Expression of vascular endothelial growth factor-A in human lymph node metastases of prostate cancer

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Introduction: *Vascular endothelial growth factors (VEGF) figure among the most potent angiogenic factors identified. Local as well as serum VEGF-A over-expression has been correlated with metastasis in prostate cancer. However, little is known on VEGF-A expression in prostate cancer metastases themselves. Our objective was to assess VEGF-A expression in relation with angiogenesis in prostate cancer lymph node metastases.*

Methods: *Fifty-four lymph node metastatic specimens obtained from 32 patients were included in this study. All nodes were of prostate cancer origin as confirmed by positive PSA staining. Immunohistochemistry was performed to identify VEGF-A expressing cancer cells. Consecutive sections were stained for angiogenesis using von willebrand factor (vWF) as a maker and neuroendocrine (NE) differentiation using chromogranin-A and serotonin antibodies. VEGF-A expressing cells were recorded (at 100x magnification) and expression was classified as: 1- Negative (0 cells), 2-Low (1-5 positive cells/high power field (HPF)), and 3- High (> 5 cells/HPF).*

Results: *VEGF staining was identified mainly in NE cells and endothelial cells. Of the 32 patients, VEGF expression was positive in 22 (69%), with 2 patients (6%) showing high levels and 20 patients (63%) classified as low. No VEGF expression was noted in 10 patients (31%). Of the 54 metastatic lymph node specimens examined, VEGF expression was positive in 31 (57%). VEGF-A stained NE cells did not correlate with blood vessels as revealed by vWF expression in consecutive sections (p=0.9).*

Conclusion: *VEGF-A is preferentially expressed in NE cells of prostate cancer lymph node metastases and appears unrelated to angiogenesis. These observations support the role played by NE-expressing VEGF-A in the development of metastatic prostate cancer.*

Key Words: VEGF-A, human, prostate cancer, lymph nodes, metastases

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Introduction

It is well established that the growth and dissemination of solid tumors depends on angiogenesis.[1-3] In prostate cancer, microvessel density has been shown to correlate with clinical stage,[4-6] progression after radical prostatectomy,[7] development of metastasis,[5] and patient survival.[6,8] A number of angiogenic factors including fibroblast growth factors, platelet-derived growth factor, transforming growth factors, and

vascular endothelial growth factors (VEGFs) have been identified.[9-12] Of these factors, the VEGF family figures as the most potent facilitators of angiogenesis to date with effects reported on endothelial cell proliferation, motility, and vascular permeability.[12] Four alternatively spliced VEGF isoforms, which differ in cellular location and in affinity for heparin, have been described (VEGF 121, 165, 189, and 206).[13] In addition, a variety of VEGF-related peptides have been identified by alternative splicing of the VEGF-A gene or as products of distinct genes (placental growth factor-1 and -2, VEGF-B, -C, –D and -E).[13]

Most studies in human prostate cancer deal with VEGF-A expression and its detection in tumor cells as well as normal epithelial cells including the neuroendocrine (NE) phenotype, and infiltrating lymphocytes from the prostate.[14-16] Local VEGF expression in tumor cells was shown to correlate positively with tumor stage, grade, microvessel density (MVD), and clinical outcome.[17] Apart from its well documented angiogenic role, VEGF has recently been reported to exert a paracrine function on prostate tumor cells expressing VEGF receptors, and resulting in enhanced motility and/or growth *in-vitro*.[18,19] Moreover, the effect of VEGF-A on the aggressive phenotype of prostate cancer cells was associated with activation of Flt-1 and downstream signaling through focal adhesion kinase.[19] In plasma, VEGF levels have been reported to be higher in patients with metastatic prostate cancer than in patients with localized disease or healthy controls,[20] and expression in primary tumors has been reported to be increased in metastatic disease.[21,22] These data suggest that VEGF expression might have an important role in the development of metastatic prostate cancer. However to the best of our knowledge, little is known on VEGF expression in prostate cancer lymph node metastases. Our objective was to assess VEGF-A expression and angiogenesis in lymph nodes of patients with metastatic prostate cancer.

Materials and methods

Tissues

Lymph node specimens were initially obtained from sixty-five patients with metastatic prostate cancer who did not receive prior hormonal treatment. Prostate cancer metastasis was confirmed by positive PSA staining. Of the 65 patients, 32 were found to have sufficient metastastic prostate cancer in lymph nodes (>10% of volume) for study and hence were included. From the 32 patients, 54 lymph node metastases were evaluated.

Immunohistochemistry

Paraffin-embedded lymph node sections (4 μm) were initially deparaffinized with toluene and rehydrated through graded ethanol. All steps were performed at room temperature. Following each step, sections were washed with 0.01M TTBS (Tween/Tris buffered saline solution) for 10 minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide/50% methanol for 15 minutes. The procedure for VEGF-A staining was essentially as previously reported.[19] Briefly, an antigen retrieval technique was applied through boiling slides for 15 minutes in a water bath at 95°C in a 0.01M sodium citrate buffer, pH 6.0. Tissue sections were incubated with normal goat serum for 15 minutes to block non-specific binding. VEGF expression was studied using a polyclonal VEGF-A antibody (Santa Cruz Biotech, Santa Cruz, Ca) that recognizes isoforms 121, 165, and 189. The antibody was applied on tissue sections at a concentration of 1:400 in TTBS and slides were incubated over night at room temperature. Immune complexes were revealed using a biotin-conjugated anti-rabbit secondary antibody (1hr), followed by a streptavidin-peroxidase conjugate for 15 minutes (Zymed, San Francisco, CA), and finally by the chromogenic substrate (0.06% 3,3'-diaminobenzidine tetrahydrochloride/0.01% hydrogen peroxide in TBS). Sections were counter stained with Mayer's haematoxylin, dehydrated and then mounted. Negative controls consisted of omitting the primary antibody and positive controls employed prostate tissue sections, as reported.[19]

Blood vessels were stained on consecutive sections using rabbit polyclonal von Willibrand factor (vWF) antibody (1:800 dilution, 1hr; Dako Corp.), according to the manufacturer's instructions. An antigen retrieval technique was first applied by using Pronase digestion (Boehringer Mannheim, Germany) in 0.1% TBS for 15 min, and followed by incubation with 10% normal goat serum and subsequent steps, as mentioned above.

Consecutive sections from lymph nodes showing high VEGF expression (n=5) were further stained for chromogranin A and serotonin to identify NE cells and correlate them to the VEGF-expressing cancer cells. Chromogranin A staining was done using a monoclonal antibody (Boehringer Mannheim, Germany), while serotonin was stained using a polyclonal antibody (Zymed, San Francisco, Ca) in a procedure using pronase as an antigen retrieval technique (0.2% in TBS for 15 min). Other steps were essentially similar to the VEGF staining method and were performed using Histostain-SP Bulk Kit (Zymed). Neuroendocrine-positive controls were included using prostate tissue sections, as reported.[19,23,24]

Quantification

Lymph node sections were evaluated at high power field microscopy (100x). The number of VEGF-positive cells was assessed. Chromogranin and serotonin-positive cells were not quantified *per se* but were used for identification of NE cells. A minimum of five areas were evaluated for each section and the mean was calculated. Three categories were predefined: 1- negative (0 cells), 2- Low positive (1-5 VEGF-stained cells/ high power field (HPF)), and 3- High positive (>5 cells/ HPF). For vWF, the number of stained blood vessels was counted at 100x magnification in five different areas for each specimen. Means were calculated and results were analyzed using the student t-test.

Results

VEGF immunostained cancer cells were identified in 31 of the 54 metastatic lymph nodes examined (57%), while 23 lymph nodes (43%) showed no staining. Figure 1a shows a representative specimen exhibiting strong VEGF-A immunoreactivity. The staining was preferentially associated with: 1) neuroendocrine prostate cancer cells, as confirmed by staining 5 consecutive sections for chromogranin-A and serotonin (not shown), as well as 2) endothelial cells in some blood vessels. A closer view of VEGF-positive neuroendocrine cells is shown in Figure 1b where VEGF-expressing NE cells in tumors were isolated and not found in clusters or colonies. Immunoreactivity was exclusively cytoplasmic and intense. Neuroendocrine cells also commonly possessed cytoplasmic processes extending laterally around adjacent cells or toward the lumen of malignant gland-like structures. Overall VEGF-A stained neuroendocrine cells comprised the minority of tumor cells in all metastatic specimens examined. This was also the case for chromogranin-A and/or serotonin in specimens used for NE cell identification.

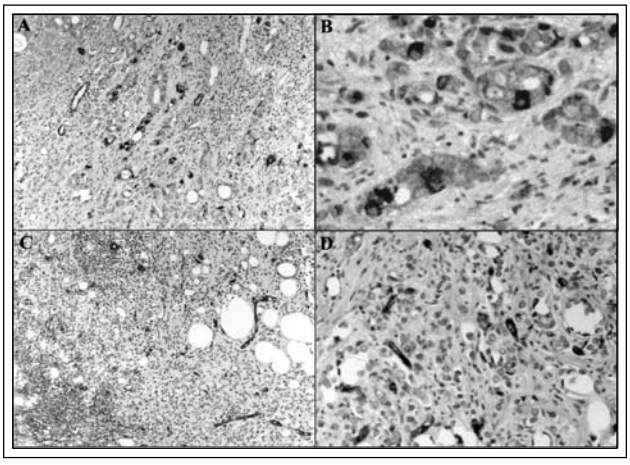

Figure 1a,b,c,d. Photomicrographs showing VEGF-A-positive cells in prostate cancer lymph node metastases at 100x a) and 200x b) magnification, as well as vWF-stained blood vessels at 100x c) and 200x d).

TABLE 1. **Vascular endothelial growth factor (VEGF) expression in prostate cancer lymph node metastasis**

VEGF expression	Patients (n=32)	Proportion (%)
Negative (0 cells/HPF)	10	31%
Low + ve (1-5 cells/HPF)	20	63%
High + ve (>5 cells/HPF)	2	6%

TABLE 2. **Correlation between vascular endothelial growth factor (VEGF) expression in prostate cancer lymph node metastasis and angiogenesis (number of blood vessels in 100x magnification field) using vWF as a marker**

VEGF expression	VWF stained blood vessels (mean ± SD)	P value
Negative (0 cells/HPF)	32 ± 10	
Low + ve (1-5 cells/HPF)	58 ± 11	0.9
High + ve (>5 cells/HPF)	34 ± 6	

Table 1 summarizes the differential lymph node-expression of VEGF-A in NE cells of the 32 patients included in this study. A majority (approximately 70%) had VEGF-positive NE cells in metastatic prostate cancer lymph nodes: 2 patients (6%) were in the group classified as high (> 5 cells/HPF) and 20 patients (63%) in the low VEGF expression group (1-5 cells/HPF). Approximately one third (31%; 10 patients) showed no VEGF expression.

The vWF-stained blood vessels and their correlation with VEGF expression in NE cells are illustrated in Figure 1c and Figure 1d and Table 2, respectively. In general, VEGF-A expression in endothelial cells (Figure 1a and Figure 1b) only represented a minority of vWF-stained blood vessels (Figure 1c and Figure 1d). Moreover, the number of VEGF-A stained NE cells in tumors showed no correlation with angiogenesis, as

the mean number of stained blood vessels in patients with high VEGF expression was 34 ± 6 compared to 58 ± 11 and 32 ±10 in patients with low and no VEGF expression; respectively (p = 0.9; Table 2).

Discussion

In the present study we expanded our initial observation on VEGF-A expression in prostate cancer lymph node metastasis[19] and demonstrated that a majority of prostate cancer lymph node metastases express this growth factor. VEGF-expressing cells showed a dispersed and focal pattern similar to that observed in primary tumors.[19] Our data indicate that in prostate cancer lymph node metastasis, VEGF-A immunoreactivity was preferentially distributed in prostate cancer NE cells, as confirmed by staining consecutive sections for chromogranin-A and serotonin, together with vascular endothelial cells seen in isolated blood vessels. VEGF-positive NE cells were detected in approximately 70% of patients (22 out of 32 patients) as well as in 57% of metastatic lymph nodes (31 out of 54). Of interest, these findings are in-agreement with a report on neuroendocrine differentiation in metastatic prostatic adenocarcinoma, where chromogranin-A positive NE cells were identified in 19 of 41 lymph node metastasis (46%), while serotonin-immunoreactive NE cells were observed in 7 of 12 node metastasis (58%).[23] Neuroendocrine cell differentiation in primary prostatic tumors has been previously correlated with progression and poor prognosis.[24-26] Furthermore, some reports have postulated that NE cells may exert a paracrine growth regulatory role on prostate cancer cells.[18,19,23,26] The present observation confirming the presence of NE cells in prostate lymph node metastasis expressing VEGF-A, with its documented angiogenic and possible paracrine effects, therefore further supports the possible growth regulatory role played by NE cells in the development of metastatic prostate cancer.

Tissue VEGF expression has been reported to be associated with the metastatic potential of prostate tumor cells,[27,28] while circulating VEGF levels were higher in patients with disseminated, as well as hormone-resistant prostatic tumors.[20,29,30] The action of VEGF is mediated by well-known high affinity VEGF signaling receptors activated by autophosphorylation and resulting in various biological responses that include changes in morphology, chemotaxis, mitogenicity and cellular protein expression. [12,13,31-33] On the other hand, inhibition of tumor-secreted VEGF by neutralizing antibodies has been shown to suppress primary tumor growth and inhibit metastatic dissemination.[34] Accordingly, VEGF-A expression in metastatic prostate cancer, as identified in the present study on lymph nodes of prostate cancer patients, may be helpful in identifying tumors with more aggressive phenotypes.

Tumor growth and metastasis depend on the induction of angiogenesis,[1-3] which is mediated by several angiogenetic factors, especially VEGFs.[11-13] In the present study, VEGF-A expression in endothelial cells of blood vessels and in malignant NE-cells of lymph node metastases did not correlate with angiogenesis. While this can be partly explained by an inadequate sample size, the contribution of other angiogenic factors is also conceivable.[9-11] Accordingly, VEGF-A expression in endothelial cells likely reflect only part of the angiogenic process. Moreover, vWF may not be the ideal marker for detecting angiogenesis in metastatic lymph nodes of the prostate, especially since to our knowledge angiogenesis in prostate cancer lymph node metastases has not been well documented. The lack of correlation between VEGF-A expression in NE cells and the number of blood vessels may also be attributed to a non-angiogenic but direct paracrine role played by NE cell products on motility and/or growth of surrounding tumour cells in lymph node metastasis.[19,26] Unfortunately, the corresponding primary tumor samples and clinical information were not available in our study and therefore no conclusions can be drawn regarding the clinical significance of our findings. Despite this limitation, this study represents a detailed histologic and immunophenotypic characterization of a relatively large set of prostate cancer lymphatic metastases. The relationship between VEGF-A expression in NE cells and progression or outcome needs to be established in further studies on a larger cohort of lymph node metastases and primary tumors from individual patients with known clinical characteristics.

Conclusion

Prostate cancer lymph node metastases commonly express VEGF-A in focal neuroendocrine tumor cells as well as a few endothelial cells. No correlation was observed between VEGF expression and the number of blood vessels. Our observations support the possible role played by VEGF-A and neuroendocrine cells in the development of metastatic prostate cancer. Further studies are required to correlate VEGF expression in lymph node metastases to clinical outcome. □

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