Mechanistic Investigation and Implications of Photodynamic Therapy Induction of Vascular Endothelial Growth Factor in Prostate Cancer


Abstract
Photodynamic therapy (PDT) is now an approved therapeutic modality, and induction of vascular endothelial growth factor (VEGF) following subcurative PDT is of concern as VEGF may provide a survival stimulus to tumors. The processes that limit the efficacy of PDT warrant investigation so that mechanism-based interventions may be developed. This study investigates VEGF increase following subcurative PDT using the photosensitizer benzoporphyrin derivative (BPD) both in an in vitro and in an orthotopic model of prostate cancer using the human prostate cancer cell line LNCaP. The two subcurative doses used, 0.25 and 0.5 J/cm², mimicked subcurative PDT and elicited a 1.6- and 2.1-fold increase, respectively, in secreted VEGF 24 hours following PDT. Intracellular VEGF protein measurement and VEGF mRNA showed a 1.4- and 1.6-fold increase only at 0.5 J/cm². In vivo subcurative PDT showed an increase in VEGF by both immunohistochemistry and ELISA. In vitro analysis showed no activation of hypoxia-inducible factor-1α (HIF-1α) or cyclooxygenase-2 (COX-2) following subcurative PDT; furthermore, small interfering RNA inhibition of HIF-1α and COX-2 inhibitor treatment had no effect on PDT induction of VEGF. PDT in the presence of phosphatidylinositol 3-kinase/AKT inhibitor or mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase inhibitor still induced VEGF. However, subcurative PDT increased phosphorylated p38 and stress-activated protein kinase/c-Jun NH2-terminal kinase. The p38 MAPK inhibitor abolished PDT induction of VEGF. The results establish the importance of VEGF in subcurative BPD-PDT of prostate cancer and suggest possible molecular pathways for its induction. These findings should provide the basis for the development of molecular-based interventions for enhancing PDT and merit further studies. (Cancer Res 2006; 66(11): 5633-40)

Introduction
Photodynamic therapy (PDT) is an evolving technology that is approved as a first line treatment for age-related macular degeneration and for a variety of cancers (1). PDT consists of the systemic or local administration of a photosensitizer, its preferential accumulation in malignant tissues, and its subsequent activation by visible light. In the presence of oxygen, this activated photosensitizer can generate reactive oxygen species that are toxic to the tumor (2, 3). With the use of modern fiber-optic systems and various types of endoscopy, light can now be targeted accurately to almost any part of the body for the treatment of tumors. Several thousand patients have already been treated with PDT for a variety of advanced neoplasms and have shown an improvement in their quality of life and a lengthened survival (3, 4). For early and localized disease, PDT has also been shown to be a selective and curative therapy. Porfimer sodium (Photofrin) is approved for use in advanced and early stage lung cancers, superficial gastric cancer, esophageal adenocarcinoma, cervical cancer, bladder cancer, and Barrett’s esophagus. Temoporfin, another photosensitizer, is approved in Europe for the palliative treatment of head and neck cancer. Although no other systemically administered photosensitizers are currently approved for the treatment of neoplasms, topically applied photosensitizers are approved for the treatment of actinic keratosis and basal cell carcinomas.

Today, PDT is being considered not only as palliative therapy but also as a treatment option for early lung cancer, actinic keratosis, and basal cell carcinoma. Currently, the use of PDT for localized disease and precancerous lesions is under investigation for bladder cancer, pituitary tumors, and glioblastoma (3, 4). The feasibility of using PDT for the treatment of localized recurrent prostate cancer has also been shown previously (5, 6). Furthermore, numerous ongoing clinical studies have been designed for the optimization of PDT conditions. As PDT becomes more of a mainstream treatment option for early cancers, it is important to understand factors that might counteract its tumoricidal effect. Our group is interested in studying the molecular responses of cancer cells that have been exposed to both photosensitizer and light but not in sufficient quantities to kill them. An understanding of these molecular responses will help in the design of new mechanism-based interventions and potentially improve long-term survival of PDT-treated patients.

An inherent consequence of PDT is local hypoxia. This condition can arise directly from oxygen consumption during treatment (7–9) or indirectly from the destruction of the tumor vasculature as a result of effective treatment (10, 11). Hypoxia is the major stimulus for angiogenesis through its stabilization of the transcription factor hypoxia-inducible factor-1α (HIF-1α; ref. 12), which is then able to bind to the HIF-1α response element (HRE; ref. 13) in the promoter of numerous genes, including in the promoter of the vascular endothelial growth factor (VEGF) gene, a potent angiogenic molecule, resulting in an increase in VEGF production and secretion (14). Following PDT, an increase in VEGF secretion (15, 16) as well as an angiogenic response has been documented (17, 18) in vivo. Ferrario et al. have shown an increase in HIF-1α following Photofrin-mediated PDT of a s.c. BA mouse mammary carcinoma (16) as well as an increase in cyclooxygenase-2 (COX-2) following PDT (19), leading to an increase in VEGF. However, the
molecular mechanism of this PDT-induced VEGF increase may involve multiple pathways and may be system dependent (cell, tumor type, tumor model, or photosensitizer). Importantly, the host microenvironment can have profound effects on tumor physiology and expression of cellular molecules (20). A recent report by Chen et al. (21) of a rat prostate cancer model showed that s.c. tumors had reduced vascular density, VEGF secretion, and uptake of photosensitizer when compared with orthotopic tumors. In general, although sometimes difficult to generate, orthotopic models more adequately mimic physiologic conditions and are thought to be of more clinical relevance (22).

In the current study, we investigated the subcurative benzoporphyrin derivative (BPD)-PDT-based induction of VEGF in vitro and in vivo in an orthotopic model of prostate cancer using the human prostate cancer cell line LNCaP. Our results indicate that subcurative treatment induces VEGF synthesis and release both in vitro and in vivo. Somewhat surprisingly, our in vitro study shows that, in contrast to previously published data (16, 19, 23), this increase is independent of HIF-1α and COX-2 but was induced by the p38 mitogen-activated protein kinase (MAPK) signaling pathway. Due to the complexity of the disease process in cancer, single treatment modalities may not be highly effective; it is likely that rationally designed, mechanism-based combinations will offer greater chances of success. The results in this study establish the molecular pathway for subcurative BPD-PDT induction of VEGF in prostate cancer cells and should be useful in the development of molecular-based interventions for enhancing photodynamic treatment response. Furthermore, these results suggest that the molecular responses elicited by PDT are system specific and determined by many factors, such as the photosensitizer, cell type, tumor physiology, and photosensitizer localization at the time of treatment.

Materials and Methods

Cell culture and reagents. LNCaP cells, human prostate carcinoma cells, were obtained from the American Type Culture Collection (Rockville, MD). Monolayer cultures were incubated in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% FCS (Invitrogen, Carlsbad, CA), 100 U/mL penicillin, 100 μg/mL streptomycin (Mediatech), and 10 mmol/L HEPEs. BPD (140 nmol/L) was used in all in vitro assays, and liposomal BPD (0.25 mg/kg body weight) was used in all in vivo studies. BD and liposomal BPD were donated by QLT, Inc. (Vancouver, British Columbia, Canada). NS-398 (COX-2 inhibitor) was purchased from Calbiochem (San Diego, CA). BPD were donated by QLT, Inc. (Vancouver, British Columbia, Canada). BPD localization. In vivo imaging: liposomal BPD localization in prostate tumors was imaged 1 hour after injection of 1 mg/kg BPD using a microscope coupled to a high-sensitivity CCD camera (Cascade:512F, Photometrics, Tucson, AZ). The microscope is composed of (a) 455-nm blue light-emitting diode (Lumonix XLH-MRB, Lumileds Lighting, San Jose, CA), (b) exciter filter (455/70, Chroma Technology, Rockingham, VT), (c) long-distance objective (Mitutoyo M Plan Apo 90×, Mitutoyo, Kawasaki, Japan), and (d) emitter filter (HQ700/75, Chroma Technology). Images were acquired with an exposure time of 200 ms/frame.

VEGF immunohistochemistry. Tumors were fixed in 10% formalin and embedded in paraffin. Tissue sections were deparaffinized, subjected to heat-induced epitope retrieval, immersed 30 minutes in 0.3% H2O2 to quench endogenous peroxidase activity, and blocked with normal mouse serum for 20 minutes (Vectastain avidin-biotin complex method kit, Vector Laboratories, Burlingame, CA). Sections were then incubated overnight at 4°C with VEGF antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), incubated with biotinylated secondary antibody for 30 minutes, incubated with avidin-peroxidase conjugate for 30 minutes, and stained with 3,3′-diaminobenzidine (DakoCytomation, Carpinteria, CA) for 3 minutes.

ELISA and reverse transcription-PCR. For intracelullar VEGF measurements, proteins were extracted from orthotopic prostate tumors or from LNCaP cells. Briefly, frozen tumors were pulverized to powder in a tissue homogenizer and thawed in 1 mL/mg lysis buffer. LNCaP cells were resuspended directly in the lysis buffer. The protein concentration was determined using a standard Lowry method. For secreted VEGF measurements, cell medium was collected and centrifuged to remove cell debris. Viable cells were then counted using trypan blue. A human VEGF DuoSet ELISA Development System (R&D Systems, Minneapolis, MN) was used to quantify human VEGF levels. Results were normalized to protein concentrations or cell numbers.

Total RNA was extracted from LNCaP cells using the RNeasy Protect Mini kit (Qiagen, Inc., Valencia, CA) according to the manufacturer’s instruction. Possible genomic DNA contamination was removed by RNase-free DNase I treatment (Qiagen). RNA concentration was estimated by reading the absorbance at 260 nm, and RNA integrity was shown by 1% agarose gel electrophoresis.

First-strand cDNAs were synthesized from 1 μg total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and oligo(dT)15 Primer (Promega, Madison, WI) according to the manufacturer’s instructions. Human VEGF-specific primers 5′-TCCGGGCTTCCAAGACCATG-3′ (forward) and 5′-CCTGTTGAGAGATCTGGTTC-3′ (reverse) were custom synthesized (Invitrogen) and used at 1 μmol/L each. The forward primer was located in the 5’-flanking region of exon 1 and the reverse primer in the 3’-open frame flanking region. PCR amplification with these primers could yield products of 772 bp (VEGF206), 721 bp (VEGF189), 649 bp (VEGF165), 580 bp (VEGF164), and 517 bp (VEGF154). The following conditions were used: 94°C for 5 minutes followed by 30 cycles of amplification (94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds) and a final 72°C extension for 7 minutes. PCR products were electrophoresed through an ethidium bromide-stained 3% agarose gel. The bands were visualized by densitometry.

Western blotting. LNCaP cells were incubated in ice for 30 minutes with vortexing every 5 minutes and then centrifuged at 14,000 rpm for 10 minutes at 4°C. The protein concentration was then determined using the standard Lowry method. Equal amounts of protein were separated by SDS-PAGE, blotted on polyvinylidene fluoride membrane, and probed with phosphorylated MAPK family antibody sampler kit (Cell Signaling Technology, Danvers, MA) or MAPK family antibody sampler kit (Cell Signaling Technology). BPD localization. In vitro imaging: liposomal BPD localization in prostate tumors was imaged 1 hour after injection of 1 mg/kg BPD using a microscope coupled to a high-sensitivity CCD camera (Cascade:512F, Photometrics, Tucson, AZ). The microscope is composed of (a) 455-nm blue light-emitting diode (Lumonix XLH-MRB, Lumileds Lighting, San Jose, CA), (b) exciter filter (455/70, Chroma Technology, Rockingham, VT), (c) long-distance objective (Mitutoyo M Plan Apo 90×, Mitutoyo, Kawasaki, Japan), and (d) emitter filter (HQ700/75, Chroma Technology). Images were acquired with an exposure time of 200 ms/frame.

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before irradiation. Before irradiation, a laparotomy was done and the prostate tumor was exposed. The tumor was irradiated at a fluence of 50 J/cm², with a fluence rate of 100 mW/cm². The incision was then closed. Twenty-four hours after treatment, the animals were euthanized and the tumors were collected.

**Transfection and luciferase assay.** For all transfections, 0.25 × 10⁶ LNCaP cells in 35-mm dishes were transfected using Lipofectin (Invitrogen). At 24 hours after transfection, the cells were PDT treated. Twenty-four hours after treatment, cell medium was collected and analyzed by ELISA or cells were lysed and luciferase was measured using the Luciferase Assay System (Promega). Duplex HIF-1α RNA interference (RNAi) was designed using BLOCK-iT RNAi Designer (Invitrogen) as follows: 5′-CCAUGAG-GAAAGAGAGAAAGCUU-3′ recognizes the open reading frame (ORF) of HIF-1α at position 704, a control duplex RNAi, based on the scrambled sequence of HIF-1α RNAi (5′-CCAAGGAGUAAGGAAAGGCUU-3′) was also designed. Both RNAi were ordered from Invitrogen, and 100 pmol were used for transfection.

**Statistical evaluation.** Data represented as mean ± SE of three independent experiments. A comparison of VEGF production by ELISA between PDT and light only or BPD only was calculated by unpaired Student’s t test, and a mixed effects model for repeated measures analysis was used for in vivo measurements comparisons. P < 0.05 was considered statistically significant.

**Results**

**VEGF Secretion and Transcription Are Increased by Sublethal PDT**

In the LNCaP cells, BPD is localized in the mitochondria and also in the cytosol (data not shown). This extramitochondrial localization suggests that PDT could also affect cytoplasmic molecules. PDT of LNCaP cells with different light doses showed that LNCaP cells are highly susceptible to PDT killing. The very low light dose, 0.25 J/cm², kills ~10% of cells, whereas the 1.25 J/cm² dose kills ~90% of cells. To study the molecular response of cells that have been subjected to PDT but not enough to kill them, we chose the two subcurative doses, 0.25 and 0.5 J/cm². These doses kill ~10% and 40% of cells, respectively (data not shown). The time course analysis of VEGF release at 8, 16, and 24 hours following PDT with the two subcurative doses is presented in Fig. 1A; results were normalized to cell number. Treatment with 0.25 and 0.5 J/cm² led to a 1.6- and 2.1-fold increase (P < 0.01; Fig. 1A) in VEGF, respectively, when compared with light only or to BPD only 24 hours after treatment. Viability assay showed that cell death following BPD-PDT occurs before 8 hours and that the number of cells for each group does not significantly vary between 8 and 24 hours (data not shown). Furthermore, because there is no increase in VEGF after 8 or 16 hours (Fig. 1A), this suggests that the observed increase in VEGF following PDT is not due to the release of intracellular VEGF from dead cells.

To determine the mechanism of this increase, PDT-treated LNCaP cells were collected 24 hours following treatment, and intracellular VEGF levels were measured by ELISA. The results were normalized to protein concentration (Fig. 1B). A significant increase (P < 0.05) in intracellular VEGF at 0.5 J/cm² (1.4-fold) was observed (Fig. 1B). Surprisingly, despite an increase of VEGF in the cell-conditioned medium after the lower dose treatment (0.25 J/cm²), there was no significant increase in the intracellular VEGF protein levels. To establish the mechanism of this increase, we used primers specific for exons 1 and 8 of the VEGF gene to determine VEGF mRNA levels following PDT. As described previously (25), these primers can amplify all possible isoforms of VEGF. Figure 1C shows a representative picture of a reverse transcription-PCR (RT-PCR) experiment following PDT. Only three isoforms of VEGF are expressed in LNCaP cells: VEGF121, VEGF145, and VEGF165. With VEGF121 being the most abundant and VEGF145 being the least abundant. With the 0.5 J/cm² treatment, all three VEGF isoforms are increased (Fig. 1C). Figure 1D shows the average fold induction of each VEGF isoform following GAPDH normalization. All three isoforms detected are increased but only following the 0.5 J/cm² PDT. Concordant with intracellular protein levels, there is a significant (P < 0.01) increase in mRNA levels of VEGF121, VEGF145, and VEGF165 (1.5-, 1.5-, and 1.6-fold increase, respectively, when compared with no treatment). However, because it is known that VEGF can be regulated at both transcriptional (26, 27) and post-transcriptional levels (27–29) from these experiments, we cannot exclude either mechanism.

**In vivo Effects of PDT**

To study the in vivo effect of subcurative PDT, we have used an orthotopic prostate cancer model that was shown to more reliably mimic pathologic conditions than ectopic models (21, 30, 31). Three weeks after LNCaP injection, a 0.1- to 0.2-cm³ tumor will develop in 90% of cases. For in vivo studies, we have used the Food...
and Drug Administration–approved liposomal formulation of BPD (verteporfin) because tumor accumulation was shown to be increased in vivo when compared with its nonliposomal formulation (1). For subcurative treatment, light was delivered with a fluence rate of 100 mW/cm² and a total fluence of 50 J/cm². This treatment was shown to be subcurative but still causes significant tumor damage (data not shown). Therefore, it is ideal for the study of the response of tumors that have been exposed to both photosensitizer and light but not at sufficient levels to kill them. Immunohistochemical analysis of tumors collected 24 hours after treatment showed a more intense VEGF staining following PDT (Fig. 2, bottom; compare Fig. 2C to Fig. 2A and B). Figure 2 (top) shows the H&E staining of tumor sections. There were numerous necrotic areas observed after PDT (Fig. 2C, top, arrows) and a significant infiltration of RBC indicative of effective treatment. To have a more quantitative approximation of the VEGF increase, we collected proteins from tumors 24 hours after treatment. VEGF ELISA was done, and all results were normalized to protein concentration. There was a significant (P < 0.05) increase in VEGF levels following PDT (1.9-fold increase when compared with BPD only) in orthotopic prostate tumors (Fig. 3A), consistent with the immunohistochemical observations. In vivo imaging of BPD 1 hour after injection showed both a vascular and intratumoral localization (Fig. 3C, right) of BPD. Therefore, with the experimental conditions used in this study, it is possible that hypoxia (vascular damage) and/or a direct effect of BPD maybe responsible for the increase in VEGF following PDT.

Molecular Mechanisms Underlying PDT Induction of VEGF

Sublethal PDT increases transcription through a HIF-1α–independent mechanism. Hypoxia-induced stabilization of HIF-1α followed by its binding to the HRE in the VEGF promoter is a major regulator of VEGF gene expression (14). Because it is well documented that PDT consumes oxygen and can therefore generate hypoxic conditions in vivo (7–9, 11), we decided to evaluate the contribution of HIF-1α in the PDT induction of VEGF. LNCaP cells were transiently transfected with a luciferase-expressing plasmid under the control of the HRE (p5HRE-Luc). An increase in luciferase following PDT would indicate activation of HIF-1α; however, we did not measure any increase in luciferase 24 hours after treatment (data not shown). We have also measured luciferase activity at 4, 8, and 16 hours after PDT, but we did not detect any increase in luciferase at these various times either (data not shown). However, treatment with cobalt chloride, which has been shown to stabilize HIF-1α (12), induced a 2-fold increase in luciferase (data not shown). It is well established that HIF-1α activation can be rapid and transient (32), and it is possible that...
PDT activation of HIF-1α occurred transiently and below our threshold of detection. Consequently, we designed a RNAi to inhibit endogenous HIF-1α. HIF-1α RNAi recognizes the ORF of HIF-1α at position +704. We also designed a control RNAi based on the scrambled sequence of HIF-1α RNAi. Because the detection of the HIF-1α protein is difficult in LNCaP cells, we cotransfected LNCaP cells with p5HRE-Luc together with a HIF-1α expression plasmid and HIF-1α RNAi or control RNAi. We measured a 10-fold increase in luciferase activity with the HIF-1α expression vector and no effect of the control RNAi. On the other hand, this induction is abolished in the presence of HIF-1α RNAi, confirming the efficacy of our RNAi. No effect was observed with the HRE-independent plasmid pSV40-Luc. Next, we did PDT 24 hours after transfection of HIF-1α RNAi. Twenty-four hours after PDT, cell-conditioned medium was collected and assayed for VEGF. Figure 4A shows an increase in VEGF after PDT even in the presence of HIF-1α RNAi. Transfection efficiency in the presence of HIF-1α RNAi is ~90% (data not shown). Together, these results suggest a HIF-1α-independent mechanism of VEGF increase after PDT.

Subcurative PDT activation of VEGF through a MAPK pathway. It was recently shown that PDT could induce COX-2 and subsequently lead to an increase in VEGF (19, 23). However, Western blot analysis of COX-2 levels following subcurative PDT in the LNCaP cell lines did not show any induction (data not shown). We have also used the COX-2 inhibitor, NS-398, to test the contribution of COX-2 in VEGF induction after PDT. As shown in Fig. 4B, there is an increase in VEGF after PDT even in the presence of the COX-2 inhibitor, suggesting a different pathway for VEGF induction. It has also been shown that PDT can activate the PI3K/AKT pathway (33). Western blot analysis did not show any activation of the PI3K/AKT pathway. Furthermore, this pathway can also lead to VEGF induction. It has also been shown that PDT can activate the PI3K/AKT pathway. Furthermore, inhibition of the PI3K/AKT pathway with the specific inhibitor LY 294002 did not inhibit PDT induction of VEGF (Fig. 4C). Next, we evaluated the contribution of the MAPK pathways in PDT induction of VEGF. Western blot analysis using phosphorylation-specific MAPK antibodies showed an activation of the PI3K/JNK pathway and the p38 MAPK pathway at 30 minutes and 1 and 2 hours following treatment (Fig. 5A). No activation of the p44/42 MAPK pathway could be detected at any time point (data not shown). Finally, we used specific MAPK inhibitors to study VEGF induction after PDT. As expected, the p44/42 inhibitor (PD 98059) had no effect on VEGF synthesis (Fig. 4D). However, only the p38 MAPK inhibitor (SB 202190) inhibited VEGF synthesis after PDT (Fig. 5C), whereas the JNK inhibitor (SP 600125) had no effect on PDT induction of VEGF (Fig. 5B).

Discussion

PDT is an emerging modality for the treatment of various neoplastic and nonneoplastic pathologies. The feasibility of using PDT for the treatment of recurrent prostate cancer has previously been established (5, 6) and is now in early phase clinical trials (34). Most initial PDT-prostate cancer studies were interested in feasibility and efficacy (35). Due to the limited penetration depth of light in tissue and to the nonhomogenous distribution of the photosensitizer in the tumor, some areas receive suboptimal PDT (either not enough light or not enough photosensitizer or both). The relevance of the current study concerns suboptimal PDT, with our investigation of the biological response of tumor cells that have received sublethal PDT. Consistent with the findings of previous studies that showed that PDT induces VEGF in s.c. models (16), this study shows that sublethal and subcurative PDT induces VEGF secretion in LNCaP cell cultures as well as in an orthotopic model of prostate cancer.

In vivo experiments were done 1 hour after injection of liposomal BPD. At this specific time, the photosensitizer is localized in the...
vascularity but also starts to accumulate in the tumors (Fig. 3C, right). The localization of the photosensitizer at the time of irradiation is an important determinant of the mode of tumor destruction. In a recent study, Chen et al. (36) showed that BPD-PDT 15 minutes after injection of the photosensitizer induced endothelial cell damage, causing vascular leakage, thrombi formation, and, eventually, vascular shutdown. Therefore, a vascular photosensitizer at the time of treatment will induce vascular shutdown, efficiently starving the tumor, whereas an intratumoral photosensitizer will cause tumor cell apoptosis or necrosis (37). Consequently, subcurative PDT with a vascular photosensitizer could induce tumor hypoxia, leading to an increase in VEGF production. On the other hand, subcurative PDT with an intratumoral photosensitizer could induce signaling pathways, leading to VEGF increase. Therefore, PDT 1 hour following injection would lead to both direct tumor destruction and indirect destruction through vascular shutdown.

Studies by Ferrario et al. showed that tumoricidal action of PDT was enhanced by antiangiogenic treatment (16) and COX-2 inhibition (19, 23) initiated at the time of PDT. Inhibition of VEGF action was investigated in a separate study (3), where the angiogenic pathway under hypoxic conditions (14). Furthermore, because PDT is an \( \text{O}_2 \)-consuming modality (7, 8) and an increase in HIF-1 \( \alpha \) pathway has been previously reported by Ferrario et al. (16), this seemed like the most logical choice with which to start our investigation. Somewhat to our surprise, negative data were obtained with the

On the mechanistic side, at a low PDT dose, VEGF increase is independent of protein synthesis, whereas, at the higher light dose, an increase in VEGF mRNA is observed (Fig. 1B and D). This suggests that the increase at the low light dose could be caused by the release of VEGF isoforms bound to the cell surface. Although there are no reports on the direct effect of PDT on proteoglycan, it is well documented that PDT can affect the cell membrane (38, 39). Subcellular localization of BPD showed not only a mitochondrial accumulation but also a cytosolic accumulation; it is therefore possible that activated BPD releases membrane-bound VEGF.

A single VEGF gene encodes multiple isoforms generated from alternative splicing (40). The VEGF gene contains 8 exons, and the various isoforms differ by the presence or absence of sequences encoded by exons 6 and 7. These isoforms differ in their ability to bind heparan sulfate proteoglycan found on cell surfaces as well as in the extracellular matrix (41). VEGF\(_{121}\) does not bind to heparan sulfate proteoglycan and is freely secreted (42), whereas VEGF\(_{145}\) and VEGF\(_{165}\) are able to bind heparan sulfate proteoglycan and can be associated with the cell surface and extracellular matrix (41). We have shown that LNCaP cells express three of the VEGF isoforms: VEGF\(_{121}\), VEGF\(_{145}\), and VEGF\(_{165}\) (Fig. 1C). It is therefore probable that some of the secreted VEGF remains bound to the surface of LNCaP cells.

To further probe the mechanism of VEGF induction and secretion, we investigated various cell signaling pathways that could contribute to an increase in VEGF. The first pathway investigated was the hypoxia-inducible pathway mediated by HIF-1\( \alpha \). It is well documented that VEGF can be regulated by this pathway under hypoxic conditions (14). Furthermore, because PDT is an \( \text{O}_2 \)-consuming modality (7, 8) and an increase in HIF-1\( \alpha \) has been previously reported by Ferrario et al. (16), this seemed like the most logical choice with which to start our investigation. Somewhat to our surprise, negative data were obtained with the

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**Figure 5.** Subcurative PDT increases VEGF through a p38 MAPK pathway. Proteins were extracted 30 minutes and 1 and 2 hours following subcurative PDT of LNCaP cells. Equal amounts of protein lysates were assayed for the levels of phosphorylated p38 MAPK (top) or for phosphorylated SAPK/JNK. The levels of p38 MAPK and SAPK/JNK were also measured for total protein. A, there is an increase in phosphorylated p38 and in SAPK/JNK following PDT, indicating activation of the p38 MAPK and of the SAPK/JNK MAPK pathways. LNCaP cells were incubated with 1, 5, or 10 \( \mu \text{mol/L} \) of the SAPK/JNK inhibitor SP 600125 (B) or with 5, 10, or 30 \( \mu \text{mol/L} \) of the p38 MAPK inhibitor SB 202190 (C) for 24 hours before PDT. Twenty-four hours after PDT, VEGF was measured using an ELISA. PDT induction of VEGF was abolished only in the presence of the p38 MAPK inhibitor. Columns, mean of three independent experiments; bars, SE. *, \( P < 0.05 \), statistically significant difference when compared with light only or to BPD only.
luciferase reporter plasmid and with HIF-1α RNAi. This excludes the possibility of the activation of HIF-1α by PDT, suggesting a HIF-1α–independent mechanism of VEGF secretion in LNCaP cells in vitro. A PDT induction of COX-2 that subsequently led to an increase in VEGF has previously been reported (19, 23). In contrast to these studies, we were not able to detect any COX-2 activation following PDT in the LNCaP cell line, and the use of a COX-2 inhibitor had no effect on the induction of VEGF following PDT. Our results suggest that, in the LNCaP cell line, the induction of VEGF is independent of COX-2. It is important to note that the study by Ferrario et al. used the photosensitizer Photofrin to treat a mouse mammary carcinoma, whereas, in our study, we used the photosensitizer BPD to treat a human prostate cancer. It is likely that different photosensitizer and different cell types induce VEGF via different pathways. These differences underscore the importance of the fact that PDT responses cannot be viewed as generic but are instead system specific. In fact, the specificity of the mechanistic pathways that lead to VEGF induction was further shown when the MAPK pathways were investigated.

VEGF is also under the control of MAPKs (26, 27), and because PDT has been shown to activate MAPKs (43, 44), we evaluated the activation of various MAPK family members following PDT in LNCaP cells. An increase in phosphorylated p38 and stress-activated protein kinase (SAPK)/JNK was measured following treatment but not in the p44/42 MAPK pathway. The use of specific MAPK inhibitors showed the involvement of the p38 MAPK pathway in the induction of VEGF following PDT. It has previously been shown that the p38 MAPK pathway as well as the SAPK/JNK pathway can increase VEGF mRNA stability (26, 27). Therefore, it is possible that the measured increase in VEGF mRNA is due to an increase in its stability.

Numerous studies have reported a biological response of cells following PDT, such as a decrease in cell adhesion (45, 46), an increase in cytokines production (47), and an increase in heat shock proteins (48). This study describes the effect of subcurative PDT on prostate cancer cells and reports an increase in VEGF both in vitro and in vivo in an orthotopic prostate cancer model. It was previously reported that COX-2 induces VEGF secretion (19, 23). However, in the prostate cancer cell lines, this increase is HIF-1α, COX-2, extracellular signal-regulated kinase, and AKT independent. On the other hand, subcurative PDT activates both the p38 MAPK and the SAPK/JNK pathway, but only inhibition of p38 MAPK abrogates PDT induction of VEGF secretion. The results shown establish the molecular pathway for subcurative PDT induction of VEGF in prostate cancer cells and should be useful in the development of molecular-based intervention for enhancing PDT. The best treatment outcomes from cancer treatments are increasingly recognized as resulting from combination treatments based on an understanding of molecular pathways that promote tumorigenesis (49). The details of the clinical relevance of the induction of VEGF by PDT are currently under investigation, but this induction could contribute to tumor survival and regrowth and therefore could be one of the factors impairing PDT from achieving its full tumorcidal potential. This deduction is supported by the improved tumor treatment response to PDT in combination with antiangiogenic agents (16, 50). In conclusion, rational combinations with appropriate mechanism-based interventions specific to the system being treated with PDT could significantly improve therapeutic outcomes.

Acknowledgments

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p38 MAPK Activation by Sublethal PDT Induces VEGF

In the article on how p38 MAPK activation by sublethal PDT induces VEGF in the June 1, 2006 issue of Cancer Research (1), the correct spelling of the second author’s name is Pål K. Selbo, the correct spelling of the third author’s name is Alok K. Sinha, and the correct spelling of the fourth author’s name is Sung K. Chang.

Mechanistic Investigation and Implications of Photodynamic Therapy Induction of Vascular Endothelial Growth Factor in Prostate Cancer


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