Expression of Different Vascular Endothelial Markers in Prostate Cancer and BPH Tissue: An Immunohistochemical and Clinical Evaluation

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Abstract. Background: The microvessel density (MVD) is often used as a quantitative parameter for angiogenesis. The aim of this study was to evaluate the three most commonly used endothelial markers of microvessels, CD31, CD34 and vWF, in benign and malignant prostatic tissue. Materials and Methods: Fifteen benign hyperplastic prostate and 54 prostate cancer specimens were immunohistochemically stained for CD31/CD34/vWF. The MVD obtained by each marker was quantified by a vessel count in standardized grids within the area of maximum angiogenesis. The data on MVD was interrelated and compared to tumor staging, grading and clinical follow-up. Results: A significant correlation of the CD31/CD34/vWF-MVD data was observed in BPH tissue, but not in PCa. In PCa, the most sensitive marker for newly derived blood vessels was CD34. While CD34-MVD demonstrated a significant association with tumor grading and PSA follow-up, CD31- or vWF-MVD did not. Conclusion: CD34 is a suitable marker for the immunohistochemical visualization of microvessels in benign and malignant prostate tissue.

Angiogenesis has been a major focus of oncological research over the past 30 years. With regard to solid tumors in particular, the development of new blood vessels has been investigated extensively. New therapeutic strategies targeting angiogenesis have emerged, such as antibodies on the protein level and oligonucleotides on the RNA level, which can have an effect on receptors and pathways involved in the regulation of endothelial proliferation (1,2). In addition, specific drug delivery systems have been developed that target immature endothelial cells such as cationic liposomes (3).

The basis of most research on angiogenesis, past and present, is the assessment of blood vessels in the tissue under investigation. To date, the immunohistochemical analysis and quantification of the microvascular structure within the tumor is the basis for understanding the effects of anti-angiogenic treatment and for monitoring its efficiency. Several different concepts exist. The most common used is the quantitative assessment of microvessels which was first described by Weidner et al. (4). Sections of tissue are immunohistochemically-stained with different endothelial markers such as CD31, CD34 or the von Willebrand factor (vWF, factor IIIV). The microvessels/endothelial cells are then counted in a standardized grid using light microscopy and expressed as microvessel density (MVD). Further development was directed towards a semi-automatic assessment of microvessels using morphometry (5).

Previous reports on the expression of endothelial markers have shown an inconsistent expression pattern in different tissues (6,7). The data on MVD in benign and malignant tissue quoted in different publications is often compared in literature, even though different endothelial markers were used. It is unknown, however, whether the data on MVD in benign and malignant prostate tissue obtained by different endothelial markers can be compared. In addition, some studies have shown that MVD (obtained by different endothelial markers) is a prognostic marker for prostate cancer in some studies (4), whereas others have failed to demonstrate the predictive value of MVD (8).

Therefore, our aim was to quantify MVD by using the three most common endothelial markers, CD31, CD34 and vWF, in serial sections of benign and malignant prostatic specimens. The data obtained for MVD were interrelated and, in the case of prostate cancer, compared to tumor
Serial sections of BPH and PCa tissue, stained by three different endothelial markers. BPH (1A-C) and PCa tissue (1D-E) stained by vWF (A+D), CD31 (B+E) and CD34 (C+F). In BPH tissue, mature microvessels are visible and are stained by all three markers. In contrast to benign tissue, vWF, CD31 and CD34 display a different staining pattern in malignant tissue. Some microvessels with a mature endothelial layer and a lumen are detected by all three markers (arrowheads), whereas some immature vessels are detected only by CD34 (arrows).

Figure 1. Endothelial staining of BPH and PCa (vWF, CD31, CD34).
staging and grading. We also investigated the prognostic relevance of MVD for biochemical failure after PCa treatment.

Materials and Methods

Patients and specimens. The study included a total of 54 prostate cancer specimens obtained by radical prostatectomy due to clinically localized prostate cancer. Fifteen specimens of benign hyperplastic tissue of the prostate (BPH) were obtained by transurethral resection (Department of Urology, University Hospital Mannheim, Germany). Specimens were not selected by any particular means. The mean age on the date of surgery was 65.5±5.7 (PCa) and 66.3±8.7 (BPH) years.

Tissues were fixed in 10% buffered paraformaldehyde (Sigma Chemical, St. Louis, USA) for 24 h and embedded in paraffin. After hematoxylin/eosin staining, routine staining and grading diagnostics were performed in accordance with the UICC TNM classification (1997) and the Gleason method (9) at the Department of Pathology. Twenty-five of the investigated cancer specimens were organ-confined (T1/2) and 29 were staged T3/4. Twenty-seven of the specimens were regarded as low-grade tumors, a further 27 as high-grade tumors following the method of Gleason (Gleason sum score: 2-6 and 7-10, respectively). Using histomorphological grading, 25 of the specimens were graded as G1 or G2a and another 29 as G2b or G3. In the case of PCa, PSA follow-up was performed. PSA >0.1 ng/ml was considered as a biochemical failure of treatment. The major tumor-containing block of each prostate cancer specimen was used for further investigations.

Immunohistochemical staining and analysis. Serial sections of 3 μm were mounted onto Superfrost/Plus slides (Menzel, Braunschweig, Germany) and dried at 30°C overnight. The sections were deparaffinized in Neoclear (3 x 5 min, Merck) and rehydrated in graded alcohol, then washed twice in PBS (phosphate-buffered saline, pH 7.4; Sigma, Taufkirchen, Germany) for 5 min.

The sections were stained using the standard ABC method (Vector Laboratories, Burlingame, USA). Endogenous peroxidase activity was blocked by incubation with 1% hydrogen peroxide in methanol for 30 min, followed by double-washing (PBS). The sections were power-cooked in citrate buffer (pH 6.0; DAKO, Glostrup, Denmark) three times at 680W in a microwave oven for antigen retrieval. Non-specific binding was blocked by incubation with horse non-immune serum (Vector Laboratories) for 20 min. All primary and secondary immunoglobulins were diluted in PBS and incubated in a humid chamber. In each series, primary antibodies were omitted as a negative control.

CD31, CD34 and vWF. Specific staining for endothelial cells was conducted by using different monoclonal mouse anti-human immunoglobulins as primary antibodies, raised against the PECAM-1 molecule (CD31, DAKO; dilution: 1:25; temperature and time of incubation: 4°C, overnight), the Q8End10 (CD34, DAKO; dilution: 1:50; temperature and time of incubation: room temperature, 1 h) or the von-Willebrand factor (DAKO, dilution: 1:25; temperature and time of incubation: 4°C, overnight).

After being washed twice with PBS, the sections were incubated with a biotinylated horse anti-mouse antibody (AB) at room temperature (RT) for 30 min and afterwards incubated with the ABC peroxidase complex (Staining Kit, Vector Laboratories). Diaminobenzidine (DAB, Vector Laboratories) served as a chromogene. The tissue sections were briefly counterstained with Mayer’s Hämalaun (Merck), rinsed in water and dehydrated in graded ethanol. They were then washed in Neoclear (2x2 min) and mounted (Neo-Mount, Merck).

Assessment of microvessel density. Assessment of microvessel density (MVD) in benign and malignant prostatic tissue was performed by light microscopy as described before with slight modifications (4). Briefly, areas of high MVD were identified by screening the tumor (Gleason sum score: 2-6 and 7-10, respectively). Using histomorphological grading, 25 of the specimens were graded as G1 or G2a and another 29 as G2b or G3. In the case of PCa, PSA follow-up was performed. PSA >0.1 ng/ml was considered as a biochemical failure of treatment. The major tumor-containing block of each prostate cancer specimen was used for further investigations.

Statistical analysis. All statistical analysis were performed using SAS for Windows (Version 8.02; SAS Institute Inc., Cary, NC, USA). The Pearson correlation coefficient was used for the inter-correlation of MVD data obtained by different endothelial markers. The correlation of data on MVD to staging, grading and PSA follow-up was performed with the Wilcoxon rank sum test. A two-sided p value <.05 was considered to be significant.

Results

MVD was assessed by different endothelial markers (CD31, CD34 and vWF) in benign and malignant prostatic tissue. The expression of the three endothelial cell antigens on microvascular structures was observed in all BPH and PCa specimens. Representative serial sections of benign and malignant tissue stained by the different endothelial markers are displayed in Figure 1.

The analysis of MVD by CD31 and CD34 showed a significant increase in the number of microvessels in prostate cancer compared to BPH tissue. The highest MVD was observed with CD34 as the endothelial marker in PCa tissue. vWF-MVD showed a somewhat different picture:
there was a significant decrease in MVD in prostate cancer tissue compared to BPH specimens when vWF was used as an endothelial marker. The data on MVD, including the statistical evaluation, is summarized in Table I.

Interrelation of data. The interrelation of the data on MVD obtained by the three different endothelial markers is dependent on the investigated tissue. In BPH tissue, there was a strong and significant correlation between the MVD data with the three markers. In contrast, PCa tissue showed only a weak, although significant, correlation between vWF and CD31. No correlation was found between CD34 and CD31/vWF. The data is summarized in Table II.

MVD and tumor staging and grading. The data on MVD was compared to tumor staging (as defined by organ-confined vs. non-organ-confined tumors) and grading (as defined by low-grade vs. high-grade tumors). MVD, as assessed by any of the markers, was not dependent on tumor staging (vWF: $p=0.464$; CD34: $p=0.782$; CD31: $p=0.971$). The Gleason sum score showed a trend towards an association with MVD (CD34 marker): an association of high-grade tumors (Gleason score 7-10) with a high MVD was observed, but remained statistically insignificant ($p=0.119$). No correlation was observed when the other two markers were used (CD31: $p=0.957$; vWF: $p=0.931$).

The histomorphological grading was associated with MVD (CD34 marker). High-grade tumors showed a significantly higher MVD than low-grade tumors ($p=0.0245$). No correlation was seen with the other two markers (vWF: $p=0.671$; CD31: $p=0.898$).

MVD and clinical follow-up. In 53 out of 54 cases of PCa, a PSA follow-up after surgical treatment was available. Seventeen of the patients showed a PSA $>$0.1 ng/ml, which was considered as a biochemical failure of treatment. Biochemical failure was correlated to MVD (obtained by CD31, CD34 and vWF), tumor staging and grading.

When CD34 was the endothelial marker for blood vessels, MVD was significantly associated with the biochemical failure of PCa patients. No correlation was seen between CD31- or vWF-MVD and biochemical failure.

Additionally, there was a significant correlation between biochemical failure and the Gleason sum score as well as histopathological grading. No association of biochemical failure with tumor staging was observed in our collective. The data is summarized in Table III.

Discussion

Various studies have reported on the concept of angiogenesis in prostate cancer. It is indeed a widely accepted concept. The immunohistochemical evaluation of microvascular structures within benign and malignant tissues is important, not only for the study of pathological alterations in cancer specimens, but also for documenting the effects of different anti-angiogenic treatment strategies. In addition, the quantification of angiogenesis is of great significance, since it provides prognostic information on a wide variety of solid cancers (4, 10, 11).

In prostate cancer, it has been shown that angiogenesis correlates with tumor progression (12, 13). By counting microvessels/capillaries, angiogenesis can be quantified and expressed as an angiogenesis index, defined as microvessel density [MVD, (4)]. Different endothelial markers, including CD31, CD34 and vWF, are used for staining vascular endothelial cells. This study involved a quantitative comparison of the expression of three different endothelial markers in prostate cancer and BPH tissue, and a comparison of the obtained data to tumor characteristics and clinical follow-up.

In benign prostate tissue, different endothelial markers show a homogeneous staining pattern with a high correlation to MVD data. The range in the mean number of microvessels (MVD) was assessed to be between 39.09 (vWF) and 60.71 (CD34).

### Table II. Interrelation of data on MVD in BPH and PCa tissue.

<table>
<thead>
<tr>
<th></th>
<th>BPH CD34</th>
<th>vWF</th>
<th>PCa CD34</th>
<th>vWF</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31</td>
<td>$r=0.69^*$</td>
<td>$p=0.0047^*$</td>
<td>$r=0.20$</td>
<td>$p=0.1536$</td>
</tr>
<tr>
<td>CD34</td>
<td>$-\cdot$</td>
<td>$r=0.55^*$</td>
<td>$-\cdot$</td>
<td>$r=0.11$</td>
</tr>
<tr>
<td></td>
<td>$p=0.0353^*$</td>
<td>$p=0.4444$</td>
<td>$p=0.0233^*$</td>
<td>$p=0.0004^*$</td>
</tr>
</tbody>
</table>

Data is expressed as correlation coefficient ($r$; Pearson correlation coefficient) and statistical $p$ value. Significant correlations are indicated by $^*$.

### Table III. Correlation of PSA recurrence with MVD (CD31, CD34 and vWF) as well as tumor staging and grading.

<table>
<thead>
<tr>
<th>PSA recurrence</th>
<th>MVD (CD34)</th>
<th>MVD (CD31)</th>
<th>MVD (vWF)</th>
<th>Gleason sum score</th>
<th>Histopathological grading</th>
<th>Staging</th>
</tr>
</thead>
<tbody>
<tr>
<td>$&gt; 0.1$ ng/ml</td>
<td>$p=0.0169^*$</td>
<td>$p=0.4571$</td>
<td>$p=0.3897$</td>
<td>$p=0.0263^*$</td>
<td>$p=0.0350^*$</td>
<td>$p=0.1202$</td>
</tr>
</tbody>
</table>

Significant correlation are indicated by $^*$.
A quite heterogeneous staining pattern of endothelial cells for the three markers was observed in prostate cancer tissue: a significantly increased MVD was only reflected when the two endothelial markers, CD31 and CD34, were used whereas vWF showed a significantly decreased number of microvessels in prostate cancer compared to benign BPH tissue. This points to the conclusion that angiogenesis of prostate cancer is not monitored by vWF. These results are partly consistent with previous reports on endothelial markers in angiosarcomas: vWF has been shown to lack staining in undifferentiated endothelial cells from malignant tumors (14). In prostate cancer specimens, benign tissue is displaced by invasive tissue, resulting in a decreased number of pre-existing mature microvessels, which are visualized by vWF. Therefore, vWF is a suitable marker for endothelial cells in benign tissue, but would seem to be an inferior marker for assessing newly developed microvessels in prostate cancer. The lack of staining of undifferentiated endothelial cells in angiosarcomas, seen in conjunction with our own results, is also the explanation for the missing correlation of MVD data in prostate cancer.

In benign prostate and prostate cancer tissue, CD34 detected the highest number of microvessels compared to CD31 and vWF, with only minor differences in benign tissue as opposed to large differences in malignant tissue. Previous investigations on MVD in prostate cancer have demonstrated remarkable differences between vWF-MVD and CD34-MVD (15) as well as CD31-MVD and CD34-MVD (16) and therefore underline our results. The investigations on astrocytoma and oligodendroglioma are in accordance with our findings in PCa: CD34 showed the highest sensitivity for vascular endothelial cells compared to CD31 and vWF. Accordingly, the specificity of CD34 for endothelial cells is higher in malignant tumors than in more differentiated tumor entities (6).

A correlation between angiogenesis and tumor grading has previously been reported in breast and gastric cancer (17, 18). Our data on the expression of CD34, the most specific marker for endothelial cells in malignant prostatic tissue, reflected a similar, significant correlation between histomorphological grading and MVD as an index for angiogenesis. In addition, the Gleason sum score shows a trend towards an association with angiogenesis in prostate cancer. Thus, tumor dedifferentiation would seem to be associated with an increased pro-angiogenic activity. Even though a correlation between angiogenesis and tumor staging has been demonstrated in other types of cancer (18), no correlation between these two parameters was observed in our study.

It is widely accepted that angiogenesis is a prognostic factor in several solid tumors, including prostate cancer (4, 10-12). Our data clearly showed that the prognostic relevance of angiogenesis was reflected only by the use of CD34 as an endothelial marker. MVD, as assessed by CD34, was significantly associated with PSA failure after curative treatment. Our observation is underlined by previous reports: CD34-MVD but not vWF-MVD has been demonstrated to predict prognosis of prostate cancer (15). Since CD34 is the most sensitive of the three investigated markers for angiogenesis in prostate cancer, this may well explain the lack of association between CD31- and vWF-MVD and PSA failure.

The assessment of angiogenesis is still experimental and not used in routine clinical diagnostics. Further data on angiogenesis in prostate cancer may well underline the importance of MVD assessment as a prognostic factor. With regards to both experimental and clinical application, we can conclude from our data that CD34 is a suitable marker for immunohistochemically visualizing microvessels in benign and malignant prostatic tissue. The use of this marker enables us to document angiogenesis in prostate cancer. It also supports the concept of a link between angiogenesis and prognosis in patients suffering from prostate cancer.

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