Reduction of Liver Metastasis of Intraocular Melanoma by Interferon-β Gene Transfer

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Purpose. This study determined whether adenovirus-mediated transfer of the murine interferon-β (AdCMVIFN-β) gene protects against liver metastases arising from intraocular melanomas in mice.

Methods. A replication-deficient adenovirus vector (AdCMV-IFN-β) was used for the in vivo transfer of the murine IFN-β gene into intraocular melanoma–bearing mice. AdCMVIFN-β was injected either intravenously or directly into the intraocular melanomas. The effect of gene transfer on liver metastases was ascertained by histopathologic analysis of the livers and by measuring serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are two enzymes associated with liver metastases in patients with uveal melanoma.

Results. Mice treated with two intratumoral injections of AdCMVIFN-β had a 68% reduction in metastatic liver lesions ($P = 0.016$) and a 51% reduction in liver enzyme levels compared with control mice ($P = 0.02$). However, the antimetastatic effect of AdCMVIFN-β was not directly attributable to the adenovirus vector or virus-mediated cytolysis of tumor cells. Intravenous treatment with AdCMVIFN-β resulted in an 86% reduction in the number of metastatic foci in the liver ($P = 0.014$) and a 61% reduction of serum AST levels compared with mice treated with AdCMVLacZ ($P = 0.015$). AdCMVIFN-β treatment produced a sharp increase in the NK cell activity that was demonstrable in vivo and in vivo. In vivo depletion of NK cells by anti-asialo GM1 antibody abrogated the antimetastatic effects of AdCMVIFN-β.

Conclusions. The results support the feasibility of activation of NK cell function through gene transfer as one possible therapeutic strategy for reducing hepatic metastases of uveal melanomas. (*Invest Ophthalmol Vis Sci.* 2003;44:3042–3051) DOI:10.1167/iovs.02-1147

Uveal melanoma is the most common intraocular malignancy in adults, with an incidence of six to seven cases per 1 million adults per year.[1] These neoplasms are eight times more common in whites than in African Americans and three times more common than in Asians. The 5-year survival rate in studies of uveal melanoma is 75%, comparable to that of cutaneous melanoma.[2] Unlike cutaneous melanoma, which can metastasize by lymphatic and hematogenous routes to multiple organs, including the lung and lymph nodes, uveal melanomas metastasize by the hematogenous route and preferentially localize in the liver.[3] Approximately half of patients die of metastatic disease 10 to 15 years after diagnosis.[4] Despite diagnostic advances and introduction of new treatment modalities during the past several decades, the rate of metastatic disease from these tumors has not been substantially reduced.[5] Advances in the treatment of primary uveal melanoma have been substantial. For the present, however, reduction of metastasis may be the only effective means of mitigating the mortality associated with uveal melanoma.

Type I and II interferons (IFNs) are cytokines with pleiotropic properties that can have salutary effects in various tumor models. IFN-β, a type I IFN, affects tumor regression in two ways: directly, by regulation of cell proliferation and differentiation, and apoptosis,[6–8] and indirectly, by activating natural killer cells (NK) and macrophages.[7–11] Moreover, IFN-β is a potent stimulator of immune responses and a modulator of tumor angiogenesis.[12–15] However, in clinical trials, IFN-β protein used on a variety of solid tumors has failed to exhibit a sustained beneficial effect, suggesting that the poor performance of IFN-β in cancer therapy may be due to the insufficient delivery or lack of sustained delivery of the cytokine to the tumor site. Pharmacokinetic studies have demonstrated that the half-life of recombinant IFN-β in the blood stream of patients is less than 5 minutes.[16] It has been shown that 1 hour after a bolus intravenous (IV) injection of 6×10^6 U of IFN-β, the serum concentrations decline to less than 8 U/mL. This concentration is well below that necessary to suppress significant tumor cell proliferation or NK cell activation.[16]

An alternative approach to overcoming this problem is to increase the exposure of tumor cells to IFN-β by gene transfer. Several studies in animal models have demonstrated the feasibility of IFN-β gene therapy.[17–20] Although recombinant adenoviruses display a strong predilection for gene transfer into the liver after IV injection, they localize to a relatively small focus after direct injection into various organs.[21,22] Intraocular gene transfer with adenovirus vectors has been reported, with gene expression persisting for more than 100 days in some cases.[23–26] Moreover, we have demonstrated that intraocular or IV injection of an adenovirus vector containing plasminogen activator inhibitor type I results in a sharp reduction in the incidence of metastases and a significant prolongation of host survival.[21] This study also showed that adenovirus vectors have a predilection to transduce cells of the liver, the target organ most commonly affected by uveal melanoma metastases. These findings suggest that adenovirus vectors are attractive candidates for gene targeting in the treatment of intraocular melanoma. In this study, we used adenovirus-mediated IFN-β gene transfer to activate host effector cells as a means of reducing the metastasis of murine intraocular melanoma.

The rationale for this study is based on the following principles: (1) adenovirus vectors have a predilection for transferring genes to the liver after IV injection,[22] (2) IFN-β is a potent

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activator of NK cells,[11,16,22,27] (3) the liver has the highest concentration of NK cells in the body,[28] and (4) human uveal melanomas and the B16LS9 melanoma used in this study are susceptible to NK cell-mediated lysis.[29–32]

Materials and Methods

Murine Melanoma Cell Line

The murine cutaneous melanoma cell line B16LS9 has been used by other laboratories as a model for uveal melanoma because of its strong predilection to metastasize from the eye to the liver in syngeneic C57BL/6 mice.[17–20,30,33–37] B16SL9 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GibcoBRL, Grand Island, NY) containing 10% heat-inactivated fetal calf serum, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 1% HEPES buffer, and 1% antibiotic–antimycotic solution.

Intraocular Tumor Transplantation

B16SL9 melanoma cells were transplanted transclerally, immediately posterior to the ciliary body of C57BL/6 mice as described previously.[34] This technique produces intraocular melanoma that invades the retina and choroid and produces liver metastasis.[30,34,35,38] Briefly, mice were deeply anesthetized with 0.66 mg/kg of ketamine hydrochloride (Vetalar; Parke-Davis and Co, Detroit, MI) given intramuscularly. Using a syringe (Hamilton Co., Reno, NV) fitted with a 35-gauge glass needle, a tunnel was prepared from the cornea at the limbus, along the sclera and ciliary body to the choroid aided by a dissection microscope. Tumor cells (10^5/2.5 μL) were injected into the choroid and subretinal space. Eyes were examined two or three times per week, and tumor growth was examined under a dissecting microscope. Tumor-containing eyes were enucleated when the intraocular tumor invaded the anterior chamber from the ciliary body/choroid injection site (usually between days 10 and 13 after tumor inoculation). Mice were anesthetized with ketamine hydrochloride and the tumor-bearing eyes were enucleated with sterile curved scissors. Mild hemorrhage was arrested by gentle tamponade. All mice were killed by CO_2 inhalation 13 days after enucleation.

Mice

Female C57BL6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were incorporated into the experiments at 8 to 10 weeks of age. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Adenovirus Vectors

The adenovirus virus–encoding murine IFN-β cDNA driven by a human cytomegalovirus promoter (AdCMVIFN-β) was kindly provided by Zhongyun Dong (M. D. Anderson Center, University of Texas, Houston, TX). AdCMVLacZ (a replication-deficient recombinant adenovirus encoding the *Escherichia coli* β-galactosidase gene) was generously provided by Karl G. Csaky (National Institutes of Health, Bethesda, MD).

AdCMVIFN-β and AdCMVLacZ had deletion of early region 1, which makes them replication deficient and restricts their propagation in 293 cells (human embryonic kidney cells), which supply the Ad5 E1 gene products.[39] These adenoviruses were propagated in 293 cells (kindly provided by George M. Smith, University of Arkansas, Little Rock, AR) grown in MEM containing 10% fetal calf serum and purified by two-step CsCl gradient centrifugation. They were shown to be negative for the presence of wild-type adenovirus.[20] After dialysis at 4°C against 10 mM Tris/HCl (pH 8.0), the vectors were stored in 10% glycerol at −80°C. The viral particle plaque-forming unit (pfu) ratio for each experiment was determined as described previously.[40]

In Vivo Gene Transfer with Adenoviral Vectors

Tumor-bearing mice were treated at different time points after tumor transplantation, with intratumoral injections of 6×10^7 pfu/2 μL of either AdCMVIFN-β or AdCMVLacZ. For IV injection, 5×10^8 pfu of either AdCMVIFN-β or AdCMVLacZ was suspended in 100 μL Hank's balanced salt solution (HBSS) and injected into lateral tail veins with a 30-gauge needle mounted on a tuberculin syringe.

Assessment of Hepatic Metastases

Liver metastases arising from intraocular B16LS9 melanomas are readily demonstrable by histologic examination of the liver, as previously described.[30,35] Hepatic micrometastases appear as small clusters (approximately 40 μm in diameter) of intrasinusoidal cells with hyperchromatic nuclei.[30] Standard hematoxylin and eosin (H&E)–stained liver sections were observed microscopically in a blind fashion by three independent observers. The results were reported as the mean number of metastases per 10 low-power fields (×10) as calculated from the scores of the three observers. The variation between the observers in the number of micrometastases per specimen was routinely less than 10%.

Serum Levels of Aspartate Aminotransferase and Alanine Aminotransferase

It has been demonstrated that metastatic disease from human melanomas can be effectively monitored by liver function assays.[41] Metastatic tumor burden was also assayed by measuring aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the serum of tumor-bearing and naive mice on the day of necropsy. Serum AST and ALT levels were assessed on a chemistry analysis system (model AU400; Olympus America, Lake Success, NY) at the Aston Ambulatory Care Center (Dallas, TX) and reported as units per liter.

In Vivo IFN-β Activity

A bioassay for IFN-β activity was performed as described previously.[20] Briefly, peritoneal exudate cells (PECs) were obtained from mice, as reported previously.[42] Cell suspensions were incubated for 2 hours at 37°C in plastic Petri dishes to allow for adherence of macrophages. Nonadherent cells were removed by vigorous washing with RPMI 1640 medium. The resultant macrophage cultures were 98% pure as determined by acid phosphatase staining, as described previously.[42] Macrophages (1×10^5 cells/mL) in 96-well plates were incubated with adenoviruses containing either AdCMVIFN-β or AdCMVLacZ in the presence or absence of 1 μg/mL lipopolysaccharide (LPS; Sigma Chemical, Co., St. Louis, MO). As a positive control, naive macrophages were activated with 1000 U of recombinant IFN-β (Access Biomedical, San Diego, CA), for 20 hours. Because activated macrophages produce nitric oxide (NO),[31] the synthesis and release of NO were assessed by staining cultured supernatants for nitrite content (NO is not stable and is converted to nitrite and nitrate within a short period) with Griess regent (ICN, Costa Mesa, CA), as described elsewhere.[43] Briefly, supernatants from activated and nonactivated macrophages were collected and centrifuged at 1000*g* for 10 minutes and concentrated fourfold by centrifugation through a 10-kDa microconcentrator (Pal-Filtron, Northborough, MA). Concentrated and nonconcentrated supernatants (100 μL) were mixed with an equal volume of Griess reagent. The optical density at 570 nm was determined on a microtiter plate reader (Molecular Devices, Menlo Park, CA). The nitrite content of supernatants was quantified by comparison with a standard curve generated with nitrite ($NaNO_2$; Sigma Chemical Co.) in the range of 0 to 400 μM.

IFN-β Cytotoxicity Assays

Cytotoxicity assays were performed in 12-well plates containing 5×10^4 B16LS9 melanoma cells per well. Twenty-four hours later, various concentrations of AdCMVIFN-β or AdCMVLacZ were added to each well, and the plates were incubated for 6 days at 37°C. For the final 2 hours of incubation, 50 mg/mL of MTT (Sigma Chemical Co.) in

phosphate-buffered saline (PBS) was added. Additional control experiments were conducted in tumor cells incubated in medium alone. After incubation, each well was washed with PBS, and 500 μL of 10% SDS added to each well and allowed to complete cell lysis. Each well was mixed, 100 μL of lysate was transferred to a 96-well plate, and the optical density (OD) at 562 nm was determined on a microtiter plate reader (Molecular Devices). All experiments were performed in triplicate. The results are presented as the mean OD.

In Vivo NK Assay

Splenic NK cell–mediated cytolysis was evaluated by a conventional 4-hour ^{51}Cr-release assay using murine YAC-1 lymphoma target cells.[44] Spleen cells were enriched for NK cells using a murine NK cell enrichment antibody cocktail (StemSep; Stemcell Technologies, Inc., Vancouver, British Columbia, Canada) according to the manufacturer's instructions.[45] Effector-to-target ratio was 25:1.

In Vivo Clearance Assay

Assessment of NK cell activity was performed by an in vivo clearance assay, as described previously.[31] The elimination of intravenously injected radiolabeled tumor cells is a well-established method for evaluating NK cell-mediated lysis in vivo. Therefore, the in vivo elimination of B16LS9 cells was evaluated by determining the residual radioactivity in lungs removed from mice 4 hours after IV injection of radiolabeled cells. In some mice, NK cell function was stimulated by three IV injections of 5×10^8 pfu of AdCMVIFN-β on days 0, 5, and 10. In another group of mice, NK cell activity was impaired by intraperitoneal (IP) injection of anti-asialo GM1 antibody during adenovirus treatments. Mice injected with HBSS served as the negative control group. NK activity was assessed 2 days after the third injection of AdCMVIFN-β. B16LS9 cells were labeled in vivo with 100 μCi of Na_2 ^{51}CrO ^{4} for 1 hour (37°C), washed three times with HBSS, and resuspended in HBSS at a concentration of 1×10^7 cells/mL.^{51}Cr labeled cells (1×10^6 cell /0.1 mL) were injected into the lateral tail veins of C57BL/6 mice. Clearance of ^{51}Cr labeled tumor cells was determined by counting the radioactivity (gamma emission) in the lungs, removed from each animal (five per group) 4 hours after IV tumor injection.

Anti-asialo GM1 Antibody Treatment

Anti-asialo GM1 antibody was purchased from Wako Chemicals (Dallas, TX) and has been used extensively for the in vivo deletion of NK cells. In vivo treatment with doses of anti-asialo GM1 antibody similar to those used in this study eliminates more than 95% of NK cell–mediated cytolytic activity, profoundly suppresses NK cell-mediated clearance of NK-sensitive melanoma cells in vivo, and results in a steep increase in the metastasis of intraocular B16LS9 melanomas in mice.[30,31,46] Accordingly, C57BL/6 mice bearing intraocular tumors were treated intravenously with anti-asialo GM1 antibody (0.2 mL of 1:10 dilution per mouse per injection) on days −2, 2, 6, 10, and 15 after tumor injection.

Infection of B16LS9 Cells with Adenovirus Containing IFN-β

B16LS9 cells (5×10^5 cells) were plated into tissue culture flasks. After 24 hours, adenoviruses containing either AdCMVIFN-β or AdCMVLacZ (1×10^7 pfu) were added to the cell cultures. The cultured cells were incubated for 24 hours and washed three times, and fresh medium was added. The supernatants were collected 48 hours after incubation. The supernatants were centrifuged at 2000*g* for 15 minutes and concentrated threefold through a 10-kDa microconcentrator (Pal-Filtron). Total DNA was extracted and subjected to polymerase chain reaction (PCR) to detect IFN-β. Supernatants collected from cells cultured in medium alone served as a control.

PCR Detection of AdCMVIFN-β in B16LS9 Culture Supernatants

Ten microliters of culture supernatant was used in a 50-μL PCR reaction containing 0.5 μM of each primer, 200 μM of each dNTP, 2 mM $MgCL_2$, 10× reaction buffer, and 1 U *Taq* DNA polymerase (Roche Diagnostics Corp., Indianapolis, IN). The primer sequences for AdCMVIFN-β are described elsewhere.[20] The samples were amplified for 30 cycles using a PCR system (Gene Amp 9700; Applied Biosystems, Foster City, CA). The program consisted of one cycle at 94°C for 5 minutes and 30 cycles of 94°C for 1 minute, 52°C for 1 minute, 72°C for 1 minute, and 72°C for 10 minutes. PCR products were run on a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide and visualized by UV light.

Quantitation of Serum Levels of IFN-β by ELISA

The levels of IFN-β in the serum were quantified by ELISA, as described previously.[19] Serum samples from AdCMVIFN-β–treated, AdCMVLacZ-treated, and untreated mice (5 mice/group) were collected. The flat-bottomed 96-well microtiter plates were coated with 50 μL of the serum samples and incubated overnight. Purified IFN-β (R&D Systems, Minneapolis, MN) was used as a standard curve. Plates were washed four times with PBS containing 0.05% Tween 20 (Sigma Chemical Co.), then blocked with 5% casein (Sigma Chemical Co.) in PBS for 2 hours at 37°C. Plates were then washed with PBS-Tween 20 and 100 μL of rabbit anti-IFN-β (1:500; R&D Systems) was added to each well. The plates were incubated for 1 hour at room temperature and then washed with PBS-Tween 20. One hundred microliters of goat anti-rabbit IgG conjugated to horseradish peroxidase (Accurate Chemical, San Diego, CA) at a dilution of 1: 2000 was added to each well and incubated for 1 hour at room temperature. Plates were washed and developed with 100 μL of 1 mg/mL 2,2′-azino-bis(3-ethylbenziothiazoline-6-sulfonic acid diammonium salt (ABTS; Sigma Chemical Co.) and 0.003% H_2O_2. Plates were allowed to develop, and 0.1 mL 5% sodium dodecyl sulfate (SDS; Sigma Chemical Co.) was added to stop the reaction before optical density was determined on a microplate reader (Molecular Devices, Menlo, CA) at 405 nm. The results are expressed as nanograms of IFN-β per milliliter. The detection limit of the ELISA is 3 ng/mL.

Reverse Transcription–Polymerase Chain Reaction

The expression of IFN-β mRNA in the livers of animals treated with AdCMVIFN-β was analyzed by RT-PCR. Briefly, total RNA was isolated from 30 mg of liver from each animal with a kit (RNeasy Mini Kit; Qiagen, Valencia, CA). The first-strand of cDNA was synthesized with reverse transcriptase (Omniscript, Qiagen) and oligo (dT) primers (Qiagen). From the resultant cDNA, 2 μL was used in a 50-μL reaction containing 0.5 μM of each primer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 1× reaction buffer, and 1.25 U of *Taq* DNA polymerase (Hot Star, Qiagen). The primer sequences for murine IFN-β were as follows:forward 5′-CCTCACCTACAGGGCGGACTT-3′, reverse 5′-ATACCAGTCCCAGAGTCCGC-3′ (IDT, Coralville, IA). Murine β-actin was used as an internal control, and the sequences were as follows: forward 5′-TGTGATGGTGGGAATGGGTCAG-3′, reverse 5′-TTTGATGTCACGCACGATTTCC-3′ (Stratagene, La Jolla, CA). The samples were amplified for 30 cycles with the PCR system (9700 Gene Amp; Applied Biosystems). The program consisted of 1 cycle at 94°C for 5 minutes and 30 cycles at 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 45 seconds, and 72°C for 10 minutes. PCR products were run on a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide and visualized by UV light. Control consisted of liver RNA obtained from normal animals and animals treated with AdCMVLacZ adenovirus.

Statistical Analysis

Statistical significance between the various experimental groups were determined by the Mann-Whitney test. Student's *t*-test was used to evaluate results from the in vivo and in vitro NK cell assays. Results were considered significant if $P < 0.05$.

RESULTS

In Vivo Expression of IFN-β by B16LS9 Cells Infected with AdCMVIFN-β

To evaluate the ability of AdCMVIFN-β and AdCMVLacZ to infect B16LS9 cells in vivo, B16LS9 cells were infected with either adenovirus vectors containing IFN-β or LacZ genes and the expression of IFN-β and LacZ was evaluated by PCR and β-galactopyranoside (X-Gal), respectively. As shown in Figure 1, a specific 900-bp PCR product was detected in B16LS9 cells (Fig. 1A, IFN-B) infected with AdCMVIFN-β. In contrast, IFN-β was not detected in either wild-type (WT) or AdCMVLacZ-infected (LacZ) B16LS9 cells. Successful expression of the LacZ gene was demonstrated by the presence of the LacZ product β-galactosidase, which was detectable by X-Gal staining (data not shown). These results indicate that the AdCMVIFN-β–infected B16SL9 cell line expressed IFN-β in culture.

Assessment of IFN-β Activity

A well-characterized assay was implemented to confirm that gene transfer with the adenovirus-containing IFN-β gene could induce the production of IFN-β.[20] Peritoneal macrophages were transduced with either AdCMVLacZ or AdCMVIFN-β and stimulated with LPS, and NO production was assessed. As shown previously, macrophages exposed to IFN-β alone did not produce increased quantities of NO.[20] However, macrophages stimulated with IFN-β and LPS were induced to produce significant amounts of nitrite (Fig. 1B). These results indicate that the AdCMVIFN-β vector is capable of transferring the IFN-β gene and inducing the production of biologically active IFN-β.

In Vivo Cytotoxicity of B16LS9 Cells to Recombinant Adenovirus Infection

To test whether infection with adenovirus containing IFN-β or LacZ genes has a direct cytopathic effect on B16LS9 melanoma cells in vivo, B16LS9 cells were infected with various concentrations of either AdCMVIFN-β or AdCMVLacZ and cell proliferation was examined 6 days after adenovirus infection. The MTT assay revealed that the viability of B16LS9 cells infected with AdCMVIFN-β was similar to those infected with AdCMVLacZ or uninfected cells (Fig. 1C).

Effect of Intratumoral Gene Transfer of IFN-β on Liver Metastasis of Intraocular Melanoma

Intraocular melanoma–bearing mice treated with two intratumoral injections of AdCMVIFN-β had a significantly lower number ($P = 0.014$) of metastatic foci than did the AdCMVLacZ-treated control (Fig. 2A). Moreover, AdCMVIFN-β injection resulted in a significant reduction of serum AST ($P = 0.015$) and ALT ($P = 0.048$) levels compared with the AdCMVLacZ-treated control (Fig. 2B). However, there was no discernible effect of intratumoral gene transfer on the growth of the primary intraocular tumors (data not shown).

Effect of IV Transfer with AdCMVIFN-β on Liver Metastasis of Intraocular Melanoma

An important advantage of adenovirus-mediated gene transfer in the context of uveal melanoma is the propensity of recombinant adenovirus to target the liver after IV injection.[21,22] This is especially desirable in the treatment of uveal melanoma, because the liver is the primary organ for the development of metastases. The antimetastatic effects of INF-β gene transfer were evaluated by IV injection of either AdCMVIFN-β or AdCMVLacZ into mice bearing intraocular B16LS9 tumors. Three IV injections of AdCMVIFN-β resulted in an 86% reduction ($P = 0.016$) in the number of metastatic tumor foci in the liver (Fig. 2C) and a 61% reduction in the serum AST levels ($P = 0.026$) compared with mice treated with AdCMVLacZ (Fig. 2D).

Effect of Gene Transfer on Systemic Levels of IFN-β

The salutary effect of either intracameral (IC) or IV gene transfer in controlling liver metastases suggests that IFN-β was systemically disseminated in the treated mice. Accordingly, serum levels of IFN-β were measured in intraocular tumor-bearing mice treated with either IC or IV injections of AdCMVIFN-β. Gene transfer using AdCMVIFN-β delivered by either the IC or the IV route resulted in a steep increase in the serum level of IFN-β (Fig. 3). By contrast, transferring the LacZ gene did not produce a significant elevation in the serum levels of IFN-β compared with the untreated control.

Effect of IFN-β Gene Transfer on NK Cell–Mediated Clearance of Blood-Borne B16LS9 Melanoma Cells and In Vivo NK Cytolytic Activity

Type I IFNs can inhibit tumor growth directly by suppressing tumor cell proliferation and indirectly by activating NK cell–mediated tumor cell lysis.[11,17,47] The possibility that the antimetastatic effect of AdCMVIFN-β treatment is associated with increased NK activity was tested with a previously described NK cell lung clearance assay, which has been widely used to quantify in vivo NK cell activity against blood-borne tumor cells.[48] The elimination of IV injected radiolabeled tumor cells is a well-established method for evaluating NK cell-mediated lysis in vivo. The results of a typical experiment are shown in Figure 4A and demonstrate that NK-sensitive, blood-borne B16LS9 cells were eliminated more efficiency from mice injected with AdCMVIFN-β than from mice injected with medium alone. Moreover, disruption of NK cell function with anti-asialo GM1 antibody significantly impaired the lung clearance of B16LS9 cells. Thus, the antimetastatic effect produced by gene transfer with AdCMVIFN-β is correlated with enhanced NK cell–mediated clearance of blood-borne melanoma cells.

Additional experiments were performed to confirm that AdCMVIFN-β treatment stimulated NK cell activity that was detectable in vivo. Mice were treated with IV injections of AdCMVIFN-β and their spleen cells evaluated for NK cell activity in vivo. The results of a typical experiment demonstrate that AdCMVIFN-β treatment produced a remarkable enhancement of NK cell–mediated cytolysis compared with mice treated with the control vector (Fig. 4B).

Role of NK Cells in the Inhibition of Liver Metastasis in Mice Treated with AdCMVIFN-β

The results from the in vivo and in vivo NK assays indicated that AdCMVIFN-β treatment stimulated the host's NK cell activity. Additional experiments were performed to confirm the association between elevated NK activity and heightened resistance to metastasis of intraocular melanomas. Intraocular tumor–bearing mice were treated with IV injections of either AdCMVIFN-β or AdCMVLacZ and the burden of liver metastasis was assessed. In some mice, NK cell function was disrupted by IP injection of anti-asialo GM1 antibody during the course of adenovirus treatment. The results showed that IV gene transfer with AdCMVIFN resulted in a significant reduction in the number of metastatic tumor foci (Fig. 5A) and serum AST or ALT levels (Figs. 5B, 5C, respectively) compared with mice treated with AdCMVLacZ. Depletion of NK cells with anti-asialo GM1 antibody during the course of AdCMVIFN-β treatment signifi-

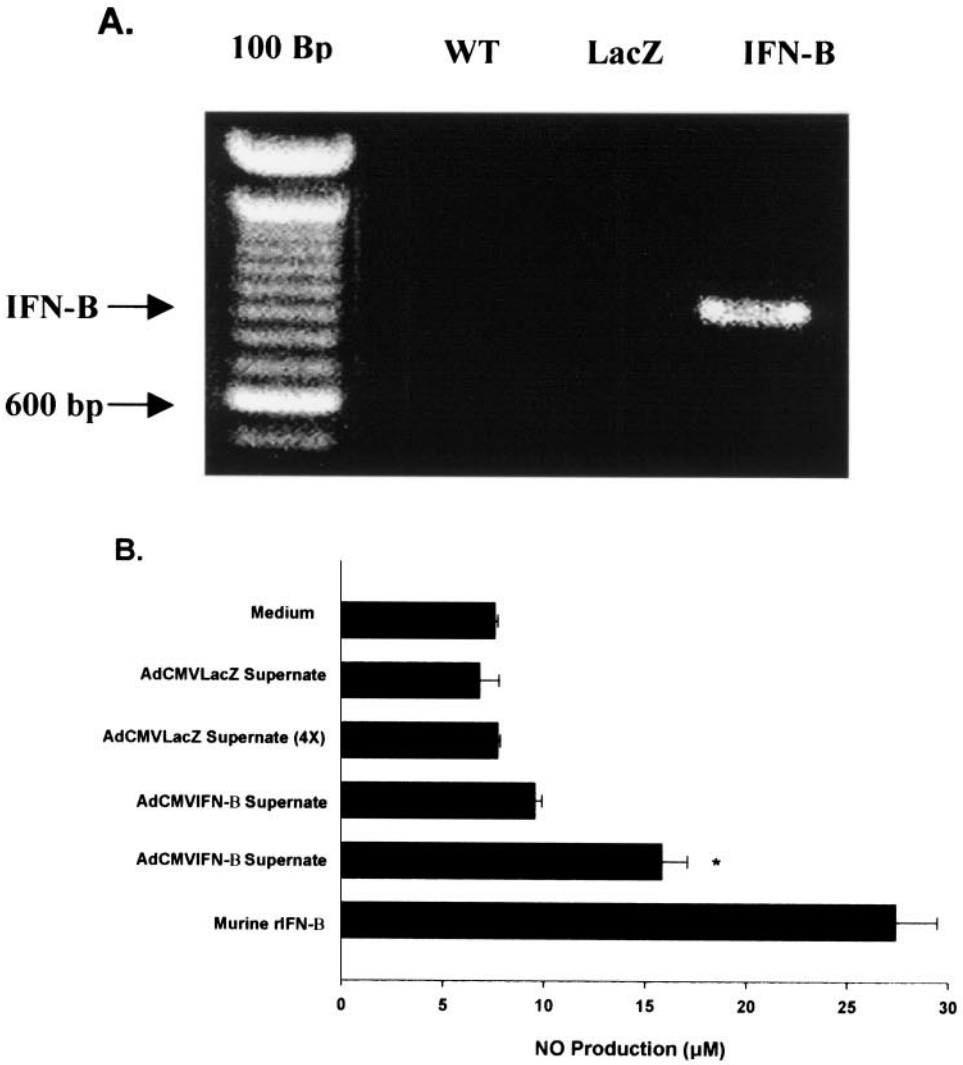

FIGURE 1. Detection of AdCMVIFN-β gene transfer in B16LS9 melanoma cells after infection with AdCMVIFN-β and its effect on melanoma cell proliferation. (**A**) IFN-β gene expression in transduced melanoma cells as detected by PCR. *Lane 1*: 1-kb DNA marker; *lane 2*: PCR from wild-type cells without addition of adenovirus; *lane 3*: PCR from cells infected with AdCMVLacZ; *lane 4*: PCR from cells infected with AdCMVIFN-β. (**B**) Production of biologically active IFN-β by transfected melanoma cells as measured by NO production by macrophages exposed to melanoma supernatants. (**C**) Effect of IFN-β gene transfer on the viability of B16LS9 melanoma cells. The data presented were representative of three experiments. The results are presented as the mean OD $\pm$ SD. $^*P = 0.002$ compared with 4 injections of AdCMVLacZ supernatant control.

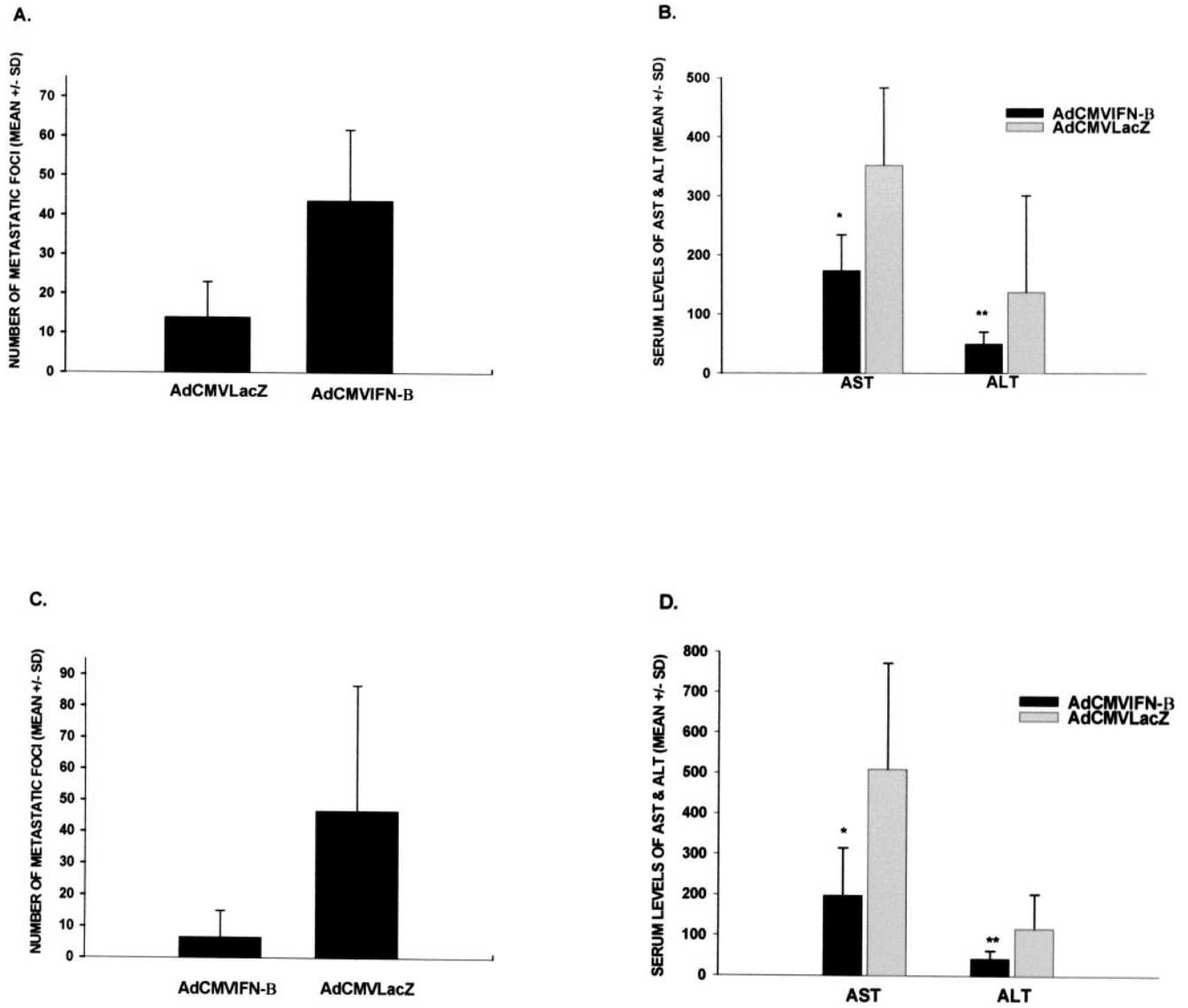

FIGURE 2. Effect of in vivo transfer of AdCMVIFN-β on liver metastases. (**A**) Liver metastases ($P = 0.014$) and (**B**) liver enzyme levels in intraocular melanoma–bearing mice after intratumoral transfer of AdCMVIFN-β ($^*P = 0.015$; $^{**}P = 0.048$). (**C**) Liver metastases ($P = 0.016$) and (**D**) liver enzyme levels in intraocular melanoma–bearing mice after IV transfer of AdCMVIFN-β ($^*P = 0.026$; $^{**}P = 0.042$). The results are expressed as mean number of metastatic lesions per 10 low-power fields (10×) ± SD and mean ALT and AST serum enzyme levels are expressed in units per liter.

cantly reduced the antimetastatic effects of IFN-β. These experiments suggest that inhibition of liver metastases arising from the intraocular melanomas is mediated by IFN-β–activated NK cells.

Expression of IFN-β and LacZ Genes in the Liver

One of the attractive features of adenovirus-mediated gene transfer is the predilection of these vectors to transduce hepatocytes.[21,22] Experiments were performed to confirm that IFN-β and LacZ genes are expressed in the livers of IV treated mice. Intraocular tumor–bearing mice were treated with IV injections of either IFN-β or LacZ genes and the livers were subsequently assessed by RT-PCR for IFN-β gene expression. A band of 600 bp corresponding to murine IFN-β was detected in the livers of mice injected with AdCMVIFN-β (Fig. 6A). However, no IFN-β PCR product was detected in the livers of mice injected with AdCMVLacZ. Similarly, no band corresponding to murine IFN-β product was detected. Successful gene transfer and expression of LacZ gene was confirmed by histochemical analyses of the livers of tumor-bearing mice (Figs. 6B, 6C).

DISCUSSION

The prudent use of animal models is a logical strategy for developing and evaluating new therapeutic modalities for controlling liver metastases that arise from intraocular melanomas. Intraocularly transplanted cutaneous melanomas, such as the B16LS9 murine melanoma, have significant disadvantages as well as advantages. Cutaneous melanomas differ from uveal melanomas in their cytogenetics, epidemiology, and growth kinetics. Moreover, uveal melanomas grow slowly within the eye and can remain quiescent for years. By contrast, the B16LS9 murine melanoma grows rapidly and disseminates to the liver within days of intraocular implantation. Despite these shortcomings, B16LS9 melanoma possesses key attributes that make it a useful model for evaluating the therapeutic efficacy of

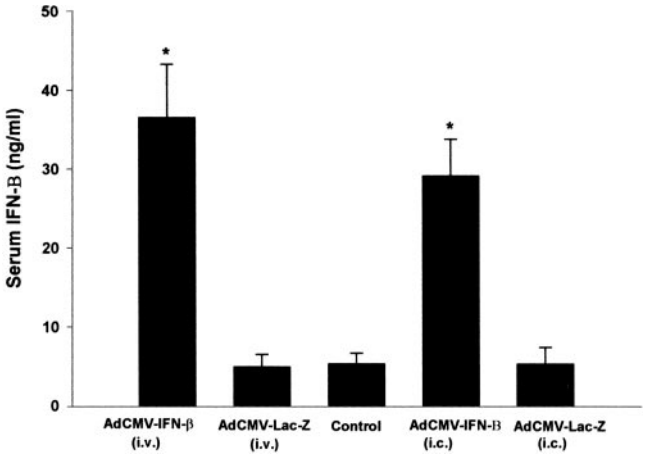

FIGURE 3. Effect of gene transfer on serum levels of IFN-β in tumor-bearing mice. Intraocular tumor–bearing mice were treated with either intracameral (IC) or IV injections of AdCMVIFN-β, and serum was assessed for the presence of IFN by ELISA. Results are expressed as the mean ± SD. *P = 0.0001.

biological response modifiers such as IFN-β. These include: (1) B16LS9, as does uveal melanoma, preferentially metastasizes from the eye to the liver; (2) B16LS9 melanoma cells and many human uveal melanoma cells are susceptible to NK cell–mediated cytolysis in vitro; and (3) there is compelling evidence that NK cells affect the metastasis of uveal melanomas in patients and B16LS9 melanomas in mice.[49,50]

The B16LS9 melanoma displays a strong propensity for metastasizing from the eye to the liver in syngeneic C57BL/6 mice and has been used by others as a model of human uveal melanoma.[33] Dithmar et al.[35] demonstrated that daily bolus injections of IFN-α, a type I IFN, produces a steep reduction in the development of liver metastases in mice harboring intraocular B16LS9 melanomas. We wanted to extend these findings by exploring another type I IFN, IFN-β, delivered by gene transfer. An important advantage of gene transfer is the ability to sustain high serum levels of the immune modulating cytokine. This is in sharp contrast to the extraordinarily short serum half-life of type I IFNs after IV administration. In human subjects, the serum half-life of IFN-β is less than 5 minutes and decreases to control levels in 1 hour after a bolus IV injection.[16]

There is a growing interest in the use of IFN-β in the treatment of various malignancies. It has been reported that murine fibrosarcoma cells, as well as several other tumor cell lines transfected with IFN-β, display reduced growth and metastasis compared with nontransfected parental tumor cells.[51–54] IFN-β gene therapy delivered by adenoviral vectors is effective in eradicating primary murine fibrosarcomas and reducing the growth of mesotheliomas in mice.[19,20,51] The remarkable success of IFN-β gene therapy in treating liver metastases in a nude mouse xenograft model of colorectal cancer prompted us to explore this therapeutic modality in the treatment of liver metastases arising from intraocular melanomas in immunocompetent, syngeneic mice.

The present study shows that intraocular injection of adenovirus containing IFN-β inhibited liver metastasis of progressively growing intraocular melanomas. However, the similarity in intraocular tumor growth in AdCMVIFN-β– and AdCMVLacZ-treated mice rules out the possibility that the antimetastatic effect of intraocularly delivered AdCMVIFN-β is due to direct cytotoxic effects of IFN-β. This conclusion is supported by the fact that B16LS9 cells infected in vivo by AdCMVIFN-β produce high levels of IFN-β. However, the melanoma cells had the same in vivo growth rate as melanoma cells transduced with AdCMVLacZ.

The salutary effect of IFN-β gene transfer in limiting the development of liver metastases is attributable in large part to the activation of the host's NK cell repertoire. We have previously shown that NK cells are effective at killing human uveal melanoma cells in vivo and at limiting the metastasis of human uveal melanoma cells transplanted into the eyes of nude mice.[31,32] These studies also showed a strong correlation between high expression of major histocompatibility complex (MHC)-I antigens and resistance to NK cell–mediated cytolysis.[31,32] Subsequent retrospective studies have supported this hypothesis and have reported that the expression of MHC class I antigen expression on primary uveal melanomas correlates with a poor prognosis in patients with uveal melanoma.[49,50]

The inability of intratumoral IFN-β gene transfer to affect the growth of intraocular melanomas is probably due to the inhibitory effects of the aqueous humor on NK cell activity in situ. Aqueous humor contains TGF-β and macrophage migra-

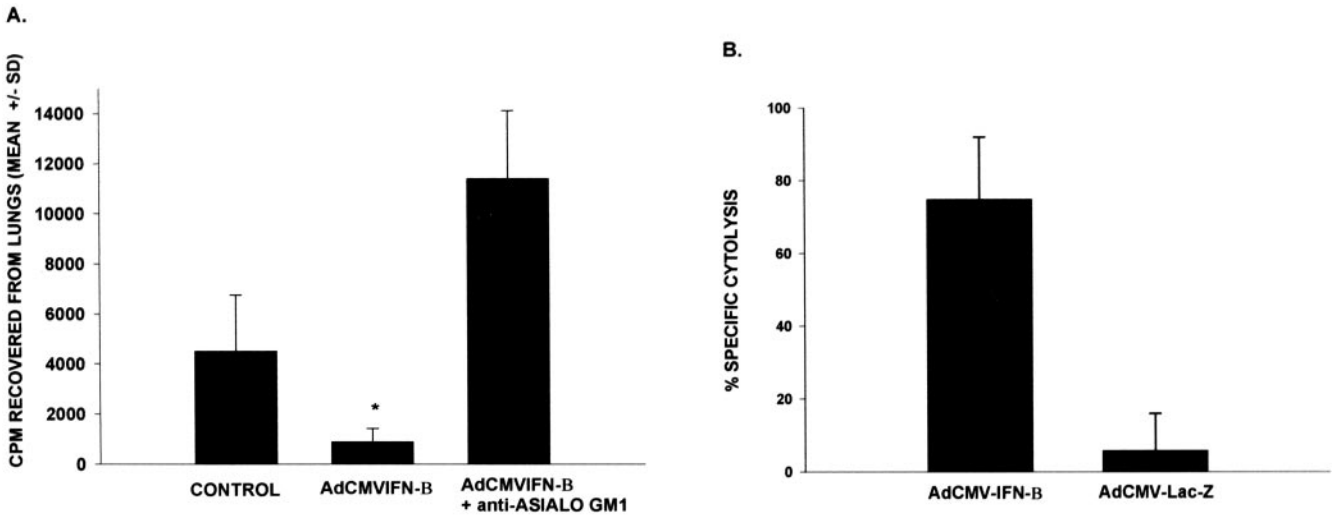

FIGURE 4. Effect of gene transfer with AdCMVIFN-β on in vivo NK cell activity. Mice were injected IV with AdCMVIFN-β on days 0, 5, and 10. NK activity was assessed on day 12. (**A**) In vivo lung clearance results. *P = 0.04 compared with control group and P = 0.0001 compared with the anti-asialo GM1–treated group. (**B**) In vitro NK activity. P = 0.0004. Results are expressed as the mean ± SD.

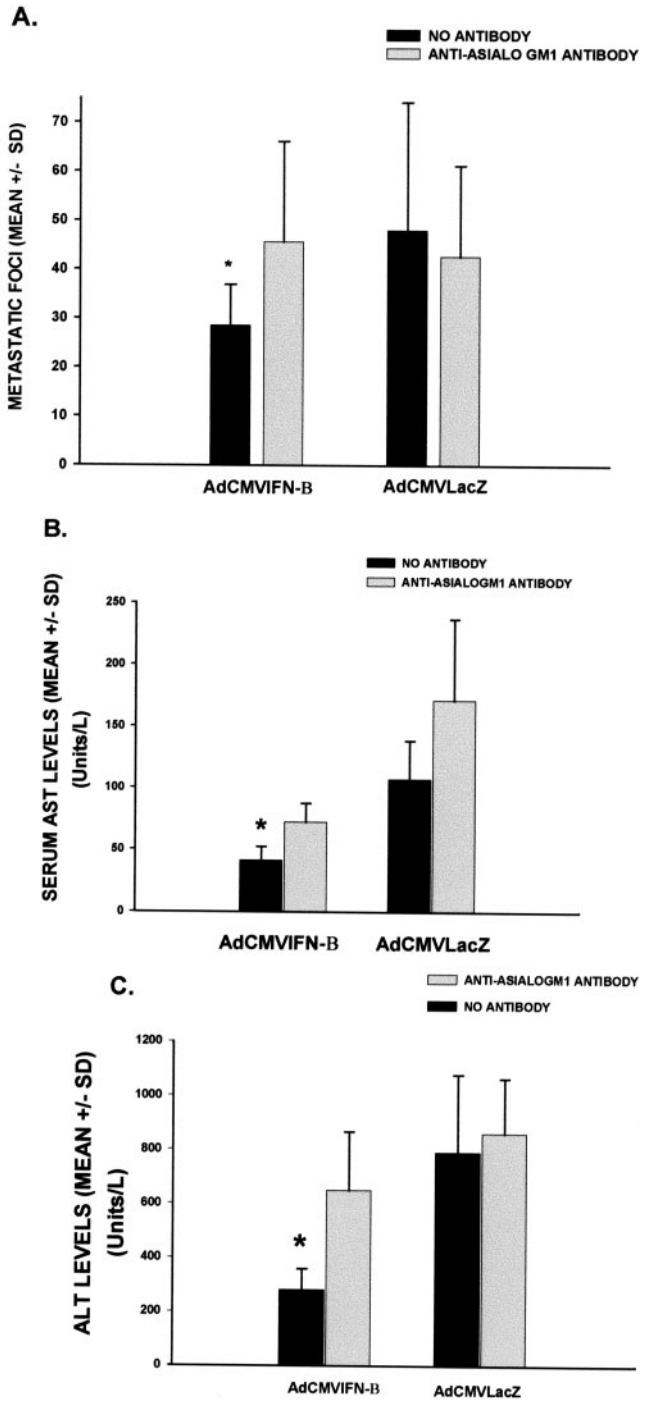

FIGURE 5. Effect of NK depletion on the antimetastatic effect of AdCMVIFN-β gene transfer. Intraocular tumor–bearing mice received IV injections of either AdCMVIFN-β or AdCMVLacZ. Mice were killed on day 26, and the number of liver foci were counted microscopically and liver enzyme levels measured. (**A**) Number of liver metastases. $^*P = 0.021$. (**B**) Serum AST levels. $^*P = 0.012$. (**C**) Serum ALT levels. $^*P = 0.014$. The results are expressed as mean number of metastases per 10 low-power fields (10×) ± SD and serum ALT and AST levels.

tion inhibitory factor (MIF), both of which strongly suppress NK cell–mediated lysis in vivo and in the eye.[55–58]

IFN-α and IFN-β (type I IFNs) can inhibit tumor growth directly, by suppressing cell proliferation and inducing apoptosis,[6,7] and indirectly, by activation of macrophages and NK cells and suppression of angiogenesis.[11,16,27,42,59] Because NK cells are involved in restraining liver metastases of intraocular B16LS9 melanomas,[30] we hypothesized that the antimetastatic effect of IFN-β is mediated by the indirect activation of NK cells. Our studies show that NK cells play an important role in the IFN-β gene therapy for intraocular melanoma metastases. This conclusion is based on the following results: (1) Depletion of NK activity through the administration of anti-asialo GM1 antibody significantly increased the severity of hepatic metastases and elevated the liver enzymes in tumor-bearing mice treated with AdCMVIFN-β compared with untreated and

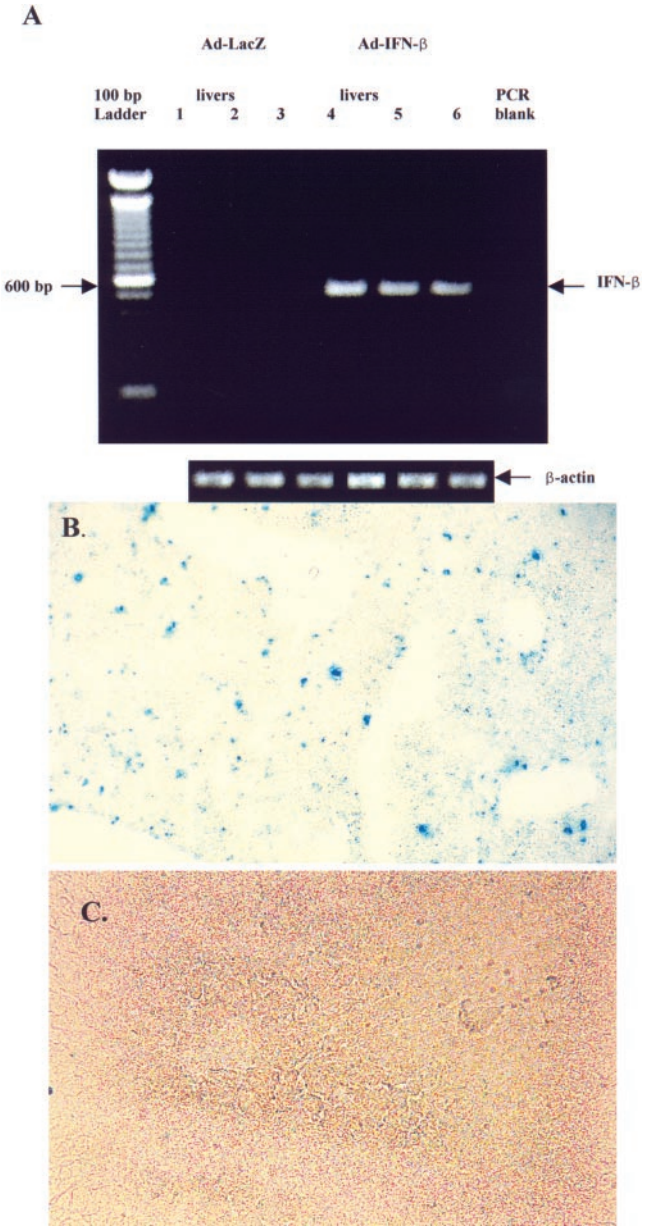

FIGURE 6. IFN-β gene expression in the livers of mice bearing intraocular tumors after IV gene transfer with either AdCMVIFN-β or AdCMVLacZ. (**A**) RT-PCR analysis. *Lanes 1*, *2*, and *3*: RT-PCR of total RNA isolated from the livers of mice injected with AdCMVLacZ. *Lanes 4*, *5*, and *6*: RT-PCR of total RNA isolated from the livers of mice injected with AdCMVIFN-β. RT-PCR containing no RNA served as a negative control (PCR blank). Mouse β-actin was used as a control. (**B**) β-Galactosidase reporter gene expression in the livers of mice bearing intraocular tumors after IV gene transfer with the AdCMVLacZ reporter gene. (**C**) Absence of β-Galactosidase staining in control liver. Original magnification, ×250.

AdCMVLacZ-treated animals; and (2) anti-asialo GM1 significantly impaired the in vivo clearance of radiolabeled B16 LS9 melanoma cells in the liver. These observations support the hypothesis that NK cells restrict intraocular melanoma metastases. Moreover, the steep increase in splenic NK cell activity further supports the notion that NK cells contribute to the antimetastatic effects of IFN-β gene therapy. Although these results are derived from a murine cutaneous melanoma, we strongly suspect that they will be applicable to human uveal melanoma metastasis, in that many human uveal melanomas are susceptible to NK cell–mediated lysis in vivo[30–32] and in vivo.[56] Although not examined in our study, it is possible that IFN-β affects other immune responses, such as antibody production, delayed hypersensitivity, T-cell activation, macrophage function, and class I MHC antigen expression.[19,56,60–62]

IFN-β exerts pleiotropic effects that restrict tumor progression by direct and indirect mechanisms. Yagi et al.[61] showed that antitumor effects of liposome-encapsulated human IFN-β is mediated by direct inhibition of human glioma cell growth in vivo. Studies performed by Xie et al.[53] and Xu et al.[62] demonstrated that the antitumor effects of IFN-β are attributable to upregulation of inducible NO by murine macrophages. In other experiments, Lu et al.[20] showed that administration of adenovirus containing IFN-β inhibits murine fibrosarcoma growth by its direct inhibitory effects on tumor cell proliferation and its indirect effects in activating NK cells, macrophages, and T cells and in inhibiting angiogenesis.

In summary, in the present study, B16LS9 melanoma cells differed in their susceptibility to in vivo IFN-β–meditated cytolysis compared with other tumor cells. IFN-β gene therapy delivered by intraocular or IV injection limited the development of hepatic metastases of intraocular melanomas in mice. Disruption of NK cell function impaired NK cell–mediated clearance of blood-borne melanoma cells and abrogated the antimetastatic effects of AdCMVIFN-β. Although the present results support the feasibility of IFN-β gene transfer as a means of reducing hepatic metastases of intraocular melanomas, direct extrapolation to human uveal melanoma is premature. Gene therapy is still in its infancy and a myriad of technical and safety issues must be resolved before it becomes a practical therapeutic option. The present study was not designed to translate the results directly to humans, rather it was an effort to prove the principle of IFN-β gene transfer as a feasible strategy for controlling liver metastases arising from intraocular melanomas. Refinement in vector development and broader experience in gene transfer may make gene transfer a feasible modality for a variety of genetic disorders and disease conditions. Advancements in the treatment of uveal melanoma metastases by whatever means are eagerly awaited, as the 5-year survival rate in patients with uveal melanoma has not improved in the past three decades.

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