Expression of the Angiogenic Factor Thymidine Phosphorylase/Platelet-derived Endothelial Cell Growth Factor in Primary Bladder Cancers

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ABSTRACT

Thymidine phosphorylase (TP), also known as platelet-derived endothelial cell growth factor, has been implicated in bladder cancer angiogenesis. To examine its role more clearly, we have quantified and localized its expression using Western analysis and immunohistochemistry in a series of 105 bladder cancers. We have also assessed the relationship between TP expression and other tumor parameters including quantitative angiogenesis, p53 status, ploidy, and survival.

By Western analysis, TP expression was 5-fold higher in tumors than in normal bladder samples (P < 0.02). Expression was 15-fold higher in invasive tumors than in normal bladder (P < 0.001) and 8-fold higher than in superficial tumors (P < 0.005). Immunohistochemistry of the tumors showed TP was present in the neoplastic epithelium in 27% of the tumors, with paired normal bladder tissue in 34% of the tumors. Immunohistochemistry of the tumors was significantly up-regulated in tumors compared with normal bladder (P < 0.05). Tumor cell TP expression correlated with tumor grade (P < 0.02), but there was no correlation between tumor cell TP expression and tumor stage (P = 0.46), ploidy (P = 0.52), p53 expression (P = 0.9), nuclear variability (P = 0.8), relapse-free survival (P = 0.57), or overall survival (P = 0.94).

TP protein is expressed in bladder cancers, and expression is associated with an aggressive phenotype. Because TP can activate a number of cytotoxic agents, it provides a potential therapeutic target in bladder cancer.

INTRODUCTION

Angiogenesis is the process leading to the formation of new blood vessels and is a prerequisite for normal growth and development (1). Angiogenesis is a feature of various diseases, including cancer, and there is now strong evidence that the growth rate of tumors is dependent on angiogenesis (2). Furthermore, metastasis is more likely from highly vascularized tumors because abundant new leaky vessels allow tumor cells more ready access to the circulation. This assertion is supported by clinical studies in which the intensity of angiogenesis, as determined by vascular density, has been shown to correlate with a higher incidence of metastases and a worse prognosis in tumors of the bladder (3–5) and prostate (6–8) in addition to other nonurological tumors including those of the breast (9, 10) and skin (11).

Understanding the events controlling tumor angiogenesis should allow the development of new therapeutic strategies. By manipulating the host microenvironment, inhibition of angiogenesis presents a new and far more general approach to cancer treatment. Antiangiogenic strategies are particularly exciting because of their potential selectivity for tumor tissue; in the normal adult, active angiogenesis occurs very infrequently, except during the monthly changes in the female reproductive tract, placenta, and in wounds.

Tumor angiogenesis involves interactions between endothelial cells, soluble growth factors, tumor cells, tumor-infiltrating leukocytes, and the extracellular matrix. The mechanisms regulating these interactions are incompletely understood but seem to depend upon a balance between the competing actions of molecules that stimulate and those that inhibit angiogenesis. A number of factors that stimulate angiogenesis have been identified, one of which is PDECGF (12, 13).

PDECGF was originally identified in 1987 as the factor in platelet lysates that stimulated proliferation of aortic endothelial cells in vitro (12). In 1992, PDECGF was identified as being homologous to TP (14–16), the enzymatic activity being crucial to its angiogenic activity. TP catalyzes the reversible breakdown of thymidine to thymine in the presence of inorganic orthophosphate.

The metabolic role of TP is almost certainly primarily catabolic, controlling thymidine levels in cells. Accumulation of thymidine is toxic to cells and causes errors in DNA replication, integrity, and repair. However, high levels of TP have been identified in macrophages (17), suggesting that it provides other functions such as angiogenic activity.

TP differs from many angiogenic factors in lacking both heparin-binding domains and a secretion peptide. Indeed, the exact mechanism of its angiogenic action is unknown, but it is chemotactic for endothelial cells (18), and the hydrolytic breakdown product of deoxyribose-1-phosphate is reported to be angiogenic (19).

Direct evidence of a role for TP in angiogenesis comes from experiments in which xenografts from transformed fibroblasts transfected with TP form more highly vascularized tumors than controls (13). In addition, xenografts of the breast carcinoma line MCF-7, when transfected with TP, form more rapidly growing tumors than controls (18). The normal tissue distribution of immunohistochemically identified TP has been recently reported, and the molecule is most strongly expressed by macrophages, stromal cells, glial cells, and some epithelia (17). Increased expression of TP has been reported in a number of tumor types (20, 21), and Reynolds et al. (22) recently found that elevated PDECGF/TP expression is associated with increased blood flow and a malignant phenotype in ovarian tumors.

TP is also of therapeutic interest because a number of thymidine analogues are metabolized by TP, including 5'-deoxy-5-fluorouridine (Furtulon; Ref. 23). This latter compound is of interest because it is a prodruk of 5-FU and is converted to 5-FU by TP. Elevated expression of TP in tumor cells would mean that Furtulon would be differentially activated in tumor cells.

In a pilot study of TP mRNA expression in bladder cancer, we have recently demonstrated a marked up-regulation of TP mRNA in invasive bladder cancers (24). Expression was 260-fold higher in invasive cancers than in normal bladder samples and 40-fold higher in invasive cancers than superficial cancers. In light of these findings, we have explored the role of TP in bladder cancer in more detail.

We used Western analysis and immunohistochemistry to quantify TP protein expression in bladder cancers and determine the cellular
TP IN BLADDER CANCER

MATERIALS AND METHODS

Tumors and Patients. A total of 105 primary bladder carcinomas treated by transurethral resection between 1987 and 1991 were taken from the historical archive of the Bristol Royal Infirmary and Southmead Hospital. Patients' ages ranged from 36–91 years (median age, 72 years); 73 (71%) patients were male and 32 (29%) were female. Two tumors were well differentiated (G1), 28 tumors (26%) were moderately differentiated (G2), and 75 tumors (72%) were poorly differentiated (G3). Forty-six (44%) tumors were papillary, 44 tumors (42%) were solid, and 15 tumors (14%) were of mixed morphology. Fifty-seven tumors (54%) were superficial to the detrusor muscle (pTa or pT1), and 48 tumors (46%) were muscle-invasive (pT2, pT3, and pT4). Overall, 73 (71%) patients were male and 32 (29%) were female. Two tumors were well differentiated (G1), 28 tumors (26%) were moderately differentiated (G2), and 75 tumors (72%) were poorly differentiated (G3). Forty-six (44%) tumors were papillary, 44 tumors (42%) were solid, and 15 tumors (14%) were of mixed morphology. Fifty-seven tumors (54%) were superficial to the detrusor muscle (pTa or pT1), and 48 tumors (46%) were muscle-invasive (pT2, pT3, and pT4). Seven normal bladder samples obtained from patients at the time of radical prostatectomy were used as controls.

Median length of follow-up in this group of patients is 26 months (range 1–102 months). Patients with tumors superficial to the detrusor muscle had been treated by transurethral resection and cystoscopic review. Patients with muscle-invasive tumors had been treated with transurethral resection, adjuvant radiotherapy, and salvage cystectomy in the event of relapse postradiotherapy.

Forty-five of the primary tumors formed the basis of the Western analyses, with seven normal bladder samples used as controls.

Immunohistochemistry. This was performed on formalin-fixed paraffin-embedded sections. The antibody PGF-44C (17) was used to identify TP, and the antibody JC70 (25) was used to highlight endothelium. Sections were incubated for 1 h in four aliquots of blocking buffer before addition of the detection reagent. The enhanced chemiluminescence system (Amersham Life Sciences, Amersham, United Kingdom) was used for the detection of bound antibody. Primary antibody-bound filters were incubated for 1 h with horseradish peroxidase conjugated rabbit anti-mouse immunoglobulins (1:400 dilution with blocking buffer; Dako, Ltd., High Wycombe, United Kingdom). The filters were washed in blocking buffer, immersed in PBS, and treated with enhanced chemiluminescence reagents according to the manufacturer’s protocol. Filters were exposed to X-ray film for 3–60 s.

Protein quantification was achieved by scanning laser densitometry. The magnitude of each signal was expressed as a ratio to the size of the signal from the positive control. The positive control was then given a notional value of 100, and tumor signals were expressed relative to this.

Statistical Analysis. Values for expression of TP, as determined by Western blotting, in normal bladder and tumors were compared by the Mann-Whitney U test (two-tailed). A P value of <0.05 was judged to be statistically significant. The relationships between the categorical immunohistochemical data and other tumor variables were assessed using χ² tests. Survival curves were determined using the method of Kaplan and Meier (29), with a log-rank test used to investigate the differences between life tables. The analysis was performed using Stata release 3.1 (Stata Corporation, TX).

RESULTS

Western Analysis for PDECGF/TP Expression. Fifty-two cytosol samples were analyzed for TP expression (Fig. 1): 7 samples of normal bladder; 7 pTa tumors; 19 pT1 tumors; and 19 invasive tumors (pT2, pT3, and pT4). Overall, TP expression was 5-fold higher in tumors than in normal bladder samples (P < 0.02). Expression was 15-fold higher in invasive tumors than in normal bladder (P < 0.001) and 8-fold higher than in superficial tumors (P < 0.005). There was no statistical difference in expression between normal bladder and superficial tumors (P > 0.05).

Immunohistochemical Analysis of PDECGF/TP Expression. TP immunoreactivity was not detected in normal transitional epithelium but was expressed in stromal and inflammatory cells. There was...
significant up-regulation of TP in tumors. TP was expressed in the neoplastic epithelium in 28 of 105 (27%) tumors. Immunoreactivity was nuclear and/or cytoplasmic. Immunoreactivity was also present in inflammatory cell elements of the tumors in 72% of the cases, in the stromal cells in 30% of the cases, and in endothelial cells in 12 of 105 cases (11%). This was usually focal and at the tumor edge (Fig. 2).

The total TP expression determined immunohistochemically correlated with that determined by Western analysis ($r = 0.53; P < 0.06$).

**Vascular Counts.** Vascular counts were performed on 55 of the invasive tumors (3). The vascular staining within individual tumors was homogeneous. The total vascular counts ranged from 16–32 (median, 24). Because prognostic factors are usually considered di-

Fig. 2. A, an invasive bladder carcinoma with heterogeneous TP expression in both tumor and stromal cell elements (+, positive); B, TP-positive stromal cells (arrows) but negative tumor cells; C, tumor cell nuclear and cytoplasmic immunoreactivity; D, intense basal TP immunoreactivity in a papillary carcinoma (arrows); E, focal accentuation for TP at the infiltrating tumor margin (arrows); F, strong staining for TP in macrophages and endothelial cells (arrows).
chotomized discontinuous variables (30), a cut-point analysis was performed, which revealed a significant difference in survival times between patients with counts < 21 and those with counts ≥ 21 (P = 0.019; Ref. 3). Of the tumors, 38 of 55 (69%) had high vascular counts (vascular count > 21), whereas 17 of 55 (31%) had low vascular counts (vascular count < 21). Overall survival was significantly reduced in those patients with highly vascular tumors compared to those with tumors of low vascularity (P < 0.05; Ref. 3).

Ploidy Analysis. Ploidy analysis was performed on 69 cases. Of these cases, 30 (43%) were diploid, and 39 (57%) were aneuploid (including 4 cases that were tetraploid). Neither overall survival nor relapse-free survival was related to ploidy (P = 0.14 and P = 0.15, respectively).

p53 Status. p53 staining was performed on 74 cases. Of these, 28 (38%) tumors were negative for p53, and 46 (62%) were positive. Overall survival was worse in those patients with p53-positive tumors, but the difference did not reach statistical significance (P < 0.06).

Relationship of Immunohistochemical TP Expression to Clinopathological Variables. Tumor cell TP expression correlated with tumor grade (P < 0.02) but not tumor stage (P = 0.46), p53 expression (P = 0.9), or tumor vascularity (P = 0.8). No correlation was observed between tumor cell TP expression and endothelial cell TP expression, but there was a significant inverse correlation between endothelial cell TP expression and tumor vascularity when summing the scores for each tumor element (P < 0.01; Table 1).

Pathological Status and Survival. Survival correlated closely with the stage and grade of the primary tumor. Survival among patients with T2/T4 tumors was significantly worse than in those with Ta/T1 tumors (P < 0.0001), as was survival in patients with high-grade tumors (G3) compared to those with low- or moderate-grade tumors (P < 0.0001). There was no relationship between tumor cell TP and overall survival or relapse-free survival (Fig. 3; Table 2).

### DISCUSSION

In this study, we have demonstrated increased expression of TP protein in bladder cancers by both Western blotting and immunohistochemistry. In addition, TP protein has been shown to be up-regulated in neoplastic cells but is also expressed by inflammatory, stromal, and endothelial cell elements of bladder cancers.

By Western analysis, TP protein expression in invasive cancers was shown to be elevated 15-fold compared to that of normal bladder and 8-fold compared to that of superficial cancers. No significant difference in expression was demonstrated between superficial tumors and normal bladder. These results confirm our previous studies, which demonstrated elevated TP mRNA expression in invasive bladder cancers but not superficial cancers, and suggest that TP is regulated at the mRNA level rather than posttranscriptionally. Bladder tumors are thought to develop along two different genetic pathways, with most invasive cancers arising from areas of carcinoma in situ. It may be that TP facilitates the transition from carcinoma in situ to invasive cancer (24).

TP immunoreactivity in the neoplastic cells was heterogeneous, being seen in some cases in both the nuclear and cytoplasmic elements, whereas in other tumors, the immunoreactivity was confined to either the nucleus or cytoplasm. The significance of these differences is unclear but could imply that TP has a variety of roles in the regulation of cell metabolism. The immunoreactivity was often maximal at the infiltrating margin of the tumor and in areas of tumor necrosis. In each of these situations, expression of an angiogenic factor would be logical, and angiogenic factors are induced by hypoxia in areas of necrosis within a tissue. It will be of interest to investigate the induction of TP by hypoxia. It is also possible that inflammatory cells in the region of areas of necrosis are inducing TP expression in the tumor cells because tumor necrosis factor α and IFN-γ are known to induce TP in tumor cell lines (31).

TP immunoreactivity was also striking in the inflammatory cell elements of the tumors, with over 70% of tumor inflammatory ele-
expressed by macrophages in normal tissues (17, 32). The density does not necessarily indicate that TP plays no role in bladder cancer angiogenesis. Our findings concur with several different in vivo models of TP-induced angiogenesis, including the rat freeze-injured skin graft model, the rat sponge model, and xenografts, in which TP has been demonstrated to increase the rate of vascularization but not microvessel density in the tissue concerned (18). An explanation might be that TP is chemoattractant but not a significant mitogen for endothelium. Interestingly, there was an inverse correlation between endothelial cell staining of TP and vascular density. The explanation for these findings may be that TP is required in the early phase of angiogenesis before vascular density becomes maximal and that TP expression is down-regulated once satisfactory angiogenesis is established. Almost certainly, multiple angiogenic factors will prove to be involved in the vascularization of bladder cancer and may be operational at different times during tumor evolution. The situation is clearly complex; the interrelationships between various angiogenic factors are unclear and worthy of further study.

Immunohistochemically determined tumor cell TP expression and total TP activity correlated with high tumor grade. In light of the mRNA and Western analyses, a correlation of TP immunoreactivity with stage would also be expected. However, this has not been demonstrated in our study. This notwithstanding, the Western and immunohistochemical data and our published mRNA data all point to an association of TP expression with an aggressive phenotype in bladder cancer.

TP immunoreactivity also showed no correlation with other established prognostic parameters in bladder cancer (e.g., ploidy and p53). In addition, TP immunoreactivity gave no prognostic information for relapse-free or overall survival. Therefore, routine examination of tissue sections in the clinical evaluation of patients cannot be recommended.

Nevertheless, TP is of interest from two potential therapeutic standpoints. TP activates Furtulon, the produg of 5-FU. The TP in invasive bladder cancers would necessarily mean that Furtulon would be important in determining the sensitivity to chemotherapy should be further evaluated.

### Table 2: Relationship between tumor clinicopathological variables and TP and relapse-free and overall survival

<table>
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<tr>
<th>Variable</th>
<th>Relapse-free survival</th>
<th>Overall survival</th>
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<tr>
<td>TP</td>
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<tr>
<td>Positive</td>
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<tr>
<td>Negative</td>
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<td>Grade</td>
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<td>0.0001</td>
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<tr>
<td>p53</td>
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**REFERENCES**

21. Pauly, J. L., Paulini, N. S., Ehrbar, R. L., and Germain, M. J. Elevated thymidine phosphorylase activity as part of methotrexate, vinblastine, Adriamycin, and cisplatinum regimens, relies for its effectiveness on indirectly inhibiting production of TMP, a key substrate for DNA biosynthesis. Cells that cannot generate this main source of TMP must rely on salvage of thymidine from other pathways. Tumors that overexpress TP will degrade salvaged thymidine and will therefore lack an essential nucleotide. Thus, tumors that overexpress TP might be more sensitive to thymidylate synthase inhibition, an observation that has been demonstrated in vivo. Moreover, in TP-poor tumors, expression of TP might be enhanced by cytokine induction (31). The role of TP in determining sensitivity to chemotherapy should be further evaluated.


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