Inhibition of Tumorigenicity and Metastasis of Human Bladder Cancer Growing in Athymic Mice by Interferon-β Gene Therapy Results Partially from Various Antiangiogenic Effects Including Endothelial Cell Apoptosis

Jonathan I. Izawa, Paul Sweeney, Paul Perrotte, Daniel Kedar, Zhongyun Dong, Joel W. Slaton, Takashi Karashima, Keji Inoue, William F. Benedict, and Colin P. N. Dinney


ABSTRACT

We determined whether the IFN-β gene could suppress angiogenesis, tumor growth, and metastasis of human bladder transitional cell carcinoma. The highly tumorigenic and metastatic 253J B-VR human bladder transitional cell carcinoma (TCC) cell line (resistant to the antiproliferative effects of IFN-β) was infected in vitro with adenoviral β-galactosidase (Ad-LacZ), murine adenoviral IFN-β (Ad-mIFN-β), or human adenoviral IFN-β (Ad-hIFN-β) and implanted into the bladders of athymic nude mice. Ad-mIFN-β and Ad-hIFN-β were used because of the species specificity of IFN-β. The transient production of mIFN-β and hIFN-β from the infected 253JB-VR tumor cells significantly inhibited tumorigenicity and spontaneous lymph node metastasis. Subsequently, the 253J B-VR cells were implanted into the subcutis of athymic nude mice, and established tumors were treated by direct intratumoral injection with Ad-mIFN-β, Ad-hIFN-β, Ad-LacZ, or PBS. By in situ hybridization (ISH) and immunohistochemical analysis (IHC), expression of hIFN-β and mIFN-β mRNA and protein within the tumors was demonstrated after Ad-hIFN-β and Ad-mIFN-β gene therapy, respectively. The therapy also induced necrosis in both the Ad-mIFN-β- and Ad-hIFN-β-treated tumors. IHC revealed decreased tumor cell proliferation and the sequestration of activated macrophages within the tumors after Ad-mIFN-β therapy. In addition, the expression of the proangiogenic factors bFGF, and MMP-9 protein (by IHC) was significantly down-regulated by Ad-hIFN-β gene therapy. Double-immunofluorescent IHC for terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) and CD-31 demonstrated tumor and endothelial cell apoptosis in those tumors treated with Ad-hIFN-β gene therapy. Tumor-induced angiogenesis, as determined by the microvessel density, was decreased in tumors treated with both Ad-mIFN-β and Ad-hIFN-β. These data suggest that the inhibition of tumorigenicity and the metastasis of the 253J B-VR cells after infection with Ad-IFN-β is caused by the inhibition of angiogenesis and the activation of host effector cells.

INTRODUCTION

TCC is the fifth most common solid malignancy in the United States. Over 52,000 cases will be diagnosed and more than 12,000 deaths will be secondary to this cancer in the year 2000 (1). Seventy to 80% of patients with bladder cancer present with low-grade, noninvasive tumors confined to mucosa, with these cases initially managed by endoscopic resection. Superficial tumors recur in 60–70% of patients, and about 30% of these tumors progress to a higher grade or stage (2). The high recurrence rate and the unpredictability of the progression patterns have led to the widespread use of various intravesical therapies in an attempt to qualitatively change the ongoing urothelial...

Received 9/18/01; revised 12/28/01; accepted 1/2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported in part by NIH Core Grant CA-16672, CA71861-01 (to C. P. N. D.), NIH SPORE Grant 1P50 CA91846 tobacco settlement funds as appropriated by the Texas State Legislature (to W. F. B.), American Cancer Society Grant RPG-98-332-01 (to Z. D.), the Detweiler Traveling Fellowship from the Royal College of Physicians and Surgeons of Canada (to J. I. I.), and the Royal College of Surgeons in Ireland Postgraduate Travelling Fellowship and Trinity College Dublin Postgraduate Travelling Scholarship (to P. S.).

2 To whom requests for reprints should be addressed, at Department of Urology, Box 446, the University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-3250; Fax: (713) 794-4824; E-mail: cdinney@mdanderson.org.

3 The abbreviations used are: TCC, transitional cell carcinoma of the bladder; Ad-IFN-β, adenoviral vector carrying IFN-β; Ad-hIFN-β, human adenoviral IFN-β; Ad-mIFN-β, murine adenoviral IFN-β; Ad-LacZ, adenoviral β-galactosidase; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; MMP-9, matrix metalloproteinase-9; mIFN-β, murine IFN-β; hIFN-β, human IFN-β; IL-8, interleukin-8; NK, natural killer; CMV, cytomegalovirus; MOI, multiplicity/multiplicities of infection; IHC, immunohistochemical analysis; PCNA, proliferative cell nuclear antigen; Tdt, terminal deoxynucleotidyl transferase; TUNEL, Tdt-mediated dUTP-biotin nick-end labeling; ISH, in situ hybridization; MLEC, murine lung endothelial cell, HMEC, human microvascular endothelial cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pfu, plaque-forming unit(s); poly(dT), polydeoxythymidylic acid; iNOS, inducible nitric oxide synthase.
carcinogenesis (3). Intravesical therapy is an attractive option because relatively high doses of therapeutic agents can be delivered, establishing direct contact with the bladder tumor, with minimal systemic side effects. Intravesical chemotherapy, or more successfully intravesical Bacille Calmette-Guérin, often can prolong the progression-free interval after initial endoscopic resection. However, up to 50% of these patients will have disease progression despite Bacille Calmette-Guérin, and up to 30% of patients with recurrent superficial TCC will die of metastatic bladder cancer within 15 years (3). The standard therapy for patients who progress to invasive TCC is radical cystectomy. Unfortunately, disease recurs in up to 50% of these patients despite surgery (4).

The growing awareness of the critical role of angiogenesis in tumor growth and metastasis has prompted a major effort to develop antiangiogenic therapies. Preclinical studies indicate that gene transfer strategies are a means to apply antiangiogenic therapy against solid tumors (5–7). TCC appears to be an ideal target to evaluate gene therapy because of the relative ease with which the vector can be delivered directly into the bladder (8). The profound bystander effect induced by Ad-IFN-β makes this vector and cytokine an attractive choice for intravesical gene therapy (9).

The IFNs are a family of regulatory natural glycoproteins that regulate cell growth and differentiation; inhibit the expression of oncogenes; up-regulate apoptosis; and activate lymphocytes, NK cells, and macrophages (10–13). The continuous exposure of various human cancer cells to noncytostatic concentrations of human IFN down-regulated the expression of bFGF (8, 14–16), IL-8 (17, 18), and MMP-9 (19–21). Furthermore, chronic administration of IFNs can inhibit the in vivo expression of bFGF, VEGF, and MMP-9 by human TCC, with subsequent inhibition of tumor-induced neovascularization and tumor growth (22). Although IFNs have been clinically effective against certain hematopoietic neoplasms (23, 24), and although chronic systemic administration of low-dose IFN has produced regressions of such vascular tumors as pulmonary hemangiomas (25), infantile hemangiomas (26), Kaposi’s sarcoma (27), and malignant hemangiopericytoma (28), IFNs have not been clinically effective against most solid neoplasms (25). Chronic systemic administration of low-dose IFN has produced regressions of such vascular tumors as pulmonary hemangiomas (25), infantile hemangiomas (26), Kaposi’s sarcoma (27), and malignant hemangiopericytoma (28), IFNs have not been clinically effective against most solid neoplasms (25–28). Pharmacokinetic studies have demonstrated that the half-life of IFNs in the circulation of patients is in the order of minutes (33–35). The resulting lack of sustained IFN levels may be responsible for the failure of IFN to inhibit tumor growth, in part, by its antiangiogenic effect.

The poor outcome of many patients with superficial TCC is a sobering testimony to the double-edged sword of attempted therapy. Because IFN-β appears to be more stable than IFN-α in an acidic environment, we focused our study on IFN-β gene therapy (37–39). Because of the species specificity of IFN-β, we used both mIFN-β and hIFN-β to investigate the host response as well as the direct effects of therapy on the human tumor cells. We found that the orthotopic growth and metastatic potential of 253J-B-V R cells engineered to transiently and constitutively produce either hIFN-β or mIFN-β was suppressed, and that the direct intratumoral delivery of IFN-β gene therapy inhibited tumor growth by activating tumor-specific host effector cells with mIFN-β and by suppressing tumor-induced angiogenesis with hIFN-β.

**MATERIALS AND METHODS**

**Mice.** Male athymic BALB/c nude mice (age, 6 to 8 weeks) were obtained from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). All of the mice were maintained in a laminar air-flow cabinet under pathogen-free conditions and used at 10–12 weeks of age. Mice were housed in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, the Department of Health and Human Services, and NIH.

**Tumor Cell Culture.** 253J-B-V R human TCC tumor cells were maintained as a monolayer culture in MEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, sodium pyruvate, non-essential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, and vitamins (CMEM). MLECs were immortalized by SV40 transformation and maintained in CMEM. HMECs (Cascade Biologicals, Portland, Or) were maintained in medium 131 with microvascular growth supplements (Cascade Biologicals). The 253J B-V R cells were harvested by a 1-min treatment with 0.025% trypsin-0.01% EDTA solution. The culture flask was tapped to detach the cells. The cells were washed in CMEM and resuspended in HBSS in preparation for implantation into the mice.

**Production of Adenoviral Vectors.** The full coding region of the mIFN-β cDNA (provided by Dr. T. Taniguchi, University of Osaka, Osaka, Japan) was subcloned into the plasmid pxCMV to derive the shuttle vector pECIFN-β. The shuttle vector and plasmid pJM17 were cotransfected into 293 cells (American Type Culture Collection) by liposome-mediated transfection with Lipofectin (Life Technologies, Inc.) to generate the replication-deficient adenoviral vector AdCMVmIFN-β. Dr. W. Zhang (Baxter Healthcare Co., Round Lake, IL) provided the plasmids pxCMV, pJM17, and Ads-LacZ (a recombinant adenovirus encoding the Escherichia coli lacZ gene was used as a control). The human CMV promoter drives both the lacZ and IFN genes. AdCMVmIFN-β (Ad-mIFN-β) was identified by PCR and infection of cells. The sequences of the PCR primers were 5’-CTTTGTTCCTATCAGGCGG-3’ and 5’-CCACAACTGATAGCAGTGA-3’, which are located outside the two ends of the insert on the shuttle vector pECIFN-β. One
clone of Ad-mIFN-β was subcloned three times, resulting in a clone of wild-type virus-free Ad-mIFN-β. The presence of wild-type adenovirus was identified by plaque assay on HeLa cells at 10 MOI and by PCR using primers specific for the E1a region (U: 5′-TGA GAC ATA TTA TCT GCC ACG-3′ and L: 5′-CCT CTT CAT CCT CGT CAC-3′) that were deleted in the recombinant adenovirus. Only preparations that did not contain wild-type virus were used.

Ad-LacZ, and Ad-mIFN-β were propagated in 293 cells grown in CMEM. The viruses were released by three freeze/thaw cycles and purified by a two-step CsCl gradient centrifugation. After dialysis at 4°C against 10 mM Tris/HC1 (pH 7.5), 1 mM MgCl2, and 10% glycerol, aliquots of the vectors were stored at −80°C. The titers of the vectors were assessed on 293 cells by plaque assay and were 5 × 2 × 1010 pfu/ml for most preparations. Ad-hIFN-β (provided by Dr. Arbans K. Sandhu, University of Pennsylvania, Philadelphia, PA) was propagated in the same manner.

Susceptibility of 253J B-VR Cells to Adenoviral Infection. The 253J B-VR cells were trypsinized and harvested, and 1 × 105 cells were plated in each well of a 24-well polystyrene culture plate. After overnight incubation, the 253J B-VR cells were infected with Ad-mIFN-β, Ad-hIFN-β, or Ad-LacZ as described previously (40). Staining for β-galactosidase protein, which indicated successful infection with Ad-LacZ, was performed by aspirating the medium, then rinsing gently with cold PBS using 1 ml/well. The cells were overlayed with a fixative containing 0.5% glutaraldehyde in PBS. They were then incubated at 4°C for 5 min. The fixative was then rinsed gently twice with PBS at room temperature. After the PBS was rinsed off, the cells were exposed to the β-galactosidase stain, as described previously (40). A MOI of 10:1 was shown to be optimal for both Ad-mIFN-β and Ad-LacZ, demonstrating minimal cytotoxicity and maximal effect on IFN-β production. The optimal MOI for the Ad-hIFN-β was 5:1. No increase in cytotoxicity was seen when the MOI was increased to 10:1.

ELISA Analysis. The 253J B-VR cells were trypsinized and harvested, and 1 × 106 cells were plated in six-well polystyrene culture plates. After overnight incubation, the 253B-VR cells were infected with 0, 5, 10, 20, 25, 30, and 40 MOI of Ad-hIFN-β. Ad-LacZ was used as a control using the same titers. After incubation for 24, 48, 72, 96, and 120 h, the cell supernatant was collected and analyzed by ELISA (Fujirebio Inc., Tokyo, Japan) for accumulated hIFN-β protein concentration.

ELISA for Angiogenic and Metastatic Factor Expression. 253J B-VR, MLEC, and HMEC cells were plated in six-well plates. Each cell type was infected with Ad-LacZ, Ad-mIFN-β, and Ad-hIFN-β at a MOI of 10:1. Cell supernatants were collected 24, 48, and 72 h after viral infection and analyzed by ELISA (Quantikine immunoassay kits; R&D Systems, Minneapolis, MN) for expression of bFGF, IL-8, and VEGF protein. Cell numbers in each group were determined by MTT assay.

RNA Isolation and Northern Blot Analysis. The 253J B-VR cells were trypsinized and harvested, and 1 × 107 cells were placed in 50-mm polystyrene culture plates and incubated overnight. Ad-hIFN-β at 10:1 MOI was used to infect the cells. Ad-LacZ was used as a control at 10 MOI. The mRNA was extracted from these cells at 24, 48, and 72 h using the Fast-Track kit (Invitrogen, San Diego, CA). For Northern blot analysis, 1 μg of mRNA was fractionated on 1% denaturing formaldehyde/agarose gels, capillary-transferred to GeneScreen nylon membrane (DuPont Co., Boston, MA), and UV cross-linked with 120,000 μJ/cm2 using a UV Stratlinker 1800 (Stratagene). Hybridization using cDNA probes was performed as described previously (16). The DNA probes used were cDNA fragments corresponding to hIFN-β, bFGF, and MMP-9. Rat glyceraldehyde-3-phosphate dehydrogenase cDNA fragments were used as a loading control.

Growth-inhibitory Effects of Adenoviral Vectors on 253J B-VR Cells. The 253J B-VR tumor cells were plated at 5 × 103 cells/well in a 96-well plate. Cells were treated with Ad-hIFN-β and Ad-LacZ, both at a 10:1 MOI, and with PBS using equal volumes. After incubation for 1–5 days, the cells were treated with 50 μl of 1% MTT solution and incubated for 2 h. After soaking of the MTT solution, 100 μl of 10% DMSO was added to each well; and after the colorimetric reaction had occurred, the absorbance of the plates was read on the spectrophotometer at 450 μm. There was insignificant growth inhibition of the 253J B-VR cells when exposed to either Ad-mIFN-β or Ad-hIFN-β compared with Ad-LacZ (data not shown).

In Vitro Infection of 253J B-VR Cells for Orthotopic Implantation. The 253J B-VR cells were trypsinized and harvested, and subsequently 1 × 107 cells were plated in 50-mm culture flasks. After a 12-h incubation, the tumor cells were infected using Ad-hIFN-β, Ad-mIFN-β, and Ad-LacZ at a MOI of 10:1. After a 12 h incubation, the cells (253J B-VR-mIFN-β, 253J B-VR-hIFN-β, and 253J B-VR-LacZ) were trypsinized and harvested in preparation for orthotopic implantation.

Orthotopic Implantation of 253J B-VR and in Vivo-infected 253J B-VR Cells. Each mouse was anesthetized with sodium pentobarbital (25 mg/kg) i.p. and placed in the supine position. A lower midline incision was performed, and the bladder was exposed. The 253J B-VR, 253J B-VR-mIFN-β, 253J B-VR-hIFN-β, and 253J B-VR-LacZ cells (1 × 106 cells in 50 μl of HBSS) were implanted into the wall of the bladder in the area of the bladder dome using 30-gauge needles on disposable 1-ml syringes. A successful implantation was indicated by a bleb in the bladder wall serosa. The abdominal wound was closed in one layer with metal wound clips (Autoclips; Clay Adams, Parsippany, NJ). Twenty-eight days later, the mice were killed by cervical dislocation after anesthesia with sodium pentobarbital, and the bladders and lymph nodes were excised and placed into formalin or OCT compound (Miles Laboratories, Elkhort, IN).

Heterotopic Implantation of 253J B-VR Cells and in Vivo Therapy. Mice were anesthetized with sodium pentobarbital (25 mg/kg) i.p. The 253J B-VR cells (1 × 108 cells in 200 μl of HBSS) were subsequently injected s.c. into each mouse’s right flank using 30-gauge needles on disposable 1-ml syringes. A successful injection was indicated by a raised skin bleb. Once the tumors reached 5 mm in diameter, they were intratumorally treated weekly for 3 weeks with Ad-mIFN-β, Ad-hIFN-β, and Ad-LacZ using 1.0 × 109 pfu/injection or with PBS. Assuming tumor cell number is 1 × 109 in a 1 cm3 tumor, we estimated tumor cell number to be 1.25 × 106 in a 5 × 5 × 5-mm tumor, also assuming that this volume would approximate our tumors.
when measured at a 5-mm diameter at the skin surface. A 30-gauge needle on a disposable 1-ml syringe was used to instill 200 µl per treatment. Tumor diameters were measured weekly with calipers. Mice in all of the treatment groups maintained stable body weight throughout the entire treatment, without any obvious toxic effects. Mice were killed by using sodium pentobarbital (25 mg/kg) i.p. and subsequent cervical dislocation. The s.c. tumors were excised and weighed. The tumor samples were analyzed by H&E staining, IHC, and ISH.

IHC. Frozen tissue sections were used by placing harvested tumors in OCT compound and snap-frozen in liquid nitrogen. Frozen sections (8–10 µm) were subsequently placed on Superfrost-plus slides (Fisher Scientific) and fixed in cold acetone. Paraffin-embedded tissue sections were obtained by placing harvested tumors in 10% formalin with subsequent paraffin embedding after 48 h. Paraffin sections (3–5 µm) were subsequently placed on Superfrost-plus slides (Fisher Scientific) and were deparaffinized in xylene, rehydrated in graded ethanol, and transferred to PBS. The slides were rinsed twice with PBS, and endogenous peroxidase was blocked using 3% hydrogen peroxide in PBS for 12 min. The tissues were 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. Frozen tissue sections were incubated for 18 h at 4°C with a 1:100 dilution of a monoclonal rat anti-CD31 antibody (Phar-Mingen, San Diego, CA) or a 1:70 dilution of rat antiscavenger macrophage receptor antibody (Serotec, Raleigh, NC). Paraffin-embedded tissue samples were incubated for 18 h at 4°C with a 1:200 dilution of rabbit-polyclonal anti-bFGF antibody (Sigma Chemical Co., St. Louis, MO), a 1:750 dilution of rabbit polyclonal anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA), a 1:50 dilution of rabbit polyclonal anti-IL-8 antibody (Biosource International, Camarillo, CA), a 1:50 dilution of rabbit polyclonal anti-MMP-9 antibody (Calbiochem, La Jolla, CA), a 1:400 dilution of rabbit anti-hIFN-β antibody (Lee Biomolecular Research Laboratories, San Diego, CA), a 1:400 dilution of rabbit anti-mIFN-β antibody, a 1:50 dilution of monoclonal mouse anti-PCNA antibody (Dako, Carpinteria, CA), or a 1:200 dilution of rabbit polyclonal anti-iNOS antibody (BD Transduction Laboratories, Chino, CA).

The samples were rinsed four times with PBS and incubated for 60 min at room temperature with the appropriate dilution of peroxidase-conjugated antirabbit IgG or antirat IgG. The slides were rinsed with PBS and incubated for 5 min with dianamobenzidine (Research Genetics). The sections were then washed three times with distilled water. A positive reaction was indicated by a brown staining. The stained sections were examined in a Zeiss times with distilled water. A positive reaction was indicated by a brown staining. The stained sections were examined in a Zeiss microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a three-chip charge-coupled device color camera (model DXC-960 MD; Sony Corp., Tokyo, Japan). The intensity of the staining was quantified in six different areas of tumor samples for bFGF, VEGF, IL-8, MMP-9, hIFN-β, mIFN-β, and iNOS by an image analyzer using the Optimas software program (version 5.2; Media Cybernetics, Carlsbad, CA) to yield an average measurement (22). Images covering a 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 of the subsequent images were quantified based on this threshold. The integrated absorbance of the selected fields was determined based on the equivalence to the mean log inverse gray-scale value multiplied by the area of the field. The samples were not counterstained for this analysis; therefore, absorbance was attributable solely to the product of the IHC reaction. For PCNA, densities of proliferative cells were expressed as the average of the five highest areas identified within a single ×200 field. The area of highest neovasularity on the slides analyzed for CD31 was identified under low power (×40). Individual microvessels were quantified according to the procedure described by Weidner et al. (41) The image was projected and recorded by digitizing the image in a cooled charge-coupled device Optotronics Tec 470 camera (Optotronics Engineering, Goleta, CA) linked to a computer and a digital printer (Sony Corporation). The results were expressed as the highest number of microvessels identified within a single ×200 field. After image analysis, the slides were counterstained with Gill’s hematoxylin (Biogenex Laboratories, San Ramon, CA).

TUNEL Assay. Cell death in tumors was determined by the TUNEL method (42). Paraffin-embedded tissue sections (5 µm thick) were deparaffinized and rehydrated as above. The slides were rinsed twice in distilled water with BRJ1 (DW/BRIJ) and treated with proteinase K (1:500, 20 µg/ml) for 15 min; endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 12 min. The tissue sections were then washed three times with DW/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 rinsed four times with TB buffer (0.03 m sodium chloride, 0.03 m sodium citrate) and incubated for 30 min at 37°C in a 1:400 dilution of peroxidase-conjugated streptavidin. The slides were rinsed with PBS and incubated for 5 min with dianamobenzidine (Research Genetics). The sections were washed three times with PBS, counterstained with Gill’s hematoxylin, and again washed three times with PBS. For TUNEL, densities of apoptotic cells were expressed as the average of the five highest areas of greatest intensity identified within a single ×200 field.

ISH. A specific antisense oligonucleotide cDNA probe, complementary to the mRNA transcripts of hIFN-β identified on the basis of published reports of the cDNA sequence, was designed as described previously (43). The cDNA probe was synthesized with six biotin molecules at the 3’ end via direct coupling using standard phosphoramidine chemistry as described previously (Research Genetics; 44, 45). The lyophilized probes were reconstituted to a 1-µg/µl stock solution in Brigati probe diluent (Research Genetics).

ISH was performed as described previously (46). The Microprobe manual staining system (Fisher Scientific) was used to stain tissue sections (4 µm) of formalin-fixed, paraffin-embedded tumor specimens mounted on ProbeOn slides (Fisher Scientific). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics), followed by enzymatic digestion with pepsin. Hybridization of the probe was performed for 90 min at 45°C, and the samples were then washed three times with 2× SSC (0.15 m NaCl and 0.015 m sodium citrate) for 2 min at 45°C. The samples were incubated with alkaline phosphatase-linked enhancer (Biomedta Corp., Foster City, CA) for 1 min at 45°C and then incubated at 45°C for 40 min with chromagen...
Inhibition of Tumorigenicity and Metastasis of 253J B-V<sup>R</sup>

In vitro infection of the 253J B-V<sup>R</sup> cells with Ad-LacZ or Ad-mIFN-β indicated the presence of β-galactosidase and demonstrated the susceptibility of these cells to adenoviral infection. With increasing titers of Ad-LacZ, more cells underwent cell death—observed detachment from the polystyrene plates or clumping of nuclei. The optimal MOI for Ad-LacZ was 10:1. At these MOI, minimal toxicity of the adenovirus was observed. Areas of the cells within the culture plates were photographed at ×200. Results shown represent one of 2 experiments.

Production of hIFN-β after in Vitro Infection. The optimal MOI for infection of the 253J B-V<sup>R</sup> cells with Ad-LacZ or Ad-mIFN-β was 10:1 (Fig. 1), whereas it was 5:1 for Ad-hIFN-β. At this MOI, minimal toxicity of the adenovirus was observed (<10%; data not shown). After in vitro infection, the hIFN-β protein expression within the culture supernatant was measured by ELISA, and detectable levels of hIFN-β were present with infection at a MOI ranging from 5 to 40 (Fig. 2). At the MOI of 5:1, optimal hIFN-β protein production was determined because minimal toxicity was observed at this titer in the susceptibility experiments. The level expression was seen at 72 h and persisted for at least 7 days. Untreated and Ad-LacZ-treated 253 J B-V<sup>R</sup> cells were used as controls. Data shown are one representative experiment of two; bars, range.

**RESULTS**

The significance of the differences in the in vivo data were evaluated by the Mann-Whitney U test (47).

**Statistical Analysis.** The significance of the differences in the in vivo data were evaluated by the Mann-Whitney U test (47).
There was a significant difference between the tumor weights of lesions treated with intralesional PBS, Ad-LacZ, or Ad-hIFN-β commenced and continued weekly for 3 weeks. The weekly tumor diameters of lesions treated with intralesional PBS, Ad-LacZ, or Ad-hIFN-β are shown in Figs. 4 and 5. Over the course of therapy, the tumors treated with Ad-hIFN-β regressed in size, whereas those treated with Ad-mIFN-β remained static. At the completion of therapy, the median tumor weight of the Ad-mIFN-β treated mice was significantly less than that of tumors treated with PBS or Ad-LacZ (P < 0.001; Table 2). There was no significant difference in median tumor weight between the mice treated with Ad-LacZ and PBS control groups (P = 0.10), although those treated with Ad-LacZ were
Inhibition of Tumorigenicity and Metastasis of 253J B-V R

This corresponded with the presence of extensive tumor necrosis shown on H&E staining (Fig. 6). The degree of proliferation within the tumors treated with Ad-mIFN-β was less than that of tumors treated with PBS (P = 0.011), demonstrating the nonspecific antitumor effect of adenoviral infection, which was independent of IFN-β gene expression.

Analysis of the in Vitro and in Vivo Effects on Various Markers with Ad-mIFN-β Therapy. By IHC, strong expression of mIFN-β was demonstrated within the tumors treated with Ad-mIFN-β but not in PBS- or Ad-LacZ-treated controls. This corresponded with the presence of extensive tumor necrosis shown on H&E staining (Fig. 6). The degree of proliferation as determined by PCNA, which is expressed in the late G1 and M phases of the cell cycle (48), was significantly lower in the tumors treated with Ad-mIFN-β compared with those treated with either Ad-LacZ or PBS (P = 0.001; Fig. 6). By TUNEL, apoptosis was increased in the tumors treated with Ad-mIFN-β compared with either PBS or Ad-LacZ (P = 0.001 and P = 0.014, respectively; Fig. 6). IHC, using an antibody against the macrophage-specific scavenger receptor, indicated that only the Ad-mIFN-β–treated tumors were heavily infiltrated with macrophages, and increased iNOS was detected in these same tumors (Fig. 6). Anti-CD-31 IHC demonstrated that tumor-induced angiogenesis microvessel density at ×200 was significantly less within the tumors treated with Ad-mIFN-β (21 ± 3 vessels) than with those treated with PBS (49 ± 6 vessels) or Ad-LacZ (43 ± 5 vessels) therapy (P = 0.050; Fig. 6). Using IHC, we did not observe down-regulation of bFGF, VEGF, IL-8, or MMP-9 protein expression by the human TCC cells after in vitro therapy with Ad-mIFN-β (Fig. 7), which correlated with the in vitro ELISA findings (Fig. 8). Ad-hIFN-β infection in vitro did not alter production of bFGF, IL-8, or VEGF in 253J B-V R cells. In support of these findings we did not identify apoptotic endothelial cells in the tumors of mice treated with Ad-mIFN-β alone after double-staining immunofluorescence.

Analysis of the in Vitro and in Vivo Effects on Various Markers with Ad-hIFN-β Therapy. By ISH, intense hIFN-β gene expression was observed within the tumors treated with Ad-hIFN-β compared with either PBS or Ad-LacZ (Fig. 9), and this result corresponded with necrosis shown by H&E staining, which was not as marked as that observed after Ad-mIFN-β therapy (Fig. 10). In contrast to therapy with Ad-mIFN-β, PCNA, macrophage activation, or iNOS expression did not change significantly with hIFN-β gene therapy. However, we did observe a significant antiangiogenic effect of Ad-hIFN-β therapy mediated by the down-regulation of angiogenesis factor expression by the human TCC cells. The expression of bFGF (P = 0.041; Ad-hIFN-β versus Ad-LacZ and PBS) and MMP-9 (P = 0.009, Ad-hIFN-β versus Ad-LacZ; and P = 0.002, Ad-hIFN-β versus PBS) was significantly lower after therapy with Ad-hIFN-β compared with either PBS or Ad-LacZ (Fig. 11), whereas VEGF and IL-8 levels were unchanged after therapy. These findings are in agreement with the in vitro ELISA findings in which bFGF production by 253J B-V R cells was reduced by almost 50% after infection with Ad-hIFN-β, but not after infection with Ad-mIFN-β (Fig. 8). Likewise, VEGF and IL-8 production was reduced although to a lesser extent after in vitro infection of 253JB-V R cells with Ad-hIFN-β, but not with Ad-mIFN-β. Tumor-induced angiogenesis microvessel density, as determined by anti-CD-31 IHC at ×200 (Fig. 10), was significantly lower in the tumors treated with Ad-hIFN-β (33 ± 4 vessels) compared with either PBS (45 ± 5 vessels; P = 0.010) or Ad-LacZ (33 ± 4 vessels; P = 0.021) therapy. Using double-staining immunofluorescence, significant tumor cell (green) and endothelial cell (yellow) apoptosis was observed only after Ad-hIFN-β treatment (Fig. 10).

DISCUSSION

Even with expert surveillance and follow-up at referral centers, at least 50% of patients who present with superficial bladder cancer will eventually undergo a cystectomy, and fully 30% will die of metastatic bladder cancer, despite originally presenting with “only” carcinoma in situ (49). Intravesical gene therapy provides a promising approach for patients with refractory superficial bladder cancer. TCC is an ideal target to evaluate gene therapy because of the relative ease with which the vector can be delivered directly into the bladder and potentially provide transgene expression in the target organ. The profound bystander effect induced by Ad-IFN-β makes it an attractive choice for gene therapy (9, 50).

Our results demonstrated that the 253J B-V R TCC cells were susceptible to adenoviral infection. The transient constitutive production of mIFN-β and hIFN-β in these cells after in vitro infection with either Ad-hIFN-β or Ad-mIFN-β resulted in their decreased tumorigenicity and metastasis after implantation within the bladders of athymic nude mice. This is in contrast to the fact that no discernible differences in in vitro growth were observed after the infection of 253J B-V R with either Ad-hIFN-β or Ad-mIFN-β.

**Table 2** Therapy of 253J B-V R with intrulesional Ad-mIFN-β

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Median tumor weight, mg (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>1180 (332–3966) a,b</td>
</tr>
<tr>
<td>Ad-LacZ</td>
<td>1017 (132–4468) a,c</td>
</tr>
<tr>
<td>Ad-mIFN-β</td>
<td>188 (59–264) a,b,c</td>
</tr>
</tbody>
</table>

a P = 0.10.  
b P < 0.001.  
c P < 0.001.

**Table 3** Therapy of 253J B-V R with intrulesional Ad-hIFN-β

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Median tumor weight, mg (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>792 (181–2326) a,b</td>
</tr>
<tr>
<td>Ad-LacZ</td>
<td>170 (30–1494) a,b,c</td>
</tr>
<tr>
<td>Ad-hIFN-β</td>
<td>53 (23–115) b,c</td>
</tr>
</tbody>
</table>

a P = 0.11.  
b P < 0.0001.  
c P = 0.042.
compared with Ad-β-gal, confirming that these cells were resistant to the antiproliferative effects of IFN and that these antiproliferative effects are species specific (51, 52).

Because of the species specificity of IFN, it was necessary to treat the tumors with both Ad-mIFN-β and Ad-hIFN-β to activate host and tumor cell responses. Although the mechanisms of action were very different, both therapies caused the regression of established TCC. Therapy of 253J B-V R with intralesional Ad-mIFN-β initiated a host response that was characterized by decreased cellular proliferation (as measured by PCNA), and enhanced apoptosis (as indicated by the TUNEL assay). We propose that the therapeutic effect of
Ad-mIFN is mediated via interactions with the host stroma. These as-yet-undefined interactions upset the critical homeostasis that exists between the host stroma and tumor cell population resulting in the inhibition of tumor growth.

An important component of the host response was the activation of macrophages to up-regulate iNOS (9). We have previously demonstrated that activated macrophages produced significant cytostasis of 253J B-V cells (53), and we hypothesize that the decrease in the PCNA positive tumor cells is attributable to the activation of macrophages. IFN-β also enhances the maturation of NK cells from a pre-NK cell pool (54) and stimulates the secretion of pore-forming protein (55). Because there is no suitable antibody for IHC staining of NK cells in vivo, we have no direct evidence for the infiltration of NK cells into these tumors. However, Dong et al. (9) showed that NK cells mediated the tumoricidal activity of Ad-mIFN-β against

Fig. 8 Angiogenic factor expression (bFGF, IL-8, and VEGF) measured by ELISA 24, 48, and 72 h after in vitro infection of 253J B-V\textsuperscript{R} cells with Ad-LacZ, Ad-mIFN-β, and Ad-mIFN-β. The reduced expression of bFGF, VEGF, and IL-8 occurs only in response to Ad-hIFN-β infection.
human prostate cancer xenografts growing in athymic nude mice; therefore, it is likely that activated NK cells contribute to
the enhanced tumoricidal effect observed within the bladder
tumors treated with Ad-mIFN-β.

In contrast, the primary effects of Ad-hIFN-β were
secondary to an indirect antiangiogenic effect caused by the
down-regulation of the expression of the angiogenesis factors
bFGF and MMP-9 by the tumor cells, with no significant
inhibition of cellular proliferation (PCNA), macrophage ac-
tivation, or iNOS production. It is well established that
angiogenesis plays a crucial role in tumor growth and me-
tastasis. The development of the neovasculature is promoted
by the production of proangiogenic factors by the tumor or
microenvironment (56–59) or by the decrease in the expres-
sion of endogenous angiogenic inhibitors such as angiostatin
(58, 60) or IFN-β. IFN-β or -α are known to down-regulate
the expression of a number of proangiogenic factors that are
crucial for the induction and maintenance of the vasculature
(16, 22), such that the down-regulation of these factors by
IFN may induce the regression of blood vessels through
endothelial cell apoptosis. This hypothesis is consistent with
our previous report that the regression of established TCC
growing in the bladders of nude mice after therapy with the
EGFR inhibitor IMC-C225 was caused by a reduction in

**Fig. 9** *In situ* hybridization mRNA expression of hIFN-β after therapy with Ad-hIFN-β (×40). The poly(dt) staining confirmed the mRNA integrity of the tumor samples.

**Fig. 10** IHC of 253J B-Vβ after therapy with Ad-hIFN-β. Tissue sections analyzed by H&E, and CD-31 were photo-
graphed at ×40, and dual immuno-fluorescence (TUNEL/CD-31) were photographed at
×200. By IHC, significant necrosis was observed within the
Ad-hIFN-β tumors. Tumor-induced angiogenesis (CD-31)
was significantly inhibited, and by dual immuno-fluorescence
endothelial (yellow) and tumor cell (green) apoptosis was seen
only after Ad-IFN-β therapy.
tumor-induced neovascularization secondary to the down-regulation of tumor cell expression of the angiogenic factors VEGF, IL-8, and bFGF, leading to endothelial and tumor cell apoptosis and ultimately to the regression of established tumors (61). Thus, the presence of these proangiogenic factors were necessary to maintain a viable microcirculation and maintain tumor growth. The withdrawal of the angiogenic stimulus by IMC-C225 therapy preceded the regression of the tumor’s neovascularity and, ultimately, tumor cell death. Similarly, in the current study, we demonstrated that Ad-hIFN-β therapy resulted in the down-regulation of angiogenesis factor expression, endothelial cell apoptosis, inhibition of the neovasculature, and the regression of established tumors. Thus, it appears that IFN-β has an indirect apoptotic effect mediated through the down-regulation of endothelial cell survival factors. This important observation that down-regulation of the angiogenic stimulus of the tumor cells inhibits the host angiogenic responses emphasizes the complexity of tumor-host interactions. In the current animal models, we have delivered therapy (intravesional) once a week which is in keeping with the current schedule for human intravesical therapy and will be used in our human intravesical gene therapy program.

In conclusion, IFN-β inhibits tumor growth and metastasis in highly tumorigenic and metastatic human TCC growing in athymic nude mice. The effect appears to be secondary attributable to the direct effect of Ad-hIFN-β on the infected tumor cells and by a bystander effect of the mIFN-β on the host microenvironment. The exact mechanism of the antiangiogenic effect of IFN-β is not fully elucidated; however, endothelial cell antiproliferation and apoptosis appear to characterize the antiangiogenic activity of IFN-β. These studies indicate that IFN-β gene therapy may be a potential intravesical therapy for TCC. However, despite the perception that intravesical gene therapy is feasible because of the ease of intravesical administration, a number of important issues need to be clarified. It is recognized that bladder cancer cells express variable levels of the Coxsackievirus and adenovirus receptors, which mediate viral entry into the target cell (62). However, the effect of this variability in receptor expression on tumor cells for successful intravesical gene therapy is unknown. It is also recognized that the glycoaminoglycan layer of the bladder may be an effective barrier that limits effective intravesical gene therapy. For this reason, there is considerable interest in developing strategies to alter the glycoaminoglycan layer and allow for gene transfer across the

Fig. 11 IHC of the angiogenic factors bFGF, VEGF, IL-8, and MMP-9 after therapy of 253J B-VR with Ad-hIFN-β (×40). Both bFGF and MMP-9 were significantly decreased after therapy with Ad-hIFN-β.
urothelium (63). Such issues need to be resolved before successful intravesical gene therapy will become a reality.

REFERENCES


Inhibition of Tumorigenicity and Metastasis of Human Bladder Cancer Growing in Athymic Mice by Interferon-β Gene Therapy Results Partially from Various Antiangiogenic Effects Including Endothelial Cell Apoptosis


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/8/4/1258

Cited articles
This article cites 59 articles, 19 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/8/4/1258.full#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/8/4/1258.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.