KDR (VEGF Receptor 2) Is the Major Mediator for the Hypotensive Effect of VEGF

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Abstract—Vascular endothelial growth factor (VEGF) exerts vasodilation-induced hypotension as a major side effect for treatment of ischemic diseases. VEGF has 2 receptor tyrosine kinases, KDR and Flt-1. Little is known about which receptor mediates VEGF-induced hypotension. To elucidate the role of each receptor in mediating hypotension, KDR-selective and Flt-1–selective mutants were used for in vitro and in vivo studies. The KDR-selective mutant induced vascular endothelial cell proliferation comparable to VEGF, whereas the Flt-1–selective mutant had no effect on proliferation. Intravenous injection of KDR-selective mutant, Flt-selective mutant, or VEGF caused a dose-related decrease in mean arterial pressure in conscious rats. The hypotensive response to KDR-selective mutant was significantly less than that to VEGF ($P<0.01$) but was greater than that to Flt-selective mutant ($P<0.01$). Similarly, VEGF and KDR-selective mutant induced more potent vasorelaxation than Flt-selective mutant or placenta growth factor that binds Flt-1 only ($P<0.01$), and the vasorelaxation to KDR-selective mutant was not significantly different at low concentrations but less than that to VEGF at high concentrations. The results indicate that the vasodilation and hypotensive effect of VEGF may involve both receptors, but KDR is the predominant receptor mediating this effect. Because KDR-selective mutant induced proliferation and angiogenesis similar to VEGF but was associated with 36% attenuation in hypotension, the data suggest that the KDR-selective mutant may represent an alternative treatment for ischemic diseases. (Hypertension. 2002;39:1095-1100.)

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Vascular endothelial growth factor (VEGF), a fundamental regulator of physiological and pathological angiogenesis, is an endothelial cell–specific mitogen. VEGF has been shown to promote endothelial cell proliferation and migration in vitro and in vivo. Systemic administration of VEGF causes hypotensive and tachycardic responses in a dose-dependent fashion in rats, rabbits, and pigs. Intracoronary injection also induces a dose-related decrease in arterial pressure in pigs and the hypotensive and tachycardic responses to both intravenous and intracoronary administration at the same dose are similar. The VEGF-induced hypotension is due to vasodilation, which is likely mediated by nitric oxide and prostaglandin I2 (PGI2), whereas the tachycardia may be a result of reflex responses to hypotension. The hypotension induced by acute administration of VEGF is transient and dose-rate-dependent and usually can be tolerated at therapeutic doses in normal animals. The side effects, however, could be life threatening in the setting of severe heart diseases. It has been reported that a single intracoronary injection of VEGF (2 mg) improves myocardial blood flow but produces severe hypotension resulting in a 50% death rate in pigs with chronic myocardial ischemia. Thus, hypotension as a major side effect may limit clinical use of VEGF. Clinical studies in patients with coronary artery disease have shown that the hypotensive effect is dependent on the VEGF infusion rate over the dose range studied (5 to 100 ng/kg per minute). The maximal tolerated infusion rate is 50 ng/kg per minute for a 20-minute intracoronary infusion and a 4-hour intravenous infusion.

VEGF exerts multiple biological effects through its interaction with 2 receptor tyrosine kinases, Flt-1 (VEGF receptor 1) and KDR (VEGF receptor 2). Based on knockout studies in mice, the 2 receptors play an essential role in survival.
However, it is still not entirely clear which biological effect is mediated by each receptor. Although several natural receptor-selective VEGF variants, such as placental growth factor (PIGF), VEGF-B, and VEGF<sub>165</sub>, have been used to examine the role of the VEGF receptors, some of the results may be controversial or difficult to interpret. The disadvantages of these natural VEGF variants may include (1) additional domains of unknown function, (2) low sequence similarity to the wild-type proteins, (3) the wide range of receptor affinities, and/or (4) possible immunogenic responses. Recently, we generated novel, highly receptor-selective VEGF mutants by phage-display technology. These mutants have been shown to be especially valuable in selectively defining the biological role of the VEGF receptors. Both in vitro and in vivo studies using these VEGF mutants have demonstrated that KDR is the sole mediator of endothelial cell migration, angiogenesis, and vascular permeability. However, the VEGF receptor responsible for hypotension has yet to be identified.

The present study was designed to determine the role of individual VEGF receptors in mediating proliferation of endothelial cells, vasorelaxation, or vasodilation in vitro and hypotension in conscious animals. We confirm that KDR is the receptor that mediates VEGF-induced endothelial cell proliferation, whereas hypotension induced by VEGF is mediated by both receptors, but primarily by KDR.

**Methods**

**Receptor-Selective VEGF Mutants**

Human recombinant VEGF<sub>165</sub> and receptor-selective VEGF mutants are produced by Genentech, Inc (South San Francisco, Calif). The construction and characterization of the VEGF mutants are as described previously. The mutations of KDR-selective and Flt-selective VEGF are D63S/G65M/L66R and I43A/I46A/Q79A/I83A, respectively. The mutations were introduced into VEGF<sub>165</sub>, and the receptor-selective mutants and wild-type VEGF<sub>165</sub> were expressed in Escherichia coli. After purification, the endotoxin content of the material was <0.2 endotoxin units per milligram (EU/mg).

**BIAcore Competitive Analysis**

Binding affinities of the receptor-selective VEGF mutants were determined by use of a BIAcore-2000 surface plasmon resonance system (BIAcore, Inc). A biosensor chip was activated for covalent coupling of VEGF using N-ethyl-N-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). VEGF<sub>165</sub> was injected at a flow rate of 2 μL/min to achieve about 825 response units (RU) of coupled protein. For affinity measurements, IgG Fc fusions of the extracellular portion of KDR (at 1 nmol/L) or Flt-1 (at 0.1 nmol/L) were mixed with 3-fold serial dilutions of each VEGF mutant and incubated for 1 hour at room temperature. The mixtures were then injected at a flow rate of 10 μL/min. Titration curves were plotted with a 4-parameter nonlinear regression curve-fitting program (KaleidaGraph) for determination of the dissociation binding constants (Kd) for the VEGF mutants and their receptors.

**Endothelial Cell Proliferation Assay**

The cell assay was performed as previously described. Briefly, human umbilical vein endothelial cells (HUVECs) were seeded in 96-well plates (about 2000 cells per well). The cells were fasted in CS-C medium (Cell Systems) without growth factors and supplemented with 2% diaphragmed fetal bovine serum for 24 hours at 37°C with CO<sub>2</sub> before replacing the medium with fresh fasting medium. VEGF mutants with 3-fold serial dilution were added to the same fixing medium. The cells were incubated for 18 hours, pulsed with [3H] thymidine (0.5 μCi per well) for 24 hours, and then harvested and counted with a Topcount microplate scintillation gamma counter (Packard Instrument Co).

**Animals**

Male Sprague-Dawley rats (n=140), aged 8 to 9 weeks (Charles River Breeding Laboratories, Wilmington, Mass), were used for this in vitro and in vivo study. The experimental procedures were approved by the Animal Experimentation Committee of the Oregon Health Sciences University and Genentech’s Institutional Animal Care and Use Committee.

**Determination of Vasorelaxation In Vitro**

Thoracic aortic rings were prepared, and organ bath technique was used as previously described. Briefly, thoracic aortic ring segments were mounted on 2 thin parallel stainless steel wire hooks in a 25-ml glass organ bath containing Krebs solution gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 37°C. The lower wire hook was attached to a micrometer-adjustable support leg and the upper to an isometric force transducer (Model FT03, Grass Instruments) to record changes in isometric force, which were amplified and recorded on a polygraph chart recorder (Model 79, Grass Instruments). A normalization technique using iterative-fitting software (VESTAND 2.1: Princeton University, Princeton, NJ) was performed to set the vascular ring segments at a pressure (100 mm Hg) comparable to that in vivo.

Ring segments were precontracted with U46619 at a concentration of 10<sup>–6</sup> mol/L. Our preliminary study showed that U46619 at 10<sup>–7</sup> mol/L induced ~60% to 80% of maximal contractile responses in the rat aortic ring segments. When the contraction reached a stable plateau (about 10 minutes), cumulative concentration-relaxation curves to VEGF<sub>165</sub>, the KDR-selective mutant (KDR-SM), Flt-1–selective mutant (Flt-SM), and placenta growth factor (PIGF) (Sigma) were established. The group of endothelium-denuded ring segments was also tested by VEGF<sub>165</sub>.

**Hemodynamic Measurement in Conscious Rats**

Rats were anesthetized with an intraperitoneal injection of ketamine 80 mg/kg and xylazine 10 mg/kg. Catheters filled with heparin-saline were implanted into the right femoral artery and vein for measurement of mean arterial pressure (MAP) and heart rate (HR), and for intravenous administration as described previously.

One day after catheterization, MAP and HR were measured in conscious, unrestrained rats with a Model P23XL pressure transducer (Viggo Spectramed) coupled to a polygraph. After a 45-minute stabilization period, rats received intravenous injection of VEGF<sub>165</sub>, KDR-SM, or Flt-SM at 15, 50, or 250 μg/kg in 200 μL saline, and MAP and HR were monitored for 1 hour after injection. The dose of 250 μg/kg is an effective dose as a single bolus for stimulation of angiogenesis in an animal model of limb ischemia.

**Statistical Analysis**

Results are expressed as mean±SEM. For binding affinity and cell proliferation assay, an F-test was performed to compare parameters in the 3 groups in a 4-parameter logistic model estimated by nonlinear regression. In vasorelaxation experiments, EC<sub>50</sub>, the effective concentration causing 50% of maximal relaxation, was determined from each concentration-relaxation curve by a sigmoid logistic curve-fitting equation: E=MA/(A+K<sub>P</sub>), where E is response, M is maximal contraction or relaxation, A is concentration, K is EC<sub>50</sub> concentration, and P is the slope parameter. A computer program was used for the curve fitting, and EC<sub>50</sub> values were determined and expressed as log<sub>10</sub> M. Statistical comparisons of the percentage relaxation under different treatments were tested by 2-way ANOVA with repeated measures, followed by post hoc Bonferroni test. Differences of the EC<sub>50</sub> and the maximal response values between groups were determined by 1-way ANOVA followed by post hoc Bonferroni test. In the in vivo study, 1-way ANOVA was performed to assess differences in parameters among groups at the
same time-point and to compare changes over time within each group. \(P<0.05\) was considered to be statistically significant.

**Results**

**Binding Affinities of Receptor-Selective VEGF Mutants**

The relative receptor binding affinities of the VEGF mutants were measured through competitive binding on a BIAcore system (see Methods). KDR-SM had similar wild-type VEGF binding affinity for KDR (48 pmol/L versus 41 pmol/L, Figure 1A), but bound Flt-1 receptors with 1800-fold lower affinity (18 nmol/L versus 10 pmol/L, Figure 1B). Flt-SM bound with wild-type affinity to Flt-1 (9 pmol/L versus 10 pmol/L, Figure 1B) but was 200-fold reduced in binding to KDR (8.3 nmol/L versus 41 pmol/L, Figure 1A).

**Effects of Receptor-Selective VEGF Mutants on Endothelial Proliferation**

Cell-proliferation assays were used to measure the mitogenic activity of VEGF on HUVEC cells. KDR-SM showed activity of vascular endothelial cell proliferation equivalent to wild-type VEGF165, whereas Flt-SM had no effect on proliferation of endothelial cells (Figure 1C). The affinities for the wild-type protein are in good agreement with those previously measured using a radioimmunoassay.32

**Effects of Receptor-Selective VEGF Mutants on Vasorelaxation**

The internal diameters of the aortic ring segments at an equivalent transmural pressure of 100 mm Hg (D100, millimeters) determined from the normalization procedure were similar in the five experimental groups (3.01 ± 0.08, 2.96 ± 0.07, 3.00 ± 0.09, 3.04 ± 0.03, and 2.78 ± 0.05 in the VEGF, KDR-SM, Flt-SM, PlGF, and endothelium-denuded groups, respectively). The equivalent transmural pressures of the aortic ring segments set at a resting diameter of 90% D100 (P90, mm Hg) were not different among the groups (75.3 ± 0.4, 74.7 ± 0.6, 74.2 ± 0.5, 75.6 ± 0.5, and 73.5 ± 0.6, respectively). The U46619-induced precontraction forces (g) were also similar (3.41 ± 0.09, 3.26 ± 0.09, 3.27 ± 0.10, 3.48 ± 0.07, and 3.03 ± 0.07 in the respective groups). The results indicated that the experimental conditions before adding reagents and/or handling endothelium were the same in the 5 groups.

Wild-type VEGF165, KDR-SM, Flt-SM, and PlGF induced a concentration-dependent relaxation in endothelium-intact aortic ring segments, but VEGF165 had almost no relaxing effect in endothelium-denuded aortic ring segments (Figure 2A). The relaxation induced by VEGF165 or KDR-SM was substantially more potent than that induced by Flt-SM or PlGF (\(P<0.01\), Figure 2A). Flt-MS and PlGF produced similar relaxation. Although there was no significant difference between the relaxation curves induced by VEGF165 and KDR-SM at low concentrations, the 2 relaxation curves separated at higher concentrations (Figure 2A). The KDR-SM–induced maximal relaxation was significantly reduced compared with that induced by wild-type VEGF165 at \(-8.5\) log10 M (73.2 ± 4.3% versus 87.2 ± 2.5%, \(P<0.05\), Figure 2B). In addition, for sensitivity, EC50 (−9.90 ± 0.13, −9.90 ± 0.19, −9.44 ± 0.09, and −9.59 ± 0.14 in the VEGF, KDR-SM, Flt-SM, and PlGF groups, respectively) was not significantly different in the endothelium-intact aortic ring segments among these groups.

**Effects of Receptor-Selective VEGF Mutants on Hypotension In Vivo**

Basal levels of MAP (106.3 ± 2.7, 106.3 ± 1.2, 106.8 ± 1.2, and 103.7 ± 1.7 mm Hg) and HR (376.0 ± 7.3, 373.7 ± 10.8, 376.9 ± 4.6, and 382.1 ± 3.3 bpm) in conscious rats were not significantly different among the vehicle (n = 10), Flt-SM (n = 30), KDR-SM (n = 37), and VEGF (n = 43) groups.
venous injection of \( \text{VEGF}_{165} \), KDR-SM, or Flt-SM resulted in a dose-related decrease in MAP and an increase in HR, whereas saline vehicle had no effect on MAP and HR (Figure 3). The hypotensive response began almost immediately, reached a nadir at 3 to 5 minutes, and lasted for 20 minutes (Figure 2C). The time course of the tachycardic response was similar to that of the hypertensive response, but lasted longer (Figure 2D). The hypotension induced by KDR-SM was greater than that induced by Flt-SM at the same dose (\( \text{Figure 2D} \)). The hypotension induced by KDR-SM-GP was similar to that of the hypertensive response, but lasted longer (Figure 2D). The hypotension induced by KDR-SM was greater than that induced by Flt-SM at the same dose (\( \text{Figure 2D} \)). The hypotension induced by KDR-SM was greater than that induced by Flt-SM at the same dose (\( \text{Figure 2D} \)).

**Discussion**

The present study showed that wild-type VEGF, KDR-selective, and Flt-1–selective mutants induced a concentration-dependent relaxation or vasodilation in arterial ring segments and a dose-related decrease in arterial pressure in conscious rats. However, the vasodilatory and hypotensive responses to the KDR-selective mutant was significantly less than those to wild-type VEGF but greater than those to the Flt-1–selective mutant. This is the first to demonstrate that both vasodilation and hypotension induced by VEGF are mediated by both KDR and Flt-1 receptors, but primarily by the KDR receptor.

It is known that vascular endothelial cells express both high-affinity VEGF receptors. To determine the role of each receptor in mediating the vasodilatory and hypotensive effects of VEGF, we used VEGF\(_{165}\) as wild-type VEGF and 2 mutants thereof engineered by using the competitive phage display technique to bind selectively to either the KDR or Flt-1 receptor. Using BIAcore competitive binding analysis, we found that, compared with wild-type VEGF, binding affinity of the KDR-selective VEGF mutant to the Flt-1 receptor was reduced by 1800-fold, whereas the Flt-selective mutant bound the KDR receptor 200-fold less tightly. The results are consistent with previous data on the binding affinity of the 2 mutants by using radiommunooassay and further confirm the high receptor selectivity of the mutants.29 The present study also demonstrated that the KDR-selective mutant caused proliferation of endothelial cells similar to wild-type VEGF, but the Flt-selective mutant had no effect on endothelial cell proliferation, indicating that KDR is the receptor that mediates VEGF-induced endothelial cell proliferation. The finding is in agreement with previous observations that KDR activation alone is sufficient to induce angiogenesis in vivo.29

In the present study, wild-type VEGF initiated a substantial vasodilation in arterial ring segments with intact endothelium, which was almost completely abolished when endothelium was denuded. This is consistent with the finding that VEGF-induced vasodilation is endothelium-dependent.6,9,30 The concentration-related relaxant response to the KDR-selective mutant was not different at lower concentrations from that to wild-type VEGF. At higher concentrations, which were equivalent to the in vivo dose range, the relaxant effect of the KDR-selective mutant was significantly less than that of wild-type VEGF. In contrast, the Flt-selective VEGF mutant or PlGF induced much less relaxant responses than wild-type VEGF or the KDR-selective mutant at all concentrations. PlGF shares 53% identity with the platelet-derived growth factor-like domain of VEGF.24 It has high binding affinity for Flt-1 but is unable to interact with KDR.25 It is noted that both the Flt-selective mutant and PlGF exerted a similar relaxant effect. Taken together, the data indicate that KDR is the major receptor for mediating VEGF-induced vasorelaxation or vasodilation, although both KDR and Flt-1 contribute to the biological effect.
Consistent with the in vitro findings, intravenous administration of wild-type VEGF and the mutants caused a dose-related decrease in arterial pressure in conscious rats. The pattern of hypotensive response to VEGF is similar to that observed in our previous studies.\textsuperscript{11,12} The present study showed that the hypotensive response to the KDR-selective mutant was significantly less than that to wild-type VEGF but greater than that to the Flt-selective mutant. After administering wild-type VEGF, the KDR-selective mutant, and the Flt-selective mutant at the therapeutic dose (250 $\mu$g/kg), the maximal reduction in MAP was $-22.0 \pm 2.7$ mm Hg, $-14.1 \pm 0.7$ mm Hg, and $-6.7 \pm 0.5$ mm Hg, respectively. The results indicate that the hypotensive effect of VEGF may be mediated by both receptors, but primarily by KDR. The greater potency of wild-type VEGF suggests that the maximal hypotensive effect may require heterodimerization of KDR and Flt-1. In addition, we cannot rule out the possibility of differences in pharmacokinetics contributing to the differences in hemodynamics between KDR-SM and wild-type VEGF. However, this is unlikely based on the similarity in structure and molecular weight of the 2 proteins and the fact that these differences were also directly observed in the in vitro vasorelaxation experiments.

It is known that hypotension is a major side effect of VEGF for treatment of ischemic diseases. The VEGF-induced hypotension may limit clinical use of VEGF and could be life-threatening in the setting of severe heart diseases. Because the KDR-selective mutant induces proliferation of vascular endothelial cells in vitro and angiogenesis in vivo similar to wild-type VEGF but with a hypotensive effect reduced by at least 36% ($-14$ versus $-22$ mm Hg at the therapeutic dose), the KDR-selective mutant may pose an alternative to wild-type VEGF for the treatment of ischemic diseases.

**Perspectives**

Based on structure-function studies that elucidate the 3D structure of VEGF and define its receptor-binding site, we...
have recently generated novel, highly receptor-selective VEGF mutants by phage-display technology.28 These mutants are valuable reagents for determining the relative importance of KDR and Flt-1 for biological effects of VEGF. Previous studies using the mutants have demonstrated that vascular endothelial migration, angiogenesis, and vascular permeability are mediated by KDR.29 The present study not only confirms that KDR mediates endothelial proliferation, but also shows that VEGF-induced vasorelaxation and hypotension are mediated primarily by KDR. In addition, given the limited changes from wild-type VEGF, these mutants may have the potential of therapeutic agents with fewer side effects than the native protein. Our finding suggests that KDR-SM may be considered an appropriate candidate for therapeutic angiogenesis because it induces proliferation and angiogenesis similar to wild-type VEGF but with attenuated hypotension. Further studies are under way to determine the relaxation of human arteries in response to the VEGF mutants. Clinical studies will be required to evaluate the angiogenic and hypotensive effects of KDR-SM compared with those of VEGF. Furthermore, because the function of Flt-1 receptor is largely unclear, more studies may be necessary to further explore the Flt-1 receptor biology.

References


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