A Role for Cadherin-5 in Regulation of Vascular Endothelial Growth Factor Receptor 2 Activity in Endothelial Cells

Nader Rahimi* and Andrius Kazlauskas†‡

*Boston University, School of Medicine, Boston, Massachusetts 02028; and †Schepens Eye Research Institute, Harvard Medical School, Boston, Massachusetts 02114

Submitted May 10, 1999; Accepted July 13, 1999

Monitoring Editor: Carl-Henrik Heldin

FLK-1/vascular endothelial growth factor receptor 2 (VEGFR-2) is one of the receptors for VEGF. In this study we examined the effect of cell density on activation of VEGFR-2. VEGF induces only very slight tyrosine phosphorylation of VEGFR-2 in confluent (95–100% confluent) pig aortic endothelial (PAE) cells. In contrast, robust VEGF-dependent tyrosine phosphorylation of VEGFR-2 was observed in cells plated in sparse culture conditions (60–65% confluent). A similar cell density-dependent phenomenon was observed in different endothelial cells but not in NIH-3T3 fibroblast cells expressing VEGFR-2. Stimulating cells with high concentrations of VEGF or replacing the extracellular domain of VEGFR-2 with that of the colony-stimulating factor 1 receptor did not alleviate the sensitivity of VEGFR-2 to cell density, indicating that the confluent cells were probably not secreting an antagonist to VEGF. Furthermore, in PAE cells, ectopically introduced platelet-derived growth factor α receptor could be activated at both high and low cell density conditions, indicating that the density effect was not universal for all receptor tyrosine kinases expressed in endothelial cells. In addition to lowering the density of cells, removing divalent cations from the medium of confluent cells potentiated VEGFR-2 phosphorylation in response to VEGF. These findings suggested that cell–cell contact may be playing a role in regulating the activation of VEGFR-2. To this end, pretreatment of confluent PAE cells with a neutralizing anti-cadherin-5 antibody potentiated the response of VEGFR-2 to VEGF. Our data demonstrate that endothelial cell density plays a critical role in regulating VEGFR-2 activity, and that the underlying mechanism appears to involve cadherin-5.

INTRODUCTION

Regulation of angiogenesis is required for many pathological conditions. Recent studies have revealed that vascular endothelial growth factor (VEGF) is an important element for many angiogenic processes under normal and abnormal conditions (Risau and Flamme 1995; Risau 1997). The receptors for VEGF include the tyrosine kinases VEGF receptor 1 (VEGFR-1 [FLT-1]) and VEGFR-2 (FLK-1), whose expression is restricted to endothelial cells, their precursors, and monocytes (Terman et al., 1991; de Vries et al., 1992; Fong et al., 1995; Folkman et al., 1996). Although the role VEGFR-2 in angiogenesis and vasculogenesis has been firmly established, activation of VEGFR-2 appears to involve more than just the availability of VEGF (Ortega et al., 1999).

Angiogenesis and vasculogenesis require precisely regulated programs, which include cell migration, proliferation, and differentiation. Two types of instructive signals are required for these processes: secreted or diffusible factors and physical interactions with other cells and the extracellular matrix (Brooks et al., 1994; Skobe et al., 1997). Both type of signals are mediated by cell surface growth factor receptors, cadherins, and integrins. Many growth factor-induced signal transduction pathways are well characterized. There is growing evidence that growth factor receptors can modulate cadherin-associated signaling molecules (Pollack et al., 1997; Esser et al., 1998). Whether cadherin-induced signals also can also modulate growth factor receptor activity has not yet been established.

Cadherins are transmembrane glycoproteins that mediate calcium-dependent cell–cell adhesion and play important roles in both development and disease processes (Yap et al., 1997). There are two major cadherins in endothelial cells, vascular endothelial cadherin, called VE-cadherin or cadherin-5, and N-cadherin (Breier et al., 1996; Lampugnani et al., 1997). Cadherin-5 is preferentially localized at interendothelial cell junctions (Salomon et al., 1992; Navarro et al.,...
Reduction of the extracellular calcium concentration leads to its rapid redistribution and loss of endothelial cell function, as measured by the integrity of a barrier to permeability (Lampugnani et al., 1992). N-cadherin is also clustered at cell–cell junctions; however it can also be found distributed diffusely across the cell membrane of endothelial cells. This difference in cellular localization suggests non-identical roles for these two types of cadherins in regulating cellular properties of endothelial cells. In the current study we demonstrate that endothelial cell interactions play a critical role in regulating VEGFR-2 activity, and that cadherin-5 may contribute to activation of VEGFR-2.

MATERIALS AND METHODS

Reagents and Antibodies

Anti-mouse and anti-rabbit immunoglobulin G conjugated to HRP were purchased from Amersham (Arlington Heights, IL). Rabbit anti-colony-stimulating factor 1 receptor (CSF-1R) antibody and mouse anti-phosphotyrosine (PY-20) antibody and anti-cadherin-5 antibody were purchased from Transduction Laboratories (Lexington, KY) or from Pharmingen (catalog no. 28091D; San Diego, CA). The anti-VEGFR-2 antibody was raised against a glutathione S-transferase fusion protein including the kinase insert or carboxyl terminus of mouse VEGFR-2.

Construction of the Chimeric CSF-VEGFR-2

A full length cDNA of human CSF-1R was used as a template to generate the extracellular domain of the CSF-1R by PCR. Two oligonucleotides, corresponding to nucleotides –267 to –294 (CCT-GCACCTGGGGGCAGGGGTCCACCAC and +1841 to +1862 (ACGCCATGCCATGATGTTCC) in CSF-1R sequence, were designed to generate a new NotI site at the 5′ end and ClaI site at the 3′ end. The VEGFR-2 transmembrane and cytoplasmic domain (amino acids 759-1367) were cloned from mouse aortic endothelial cells by RT-PCR. Two oligonucleotides corresponding to nucleotides 2266 –2296 (GAAAAGACCATCGATGAAGTCATTATCCTC) and 4296 – 4321 (GCCTTGAATTCATGCTTCTCTC) in the mouse VEGFR-2 sequence were designed to generate a new ClaI site at the 5′ end and a SalI site at the 3′ end. The resulting cDNA was cloned into pGEMT vector. To generate the chimeric CSF-VEGFR-2, the CSF-1R PCR product was digested with ClaI and NotI and ligated with VEGFR-2 by ClaI and NotI sites. The resultant hybrid CSF-VEGFR-2 cDNA was then subcloned into the retrovirus vector, pLNCl2.

Cell Lines

Pig aortic endothelial (PAE) cells expressing wild-type VEGFR-2 or platelet-derived growth factor receptor α (PDGFR-α) were kindly provided by Lena Claesson-Welsh (Uppsala University, Uppsala, Sweden). Adrenal microvascular endothelial (AEC) cells are primary endothelial cells and were kindly provided by Patricia D’Amore (Scheepens Eye Research Institute). A retroviral expression system was used to establish PAE cells expressing the chimeric VEGFR-2 (hereby referred to as CK) or NIH-3T3 cells expressing VEGFR-2. Briefly, the cDNA for CK or VEGFR-2 was cloned into a retroviral vector, pLNCl2, and transfected into 293GFP cells. The viral supernatant was collected for 7 d, concentrated by centrifugation, and used as previously described (Ory et al., 1996). Equal amounts of colony-forming units from the concentrated vectors were used to infect target cells.

In Vitro Kinase Assay

The kinase activity of VEGFR-2 was analyzed exactly as described before (Rahimi et al., 1998). Briefly, VEGFR-2 immunoprecipitates were incubated in 10 μCi of [γ-32P]ATP for 10 min at 30°C. The reaction was stopped by adding 2× sample buffer, samples were resolved by SDS-PAGE, and radiolabeled VEGFR-2 was detected by autoradiography.

Immunoprecipitation and Western Blot Analysis

To make cells sparse or confluent, PAE or NIH3T3 cells expressing wild-type VEGFR-2 or CK were grown in media containing 10% FBS. The cells were trypsinized, washed, and counted, and equal numbers of cells were plated into either 10- or 15-cm tissue culture dishes. The cells were then incubated for at least 18 h in media containing 0.1% calf serum. Cells were left resting or stimulated with 100 ng/ml VEGF or 40 ng/ml CSF-1 for 5 min at 37°C. Cells were washed twice with H/S buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, and 2 mM Na3VO4) and lysed in lysis (EB) buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 1 mM PMSF, 2 mM Na3VO4, and 20 μg/ml apronin), and VEGFR-2 was immunoprecipitated using an anti-VEGFR-2 antibody. Immune complexes were bound to formalin-fixed Staphylococcus aureus membranes, spun through EB supplemented with 10% sucrose, and washed twice with 1.0 ml of EB, twice with 1.0 ml of PAN buffer (containing 10 mM 1,4-piperazinedithanesulfonic acid, pH 7.0, 100 mM NaCl, and 20 μg/ml apronin) plus 0.5% NP-40, and twice with 1.0 ml of PAN.

Immunoprecipitates were resolved on a 7.5% SDS-PAGE gel, and the proteins were transferred to Immobilon (Millipore, Bedford, MA). For anti-phosphotyrosine Western blot analysis, the membranes were incubated for 60 min in Block containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mg/ml BSA, 10 mg/ml ovalbumin, 0.05% Tween 20, and 0.005% NaN3 and then incubated for 60 min with primary antibody diluted in Block. The membranes were then washed and incubated for 60 min with an HRP-conjugated goat anti-mouse antibody. Finally, the membranes were washed and developed using ECL (Amersham). On some occasions, the membranes were stripped by incubating for 30 min at 50°C in a buffer containing 6.25 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol and then reprobed.

RESULTS

VEGFR-2 Activity Is Regulated by Endothelial Cell Density

Under normal conditions, endothelial cells are quiescent and can be induced to rapidly proliferate by factors such as injury, oxidant, and shear stress and tumor growth (Augustin et al., 1994; Cines et al., 1998). Because VEGFR-2 is a major growth regulator of endothelial cells, it is conceivable that endothelial cell–cell interaction may play a role in regulating VEGFR-2 activity. To examine whether cell density plays a role in VEGFR-2 activity, PAE cells were plated at high (100% confluent) or low (60% confluent) cell density and stimulated with VEGF for 5 min. The cells were lysed, the receptors were immunoprecipitated, and the extent of VEGFR-2 tyrosine phosphorylation was evaluated. In sparse conditions VEGF induced robust tyrosine phosphorylation of VEGFR-2, whereas little or no tyrosine phosphorylation of VEGFR-2 was observed in cells plated in confluence (Figure 1A). Essentially, the same results were obtained when VEGFR-2 immunoprecipitates were subjected to an in vitro kinase assay (Figure 1C).

N. Rahimi and A. Kazlauskas
To determine whether VEGFR-2 activity is regulated in other endothelial cells by cell density, AEC cells were also examined. As with the PAE cells, VEGF induced only a slight increase in tyrosine phosphorylation of VEGFR-2 in confluent AEC cells (Figure 1, D and E). In contrast, reducing the cell density markedly increased VEGF-dependent tyrosine phosphorylation of VEGFR-2. Collectively these observations strongly suggest that VEGFR-2 activity is regulated by endothelial cell density.

To examine whether longer exposure of VEGF is required for substantial tyrosine phosphorylation of VEGFR-2, confluent PAE cells overexpressing VEGFR-2 were stimulated with VEGF (100 ng/ml) for 5–180 min. Tyrosine phosphorylation of VEGFR-2 gradually increased, reaching a maximum 1–2 h after VEGF, and this level was maintained at least for 7 h (Figure 2, A and B). Although the level of VEGFR2 tyrosine phosphorylation could be increased by prolonging the exposure to VEGF, it never reached the level seen in the sparse condition. Hence it is unlikely that the reason that the receptor responds poorly is because the time of exposure to VEGF is too short. To determine whether increase in concentration of VEGF overcomes the slow or delayed tyrosine phosphorylation of VEGFR-2, confluent PAE cells were stimulated for 5 min with increasing (100–400 ng/ml) or decreasing (100–5 ng/ml) concentrations of VEGF. The result showed that stimulation of confluent PAE cells with higher or lower concentrations of VEGF did not substantially improve tyrosine phosphorylation of VEGFR-2 (Figure 2, C and D; our unpublished data).

To determine whether cell density-dependent activation of VEGFR-2 in endothelial cells is an intrinsic feature of the receptor itself or an endothelial cell-specific phenomenon, we introduced VEGFR-2 into NIH-3T3 cells (which normally do not harbor VEGFR-2). NIH-3T3 cells overexpressing VEGFR-2 were plated in confluent or sparse conditions, similar to PAE cells, and stimulated with VEGF for 5 min. VEGF stimulation of NIH-3T3 cells resulted in comparable robust tyrosine phosphorylation of VEGFR-2 in both confluent and sparse conditions (Figure 3). Thus, the density of NIH-3T3 cells had little or no effect on tyrosine phosphorylation of VEGFR-2. These data suggest that the cell density-dependent activation of VEGFR-2 in endothelial cells is not an intrinsic feature of receptor but, rather, that it is cell type dependent.

To test whether other growth factor receptors are also subject to the same regulation in endothelial cells, PAE cells overexpressing PDGFR-α were plated in confluent or sparse conditions and stimulated with PDGF-AA for 5 min.
PDGF-AA stimulation of cells resulted in enhanced tyrosine phosphorylation of PDGFR in both dense and sparse conditions (Figure 4). Hence cell density did not greatly affect PDGF-induced tyrosine phosphorylation of PDGFR-α, indicating that not all receptor tyrosine kinases are subject to density-dependent regulation.

The Extracellular Domain of VEGFR-2 Is Not Required for Cell Density-dependent Inhibition of VEGFR-2

Two complementary approaches were taken to address whether confluent PAE cells secrete a VEGF antagonist.

First, conditioned medium was collected from confluent PAE cells and tested for its ability to inhibit VEGF-induced tyrosine phosphorylation of VEGFR-2 in the sparse condition. Pretreatment of sparse PAE cells with conditioned medium from a confluent culture of cells did not diminish VEGF-stimulated tyrosine phosphorylation of VEGFR-2 (our unpublished data). The second approach was to construct a chimeric VEGFR-2 in which extracellular domain of VEGFR-2 is replaced by the extracellular domain of human CSF-1R (CK). The CK chimera was introduced into PAE cells, which were plated in both sparse and dense conditions. Cells were stimulated with human CSF-1 for 5 min, and tyrosine phosphorylation of VEGFR-2 was evaluated. Like VEGFR-2, CK was poorly tyrosine phosphorylated in confluent cells; reducing the cell density markedly increased the response to CSF-1 (Figure 5). Taken together these data suggest that inhibition of tyrosine phosphorylation of VEGFR-2 in confluent PAE cells is not due to secretion of an inhibitory factor.

EGTA Augments VEGF-induced Tyrosine Phosphorylation of VEGFR-2 in the Confluent Condition

The observations described above indicate that factors other than the availability of ligand regulate tyrosine phosphorylation.

Figure 3. VEGFR-2 activation is not affected by cell density when expressed in NIH-3T3 cells. NIH-3T3 cells expressing VEGFR-2 were cultured in the confluent or sparse condition as described in the text, serum-starved overnight, and then stimulated with VEGF for 5 min. Cells were lysed and immunoprecipitated with an anti-VEGFR-2 antibody and immunoblotted with an anti-phosphotyrosine (pY) antibody (A). The same membrane was reprobed with an anti-VEGFR2 antibody (B).

Figure 4. PAE cell density does not alter PDGFR-α activation. PAE cells expressing PDGFR-α were cultured in the confluent or sparse condition, serum starved overnight, and then stimulated with PDGFAA (40 ng/ml) for 5 min. Cells were lysed and immunoprecipitated with an PDGFR-α antibody and immunoblotted with an anti-phosphotyrosine (pY) antibody (A). The same membrane was reprobed with PDGFR-α antibody (B).

Figure 5. Replacement of the extracellular domain of VEGFR-2 with the extracellular domain of human CSF receptor-1 does not alter the behavior of VEGFR-2 when expressed in PAE cells. PAE cells expressing an empty vector (X²) or chimeric VEGFR-2 (CK) were cultured in the confluent or sparse condition, serum starved overnight, and stimulated with CSF (40 ng/ml) for 5 min. The cells were lysed and immunoprecipitated with an anti-VEGFR-2 antibody and immnunoblotted with an anti-phosphotyrosine (pY) antibody (A). The same membrane was reprobed with an anti-VEGFR-2 antibody (B).
of VEGFR-2. Furthermore, it appears that cell–cell interactions are important, and so perhaps the molecules that mediate intercellular relationships play a role in activation of VEGFR-2. One such candidate molecule is cadherin, the primary determinant of calcium-dependent homophilic interactions between cells. To begin to test whether cadherins are involved in the cell density-dependent inhibition of VEGFR-2 activity, we pretreated PAE cells with EGTA, a chelator that interrupts cadherin-mediated cellular interactions (Takeichi, 1991). Pretreatment of the confluent PAE cells with 5 mM EGTA for 1–10 min greatly increased VEGF-dependent tyrosine phosphorylation of VEGFR-2 (Figure 6). The same result was obtained when PAE cells were pretreated with EDTA (our unpublished data). These data suggest that cadherins, or some other divalent cation-dependent system, negatively regulate tyrosine phosphorylation of VEGFR-2.

The Density-dependent Inhibition of VEGFR-2 Is Mediated by Cadherin-5

The data presented thus far indicate that in endothelial cells there is an inverse correlation between cadherin function and VEGF-dependent tyrosine phosphorylation of VEGFR-2. Preventing cadherin function by keeping the cells at low density or by removing extracellular calcium potentiated VEGF-dependent tyrosine phosphorylation of VEGFR-2. To more directly test the idea that cadherins modulate VEGF-2 tyrosine phosphorylation, we determined the effect of a neutralizing cadherin-5 antibody on VEGF-dependent tyrosine phosphorylation of VEGFR-2 (Figure 6). The same result was obtained when PAE cells were pretreated with EDTA (our unpublished data). These data suggest that cadherins, or some other divalent cation-dependent system, negatively regulate tyrosine phosphorylation of VEGFR-2.

To further test this idea, we coexpressed cadherin-5 and VEGFR-2 in NIH 3T3 cells and tested the effect of cell density on VEGF-dependent activation of VEGFR-2. Despite readily detectable levels of both proteins, activation of VEGFR-2 remained independent of cell density in these cells (our unpublished data). It is possible that the functional interaction between cadherin-5 and VEGFR-2 involves proteins that are expressed in endothelial cells but not NIH 3T3 cells. Alternatively, because the localization of cadherin-5 within adherens junctions appears to be required for its function (Lampugnani et al., 1997), it is possible that cadherin-5 is not suitably organized in 3T3 cells to mediate its suppressive effect on VEGFR-2.

DISCUSSION

Vascular endothelial cells are major components of the vascular system and form cell–cell contacts in vivo and in vitro. Such cell–cell contact is essential for blood circulation and also is associated with many pathological conditions, including hemorrhage disorders, thrombosis, and tumor metastasis (Dejana et al., 1996; Folkman and D’Amore, 1996). Under normal conditions, endothelial cells are quiescent, and they rapidly proliferate in response to pathological conditions such as those described above. At least a partial explanation for these observations is that endothelial cells undergo an angiogenic switch, which enables them to respond to angiogenic agents.
such as VEGF (Ortega et al., 1999). Thus there appears to be negative control mechanisms that block activation of growth factor receptors in endothelium with cell–cell contact.

In this study we examined the effect of endothelial cell density on activation of VEGFR-2. VEGF induced only very slight tyrosine phosphorylation of VEGFR-2 in confluent PAE cells, whereas robust tyrosine phosphorylation of VEGFR-2 was observed only in PAE cells cultured in the sparse condition. Notably, PAE cell density had no significant effect on tyrosine phosphorylation of PDGFR-α. The basis for this density-dependent regulation of VEGFR-2 phosphorylation may be due to cadherin-5.

The lack of response for VEGF in the confluent condition was not due to down-regulation of VEGFR-2 protein. Equal amounts of VEGFR-2 protein were detected in sparse and confluent culture conditions. Furthermore, the lack of response was not due to the concentration of VEGF used. For example, increasing the dose of VEGF to up to 400 ng/ml did not result in an increase in VEGFR-2 response. It is notable that 5–100 ng/ml VEGF stimulated a robust tyrosine phosphorylation of VEGFR-2 in PAE cells plated in the sparse condition. Remarkably, cell density of NIH-3T3 fibroblast cells had little or no effect on tyrosine phosphorylation of VEGFR-2. These data suggest that cell density-dependent activation of VEGFR-2 in endothelial cells is not an intrinsic feature of the receptor, but rather, it may represent an endothelial cell-specific occurrence. These results suggest the existence of an endothelial-specific negative control mechanism for VEGFR-2. Our findings suggest that this negative control mechanism involves cadherin-5. Calcium-dependent intercellular contacts between endothelial cells are mainly mediated by cadherin-5 (Lampugnani et al., 1997; Navarro et al., 1998), and interfering with cadherin-5 function potentiated VEGF-induced tyrosine phosphorylation of VEGFR-2.

There is now growing evidence that cadherins play a role in the control of growth and migration of both epithelial and endothelial cells. A role of E-cadherin in the epithelial cell contact-dependent growth inhibition is well documented (Semb et al., 1998), and a recent study suggests that cadherin-5, like E-cadherin, is also involved in cell contact-dependent inhibition of growth (Caveda et al., 1996). Given the ability of cadherins to suppress growth and migration of many cell types, our observations suggest that contact inhibition by cadherins in endothelial cells is in part mediated by blocking VEGFR-2 activity. Further work is required to establish the nature of the cross-talk between cadherin-5 and VEGFR-2 and how endothelial cell density modulates this interaction.

ACKNOWLEDGMENTS

We thank David Lyons (Amgen, Thousand Oaks, CA) for providing VEGF, Karen Symes for human CSFR-1 cDNA, Lena Claesson-Welsh for PAE cells expressing FLK-1 and PDGFR, and Patricia D’Amore for AEC cells. N.R. is a recipient of a research training award from the National Eye Institute (T32 EY07145-01). This project was also supported by a grant from the Massachusetts Lions Club.

REFERENCES


Takeichi, M. (1991). Cadherin cell adhesion receptors as a morpho-