Abrogation of Tumorigenicity and Metastasis of Murine and Human Tumor Cells by Transfection with the Murine IFN-β Gene: Possible Role of Nitric Oxide

Keping Xie, Diane Bielenberg, Suyun Huang, Lei Xu, Thomas Salas, Shin-Hun Juang, Zhongyun Dong, and Isaiah J. Fidler

Department of Cell Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

The purpose of this study was to determine whether sustained local production of murine IFN-β (mIFN-β) could inhibit the tumorigenicity and metastasis of human and murine tumor cells implanted into nude mice. Human melanoma cells (A375SM), renal carcinoma cells (SN12PM6), and colon carcinoma cells (KM12SM) were transfected with mIFN-β or a control neomycin resistance vector. All cell lines grew well in culture. Tumor cells were injected into the subcutis, kidney, spleen, or lateral tail vein of nude mice. Parental or control transfected cells produced local tumors and experimental or spontaneous lung metastases, whereas mIFN-β-transfected cells did not.

In vivo survival experiments using [125]IIdUrd-labeled cells showed that by day 7 after s.c. implantation, all IFN-β-transfected cells died. IFN-β transfection prevented the outgrowth of parental or control-transfected cells only when they were injected together with transfected cells into one site, suggesting that IFN-β promoted a local lysis of the bystander cells. Similar indirect antitumor activity was demonstrated in various human (KM12SM and SN12PM6) and murine (CT-26 colon carcinoma, RENCA renal cell carcinoma, and 3LL Lewis lung carcinoma) tumors. The IFN-β-transfected tumor cells stimulated a high level of nitric oxide production by murine macrophages under in vitro and in vivo conditions, which correlated with the vigorous nonspecific antitumor activity. Collectively, these results demonstrate that local production of IFN-β can eradicate tumor cells of different histology by inducing inducible nitric oxide synthase expression in infiltrating cells.

INTRODUCTION

The IFNs are a family of natural glycoproteins that consist of IFN-α, IFN-β, and IFN-γ. IFNs were first considered antiviral agents (1), but later data revealed that they also control cell growth and differentiation (2), inhibit expression of oncogenes, and modulate host T lymphocytes, natural killer cells, and macrophages, and hence, various aspects of immune reactivity (3–5).

The efficacy of IFN therapy for various malignancies has been investigated for many years (5–13). Extensive clinical trials have concluded that the IFNs can be efficacious against many hematopoietic neoplasms but not against most solid tumors (11–15). Pharmacokinetic studies have demonstrated that the half-life of IFNs in the circulation of patients is on the order of minutes (13–17). The lack of sustained levels (14–18) may have been responsible for the failure to inhibit or eradicate tumors.

The systemic administration of IFN-α or IFN-β has been shown to produce regression of vascularized tumors in rodents by inducing damage to endothelial cells (19, 20). More recent clinical data demonstrated that the systemic chronic administration of low-dose IFN-α can produce regression of vascular tumors, such as pulmonary hemangiomatosis (21), infantile hemangiomas (22), Kaposi sarcoma (23), and malignant hemangioendothelioma (24). Work from our laboratory and others has suggested that regression was due to inhibition of angiogenesis (25). 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 bFGF (26, 27), IL-8 (28, 29), and collagenase type IV (30, 31), all of which are involved in the angiogenic response. This study was designed to determine whether the local, continuous production of IFN-β in and around tumor lesions would compensate for the short half-life of IFN and inhibit tumor growth and metastasis. We show that transfection of different murine and human tumor cells by the mIFN-β gene can indeed accomplish these tasks by a mechanism that involves the production of NO by host macrophages.

MATERIALS AND METHODS

Reagents. Eagle’s MEM, Ca2+ - and Mg2+ -free HBSS, and fetal bovine serum were purchased from M. A. Bioproducts (Walkersville, MD). [3H] thymidine (specific activity, 20 Ci/

Received 5/30/97; revised 9/9/97; accepted 9/11/97.

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

1 This work was supported in part by the University Cancer Foundation and by Cancer Center Support Core Grant CA 16672 and Grant R35-CA 42107 from the National Cancer Institute. NIH.

2 To whom requests for reprints should be addressed. at Department of Cell Biology, Box 173, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030.
mmol) and \([^{125}\text{I}]\text{UdR}\) (specific activity, 2000 Ci/mmol) were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). NMA was purchased from Sigma Chemical Co. (St. Louis, MO), and murine IFN-\(\beta\) (specific activity, 10\(^6\) units/mg protein) was purchased from Lee BioMolecular Co. (San Diego, CA). Murine recombinant IFN-\(\gamma\) (specific activity, 5.2 \times 10\(^8\) units/mg protein) was the generous gift of Genentech, Inc. (South San Francisco, CA). Phenol-extracted \textit{Salmonella} LPS and MTT were purchased from Sigma. All reagents used in tissue culture, except LPS, were free of endotoxin as determined by \textit{Limulus} amoebocyte lysate assay kit (sensitivity limit, 0.125 ng/ml) purchased from Associates of Cape Cod (Falmouth, MA).

**Cell Lines and Culture Conditions.** The A375SM metastatic melanoma cell line was established in culture from lung metastases produced by A375P cells growing s.c. in nude mice (32). A375P was originally established in culture from a lymph node metastasis from a melanoma patient (33). The highly metastatic KM12SM cell line was isolated from a liver metastasis produced by parental KM12C cells growing in the cecal wall of a nude mouse. The KM12C cell line was originally isolated from a primary colon carcinoma classified as Dukes’ stage B2 (34). The metastatic SN12PM6 was established in culture from a spontaneous pulmonary metastasis produced in a nude mouse by parental SN12C cells growing in the subcapsule of kidneys of nude mice. SN12C cells were originally derived from a primary human renal cell carcinoma (35). B16-BL6 is an invasive variant of B16 murine melanoma syngeneic to C57BL/6 mice (36). CT-26 is a murine colon carcinoma cell line syngeneic to BALB/c (37), 3LL is Lewis lung carcinoma syngeneic to C57BL/6 mice (38), and RENCA is a murine renal cell carcinoma syngeneic to BALB/c mice (39). All cell lines were maintained as adherent monolayers in Eagle’s MEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and 2-fold vitamin solution (MEM; Flow Laboratories, Rockville, MD). Cultures were maintained on plastic and incubated in 5% CO\(_2\)-95% air at 37\(^\circ\)C. All cultures were free of \textit{Mycoplasma} and the following murine viruses: reovirus type 3, pneumonia virus, K virus, Thieller’s encephalitis virus, Sendai virus, minute virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by M. A. Bioproducts).

**Mice.** Specific pathogen-free male athymic nude mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). The mice were used when they were 6 to 8 weeks of age. The animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and NIH.

**Transfection of mIFN-\(\beta\) Gene.** Tumor cells were plated onto 100-mm dishes at a density of 2 \times 10\(^5\)/dish. The monolayers were transfected with pcDNA3/mIFN-\(\beta\) or control pcDNA3 plasmids using a stable mammalian transfection kit from Stratagene (La Jolla, CA). The cultures were placed in a 37\(^\circ\)C incubator for 12 h and then washed and fed with CMEM. After 24 h, 500–800 \(\mu\)g/ml G418 (Life Technologies, Inc.) was added. The CMEM/G418 medium was replaced every 3 days until individual resistant colonies were isolated and established in culture as individual lines. All of the lines were maintained in CMEM/G418 and frozen after one to three \textit{in vitro} passages. The expression of mIFN-\(\beta\) in individual clones was identified by Northern (mRNA) blot. The mRNA/IFN-\(\beta\) activity was determined by measuring NO induction in murine macrophages according to procedures as described below. To avoid clonal variation, the successful transfectants of more than 20 clones were pooled for additional studies.

**Northern Blot Analysis.** Cellular mRNA was prepared by using the FastTrack mRNA isolation kit (Invitrogen Co., San Diego, CA). mRNA (2 \(\mu\)g) was separated electrophoretically on 1.0% denaturing formaldehyde agarose gel, transferred to a GeneScreen nylon membrane (DuPont NEN, Boston, MA) in 20\(\times\) SSC, and UV-cross-linked with a UV-Stratalinker 1800 (Stratagene, La Jolla, CA). The 700-bp mIFN-\(\beta\) cDNA probe was labeled with \([\text{\(^{32P}\)}\text{J}]\text{dCTP}\) using a random labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Equal loading of mRNA samples was monitored by hybridizing the same membrane filter with a rat glyceraldehyde-3-phosphate dehydrogenase cDNA probe.

**Collection and Cultivation of Mouse PEMs.** PEMs were collected by peritoneal lavage from mice given an i.p. injection of 2 ml of thioglycollate broth (Baltimore Biological Laboratories, Cockeysville, MD) 4 days before harvest or as indicated otherwise in the text. The PEMs were washed with Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS and suspended in serum-free CMEM, and 1 \times 10\(^5\) cells in 0.2 ml CMEM were plated into a 38-mm\(^2\) well of 96-well, flat-bottomed Microtest III plates (Falcon Plastics, Oxnard, CA). After 90 min, all nonadherent cells were removed by washing with CMEM. The resultant macrophage monolayer was >98% pure according to morphological and phagocytic criteria (40).

**\textit{In Vitro} Macrophage-mediated Cytolysis.** Macrophage-mediated cytosis was assessed by measuring the release of radioactivity from DNA of target cells as described previously (40). In brief, tumor target cells in their exponential growth phase were incubated for 24 h in CMEM containing 0.1 \(\mu\)Ci/ml \([\text{\(^{3}H\)}]\text{thymidine}\). The cells were washed twice with HBSS to remove unbound radioisotope and then harvested by trypsinization (0.25% trypsin and 0.02% EDTA). The cells were washed and resuspended in CMEM, and 1 \times 10\(^4\) viable cells were plated into wells containing macrophages (1 \times 10\(^5\)/well) to achieve a population density of 2500 macrophages and 250 tumor cells/mm\(^2\). At this population density, normal (untreated) macrophages are not cytotoxic to tumor cells (40). Radiolabeled target cells were also plated alone as an additional control group. After a 72-h incubation, the cultures were washed twice with PBS, and adherent viable cells were lysed with 0.1 ml of 0.1 \(n\) NaOH. The lysates were harvested with a Haverster 96 (Tomec, Orange, CT) and counted in a beta counter. Maximal \textit{in vitro} macrophage-mediated cytotoxicity in this assay was obtained after 3 days of incubation with target cells. The cytotoxic activity of macrophages was calculated as follows:

\[
\text{Cytolysis (\%) = } \left(\frac{A - B}{A}\right) \times 100
\]

Downloaded from clincancerres.aacrjournals.org on November 11, 2017. © 1997 American Association for Cancer Research.
where $A = \text{cpm in cultures of untreated macrophages in medium}$ and parental target cells and $B = \text{cpm in cultures of treated macrophages and transfected target cells}$.  

**Bioassay for IFN-β Activity.** PEMs plated at the density of $1 \times 10^5$ cells/well of 96-well plates were incubated for 24 h with test samples or with increasing concentrations of mIFN-β in the presence of $1 \mu$g/ml LPS. The bioassay for mIFN-β was based on previous findings that this cytokine induces NO production by LPS-primed PEMs (41). We, therefore, measured NO$_2$ levels in the culture supernatants of control, LPS-primed, and LPS-primed IFN-β-treated PEMs. NO$_2$ levels were determined as described previously (42). 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% $\text{H}_2\text{PO}_4$) at room temperature for 10 min. The absorbance at 540 nm was monitored with a microplate reader (Dynatech, Chantilly, VA). Nitrite concentrations were determined using sodium nitrite as a standard. Recombinant mIFN-β induced NO$_2$ production in a dose-dependent manner (10–1000 units/ml). To confirm that the induction of NO$_2$ by LPS-primed supernatant-treated PEMs was due to mIFN-β (in the supernatants), we used a rat monoclonal antibody against mIFN-β (Yanasy Shoru Co., Japan).

**Western Blotting for iNOS Protein.** Macrophages (1.5–2.5 $\times 10^7$ in 10 ml medium) were seeded on 100-mm Petri dishes and incubated for 2 h at 37°C. Nonadherent cells were removed by HBSS wash, and the macrophages were scraped off and washed in 4°C PBS containing 5 mM EDTA. The cell pellet was lysed in 0.25–0.5 ml lysis buffer. The soluble protein in the lysates was separated by centrifugation at 15,000 $\times g$ for 30 min at 4°C. Protein concentration in the lysates was determined and diluted in the lysis buffer to 1 mg/ml. After boiling, a 20-μg sample was loaded onto and separated on 7.5% SDS-PAGE and then electrophoretically transferred to 0.45 μm nitrocellulose membrane (Bio-Rad). The membrane was washed in a blocking buffer, incubated in 1:50 diluted first antibody (anti-mouse iNOS monoclonal antibody; Transduction Laboratories, Lexington, KY), followed by treatment with 1:3000 diluted second antibody [anti-mouse immunoglobulin, horseradish peroxidase-linked F(ab')₂, fragment from donkey]. The probed proteins were detected with an ECL system (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

**Immunostaining of Macrophages with Specific Anti-iNOS Antibody.** PEMs grown on glass coverslips for 2–3 h, and nonadherent cells were removed by washing as described above. The cells were then fixed by immersing coverslips in ice-cold acetone for 10 min. Frozen tissue sections were fixed in ice-cold acetone-chloroform for 5 min and then in ice-cold acetone for 5 min. The coverslips or slides were then briefly rinsed in PBS. The endogenous peroxidase was inactivated by incubating the cells with 3% hydrogen peroxide diluted in methanol for 20 min and then rinsed with PBS. The samples were then incubated with blocking buffer containing 1% BSA and 1% NGS in PBS for 10–20 min to minimize nonspecific adsorption of the antibodies to the coverslips. The samples were then incubated with rabbit anti-iNOS polyclonal antibody (Transduction Laboratories) or rat anti-mouse antibody F4/80 (4 μg/ml; Ref. 43) diluted in the same blocking buffer overnight at 4°C. The coverslips were washed three times in PBS and incubated with blocking buffer solution for 5–10 min. After the blocking solution was removed, the cells were incubated for 30–60 min at room temperature with peroxidase-conjugated secondary antibodies [goat anti-rabbit IgG (H + L) antibody, peroxidase-conjugated; Boehringer Mannheim, Indianapolis, IN] and diluted 1:250 in blocking buffer. At the end of the incubation, the samples were washed and then incubated with 3,3'-diaminobenzidine (Research Genetics, Huntsville, AL) for 5–20 min, rinsed with distilled water, counterstained with aqueous hematoxylin for 5–10 s, and finally washed three times with PBS and once with water. The cells were observed under light microscopy.

**In Vitro Cytolysis Assay.** Tumor cells were plated at a density of $3 \times 10^4$ cells/well in 96-well plates in medium. After 72 h, the cell number was determined by the MTT assay (Sigma). After incubation for 2 h in medium containing MTT at 0.42 mg/ml, the medium was removed, and the cells were lysed in DMSO. The conversion of MTT to formazan by metabolically viable cells (44) was monitored by a 96-well microtiter plate reader at 570 nm. The percentage of cytostasis was calculated by the formula:

$$\text{Cytostasis (%)} = \frac{1 - (B/A)}{100}$$

where $A$ is the absorbance of parental cells and $B$ is the absorbance of the transfected cells incubated in medium (44).

**In Vitro Cytolysis Assay.** For in vitro cytolysis assay, tumor cells in their exponential growth phase were incubated for 18 h in medium containing 0.1 μCi/ml [³H]thymidine. The cells were washed twice with CMEM and harvested by trypsinization, and $3 \times 10^4$ viable cells were seeded in triplicate into wells in 96-well plates and incubated for 72 h. Supernatants were removed, and the monolayers were washed three times with PBS containing Ca²⁺ and Mg²⁺. The adherent viable cells were lysed by 0.1 N NaOH, and the radioactivity was monitored in a beta counter. The cytotoxicity was calculated according to the formula:

$$\text{Cytolysis (%)} = \frac{1 - (B/A)}{100}$$

where $A$ is the cpm of the parental cells and $B$ is the cpm of viable transfected cells (44).

**Cell Proliferation Assay.** Cell proliferation was measured by [³H]thymidine incorporation. Briefly, tumor cells (3 $\times 10^4$ cells/well) were seeded in a 96-well plate and incubated at 37°C for 60 h and then pulse treated by the addition of 0.1 μCi/well [³H]thymidine. Twelve hours later, free [³H]thymidine was removed, cells were lysed by 0.1 N NaOH, and the radioactivity was monitored in a beta counter.

**Tumorigenicity and Metastasis Assays.** For all in vivo experiments, tumor cells in exponential growth phase were harvested by a brief exposure to 0.25% trypsin-0.02% EDTA solution (w/v). The flask was tapped sharply to dislodge the cells, CMEM was added, and the cell suspension was pipetted to produce single-cell suspensions. The cells were washed, resuspended in Ca²⁺- and Mg²⁺-free HBSS, and diluted to the desired cell number/inoculum. Cell viability was determined by trypsin blue exclusion, and only single-cell suspensions of greater than 95% viability were used for in vivo experiments. To
produce s.c. tumors, suspensions of cells in 0.1 ml HBSS were injected s.c. into the flank proximal to the midline. The mean diameter of the tumors was determined twice a week by measuring tumor length and width. In some experiments, tumors were removed and weighed.

Lung and kidney tumors were produced in mice. To produce experimental lung metastases, viable cells in 0.2 ml HBSS were injected into the lateral tail veins of unanesthetized mice. The mice were monitored daily and killed when moribund or by the indicated day. The lungs were weighed and then fixed in Bouin’s solution for 24 h to differentiate the neoplastic lesions from the organ parenchyma. The lung metastases were counted with the aid of a dissecting microscope. Spontaneous metastasis of the SN12PM6 cells was examined by injecting 1 × 10^6 cells in 50 µl into the subcapsule of the kidney of nude mice (n = 5). The kidney tumors were measured, and spontaneous lung metastases were counted on day 60 (35). KM12SM (1 × 10^6/mouse in 50 µl) cells were injected into the spleen of nude mice. The splenic tumors were measured, and experimental liver metastases were counted on day 60 (34).

 Fate of [3H]TdUrd-labeled Tumor Cells after s.c. Injection. Tumor cells were seeded into 150-cm² tissue culture flasks at a density of 4 × 10^5 cells/flask. Twenty-four h later, 0.3 µCi/ml of [3H]TdUrd (specific activity, 2000 Ci/mmole; DuPont NEN, Boston, MA) was added to the CMEM. Twenty-four h later, the monolayers were rinsed twice with excess Ca²⁺- and Mg²⁺-free HBSS to remove unbound radioiodine. The cells were then harvested with 0.25% trypsin/0.02% EDTA (Lanes 1): tumor cells, Neo-transfected cells, and mIFN-3-transfected cells (1 × 10^5/mouse for human tumors or otherwise as indicated) were injected s.c. into the skin of the dorsal side of the hind foot of nude mice. At various intervals after the injection, groups of mice (n = 5) were killed. The feet, lung, liver, kidneys, and spleen were removed and placed in 50-mi test tubes containing 70% ethanol. The ethanol was replaced daily for 3 days to remove all soluble [3H] released from dead cells. The radioactivity in all samples was determined in a gamma counter (TM Analytic, Elk Grove Village, IL). Triplicate tubes containing the inoculum dose were corrected for radioactive decay of input cells.

Statistical Analysis. The significance of the in vitro results was determined by Student’s t test (two-tailed). The results of the in vivo studies were analyzed by the Mann-Whitney test.

RESULTS

Transfection of Human Tumor Cells with Murine IFN-β. Full-length cDNA of the murine IFN-β was introduced into the mammalian expression vector pcDNA3 containing a drug-selectable marker for neomycin resistance and a strong cytomegalovirus early promoter. A375SM-P, KM12SM-P, and SN12PM6-P cells were transfected with either pcDNA/mIFN-β (A375SM-β, KM12SM-β, and SN12PM6-β) or control pcDNA3 (A375SM-Neo, KM12SM-Neo, and SN12PM6-Neo) using a mammalian transfection kit (Stratagene, San Diego, CA). Individual G418-resistant (500–800 µg/ml) colonies were established as separate adherent cultures. The expression of mIFN-β in the tumor cells was initially screened by a Northern blot analysis, and the IFN-β activity was determined in 48-h culture supernatants by measuring NO production in LPS-primed murine macrophages. To avoid clonal variations, positive clones (>10) were then pooled for the in vitro and in vivo studies. The IFN-β mRNA expression in A375SM KM12SM and SN12PM6 cells was determined in Fig. 1. IFN-β activity of the transfected A375SM-β, KM12SM-β, and SN12PM6-β cells was 1 × 10^6, 2 × 10^6, and 3 × 10^6 units/ml/10^6 cells per 48 h, respectively.

Growth and Metastasis of IFN-β-transfected Human Tumors in Nude Mice. To determine tumorigenicity, parental cells, Neo-transfected cells, and mIFN-β-transfected cells (1 × 10^6/inoculum) were injected s.c. into groups of nude mice (n = 5). All control cells (A375SM-P, A375SM-Neo, KM12PM6, SN12PM6-Neo, KM12SM-P, and KM12SM-Neo) produced progressively growing tumors, whereas IFN-β-transfected cells (A375SM-β, SN12PM6-β, and KM12SM-β) did not, even by day 60 after implantation (Table 1).

The metastatic potential of the A375SM cell lines was determined by the i.v. injection of 1 × 10^6 cells into groups of nude mice (n = 5). Control A375SM-P and A375SM-Neo cells produced numerous lung metastases, whereas the A375SM-β cells produced few or no lung metastases (Table 1). SN12PM6-P, SN12PM6-Neo, and SN12PM6-β cells (1 × 10^6/mouse) were injected orthotopically into the subcapsule of the kidney of nude mice (n = 5). By day 60, the SN12PM6-β cells produced no visible kidney tumors and no spontaneous lung metastases, whereas the control SN12PM6-P and SN12PM6-Neo cells produced large kidney tumors and a high number of spontaneous lung metastases (Table 1). KM12SM-P, KM12SM-Neo, and KM12SM-β cells (1 × 10^6/mouse) were injected into the spleen of nude mice (n = 5). By day 60, the IFN-β-
transfected KM12SM-β cells produced no splenic tumors and no liver metastases, whereas the control KM12SM-P and KM12SM-Neo cells produced large tumors in the spleen and many experimental liver metastases (Table 1).

**In Vivo Growth Inhibition of Bystander Tumor Cells.**

Next, we determined whether the IFN-β-producing tumor cells can inhibit the growth of bystander (nontransduced) tumor cells. We injected a mixture of 1 × 10^6 B16-BL6 melanoma cells with either 1 × 10^6 A375SM-β, KM12SM-β, or SN12PM6-β cells into the subcutis of groups of nude mice (n = 5). B16-BL6 tumor cells (1 × 10^5) were also injected alone or in a mixture with 1 × 10^6 A375SM-Neo, KM12SM-Neo, or SN12PM6-Neo cells serving as controls. B16-BL6 melanoma cells injected alone or together with control-transduced human cells produced rapidly growing melanotic tumors (Fig. 2A). In contrast, the growth of the B16-BL6 melanoma cells was totally inhibited by either A375SM-β, KM12SM-β, or SN12PM6-β cells (Fig. 2A). These data indicate that all IFN-β-producing human tumor cells were equally effective inhibitors of the growth of bystander tumor cells. Further studies, therefore, used only A375SM-β cells.

A375SM-β cells (1 × 10^6) were coinjected s.c. with either 2 × 10^4 murine CT-26 colon carcinoma cells, 5 × 10^4 murine 3LL cells, 5 × 10^4 RENCA cells, or human A375SM cells. The data summarized in Fig. 2B show that the A375SM-β cells inhibited the growth of the three mouse tumor cell lines (Fig. 2B) and the human tumor cells (Fig. 2C).

Growth inhibition of the bystander mouse tumor cells was not organ specific. We reached this conclusion because the injection of RENCA cells admixed with A375SM-β cells into the kidney of nude mice did not yield tumors (Fig. 3A) or spontaneous lung metastases (Fig. 3B), whereas the injection of RENCA cells admixed with A375SM-Neo cells produced large kidney tumors and numerous spontaneous lung metastases (Fig. 3).

To determine whether the inhibition of tumorigenicity by the IFN-β-transfected human tumor cells was a local or systemic event, we injected 1 × 10^5 B16-BL6 cells into the subcutis of nude mice (n = 5) at the same site or in a different site with A375SM-β or A375SM-Neo cells. As shown in Fig. 4A, the growth of B16-BL6 cells was totally inhibited by the coinjection of mIFN-β-producing A375SM-β cells but not their coinjection with control A375SM-Neo cells. However, when B16-BL6 cells were implanted at one s.c. site and the A375SM-β cells were implanted at a collateral site, the growth of the B16-BL6 tumor was unimpeded (Fig. 4B).

**Growth of Tumor Cells Transfected with IFN-β in Vitro.**

The effect of IFN-β transfection on tumor growth *in vitro* was determined by three different assays: (a) A375SM-β, A375SM-Neo, or A375SM-P tumor cells were plated at the density of 3 × 10^3 cells per 38-mm^2 well in 96-well plates. After 72-h incubation in medium, the cell number was determined by the MTT assay (Fig. 5A); (b) A375SM-β, A375SM-Neo, or A375SM-P cells (3 × 10^3 cells/38-mm^2 well) were seeded in 96-well plates, incubated for 60 h at 37°C, and then pulse treated with 0.1 μCi/well [3H]thymidine. Twelve h later, [3H]thymidine incorporation was monitored (Fig. 5B); and (c) [3H]thymidine-labeled A375SM-β, A375SM-Neo, or A375SM-P cells (3 × 10^3 cells/38-mm^2 well) were incubated in triplicate in 96-well plates. Seventy-two h later, cytosis was determined by measuring the [3H]thymidine release (data not shown). None of the assays demonstrated discernible differences in *in vitro* growth among the three tumor cell lines, suggesting that the *in vivo* differences in tumorigenicity were likely due to indirect effects rather than to antiproliferative effects of the IFN-β.

**In Vivo Eradication of IFN-β-transfected Human Tumor Cells and Bystander Murine Tumor Cells.**

To determine whether the suppression of tumor growth of both human tumor cells transfected with IFN-β and bystander murine tumor cells was due to cytosis or cytostasis, both A375SM-Neo and A375SM-β cells were labeled with [125]IIdUdR and injected s.c. into nude mice. Cell survival was determined by measuring the radioactivity remaining at the injection site. The data shown in Fig. 6A suggest that IFN-β-transfected cells underwent cytosis. We base this conclusion on the results of cell survival by day 7 after injection: 20% and <1% for A375SM-Neo and A375SM-β cells, respectively.

Next, we determined whether A375SM-β cells produced lysis of B16-BL6 cells prelabeled with [125]IIdUdR and coinjected with either A375SM-Neo or A375SM-β into the subcutis of nude mice. The bystander B16-BL6 cells were also eradicated by the A375SM IFN-β-producing cells (Fig. 6B), suggesting that the production of mIFN-β is associated with the lysis of both IFN-β-producing cells and bystander (nonproducing) cells.

---

**Table 1.** Suppression of tumorigenicity and metastasis by human tumors transfected with IFN-β in nude mice.

<table>
<thead>
<tr>
<th>Cell line*</th>
<th>Tumor weight (g)</th>
<th>P*</th>
<th>Metastasis</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375SM-P</td>
<td>0.6, 1.3, 1.4, 1.7, 2.0</td>
<td>&lt;0.01</td>
<td>98, 111, 145, 200, 200</td>
<td></td>
</tr>
<tr>
<td>A375SM-Neo</td>
<td>1.1, 1.6, 1.8, 2.3</td>
<td>&lt;0.01</td>
<td>96, 102, 168, 176, 200</td>
<td></td>
</tr>
<tr>
<td>A375SM-β</td>
<td>0, 0, 0, 0, 0</td>
<td>&lt;0.01</td>
<td>0, 0, 0, 0, 3</td>
<td></td>
</tr>
<tr>
<td>KM12SM-P</td>
<td>0.8, 1.0, 1.2, 1.4, 2.4</td>
<td>&lt;0.01</td>
<td>6, 12, 15, 21, 34</td>
<td></td>
</tr>
<tr>
<td>KM12SM-Neo</td>
<td>0.9, 1.2, 1.4, 1.7, 1.9</td>
<td>&lt;0.01</td>
<td>11, 18, 24, 36, 67</td>
<td></td>
</tr>
<tr>
<td>KM12SM-β</td>
<td>0, 0, 0, 0, 0</td>
<td>&lt;0.01</td>
<td>0, 0, 0, 0, 0</td>
<td></td>
</tr>
<tr>
<td>SN12PM6-P</td>
<td>0.4, 0.7, 0.8, 1.4, 1.7</td>
<td>&lt;0.01</td>
<td>0, 12, 31, 78</td>
<td></td>
</tr>
<tr>
<td>SN12PM6-β</td>
<td>0.6, 0.6, 1.3, 1.6, 1.8</td>
<td>&lt;0.01</td>
<td>0, 2.4, 6.16</td>
<td></td>
</tr>
</tbody>
</table>

* A375SM-P, A375SM-Neo, or A375SM-β cells (1 × 10^6/inoculum) were injected s.c. or i.v. into groups of nude mice (n = 5); primary tumors and experimental lung metastasis were determined on day 60. KM12SM-P, KM12SM-Neo, or KM12SM-β cells (1 × 10^6/inoculum) were injected into the spleen of nude mice (n = 5); primary tumors and spontaneous liver metastases were determined on day 60. SN12PM6-P, SN12PM6-Neo, or SN12PM6-β cells (1 × 10^6/inoculum) were injected into the subcapsule of the kidney of nude mice (n = 5); primary tumors and spontaneous lung metastasis were determined on day 60.

* As compared to controls, i.e., mIFN-β-transfected cells versus Neo-transfected or parental cells.
Fig. 2 Human tumor cells transfected with murine IFN-β gene inhibit the tumorigenicity of bystander murine tumor cells. In A, B16-BL6 cells (2 × 10^6) were injected s.c. into the flank region of nude mice either alone or mixed with A375SM-β cells (1 × 10^6), KM12SM-β cells (1 × 10^6), or SN12PM6-β cells (1 × 10^6). Tumor incidence and size were determined twice weekly. Note significant suppression of tumor formation of B16-BL6 by mIFN-β-producing cells. In B, CT-26 (5 × 10^6), 3LL (1 × 10^6), or RENCA (5 × 10^6) cells were injected s.c. into the flank region of nude mice either alone or mixed with A375SM-β cells (1 × 10^6). Tumor incidence and size were determined twice weekly. Note significant suppression of tumor formation of all murine tumor cells by A375SM-β cells. *, P < 0.01 as compared to appropriate controls. This is one representative experiment of two.

Fig. 3 Suppression of bystander murine RENCA cells by mIFN-β-producing A375SM cells implanted into the kidneys of nude mice. RENCA cells (5 × 10^6) were injected into the kidneys of nude mice mixed alone or in a mixture with A375SM-P, A375SM-Neo, or A375SM-β cells (1 × 10^6). Tumor formation (A) and production of spontaneous lung metastasis (B) were determined 4 weeks later. Note significant suppression of tumorigenicity and metastasis of RENCA by A375SM-β cells. *, P < 0.01 as compared to appropriate controls. This is one representative experiment of two.

[^125]IIdUdR-labeled B16-BL6 cells injected at a different s.c. site from that of A375SM-β cells remained viable (Fig. 6C), suggesting that IFN-β can induce cell lysis only in nearby cells.

Modulation of Tumoricidal Activity of Murine Macrophage by IFN-β-producing Cells. In the final set of experiments, we focused on the possible role of macrophages in suppressing the in vivo growth of both the human and murine tumor cells: (a) we determined the sensitivity of IFN-β-transfected human tumor cells to lysis mediated by mouse macrophages. A375SM-P, A375SM-Neo, and A375SM-β cells labeled with [3H]thymidine were incubated for 72 h with macrophages in medium or medium containing IFN-γ, LPS, or LPS plus IFN-γ, which are known to activate iNOS in macrophages (40). Target cell lysis was determined by a [3H]thymidine 72-h release assay. As shown in Fig. 7A, mIFN-β-producing A375SM-β cells were more sensitive to macrophage-mediated cytotoxicity, especially to LPS-primed macrophages; and (b) we determined the sensitivity of B16-BL6 cells to macrophages...
cocultured with mIFN-β-transfected A375SM-β or control A375SM-Neo or A375SM-P cells. As shown in Fig. 7B, A375SM-β cells activated tumoricidal properties in macrophages to kill B16-BL6 cells. Target B16-BL6 tumor cells were not killed when they were cocultured with A375SM-β tumor cells in the absence of macrophages (data not shown).

Macrophage-mediated lysis of B16-BL6 cells is primarily mediated by NO (40). We, therefore, determined whether mIFN-β-producing A375SM-β cells induced NO production in murine macrophages. Mouse macrophages were incubated with supernatants of different transfectants in the presence of LPS, IFN-γ, or both. As reported previously, the combination of LPS and IFN-γ led to maximal induction of NO and cytolysis of tumor targets, whereas treatment of macrophages with only IFN-γ or LPS produced low levels of NO and cytolysis. As shown in Fig. 8A, supernatants of mIFN-β-transfected cells alone did not induce NO production in murine macrophages. However, in LPS-primed macrophages, the mIFN-β supernatant induced maximum NO production with a peak at 72 h (Fig. 8B), which directly correlated with induction of tumoricidal activity in the macrophages (Fig. 7). Both NO induction (data not shown) and cytolysis (Fig. 7B) were inhibited by the specific iNOS inhibitor NMA.

Finally, we determined whether iNOS could also be induced in vivo. Nude mice were injected s.c. with A375SM-β or A375SM-Neo cells. Seven days later, the mice were killed, and the injection sites were resected and processed for immunohistochemical analyses. Infiltrating macrophages were identified by a specific anti-macrophage antibody, F4/80, and iNOS protein expression was determined by the use of a specific anti-mouse iNOS antibody. A375SM-Neo cells produced tumor lesions that were surrounded by macrophages (Fig. 9A1) with no detectable iNOS expression (Fig. 9A2). In contrast, only very...
few A375SM-β cells survived among numerous infiltrating macrophages (Fig. 9B1), which were clearly stained with the anti-iNOS antibody (Fig. 9B2).

To further determine whether iNOS could also be induced in vivo, nude mice were injected i.p. with A375SM-β or A375SM-Neo cells. Four days later, peritoneal macrophages were harvested and plated into chamber slides for immunocytochemical analysis, plated into 96-well plates (1 x 10^5/well) for measurement of NO production, or lysed for Western blot analysis of iNOS expression. Macrophages isolated from mice injected i.p. with A375SM-β cells (Fig. 9D1) were strongly positive for iNOS protein, whereas peritoneal macrophages from mice injected i.p. with A375SM-Neo cells were not (Fig. 9D2).

Fig. 6 In vivo autolysis of mIFN-β-producing cells and cytolysis of bystander tumor cells. In A, [125I]IdUrd-labeled A375SM-P, A375SM-Neo, and A375SM-β cells (1 x 10^6) were injected s.c. into the flank region of nude mice. The presence of viable cells was determined 3 and 7 days after tumor injection by measuring the radioactivity remaining at the injection site. In B, [125I]IdUrd-labeled B16-BL6 cells (2 x 10^6) were injected s.c. into the flank of nude mice admixed with A375SM-P (1 x 10^6), A375SM-Neo (1 x 10^6), or A375SM-β (1 x 10^6) cells. The percentage of viable B16-BL6 cells was determined 3 and 7 days after tumor injection by measuring the radioactivity remaining at the injection site. In C, [125I]IdUrd-labeled B16-BL6 cells (2 x 10^6) were injected s.c. into the flank region of nude mice admixed with A375SM-P cells (1 x 10^6), A375SM-Neo cells (1 x 10^6), or A375SM-β cells (1 x 10^6). In another group of mice, [125I]IdUrd-labeled B16-BL6 tumor cells (2 x 10^6) were injected s.c. into the flank region of nude mice, and A375SM-P cells (1 x 10^6), A375SM-Neo cells (1 x 10^6), or A375SM-β cells (1 x 10^6) were injected into the contralateral flank. The percentage of viable B16-BL6 cells was determined 3 days after tumor cell injection by measuring the radioactivity remaining at the injection site. * P < 0.01 as compared to appropriate controls. This is one representative experiment of two; bars, SD.

Fig. 7 Activation of tumoricidal properties in murine peritoneal macrophages by mIFN-β-producing tumor cells. In A, mouse macrophages plated into 96-well plates (1 x 10^5/well) were cocultivated for 3 days with [3H]thymidine-labeled A375SM-P, A375SM-Neo, or A375SM-β tumor cells in the presence or absence of 10 units/ml mouse IFN-γ, 1 μg/ml LPS, or 10 units/ml IFN-γ plus 1 μg/ml LPS. Lysis of target cells was determined by measuring the radioactivity remaining in viable target cells. Note that mIFN-β-transfected cells were more sensitive to macrophage-mediated cytotoxicity. In B, mouse macrophages plated into 96-well plates (1 x 10^5/well) were cocultivated for 3 days with [3H]thymidine-labeled B16-BL6 tumor cells and either A375SM-P, A375SM-Neo, or A375SM-β tumor cells in medium (control) or medium containing 1 μg/ml LPS in the presence or absence of 3 mM NMA. Lysis of radiolabeled B16 cells was determined by measuring radioactivity associated with the adherent viable cells. Note that cocultivation of LPS-primed mouse macrophages and mIFN-β-transfected cells produced significant lysis of B16-BL6 tumor cells. * P < 0.01 as compared to appropriate controls. This is one representative experiment of three; bars, SD.
DISCUSSION

The present results demonstrate that transfection of the mIFN-β gene into three different human tumor cells inhibits their tumorigenic and metastatic properties in nude mice. Moreover, the mIFN-β-transfected human tumor cells inhibited the tumorigenicity of different bystander murine and human tumor cells when the cells were implanted into the same site (s.c. or kidney). The mechanisms responsible for the suppression of tumorigenicity involve the production of NO by infiltrating macrophages and associated cytostasis and cytolyis of the transfected and bystander cells.

IFNs have been shown to influence the proliferation, differentiation, and death of tumor cells (5–15). These end points, however, have not been translated into clinical reality and therapy of solid tumors (11, 15), probably because of a lack of sustained levels of IFN in the tumor tissue (15–18). Several pharmacokinetic studies have demonstrated that the half-life of recombinant human IFN-β in the circulation is about 5 min; the serum concentration falls to <8 units/ml 1 h after i.v. injection of 6 × 10⁴ units, and its s.c. or i.m. injection produces a maximal serum concentration of <2 units/ml (15–17). These concentrations of IFN-β are well below those required to inhibit cell proliferation (2–4), down-regulate collagenase type IV (30, 31), down-regulate bFGF (26, 27), or activate host macrophages (41, 46). To achieve high levels of IFN-β in tumor tissues requires the systemic administration of high doses, which are toxic (11, 15). Alternatively, a high dose of IFN-β could be delivered into localized sites to produce antitumor effects. We, therefore, tested the possibility that the sustained presence of IFN-β in and around tumor lesions would suppress tumorigenicity and hence the metastatic potential of various neoplasms.

The antitumor effects mediated by IFN-β could be due to direct or indirect mechanisms. IFNs have clearly been shown to produce antiproliferative effects by blocking G1 progression, lengthening the S phase, or lengthening all phases of the cell cycle (47, 48). IFNs may antagonize the function of various growth factors or inhibit genes that are regulated by the growth factors, e.g., platelet-derived growth factor (49, 50). The direct effects of IFN-β, however, are species specific, i.e., murine IFN-β does not inhibit the proliferation of human tumor cells (2–3). Indeed, recombinant mouse IFN-β did not influence the in vitro growth of the transfected human tumor cells. In fact, the mIFN-β-producing human cells grew in vitro at a rate identical to control transfected or nontransfected cells. Once implanted into nude mice, however, the mIFN-β-producing human tumor cells failed to produce tumors, suggesting that mIFN-β produced antitumor activity by an indirect mechanism.

There are several indirect mechanisms by which IFN-β could inhibit the growth and metastasis of tumor cells. IFNs have been shown to regulate cell surface molecules, such as major histocompatibility complex class I and II molecules (51, 52) and adhesive intercellular adhesion molecule-1 and leukocyte function antigen-1 molecules, that can affect cell growth by regulating the adhesion of malignant cells to extracellular matrix and stroma (53, 54). IFN-β can inhibit angiogenesis (20, 55) by down-regulating the expression of bFGF (26, 27), collagenase type IV (30, 31), and IL-8 (28, 29). Finally, IFN-β can modulate host immune response; it activates natural killer cells and can render macrophages cytotoxic against target cells (41, 46).

The mIFN-β-transfected human tumor cells not only failed to grow in vivo (at any site of implantation) but also inhibited the local growth of highly tumorigenic bystander human and...
murine cells. This finding was not linked to the histological type of the tumors. All three human tumor cell lines (melanoma, renal carcinoma, and colon carcinoma) transfected with mIFN-β produced these effects in murine melanoma, renal cancer, or colon cancer cells. The inhibition of tumor growth was not restricted to the subcutis of nude mice; the orthotopic implantation of mIFN-β-producing cells and bystander cells into the kidney of nude mice also failed to produce growing tumors. Regardless of the tumor types, the suppression of tumorigenicity was a local event. When we injected the mIFN-β-producing human tumor cells and the murine cells into two sites, the former did not grow, but the latter did. Our data agree with a recent observation using IFN-α-transduced B16 melanoma cells (56).

The use of [125I]IdUrd-labeled cells allowed us to determine the survival rate of injected cells, which in turn suggested a mechanism for clearance (44, 45). These in vivo cell survival experiments showed that by day 7 after s.c. implantation, a large percentage of control cells survived to yield a tumor, whereas the majority of the mIFN-β-transfected cells died. Similar data were obtained when the bystander cells were labeled with [125I]IdUrd and injected together with the mIFN-β-producing cells. These data suggest that the kinetics of cell kill were similar for the mIFN-β-producing cells (autolysis) and bystander cells (cytolysis).

IFN-β can induce the production of NO in murine macrophages (41, 46). NO is derived from the oxidation of one of the terminal quinidine nitrogens of L-arginine (57), which is itself catalyzed by several isoforms of the enzyme NOS, i.e., constitutive NOS and iNOS. iNOS is activated in macrophages and endothelial cells in response to cytokines and endotoxins (57) to generate large amounts of NO, which kill tumor cells via NO-dependent pathways (40, 57, 58). NO can also influence metastasis through inducing vasodilatation and through inhibiting platelet aggregation (59). Our present study provides the first evidence that the nonspecific antitumor activity of IFN-β is associated with the production of NO. Although recombinant IFN-β alone did not induce NO production in macrophages
under in vitro conditions, under in vivo conditions, IFN-β released from transfected tumor cells induced iNOS expression and NO production from host macrophages. This difference may be due to the in vivo presence of other cytokines, e.g., IL-1 and TNF, that could synergize with IFN-β to activate iNOS (57, 59). The IFN-β-mediated eradication of tumors was not accompanied by systemic side effects, perhaps because induction of NO was local. NO is an extremely unstable free radical that is oxidized into the stable nontoxic products nitrite/nitrate within a few seconds (57, 59, 60).

In summary, we demonstrate that localized high concentrations of IFN-β eradicate tumors of different tissue origin, presumably through activation of iNOS in host cells such as macrophages. These data demonstrate that the production of IFN-β in tumor tissue can eradicate the lesions. The potential use of the IFN-β gene in therapy of established disease is now under investigation.

ACKNOWLEDGMENTS

We gratefully acknowledge Walter Pagel for critical editorial review and Lola López for the excellent preparation of the manuscript.

REFERENCES


Abrogation of tumorigenicity and metastasis of murine and human tumor cells by transfection with the murine IFN-beta gene: possible role of nitric oxide.

K Xie, D Bielenberg, S Huang, et al.


Updated version  Access the most recent version of this article at:  
[http://clincancerres.aacrjournals.org/content/3/12/2283](http://clincancerres.aacrjournals.org/content/3/12/2283)