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

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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-INF-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...
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 nontargeted chemotherapeutic 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 (29–32). 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 bladder preservation in the absence of a truly effective intravesical therapy. Intravesical gene therapy provides a promising approach for patients with refractory superficial bladder cancer, and adenoviral-mediated IFN-β gene therapy would appear to be a suitable agent for this purpose (2, 3, 5–10, 13, 15–18, 20–22, 36, 37). However, our preliminary data and early clinical experience indicate that the removal of the glycoaminoglycan layer is necessary for the effective treatment of superficial (and perhaps invasive) disease by intravesical gene therapy approaches, and we are exploring methods for enhancing gene transfer after intravesical instillation. The present study tested the hypothesis that the growth and metastasis of human TCC can be inhibited by increasing the local expression of IFN-β within the tumor by gene transfer of either mIFN-β or hIFN-β. Demonstrating this principle provides the rationale for intravesical IFN-β gene 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-VR 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 tumoricidal 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 the 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-VR 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-VR 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 Ad5-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′-CTTGGTTCTTATGCGAGCGG-3′ and 5′-CCACAACTAGAATGCAGTG-3′, which are located outside the two ends of the insert on the shuttle vector pECIFN-β.
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 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 CEMEM. 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/HCl (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 to 20 × 10⁹ 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 253J B-VR cells were infected with 0, 5, 10, 25, 30, and 40 MOI of Ad-hIFN-β. Ad-LacZ was used as a control with 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 × 10⁶ 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 Stratilinker 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 × 10⁵ 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 suctioning 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 × 10⁵ 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, Ad-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).
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 samples were incubated for 18 h at 4°C with a 1:100 dilution of a monoclonal rat anti-CD31 antibody (PharMingen, 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 diaminobenzidine (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 photomicroscope (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 neovascularity 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 BRJ (DW/BRJ) 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/BRJ and incubated for 15 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 diaminobenzidine (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 Bratig 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 (Biomedra 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-VR

In vitro infection of the 253J B-VR cells with Ad-LacZ or Ad-mIFN-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 infection of the 253J B-VR cells with Ad-LacZ, more cells underwent cell death—observed detachment from the culture plates were photographed at ×200. Results shown represent one of two experiments.

substrate FastRed (Research Genetics). In this assay a red stain indicated a positive reaction. The integrity of mRNA in each sample was verified by using a poly(dT)20 probe. All of the specimens analyzed produced an intense histochemical reaction, which indicated that the mRNA was well preserved. To avoid variability in probe concentration, all specimens were stained in a single session for each probe.

Stained sections were examined in the same manner as for IHC above. Measured areas of 1 mm² were located at the edge or center of the tumor. In each area, the cytoplasmic staining was quantified to derive an average value (13). The intensity of staining was compared by integrated absorbance of poly(dT)20.

Immunofluorescence Double Staining of Endothelial Cells and Apoptotic Cells. For IHC, frozen tissue sections (8 μm thick) were fixed and treated as above for CD31 IHC. Samples were washed four times with PBS and incubated for 1 h with a fluorescent-labeled antirat secondary antibody, Texas Red (1:200; Jackson ImmunoResearch Laboratory, Inc., West Grove, PA). After sections were washed twice 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% Triton X-100 in PBS for 5 min at room temperature. After the slides were rinsed twice with PBS for 5 min, they were incubated with equilibration buffer from kit for 10 min. The equilibration buffer was removed, and reaction buffer containing equilibration buffer, nucleotide mix, and Tdt enzyme was added to the tissue sections and incubated in a humid chamber at 37°C for 1 h, although avoiding exposure to light. EDTA was added to the slides for 5 min to stop the reaction. To identify all nuclei, the slides were soaked with a 1:2000 dilution of Hoechst solution in a Coplin jar for 10 min. The slides were examined under an Olympus Inverted System IX70 microscope (Melville, NY) and 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. The apoptotic endothelial cells had a yellow fluorescence because of the overlapping green and red.

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

RESULTS

Production of hIFN-β after in Vitro Infection. The optimal MOI for infection of the 253J B-VR 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 with minimal toxicity of the adenovirus toward the tumor cells was observed. Minimal cytotoxicity was also observed with a MOI of 10:1. High levels of IFN-β could be detected in the culture supernatants at 72 h after infection, and measurable levels persisted for at least 7 days. Infection of the 253J B-VR cells with Ad-mIFN-β was confirmed by Northern blot analysis (Fig. 3).
significant difference in tumor weight between the 253J B-V R \(P\)-/H11005 \(P\) groups (Ad-LacZ group and both the Ad-mIFN-\(\beta\) also significant differences between the tumor weights of the 253J B-V R cells. PBS and Ad-hIFN-\(\beta\) treatments were used as controls. This is one representative experiment of two.

Table 1  Inhibition of tumorigenicity and metastasis after \textit{in vitro} infection with Ad-IFN-\(\beta\)

Cells (1 \(\times\) 10\(^6\)) were implanted into the bladder (six mice per group). Tumorigenicity and metastasis were determined 28 days later. Results shown represent one of two experiments. \(P\) relates to the statistical comparison between the two groups designated by that letter.

<table>
<thead>
<tr>
<th>Group</th>
<th>Median tumor weight (range), mg</th>
<th>Lymph node metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>253J B-V(^R)</td>
<td>431 (137–744) (^{a,b,c})</td>
<td>3/6</td>
</tr>
<tr>
<td>253J B-V(^R)-LacZ</td>
<td>149 (31–584) (^{d,e})</td>
<td>5/6</td>
</tr>
<tr>
<td>253J B-V(^R)-mIFN-(\beta)</td>
<td>29 (20–60) (^{f})</td>
<td>0/6</td>
</tr>
<tr>
<td>253J B-V(^R)-hIFN-(\beta)</td>
<td>41 (22–53) (^{g})</td>
<td>0/6</td>
</tr>
</tbody>
</table>

\(^{a} P = 0.59; \)  \(^{b} P = 0.025; \)  \(^{c} P = 0.045; \)  \(^{d} P = 0.050; \)  \(^{f} P = 0.011; \)  \(^{g} P = 0.101.\)

**In Vitro Endothelial Cell Response after Infection with Ad-LacZ, Ad-mIFN-\(\beta\), and Ad-hIFN-\(\beta\).** With a MOI of 10:1 the HMECs were very susceptible to viral-induced cytotoxicity. Neither endothelial cells of murine or human origin used in this study produced IL-8 or VEGF in sufficient amounts to allow detection using ELISA. Likewise, negligible amounts of bFGF were produced by these cells.

Orthotopic Implantation of \textit{in Vitro}-infected 253J B-V\(^R\) Cells. To determine tumorigenicity and production of metastasis, the 253J B-V\(^R\), 253J B-V\(^R\)-mIFN-\(\beta\), 253J B-V\(^R\)-hIFN-\(\beta\), and 253J B-V\(^R\)-LacZ cells were implanted into the bladders of athymic mice. After 28 days, the mice were killed and necropsied, and tumor growth and metastasis was evaluated (Table 1). There was a significant difference between the tumor weights of the 253J B-V\(^R\) group and both the Ad-mIFN-\(\beta\) and Ad-hIFN-\(\beta\) groups \((P = 0.025 \text{ and } P = 0.045, \text{ respectively})\). There were also significant differences between the tumor weights of the Ad-LacZ group and both the Ad-mIFN-\(\beta\) and the Ad-hIFN-\(\beta\) groups \((P = 0.05 \text{ and } P = 0.011, \text{ respectively})\). There was no significant difference in tumor weight between the 253J B-V\(^R\) and 253J B-V\(^R\)-Ad-LacZ groups \((P = 0.59)\). No metastases were present in the regional retroperitoneal lymph nodes in mice implanted with either Ad-mIFN-\(\beta\)- or Ad-hIFN-\(\beta\)-treated cells, whereas regional lymph node metastases were present in three of the mice with 253J B-V\(^R\)-Ad-LacZ or 253J B-V tumors (Table 1).

**Therapy of Established Tumors with Ad-mIFN-\(\beta\) and Ad-hIFN-\(\beta\) Gene Therapy.** The 253J B-V\(^R\) cells were implanted s.c. into the flank of athymic nude mice. When the tumors reached 5 mm in their greatest diameter, therapy with intralesional PBS, Ad-LacZ, or Ad-IFN-\(\beta\) commenced and continued weekly for 3 weeks. The weekly tumor diameters of lesions treated with intralesional PBS, Ad-LacZ, or Ad-IFN-\(\beta\) are shown in Figs. 4 and 5. Over the course of therapy, the tumors treated with Ad-hIFN-\(\beta\) regressed in size, whereas those treated with Ad-mIFN-\(\beta\) remained static. At the completion of therapy, the median tumor weight of the Ad-mIFN-\(\beta\) treated mice was significantly less than that of tumors treated with PBS or Ad-LacZ \((P < 0.001; \text{ Table 2})\). 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...
consistently smaller. Similarly, the median tumor weight of the Ad-hIFN-β-treated mice was significantly less than the weight of the tumors from mice treated with PBS or Ad-LacZ ($P < 0.0001$ and $P = 0.042$, respectively; Table 3). In this experiment, the weight of the tumors treated with Ad-LacZ 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 $\times 200$ 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 vivo infection with Ad-mIFN-β (Fig. 7), which correlated with the in vitro ELISA findings (Fig. 8). Ad-mIFN-β infection in vitro did not alter production of bFGF, IL-8, or VEGF in 253J B-VR 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-VR 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 253J B-VR cells with Ad-hIFN-β, but not with Ad-mIFN-β. Tumor-induced angiogenesis microvessel density, as determined by anti-CD-31 IHC at $\times 200$ (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-VR 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-VR with either Ad-hIFN-β or Ad-mIFN-β.
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-VR with intralesional Ad-mIFN-β initiated a host response that was characterized by decreased cellular proliferation (as measured by PCNA), and enhanced tumor cell apoptosis (as indicated by the TUNEL assay). We propose that the therapeutic effect of
Ad-miFI 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-miFI against
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 activation, or iNOS production. It is well established that angiogenesis plays a crucial role in tumor growth and metastasis. 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 expression 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
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 neovascularature 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 glycosaminoglycan 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 glycosaminoglycan 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.
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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


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