Receptor Chimeras Indicate That the Vascular Endothelial Growth Factor Receptor-1 (VEGFR-1) Modulates Mitogenic Activity of VEGFR-2 in Endothelial Cells*

Vascular endothelial growth factor (VEGF) provokes angiogenesis in vivo and stimulates growth and differentiation of endothelial cells in vitro. Although VEGF receptor-1 (VEGFR-1) and VEGFR-2 are known to be high affinity receptors for VEGF, it is not clear which of the VEGFRs are responsible for the transmission of the diverse biological responses of VEGF. For this purpose we have constructed a chimeric receptor for VEGFR-1 (CTR) and VEGFR-2 (CKR) in which the extracellular domain of each receptor was replaced with the extracellular domain of human colony-stimulating factor-1 receptor (CSF-1R), and these receptors were expressed in pig aortic endothelial (PAE) cells. We show that CKR individually expressed in PAE cells is readily tyrosine-phosphorylated in vivo, autophosphorylated in vitro, and stimulates cell proliferation in a CSF-1-dependent manner. In contrast, CTR individually expressed in PAE cells showed no significant in vivo, in vitro tyrosine phosphorylation and cell growth in response to CSF-1 stimulation. The kinase activity of CKR was essential for its biological activity, since mutation of lysine 866 to arginine abolished its in vivo, in vitro tyrosine phosphorylation and mitogenic signals. Remarkably, activation of CTR repressed CKR-mediated mitogen-activate protein kinase activation and cell proliferation. Similar effects were observed for VEGFR-2 co-expressed with VEGFR-1. Collectively, these findings demonstrate that VEGFR-2 activation plays a positive role in angiogenesis by promoting endothelial cell proliferation. In contrast, activation of VEGFR-1 plays a stationary role in angiogenesis by antagonizing VEGFR-2 responses.

Vascular endothelial growth factor receptor-1 (VEGFR-1/Flt-1) and VEGFR-2/Flk-1 belong to a family of receptor tyrosine kinases whose expression is mainly restricted to endothelial cells. Recent studies have shown that these receptors play key roles in vasculogenesis and angiogenesis. VEGFR-1 and VEGFR-2 seem to have distinct functions in these cellular processes, as illustrated by the non-identical phenotypes of mice expressing homozygous knock-outs of each of the genes. The VEGFR-2 knock-out mice failed to develop endothelial cells, whereas knock-out mice for VEGFR-1 gene contained endothelial cells, but they were poorly organized (1, 2).

VEGF is known to be a ligand for VEGFR-1 and VEGFR-2 (3). VEGF is a dimeric glycoprotein with at least five isoforms due to alternative splicing, VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206. All of these isoforms have been shown to bind to both VEGFR-1 and VEGFR-2 (3, 4). On the other hand, placenta growth factors (PIGFs) have been shown to bind VEGFR-1 only (4, 5). VEGF is a potent stimulator of angiogenesis in vivo and growth, migration, and differentiation of endothelial cells in vitro (6–8). To date, it is not clear which of the VEGFRs is responsible for the transmission of the diverse biological responses of VEGF. The study of the role of individual VEGFRs in VEGF-induced endothelial functions is complicated in part by the observation that most endothelial cells express both VEGFR-1 and VEGFR-2 (9, 10). On the other hand, the activity of VEGFRs is affected by the cell type in which they are expressed. For example, activation of VEGFR-1 or VEGFR-2 ectopically expressed in NIH-3T3 fibroblast cells resulted in no significant biological responses (11, 12). In an attempt to address the role of individual VEGFRs in endothelial functions, Waltenberger et al. (13) over-expressed VEGFR-1 and VEGFR-2 individually in PAE cells that already express a detectable amount of VEGFR-1. Stimulation of PAE cells over-expressing VEGFR-2 with VEGF resulted in growth and migration (13). Since PAE cells naturally express VEGFR-1, contribution of VEGFR-1 to these responses cannot be excluded (14). The role of VEGFR-1 in endothelial cell functions is even less clear and controversial. Stimulation of PAE cells over-expressing VEGFR-1 with VEGF resulted in no biological responses (14). Additionally, Keyt et al. (15) show that mutant forms of VEGF that lack binding to VEGFR-1 are able to stimulate growth, suggesting no significant biological role for VEGFR-1 (15). In contrast, other laboratories show that activation of VEGFR-1 correlates with cell migration and growth in vitro and angiogenesis in vivo (16, 17). The apparent differences in the role of VEGFR-1 in endothelial cell growth is not known. This discrepancy could be due to the inherent problems associated with the above cellular systems, such as the level of expression of individual VEGFRs and the type of ligands that were used for stimulation. Therefore, the current literature does not provide a clear and adequate explanation of the role of individual VEGFRs in transmission of the diverse biological responses of VEGF in endothelial cells. For this purpose, we have constructed a chimeric receptor for VEGFR-1

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The abbreviations used are: VEGF, vascular endothelial growth factor receptor; CSF-1R, colony-stimulating factor-1 receptor; PAE cells, pig aortic endothelial cells; PIPES, 1,4-piperazinediethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; MAP, mitogen-activated protein.

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VEGF Receptors and Endothelial Cells

(CTR) and VEGFR-2 (CKR) in which the extracellular domain of the receptor is replaced with the extracellular domain of human CSF-1R. In this report we show that activation of CTR but not CTR elicits mitogenic responses in the endothelial cells and that tyrosine kinase activity of CTR is required for its mitogenic signals, since kinase-inactive CTR fails to stimulate mitogenic responses. Furthermore, simultaneous co-activation of CTR with CTKR co-expressed in PAE cells results in inhibition of mitogenic signals by CTKR, suggesting a stationary role for VEGFR-1 in endothelial cells functions.

MATERIALS AND METHODS

Reagents and Antibodies—Rabbit anti-CSF-1R antibody and mouse anti-phosphotyrosine (4G10) were purchased from Upstate Biotechnology, Inc. Mouse anti-phosphotyrosine (PY-20) antibody was purchased from Transduction Laboratories. Rabbit anti-VEGFR-1 and VEGFR-2 antibodies are made to amino acids corresponding to kinase insert of VEGFR-1 and VEGFR-2.

Cell Lines—Pig aortic endothelial (PAE) cells expressing the chimeric VEGFR-1 and VEGFR-2 (herein referred to as CTR and CTKR, respectively) were established by a retroviral system. Briefly, the cDNA for CTR or CTKR were cloned into a retroviral vectors (pLNCX2 and pLNCX2-FLK1 receptor). They contain the five immunoglobulin-like repeats of VEGFR-2 by cDNA and NotI, respectively (Fig. 1).

NotI site at the 5′ end and ClaI site at the 3′ end. The resulting cDNA was cloned into pGEMT vector. To generate the chimeric CSF-VEGFR-2, the CSF-1R polymerase chain reaction product was digested with ClaI and NotI and ligated with VEGFR-2 by ClaI and NotI sequences. The resultant hybrid CSF-VEGFR-2 cDNA was then subcloned into the retrovirus vector, pLNCX2. VEGFR-1 chimera was constructed in similar manner by using two oligonucleotides, 2202–2229 (GTCTAATTGACACGAAGCCTTT) and 3948–39975 (CCCATCTAGAGTGGAGCTGATCGATCTAAC) and 3′-end and ClaI site at the 3′ end. The VEGFR-2 transmembrane and cytoplasmic domains (amino acids 759–1367) were cloned from mouse aortic endothelial cells by reverse transcriptase-polymerase chain reaction (19). Two oligonucleotides corresponding to nucleotides 2266 to 2296 (GAAAAGACCATCGATGAGTTC) in the mouse VEGFR-2 sequence were designed to generate a new NotI site at the 5′ end and ClaI site at the 3′ end. The viral supernatant was collected for 7 days, concentrated by centrifugation, and used as described previously (18). Equal amounts of colony-forming units from the concentrated virus were used to infect target cells.

Construction of the Chimeric CSF-VEGFR-1 and 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 polymerase chain reaction. Two oligonucleotides, corresponding to nucleotides 267 to 294 (CCTGGAGCTGATCGATCTAAC) and 3′-end. The resulting cDNA was cloned into pGEMT cloning vector, and site-directed mutagenesis was carried out by using the Stratagene site-directed mutagenesis kit. To construct receptor chimeras containing the extracellular domain of human CSF-1R, fused with the transmembrane and cytoplasmic domains of VEGFR-1 and VEGFR-2. The resultant chimera receptors are called CTR (CSF-1R-FLK-1 receptor) and CTKR (CSF-1R-FLK-1 receptor). They contain the five immunoglobulin-derived sequences from the extracellular domain of the CSF-1R that are essential for ligand binding (22). The resultant chimera receptors were transfected into 293GPG cells. The viral supernatant was collected for 7 days, concentrated by centrifugation, and used as described previously (18). Equal amounts of colony-forming units from the concentrated virus were used to infect target cells.

Solid-Directed Mutagenesis—The VEGFR-2 chimera (CKR) was subcloned into pGEMT cloning vector, and site-directed mutagenesis was carried out by using the Stratagene site-directed mutagenesis kit. To mutate lysine (K) to arginine (R) the oligonucleotide corresponding to VEGFR-2 (′-ACGAGTAGCCGTCCGAATGTTGAAAGAA-3′) was used. The Amp gene primer, Scol to M13I (Stratagene), was used as a selection primer. The resultant mutation on CKR was verified by sequence analysis and subsequently subcloned into pLNCX2 vector by NotI and Sall sites.

Immunoprecipitation and Western Blot Analysis—PAE cells expressing CTR or CTR were grown in sparse conditions in 10% fetal bovine serum in 24-well plates and incubated at 37 °C for 12 h. Cells were then washed twice in phosphate-buffered saline and growth-arrested in Dulbecco’s modified Eagle’s medium containing 1 mg/ml bovine serum albumin and 0.1% calf serum for 24 h at 37 °C. Various amounts of CSF-1 or VEGF were added and incubated for 18–20 h at 37 °C. Cells were pulsed with 1.0 Ci [3H]thymidine (0.2 μCi/ml) and harvested. Triplicate samples were performed for each group. Three independent experiments were performed, and essentially the same results were obtained. The data are presented as a fold increase over control.

In Vitro Kinase Assay—Kinase activity of CTR and CTKR were analyzed exactly as described before (20). Briefly, CTR and CTKR immunoprecipitates were washed twice with 1.0 ml of EB buffer, twice with 1.0 ml of PAN buffer, and washed twice with 1.0 ml of PH buffer. The kinase reaction was stopped by adding 2× sample buffer, samples were resolved by SDS-PAGE, and radiolabeled CKR and CTKR were detected by autoradiography.

RESULTS

Construction of the VEGFR-1 and VEGFR-2 Chimeras—There is compelling evidence for involvement of both VEGFR-1 and VEGFR-2 in angiogenesis (8). However, it is not clear whether all of the biological responses seen in endothelial cells in response to VEGF are mediated by activation of either VEGFR-1 or VEGFR-2 or whether co-stimulation of both receptors is required. To establish whether stimulation of VEGFR-1 and VEGFR-2 mediates endothelial cell growth and whether there is cross-talk between VEGFR-1 and VEGFR-2, we constructed receptor chimeras containing the extracellular domain of human CSF-1R, fused with the transmembrane and cytoplasmic domains of VEGFR-1 and VEGFR-2. The resultant chimera receptors are called CTR (CSF-1R-FLK-1 receptor) and CTKR (CSF-1R-FLK-1 receptor). They contain the five immunoglobulin-derived sequences from the extracellular domain of the CSF-1R that are essential for ligand binding (22). The VEGFR-1 and VEGFR-2 regions contain cytoplasmic and transmembrane domains of VEGFR-1 and VEGFR-2, including four amino acids of the VEGFR-1 extracellular domain and three amino acids of the VEGFR-2 extracellular domain, respectively (Fig. 1A).

Selection of PAE Cell Lines Expressing CTR and CTKR—Although most endothelial cells express both VEGFR-1 and VEGFR-2, their level of expression is different. PAE cells were used as a cellular model to study VEGF-induced biological responses in endothelial cells by over-expressing them with VEGFR-1 or VEGFR-2 (13). Since PAE cells express low levels of VEGFR-1, it is not clear whether all the biological responses observed in PAE cells in response to VEGF are attributed directly to activation of either VEGFR-1 or VEGFR-2 alone or whether co-stimulation of both of receptors is required. To investigate the effects of stimulation of VEGFR-1 and VEGFR-2 individually or jointly in the endothelial cell function, we...
expressed CTR and CKR in PAE cells by a retroviral system. This type of gene expression results in mass populations of cells expressing similar levels of the introduced gene (18, 19). To avoid clonal variations, all experiments were performed on pooled G418- or histidinol-resistant clones rather than on isolated clones. PAE cells expressing either an empty vector, CTR, or CTR were lysed, and equal amount of proteins of total cell lysates were subjected to Western blot analysis by using an anti-CSF-1R antibody, which specifically recognizes the extracellular domain of human CSF-1R. As Fig. 1A shows both CTR and CKR are expressed in PAE cells in comparable levels. The chimeric proteins were migrated according to their expected apparent molecular mass of 180 kDa in SDS-polyacrylamide gel. Reprobing of the same membrane with an anti-VEGFR-2 antibody confirmed CTR expression, as expected (Fig. 1C). Cells expressing an empty vector, pLNClX2 (X2), were negative for both of the antibodies.

CSF-1-dependent Kinase Activity of CTR and CKR in Vitro and Tyrosine Phosphorylation in Vivo—VEGF has been shown to stimulate autophosphorylation of VEGFR-2 (13, 19). In contrast, no significant autophosphorylation is seen for VEGFR-1 in response to VEGF (13). Therefore, we have examined in vivo and in vitro tyrosine phosphorylation of CTR and CKR expressed in PAE cells. To establish whether CKR and CTR chimeras are functional, we tested the ability of CSF-1 to stimulate the tyrosine phosphorylation in vivo and kinase activity in vitro. PAE cells expressing either an empty vector, CTR, or CTR were stimulated with CSF-1 (40 ng/ml) for 10 min, and cells were lysed and immunoprecipitated with an anti-CSF-1R antibody. The immunoprecipitated proteins were subjected into an anti-phosphotyrosine Western blot analysis or to an in vitro kinase assay. CTR was found to be readily tyrosine-phosphorylated in response to CSF-1 stimulation both in vivo and in vitro (Fig. 2, A and E). However, CSF-1 stimulation of cells expressing CTR showed no significant tyrosine phosphorylation in an anti-phosphotyrosine Western blot analysis. A long exposure (30 min) of the blot showed trace evidence of tyrosine phosphorylation (Fig. 2C). The lack of tyrosine phosphorylation of CTR was not due to level of CTR protein, since reprobing the membrane with an anti-VEGFR-1 antibody showed that CTR was present (Fig. 2D). Additionally, CTR showed no robust tyrosine phosphorylation in an in vitro kinase assay (E). To determine the protein levels in each lane, the same membranes were reprobed with either an anti-VEGFR-2 or anti-VEGFR-1 antibodies (B and D).

CSF-1-dependent Activation of CKR but Not CTR Results in Growth of PAE Cells—Although activation of VEGFR-2 is correlated with growth and migration of endothelial cells (13), it is not clear whether activation of VEGFR-2 alone is sufficient for induction of these biological responses. Most of the studies to date have used VEGF to activate VEGFR-2, which binds not only to VEGFR-1 but to VEGFR-2 as well (23, 24). To establish whether stimulation of VEGFR-2 and VEGFR-1 alone is sufficient to mediate the mitogenic responses in PAE cells, we subjected PAE cells expressing CTR and CTR to a cell proliferation assay. Stimulation of CTR/PAE cells with CSF-1 stimulated growth of PAE cells in a dose-dependent manner. Maximum growth stimulation was observed with 1 ng/ml, and increasing doses of CSF-1 above the 2 ng/ml resulted in no additional growth (Fig. 3A). Stimulation of PAE cells expressing an empty vector (X2) with CSF-1 did not result in any growth response above base line. Higher concentrations of CSF-1 (5–20 ng/ml) resulted in a profound effect on the morphology of PAE cells expressing CKR after a 24-h stimulation. The CSF-1-induced morphological changes were associated with elongation and acquisition of a dendrictic shape. After 2–3 days of stimulation with CSF-1, these cells organized into short...
branching cords that formed a discontinuous network (data not shown). However, stimulation of PAE cells expressing CTR with CSF-1 showed that CSF-1-dependent activation of CTR results in no significant growth (Fig. 3B). Stimulation of PAE cells expressing CTR with high doses of CSF-1 also did not result in any obvious morphological changes (data not shown). Collectively, these results demonstrate competence of VEGFR-2 activation in mediating endothelial cell growth, whereas VEGFR-1 activation may not be involved in cell growth. MAP kinase/Erk phosphorylation is a mandatory signal for mitogenic signals induced by many growth factor receptors (25, 26). In an alternative approach to investigate the function of CTR, we measured MAP kinase/Erk activation on PAE cells expressing CTR. Activation of CTR with CSF-1 resulted in a modest activation of MAP kinase in a time-dependent manner (5–30 min). However, in a similar assay condition, CTR activation resulted in a robust activation of MAP kinase (data not shown and Fig. 7, A and C).

**Kinase Activity of CRK Is Required for CRK-mediated Mitogenic Signals in Endothelial Cells**—To establish whether kinase activity of CRK is essential for its ability to induce biological responses in endothelial cells, we constructed a kinase-inactive CRK (herein referred to as CRK/R866) by replacing lysine 866, an ATP binding site, to arginine. The resulting construct was cloned into pLNCX² retroviral vector and was stably expressed in PAE cells, and its expression was analyzed by a Western blot using anti-VEGFR-2 antibody. As Fig. 4A shows, CRK/R866 was expressed at levels comparable with wild type CRK. Following stimulation with CSF-1, the wild type CRK showed both increased tyrosine phosphorylation in vivo (Fig. 4B) and kinase activity in vitro (Fig. 4D), whereas the CRK/R866 showed no in vitro kinase activity (Fig. 4B) or increased tyrosine phosphorylation in vivo (Fig. 4D). We also analyzed the ability of CRK/R866 to stimulate mitogenic responses in PAE cells. Treatment of PAE cells expressing CRK with 1 ng/ml CSF-1 resulted in growth, whereas no mitogenic responses were observed for CRK/R866 (Fig. 4E), demonstrating that kinase activity of CRK is essential in mediating the biological responses in the endothelial cells.

**Effect of Co-stimulation of CTR and CRK in Endothelial Cell Growth**—The observation that activation of CTR alone is not sufficient to stimulate growth of endothelial cells raises the question of whether CTR activation requires the presence of CRK as a partner/co-receptor for its activation. For this purpose, we generated PAE cells co-expressing CRK and CTR at two different titers, respectively. Fig. 5, A and B, shows expression levels of CRK and CTR in PAE cells. We first evaluated tyrosine phosphorylation of CRK and CRK co-expressed in PAE cells by immunoprecipitation with an anti-VEGFR-2 antibody and Western blot analysis with an anti-phosphotyrosine antibody. Surprisingly, CRK was found tyrosine-phosphorylated before CSF-1 treatment and CSF-1 stimulation resulted in a further increase in tyrosine phosphorylation (Fig. 5C). The increased tyrosine phosphorylation of CRK was not due to more CRK protein, since PAE cells expressing CRK were used to express CTR into them, and furthermore, reprobing of the same membrane with an anti-VEGFR-2 antibody showed relatively similar amounts of CRK in each group (Fig. 5D). In fact, CSF-1 treatment may have caused receptor down-regulation or internalization, thus reducing the signal. Surprisingly, tyrosine phosphorylation of CRK in response to CSF-1 was not affected by its co-expression with CRK (Fig. 5E). These results suggest that CSF-1 treatment may have caused CRK to form a heterodimer complex with CRK and its transphosphorylation in a unidirectional manner.

To gain insight into the biological consequences of CTR effect on the CRK activation, we subjected PAE cells co-expressing CTR and CRK to proliferation assay. Fig. 6A shows that co-stimulation of CTR with CRK greatly reduces CRK-mediated PAE cell growth. To demonstrate that the observed effect of CTR modulation of CRK activity, is not due to chimera system, we also co-expressed VEGFR-1 and VEGFR-2 in PAE cells and subjected them to a proliferation assay in a similar manner. Essentially the same results were obtained when VEGFR-1 was co-stimulated with VEGFR-2 with VEGF (Fig. 6B). Fig. 6, C and D, shows that both VEGFR-1 and VEGFR-2 are expressed at comparable levels. As expected, a trace amount of endogenous VEGFR-1 is also evident (Fig. 6D). Thus, it appears that VEGFR-2-mediated mitogenic signals are regulated.
In an alternative approach, we analyzed the capacity of CKR and CTR expressed individually or jointly in PAE cells to stimulate MAP kinase phosphorylation. PAE cells expressing CKR showed a significant phosphorylation of both p42 and p44 MAP kinase (Fig. 7C). CTR activation also induced phosphorylation of MAP kinase but less significantly than CKR (Fig. 7A). Consistent with proliferation data, CSF-1 stimulation of cells co-expressing CKR and CTR also resulted in a significant inhibition of MAP kinase phosphorylation. This effect was much profound in the cells expressing higher levels of CTR (Fig. 7E). The MAP kinase Western blot of the same membrane indicates that these differences are not due to differences in the level of protein present in each lane (Fig. 7F). These findings suggest that the inability of CTR to stimulate mitogenic signals may be due to its inability to drive maximal activation of MAP kinase. Furthermore, the data suggest that VEGF-induced biological responses are subject to type and amount of VEGFRs expressed by endothelial cells and VEGFR-1 may play a central role in regulating these biological responses.

FIG. 4. Kinase activity of CKR is required for in vitro autophosphorylation and in vivo tyrosine phosphorylation. PAE cells expressing CKR or CKR/R866 (kinase-dead) were cultured in semi-confluent condition and lysed, and equal amounts of proteins were resolved in SDS-PAGE and blotted with an anti-VEGFR-2 antibody (A) or serum-starved overnight and then stimulated with CSF-1 (40 ng/ml) for 10 min. Cells were lysed, immunoprecipitated with an anti-CSF-1R antibody, and immunoblotted with an anti-photophosphorysine (pY) antibody (B) or subjected into an in vitro kinase assay (D). The same membrane was reprobed with an anti-VEGFR-2 antibody (C). Serum-starved PAE cells expressing CKR or CKR/R866 were treated with the indicated concentrations of CSF-1, and DNA synthesis was measured by [3H]thymidine uptake as described under “Material and Methods.” The data are expressed as a ratio of stimulated over unstimulated samples (E).

FIG. 5. Co-expression of CTR and CKR in PAE cells. PAE cells expressing CKR were infected with two different titers of virus (CTR-25 and CTR-75) and selected in histidinol. PAE cells co-expressing CKR and CTR were cultured in semi-confluent condition and lysed, and equal amounts of proteins were resolved in SDS-PAGE and blotted with an anti-VEGFR-1 antibody (A) or anti-VEGFR-2 antibody (B). Cells were serum-starved overnight and then stimulated with CSF-1 (40 ng/ml) for 10 min. Cells were lysed and immunoprecipitated with an anti-VEGFR-2 antibody and immunoblotted with an anti-photophosphorysine (pY) antibody (C). The same membrane was reprobed with an anti-VEGFR-2 antibody (D). In a similar manner, cells were lysed and immunoprecipitated with an anti-VEGFR-1 antibody and immunoblotted with an anti-photophosphorysine (pY) antibody (E). The same membrane was reprobed with an anti-VEGFR-1 antibody (F).
DISCUSSION

VEGF is a multifunctional cytokine that acts as a mitogenic, motogenic, permeability, and angiogenic factor for endothelial cells (6–9). VEGFR-1 and VEGFR-2 have been identified as high affinity receptors for VEGF. However, it is not clear which of the VEGFRs are responsible for the transmission of diverse biological responses of VEGF. Many endothelial cells in culture express both VEGFR-1 and VEGFR-2. In addition, proteoglycans and neuropilin-1 may act as receptor/co-receptors for VEGF and mediate its various biological signals (24, 27). To first determine whether stimulation of VEGFR-1 and VEGFR-2 alone or jointly are sufficient to mediate the mitogenic responses in endothelial cells, we generated a chimeric receptors containing the extracellular domain from human CSF-1R fused to the transmembrane as well as the cytoplasmic domains of VEGFR-1 and VEGFR-2. This unique strategy allowed us to investigate the consequence of stimulation of VEGFR-1 and VEGFR-2 alone or together in endothelial cells that normally respond to VEGF but not to CSF-1.

We demonstrated that VEGFR-1 (CTR) and VEGFR-2 (CKR) chimeras in PAE cells were expressed as stable proteins and appear to be fully functional. CSF-1 treatment of PAE cells expressing CKR alone resulted in autophosphorylation of CKR in vivo and its kinase activity in vitro. Stimulation of CKR in PAE cells results in growth with CSF-1 (0.5–2 ng/ml). However, at higher concentrations (5–10 ng/ml), it results in growth arrest associated with cellular differentiation/tube formation. Unlike CKR activation, CSF-1 treatment of PAE cells expressing CTR alone resulted in only a slight increase in autophosphorylation of CTR in vivo and negligible kinase activity in vitro. Also, stimulation of CTR with CSF-1 did not result in a measurable growth response. Similarly, higher concentrations of CSF-1 (5–10 ng/ml) did not result in apparent cellular differentiation/tube formation.

These results are in agreement with previous studies and clearly demonstrate a key role for VEGFR-2 activation in endothelial cell growth (1, 3, 8). More importantly, they demonstrate that kinase activity of VEGFR-2 is essential for its mitogenic signals. The role of VEGFR-1 in endothelial cell functions is not settled. Genetic approaches such as knock out of VEGFR-1 (2), along with in vivo and in vitro role of the placenta growth factor, PIGF, which binds VEGFR-1 only (10, 24), suggest that VEGFR-1 activation plays a key role in angiogenesis. In contrast, several other laboratories have shown no biological responses for VEGFR-1 activation in endothelial cells (16, 17). Our results are consistent with previous reports showing that VEGF-1 does not efficiently undergo autophosphorylation upon ligand stimulation (13), and its activation does not stimulate endothelial cell growth (13, 14). More importantly, these results show that VEGFR-1 can modulate VEGF-2-induced cell growth when it is co-expressed with...
VEGFR-2. Simultaneous co-activation of VEGFR-1 and VEGFR-2 chimeras strongly suggest that VEGF may have distinct functions that are dependent on the combination of receptors expressed on the endothelial cells. Since both VEGF receptors are expressed during embryonic development and tumor-induced angiogenesis, VEGF-1 expression and activation may play a mandatory role in angiogenesis by regulating VEGF-2-induced cell growth. In agreement with this hypothesis, Hiratsuka et al. (34) demonstrated that the cytoplasmic domain of VEGFR-1 is not required for vasculogenesis during development (34), and very recent work by Fong et al. (21) showed that embryos lacking VEGFR-1 displayed an increase in the growth of endothelial cells and hemorrhagic blast commitment, suggesting a negative role for VEGFR-1 in angiogenesis.

Modulation of VEGFR-2-induced cell growth by VEGFR-1 activation is not clear. This effect in part might be mediated by heterodimerization of VEGFR-1 with VEGFR-2, therefore altering recruiting and activation of signaling molecules involved in cell proliferation. This type of cross-talk has been demonstrated for the EGFR family (28, 29). VEGFR-1 may also elicit in cell proliferation. This type of cross-talk has been demonstrated for fibroblast growth factor receptors (31, 32). Some spliced variant forms of VEGF have been shown to bind to heparan sulfate proteoglycans, suggesting a potential role for heparan sulfate proteoglycans in VEGF-induced biological functions. Our results suggest that heparan sulfate proteoglycans may not be directly involved in VEGF-mediated responses of endothelial cells. However, the heparan sulfate proteoglycans may still play a role in the storage of the VEGF isoforms in the extracellular matrix or their presentation to the VEGFRs. Such a role for heparan sulfate proteoglycans has been demonstrated for fibroblast growth factor receptors (31, 32), and a recent study on the VEGF suggests a similar role for heparan (33).

Coordinated movement, growth, and differentiation of cells are essential prerequisites for vasculogenesis and angiogenesis. A recurring picture in this process is that both cooperative and antagonistic signals are most likely involved among the factors that influence endothelial cell functions. In conclusion, this study represents the first direct analysis of VEGF-1 and VEGF-2 function in endothelial cell growth and demonstrates the remarkable cross-talk between VEGFR-1 and VEGFR-2 and the hierarchy of signals involved in regulation of angiogenesis. Further work is required to determine the underlying mechanism involved in this process.

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