Vascular Endothelial Growth Factor-C Protects Prostate Cancer Cells from Oxidative Stress by the Activation of Mammalian Target of Rapamycin Complex-2 and AKT-1

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Abstract
Recurrence and subsequent metastatic transformation of cancer develops from a subset of malignant cells, which show the ability to resist stress and to adapt to a changing microenvironment. These tumor cells have distinctly different growth factor pathways and antiapoptotic responses compared with the vast majority of cancer cells. Long-term therapeutic success can only be achieved by identifying and targeting factors and signaling cascades that help these cells survive during stress. Both microarray and immunohistochemical analysis on human prostate cancer tissue samples have shown an increased expression of vascular endothelial growth factor-C (VEGF-C) in metastatic prostate cancer. We have discovered that VEGF-C acts directly on prostate cancer cells to protect them against oxidative stress. VEGF-C increased the survival of prostate cancer cells during hydrogen peroxide stress by the activation of AKT-1/protein kinase B. This activation was mediated by mammalian target of rapamycin-complex-2 and was not observed in the absence of oxidative stress. Finally, the transmembrane nontyrosine kinase receptor neuropilin-2 was found to be essential for the VEGF-C-mediated AKT-1 activation. Indeed, our findings suggest a novel and distinct function of VEGF-C in protecting cancer cells from stress-induced cell death, thereby facilitating cancer recurrence and metastasis. This is distinctly different from the known function of VEGF-C in inducing lymphangiogenesis. (Cancer Res 2009;69(15):6042–8)

Introduction
The increased ability of tumor cells to survive under stress is important for cancer progression, subsequent metastatic transformation, and therapy resistance (1). The stress can be incurred by the microenvironment surrounding the cancer cells and/or by therapeutic interventions used to treat cancer (2). Alternative growth factor pathways and adaptive up-regulation of antiapoptotic mechanisms are the primary causes for cancer cells to evade death during unfavorable conditions (3). Reactive oxygen species (ROS) and cellular oxidative stress have long been understood to be associated with cancer, although this association is often complex and paradoxical (4). Oxidative stress may induce cancer, and receptor tyrosine kinase–activated cell cycle progression often involves an increase in ROS signaling (3, 5). On the other hand, the antioxidant system in cancer cells increases paradoxically as transformed cells generate higher level of ROS compared with normal cells (6). Indeed, several therapeutic agents promote cell death by increasing oxidative stress (7, 8). Several agents, including radiotherapy, chemotherapeutic drugs such as paclitaxel, histone deacetylase inhibitors, proteasome inhibitors, as well as redox cycling agents, increase oxidative stress (6, 9). This common effect suggests that cancer cells are more vulnerable to oxidant stress because they function with an enhanced basal level of ROS-mediated signaling (10). Therefore, by further increasing the ROS level by these therapeutic agents, cancer cells are pushed beyond the breaking point of damaging cellular organelle and DNA and undergo apoptosis. Thus, a recurrence of the tumor after therapy likely results from a subset of cells that have developed the ability to overcome oxidative damage (3, 9). These cells also acquire a metastatic phenotype, which becomes the major cause of death due to the cancer (1, 2).

The tumor microenvironment, which is highly heterogeneous in terms of nutrient supply, pH, and oxygenation, plays a major role in the ability of tumor cells to resist stress by altering gene expression and cellular functions of cancer cells (11, 12). The presence of inflammatory cells, myofibroblasts, and endothelial cells in the tumor microenvironment supports not only the growth of tumor cells but also its ability to resist stress and therefore facilitates metastasis (11). The metastatic cascade is thus orchestrated by signals from both a tumor and its microenvironment. In this context, the growth factors and cytokines that facilitate communication between the tumor cells and the stromal compartment are particularly important. For instance, members of the vascular endothelial growth factor (VEGF) gene family are known to execute a functional communication between the tumor cell and its surrounding environment (13, 14).

VEGF-C seems to be particularly unique among the VEGF family members (15, 16) because of its involvement in the lymph node metastasis (17). Several studies have reported a significant correlation between the expression of VEGF-C and lymph node metastases in human prostate carcinoma (18–21). The cancer microarray profile database Oncomine also describes a significant up-regulation of VEGF-C mRNA in human metastatic prostate cancer tissue specimens (22, 23). VEGF-C functions by activating its cognate tyrosine kinase receptors VEGF-R3 (Flt4; ref. 24) and VEGF-R2 (KDR) and the nontyrosine kinase receptor neuropilin-2 (NRP-2; ref. 25). These receptors were identified initially on lymphatic endothelial cells (26), and one of the known
functions of VEGF-C is to promote the formation of new lymphatic vessels by inducing the proliferation, migration, and sprout formation of existing lymphatic endothelial cells, a process called lymphangiogenesis (27). It has been postulated that, by inducing lymphangiogenesis, VEGF-C facilitates lymph node metastasis (28). Furthermore, several reports now point to functions for VEGF-C, which are independent of lymphangiogenesis and instead are important for cancer progression. For example, VEGF-C can stimulate the proliferation and migration of Kaposi’s sarcoma cells and also the proliferation and survival of leukemia cells (29). Lack of lymphangiogenesis has been reported in uveal melanoma despite high VEGF-C expression (15). More importantly, VEGF-C is often overexpressed in glioblastoma patients, although brain tissue is void of lymphatics (30). In a recent report, it has been postulated that the risk of childhood neuroblastoma treatment failure (progression or relapse) as well as tumor-related death was found to be significant in VEGF-C–positive patients (31). VEGF-C has also been suggested to be a trophic factor for neural progenitors in the vertebrate embryonic brain (32). An autocrine function of VEGF-C to promote the invasion and metastasis of lung, breast, and gastric cancers has also been reported (33–35).

In this study, we have identified a survival-promoting function of VEGF-C on prostate cancer cells under severe oxidative stress conditions. We have also delineated the underlying molecular mechanism for this stress-resistant function of VEGF-C, which involves the activation of mammalian target of rapamycin complex-2 (mTORC-2) and AKT-1. The nontyrosine kinase VEGF-C receptor NRP-2 was identified as an upstream component in this pathway. Our findings therefore provide a novel mechanism by which VEGF-C protects cancer cells from stress-induced cell death. It also sheds new light on the upstream events of mTORC-2 activation.

Figure 1. A, Western blot of LNCaP, LNCaP C4-2, and PC3 whole-cell lysates for the protein expression of NRP-2 and VEGF-R3. B and C, apoptosis assays with H2O2 (3 mmol/L)–treated LNCaP C4-2 cells for 5 h with prior addition of increasing concentrations of VEGF-C. Cells were serum starved overnight before the addition of VEGF-C and H2O2. B, graphical representation of the apoptotic experiment after evaluation of three independent results. Dead cells were counted in 10 randomly selected high-power (40 ×) fields (HPF) for each experimental condition. We calculated the average of all the fields for each experimental condition. C, cell death was measured by PI (red) and YO-PRO (green). Hoechst staining was used to visualize the nucleus.
Materials and Methods

Cell culture. Human prostate cancer cell lines LNCaP (American Type Culture Collection), LNCaP C4-2 (ViroMed Laboratories), and PC3 (American Type Culture Collection) were cultured at 37°C in RPMI 1640 with 1-glutamine (Mediatech, Inc.) supplemented with penicillin/streptomycin and containing either 10% fetal bovine serum (HyClone Laboratories, Fisher Scientific). Cells were serum starved overnight before adding recombinant wild-type VEGF-C (R&D Systems, Inc.) for 9 h. After 4 h of VEGF-C incubation, hydrogen peroxide (Sigma-Aldrich) at different concentration was added for another 5 h.

Immunoprecipitation and Western blot assay. Immunoprecipitation was performed with 0.75 mg of total cellular protein from whole cell extracts with antibody (1 μg) directed against mTOR (Cell Signaling Technology) and pulled down by protein A-agarose beads (Pfizer-Pharmacia). Western blots were conducted using antibodies against phospho-AKT-1 (Ser473; Upstate-Millipore), phospho-AKT (Thr308), AKT-1, phospho–glycogen synthase kinase-3β (GSK-3β), phospho–FOXO-1, 4EBP-1, S6, mTOR, rictor, phospho–4EBP-1, phospho–S6 (Cell Signaling Technology), GSK-3β (BD Biosciences), NRP-2 (R&D Systems), rho-GDI (Santa Cruz Biotechnology, Inc.) and β-actin (Sigma-Aldrich).

Apoptosis assay. Vybrant Apoptosis Assay Kit #7, purchased from Molecular Probes-Invitrogen Detection Technologies, was used according to the manufacturer’s protocol. Briefly, LNCaP and LNCaP C4-2 cells (2 × 10^6 per well, six-well plate) were serum starved overnight followed by recombinant VEGF-C and hydrogen peroxide addition as mentioned in "Cell Culture" section of Materials and Methods. PC3 cells were transfected with VEGF-C–specific small interfering RNA (siRNA) for 72 h before collection of whole-cell extract or total RNA.

Development of VEGF-C–expressing stable clones of LNCaP C4-2. Mature form of VEGF-C–expressing plasmid (pSecTag2BVEGF-C, a kind gift from Dr. Mihaela Skobe, Mount Sinai School of Medicine, New York, NY) was transfected to LNCaP C4-2 cells. For vector-only control, pSecTag2B plasmid with no insert was transfected in parallel to another set of LNCaP C4-2 cells. Cells were selected with zeocin. Multiple stable clones of LNCaP C4-2 cell line expressing varying levels of VEGF-C will be isolated and characterized.

Results and Discussion

VEGF-C receptors are expressed in prostate cancer cells. Receptors for VEGF-C have been characterized on several cancer cells (34, 36), suggesting the possibility of cancer cell–specific functions of VEGF-C. We have detected the VEGF-C receptor NRP-2 in prostate cancer cell lines such as LNCaP, LNCaP C4-2, and PC3 (Fig. 1A). LNCaP C4-2 is a hormone-refractory or castration-recurrent prostate cancer cell and is more metastatic than its syngeneic parental cell line LNCaP. This cell line was developed by isolating tumor cells from the regional lymph nodes after injecting LNCaP cells orthotopically into castrated mice (37). LNCaP C4-2 cells are not dependent on androgen for their growth, although they express androgen receptor. LNCaP C4-2 cells are not dependent on androgen for their growth, although they express androgen receptor.
were isolated from a site of bone metastasis. These cells do not express androgen receptor. Both PC3 and LNCaP C4-2 express higher levels of NRP-2 compared with LNCaP. The other known VEGF-C receptors, VEGF-R3 and VEGF-R2 (data not shown), were detectable in significantly lower levels in LNCaP and LNCaP C4-2.

**VEGF-C increases prostate cancer cell survival under oxidative stress.** The presence of VEGF-C receptors on prostate cancer cells led us to look for a cancer cell–specific function of VEGF-C. LNCaP C4-2 was selected initially because it is an androgen-responsive and androgen receptor–expressing prostate cancer cell line that recapitulates many features of hormone-refractory human prostate cancers. This cell line also expresses high levels of the VEGF-C receptor NRP-2, which we subsequently identified to be critical for VEGF-C function. We have observed an increase in apoptosis of LNCaP C4-2 cells when incubated for 5 hours with increasing concentrations of H2O2 (Supplementary Fig. S1). To investigate the survival-promoting role of VEGF-C, we first incubated serum-starved LNCaP C4-2 cells with increasing concentrations of recombinant VEGF-C (9 hours; R&D Systems). After 4 hours of VEGF-C addition, cells were treated with a fixed dose of H2O2 (3 mmol/L) for the remaining 5 hours. Cell death was measured by the YO-PRO-1/PI apoptosis assay using fluorescence microscopy. Our results (Fig. 1B and C) suggested that prior addition of VEGF-C to these cells protects them from H2O2-induced cell death in a dose-dependent manner. In this respect, it should be noted that LNCaP C4-2 and its syngeneic parental line LNCaP express low level of endogenous VEGF-C, which showed moderate protection ability against a much lower (0.25 mmol/L) concentration of H2O2. Therefore, the total level of VEGF-C should be higher than that secreted by the cancer cells alone. Equivalent concentrations of VEGF-C have been used by other investigators (39), who showed that these concentrations of VEGF-C are physiologically relevant.

**VEGF-C restores activation of AKT-1 in prostate cancer cells under oxidative stress.** To evaluate the signaling pathways important for VEGF-C–mediated survival in prostate cancer cells under oxidative stress, we tested the regulation of AKT as a downstream event. We observed a decrease in the phosphorylation status of AKT-1 (at Ser473 and Thr308) in LNCaP C4-2 cells treated for 5 hours with increasing concentrations of H2O2 (Fig. 2A and B; Supplementary Fig. S2). Prior incubation with VEGF-C abrogated the AKT-1 inactivation (Fig. 2A), supporting the survival-promoting function of VEGF-C as described in the previous result. No decrease in phospho–AKT-1 levels by H2O2 treatment was also observed when mature form of VEGF-C was stably overexpressed in LNCaP C4-2 cells (Fig. 2B; relative expression levels of VEGF-C in the stable clones of C4-2 are described in Supplementary Fig. S3). These results emphasize the importance of VEGF-C in up-regulating AKT-1 phosphorylation in prostate cancer cells during oxidative stress. It is important to note that VEGF-C–mediated AKT-1 phosphorylation was observed only under oxidative stress conditions, as we detected no increase.
in phosphorylation of AKT-1 in the absence of H$_2$O$_2$ in LNCaP C4-2 cells when incubated with either recombinant VEGF-C or stably overexpressing mature VEGF-C (Fig. 2 A and B). The importance of this finding is confirmed by the fact that the AKT-signaling cascade is considered a key determinant of tumor aggressiveness and an attractive target for therapeutic intervention (40). In addition, cancer cells expressing constitutively active AKT are more resistant to chemotherapeutic drugs such as paclitaxel and cisplatin than cancer cells expressing low levels of AKT (41).

Phosphorylation of FOXO-1 (at Thr$^{24}$) and GSK-3$\beta$ (at Ser$^{9}$), both downstream targets of AKT-1, was also retained in VEGF-C–treated cells (Fig. 2 A). FOXO-1, a known inducer of apoptosis, loses its activity on phosphorylation by AKT-1 (42). GSK-3$\beta$ is a mediator of c-Flip–mediated apoptosis (43), and thus, inactivation of GSK-3$\beta$ by phosphorylation suggests an antiapoptotic function of VEGF-C.

Interestingly, VEGF-C could not restore the decrease in phosphorylation of S6 and 4EBP-1, two downstream targets of AKT-mTOR.
complex-1 (mTORC-1) pathway in H2O2-treated prostate cancer cells (Fig. 2C). Although it is not clear why those proteins are not phosphorylated by VEGF-C under oxidative stress despite the presence of active AKT-1, an explanation might come from the observation that oxidative stress can block mTORC-1 activity downstream of AKT-1 (44). Therefore, the inactivation of mTORC-1 during severe stress may have biological significance. The major functions of mTORC-1 are protein synthesis and cellular growth (45). It is possible that during severe stress, cells prefer to shut off the energetically expensive processes such as protein synthesis and growth and enhance cellular processes that mediate cell survival. Again, one of the downstream targets of mTORC-1 is S6 kinase, which apart from activating its substrate S6 is also involved in a negative feedback loop to inactivate AKT-1 (46). Therefore, the absence of mTORC-1 activity facilitates prolonged activation of AKT-1 during severe stress and thus provides a better survival advantage.

mTORC-2 is responsible for AKT-1 activation under oxidative stress. To understand the molecular pathway of VEGF-C–mediated stress resistance in prostate cancer cells, we studied the upstream events required for Ser473 phosphorylation of AKT-1. Ser473 phosphorylation of AKT was previously reported to be an excellent predictor of poor clinical outcome in prostate cancer (47). One of the important upstream candidates is the multiprotein complex mTORC-2, which has shown to be necessary for prostate cancer development (46, 48). Accordingly, we tested the involvement of mTORC-2 in the VEGF-C–induced restoration of AKT-1 activation under ROS stress. We studied the association of mTOR and rictor, two important components of mTORC-2, in C4-2 cells treated with 3 mmol/L H2O2 alone or in the presence of increasing doses of VEGF-C. A decrease in association of mTOR with rictor in prostate cancer cells was observed when treated with H2O2 alone (Fig. 3A). Interestingly, prior incubation with VEGF-C restored the complex formation even in the presence of H2O2, suggesting a role of VEGF-C in maintaining mTORC-2 under stress (Fig. 3A). The total expression level of mTOR and rictor was not influenced by the addition of H2O2 and/or VEGF-C (Fig. 3A, bottom). Next, we knocked down the expression of rictor using specific siRNA to inactivate mTORC-2 in prostate cancer cells and monitored VEGF-C–induced Ser473 phosphorylation of AKT-1 under ROS stress. Recombinant VEGF-C failed to restore phospho–AKT-1 levels when rictor was knocked down (Fig. 3B). These results together with the results in Fig. 3A suggest the involvement of mTORC-2 in this pathway and rule out any significant contribution of other signaling pathways for VEGF-C–mediated Ser473 phosphorylation of AKT-1 in prostate cancer cells during oxidative stress. Recent report also indicated the involvement of mTORC-2 for the progression of prostate cancer (48), underscoring the significance of our study.

NRP-2 mediates VEGF-C–induced AKT-1 activation. To identify the VEGF-C receptor/s in this pathway, we showed interest in NRP-2 because of its involvement in resisting metabolic stress (49). Furthermore, the Oncamine database showed an increased expression of NRP-2 in human metastatic prostate cancer tissues (23). Knocking down NRP-2 in LNCaP C4-2 cells failed to rescue phospho–AKT-1 levels by VEGF-C when treated with 3 mmol/L H2O2 (Fig. 3C and D). Thus, our results show the involvement of NRP-2 in VEGF-C–mediated AKT-1 activation. Because NRP-2 is a nontyrosine kinase receptor and has a very short cytoplasmic tail, it is widely believed that it cooperates with other tyrosine kinase receptors to induce downstream signaling events (50). Currently, the identity of the tyrosine kinase receptor, if any, in this process is unknown.

VEGF-C restores activation of AKT-1 in LNCaP and PC3 cells under oxidative stress. Finally, we also checked the VEGF-C-AKT-1 axis in other prostate cancer cells such as LNCaP and PC3. Similar to LNCaP C4-2 cells, a dose-dependent recovery of LNCaP cells from H2O2-induced apoptosis was observed with increasing concentrations of VEGF-C (Fig. 4A, right; Supplementary Figs. S4 and S5). We also observed the phosphorylation of AKT-1 and its downstream target GSK-3β in LNCaP cells by the addition of recombinant VEGF-C during oxidative stress (Fig. 4A, left). Interestingly, unlike LNCaP C4-2, we only observed this restoration of phosphorylation at the lower concentrations of H2O2 (1 mmol/L; Fig. 4A; Supplementary Fig. S6). The differences between LNCaP and C4-2 cells might be due to different NRP-2 expression levels in these two cell lines (Fig. 1A). NRP-2 expression is significantly higher in C4-2 cells and therefore should be more potent in resisting stress by VEGF-C.

Finally, we tested the stress-resistant function of VEGF-C in a highly metastatic prostate cancer cell line, PC3, which not only expresses high level of NRP-2 but also synthesizes significant levels of endogenous VEGF-C. Decrease in phospho–AKT-1 levels in PC3 cells by H2O2 was observed only at higher concentrations (7.5 and 10 mmol/L; Fig. 4B, left). As expected, a rapid decrease in AKT-1 phosphorylation in PC3 cells with increasing concentrations of H2O2 occurred (Fig. 4B, left) when endogenous expression of VEGF-C was knocked down by siRNA (10 mmol/L). A significant increase in apoptosis in PC3 cells was also observed when endogenous VEGF-C was knocked down (Fig. 4B, right; Supplementary Fig. S7) along with the decrease in phosphorylation of FOXO-1 and GSK-3β (Fig. 4C), confirming the requirement of VEGF-C in resisting ROS stress in PC3 cells (Supplementary Fig. S8 for the efficiency of VEGF-C knocking down in PC3 by siRNA).

In conclusion, we have determined a novel function of VEGF-C, which promotes survival of prostate cancer cells under oxidative stress, as schematically represented in Fig. 4D. Involvement of NRP-2 in this function of VEGF-C is intriguing because of its higher expression in metastatic prostate cancer. Because other cancers such as glioblastoma, osteosarcoma,1 and several epithelial cancers also express higher levels of VEGF-C and NRP-2, it is possible that the VEGF-C/NRP-2/AKT-1 axis is involved in the recurrence of those cancers as well, a possibility that should be tested.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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