantitation of tube formation and its inhibition is shown in Figure 1D. Anti-PECAM-1 antibody (anti-PECAM-1) when present for the entire 4-day culture period inhibited tube formation by approximately 40% ($P < 0.0001$). In contrast, normal rabbit sera (data not shown) and angiotensin-converting enzyme antisera (anti-ACE) had no significant effect on *in vitro* tube formation compared with control (P was not significant). Antibody against β1 integrin (anti-β1) inhibited tube formation by 47% compared with the control ($P < 0.0001$), which was not significantly different from the levels of inhibition noted for anti-PECAM-1. Incubation with GRGDSP-albumin inhibited tube formation by 76%, an effect not seen with control peptide. Taken together these data suggest that PECAM-1 interactions have an important role during *in vitro* tube formation in this model.

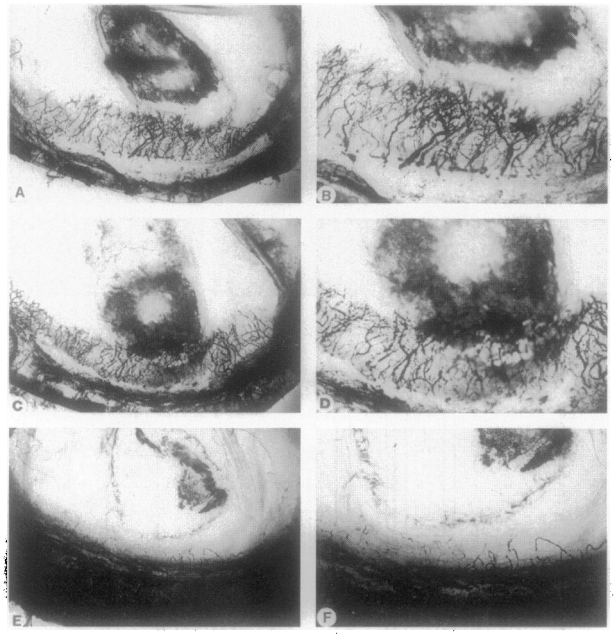

Figure 2. Effect of anti-PECAM-1 antibody on rat cornea neovascularization. Shown are representative photomicrographs of rat corneas with pellets containing bFGF alone (**A** and **B**), with nonimmune rabbit IgG (**C** and **D**), or anti-PECAM-1 antibody (**E** and **F**). **Panels A**, **C**, and **E**, ×25 views and **panels B**, **D**, and **F**, ×50 views. Anti-PECAM-1 completely inhibited neovascularization. Numbers were as follows: bFGF alone, 6 of 6 corneas with a positive neovascularization response; bFGF with nonimmune rabbit serum, 6 of 6 corneas with a positive neovascularization response; and bFGF with rabbit polyclonal anti-PECAM-1 antibody, 0 of 6 corneas with a positive neovascularization response.

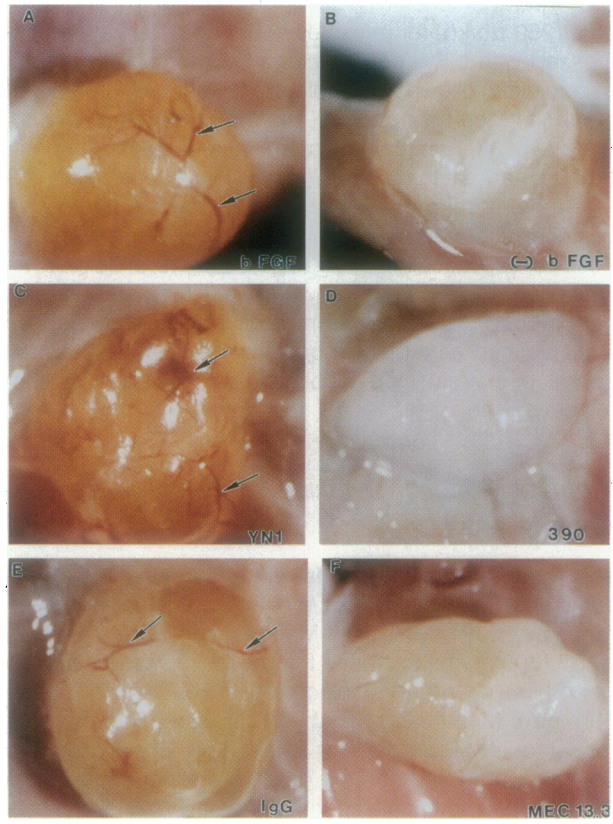

Figure 3. Effect of anti-murine PECAM-1 antibodies on bFGF-induced murine angiogenesis. Shown are subcutaneously implanted Matrigel pellets supplemented with bFGF that have been removed from animals injected intraperitoneally with BSA (**A**), anti-ICAM-1, mAb YN1 (**C**) anti-PECAM-1, mAb 390 (**D**), nonimmune rat IgG (**E**), and anti-PECAM-1, mAb Mec 13.3 (**F**). A pellet without bFGF from a mouse given BSA intraperitoneally is presented in **panel B**. The **arrows** indicate vessels that are visible in the pellets. Few if any vessels are visible in the pellets recovered from mice treated with mAbs 390 or Mec 13.3. These data are representative of three separate experiments.

A Cross-Reacting Polyclonal Antibody Against Human PECAM-1 Inhibits Cytokine-Induced Rat Corneal Angiogenesis

The experiments described above demonstrated that blockade of PECAM-1-inhibited *in vitro* rat endothelial tube formation. To determine whether this was also true for *in vivo* angiogenesis of the rat microvasculature, the effect of blocking PECAM-1 function was assessed in a well characterized rat corneal micropocket model of neovascularization.[33,34] After 6 days Hydron pellets containing bFGF (50 ng/pellet) implanted in rat corneas induced a brisk neovascular response in this normally avascular site (Figure 2A and B). Inclusion of nonimmune rabbit IgG in the pellet had no effect on new vessel formation (Figure 2, C and D). However, this corneal angiogenic response was completely blocked if antibody against PECAM-1 (500 ng/pellet) that inhibited *in vitro* rat endothelial tube formation (see above) was added to the pellet (Figure 2, E and F). Corneal neovascularization induced by ENA-78 was also completely blocked by this anti-PECAM-1 antibody (data not shown).

Antibodies to Murine PECAM-1 Inhibit bFGF-Induced Murine Angiogenesis

The observation that *in vivo* rat angiogenesis was blocked by an antibody to PECAM-1 raised the question of whether this finding was also true for other *in vivo* models of angiogenesis. We therefore studied the effect of two monoclonal antibodies against murine PECAM-1 (mAbs 390 and Mec 13.3) administered daily via the intraperitoneal route (50 μg/day for 7 days) in a model of murine angiogenesis in which vessels form within Matrigel plugs supplemented with bFGF implanted subcutaneously for 7 days in C57BL/6 mice. Compared with control animals treated with BSA, nonimmune rat IgG or the rat anti-mouse ICAM-1 mAb YN1, the mAbs 390 and Mec 13.3 completely inhibited the angiogenic response to bFGF (n = 4 or 5) (Figure 3). Immunohistochemical staining demonstrated reduced numbers of vessels in gels recovered from animals treated with anti-murine PECAM-1 antibodies compared with those taken from control animals (data not shown). As assessed by the hemoglobin concentration in the recovered Matrigel plugs, mAb 390 and Mec 13.3 reduced the vascularization of the plugs to background levels (Figure 4). No statistically significant differences between BSA and rat IgG or anti-ICAM-1 antibody were noted.

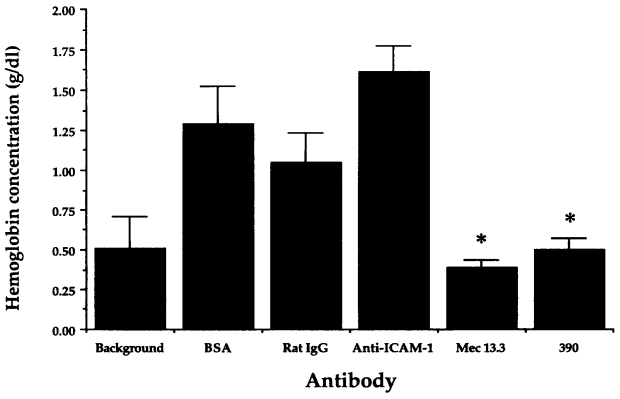

Figure 4. Quantitation of the effect of anti-murine PECAM-1 antibodies on bFGF-induced murine angiogenesis. Hemoglobin concentration of the recovered Matrigel pellets for the various antibody conditions is presented ($n = 4$ or 5). Background represent hemoglobin in pellets without bFGF that were removed from animals treated with BSA. Anti-murine monoclonal antibodies 390 and Mec 13.3 completely inhibited the angiogenic response to bFGF. Data are presented as means ± SEM. Statistical differences were calculated by analysis of variance. *, $P < 0.05$ when compared to BSA and not significant when compared to background.

Discussion

The importance of endothelial cell-substratum adhesion receptors in the process of angiogenesis has become increasingly apparent.[5,6] However, the formation of new capillary structures also depends on the ability of the endothelial cell to first disrupt its normal cell-cell attachments, migrate, and then form new endothelial cell-cell interactions that allow blood flow to occur. Because of the strategic location of PECAM-1 at the endothelial cell-cell border in both large and small vessel endothelium[18,19,39] and in three-dimensional capillary networks and the role of PECAM-1 in initiation of cell-cell contacts in *in vitro* experiments,[19] we hypothesized that PECAM-1 was important in angiogenesis and evaluated the consequences of blocking PECAM-1 function with bioactive antibodies in various models of angiogenesis.

To study the role of PECAM-1 in angiogenesis, we first used an *in vitro* model. We chose to use a three-dimensional culture system in which rat fat capillary cells grown in collagen gels are induced to form capillary-like structures after the addition of TGF-β.[32] Although no *in vitro* system exactly mimics *in vivo* angiogenesis, this model has been well characterized, can be quantitated, and the capillary tubes that form bear a strong morphological resemblance to normal microvessels exhibiting luminal and abluminal differentiation including abluminal deposition of basal lamina components, tight junction formation between endothelial cell processes, and the formation of lumina. More importantly, a well-characterized polyclonal antibody to human PECAM-1 that cross-reacted with rat PECAM-1 was found to block *in vitro* tube formation by rat capillary endothelial cells (Figure 1).

The role of PECAM-1 was next studied in two *in vivo* model systems. The rodent corneal pellet model has been used for many years to analyze the ability of substances to induce or to inhibit angiogenesis.[33,34] Using the same cross-reacting polyclonal anti-PECAM-1 antibody as was used in the *in vitro* studies, growth factor-induced (bFGF) and chemokine-induced (ENA-78) rat corneal neovascularization were strongly inhibited (Figure 2).

To extend these findings to another species as well as to a second set of independently derived anti-PECAM-1 reagents, experiments were performed in mice using two distinct rat anti-murine PECAM-1 mAbs. These antibodies bind to separate epitopes in the second extracellular immunoglobulin-like domain (H. DeLisser and S. Albelda, unpublished observations) but have been found to have differential effects on *in vitro* PECAM-1-dependent adhesion.[40] Mec 13.3 blocks PECAM-1-dependent homophilic and heterophilic aggregation in mouse L-cells transfected with murine PECAM-1, whereas 390 only inhibits the heterophilic interaction in these transfectants. For these experiments another well-characterized *in vivo* model of angiogenesis was used; migration of new vessels into a growth-factor-laced Matrigel plug.[35] Both monoclonal antibodies against murine PECAM-1 prevented vessel growth into subcutaneously placed gels supplemented with bFGF, whereas nonimmune rat IgG and an endothelial cell-binding control rat mAb against murine ICAM-1 had no effect (Figures 3 and 4).

These data show that blockade of PECAM-1 can inhibit angiogenesis in two very different animal models of growth factor/chemokine-induced angiogenesis. Studies are currently ongoing to define the role of PECAM-1 in the more complex process of tumor-induced angiogenesis[39] in which neovascularization arises from the interactions of multiple stimulatory factors (ie, vascular endothelial cell growth factor, bFGF, interleukin-8, angiogenin, and others) as well as inhibitors of angiogenesis (ie, angiostatin, thrombospondin, and others).[41]

Although our data are consistent with the studies of Matsumura et al,[23] they differ from their report in that they found that vascular tube formation was inhibited only when antibodies against either human vascular endothelial cadherin or human PECAM-1 were given together but not when they were administered singly. The reasons for this difference are not clear but may be caused by differences in the assay systems used and the species studied.

However, the precise role of PECAM-1 in this process remains uncertain but is under active investigation. There are data from previous studies to suggest at least two possibilities. First, PECAM-1 concentrates at endothelial intercellular junctions[18,19] and endothelial cell plated in the presence of antibodies to PECAM-1 fail to form tight monolayers, an effect not seen when antibodies were added to confluent cultures.[19] Antagonism of PECAM-1 may therefore interfere with the initial stabilization of endothelial cell-cell contacts and thus prevent tube formation. The finding that PECAM-1 localizes to areas of cell-cell contact is consistent with this suggestion. Second, integrin-mediated adhesion is crucial for endothelial cell migration on extracellular matrix proteins.[6,41] Although data are currently lacking for endothelial cells, engagement of surface PECAM-1 up-regulates the adhesive function of β1 and β2 integrins on a variety of leukocytes.[23,42–46] Therefore, it is possible that for endothelial cells binding of endothelial PECAM-1 to its ligand facili-

tates or triggers endothelial cell migration by augmenting integrin-dependent adhesion. Given the complexity of angiogenesis, it is conceivable that both processes may be involved.

Another layer of complexity with regard to mechanistic studies is provided by the multiple ligand partners of PECAM-1. The ligand interactions of PECAM-1 are still being defined. The molecule has been reported to interact with itself (homophilic adhesion)[23,24,47–50] and with an ever-growing number of heterophilic ligands including the integrin $\alpha v\beta 3$,[51,52] an unidentified proteoglycan,[47,51,53] and an unidentified ligand on the surface of activated T cells.[54] Whether PECAM-1-dependent homophilic or heterophilic interactions are required in angiogenesis remains to be determined. It is possible that both are involved. For example, engagement of PECAM-1 with its putative proteoglycan ligand in the surrounding matrix may induce intracellular signals that up-regulate the function of integrins involved in endothelial cell migration as has been demonstrated for leukocytes,[55] whereas PECAM-1-PECAM-1 binding may facilitate the stabilization of cell-cell contacts.

An expanding number of functions have been defined or proposed for PECAM-1.[21] Data indicate that PECAM-1 may play important roles in the recruitment of leukocytes at inflammatory sites,[37,56–58] cardiovascular development,[26] release of bone marrow leukocytes,[44] and T-cell-mediated immune responses.[54,58,59] The studies described in this report provide additional evidence that PECAM-1 is also involved in angiogenesis and suggest that endothelial cell-cell adhesion molecules are important in the formation of new vessels. Ultimately, as the role of PECAM-1 in angiogenesis is better defined, it may emerge as a possible therapeutic target in pathological processes dependent on the formation of new blood vessels.

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