Advances in Brief

Tumor Angiogenesis Is Associated with MUC1 Overexpression and Loss of Prostate-specific Antigen Expression in Prostate Cancer

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Abstract

The biological potential of prostate cancer is highly variable and cannot be satisfactorily predicted by histopathological criteria alone. Therefore, additional and more precise information is desirable. Although angiogenesis has been suggested as being of prognostic importance in many human cancers, and MUC1, also known as episialin, was thought to be responsible for the development of metastasis, the role of these parameters in prostate cancer remains unclear. The aim of this study was to investigate whether angiogenesis, assessed as microvessel density (MVD), was correlated with the expression of prostate tumor MUC1 and prostate-specific antigen (PSA) or with histopathological grade at diagnosis, and to determine whether any of these factors might provide additional information with regard to prostate tumor biology. Paraffin-embedded material from 60 patients with prostate carcinoma was examined immunohistochemically, using the monoclonal antibody CD31 to determine MVD, and the monoclonal antibodies CCE831 and ER-PR8 to assess MUC1 and PSA expression, respectively. The tumors were categorized according to the Gleason grading system. MUC1 overexpression was significantly related to a high intratumoral angiogenesis ($P = 0.02$). By contrast, a high PSA expression by prostate cancer cells was associated with low MVD ($P = 0.03$). No correlation was found between MUC1 and PSA expression. Usually, high-grade tumors were not PSA-expressive and tended to display increased angiogenesis. These differences, however, were not of statistical significance. Similarly, there was no statistically significant association between histological grade and MUC1 expression or angiogenesis. It is suggested that PSA may have a direct suppressive effect on new blood vessel formation in prostate cancer, whereas the expression of MUC1 in this tumor may be connected with an angiogenic phenotype. Additional studies are obviously needed to clarify the precise role of these proteins in prostate cancer.

Introduction

The biology of prostate cancer has not been fully understood and its clinical behavior remains unpredictable. In both incidental and advanced prostate cancer, the identification of "high-risk" groups of patients, and the prediction of tumor aggressiveness are of great importance in planning therapeutic strategies. A number of prognostic factors have been studied up to now, yet most of them are either of ambiguous prognostic value or are not applicable to the everyday clinical practice. At present, tumor stage and tumor grade are considered to be the standard prognostic criteria for prostate cancer. Tumor volume seems to be significantly related with the incidence of metastases and the concentration of serum PSA[2] (1). In this context, PSA may serve as an early indicator of an ongoing neoplastic process within the prostate gland, although it does not provide any information on the nature of this process (2). By contrast, after radical prostatectomy for cancer, a new rise in PSA levels is a sensitive indicator of local recurrence or metastatic disease (3). Histopathological grading has also been found to influence the course of prostate cancer in cases with similar tumor stage (4, 5), but the existence of wide deviations in the clinical evolution of the disease imposes limitations on the standard histopathological criteria of prognosis.

Angiogenesis, *i.e.*, the formation of new blood vessels from preexisting blood vessels, is thought to play an important role in tumor progression and the development of metastases and may prove to be a useful prognostic marker for prostate cancer (6, 7). MUC1, also known as episialin, is a glycoprotein expressed at the apical side of normal glandular epithelial cells. In cancer cells, depolarized expression through the entire cell cytoplasm has been observed (8). In several neoplasms, the detection of MUC1 has been related to the simultaneous expression of multiple angiogenic factors and with an aggressive tumor behavior (9–12). In prostate cancer, a significant correlation between MUC1 expression and high grade, and high stage (13) and patient survival, has been shown (14). However, the role of both angiogenesis (15) and MUC1 expression (16) in prostate cancer is still unclear for there are, indeed, studies in which the presence of these molecules are deprived of any prognostic significance.

Interestingly, in a recent *in vitro* investigation (17), it was revealed that PSA converts Lys-plasminogen to biologically active angiostatin-like fragments which, similarly to angiostatin,

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[2] The abbreviations used are: PSA, prostate-specific antigen; MVD, microvessel density; MoAb, monoclonal antibody; TBS, Tris-buffered saline; DAB, 3,3′-diaminobenzidine; VEGF, vascular endothelial cell factor; bFGF, basic fibroblastic growth factor.

suppress angiogenesis and, therefore, tumor growth and tumor metastases.

The present study examines the relationship of intratumoral angiogenesis with PSA and MUC1 in prostate cancer, and compares the expression of these proteins with the standard prognostic parameter of the disease, the Gleason grading system. The ultimate question was which, if any, of these factors could provide additional information regarding the biology of prostate cancer.

Materials and Methods

The material comprised 60 surgical specimens of prostate cancer. All of the tissues had been fixed in 10% formol saline and processed routinely through graded alcohols to paraffin blocks. Histological diagnosis of prostate cancer was based on H&E-stained sections. The Gleason system (18) was used for histological grading. A primary and secondary Gleason score (1 through 5) was determined for every tumor, and the combined score (Gleason sum) was then calculated. To obtain sufficient quantities for statistical analysis, the tumors were grouped in three categories: low grade (well differentiated) if the combined Gleason score was 4 or less; intermediate grade (moderately differentiated) if combined Gleason score was 5, 6, or 7 [3 + 4, with a majority of Gleason 3 areas and a small proportion (<20%) of Gleason 4 components]; and high grade (poorly differentiated) if the Gleason sum was ≥7 (4 + 3). For immunohistochemical staining, the histological sections were cut serially at 3 μm.

Assessment of PSA Expression. PSA was detected using the ER-PR8 MoAb (IgG1; Immunon, Shandon, Pittsburgh). Sections were deparaffinized, and peroxidase was quenched with methanol and 3% H_2O_2 for 15 min. Microwaving for antigen retrieval was used (three times for 5 min each). Samples were then washed three times in TBS (pH 7.4), and nonspecific binding was blocked in normal rabbit serum for 10 min. (Immunon) in TBS. The primary antibody was applied for 75 min. After washing with TBS, sections were incubated with a secondary antirabbit antimouse antibody (Kwik biotinylated secondary; Immunon) for 15 min and washed in TBS. Kwik streptavidin peroxidase reagent (Immunon) was applied for 15 min, and sections were again washed in TBS. The color was developed by 15-min incubation with DAB solution, and sections were weakly counterstained with hematoxylin. Prostate cancer tissue sections with strong PSA expression were used as positive controls. Normal rabbit IgG was substituted for primary antibody as the negative control (same concentration as the test antibody).

Assessment of MVD. The JC70 MoAb (DAKO) recognizing the CD31 pan-endothelial antigen (platelet/endothelial cell adhesion molecule) was used for microvessel staining on 3-μm paraffin embedded sections. Sections were deparaffinized, and peroxidase was quenched with methanol and 3% H_2O_2 for 15 min. Microwaving for antigen retrieval was used (three times for 5 min each). Samples were subsequently washed three times in TBS (pH 7.4), and nonspecific binding was blocked in normal rabbit serum for 10 min (Immunon) in TBS. The primary antibody (1:20) was applied for 75 min. After washing with TBS, sections were incubated with a secondary antirabbit antimouse antibody (Kwik biotinylated secondary, 030A; Immunon) for 15 min and washed in TBS. Kwik streptavidin peroxidase reagent (Immunon) was applied for 15 min, and sections were again washed in TBS. The color was developed by 15-min incubation with DAB solution, and sections were weakly counterstained with hematoxylin.

Microvessel counting was used for angiogenesis assessment. For eye appraisal, sections were scanned at low power (×40 and ×100) and afterward at ×200 so as to group cases in three vascular grade categories (low, medium, and high). The areas of the highest vascularization were chosen at low power (×100), and microvessel counting followed on three chosen ×200 fields of the highest density. The microvessel score (*MS*) was the mean of the vessel counts obtained in these three fields. Vessels with a clearly defined lumen or well-defined linear vessel shape, but not single endothelial cells, were taken into account for microvessel counting.

Episialin Immunohistochemistry. The glycosylated form of episialin expression was assessed on paraffin-embedded material using the MoAb Muc1 (IgG1, CCE831, YLEM; Rome, Italy), recognizing a carbohydrate epitope of the MUC1 glycoprotein. The avidin-biotin complex immunoperoxidase technique was used. Sections were dewaxed and rehydrated, treated for 10 min with 3% H_2O_2 to limit endogenous peroxidase activity. Samples were then washed three times in TBS (pH 7.4) and nonspecific binding was blocked in normal rabbit serum for 10 min. (Immunon) in TBS, and incubated with the MoAb Muc1 (IgG1 mouse, CCE831, diluted 1:100) for 30 min. The sections were then washed thoroughly in TBS and incubated with biotin-conjugated rabbit antimouse immunoglobulin antibody for 10 min. (Immunon), followed by an avidin-biotin-peroxidase complex for 30 min. (Immunon). Finally, the sections were incubated with DAB as chromogen for 15 min and counterstained with hematoxylin. Omission of the primary antibody was used for negative control.

In normal epithelium, episialin shows a polarized pattern of immunoreactivity. The patterns of expression of episialin in the normal glands show localization in the cytoplasmic vacuoles and/or in the cell membrane at the apical site of cells. The circumferential cytoplasmic and membrane immunoreactivity, never seen in normal cells, is recorded as overexpression of episialin. The percentage of cancer cells with episialin overexpression was recorded. This allowed analysis using MUC1 expression as a continues variable. Cases were also divided into two groups using as a cutoff point the mean percentage of cells with depolarized expression.

Statistical Analysis. Statistical analysis and graphic presentation were performed using the GraphPad Prism 2.01 package (GraphPad, San Diego CA).[3] The Fisher's exact test or the unpaired two-tailed *t* test was used for testing relationships between categorical variables as appropriate. Linear regression analysis was used to assess correlation between continues variables. $P < 0.05$ was considered significant.

[3] Internet address: www.graphpad.com.

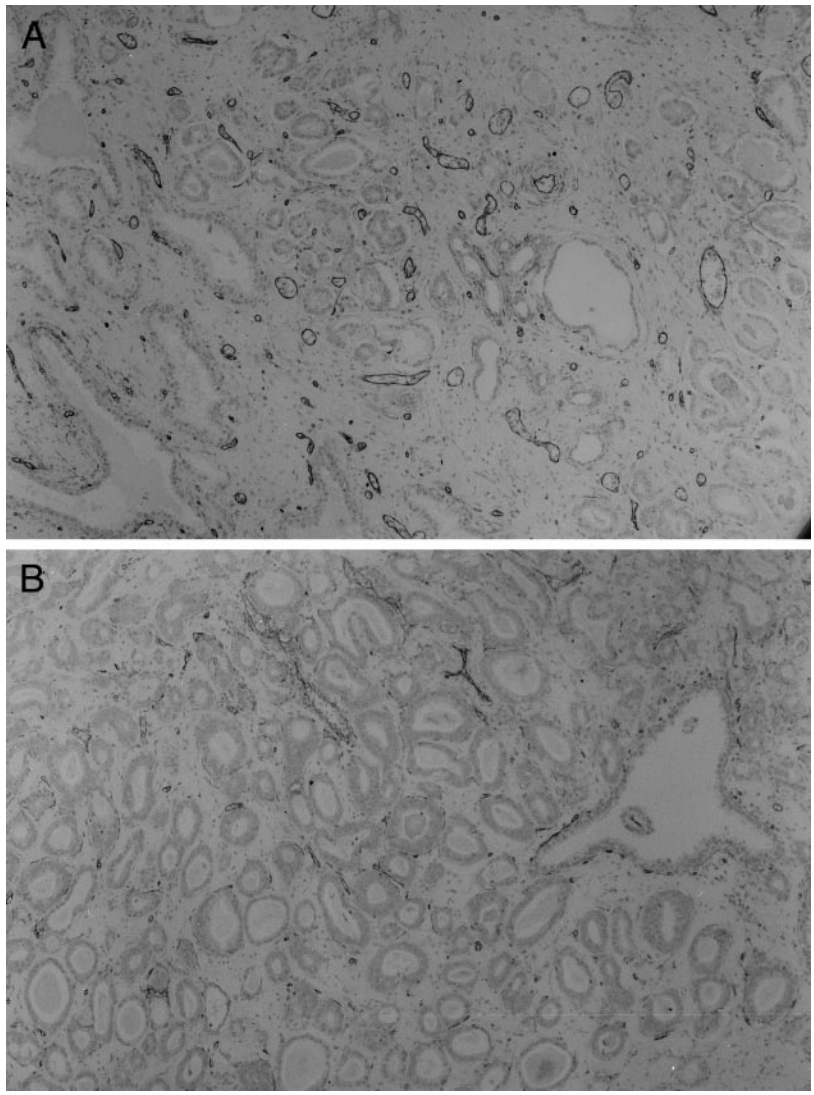

Fig. 1 *A*, prostate cancer with high intratumoral MVD in the surrounding tumor stroma (CD31 MoAb). *B*, prostate cancer with low intratumoral MVD in the supporting tumor stroma (CD31 MoAb).

Results

The mean values (less than mean *versus* mean) were used to define two groups of high and low MVD (Fig. 1, *A* and *B*) and of high or low MUC1 and PSA (Figs. 2 and 3) expression. The mean MVD was 26 microvessels per ×200 optical field (range, 6–69; median, 22). The mean percentage of cells with overexpression of MUC1 and PSA proteins was 21% (range, 0–90%; median, 10%) and 47% (range, 0–100%; median, 50), respectively.

Table 1 shows the association of MVD with tumor PSA and MUC1 expression, and also with tumor histological grade. The mean MVD was 21 ± 12 for the group of low MUC1 expression and 29 ± 14 for the group of high MUC1 expression. This difference was statistically significant ($P = 0.02$), which suggests that a high MVD is related to MUC1 overexpression in prostate cancer. On the other hand, the mean MVD was 31 ± 17 for the group of low-PSA expression and 21 ± 11 for the group of high-PSA expression. This difference was also statistically significant ($P = 0.03$), which indicated that a high MVD is associated with loss of PSA expression in malignant prostate tissue. There was, however, no significant association between histological grade and MVD (>0.40). Fig. 4, *A* and *B*, shows schematically the association of MVD with PSA and MUC1 expression.

Table 2 shows the relation of the histological grade (1, 2, and 3) with the MUC1 and PSA expression in prostate cancer. Histological grade was not significantly associated with low- or high-MUC1 expression ($P = 0.63$) and the same was true with reference to low- or high-PSA expression ($P = 0.24$).

Discussion

The biological potential of prostate cancer is highly variable and cannot be satisfactorily predicted by histopathological criteria alone. Therefore, additional and, apparently, more precise prognostic markers are desirable. Tumor angiogenesis is generally considered an important factor for continued tumor growth and progression. Angiogenic activity seems to be the result of a dynamic balance between angiogenic stimulators and

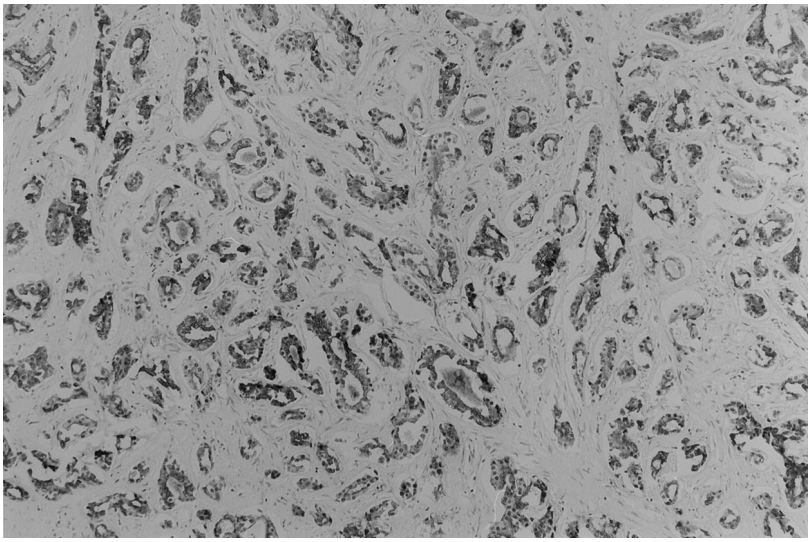

Fig. 2 Prostate cancer with high and strong PSA expression in the cytoplasm of the neoplastic cell population (ER-PR8 MoAb).

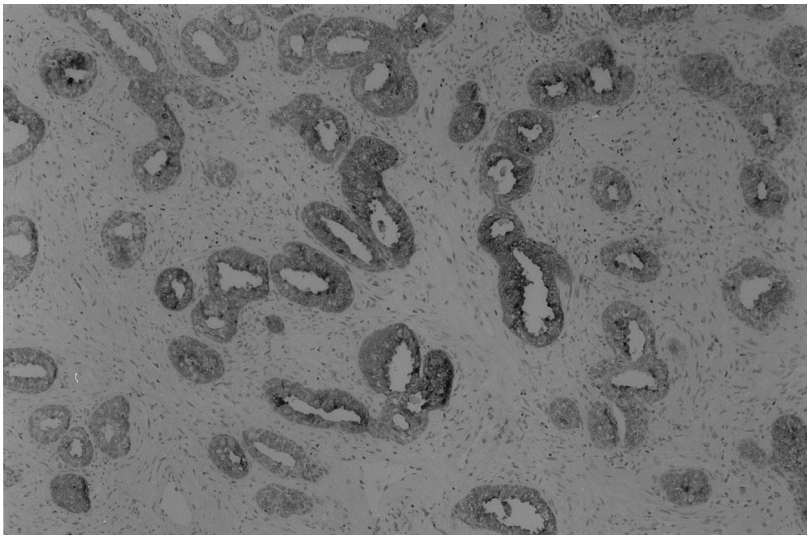

Fig. 3 Prostate cancer with high and strong MUC1 expression in the cytoplasm of the neoplastic cells (Muc1 MoAb).

inhibitors, produced by tumor cells and by benign host cells (mainly macrophages and stromal components). Cancer metastasis appears to be angiogenesis dependent. Although a quantitative relationship between angiogenesis and metastasis, and angiogenesis and prognosis, has been reported for prostate cancer (6, 19, 20), the significance of angiogenesis with respect to the particular features of this carcinoma, such as slow proliferation and low progression rate, has not been fully investigated.

Angiostatin is a known inhibitor of angiogenesis. Recently, it was shown in an *in vitro* study that the PSA, a serine proteinase secreted by human prostate and human prostate cancer cells, is able to convert Lys-plasminogen to biologically active angiostatin-like fragments, containing kringels 1–4, by limited proteolysis of peptide bond Glu439-Ala 440 (16). In a further *in vitro* morphogenesis assay, it was shown that the purified angiostatin-like fragments inhibited proliferation and tubular formation of human umbilical vein endothelial cells with the same efficacy as angiostatin. In the present investigation, it was found that high PSA expression by prostate cancer cells is accompanied by low intratumoral angiogenesis. This inverse relation between angiogenesis and PSA is in accord with the *in vitro* studies, revealing a similar relationship between these two parameters. This fact could be the result of either a direct suppressive action of PSA toward angiogenesis, or of a simple coexistence of PSA with a nonangiogenic phenotype. This relationship between PSA expression and tumor angiogenesis may help in a better understanding of the general observation that prostate cancer is usually characterized by a very low progression rate.

Episialin, also known as MUC1 (or PEM, CA-15–3 antigen, and EMA) is a transmembrane protein shown to reduce E-cadherin-mediated cell-cell adhesion *in vitro* by steric hindrance (21). Several *in vitro* studies suggest that MUC1 expression by cancer cells is an important component of biochemical

Table 1 Correlation of MVD with MUC1, PSA, and histological grade in 60 patients with prostate cancer

	MVD (mean ± SD)	*P*
MUC1 expression		
Low	21 ± 12	0.02
High	29 ± 14	
PSA expression		
Low	31 ± 17	0.03
High	21 ± 11	
Histological grade		
1	24 ± 11	
2	25 ± 15	>0.40
3	27 ± 15	

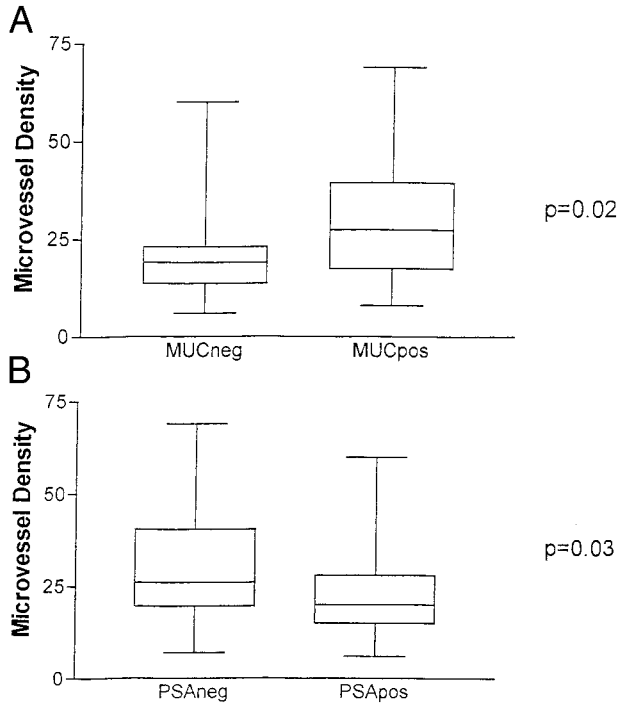

Fig. 4 Association between MVD and MUC1 and PSA expression in prostate cancer. *A,* MUC1 cytoplasmic status; *B,* PSA status.

events that promote metastasis. Although many reports show a correlation of MUC1 expression with survival in breast, colon, and lung cancer (9–11), the role of MUC1 in prostate cancer has not been clarified. In a recent investigation, MUC1 expression was correlated with advanced Gleason grade and advanced pathological stage (13), whereas another study demonstrated a high prognostic relevance of MUC1 overexpression in prostate cancer (14). In the present work, overexpression of MUC1 protein was independent of PSA expression and of histological grade. However, a significant association between MUC1 overexpression and a high intratumoral neoangiogenesis was noted. These discrepancies may reflect variations in methodology, mainly differences in the specificity of antibodies and the dilutions used, or the small number of cases included in the earlier studies.

Table 2 Correlation between histological grade and MUC1 and PSA expression in 60 patients with prostate cancer

	Grade			
	1	2	3	*P*
MUC1 expression				
Low	16	12	11	0.63
High	6	8	7	
PSA expression				
Low	6	4	8	0.24
High	16	16	10	

Although there is no evidence that MUC1 protein is directly involved in the regulation of tumor angiogenesis, a recent report indicates a striking correlation of MUC1 expression with multiple angiogenic factors (VEGF, platelet derived-endothelial cell factor, and bFGF) and angiogenic factor receptors (KDR, bFGF receptor-2; Ref. 12). In that study, it was suggested that in the context of a primordial genetic event, both migration (like MUC1) and angiogenic pathways are "switched on" in human cancers. The present study, showing a direct association of MUC1 expression with angiogenesis in prostate cancer, further supports this hypothesis. Verification of the clinical significance of MUC1 expression in prostate carcinoma, on the basis of clinicopathological studies, is incomplete up to now and additional retrospective and prospective studies will be necessary to determine with greater accuracy the prognostic value of this parameter.

In conclusion, the high PSA expression in prostate cancer cells that is accompanied by low intratumoral angiogenesis, could be interpreted as the result of either a direct vascular suppressive action of PSA, or the coexistence with a nonangiogenic phenotype. The first hypothesis seems more likely and confirms the *in vitro* observation that PSA is able to convert Lys-plasminogen to biologically active angiostatin-like fragments that inhibit angiogenesis. The vascular suppressive action of PSA could explain some of the growth characteristics of prostate cancer, *i.e.,* the low MVD and the slow proliferation rate. PSA could, theoretically, play the role of a regulator of the proliferative activity of prostate cancer in early stages of the disease. A loss of this controlled balance, that is apparently maintained by the contribution of other growth factors, like the integrins and adhesion molecules, could lead to an increase in PSA levels, tumor growth, and tumor metastasis. It would be certainly interesting to examine by retrospective analyses in the future whether serum PSA is of any significance in predicting tumor response to therapy, knowing that an increased therapeutic efficacy would be theoretically anticipated because of its vascular suppressive action. With regard to MUC1, this is an important molecule, the depolarized expression of which relates to a high intratumoral angiogenesis in prostate cancer, a fact that may suggest coactivation of angiogenic and migration pathways of these parameters in human cancers.

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