Molecular Mediators of Angiogenesis in Bladder Cancer

Steven C. Campbell, Olga V. Volpert, Marina Ivanovich, and Noël P. Bouck

ABSTRACT

Bladder tumors are characterized by markedly increased angiogenesis when compared to the normal urothelium (NU) from which they are derived. Here, we use both cultured cells and immunohistochemistry to demonstrate a primary regulatory role for thrombospondin-1 (TSP-1), a potent inhibitor of angiogenesis, in the development of bladder tumor angiogenesis. Secretions from bladder cancer (CA) cells stimulated endothelial cell migration and corneal neovascularization, whereas those from NU cells were inhibitory. The antiangiogenic activity of NU cells was primarily due to secreted TSP-1 because neutralizing antibodies completely relieved the inhibition. Neutralizing antibodies to several putative angiogenesis inducers identified vascular endothelial growth factor (VEGF) and, to a lesser extent, basic fibroblast growth factor as the primary inducers secreted by bladder cancer cells. The secretion of TSP-1 by low- and high-grade cancer cells was reduced >94% when compared to NU cells, and this loss of inhibitory TSP-1 accounted for the development of an angiogenic phenotype because both NU cells and cancer cells secreted similar levels of total stimulatory activity and VEGF. Immunohistochemistry showed that TSP-1 was significantly reduced in all grades of bladder cancer when compared to NU, whereas VEGF staining remained relatively constant. Taken together, these data suggest that down-regulation of TSP-1 secretion is a key event in the switch from an antiangiogenic to an angiogenic phenotype, which occurs early in the development of bladder cancer.

INTRODUCTION

Transitional cell carcinoma of the bladder accounts for 10% of all cancer cases in men and 4% in women (1, 2). Recurrence is common for tumors confined to the the urothelium, and 10–15% of tumors progress to invade the underlying muscle layer (2, 3). Patients with muscle-invasive disease are at high risk for metastases, and recurrence rates are high (~50%), even for patients who appear to be cancer free after radical cystectomy (3, 4). Unfortunately, chemotherapy has provided only modest results for patients with metastatic disease, yielding only 10% long-term survivors (3, 5). There is clearly a need for better markers to identify patients at risk for progression and metastasis and to develop improved systemic treatment modalities.

Bladder cancer, like all solid malignancies (6), is dependent on angiogenesis to grow progressively and metastasize efficiently (7). In patients with muscle-invasive disease, a strong correlation has been established between microvesSEL density and lymph node metastases, disease recurrence, and reduced survival (8–10). The ability of antiangiogenic agents such as TNP-470 to inhibit the development of carcinogen-induced bladder tumors in animal models suggests that the switch to an angiogenic phenotype is an early and essential step in bladder tumorigenesis (11).

The angiogenic response is determined in part by the balance between angiogenesis inducers and inhibitors within a given microenvironment (6, 12, 13). Cells from most healthy adult tissues secrete high levels of angiogenesis inhibitors and low levels of inducers, accounting for the vascular quiescence observed under ambient conditions (12). As normal cells progress to malignancy, they acquire the ability to induce angiogenesis, in part by altering the milieu of secreted angiogenesis mediators to favor a net inductive activity (12). In many systems, these changes are mediated by activation of oncoproteins in the cytoplasm, leading to inactivation of tumor suppressor genes (12, 14–16). Although the sequence of genetic changes that underlies the development of bladder tumors is not fully understood, loss of genetic material on chromosome 9 appears to be an early event, whereas loss or mutation of the p53 and RB-1 tumor suppressor genes are found primarily in advanced tumors, suggesting that they are relatively late events in this process (2, 17–22). None of these genetic alterations have been directly linked to changes in the angiogenic phenotype of bladder tumors.

A number of angiogenesis inducers have been found to be increased in bladder cancer at the protein or transcript levels, including aFGF (23), bFGF (24, 25), VEGF (26), TP (26, 27), HGF (scatter factor; Refs. 28 and 29), midkine (30), IL-8 (31), and TGF-β (32). Increased expression of TP, HGF, and midkine has been found in invasive tumors (26–30), whereas up-regulation of VEGF appears to be more characteristic of superficial disease (26). Elevated levels of transcript for TP and midkine have correlated inversely with outcome (27, 30), and both bFGF and small angiogenic fragments of hyaluronic acid have been found at increased levels in the urine of patients with advanced bladder cancer (24, 25, 33). Although there is correlative evidence that each of these known angiogenic factors contributes to the pathogenesis of bladder cancer, most have pleiotropic effects, and it has not yet been possible to directly test their physiological relevance with respect to the angiogenic phenotype of the tumor (7).

One endogenous inhibitor, TSP-1, has been associated with bladder cancer: an inverse relationship between TSP-1 staining and microvessel density has been reported for patients with invasive bladder cancer (10). TSP-1 is a multifunctional, M, 450,000 trimeric glycoprotein that can prevent the induction of neovascularity in the rat cornea (34) and, when overexpressed in cancer cells, can block angiogenesis, tumor growth, and metastasis (14, 35–37).

Here, we use benign and malignant human epithelial cells cultured from the bladder, along with immunohistochemistry of normal and malignant tissues, to identify VEGF and bFGF as the major secreted inducers of angiogenesis and to identify loss of inhibitory TSP-1 as the event responsible for the switch from an antiangiogenic to an angiogenic phenotype during the development of bladder cancer.

MATERIALS AND METHODS

Reagents. Recombinant human aFGF, bFGF, TGF-β1, TP, PDGF, HGF, and IL-8 were obtained from R&D Systems (Minneapolis, MN), as were neutralizing polyclonal antibodies to aFGF, bFGF, PDGF, and TP and mono-
clonal antibodies to pan-TGF-β, IL-8, and HGF. Recombinant VEGF and its polyclonal neutralizing antibody were purchased from R&D Systems and Genzyme Diagnostics (Cambridge, MA), respectively. Neutralizing antibodies were used at 1–20 μg/ml in the migration assays. TP was assayed in the presence of 100 nM thymidine, as described previously (38).

Murine monoclonal antibody A4.1, which is specific for TSP-1 (39, 40), was purified from ascites and used at 30 μg/ml in the migration assays. Murine monoclonal antibody MA-1, which is specific for human TSP-1 (a kind gift of Dr. Jack Lawler, Harvard University, Cambridge, MA), was used for immuno-precipitation. Specificity of A4.1 and MA-1 for human TSP-1 but not TSP-2 which also has antiangiogenic activity, was confirmed by Western blot analysis. Human TSP-1 was purified from platelets, as described previously (41), and human TSP-2 was received as a gift from Drs. Pat Tooney and Deane Mosher (University of Wisconsin, Madison, WI). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cells. NU cells were grown from explants derived from the normal urothelium of healthy renal transplant donors using a modification of previously described techniques (42, 43). Briefly, the mucosal layer was dissected from the underlying stroma, the uroepithelium was cut into 1–2-mm fragments, and explants were plated in a minimal amount of Ham’s F-12 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS, 1.0 μg/ml hydrocortisone (Sigma), 5 μg/ml transferrin (Life Technologies, Inc.), 10 μg/ml insulin (Life Technologies, Inc.), 10 μM nonessential amino acid solution (Life Technologies, Inc.), 10 ng/ml epidermal growth factor (Life Technologies, Inc.), and 2.7 mg/ml dextrose. After 3 days, the cells were maintained in keratinocyte growth medium containing epidermal growth factor and bovine pituitary extract (Life Technologies, Inc.). Cell morphology and immunostaining for cytokeratin and vimentin were consistent with epithelial origin (data not shown). Cells were used prior to the fourth passage in all instances.

Bladder cancer cell lines derived from low-grade (RT4) and high-grade (HT1376, UMUC-3, and TCC-S) transitional cell carcinomas were obtained from American Type Culture Collection (Rockville, MD) and maintained in DMEM with 10% FCS.

Serum-free conditioned medium was harvested from confluent cells after 48–64 h and concentrated and dialyzed against PBS in an Ultrafree filter with a nominal molecular weight exclusion of M, 5000 (Millipore, Bedford, MA).

Angiogenesis Assays. The endothelial cell migration assay was used for in vitro assessment of angiogenic activity because it has been found to most closely reflect angiogenic activity in vivo, particularly angiogenic activity (44). All migration assays were performed in a modified Boyden chamber using cultured bovine capillary endothelial cells (a gift from J. Folkman), as described previously (44). Briefly, endothelial cells were plated in the lower wells of the chamber and allowed to attach to the undersurface of a gelatinized membrane (5-μm pore size; Nucleopore Corp., Pleasanton, CA). Serum-free conditioned medium (20 μg/ml) in DMEM with 0.1% BSA and other test substances or neutralizing antibodies was added to the upper wells of the chamber and incubated for 4 h at 37°C in 8% CO2. The filters were stained, and the number of cells migrating to the top of the membrane per 10 high-power fields was counted. Results are reported as percentage of induced migration compared to standard inducer (bFGF at 10 ng/ml) after subtraction of background migration observed in the presence of DMEM with 0.1% BSA.

RESULTS

Angiogenic Activity of Bladder Cancer and NU Cells. To evaluate the angiogenic activity of bladder cancer and NU cells, serum-free conditioned media were obtained and tested for their effect on endothelial cell migration and corneal neovascularization. All four bladder cancer cell lines strongly induced endothelial cell migration in the in vitro assay (Fig. 1A), and medium from high-grade bladder cancer cells consistently stimulated neovascularity in the rat cornea (Fig. 2 and Table 1). In contrast, NU cells failed to induce endothelial cell migration (Fig. 1, A and B) or corneal neovascularity (Fig. 2 and Table 1). Four independently derived NU cell strains were all strongly antiangiogenic: their media effectively blocked endothelial cell migration and corneal neovascularization induced by bFGF, VEGF, or medium conditioned by high-grade bladder cancer cells (Figs. 1B and 2 and Table 1).

TSP-1 Was Responsible for the Antiangiogenic Effect of NU Cells. Antibody A4.1, known to neutralize the antiangiogenic activity of TSP-1 (40), also blocked the antiangiogenic activity of medium conditioned by NU cells. In the presence of A4.1, medium from NU
cells no longer inhibited endothelial cell migration or corneal neovascularity induced by either bFGF or medium from high-grade bladder cancer cells (Figs. 1B and 2 and Table 1). Moreover, medium derived from NU cells became angiogenic when TSP-1 was inactivated (Figs. 1B and 2 and Table 1), demonstrating that these cells secrete angiogenic activity that is normally neutralized by TSP-1. Taken together, these results identify TSP-1 as the primary functional inhibitor secreted by cultured NU cells.

Controls evaluating the effect of A4.4 on endothelial cell migration and corneal neovascularization showed that A4.4 relieved inhibition due to exogenous TSP-1 but had no effect when tested alone or in the presence of bFGF (Fig. 1C and Table 1).

**VEGF and bFGF Were the Primary Inducers Secreted by Bladder Cancer Cells.** A number of molecules capable of inducing angiogenesis have been found at increased levels in bladder cancer, but their functional significance has not been defined (7). To determine which of these inducers are major contributors to the angiogenic phenotype, a panel of neutralizing antibodies was evaluated for ability to inhibit endothelial cell migration induced by bladder cancer cells. Neutralizing antibody to VEGF and, to a lesser extent, bFGF significantly reduced the angiogenic activity of the HT1376 bladder cancer cells (P < 0.05), demonstrating that these two factors are the primary inducers secreted by these cells (Fig. 3). In contrast, neutralizing antibody to αFGF, pan-TGF-β, PDGF, HGF, and IL-8 had no effect on migration levels (Fig. 3), nor did antibodies that neutralized the angiogenic activity of TP when tested in the presence of added thymidine (data not shown; Ref. 38). In all instances, the neutralizing antibodies had no effect on endothelial cell migration when tested alone, and each efficiently blocked migration induced by its respective inducer, which was run as a positive control (data not shown). When neutralizing antibodies to VEGF were combined with those against bFGF, all of the stimulatory activity secreted by the HT1376 bladder cancer cells was eliminated (Fig. 4). Similar results were obtained with medium from the low-grade RT4 and from two additional high-grade bladder cancer cell lines, UMUC-3 and TCC-S (data not shown).

**High Levels of VEGF Were Also Secreted by NU Cells.** When the angiogenic activity of medium derived from NU cells was tested in the presence of neutralizing antibody to TSP-1, VEGF was found to be the major inducer, and as was the case with the tumor cell-conditioned medium, antibodies to both VEGF and bFGF eliminated all stimulatory activity (Fig. 4).

When the amounts of secreted VEGF were quantified by ELISA, the levels present in medium derived from NU cells (average = 335 pg/μg protein) were as high as those in medium from bladder cancer cells (average = 297 pg/μg protein; Table 2). These concentrations are approximately 20-fold greater than the ED₅₀ for VEGF for stimulation of endothelial cell migration in the in vitro assay. In comparison, secreted levels of bFGF were much lower, approximately 2–3 orders of magnitude lower than the ED₅₀ for bFGF when tested alone in the migration assay. The modest inhibitory effect of bFGF antibodies in the migration assay likely reflects synergism between VEGF and bFGF, as has been seen by others (45, 46).

To further confirm that angioinductive activity did not increase during tumor progression, the total inducing activity was estimated by defining the ED₅₀ for the stimulation of endothelial cell migration in the presence of anti-TSP-1. The ED₅₀ of medium derived from NU

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**Table 1 In vivo angiogenic activity of NU and bladder cancer cells**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Positive corneas/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM from high-grade tumor cells (HT1376)</td>
<td></td>
</tr>
<tr>
<td>Alone</td>
<td>3/3</td>
</tr>
<tr>
<td>+ anti-VEGF</td>
<td>0/6</td>
</tr>
<tr>
<td>CM from NU cells</td>
<td></td>
</tr>
<tr>
<td>Alone</td>
<td>0/3</td>
</tr>
<tr>
<td>+ VEGF</td>
<td>0/3</td>
</tr>
<tr>
<td>+ anti-TSP-1</td>
<td>3/3</td>
</tr>
<tr>
<td>+ tumor CM</td>
<td>0/3</td>
</tr>
<tr>
<td>+ tumor CM + anti-TSP-1</td>
<td>0/3</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>0/2</td>
</tr>
<tr>
<td>anti-TSP-1</td>
<td>0/3</td>
</tr>
<tr>
<td>VEGF</td>
<td>6/7</td>
</tr>
<tr>
<td>Anti-VEGF</td>
<td>1/5</td>
</tr>
<tr>
<td>VEGF + anti-VEGF</td>
<td>0/6</td>
</tr>
</tbody>
</table>

* Serum-free conditioned medium from NU and bladder cancer cells (HT1376) was incorporated into a Hydron pellet and implanted into the rat cornea. Neutralizing antibodies to TSP-1 and/or VEGF were also included, where indicated. Induction of vigorous neovascularization after 7 days was scored as a positive response.  

* CM, conditioned medium.

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**Fig. 1.** Rat corneal assay to evaluate the in vitro angiogenic activity of NU and bladder cancer cells. Media conditioned by NU or high-grade HT1376 cells were formulated into pellets and implanted into the normally avascular cornea of a rat and neovascularization was assessed 7 days later. Neutralizing antibody to TSP-1 and/or VEGF was also included, where indicated.

**Fig. 2.** Rat corneal assay to evaluate the in vivo angiogenic activity of NU and bladder cancer cells. Media conditioned by NU or high-grade HT1376 cells were formulated into pellets and implanted into the normally avascular cornea of a rat and neovascularization was assessed 7 days later. Neutralizing antibody to TSP-1 and/or VEGF were also included, where indicated.

**Fig. 3.** Angiogenesis inducers secreted by bladder cancer cells include VEGF and bFGF. Media from high-grade bladder cancer line HT1376 (CA) was tested for the ability to stimulate endothelial cell migration in the presence of anti-TSP-1 antibody. Neutralizing antibodies to a variety of angiogenesis inducers were tested for inhibitory effect. Columns, percentage maximal migration when compared to standard inducer, bFGF; bars, SE. *, differs from control (CA + anti-TSP-1), P < 0.05.
cells (2.5 ± 1.1 μg/ml) was similar to that of the low-grade RT4 (2.4 ± 0.3 μg/ml) and the high-grade HT1376 bladder cancer cells (2.4 ± 0.5 μg/ml). The ED₅₀ of medium derived from the high-grade UMUC-3 and TCC-S bladder cancer cell lines were 4.9 ± 0.1 μg/ml and 9.4 ± 0.85 μg/ml, respectively, which represent even lower levels of intact TSP-1. RT4, a low-grade bladder cancer cell line, secreted a protein of approximate Mr, 130,000, which was recognized by A4.1 and may represent a breakdown product. Densitometric analysis estimated that the level of TSP-1 secreted by the bladder cancer cells was ≤5.8% of that secreted by NU cells in all instances. Significantly reduced levels of intracellular TSP-1 protein and mRNA were also found within bladder cancer cell lysates when compared to NU cell lysates (data not shown).

TSP-1 and VEGF Expression in Normal and Malignant Bladder Tissue. To evaluate TSP-1 and VEGF expression in vivo, frozen sections of human bladder tissue were stained with antibody MA-1 specific for TSP-1 or with a polyclonal antibody specific for human VEGF (Fig. 6 and Table 3). Intense staining for TSP-1 was characteristic of the NU but was not found in any of the bladder tumors tested, consistent with the in vitro data. Staining could be seen in tumor stroma and basement membrane but was weak or absent within the tumor cells themselves. Positive staining of endothelial cells for TSP-1 served as an internal control for these experiments. In contrast, immunostaining for VEGF was equally strong in both benign and malignant bladder epithelial cells, suggesting no significant modulation of the expression of this factor. All NU samples and 14 of 16 tumors stained strongly for VEGF.

DISCUSSION

The study of tumor-associated angiogenesis provides one promising approach for the discovery of much needed novel therapeutic agents and treatment strategies. However, before antiangiogenic therapy can be rationally integrated into the management of patients with bladder cancer, improved knowledge of the molecular mechanisms regulating angiogenesis in this disease will be required (7). This study takes a first step in this process by identifying TSP-1 as the protein responsible for the angiogenic activity secreted by NU cells and VEGF as the angiogenic factor principally responsible for the angiogenic activity of cultured malignant uroepithelial cells. In addition, we have identified down-regulation of TSP-1 as a primary factor contributing to the switch from an angiogenic to an angiogenic phenotype during the development of bladder cancer.

This study adds bladder uroepithelial cells to a growing list of normal cell types, including fibroblasts, glial cells, and breast epithelial cells, that produce TSP-1 at levels high enough to avidly inhibit angiogenesis (12). In the case of bladder urothelium, the high levels of active VEGF expressed by normal cells, the secretions of which were nonetheless angioinhibitory, and tissues suggests that TSP-1 plays an...
Fig. 6. TSP-1 and VEGF expression in NU and bladder cancer tissue. Frozen sections of human tissue from NU (A and E); low-grade, superficial bladder cancer (B and F); high-grade, superficial bladder cancer (C and G); and high-grade, muscle-invasive bladder cancer (D and H) were stained for TSP-1 (left) or VEGF (right). Magnification, ×100.
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Table 3 Summary of staining for TSP-1 and VEGF in NU and bladder cancer

<table>
<thead>
<tr>
<th>Tissue or tumor type</th>
<th>TSP-1 Cases (n)</th>
<th>Stain*</th>
<th>VEGF Cases (n)</th>
<th>Stain*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NU</td>
<td>5</td>
<td>++</td>
<td>6</td>
<td>++</td>
</tr>
<tr>
<td>Papillary, grade 1–2 bladder cancer</td>
<td>1</td>
<td>++</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>Muscle-invasive, high-grade bladder cancer</td>
<td>1</td>
<td>0</td>
<td>5</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>++</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0</td>
<td>4</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>4</td>
<td>+</td>
</tr>
</tbody>
</table>

* Frozen sections of human tissue.

** Intensity of staining was graded from 0 (undetectable) to +++++ (maximal).

Stage TA transitional cell carcinoma.

Stage T2 or higher, grade 3/3 transitional cell carcinoma.

active role in maintaining vascular quiescence in the absence of disease. Expression of TSP-1 in culture can be regulated by a variety of factors such as the concentration of serum, proliferative status of the cells, and level of confluence, raising the question of its in vivo relevance (47, 48). However, we collected conditioned media under conditions designed to conform to those in vivo, normoxia and no serum, and with the cells close to confluence, and changes in TSP-1 seen in vitro did correlate well with differential staining of NU and cancers seen in vivo.

This study also identifies VEGF and, to a lesser extent, bFGF as the relevant inducers secreted by bladder cancer cells in vitro. Despite the relatively low concentrations of bFGF, a significant effect was seen when it was neutralized, suggesting a synergistic interaction between bFGF and VEGF, as has been observed previously in both in vitro and in vivo systems (45, 46, 49). Although VEGF, as measured by RNase protection, has been reported by others to be increased in bladder tumors when compared to normal tissue, the observed increases were modest (2–3-fold) in most instances (26), and as VEGF expression is known to be regulated at least in part by posttranscriptional processes, protein levels may be the more reliable indication of activity (50). We found that the levels of VEGF in vitro and in vivo as well as total angiostimulatory substances found in bladder tumors, such as TP, HGF, midkine, and angiogenic fragments of hyaluronic acid might contribute to the angiogenic phenotype of bladder tumors (26, 28, 30, 33). Relatively weak staining for VEGF was observed in 2 of 10 high-grade tumors in this study, suggesting that other inducers may not play a significant role in the switch to an angiogenic phenotype in this system. Other normal cell types, such as the epithelial cells of lung alveoli, renal glomeruli, and adrenal cortex and the islet cells of the pancreas, also express high ambient levels of VEGF, whether or not it is further modulated during tumor progression is not known (51, 52). These in vitro experiments cannot exclude the possibility that other angiostimulatory substances found in bladder tumors, such as TP, HGF, midkine, and angiogenic fragments of hyaluronic acid might contribute to the angiogenic phenotype of bladder tumors (26, 28, 30, 33). Relatively weak staining for VEGF was observed in 2 of 10 high-grade tumors in this study, suggesting that other inducers may play a prominent role for a small but substantial subset of bladder cancers. Our experimental protocol does not allow assessment of a number of additional factors, including the roles of: (a) stromal/epithelial interactions, which may be involved in the production of HGF (28); (b) tumor-infiltrating macrophages or lymphocytes, which can secrete or activate angiogenic mediators or induce their production from the tumor cells themselves (6, 12); or (c) small fragments of hyaluronic acid, which can be angiogenic and were recently found in the urine of patients with advanced bladder cancer (33). Such small molecules would have been lost during concentration and dialysis of the conditioned media we tested.

The genetic elements controlling TSP-1 expression and angiogenesis in bladder cancer are currently under investigation in our laboratory. In fibroblasts and breast epithelial cells, TSP-1 can be regulated by p53 (12, 14), and Grossfeld and colleagues (10) recently reported a correlation between p53 mutation, as determined by immunohistochemistry, and reduced TSP-1 staining in stroma adjacent to bladder tumors. However, p53 is lost late in bladder tumor progression, whereas our staining of low-grade tumors indicates that loss of TSP-1 occurs early and correlates more closely with the loss of unidentified tumor suppressor genes on chromosome 9, an early event in both superficial and invasive bladder cancer (2).

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REFERENCES


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