

Adenovirus-mediated interferon- β gene transfer inhibits angiogenesis in and progression of orthotopic tumors of human prostate cancer cells in nude mice

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Abstract. Interferon (IFN)- β is a multifunctional cytokine. Our previous studies revealed that intratumoral transfer of the murine interferon (IFN)- β gene inhibited the growth of human and mouse prostate cancer cells in mice. Since IFN- β activity is species-restricted, we investigated the efficacy and mechanisms of forced expression of human IFN- β in suppressing the growth of human prostate cancer cells in mice. Orthotopic tumors of PC-3MM2 human prostate cancer cells were forced to express human IFN- β by intratumoral injection of an adenoviral vector (AdhIFN- β). Tumor growth and survival of tumor-bearing mice were determined. Cell proliferation and apoptosis were evaluated by immunohistochemistry (IHC) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). Angiogenesis and angiogenic molecule expression were evaluated by IHC and quantitative real-time reverse-transcriptional PCR (qRT-PCR). We found that forced expression of human IFN- β inhibited tumor growth in a dose-dependent manner. An injection of 2×10^9 PFU (plaque-forming units) of AdhIFN- β retarded tumor growth by 90% and prolonged the survival of tumor-bearing mice. Control tumors contained more proliferating cells (PCNA⁺) and fewer apoptotic cells (TUNEL⁺) than did AdhIFN- β treated-tumors. Treatment with AdhIFN- β downregulated the expression of interleukin-8 and vascular endothelial cell growth factor-A. Taken together, our data indicated that forced expression of human IFN- β in human prostate cancer cells significantly inhibited their prostatic growth, which correlated with downregulation of angiogenic molecules and suggested that adenoviral vector-mediated IFN- β gene therapy could be an effective approach for the management of human prostate cancer.

Introduction

Prostate cancer is the most common cancer and the second most common cause of cancer death among men in the United States (1). It was expected that 232,090 men would be diagnosed of and 30,350 men would die from this disease in 2005 (1). As cancer detection techniques improve, more patients are diagnosed with clinically localized disease, whereas the number of patients with disseminated disease is on the decline (2). Although most patients with clinically localized prostate cancer can be cured by radical prostatectomy or radiation therapy, a significant number of them (~30%) will develop local recurrence within the prostate, and some ultimately will develop disseminated disease (2). Therefore, more effective therapies that can cure localized tumors and prevent their metastasis are urgently needed.

IFN- β , a type I IFN, is a cytokine that can stimulate immune response, inhibit tumor cell growth, and induce cell differentiation and apoptosis (3-7). Moreover, compelling evidence indicates that IFN- β is a potent anti-angiogenic and anti-metastatic molecule. Not only does IFN- β inhibit the growth and migration of endothelial cells (8), as other angiogenic inhibitors do, but it also downregulates the expression of molecules that mediate angiogenesis and invasion in tumor cells (9,10). Clinical trials using type I IFNs have shown a limited response for most solid tumors, possibly because of insufficient accumulation of biologically active IFN- β within the tumors (11). Indeed, it has been shown that intralesional delivery of the IFN- β gene, which allows for the accumulation of higher intratumoral concentrations of IFN- β , is able to suppress primary tumors and prolong the survival of tumor-bearing mice (12-21). Moreover, IFN- β gene therapy in immune-competent mice has been shown to induce systemic immune responses and confer tumor-specific immune protection against a second challenge (14,20,22,23).

Our previous studies concluded that: i) human prostate cancer cells overexpressing murine IFN- β lost their tumorigenicity and metastasis potential in mice (24); ii) intratumoral delivery of murine IFN- β gene using an adenoviral vector inhibits progression of orthotopic tumors of human prostate cancer cells and significantly prolonged the survival of tumor-bearing mice (25,26); iii) murine IFN- β gene therapy inhibits the growth of mouse prostatic tumors in immune-competent

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mice; and iv) murine IFN- β mediates its inhibitory effects on tumor growth and metastasis by activation of macrophages and natural killer cells and by suppression of angiogenesis (25,26). Because the action of IFN- β is species-restricted (27,28), we further investigated the effects of intratumoral delivery of the human IFN- β gene on the orthotopic growth of human prostate cancer cells in the present study. We found that forced expression of human IFN- β significantly inhibited prostatic tumor growth, which correlated with the down-regulation of angiogenic molecules. These data strongly suggest that adenoviral vector-mediated IFN- β gene therapy could be an alternative approach for management of human prostate cancer, especially locally advanced disease.

Materials and methods

Mice. Specific pathogen-free male athymic nude mice were purchased from Harlan Sprague-Dawley, Inc., Indianapolis, IN. Mice were maintained in a facility 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 Health and Human Services, and NIH. The mice were used according to institutional guidelines when they were 8-10 weeks of age.

Reagents. Eagle's minimal essential medium (MEM), Ca²⁺, Mg²⁺-free HBSS, and FBS were purchased from Hyclone (Logan, UT). RNA Later was from Ambion (Austin, TX). A TRIzol RNA extraction kit was purchased from Invitrogen (Carlsband, CA). Antibodies to PCNA and CD31 were purchased from BD Biosciences (San Jose, CA). Antibodies against IL-8 and VEGF were purchased from R&D Systems (Minneapolis, MN) and Chemicon International (Temecula, CA), respectively. A TUNEL kit was purchased from Promega (Madison, WI). An enzyme-linked immunosorbent assay (ELISA) kit for human IFN- β was obtained from Fujirebio Inc. (Tokyo, Japan). AdhIFN- β , an adenoviral vector encoding the human IFN- β gene, and AdE/1, a control adenoviral vector with no expression cassette, were generously provided by Drs Colin Dinney and Bingliang Fang at the University of Texas M.D. Anderson Cancer Center, Houston, respectively. The adenoviral vectors were propagated in 293 cells and purified by the two-step CsCl₂ gradient centrifugation protocol (29).

Tumor cell inoculation. The well-characterized metastatic PC-3MM2 human prostate carcinoma cell lines (24,30-32) were used. PC-3MM2 cells were maintained as a monolayer culture in MEM supplemented with 5% FBS, non-essential amino acids, sodium pyruvate, vitamin A, and glutamine. Cells in their exponential growth phase were harvested by a 1-min treatment with a 0.25% trypsin-0.02% EDTA solution, detached into MEM-10% FBS. The cell suspension was agitated gently to produce a single-cell suspension and resuspended in HBSS. Only suspensions of single cell with viability exceeding 95% (ascertained by trypan-blue exclusion) were used in the studies.

Mice were anesthetized with Nembutal and placed in the supine position. The surgical procedure was performed as detailed in our previous study (26). Briefly, a lower midline

incision was created and the prostate exposed. A tumor cell suspension (10⁵ cells in 20 μ l HBSS) was injected into the dorsal prostatic lobes using a 30-gauge needle, a 1-ml disposable syringe, and a calibrated push button-controlled dispensing device (Hamilton Syringe Company, Reno, NV). The abdominal wound was closed in two layers with suture and wound clips (Autoclip; Clay Adama, Parsippany, NJ).

Treatment procedure. Mice were anesthetized with Nembutal, the incision reopened, and the prostate tumor exposed. PBS, AdE/1, or AdhIFN- β in 40 μ l of buffer was injected into the center of each tumor with a Hamilton syringe and a 30-gauge needle over 3 min. The needle was removed slowly after a 30-sec delay. Prostate tumors and lymph node metastases were determined at the end of the experiments (28-32 days after tumor cell inoculation). Primary tumors (including the entire prostate) were weighed when the experiments were terminated; regional lymph node metastasis was assessed by microscopic examination of H&E-stained serial paraffin sections (26).

Immunohistochemical staining. Immunohistochemical analyses were performed as described previously (24,26). Briefly, tumor tissues were placed in OCT compounds and snap frozen in liquid nitrogen. Frozen sections (8-10 μ m) were fixed in cold acetone and treated with 3% hydrogen peroxide (H₂O₂) in methanol (v/v). The treated slides were incubated in blocking solution and then treated with antibody to CD31 (to assess tumor vascularity). The sections were rinsed and incubated with peroxidase-conjugated secondary antibodies. A positive reaction was visualized by incubating the slides with stable 3,3'-diaminobenzidine and counterstaining with Mayer's hematoxylin. For immunohistochemical staining using the antibodies to the proliferative cell nuclear antigen (PCNA), IL-8, and VEGF, paraffin sections of the tumor samples were dewaxed and stained as described for the frozen sections.

TUNEL assay. The TUNEL assay kit was used to identify apoptotic cells in tumor lesions following the manufacturer's instruction. Briefly, paraffin sections were dewaxed in xylene and rehydrated and then treated with 20 μ g/ml proteinase K and incubated with 3% H₂O₂ in methanol. The slides were incubated first in terminal deoxynucleotidyl transferase buffer, then with terminal deoxynucleotidyl transferase, and finally with a streptavidin-peroxidase conjugate and stained with 3-amino-9-ethyl carbazole.

RNA isolation and quantitative real-time reverse-transcriptional PCR (qRT-PCR). After removal from mice, tumor tissues were treated with RNA Later, transferred into TRIzol at 100 mg tissue/ml, homogenized using a Polytron homogenizer. Total tissue RNA was isolated following the manufacturer's instructions. Genomic DNA potentially present in the RNA samples was eliminated by treatment for 30 min at 37°C with 10 U of RNase-free DNase I (Promega), followed by 5-min heat inactivation at 95°C. Two μ g of total RNA was reverse-transcribed in a 20 μ l reaction containing 200 units of reverse transcriptase (Stratagene, La Jolla, CA), 500 ng of Oligo d(T) primer, 500 nM dNTP mix, for 60 min at 42°C, followed by 5-min heat inactivation at 95°C. Twenty-five ng of the obtained cDNA were amplified in a 7300 real-time PCR

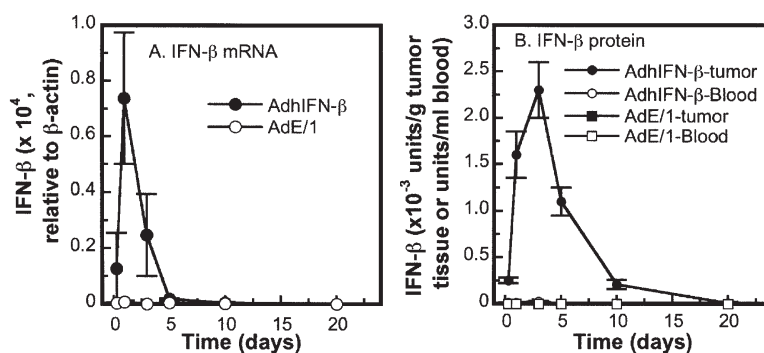


Figure 1. Adenoviral vector-mediated IFN-β gene transfer in mice. PC-3MM2 cells were inoculated into the prostates of nude mice. Seven days later, the prostatic tumors were injected with 2×10^9 PFU of either AdE/1 or AdhIFN-β. Expression of human IFN-β mRNA (A) and protein (B) in the treated tumors was analyzed at various times after the injection by qRT-PCR and ELISA, respectively.

system (Applied Biosystems, Foster City, CA) in a reaction containing the primer pairs and the Brilliant SYBR-Green QPCR Master Mix (Stratagene). After an initial step at 95°C for 10 min, temperature cycling was started (denaturation at 95°C for 30 sec, hybridization at 60°C for 30 sec, and elongation at 72°C for 30 sec) for 40 cycles. The cycle threshold values were used to calculate the normalized expression of target genes against β-actin using Q-Gen software (33).

Cell growth assay. PC-3MM2 cells were plated in triplicate at a density of 10^3 cells/well in 96-well plates. Twenty-four hours later, adenoviral vectors or IFN-β was added, and the plates were incubated for 4 days. During the final 2 h of incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemicals, St. Louis, MO) was added at 0.42 mg/ml. The medium was then removed, and dark-blue formazan was dissolved in dimethyl sulfoxide and the absorbance was measured with a FLUOstar Optima microplate reader at 570 nm. The percentage of cell growth inhibition was calculated according to the following formula: growth inhibition (%) = $(1 - A570 \text{ of treated group} / A570 \text{ of control group}) \times 100$.

ELISA. Fresh tumor samples were homogenized in PBS containing proteinase inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Protein concentrations of the lysates were measured using a protein assay kit (Bio-Rad Laboratory, Hercules, CA). Human IFN-β in the lysate was measured using an ELISA kit according to the manufacturer's instructions.

Statistical analysis. The gene therapy experiments were performed with 4-8 mice/group and were repeated at least once. Differences between treatment and control groups of tumor incidences and cell growth were analyzed with the t-test. Differences in tumor weight among groups were compared by ANOVA. Survival data were analyzed using the Kaplan-Meier plot, and their statistical significance was determined using the Mantel-Cox log-rank test.

Results

Efficiency of adenoviral vector-mediated gene transfer in prostatic tumors. In the first set of experiments, we evaluated

the efficiency of adenovirus-mediated gene transduction in prostatic tumors. PC-3MM2 cells (10^5 /mouse) were inoculated into the prostates of nude mice. On day 7 after the inoculation, tumors were injected with PBS or 2×10^9 PFU of AdLacZ. Three days later, frozen sections of tumor samples were stained to reveal AdLacZ-transduced cells. We found that >80% of tumor cells in lesions injected with AdLacZ but not PBS expressed β-galactosidase (data not shown). Next, we determined the AdhIFN-β-induced expression of human IFN-β in a similar setting. Tumors, injected with 2×10^9 PFU of either AdE/1 or AdhIFN-β, were sampled at different times up to 20 days and IFN-β mRNA and protein were analyzed by qRT-PCR and ELISA, respectively. Human IFN-β mRNA was not detected in tumors injected with AdE/1 (Fig. 1A). In contrast, in AdhIFN-β-injected tumors, IFN-β mRNA was detected at 8 h, reached a peak on day 1, decreased thereafter, and became undetectable 5 days later (Fig. 1A). IFN-β protein was found only in tumors injected with AdhIFN-β, which was detected 8 h (125 units/g tumor tissue, $\sim 1 \text{ cm}^3$) after the treatment and peaked on day 3 (1,150 U/g tumor tissue). Intratumoral IFN-β reduced to 110 U/g tumor tissue on day 10 and was not detected in samples collected on day 20, respectively. Low levels of IFN-β, which followed the same kinetics as that in tumor lesions, were found in the blood of mice bearing tumors injected with AdhIFN-β, but not AdE/1. The highest concentration of IFN-β in the blood was 23 U/ml on day 3 after intratumoral injection of AdhIFN-β. Taken together, our data show that intralesional injection of adenoviral vector efficiently transduced prostatic tumors.

Effects of AdhIFN-β therapy on the growth and metastasis of orthotopic PC-3MM2 tumors. We next investigated the efficacy of adenovirus-mediated IFN-β gene transfer in suppressing prostatic tumor progression. On day 7 after tumor cell inoculation, the lesions were injected with PBS or 2×10^9 PFU of AdE/1 or AdhIFN-β. As shown in Fig. 2A, treatment with AdhIFN-β suppressed growth of PC-3MM2 tumors by up to 90%. In contrast, injection with PBS or AdE/1 did not affect the growth of PC-3MM2 tumors (Fig. 2A). Regional lymph node metastases, identified on H&E-stained slides (not shown), were observed in 100% of mice treated with PBS or AdE/1, and none of the mice treated with AdhIFN-β (Fig. 2A). We further determined the dose-dependent effects of AdhIFN-β on the growth of orthotopic PC-3MM2 tumors. PC-3MM2

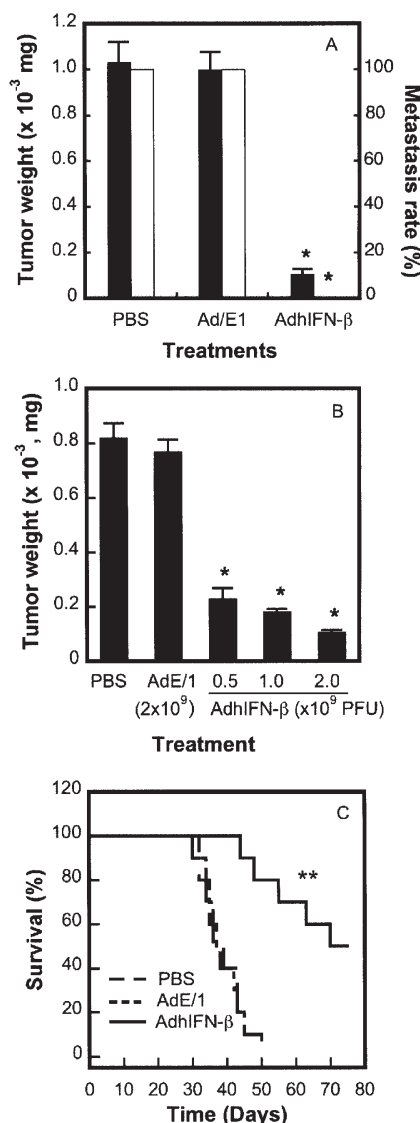


Figure 2. Therapeutic effects of AdhIFN- β against orthotopic PC-3MM2 tumors. PC-3MM2 cells were inoculated into the prostates of nude mice. Seven days later, the tumors were injected with PBS or 2×10^9 PFU of either AdE/1 or AdhIFN- β . Experiments were terminated 3 weeks after the therapy. Prostatic tumors were weighed and aortic lymph node metastases were determined on paraffin-embedded sections (A); solid bar, tumor weight; open bar, metastasis rate. On day 7 after tumor cell inoculation, tumors were injected with PBS, 2×10^9 of AdE/1, or different doses of AdhIFN- β . Experiments were terminated 3 weeks after the therapy and prostatic tumors were weighed (B). Seven days after tumor cell inoculation, the tumors were injected with PBS or 2×10^9 PFU of either PBS or AdhIFN- β . Mice were sacrificed when they were moribund and the survival times were recorded (C).

tumors, on day 7 after tumor cell inoculation, were injected with PBS, AdE/1, or increasing doses of AdhIFN- β . Data in Fig. 2B show that AdhIFN- β inhibited tumor growth in a dose-dependent manner: tumor growth was reduced by 72, 78, and 87% in mice injected with 0.5, 1, and 2×10^9 of AdhIFN- β , respectively. To determine whether AdhIFN- β could improve the survival of tumor-bearing mice, tumors were injected with PBS or 2×10^9 PFU of either AdE/1 or AdhIFN- β . Mice were sacrificed when they were moribund. The data in Fig. 2C show that therapy with AdhIFN- β significantly prolonged the survival of tumor-bearing mice as compared to those injected

with PBS or the control vector ($p < 0.01$); median survival for mice treated with PBS, AdE/1, and AdhIFN- β were 37, 38, and 70 days, respectively.

Effects of AdhIFN- β therapy on cell growth and apoptosis in tumors. Cell replication and death are the two parameters that determine tumor growth rate. We, therefore, examined PCNA expression, which is expressed mainly in the late G1 and M phase of the cell cycle and indicates cell replication (34), by immunohistochemical staining and apoptosis by TUNEL staining, respectively. On day 5 after the intralesional injection of PBS or 2×10^9 PFU of either AdE/1 or AdhIFN- β , tumors were sampled for *in vitro* analyses. As shown in Fig. 3, most cells ($>85\%$) in tumors injected with PBS (Fig. 3a) or AdE/1 (Fig. 3b) were stained strongly by the PCNA antibody and very few cells ($<0.1\%$) stained positively in the TUNEL assay (Fig. 3d and e). Although most cells ($>50\%$) in tumors injected with AdhIFN- β were also stained positively by the PCNA-specific antibody (Fig. 3c), both the intensity of the staining and the number of positively stained cells were significantly reduced in comparison with those in tumors injected with PBS or AdE/1. In contrast, the number of cells positively stained by the TUNEL assay increased by ~ 3 -5-fold in tumors injected with AdhIFN- β (Fig. 3f) compared with that in PBS or AdE/1 treated tumors. Comparing the effects of AdhIFN- β therapy on cell proliferation apoptosis, these data suggest that the suppression of tumor growth by AdhIFN- β is due mainly to the reduction of tumor cell replication.

Tumor growth and metastasis require angiogenesis (35). Since IFN- β is a potent angiogenesis inhibitor (8-10), we examined microvessels in the tumors by immunohistochemical staining using an antibody against CD31, which is expressed on microvessel endothelial cells. Interestingly, we did not observe significant differences in microvessel density among tumors injected with PBS (Fig. 3g), AdE/1 (Fig. 3h), and AdhIFN- β (Fig. 3i). IL-8 and VEGF-A are overexpressed in human prostate cancer, especially in advanced diseases (36-39). Overexpression of these two proangiogenic molecules is shown to be involved in angiogenesis and progression of PC-3 tumors in mice (40-43). We, therefore, determined the effects of AdhIFN- β therapy on the expression of IL-8 and VEGF-A in orthotopic PC-3MM2 tumors. Data in Fig. 3l and o show that IL-8 and VEGF-A in tumors injected with AdhIFN- β were significantly lower than in those injected with PBS (Fig. 3j and m) or AdE/1 (Fig. 3k and n). We further analyzed the expression levels of IL-8 and VEGF-A during the course of the therapy by qRT-PCR. As shown in Fig. 4, the down-regulation of both IL-8 (Fig. 4A) and VEGF-A (Fig. 4B) was detected 1 day after intratumoral injection of AdhIFN- β , reached its maximum on day 3 and lasted for up to 10 days.

Effects of AdhIFN- β on cell growth and expression of IL-8 and VEGF-A. To investigate whether the therapeutic effects of AdhIFN- β correlate with its direct effects on cells and to exclude the possibility that the apparent downregulation of IL-8 and VEGF-A mRNA was an artifact caused by the contamination of mouse tissue RNA in the samples, we determined the effects of AdhIFN- β on the growth and expression of IL-8 and VEGF-A in PC-3MM2 cells in cell culture. PC-3MM2 cells in a 96-well plate at 1,000 cells/well

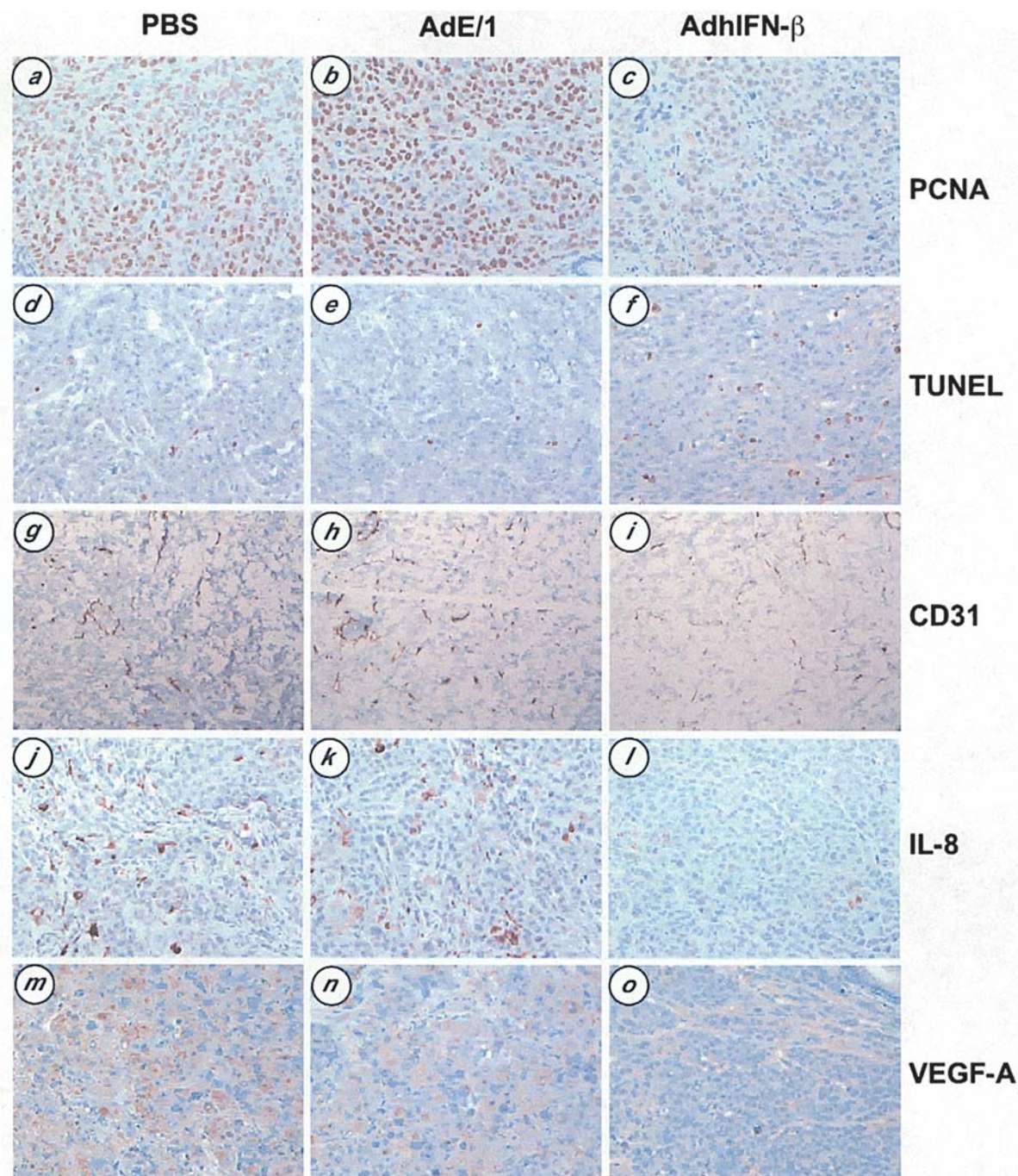


Figure 3. Immunohistochemical staining of tumors. PC-3MM2 tumors were inoculated into the prostates of mice. On day 7 after tumor cell inoculation, tumors were injected with PBS or 2×10^9 of either AdE/1 or AdhIFN- β . The treated tumors were sampled 5 days after the therapy for immunohistochemical staining or TUNEL assay.

were cultured for 96 h in normal medium or infected with 3×10^4 plaque-forming U/ml of either AdE/1 or AdhIFN- β . Cells treated with 1,000 U/ml of recombinant human IFN- β served as a positive control. As shown in Fig. 5A, the growth of PC-3MM2 cells was significantly inhibited by AdhIFN- β and, to a less extent, by IFN- β , but not by the same concentration of AdE/1. Similarly, expression of both IL-8 (Fig. 5B) and VEGF-A (Fig. 5C) was downregulated by transduction of PC-3MM2 cells for 48 h with 30 multiplicity of infection (MOI) of AdhIFN- β but not AdE/1. The expression of these two angiogenic molecules was also reduced by treatment with human IFN- β (Fig. 5B and C).

Discussion

Our previous studies have shown that intralesional delivery of the murine IFN- β gene inhibits the growth of PC-3MM2 human prostate cancer cells implanted in the prostates of nude mice (25,26). Because of the species-restricted action of IFN- β , we further investigated the effects of the adenovirus-mediated transfer of the human IFN- β gene on growth and angiogenesis in an orthotopic model of human prostate cancer. Our data show that the adenoviral vector efficiently transduced human prostate cancer cells in both cell culture and tumor lesions. After a single intratumoral injection of AdhIFN- β ,

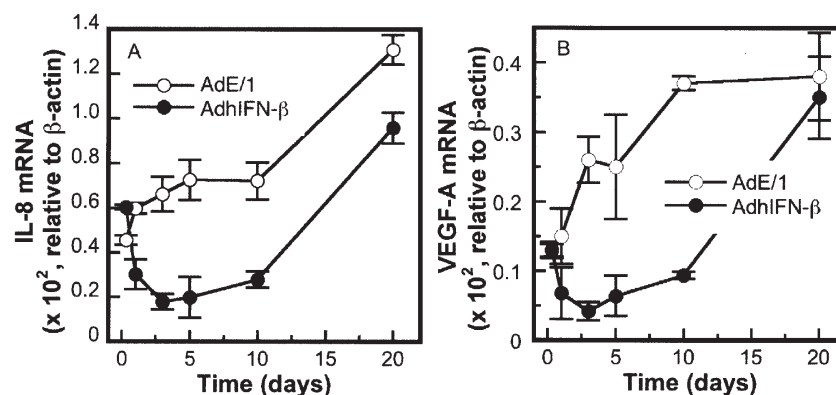


Figure 4. Expression of IL-8 and VEGF-A in PC-3MM2 tumors. PC-3MM2 cells were inoculated into the prostates of nude mice. Seven days later, the prostatic tumors were injected with 2×10^9 PFU of either AdE/1 or AdhIFN- β . Expression of IL-8 (A) and VEGF-A (B) in the treated tumors was analyzed at various times after the injection by qRT-PCR.

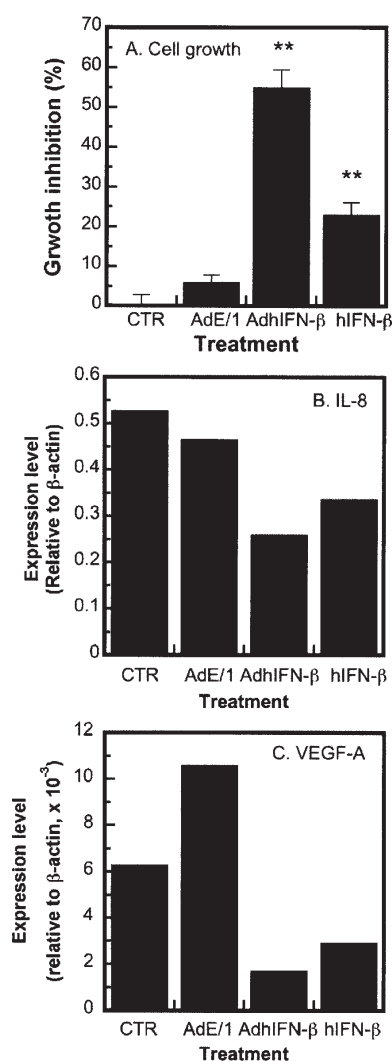


Figure 5. Effects of AdhIFN- β on cell growth and angiogenic molecule expression in culture. PC-3MM2 cells were plated into a 96-well plate at 1,000 cells/well and incubated for 4 days with adenoviral vectors or recombinant IFN- β . Viable cells in the wells were stained with MTT and the effects of the vectors or IFN- β on cell growth were determined (A). PC-3MM2 cells were plated into 60-mm plates at 10^6 /plate and incubated for 48 h in 5% FBS-MEM or the medium containing 30 MOI of either AdE/1 or AdhIFN- β . Total cellular RNA was then extracted and the presence of IL-8 mRNA (B) and VEGF-A mRNA (C) was determined by qRT-PCR.

human IFN- β protein was detected in tumor lesions for up to 10 days. The intratumoral delivery of AdhIFN- β significantly retarded tumor growth, prevented metastasis, and prolonged the survival of tumor-bearing mice.

Analyses of cell growth and apoptosis suggest that inhibition of tumor growth by AdhIFN- β gene therapy is due mainly to the suppression of tumor cell replication. We based this conclusion on the following observations. First, AdhIFN- β injection significantly reduced cell replication in tumors revealed by PCNA staining. Second, AdhIFN- β therapy only moderately enhanced apoptosis in tumors. Third, infection of cells in culture with AdhIFN- β or treatment of cells with recombinant human IFN- β significantly inhibited cell growth.

Intratumoral injection of AdhIFN- β downregulated the expression of angiogenic molecules IL-8 and VEGF-A by PC-3MM2 cells both in mice and in tissue culture, suggesting that the therapy suppressed tumor angiogenesis. In contrast to the therapy against PC-3MM2 tumors using an adenoviral vector encoding the murine IFN- β gene that leads to a reduction of microvessel density (25,26), we did not observe significant alteration of the microvessel density in tumors treated by AdhIFN- β . This difference is very possibly caused by the species-restricted action of IFN- β (27,28). Murine IFN- β has no direct effects on PC-3MM2 cells (24). It activates host macrophages to express inducible nitric oxide synthase and, hence, to generate cytotoxic nitric oxide (25,26). Therefore, a therapy using the murine IFN- β gene induced apoptosis of endothelial cells, leading to a reduction of microvessel density, and massive tumor cell death in the lesions (25,26). On the other hand, human IFN- β has no direct effects on host cells in the tumor environment. It inhibits tumor cell replication and expression of angiogenic molecules by the tumor cells. Under these conditions, blood vessel formation, i.e., angiogenesis, is blocked, resulting in the retardation of tumor growth, but vessel density is not significantly altered. The significant reductions of cell replication and moderate increase in apoptosis observed in the present study also support this notion. Therefore, therapies against human prostate cancer in patients using an adenoviral vector encoding human IFN- β may suppress tumor growth by the following mechanisms: a) inhibition of angiogenesis as well as tumor cell replication as observed in the present study, b) microvessicular damage

as reported in our previous studies (25,26), and c) stimulation of host immune response (14,20,22,23), and will produce much superior therapeutic effects than do using either murine or human IFN- β genes in animal models of human prostate cancer cells.

In conclusion, we report that intratumoral delivery of an adenoviral vector encoding the human IFN- β gene suppressed tumor growth and prolonged the survival of tumor-bearing mice, which correlated with the downregulation of the expression of IL-8 and VEGF-A. Together with our previous studies showing the therapeutic effects of murine IFN- β gene against both human tumor cells in nude mice (25,26) and mouse prostatic tumors in immune-competent mice (44), these data strongly support the notion that IFN- β gene therapy could be an effective alternative therapy for locally advanced prostate cancer.

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References

- Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ and Thun MJ: Cancer statistics, 2005. *CA Cancer J Clin* 55: 10-30, 2005.
- Steele GS and Richie JP: Management of local failure following treatment of localized prostate cancer. In: *Advanced Therapy of Prostate Disease*. Resnick MI and Thompson IM (eds). B.C. Decker Inc., Hamilton, Ontario, pp365-377, 2000.
- Lokshin A, Mayotte JE and Levitt ML: Mechanism of interferon beta-induced squamous differentiation and programmed cell death in human non-small-cell lung cancer cell lines. *J Natl Cancer Inst* 87: 206-212, 1995.
- Belhumeur P, Lanoix J, Blais Y, Forget D, Steyaert A and Skup D: Action of spontaneously produced beta interferon in differentiation of embryonal carcinoma cells through an auto-induction mechanism. *Mol Cell Biol* 13: 2846-2857, 1993.
- Qin XQ, Runkel L, Deck C, De Dios C and Barsoum J: Interferon-beta induces S phase accumulation selectively in human transformed cells. *J Interferon Cytokine Res* 17: 355-367, 1997.
- Clemens MJ: Interferons and apoptosis. *J Interferon Cytokine Res* 23: 277-292, 2003.
- Biron CA: Interferons alpha and beta as immune regulators - a new look. *Immunity* 14: 661-664, 2001.
- Albini A, Marchisone C, Del Grosso F, Benelli R, Masiello L, Tacchetti C, Bono M, Ferrantini M, Rozera C, Truini M, Belardelli F, Santi L and Noonan DM: Inhibition of angiogenesis and vascular tumor growth by interferon-producing cells: a gene therapy approach. *Am J Pathol* 156: 1381-1393, 2000.
- Singh RK, Gutman M, Bucana CD, Sanchez R, Llansa N and Fidler IJ: Interferons alpha and beta down-regulate the expression of basic fibroblast growth factor in human carcinomas. *Proc Natl Acad Sci USA* 92: 4562-4566, 1995.
- Yamamoto S, Yasui W, Kitadai Y, Yokozaki H, Haruma K, Kajiyama G and Tahara E: Expression of vascular endothelial growth factor in human gastric carcinomas. *Pathol Int* 48: 499-506, 1998.
- Einhorn S and Grandner D: Why do so many cancer patients fail to respond to interferon therapy? *J Interferon Cytokine Res* 16: 275-281, 1996.
- Ferrantini M and Belardelli F: Gene therapy of cancer with interferon: lessons from tumor models and perspectives for clinical applications. *Semin Cancer Biol* 10: 145-157, 2000.
- Alizadeh H, Howard K, Mellon J, Mayhew E, Rusciano D and Niederkorn JY: Reduction of liver metastasis of intraocular melanoma by interferon-beta gene transfer. *Invest Ophthalmol Vis Sci* 44: 3042-3051, 2003.
- Odaka M, Sterman DH, Wiewrodt R, Zhang Y, Kiefer M, Amin KM, Gao GP, Wilson JM, Barsoum J, Kaiser LR and Albelda SM: Eradication of intraperitoneal and distant tumor by adenovirus-mediated interferon-beta gene therapy is attributable to induction of systemic immunity. *Cancer Res* 61: 6201-6212, 2001.
- Krukltis RJ, Singhal S, Delong P, Kapoor V, Sterman DH, Kaiser LR and Albelda SM: Immuno-gene therapy with interferon-beta before surgical debulking delays recurrence and improves survival in a murine model of malignant mesothelioma. *J Thorac Cardiovasc Surg* 127: 123-130, 2004.
- Kobayashi N, Kuramoto T, Chen S, Watanabe Y and Takakura Y: Therapeutic effect of intravenous interferon gene delivery with naked plasmid DNA in murine metastasis models. *Mol Ther* 6: 737-744, 2002.
- Hendren SK, Prabakaran I, Buerk DG, Karakousis G, Feldman M, Spitz F, Menon C and Fraker DL: Interferon-beta gene therapy improves survival in an immunocompetent mouse model of carcinomatosis. *Surgery* 135: 427-436, 2004.
- Streck CJ, Ng CY, Zhang Y, Zhou J, Nathwani AC and Davidoff AM: Interferon-mediated anti-angiogenic therapy for neuroblastoma. *Cancer Lett* 228: 163-170, 2005.
- Streck CJ, Zhang Y, Miyamoto R, Zhou J, Ng CY, Nathwani AC and Davidoff AM: Restriction of neuroblastoma angiogenesis and growth by interferon-alpha/beta. *Surgery* 136: 183-189, 2004.
- Lu W, Fidler IJ and Dong Z: Eradication of primary murine fibrosarcomas and induction of systemic immunity by adenovirus-mediated interferon beta gene therapy. *Cancer Res* 59: 5202-5208, 1999.
- Lu W, Su J, Kim LS, Bucana CD, Donawho C, He J, Fidler IJ and Dong Z: Active specific immunotherapy against occult brain metastasis. *Cancer Res* 63: 1345-1350, 2003.
- Odaka M, Wiewrodt R, De Long P, Tanaka T, Zhang Y, Kaiser L and Albelda S: Analysis of the immunologic response generated by Ad.IFN-beta during successful intraperitoneal tumor gene therapy. *Mol Ther* 6: 210-218, 2002.
- Natsume A, Mizuno M, Ryuke Y and Yoshida J: Antitumor effect and cellular immunity activation by murine interferon-beta gene transfer against intracerebral glioma in mouse. *Gene Ther* 6: 1626-1633, 1999.
- Dong Z, Greene G, Pettaway C, Dinney CP, Eue I, Lu W, Bucana CD, Balbay MD, Bielenberg D and Fidler IJ: Suppression of angiogenesis, tumorigenicity, and metastasis by human prostate cancer cells engineered to produce interferon-beta. *Cancer Res* 59: 872-879, 1999.
- Zhang F, Lu W and Dong Z: Tumor-infiltrating macrophages are involved in suppressing growth and metastasis of human prostate cancer cells by INF-beta gene therapy in nude mice. *Clin Cancer Res* 8: 2942-2951, 2002.
- Cao G, Su J, Lu W, Zhang F, Marteralli D, Zhao G and Dong Z: Adenovirus-mediated interferon-beta gene therapy suppresses growth and metastasis of human prostate cancer in nude mice. *Cancer Gene Ther* 8: 497-505, 2001.
- Constantinescu SN, Croze E, Wang C, Murti A, Basu L, Mullersman JE and Pfeffer LM: Role of interferon alpha/beta receptor chain 1 in the structure and transmembrane signaling of the interferon alpha/beta receptor complex. *Proc Natl Acad Sci USA* 91: 9602-9606, 1994.
- Keay S and Grossberg SE: Interferon inhibits the conversion of 3T3-L1 mouse fibroblasts into adipocytes. *Proc Natl Acad Sci USA* 77: 4099-4103, 1980.
- Graham FL and Prevec L: Manipulation of adenovirus vectors. In: *Gene Transfer and Expression Protocols*. Murray EJ (ed). Humana Press, NJ, pp109-128, 1991.
- Kozlowski JM, Fidler IJ, Campbell D, Xu ZL, Kaighn ME and Hart IR: Metastatic behavior of human tumor cell lines grown in the nude mouse. *Cancer Res* 44: 3522-3529, 1984.
- Stephenson RA, Dinney CP, Gohji K, Ordonez NG, Killion JJ and Fidler IJ: Metastatic model for human prostate cancer using orthotopic implantation in nude mice. *J Natl Cancer Inst* 84: 951-957, 1992.
- Greene GF, Kitadai Y, Pettaway CA, von Eschenbach AC, Bucana CD and Fidler IJ: Correlation of metastasis-related gene expression with metastatic potential in human prostate carcinoma cells implanted in nude mice using an *in situ* messenger RNA hybridization technique. *Am J Pathol* 150: 1571-1582, 1997.

33. Simon P: Q-gene: processing quantitative real-time RT-PCR data. *Bioinformatics* 19: 1439-1440, 2003.
34. Wilson GD, Camplejohn RS, Martindale CA, Brock A, Lane DP and Barnes DM: Flow cytometric characterisation of proliferating cell nuclear antigen using the monoclonal antibody PC10. *Eur J Cancer* 12: 2010-2017, 1992.
35. Folkman J: The role of angiogenesis in tumor growth. *Semin Cancer Biol* 3: 65-71, 1992.
36. Balbay MD, Pettaway CA, Kuniyasu H, Inoue K, Ramirez E, Li E, Fidler IJ and Dinney CP: Highly metastatic human prostate cancer growing within the prostate of athymic mice overexpresses vascular endothelial growth factor. *Clin Cancer Res* 5: 783-789, 1999.
37. Uehara H, Troncoso P, Johnston D, Bucana CD, Dinney C, Dong Z, Fidler IJ and Pettaway CA: Expression of interleukin-8 gene in radical prostatectomy specimens is associated with advanced pathologic stage. *Prostate* 64: 40-49, 2005.
38. Murphy C, McGurk M, Pettigrew J, Santinelli A, Mazzucchelli R, Johnston PG, Montironi R and Waugh DJ: Nonapical and cytoplasmic expression of interleukin-8, CXCR1, and CXCR2 correlates with cell proliferation and microvessel density in prostate cancer. *Clin Cancer Res* 11: 4117-4127, 2005.
39. Ferrer FA, Miller LJ, Andrawis RI, Kurtzman SH, Albertsen PC, Laudone VP and Kreutzer DL: Angiogenesis and prostate cancer: *in vivo* and *in vitro* expression of angiogenesis factors by prostate cancer cells. *Urology* 51: 161-167, 1998.
40. Zhang F, Lee J, Lu S, Pettaway CA and Dong Z: Blockade of transforming growth factor-beta signaling suppresses progression of androgen-independent human prostate cancer in nude mice. *Clin Cancer Res* 11: 4512-4520, 2005.
41. Moore BB, Arenberg DA, Stoy K, Morgan T, Addison CL, Morris SB, Glass M, Wilke C, Xue YY, Sitterding S, Kunkel SL, Burdick MD and Strieter RM: Distinct CXC chemokines mediate tumorigenicity of prostate cancer cells. *Am J Pathol* 154: 1503-1512, 1999.
42. Kitagawa Y, Dai J, Zhang J, Keller JM, Nor J, Yao Z and Keller ET: Vascular endothelial growth factor contributes to prostate cancer-mediated osteoblastic activity. *Cancer Res* 65: 10921-10929, 2005.
43. Liu XH, Kirschenbaum A, Yao S, Stearns ME, Holland JF, Claffey K and Levine AC: Upregulation of vascular endothelial growth factor by cobalt chloride-simulated hypoxia is mediated by persistent induction of cyclooxygenase-2 in a metastatic human prostate cancer cell line. *Clin Exp Metastasis* 17: 687-694, 1999.
44. Olson MV, Lee J, Zhang F, Wang A and Dong Z: Inducible nitric oxide synthase activity is essential for inhibition of prostatic tumor growth by interferon-beta gene therapy. *Cancer Gene Ther* 13: 676-685, 2006.