

# Tumor-infiltrating Macrophages Are Involved in Suppressing Growth and Metastasis of Human Prostate Cancer Cells by IFN- $\beta$ Gene Therapy in Nude Mice<sup>1</sup>

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## ABSTRACT

**Purpose:** This study was to determine the role of tumor-infiltrating macrophages in IFN- $\beta$ -induced host defense against prostate cancer.

**Experimental Design:** Efficacy of adenovirus-mediated IFN- $\beta$  gene therapy against orthotopic xenografts of human prostate cancer was tested in macrophage-compromised nude mice. Immunohistochemistry and Northern blotting were used to elucidate mechanisms responsible for the IFN- $\beta$  gene therapy.

**Results:** PC-3MM2 human prostate cancer cells were inoculated into the prostates of nude mice. Intralesional injection of an adenoviral vector-encoding murine IFN- $\beta$  (AdmIFN- $\beta$ ) but not control vector AdE1 suppressed growth of PC-3MM2 tumors in a dose-dependent manner, with a maximal reduction of tumor weight by  $\sim 85\%$  at  $2 \times 10^9$  plaque-forming units. The therapy prevented metastasis, eradicated established metastases in some mice, and prolonged the survival of tumor-bearing mice. The efficacy of AdmIFN- $\beta$  therapy was reduced significantly in mice treated with macrophage-selective anti-Mac-1 and anti-Mac-2 antibodies. Moreover, the i.p. injection of the antibodies restored the tumorigenicity of PC-3MM2 cells stably engineered with murine IFN- $\beta$  gene. Tumor-infiltrating macrophages, significantly increased in AdmIFN- $\beta$ -injected lesions, were depleted by the antibodies. The therapy stimulated expression of the inducible nitric oxide synthase, down-regulated transforming growth factor- $\beta 1$  and interleukin-8, reduced microvessel density, and resulted in apoptosis of endothelial cells in the lesions. These effects of AdmIFN- $\beta$  were partially diminished in mice treated with the antibodies.

**Conclusions:** These data suggest that macrophages play an important role in IFN- $\beta$  gene therapy and that intralesional delivery of the IFN- $\beta$  gene could be an effective therapy for clinically localized 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). 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). Currently, no optimal treatment exists for clinically localized prostate cancer. Whereas many patients in this group can be cured by radical prostatectomy or radiation therapy, a significant number will develop local recurrence within the prostate, and some ultimately will develop disseminated disease (2). Moreover, both surgery and radiotherapy can have short- and long-term side effects, including injury to the bladder, bowel, and interference with sexual functioning; surgical mortality is  $\sim 1\%$  (3). Therefore, more effective therapies that can cure localized tumors and prevent their metastasis are urgently needed.

IFN- $\beta$ , a type I IFN together with IFN- $\alpha$ , can directly inhibit the growth of tumor cells by inducing differentiation, S phase accumulation, and apoptosis (4–6). Whereas IFN- $\beta$  and IFN- $\alpha$  function through the same receptor (7), prostate cancer cells and cells of several other types of tumor are more sensitive to IFN- $\beta$  than to IFN- $\alpha$  (5, 8–10). IFN- $\beta$  is a potent antiangiogenic and antimetastatic molecule. Not only does IFN- $\beta$  inhibit growth and migration of endothelial cells (11, 12), as other angiogenic inhibitors do, but it also down-regulates expression of molecules for angiogenesis and invasion (13–19). However, clinical trials with type I IFNs for most solid tumors showed limited response, possibly because of insufficient accumulation of biologically active IFN- $\beta$  in tumors (20). Indeed, serum contained  $< 8$  units/ml 1 h after a bolus i.v. dose of  $6 \times 10^6$  units of IFN- $\beta$  and  $< 2$  units/ml after an i.m. or s.c. injection. These concentrations are far below those required to suppress tumor cell growth and angiogenesis or to activate the host defense systems. This hypothesis is additionally validated by our previous studies in several murine and human tumor models showing that tumor cells engineered to generate IFN- $\beta$  lost their tumorigenicity and abrogated tumor formation by parental cells (21, 22). Moreover, we and others have shown that intralesional delivery of the IFN- $\beta$  gene suppressed primary tumors, prolong the survival of tumor-bearing mice, and protect mice from a second challenge in syngeneic mice (12, 23–26).

Tumor-infiltrating macrophages can constitute up to 80% of the stroma in solid tumors (27–29). Nonactivated tumor-infiltrating macrophages may promote tumor growth by providing a variety of angiogenic molecules (28, 30, 31). In contrast,

Received 4/4/02; revised 5/22/02; accepted 5/24/02.

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<sup>1</sup> Supported in part by Cancer Center Support Core Grant CA16672 and Grant RPG-98-332 from the American Cancer Society (to Z. D.).

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activated macrophages can discriminate between “self” and “altered self” and, thus, participate in host defense against tumors (32). Our previous studies suggested that macrophages, by expressing the iNOS,<sup>3</sup> play an important role in IFN- $\beta$ -induced host defense against tumors (22, 23, 25, 26). Furthermore, recent studies by others demonstrated that reduced infiltration of tumor-associated macrophages in human prostate cancer is associated with cancer progression (33), and iNOS from tumor-infiltrating macrophages is of major importance in adenovirus-mediated IL-12 *in situ* gene therapy against orthotopic murine prostate cancer (34). These findings prompted us to additionally investigate effects of IFN- $\beta$  gene therapy on growth and metastasis of human prostate cancer cells in macrophage-compromised mice. We show that depletion of macrophages restored the tumorigenicity of IFN- $\beta$ -transduced cells and reduced the efficacy of the IFN- $\beta$  gene therapy. These data provide direct evidence that tumor-infiltrating macrophages are involved in IFN- $\beta$ -induced suppression of tumor growth and metastasis, and suggest that intralesional delivery of the IFN- $\beta$  gene could be an effective therapy for clinically localized human prostate cancer.

## MATERIALS AND METHODS

**Mice.** Specific pathogen-free male athymic BALB/c nude mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). The 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 Agriculture, 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, Ca<sup>2+</sup>, Mg<sup>2+</sup>-free HBSS, and FBS were purchased from M. A. Bioproducts (Walkersville, MD). An mRNA isolation kit was purchased from Invitrogen (San Diego, CA). The AdmIFN- $\beta$  was constructed in our laboratory as described previously (23, 26). AdE/1 (an E1-deleted adenovirus that does not contain an expression cassette) was generously provided by Dr. B. Fang (The University of Texas M. D. Anderson Cancer Center, Houston, TX). AdE/1 and AdmIFN- $\beta$  were propagated in 293 cells and purified by the two-step CsCl<sub>2</sub> gradient centrifugation protocol (35).

**Tumor Cell Inoculation.** The well-defined parental control vector (PC-3MM2-Neo) and murine IFN- $\beta$  gene-transduced (PC-3MM2-mIFN- $\beta$ ) PC-3MM2 human prostate carcinoma cell lines (25, 36–38) were used. The cells were maintained as a monolayer culture in MEM supplemented with 10% FBS, non-essential amino acids, sodium pyruvate, vitamin A, and glutamine. PC-3MM2, PC-3MM2-Neo, and PC-3MM2-mIFN- $\beta$

cells in exponential growth phase were harvested by a 1-min treatment with a 0.25% trypsin-0.02% EDTA solution. The flask was tapped to detach the cells, MEM-10% FBS was added, and the cell suspension was agitated gently to produce a single-cell suspension. The cells were washed and resuspended in HBSS. Only suspensions of single cells with viability exceeding 95% (ascertained by trypan blue exclusion) were used.

Mice were anesthetized with Nembutal and placed in the supine position. The surgical procedure was performed as detailed in our previous study (25, 26). Briefly, a lower midline incision was created and the prostate exposed. A tumor cell suspension ( $2 \times 10^5$  cells in 20  $\mu$ 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 one layer with 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 AdmIFN- $\beta$  in 40  $\mu$ 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-s 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.

**Depletion of Macrophages.** TIB-128 and TIB-166 hybridoma cells, which produce monoclonal antibodies against macrophage-selective Mac-1 and Mac-2 antigens, respectively (39–41), were purchased from American Type Culture Collection (Manassas, VA). We tested whether *i.p.* injections of the antibodies can deplete peritoneal inflammatory macrophages of nude mice. The antibodies were *i.p.* injected at 125  $\mu$ g/injection each (two consecutive injections 3 days apart). One day after the first antibody injection, mice were *i.p.* injected with 1.5 ml of thioglycollate broth (Baltimore Biological Laboratories, Cockeysville, MD), and macrophages in the peritoneal cavity were collected by peritoneal lavage 4 days later. We found that the antibody treatment reduced the number of peritoneal macrophages by ~76% (76%  $\pm$  6%; mean  $\pm$  SE of three experiments). In experiments detailed in “Results,” anti-Mac-1 and anti-Mac-2 antibodies (125  $\mu$ g each/injection) were injected (*i.p.*) 5 days after tumor cell inoculation and twice a week thereafter. For determination of the tumorigenicity in macrophage-compromised mice, tumor cells were inoculated into the prostates of mice 2 days after the first injection of the antibodies.

**Immunohistochemical Staining.** Immunohistochemical analyses were performed as described previously (25, 26). Briefly, the tumor tissues were placed in OCT compounds and snap frozen in liquid nitrogen. Frozen sections (8–10  $\mu$ m) were fixed in cold acetone and treated with 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in methanol (v/v). The treated slides were incubated in blocking solution and then treated with antibodies to the macrophage-specific scavenger receptor (Scav R), iNOS, TGF- $\beta$ 1, and IL-8. The sections were rinsed and incubated with peroxidase-conjugated secondary antibodies. A positive reaction was

<sup>3</sup> The abbreviations used are: iNOS, inducible nitric oxide synthase; AdmIFN- $\beta$ , adenoviral vector-encoding murine IFN- $\beta$ ; FBS, fetal bovine serum; TGF, transforming growth factor; IL, interleukin; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling; PFU, plaque-forming unit(s); MMP, metalloproteinase.

visualized by incubating the slides with stable 3,3'-diaminobenzidine and counterstaining with Mayer's hematoxylin. For immunohistochemical staining using an antibody to the proliferative cell nuclear antigen, paraffin sections of the tumor samples were dewaxed and stained as described for the frozen sections.

**Immunofluorescence Double Staining.** Apoptotic endothelial cells in tumor tissues were examined following a protocol described in our previous study (26). Briefly, frozen tissue sections (8–10  $\mu$ m) were fixed in cold acetone for 5 min, acetone/chloroform (1:1) for 5 min, and acetone for another 5 min. They were then washed with PBS, incubated with protein-blocking solution (PBS-5% normal horse serum and 1% normal goat serum) for 20 min at room temperature, and incubated with a rat antimouse CD31 antibody over 18 h at 4°C. After the slides were rinsed with PBS, they were incubated with Texas Red-conjugated goat antirat antibody for 1 h in the dark. Samples were washed with PBS containing 0.1% Brij and then with PBS. The sections were then stained by the TUNEL method using a kit (Promega, Madison, WI). Briefly, sections were fixed with 4% paraformaldehyde (methanol-free), washed with PBS, and then incubated with 0.2% Triton X-100 for 15 min at room temperature. After the samples were washed twice with PBS, they were incubated in equilibration buffer (from the kit) for 10 min. The equilibration buffer was drained, and reaction buffer containing equilibration buffer, nucleotide mix, and TdT enzyme was added and incubated in a humid atmosphere at 37°C for 1 h in the dark. The reaction was terminated by immersing the samples in 2 $\times$  SSC for 15 min. Samples were washed with PBS to remove unincorporated fluorescein-dUTP.

**Image Analysis.** Stained sections were examined under a Zeiss photomicroscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a three-chip-charged coupled device color camera (model DXC-960 MD; Sony Corp., Tokyo, Japan). The images were analyzed using Optimas image analysis software (version 5.2; Bothell, WA). The slides were prescreened to determine the range in staining intensity of the slides to be analyzed. Images covering the range of staining intensities were captured electronically. A color bar (montage) was created, and a threshold value was set in the red, green, and blue modes of the color camera. All of the subsequent images were quantified based on this threshold. Positively stained cells in five areas of 1-mm<sup>2</sup> at the center or edge of the tumor were counted from each slide, calculated, and presented as mean  $\pm$  SD.

**mRNA Isolation and Northern Blot Analyses.** The mRNA was isolated from tumor tissue using a Fast Track kit (Invitrogen) and analyzed by Northern blotting as described in our previous studies (25, 26, 42). One  $\mu$ g of mRNA was fractionated on 1% denaturing formaldehyde/agarose gels, transferred to Gene Screen nylon membrane (DuPont Co., Boston, MA), and UV cross-linked with 120,000  $\mu$ J/cm<sup>2</sup> using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Hybridization with cDNA probes was performed as described. Filters were washed two to three times at 50° to 60°C with 30 mM NaCl/3 mM sodium citrate (pH 7.2)/0.1% SDS. The DNA probes used were cDNA fragments corresponding to murine IFN- $\beta$ , human IL-8, human TGF- $\beta$ 1, and human  $\beta$ -actin.

**Statistical Analysis.** The gene therapy experiments were performed with 5–10 mice/group and were repeated at least once. Differences in tumor incidence between treatment and control groups were analyzed with the  $\chi^2$  test. Differences in tumor weight among study and control 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.

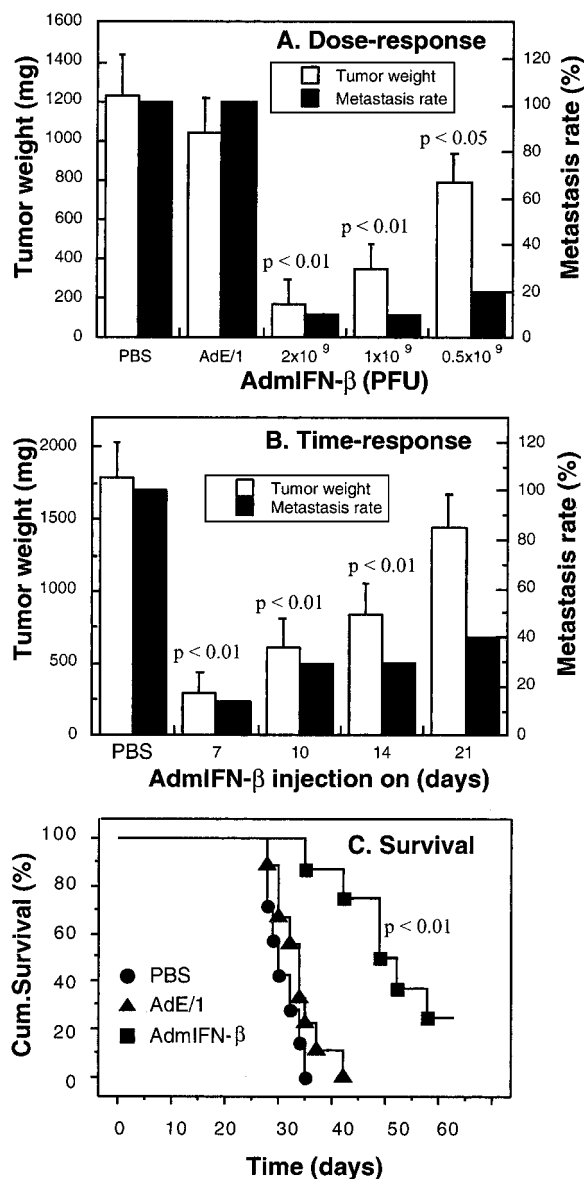
## RESULTS

**Efficacy of IFN- $\beta$  Gene Therapy for Orthotopic PC-3MM2 Tumors in Nude Mice.** Our previous study shows that intratumoral injection of AdmIFN- $\beta$  significantly suppresses growth of PC-3MM2 tumors in the subcutis and prostate of nude mice (26). We additionally investigated efficacy of AdmIFN- $\beta$  therapy in the orthotopic model, focusing on dose-dependent responses of the therapy and effects of the therapy on large tumors, existing metastases, and survival of tumor-bearing mice.

PC-3MM2 cells ( $2 \times 10^5$ ) were implanted into the dorsal prostatic lobes. Seven days later, prostate tumors were treated by a single intralesional injection with PBS or AdE/1 ( $2 \times 10^9$  PFU), or different doses of AdmIFN- $\beta$ . As shown in Fig. 1A, a single injection with AdE/1 did not affect the growth of PC-3MM2 tumors. In contrast, progression of PC-3MM2 tumors was suppressed by 85, 70, and 36% in mice treated with  $2 \times 10^9$ ,  $1 \times 10^9$ , and  $0.5 \times 10^9$  PFU of AdmIFN- $\beta$ , respectively. In addition, the injection with  $2 \times 10^9$  PFU of AdmIFN- $\beta$  eradicated tumors in 20% of mice (2 of 10), confirming the finding in our previous report (26). Regional lymph node metastases, identified on H&E-stained slides, were observed in 10% of mice treated with PBS or AdE/1, and in 10, 15, and 25% of mice treated with  $2 \times 10^9$ ,  $1 \times 10^9$ , and  $0.5 \times 10^9$  PFU of AdmIFN- $\beta$ , respectively (Fig. 1A).

To determine whether AdmIFN- $\beta$  therapy affects growth of large tumors and established metastases, AdmIFN- $\beta$  ( $2 \times 10^9$  PFU) was injected on days 7, 10, 14, or 21 after tumor cell inoculation. At each time point, 10 mice were killed, and prostatic tumors and lymph node metastases examined (Table 1). Lymph node metastases were observed on day 7 and mostly occurred between days 14 and 21 after the tumor cell inoculation. Data in Fig. 1B show that growth of PC-3MM2 tumors was suppressed by 80, 65, 55, and 20%, and the incidence of lymph node metastasis rate was 1 of 10, 3 of 10, 3 of 10, and 4 of 10 in mice treated with AdmIFN- $\beta$  on days 7, 10, 14, and 21, respectively. Therefore, the therapy was effective for both small and large tumors. More interestingly, the therapy not only prevented metastasis, but it also eradicated established metastatic lesions in some mice (Fig. 1B).

Next we determined whether AdmIFN- $\beta$  alters the survival of tumor-bearing mice. PC-3MM2 cells were inoculated into the prostates of nude mice. Seven days later, PBS, AdE/1 ( $2 \times 10^9$  PFU), or AdmIFN- $\beta$  ( $2 \times 10^9$  PFU) was injected into the tumors. The mice were killed when they were moribund. The data in Fig. 1C demonstrate that therapy with AdmIFN- $\beta$  significantly prolonged the survival of mice with PC-3MM2 prostate tumors as compared with mice treated with PBS or AdE/1. The 50% of survival for mice receiving PBS, AdE/1, and AdmIFN- $\beta$  were 31, 35, and 50 days, respectively.



**Fig. 1** AdmIFN- $\beta$  therapy against PC-3MM2 tumors in the prostate of nude mice. PC-3MM2 cells ( $2 \times 10^5$ /mouse) were inoculated into the prostate of nude mice. **A**, 7 days later, mice were treated by an intralethral injection with PBS; or  $2 \times 10^9$  PFU of AdE/1; or 2, 1, or 0.5  $\times 10^9$  PFU of AdmIFN- $\beta$ . **B**,  $2 \times 10^9$  PFU of AdmIFN- $\beta$  was intralethally injected on days 7, 10, 14, or 21 after the tumor cell inoculation. **C**, PBS or  $2 \times 10^9$  PFU of AdE/1 or AdmIFN- $\beta$  were injected into PC-3MM2 tumors 7 days after the inoculation of tumor cells. On days 30–35 (**A** and **B**) or when they were moribund (**C**), the mice were killed. The prostate tumors were removed and weighed, and lymph node metastases were determined in H&E-stained paraffin sections. Tumor weight included prostate weight; normal prostate weights  $45 \pm 8$  mg. Data shown are mean of 10 mice (**A** and **B**) and 7 mice (**C**) from one representative experiment of two; bars,  $\pm$  SD.

**Macrophages Are Involved in IFN- $\beta$ -induced Suppression of Growth and Metastasis of PC-3MM2 Cells in the Prostate of Nude Mice.** Next, we studied the role of macrophages in suppressing the orthotopic tumors by AdmIFN- $\beta$

**Table 1** Growth and metastasis of PC-3MM2 tumors in the prostate of nude mice

Days after inoculation	Prostate tumor weight (mg)	Lymph node met incidence (%)
7	$60 \pm 10^{a,b}$	10
10	$140 \pm 20$	20
14	$215 \pm 55$	40
21	$755 \pm 150$	70

<sup>a</sup> Including prostate weight; normal mouse prostate weights  $45 \pm 5$  mg.

<sup>b</sup> Data shown are mean  $\pm$  SD of 10 mice from one representative experiment of two.

therapy. Because the control adenoviral vector did not significantly alter tumor growth and angiogenesis (23, 26), we compared effects of AdE/1 and AdmIFN- $\beta$  on PC-3MM2 tumors only. Seven days after the implantation of PC-3MM2 cells,  $2 \times 10^9$  PFU of AdE/1 or AdmIFN- $\beta$  were injected into the tumors. Macrophage-selective anti-Mac-1 and anti-Mac-2 antibodies (125  $\mu$ g each/mouse) or IgG were injected (i.p.) 2 days before the therapy and twice a week thereafter. Mice were necropsied on day 28 after the tumor cell inoculation. Data in Table 2 show the therapeutic effects of AdmIFN- $\beta$  were significantly reduced in mice treated with the antibodies. AdmIFN- $\beta$ -injected tumors in IgG- and anti-Mac-1/anti-Mac-2-treated mice were  $280 \pm 98$  mg versus  $685 \pm 105$  mg, and the incidences of lymph node metastasis were 10% versus 40%, respectively.

We reported previously that the injection site of PC-3MM2-mIFN- $\beta$  cells is densely infiltrated by macrophages and that PC-3MM2 cells are susceptible to macrophage-mediated cytotoxicity, suggesting that macrophages are involved in abrogating the tumorigenicity of PC-3MM2-mIFN- $\beta$  cells (25). To establish whether macrophages are necessary for suppressing the tumorigenicity of PC-3MM2-mIFN- $\beta$  cells, growth and metastasis of PC-3MM2-mIFN- $\beta$  cells were evaluated in macrophage-compromised mice. Because there is no significant difference in tumorigenicity between PC-3MM2 parental cells and PC-3MM2-Neo cells (25), we used PC-3MM2-Neo and PC-3MM2-mIFN- $\beta$  cells in the present study. PC-3MM2-Neo and PC-3MM2-mIFN- $\beta$  cells ( $2 \times 10^5$ /mouse) were injected into the prostates of nude mice. Two days before the tumor cell inoculation, mice were injected i.p. with normal rat IgG or anti-Mac-1 and anti-Mac-2 antibodies (125  $\mu$ g each/mouse); injections were given twice a week thereafter. As shown in Table 3, PC-3MM2-Neo cells produced large tumors and regional lymph node metastases in all of the mice, whereas PC-3MM2-mIFN- $\beta$  cells did not. Treatment of mice by i.p. injection of macrophage-selective Mac-1 and Mac-2 antibodies, but not normal rat IgG, significantly increased the tumorigenicity of PC-3MM2-mIFN- $\beta$  cells. The cells produced prostatic tumors and lymph node metastases in 80% and 20% of mice, respectively (Table 3). Tumors formed by PC-3MM2-mIFN- $\beta$  cells were smaller than those produced by PC-3MM2-Neo cells (Table 3), indicating that mechanisms independent of macrophages are also involved in the IFN- $\beta$ -induced suppression of tumorigenicity. Depletion of macrophages moderately reduced the weight of tumors formed by PC-3MM2-Neo cells, suggesting that tumor-infiltrating macrophages promote tumor growth (43–45).

Table 2 Effects of anti-Mac-1 and anti-Mac-2 antibodies on the therapeutic effects of AdmIFN- $\beta$ 

Vectors	Antibodies	Primary tumors		
		Incidence (%)	Weight (mg) <sup>a</sup>	Lymph node metastasis incidence (%)
AdE/1	IgG	100	1370 $\pm$ 225	100
AdE/1	Anti-Mac-1/2	100	1120 $\pm$ 250	100
AdmIFN- $\beta$	IgG	80	280 $\pm$ 98 <sup>b</sup>	10
AdmIFN- $\beta$	Anti-Mac-1/2	100	685 $\pm$ 112 <sup>c</sup>	40

<sup>a</sup> PC-3MM2 cells ( $2 \times 10^5$ /mouse) were inoculated into the prostates of nude mice. Seven days later, mice were treated by an intrasplenic injection with  $2 \times 10^9$  PFU of AdE/1 or AdmIFN- $\beta$ . Anti-Mac-1 and anti-Mac-2 antibodies (125  $\mu$ g/mouse) or IgG were injected (i.p.) on day 5 after the tumor cell inoculation and twice a week thereafter. The experiment was terminated on day 28. The prostate tumors were removed and weighed, and lymph node metastases were determined in H&E-stained paraffin section. Normal mouse prostate weighs  $45 \pm 8$  mg. Data shown are mean  $\pm$  SD of 10 mice from representative experiment of three.

<sup>b</sup>  $P < 0.001$ , in comparison with AdE/1-treated tumors in mice injected with IgG.

<sup>c</sup>  $P < 0.05$ , in comparison with AdmIFN $\beta$ -treated tumors in mice injected with IgG.

Table 3 Tumorigenicity of PC-3MM2 cells in mice treated with anti-Mac-1 and anti-Mac-2 antibodies

Tumors	Treatment	Primary tumors		
		Incidence (%)	Weight (mg) <sup>a</sup>	Lymph node metastasis incidence (%)
PC-3MM2-Neo	IgG	100	1326 $\pm$ 205	100
PC-3MM2-Neo	Anti-Mac-1/2	100	1043 $\pm$ 180 <sup>b</sup>	90
PC-3MM2-mIFN- $\beta$	IgG	0 <sup>c</sup>	43 $\pm$ 12 <sup>c</sup>	0
PC-3MM2-mIFN- $\beta$	Anti-Mac-1/2	80 <sup>d</sup>	155 $\pm$ 105 <sup>d</sup>	20

<sup>a</sup> PC-3MM2-Neo or PC-3MM2-mIFN- $\beta$  cells ( $2 \times 10^5$  cells/mouse in 40  $\mu$ l of HBSS) were inoculated into the prostates of nude mice. Control IgG or anti-Mac-1 and anti-Mac-2 antibodies (125  $\mu$ g/injection) were i.p. injected 2 days before the tumor cell inoculation and twice a week after the inoculation. The tumor incidence, prostate weight, and incidence of aortic lymph node metastasis were determined 28 days later. Data shown are mean  $\pm$  SD of 10 mice from one representative experiment of two. Including prostate weight; normal prostate weighs  $45 \pm 8$  mg.

<sup>b</sup>  $P < 0.05$ , in comparison with PC-3MM2-neo tumors in IgG injected mice.

<sup>c</sup>  $P < 0.001$ , in comparison with PC-3MM2-neo tumors in IgG injected mice.

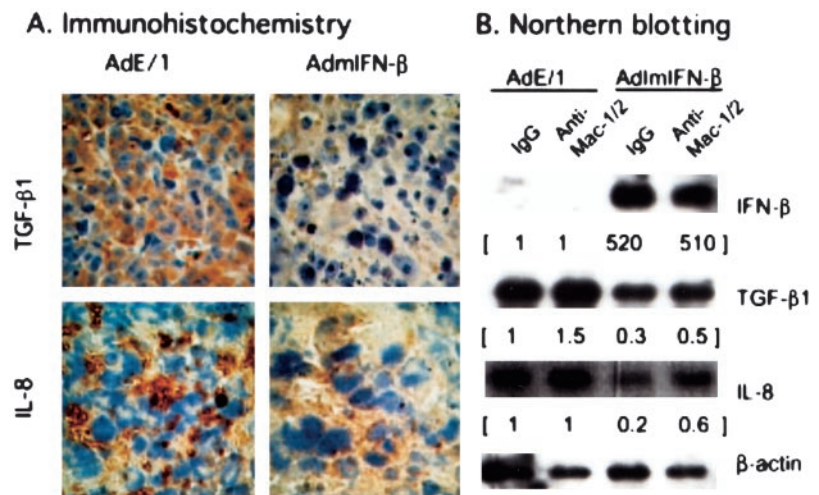
<sup>d</sup>  $P < 0.01$ , in comparison with PC-3MM2-mIFN- $\beta$  tumors in IgG injected mice.

### Macrophages and iNOS in AdmIFN- $\beta$ -induced Suppression of Tumor Angiogenesis.

AdmIFN- $\beta$  therapy inhibits expression of TGF- $\beta$ 1 and bFGF in PC-3MM2 tumors (26). Because of the importance of IL-8 in progression of prostate cancer (46–48), we additionally examined effects of the therapy on expression of TGF- $\beta$ 1 and IL-8. PC-3MM2 cells ( $2 \times 10^5$ ) were implanted into the prostates of nude mice and injected with AdE/1 or AdmIFN- $\beta$  ( $2 \times 10^9$  PFU) on day 7 after the tumor cell implantation. On days 5, 7, and 10 after the tumor cell implantation, mice were i.p. injected with either IgG or the anti-Mac-1 and anti-Mac-2 antibodies. Tumor samples were collected 5 days after the virus injection and analyzed by immunohistochemical staining and Northern blotting. As shown in Fig. 2A (immunohistochemical staining), AdE/1-treated PC-3MM2 tumors expressed high levels of TGF- $\beta$ 1 and IL-8, which was significantly reduced by AdmIFN- $\beta$ . Fig. 2B (Northern blotting) shows that murine IFN- $\beta$  mRNA was detectable only in tumors injected with AdmIFN- $\beta$ , which was not affected by the administration of the anti-Mac-1/anti-Mac-2 antibodies. Consistent with the data in immunohistochemical staining, high levels of TGF- $\beta$ 1 and IL-8 mRNA were detected in tumors injected with AdE/1. The expression of TGF- $\beta$ 1 and IL-8 was reduced by 70% and 80%, respectively, in AdmIFN- $\beta$ -injected tumors (Fig. 2B). Treatment with the antibodies partially blocked the inhibitory effect of AdmIFN- $\beta$  on the expression of TGF- $\beta$ 1 and IL-8 (Fig. 2B). H&E staining revealed some necrotic

areas in tumors treated with AdmIFN- $\beta$  but not in those treated with AdE/1 (Fig. 3). Tumors injected with AdmIFN- $\beta$  contained more infiltrating macrophages revealed by immunohistochemical staining using the macrophage-specific scavenger receptor antibody (49). Tumors from mice i.p. injected with the anti-Mac-1 and anti-Mac-2 antibodies contained no cells that could be stained by the scavenger receptor antibody (Fig. 3). The expression of iNOS was found only in tumors injected with AdmIFN- $\beta$ , and the intensity of iNOS staining was reduced significantly in tumors from anti-Mac-1/anti-Mac-2-treated mice (Fig. 3). Tumors injected with AdE/1 contained more CD31<sup>+</sup> cells (stained red) than did those injected with AdmIFN- $\beta$ . Tumors injected with AdmIFN- $\beta$  but not AdE/1 stained positive by the TUNEL methods (green reaction). Some cells in AdmIFN- $\beta$ -treated tumors were stained by both the TUNEL (green color) and anti-CD31 antibody (red color), yielding the yellow color in a superimposed image (Fig. 3), which suggests that they were apoptotic endothelial cells. The microvessel density was lower in AdmIFN- $\beta$ -injected tumors than in the AdE/1-injected ones, and this effect of AdmIFN- $\beta$  was also reduced in mice treated with the anti-Mac-1/anti-Mac-2 antibodies (Fig. 3). The microvessel densities in tumors injected with AdE/1 and AdmIFN- $\beta$  from normal mice were  $261 \pm 111/\text{mm}^2$  and  $79 \pm 26/\text{mm}^2$ , respectively, and the densities in tumors injected with AdE/1 and AdmIFN- $\beta$  from the antibody-

**Fig. 2** Expression of TGF- $\beta$ 1 and IL-8 in PC-3MM2 tumors. PC-3MM2 cells ( $2 \times 10^5$ /mouse) were inoculated into the prostate of nude mice. On days 5 and 8 after the tumor cell inoculation, 125  $\mu$ g/mouse of anti-Mac-1/2 antibodies or IgG were i.p. injected. On day 7 after the tumor cell implantation, the tumors were injected with  $2 \times 10^9$  PFU of AdE/1 or AdmIFN- $\beta$ . On day 10 after the tumor cell inoculation, tumor samples were collected. **A**, frozen sections were prepared for immunohistochemical staining using antibodies to human TGF- $\beta$ 1 and IL-8 (Santa Cruz Biotechnologies, Santa Cruz, CA). **B**, mRNA was prepared and analyzed by Northern blotting using cDNA probes specific to murine IFN- $\beta$ , human TGF- $\beta$ 1, human IL-8, and human  $\beta$ -actin.



treated mice were  $232 \pm 156/\text{mm}^2$  and  $177 \pm 70/\text{mm}^2$ , respectively.

## DISCUSSION

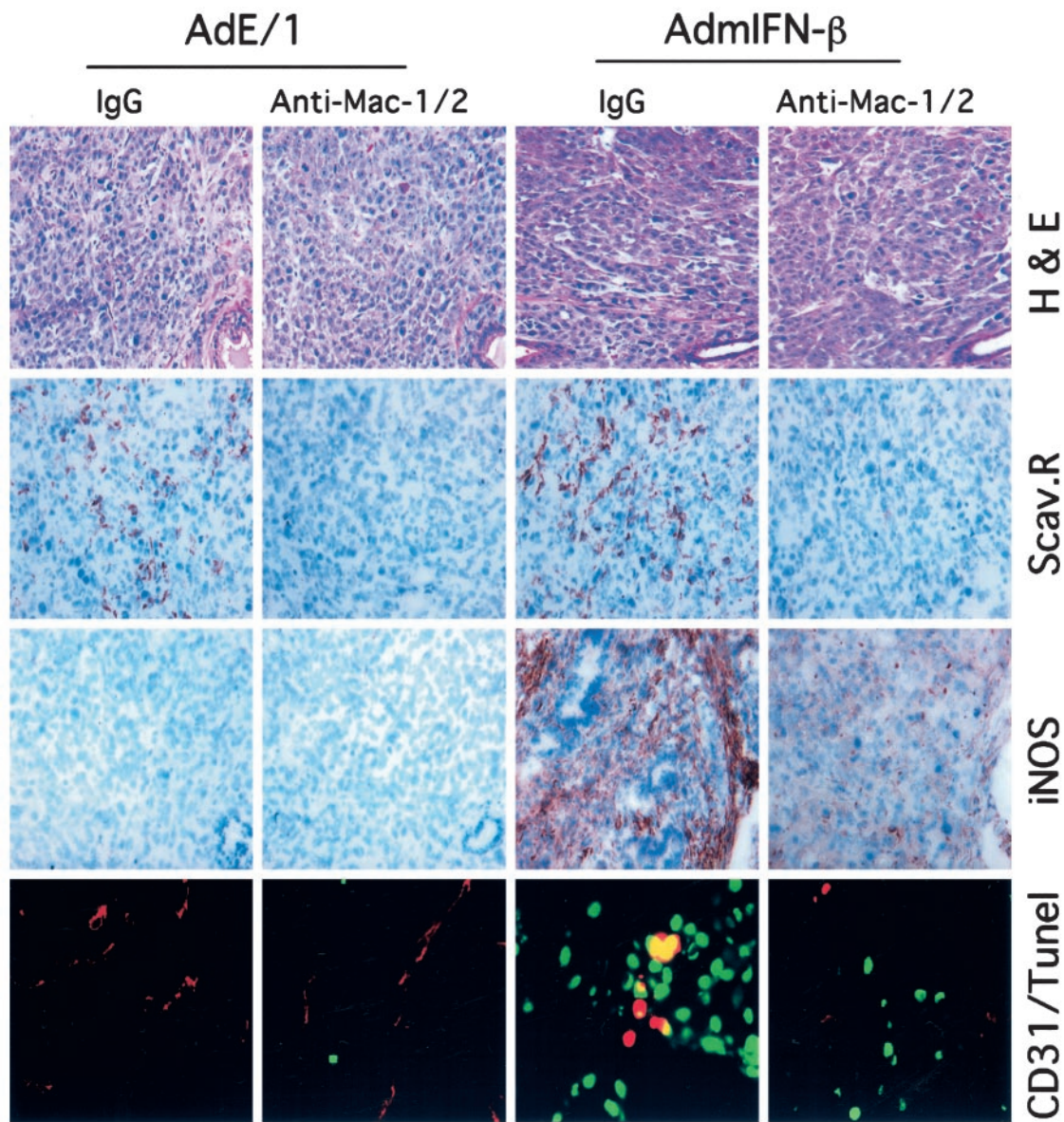
Our previous study showed that intralesional delivery of AdmIFN- $\beta$ , but not control vector AdLacZ, suppresses the growth of PC-3MM2 tumors in the prostates of nude mice. Data presented here confirm and extend that finding. We have demonstrated that AdmIFN- $\beta$  suppressed prostatic PC-3MM2 tumors in a dose-dependent manner. Although the therapy was most effective for smaller tumors, it also suppressed the growth of larger tumors. Furthermore, the therapy not only prevented metastasis to the regional lymph nodes but also eradicated established metastatic lesions in some mice. Finally, AdmIFN- $\beta$  significantly prolonged the survival of tumor-bearing mice. These data suggest that intralesional delivery of the IFN- $\beta$  gene could be an effective therapy for clinically localized human prostate cancer, especially for those patients with a high risk of the presence of micrometastases, which cannot be accurately diagnosed by currently available technique, or cured by radical prostatectomy or radiation therapy.

IFN- $\beta$  is a potent macrophage activator that induces iNOS expression and tumoricidal function of the cells (50, 51). Our previous studies indicated that the amounts of IFN- $\beta$  released by IFN- $\beta$  gene-engineered UV-2237m fibrosarcoma cells (22) and PC-3MM2 cells (25) were sufficient to activate macrophage-mediated cytotoxicity *in vitro*. In addition, indirect evidence from the therapy studies in mice suggests that macrophages and iNOS play important roles in AdmIFN- $\beta$ -induced suppression of tumor progression in both immune-competent mice (23) and T-cell-deficient nude mice (26). In the present study, we additionally examined the role of macrophages in IFN- $\beta$ -induced suppression of tumor growth and metastasis in mice in which macrophages were depleted by using macrophage-selective monoclonal antibodies. The data show that the tumorigenicity of IFN- $\beta$ -transduced cells is significantly increased, and the therapeutic effects of AdmIFN- $\beta$  are reduced in macrophage-compromised mice. These findings are the first direct evidence

that tumor-infiltrating macrophages play important roles in IFN- $\beta$ -induced host defense against tumors.

Immunofluorescence double staining showed that AdmIFN- $\beta$  therapy induces apoptosis in endothelial cells and reduces microvessel density in PC-3MM2 tumors. Therefore, suppression of tumor angiogenesis appears to be one of the mechanisms by which AdmIFN- $\beta$  inhibits the progression of PC-3MM2 tumors in nude mice. Suppression of tumor angiogenesis may result from the down-regulation of TGF- $\beta$ 1 expression by AdmIFN- $\beta$ . Up-regulation of TGF- $\beta$ 1 in prostate cancer tissues and high urinary and serum levels of TGF- $\beta$ 1 are associated with enhanced tumor angiogenesis and tumor metastasis, and, in turn, with poor clinical outcome (52–56). Tumor cells enforced overexpression with TGF- $\beta$ 1 are found to be growth inhibited *in vitro* but produce larger, less necrotic, and more metastatic tumors than do control cells in mice (56, 57). TGF- $\beta$ 1 has been shown to promote angiogenesis in variety of murine models (52, 57–59). The mechanisms by which TGF- $\beta$ 1 promotes angiogenesis remain obscure. However, it has been noted that TGF- $\beta$ 1-induced angiogenesis is often associated with inflammatory infiltrations, and blockade of TGF- $\beta$  function simultaneously inhibits the inflammation and the angiogenesis (60).

IL-8, originally discovered as a chemokine (61), is a multifunctional cytokine that promotes tumor angiogenesis. The promotion of angiogenesis is mediated by inducing MMPs in human prostate cancer cells (62) and by recruiting inflammatory cells, which in turn produce a plethora of angiogenic molecules (30, 63, 64) and, hence, promote tumor growth and metastasis (38, 48). In human prostate cancer, IL-8 serum concentrations have been correlated with increasing prostate cancer stage, and they can differentiate benign prostatic hyperplasia from clinical stages A, B, C, or D (47). IL-8 is overexpressed in PC-3MM2 prostate cancer cells and is correlated with their metastatic potential (38). Prostate cancer cells enforced to express IL-8 produce higher levels of MMPs, are more invasive in the Matrigel assay, and are more tumorigenic in mice. In contrast, antisense IL-8-transfected cells express lower levels of IL-8 and



**Fig. 3** Potential role of macrophages and iNOS in tumor angiogenesis. PC-3MM2 cells ( $2 \times 10^5$ /mouse) were inoculated into the prostate of nude mice. On days 5 and 8 after the tumor cell inoculation, 125  $\mu$ g/mouse of anti-Mac-1/2 antibodies or IgG were i.p. injected. On day 7 after the tumor cell implantation, the tumors were injected with  $2 \times 10^9$  PFU of AdE/1 or AdmIFN- $\beta$ . On day 10 after the tumor cell inoculation, tumor samples were collected. Paraffin sections were prepared for H&E staining. Snap-frozen sections were prepared for immunohistochemical staining using antibodies to scavenger receptor (*Scav R*) and iNOS and for the immunofluorescent double staining with CD31 antibody and the TUNEL method.

MMPs, and become less invasive *in vitro* and less tumorigenic in mice (62). Moreover, neutralizing antibody to IL-8 inhibits the angiogenic activity of prostate cancer homogenates and reduces progression of prostate cancer cells in mice (65). AdmIFN- $\beta$  therapy down-regulates IL-8 expression in PC-3MM2 cells, and this also may contribute to its therapeutic effects. However, additional studies are needed to elucidate how AdmIFN- $\beta$  suppresses IL-8 expression.

The iNOS is the major cytotoxic molecule generated by iNOS in activated macrophages (66, 67). Our previous study

(26) and data presented here suggests that nitric oxide may play an important role in suppressing the expression of TGF- $\beta$ 1, bFGF, and IL-8 in AdmIFN- $\beta$  therapy. The administration of anti-Mac-1/anti-Mac-2 antibodies depleted cells that can be recognized by macrophage-selective scavenger receptor antibody in PC-3MM2 tumors. However, iNOS was still detected, albeit at much lower levels. Moreover, the treatment only moderately blocked the inhibitory effects of IFN- $\beta$  on the expression of TGF- $\beta$ 1 and IL-8. Finally, the antibody treatment depletes  $\sim$ 75% of peritoneal inflammatory macro-

phages (see "Materials and Methods"). These data suggest that the anti-Mac-1/anti-Mac-2 antibodies may destroy only a subpopulation of scavenger receptor-positive tumor-infiltrating macrophages. Alternatively, IFN- $\beta$  may induce iNOS expression in other tumor stroma cells. Finally, the AdmIFN- $\beta$  therapy may inhibit the expression of these angiogenic molecules by some macrophage- and nitric oxide-independent mechanisms.

In this orthotopic xenograft model, we demonstrated that macrophages are involved in the *in situ* IFN- $\beta$  gene therapy against tumors of human prostate cancer. However, it has been suggested that nude mice are not only deficient in T-cell activity, but they also have exaggerated and potentially non-physiological responses of natural killer cells and macrophages. In addition, the potential of macrophages to serve as antigen-presenting cells and thus to elicit a more robust specific adaptive immune response cannot be appreciated in this model. These parameters should be additionally tested in models of murine prostate cancer in immune-competent mice.

It is noteworthy that intralesional injection of AdmIFN- $\beta$  eradicated existing lymph node metastases in some mice. This could be because of the circulation of AdmIFN- $\beta$ , AdmIFN- $\beta$ -transduced-PC-3MM2 cells, and/or IFN- $\beta$  from primary tumors into the metastatic lesions in the drainage lymph nodes. Whatever the mechanisms are, this result suggests that IFN- $\beta$  gene therapy has the potential to eliminate small distant tumors, an outcome that would be superior to the results obtained from radical prostatectomy or radiation therapy when applied to clinically localized prostate cancer.

## ACKNOWLEDGMENTS

We thank Dr. Isaiah J. Fidler for advices on our research, Dr. Corazon D. Bucana, Donna Reynolds, and Yunfang Wang for technical assistance with immunohistochemical staining, Walter Pagel for editorial comments, and Patherine Greenwood for excellent preparation of the manuscript.

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