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Eradication of Primary Murine Fibrosarcomas and Induction of Systemic Immunity by Adenovirus-mediated Interferon β Gene Therapy[1]

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ABSTRACT

We determined whether an adenoviral vector-mediated murine *IFN-β* gene therapy could eradicate established s.c. tumors produced by murine UV-2237m fibrosarcoma cells. The tumor cells were highly susceptible to infection by adenoviral vectors. Cells infected with 10 or 100 multiplicity of infection of AdCIFN-β, an adenoviral vector encoding murine IFN-β driven by the human cytomegalovirus promoter, expressed high levels of steady-state IFN-β mRNA and produced 500 or 7,000 units of IFN-β activity/10^6 cells/24 h, respectively. Infection of tumor cells with 30 multiplicity of infection of AdCIFN-β (but not control AdC*Lac*Z vector) inhibited *in vitro* tumor cell proliferation by 40–45%.

Intralesional injection of 5×10^8 plaque-forming units of AdCIFN-β (but not Ad*Lac*Z) eradicated established s.c. fibrosarcomas in syngeneic mice but not fibrosarcomas in nude mice. Mice cured of the disease developed systemic immunity against rechallenge with UV-2237m cells but not against another syngeneic tumor, the K-1735 M2 melanoma. Immunohistochemical analysis revealed that tumors injected with AdCIFN-β contained more macrophages and $CD4^+$ and $CD8^+$ cells than did tumors injected with AdC*Lac*Z or saline. Most cells in the PBS- and AdC*Lac*Z-treated tumors stained positive for proliferating cell nuclear antigen, and few cells stained for terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling. In sharp contrast, AdCIFN-β-treated tumors contained few proliferating cell nuclear antigen-positive cells and many terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling-positive cells. Taken together, our data demonstrate that IFN-β gene therapy delivered by adenoviral vectors can be effective against fibrosarcomas.

INTRODUCTION

IFN-β, a type I IFN, is a multifunctional glycoprotein that can inhibit tumor growth both directly, by suppressing cell replication and inducing differentiation or apoptosis (1–3), and indirectly, by activating tumoricidal properties of macrophages (4, 5) and NK[3] cells (6, 7), by suppressing tumor angiogenesis (8–10), and by stimulating specific immune response (11–14). Extensive clinical trials, however, demonstrated that IFN-β, used either alone or in combination with other anticancer agents, was ineffective in treating most solid tumors (15–17). This failure could have been attributable to the lack of a sustained level of IFN-β in the tumor lesions. Pharmacokinetic studies have shown that the half-life of IFN-β in the circulation of patients is ~5 min. One h after a bolus i.v. dose of 6×10^6 units of IFN-β, serum concentrations are <8 units/ml, and after an i.m. or s.c. injection, serum concentrations are <2 units/ml (18). These concentrations are far below those required to suppress tumor cell growth, down-regulate angiogenesis, and activate macrophages, NK cells, and specific T-cell responses (1, 3, 5, 7, 11–14).

One way to increase tumor cell exposure to IFN-β could be *IFN-β* gene therapy. Our recent studies using the murine UV-2237m fibrosarcoma, A375 human melanoma, KM12 human colon cancer, and PC3M human prostate cancer have shown that both tumorigenicity and production of metastasis were reduced significantly when the cells were engineered to constitutively express murine IFN-β (19–21). Moreover, these *IFN-β* gene-transduced or -transfected tumor cells significantly suppressed tumorigenicity of IFN-β-nonproducing cells in both immune-competent mice and T cell-deficient nude mice (19–21). The antitumor activity of IFN-β in these systems was mainly mediated by stimulation of host effector cells and by suppression of tumor angiogenesis. Overall, early results suggest that IFN-β effectiveness against solid tumors could be realized if sustained production of IFN-β were achieved in the lesions.

Nevertheless, these earlier studies had not demonstrated whether expression of the *IFN-β* gene in an established tumor could eradicate the tumor and/or confer systemic immune protection in mice. In the present study, we investigated whether *IFN-β* gene therapy using an adenoviral vector could produce regression of established murine fibrosarcomas. We show that adenoviral vectors efficiently transduced the murine fibrosarcoma cells both *in vitro* and *in vivo* and that intralesional injection of adenoviral vector containing *IFN-β* gene, but not a control vector, eradicated established UV-2237m tumors in syngeneic mice and conferred systemic immunity.

MATERIALS AND METHODS

Mice. Specific pathogen-free female C3H/HeN mice and female athymic nude mice were purchased from the Animal Production Area, National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). 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. Mice were used when they were 6–8 weeks of age, except where otherwise indicated.

Reagents. EMEM, DMEM, 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). LPS (*Escherichia coli* 0111:B4) and 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, 0.125 ng/ml) purchased from Associates of Cape Cod (Woods Hole, MA).

Cells and Culture Conditions. The UV-2237m fibrosarcoma and K-1735 M2 melanoma cell lines were derived from a spontaneous lung metastasis produced by parental UV-2237 fibrosarcoma cells (22) and K-1735 melanoma cells (23) originally induced in a C3H/HeN mouse by UVB irradiation (24, 25). The cells were maintained as a monolayer culture in EMEM supplemented with 10% FBS, nonessential amino acids, sodium pyruvate, vitamin A, and glutamine, 100 units/ml penicillin, and 100 μg/ml of streptomycin (CMEM-10% FBS). 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, EMEM 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 single-cell suspensions with a

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[3] The abbreviations used are: NK, natural killer; EMEM, Eagle's minimal essential medium; HBSS, Hanks' balanced salt solution; FBS, fetal bovine serum; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CMV, cytomegalovirus; PFU, plaque-forming unit; MOI, multiplicity of infection; PCNA, proliferative cell nuclear antigen; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP-biotin nick-end labeling; IL, interleukin.

viability exceeding 90% were used. The 293 human embryonic renal cell line, which was engineered to provide *E1* gene products of an adenoviral vector, was maintained in DMEM supplemented with 10% FBS (CDMEM-10% FBS).

Recombinant Adenoviral Vectors. Replication-deficient adenoviral vectors have been used in numerous preclinical studies and are considered the "vector of choice" for gene therapy against tumors (26). The advantages of adenoviral vectors over other gene delivery systems include their ability to transduce both proliferating and quiescent cells, wide tissue tropism, relative stability during production and purification, their ability to produce high titers of clinical-grade materials, and the capacity to deliver large genes or multiple genes in a single vector (25, 27).

The full coding region of the murine IFN-β cDNA (kindly provided by Dr. T. Taniguchi, Osaka University, Osaka, Japan) was subcloned into plasmid pxCMV to derive shuttle vector pAdCIFN-β. The shuttle vector and plasmid pJM17 were cotransfected into 293 cells by liposome-mediated transfection with Lipofectin (Life Technologies, Grand Island, NY) to generate replication-defective adenoviral vector AdCIFN-β. The plasmids pxCMV, pJM17, and AdCLacZ (a replication-defective recombinant adenovirus encoding the *E. coli β-galactosidase* gene) were generously provided by Dr. W. Zhang (Baxter Healthcare Co., Rojnd Lake, IL). Both the *β-galactosidase* gene and the *IFN-β* gene in the adenoviral vectors are driven by the human CMV promoter. AdCIFN-β was isolated from a single plaque and identified by PCR and infection of cells. The sequences of the PCR primers were: sense, 5′-CTT GGC TTC TTA TGC GAC GG-3′, and antisense, 5′-CCA CAA CTA GAA TGC AGT G-3′, which are located outside the two ends of the insert on the shuttle vector pAdCIFN-β. One clone of AdCIFN-β was plaque-purified three times, resulting in a clone of wild-type, virus-free AdCIFN-β. Wild-type adenovirus was identified by plaque assay on HeLa cells at 10 MOI and by PCR using primers specific for the E1a region (sense, 5′-TGA GAC ATA TTA TCT GCC ACG-3; and antisense, 5′-CCT CTT CAT CCT CGT CGT CAC-3′) that were deleted from the recombinant adenovirus. Only the preparations that did not contain wild-type virus were used in our studies.

AdC*Lac*Z and AdCIFN-β were propagated in 293 cells grown in CDMEM-10% FBS and purified by two-step CsCl gradient centrifugation. After dialysis at 4°C against 10 mM Tris/HCl (pH 7.5), 1 mM $MgCl_2$, and 10% glycerol, aliquots of the vectors were stored at −80°C. The titers of the vectors, assessed on 293 cells by plaque assay, usually were 5–20 × 10^{10} PFUs/ml for most preparations.

***In Vitro* Cytotoxicity Assay.** UV-2237m cells were plated into 12-well plates at a density of 5 × 10^4 cells/well. Twenty-four h later, various concentrations of AdC*Lac*Z or AdCIFN-β were added and incubated for 6 days with one medium change on day 3. During the final 2 h of incubation, MTT (Sigma) was added at 0.42 mg/ml. The medium was removed, and dark-blue formazan was dissolved in DMSO. The solution was transferred into a 96-well plate, and the absorbance was measured with a 96-well microtiter plate reader (BenchMark; Bio-Rad Laboratory, Hercules, CA) at 570 nm. The percentage of inhibition of cell growth was calculated according to the following formula: inhibition (%) = (1− A_{570} of treated group/A_{570} of control group) × 100.

Treatment of Tumor-bearing Mice with Adenoviral Vectors. To produce s.c. tumors, UV-2237m cultures in exponential growth phase were harvested by a brief treatment with 0.25% trypsin and 0.02% EDTA. Cell viability was determined by trypan blue exclusion assay. UV-2237m cells (2 × 10^5 in 100 μl of HBSS) were injected s.c. into the right lateral flank proximal to the midline of C3H/HeN mice or nude mice. When the tumors reached 3–6 mm in diameter (8–10 days after inoculation), the lesions were injected with PBS or adenoviral vectors at doses and schedules specified in "Results." The tumor size in two perpendicular diameters was measured with calipers every 5 days and just prior to each injection. Nonpalpable lesions were considered eradicated.

Systemic Immunity against UV-2237m Cells. The mice in which the primary tumors had been eradicated were maintained for 2 months and then challenged on the left lateral flank with 5 × 10^5 UV-2237m or K-1735 M2 cells. The mice were monitored once a week, and the tumor incidence and size were measured 3 weeks later.

RNA Isolation and Northern Blot Analyses. Cultures or tumors were harvested, and mRNA was extracted using a FastTrack kit (Invitrogen, San Diego, CA). 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, MA), and UV cross-linked at 120 000 μJ/cm^2 using a UV Stratalinker 1800 (Stratagene). Hybridization using [^{32}P]dCTP-labeled cDNA probes of mouse IFN-β or rat glyceraldehyde-3-phosphate dehydrogenase was performed as described previously (21). 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.

Bioassay for IFN-β Activity. IFN-β activity was determined as described previously based on induction of nitric oxide production by murine macrophages (19). Briefly, mouse peritoneal exudate macrophages were plated at a density of 10^5 cells/38-mm^2 well of 96-well plates and incubated for 24 h with test samples or with increasing concentrations of recombinant mouse IFN-β in the presence of 1 μg/ml LPS. NO_2^- concentration was determined in the culture supernatants by its reaction at a volume of 1:1 with Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, and 2.5% H_3PO_4). The absorbance at 540 nm was monitored with a BenchMark microplate reader (Bio-Rad Laboratory). To confirm the induction of NO_2 by IFN-β, we used a rat monoclonal antibody that neutralizes murine IFN-β activity (Yamasa Shoyu Co., Tokyo, Japan). The IFN-β activity measured by this assay is comparable with the international reference units defined by antiviral activity as determined by Access Biomedical (San Diego, CA).

Immunohistochemistry. At necropsy, tumors were harvested, 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 blocked in PBS containing 5% normal horse serum/1% normal goat serum and incubated with antibodies to macrophage-specific scavenger receptor (Serotec Ltd., Kidlington, MA), CD4 (American Type Culture Collection), or CD8 (PharMingen, San Diego, CA) antigen 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 3,3′-diaminobenzidine (Research Genetics, Huntsville, AL) and counterstaining 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 Corporation, Bothell, WA).

For immunohistochemical staining using an antibody against PCNA, tumor sections were fixed in 10% buffered formalin and embedded in paraffin. Sections (3–5 μm) were placed on ProbeOn slides (Fischer Scientific) and stained as described for the frozen sections after deparaffinization and rehydration (21).

TUNEL Assay. DNA fragmentation in tumor lesions was determined by the TUNEL method (28). Briefly, 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 TdT buffer [30 mM Trizma base (pH 7.2), 140 mM sodium cacolydate, and 1 mM $CoCl_2$] containing biotinylated 16-dUTP and terminal transferase (Boehringer Mannheim) for 1 h at 37°C and then incubated with TdT. The reactions 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.

Statistical Analysis. The significance of the *in vitro* results was determined by Student's *t* test (two-tailed). The significance of the tumor incidence and tumor size was analyzed by the χ^2 test and ANOVA, respectively.

RESULTS

***In Vivo* Treatment of UV-2237m Cells *in Vitro* and UV-2237m Tumors *in Vivo* with Adenoviral Vectors.** Because UV-2237m cells are susceptible to the direct antiproliferative effects of IFN-β (19), we first examined whether infection with AdCIFN-β suppressed the growth of UV-2237m cells. UV-2237m cells infected with 30 and 100 MOI of AdCIFN-β produced 3000 and 7000 units of IFN-β/10^6 cells/24 h, respectively. As shown in Fig. 1, cell growth in cultures transduced by 30 and 100 MOI of AdCIFN-β was reduced by >40% ($P < 0.01$).

In the second set of *in vivo* experiments, we determined the minimal

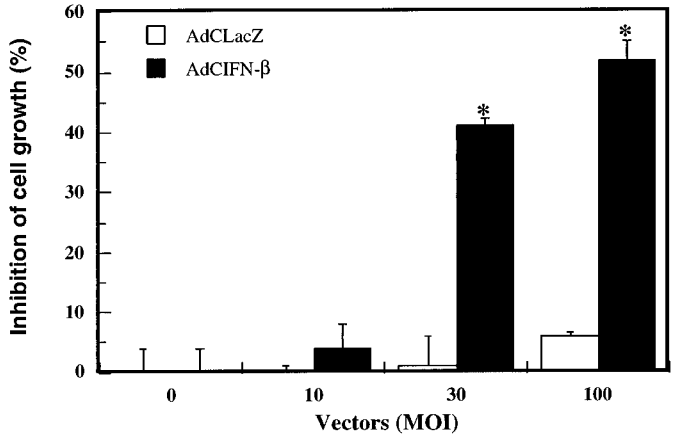

Fig. 1. Inhibition of UV-2237m growth *in vitro* by AdCIFN-β. UV-2237m cells were plated at a density of 5×10^4 cells/well. Twenty-four h later, various MOIs of Ad*CLacZ* or AdCIFN-β were added. The cells were cultured for 6 days with one medium change on day 3. Viable cells in the wells were stained with MTT as described in "Materials and Methods." The data shown are the means of two independent experiments; *bars*, SE. *, $P < 0.05$.

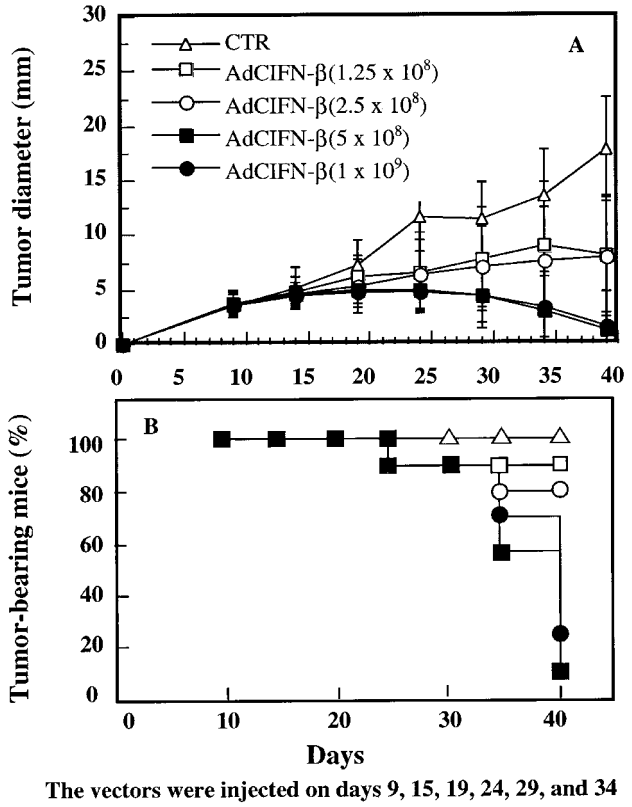

Fig. 2. *AdCIFN-β* gene therapy of s.c. tumors. UV-2237m tumors in C3H/HeN mice (10 mice/group) were treated every 5 days by intralesional injections using escalating doses of AdCIFN-β. *CTR*, untreated control. Tumor diameter (*A*) and percentage of palpable tumors (*B*) were determined prior to each injection. *Bars*, SE.

and optimal dose of AdCIFN-β required to suppress or eradicate established UV-2237m s.c. tumors. C3H/HeN mice were inoculated s.c. with UV-2237m cells. When s.c. tumors reached 4–6 mm in diameter, the lesions were injected every 5 days for six consecutive injections with different doses of AdCIFN-β (1.25×10^8 to 1×10^9 PFU/mouse). Fig. 2 shows that the treatment with AdCIFN-β produced regression of the tumors in a dose-dependent manner. Tumor growth in all treated groups was also significantly suppressed (Fig. 2*A*). Complete eradication of s.c. tumors occurred in 80–90% (seven or eight of nine) of mice receiving 5×10^8 or 1×10^9 PFUs of AdCIFN-β/injection (Fig. 2*B*). Because the therapeutic benefit for mice treated with 1×10^9 PFUs of AdCIFN-β and those receiving 5×10^8 PFUs/injections of the vector were similar (Fig. 2), all additional studies used the lower dose (5×10^8 PFUs/injection).

In the third set of experiments, we investigated whether the frequency of vector injection influenced eradication of s.c. UV-2237m tumors. AdCIFN-β or Ad*LacZ* (5×10^8 PFUs/injection) was injected into s.c. tumors every 5 or 10 days. Another two groups of tumor-bearing mice were treated with Ad*CLacZ* at the same dose and schedule. As shown in Fig. 3*A*, treatment of mice with Ad*CLacZ* every 5 or 10 days did not affect tumor growth. AdCIFN-β injected every 5 days (six consecutive injections) eradicated tumors in 80% of mice, whereas injections given every 10 days eradicated tumors in only 20% (Fig. 3*B*). i.v. (10^4 units/injection) or intratumoral administration of murine IFN-β (10^3 units/injection) every 5 days did not eradicate s.c. UV-2237m tumors (data not shown).

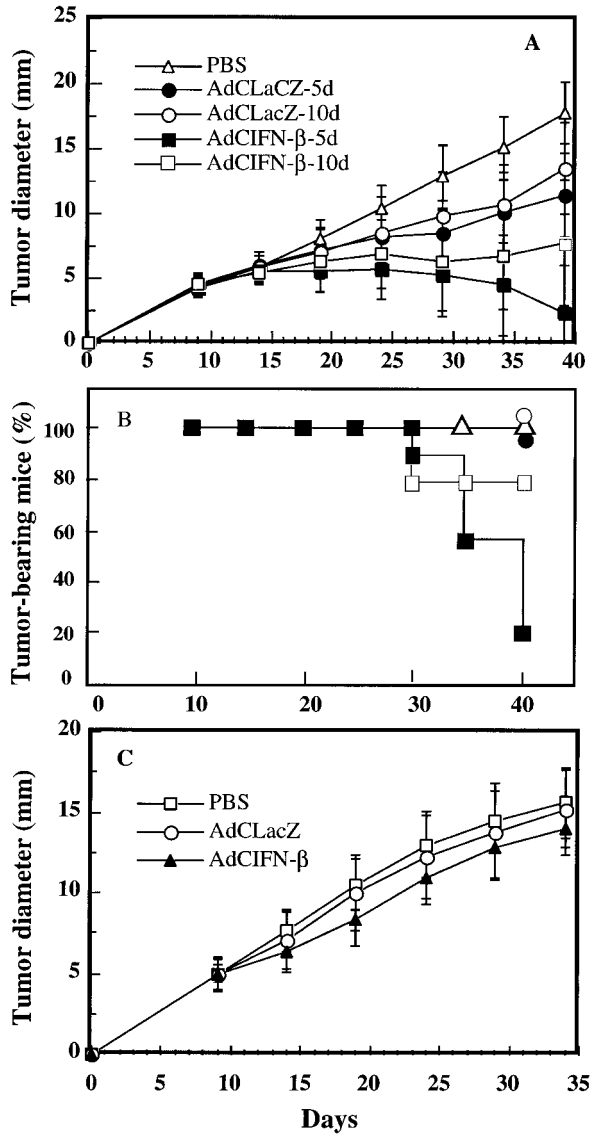

Fig. 3. *IFN-β* gene therapy against tumors grown in immune competent and nude mice. UV-2237m tumors were established in C3H/HeN mice (*A* and *B*) or nude mice (*C*) and treated every 5 days or every 10 days with intralesional injections of Ad*CLacZ* or AdCIFN-β. *CTR*, untreated control. Tumor diameter (*A* and *C*) and percentage of palpable tumors (*B*) were determined prior to each injection. This is one representative experiment (10 mice/group) of three (*A* and *B*) and two (*C*); *bars*, SE.

It is important to note that all s.c. tumors continued to grow after the first two injections with AdCIFN-β and started to regress after the fourth intratumoral injection (Fig. 3*A*), a duration suggesting the development of an immune response. To determine this possibility, we repeated the study using UV-2237m tumors in nude mice. The data shown in Fig. 3*C* indicate that AdCIFN-β or AdC*Lac*Z given at the dose and schedule described above did not affect the growth of UV-2237m tumors, suggesting that T cell-mediated immunity was important for the eradication of UV-2237m tumors.

Tumor Regression Is Associated with Systemic Immunity. Two months after the regression of the primary tumors, we tested whether AdCIFN-β treatment had produced systemic tumor-specific immunity. Animals in which primary tumors had been cured by repeated intralesional injections of AdCIFN-β were divided into two groups ($n = 7$) and challenged s.c. with a high number (5×10^5) of UV-2237m cells or with K-1735 M2 melanoma cells on the opposite flank. As shown in Table 1, all normal control mice injected with UV-2237m or K-1735 M2 developed tumors. All mice in which the primary tumors had regressed were protected from growth of a secondary UV-2237m tumor but not from growth of the K-1735 M2 tumor (Table 1).

Expression of Murine IFN-β by s.c. Tumors. s.c. UV-2237m tumors were treated by intralesional injection with PBS or PBS containing 5×10^8 PFUs of AdCIFN-β or AdC*Lac*Z. Three days later, the tumors were resected, and mRNA was extracted and analyzed by Northern blotting. IFN-β mRNA was not detected in UV-2237m tumors injected with PBS or AdC*Lac*Z. In contrast, a high level of IFN-β mRNA was found in UV-2237m tumor treated with AdCIFN-β (Fig. 4).

Immunohistochemical Analysis. UV-2237m s.c. tumors (5 mm in diameter; C3H/HeN) were treated by an intralesional injection of PBS, 5×10^8 PFU AdCIFN-β, or 5×10^8 PFU AdC*Lac*Z. One week later, the tumors were harvested and prepared for immunohistochemistry (Fig. 5). Most cells in the PBS- and AdC*Lac*Z-injected tumors were stained intensively by a monoclonal antibody against PCNA, a nuclear protein exclusively expressed in cells that are in the late G_1 and M phase of the cell cycle (29). In contrast, only a few cells in the AdCIFN-β-treated tumors were PCNA positive (Fig. 5). TUNEL staining revealed only a few positive cells in the PBS- and AdC*Lac*Z-treated tumors, whereas in the AdCIFN-β-treated tumors, many cells stained positive. Immunohistochemistry using an antibody against macrophage-specific scavenger receptor (30) revealed that the AdCIFN-β-treated tumors contained a higher number of infiltrating macrophages than that found in the PBS- and AdC*Lac*Z-treated tumors. Similarly, tumors treated with AdCIFN-β were infiltrated by many more $CD4^+$ and $CD8^+$ T cells, as evidenced by positive staining with antibodies to murine CD4 and CD8 antigens, respectively (Fig. 5).

DISCUSSION

In previous studies, we demonstrated that UV-2237m murine fibrosarcoma cells, as well as several other tumor cell lines engineered to constitutively release IFN-β, had reduced tumorigenicity and metastatic potential and that IFN-β-producing tumor cells suppressed tumorigenicity of bystander tumor cells (19–21). The present study investigated the effectiveness of *IFN-β* gene therapy delivered by an adenoviral vector against established UV-2237m fibrosarcoma. The data demonstrate that UV-2237m cells were susceptible to infection by adenoviruses both *in vitro* and *in vivo* and that repeated intralesional injections of an adenoviral vector encoding IFN-β eradicated s.c. fibrosarcomas in syngeneic but not in nude mice. Moreover, cured mice developed systemic immunity to further challenge with UV-2237m. These results demonstrate the effectiveness of *IFN-β* gene therapy in treating solid tumors in mice.

Table 1 *Development of tumors in cured mice*

	UV-2237m		K-1735 M2	
Mice[a]	Incidence	Tumor diameter	Incidence	Tumor diameter
Control	10/10	13.0 ± 3.0	10/10	13.4 ± 3.1
Cured	0/7	0	7/7	12.5 ± 2.5

[a] Normal control mice or mice in which primary tumors cured by intralesional injection of AdCIFN-β were maintained for 2 months and then challenged s.c. with 5×10^5/mouse of UV-2237m or K-1735 M2 cells. The mice were monitored once a week. The tumor incidence and diameter (mm) were recorded 4 weeks later. The data shown are from one representative experiment of two.

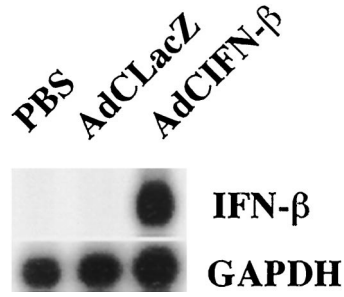

Fig. 4. Adenovirus-mediated IFN-β expression in UV-2237m tumors. UV-2237m cells were injected s.c. into C3H/HeN mice. When tumors reached 5 mm in diameter, PBS, AdC*Lac*Z, or AdCIFN-β were injected intralesionally. Three days later, tumors were harvested, and mRNA was extracted and analyzed by Northern blotting. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

Recently, Qin *et al.* (31) reported that *ex vivo IFN-β* gene transduction by a replication-defective adenovirus in as few as 1% of implanted cells blocked tumor formation, and direct *in vivo IFN-β* gene delivery into established tumors using the adenoviral vector generated high local concentrations of IFN-β, inhibited tumor growth, and in many cases produced complete tumor regression. Because the mice used in the study were immune deficient and the action of IFN-β is species specific (32), the data suggested that the antitumor effect of IFN-β was primarily mediated through inhibition of tumor cell proliferation (31). In our study, UV-2237m cells infected *in vitro* by AdCIFN-β produced high levels of IFN-β, which was associated with >40% cytostasis. *IFN-β* gene therapy against UV-2237m tumors in nude mice, however, was less effective, possibly because of the low efficacy of gene transfer *in vivo* due to the host's natural antivirus response (33) or a short diffusion distance of the vector injected.

T-cell-mediated, tumor-specific immune response plays an important role in the *IFN-β* gene therapy presented here. We base this conclusion on the observations that: (*a*) the therapy conferred systemic tumor-specific protection in the cured mice; (*b*) the therapy was less effective in T cell-defective nude mice; and (*c*) the tumors exposed to the adenoviral vector encoding IFN-β were densely infiltrated by $CD4^+$ and $CD8^+$ T cells as revealed by immunohistochemical analysis. We did not explore the mechanisms by which the immune response was induced, but many possibilities exist. IFN-β may stimulate T-cell-mediated immune responses by increasing $CD4^+$ T-cell infiltration (34, 35), up-regulating IFN-γ production (36), stimulating IL-2 production and IL-2-induced immune responses (37, 38), stimulating IL-12 production (39), enhancing the terminal differentiation of dendritic cells (40), increasing the sensitivity of tumor cells to macrophage- and $CD8^+$ T-cell-mediated cytotoxicity (41), down-regulating T-suppressor cell function (42), and keeping activated T cells alive (43).

Immunohistochemical staining indicated that UV-2237m tumors treated with AdCIFN-β were densely infiltrated by macrophages and contained significantly more TUNEL-positive cells and few PCNA-

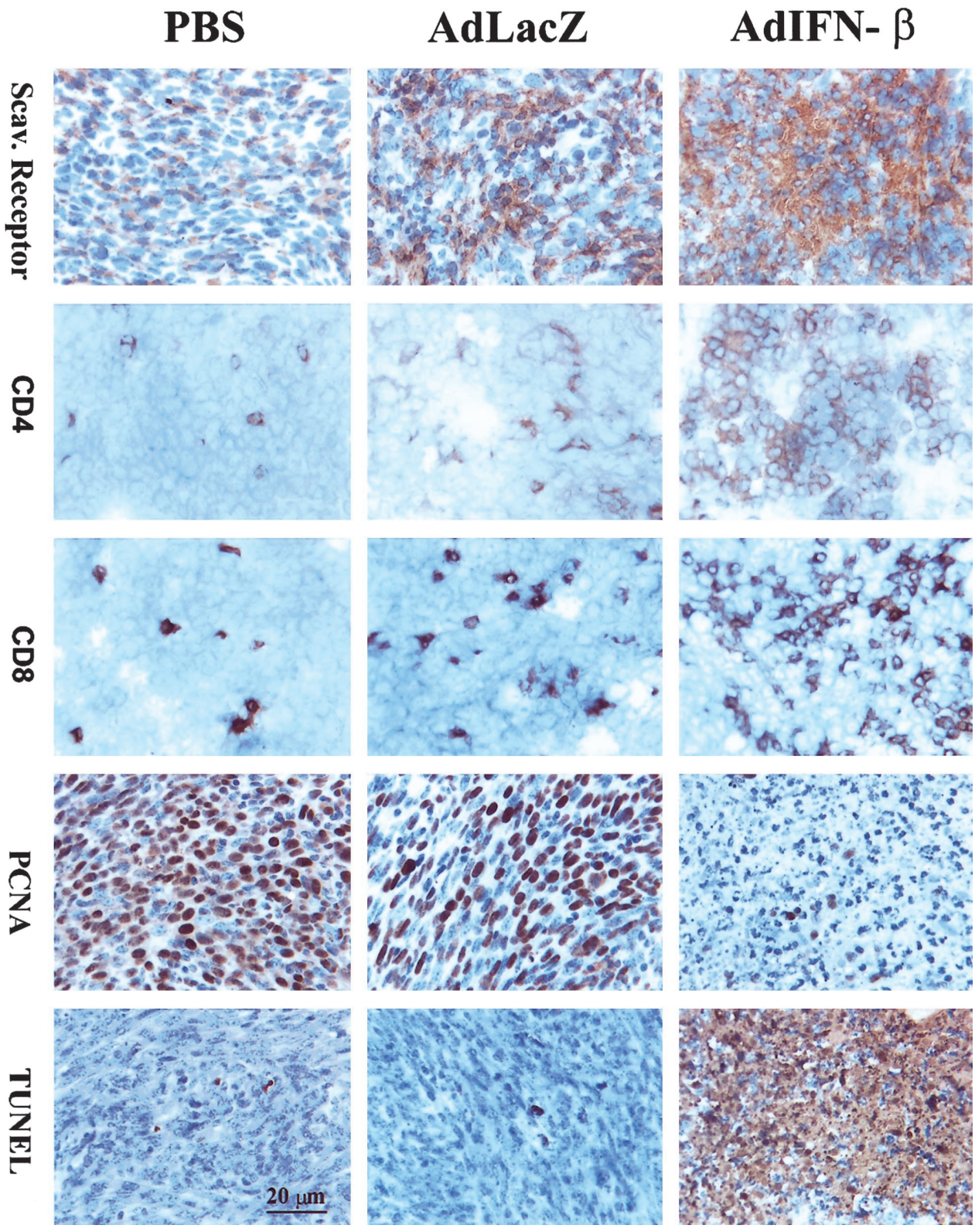

Fig. 5. Immunohistochemical analyses of s.c. tumors. UV-2237m cells were inoculated s.c. Tumors of 6 mm in diameter were injected once with PBS, AdC*LacZ*, or AdCIFN-β. One week later, the tumors were harvested and prepared for immunohistochemical analyses as described in "Materials and Methods." *Scav.*, Scavenger.

positive cells. These observations confirm and extend findings reported previously for both murine UV-2237m and human prostate cancer cells (PC-3M) transduced *ex vivo* with IFN-β (21, 44). Collectively, these data suggest that the eradication of UV-2237m s.c. tumors in immune-competent mice was attributable to multiple mechanisms, including stimulation of T-cell-mediated immune responses, activation of tumoricidal properties of macrophages (19–21, 45), stimulation of NK cells (19, 21, 46, 47), suppression of tumor angiogenesis (10, 48–50), and direct inhibition of tumor cell proliferation.

The UV-2237m fibrosarcoma is an immunogenic tumor in synge-

neic C3H/HeN mice (51). Whether the tumor-specific immune protection conferred by the *IFN-β* gene therapy is limited to immunogenic tumors remains unclear. Preliminary results, however, indicated that this *IFN-β* gene therapy can also eradicate weakly immunogenic K-1735 M2 melanoma and confer tumor-specific protection (data not shown). Similarly, IFN-α, the other member of type I IFN family that shares receptor with IFN-β, has been shown to modulate tumor immune responses in several weak or nonimmunogenic tumor systems. For example, inoculation with IFN-α-treated B16 melanoma cells, which are weakly antigenic (at best; Ref. 51), confers tumor-specific immunity in syngeneic mice challenged with parental B16 cells in the lungs and the subcutis (52, 53). *IFN-α* gene therapy mediated by i.m. injection of a plasmid DNA encoding IFN-α can also suppress growth and metastasis of B16 melanoma, Cloudman melanoma, and glioma 261 tumors as well (54).

Taken together, the present data demonstrate that IFN-β can suppress progressive growth of tumors by both direct and indirect mechanisms. The direct effects, *i.e.*, inhibition of tumor cell proliferation (2, 3) and induction of apoptosis (1), may be of major importance in suppressing IFN-β-sensitive tumors. The indirect antitumor effects of IFN-β include inhibition of angiogenesis (8–10, 21), stimulation of macrophage- and NK cell-mediated antitumor activity (4–7, 19–21), and enhancement of T-cell-mediated tumor-specific immune responses (11–14, 34–43).

In summary, we report that adenovirus-mediated *IFN-β* gene therapy delivered by intralesional injections can eradicate primary fibrosarcomas and confer systemic protection in syngeneic mice. *IFN-β* gene therapy could provide an effective and conservative therapy for treatment of solid tumors.

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