The Angiogenic Switch for Vascular Endothelial Growth Factor-A and Cyclooxygenase-2 in Prostate Carcinoma: Correlation with Microvessel Density, Androgen Receptor Content and Gleason Grade

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Abstract

Objective: Angiogenesis is essential for tumor growth and metastasis; however, angiogenic factors are not uniformly expressed in prostate carcinoma. Our aim was to determine the expression of vascular endothelial growth factor-A (VEGF-A) and cyclooxygenase-2 (COX-2) in prostate carcinomas in relation to intratumoral microvessel density (MVD), tumor grade and androgen receptor (AR) status. Materials and Methods: The expression of AR, VEGF-A and COX-2 was immunohistochemically evaluated in 24 benign prostatic hyperplasia (BPH) and 139 prostate carcinoma cases. MVD was evaluated by CD34 immunostaining. Results: Nuclear AR expression was inversely related to tumor grade (p < 0.001), MVD was strongly related to tumor grade, VEGF-A and COX-2 (p < 0.001 in all comparisons), VEGF-A expression increased with tumor grade (p < 0.01) and was inversely related to stromal AR expression. COX-2 was present in both BPH and prostate carcinoma, but its expression increased with tumor grade (p < 0.01). High-grade neoplasms presented low-to-moderate VEGF staining intensity compared to strong COX-2 expression. Conclusions: Both VEGF-A and COX-2 expression is positively correlated with tumor grade and MVD. However, in Gleason 8–10 tumors, VEGF expression is moderate while COX-2 immunostaining is intense, suggesting a possible switch in the role of these two angiogenic factors in poorly differentiated neoplasms.

Introduction

Angiogenesis, a procedure essential for tumor growth and metastasis, is a result of complex interplay of positive and negative factors. One of the most important inducers of angiogenesis is vascular endothelial growth factor (VEGF), which represents a family of cytokines including isoforms VEGF-A, VEGF-B, VEGF-C and VEGF-D. The different VEGF family members have a variety of functions and effects. In prostate carcinoma, VEGF-A has been shown to play a central role; overexpression of this angiogenic factor has been found to correlate with a higher tumor stage and poor prognosis. However, expression of VEGF-A is not uniform among neoplasms of different grades, suggesting that other proangiogenic factors may also play a critical role during prostate cancer progression [1].

Cyclooxygenase-2 (COX-2) is the inducible isofrom of COX, a rate-limiting enzyme in prostaglandin biosyn-
thesis. Enhanced expression of COX-2 has been found in many tumors, such as breast, lung, colon and prostate cancer. Recent studies have demonstrated that COX-2 could affect carcinogenesis via several mechanisms, including angiogenesis. However, the expression of COX-2 in human prostate carcinoma remains controversial. Although most studies agree that, compared to normal or hyperplastic tissue, prostate carcinoma cells express higher amounts of COX-2, a positive relationship with tumor grade has not been clearly established [2].

An established method to quantitatively assess angiogenesis is detection of CD34 antigen expression. CD34, a myeloid progenitor cell antigen, is a surface glycoprotein detectable in all types of endothelium. CD34 is preferred to older markers (e.g., factor VIII) to detect endothelial cells of the tumor vasculature, and the quantitative expression is determined by intratumoral microvessel density (MVD) [3]. MVD has been shown to correlate well with tumor grade in prostate carcinoma and can be used as a prognostic marker for cancer progression and metastasis even after radical prostatectomy [4].

The aim of this study was to immunohistochemically evaluate the expression of VEGF-A and COX-2 in benign prostatic hyperplasia (BPH) and prostate carcinoma in relation to MVD and the Gleason grade of the neoplasms. Moreover, keeping in mind that in hormone-related neoplasms, such as prostate cancer, sex steroids upregulate angiogenic factors, the relationship between androgen receptor (AR) status and VEGF-A and COX-2 expression was further investigated.

**Materials and Methods**

**Tissue Samples**

A total of 139 cases of primary prostate carcinoma and 24 cases of BPH (patients between 52 and 95 years of age, mean 74) were included in the study. Specimens were selected randomly from archival material available at the Pathology Department of Patras University Hospital. Prostatic tissue was obtained from transurethral resections and radical prostatectomies for previously untreated prostate carcinoma. Tumors were graded according to the updated Gleason grading system [5] and further divided into 3 subgroups (grade I: Gleason score 2–5; grade II: Gleason score 6–7 (when 7 = 3 + 4); grade III: Gleason score 7–10 (when 7 = 4 + 3)). The distribution of cases in each group was as follows: grade I = 38, grade II = 72 and grade III = 29. Tissues were routinely fixed in formalin and embedded in paraffin blocks. At the review of the hematoxylineosin stained sections, paraffin blocks were selected for immunostaining and serial sections were cut at a thickness of 4 μm.

**Immunohistochemistry**

Immunostaining was performed according to the streptavidin–biotin complex peroxidase method using the commercially available UltraVision Large Volume detection system (NeoMarkers, LABVISION Corporation) according to the manufacturer’s instructions. Briefly, after deparaffinization and rehydration through a series of xylene and ethanol, sections were treated with the microwave antigen unmasking buffer technique [6]. Enzyme peroxidase inhibition was accomplished by incubation in 3% hydrogen peroxide and sections were rinsed in Tris-buffered saline. Nonspecific binding was blocked by treating slides with 1% bovine serum albumin. The commercially available antibodies for VEGF-A (mouse monoclonal), CD34 (NeoMarkers, LABVISION Corporation), COX-2 (Santa Cruz Biotechnology Inc., Santa Cruz Calif., USA) and AR (monoclonal, MCR 205, YLEM, Italy) were used at dilutions of 1:40, 1:200, 1:200 and 1:20, respectively. Color development was done by using 3,3′-diaminobenzidine substrate system (NeoMarkers, LABVISION Corporation). Finally, tissue sections were counterstained with hematoxylin and dehydrated through graded ethanol and xylene, and mounted under glass cover-slips.

**Analysis of Staining**

Sections were scored under light microscopy for VEGF and COX-2 expression using a semiquantitative method with a 0–3 scale (0: no immunoreactivity, 1+: weak intensity; 2+: moderate intensity; 3+: strong intensity) [7]. Evaluation of staining was performed independently by two investigators (K.V. and E.P.), who were blinded to the clinicopathological findings. The allocation of tumors and staining scores of the two investigators were similar in most cases. In cases of disagreement, slides were reevaluated and discussed until consensus was achieved. MVD was assessed after initial identification of three vascular ‘hot spots’ (areas of maximum endothelial cell staining of microvessels at low magnification) viewed under high power (×200). Any dark stained endothelial cell or cell cluster separate from adjacent structures was counted as a single vessel. The final MVD score was expressed as the mean count of positive vessels per visual field [3]. Nuclear AR staining was expressed as percentage of positive tumor cells, whereas stromal AR expression was assessed using a semiquantitative method, as described above.

**Statistical Analysis**

Overall variations in the percentages of nuclear staining for AR and MVD (CD34) and their relationship with tumor grade, COX-2 and VEGF-A were evaluated by ANOVA; a Bonferroni test was used for comparisons between grade subgroups. Stromal AR expression was correlated to tumor grade, VEGF-A and COX-2 expression using Spearman’s rank correlation coefficient. Analysis of concordance between tumor grade and VEGF-A and COX-2 expression was estimated using Kendall’s tau. All statistical analysis was performed using commercially available software (SPSS v.14. 0 for Windows).

**Results**

AR was localized almost exclusively in the nuclei of prostate epithelial cells in 97% of cases (fig. 1a, b). The comparison of AR staining with tumor grade revealed an inverse relationship between these two parameters.
(ANOVA, p < 0.0001; fig. 2a). Stromal cells also exhibited positivity for AR staining and there was also an inverse relationship with grade (Spearman r = −0.478, p < 0.0001). When nuclear AR expression in epithelial cells was correlated with VEGF-A and COX-2 immunostaining, no statistically significant correlation was found; however, stromal AR expression presented a strong inverse relationship with VEGF-A expression (Spearman r = −0.312, p < 0.001).

MVD was strongly related to tumor grade, VEGF-A and COX-2 histoscore (ANOVA, p < 0.001 in all comparisons; fig. 1c, d and fig. 2b). All prostate cancer specimens were stained positive for VEGF-A, while BPH cases were negative in 83% of cases (20/24). Immunostaining for VEGF-A was diffuse and cytoplasmic in malignant glandular epithelial cells (fig. 1c, f), and the expression progressively increased with tumor grade (Kendall’s tau-b = 0.344, p < 0.01; fig. 2c). VEGF-A immunoreactivity was also present in peritumoral stromal cells.

COX-2 was present in both BPH and prostate cancer, but 54% of BPH cases were negative (13/24). One case of grade I prostate carcinoma was negative despite repeated...
immunostaining attempts (1/139). COX-2 staining in prostate cancer cells was mainly cytoplasmic, intense and uniform although many cases demonstrated intense membrane staining (fig. 1g, h). The enzyme was also strongly expressed in smooth muscle cells of the stromal compartment. Expression generally increased with tumor grade (Kendall's tau-b = 0.551, p < 0.01; fig. 2d). Interestingly, when VEGF-A and COX-2 expression were compared for every grade category, high-grade neoplasms (grade III) presented low-to-moderate VEGF staining intensity (55% of cases) compared to COX-2 expression, which was strongly positive in 93% of cases.

Discussion

The majority of deaths from prostate cancer occur due to lymphatic and hematogenous spread of the primary tumor and formation of distant metastases. The critical role of tumor angiogenesis in tumor growth and progression was postulated more than three decades ago [8]. Since then, extensive studies have elucidated numerous molecules that act as angiogenic promoters in several neoplasms as well as in prostatic adenocarcinoma [9]. However, many studies report conflicting results in relation to the extent of expression of different angiogenic promoters in prostate cancer. Moreover, the role of these promoters is not uniform along all types of tumors; tumor grade may significantly alter the angiogenic milieu [10, 11].

VEGF is a fundamental regulator of angiogenesis in prostate cancer. This glycoprotein stimulates endothelial cell proliferation, prevents regression of newly formed vessels and increases vascular permeability [12]. Previous reports implicate a strong role for VEGF-A-mediated angiogenesis in prostate carcinoma; however, contradictory results have been reported on the expression of VEGF in normal, hyperplastic and carcinomatous prostate glands in relation to tumor grade [13, 14]. In our study no VEGF-A expression was detected in 83% of BPH cases, but all prostate cancer specimens demonstrated some degree of immunoreactivity. The expression pattern was diffuse and multifocal, with foci of VEGF-A-positive tumor cells among negative normal or tumoral epithelium. This multifocal pattern has been previously reported in prostate as well as in other neoplasms. This finding possibly represents differential local expression due to gene amplification, local upregulation or altered VEGF turnover [14]. In the case of prostate glandular epithelium, it may also reflect different secretory activity of epithelial cells [15].

In our study, VEGF-A immunostaining at the peritumoral stromal cells was variable, with occasionally strong positivity in smooth muscle cells and neutrophils. Previous studies have reported VEGF immunoreactivity in stromal cells in colon and ovarian cancers [16, 17]. However, the biological role of stromal VEGF remains unclear. In the case of BPH, this finding may represent a highly vascularized stroma due to active proliferation. It has been postulated that VEGF is produced in cancer cells and subsequently sequestered in adjacent stroma cells in some tumor types [17]. Nevertheless, the presence of VEGF in peritumoral stroma may be associated with degradation of the extracellular matrix and facilitation of new blood vessel formation [18].

Another interesting finding was the inverse relationship of stromal AR expression in relation to VEGF-A immunoreactivity. As it has been previously shown that an-
drogens upregulate VEGF expression both at the epithelial and stromal level, one would expect that increased AR expression, particularly in the epithelial cells, would translate into high levels of VEGF [19, 20]. The effect of androgens on VEGF expression is mainly mediated through the hypoxia factors HIF-1a and HIF-2a [21]. However, other factors (e.g., peptide growth factors) may regulate VEGF expression independently of AR [21]. It has also been also shown that vascular regulators in the stroma are not decreased after castration in a rat prostate cancer model, suggesting that the stroma is androgen insensitive [22]. Moreover, VEGF-A is not the sole VEGF isoform regulated by AR. Androgen depletion through blockade of AR has been shown to upregulate VEGF-C through activation of the small GTPase, RaIA [23]. It is therefore possible that this unexpected inverse relationship between stromal AR content and VEGF-A represents regulation of VEGF-A by factors independent of androgens at the stromal level.

COX-2 is a key enzyme involved in the production of proinflammatory prostaglandins and plays an important role in tumorigenesis in many cancer tissues through a number of mechanisms which include increased proliferation [24], reduced apoptosis [25], and stimulation of angiogenesis and metastasis [26, 27]. The recent progress in the understanding of the key role of COX-2 in tumorigenesis and angiogenesis has led to an increased interest in the clinical use of selective COX-2 inhibitors as chemopreventive and curative agents in several tumors. The reports on COX-2 expression in human prostate cancer, however, are still controversial as some authors describe limited presence of COX-2 in prostate carcinoma [2, 28, 29].

In our study, COX-2 immunopositivity was present in 46% of BPH specimens and in 99% of primary prostate carcinomas. Only one case of grade I tumor was negative, despite repeated immunostaining attempts. The increased COX-2 expression correlated significantly with MVD and Gleason grade. Several studies have shown that COX-2 is overexpressed in prostate carcinoma, and in most reports the upregulation of COX-2 is related to tumor differentiation [30–34]. In the present study, the COX-2 positive rate (99%) is one of the highest between similar reports. Wang et al. [35] have also noted a 93% positive COX-2 rate in prostate carcinoma cases, with the expression increasing in cancer specimens with high Gleason scores. The discrepancy in COX-2 expression in other studies may be explained by the heterogeneity of tumor specimens, the use of cell lines (LNCaP, DU145, PC3) instead of tissue specimens, the presence of adjacent proliferative inflammatory atrophy and the methodology used in the quantitative analysis of staining [28, 35].

Although VEGF-A and COX-2 are both increasingly expressed in prostate carcinoma cases in relation to tumor grade, a discrepancy exists when their expression is compared between different grade categories. In our study, high-grade neoplasms (grade III) presented low-to-moderate VEGF staining intensity (1+–2+) in 55% of cases) compared to COX-2 expression, which was strongly positive (3+) in 93% of cases. These results suggest the activation of an angiogenic switch in poorly differentiated neoplasms, where COX-2 may play a crucial role compared to VEGF. Data from studies in other human cancers, i.e., colorectal cancer, suggest a significant role of VEGF-A at early stages of the normal-adenoma-carcinoma sequence [36]. Similar reports underline that the role of VEGF is critical during the initial stages of neoplastic progression and in different, less aggressive tumor types [1, 37].

Our findings of strong COX-2 expression in prostate carcinoma of high Gleason grade suggest that COX-2 may play a key role in poorly differentiated cancers, and this should be taken into account when designing antiangiogenic treatment. So far, antiangiogenic treatment studies, both in vitro and in vivo, do not make stratification according to tumor grade [25, 38–41]. Although simplistic, we propose that according to these findings, anti-VEGF therapy would be more beneficial in patients with low-grade disease, while patients with high-grade prostate carcinoma are more likely to respond to selective COX-2 inhibitors. Immunohistochemical determination of VEGF-A and COX-2 (and possibly other angiogenic factors) content might prove a useful tool in the design of patient-tailored antiangiogenic treatments.

Conclusions

The results of the present study demonstrate a strong correlation between mean MVD, VEGF and COX-2 expression in prostate carcinoma. AR content of the peritumoral stroma (but not the epithelial compartment) is inversely related to VEGF-A expression. Although both VEGF and COX-2 expression is positively correlated with tumor grade, COX-2 is intensely expressed in cases with high Gleason grades, suggesting a possible switch in the role of these two angiogenic factors in poorly differentiated neoplasms. Profiling individual patients for angiogenic factors like VEGF-A and COX-2 might help to determine the timing and type of antiangiogenic therapies of optimal benefit.
References


