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Suppression of tumorigenicity and metastasis in murine UV-2237 fibrosarcoma cells by infection with a retroviral vector harboring the interferon-beta gene

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Abstract In this study, we endeavored to determine the effectiveness of interferon β (IFNβ) gene therapy against highly metastatic murine UV-2237m fibrosarcoma cells. UV-2237m cells were engineered to produce murine IFNβ constitutively following infection by a retroviral vector harboring the murine IFNβ gene. Parental (UV-2237m-P), control-vector-transduced (UV-2237m-Neo), and IFNβ-transduced (UV-2237m-IFNβ) cells were injected subcutaneously (s.c.) or intravenously (i.v.) into syngeneic mice. Parental and control-transduced cells produced rapidly growing tumors, whereas IFNβ-transduced cells did not. The tumorigenicity of IFNβ-sensitive or -resistant parental cells was significantly suppressed when they were injected s.c. together with IFNβ-transduced cells. The IFNβ-transduced cells did not inhibit growth of parental cells injected s.c. at a distant site. UV-2237m-IFNβ cells produced s.c. tumors in nude, SCID/Beige, and natural killer(NK)-cell-compromised syngeneic mice. The IFNβ-transduced cells were more sensitive to in vitro splenic cell-mediated lysis than were the parental or control-transduced cells. Pretreatment of C3H/HeN mice with the NK-cell-selective antiserum (anti-asialoGM1) partially abrogated the cytotoxic activity of the cells. Cytotoxic activity was not observed in mixed culture of UV-2237m-IFNβ cells and splenic cells from SCID/Beige mice. Significant cytotoxicity against UV-2237m-IFNβ cells was mediated by macrophages activated by either IFNγ, lipopolysaccharide, or a combination of both. Our data led us to conclude that the constitutive expression of IFNβ can suppress tumorigenicity and metastasis of UV-2237m cells, which is due, in part, to activation of host effector cells.

Key words Interferon β · NK cells · Macrophages · Fibrosarcoma · Gene therapy

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Introduction

Interferon β (IFNβ), a member of the IFN family, which also includes IFNα and IFNγ, is a glycoprotein with an apparent mass of 20–25 kDa [24]. Although IFNα can be encoded by many genes, IFNβ and IFNγ are encoded by single genes only. IFNα and IFNγ are mainly expressed in leukocytes and lymphocytes, whereas IFNβ can be induced in many types of cells, including fibroblasts, macrophages, and epithelial cells [32]. Although IFNβ shares the type I IFN receptor with IFNα and induces a similar pattern of cellular responses [39], certain cellular reactions can be stimulated only by IFNβ, probably by the phosphorylation of a receptor-associated protein that is uniquely responsive to IFNβ [39].

IFNβ was discovered in the 1950s on the basis of its antiviral activity [16]. Subsequent investigation, however, revealed that it was a multifunctional regulatory cytokine involved in the homeostatic control of cellular function and replication [38]. IFNβ directly inhibits the proliferation of tumor cells of different histological origin [14, 17, 21, 33, 34] at a higher efficiency than IFNα or IFNγ in some experimental systems [17, 33]. Recent studies indicate that IFNβ can also down-regulate the expression of angiogenic molecules, such as interleukin-8 [25, 40], basic fibroblast growth factor [35], and matrix metalloproteinases [9, 13], that are necessary for tumor growth and metastasis [11]. Moreover, IFNβ can activate tumoricidal properties in macrophages and natural killer (NK) cells [26, 28, 42]. Although these data suggest a great potential for IFNβ in tumor therapy, preclinical studies and clinical trials with IFNβ have been disappointing [1, 8, 31], probably because

of a lack of sustained high levels of IFNβ in the tumor lesions [8, 35]. Pharmacokinetic studies have indeed concluded that the half-life of IFNβ in the circulation of humans is of the order of 5 min with serum concentrations below 8 U/ml 1 h after a bolus intravenous (i.v.) administration of 6×10^6 IU recombinant human IFNβ. Intramuscular or subcutaneous (s.c.) injections of IFNβ produce a maximal serum concentrations less than 2 U/ml [29], which are below those required for suppression of tumor cell growth, down-regulation of angiogenesis, and activation of macrophages and NK cells.

In the present study we tested the hypothesis that the local, continuous production of IFNβ in growing tumors would prevent tumorigenicity and metastasis. We demonstrate that s.c. growth of murine fibrosarcoma cells engineered to produce IFNβ was significantly reduced in syngeneic mice. We also show a bystander effect by the IFNβ-secreting cells on the tumorigenicity of parental cells and IFNβ-resistant cells. The inhibitory effect of IFNβ on the tumorigenicity was at least partially mediated by tumoricidal activation of host effector cells.

Materials and methods

Mice

Specific-pathogen-free female C3H/HeN mice and female athymic nude mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, Md.). Female SCID/Beige mice were purchased from the Jackson Laboratory (Bar Harbor, Me.). Inducible nitric oxide synthase knockout (iNOS$^{-/-}$) mice [22] were the generous gift of Dr. Carl Nathan (Cornell University, New York, N.Y.). All the mice were used when they were 6- to 8-weeks old. 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 National Institutes of Health.

Reagents

Eagle's minimal essential medium (EMEM), Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS), and fetal bovine serum were purchased from M. A. Bioproducts (Walkersville, Md.). Mouse IFNβ was purchased from Lee BioMolecular Co. (San Diego, Calif.). Mouse recombinant IFNγ (sp. act., 5.2×10^6 U/mg protein) was the generous gift of Genentech Inc. (South San Francisco, Calif.). Phenol-extracted *Salmonella* lipopolysaccharide (LPS), N^G-L-monomethyl-L-arginine (N^G-MeArg), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were 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 Co. (Woods Hole, Mass.).

Cells and culture conditions

The UV-2237m line [27] was derived from a spontaneous lung metastases produced by parental UV-2237 fibrosarcoma cells originally induced in a C3H/HeN mouse by ultraviolet (UV)-B radiation [20]. The cells were maintained as a monolayer culture in EMEM supplemented with 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, vitamin A, and glutamine (CMEM). The UV-2237m^R subline was established by cultivation of UV-2237m cells in vitro in increasing concentrations of IFNβ. UV-2237m^R cells used in the present study were resistant to the antiproliferative effects of 5000 U/ml IFNβ. UV-2237m cells in the exponential phase of growth 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. Only suspensions of single cells with viability exceeding 90% were used.

Generation of retroviral vector encoding IFNβ

The full coding region of mouse IFNβ cDNA (generously provided by Dr. T. Taniguchi, Osaka University, Japan) was subcloned into retroviral vector pLXSN (generously provided by Dr. A. D. Miller, Fred Hutchinson Cancer Research Center, Seattle, Wash.) [23] to generate pLXSN-IFNβ. The control vector (pLXSN-Neo) or pLXSN-IFNβ was transfected into PA317 cells (American Type Culture Collection, Rockville, Md.) by a calcium/phosphate procedure using a mammalian cell transfection kit (Strategene, La Jolla, Calif.). PA317 cells, expressing the control vector and the IFNβ-encoding vector, were isolated subsequent to selection in medium containing G418 (Gibco-BRL, Long Island, N.Y.). For collection of viral vectors for transduction, the packaging cells (2×10^6) were plated into T75 flasks, and the culture medium was replaced after an overnight incubation. Culture supernatants containing viruses were harvested 48 h later, filtered through a 0.45-μm membrane, and used for infection of the murine tumor cells.

Infection of UV-2237m cells

UV-2237m cells (2×10^5) were plated into T75 flasks. After 24 h, the cells were incubated for 6 h with viral vectors in the presence of 8 μg/ml polybrene. The cells were cultured for 48 h in CMEM and then were selected in CMEM containing 800 μg/ml G418. Expression of IFNβ was identified by Northern blot analysis and a bioassay as described below.

Tumorigenicity and experimental lung metastasis

To produce subcutaneous tumors, suspensions of UV-2237m, UV-2237_m-Neo, or UV-2237_m-IFNβ cells (1×10^6 or 1×10^5/inoculum for C3H/HeN or athymic nude mice respectively) in 0.1 ml HBSS were injected s.c. into the lateral flank proximal to the midline. The mean diameter of the tumors was determined twice a week by measuring tumor length and width. To produce experimental lung metastases, 2×10^5 viable cells in 0.2 ml HBSS were injected into the lateral tail vein of unanesthetized mice. The mice were monitored daily and killed when moribund or by 28 days after tumor cell injection. The lungs were weighed and then fixed in Bouin's solution. The number of tumor nodules was determined under a dissecting microscope.

In vitro sensitivity to antiproliferative effects of IFNβ

UV-2237m cells in 0.1 ml EMEM were plated at a density of 400 cells/38-mm^2 well of a 96-well plate. After an overnight incubation culture period, different concentrations of murine IFNβ were added. The cultures were maintained at 37 °C in 5% CO_2 for 96 h. During this period, the cells grew exponentially without change of medium. MTT (2 mg/ml in phosphate-buffered saline, PBS) was added to the cultures at 0.05 ml/well during the final 2 h of incubation. The medium was then carefully removed, and the dark blue formazan was dissolved in 100 μl dimethylsulfoxide. The absorbance of each well was measured with a Dynatech 5000 microplate reader at 570 nm (Dynatech Inc., Chantilly, Va.) [2]. The percentage cytostasis was calculated according to the following formula: cytostasis (%) = (1 $-A_{570}$ of treated group/A_{570} of control group) $\times100$.

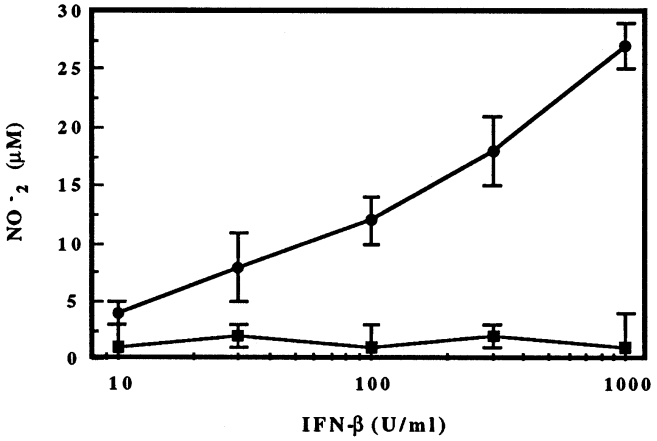

Fig. 1 Neutralization of interferon β (*IFN*β) activity by a monoclonal antibody to mouse IFNβ. Macrophages in a 96-well plate were incubated for 24 h with 100 ng/ml lipopolysaccharide and various concentrations of IFNβ in the control nonspecific IgG (●) or in the presence of 100 NU/ml of anti-IFNβ mAb (■). NO^{-}_{2} in the culture supernatant was then determined

Collection and cultivation of mouse peritoneal exudate macrophages

Peritoneal exudate macrophages were collected by peritoneal lavage of mice given an intraperitoneal (i.p.) injection of 1.5 ml thioglycollate broth (Baltimore Biological Laboratories, Cockeysville, Md.) 4 days before harvest [3]. The cells were washed with Ca^{2+}- and Mg^{2+}-free PBS and resuspended in serum-free EMEM, and 1×10^5 cells in 0.2 ml EMEM were plated into 38-mm^2 wells of 96-well flat-bottom Microtest III plates (Falcon Plastics, Oxnard, Calif.). After 90 min, the wells were washed with EMEM to remove nonadherent cells. The resultant macrophage monolayer was more than 98% pure according to morphological and phagocytic criteria. These cultures were then given supplemented medium containing macrophage activators and other reagents as described below.

In vitro assay of macrophage-mediated cytotoxicity

Macrophage-mediated cytotoxicity was assessed by measuring the release of radioactivity from DNA of target cells as described previously [4]. UV-2237m cells in their exponential growth phase were incubated for 24 h in CMEM containing 0.2 μCi/ml [^{3}H]dT (ICN, Costa Mesa, Calif., sp. act. 2 mCi/mmol). The cells were washed twice with HBSS to remove unbound radioisotope, harvested by a brief trypsinization, washed, and resuspended in supplemented EMEM. Then 1×10^4 viable cells were plated into wells containing macrophages 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 neoplastic cells. 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 0.1 M NaOH. The lysates were harvested with Harvester 96 (Tomtec, Orange, Conn.) and counted in a liquid scintillation counter. The cytotoxic activity of the macrophages was calculated as follows: cytotoxicity (%) = $100\times(A-B)/A$, where A is the radioactivity (cpm) in cultures of control macrophages and target cells, and B is the radioactivity (cpm) in cultures of test macrophages and target cells.

Spleen-cell-mediated cytotoxicity

Spleen cells from untreated C3H/HeN mice, C3H/HeN mice pretreated by anti-asialoGM1 serum (40 μl/mouse, 48 h prior to harvesting cells) or by normal rabbit serum, and SCID/Beige mice were incubated for 24 h with 1×10^4 [^{3}H]dT-labeled UV-2237m/38-mm^2 well of a 96-well plate at different ratios (target:effector = 1:10 to 1:200) [36]. After a brief spinning, 100 μl culture supernatants was collected and counted in a liquid scintillation counter. The specific cytolytic activity of the spleen cells was calculated as follows: cytolysis (%) = $(A-B)/(T-B)\times100$, where A is the radioactivity (cpm) in cultures of spleen cells and target cells, B the radioactvity (cpm) in cultures target cells only, and T the total radioactivity of target cells added to each well.

RNA isolation and Northern blot analyses

The mRNA was extracted using a Fast Track kit (Invitrogen, San Diego, Calif.). For Northern blot analysis, 1 μg of mRNA was fractionated on 1% denaturing formaldehyde/agarose gels, electrotransferred to a GeneScreen nylon membrane (DuPont Co., Boston, Mass.), and UV cross-linked with 120000 μJ/cm^2 using a UV Stratalinker 1800 (Stratagene). Hybridization using cDNA probes was performed as described previously [5]. Filters were washed two to three times at 50–60 °C with 30 mM NaCl/3 mM sodium citrate pH 7.2/0.1% sodium dodecyl sulfate. The DNA probes used were the cDNA fragment corresponding to rat glyceraldehyde-3-phosphate dehydrogenase or mouse IFNβ.

Western blot analysis

Samples of culture supernatant (40 μl/lane) were mixed with sample buffer (62.5 mM TRIS/HCl (pH 6.8), 2.3% sodium dodecylsulfate (SDS), 100 mM dithiothreiol, and 0.05% bromophenol blue), boiled, and separated by electrophoresis on a 10% SDS/polyacrylamide gel. The protein was transferred to 0.45-μm nitrocelullose membranes. The filter was blocked with 3% bovine serum albumin in TRIS-buffered saline (20 mM TRIS/HCl pH 7.5, 150 mM NaCl), probed with an antibody against mouse IFNβ (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 [3].

Immunohistochemical staining

Macrophages in tumors were identified by immunohistochemistry using the macrophage-specific scavenger receptor antibody [7, 15). At necropsy, the tumor tissues were cut into 5-mm pieces, placed in OCT compound (Miles Laboratories, Elkhart, Ind.) and snap-frozen in liquid nitrogen. Frozen sections (8–10 μM) were air-dried, fixed with cold acetone, rinsed with PBS, 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 serumin PBS, v/v), and then with a rat polyclonal antibody against Scavenger receptor (Serotec Ltd., Kidlington, Mass.) at a 1:70 dilution for 18 h at 4 °C in a humidified chamber. The sections were rinsed, incubated with the blocking solution and then with a peroxidase-conjugated anti-(rat Ig) antibody (Jackson Immuno Research Laboratories Inc.) at a 1:100 dilution. Positive reaction was visualized by incubating the slides with stable diaminobenzidine (Research Genetics, Huntsville, Ala.) for 1–5 min, followed by counterstaining with Mayer's hematoxylin (Research Genetics, Huntsville, Ala.). The slides were dried and mounted with Universal mount (Research Genetics, Huntsville, Ala.) and images were digitized using a Sony 3CCD color video camera (Sony Corporation, Japan) and a personal computer equipped with an Optimas Analysis software (Optimas Corporation, Bothell, Wash.).

Bioassay for IFNβ activity

The bioassay for IFNβ was based on the finding that this cytokine generates nitric oxide production by LPS-primed peritoneal exudate macrophages [42]. These cells, plated at the density of 1×10^5 cells/38-mm^2 well of 96-well plates, were incubated for 24 h with test

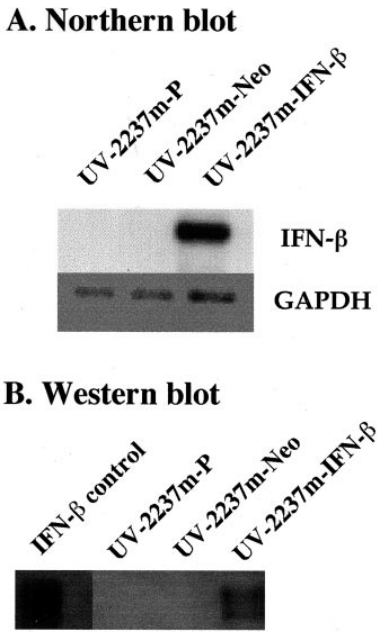

Fig. 2A,B Expression of IFNβ-transduced UV-2237m cells. **A** Total cellular RNA was extracted from parental (UV-2237m-P), control-vector-transduced (UV-2237m-Neo), and IFNβ-transduced (UV-2237m-IFNβ) cells and analyzed by Northern blotting as described in Materials and methods. **B** UV-2237m-P, UV-2237m-Neo, or UV-2237m-IFNβ cells in 5% fetal bovine serum/minimal essential medium were plated into 24-well plates at 10^6/well. After 24 h, the cells were rinsed with Hanks' balanced salt solution (HBSS), fed with serum-free DMEM/F12 (1 ml/well), and cultured for 72 h. A 40-μl sample of culture supernatant from each well was analyzed by Western blotting. The positive control contained 5 ng purified IFNβ. This is one representative experiment of three. *$GAPD_H$* glyceraldehyde-3-phosphate-dehydrogenase

samples or with increasing concentrations of IFNβ in the presence of 1 μg/ml LPS. NO^-_2 levels were determined as described previously [6].

Assay of dose-dependent induction of NO^-_2 production (10–1000 U/ml)

To confirm that the induction of NO^-_2 production was due to IFNβ (in the supernates), we used a rat monoclonal antibody against mouse IFNβ (Yamasa Shoyu Co., Tokyo, Japan). As shown in Fig. 1, addition of 100 neutralization units/ml antibody completely blocked NO^-_2 production induced by 10–1000 U/ml IFNβ.

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

Expression of mouse IFNβ in UV-2237m cells

Total cellular RNA was extracted from UV-2237m cells and analyzed by Northern blotting. IFNβ mRNA was not detected in UV-2237m-P cells (parental) or UV-2237m-Neo (transduced with control retroviral vector) cells. In contrast, a high level of IFNβ mRNA was found in UV-2237m-IFNβ cells transduced with the mouse IFNβ gene (Fig. 2A). Consistently, IFNβ protein was detected in the culture supernatant of UV-2237m-IFNβ, but not UV-2237m-P and UV-2237m-Neo, cells by Western blot analysis (Fig. 2B). UV-2237m-IFNβ cells produced IFNβ activity in a cell-density-dependent manner. At 10^6 cells/well (in a 24-well dish), UV-2237m-IFNβ cells secreted up to 3000 U IFNβ in 24 h (Fig. 3). The expression of IFNβ in UV-2237m-IFNβ cells was relatively stable. Similar levels of IFNβ activity were found in supernatants of cells cultured without G418 for 3–4 weeks.

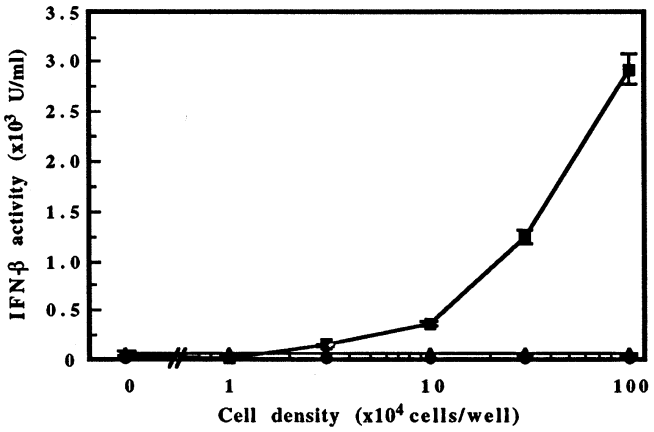

Fig. 3 Production of IFNβ by UV-2237m cells. Cells were plated into 24-well plates at different densities. After overnight adherence, the cells were refed with fresh medium (0.5 ml/well) and incubated from 24 h. IFNβ activity in the culture supernatant of UV2237m-P (●), UV-2237m-Neo (▲), or UV-2237m-IFNβ (■) was then determined

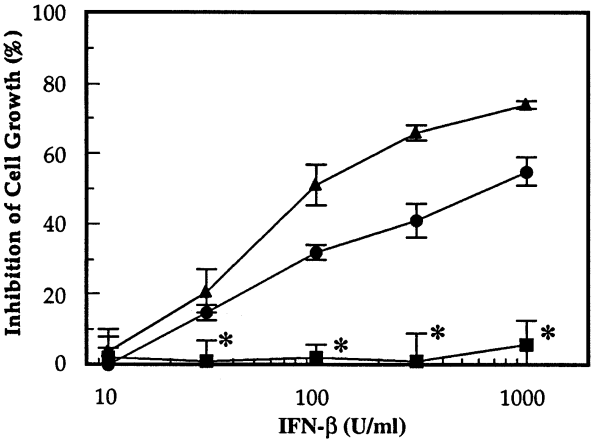

Fig. 4 Cytostatic effect of IFNβ on UV-2237m cells. UV-2237m-P (●), UV-2237m-Neo (▲), and UV-2237m-IFNβ (■) cells were plated into 96-well plates at the density of 400 cells/38-mm^2 well. After a 20-h attachment, the cells were incubated for 96 h with increasing concentrations of IFNβ. The number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Absorbance was read with a microspectrophotometer at 570 nm. $*P<0.05$, compared with UV-2273m-P and UV-2237m-Neo. This is one representative experiment of three

Table 1 Tumorigenicity and bystander inhibitory effect of UV-2237m-IFNβ cells. Parental (UV-2237m-P), control-vector-transduced (UV-2237m-Neo), and interferon-β-transduced (UV-2237m-IFNβ) cells (1×10^6/mouse) or cell mixtures ($1\times10^6+1\times10^6$/mouse) were injected s.c. into the lateral flank of C3H/HeN mice. Tumor incidence and size were determined 3 weeks later. This is one representative experiment of three

Cell line	Tumor incidence	Tumor diameter (mm) (mean ± SD)
UV-2237m-P	5/5	11.1 ± 0.7
UV-2237m-Neo	5/5	14.8 ± 1.9
UV-2237m-IFNβ	2/10	0.4 ± 0.4*
UV-2237m-P + UV-2237m-Neo		
Mix (left flank)	5/5	13.0 ± 0.8
UV-2237m-P (left flank)	5/5	13.4 ± 1.0
UV-2237m-Neo (right flank)	5/5	11.2 ± 0.8
UV-2237m-P + UV-2237m-IFNβ		
Mix (left flank)	5/5	3.9 ± 1.5*
UV-2237m-P (left flank)	5/5	11.4 ± 0.9
UV-2237m-IFNβ (right flank)	1/5	0.7 ± 0.4

* $P < 0.05$, compared with UV-2237m-P tumors

In vitro growth of UV-2237m cells

No discernible differences in morphology were found among the UV-2237m-P, UV-2237m-Neo, and UV-2237m-IFNβ cells (data not shown). Cell doubling times were 16, 16.3, and 17.5 h for the UV-2237m-P, UV-2237m-Neo, and UV-2237m-IFNβ cells respectively, suggesting that the transduction of IFNβ did not affect in vitro proliferation. Next, the three cell lines were cultured for 4 days in medium containing increasing concentrations of IFNβ. The UV-2237m-IFNβ cells were significantly more resistant ($P<0.01$) to the antiproliferative activities of IFNβ than were the UV-2237m-P or UV-2237m-Neo cells (Fig. 4). In additional experiments, we found that UV-2237m-IFNβ cells could divide in the presence of 5000 U/ml IFNβ (data not shown). This is probably due to selection for IFNβ-resistant subclones as IFNβ-inhibited clones would not grow during the transduction procedures.

Growth in vivo

Next we investigated the effect of IFNβ expression on tumorigenicity. The three lines of UV-2237m cells were inoculated s.c. into syngeneic C3H/HeN mice. UV-2237m-P, and UV-2237m-Neo cells produced rapidly growing tumors in nine of ten and seven of seven mice respectively. By day 21, the tumors were 11.9 ± 1.6 mm and 9.4 ± 1.4 mm in diameter respectively. In contrast, UV-2237m-IFNβ cells produced a tumor in only one of ten mice. By day 21, this tumor was only about 2 mm in diameter. These results demonstrate an inverse correlation between expression of IFNβ and tumorigenicity.

Tumor cells genetically engineered to produce IFNβ inhibit tumorigenicity of bystander cells

In the next set of experiments, we injected C3H/HeN mice (n = 5) with UV-2237m-P, UV-2237m-Neo, and UV-2237m-IFNβ cells alone at one s.c. site, alone at two different s.c. sites, or in combination at one s.c. site (Table 1). Here again, the UV-2237m-P or UV-2237m-Neo cells produced rapidly growing tumors in all injected mice, whereas UV-2237m-IFNβ cells did not. The mixture of UV-2237m-P and UV-2237m-Neo cells also produced rapidly growing s.c. tumors (Table 1). The growth of UV-2237m-P cells was significantly inhibited by the coinjection of UV-2237m-IFNβ cells into the same s.c. site. The growth of parental cells injected into the flank was not inhibited by IFNβ-transduced cells injected into a collateral s.c. site (Table 1), suggesting that IFNβ produced by the UV-2237m-IFNβ cells reduced the tumorigenicity of parental cells through a local bystander effect. To gain insight into the mechanisms responsible for the bystander effect, immunohistochemical staining was performed on tumors collected 1 week after implantation of the cells. As shown in Fig. 5, tumors developed from both UV-2237m-P cells and mixtures of UV-2237m-P plus UV-2237m-IFNβ

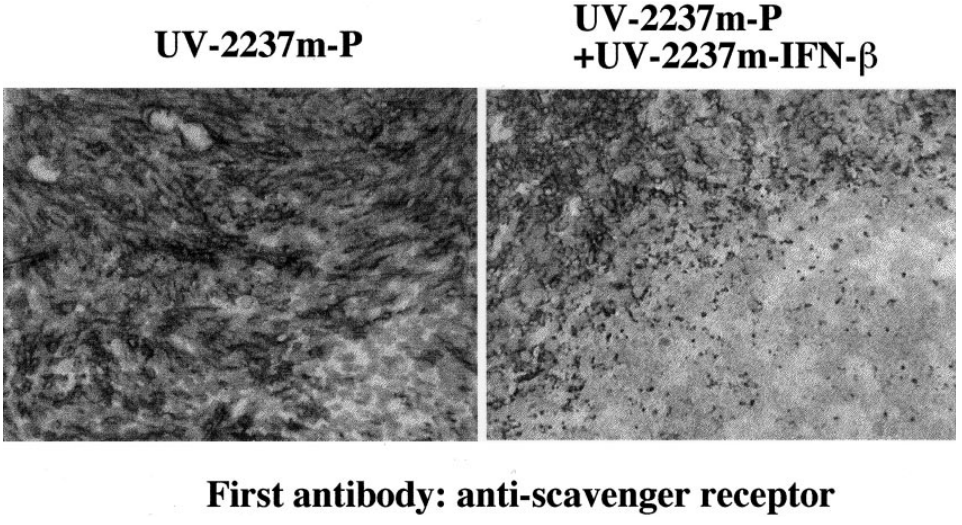

Fig. 5 Macrophage infiltration in UV-2237m tumors. Subcutaneous tumors (6–8 mm in diameter) were resected. Tumor samples were fixed in liquid nitrogen. Frozen sections (8–10 μm) were prepared and stained with an antibody against scavenger receptor and a peroxidase-conjugated secondary antibody

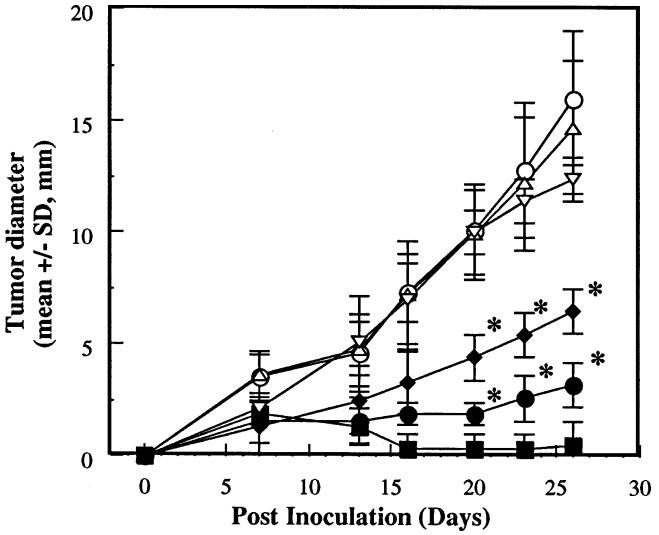

Fig. 6 UV-2237m-IFNβ cells inhibit tumorigenicity of UV-2237m-P and UV-2237m^R cells. UV-2237m-P (○), UV-2237m-Neo (Δ), UV-2237m^R (▽), and UV-2237m-IFNβ (■) cells (1×10^6/mouse), a mixture of UV-2237m-P and UV-2237m-IFNβ (◆) or UV-2237m^R and UV-2237m-IFNβ (●) cells in 0.1 ml HBSS were injected s.c. into C3H/HeN mice. The mean diameter of the tumors was determined twice a week by measuring tumor length and width. *$P<0.05$, compared with UV-2237m-P and UV-2237m^R tumors respectively. This is one representative experiment of two

Table 2 Experimental lung metastases. UV-2237m-P, UV-2237m-Neo, and UV-2237m-IFNβ cells (2×10^5/mouse) were injected intravenously into the tail vein of C3H/HeN mice. The mice were killed 4 weeks later. The lungs were weighed and fixed in Bouin's solution, and number of experimental metastases were counted under a dissecting microscope. This is one representative experiment of two

Cell line	Incidence	Median	Range	Lung weight (mg)
UV-2237m-P	10/10	41	10–200+	575 ± 106
UV-2237m-Neo	10/10	93	17–200+	775 ± 130
UV-2237m-IFNβ	10/10	14	1–45	318 ± 54*

*P <0.05, compared with UV-2237m-P and UV-2237m-Neo lung weight

cells were heavily infiltrated by macrophages, as revealed by staining with macrophage-specific scavener receptor antibody [15]. However, central necrosis was observed only in the tumors formed by the mixture of UV-2237m-P and UV-2237m-IFNβ cells (Fig. 5).

In the next set of experiments, we determined whether the bystander effect was due to a direct antiproliferative effect of IFNβ on the parental cells. We therefore selected UV-2237m-P cells in vitro for resistance to the antiproliferative effects of IFNβ by culturing the cells in increasing concentrations of IFNβ. The isolated cells, designated as UV-2237m^R, were resistant to the antiproliferative effects of up to 5000 U/ml IFNβ. The s.c. growth of these IFNβ-resistant cells was also inhibited by the coinjection of UV-2237m-IFNβ cells (Fig. 6), suggesting that the bystander effect was not due to direct antiproliferative activity of IFNβ.

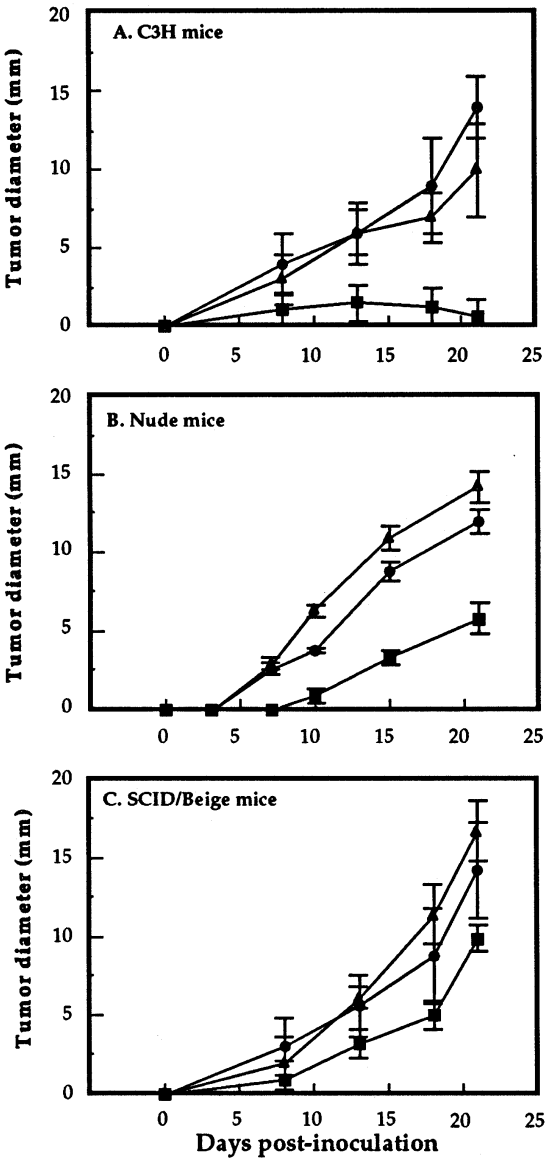

Fig. 7A–C Comparison of the growth of UV-2237m cells in the subcutis of C3H/HeN, nude, and SCID/Beige mice. UV-2237m-P (●), UV-2237m-Neo (▲), and UV-2237m-IFNβ (■) cells were injected s.c. into C3H/HeN mice at 1×10^6 cells/mouse (**A**), into nude (**B**) and into SCID/Beige (**C**) mice at 1×10^5 cells/mouse. The mean diameter of the tumors was determined twice a week by measuring tumor length and width. *$P<0.05$, compared with UV-2237m-P and UV-2237m-Neo tumors. This is one representative experiment of two

Experimental lung metastasis

UV-2237m-P, UV-2237m-Neo, and UV-2237m-IFNβ cells injected i.v. into C3H/HeN mice produced lung metastases in all the mice (Table 2). The median number of lung metastases was smaller in mice injected with UV-2237m-IFNβ cells than in those injected with parental or neo-transfected cells, which translated to differences in lung weight (Table 2). The size distribution of individual lung colonies, however, did not differ among the groups (data

Table 3 Tumorigenicity of UV-2237m cells in natural-killer-cell-compromised mice. C3H/HeN mice were treated, by i.p. injection of 40 µl/mouse, with normal rabbit serum or anti-asialoGM1 serum on days –2, 0, +2, +4, +7, +10, +14 relative to tumor cell inoculation. UV-2237m cells (10^6/mouse) were inoculated into the subcutis of groups of mice ($n = 5$). Tumor diameter was determined 3 weeks later. The data are the mean diameters ± SD. This is one representative experiment of two

Cell line	Mean tumor diameter (mm) in mice treated with:		
	None	Normal rabbit serum	Anti-asialoGM1 serum
UV-2237m-P	11.3 ± 1.1	ND	12.0 ± 1.0
UV-2237m-Neo	10.3 ± 1.6	ND	12.5 ± 0.8
UV-2237m-IFNβ	0.7 ± 0.7	2.0 ± 0.6	8.8 ± 2.0*

* $P < 0.05$, compared with UV-2237m-IFNβ cells in control mice

not shown). Northern blot analysis revealed that lung metastases (harvested from lungs of all three groups) did not express IFNβ mRNA (data not shown), suggesting that the metastases are the progeny of untransduced cells. These results further indicate that suppression of tumorigenicity by IFNβ in this tumor system was indeed due to a local bystander effect.

Growth of UV-2237m cells in syngeneic and immuneodeficient mice

In the next set of experiments, we injected the parental cells into the subcutis of syngeneic C3H/HeN, athymic nude, and SCID/Beige mice. Enhanced tumorigenicity was found in nude mice. The production of 10- to 15-mm tumors in syngeneic mice required implantation of 1×10^6 cells, whereas, in nude mice, the required inoculum was 1×10^5 cells (compare Fig. 7A and B). More significantly, UV-2237m-IFNβ cells produced tumors in all the injected nude mice (data not shown). The tumorigenicity of UV-2237m-IFNβ cells was further increased when the cells were injected into the subcutis of SCID/Beige mice, which lack T, B, and NK cells (Fig. 7C), suggesting that a T-cell-mediated immune response and an NK-cell-related natural defense may be involved in the suppression of tumorigenicity of the IFNβ-transduced cells. To further determine whether NK cells suppressed the tumorigenicity of UV-2237m-IFNβ cells, we treated C3H/HeN mice with antibody against asialoGM1 [19] or control normal rabbit serum. The growth of UV-2237m-IFNβ but not UV-2237m-P or UV-2237m-Neo cells was enhanced in the mice treated with the antibody against anti-asialoGM1 (Table 3). It is noteworthy, however, that UV-2237m-IFNβ tumors in both nude mice and SCID/Beige mice were still smaller than those produced by UV-2237m-P and UV-2237m-Neo cells, suggesting that there must be other host factors that can inhibit the growth of the IFNβ-transduced cells.

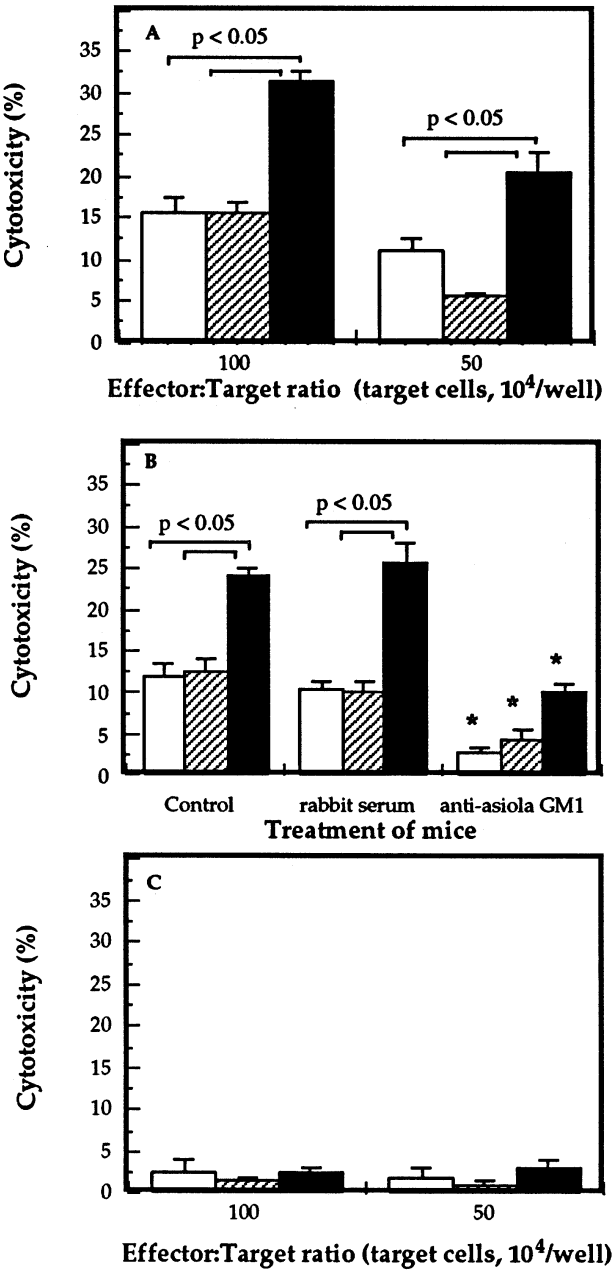

Fig. 8A–C Spleen cell-mediated killing of UV-2237m cells. [^{3}H]dT-labeled UV-2237m-P (□), UV-2237m-Neo (■), or UV-2237m-IFNβ (■) cells were incubated with spleen cells of C3H/HeN mice (**A**), C3H/HeN mice treated with anti-asialoGM1 serum (**B**), and SCID/Beige mice (**C**). After 24 h, spleen-cell-mediated cytolysis was measured. *$P < 0.05$, compared with the cytolysis of spleen cells against UV-2237m-P, UV-2237-Neo, and UV-2237-IFNβ respectively. This is one representative experiment of three

Spleen-cell-mediated in vitro cytotoxicity against UV-2237m cells

To determine whether NK cells can mediate cytotoxicity of UV-2237m cells, we incubated [^{3}H]dT-labeled tumor cells for 24 h with spleen cells from C3H/HeN mice. We found that incubation with spleen cells from C3H/HeN mice lysed

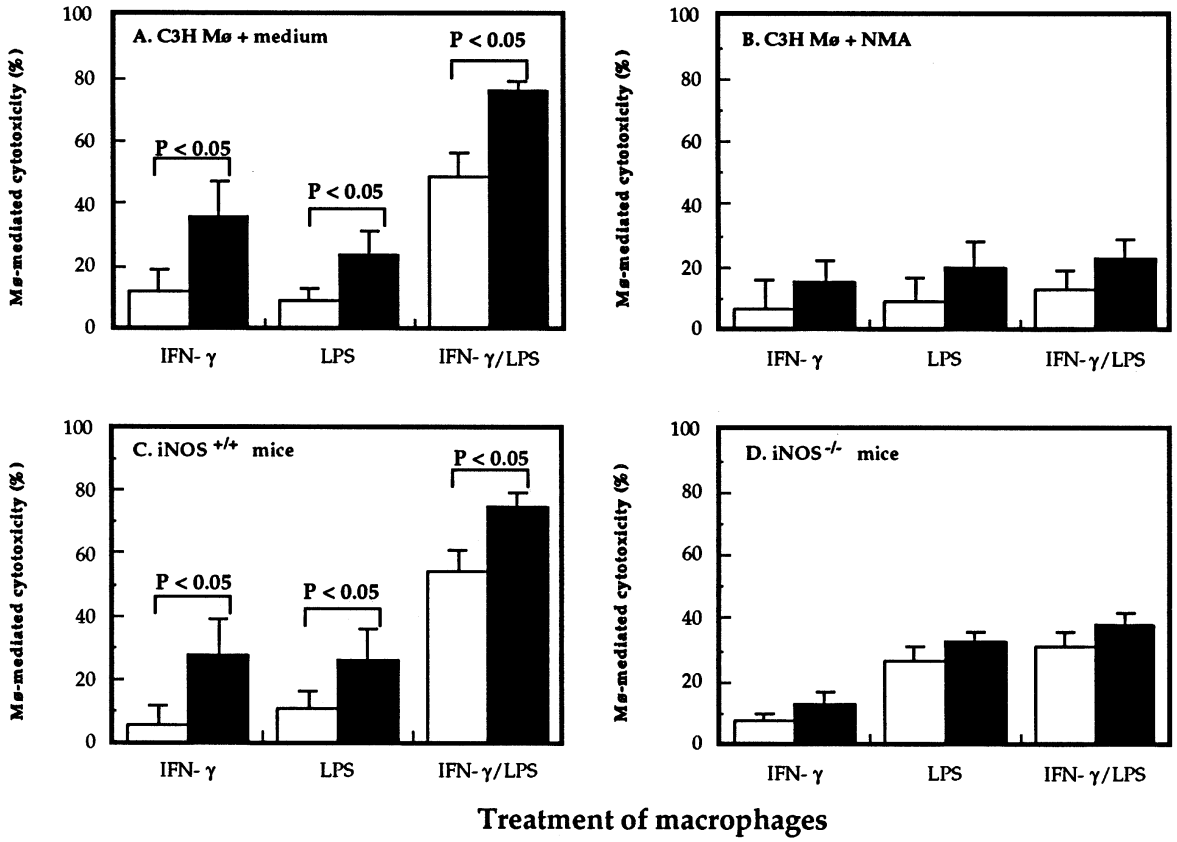

Fig. 9A–D Macrophage(*MØ*)-mediated cytotoxicity against UV-2237m-Neo and UV-2237m-IFNβ cells. Macrophages (1×10^5/well in 96-well plates) from C3H/HeN (**A**, **B**) mice or wild-type (iNOS$^{+/+}$) (**C**) and iNOS$^{-/-}$ (**D**) mice were incubated with IFNγ (10 U/ml), lipopolysacccharide (*LPS*; 100 ng/ml), or both for 20 h. The cells were washed and incubated with [^{3}H]dT-labeled UV-2237m-Neo (□) and UV-2237m-IFNβ (■) cells for 72 h. After washing, the labeled cells remaining in the wells were harvested and their radioactivity was measured in a beta counter. The difference between radioactivities in wells containing tumor cells alone and tumor cells with control macrophages was less than 10%. The data shown are the mean ± SEM of four independent experiments

more UV-2237m-IFNβ cells than UV-2237m-P and UV-2237m-Neo cells (Fig. 8A). Pretreatment of C3H/HeN mice with NK-cell-selective anti-asialoGM1 serum (40 µl/mouse, injected i.p. 48 h before isolation of spleen cells) but not with preimmune normal rabbit serum, reduced the in vitro cytotoxicity against all three lines (Fig. 8B). The spleen cells from SCID/Beige mice were not cytotoxic (Fig. 8C).

Macrophage-mediated cytotoxicity against UV-2237m cells

Because IFNβ is an endogenous macrophage activator [42], we determined whether the amount of IFNβ released by UV-2237m-IFNβ cells was sufficient to activate macrophage-mediated cytotoxicity. Peritoneal exudate macrophages were incubated 20 h with medium alone or medium containing 10 U/ml IFNγ, 100 ng/ml LPS, or both. After activation, the treated macrophages were washed and incubated with [^{3}H]dT-labeled UV-2237m-Neo or UV-2237m-IFNβ cells. Cell lysis was determined 72 h later. The data in Fig. 9A indicate that control macrophages did not lyse the tumor cells. Low levels (20%–40%) of cytotoxicity were observed in cultures with macrophages exposed to LPS or IFNγ. Significant cytotoxicity (up to 80%) was mediated by macrophages exposed to both LPS and IFNγ. This cytotoxicity was partially blocked by the addition of 1 mM N^R-MeArg during incubation of the activated macrophages with UV-2237m cells (Fig. 9B). The contribution of NO in macrophage-mediated cytotoxicity against the UV-2237m cells was further tested with macrophages from wild-type (Fig. 9C) and iNOS$^{-/-}$ mice (Fig. 9D). Interestingly, significantly more UV-2237m-IFNβ cells than UV-2237m-Neo cells were killed by macrophages activated by IFNγ and LPS alone or by the two reagents in combination (Fig. 9A, C). The elevated cytotoxicity against UV-2237m-IFNβ cells appeared to be mediated by nitric oxide, because a difference in the cytotoxicity was not observed when N^G MeArg was added (Fig. 9B) or when the UV-2237m-Neo and UV-2237m-IFNβ cells were incubated with macrophages from iNOS$^{-/-}$ mice (Fig. 9D). These results suggest that macrophages may also contribute to the reduced tumorigenicity of UV-2237m-IFNβ cells.

Discussion

We tested the hypothesis that the sustained presence of IFNβ in a tumor lesion would inhibit tumorigenicity and progression and studied the feasibility of IFNβ gene therapy with a syngeneic mouse fibrosarcoma as a model. The results show that transduction of UV-2237m cells with IFNβ cDNA resulted in the constitutive secretion of IFNβ, associated with a significant reduction of tumorigenicity and metastatic potential in syngeneic mice. The IFNβ-transduced cells did not form s.c. tumors in syngeneic mice but did produce lung metastases subsequent to i.v. inoculation. Because metastases can be clonal in origin [37, 41], the finding that none of the metastases expressed IFNβ mRNA suggests that the metastases developed from untransduced cells within the UV-2237m-IFNβ line.

The IFNβ-transduced cells could inhibit the local growth of bystander tumor cells. The suppression of tumorigenicity was not due to the direct antiproliferative effects of IFNβ. We base this conclusion on results of experiments in which IFNβ-transduced cells inhibited the growth of tumor cells that are resistant to the antiproliferative effects of IFNβ, suggesting that this effect was local and not systemic and that it was due to indirect effects of IFNβ. Our data agreed with and extended recent observations in a syngeneic tumor model using an IFNα-secreting B16 melanoma [10, 18, 30].

The present data show that the reduced tumorigenicity and bystander effects of the IFNβ-transduced cells was mediated by activated NK cells, macrophages and T cells. We base this conclusion on the following findings. First, IFNβ-transduced cells had the same in vitro growth rate as control cells. In fact, the UV-2237-IFNβ cells were highly resistant to the antiproliferative effects of IFNβ. Second, UV-2237-IFNβ cells inhibited the tumorigenicity of both IFNβ-sensitive and IFNβ-resistant tumor cells. Third, the IFNβ-secreting cells formed s.c. tumors in NK-cell-compromised syngeneic mice, nude (defective in T cells), and SCID/Beige (defective in T, B, and NK cells) mice. Fourth, NK-cell-mediated killing of IFNβ-secreting cells was significantly higher than that of the parental and control vector-transduced cells. Fifth, significantly more UV-2237m-IFNβ cells than UV-2237m-Neo cells were killed by macrophages activated by either IFNγ and LPS alone or by the two reagents in combination.

In addition to stimulating of the host's natural defense system, IFNβ is known to down-regulate expression of angiogenic molecules [9, 13, 25, 35, 40]. Because tumor angiogenesis is crucial for tumor growth and metastasis [12, 34], the suppression of local tumor growth observed in UV-2237m-IFNβ cells and the bystander effect could also be partially mediated by inhibition of blood vessel development in the lesions, and this possibility is under current investigation.

In summary, we demonstrate that the expression of IFNβ in UV-2237m cells significantly suppresses tumorigenicity and metastasis. The IFNβ-secreting cells also cause suppression of tumorigenicity on the parental cells. The suppression is not due to the direct antiproliferative effects of IFNβ but, in part, to activation of NK cells, macrophages, and T cells. These data suggest that a sustained presence of IFNβ in tumor lesions can be of therapeutic benefit. Hence, IFNβ gene therapy may have the potential of treating localized disease.

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