VEGF Exerts an Angiogenesis-Independent Function in Cancer Cells to Promote Their Malignant Progression

Ying Cao1, Guangqi E1, Enfeng Wang1, Krishnendu Pal1, Shamit K. Dutta1, Dafna Bar-Sagi2, and Debabrata Mukhopadhyay1

Abstract
VEGF/vascular permeability factor (VEGF/VPF or VEGF-A) is a pivotal driver of cancer angiogenesis that is a central therapeutic target in the treatment of malignancy. However, little work has been devoted to investigating functions of VEGF that are independent of its proangiogenic activity. Here, we report that VEGF produced by tumor cells acts in an autocrine manner to promote cell growth through interaction with the VEGF receptor neuropilin-1 (NRP-1). Reducing VEGF expression by tumor cells induced a differentiated phenotype in vitro and inhibited tumor forming capacity in vivo, independent of effects on angiogenesis. Autocrine activation of tumor cell growth was dependent on signaling through NRP-1, and Ras was determined to be a critical effector signaling molecule downstream of NRP-1. Our findings define a novel function for VEGF in dedifferentiation of tumor cells expanding its role in cancer beyond its known proangiogenic function. Cancer Res; 72(16); 1–7. ©2012 AACR.

Introduction
VEGF/vascular permeability factor (VEGF/VPF, VEGF-A) is a central regulator of physiologic and pathologic angiogenesis such as tumor angiogenesis (1, 2). The tumor cells secrete a high level of VEGF and other proangiogenic factors and promote tumor angiogenesis to maintain the oxygen and nutrition supply in the tumor microenvironment. Treatments involving blocking VEGF to inhibit tumor angiogenesis have gained advances in cancer therapeutics (2). The anti-VEGF monoclonal antibody bevacizumab inhibits tumor angiogenesis by blocking VEGF binding to the VEGF receptor 1/2 (VEGFR-1/2) and shows improvement in metastatic colorectal cancer, non–small cell lung cancer, metastatic renal cell carcinoma (RCC), and others (3).

Clear cell RCC is typically highly vascular in nature due to VHL gene mutation. VHL mutation stabilizes HIF1α/2α, which are transcription factors that can induce hypoxia-related gene expression, including constitutive VEGF expression. This formed the rationale for choosing RCC to test the efficacy of anti-VEGF therapy. In fact that despite being approved by the U.S. Food and Drug Administration for RCC treatment, bevacizumab showed only a marginal improvement in median progression-free survival (PFS) but not a statistically significant advantage in overall survival (4). In addition, conflicting reports suggested that bevacizumab could accelerate the tumorigenicity of RCC, which also compounds the problem (5).

To date, 3 VEGF receptors have been discovered that include VEGFR-1/2 and neuropilin-1 (NRP-1). VEGFR-1/2 are both receptor tyrosine kinases. Unlike VEGFR-1/2, NRP-1 lacks kinase domain and is a less defined multifunctional receptor for several ligands including VEGF (6, 7). Bevacizumab inhibits VEGF interactions with VEGFR-1/2, but not with NRP-1. Contrary to the endothelial cells, VEGFR-1/2 expression is not always detectable in certain types of tumor cells (6, 8, 9). In contrast, NRP-1 is the predominantly expressed VEGF receptor expressed in tumor cells, and its expression correlates closely with advanced tumor stages and poor patient prognosis in specific tumor types (7). There are reports that showed VEGF controls tumorigenesis properties through an autocrine fashion (9–11) but the mechanisms were still not clear, especially in the case of tumor cells, as they do not express VEGFR-1/2. In the present study, we intend to test our hypothesis that VEGF acts through an autocrine signaling pathway mediated by NRP-1 in cancer cells to promote tumorigenesis.

Materials and Methods

Cell culture
Human RCC cell lines 786-O and A-498 were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) + 10% FBS and minimum essential medium + 10% FBS, respectively. Breast cancer cell line MCF7 was maintained in DMEM + 10% FBS. All cell lines were purchased from American Type Culture Collection.

Plasmids
Retroviral pZIP-KRas (G12V) and pMMp-NRP-1 plasmid vectors were used in this experiment. Retrovirus was prepared.
by transfecting the retroviral plasmid together with packaging plasmids into 293T cells using PolyFect (QIAGEN).

**siRNA and short hairpin RNA transfection**

siRNAs for human NRP-1, VEGF, and control were from QIAGEN. siRNA transfection was conducted with HiPerFect (QIAGEN) following the manufacturer’s instructions. pLKO.1 and TRIPZ lentiviral shRNA vectors for human VEGF, SOS1/2, and nontargeting control were from Open Biosystems and were prepared as previously described (8). Cells were infected with lentiviral shRNA and after puromycin selection, stable selected cells were used for the experiments. The sequences of siRNA and shRNA were listed in Supplementary Table S1.

For the Tet-On-inducible pTRIPZ shRNA, 0.2 µg/ml of doxycycline was used to induce shRNA expression in the stable infected cells.

**Subcutaneous tumor model**

Subcutaneous tumor models were established in nude mice as described before (8). All animal work was conducted under the protocols approved by the Mayo Clinic Institutional Animal Care and Use Committee. Briefly, 2 × 10^6 cells were injected into the right flank of 6- to 8-week-old female nude mice (purchased from the NIH, Bethesda, MD). Tumor size was measured every week. In the case of GFP- or red fluorescent protein (RFP)-labeled cells, the GFP and RFP fluorescence signals were monitored and quantified using the IVIS system 200 series (Xenogen Corp).

**Ras activity assay**

Cells were lysed and Raf-1 RBD agarose bead pull-down assay was conducted. Samples were run on an SDS-PAGE and immunoblotting was conducted with the anti-Ras antibody (Millipore).

**Statistical analysis**

The independent samples t test was used to test the probability of significant differences between 2 groups. Values of P < 0.05 were considered significant.

Additional experimental details are available in the Supplementary Methods.

**Results and Discussion**

We previously showed that RCC cells express NRP-1, but not VEGFR-1/2, by reverse transcription PCR (8) and confirmed this again by Western blot in this experiment (Supplementary Fig. S1A). Two RCC cell lines A-498 and 786-O, which harbor the VHL mutation and therefore express a high level of VEGF, were chosen for this study. VEGF was knocked down via lentiviral shRNA. Two shRNA pools (VEGFsh3 and VEGFsh4) with knockdown efficiencies greater than 90% were selected for the studies (Supplementary Fig. S1B and S1C). Control- and VEGF shRNA–transfected A-498 and 786-O cells were injected subcutaneously into nude mice. Eight weeks later, the tumors were analyzed. VEGF knockdown significantly decreased tumor development (1,298.8 ± 244.6 vs. 35.9 ± 34.3; P < 0.001 for 786-O-Consh vs. 786-O-VEGFsh4 respectively; 625.3 ± 473.3 vs. 16.0 ± 9.2; P = 0.02 for A-498-Consh vs. A-498-VEGFsh3, respectively; mean ± SD, n = 5; Fig. 1A). As expected, the VEGF-knockdown tumors showed significantly less vascular density and less proliferation than control tumors [by Von Willebrand factor (vWF) and proliferating cell nuclear antigen (PCNA) staining (Fig. 1B and Supplementary Fig. S1D)]. Interestingly, the VEGF shRNA–expressing tumors showed increased Ksp-cadherin expression, a marker of mature kidney epithelial cells, suggesting that the tumors were undergoing differentiation (Fig. 1B, Supplementary Fig. S1E).

We further examined the consequences of reduced endogenous VEGF expression in cancer cells with respect to differentiation phenotypes (also was known as mesenchymal-to-epithelial transition) under in vitro conditions. Upon VEGF knockdown, cancer cells showed increased Ksp-cadherin and E-cadherin expression whereas Snail and α-smooth muscle actin (α-SMA) levels decreased (Fig. 1C). This profile corresponds to a typical differentiation phenotype of kidney epithelial cells. VEGF knockdown also decreased cancer cell invasion ability (35.4% and 57.9% for 786-O-VEGFsh and A-498-VEGFsh, respectively, as compared with each control; both P < 0.01) and in vitro anchorage-independent colony formation ability (36.6% and 35.9% for 786-O-VEGFsh and A-498-VEGFsh, respectively, as compared with each control; both P < 0.05; Fig. 1D, Supplementary Fig. S2). However, loss of VEGF did not significantly affect cancer cell proliferation or survival in vitro (Supplementary Fig. S3).

As described earlier, the loss of VEGF in cancer cells led to reduced tumor formation in vivo. One intuitive explanation is that inhibition of angiogenesis limited tumor growth and created a “dormant” tumor. However, based on our in vitro data, which suggested the cancer cells were less tumorigenic after VEGF knockdown, we sought to investigate whether VEGF had a tumorigenic function in addition to its proangiogenic role in vivo. A major obstacle in this line of inquiry was maintaining normal angiogenesis during tumor growth. To overcome this obstacle and investigate the possibility that VEGF has a tumorigenic role, we designed 2 independent in vivo experimental approaches.

First, we used a Tet-On–inducible VEGF shRNA system (Fig. 2A). Upon doxycycline-inducing expression of VEGF shRNA, endogenous VEGF mRNA level was downregulated significantly within 3 days. When doxycycline was removed at day 3, VEGF mRNA level was restored by day 7 (Fig. 2A). In accordance with regular VEGF shRNA results, doxycycline-induced shRNA-expressing cells [referred to as (+)Dox] exhibited less anchorage-independent growth ability (Supplementary Fig. S4) as well as a greater differentiation phenotype compared with noninduced cells [referred to as (−)Dox; Fig. 2B]. Despite restoration of VEGF levels by day 7 after removing doxycycline (referred to as post-Dox), the cells were still in a differentiated stage (Fig. 2B). Therefore, (−)Dox cells and post-Dox cells expressed the same level of VEGF, but post-Dox cells had already started differentiating during the 3 days of doxycycline-induced VEGF shRNA expression and could not completely revert back to an undifferentiated state after withdrawal of the doxycycline. Thus, (−)Dox and post-Dox cells would have different tumorigenic ability (Fig. 2C; schematic illustration). As expected, tumors formed by post-Dox A-498 cells were
much smaller than those formed by (-)Dox cells [799.6 ± 121 vs. 265.3 ± 264.5, P = 0.003, for A-498 (-)Dox vs. A-498 post-Dox at 8 weeks, respectively; mean ± SD, n = 5; Fig. 2D]. This result suggests that the loss of VEGF in tumor cells reduced their tumorigenic ability. As doxycycline can be used as an anticancer drug, we tested the effect of doxycycline on wild-type A-498 cells. When cells were treated with a relatively low concentration of 0.2 µg/mL doxycycline, which we used for shRNA induction, A-498 cells did not show any significant differences in cell proliferation, apoptosis, and differentiation marker expression in vitro and tumor growth in vivo. (Supplementary Fig. S5). This ruled out the possibility that the less tumor formation by post-Dox cells was due to doxycycline treatment.

In the second in vivo experiment, we labeled A-498 cells with GFP or RFP (referred to as A-498-GFP and A-498-RFP cells) and then knocked down VEGF in the A-498-RFP cells using VEGF shRNA-expressing lentivirus. Equal numbers of A-498-GFP and A-498-RFP-VEGFsh cells were mixed and injected subcutaneously into nude mice (n = 12). A-498-GFP and A-498-RFP-Consh cells were also mixed and injected as a control group (n = 12). Theoretically, in the control group, the A-498-GFP and A-498-RFP-Consh cell numbers should be similar as they have the same tumorigenic ability. In the experimental group, if VEGF knockdown does not affect the cancer cell tumorigenic ability in vivo, the cell number of A-498-RFP-VEGFsh should be similar to A-498-GFP; conversely, if the numbers of A-498-GFP and A-498-RFP-VEGFsh cells were not equal, then they would not have the same tumorigenic ability (Fig. 3A, schematic illustration). After 5 weeks of tumor formation, we used a Xenogen fluorescent imaging system to quantify GFP and RFP signal intensities in the whole tumor. As shown (Fig. 3B and Supplementary Fig. S6), GFP signals in both groups have similar intensities (1,144.8 ± 170.6 vs. 999.75 ± 255.93; P = 0.340 for A-498-GFP/A-498-RFP-Consh vs. A-498-GFP/A-498-RFP-VEGFsh, mean ± SD), but RFP signal intensity was significantly less in the A-498-GFP/A-498-RFP-VEGFsh group (7,377.5 ± 3,681.9) as compared with the A-498-GFP/A-498-RFP-Consh group (19,820 ± 4,997.2; P =
By sectioning the tumors, we observed that the number of the RFP cells was similar to the GFP cells in the A-498-GFP/A-498-RFP-Consh groups, but the number of RFP cells was much less than the GFP cells in the A-498-GFP/A-498-RFP-VEGFsh group (Supplementary Fig. S7A). Moreover, in both groups, the GFP- and RFP-labeled tumor cells were homogeneously distributed in the later stages of tumor growth and that the vascular density displayed was well distributed in both groups (Supplementary Fig. S7A and S7B), and this observation ruled out the possibility that the GFP cells were not uniformly providing VEGF to the VEGF-deficient RFP cells within the tumor microenvironment. These results suggest that the A-498-RFP-VEGFsh cells were less tumorigenic than the A-498-RFP-Consh cells and A-498-GFP cells. This evidence supports the hypothesis that VEGF promotes the tumorigenic phenotype within tumor cells through autocrine signaling independently of its proangiogenic function.

NRP-1 is a multifunctional receptor for several ligands including SEMA3, VEGF, and TGF-β (6, 7, 12). Previous studies by our group proved that NRP-1 mediated the migration and survival signaling of VEGF in endothelial cell (13, 14). We have also shown that NRP-1 controls renal cancer cell differentiation and tumorigenesis (8). We propose that NRP-1 mediates VEGF-induced tumorigenesis in cancer cells. NRP-1 does not have a kinase function, and several small GTPases including RhoA and Rac-1 have been reported to be the downstream effectors of NRP-1 (8, 13). To elucidate the effectors through which VEGF promotes tumorigenesis downstream of NRP-1, we examined whether Ras, the classical small GTPase and the connector of multiple signaling pathways, is involved in the VEGF/NRP-1 signaling transduction. We observed that VEGF can activate Ras in 786-O cells, which do not express VEGFR-1/2, in a time-dependent manner (Fig. 4A, left). Furthermore, overexpression of NRP-1 or addition of VEGF increased Ras activity in 786-O cells, whereas knockdown of either NRP-1 or VEGF was sufficient to decrease Ras activity (Fig. 4A, right). The phosphorylation of ERK1/2 and AKT proteins, which are the downstream of active Ras signaling, were upregulated by VEGF stimulation and downregulated by NRP-1/VEGF siRNA knockdown (Supplementary Fig. S8A and S8B). Next, we sought to determine whether introducing the dominant active mutant k-Ras (G12V) to VEGF shRNA–expressing cells could override the differentiation phenotype. Indeed, as shown in Fig. 4C, k-Ras (G12V) expression was sufficient to reverse the
differentiation effect of VEGF knockdown in 786-O cells (Fig. 4B). MEK1 inhibitor PD98059 could also induce the 786-O cells to express the differentiation markers (Supplementary Fig. S8C). To further validate the role of the NRP-1 in the Ras activation by VEGF, we showed in a loss of function experiment that knockdown of NRP-1 in 786-O cells eliminated the Ras activation by VEGF (Fig. 4C left). In comparison, in a gain-of-function experiment, we used the low NRP-1 and VEGF-expressing breast cancer cell line MCF-7 and showed that both overexpression of NRP-1 and VEGF were required to activate Ras (Fig. 4C; right). Taken together, our data suggest that VEGF functions as a tumor-promoting gene through NRP-1 in a VEGFR-1/2–independent manner and signals through the downstream effector Ras.

Ras activity is controlled by guanine nucleotide exchange factors (GEF) and GTPase-activating proteins. We used an shRNA capable of knocking down both SOS1 and 2, which are RasGEFs, and showed that VEGF could not activate Ras in the absence of SOS1/2 (Fig. 4D). In addition, knockdown of SOS1/2-induced cancer cells expressed differentiation markers consistent with VEGF knockdown (Supplementary Fig. S9). These results indicate that SOS proteins may be required for the VEGF-induced Ras activation. It is still not clear whether NRP-1 mediates Ras activation directly or not, and the exact mechanism requires further study. Intracellular and extracellular domains are both important for NRP-1 function. It is known that the intracellular domain of NRP-1 does not possess a kinase function, and GAIP interacting protein, C terminus (GIPC) is the only known NRP-1 intracellular domain–interacting protein so far described (15). Also, evidence exists that the extracellular domain of NRP-1 is responsible for binding with several transmembrane proteins (e.g., plexins, integri-
α5) and is crucial for NRP-1 function (16, 17). Given that these NRP-1 association proteins have been linked to Ras activation (18, 19), it is presumable that NRP-1 may need adapters or partner proteins to activate Ras.

In this report, we show that VEGF produced by tumor cells can act in an autocrine manner on the tumor cells themselves. This novel finding extends our understanding about the role of VEGF in tumorigenesis, which may translate into modified cancer treatments in the near future. Furthermore, our recent research results showed that the way of autocrine/endogenous VEGF acting in the cells differed from the paracrine/exogenous VEGF (20). The paracrine VEGF can be more easily blocked by the antibodies than the autocrine VEGF. A future goal is to develop a more efficient method of blocking VEGF and its associated mediators and prevent VEGF from acting in an autocrine fashion on tumor cells to promote tumorigenesis.

Disclosure of Potential Conflicts of Interest

D. Mukhopadhyay is a visiting professor of King Saud University. While preparing this manuscript, another group reported that VEGF could regulate the initiation and stemness of skin tumors (11). No potential conflicts of interest were disclosed by other authors.

Authors’ Contributions

Conception and design: Y. Cao, Guangqi E, D. Mukhopadhyay

Development of methodology: D. Bar-Sagi, D. Mukhopadhyay

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Cao, K. Pal, D. Mukhopadhyay

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Cao, D. Mukhopadhyay

Writing, review, and/or revision of the manuscript: Y. Cao, Guangqi E, D. Mukhopadhyay

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Wang, S.K. Dutta, D. Mukhopadhyay

Study supervision: D. Mukhopadhyay

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