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Suppression of Angiogenesis, Tumorigenicity, and Metastasis by Human Prostate Cancer Cells Engineered to Produce Interferon-β[1]

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ABSTRACT

We determined whether the *IFN-β* gene can be used to suppress angiogenesis, tumor growth, and metastasis of human prostate cancer cells growing in the prostate of nude mice. Highly metastatic PC-3M human prostate cancer cells were engineered to constitutively produce murine IFN-β subsequent to infection with a retroviral vector containing murine IFN-β cDNA. Parental (PC-3M-P), control vector-transduced (PC-3M-Neo), and IFN-β-transduced (PC-3M-IFN-β) cells were injected into the prostate (orthotopic) or subcutis (ectopic) of nude mice. PC-3M-P and PC-3M-Neo cells produced rapidly growing tumors and regional lymph node metastases, whereas PC-3M-IFN-β cells did not. PC-3M-IFN-β cells also suppressed the tumorigenicity of bystander nontransduced prostate cancer cells. PC-3M-IFN-β cells produced small tumors (3–5 mm in diameter) in nude mice treated with anti-asialo G_{M1} antibodies and in severe combined immunodeficient/Beige mice. Immunohistochemical staining revealed that PC-3M-IFN-β tumors were homogeneously infiltrated by macrophages, whereas control tumors contained fewer macrophages at their periphery. Most tumor cells in the control tumors were stained positive by an antibody to proliferative cell nuclear antigen; very few were positively stained by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling. In sharp contrast, PC-3M-IFN-β tumors contained fewer proliferative cell nuclear antigen-positive cells and many terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling-positive cells. Staining with antibody against CD31 showed that control tumors contained more blood vessels than PC-3M-IFN-β tumors. PC-3M-IFN-β cells were more sensitive to lysis mediated by natural killer cells *in vitro* or to cytostasis mediated by macrophages than control transduced cells. Conditioned medium from PC-3M-IFN-β cells augmented splenic cell-mediated cytolysis to control tumor cells, which could be neutralized by antibody against IFN-β. Collectively, the data suggest that the suppression of tumorigenicity and metastasis of PC-3M-IFN-β cells is due to inhibition of angiogenesis and activation of host effector cells.

INTRODUCTION

Prostate cancer is the leading cause of death in men in North America. Over 317,100 newly diagnosed cases and 41,400 cancer-related deaths are now reported annually (1). Improvements in early diagnosis have identified an increasing number of patients with organ-confined lesions, which has led to increases in the number of radical prostatectomies. Whether radical prostatectomies or other forms of local therapy have improved overall survival is not clear, however, because most deaths from this cancer are due to metastases that are resistant to conventional therapy (2).

The progressive growth and metastasis of neoplasms is dependent on the development of adequate vasculature, *i.e.*, angiogenesis (3–5). The extent of angiogenesis is determined by the local balance between positive and negative regulating molecules (3–5); the major positive angiogenic molecules are basic fibroblast growth factor (3), vascular endothelial growth factor, and interleukin 8 (3–8). Major negative regulators of angiogenesis include thrombospondin (9), angiostatin (10–12), and the IFNs α and β (13).

The IFNs are a family of natural glycoproteins that consist of IFN-α, -β, and -γ. The antiviral activity of IFNs led to their discovery (14), but later data revealed that they also control cell growth and differentiation (15), inhibit expression of oncogenes (16), and activate T lymphocytes, natural killer cells, and macrophages (17, 18).

The efficacy of IFN therapy for various malignancies has been investigated for many years. Extensive clinical trials have concluded that the IFNs can be efficacious against many hematopoietic neoplasms (19–23) and some vascular tumors, such as pulmonary hemangiomatosis (24), infantile hemangiomas (25), Kaposi's sarcoma (26), and malignant hemangiopericytoma (27). Work from our laboratory and others has suggested that regression was due to inhibition of angiogenesis (28). Specifically, the continuous incubation of different human carcinoma cells with noncytostatic concentrations of IFN-α or IFN-β down-regulated transcription and protein production of basic fibroblast growth factor (13, 28, 29), interleukin 8 (30, 31), and collagenase type IV (32, 33), all of which are involved in the angiogenic response. However, IFNs are not effective in treatment of most solid tumors (19–23). Pharmacokinetic studies have demonstrated that the half-life of IFNs in the circulation of patients is on the order of 5 min (34). The resulting lack of sustained levels (21, 34) may have been responsible for the failure to inhibit or eradicate tumors.

In the present study, we tested the hypothesis that the local, continuous production of IFN in growing tumors would suppress growth and metastasis of human prostate cancer cells in mice. Suppression of tumor growth and metastasis by IFN-α has been documented in many tumor models (24–28, 35–37). Because IFN-β is more potent than IFN-α in inhibiting the proliferation of human prostate cancer cells (38), we focused our study on the inhibition of growth and metastasis of human prostate cancer cells by IFN-β. We demonstrated that both orthotopic and ectopic growth of PC-3M cells engineered to produce IFN-β were significantly reduced. We also showed a bystander effect of IFN-β cells on the tumorigenicity of parental cells. The inhibitory effect of IFN-β on tumor growth and metastasis was mediated by activation of tumoricidal host effector cells and by suppression of tumor angiogenesis.

MATERIALS AND METHODS

Mice

Specific, pathogen-free, male athymic Ner-*nu/nu* mice were purchased from the Animal Production Area, National Cancer Institute-Frederick Cancer Re-

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search Facility (Frederick, MD). Male SCID[3]/Beige mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were maintained under specific pathogen-free conditions in a facility approved by the American Association for Accreditation of Laboratory Animal Care. The care and experimental procedures were in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and NIH. The mice were used according to institutional guidelines when they were 8–12 weeks of age.

Reagents

EMEM, Ca^{2+}- and Mg^{2+}-free HBSS and FBS were purchased from M. A. Bioproducts (Walkersville, MD). Murine IFN-β was purchased from Lee BioMolecular Co. (San Diego, CA). Murine recombinant IFN-γ (specific activity, 5.2×10^6 units/mg protein) was the generous gift of Genentech, Inc. (South San Francisco, CA). Phenol-extracted *Salmonella* LPS was purchased from Sigma Chemical Co. (St. Louis, MO). All reagents used in tissue culture except LPS were free of endotoxin as determined by Limulus amebocyte lysate assay (sensitivity limit of 0.125 ng/ml) purchased from Associates of Cape Cod (Falmouth, MA). [^{3}H]Thymidine (specific activity, 2 Ci/mmol) was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA).

Cells and Culture Conditions

PC-3M human prostate carcinoma cells (39, 40) were maintained as adherent monolayers in EMEM supplemented with 10% FBS, nonessential amino acids, sodium pyruvate, vitamin A, and glutamine (CMEM). PC-3M cells in their exponential growth phase were harvested by a 1-min treatment with a 0.25% trypsin-0.02% EDTA solution (v/v). The flask was tapped to detach the cells, CMEM was added, and the cell suspension was gently agitated to produce a single-cell suspension. The cells were washed in CMEM and resuspended in HBSS. Viability was determined by staining with trypan blue, and only suspensions of single cells with viability exceeding 90% were used.

Construction of Retroviral Vector Encoding Murine IFN-β

The full coding region of murine IFN-β cDNA (generously provided by Dr. T. Taniguchi, Osaka University, Osaka, Japan) was subcloned into retroviral vector pLXSN (generously provided by Dr. A. D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA; Ref. 41) to generate pLXSN-IFN-β. The retroviral vectors harboring IFN-β or neo genes were introduced into the amphotropic Moloney murine leukemia virus producer cell line PA317 (41) by the calcium phosphate procedure. Transfectant cells were maintained in medium containing 800 μg/ml G418. For virus production, medium was harvested from confluent monolayers of the virus-producing cell lines 24–48 h after a medium change. The medium was filtered through 0.45-μm pore filters and used immediately for infection.

Infection and Selection of Clones

PC-3M cells were infected for 6 h with 2 ml of pLXSN-IFN-β or pLXSN-Neo retroviral supernatants in the presence of Polybrene (final concentration, 8 μg/m1). Fresh CMEM (6 ml) was then added, and the cells were incubated at 37°C for 18 h. Infected cells were selected in medium containing 800 μg/ml G418. After 2 weeks of continuous culture, drug-resistant clones were isolated and expanded. Cells pooled from >20 individual drug-resistant clones were used in the present study. All retrovirus-infected cells tested negative for replication-competent virus. Expression of IFN-β was determined by a bioassay, Western blot, and Northern blot analyses.

Doubling Time

Tumor cells were plated at 5×10^3 cells/well of 24-well plates. After incubation for various lengths of time (1–5 days), the cells were harvested by trypsinization and counted. Doubling time was calculated from the growth curve of the cultures.

Tumor Cell Inoculation

s.c. injection. PC-3M parental (PC-3M-P), PC-3M-Neo, and PC-3M-IFN-β cells ($1–20 \times 10^5$ cells in 100 μl of HBSS) were injected s.c. into each mouse's left and/or right lateral chest wall near the axilla. Tumor diameters were measured with a caliper.

Orthotopic injection. Each mouse was anesthetized with methoxyflurane and placed in the supine position (40). A lower-midline incision was made, and the prostate was exposed. Tumor cell suspensions ($1–20 \times 10^5$ cells in 40 μl of HBSS) were injected into the dorsal prostatic lobes with a 30-gauge needle in 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 (Autoclips; Clay Adams, Parsippany, NJ). Primary tumors (including the prostate) were excised and weighed after the mice were killed by cervical dislocation at days 21–28 after tumor inoculation. Regional lymph node metastasis were assessed by microscopic examination of H&E-stained serial paraffin sections. The tumor samples were collected for H&E staining, mRNA extraction, and immunohistochemical analysis.

Macrophage-mediated Cytostasis Assay

PEMs were collected by peritoneal lavage from mice given an i.p. injection of 1.5 ml of thioglycollate broth (Baltimore Biological Laboratories, Cockeysville, MD) 4 days before harvest. The cells were washed with Ca^{2+}- and Mg^{2+}-free PBS and resuspended in serum-free EMEM; 1×10^5 cells in 0.2 ml of EMEM were plated into 38-mm^2 wells of 96-well, flat-bottomed Microtest III plates (Falcon Plastics, Oxnard, CA). After 90 min, the cells were washed with EMEM to remove nonadherent cells. The resultant macrophage monolayer, which was >95% pure according to morphological and phagocytic criteria, was treated as described in "Results." Macrophage-mediated cytostasis was assessed by measuring the uptake of [^{3}H]thymidine into the tumor cells grown with macrophages. PC-3M-P, PC-3M-Neo, or PC-3M-IFN-β cells (5×10^3) were added onto macrophage monolayers and incubated for 48 h. During the last 20 h, [^{3}H]thymidine was added into each well (0.1 μCi/well). The cells were lysed with 0.1 ml of 0.1 N NaOH. The lysates were harvested with Harvester 96 (Tomtec, Orange, CT) and counted in a liquid scintillation counter. The cytostatic activity of macrophages was calculated as follows: Cytostasis (%) = $(A - B)/A \times 100$, where A = cpm in cultures of tumor cells alone and B = cpm in cultures of macrophages and target cells.

Spleen Cell-mediated Cytotoxicity

Spleen cells from nude mice, nude mice pretreated with anti-asialo G_{M1} serum (40 μl/mouse 48 h before harvesting cells) or normal rabbit serum, and SCID/Beige mice were incubated with [^{3}H]thymidine-labeled PC-3M cells (10^4/well) in 96-well plates for 24 h (42). After a brief spin, 100 μl of the culture supernatants were collected and counted in a liquid scintillation counter. The specific cytolytic activity of the spleen cells was calculated as follows: Cytolysis (%) = $(A - B)/(T - B) \times 100$, where A = cpm in cultures of spleen cells and target cells, B = cpm in cultures of target cells only, and T = total cpm of target cells added into each well.

RNA Isolation and Northern Blot Analyses

The mRNA was extracted using FastTrack kit (Invitrogen, San Diego, CA). For Northern blot analyses, 1 μg of mRNA was fractionated on 1% denaturing formaldehyde/agarose gels, electrotransferred to GeneScreen nylon membrane (DuPont Co., Boston, MA), and UV cross-linked with 120,000 μJ/cm^2 using a UV Stratalinker 1800 (Stratagene). Hybridization using cDNA probes was performed as described (12). Filters were washed two or three times at 50–60°C with 30 mM NaCl/3 mM sodium citrate, pH 7.2/0.1% SDS. The DNA probes used were cDNA fragments corresponding to rat glyceraldehyde-3-phosphate dehydrogenase or IFN-β.

Western Blot Analysis

Culture supernatants (5 μg protein/sample) of PC-3M cells were mixed with sample buffer [62.5 mM Tris/HCl (pH 6.8), 2.3% SDS, 100 mM DTT, and

[3] The abbreviations used are: SCID, severe combined immune deficiency; EMEM, Eagle's minimal essential medium; FBS, fetal bovine serum; LPS, lipopolysaccharide; CMEM, complete minimal essential medium; PEM, peritoneal exudate macrophage; NK, natural killer; PCNA, proliferative cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling.

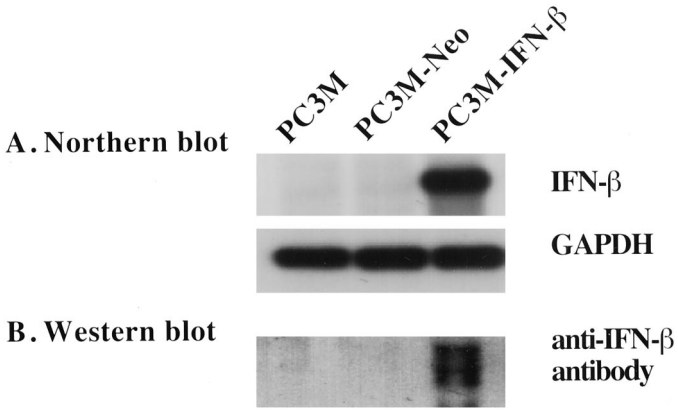

Fig. 1. Expression of IFN-β in PC-3M cells. *A*, total cellular RNA was extracted from PC-3M-P, PC-3M-Neo, and PC-3M-IFN-β cells and analyzed by Northern blotting as described in "Materials and Methods." *B*, PC-3M-P, PC-3M-Neo, or PC-3M-IFN-β cells in 5% FBS-MEM were plated into 24-well plates at 5×10^5/well. Twenty-four h later, the cells were rinsed with HBSS, fed with serum-free DMEM/F12 medium (0.5 ml/well), and incubated for 48 h. Forty μl of the culture supernatant from each well was analyzed by Western blotting. This is one representative experiment of two.

0.05% bromphenol blue], boiled, and separated on 10% SDS PAGE. The protein was transferred onto 0.45-μm nitrocellulose membranes. The filter was blocked with 3% BSA in Tris-buffered saline (20 mM Tris/HCl, pH 7.5, 150 mM NaCl), probed with antibody against murine IFN-β (1 μg/ml) in Tris-buffered saline containing 0.1% Tween 20, incubated with a second antibody in the buffer, and visualized by the enhanced chemiluminescence Western blotting detection system (43).

Immunohistochemistry

At necropsy, the tumor tissues were cut into 5-mm pieces, placed in OCT compound (Miles Laboratories, Elkhart, IN), and snap-frozen in liquid nitrogen. Frozen sections (8–10 μm) were fixed in cold acetone and treated with 3% hydrogen peroxide in methanol (v/v). The treated slides were incubated in blocking solution (5% normal human serum/1% normal goat serum in PBS) and then treated with an antibody to macrophage-specific scavenger receptor (Serotec Ltd., Kidlington, MA) or to CD31 antibody to assess vascularity (PharMingen, San Diego, CA) for 18 h at 4°C in a humidified chamber. The sections were rinsed and incubated with peroxidase-conjugated secondary antibodies. A positive reaction was visualized by incubating the slides with Stable DAB (Research Genetics, Huntsville, AL) and counterstained with Mayer's hematoxylin (Research Genetics). The slides were dried and mounted with Universal mount (Research Genetics), and images were digitized using a Sony 3CD color video camera (Sony Corp., Tokyo, Japan) and a personal computer equipped with Optimas Image Analysis Software (Optimas Corp., Bothell, WA).

For immunohistochemical staining using an antibody to PCNA, paraffin sections (3–5 μm) of the tumor samples were placed on ProbeOn slides (Fischer Scientific) and stained as described for the frozen sections.

TUNEL Assay

Cell death in tumor lesions was determined by the TUNEL method (44). Paraffin sections were dewaxed in xylene and rehydrated. The slides were treated with 20 μg/ml of proteinase K in distilled H_2O for 15 min at room temperature, rinsed with distilled H_2O, and incubated in 3% H_2O_2 in methanol for 5 min. The treated slides were incubated in terminal deoxynucleotidyl transferase buffer [30 mM Trizma base (pH 7.2), 140 mM sodium cacolydate, and 1 mM $CoC1_2$] containing biotinylated 16-dUTP and terminal transferase (Boehringer Mannheim) for 1 h at 37°C; then the slides were incubated with terminal deoxynucleotidyl transferase. The reaction were stopped with a buffer containing 300 mM NaCl and 30 mM sodium citrate. The slides were then incubated with a streptavidin-peroxidase conjugate for 30 min at 37°C, stained with 3-amino-9-ethyl carbazole (Biomeda, Foster City, CA), and evaluated under a microscope.

Bioassay for IFN-β Activity

The bioassay for murine IFN-β was based on previous findings that this cytokine induces NO production by LPS-primed PEMs (45). PEMs plated at the density of 1×10^5 cells/38-mm^2 well of 96-well plates were incubated for 24 h with test samples or with increasing concentrations of murine IFN-β (10–1000 μ/ml) in the presence of 1 μg/ml LPS. NO_2^- levels were determined as described previously (46). Briefly, 50-μl samples were harvested from conditioned medium and allowed to react with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, and 2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was monitored with a microplate reader (Dynatech, Inc., Chantilly, VA). Nitrite concentrations were determined using sodium nitrite as a standard. To confirm the induction of NO_2^- by IFN-β, we used a rat monoclonal antibody that neutralizes murine IFN-β activity (Yamasa, Shoyu, Chiba-Ken, Co., Japan).

Statistical Analysis

The significance of the results was determined by Student's *t* test (two-tailed).

RESULTS

Transduction of Human PC-3M Cells with Murine IFN-β. Full-length cDNA of the murine IFN-β was introduced into the retroviral vector pLXSN containing a drug-selectable marker for neomycin resistance. PC-3M cells were transduced with either pLXSN-IFN-β (PC-3M-IFN-β) or control pLXSN-Neo (PC-3M-Neo). No discernible difference in the number of G418-resistant colonies was found between PC-3M cells transduced by pLXSN-IFN-β or the control pLXSN-Neo vectors. To avoid clonal variations, pooled (>20) G418-resistant (800 μg/ml) colonies were established as adherent monolayers and used in the studies. The expression of murine IFN-β in the tumor cells was initially screened by a Northern blot analysis. IFN-β activity was determined in 48-h culture supernatants by measuring NO production in LPS-primed murine macrophages.

A high level of IFN-β mRNA was found in cells transduced by pLXSN-IFN-β (Fig. 1*A*). Western blot analysis revealed the presence of IFN-β protein in culture supernatants of PC-3M-IFN-β cells (Fig. 1*B*). IFN-β activity, detected only in culture supernatants of PC-3M-IFN-β cells, ranged from 7,000 to 10,000 units/10^6 cells/24 h. The expression of IFN-β in PC-3M-IFN-β cells was relatively stable; identical levels of IFN-β activity were found in the supernatant of cells cultured in the absence of G418 for 7 weeks. The morphology of the three lines of PC-3M cells were identical (data not shown). The doubling times were 20, 21, and 20 h for PC-3M-P, PC-3M-Neo, and PC-3M-IFN-β cells, respectively. Therefore, neither the transduction procedure nor the expression of IFN-β altered the *in vitro* growth of PC-3M cells.

Table 1 *Growth and metastasis of PC-3M cells in the prostate of nude mice*

Cell lines[a]	Primary tumors		Lymph node metastasis
	Incidence	Prostate weight (mg)	
PC-3M-P	10/11	751 ± 571[b]	4/11
PC-3M-Neo	14/14	1294 ± 783[c]	13/14
PC-3M-IFN-β	0/11[c]	48 ± 13[d,e]	0/11

[a] PC-3M-P, PC-3M-Neo, or PC-3M-IFN-β cells (10^5 cells/mouse in 40 μl of HBSS) were implanted into the prostates of nude mice. The tumor incidence, prostate weight, and incidence of aortic lymph node metastases were determined 28 days later. This is one representative experiment of three.
[b] Prostate weight of mean ± SD.
[c] $P > 0.05$, in comparison with PC-3M-P tumors.
[d] $P < 0.05$, in comparison with PC-3M-P tumors.
[e] Normal prostate weight: 45 ± 7 mg.

Table 2 *Growth of PC-3M cells in the subcutis of nude mice*

Cell lines[a]	Tumor incidence	Tumor diameter (mm)
PC-3M-P	13/15	14 ± 7
PC-3M-Neo	13/14	17 ± 6
PC-3M-IFN-β	1/15[b]	0.2 ± 8[b]

[a] PC-3M-P, PC-3M-Neo, or PC-3M-IFN-β cells (1×10^5/mouse) were injected s.c. into the lateral flanks of nude mice. Tumor incidence and diameter were determined 28 days later. This is one representative experiment of two.
[b] $P < 0.05$, in comparison with PC-3M-P tumors.

Table 3 *Tumorigenicity and bystander inhibitory effect of PC-3M-IFN-β cells*

	Inoculum (cells/mouse)	Primary tumors: Incidence	Primary tumors: Prostate weight[b]	Lymph node metastasis incidence
Cell lines[a]				
PC-3M-P	1×10^5	6/6	530 ± 438	3/6
PC-3M-IFN-β	1×10^5	0/6	48 ± 6[c]	0/5
	2×10^5	0/6	53 ± 6[c]	0/5
	5×10^5	0/6	50 ± 6[c]	0/5
Cell mixtures				
PC-3M-P	1×10^5			
+PC-3M-IFN-β	1×10^5	3/6	71 ± 33[c]	0/5
	2×10^5	0/6	54 ± 9[c]	0/6
	5×10^5	0/6	58 ± 7[c]	0/6

[a] PC-3M-P, PC-3M-IFN-β, or cell mixtures in 40 μl of HBSS were inoculated into the prostates of nude mice. The tumor incidence, prostate weight, and incidence of aorta lymph node metastasis were determined 28 days later. This is one representative experiment of two.
[b] Normal prostate weight: 45 ± 7 mg.
[c] $P < 0.05$, in comparison with PC-3M-P tumors.

Growth and Metastasis of IFN-β-transfected PC-3M Cells. To determine tumorigenicity and production of metastasis, PC-3M-P, PC-3M-Neo, and PC-3M-IFN-β cells (1×10^5/inoculum) were injected into the prostate of nude mice (40). The mice were killed 28 days later and necropsied. Tumor incidence and lymph node metastasis were recorded (Table 1). In contrast to PC-3M-P and PC-3M-Neo cells, PC-3M-IFN-β cells failed to produce any local tumors or lymph node metastases (Table 1). The tumorigenic potential of the PC-3M-IFN-β cells was also significantly reduced at ectopic s.c. sites (Table 2), indicating that IFN-β secreted by the PC-3M cells was associated with abrogation of tumorigenicity and hence metastasis.

***In Vivo* Growth Inhibition of Bystander PC-3M Cells.** Next, we determined whether the IFN-β producing PC-3M cells could inhibit the growth of bystander (nontransduced) PC-3M-P cells. We injected a mixture of PC-3M-P and PC-3M-IFN-β cells into the prostate of nude mice ($n = 6$). PC-3M-P or PC-3M-IFN-β cells were also injected alone as controls. The mice were necropsied 28 days later. The incidence of local tumors and lymph node metastases is summarized in Table 3. PC-3M-P cells (1×10^5/inoculum) were highly tumorigenic and produced metastases in 50% of the mice. In contrast, the PC-3M-IFN-β did not produce any local tumors or metastases (even at the inoculum of 5×10^5 cells), and the mixture produced three small tumors at the lowest of three PC-3M-IFN-β concentrations and none at the higher two concentrations (Table 3).

Growth of PC-3M Cells in NK Cell-compromised Nude Mice. Because IFN-β is a potent NK cell activator (47, 48), we next determined whether NK cells participated in tumorigenicity of the PC-3M-IFN-β cells. Nude mice were treated with NK cell-selective anti-asialo G_{M1} serum or control rabbit serum using a protocol proven effective in depleting NK cells in mice (49). This treatment with anti-asialo G_{M1} did not significantly alter the growth of PC-3M-Neo tumors in the subcutis of nude mice (Fig. 2*A*; $P > 0.05$) but did significantly increase the tumorigenicity of PC-3M-IFN-β cells ($P < 0.05$). Ten of 10 mice injected with PC-3M-IFN-β cells developed s.c. tumors. The PC-3M-IFN-β s.c. tumors in the anti-asialo G_{M1} serum-treated mice grew only up to and stabilized at 3–5 mm in diameter (Fig. 2*B*). The same results were observed when PC-3M-Neo and PC-3M-IFN-β cells were implanted into the subcutis of SCID/Beige mice that are deficient in T, B, and NK cells (Fig. 2*C*). In contrast to PC-3M-P and PC-3M-Neo tumors, the PC-3M-IFN-β tumors in the NK cell-compromised mice were nonvascularized (data not shown). Similarly, PC-3M-IFN-β cells produced smaller, poorly vascularized tumors (weight of 254 ± 37 mg *versus* 897 ± 131 mg of PC-3M-Neo tumors; $P < 0.05$) in the prostate of SCID/Beige mice.

Immunohistochemical Analysis. PC-3M cells (10^5 cells/mouse for PC-3M-P and PC-3M-Neo and 2×10^6 cells/mouse for PC-3M-IFN-β) were implanted into the prostate of nude mice. One week later, samples were collected for immunohistochemistry (Fig. 3). Most cells

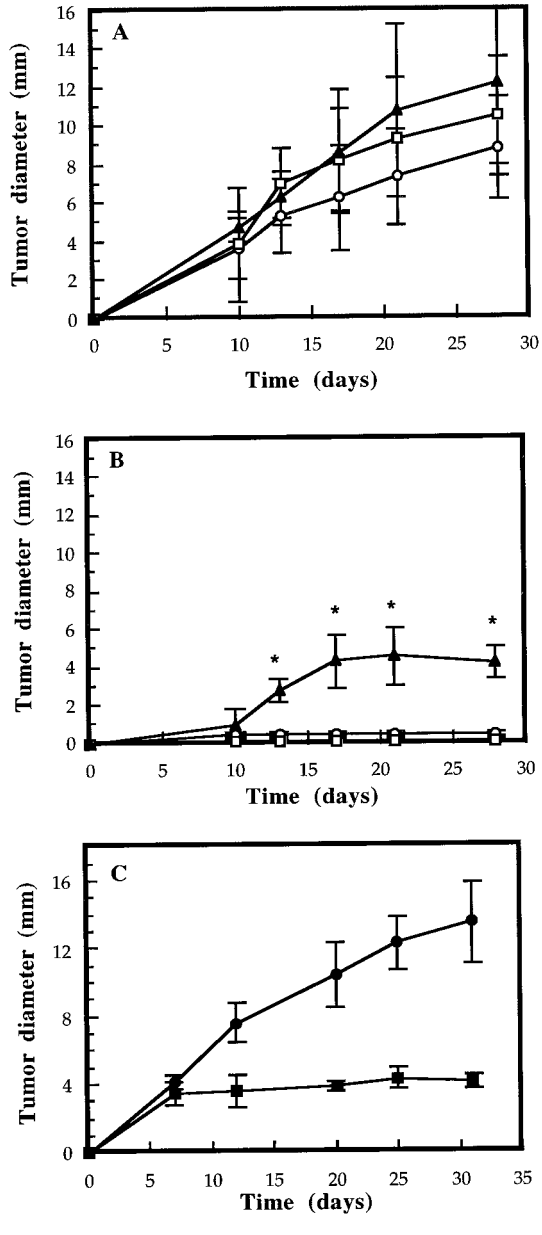

Fig. 2. Growth of PC-3M cells in the subcutis of NK cell-compromised nude mice. *A* and *B*, nude mice were not treated (□), treated by i.p. injection of 40 μl/mouse of normal rabbit serum (○), or anti-asialo G_{M1} serum (▲) on days −2, 0, 2, 4, 7, 10, and 14 relative to inoculation of PC-3M-Neo (*A*) and PC-3M-IFN-β (*B*) cells. PC-3M-Neo or PC-3M-IFN-β cells (1×10^5/mouse) were inoculated into the subcutis of nude mice (10 mice/group). *C*, PC-3M-Neo (●) or PC-3M-IFN-β (■) cells were injected into the subcutis of SCID/Beige mice (five mice/group). Tumor diameter (mm) was determined using a caliper. The data are the mean diameter; *bars*, SD. This is one representative experiment of two. *, $P < 0.05$.

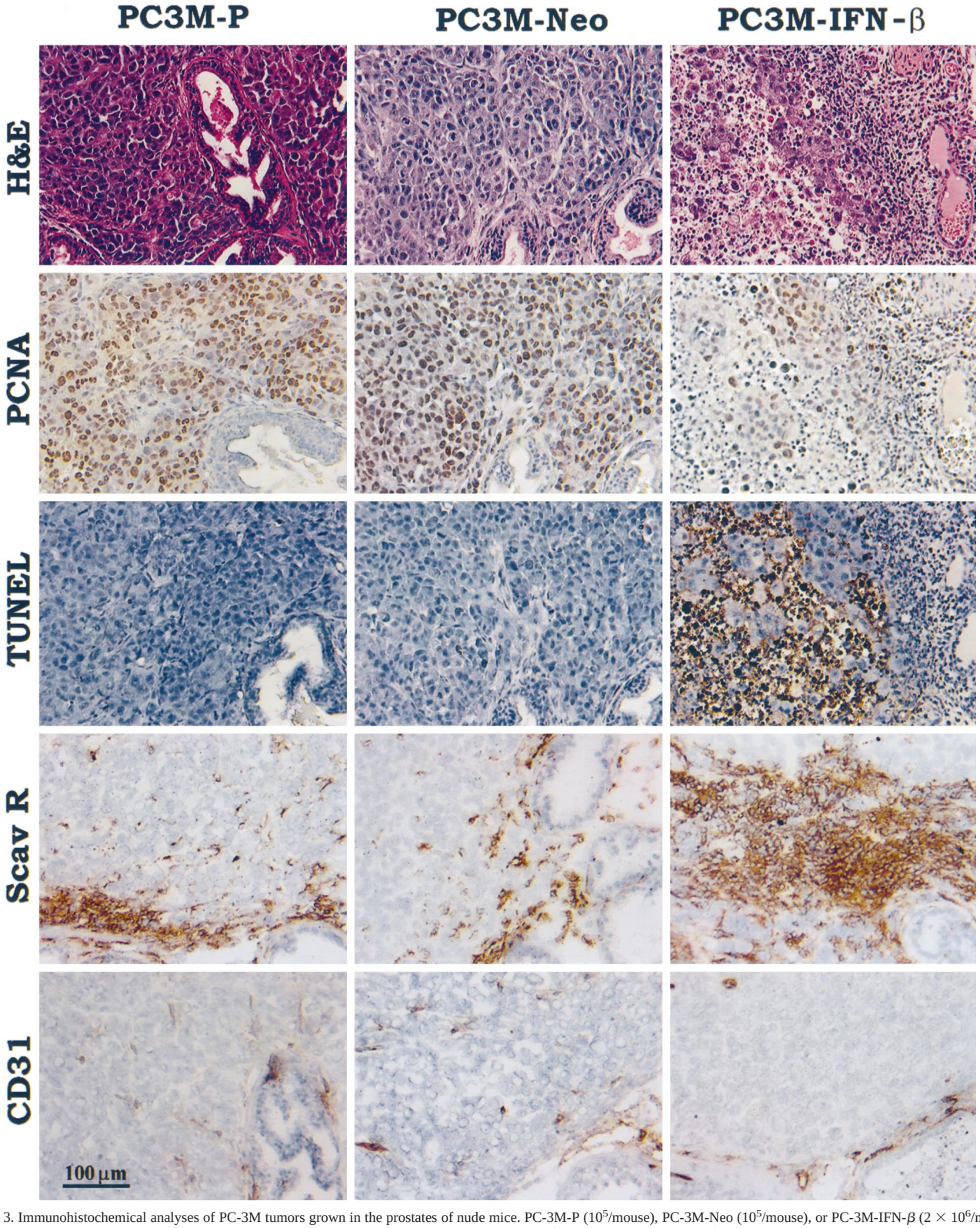

Fig. 3. Immunohistochemical analyses of PC-3M tumors grown in the prostates of nude mice. PC-3M-P (10^5/mouse), PC-3M-Neo (10^5/mouse), or PC-3M-IFN-β (2×10^6/mouse) cells were implanted into the prostates of nude mice. Seven days later, the tumors were collected. Paraffin sections were prepared for H&E staining and immunohistochemical staining using antibody to PCNA or TUNEL method. Snap-frozen sections were prepared for immunohistochemical staining using anti-scavenger receptor (Scav R) or anti-CD31 antibodies. This is one representative experiment of two.

in the PC-3M-P and PC-3M-Neo tumors stained intensively by a monoclonal antibody against PCNA, which is expressed in the late G_1 and M phase of the cell cycle (50). In contrast, only a few cells in the PC-3M-IFN-β tumors were PCNA positive. The TUNEL method revealed only a few positive cells in the PC-3M-P and PC-3M-Neo tumors, whereas most cells in PC-3M-IFN-β tumors stained positively. Immunohistochemistry using an antibody against macrophage-specific scavenger receptor (51) revealed that macrophages in the

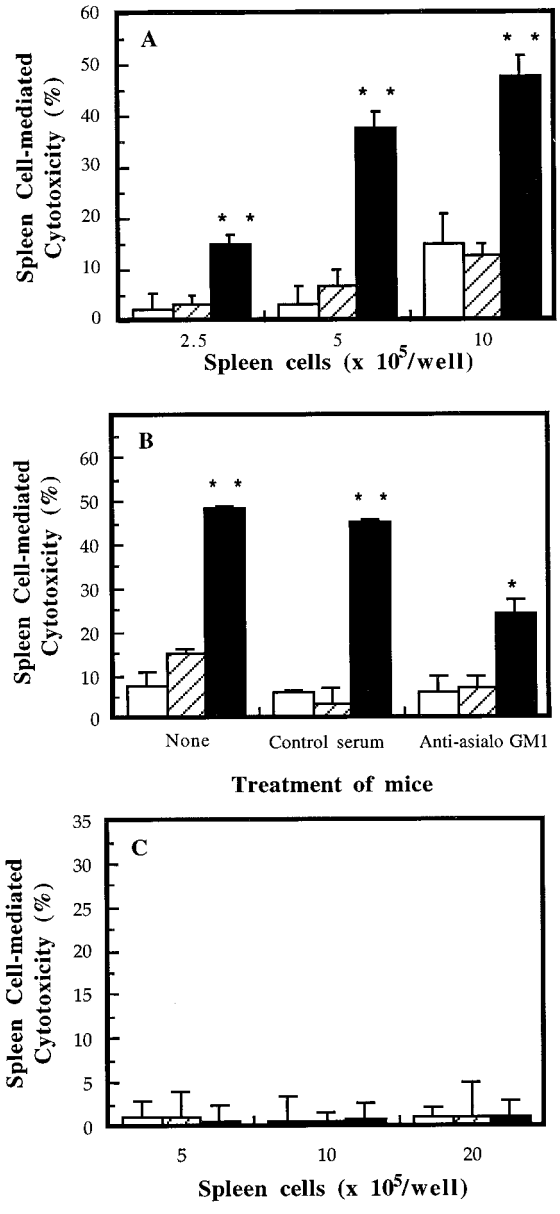

Fig. 4. Splenic cell-mediated tumor cell killing of PC-3M cells. *A*, [^{3}H]thymidine-labeled PC-3M-P (□), PC-3M-Neo (▨), PC-3M-IFN-β (■) cells (1×10^4/well in 96-well dishes) were incubated for 24 h with 2.5, 5, or 10×10^5 spleen cells of nude mice. *B*, [^{3}H]thymidine-labeled PC-3M-P (□), PC-3M-Neo (▨), PC-3M-IFN-β (■) cells (1×10^4/well in 96-well dishes) were incubated for 24 h with splenic cells (5×10^5/well) from untreated, control serum-treated, or anti-asialo G_{M1} serum-treated nude mice. *C*, [^{3}H]thymidine-labeled PC-3M-P (□), PC-3M-Neo (▨), PC-3M-IFN-β (■) cells (1×10^4/well in 96-well dishes) were incubated for 24 h with 2.5, 5, or 10×10^5/well of splenic cells from SCID/Beige mice. Spleen cell-mediated cytolysis was determined as described in "Materials and Methods." *, $P < 0.05$ compared with the killing mediated by splenic cells from untreated or control serum treated mice; **, $P < 0.05$ compared with the killing of PC-3M-P or PC-3M-Neo cells. Data shown are one representative experiment of three (*A* and *B*) and two (*C*); *bars*, SD.

PC-3M-P and PC-3M-Neo tumors were located on the periphery of the lesions, whereas the PC-3M-IFN-β tumors were heavily infiltrated by macrophages (Fig. 3). Anti-CD31 antibody staining revealed that the PC-3M-P and PC-3M-Neo prostate tumors were well vascularized, whereas the PC-3M-IFN-β tumors were not (Fig. 3).

Splenic Cell-mediated Cytolysis against PC-3M Cells. Because the *in vivo* data suggested that NK cells contribute to the suppression of the tumorigenicity of PC-3M-IFN-β cells, we examined the relative susceptibility of PC-3M cells to NK cell-mediated cytotoxicity. The [^{3}H]thymidine-labeled PC-3M cells were incubated with the spleen cells from nude mice, and cytotoxicity was determined 24 h later. Significantly more PC-3M-IFN-β cells were lysed by splenic cells than PC-3M-P or PC-3M-Neo cells (Fig. 4*A*). i.p. injection of 40 μl of anti-asialo G_{M1} (but not control normal rabbit serum) 48 h before harvest of spleen cells reduced splenic cell-mediated cytotoxicity by 40–50% (Fig. 4*B*). No cytotoxic activity against PC-3M cells was detected in spleen cells from SCID/Beige mice (Fig. 4*C*).

We next determined the effects of CM from PC-3M cells on splenic cell-mediated cytotoxicity. The addition of CM from PC-3M-IFN-β cells tripled the splenic cell-mediated killing of PC-3M-P cells (Table

Table 4 *Stimulation of NK cell activity by supernatant of PC-3M-IFN-β cells*[a]

Target cells[b]	Treatment	anti-IFN-β (100 NU/ml) −	+
PC-3M-P	None	14 ± 3	15 ± 2
	IFN-β	31 ± 3[b]	16 ± 3
	CM of PC-3M-IFN-β	47 ± 2[b]	16 ± 3
PC-3M-IFN-β	None	46 ± 1[b]	39 ± 6[b]
	IFN-β	48 ± 1[b]	41 ± 1[b]
	CM of PC-3M-P	48 ± 1[b]	42 ± 2[b]

[a] [^{3}H]Thymidine-labeled PC-3M-P or PC-3M-IFN-β cells (1×10^4/well) were incubated with spleen cells (5×10^5/well) for 24 h in CMEM, or the CMEM containing 100 units/ml of IFN-β, or conditioned media (CM) of PC-3M-P or PC-3M-IFN-β cells (1:10 dilution) in the absence or presence of anti-IFN-β monoclonal antibody. This is one representative experiment of three.

[b] $P < 0.05$, in comparison with cytotoxicity against PC-3M-P cells in the absence of treatment.

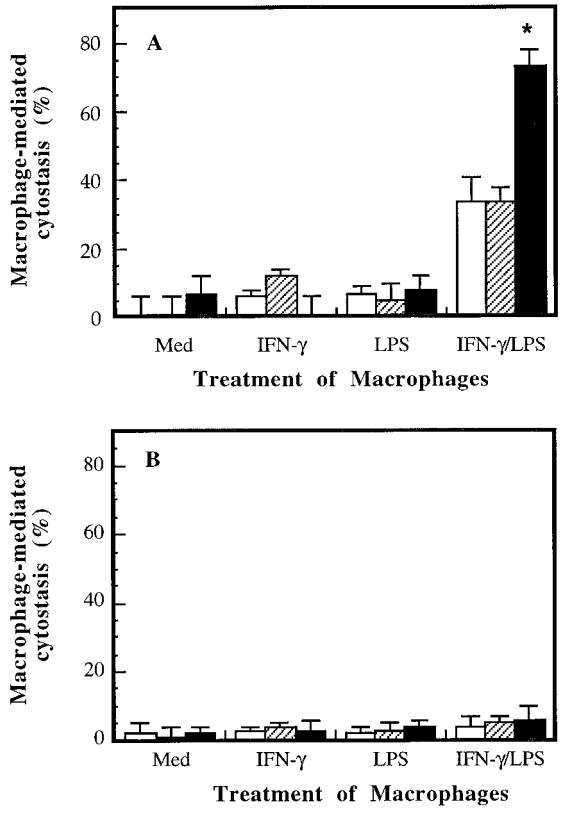

Fig. 5. Macrophage-mediated cytostasis against PC-3M cells. Macrophages (5×10^4/well in 96-well dishes) were incubated for 24 h in CMEM or CMEM containing 10 units/ml of IFN-γ, 100 ng/ml of LPS, or IFN-γ plus LPS. In *A*, after a wash with HBSS, the treated macrophages were incubated for 48 h with 5×10^3/well of PC-3M-P (□), PC-3M-Neo (▨), or PC-3M-IFN-β (■) cells. In *B*, the treated macrophages were incubated for 48 h with PC-3M-P (□), PC-3M-Neo (▨), or PC-3M-IFN-β (■) in the presence of *N*-methyl-arginine at a concentration of 2 mM. During the last 20 h, [^{3}H]thymidine (0.1 μCi/well) was added. The cytostatic effect of macrophages on the tumor cells was determined as described in "Materials and Methods." *, $P < 0.05$ as compared with the cytostasis against PC-3M-P or PC-3M-Neo cells. This is one representative experiment of three; *bars*, SD.

4). The enhancing effect of CM from the PC-3M-IFN-β cells was completely blocked by a monoclonal antibody against mouse IFN-β (Table 4). As a positive control, we added 100 units/ml mouse IFN-β to the cultures. The cytotoxicity against PC-3M cells enhanced by mouse IFN-β was also neutralized by the antibody (Table 4). In contrast, CM from PC-3M-P cell culture did not alter the cytotoxicity against PC-3M-P or PC-3M-IFN-β cells, suggesting that the bystander effect observed *in vivo* (Table 3) may have involved the activation of NK cells by IFN-β released from PC-3M-IFN-β cells.

Macrophage-mediated Cytostasis. Because recombinant IFN-β can activate tumoricidal properties in macrophages (45, 52), we investigated whether the amount of IFN-β released from PC-3M-IFN-β cells is sufficient to activate macrophage-mediated tumor cytostasis. PEMs from nude mice were incubated for 24 h in medium (control) or in medium containing IFN-γ (10 units/ml), LPS (100 ng/ml), or IFN-γ plus LPS, and then the PC-3M cells were added. Tumor cytostasis was determined 48 h later. The *in vitro* proliferation of the PC-3M cells was not affected by control, IFN-γ- or LPS-treated PEMs (Fig. 5); however, the cells were susceptible to cytostatic effects mediated by IFN-γ and LPS-treated PEMs. The PC-3M-IFN-β cells were more sensitive to the IFN-γ plus LPS-activated PEMs than the PC-3M-P and PC-3M-Neo cells (Fig. 5*A*). The cytostatic effect of activated macrophages was mediated by nitric oxide, a molecule generated by activated PEM that is toxic to a variety of cells (53, 54), because it could be blocked by the nitric oxide synthase inhibitor *N*-methyl-arginine (Fig. 5*B*).

DISCUSSION

Our results demonstrate that the transduction of the *IFN-β* gene into human prostate cancer cells inhibits their tumorigenic and metastatic properties in nude or SCID mice by suppressing angiogenesis and activating NK cells and macrophages. Moreover, the IFN-β-transduced human prostate cancer cells inhibited the tumorigenicity of bystander (nontransduced) human prostate cancer cells implanted into the prostates of nude mice. These data agree with our recent findings in different human and murine tumors (46, 55) and reports by others using IFN-α-secreting B16 melanoma (35–37).

No discernible differences in *in vitro* growth rate were found among the PC-3M-P, PC-3M-Neo and PC-3M-IFN-β cells, and these data confirm the well-established finding that the antiproliferative effects of IFN-β are species specific. The suppression of tumorigenicity and metastasis of the PC-3M-IFN-β cells (in nude or SCID mice) was therefore due to an indirect mechanism.

IFN-α or IFN-β are known to enhance the maturation of NK cells from a pre-NK cell pool (56) and to stimulate the secretion of pore-forming protein (57). Because of the lack of an antibody suitable for immunohistochemical staining of NK cells, we do not have direct evidence for the infiltration of NK cells into the PC-3M tumors. Our data, however, suggest that the cells play an important role in the suppression of the tumorigenic properties of the PC-3M-IFN-β cells. We base this conclusion on the following results: (*a*) PC-3M-IFN-β cells produced tumors in SCID/beige mice and in nude mice pretreated with anti-asialo G_{M1} antibody; (*b*) spleen cells from the NK cell-compromised mice (SCID/beige and anti-asialo G_{M1}-treated nude mice) failed to kill the PC-3M-IFN-β cells, whereas spleen cells from control mice did. The *in vivo* death of the PC-3M-IFN-β cells (in the prostate of nude mice) occurred by apoptosis, as demonstrated by the TUNEL staining method; (*c*) culture supernatants of PC-3M-IFN-β cells enhanced NK-cell mediated cytotoxicity against the PC-3M-P cells, and the enhanced cytotoxicity could be abrogated by the antibody against mouse IFN-β.

The *in vivo* suppression of tumorigenic potential of the PC-3M-IFN-β cells as evidenced by the decrease in PCNA-positive cells within the tumors could have been due to activation of macrophages: (*a*) immunohistochemical staining using an antibody against macrophage-specific scavenger receptor (51) revealed that the PC-3M-IFN-β prostate tumors were homogeneously infiltrated by macrophages, whereas the control tumors were not; and (*b*) incubation with activated macrophages produced significant cytostasis of PC-3M-IFN-β cells, suggesting that macrophage inhibition of tumorigenicity could be important, especially in NK cell-compromised mice.

Angiogenesis characterized by the formation of new blood vessels from established microvasculature (58) plays a crucial role in the progressive growth and metastasis of solid tumors (59, 60). In the absence of neovascularization, solid tumors cannot grow beyond 1–2 mm in diameter (about 10^6 cells; Refs. 61 and 62). The newly formed microvasculature is due to a concurrent increase in positive angiogenic molecules released by tumor cells (63, 64) and by infiltrating lymphoid cells (65) and a decrease in angiogenesis inhibitors, such as angiostatin (10, 12) or IFN-β. IFN-β is known to down-regulate expression of a variety of angiogenic molecules *in vitro* (13, 30–33, 66) that are crucial for formation of vasculature. One striking finding of our study was that PC-3M-IFN-β cells formed small avascularized tumors (3–5 mm in diameter) in T-cell and NK cell-compromised mice (nude mice-treated by anti-asialo G_{M1} antibody) or T-cell, B-cell, and NK-cell deficient (SCID/beige) mice. Moreover, immunostaining with anti-CD31 antibody staining revealed that blood vessel density in tumors produced by PC-3M-IFN-β cells was significantly lower than that in tumors produced by PC-3M-P or PC-3M-Neo cells. These data suggest that the decreased tumorigenicity of PC-3M-IFN-β cells was due, in part, to inhibition of tumor angiogenesis.

In summary, we demonstrate that the expression of IFN-β in PC-3M human prostate cancer lesions suppresses tumorigenicity and metastasis of the transduced cells and bystander cancer cells. The suppression of tumor growth and metastasis by IFN-β is mediated by multiple mechanisms that include activation of host effector cells and inhibition of tumor angiogenesis. These data support the hypothesis that the sustained presence of IFN-β in tumor lesions can produce regression (21) and imply that IFN-β or its encoding gene have great potential in the therapy of prostate cancer.

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REFERENCES

1. Wingo, P. A., Tong, T., and Bolden, S. Cancer statistics, 1995. CA Cancer J. Clin., *45:* 8–30, 1995.
2. Fidler, I. J. Critical factors in the biology of human cancer metastasis. Twenty-eighth Clowes Memorial Award Lecture. Cancer Res., *50:* 6130–6138, 1990.
3. Folkman, J. Clinical applications of research on angiogenesis. N. Engl. J. Med., *333:* 1757–1763, 1995.
4. Fidler, I. J., and Ellis, L. M. The implications of angiogenesis to the biology and therapy of cancer metastasis. Cell, *79:* 185–188, 1994.
5. Hanahan, D., and Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell, *86:* 353–364, 1996.
6. Singh, R. K., Bucana, C. D., Gutman, M., Fan, D., Wilson, M. R., and Fidler, I. J. Organ site-dependent expression of basic fibroblast growth factor in human renal cell carcinoma cells. Am. J. Pathol., *145:* 365–374, 1994.
7. Dvorak, H. F., Brown, L. F., Detmar, M., and Dvorak, A. M. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am. J. Pathol., *146:* 1029–1039, 1995.
8. Singh, R. K., Gutman, M., Radinsky, R., Bucana, C. D., and Fidler, I. J. Expression of interleukin-8 correlates with the metastatic potential of human melanoma cells in nude mice. Cancer Res., *54:* 3242–3246, 1994.
9. Koch, A. E., Friedman, J., Burrows, J. C., Haines, G. K., and Bouch, N. P. Localization of the angiogenesis inhibitor thrombospondin in human synovial tissues. Pathobiology, *61:* 1–6, 1993.

10. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. Angiostatin: a novel angiogenesis inhibitor that meditates the suppression of metastases by a Lewis lung carcinoma. Cell, *79:* 315–328, 1994.
11. Sim, B. K., O'Reilly, M. S., Liang, H., Fortier, A. H., He, W., Madsen, J. W., Lapcevich, R., and Nacy, C. A. A recombinant human angiostatin protein inhibits experimental primary and metastatic cancer. Cancer Res., *57:* 1329–1334, 1997.
12. Dong, Z., Kumar, R., Yang, X., and Fidler, I. J. Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma. Cell, *88:* 801–810, 1997.
13. Singh, R. K., Gutman, M., Bucana, C. D., Sanchez, R., Llansa, N., and Fidler, I. J. Interferons alpha and beta downregulate the expression of basic fibroblast growth factor in human carcinomas. Proc. Natl. Acad. Sci. USA, *92:* 4562–4566, 1995.
14. Baron, S., and Dianzani, F. The interferons: a biological system with therapeutic potential in viral infections. Antiviral Res., *24:* 97–110, 1994.
15. Hertzog, P. J., Hwang, S. Y., and Kola, I. Role of interferons in the regulation of cell proliferation, differentiation, and development. Mol. Reprod. Dev., *39:* 226–232, 1994.
16. Brzoska, J., and Obert, H-J. Immunomodulating effects of interferons: conclusions for therapy. *In:* G. Gross, S. Jablonska, H. Pfister, and H. E. Stegner (eds.), Genital Papillomavirus Infections, pp. 379–391. Berlin: Springer-Verlag, 1990.
17. Krown, S. E. Interferons in malignancy: biological products or biological response modifiers? J. Natl. Cancer Inst., *80:* 306–309, 1988.
18. Thomas, H., and Balkwill, F. R. Effects of interferons and other cytokines on tumors in animals: a review. Pharmacol. Ther., *52:* 307–314, 1991.
19. Bulbul, M. A., Huben, R. P., and Murphy, G. P. Interferon-β treatment of metastatic prostate cancer. J. Surg. Oncol., *33:* 231–233, 1986.
20. Schiller, J. H., Storer, B., Bittner, G., Wilson, J. K., and Bordon, E. C. Phase II trial of a combination of interferon-β ser and interferon-γ in patients with advanced malignant melanoma. J. Interferon Res., *8:* 581–589, 1988.
21. Einhorn, S., and Grander, D. Why do so many cancer patients fail to respond to interferon therapy? J. Interferon Cytokine Res., *16:* 275–281, 1996.
22. Ozzello, L., Habif, D. V., and DeRosa, C. M. Antiproliferative effects of natural interferon β alone and in combination with natural interferon γ on human breast carcinomas in nude mice. Breast Cancer Res. Treat., *16:* 89–96, 1990.
23. Gutterman, J. U. Cytokine therapeutics: lessons from interferon-α. Proc. Natl. Acad. Sci. USA, *91:* 1198–1205, 1994.
24. Wu, J. M., Lin, C. S., Wang, J. N., Luo, C. Y., Yu, C. Y., and Yang, H. B. Pulmonary cavernous hemangiomatosis treated with interferon α-2a. Pediatr. Cardiol., *17:* 332–334, 1996.
25. Castello, M. A., Ragni, G., Antimi, A., Todini, A., Patti, G., Lubrano, R., Clerico, A., and Calisti, A. Successful management with interferon α-2a after prednisone therapy failure in an infant with a giant cavernous hemangioma. Med. Pediatr. Oncol., *28:* 213–215, 1997.
26. Shepherd, F. A., Beaulieu, R., Gelmon, K., Thuot, C. A., Sawka, C., Read, S., and Singer, J. Prospective randomized trial of two dose levels of interferon α with zidovudine for the treatment of Kaposi's sarcoma associated with human immunodeficiency virus infection: a Canadian HIV clinical trials network study. J. Clin. Oncol., *16:* 1736–1742, 1998.
27. Kirn, D. H., and Kramer, A. Long-term freedom from disease progression with interferon α therapy in two patients with malignant hemangiopericytoma. J. Natl. Cancer Inst., *88:* 764–765, 1996.
28. Dinney, C. P. N., Bielenberg, D. R., Perrotte, P., Reich, R., Eve, B. Y., Bucana, C. D., and Fidler, I. J. Inhibition of basic fibroblast growth factor expression, angiogenesis, and growth of human bladder carcinoma in mice by mice by systemic interferon-α administration. Cancer Res., *58:* 808–814, 1998.
29. Vermeulen, P. B., Diriz, L. Y., Martin, M., Lemmens, J., and Van Oosterom, A. T. Serum basic fibroblast growth factor and vascular endothelial growth factor in metastatic renal cell carcinoma treated with interferon α-2b. J. Natl. Cancer Inst., *89:* 1316–1317, 1997.
30. Oliveira, I. C., Sciavolino, P. J., and Lee, T. H. Downregulation of interleukin 8 gene expression in human fibroblasts: unique mechanism of transcriptional inhibition by interferon. Proc. Natl. Acad. Sci. USA, *89:* 9049–9053, 1992.
31. Van Damme, J., and Opdenakker, G. Interaction of interferons with skin reactive cytokines: from interleukin-1 to interleukin-8. J. Invest. Dermatol., *95:* 90S–93S, 1990.
32. Gohji, K., Fidler, I. J., Tsan, R., Radinsky, R., von Eschenbach, A. C., Tsuruo, T., and Nakajima, M. Human recombinant interferons-β and γ decrease gelatinase production and invasion by human KG-2 renal carcinoma cells. Int. J. Cancer, *58:* 380–384, 1994.
33. Fabra, A., Nakajima, M., Bucana, C. D., and Fidler, I. J. Modulation of the invasive phenotype of human colon carcinoma cells by organ-specific fibroblasts of nude mice. Differentiation, *52:* 101–110, 1992.
34. Salmon, P., Le Cotonnec, J. Y., Galazka, A., Abdul-Ahad, A., and Darragh, A. Pharmacokinetics and pharmacodynamics of recombinant human interferon-β in healthy male volunteers. J. Interferon Cytokine Res., *16:* 759–764, 1996.
35. Kaido, T., Bandu, M. T., Ferrrantini, M., Belardelli, F., and Gresser, I. IFN-α gene transfection completely abolishes the tumorigenicity of murine B16 melanoma cells in allogeneic DBA/2 mice and decreases their tumorigenicity in syngeneic C57BL/6 mice. Int. J. Cancer, *60:* 221–227, 1995.
36. Sarkar, S., Flores, I., De Rosa, C., Ozzello, L., Ron, Y., and Pestak, S. Injection of irradiated B16 melanoma genetically modified to secrete IFN-α causes regression of an established tumor. Int. J. Oncol., *7:* 17–24, 1995.
37. Fleischmann, C. M., Stanton, G. J., and Fleischmann, W. R., Jr. Enhanced *in vivo* sensitivity to interferon with *in vitro* resistant B16 tumor cells in mice. Cancer Immunol. Immunother., *39:* 148–154, 1994.
38. Sica, G., Fabbroni, L., Gastagnetta, L., Cacciatore, M., and Pavone-Macaluso, M. Antiproliferative effect of interferons on human prostate carcinoma cell lines. Urol. Res., *17:* 111–115, 1989.
39. Kozlowski, J. M., Fidler, I. J., Campbell, D., Xu, Z. L., Kaighn, M. E., and Hart, I. R. Metastatic behavior of human tumor cell lines grown in the nude mouse. Cancer Res., *44:* 3522–3529, 1984.
40. Stephenson, R. A., Dinney, C. P. N., Gohji, K., Ordonez, N. G., Killion, J. J., and Fidler, I. J. Metastatic model for human prostate cancer using orthotopic implantation in nude mice. J. Natl. Cancer Inst., *84:* 951–957, 1992.
41. Miller, A. D., and Buttimore, C. Redesign of retrovirus packaging cell lines to avoid recombination leading helper virus production. Mol. Cell. Biol., *6:* 2895–2902, 1986.
42. Talmadge, J. E., Meyers, K. M., Prieur, D. J., and Starkey, J. R. Role of natural killer cells in tumor growth and metastasis: C57BL/6 normal and beige mice. J. Natl. Cancer Inst., *65:* 929–935, 1980.
43. Dong, Z., Qi, X., and Fidler, I. J. Tyrosine phosphorylation of mitogen-activated protein kinases is necessary for activation of murine macrophages by natural and synthetic bacterial products. J. Exp. Med., *177:* 1071–1077, 1993.
44. Gavrieli, Y., Shermen, Y., and Ben-Sasson, S. A. Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. J. Cell. Biol., *119:* 493–501, 1992.
45. Fujihara, M., Ito, N., Pace, J. L., Watanabe, Y., Russell, S. W., and Suzuki, T. Role of endogenous interferon-β in lipopolysaccharide-triggered activation of the inducible nitric-oxide synthase gene in a mouse macrophage cell line, J774. J. Biol. Chem., *269:* 12773–12778, 1994.
46. Dong, Z., Juang, P., S. H., Kumar, R., Eue, I., Xie, K., Bielenberg, D., Yang, X., and Fidler, I. J. Expression of interferon-β in UV2237m mouse fibrosarcoma cells suppressed their tumorigenicity and metastasis in syngeneic mice. Cancer Immunol. Immunother., *46:* 137–146, 1998.
47. Fujimiya, Y., Wagner, R. J., Groveman, S., Sielaff, K., Kohsaka, T., and Nakayama, M. *In vivo* priming effects of interferon-β ser on NK activity of peripheral blood mononuclear cells in cancer patients. Ther. Immunol., *2:* 15–22, 1995.
48. Senik, A., Gresser, I., Maury, C., Gidlund, M., Orn, A., and Wigzell, H. Enhancement by interferon of natural killer cell activity in mice. Cell Immunol., *44:* 186–200, 1979.
49. Kasai, M., Yoneda, T., Habu, S., Maruyama, Y., Okumura, K., and Tokunaga, T. *In vivo* effects of anti-asialo GM1 antibody on natural killer activity. Nature (Lond.), *291:* 334–337, 1981.
50. Wilson, G. D., Camplejohn, R. S., Martindale, C. A., Brock, A., Lane, D. P., and Barnes, D. M. Flow cytometric characterization of proliferating cell nuclear antigen using the monoclonal antibody PC10. Eur J. Cancer, *12:* 2010–2017, 1992.
51. Hughes, D. A., Fraser, I. P., and Gordon, S. Murine macrophage scavenger receptor: *in vivo* expression and function as receptor for macrophage adhesion in lymphoid and non-lymphoid organs. Eur. J. Immunol., *25:* 466–473, 1995.
52. Riches, D. W., and Underwood, G. A. Expression of interferon-β during the triggering phase of macrophage cytocidal activation: evidence for an autocrine/paracrine role in the regulation of this state. J. Biol. Chem., *266:* 24785–24792, 1991.
53. Steuhr, D. J., and Nathan, C. F. Nitric oxide. A macrophage produce responsible for cytostasis and respiratory inhibition in tumor target cells. J. Exp. Med., *169:* 1543–1555, 1989.
54. Xie, K., Dong, Z., and Fidler, I. J. Activation of nitric oxide synthase gene for inhibition of cancer metastasis. J. Leukocyte Biol., *59:* 797–803, 1996.
55. Xie, K., Bielenberg, D., Huang, S., Xu, L., Salas, T., Juang, S., Dong, Z., and Fidler, I. J. Abrogation of tumorigenicity and metastasis of murine and human tumor cells by transfection with murine interferon β gene: possible role of nitric oxide. Clin. Cancer Res., *3:* 2283–2294, 1997.
56. Targan, S., and Dorey, F. Interferon activation of "pre-spontaneous killer" (Pre-Sk) cells and alteration in kinetics of lysis of both "pre-SK" and active SK cells. J. Immunol., *124:* 2157–2167, 1980.
57. Liu, C. C., Perussia, B., Cohen, Z. A., and Young, J. D-E. Identification and characterization of a pore-forming protein of human peripheral blood natural killer cells. J. Exp. Med., *164:* 2061–2071, 1986.
58. Folkman, J. The role of angiogenesis in tumor growth. Semin. Cancer Biol., *3:* 65–71, 1992.
59. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat. Med., *1:* 27–31, 1995.
60. Folkman, J., Merler, E., Abernathy, C., and Williams, G. Isolation of a tumor factor responsible for angiogenesis. J. Exp. Med., *133:* 275–288, 1971.
61. Dvorak, H. F. Tumor wounds that do not heal: similarities between tumor stroma generation and wound healing. N. Engl. J. Med., *315:* 1650–1659, 1986.
62. Klagsbrun, M., and D'Amore, P. A. Regulators of angiogenesis. Annu. Rev. Physiol., *53:* 217–239, 1991.
63. Polverini, P. J., and Leiboviach, S. J. Induction of neovascularization *in vivo* and endothelial proliferation *in vitro* by tumor-associated macrophages. Lab. Invest., *51:* 635–642, 1984.
64. Folkman, J., and Klagsbrun, M. Angiogenic factors. Science (Washington DC), *235:* 442–447, 1987.
65. Sunderkotter, C., Goebeler, M., Schulze-Osthoff, K., Bhardwaj, R., and Sorg, C. Macrophage-derived angiogenesis factors. Pharmacol. Ther., *51:* 195–216, 1991.
66. Singh, R. K., Gutman, M., Llansa, N., and Fidler, I. J. Interferon-β prevents the upregulation of interleukin-8 expression in human melanoma cells. J. Interferon Cytokine Res., *16:* 577–584, 1996.