Intrapulmonary IFN-β Gene Therapy Using an Adenoviral Vector Is Highly Effective in a Murine Orthotopic Model of Bronchogenic Adenocarcinoma of the Lung


Abstract

Given previous work showing that an adenoviral vector expressing IFN-β (Ad.IFNβ) was highly effective in eradicating i.p. mesothelioma tumors, the antitumor efficacy of this agent was evaluated in an orthotopic model of bronchogenic adenocarcinoma of the lung. These transgenic mice have a conditionally expressed, oncogenic K-rasG12D allele that can be activated by intratracheal administration of an adenovirus expressing Cre recombinase (Ad.Cre). K-rasG12D mutant mice were given Ad.Cre intranasally to activate the oncogene. Mice were then given 10⁹ plaque-forming units of a control vector (Ad.LacZ) or Ad.IFNβ intranasally 3 and 4 weeks later, a time when lung tumors had been established. Cells derived from K-ras-mutated lung tumors were also grown in the flanks of mice to study mechanisms of therapeutic responses. In two separate experiments, untreated tumor-bearing mice all died by day 57 (median survival, 49 days). Ad.LacZ-treated mice all died by day 71 (median survival, 65 days). In contrast, 90% to 100% of mice treated with Ad.IFNβ were long-term survivors (>120 days; P < 0.001). In addition, immunity to re-challenge with tumor cells was induced. In vitro and flank tumor studies showed that Ad.IFNβ induced direct tumor cell killing and that depleting natural killer or CD8+ T cells, but not CD4+ T cells, with antibodies attenuated the effect of Ad.IFNβ. These studies, showing remarkable antitumor activity in this orthotopic lung cancer model, provide strong preclinical support for a trial of Ad.IFNβ to treat human non–small cell lung cancer. (Cancer Res 2005; 65(18): 8379-87)

Introduction

Lung cancer is the leading cause of cancer-related death in the U.S. and throughout most of the world. It was estimated that in 2004, there were 173,770 cases of lung cancer and 160,440 deaths related to lung cancer in the U.S. alone, with >80% being non–small cell lung cancer (NSCLC; ref. 1). Of all patients diagnosed with NSCLC, only 14% survive >5 years. Surgery has been the mainstay of treatment in early stages of lung cancer. However, most patients (>75%) are not surgical candidates due to their advanced lung cancer stage, and thus medical therapy or radiation are their only options. Unfortunately, medical therapies have been employed with little success. Clearly, novel treatments for NSCLC are desperately needed.

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A potential strategy in the treatment of lung cancer could be the use of IFNs. Type I IFNs (the IFN-α family and IFN-β) are known to inhibit tumor cell growth and stimulate the immune system (2). IFNs have immunoregulatory effects on antibody production, natural killer (NK) and T cell activation, macrophage function, delayed-type hypersensitivity, and MHC antigen expression (3–6). They have also been shown to have antiproliferative effects (4) and antiangiogenic properties (7).

Accordingly, delivery of type I IFN proteins via the i.v. or s.c. route has been explored in many types of cancers. Unfortunately, success has been limited in solid tumors. Although used at their maximally tolerated dose, the short half-life of the protein (<60 minutes) makes sustained therapeutic levels difficult to achieve. To counteract these limitations, a number of investigators have shown that in vivo gene delivery of the IFN-α or IFN-β genes using gene transfer methods such as plasmids or various viral vectors (e.g., adenovirus), could be effective in tumor models of metastatic lung cancer, breast cancer, bladder cancer, cervical cancer, renal cell carcinoma, glioma, and liver metastases from colorectal cancer (8–17). Our group has previously reported significant antitumor activity and improved survival in mouse mesothelioma models using an adenovirus vector encoding IFN-β (18–20). We are not aware of studies existing that examine the use of IFN-β gene therapy in lung cancer.

To date, however, there have been few good models of lung cancer. Most traditional lung cancer models involve injecting lung cancer cells s.c. or i.v. (21, 22). Although useful, these models have the limitation of generating tumors that grow much more rapidly than a human tumor would and in nonphysiologic sites (i.e., skin or lung vasculature). Recently, however, an orthotopic mouse model for bronchogenic lung cancer involving a conditionally expressed oncogenic K-rasG12D allele has been developed (23). After removal of a lox-flanked stop codon by intrapulmonary administration of an adenovirus expressing the Cre recombinase (Ad.Cre), mutant K-ras transcription occurs leading to the development of lung cancer in situ. The tumors progress in a controlled fashion from small diffuse adenomas to widespread sheets of lung cancer. The pathology is similar to what is seen in humans with bronchogenic lung cancer. Depending on the dose of Ad.Cre, the number lesions and the extent of tumor can be controlled.

Given the previous success using adenoviral vector expressing IFN-β (Ad.IFNβ) in the treatment of other solid tumors, we studied this new orthotopic model of advanced bronchogenic lung cancer to evaluate the safety and efficacy of Ad.IFNβ delivered via an intratracheal route. Although one potential limitation in this model would be that Ad.Cre might generate anti-adenoviral humoral antibodies after the administration of adenovirus (24–27) that...
could limit the effectiveness of subsequent Ad.IFN\(\beta\) vectors, we found that this approach led to remarkable efficacy. We also used a cell line derived from K-ras-mutated lung tumors to elucidate some of the mechanisms behind these responses.

Materials and Methods

Animals. Breeding pairs of Lox-Stop-Lox (LSL) K-rasG12D mice (from a mixed 129Sv/j and C57BL/6 background) were generously provided by Dr. David Tuveson of the University of Pennsylvania, Philadelphia, PA. The mice were initially described in a paper by Jackson et al. (23). Mice were genotyped by PCR amplification of genomic DNA obtained from tail samples (primer sequences available upon request). LSL-K-rasG12D-positive mice were used for orthotopic lung cancer experiments, whereas the wild-type littermates were used for flank tumor studies. In addition, pathogen-free female C57BL/6 J F1 hybrids were purchased from Jackson Labs (Bar Harbor, ME) for flank tumor studies. Animals used for all experiments were between 6 and 10 weeks old and were housed in the animal facility at Wistar Institute (Philadelphia, PA). All protocols were approved by the Animal Use Committees of the Wistar Institute and University of Pennsylvania and were in compliance with the guide and care and use of animals.

Cell lines. The murine lung cancer cell line “LKR” was obtained as a gift from Dr. Joseph Friedberg of the University of Pennsylvania School of Medicine. These cells were derived from an explant of a pulmonary tumor from an activated K-rasG12D mutant mouse grown in Dr. Tyler Jack’s Lab at M.I.T. (ref. 28; Boston, MA). Cells were cultured and maintained in high glucose DMEM (Mediatech, Washington, DC) supplemented with 10% fetal bovine serum (Georgia Biotechnology, Atlanta, GA), 2 mmol/L glutamine, and 1% penicillin/streptomycin.

To determine levels of MHC class 1 expression, the LKR cells were analyzed by flow cytometry. LKR cells were left untreated or exposed to 50 MOI (multiplicity of infection) of Ad.LacZ, 10 MOI of Ad.IFN\(\beta\), or 50 MOI of Ad.IFN\(\gamma\) for 48 hours. At this time, the cells were harvested and subjected to fluorescence-activated cell sorting analysis using a rat anti-mouse FITC-conjugated monoclonal antibody directed against the H-2K\(\beta\) molecule (BD PharMingen, San Diego, CA), which is reactive in C57/B6 mice and expressed on these F1 hybrid mice.

Lung tumor model. To induce tumors, 100 \(\mu\)L of saline containing 3 \(\times\) 10\(^6\) particles of Ad.Cre (see below) was given to each LSL-K-rasG12D mouse intranasally. Preliminary studies showed this route to be less traumatic and equally effective as intratracheal administration. Virus was suspended in serum-free DMEM medium (which contains 125 mg/L of NaH\(_2\)PO\(_4\) and 200 mmol/L of CaCl\(_2\)) and added to the cells. Calcium phosphate coprecipitation has been shown to improve lung gene transfer (29). Animals were closely observed daily for signs of distress. When they appeared lethargic, they were killed by carbon dioxide asphyxiation.

Flank tumor models. In pilot studies, we tested 5 \(\times\) 10\(^6\), 1 \(\times\) 10\(^6\), and 2 \(\times\) 10\(^6\) LKR cells. Although all doses formed tumors, the 2 \(\times\) 10\(^6\) dose had kinetics most consistent with our other tumor models and most consistently formed uniform tumors and was thus selected for further studies. To create peripheral tumors, mice were thus injected with 2 \(\times\) 10\(^6\) LKR cells s.c. in the flanks of C57BL/6 J F1 hybrid mice or transgene-negative transgenic mice. Tumors were measured twice weekly and volumes were estimated using the formula 3.14 \(\times\) [largest diameter \(\times\) (perpendicular diameter)]\(^2\) / 6.

Immunogenicity tests for murine tumors. The immunogenicity of the murine lung cancer cell line LKR was tested. Irradiated (50 Gy) tumor cells (2 \(\times\) 10\(^6\)) were inoculated on one flank of naive mice followed by challenge with 2 \(\times\) 10\(^6\) live tumor cells on the opposite flank 14 or 25 days later. As a control, live tumor cells (2 \(\times\) 10\(^6\)) were inoculated into the flanks of naive mice at the same time as the immunized mice received live tumor cell injections.

Recombinant adenoviral vectors. The first-generation E1/E3-deleted type 5 Adenoviral vector encoding the murine IFN-\(\beta\) (Ad.IFN\(\beta\)) gene has been described (18). A similar E1/E3-deleted Ad.LacZ control virus was purchased from the University of Pennsylvania Vector Core. The Ad.Cre virus was provided by Dr. David Tuveson (23). Vector preparations were shown to be negative for the presence of wild-type adenovirus. We ascribed the particle to the plaque-forming unit (pfu) ratio of each preparation in 293 cells; this ranged between 50:1 and 100:1.

We evaluated the amount of IFN-\(\beta\) generated by Ad.IFN\(\beta\) after infection of LKR cells in vitro. LKR cells were seeded in six-well plates and transfected with Ad.IFN\(\beta\) at various MOIs. Twenty-four hours after infection, the supernatants were collected and evaluated using a mouse IFN-\(\beta\) ELISA kit (BDI, Flanders, NJ) following the manufacturer’s protocol. In addition, flank tumors were collected 3 days after intratumoral injection with 10\(^6\) pfu of Ad.IFN\(\beta\) or Ad.LacZ. Tumors were homogenized, protein levels determined, and IFN-\(\beta\) detected using the same ELISA kit.

Treatment of tumor-bearing mice with Ad.IFN\(\gamma\). Each LSL-K-rasG12D mouse was injected intranasally at 21 and 28 days post-Ad.Cre treatment with 1 \(\times\) 10\(^6\) pfu Ad.IFN\(\gamma\) or Ad.LacZ diluted to 100 \(\mu\)L of DMEM (125 mg/L NaH\(_2\)PO\(_4\)) and mixed with 0.5 \(\mu\)L of 2 mol/L CaCl\(_2\). In flank tumor studies, mice treated with Ad.IFN\(\gamma\) received a single intratumoral dose of 1 \(\times\) 10\(^7\) pfu, when flank tumors reached a volume of at least 200 mm\(^3\).

In vitro cytotoxicity assay. We evaluated the antiproliferative effect of Ad.IFN\(\gamma\) on LKR cells in vitro. LKR cells were seeded in six-well plates and transfected with Ad.IFN\(\gamma\) at various MOIs. After 6 hours, the cells were transferred to 96-well plates and evaluated for cell number at 48 and 72 hours after virus exposure. As a positive control for virus effect, another cell line (AR12, a mouse mesothelioma cell line) known to be sensitive to Ad.IFN\(\gamma\) (18), was also used. Cancer cell viability was assessed using an MTS assay, which is a colorimetric test for the quantification of cell viability and proliferation (CellTiter 96 AQueous nonradioactive MTS cell proliferation assay, Promega Corp., Madison, WI).

To further verify that the cell-killing was due to INF-\(\beta\), rather than viral effects, the experiment was repeated, with the addition of 10 \(\mu\)g/mL of a neutralizing anti-mouse INF-\(\gamma\) antibody ( Associates of Cape Cod, Inc., East Falmouth, MA) to untreated cells and cells exposed to 50 MOI of Ad.IFN\(\gamma\). Cell proliferation was measured 24 hours after the addition of virus and antibody using the MTS assay.

Preparation of lymphokine-activated killer cells. To generate lymphokine activated killer (LAK) cells with NK cell activity, spleen cells, at a concentration of 3 \(\times\) 10\(^7\)/mL, were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, 100 \(\mu\)g/mL streptomycin, 10 mmol/L HEPEs buffer, 2 mmol/L glutamine, 10 mmol/L pyruvate, and 50 \(\mu\)mol/L L-\(\gamma\)-mercaptoethanol containing 20 ng/mL of recombinant mouse interleukin-2 (R&D Systems Inc., Minneapolis, MN) for 5 days. We added 20 ng/mL of interleukin-2 every other day to the other well.

\(^{51}\text{Cr}-\text{release assay.}\) The cytotoxic activity of LAK cells on LKR cells was determined by the 4-hour \(^{51}\text{Cr}-\text{release assay.}\) Target cancer cells, LKR and YAC-1 (positive controls for LAK/NK cell cytotoxicity), were labeled with 100 \(\mu\)Ci Na\(^{51}\)CrO\(_4\) and washed thrice with PBS.

The \(^{51}\text{Cr}-\text{labeled target cells were then cultured in triplicate with LAK/NK (effector cells) cell suspensions in round-bottomed microtiter plates for 5 hours. The effector to target ratios were 6:1 and 50:1. The percentage of cytotoxicity was calculated via the following equation (all \(^{51}\text{Cr}\) values in cpm): cytotoxicity (%) = (test \(^{51}\text{Cr} \text{release} – \text{spontaneous release}) / (\text{maximum release} – \text{spontaneous release}) \times 100.

Measurement of CTL activity (Winn assay). Winn assays were done as described previously (30). Splenocytes were isolated and CD8\(^+\) T lymphocytes were purified using the MACs system (Miltenyi Biotec, Auburn, CA). This cell population contained >90% CD8\(^+\) cells by flow cytometry (data not shown). The CD8\(^+\) T lymphocyte-enriched populations were obtained from naive mice, mice bearing LKR tumors (200–300 mm\(^3\) in size), and mice bearing LKR tumors that were treated 10 days earlier with Ad.IFN\(\gamma\). These CD8\(^+\) T cells were mixed with naive viable LKR tumor cells or tumor cells pretreated with IFN-\(\gamma\) (from BD Biosciences, San Diego, CA; 10 ng/mL for 3 days) at a ratio of three purified CD8\(^+\) splenocytes for each tumor cell, and the mixture was inoculated into the flanks of naive mice. Each mixture thus contained 0.5 \(\times\) 10\(^6\) tumor cells and 1.5 \(\times\) 10\(^6\) CD8\(^+\) T
cells. This ratio has previously been determined to be optimal for detecting positive and negative effects (21). Tumor growth was measured after 1 week and expressed as the mean ± SE of at least five mice per group.

**In vivo depletion of CD4⁺, CD8⁺ T cells and natural killer cells.** To deplete specific lymphocyte populations in our model, mice were injected i.p. with monoclonal antibodies purified from the anti-CD4 hybridoma GK1.5 or the anti-CD8 hybridoma 53-6.7 (American Type Tissue Culture Collection, Manassas, VA). Mice were given 300 µg of purified antibody i.p. dissolved in 200 µL of PBS for CD4⁺ and CD8⁺ antibodies. For NK cell depletion, 100 µg of polyclonal rabbit anti-asialo GM1 antibody (Wako Chemicals, Richmond, VA) was used. Antibodies were given 1 day before and 1 day after treatment injection. Thereafter, a maintenance dose of 300 µg for CD4⁺ and CD8⁺ cell depletion or 100 µg for NK cell depletion was delivered i.p. every sixth day to ensure depletion of targeted lymphocyte population. Cell depletion was confirmed by flow cytometry of splenic cell suspensions (data not shown). In addition, we did fluorescence-activated cell sorting to confirm that the anti-asialo-GM1 antibody did not deplete CD8⁺ T cells (data not shown).

**Statistical analysis.** Unless otherwise noted, data comparing differences between groups were assessed using one-way ANOVA with appropriate post hoc testing. Survival studies were assessed using Kaplan-Meier survival curves and analyzed with the Mantel-Cox log rank test. Differences were considered significant when P value was <0.05.

**Results**

**Establishment of orthotopic model.** Using techniques originally described by Jackson et al. (23) and described above, LSL-K-rasG12D-positive mice were injected with Ad.Cre intranasally and tumor progression and survival were followed. Under our conditions, mice started dying about 1 month after Ad.Cre administration and all of the mice were dead between 47 and 57 days (Fig. 1). Mice that were sacrificed to look at tumor progression and the amount of IFN-β generated 24 hours after transduction measured using a commercial ELISA kit. Whereas control LKR cells and cells transduced with control Ad.LacZ produced no detectable IFN-β, we found large and increasing amounts of IFN-β in the group that received Ad.IFNβ. Cells transduced with 10 MOI of IFN-β generated 9.6 × 10⁴ pg/mL/24 hours/10⁶ cells of IFN-β and 50 MOI of IFN-β generated 1.4 × 10⁵ pg/mL/24 hours/10⁶ cells of IFN-β; 50 MOI of Ad.LacZ generated 0 pg/mL/24 hours of IFN-β.

We next evaluated the direct cytotoxic effects of Ad.IFNβ on LKR cells in vitro, using the previously studied AB12 mesothelioma cell line as a sensitive cell line control. LKR and AB12 cells were infected with 0, 10, 50, or 100 MOI of Ad.IFNβ and assayed after 48 hours. Both cell lines were sensitive to the cell-killing effects of Ad.IFNβ with the LD₅₀ of both cell lines between 10 and 50 MOI (Fig. 3A). However, LKR cells infected with up to 250 MOI of Ad.LacZ showed minimal cytotoxicity. This experiment was repeated with similar results.

To confirm that this effect was due to the IFN-β produced (rather than on nonspecific viral effects), we repeated the experiment, but analyzed cell numbers 24 hours after vector transduction in the

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Ad.IFNβ treatment results in significant survival benefit in the orthotopic mouse model of bronchioalveolar lung cancer. LSL-K-rasG12D-positive mice were given Ad.Cre recombinase intranasally at time 0 (control). Ad.LacZ (n = 8) or Ad.IFNβ (n = 8) was given intranasally at 21 days, and then again, 28 days after Ad.Cre administration. One group of mice (n = 8; control) received no treatment. The mice were followed until sacrifice (see Materials and Methods for criteria). Two independent experiments were done. In the first experiment (Fig. 1A), all mice in the control group died by day 57. In the group that received Ad.LacZ, survival was significantly prolonged, but all mice were dead by day 77 (P = 0.0002 versus control). In the group that received Ad.IFNβ, all of the mice survived >120 days (P < 0.0001 versus control; P < 0.0001 versus Ad.LacZ group). In the second experiment (Fig. 1B), all mice in the control group died by day 46. In the group that received Ad.LacZ, all mice died by day 66 (P = 0.007 versus control). In the group that received Ad.IFNβ, one mouse died at day 27, the remainder survived >120 days (P < 0.0014 versus control; P < 0.001 versus Ad.LacZ group).

**Characterization of the LKR cell line.** To explore the mechanisms of this striking therapeutic effect, we took advantage of the existence of a murine lung cancer cell line (LKR) that had been derived from an explant of a pulmonary tumor arising in an activated K-rasG12D-mutated mouse (28).

We first analyzed the sensitivity of the line to Ad.IFNβ infection. LKR cells were transduced with different MOIs of Ad.IFNβ vector and the amount of IFN-β generated 24 hours after transduction measured using a commercial ELISA kit. Whereas control LKR cells and cells transduced with control Ad.LacZ produced no detectable IFN-β, we found large and increasing amounts of IFN-β in the group that received Ad.IFNβ. Cells transduced with 10 MOI of IFN-β generated 9.6 × 10⁴ pg/mL/24 hours/10⁶ cells of IFN-β and 50 MOI of IFN-β generated 1.4 × 10⁵ pg/mL/24 hours/10⁶ cells of IFN-β; 50 MOI of Ad.LacZ generated 0 pg/mL/24 hours of IFN-β.

We next evaluated the direct cytotoxic effects of Ad.IFNβ on LKR cells in vitro, using the previously studied AB12 mesothelioma cell line as a sensitive cell line control. LKR and AB12 cells were infected with 0, 10, 50, or 100 MOI of Ad.IFNβ and assayed after 48 hours. Both cell lines were sensitive to the cell-killing effects of Ad.IFNβ with the LD₅₀ of both cell lines between 10 and 50 MOI (Fig. 3A). However, LKR cells infected with up to 250 MOI of Ad.LacZ showed minimal cytotoxicity. This experiment was repeated with similar results.
We next evaluated the “immunogenicity” of the cell line using the classical prophylactic immunity assay. Two million LKR cells were irradiated and injected s.c. into the flanks of naïve mice (n = 6). Fourteen and 25 days later, the same number of nonirradiated LKR cells were injected s.c. into flanks of the mice that received the irradiated cells and into naïve mice (n = 6). The tumor cells grew equally well in the flanks of the “vaccinated” mice and control mice (data not shown). This lack of inhibition of tumor growth is characteristic of “nonimmunogenic” tumors.

Given this lack of immunogenicity, we measured MHC I expression in the LKR cell line before and after treatment with Ad.IFNβ. As shown in Fig. 3C, we found very low levels of expression of H-2Kβ at baseline or after exposure to Ad.LacZ. In contrast, Ad.IFNβ at doses of 10 and 50 MOI markedly increased MHC I expression in the LKR cells.

Finally, we evaluated the sensitivity of these cells to NK cell lysis. LKR cells and the NK-cell sensitive line, YAC-1, were labeled with radioactive chromium, combined with activated NK cells at ratios of 1:6 and 1:50, and the extent of chromium release determined. As shown in Fig. 3D, marked lysis of YAC-1 and LKR cells was seen at both NK cell/tumor ratios. Consistent with their low levels of MHC class I expression, LKR cells are extremely sensitive to NK cell lysis.

Re-challenge of cured animals with LKR tumor cells. To determine if an immunologic memory response was generated in the LSL-K-rasG12D–positive mice that were cured by Ad.IFNβ treatment, we re-challenged some of these cured mice (96 days after their initial treatment with Ad.IFNβ) with 2 million LKR cells injected into their flank. Naïve mice of the same age were also injected with the same number of LKR cells to serve as positive controls. Tumor volumes were compared over 25 days. Tumor growth was significantly inhibited (P < 0.001) in the mice previously treated with Ad.IFNβ (Fig. 4). At day 25, the naïve mice had an average tumor volume of 422 ± 35 mm³ (n = 5); compared with the cured mice who had an average tumor volume of 109 ± 18 mm³ (n = 5). This experiment was repeated with similar results.

Efficacy of Ad.IFNβ3 treatment on LKR flank tumors. To determine if the cell line grown as a flank tumor model would behave similarly to the orthotopic model, mice were injected with 2 million LKR cells in each flank. When the tumors reached ~200 mm³, mice were left untreated or given one dose of either Ad.LacZ or Ad.IFNβ (1 × 10⁹ pfu) intratumorally. We confirmed that the virus produced IFN-β within tumors by making homogenates from each group and performing ELISA assays. We found no detectable IFN-β in control tumors or tumors treated with Ad.LacZ; however, low, but clearly detectable levels (16 pg/mg of total tumor protein) of Ad.IFNβ3 was measured in homogenates of tumors treated with Ad.IFNβ3.

As shown in Fig. 5 at day 36, the untreated mice had an average tumor volume of 703 ± 33 mm³ (n = 5) compared with the Ad.LacZ-treated mice who had an average tumor volume of 470 ± 42 mm³ (n = 5; P < 0.001). However, the mice treated with Ad.IFNβ3 had complete tumor regressions with an average tumor volume of 2 ± 2 mm³ (n = 5; P < 0.001). This experiment was repeated multiple times with similar results (i.e., see controls for immune cell depletion studies below).

Re-challenge of cured flank tumor animals with LKR tumor cells. To determine if an immunologic memory response was generated using the flank tumor model, as it was in the orthotopic model, mice with “cured” LKR flank tumors were re-challenged
with $2 \times 10^6$ LKR cells injected into their contralateral flank 38 days after initial treatment with Ad.IFN-β. Naive mice (controls) were also injected with $2 \times 10^6$ LKR cells to serve as positive controls. At day 24, the naive mice had an average tumor volume of $54.9 \pm 85 \text{ mm}^3$ ($n = 5$); compared with the cured mice who showed no tumor growth at all ($n = 5; P = 0.0003$). This experiment was repeated with the same number of mice and similar results were obtained.

**Treated mice generate CTLs.** To confirm that this immunologic memory was due to the generation of CTLs, we conducted Winn assays using CD8+ T cells isolated from splenocytes from control mice, tumor-bearing mice, and tumor-bearing mice treated with Ad.IFN-β. When these CD8+ T cells were mixed with $5 \times 10^5$ LKR cells and injected into the flanks of naive animals, the tumors size after 6 days was equal in all groups (146 ± 15, 141 ± 10, and 151 ± 10 mm³, respectively) and virtually identical to tumors generated by injection of $5 \times 10^5$ LKR cells alone (150 ± 11 mm³). Because of the very low MHC class I expression on LKR cells, however, we also mixed the CD8+ T cells with $5 \times 10^5$ LKR cells that had been pretreated with 10 ng/mL of IFN-β (in vitro) to up-regulate MHC class I and injected these mixtures into naive mice. Pretreatment with IFN-β did not affect tumor growth (154 ± 10 mm³). The presence of CD8+ T cells from naive or tumor-bearing mice also did not affect tumor growth (142 ± 15 and 134 ± 15 mm³, respectively). However, addition of the CD8+ T cells from the tumor-bearing, Ad.INF-β-treated mice significantly ($P < 0.01$) inhibited the growth of the IFN-β-pretreated LKR cells (5 ± 3 mm³). This experiment shows that CTL were generated by Ad.IFN-β treatment, however, they were unable to kill LKR cells unless MHC class I was up-regulated.

**Immune cell depletion during Ad.IFN-β treatment.** To determine the immunologic mechanisms responsible for the strong Ad.IFN-β-induced antitumor response, specific immune cell subsets were depleted using antibodies. Mice were injected with $2 \times 10^6$ LKR cells in one flank. When tumors reached a size of ~200 mm³, groups of mice were given antibodies to deplete CD4+ T cells, CD8+ T cells or NK cells (see Materials and Methods). Twenty-four hours later, mice were given one dose of Ad.IFN-β ($10^9$ pfu) intratumorally.

The appropriate antibody was then given 24 hours after the Ad.IFN-β, and weekly thereafter. Adequate and specific depletion of each subset was confirmed by flow cytometry of spleen cells (data not shown).

**Figure 3.** Characterization of the LKR cell line. A, in vitro sensitivity to Ad.IFN-β. LKR cells, along with AB12 cells (a mouse mesothelioma cell line serving as a positive control) were exposed to increasing concentrations of Ad.IFN-β for 48 continuous hours of exposure. LKR cells were also exposed to Ad.LacZ to test the direct toxicity of the adenovirus. An MTS assay was done. LKR and AB12 cells were killed by relatively low concentrations of Ad.IFN-β, whereas control adenovirus resulted in minimal cytotoxicity. B, use of an antibody to IFN-β to establish specificity of the cytocidal effect of Ad.IFN-β. LKR cells were exposed to 50 MOI of Ad.IFN-β for 24 hours in the presence or absence of a neutralizing mouse antibody to IFN-β (AB). LKR cells were also exposed to Ad.LacZ to test the direct toxicity of the adenovirus. An MTS assay was done. About 50% of cells were killed by Ad.IFN-β; however, the anti-IFN-β antibody completely blocked this response. Control adenovirus resulted in minimal cytotoxicity. C, MHC I expression on LKR cells. LKR cells were exposed to increasing concentrations of Ad.IFN-β to 24 hours. The cells were then harvested and evaluated using flow cytometry. Baseline levels of MHC I expression were very low, but increased markedly with increasing concentrations of Ad.IFN-β. Control adenovirus resulted in minimal MHC I expression. MFI, mean fluorescent intensity in arbitrary units. The numbers in each tracing represent the percentage of positive cells. D, sensitivity of LKR cells to NK cell lysis. The cytotoxic activity of activated NK (LAK) cells on LKR cells was determined by the 4-hour $^{51}$Cr-release assay. Chromium-labeled target LKR cells and YAC-1 cells (positive controls for LAK cell cytotoxicity) were cultured in triplicate with LAK (effector cells) cell suspensions for 5 hours; the effector to target ratio was 6:1 and 50:1, respectively. The percentage of cytotoxicity was calculated via the following equation (all $^{51}$Cr values in cpm): cytotoxicity (%) = (test $^{51}$Cr release – spontaneous release) / (maximum release – spontaneous release) $\times$ 100. LKR cells were extremely sensitive to NK cell–mediated lysis.
Figure 6A shows that depletion of CD4+ T cells had no effect on the growth of the tumor. In addition, depletion of CD4+ T cells did not decrease the efficacy of Ad.IFNβ. The Ad.IFNβ-treated mice (n = 5) had an average tumor volume of 40 ± 7 mm³ (P < 0.001 compared with control) whereas the Ad.IFNβ + CD4+ antibody group (n = 5) had an average tumor volume of 51 ± 6 mm³ (P < 0.001 compared with control; P = 0.99 compared with Ad.IFNβ). This experiment was repeated with the same number of mice and similar results were obtained.

Figure 6B shows that depletion of CD8+ T cells had no effect on the growth rate of the tumors, an observation that is consistent with the previous finding that LKR cells are nonimmunogenic. CD8+ T cell depletion did diminish the effect of Ad.IFNβ, however, the effects were delayed. At an early time point after Ad.IFNβ treatment (i.e., day 16, 4 days after treatment), tumor size was decreased significantly (P < 0.05), but similarly in animals treated with Ad.IFNβ alone (114 ± 22 mm³) and in the Ad.IFNβ/CD8+ T cell–depleted animals (145 ± 9.2 mm³) when compared with controls (275 ± 28 mm³). However, after day 20, the tumors in the Ad.IFNβ/CD8-depleted mice began to grow rapidly, at the same rate as control tumors, whereas the mice treated with Ad.IFNβ alone continued to decrease in size. By day 28, the Ad.IFNβ-treated mice (n = 5) had an average tumor volume of 44 ± 4 mm³ whereas the Ad.IFNβ/CD8+ T cell–depleted group (n = 5) had an average tumor volume of 323 ± 25 mm³ (P = 0.045). The experiment was repeated with the same number of mice and similar results were obtained.

Figure 6C shows that depletion of NK cells did not affect the growth of the tumors. However, depletion of NK cells had an effect on the Ad.IFNβ-treated mice, but in a different pattern. In contrast to CD8+ T cell depletion that affected the later response to Ad.IFNβ, NK cell depletion led to loss of the initial response to the Ad.IFNβ. Thus, 4 days after treatment (day 20), the tumors in the Ad.IFNβ/NK cell–depleted group (324 ± 27 mm³) were not significantly different in size from control tumors (393 ± 25 mm³), whereas the tumors in Ad.IFNβ group were significantly (P < 0.001) smaller (130 ± 20 mm³). After that point, however, the tumor growth slowed and actually decreased, with a slope similar to that of the Ad.IFNβ group. By 31 days, the size of tumors in the Ad.IFNβ/NK cell–depleted group (257 ± 41 mm³) was significantly smaller (P < 0.005) than the control group (885 ± 92 mm³).

Discussion

Type I IFNs stimulate both the innate and adaptive immune system and are thus critical mediators in antiviral and antitumor activity (3, 5, 31). In addition to directly suppressing tumor cell replication and inducing differentiation or apoptosis (4, 32, 33), type I IFN's can: (a) stimulate NK cell–mediated tumor lysis (31), (b) induce NK cell proliferation (34), (c) stimulate macrophages and enhance their tumoricidal properties (8, 35, 36), (d) up-regulate MHC I expression (37), and (e) promote antitumor T cell responses via stimulation of memory phenotype subsets and prolonging survival of activated populations (38, 39). Type I IFNs have also been shown to reduce tumor growth via antiangiogenic properties (40).

Despite these impressive potential antitumor activities, administration of IFN-α and IFN-β proteins have not been very effective in the treatment of solid tumors, probably because the short half-life requires high doses that result in toxic side effects. In contrast, evidence is mounting that gene therapy approaches for the delivery of type I IFN's are both well-tolerated and have much higher efficacy. Successful preclinical experiments transferring the murine IFN-β gene using liposomes or adenovirus vectors have been reported in immunocompetent animal models of gliomas (14, 41), colorectal liver metastases (17), fibrosarcomas (13), and models of malignant mesothelioma (18–20).

The data presented here extends these findings by demonstrating remarkable in vivo antitumor activity of Ad.IFNβ in an orthotopic tumor model of bronchogenic lung cancer. In two independent studies, intrapulmonary administration of two doses of Ad.IFNβ into animals with well-established tumors led to 90% to 100% long-term survivals.

An important component of these experiments was the use of an orthotopic model of lung cancer where tumors arose in the distal airways, much like actual lung carcinomas. This can be contrasted to other lung cancer models where tumor cells are injected i.v.—models that actually more closely mimic distant metastatic disease rather than primary lung cancer. Although there are aspects of the LSL-K-rasG12D model that are challenging (i.e., generating a...
breeding colony and the difficulty in following tumor growth noninvasively), the use of an orthotopic lung cancer model such as this one, in which the pathology and anatomic localization of the tumors closely resembles the human counterpart, has a number of advantages for gene therapy studies. First, the immune environment of the lungs is distinctive. For example, the proximity of lung cancers to normal bronchial and alveolar epithelial cells and to tumor-associated alveolar macrophages is likely important. As the primary site for naturally occurring adenoviral infection, the immune response generated by the adenoviral vector is very different in the lungs than it would be in a flank tumor. Second, the physical requirements of treating lung cancer are unique. Lung cancers, especially bronchioloalveolar lung cancers are diffuse and multifocal, requiring treatment by intratracheal or intrabronchial instillation of vector. 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endogenously arising tumors, we took advantage of a cell line (LKR) that was derived from a tumor that had developed spontaneously in a K-rasG12D-positive mouse. This cell line grew well in the flanks of C57BL/6 F × 129P3/J M F1 mice, the same strain as the LSL-K-rasG12D-positive mice. We first obtained some information about the nature of the tumor cells and were able to show that: (a) Ad.IFNβ could directly inhibit LKR cell growth in vitro (Fig. 3A), (b) these cells were not "immunogenic" because vaccination of mice with irradiated tumor cells did not prevent the subsequent growth of injected tumor cells and depletion of CD8+ T cells did not enhance growth (Fig. 6B), (c) the cells expressed low levels of MHC class I antigen at baseline that could be up-regulated by IFN-β (Fig. 3C), and (d) the cells were quite sensitive to NK cell–mediated lysis (Fig. 3D).

We used these cells in a number of experiments. Cells were injected into "cured" mice to determine if an immunologic memory had been produced. As shown in Fig. 4, there was, in fact, a significant slowing of the growth of the cells when injected into previously treated lung cancer mice. The ability of the cells to grow to a limited degree in these treated mice might be due to the large number of cells used to "challenge" the mice. It is also possible that the cultured cells had some slight immunologic differences from the endogenous tumor and were thus able to escape partially from immune surveillance. We also used the cells in a flank tumor model. Like the lung tumors, the cells growing in the flanks were highly sensitive to treatment by intratumoral injection of Ad.IFNβ (Fig. 5A). By depleting specific lymphocyte populations, we were able to show that efficacy was not dependent on CD4+ T cells (Fig. 6A). Instead, we saw that early inhibition of tumor growth was mediated by NK cells (Fig. 6C), whereas late tumor growth inhibition required CD8+ T cells (Fig. 6B). We also directly showed generation of cytotoxic CD8+ T lymphocytes, although interestingly, these CTL required up-regulation of MHC class I on the LKR tumor cells to induce cell lysis. In vivo, it is likely that such MHC class I up-regulation was induced directly by IFN-β from the vector, or indirectly by IFN-γ release by NK cells.

Although it is well-established that Ad.IFNβ can have effects on both NK cells and CD8+ T cells, this is the first example, to our knowledge, of a tumor where both activities were required for full antitumor activity. In our previous studies of Ad.IFNα in mesothelioma, we found that efficacy was almost exclusively due to CD8+ T cells (18, 19). In a fibrosarcoma model, immunologic memory was observed and it was found that the antitumor effects of Ad.IFNβ were completely lost in severe combined immunodeficiency mice, suggesting complete dependence on T cells (13). Natsume et al. also saw immunity to reinjected tumor cells and found that administration of anti-CD8 monoclonal antibodies blocked the antitumor cytolytic activity of Ad.IFNβ in their glioma model (41). However, NK cell–mediated cell killing has also been observed to be dominant in other models. For example, Tada et al. (17) showed a strong NK cell–mediated component in their studies with colorectal tumors. Some of these differences may be due to the sensitivity of the tumors to NK cell lysis. For example, although we did see a marked influx of NK cells in the peritoneal fluid of tumor-bearing mice treated with Ad.IFNα, the mesothelioma cell lines used in our study were almost completely resistant to NK cell–mediated lysis, unlike the LKR cells used here (18, 19).

In conclusion, this study shows that adenovirus-mediated IFN-β gene therapy can induce a potent antitumor effect in a poorly immunogenic orthotopic model of bronchogenic NSCLC that resembles the human condition. We found that this profound antitumor effect was initially due to natural killer cells, but was then followed by the generation of cytotoxic CD8+ T cells. Blocking either of these pathways with monoclonal antibodies attenuated the therapeutic effects. Our group is currently conducting a phase I trial for malignant mesothelioma and metastatic pleural disease using intrapleural administration of Ad.human IFNβ. Early results have shown that the approach is safe and both clinical and immunologic responses have been seen. Given the results from this study and the lack of effective therapy for lung cancer, we propose that Ad.IFNβ should be further investigated in the treatment of human lung cancer. Given the lack of suitable therapeutic options and the especially favorable anatomy for intrapulmonary instillation, bronchioalveolar cell carcinoma might be an especially attractive target.

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References


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