Two Mechanisms of Basic Fibroblast Growth Factor-induced Angiogenesis in Bladder Cancer

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ABSTRACT

In the urine of patients with bladder cancer, levels of the angiogenic peptide basic fibroblast growth factor (bFGF) may be elevated 100-fold. To date, levels of expression of bFGF in bladder tumor tissue have not been determined, nor has the cellular source of the urinary bFGF been identified.

bFGF mRNA expression was quantified using RNase protection analysis in 32 primary bladder tumors and 8 normal bladder specimens. In addition, bFGF protein expression in the tumor cytosol was determined using a Quantikine ELISA, and bFGF protein expression was localized with immunohistochemistry.

bFGF mRNA expression was absent in 28 of 32 (87%) bladder cancers despite detectable expression in 7 of 8 (87%) normal bladder specimens ($P = 0.0001$). In only one tumor was bFGF mRNA expression higher than in normal bladder tissue. Median bFGF protein expression was also higher in the normal bladder specimens than in the superficial tumors (3800 pg/g protein versus 1140 pg/g protein; $P < 0.02$), but there was no statistically significant difference between protein expression in normal bladder and invasive cancers (3800 pg/g versus 3600 pg/g). Median bFGF protein expression was higher in invasive cancers than in superficial tumors ($P < 0.05$).

Intense bFGF immunoreactivity was seen in the basal lamina of normal transitional epithelium, in normal human detrusor muscle, and in vessels within tumors. Tumor cell immunoreactivity was rare and was usually weak. Only in the tumor which strongly overexpressed bFGF mRNA and protein was cytoplasmic staining detectable in the neoplastic cells.

There are two mechanisms of bFGF-induced angiogenesis in bladder cancer. Rarely, neoplastic cells synthesize bFGF but more commonly bFGF is released by degradation of epithelial basement membranes and detrusor muscle, from where it can diffuse into the tumor microenvironment and bind to blood vessels. Mechanisms of extracellular matrix degradation may be important in bladder cancer angiogenesis and progression and as such are potential therapeutic targets.

INTRODUCTION

Angiogenesis is a prerequisite for tumor growth and metastasis (1) and the intensity of angiogenesis, as estimated by quantitation of vascular density, has been shown to correlate with a higher incidence of metastases and a worse prognosis in tumors of the bladder (2, 3), breast (4, 5), skin (6), and prostate (7—9). Consequently, there is considerable interest in the molecular regulation of angiogenesis. Angiogenesis is induced by angiogenic factors which are thought usually to be produced by either the neoplastic cells themselves or the nonmalignant cells which infiltrate the tumor. Numerous angiogenic factors have been described, including aFGF, bFGF, VEGF, PDECGF, midline, and pleiotrophin (10, 11). The relative importance of individual angiogenic factors in the vascularization of most tumor types is still largely unclear.

bFGF (FGF-2) is one of a family of nine FGFs showing a wide spectrum of biological activities that include regulation of angiogenesis, cell proliferation, cell to cell adhesion, and neuronal differentiation (12, 13). All of the family members are characterized by their strong affinity for heparin, and all are functional ligands for FGF receptors which have intrinsic tyrosine kinase activity. bFGF is synthesized by several cell types including fibroblasts, macrophages, and endothelial cells. It is pleiotrophic, being a mitogen for fibroblasts, epithelial cells, vascular endothelial cells, osteoblasts, and smooth muscle cells. In vitro it is one of the most potent angiogenic factors. The molecule is unusual in that it lacks a classical signal sequence; it is however undoubtedly exported from cells but the mechanism of release is not clear. Suggested mechanisms include export bound to extracellular matrix components (14—16), release on cell death, externalization in evaginated segments of plasma membrane (17), and release in response to heat shock (18). Indirect evidence for a role for bFGF in tumor angiogenesis comes from observations that bFGF is elevated in the serum and urine of patients with a wide variety of cancers (19), and the correlation of a poor outcome in renal cell carcinoma with immunochemical evidence of elevated expression of bFGF (20). More direct evidence comes from the demonstration of inhibition of tumor growth in nude mice in vivo by neutralizing antibodies to bFGF (21). Furthermore, in a transgenic mouse model of dermal fibrosarcoma, neovascularization and sarcoma formation are associated with a change from the normal cell-associated localization of bFGF to extracellular release (17).

Reports to date suggest that angiogenesis in bladder cancer may be under the control of several different angiogenic factors. VEGF mRNA expression is elevated in bladder tumors which have not invaded detrusor muscle (superficial tumors), whereas expression of PDECGF mRNA is elevated in muscle invasive cancers (22). The VEGF is thought to be produced principally by the tumor cells, whereas PDECGF localizes most strongly to the stromal and inflammatory elements of a tumor. Increased expression of aFGF in bladder cancers compared to normal bladder has been demonstrated using immunohistochemistry, with immunoreactivity being strongest in the neoplastic cells (23). The same investigators also found that the urine of patients with bladder cancer contains aFGF (23). bFGF has also been identified in the urine of patients with bladder cancer, the concentration in cancer patients being at least 10-fold higher than in those without cancer (24). Patients with aggressive metastatic cancers were noted to have particularly high levels (around 100-fold higher than in patients with no active disease). We have confirmed these findings in our own series of 83 patients in which urinary bFGF was 4-fold higher in patients with active bladder cancers compared to controls with no active disease (25). Despite this strong association of urinary bFGF and bladder cancer, the cellular source and the mechanism by which bFGF appears in urine remain unclear. The only reported study to date is from Ravery et al. (26), who detected strong bFGF immunoreactivity on stromal vessels but no significant bFGF expression within the epithelial component of 12 transitional cell carcinomas of the bladder.

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In an attempt to determine mechanisms for bFGF-induced angiogenesis in bladder cancer, we have quantified bFGF mRNA and protein expression in a series of bladder cancers and have localized this expression with immunohistochemistry.

**MATERIALS AND METHODS**

Preparation of RNA and Construction of Plasmids to Generate Probes for RNase Protection Analysis. RNA was prepared from 32 primary bladder tumors and 8 normal bladder samples using the method of Chomczynski and Saachi (27). The tumor samples were all obtained by transurethral resection of newly diagnosed tumors. None of the patients had been previously treated with radiotherapy or chemotherapy. The normal bladder samples were obtained from patients at the time of cadaveric organ retrieval. None of the control patients had a history of bladder disease.

bFGF Antisense Probe. A 214-bp EcoRI/BamHI fragment of human bFGF cDNA in pHFL 1—7 was cloned into the EcoRI/BamHI site of pBlue-script SK*+. The resulting plasmid was linearized with EcoRV and an antisense radiolabeled probe generated with T3 RNA polymerase. Plasmid pHFL1—7 was a gift from Drs. Judith Abraham and John Fiddes (California Biotech, Mountain View, CA).

Ribonuclease Protection Analysis. Antisense probes, labeled with [α-32P]dCTP, were hybridized to 10 μg of total cellular RNA, and the free unhybridized probe was removed by digestion with RNases A and T1. Protected fragments were analyzed by electrophoresis in 6% polyacrylamide/urea-sequencing gels followed by autoradiography. In each hybridization, an antisense transcript, corresponding to glyceraldehyde-3-phosphate dehydrogenase (28), was included as an internal control. mRNA abundance was quantified using scanning laser densitometry (Bio-Image analyzer; Millipore, Bedford, MA), and signals were standardized to the glyceraldehyde-3-phosphate dehydrogenase control to provide a measure of expression. A positive control from the 253J human bladder cell line was loaded onto each gel to allow cross-gel comparisons.

Preparation of Tumor Cytosols. Tumor cytosols were prepared from 12 of the tumors (5 superficial and 7 invasive) and 5 of the normal bladder samples according to the method described by Smith et al. (29). Protein concentrations were assayed using the method of Bradford (30) with BSA as the standard.

bFGF Immunoassay. Concentrations of bFGF in the cytosolic extracts were determined using a Quantikine human bFGF immunoassay (R&D Systems, Minneapolis, MN) according to manufacturer’s instructions. The intrasay precision is ~5%, the recoverability of bFGF is ~90%, and there is no cross-reactivity with other members of the FGF family (R&D Systems; manufacturer’s data sheet). Samples were analyzed in triplicate, and the mean value was determined. Concentrations of bFGF were expressed as pg/g cytosol protein (Fig. 2).

Immunohistochemistry. Frozen tissue sections (8 μm) were cut from the same tumor blocks that were utilized for the RNA and cytosolic extractions. The sections were fixed in acetone for 10 min, dried, and stored at −20°C. The sections were washed in PBS for 5 min and then incubated for 1 h at room temperature with a 1:1000 dilution in PBS of a mouse monoclonal antibody against bovine bFGF type II (TCS Biologicals, Botolph Claydon, Buckingham, England; Ref. 31). This antibody is a neutralizing IgG1 antibody. The epitope of bFGF it recognizes is not known but the antibody does recognize heat-inactivated bFGF. Sections were rinsed twice in PBS for 10 min before incubation with a 1:100 dilution of peroxidase-conjugated rabbit anti-mouse antibody (Dako Ltd., High Wycombe, United Kingdom) in PBS containing 20% AB serum for 30 min at room temperature. Sections were washed twice in PBS for 10 min. Diaminobenzidine (Sigma, Poole, England) was used as the chromogen (12 mg of diaminobenzidine 20 ml of PBS, and 80 μl of H2O2) and was incubated for 8 min. Sections were counterstained in hematoxylin, dried in ethanol and xylene, and mounted. Two negative controls were used: the first used no primary antibody, and the second utilized a type-specific IgG1 monoclonal antibody (Coulter Corporation, Hialeah, FL). Results were confirmed with a second neutralizing mouse monoclonal IgG1 antibody against bFGF (antibody DG2; DuPont-Merck, Wilmington, DE). The epitope recognized by DG2 is not known (32).

Statistical Analysis. Expression of mRNA in the tumors and normal bladder samples was compared using the χ² test with a Yates' correction. The median levels of expression of bFGF protein in normal bladder and tumors were compared using the Mann-Whitney U test (two tailed).

**RESULTS**

Ten (32%) of the tumors were muscle invasive (T2, T3, T4) and 22 (68%) were superficial (Tα, T1). Of the superficial tumors, 4 were pTα.

![Fig. 2. bFGF protein in bladder tumor cytosols. Protein concentrations were estimated using a Quantikine bFGF ELISA. Median bFGF concentration in normal bladder was 3800 pg/g total protein, in superficial tumors it was 1100 pg/g, and in invasive tumors it was 3600 pg/g.](image-url)
tumors and 18 were pT₁ tumors. Two of the invasive cancers were graded moderately differentiated, whereas eight were poorly differentiated. Two of the superficial tumors were poorly differentiated, 12 were moderately differentiated, and 8 were well differentiated.

Representative RNase protection assays are shown in Fig. 1. Twenty-eight of 32 tumors (87%) did not express bFGF RNA. However, in only one of eight normal bladder samples (13%) was bFGF RNA undetectable ($\chi^2 = 14.5; P = 0.0001$). Seven of eight of the normal bladder samples (87%) expressed moderate amounts of bFGF RNA. One invasive cancer strongly expressed bFGF RNA (stronger than normal bladder) and two invasive and one superficial, pT₁G₂, tumor expressed moderate amounts of bFGF RNA (detectable but less intensity than the normal bladder specimens). No tumor expressed more bFGF RNA than the positive control 253J cell line.

Median bFGF protein concentration in the normal bladder cytosols was 3800 pg/g protein (range, 2130–6000); in the superficial tumors, it was 1140 pg/g protein (range, 980–1600); and in the invasive cancers, it was 3600 pg/g protein (range, 743–23,000; Fig. 2). bFGF protein concentration in one invasive tumor was approximately 10-fold higher (23,000 pg/g) than the median value and 4-fold higher than

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Fig. 3. Immunohistochemical localization of bFGF protein in normal bladder specimens and bladder cancers. Frozen tissue sections (8 μm) were incubated with a 1:1000 dilution in PBS of a mouse monoclonal antibody against bovine bFGF type II (TCS Biologicals, Botolph Claydon). The secondary antibody was peroxidase-conjugated rabbit anti-mouse (DAKO Ltd.), and the chromogen was diaminobenzidine (Sigma). A, normal bladder. Intense immunoreactivity is seen in the basement membrane of the epithelial layer and in muscle. B, normal bladder. Intense immunoreactivity is seen in the bundles of detrusor muscle. C, normal bladder. Normal bladder stained with the control IgG1 antibody. No staining of detrusor bundles is seen. D, bladder cancer. An invasive bladder cancer showing strongly positive vessels, moderately positive muscle cells, and negative neoplastic cells. E, bladder cancer. Neoplastic cells of this superficial tumor did not stain. F, bladder cancer. Positive cytoplasmic staining is seen in the invasive cancer which strongly overexpresses bFGF mRNA and protein. A–D, ×25; E and F, ×40.
the highest of the normal samples. In this tumor, bFGF mRNA was also strongly overexpressed (Fig. 1). The difference between the concentrations of bFGF protein in the normal samples and the superficial tumors was statistically significant \( P < 0.02 \), as was the difference between invasive and superficial tumors \( P < 0.05 \).

Immunoreactivity was most intense around the basal lamina of the epithelium of normal bladder and in the detrusor muscle bundles (Fig. 3A and B). Normal transitional epithelium did not stain (Fig. 3A). Staining of vessels within normal bladder tissue was very sparse. Tumor cell immunoreactivity was rare (Fig. 3, D and E) but when seen was confined to the cytoplasm and was strongest in the tumor which markedly overexpressed bFGF RNA and protein (Fig. 3F). By contrast most tumor vessels stained intensely for bFGF (Fig. 3D). Occasional weak staining of the extracellular matrix was seen in some cases.

**DISCUSSION**

The most striking findings in this study are (a) the absence of increased bFGF mRNA expression within bladder cancers despite documented markedly increased urinary levels of bFGF in patients with bladder cancer, and (b) the intense bFGF immunoreactivity of the basal lamina of the transitional epithelium and normal human detrusor muscle.

The rarity of bFGF mRNA expression in bladder cancer [4/32 (13%) of tumors] and the paucity of cytoplasmic or nuclear tumor cell staining suggest that, if bFGF is a significant contributor to angiogenesis in bladder cancer rather than direct synthesis by tumor cells, alternative mechanisms of regulation are usually involved. This pattern of bFGF mRNA expression, i.e., expression higher in normal tissue controls than in tumors, has also been described in breast cancer (33, 34) but differs from that seen in gliomas (35) and renal cell cancer (36).

The immunohistochemical findings go some way to determining the probable origin of the bFGF which is found in increased amounts in the tumor cytosol, serum, and urine of patients with aggressive bladder cancers (24). Immunoreactivity is strongest in the tissue around the basal lamina of the epithelium and in the smooth muscle of the bladder wall (Fig. 3, A and B). Although the immunoreactivity may be the result of binding to nonspecific unidentified epitopes, this seems unlikely since both of the antibodies studied result in similar patterns of staining. Furthermore, a previous study of the distribution of bFGF immunoreactivity in normal tissues demonstrated strong staining in the smooth muscle of several organs (37).

Our hypothesis is that as an aggressive tumor spreads to invade the basement membrane and involve the muscle layers of the bladder, it releases the bFGF from stores within those structures. The bFGF that is released is then available to stimulate angiogenesis at the invading margin of the tumor and can be detected bound to tumor blood vessels.

This proposed mechanism is supported by *in vitro* studies which have suggested that mobilization of bFGF from tissue stores within extracellular matrix rather than increased production may be the primary source of bFGF (15) (38, 39). bFGF was found in the extracellular matrix of vascular endothelial cells complexed to glycosaminoglycans and could be released from the complexes by the action of heparanases, plasmin, or cathepsin D (16, 38). Complexing the bFGF in this way to a glycosaminoglycan protects it from proteolytic breakdown (14) and allows large stores to be maintained in an inactive state, ready for rapid mobilization when required, e.g., after wounding. It may be that bFGF is also complexed to these same glycosaminoglycans in muscle. An invading tumor secretes a variety of enzymes, including heparanases, urokinase, and cathepsin D, which digest the host architecture to allow invasion.

Our hypothesis is that these enzymes, secreted by the tumor, release bFGF from the stores in epithelial basement membrane and detrusor muscle. Certainly our data from the ELISA studies suggest that there is abundant soluble bFGF protein in the invasive tumor biopsies, despite low levels of bFGF mRNA transcripts in these same tumors. The high level of bFGF protein in the invasive tumors is not the result of sampling artifact (i.e., invasive tumor biopsy not merely normal detrusor muscle) because the immunohistochemistry shows the biopsies to be predominantly composed of tumor tissue. In addition, if sampling artifact was the explanation, then one would expect broadly similar mRNA expression in normal bladder and invasive tumor biopsies. The bFGF identified in the invasive tumors may have diffused into the body of the tumor after enzymatic release from the basement membrane or detrusor muscle (Fig. 3D). This free soluble bFGF is also able to diffuse into the circulation before excretion in high concentration in the urine.

In superficial tumors, bFGF mRNA expression is rare and protein concentrations are low. mRNA expression is low because the biopsy is comprised primarily of neoplastic cells, and protein levels are low because the main sites of bFGF storage (smooth muscle and basal lamina of the epithelium) remain largely intact, free from enzymatic digestion. We have not examined sufficient superficial tumors to draw distinctions between the two subgroups of superficial tumors, pT\(_2\) (tumor not penetrating lamina propria) or pT\(_1\) (tumor penetrates lamina propria). However, our model would predict that bFGF protein would be lower in pT\(_1\) tumors because these tumors have not breached the basement membrane and released the bFGF stored within it.

Thus, we have demonstrated two different sources of bFGF in bladder cancer. Tissue stores in epithelial basement membranes and smooth muscle appear to be the principle sources but undoubtedly some neoplastic cells may synthesize large amounts of bFGF *de novo*. Our findings support the hypothesis that bFGF may be an important determinant of angiogenesis in bladder cancer and suggest that mechanisms of extracellular matrix degradation may be a useful therapeutic target.

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**REFERENCES**


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