Gene Therapy of Human Bladder Cancer with Adenovirus-mediated Antisense Basic Fibroblast Growth Factor

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ABSTRACT
We previously investigated the role of basic fibroblast growth factor (bFGF) as a mediator of angiogenesis, tumorigenicity, and metastasis of transitional cell carcinoma (TCC) of the bladder. In the present study, we determined whether adenoviral-mediated antisense bFGF gene transfer therapy (Ad bFGF-AS) would inhibit TCCs growing in the subcutis of nude mice. In vitro, Ad bFGF-AS inhibited endothelial cell proliferation and enhanced apoptosis. The highly metastatic human TCC cell line 253J-BV was implanted ectopically in the subcutis of athymic nude mice, and therapy was begun when the tumors reached a diameter of 5 mm. Intratrabecular therapy with Ad bFGF-AS decreased the in vivo expression of bFGF and matrix metalloproteinase type 9 mRNA and protein, and reduced microvessel density and enhanced endothelial cell apoptosis. Tumor growth was significantly inhibited by Ad bFGF-AS (mean, 58 mg) compared with controls [saline (mean, 562 mg), β-galactosidase adenovirus (mean, 586 mg), and sense bFGF adenoviral therapy (Ad bFGF-S; mean, 3012 mg)]. These results suggest that Ad bFGF-AS therapy affects endothelial cells directly and tumor cells indirectly through down-regulation of bFGF and matrix metalloproteinase type 9, resulting in endothelial cell apoptosis and significant tumor growth inhibition. Furthermore, these studies confirm that bFGF expression is a valid target for the therapy of bladder cancer.

INTRODUCTION
The growing awareness of the critical role of angiogenesis in tumor growth and metastasis has prompted a major effort toward the development of antiangiogenic therapy (1–4). Antiangiogenic therapy targets the release of angiogenic factors by the tumor or host, receptors for angiogenic mediators, or endothelial cells directly. To be effective, an agent of antiangiogenic therapy should have a biological half-life sufficient to counter the angiogenic phenotype of the tumor, must act against a broad spectrum of tumor types, and should not interfere with physiological angiogenesis (5).

The process of angiogenesis is relevant to the pathogenesis of TCC of the bladder (6). Human TCC is characterized by overexpression of various proangiogenic factors, including VEGF (7), platelet-derived endothelial growth factor (8), midkine (9), and bFGF (10, 11), and the loss of expression of endogenous inhibitors of angiogenesis such as IFN-β (12) and thrombospondin-1 (13, 14). MVD, which is a histological surrogate for angiogenesis, correlates with disease progression for patients with invasive TCC (15).

bFGF regulates the growth and metastasis of TCC, in part by regulating the process of angiogenesis (10, 16–18). Recently, we reported that therapy with either IFN-α (12) or anti-epidermal growth factor receptor monoclonal antibody Mab C225 (19) inhibited growth and metastasis of human TCC secondary to down-regulation of bFGF expression and the subsequent regression of tumor-induced neovascularization. Alternatively, the down-regulation of bFGF with either neutralizing antibodies or antisense oligonucleotides inhibited the growth of human breast cancer, glioblastoma, hepatoma, and melanoma (20–22), and liposome- or viral-mediated gene transfer with vectors containing antisense oriented bFGF or FGF receptor-1 inhibited both angiogenesis and growth of human melanoma and ovarian cancer (23, 24).

To determine whether bFGF directly regulates growth and metastasis of human TCC, we previously constructed sense and antisense recombinant adenoviral vectors containing the cDNA sequence for bFGF. Following in vitro gene transfer, we found that overexpression of bFGF increased tumorigenicity, whereas inhibition of bFGF expression decreased tumorigenicity, tumor-induced angiogenesis, and metastasis of human TCC (25). This effect was independent of cell proliferation because transduction of the cell lines 253J-B-V and 253J with either Ad bFGF-AS or Ad bFGF-S, respectively, at the titers used did not alter cell proliferation in vitro. These studies provided direct evidence that bFGF is an important mediator of tumor angiogenesis and

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Abbreviations: TCC, transitional cell carcinoma; VEGF, vascular endothelial cell growth factor; bFGF, basic fibroblast growth factor; Ad, adenovirus; IFN, interferon; MVD, microvessel density; MNP, matrix metalloproteinase; IL, interleukin; MOI, multiplicity of infection; pfu, plaque-forming unit(s); PI, propidium iodide; ISH, in situ hybridization; IHC, immunohistochemistry; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; TDT, terminal deoxynucleotidyl transferase; HUVEC, human umbilical vascular endothelial cell; PCNA, proliferating cell nuclear antigen.
virulence and suggested that adenovirus-mediated therapy with the antisense bFGF construct could be effective against human TCC (25). In the present study, we demonstrated that direct intralesional therapy of human TCC with Ad bFGF-AS inhibits tumor growth and angiogenesis by reducing bFGF and MMP-9 production by tumor cells and enhancing endothelial cell apoptosis, and validated bFGF as a therapeutic target.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The highly metastatic human TCC cell line 253J B-V was selected by orthotopic implantation within the bladders of athymic nude mice (11). 253J B-V R is a subline of 253J B-V selected for in vitro resistance to the antiproliferative effects of IFN-a or -b (12). 253J B-V R grows as a s.c. xenograft and was used for these experiments to facilitate the evaluation of response to therapy without the need for repeated laparotomy. These cell lines were grown as a monolayer culture in modified Eagle’s minimal essential medium supplemented with 10% fetal bovine serum, vitamins, sodium pyruvate, L-glutamine, nonessential amino acids, and penicillin-streptomycin (11).

Adenovirus. GenBank was searched, and a full-length gene for sense and antisense bFGF was constructed and incorporated into a replication-deficient human adenovirus as described previously (25). A MOI of 10:1 was shown to be optimal for both Ad bFGF-AS and -S, demonstrating minimal cytotoxicity and maximal effect on bFGF production (Fig. 1). The optimal MOI for the β-galactosidase adenovirus (Ad β-gal; a generous gift from Dr. J. T. Hsieh, Southwestern Medical Center, Dallas, TX) was 1:1 (11).

In Vitro Apoptosis. The in vitro dose-dependent apoptotic effect of Ad bFGF-AS was evaluated by incubating 5 × 10^5 253J B-V R cells for 24 h in conditioned medium and then exchanging the medium for 10% FBS-supplemented minimal essential medium containing increasing concentrations of Ad IL-8 AS (0–10^6 pfu/ml). Cells were harvested by centrifugation and incubated at 4°C for 24 h in PBS containing 50 μg/ml PI for staining. Quantification of DNA fragmentation was accomplished using flow cytometry with PI staining. Cells in the sub-G0 population were considered to be undergoing apoptosis.

Animals. Male athymic BALB/c nude mice (obtained from the Frederick Cancer Research Facility, National Cancer Institute, Frederick, MD) were maintained in a laminar-airflow cabinet under pathogen-free conditions and used at 8–12 weeks of age. All facilities were approved by the American Association for Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the United States Department of Agriculture, the Department of Health and Human Services, and the NIH.

In Vivo Therapy of Established Human TCC Growing s.c. in Athymic Nude Mice. Cultured 253J B-V R cells (60–70% confluent) were prepared for injection as described previously (11, 12). Mice were anesthetized with methoxyflurane. Viable tumor cells (1 × 10^6/0.5 ml) in HBSS were implanted s.c. into the flank. Formation of a bulla indicated a satisfactory injection. Therapy began when the tumors reached a diameter of 5–7 mm (designated day 0).

Groups of mice were treated with intralesional Ad bFGF-AS (5 × 10^8 pfu/20 μl/mouse), Ad bFGF-S (5 × 10^8 pfu/20 μl/mouse), Ad-β-gal (5 × 10^8 pfu/20 μl/mouse), or PBS every 10 days for three treatments, based on the time course of gene expression seen following in vitro infection with sense and antisense constructs. Mice were necropsied 10 days after completion of gene therapy. The tumors were removed and weighed, and the presence of tumor was determined grossly and microscopically. The tumors were then quickly frozen in liquid nitrogen for mRNA extraction, fixed in 10% buffered formalin, or placed in OCT compound (Miles Laboratories, Elkhart, IN).
**In Situ** mRNA Hybridization Analysis. Specific antisense oligonucleotide DNA probes were designed complementary to the mRNA transcripts based on published reports of the cDNA sequence: bFGF (5′-CCG GAA GCC GCC GCT GCC GCC-3′), 85.7% guanosine-cytosine content (27); vascular endothelial growth factor/vascular permeability factor, (5′-TGG TGA TGG ACT CTT CAG TGG GCU-3′), 57.7% guanosine-cytosine content (28); IL-8 (5′-CTC CAC AAT CCT CTG CAC CC-3′), 66% guanosine-cytosine content (29); MMP-9 (5′-CCG GTC CTC GCT GCC GCT CCG GU-3′), 80.0% guanosine-cytosine content (30). The specificity of the oligonucleotide sequence was initially determined by a Gene Bank European Molecular Biology Library database search using the Genetics Computer Group sequence analysis program (GGC, Madison, WI) based on the FastA algorithm; these sequences showed 100% homology with the target gene and minimal homology with nonspecific mammalian gene sequences. The specificity of each of the sequences was also confirmed by Northern blot analysis (31). A poly(dT)20 oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample.

All DNA probes were synthesized with six biotin molecules (hyperbiotinylated) at the 3′ end via direct coupling, with the use of standard phosphoramidite chemistry (Research Genetics, Huntsville, AL). The lyophilized probes were reconstituted to a stock solution at 1 µg/µl in 10 mmol/L Tris (pH 7.6), 1 mmol/L EDTA. Immediately before use, the stock solution was diluted with probe dilution (Research Genetics).

**In situ** mRNA hybridization was performed as described previously with minor modifications (32, 33). ISH was performed using the Microprobe Manual Staining System (Fisher Scientific, Pittsburgh, PA; Ref. 34). Tissue sections (4 µm) of formalin-fixed, paraffin-embedded specimens were mounted on silane-treated ProbeOn slides (Fisher Scientific; Refs. 32, 33). The slides were placed in the Microprobe slide holder, dewaxed, and rehydrated with Autowaxer and Autoalcohol (Research Genetics), followed by enzymatic digestion with pepsin. Hybridization of the probe was performed for 45 min at 45°C, and the samples were then washed three times with 2× SSC for 2 min at 45°C. The samples were incubated with alkaline phosphatase-labeled avidin for 30 min at 45°C, rinsed in 50 mm Tris buffer (pH 7.6), rinsed with alkaline phosphatase enhancer for 1 min, and incubated with a chromogen substrate for 15 min at 45°C. If necessary, samples were incubated a second time with fresh chromogen substrate to enhance a weak reaction. A red stain indicated a positive reaction in this assay. Controls for endogenous alkaline phosphatase included treatment of the sample in the absence of the biotinylated probe and the use of chromogen alone.

**Quantification of Color Reaction.** Stained sections were examined using a Zeiss photomicroscope (Carl Zeiss, Thornwood, NY) equipped with a three-chip, charge-coupled device color camera (model DXC-969 MD; Sony Corp., Tokyo, Japan). The images were analyzed by Optimas image analysis software (version 4.10; Bothell, WA). The slides were pre-screened by one investigator to determine the range of staining intensity of the slides to be analyzed. Images covering the range of staining intensities were captured electronically, a color bar (montage) was created, and a threshold value was set in the red, green, and blue mode of the color camera. All subsequent images were quantified based on this threshold. The integrated absorbance of the selected fields was determined based on its equivalence to the mean log inverse gray value multiplied by the area of the field. The samples were not counterstained, so the absorbance was attributable solely to the product of the ISH reaction. Three different fields in each sample were quantified to derive an average value. The intensity of staining was determined by comparison with the integrated absorbance of poly(dT)20. The results were presented as a number of each cell line compared with the control, which was set at 100 (19).

**IHC.** For immunohistochemical analysis, frozen tissue sections (8 µm thick) were fixed with cold acetone. Tissue sections (5 µm thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. The slides were rinsed twice with PBS. Antigen retrieval was performed with pepsin for 12 min. Endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 12 min. The samples were then washed three times with PBS and incubated for 20 min at room temperature with a protein-blocking solution consisting of PBS (pH 7.5) containing 5% normal horse serum and 1% normal goat serum. Excess blocking solution was drained, and the samples were incubated for 18 h at 4°C with a 1:100 dilution of rat monoclonal anti-CD31 antibody (PharMingen, San Diego, CA; Ref. 32), a 1:50 dilution of a rabbit polyclonal anti-IL-8 antibody (Biosource International, Camarillo, CA), a 1:500 dilution of rabbit polyclonal anti-bFGF antibody (Sigma Chemical Co., St. Louis, MO), a 1:500 dilution of rabbit polyclonal anti-VEGF/VAPF antibody (Santa Cruz Biotech, Santa Cruz, CA), or a 1:100 dilution of mouse monoclonal anti-MMP-9 antibody (Oncogene Research Products, Cambridge, MA). The samples were then rinsed four times with PBS and incubated for 60 min at room temperature with the appropriate dilution of the secondary antibody-peroxidase-conjugated antirat IgG (H+L; Jackson ImmunoResearch Laboratory, Inc., West Grove, PA), antirabbit IgG F(ab)2 fragment (Jackson ImmunoResearch Laboratory, Inc.), or antimouse IgG1 (PharMingen). The slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics). The sections were then washed three times with PBS, counterstained with Gill’s hematoxylin (Biogenex Laboratories, San Ramon, CA), and washed three times with PBS. The slides were mounted using Universal mount (Research Genetics; Ref. 19).

**Quantification of Intensity of Immunostaining.** The intensity of immunostaining for bFGF, VEGF, IL-8, and MMP-9 was quantified in each sample by an image analyzer using the Optimas software program (Bioscan, Edmonds, WA). Three different areas in each sample were quantified to obtain an average measurement of the intensity of immunostaining, which was normalized to the intensity for adjacent epithelium, for which the value was arbitrarily set to 100 (19).

**Quantification of MVD.** MVD was determined by light microscopy after immunostaining of tumor sections with anti-CD31 antibodies (35) according to the procedure of Weidner et al. (36). Clusters of stained endothelial cells distinct from adjacent microvessels, tumor cells, or other stromal cells were counted as one microvessel. The tissue was recorded using a cooled CCD Optotronics Tec 470 camera (Optotronics Engi-
neering, Goletha, CA) linked to a computer and digital printer (Sony). The density of microvessels was expressed as the mean of the five highest areas identified within a single \( \times 200 \) field.

**TUNEL Assay.** For the TUNEL assay, tissue sections (5 \( \mu \text{m} \)) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. The slides were rinsed twice with distilled water with BRIJ and treated with 1:500 proteinase K (20 \( \mu \text{g/ml} \)) for 15 min; endogenous peroxidase was blocked by 3% hydrogen peroxide in PBS for 12 min. The samples were washed three times with distilled water with BRIJ and incubated for 10 min at room temperature with TDT buffer. Excess TDT buffer was drained, and the samples were incubated for 18 h at 4°C with terminal transferase and biotin-16-dUTP. The samples were then rinsed four times with Tris buffer and incubated for 5 min at 37°C with a 1:400 dilution of peroxidase-conjugated streptavidin. The slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics). The sections were then washed three times with PBS, counterstained with Gill’s hematoxylin (Biogenex Laboratories), and washed three times with PBS. The slides were mounted using a Universal mount (Research Genetics).

**Quantification of Apoptosis.** The results of the TUNEL immunoreactivity assay were quantified by light microscopy and recorded using a cooled CCD Optotronics Tec 470 camera linked to a computer and digital printer (Sony). The density of apoptotic cells was expressed as the mean of the five highest areas identified within a single \( \times 200 \) field.

**Immunofluorescence Double-Staining of Endothelial Cells and Apoptotic Cells.** For IHC analysis, frozen tissue sections (8 \( \mu \text{m} \) thick) were fixed with cold acetone. The samples were washed three times with PBS and incubated for 20 min at room temperature with a protein-blocking solution consisting PBS (pH 7.5), 5% normal horse serum, and 1% normal goat serum. Excess blocking solution was drained, and the samples were incubated for 18 h at 4°C with a 1:400 dilution of rat monoclonal anti-CD31 antibody (PharMingen). The samples were then rinsed four times with PBS and incubated for 60 min at room temperature with a 1:200 dilution of secondary goat anti-rat IgG conjugated to Texas Red (Jackson Immunoresearch Laboratory). The sections were then washed two times with PBS.

TUNEL was performed using a commercial kit according to the manufacturer’s protocol (Promega, Madison, WI). The tissue sections were fixed in 4% paraformaldehyde at room temperature for 15 min. The tissue sections were then permeabilized by incubation with 0.5% Triton X-100 in PBS for 5 min at room temperature. The slides were twice with PBS for 5 min and then incubated with equilibration buffer for 10 min. The equilibration buffer was removed, and reaction buffer containing equilibration buffer, a nucleotide mixture, and TDT enzyme was then added to the tissue sections, followed by incubation in a humid atmosphere at 37°C for 1 h in the dark. EDTA was added to the slides for 5 min to stop the reaction. To identify all cell nuclei, the slides were incubated with a 1:2000 dilution of Hoechst (300 \( \mu \text{g/ml} \)) at room temperature for 10 min. The slides were examined under an Olympus Inverted System IX70 microscope (Melville, NY). Images were captured using a digital camera. CD31-positive endothelial cells were detected by localized red fluorescence. Fluorescein-12-dUTP was incorporated at the 3’-OH ends of fragmented DNA, resulting in localized green fluorescence within the nucleus of apoptotic cells. All cell nuclei were detected by blue fluorescence. The number of apoptotic endothelial cells was expressed as the ratio of apoptotic endothelial cells to the total number of endothelial cells in 10 random 0.011-mm² fields at \( \times 400 \) magnification.

**Statistical Analysis.** The statistical differences in vessel counts and staining intensity for bFGF, VEGF, IL-8, and MMP-9 in bladder tumor samples were analyzed by the Mann-Whitney test. Incidences of tumor and metastasis were statistically analyzed using the \( \chi^2 \) test. \( P < 0.05 \) was considered significant.

**RESULTS**

**In Vitro Apoptosis.** The in vitro apoptotic effect of Ad bFGF-AS (0–10⁶ pfu/ml) on HUVECs was evaluated by flow cytometry with PI staining. Apoptosis was enhanced in HUVECs from 0.8% at an MOI of 1:1 to 13.6% at an MOI of 10:1. *Bars*, SE.

**In Vivo Growth Inhibition by Ad bFGF-AS.** We evaluated the therapeutic effect of Ad bFGF-AS therapy against 253J B-VR cells growing s.c. in athymic nude mice. Therapy with intrasplenal Ad bFGF-AS, Ad bFGF-S, Ad-β-gal, or PBS was begun when the tumors reached a diameter of 5–7 mm (designated day 0). The mice were treated every 10 days for three treatments, and 10 days after the final treatment (day 30) the tumors were harvested. Mice treated with Ad bFGF-AS had significantly lower tumor weights compared with all controls.
Antisense bFGF Gene Therapy for Bladder Cancer

CD31 immunostaining. The number of CD31 microvessels counted per 200 field was reduced from 72 ± 8 to 45 ± 8 in the tumors following therapy with Ad bFGF-AS (P < 0.005; Table 2 and Fig. 4). Mice treated with Ad bFGF-S had increased tumor-induced neovascularization. In these mice, the MVD within the tumors was 127 ± 24, which was significantly greater than for either the PBS or Ad-β-gal controls (P < 0.005; Table 2 and Fig. 4).

Enhancement of Apoptosis and Inhibition of Proliferation Following Therapy with Ad bFGF-AS. We next evaluated whether endothelial cell apoptosis occurred after therapy with Ad bFGF-AS. Endothelial cell apoptosis was evaluated using double-staining immunofluorescence with anti-CD31 and TUNEL (Fig. 6). In this technique, endothelial cells appear red and the nuclei of apoptotic tumor cells appear green, whereas the nuclei of apoptotic endothelial cells appear yellow. The number of apoptotic endothelial cells counted per ×200 field was calculated as the ratio of double-labeled endothelial cells to total endothelial cells. We observed a significant increase in apoptotic endothelial cells, from 0.0 ± 0.0, 0.9 ± 1.2, and 0.4 ± 0.8 in mice treated with PBS, Ad-β-gal, and Ad bFGF-S, respectively, to 46 ± 8.7 in mice treated with Ad bFGF-AS (P < 0.005).

DISCUSSION

Gene therapy strategies targeting bFGF expression have been explored as potential treatments for cardiovascular and malignant diseases (23, 24, 37–39). Preclinical studies with adenoviral vectors have demonstrated successful gene transfer, but these studies have been hampered by toxicity, transient gene expression, and immune responses directed against the adenoviral vector. These limitations can be overcome to a certain extent by directing therapy at specific receptors on the target cells with adenoviral vectors bound to that receptor’s ligand (24, 40) or by direct instillation into the target organ (39, 41, 42).

Bladder cancer is an ideal target for gene therapy because of the relative ease with which the vector can be delivered directly into the bladder, make contact with the tumor, and provide transgene expression in the target organ with minimal dissemination to other sites (43).

Table 1 bFGF gene therapy for IFN-resistant human bladder cancer cell line (253J B-V6) growing in the subcutis of athymic nude mice

<table>
<thead>
<tr>
<th>Tumor growth beyond day 0 (%)</th>
<th>Tumor weight, median (range), mg</th>
</tr>
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<tbody>
<tr>
<td>Control (PBS) (n = 6)</td>
<td>160  246  323  562 (64–1548)</td>
</tr>
<tr>
<td>Control (β-gal) (n = 6)</td>
<td>412  683  820  586 (138–1298)</td>
</tr>
<tr>
<td>Sense bFGF (n = 6)</td>
<td>419  814  1767 3012 (1638–3984)</td>
</tr>
<tr>
<td>Antisense bFGF (n = 7)</td>
<td>81  111  121  58 (0–540)</td>
</tr>
</tbody>
</table>

a Growth on day 0 defined as 100%.
b,c Compared with controls (Mann-Whitney statistical comparison): bP < 0.01; cP < 0.05.
We previously established an orthotopic model of human TCC by directly implanting the human TCC line 253J into the bladder wall of athymic nude mice (11). We were able to select subpopulations from the parental cell line with enhanced tumorigenicity and metastasis. These highly vascular tumors overexpress the angiogenic factors bFGF, VEGF, IL-8, and MMP-9 and provide a relevant model to study the biology of human TCC (11, 44) and to evaluate novel antiangiogenic therapy for

### Table 2 In vivo mRNA and protein expression and MVD following therapy with Ad bFGF-AS

<table>
<thead>
<tr>
<th>mRNA expression index</th>
<th>Protein expression index</th>
<th>MVD (per ×200 field)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>bFGF</td>
<td>VEGF</td>
</tr>
<tr>
<td>Control (PBS)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Control (β-gal)</td>
<td>100</td>
<td>104</td>
</tr>
<tr>
<td>Sense bFGF</td>
<td>148d</td>
<td>93</td>
</tr>
<tr>
<td>Antisense bFGF</td>
<td>58d</td>
<td>90</td>
</tr>
</tbody>
</table>

*a The intensity of the cytoplasmic color reaction was quantitated by an image analyzer and compared with the intensity of poly(dT) color reaction in each sample. The results are presented as the number of each group compared with control (PBS), defined as 100.

*b The intensity of the cytoplasmic immunostaining was quantitated by an image analyzer in five different areas of each sample to yield an average measurement. The results were presented as the number of each group compared with control (PBS), defined as 100.

*c MVD was expressed as an average number of five highest areas identified within a single ×200 field.

*d P < 0.005 vs. control (PBS) and control (β-gal), using the Mann-Whitney statistical comparison.
human TCC. We previously reported that treatment of 253J-B-V with IFN-α (14) or Mab C225 (22) inhibited bladder tumor-induced angiogenesis, tumorigenicity, and metastasis. Although the expression of bFGF was down-regulated by both therapies, these studies did not provide direct evidence for the role of bFGF in tumor growth or angiogenesis.

Recently, Wood et al. (25) demonstrated that bFGF expression directly regulated the tumorigenicity and metastasis of human TCC. They transduced the highly metastatic TCC cell line 253J-B-V with Ad bFGF-AS in vitro and implanted these cells into the bladders of athymic nude mice. The production of bFGF and MMP-9 by the transduced tumor cells was down-regulated, and tumorigenicity, tumor-induced angiogenesis (MVD), bFGF, and MMP-9 expression, and metastasis were reduced after implantation into the bladder of these mice. These experiments established the rationale for antisense bFGF gene therapy for human TCC. In the present study, we demonstrated the feasibility and efficacy of adenoviral-mediated antisense gene therapy targeting bFGF expression by tumor and endothelial cells. Our results are similar to those reported by Wang and Becker (23), who successfully treated human melanoma xenografts with liposome-mediated antisense bFGF or bFGF receptor-1 gene therapy. These therapies down-regulated the expression of bFGF and MMP-9 and inhibited tumor-induced angiogenesis. The MMPs have been implicated in angiogenesis.

Both synthetic and endogenous MMP inhibitors inhibit angiogenic responses in vitro and in vivo (45). The highly metastatic 253J-BV cells express high levels of MMP-9, which facilitate invasion of both tumor and endothelial cells through the extracellular matrix. MMP-9 is regulated by various factors, including tumor necrosis factor-α (46), IL-1 (47), transforming growth factor-β (47, 48), epidermal growth factor (47, 49, 50), hepatocyte growth factor (50), and bFGF (16).

One of the limitations of therapeutically targeting specific angiogenic factors is the redundancy that exists for the expression of these factors by tumors (5). For example, the 253J-B-V TCC cell line overexpresses VEGF and IL-8 in addition to bFGF. Therapy targeting bFGF production by tumor cells could significantly inhibit bFGF expression without inhibiting angiogenesis or tumor growth because the production of VEGF and IL-8 by the tumor cells could maintain the vasculature in an activated state. However, if this therapy also targeted the tumor endothelium, it could potentially eradicate tumor growth (19). Under normal circumstances, the microvasculature is maintained in a quiescent state (3). The switch to an angiogenic phenotype is a dynamic process accomplished through an integrated process involving basement membrane degradation, endothelial cell proliferation and migration, and capillary tubule formation. Endothelial cells respond to secreted angiogenic factors from

Fig. 5 Tumors were analyzed for bFGF-, VEGF-, IL-8-, and MMP-9-specific mRNA production by ISH. mRNA expression of bFGF and MMP-9 was decreased in tumors treated with antisense bFGF, whereas it was increased in tumors treated with sense bFGF relative to tumors treated with either PBS or β-gal. Tumors treated with either sense or antisense bFGF showed the same level of mRNA expression of VEGF or IL-8 compared with controls.
the tumor or host cells (e.g., macrophages), to factors released from the extracellular matrix, or alternatively to endogenous expression and production of these factors (1–3). After induction of vascularization, the rate of tumor growth increases exponentially (3). Kumar et al. (51) demonstrated that bFGF protein directly induces the proliferation and migration of endothelial cells in vitro. Takahashi et al. (52) observed that bFGF expression by HUVECs increased after infection with an adenovirus containing the sense bFGF construct. Infection with this virus increased endothelial cell proliferation and tubular formation on reconstituted basement membrane. Conversely, in vitro infection of HUVECs with Ad bFGF-AS enhanced apoptosis in the endothelial cells. Using double-labeled immunofluorescence, we observed in vivo endothelial cell apoptosis following therapy with Ad bFGF-AS, although VEGF and IL-8 expression levels were

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**Fig. 6** Proliferation and apoptosis of cancer cells was analyzed by IHC with anti-PCNA antibody and TUNEL assay. The number of PCNA-positive cancer cells counted per ×200 field was significantly decreased, from 146 ± 22 and 127 ± 18 in PBS- and β-gal-treated animals, respectively, to 41 ± 33 after therapy with Ad bFGF-AS (P < 0.005). Ad bFGF-S significantly enhanced proliferation (173 ± 29; P < 0.05) compared with controls. The number of apoptotic cancer cells counted per ×200 field was significantly increased, from 5 ± 3 and 11 ± 2 in PBS and β-gal controls, respectively, to 19 ± 5 after therapy with Ad bFGF-AS (P < 0.005). Endothelial cell apoptosis was analyzed by double-staining immunofluorescence with anti-CD31 and TUNEL. Endothelial cells are detected by localized red fluorescence, whereas green fluorescence is detected within the nuclei of apoptotic cells. Double-labeling of endothelial cells undergoing apoptosis results in localized yellow fluorescence. The number of apoptotic endothelial cells counted per ×400 field was calculated as the ratio of double-labeled endothelial cells to total endothelial cells. We observed a significant increase in apoptotic endothelial cells, from 0.0 ± 0.0 and 0.9 ± 1.2 in PBS and β-gal controls, respectively, to 46.8 ± 8.7 after therapy with Ad bFGF-AS (P < 0.005).
unaffected by treatment. Collectively, these studies indicate the importance of endogenous bFGF production for endothelial cell proliferation, morphogenesis, and survival.

In summary, this study supports the concept that gene therapy targeting angiogenesis is a potential strategy for cancer therapy. Ad-bFGF-AS successfully inhibited the growth of established human TCC xenografts by down-regulating the expression of bFGF and MMP-9 by the tumor cells, resulting in decreased tumor cell proliferation and enhanced apoptosis in tumor and endothelial cells. By targeting endothelial cells in addition to tumor cells, this therapy was not inhibited by the redundancy of angiogenic factor expression because treatment was effective despite the local production of IL-8 and VEGF. Although the ectopic model used in these studies is not entirely relevant for human gene therapy, these studies validate bFGF as a target for novel therapeutic strategies for bladder cancer. At present, we are developing a more relevant orthotopic model for human TCC, which we will use to develop intravesical gene therapy.

REFERENCES

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### Table 3 In vivo apoptosis/PCNA ratio of 253J B-V* following therapy with Ad bFGF-AS

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Apoptosis index* (per ×200 field)</th>
<th>PCNA index* (per ×200 field)</th>
<th>Apoptosis/PCNA ratio</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>CTRL (PBS)</td>
<td>5 ± 3</td>
<td>2–9</td>
<td>146 ± 22</td>
</tr>
<tr>
<td>Control (β-gal)</td>
<td>11 ± 2</td>
<td>8–13</td>
<td>127 ± 18</td>
</tr>
<tr>
<td>Sense bFGF</td>
<td>9 ± 3</td>
<td>5–12</td>
<td>173 ± 29</td>
</tr>
<tr>
<td>Antisense bFGF</td>
<td>19 ± 5*</td>
<td>13–25</td>
<td>41 ± 33*</td>
</tr>
</tbody>
</table>

* The density of apoptosis on cancer cells by TUNEL assay and cell proliferation by IHC with PCNA antibody was expressed as an average number of five highest area identified within a single ×200 field.

** Apoptosis/PCNA ratio: mean percentage of the number of apoptotic cells divided by the number of PCNA-positive cells.

\*P < 0.005 vs. any other groups (Mann-Whitney statistical comparison).
Gene Therapy of Human Bladder Cancer with Adenovirus-mediated Antisense Basic Fibroblast Growth Factor

Keiji Inoue, Paul Perrotte, Christopher G. Wood, et al.


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