

Forum: Brief Communication**Variations of Components of the Plasminogen Activation System With the Cell Cycle in Benign Prostate Tissue and Prostate Cancer**Eugen Plas,^{1,4*} Veronica A. Carroll,² Ruth Jilch,³ Rainer Simak,¹ Judith Mihaly,² Sebastian Melchior,⁴ Joachim W. Thüroff,⁴ Bernd R. Binder,² and Heinz Pflüger¹¹Department of Urology and LBI for Urology and Andrology, Lainz Hospital Vienna, Vienna, Austria²Department of Vascular Biology and Thrombosis Research, University of Vienna, Vienna, Austria³Department of Clinical Chemistry, Lainz Hospital Vienna, Vienna, Austria⁴Department of Urology, Johannes Gutenberg University Mainz, Mainz, Germany

Background: Components of the fibrinolytic system are involved in tumor cell invasion and metastasis. Previous investigations suggested a cell cycle-dependent expression of urokinase-type plasminogen activator (u-PA) in epithelial cells. In order to determine a correlation of cell cycle phases with the fibrinolytic system, we investigated the expression of u-PA, tissue-type plasminogen activator (t-PA), and plasminogen activator inhibitor type 1 (PAI-1) in normal and tumor-containing prostate extracts and analyzed a possible relationship with flow cytometry-determined proliferative activity of the samples. Cell cycle phases were correlated with fibrinolytic parameters in prostate tissue. **Methods:** Samples were obtained from patients undergoing radical prostatectomy for prostate cancer and separated into two portions for DNA analysis and the detection of u-PA, t-PA, and PAI-1. Flow cytometric analysis was performed according to the Vindelov technique. The concentrations of u-PA, t-PA, and PAI-1 were determined from tissue extracts after homogenization by an enzyme-linked immunosorbent assay (ELISA) technique. **Results:** Correlations of u-PA and t-PA expression with the frequency of G0/G1, S, G2M, S-phase fraction (SPF), and proliferation index (PI) for normal prostate and prostate cancer revealed no significant correlation. The only significant finding was observed in normal tissue revealing a positive correlation between PAI-1 expression and G0/G1 and a negative correlation with S-phase, SPF, and PI. No dependence of PAI-1 expression on different cell phases was found in prostate cancer. Furthermore, no significant correlation of u-PA, t-PA, and PAI-1 with cell cycles in organ-confined (<pT3a) and disseminated (≥pT3a) tumors was found. No significant correlation in prostate cancer of components of the fibrinolytic system differentiated according to tumor grade or perineural tumor infiltration and cell cycle analysis was found. Only in highly differentiated G1 (Gleason 2–4) cancer, u-PA had a significant positive correlation with G2M-phase. **Conclusion:** Absence of a correlation between levels of components of the fibrinolytic system and cell cycle phases suggests that the reported association between increases of some of these components and aggressive biological behavior of prostate cancer is secondary to non-cell cycle-related mechanisms. *Cytometry (Comm. Clin. Cytometry)* 46: 184–189, 2001. © 2001 Wiley-Liss, Inc.

Key terms: prostatic neoplasms; plasminogen activators; plasminogen inactivators; cell cycle; S-phase fraction (SPF); proliferative index (PI)

Prostate cancer remains the second major cause of death in men over the age of 50. It is the most common malignancy in the United States, with an increasing incidence over the last 10 years. Despite this, progression of the disease is fairly unknown with respect to tumor initiation, promotion, penetration of the prostatic capsule, and the development of metastases. One pathway that has been consistently associated with progression of prostate cancer is the plasminogen activation system. The most well-characterized function of this pathway is the gener-

ation of plasmin from its inactive precursor, plasminogen, by plasminogen activators (PA). Two types of PAs are known, the serine protease urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator

*Correspondence to: Dr. Eugen Plas, Department of Urology, Johannes Gutenberg University Mainz, Langenbeckstr. 1, D-55131 Mainz, Mainz, Germany.

E-mail: plas@urologie.klinik.uni-mainz.de

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(t-PA). The role of u-PA in the development of tumor cell invasion and metastasis has been well established. However, t-PA has primarily a thrombolytic function and its role in tumor progression is still controversial (1,2). The activity of PAs, in turn, is regulated by binding to their receptors, most importantly the urokinase-type plasminogen activator receptor (u-PAR) and the plasminogen activator inhibitor type 1 (PAI-1). Plasmin generation allows tumor and vascular cell invasion and angiogenesis as well as activation of latent growth factors, cytokines, and metalloproteases. Apart from mediating plasmin formation, components of the fibrinolytic system have other nonproteolytic functions that may also be important for tumor progression (2,3).

Several lines of evidence implicate u-PA in the progression of prostate cancer. It is the predominant PA expressed in hyperplastic and malignant prostate tissue. Increased levels of u-PA are found in prostate cancer patients compared with patients with normal tissue and in patients with bone metastases of prostate cancer compared with patients with primary tumors (4-7). Additionally, higher plasma levels of u-PA have been detected in patients with disseminated disease compared with patients with organ-confined disease (8,9). In vitro studies on prostate cancer cell lines determined higher u-PA expression associated with the most aggressive cells (10-12). Furthermore, overexpression of u-PA resulted in increased development of metastasis in a rat model that could be inhibited by a selective inhibitor for u-PA activity (13,14). Other u-PA inhibitors have also been reported to decrease tumor growth in a human prostate cancer xenograft model (15). Despite experimental evidence of the involvement in tumor cell progression, the significance of u-PA as a prognostic marker for prostate cancer has yet to be established. Recently, elevation of u-PA and its receptor was associated with disease progression and prognosis in patients with prostate cancer (16). In contradiction, Plas et al. (6) reported no correlation of fibrinolytic parameters with disease progression after radical prostatectomy. However, components of the plasminogen activation system, especially u-PA and PAI-1, have already been reported as useful prognostic markers in breast, ovarian, gastric, and colon cancer (17-20).

During carcinogenesis, alterations of cellular proliferation and control mechanisms occur, which can be investigated by DNA analysis. The differentiation between DNA diploid and DNA aneuploid cancers was determined as a prognostic marker in prostate cancer patients. DNA ploidy has been reported as an important, but not independent, prognostic factor for tumor progression and survival in prostate cancer patients (21,22). Cancer DNA aneuploidy was associated with higher tumor stage, higher frequency of tumor metastasis, and worse prognosis whereas DNA diploid tumors were seen more frequently in low-stage tumors with better outcome (21,23). Because tumor cells have altered cell populations with accelerated proliferation rates and higher cellular turnover, the clinical value of S-phase analysis in prostate

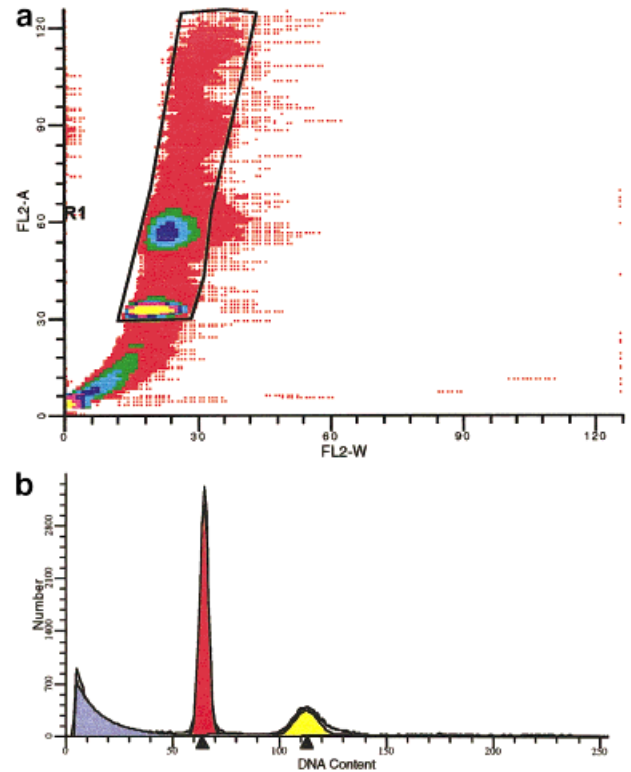


Fig. 1. **a:** Dot plot of aneuploid prostate cancer tissue (70% diploid cells, 30% aneuploid cells). **b:** DNA histogram of aneuploid prostate cancer tissue (ungated, including debris). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cancer patients has been proposed as predictive for survival although its relevance is disputed (24,25).

In patients undergoing radical prostatectomy for organ-confined prostate cancer, we have previously shown that high levels of u-PA and decreased PAI-1 expression are associated with DNA aneuploid tumors (6). A higher expression of u-PA may be one explanation for the more aggressive behavior of DNA aneuploid tumors compared with diploid tumors. Recently, a cell cycle-dependent expression of u-PA, u-PAR, and PAI-1 was observed in bovine mammary epithelial cells (26). It was the aim of our study to analyze whether levels of components of the fibrinolytic system correlate with cell cycle phases.

MATERIALS AND METHODS

Samples were obtained from 46 patients (52 benign and 42 tumor samples) undergoing radical prostatectomy for clinically organ-confined prostate cancer. The pathohistological results of the surgical specimen are listed in Table 1. Tissue samples (approximately $3 \times 8 \times 2$ mm) were taken from the peripheral zone of the prostate in each case because 80% of prostate cancer cases are primarily located in this zone. Specimens were separated into two portions for DNA analysis and for the detection of u-PA, t-PA, and PAI-1. To determine the histology of the samples, small sections of the cutting edges were sent for histology

Table 1
*Histopathological Results After Radical Prostatovesiculectomy
 for Prostate Cancer (n = 46) (TNM 1998)*

| | G1 | G2 | G3 | Perineural infiltration | Lymph nodes positive |
|------|----|----|----|----------------------------|----------------------|
| pT2a | 4 | 2 | 0 | 2 | 0 |
| pT2b | 23 | 8 | 2 | 21 | 0 |
| pT3a | 0 | 3 | 0 | 2 | 0 |
| pT3b | 2 | 2 | 0 | 4 | 0 |

to perform standard histopathology by hematoxylin-eosin staining in each case. Prostate cancer was classified according to the World Health Organization grading system (G1 tumors comparable to Gleason score 2-4; G2 comparable to Gleason score 5-7; G3 cancer comparable to Gleason 8-10). Samples were differentiated according to the histological result into a normal prostate or a prostate with cancer. A normal prostate was defined by the presence of normal and hyperplastic tissue and a lack of prostatic intraepithelial neoplasia (PIN) and/or cancer tissue, whereas sections of the prostate classified as having cancer predominantly contained cancer tissue.

Analytical Methods

Tissue preparation and DNA analysis. For the determination of cell cycle analysis and DNA ploidy, all tissue samples were analyzed according to the Vindelov technique utilizing the CycleTEST-TM PLUS DNA reagent kit (Becton Dickinson, San Jose, CA). After excision of the specimen, the tissue was immediately destructed mechanically with the MediMachine (Dako, Carpinteria, CA, pore size 50 μ m) into a 3-ml cell suspension in phosphate-buffered saline (PBS). Two aliquots containing approximately 10^6 cells were obtained by aspiration. The remaining suspension was frozen at -80°C after adding 3 ml of cell freezing medium (Sigma, St. Louis, MO, cell freezing medium C-6164). PBS (3 ml) was added to the aliquot and centrifuged at 400 times; g for 5 min at 20°C . The supernatant was discarded and trypsin buffer (250 μ l) was added to the pellet and incubated for 10 min. Following the addition of trypsin inhibitor and RNAase buffer (1 mg/ml), the probes were incubated for 10 min. Finally, propidium iodide (PI) stain solution (1 mg/ml) was added and incubated in the dark at 4°C . The suspension was then filtered through a 50- μ m nylon mesh. All flow cytometry analyses were performed on fresh tissue suspensions and on thawed frozen aliquots as controls.

A flow cytometer (FACScan, Becton Dickinson) was used to determine the DNA ploidy of each specimen and cell cycle analysis was performed with the Cell FIT software (Becton Dickinson). For each analysis, more than 40,000 cells were counted. All samples were classified as DNA diploid (0.97-1.03) or DNA aneuploid (<0.97 or >1.03) according to their DNA index. The coefficient of variation was less than 3% in all cases. Human leukocytes (1×10^6) from healthy donors were used for calibration and as a DNA diploid standard.

For the determination of S-phase fraction (SPF) and proliferation index (PI), calculations were performed according to Riley et al. (27):

$$\text{SPF} = S \times 100 / (G0/G1 + S + G2M)$$

and

$$\text{PI} = (S + G2M \times 100) / (G0/G1 + S + G2M)$$

where G0/G1, S, and G2M represent the fraction of cells in each phase of the cell cycle.

Tissue preparation for detection of u-PA, t-PA, and PAI-1. Following excision, all samples were stored at -80°C until further processing. All specimens were weighed (benign prostate, mean 0.18 g; prostate cancer, mean 0.20 g), freeze dried by lyophilization, and mechanically pulverized. The powder was resuspended with 2 ml of tris-buffered saline and centrifuged at 15,000 rpm for 60 min at 4°C , the lipid layer was discarded, and the supernatant was stored at -70°C ready for analysis. The pellet was resuspended in 2 ml Camiolo buffer with Triton-X 100, stirred for 16 h at 4°C , and centrifuged at 15,000 rpm for 60 min at 4°C . The supernatant was stored at -80°C .

Analysis of the fibrinolytic components (u-PA, t-PA, PAI-1) was performed by sandwich enzyme-linked immunosorbent assay (ELISA) techniques (Technoclone, Vienna). The catching antibody for u-PA was 5UK, which recognized both single- and two-chain urokinase, 3VPA was used to recognize all forms of t-PA, and 5PAI was used as the catching antibody to recognize active and latent PAI-1 and t-PA-PAI-1 complexes. Finally, horseradish peroxidase (HRP)-labeled monoclonal antibodies (scu-PA1 for u-PA; 10VPA for t-PA; 3PAI for PAI-1) were used for detection. The National Institute for Biological Standards and Controls (NIBSC) standard was used for standardization.

In both benign and malignant prostate tissue, the concentrations of u-PA, t-PA, and PAI-1 were expressed as nanograms (extracted protein)/gram (prostatic tissue).

Statistics

Statistical analysis was performed by a signed rank Wilcoxon test to compare the differences of the G0/G1-phase fraction, the G2M-phase fraction, SPF, PI, u-PA, t-PA, and PAI-1 in normal and tumor-containing prostate tissue. The values are given as median \pm SE. For determination of correlations of u-PA, t-PA, and PAI-1 with the G0/G1 fraction, the G2M fraction, SPF, and PI, Spearman correlation coefficients were performed. $P < 0.05$ was considered significant.

RESULTS

DNA analysis of the histologically proven 52 benign prostate samples revealed a diploid DNA index in 50 cases (96.2%) and an aneuploid DNA index in two samples (3.8%). In the tumor samples, DNA diploidy was found in 32 cases (76.7%) and tumor cell DNA aneuploidy was found in 10 cases (23.3%). Cell cycle analysis revealed

significant differences in the proliferative activity between the benign and tumor samples. In prostate cancer patients, a significantly lower percentage of cells was observed in G0/G1-phase compared with normal tissue ($89.3\% \pm 0.4$ versus $94.1\% \pm 0.2$; $P = 0.0001$). Higher syntheses (S-phase) and mitotic phases (G2M) were detected in tumor tissue ($3.3\% \pm 0.7$ and $4.4\% \pm 0.7$) compared with normal tissue ($1.8\% \pm 0.4$ and $2.8\% \pm 0.2$; $P = 0.047$ and $P = 0.0001$, respectively). The calculated SPF and PI revealed significantly higher rates (9.0 ± 1.0 and 13.0 ± 1.5) in prostate cancer patients compared with normal controls (5.0 ± 0.5 and 7.9 ± 0.8 ; $P = 0.0001$ and $P = 0.0007$, respectively).

u-PA levels in prostate cancer patients were $57.24 \text{ ng/g} \pm 5.26$ versus $46.37 \text{ ng/g} \pm 3.76$ for normal controls ($P = 0.07$). t-PA levels in prostate cancer patients were $159.5 \text{ ng/g} \pm 102.11$ versus $116.26 \text{ ng/g} \pm 35.33$ for normal controls ($P = 0.47$) and PAI-1 levels in cancer tissue were $61.50 \text{ ng/g} \pm 73.82$ compared with benign prostate $41.33 \text{ ng/g} \pm 6.37$ ($P = 0.07$). Although all fibrinolytic parameters were higher in the tumor samples compared with the normal tissue, none reached statistical significance.

Correlations of u-PA, t-PA, and PAI-1 with percent G0/G1, G2M, SPF, and PI for normal prostate controls did not show any significance. The only significant finding was observed in normal tissue revealing a positive correlation between PAI-1 expression and the frequency of G0/G1 and a negative correlation with SPF and PI ($P = 0.03$, $P = 0.02$, $P = 0.04$, respectively). In prostate cancer patients, no significant correlation of components of the plasminogen system with cell cycle analysis was found.

In order to investigate correlations of fibrinolytic proteins differentiated according to tumor stage or grade with the cell cycles, subgroups of cancer-containing samples were correlated with cell cycle phases. Again, there was no significant correlation of u-PA, t-PA, and PAI-1 with cell cycles in organ-confined ($<pT3a$) and disseminated ($\geq pT3a$) tumors. Further, no significant correlation of components of the fibrinolytic system differentiated according to tumor grade and cell cycle analysis was found. Only u-PA in highly differentiated G1 tumors showed a positive correlation with percent G2M-phase ($P = 0.034$). Furthermore, the presence of perineural tumor infiltration irrespective of tumor stage and grade did not reveal any significant correlation with percent G1, S, G2M, SPF, and PI in tumor samples.

DISCUSSION

Pericellular proteolysis is essential for the development of tumor cell invasion and metastasis. This process is largely controlled by proteolytic enzymes that are capable of disrupting peptide bonds to digest basement membrane proteins (28). The u-PA-dependent plasminogen activation system has been implicated in tumor cell invasion (1-3). The activation of the PA is modulated by PAI. The addition of inhibitors or antibodies causes a reduction in tumor invasion and metastatic potential. This emphasizes the importance of a balanced interaction of the

fibrinolytic system as a prerequisite for normal tissue and homeostasis.

In prostate cancer patients, established biological markers of disease progression include tumor stage, grade, and prostate-specific antigen (PSA). However, there are other potential markers such as proliferation markers, DNA ploidy, SPF, and u-PA. Many studies, both in prostate and other types of cancer, have shown that DNA aneuploid tumors are significantly more aggressive than DNA diploid tumors. We have recently shown in paired benign and tumor prostate samples taken from the same patient that both DNA diploid and DNA aneuploid tumors express more u-PA than the adjacent benign tissue (6). u-PA can promote pericellular proteolysis by plasmin activation and degradation of the extracellular matrix, thereby allowing cell invasion. It can also activate growth factors such as vascular endothelial growth factor (VEGF) directly and indirectly via plasmin generation, which could also contribute to the increased aggressive potential of DNA aneuploid tumors. Recently, VEGF and u-PA expression was positively correlated in patients with primary colorectal cancer, suggesting that both proteins interact in tumor progression (29). These results were supported by Mandriota et al. (30), who demonstrated that VEGF-induced angiogenesis was accompanied by increased u-PAR expression and u-PA activity on the endothelial cell surface. Although there are some hypothesized pathways such as upregulation of u-PA by growth factors or pericellular control mechanisms, the reason for the altered expression of u-PA in cancer tissue compared with normal tissue is fairly unknown. Carcinogenesis is characterized by an alteration of control mechanisms of gene expression and the cell cycle. These alterations may result in an increased production of components of the fibrinolytic system in malignant diseases. The expression of these components in relation to DNA ploidy status in prostate cancer patients was recently reported (6). Interestingly, DNA aneuploid tumors contained higher u-PA but less PAI-1 compared with DNA diploid tumors and normal prostate tissue (6). Although u-PA has been determined as one important protease in the process of tumor invasion, the role of PAI-1 in tumor progression is complex and only partly known. As the primary inhibitor of u-PA and t-PA, it inhibits proteolytic activity but may also be necessary for cell invasion and angiogenesis because regulated proteolysis is required for these processes. Furthermore, PAI-1 can also modulate integrin-dependent migration and u-PAR-dependent adhesion on vitronectin. These other nonproteolytic functions may be responsible for the observed effects of PAI-1 in metastasis dissemination (2,3). Clinically, PAI-1 has been reported as a potent predictor of metastases in lung and kidney cancer and as a predictor for poor response to tamoxifen therapy in recurrent breast cancer (31-33).

Tumor cells have a higher proliferation rate than normal cells, as shown in our results. Prostate cancer had a significantly lower frequency of G0/G1 cells, but a higher frequency of S-phase and G2M-phase than normal tissue. In addition, SPF and PI were significantly higher in tumor

samples compared with normal prostate samples. Although there was no dependence of u-PA, t-PA, and PAI-1 with different stages of the cell cycle in tumor tissue, we detected a significant positive correlation of PAI-1 with the frequency of G0/G1 and a negative correlation with the percentage of S-phase and PI in normal prostate tissue. Although there was no cell cycle-dependent regulation of u-PA and t-PA, the results of PAI-1 in normal prostate tissue are in agreement with Ryan et al. (34). They reported increased PAI-1 transcript during G1, which declined with S-phase. These data suggest that PAI-1 may be growth state regulated. Interestingly, increased expression of PAI-1 transcript and protein levels are also associated with senescent endothelial cells (35). Although there was no correlation of fibrinolytic proteins with cell cycle phases in tumor samples, a loss of control of at least PAI-1 regulation compared with normal cells can be suggested. In prostate cancer cell lines, Keer et al. (10) supported a relationship between u-PA production with cell cycle phases in PC-3 and DU-145 prostate cancer cells. However, a wide variation of u-PA expression within the same cell cycle was observed. It was concluded that the variation of u-PA represented a cell cycle-independent phenomenon that was rather dependent on cellular DNA ploidy. This is because DNA aneuploid cells had a higher u-PA expression than DNA diploid cells of the same cell line. These results are in agreement with recent reports on the expression of fibrinolytic parameters in diploid and aneuploid gastric and prostate cancer tissue (6,36).

In a limited number of patients, we have shown by immunohistochemistry that u-PA and PAI-1 are predominantly expressed in epithelial cells and tumor-associated stroma (6). Therefore, a local feedback loop for the production of fibrinolytic components between tumor and stromal cells is likely to exist, leading to an altered expression of PAs and plasminogen inhibitors by the tumor. Because the activation of these proteases does not only directly induce pericellular proteolysis, an interaction with other proteins such as plasminogen, vitronectin, integrins, and α 2-macroglobulin receptor are also involved in tumor progression (2,37,38).

CONCLUSION

A correlation for u-PA, t-PA, and PAI-1 with specific cell cycle phases in prostate cancer cells was not observed. In combination with our previous data, it is suggested that the proteolytic balance in prostate cancer is either tumor specific or results from a complex interaction among tumor cells, stromal cells, and the extracellular environment but is not related to the cell cycle.

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