Homeostatic Modulation of Cell Surface KDR and Flt1 Expression and Expression of the Vascular Endothelial Cell Growth Factor (VEGF) Receptor mRNAs by VEGF*

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Vascular endothelial cell growth factor (VEGF) is a potent angiogenic factor expressed during embryonic development, during wound healing, and in pathologies dependent on neovascularization, including cancer. Regulation of the receptor tyrosine kinases, KDR and Flt-1, to which VEGF binds on endothelial cells is incompletely understood. Chronic incubation with tumor-conditioned medium or VEGF diminished 125I-VEGF binding to human umbilical vein endothelial cells, incorporation of 125I-VEGF into covalent complexes with KDR and Flt1, and immunoreactive KDR in cell lysates. Receptor down-regulation desensitized VEGF activation of mitogen-activated protein kinase (extracellular signal-regulated kinases 1 and 2) and p38 mitogen-activated protein kinase. Preincubation with VEGF or tumor-conditioned medium down-regulated cell surface receptor expression but up-regulated KDR and Flt-1 mRNAs, an effect abrogated by a neutralizing VEGF antibody. Removal of VEGF from the medium led to recovery of 125I-VEGF binding and resensitization of human umbilical vein endothelial cells. Recovery of receptor expression was inhibited by cycloheximide, indicating that augmented VEGF receptor mRNAs, and not receptor recycling from a cytoplasmic pool, restored responsiveness. As the VEGF receptors promote endothelial cell survival, proliferation, and other events necessary for angiogenesis, the noncoordinate regulation of VEGF receptor proteins and mRNAs suggests that human umbilical vein endothelial cells are protected against inappropriate or prolonged loss of VEGF receptors by a homeostatic mechanism important to endothelial cell function.

Angiogenesis is an important component of embryonic vascular development, wound healing, and organ regeneration as well as pathological processes such as diabetic retinopathies, rheumatoid arthritis, and tumor growth and spread (1, 2). A network of growth factors and cytokines regulate angiogenesis. Some of these, such as tumor necrosis factor, transforming growth factor, angiotatin, and prostaglandin E₂, induce angiogenesis indirectly (1, 2), whereas other factors that play a role in blood vessel development, such as the acidic and basic fibroblast growth factors and platelet-derived growth factor, are mitogens for many cell types.

Vascular endothelial cell growth factor (VEGF)¹ is unique, being an endothelial cell-specific mitogen that promotes the proliferation and movement of endothelial cells, remodeling of the extracellular matrix, formation of capillary tubules, and vascular leakage (3–8). VEGF is produced by normal and transformed cells (9, 10) and plays an obligate role in the development of the fetal cardiovascular system as well as a significant role in the physiology of normal vascular and in the progression of cancer (11–15). Interference of VEGF action in vivo by administration of a monoclonal antibody that neutralizes VEGF activity or through the introduction of dominant negative VEGF receptor constructs inhibits tumorigenesis in animal models of colon cancer or glioblastoma (16–18). Thus, it is of considerable importance to understand how the VEGF/VEGFR receptor signaling system works and is regulated.

In the endothelium, VEGF exerts its actions by binding to two cell surface receptor tyrosine kinases, KDR (the human homolog of Flk1) and Flt1 (19–23). Flt1 binds VEGF with high affinity but is poorly expressed by endothelial cells, making it difficult to detect, whereas KDR binds VEGF with somewhat lower affinity but is the more highly expressed and readily detected receptor (24, 25). Mouse embryos deficient in Flk1 or Flt1 die between days 8.5 and 9.5, and the phenotypes of the knockout animals are distinct (26, 27). Endothelial and hematopoietic cell development are impaired in Flk1 null mice (26), whereas endothelial cells overgrow and blood vessels are disorganized in Flt1 null mice (27), indicating that the receptors have different functions. Consistent with this conclusion, VEGF mutants that selectively bind KDR/Flk1 induce endothelial cell proliferation and survival (28), whereas the ability of VEGF and placenta growth factor, a VEGF homolog that binds Flt1 but not KDR/Flk1, to induce chemotaxis and procoagulant activity associates these responses with signaling through Flt1 (29–31). Thus, the functions of VEGF are segregated between two structurally related receptors.

We previously demonstrated that VEGF promotes the tyrosine phosphorylation of a group of signaling molecules that contain SH2 domains and associated this process with endothelial cell proliferation (32). Subsequent studies with porcin

¹ The abbreviations used are: VEGF, vascular endothelial cell growth factor; KDR/Flk1, kinase domain-containing receptor/fetal liver kinase; Flt1, fms-like tyrosine kinase; MAPK, mitogen-activated protein kinase; HUVEC, human umbilical vein endothelial cell(s); MEM, modified Eagle’s medium; PBS, phosphate-buffered saline; bFGF, basic fibroblast growth factor; ERK, extracellular signal-regulated kinase.

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aortic endothelial cells and NIH 3T3 cells overexpressing KDR/Flk1 or Flt1, bovine aortic endothelial cells, immortalized endothelial cells, and HUVEC have begun to identify the signaling proteins downstream of KDR/Flk1 or Flt1 (25, 33–41). Thus, considerable insight into the functions and mechanisms of action of the VEGF receptors has been achieved.

The present study was aimed at defining how expression of the VEGF receptors is regulated. We demonstrate that VEGF or tumor-conditioned medium down-regulates cell surface expression of KDR and Flt1 on HUVEC. Such down-regulation diminishes the ability of VEGF to stimulate MAPK (ERK1 and ERK2) and p38 MAPK activities. Whereas VEGF or tumor-conditioned medium down-regulated cell surface receptor expression, expression of the mRNAs for KDR and Flt1 was increased by VEGF or tumor-conditioned medium. Once VEGF or tumor-conditioned medium were no longer present, HUVEC rapidly recovered receptor expression and responsiveness to VEGF, a process of resensitization that was fully dependent on protein synthesis. The demonstration that loss of cell surface VEGF receptor expression and responsiveness to VEGF occur coincidently with up-regulation of the VEGF receptor mRNAs indicates the operation of a homeostatic mechanism that replenishes cell surface receptor expression and restores HUVEC responsiveness once exogenous levels of VEGF are diminished.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human VEGF was a gift from Genentech, Inc. (South San Francisco, CA). Epidermal growth factor, bovine brain extract, and hydrocortisone were from Clonetics, Inc. (San Diego, CA). F-12, MEM, and MCDB 131 medium were from Life Technologies, Inc.

**Cell Culture**—The HeLa line of human cervical carcinoma cells was obtained from the ATCC (Manassas, VA); HM-7 and LS LiM6 human colon adenocarcinoma cells were kindly provided by Dr. Young Kim (University of California at San Francisco) (17). Cells were grown and maintained in a 1:1 mixture of F-12 and MEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 units/ml penicillin in a humidified incubator under 5% CO2 at 37°C. To isolate tumor-conditioned medium, confluent tumor cells were washed and then incubated in serum-free F-12/MEM (1:1) for 72 h. The conditioned medium was recovered from the cultures, centrifuged to remove cell debris, and stored at −70°C. To test the effects of conditioned medium, cells were grown in equal volumes of conditioned medium and F-12/MEM.

HUVEC isolated according to the method of Jaffe et al. (42) were grown on rat tail collagen type I (100 μg/ml)-coated tissue culture plates in MCDB 131 medium supplemented with 10% FBS, 12 μg/ml bovine brain extract, 10 ng/ml epidermal growth factor, and 1 μg/ml hydrocortisone in a humidified incubator under 5% CO2. Before exposure to VEGF or tumor-conditioned medium, HUVEC (passage 4–8) were washed with PBS and incubated with serum-free F-12/MEM for 8 h. The HUVEC were then incubated in serum-free F-12/MEM supplemented with human recombinant VEGF165 or a 1:1 mixture of tumor-conditioned medium and serum-free F-12/MEM.

Binding of 125I-VEGF to Receptors on the Surface of HUVEC—Human recombinant VEGF165 was coupled to 125I in an IODO-GEN-catalyzed reaction (43). Confluent HUVEC in 12- or 24-well plates were washed, incubated with serum-free F-12/MEM for 8 h at 37°C, and then treated with VEGF or tumor-conditioned medium as described in the legend to Fig. 3. The cells were washed with PBS and lysed into 200 μl of lysis buffer (1:1 mixture of radiimmune precipitation assay buffer and 2X Laemmli buffer). The lysates were boiled at 100°C for 3 min and centrifuged at 12,000 × g (4°C, 5 min) to remove debris. Equal amounts of protein from the lysates were fractionated on 7.5% polyacrylamide gels, and affinity labeled mRNAs were analyzed by the mobility shift assay. The autoradiographic bands were quantitated using a GS-700 imaging densitometer (Bio-Rad, Hercules, CA).

**RESULTS**

**VEGF and Tumor-conditioned Medium Down-regulate Cell Surface VEGF Receptor Expression**—To determine the effect of tumor-conditioned medium or VEGF on the expression of cell surface VEGF receptors, HUVEC were incubated with medium conditioned by HeLa or LS LiM6 cells or various concentrations of VEGF. The cells were then subjected to acidic washes to promote ligand dissociation, rendering cell surface VEGF receptors that had not been internalized available for interaction with subsequently applied 125I-VEGF. As illustrated by the data in Fig. 1A, pretreatment of HUVEC with tumor-condi-
tioned medium diminished the subsequent binding of $^{125}$I-VEGF 25 and 52%, respectively. Furthermore, 30 ng/ml VEGF diminished the subsequent binding of $^{125}$I-VEGF by 63% (Fig. 1), showing that VEGF down-regulates its own receptors and does so in a dose-dependent manner.

Affinity labeling assays also evaluated the effect of tumor-conditioned medium and VEGF on cell surface receptor expression. By using disuccinimydyl suberate, an organic cross-linking reagent, we covalently coupled $^{125}$I-VEGF to VEGF receptors on HUVEC, leading to the formation of a predominant complex of 245 kDa and a less abundant 195-kDa complex (Fig. 2). By subtracting the contribution of $^{125}$I-VEGF (monomeric molecular weight 14,000) to these complexes, it was determined that VEGF bound to species of 231 and 181 kDa, which correspond with the $M_r$ values of KDR and Flt1, respectively. Analysis by densitometry revealed that pretreatment of HUVEC with HeLa and LS LiM6 conditioned medium inhibited the subsequent incorporation of $^{125}$I-VEGF into the putative KDR complex by 32 and 70%, respectively, whereas 20 ng/ml VEGF decreased complex formation by 66% (Fig. 2). Such treatments produced a similar effect on the amount of Flt1 available for complex formation with $^{125}$I-VEGF. The diminished incorporation of $^{125}$I-VEGF into affinity-labeled complexes induced by tumor-conditioned medium or VEGF was comparable with the degree to which these agents diminished the specific binding of $^{125}$I-VEGF to HUVEC (Fig. 1).

Western blot analysis also evaluated the effect of VEGF or tumor-conditioned medium on cell surface VEGF receptor expression. In this procedure, lysates of control HUVEC or of HUVEC pretreated with tumor-conditioned medium or two different concentrations of VEGF were fractionated by SDS-PAGE and then transferred to nitrocellulose membranes, which were probed with an antibody directed against KDR (Fig. 3). Under basal conditions, HUVEC expressed low levels of the unglycosylated 150-kDa and higher levels of an intermediate 200-kDa form of KDR. The mature 230-kDa form of KDR was also detected at a level of expression between that of the unglycosylated and intermediate receptor forms (44). The molecular mass of the mature form of the receptor was similar to that of the KDR within the affinity-labeled complex described above (Fig. 2). Expression of the immature and mature forms of KDR was down-regulated by tumor-conditioned medium or VEGF, the latter in a dose-dependent manner (Fig. 3). We could not successfully probe Flt1 expression by Western blotting, as an antibody with the requisite sensitivity and specificity for such analysis is not available. Our results show that VEGF receptor expression is regulated by ligand-induced down-regulation.

**Effect of VEGF on MAPK Activity**—We have demonstrated that KDR transduces the VEGF signal that leads to activation of MAPK (40). This led us to conduct experiments to determine whether the down-regulation of KDR, demonstrated above, was associated with desensitization of the ability of VEGF to promote MAPK activation in HUVEC previously exposed to tumor-conditioned medium or VEGF. To address this possibility experimentally, we first treated HUVEC with various concentrations of VEGF for 10 min before proteins in cell lysates were fractionated by SDS-PAGE and transferred to nitrocellulose...
membranes, which were probed with the antibody that specifically recognizes dually phosphorylated (activated) MAPK. VEGF (1 ng/ml) increased the activity of both ERK1 and ERK2 in HUVEC. The stimulatory effect of VEGF on MAPK activation was maximal after exposure of HUVEC to 5–10 ng/ml VEGF, whereas higher mitogen concentrations elicited a diminished response. This latter effect may have been due to KDR down-regulation induced by exposure of HUVEC to the higher concentrations of VEGF (Fig. 4A).

Pretreatment of HUVEC with 0–45 ng/ml VEGF for 24 h diminished basal activity of MAPK in HUVEC in a dose-dependent manner. Furthermore, such pretreatment with 15 and 45 ng/ml VEGF diminished the ability of a second VEGF challenge to activate MAPK by 40 and 46%, respectively, when compared with cells not pretreated with VEGF (Fig. 4B). Thus, down-regulation of KDR resulted in a loss of HUVEC responsiveness to VEGF, as evidenced by desensitization of MAPK responsiveness to VEGF stimulation.

VEGF and Tumor-conditioned Medium Up-regulate Flt-1 and KDR mRNA Expression—Northern blot analysis was used to investigate the effect of tumor-conditioned medium and VEGF on the cellular content of the mRNAs for KDR and Flt-1. As illustrated by Fig. 5A, 24 h of exposure of HUVEC to medium conditioned by LS LiM6, HM-7, and HeLa cells increased the levels of Flt-1 mRNA by 2.5-, 1.7-, and 1.5-fold, respectively, whereas the level of KDR mRNA was increased 2.2-, 1.6-, and 1.8-fold over the same time course. The effects of VEGF on Flt-1 and KDR mRNA expression were also dependent on the concentration of VEGF to which HUVEC were exposed (Fig. 5B). Here, up-regulation of both mRNAs was detected after exposure of HUVEC to 10 ng/ml VEGF, and the mRNA levels increased progressively with exposure to higher (20 and 40 ng/ml) concentrations of VEGF.

Control experiments were conducted to demonstrate that the effects of VEGF on receptor modulation were specific. Thus, Northern blot analyses were conducted with human foreskin fibroblasts. These cells did not contain mRNAs for either KDR or Flt1 under basal conditions. Furthermore, exposure of these cells to VEGF did not result in the induction of mRNA for either of the VEGF receptors (data not shown). Thus, our results show that on HUVEC VEGF has opposite effects on the expression of cell surface VEGF receptors and the mRNAs that encode these receptors: the former are down-regulated in response to exposure to VEGF, whereas the effect of VEGF on the latter is quite the opposite, resulting in up-regulation.

Recovery of Cell Surface Receptor Expression—The time course over which cell surface VEGF receptor expression could be recovered after down-regulation was defined. To accomplish this, HUVEC were pretreated with 20 ng/ml VEGF for 24 h and then subjected to serum-free medium washes to remove VEGF from medium. Subsequently, the specific binding of 10 or 400 pM 125I-VEGF to the HUVEC was assayed over time. Within 2 h, half of the lost binding capacity of the HUVEC was recovered (Fig. 7A and B). Within 5 h, more than ½ of the initial cellular binding capacity had been recovered. The \( K_d \) with which Flt-1 binds VEGF has been estimated at 10–18 pM, whereas the \( K_d \) for binding to KDR is in the range of 400 pM (22, 23). Thus, binding assays conducted with 10 pM 125I-VEGF would preferentially assay Flt1 recovery, whereas binding assays conducted with 200–500 pM 125I-VEGF would assay for recovery of the more abundant VEGF receptor, KDR. The recovery of VEGF binding capacity assayed at very low and
higher concentrations of 125I-VEGF supports the conclusion that exposure of HUVEC to VEGF down-regulates both receptors, which are recovered after the agent inducing down-regulation is removed. Significantly, the protein synthesis inhibitor cycloheximide completely blocked receptor recovery, indicating that recycling from an internalized receptor pool or the presence of a preformed latent receptor population could not account for the recovery of VEGF receptor expression. Rather, such recovery is the result of new receptor synthesis from the up-regulated pools of KDR and Flt1 mRNAs. Some loss of cell surface receptor expression was observed in HUVEC treated with cycloheximide. We speculate that this phenomenon results from continued turnover of cell surface receptors during a time when replenishment from the internal mRNA pools could not occur.

**DISCUSSION**

The temporal and spatial expression of VEGF and VEGF receptors in the embryo and female reproductive system provides evidence that VEGF is involved in developmental and hormonally regulated angiogenesis (1, 2, 13, 14, 23). Indeed, the unique importance of this angiogenic factor is illustrated by the observations showing that exposure of cells to recombinant VEGF induces endothelial proliferation (40) and migration (68, 69), respectively. Moreover, such homologous receptor down-regulation desensitizes MAPK and p38 MAPK to activation by VEGF. Activation of MAPK and p38 MAPK are components of the mechanism through which VEGF induces endothelial proliferation (40) and migration (68, 69), respectively.

**Fig. 6. Effect of VEGF on KDR and Flt-1 mRNA expression.** Serum-starved HUVEC were incubated in the absence or presence of VEGF. Total RNA was isolated, and the expression of KDR and Flt-1 mRNAs was analyzed by Northern blot analysis. A, time course of the VEGF effect. B, dose response of the effect of VEGF after 24 h.

Generally, augmented VEGF expression is induced by hypoxic conditions and by growth factors, such as IGF-1 (52–55). As with VEGF, expression of the VEGF receptors is regulated. Flk1 is particularly abundant on the proliferating endothelial cells of vascular sprouts of embryonic and early postnatal brain; however, the level of Flk1 mRNA is dramatically reduced in adult brains in which endothelial cell proliferation has ceased (23). Augmented VEGF receptor expression occurs during wound healing (56), in ischemic eye disease (57), in the angiogenesis and stromal deposition associated with myocardial infarction (58), and in various cancers (17, 59–61). The conditions implicated in up-regulation of VEGF receptor expression appear the same as those responsible for up-regulation of VEGF. Thus, expression of the mRNAs for KDR and Flt1 is increased in lungs exposed to acute or chronic hypoxia (62). Gerber et al. (63) and Barleon et al. (64) found hypoxic induction of Flt1 mRNA expression in HUVEC; however, the former study assayed for but was unable to detect up-regulation of KDR. On the other hand, Waltenberger et al. (65) found that hypoxia induced KDR expression in HUVEC and in porcine aortic endothelial cells transfected so as to express KDR, which is not ordinarily present in such cells. Brogi et al. (66) have also documented up-regulation of KDR expression in HUVEC and microvascular endothelial cells exposed to medium conditioned by hypoxic myoblasts. A particularly interesting aspect of this investigation was the inability of various neutralizing antisera, including anti-VEGF, to abrogate KDR up-regulation, suggesting that an unidentified factor promoted this paracrine effect. Another report from Suzuki found that medium conditioned by hypoxic hepatoma cells was able to induce Flt1 mRNA expression but not that of KDR in HUVEC (66). VEGF itself is reported to up-regulate KDR in cultures of cerebral slices, but not in HUVEC (67), whereas homologous up-regulation of both VEGF receptors has been found in bovine adrenal cortex endothelial cells (44). The dissimilarities among research reports may be ascribed to differences in the cells and experimental conditions used for experimentation and to the technical difficulty in detecting Flt1, which is poorly expressed in endothelial cells (24, 25). However, the conclusion that exposure of normal or neoplastic cells to hypoxia induces VEGF, which modulates expression of its own receptors, appears generally consistent with most studies performed to date.

In the present work, the effects of VEGF and tumor-conditioned medium on cell surface VEGF receptor expression as well as the mRNAs for the VEGF receptors were investigated. Whereas most previous studies have presented observations suggesting unrelenting up-regulation of the VEGF/VEGF receptor system under circumstances demanding an angiogenic response from a host organism, our work suggests the operation of a homeostatic mechanism that tightly regulates cellular responsiveness to VEGF.

Support for a homeostatic regulatory view for signaling through the VEGF/VEGF receptor signaling system is found in observations showing that exposure of cells to recombinant VEGF or VEGF in tumor-conditioned medium provokes loss of cell surface receptor expression. Down-regulation of Flt1 and KDR is demonstrated by assays quantitating the specific binding of high and low concentrations of 125I-VEGF to HUVEC, affinity labeling, and, in the case of KDR (for which an adequate antibody is available), Western blot analysis. Functionally, such homologous receptor down-regulation desensitizes MAPK and p38 MAPK to activation by VEGF. Activation of MAPK and p38 MAPK are components of the mechanism through which VEGF induces endothelial proliferation (40) and migration (68, 69), respectively.
re-expressed at the surface of HUVEC. Recovery of receptor expression is complete within 3 h and reaches a level that closely approximates that present on the cell before exposure to VEGF. The recovery is not mediated by translocation of receptors from a preformed cytoplasmic pool, since it requires protein synthesis. Rather, down-regulation of cell surface VEGF receptor expression is accompanied by up-regulation of the mRNAs for KDR and Flt1. This augmented pool of mRNAs allows the rapid recovery of cell surface VEGF receptor expression once exogenous VEGF is removed from the HUVEC. Functionally, such up-regulation results in virtually completed recovery of HUVEC responsiveness to VEGF.

In addition to VEGF, a number of other peptide growth factors, including basic fibroblast growth factor (bFGF), hepatocyte growth factor, and transforming growth factor alpha, are believed to act as direct endothelial cell mitogens. bFGF is an angiogenic protein with potency comparable with that of VEGF (70). However, bFGF expression in primary human colorectal cancers is sparse, and immunoactivity resides principally in the stromal cells of these tumors (71). While bFGF lacks a signal sequence and is not likely to act as a carcinoma-derived endothelial growth factor in human colorectal cancer, bFGF can be detected in the extracellular matrix of many tissues and synergizes with VEGF in in vitro and in vivo assays of angiogenesis (72). Consequently, a contributory role of bFGF in colon cancer metastasis formation cannot be excluded, although it would seem unlikely to play a role in the assays described in the present report. Hepatocyte growth factor, also called scatter factor, stimulates endothelial cell proliferation and migration in vitro and is angiogenic in a rabbit cornea assay (73). The hepatocyte growth factor receptor, cMET, is overexpressed in colon cancers and may regulate the invasive behavior of these neoplasms (74). However, hepatocyte growth factor, the cMET ligand, appears to be expressed by stromal cells rather than the carcinoma cells in human colon cancer metastases.2 While transforming growth factor alpha is secreted by a number of human colon cancer cell lines, this growth factor and its receptor are expressed at comparable levels in benign and malignant gastrointestinal epithelium (75).

VEGF is involved in wound repair, inflammation, and tumor growth, processes in which cytokines are elaborated. TNF and transforming growth factor beta-1 modulate the expression of VEGF receptors (76–78), and TNF additionally inhibits VEGF activation of KDR and endothelial cell proliferative responses dependent on KDR by activating a protein-tyrosine phosphatase (79). However, the ability of a neutralizing antiserum to VEGF to block the ability of tumor-conditioned medium to induce up-regulation of the mRNAs for KDR or Flt1 suggests that VEGF is the predominant factor acting to modulate VEGF

2 D. Wang, D. B. Donner, and R. S. Warren, unpublished observations.
receptor expression in the isolated cell cultures studied here. VEGF is important to physiologic and pathologic processes dependent on neovascularization and is an endothelial cell survival factor (1, 2, 40, 80). Thus, it is reasonable to predict that HUVEC have mechanisms that would modulate VEGF responses, both down and up, through alterations of receptor expression. The present study demonstrates that down-regulation of receptor expression and desensitization of cellular receptor responsiveness is coupled with up-regulation of receptor mRNA expression, which endows HUVEC with the ability to rapidly replenish the cell surface with VEGF receptors and fully recover responsiveness to VEGF.

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