

# INCREASED HYPOXIA CORRELATES WITH INCREASED EXPRESSION OF THE ANGIOGENESIS MARKER VASCULAR ENDOTHELIAL GROWTH FACTOR IN HUMAN PROSTATE CANCER

DUSICA CVETKOVIC, BENJAMIN MOVSAS, ADAM P. DICKER, ALEXANDRA L. HANLON, RICHARD E. GREENBERG, J. DONALD CHAPMAN, GERALD E. HANKS, AND JAMES V. TRICOLI

## ABSTRACT

**Objectives.** To test the hypothesis that increasing levels of hypoxia are associated with increased expression of vascular endothelial growth factor (VEGF) in prostate cancer by correlating the level of median tissue oxygenation in human prostate tumors with the immunohistochemically determined level of VEGF expression.

**Methods.** Custom-made Eppendorf oxygen microelectrodes were used to quantitate the  $pO_2$  levels in prostate tumors of 13 men undergoing radical prostatectomy. All  $pO_2$  measurements were performed under fluorine-based general anesthesia. Paraffin-embedded tumor tissue from these men was analyzed to measure the level of VEGF expression by immunohistochemical staining. The significance of the associations between the  $pO_2$  levels and VEGF staining were determined by the Pearson correlations.

**Results.** The range of the median  $pO_2$  levels (based on between 97 and 129 individual measurements) among 13 prostate tumors was 0.5 to 44.9 mm Hg. The blinded comparison of  $pO_2$  levels and VEGF staining intensity demonstrated a significant correlation between increasing hypoxia and the percentage of cells staining positive for VEGF ( $r = -0.721$ ,  $P = 0.005$ ). This correlation was also significant when  $pO_2$  levels were compared with the overall immunoreactive score, which takes into account staining intensity ( $r = -0.642$ ,  $P = 0.018$ ).

**Conclusions.** To our knowledge, this is the first study demonstrating a significant association between increasing levels of hypoxia and increased expression of the angiogenesis marker VEGF in human prostate carcinoma. The results of our study further support the exploration of antiangiogenesis strategies for the treatment of human prostate cancer. *UROLOGY* 57: 821–825, 2001. © 2001, Elsevier Science Inc.

Previous studies have shown a correlation between the oxygenation status of tumors to treatment outcome after radiation therapy.<sup>1–4</sup> Hypoxia has also been associated with a poor outcome after surgery, suggesting a role for hypoxia in tumor aggressiveness or metastasis.<sup>5</sup> Studies in both humans and animals have demonstrated the existence of hypoxic cells in some prostate carcinomas.<sup>6–8</sup> Recently, we have shown that hypoxic regions exist in human prostate carcinoma and that

increasing levels of hypoxia are associated with higher clinical stages.<sup>9</sup> Previous studies have demonstrated that cancer cells adapt to hypoxia by secreting angiogenic proteins in response to the transcriptional factor, hypoxia-inducible factor 1 (HIF-1).<sup>10</sup> Vascular endothelial growth factor (VEGF) is an angiogenesis factor secreted in response to HIF-1 by a variety of human tumor cells that promotes neovascularity.<sup>11–13</sup> One study, using immunohistochemical methods, demonstrated VEGF expression in the malignant epithelial cells of 20 of 25 prostate tumors examined, with little or no expression in areas of benign prostatic hypertrophy.<sup>14</sup> Immunohistochemical studies have also demonstrated that increased VEGF expression is associated with increased tumor dedifferentiation,<sup>15</sup> and that they correlate with increased interleukin-8 staining.<sup>16</sup> Expression of VEGF receptors

From the Department of Radiation Oncology, Fox Chase Cancer Center; Department of Radiation Oncology, Jefferson Cancer Center; and Department of Surgery, Fox Chase Cancer Center, Philadelphia, Pennsylvania

Reprint requests: James V. Tricoli, Ph.D., Department of Radiation Oncology, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111

Submitted: August 4, 2000, accepted (with revisions): October 27, 2000

has also been detected in human prostate cancer, suggesting a role for paracrine stimulation of vascular endothelial cells within these tumors.<sup>17</sup> We hypothesize that increasing levels of hypoxia are associated with increased expression of VEGF in human prostate tumors. In this study, we correlated the Eppendorf pO<sub>2</sub> measurements in 13 prostate tumors with the extent of VEGF expression, as determined by immunohistochemical staining methods.

## MATERIAL AND METHODS

### OXYGEN MEASUREMENTS

Eppendorf pO<sub>2</sub> measurements were obtained from 13 patients (age range 44 to 77 years) with prostate cancer undergoing radical prostatectomy, as described previously.<sup>9</sup> In brief, all pO<sub>2</sub> measurements were obtained under general fluorine-based anesthesia using custom-made 12 to 14-cm microelectrodes with 0.3-mm stainless steel shafts manufactured by Eppendorf (Hamburg, Germany). For pO<sub>2</sub> measurements, the gas-sterilized electrodes were first calibrated in a sterile phosphate-buffered saline (PBS) solution (pH 7.8 to 8.4) immediately before and after each set of tissue measurements. The electrode was automatically moved through the tissue in precise steps of 0.7 mm. Each forward step was followed immediately by a backward step of 0.3 mm to minimize compression effects. The Eppendorf histograms (Eppendorf pO<sub>2</sub> Histogram model 6650) and the median pO<sub>2</sub>, mean pO<sub>2</sub>, and percentage of measurements less than 5 mm Hg and less than 10 mm Hg were recorded. The pO<sub>2</sub> measurements were obtained under direct visualization after nodal dissection, but before vascular disruption of the prostate. The measurements were obtained through an anterior approach from the pathologically involved side of the prostate, as determined by pretreatment sextant biopsies. Each set of measurements comprised approximately 100 separate pO<sub>2</sub> readings, which were obtained along three to four separate electrode tracks. The median of these separate readings was used as the final pO<sub>2</sub> value. Eligible patients for this study were those with histologically confirmed prostate adenocarcinoma with no evidence of distant metastatic disease. Two patients (patients 2 and 13) had received hormonal therapy before surgery. All patients signed an institutional review board-approved informed consent document before enrolling in this study.

### VEGF IMMUNOHISTOCHEMICAL STAINING

Prostate tissue was obtained from 13 patients diagnosed with prostate cancer who underwent radical prostatectomy. Representative samples of the prostatectomy specimens containing regions of both malignant prostate epithelium and benign hyperplastic epithelium from the site of the microelectrode measurements were selected. The tissue samples were fixed in 4% buffered formalin and embedded in paraffin. Serial 4- $\mu$ m sections were cut from the appropriate tumor blocks, mounted for immunohistochemical analysis, and the presence of tumor epithelium verified by hematoxylin-eosin staining. Slides were incubated at 65°C for 30 minutes and deparaffinized in xylene baths using three changes for 5 minutes each, then rehydrated through graded alcohols (100%, 90%, and 80% ethanol). Endogenous peroxidase activity was blocked by incubation for 10 minutes with 5% hydrogen peroxide. The slides were washed with 1  $\times$  PBS. An antigen retrieval method was used before immunostaining. The slides were immersed in 10 mM citrate buffer (pH 7.6), heated in a microwave oven on high power twice for 2.5 minutes each, and cooled down

for 20 minutes. Nonspecific binding was blocked by a 30-minute immersion of the slides in 3% bovine serum albumin/PBS with 0.2% Triton X-100 at room temperature.

VEGF was detected using goat affinity-purified antihuman VEGF (Santa Cruz Biotechnology, Santa Cruz, Calif) at a working dilution of 1:500. The slides were incubated with primary antibody for 30 minutes at room temperature, then rinsed with PBS. A negative control was performed by the incubation of goat IgG (Santa Cruz Biotechnology) in substitution for the primary antibody. After washing, the antigen was detected using the Universal DAKO LSAB Kit (DAKO, Carpinteria, Calif) and polyclonal reagents as per protocol, and immunolocalization of VEGF was visualized with chromogen. Sections were lightly counterstained with hematoxylin (DAKO) for 1 minute, washed in distilled deionized water, dipped in dilute ammonium-hydroxide, and mounted in Aquamount (DAKO). The analysis of VEGF staining in prostate tumor specimens was carried out by two independent observers who were unaware of the oxygen measurements. The results were scored using a semiquantitative scoring system.<sup>18,19</sup> This system assesses the percentage of positive glands (none = 0; less than 1% = 1; 1% to 10% = 2; 11% to 33% = 3; 34% to 67% = 4; and more than 67% = 5) and the staining intensity (none = 0; weak = 1; intermediate = 2; and strong = 3). The percentage and intensity scores were added together to give a final immunoreactive score (IRS) of 0 to 8. An Olympus BX50 microscope was used to examine the slides, and photographic images were produced using an Olympus PM30 35-mm camera.

### STATISTICAL ANALYSIS

The distributional forms of the outcome and independent variables were examined visually using histograms, revealing no obvious reason to doubt normality assumptions. Therefore, the relationship between the continuous outcome measures of VEGF (percentage of positive cells and IRS) and pO<sub>2</sub> levels was quantified parametrically by Pearson's correlation. All reported *P* values are two-tailed.

## RESULTS

The patient characteristics (age, PSA, Gleason grade) and the VEGF staining results and median pO<sub>2</sub> levels are shown in Table I. The median pO<sub>2</sub> levels for these 13 samples ranged from 0.5 to 44.9 mm Hg (mean 20.9). An example of VEGF immunohistochemical staining revealing staining of malignant prostate epithelium (sample 12 in Table I) using VEGF antibody is shown in Figure 1. In Figure 1B, the staining is cytoplasmic. Figure 1C and D are sections adjacent to those in Figure 1A and B that were stained with goat IgG instead of the VEGF antibody. Specimens stained with goat IgG served as the negative controls and were performed for each of the 13 samples analyzed.

The results of our blinded comparison of the median pO<sub>2</sub> levels and VEGF staining intensity demonstrated a significant correlation between increasing tumor hypoxia and the percentage of cells staining positive for VEGF ( $r = -0.721$ ,  $P = 0.005$ ). In addition, the correlation between the decreased pO<sub>2</sub> levels and an increased overall IRS, which takes into account staining intensity, was significant ( $r = -0.642$ ,  $P = 0.018$ ). The spread of

TABLE I.  $pO_2$  and VEGF values on human prostate tumors

Sample	Age (yr)	PSA (ng/mL)	Gleason Grade	PP	SI	IRS	Median $pO_2$ (mm Hg)	n	SD $pO_2$ (mm Hg)	Mean
1	65	4.7	6	5	1	6	0.5	103	14.2	5.8
2	77	3.3	7	3	1	4	4.7 (H)	104	25.6	18.1
3	44	4.1	6	5	1	6	6.1	100	10.4	9.5
4	55	5.5	6	3	1	4	10.7	104	18.3	16.4
5	67	3.9	5	5	2	7	11.1	100	21.6	19.1
6	45	3.0	7	5	2	7	15.1	97	22.0	22.5
7	68	5.5	6	3	2	5	18.6	112	39.6	33.5
8	66	6.3	6	5	2	7	19.9	105	21.3	23.5
9	65	7.8	4	4	1.5	5.5	21.3	102	14.2	22.5
10	54	22.5	7	1	1	2	44.9	106	22.2	39.6
11	44	3.3	6	0	0	0	36.0	114	18.2	31.4
12	53	4.0	6	0	0	0	38.2	129	23.6	40.3
13	62	16.0	7	3	1.5	4.5	44.6 (H)	101	19.1	45.8

KEY:  $pO_2$  = partial pressure of oxygen; VEGF = vascular endothelial growth factor; PSA = prostate-specific antigen; PP = percentage of positive glands (0–5); SI = staining intensity (0–3); IRS = immunoreactive score (0–8); SD = standard deviation; (H) = patient received hormonal therapy.

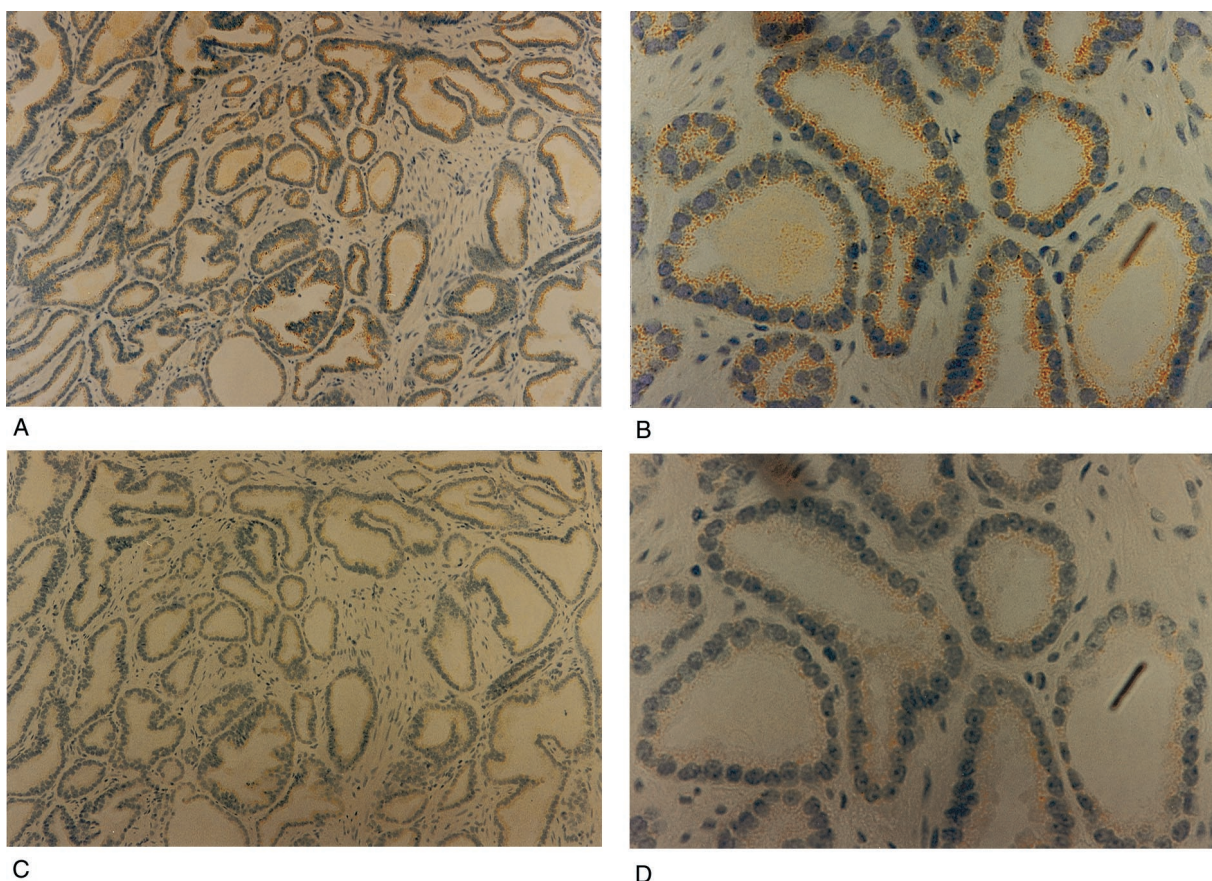


FIGURE 1. Immunohistochemical staining of human prostate carcinoma using antibodies to VEGF. (A and B) VEGF staining at original magnification  $\times 100$  and  $\times 400$ , respectively. (C and D) Serial section negative controls using goat IgG in substitution for the primary VEGF antibody at original magnification  $\times 100$  and  $\times 400$ , respectively.

the data for the  $pO_2$  values with respect to the percentage of positive glands and IRS is shown in Figure 2. The correlation between the  $pO_2$  levels or VEGF staining and age, Gleason grade, or prostate-specific antigen level was not significant. Two pa-

tients (patients 2 and 13) received hormonal therapy before surgery. Although their IRS scores were similar, the median  $pO_2$  level for these tumors was radically different. Whether hormonal therapy has any effect on the  $pO_2$  or VEGF levels in these tu-

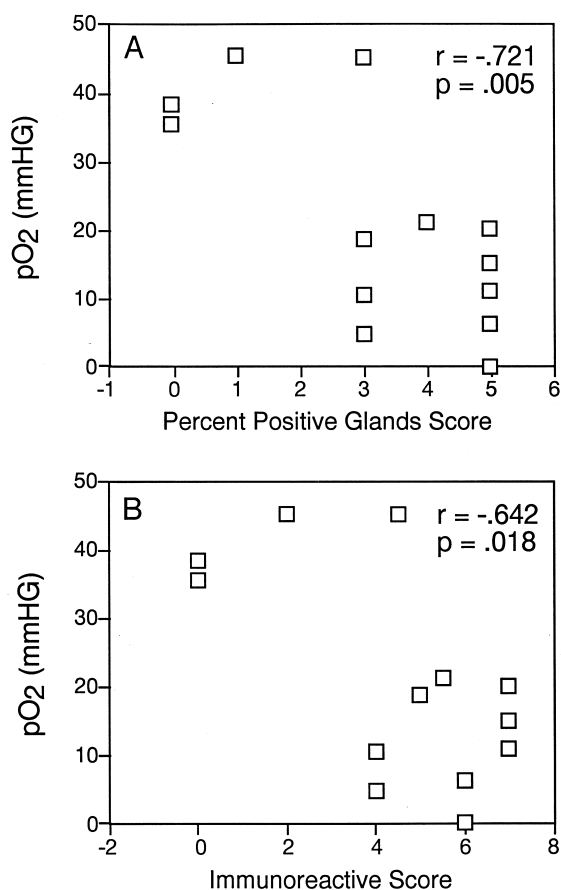


FIGURE 2.  $pO_2$  values versus (A) intensity values for percentage of positive glands for VEGF staining and (B) IRS.

mors is as yet unknown. At a median follow-up of 12 months, all patients were biochemically free of disease.

#### COMMENT

To our knowledge this is the first study demonstrating a significant association between increased levels of hypoxia, as measured in patients, and the increased expression of the angiogenesis marker, VEGF, in human prostate cancer. Because of the nature of the surgical procedure, these studies were performed under fluorine-based anesthesia. Previous studies have shown that these agents can modulate microelectrode measurements of  $pO_2$  levels.<sup>20,21</sup> Recent studies using the Dunning rat prostate carcinoma model showed that Eppendorf  $pO_2$  measurements obtained in the presence of the fluorine-based anesthesia halothane were fourfold to fivefold greater than those obtained in air-breathing, restrained animals.<sup>22</sup> Thus, the actual  $pO_2$  levels in the 13 prostate tumors of this study may be lower than reflected by our Eppendorf microelectrode measurements.

Increased microvessel density has been associated with increased aggressiveness and a worse prognosis in a number of human cancers, including breast, gastrointestinal, and bladder cancer.<sup>23</sup> In the mouse, VEGF has been shown to facilitate tumor progression in a colon cancer model.<sup>24</sup> VEGF expression and intratumoral microvessel density both correlate with decreased survival in men with prostate cancer,<sup>25</sup> with most studies demonstrating that microvessel density correlates with disease progression after radical prostatectomy.<sup>26</sup> In our study, the  $pO_2$  values did not correlate with tumor grade or prostate-specific antigen level. This may be because of the small sample number in our data set. Circulating plasma levels of VEGF are increased in patients with metastatic prostate cancer.<sup>27</sup> VEGF is known to be induced by HIF-1 in response to a decrease in cellular oxygen concentration and exerts its angiogenic effect by binding to fms-like tyrosine kinase 1 and fetal liver kinase 1.<sup>28-30</sup> These receptors are present in malignant and benign prostate epithelium. A recent study has shown that, in prostate cancer cell lines, HIF-1 expression is positively regulated by the PI3K/AKT/FRAP signal transduction pathway, resulting in enhanced VEGF production.<sup>31</sup> This suggests that, in addition to hypoxia, mutations in the tumor suppressor PTEN could contribute to the increased VEGF expression observed in these patients. In preclinical prostate tumor models, Borgstrom *et al.*<sup>32</sup> found that anti-VEGF antibodies can inhibit angiogenesis and the growth of prostate carcinoma microtumors *in vivo*.

The phenomenon of hypoxia-induced expression of VEGF has been well documented, and evidence of both transcriptional and post-transcriptional regulation of this expression has been observed.<sup>33-37</sup> Our results suggest that prostate tumor cells in patients adapt to decreased  $pO_2$  levels by secreting VEGF, possibly in response to transcriptional regulation by HIF-1. This may lead to enhanced angiogenesis and thus contribute to the growth and invasiveness of these tumors. The results of our study further support the exploration of antiangiogenesis strategies for the treatment of human prostate cancer.

ACKNOWLEDGMENT. To Corrine Stobbe for technical assistance with the oxygen measurements, Dr. Tahseen Al-Saleem for assistance with the pathologic examinations, and Ellen Ragan for assistance in preparing the manuscript.

#### REFERENCES

1. Gatenby RA, Kessler HB, Rosenblum JS, *et al*: Oxygen distribution in squamous cell carcinoma metastases and its relationship to outcome of radiation therapy. *Int J Radiat Oncol Biol Phys* 14: 831-838, 1988.
2. Höckel M, Knoop C, Schlenger K, *et al*: Intratumoral

PO<sub>2</sub> predicts survival in advanced cancer of the uterine cervix. *Radiother Oncol* 26: 45–50, 1993.

3. Nordmark M, Overgaard M, and Overgaard J: Oxygenation status predicts radiation response in advanced squamous cell carcinoma of head and neck. *Radiother Oncol* 41: 31–39, 1996.

4. Brizel DMN, Sibley GS, Prosnitz LR, *et al*: Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. *Int J Radiat Oncol Biol Phys* 38: 285–289, 1997.

5. Höckel M, Schlenger K, Aral B, *et al*: Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res* 56: 4509–4515, 1996.

6. Groshar D, McEwan AJ, Parliament MB, *et al*: Imaging tumor hypoxia and tumor perfusion. *J Nucl Med* 34: 885–888, 1993.

7. Urtasun RC, Parliament MB, McEwan AJ, *et al*: Measurement of hypoxia in human tumors by non-invasive SPECT imaging of iodoazomycin arabinoside. *Br J Cancer* 74: 209–212, 1996.

8. Rasey JS, Koh WJ, Evans ML, *et al*: Quantifying regional hypoxia in human tumors with positron emission tomography of [<sup>18</sup>F]fluoromisonidazole: a pretherapy study of 37 patients. *Int J Radiat Oncol Biol Phys* 36: 417–428, 1996.

9. Movsas B, Chapman JD, Horwitz EM, *et al*: Hypoxic regions exist in human prostate carcinoma. *Urology* 53: 11–18, 1999.

10. Zhong H, DeMarzo AM, Laughner E, *et al*: Overexpression of hypoxia-inducible factor 1 $\alpha$  in common human cancers and their metastases. *Cancer Res* 59: 5830–5835, 1999.

11. Koch AE, Polverini PJ, Kunkel SL, *et al*: Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science* 258: 1798–1801, 1992.

12. Fidler IJ, and Ellis LM: The implications of angiogenesis for the biology and therapy of cancer metastasis. *Cell* 79: 185–188, 1994.

13. Dvorak HF, Brown LF, Detmer M, *et al*: Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 146: 1029–1039, 1995.

14. Ferrer FA, Miller LJ, Andrawis RI, *et al*: Vascular endothelial growth factor (VEGF) expression in human prostate cancer: In situ and in vitro expression of VEGF by human prostate cancer cells. *J Urol* 157: 2329–2333, 1997.

15. Harper ME, Glynne-Jones E, Goddard L, *et al*: Vascular endothelial growth factor (VEGF) expression in prostatic tumours and its relationship to neuroendocrine cells. *Br J Cancer* 74: 910–916, 1996.

16. Ferrer FA, Miller LJ, Andrawis RI, *et al*: Angiogenesis and prostate cancer: in vivo and in vitro expression of angiogenesis factors by prostate cancer cells. *Urology* 51: 161–167, 1998.

17. Ferrer FA, Miller LJ, Lindquist R, *et al*: Expression of vascular endothelial growth factor receptors in human prostate cancer. *Urology* 54: 567–572, 1999.

18. Allred DC, Clark GM, Elledge R, *et al*: Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. *J Natl Cancer Inst* 85: 200–206, 1993.

19. O'Malley FP, Saad Z, Kerkvliet N, *et al*: The predictive power of semiquantitative immunohistochemical assessment of p53 and *c-erb* B-2 in lymph node-negative breast cancer. *Hum Pathol* 27: 955–963, 1996.

20. Dent J, and Netter K: Errors in oxygen tension measurements caused by halothane. *Br J Anaesth* 48: 195–197, 1976.

21. Bates M, Feingold A, and Gold M: The effects of anesthetics on an in-vivo oxygen electrode. *Am J Clin Pathol* 64: 448–451, 1975.

22. Iyer RV, Haynes PT, Schneider RF, *et al*: Comparison of  $\beta$ -D-IAZGP and (Tc-99m)HL-91 marking of hypoxia in rat prostate carcinomas with microelectrode measurements of PO<sub>2</sub>. *J Nucl Med* (in press).

23. Weidner N: Intratumor microvessel density as a prognostic factor in cancer. *Am J Pathol* 147: 9–19, 1995.

24. Warren RS, Yan H, Matli MR, *et al*: Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J Clin Invest* 95: 1789–1797, 1995.

25. Weidner N, Carroll PR, Flax J, *et al*: Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol* 143: 401–409, 1993.

26. Silberman MA, Partin AW, Veltri RW, *et al*: Tumor angiogenesis correlates with progression after radical prostatectomy but not with pathologic stage in Gleason sum 5 to 7 adenocarcinoma of the prostate. *Cancer* 79: 772–779, 1997.

27. Duque JLF, Loughlin KR, Adam RM, *et al*: Plasma levels of vascular endothelial growth factor are increased in patients with metastatic prostate cancer. *Urology* 54: 523–527, 1999.

28. Claffey KP, and Robinson GS: Regulation of VEGF/VPF expression in tumor cells: consequences for tumor growth and metastasis. *Cancer Metast Rev* 15: 165–176, 1996.

29. Terman BI, and Dougher-Vermazen M: Biological properties of VEGF/VPF receptors. *Cancer Metast Rev* 15: 159–163, 1996.

30. Herley MT, Yu Y, Whitney RG, *et al*: Characterization of the VEGF binding site on the Flt-1 receptor. *Biochem Biophys Res Comm* 262: 731–738, 1999.

31. Zhong H, Chiles K, Feldser D, *et al*: Modulation of hypoxia-inducible factor 1 $\alpha$  expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res* 60: 1541–1545, 2000.

32. Borgstrom P, Bourdon MA, Hillan KJ, *et al*: Neutralizing anti-vascular endothelial growth factor antibody completely inhibits angiogenesis and growth of human prostate carcinoma micro tumors in vivo. *Prostate* 35: 1–10, 1998.

33. Shima DT, Deutsch U, and D'Amore PA: Hypoxic induction of vascular endothelial growth factor (VEGF) in human epithelial cells is mediated by increases in mRNA stability. *FEBS Lett* 370: 203–208, 1995.

34. Shweiki D, Itin A, Stoffer D, *et al*: Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359: 843–845, 1992.

35. Levy AP, Levy NS, Wegner S, *et al*: Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *J Biol Chem* 270: 13333–13340, 1995.

36. Levy AP, Levy NS, and Goldberg MA: Post-transcriptional regulation of vascular endothelial growth factor by hypoxia. *J Biol Chem* 271: 2746–2753, 1996.

37. Ikeda E, Achen MG, Breier G, *et al*: Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. *J Biol Chem* 34: 19761–19768, 1995.