Abstract. Background: The process of ingrowth of new blood vessels is stimulated by the action of vascular endothelial growth factor (VEGF), while it may be simultaneously related to the degree of tissue hypoxia. Hypoxia-inducible factor 1α (HIF-1α) is a protein of cellular response to hypoxia. The relationship between hypoxia and angiogenesis in patients with benign prostate hyperplasia (BPH) and prostate cancer (PCa) was examined. Materials and Methods: One hundred and seventy (170) prostatic tissue samples were immunohistochemically evaluated. The microvessel density (MVD) was calculated by CD34 immunostaining; the angiogenetic profile was further assessed using a monoclonal antibody against VEGF. HIF-1α immunoreaction was recognized through nuclear staining of positive cells. Results: CD34, VEGF and HIF-1α staining reactions were significantly higher in the PCa group than in the BPH group. In both groups, an interrelationship between the immunoexpression of CD34 and HIF-1α, VEGF and HIF-1α, as well as VEGF and CD34 was detected. Conclusion: MVD, VEGF cytoplasmic immunoreactivity and HIF-1α immunoreaction were more prominent in PCa than in BPH and were also significantly associated with high-grade carcinomas.

Benign prostate hyperplasia (BPH) is a common pathology in the aging male (1). Prostate cancer (PCa) is the most common form of cancer among males and the second leading cause of cancer deaths (2). The most common denominator between BPH and PCa is angiogenesis. Angiogenesis is the generation of new blood vessels, once a primary vascular plexus has been formed. Angiogenesis is one of the critical steps in tumor growth and plays a major role in the development of metastasis in a variety of clinical malignancies (3, 4). A recent experimental study in rats provided evidence that prostatic vasculature may be regulated by androgens (5).

The microvessel density (MVD) represents the number of small blood vessels in hyperplastic or malignant prostate tissue and is frequently evaluated by CD34 immunolabeling. In certain microenvironments, non-vascular cells such as macrophages, fibroblasts and mast cells can modulate angiogenesis by producing a large number of angiogenic factors; the most prevalent amongst these are basic fibroblast growth factor (bFGF), angiogenin, transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF). Although the mechanism of VEGF overexpression is not very well known, transcriptional and post-transcriptional regulation has been postulated (6). Hypoxia-inducible factor-1α (HIF-1α) is the first factor that was demonstrated to be involved in the transcriptional regulation of VEGF. HIF-1α is a subunit of HIF-1, which is a heterodimeric basic helix–loop–helix (bHLH-PAS) transcription factor. It is critically involved in cancer cell clonal selection, hypoxic adaptation, glycolysis and angiogenesis (7). The mRNA levels of HIF-1α are equivalent in normoxia and hypoxia, but in hypoxia there is inhibition of the O2-dependent degradation of the HIF-1α protein via the ubiquitin-proteasome pathway, recently
shown to be regulated by the von Hippel-Lindau tumor-suppressor gene product (8). After heterodimerization with the protein HIF-1β (a nuclear translocator expressed constitutively and unaffected by hypoxia), HIF-1 binds to DNA at the hypoxia response elements, thereby activating the VEGF gene, one of the key angiogenic stimulators (9).

In the current study, the expressions of CD34, VEGF and HIF-1α were investigated in 170 prostate specimens from patients with the diagnosis of BPH (group A; 85 patients) and from patients with the diagnosis of PCA (group B; 85 patients). Statistical correlations amongst the three immunohistochemical markers were also investigated.

**Materials and Methods**

**Specimens.** Our material consisted of 85 radical prostatectomy specimens with adenocarcinoma of the prostate of various Gleason scores (G.S) (50 with G.S 2 to 6 and 35 with G.S 7 to 10) and BPH specimens (60 from TUR-P and 25 from suprapubic prostatectomy). The material was selected from patients treated at the Department of Urology, General Hospital of Nikea, Piraeus, Greece and at the Department of Urology, Sismanoglio Hospital, School of Medicine, National and Kapodistrian University of Athens, Greece, from 2000 to 2004.

The patients of group A had a mean (±SD) age of 67.6 (±5.6) years, whereas the patients of group B had a mean (±SD) age of 67.8 (±6.4) years (Table I).

The tissues were fixed immediately after removal in 10% buffered formalin and embedded in paraffin. All cases were immunohistochemically stained using the standard three-step streptavidin-peroxidase technique (10-12). The three immunomarkers were assessed both semi-quantitatively and quantitatively. The statistical analysis was performed using SPSS for Windows (version 10) package. A one-way analysis of variance was used to estimate the difference between continuous variable means between the two groups. For categorical variables, the chi-square test was performed. The relationship between the proportion of stained microvessels and the Gleason score was analyzed using a one-way analysis of variance. The differences of >10% between observers were resolved by reevaluation of slides. The results were in agreement in >90% of all cases.

**Assessment of microvessel density.** Angiogenesis was assessed by calculating the standard MVD. Microvessels were identified by immunostaining of endothelial cells with the specific monoclonal antibody to CD34 (Biogenex, San Ramon, CA, USA, diluted 1:150, with microwave pre-treatment and overnight incubation). Each slide was first scanned at low magnification (x 100) to identify the four hyperplastic areas with the highest density of microvessels ("hotspots"). Each hotspot was then evaluated at high-power magnification (x 400) and the number of stained microvessels per high-power field was determined [microvessel count (MVC)]. The median value of MVC with the use of CD34 antibody was 90 microvessels (range 35 to 210 microvessels). The semi-quantitative characterization was performed as follows: (a) 0 to 35 microvessels; (b) 36 to 90 microvessels; and (c) >90 microvessels. This was the cut-off point to distinguish low from high MVC.

**VEGF detection and assessment.** VEGF expression was assessed with the purified mouse anti-human VEGF monoclonal antibody (IgG2b, isotype, clone G153-694, Pharmingen, San Diego, CA, USA, diluted 1:75, with microwave pre-treatment and overnight incubation) recognizing the 165, 189 and 206 isoforms of VEGF. The percentage of cells with cytoplasmic VEGF reactivity was recorded in order to assess the VEGF reactivity. Evaluation of the VEGF staining was graded on a four-point classification as follows: (−), not detected; (+) positive staining in < 5% of cells; (++) positive staining in 5 to 25% of cells; and (++++) >25% positive-staining cells (high VEGF).

**Immunohistochemistry for HIF-1α.** Serial sections of formalin-fixed, paraffin-embedded material were stained with a mouse monoclonal antibody against HIF-1α (clone H1α 67, IgG2β isotype, Stress Gene, diluted 1:1200, with microwave pre-treatment and overnight incubation). The assessment of HIF-1α was based on previously described guidelines (13). Immunoreactivity for HIF-1α was evaluated according to nuclear staining. The percentage of positive cells was rated as follows: (−) absence of nuclear immunostaining; (i) (10-50%) 11 (12.9%) 26 (30.6%) 0.001 (ii) (5-25%) 26 (30.6%) 24 (28.2%) (iii) (>50%) 36 (42.3%) 44 (51.8%)

**Table I. Patients’ characteristics and overall study results**

<table>
<thead>
<tr>
<th></th>
<th>Group A (BPH)</th>
<th>Group B (PCA)</th>
<th>p value</th>
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<tbody>
<tr>
<td>No. of pts.</td>
<td>85</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Age; mean (yrs)</td>
<td>67.6 (54-80)</td>
<td>67.8 (50-75)</td>
<td></td>
</tr>
<tr>
<td>PSA; mean (ng/ml)</td>
<td>3.9</td>
<td>6.5</td>
<td>0.001</td>
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<tr>
<td>MVC</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>a (0-35)</td>
<td>20 (23.6%)</td>
<td>16 (18.8%)</td>
<td>0.043</td>
</tr>
<tr>
<td>b (36-90)</td>
<td>30 (35.3%)</td>
<td>23 (27.1%)</td>
<td></td>
</tr>
<tr>
<td>c (&gt;90)</td>
<td>35 (41.1%)</td>
<td>46 (54.1%)</td>
<td></td>
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<tr>
<td>VEGF</td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>−</td>
<td>13 (15.3%)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>+ (&lt;5%)</td>
<td>10 (11.8%)</td>
<td>17 (20.0%)</td>
<td></td>
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<tr>
<td>++ (5-25%)</td>
<td>26 (30.6%)</td>
<td>24 (28.2%)</td>
<td></td>
</tr>
<tr>
<td>+++ (&gt;25%)</td>
<td>36 (42.3%)</td>
<td>44 (51.8%)</td>
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<tr>
<td>HIF-1α</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
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<tr>
<td>−</td>
<td>40 (47.1%)</td>
<td>16 (18.8%)</td>
<td></td>
</tr>
<tr>
<td>i (&lt;10%)</td>
<td>26 (30.6%)</td>
<td>19 (22.4%)</td>
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</tr>
<tr>
<td>ii (10-50%)</td>
<td>11 (12.9%)</td>
<td>26 (30.6%)</td>
<td></td>
</tr>
<tr>
<td>iii (&gt;50%)</td>
<td>8 (9.4%)</td>
<td>24 (28.2%)</td>
<td></td>
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</tbody>
</table>

**BPH=benign prostate hyperplasia; PCA=prostate cancer; MVC=microvessel count; VEGF=vascular endothelial growth factor; HIF-1α=hypoxia-inducible factor-1α.**

**Statistical analysis.** The three immunomarkers were assessed both semi-quantitatively and quantitatively. The statistical analysis was performed using SPSS for Windows (version 10) package. A one-way analysis of variance was used to estimate the difference between continuous variable means between the two groups when the data followed the normal distribution. The Mann-Whitney and Kolmogorov-Smirnov non-parametric tests for two different
independent samples were used in the event that the normality assumption was violated.

The correlation between continuous variables in the same group was assessed by regression analysis (linear and non-linear), whereas the correlation between categorical variables in the same group was assessed using Spearman’s correlation coefficient and Friedman’s test.

In the cancer specimens, in order to examine if the pattern of the Gleason score was affected by CD34, VEGF and HIF-1α within the same group, a multifactor ANOVA was implemented assuming no interactions between the factors.

For all the previous tests, a significance level of 5% was used.

Results

Using the CD34 antibody, the vascular networks were easily observed within both the hyperplastic (Figure 1a) and the prostate cancer tissues (Figure 1b). In hyperplastic prostatic glands neighboring cancerous acini, CD34 immunoreactivity tended to be higher than in totally benign prostatic glands. Regarding the benign specimens, CD34-positive vessels increased at the periphery of the hyperplastic nodules in comparison to their center, especially when the nodules were of the adenocystic type. Small nodules tended to have more CD34-positive microvessels than bigger nodules. In the BPH samples 41.1% exhibited high MVC; 54.1% of prostate carcinomas also exhibited high MVC (Table I). Of the prostate carcinomas, 40% were low-grade and 74% were high-grade (Table II).
VEGF cytoplasmic immunostaining was detected in the majority of cases (Figure 2). VEGF immunoreactivity was found in 72/85 (84.7%) of BPH cases and in 85/85 (100%) of prostate cancer specimens. High VEGF expression was shown in 42.3% of the BPH, in 46% of the low-grade and in 89% of the high-grade adenocarcinomas (Table II).

In HIF-1α-positive areas of the BPH and PCa sections (Figure 3 a, b), HIF-1α immunostaining was mainly nuclear, but cytoplasmic immunoreactivity could also be observed occasionally. The results are presented in detail in Tables I and II.

When all specimens were taken into account (n=170), CD34, VEGF and HIF-1α expressions were significantly higher in the PCa group than in the BPH group (Mann-Whitney $p=0.001$ and Kolmogorov-Smirnov $p<0.001$) (Table I). Potential correlations between the expressions of the examined immunomarkers were investigated in each patient group. In the BPH group, CD34 and HIF-1α, as well as VEGF and HIF-1α and VEGF and CD34 immunoreactivities were positively correlated (Friedman test, $p=0.001$, $p<0.001$ and $p<0.001$, respectively). In the PCa group, a positive correlation only between VEGF and HIF-1α immunexpressions was found (Friedman test $p<0.001$, Spearman's correlation coefficient $p<0.001$).

**Discussion**

Angiogenesis refers to the formation of new capillaries from pre-existing blood vessels and is a requirement for the continued growth of cancer, whether primary or metastatic (14). The new microvessels that are generated in tumors differ from those of non-neoplastic tissues. They are more fragile and irregular, with increased permeability and a higher proliferation rate than that of normal endothelial cells. In current studies, angiogenesis is preferentially assessed by immunodetection of the endothelial marker CD34 (15). The angiogenic profile can be further assessed by using monoclonal antibodies against VEGF and HIF-1α.

The overexpression of HIF-1α was demonstrated in many types of human carcinoma, as well as in the regional and distant metastases, as a result of adaptation of tumor cells to hypoxia (10). Giatromanolaki et al. showed that HIF-1α overexpression is a common event in non-small cell lung carcinomas, up-regulating the angiogenic pathways and is associated with poor prognosis (16). HIF-1α was associated with unfavorable prognosis in women with cervical cancer and with poor response to radiotherapy-treated oropharyngeal and esophageal cancer. The association of HIF-1α overexpression with up-regulation of the VEGF pathway, an increase in MVD and poor outcome was also shown in endometrial and urothelial carcinoma (12). Accumulated HIF-1α was associated with a poor overall and disease-free survival (17). The intracellular stabilization and binding of HIF-1α to hypoxia response elements (HREs) of genes related to angiogenesis, glycolysis and erythropoiesis, the most important processes facilitating tumor cell survival, proliferation, progression and distant metastasis, can explain the association of the activated HIF-1α pathway with aggressive tumor behavior and poor prognosis (13).

HIF-1α is a critical transcription factor that transactivates genes the protein products of which function either to increase the O₂ availability or to allow metabolic adaptation to O₂ deprivation (8). Most of these proteins, including VEGF, are implicated in tumor angiogenesis and progression. MDV and VEGF expression were increased in high-grade prostatic intra-epithelial neoplasia and PCa compared to normal prostate tissue and BPH (18, 19). HIF-1α was up-regulated in the lesions of high-grade prostatic intra-epithelial neoplasia, suggesting that up-regulation of HIF-1α is an early molecular event in prostate carcinogenesis (20, 21). Hua Zhong et al. suggested that overexpression of HIF-1α can occur very early in carcinogenesis, before there is histological evidence of angiogenesis or invasion (20).

In the current work, HIF-1α nuclear immunostaining was demonstrated in PCa and in BPH. PCa tissue showed a significantly higher expression compared to BPH tissue. These results may suggest the potential role of the HIF-1α protein in PCa. VEGF is one of the major HIF-1 target genes. This 32-to 45-kDa homodimer specifically recruits endothelial cells into hypoxic and avascular areas and stimulates their proliferation. This growth factor interacts with its receptors (VEGF-R1 and -R2), thereby stimulating endothelial cell proliferation. Hypoxia induces the expression of VEGF mRNA and protein, up-regulating VEGF expression (17). In our study, detectable VEGF staining was observed in all carcinoma cases and in a high proportion of benign hyperplasias. Moreover, there was a remarkably higher VEGF expression in high-grade than in low- to intermediate-grade prostate carcinomas. Some investigators have proposed that VEGF-induced neovascularization begins in totally benign conditions such as BPH, but keeps

<table>
<thead>
<tr>
<th>Table II. Occurrence of high levels of the 3 markers (MVC, VEGF, HIF-1α) in low-intermediate and high grade carcinomas.</th>
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<tr>
<td></td>
</tr>
<tr>
<td>High MVC</td>
</tr>
<tr>
<td>High VEGF</td>
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<tr>
<td>High HIF-1α</td>
</tr>
</tbody>
</table>

PCa=prostate cancer; MVC=microvessal count; VEGF=vascular endothelial growth factor; HIF-1α=hypoxia-inducible factor-1α.
progressing in a step-wise fashion in premalignant and malignant states. However, not all researchers are in agreement and, thus, the exact role of angiogenesis in prostate cancer has not yet been determined (15).

The statistical analysis of our data revealed a positive correlation between the expressions of VEGF and HIF-1α in the PCa group. This reflects the impact of HIF-1α on tumor angiogenesis and progression.

Neo-angiogenesis is quantitated by the MVD (15, 17). Regarding the MVC, a higher mean value was observed in PCAs than in BPHs. However, taking into account that 41.1% of BPH cases exhibited a high MVD, we can claim that angiogenesis does play a role in the pathogenesis of the benign process of BPH. In addition, it is interesting to note that the MVD was more increased in high-grade than in low-to-intermediate-grade PCAs.

Conclusion

MVC, VEGF and HIF-1α were more prominent in PCAs than in BPH. Moreover, high-grade carcinomas displayed high immunoexpression of these three markers.

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References


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