Combined 5-Fluorouracil/Systemic Interferon-β Gene Therapy Results in Long-Term Survival in Mice with Established Colorectal Liver Metastases

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ABSTRACT
Preclinical in vitro and in vivo studies have demonstrated synergistic interactions between 5-fluorouracil (5-FU) and type I and II IFNs against human colorectal cancer cells. Despite these activities, randomized human trials have failed to identify a clinical benefit for this combination treatment. These limited clinical results may be secondary to the short half-life of recombinant IFN protein and the increased systemic toxicities of 5-FU/IFN combinations. We have previously reported an adenoviral-mediated IFN-β gene therapy strategy, which may circumvent the pitfalls of recombinant IFN therapy. However, a dose-dependent toxicity and acute inflammatory response to systemically administered adenovirus vectors may limit the clinical application of this therapy. The combination of adenoviral-mediated IFN-β gene therapy and 5-FU resulted in tumor regression, apoptosis, and improved survival in an established liver metastases model. These therapeutic effects were observed at a significantly lower vector dose than we had previously reported and with limited toxicity. This approach may allow for an effective clinical application of this therapy and warrants additional investigation.

INTRODUCTION
Type I IFNs, IFN-α and IFN-β, are known to have pleiotropic effects including the inhibition of tumor cell growth, stimulation of the immune system, and inhibition of angiogenesis and tissue remodeling (1–6). Preclinical in vitro and in vivo studies have demonstrated synergistic interactions between 5-fluorouracil (5-FU) and type I and II IFNs against human colorectal cancer cells (7–18). Despite these activities, randomized human trials have failed to identify a clinical benefit for this combination treatment (19–21). These limited clinical results may be secondary to the short half-life of recombinant IFN protein and the increased systemic toxicities of 5-FU/IFN combinations (22). These factors make it difficult, by parental administration of protein, to attain local concentrations at which antiproliferative and synergistic effects are observed.

Recombinant adenovirus vectors have many potential advantages as cancer gene therapy vectors, including the safety of transient expression, ease of producing high titers of virus, and relatively high transduction efficiency when compared with other gene therapy vectors (23). Several cancer gene therapy strategies using adenoviral vectors are being tested in human trials (23). These therapies have generally been targeted at local disease; however, more recent strategies targeting metastases have been evaluated. Because a majority of patients who succumb to colorectal cancer do so secondary to systemic metastatic disease, therapeutic strategies directed at metastatic disease are needed to significantly impact this cancer.

We have previously reported an adenoviral-mediated IFN-β gene therapy strategy, which may circumvent some of the pitfalls of recombinant IFN therapy and present gene therapy technology (24). The systemic administration of adenoviral vectors target hepatocytes and circumvents the inability to transduce a significant population of cancer cells (24–26). The transduction of hepatocytes by a recombinant adenoviral vector expressing human IFN-β resulted in sustained local secretion of IFN-β along with tumor regression and long-term survival in a colorectal liver metastases model. However, this therapy resulted in a dose-dependent systemic toxicity. Additionally, as highlighted by recent studies, the acute inflammatory response to systemically administered adenovirus vectors may lead to a substantial dose-dependent toxicity (27, 28). A dose-dependent toxicity to systemically administered adenovirus has also been observed in human trials (29). Therefore, interventions that allow for the reduction of vector dose may also reduce toxicity.

To minimize viral particle-related toxicities, we examined the synergistic interaction between 5-FU and IFN-β. We hypothesized that the combination of an adenoviral-mediated IFN-β gene therapy strategy and 5-FU treatment would result in significant antitumor activity in a colorectal cancer liver metastases model at levels of vector administration significantly lower than reported in our previous studies. Thus improving the therapeutic benefits of 5-FU while reducing risks related to vector administration.
MATERIALS AND METHODS

Drugs and Chemicals. IFN-β was kindly provided by Dr. James Barsoum (Biogen, Inc., Cambridge, MA) and 5-FU (Adrucil; 50 mg/ml) was obtained from Pharmacia and Upjohn (Kalamazoo, MI). The reagents for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay were purchased from Promega, Inc. (Madison, WI) and prepared according to provided instructions.

Cells, Virus, and Tissue Culture. The human colorectal adenocarcinoma cell line KM12L4 (kindly provided by Dr. Isaiah Fidler) was maintained in RPMI 1640 (without folate; Life Technologies, Inc.) containing 10% fetal bovine serum and antibiotics. Cells were subjected to routine testing for Mycoplasma.

The H5.001o1AHIFN-β (AdhIFN-β) virus is a replication-defective E1- and E4-deleted adenovirus vector expressing the hIFN-β gene driven by the liver-directed α1-antitrypsin promoter. It was constructed as described previously (5, 30). The H5.010CMV-βgal (Adβ-gal) virus is an E1- and E3-deleted adenovirus vector that expresses the bacterial protein β-galactosidase driven by the cytomegalovirus promoter/enhancer.

In Vitro Evaluation of Cytotoxicity. Cell proliferation and viability was evaluated with the MTT assay (Promega, Inc.). Briefly, cells were plated into a 96-well plate, 5 × 10^3 cells/100 µl of complete media/well in eighths. The plates were cultured overnight in a 37°C incubator with 5% CO2. Twenty-four hours later, the cells were treated with 100 µl of fresh complete medium containing 5-FU and/or recombinant IFN-β for 24 h. (Drug concentration tested ranged between 0 and 500 µg/ml for 5-FU and between 0 and 1000 units/ml for IFN-β.) Media was replaced, and MTT assays were performed 24 h later. Twenty µl of MTT reagent containing 333 µg/ml MTT and 25 µM phenazine methosulfate were then added to each well, and the plates were incubated for an additional 3 h, which is sufficient time for color development. The absorbance was then measured at a wavelength of 570 nm on a microplate reader. Cell survival was determined by dividing the A570 nm of the treated cells compared with saline-treated control cells.

In Vitro Evaluation of Apoptosis. The induction of apoptosis was evaluated by *in situ* terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) staining. Cells were plated in chamber slides at a density of 5 × 10^4 cells in 2 ml of complete media/well in triplicates. The plates were cultured overnight in a 37°C incubator with 5% CO2. Medium was replaced, and TUNEL analyses were performed 24 h later. The chamber slides were then fixed in 4% paraformaldehyde in PBS for 1 h at room temperature. The slides were also permeabilized with a buffer containing 0.1% Triton X-100 (Sigma, St. Louis, MO) and 0.1% sodium citrate for 2 min on ice. The slides were then incubated in 20 mg/ml proteinase K (Sigma) in 10 mM Tris-HCl buffer (pH 7.0) for 15 min at room temperature. Fifty µl of the TUNEL reagent (Roche) was placed on the slides, and the slides were covered with strips of parafilm and incubated at 37°C for 2 h. Slides were then analyzed with an Olympus IX70 fluorescence microscope with an excitation wavelength of 488 nm.

Animals. Six- to 8-week-old female NCR athymic nude mice or BALB/c mice (Charles River Laboratories, Wilmington, MA) were used in protocols approved by the Institutional Animal Care and Use committee of the University of Pennsylvania. Animals were housed in the Wistar Institute Animal facility in sterile cages and fed a diet of animal chow with free access to sterile water.

Animal Procedures. All animals were handled according to protocols that have been approved by the Institutional Animal Care and Use committee of the University of Pennsylvania. The staff of veterinarians provided veterinary care.

Intrahepatic Metastatic Tumor Model. Our liver tumor model was developed by direct subcapsular injection of cells into the liver parenchyma. This model was used primarily in experiments where quantitative effects on tumor size and volume were measured. KM12L4 were grown in monolayers as described above. On the day of injection, cells were harvested with trypsin, washed with complete medium, and counted using the trypan blue exclusion method. Cells were resuspended in complete medium at a concentration of 1 × 10^6 cells/100 µl.

Mice were anesthetized with 10% ketamine/xylazine solution by i.p. injection and their abdomens were prepared with betadine solution. A subcostal incision was made, and the left lobe of the liver was delivered into the wound. The left lobe was directly injected with 1 × 10^6 KM12L4 cells using a 27-gauge needle, and pressure was held with a sterile cotton tip applicator until the injection site was hemostatic. The left lobe was returned to the abdominal cavity, and the incision was closed in two layers with absorbable sutures. The animals were placed on warming blankets and allowed to recover in their cages with free access to food and water. This intrahepatic injection model consistently yielded sizable tumors in 95–100% of animals.

Intraspinal Metastatic Tumor Model. An intraspinal liver metastases model was developed for survival studies as described previously (31). On the day of injection, cells were harvested with trypsin, washed with complete medium, and counted using trypan blue exclusion. Cells were resuspended in complete medium at a concentration of 1 × 10^6 cells/100 µl (KM12L4).

Mice were anesthetized with 10% ketamine/xylazine solution by i.p. injection and their abdomens were prepared with Betadine solution. A subcostal incision was used to deliver the spleen for injection. The spleen was injected with 1 × 10^6 KM12L4 cells, and pressure was held with a sterile cotton tip applicator until the injection site was hemostatic. The spleen was subsequently removed by cautery using a handheld device (Fisher Scientific). The incision was closed in two layers with absorbable sutures, and the animals were allowed to recover. This intraspinal injection model consistently yielded metastases in 90–95% of animals.

Intraspinal Injection Survival Model. For the survival studies, mice were weighed three times weekly and assessed after treatment. Moribund mice were euthanized according to preestablished criteria, which were the presence of two or more of the following premorbid conditions: presence of gross ascites; palpable tumor burden > 1.5 cm; signs of dehydration, lethargy; emaciation; or weight loss > 20% of initial body weight.
In Vivo Synergy between IFN-β and 5-FU. Fourteen days after tumor injection as described in the above section, the animals were randomized into six groups. Two groups were treated with systemic administration of $2 \times 10^{10}$ AdhIFN-β, Ad-β-gal, or given an equal volume of phosphate-buffered solution via tail vein injection. Virus stocks were prepared as previously described and stored at $-70^\circ$C at a concentration of $4 \times 10^{12}$ particles/ml (30). Viral stocks were rapidly thawed, diluted in PBS to an appropriate concentration, and used immediately. On the day after administration of virus, half the animals from each treatment group received 3 consecutive daily injections of 5-FU (40 mg/kg) beginning 24 h after virus administration.

In a separate set of experiments, animals were given i.p. injections of recombinant IFN-β (10,000 units/ml) daily for 7 consecutive days, starting 14 days after tumor inoculation and given 3 consecutive daily doses of 5-FU (40 mg/kg) days 15–17.

In Vivo Gene Therapy: Tumor Measurement Studies. Liver tumors were measured 14 days after the mice received vector. Animals were euthanized by CO₂ asphyxiation, and blood was collected by thoracotomy and cardiac puncture. The livers were subsequently removed intact and bisected in the horizontal plane through the middle of the tumors. The two halves were fixed in 10% formalin overnight and stored in 70% ethanol. The tumors were subsequently measured in two planes with a caliper, and the tumor volumes calculated assuming an ellipsoid shape. One half of each of the tumors was subsequently embedded in paraffin, sectioned, and stained with H&E.

In Vivo Apoptosis Assays. The intrahepatic gross disease model described above generated specimens for the apoptosis studies. Mice were treated with vector 14 days after intrahepatic tumor inoculation. On the day after administration of virus, half the animals from each treatment group received 3 consecutive daily injections of 5-FU (40 mg/kg) beginning 24 h after virus administration. The livers were harvested from mice 3 days after virus administration. The liver tumors were bisected in the horizontal plane and fixed in 10% neutral-buffered formalin for 24 h, then routinely processed, embedded in paraffin, and sectioned at 6 µm. Apoptotic cells were detected by in situ TUNEL staining as described above.

Measurement of Serum and Liver IFN-β Expression. Nontumor bearing nude mice were given tail vein injections of AdhIFN-β at $2 \times 10^{9}$ particles. Half the virus-injected animals ($n = 9$) were given 3 consecutive daily doses of 40 mg/ml 5-FU. At days 3, 7, and 14 days after virus injection, 3 animals from each group were sacrificed for blood and liver collection.

Serum hIFN-β assays were performed by ELISA as described previously (22). Briefly, assays were performed in 96-well plates coated overnight with hIFN-β antibody. Plates were subsequently blocked at room temperature with 1% casein in PBS for 1 h. hIFN-β samples and standards were added, and the samples were successively incubated with the primary and horseradish peroxidase-conjugated secondary antibodies and the colorimetric substrate. Absorbance was measured at 450 nm. Human IFN-β levels are expressed as ng/ml of serum. Serum was also analyzed for liver transaminase levels [aspartate aminotransferase and alanine aminotransferase (ALT)].

The livers were halved, with one-half saved in 4% formalin and the other half snap frozen. The formalin half of each of the tumors was subsequently embedded in paraffin, sectioned, and stained with H&E. Frozen livers were thawed, and 2 volumes of cold PBS containing a mixture of protease inhibitors were added to the liver. The liver was homogenized using 8 strokes of a glass homogenizer. The homogenate was spun in an Eppendorf centrifuge at maximum speed. The supernatant was removed and assayed for hIFN-β levels using the ELISA described above. Human IFN-β levels are expressed as ng/ml of original liver volume.

Histopathological Analysis. Histopathological analysis of liver specimens was performed by a histopathologist (Q.-c. Y.) blinded to treatment group. Histological specimens were graded for evidence of vascular congestion, cellular degeneration, and periportal inflammation. Specimens were graded as 0 for absent, 1 for mild, 2 for moderate, 3 for severe.

Statistical Analysis

In Vitro Synergy Studies. The combination index (CI) for dose combinations of 5-FU/IFN was calculated based on the multiple drug-effect equation of Chou-Talalay (Calcusyn, Biosoft, MO; Ref. 32). Synergism is defined as a more than expected additive effect. A CI < 1 for a given drug combination is considered to be a synergistic interaction.

Nonparametric response surface methods were used to analyze the data from a series of IFN-β/5FU interaction studies (33). A three-dimensional (x, y, and z axes) response surface was defined by dose levels of cytotoxic agent (5-FU) and cytokine agent (IFN) and by the resulting outcome (percent control), respectively. A nonparametric response surface was fit with bivariate splines without smoothing using SAS PROC G3GRID and plotted with SAS PROC G3D. Isobologram analysis was performed based on the fitted response surface by SAS PROC GCONTOUR. The interaction index, as proposed by Bermanbaum (34, 35), was estimated from the selected Isobole. Synergy, antagonism, or no interaction (additivity) are demonstrated if the interaction index is <1, >1, or =1, respectively. A mathematical model was used to assess the statistical significance of the interaction. Piecewise linear models are fit using bivariate splines. The fit of the model without interaction terms will be compared with the fit of a model with interaction terms by the likelihood ratio test, which allows statistical significance of the interaction to be determined (P; Ref. 36). The models were constructed using Stata software with the xtregr procedure, which accounts for the correlation among replicate measures at each dose level of the cytotoxic agent-cytokine agent combination. All Isobologram analyses were with SAS software (release 6; SAS, Cary, NC) or Stata software (release 5; Stata Corp., College Station, TX).

For the tumor volume data, Kruskal-Wallis tests were performed to test the effect of treatment on total volume. For the survival data, log-rank tests were performed to test the effect of treatment and Kaplan-Meier curves were plotted. $P < 0.05$ was considered significant.

RESULTS

In Vitro Synergy of Combination Therapy with 5-FU and IFN-β. We investigated combination treatment of 5-FU and IFN-β on a colorectal cancer cell line in vitro. KM12L4
cells were treated with recombinant human IFN-β in combination with 5-FU (Fig. 1A). The CI for dose combinations of 5-FU/IFN was calculated based on the multiple drug-effect equation of Chou-Talalay (Calcusyn, Biosoft, MO; Ref. 32). As demonstrated in Fig. 1B, the CIs calculated for the various dose combinations were consistent with very strong or strong synergy (CI < 1). Additionally, Isobologram analysis was performed. The interaction index was 0.30, which implies a strong synergistic interaction. Mixed effects piecewise linear models were fit and also were consistent with very strong synergy (χ² = 44.01, df = 12, P = 0.000015).

5-FU/IFN-β Synergy Is Associated with Induction of Apoptosis in Vitro. In an effort to evaluate potential mechanisms for this interaction, we performed in situ TUNEL staining of cells treated with 5-FU/IFN combinations. As demonstrated in Fig. 2, neither treatment with IFN-β or 5-FU alone resulted in a significant apoptotic response. However, the combination treatment resulted in an apoptotic response, indicating enhancement of the apoptotic response may be contributing to this synergistic interaction.

Adenovirus-Mediated IFN-β Gene Therapy in Combination with 5-FU Results in Tumor Regression. The AdhIFN-β (H5.001α1AThIFN-β) virus is a replication-defective E1- and E4-deleted adenovirus vector expressing the hIFN-β gene driven by the liver-directed α1-antitrypsin promoter. We evaluated this vector in an in vivo gross disease nude mouse xenograft model for liver metastases. Adenoviral vector (2 × 10¹⁰ particles) in combination with 5-FU was delivered systemically to nude mice 14

Fig. 1 Combination therapy of recombinant IFN-β and 5-fluorouracil (5-FU) results in a synergistic effect in vitro. A, in vitro cell viability (3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide). Cells were treated with rIFN-β and/or 5-FU. The results are reported as percent effect (the percent reduction in cell viability relative to PBS control-treated cells); n = 8/treatment group. B, the combination index for dose combinations of 5-FU/IFN were calculated based on the multiple drug-effect equation of Chou-Talalay. Combination index < 1 for a given drug combination is considered to be a synergistic interaction.
days after tumor cell inoculation. Previous tumor modeling experiments had demonstrated the appearance of measurable tumors of 3–5 mm in diameter 14 days after tumor inoculation (data not shown). As demonstrated in Fig. 3, mice treated with 5-FU or AdhIFN-β/H9252 alone experienced a moderate reduction in tumor size, whereas the combination of 5-FU and AdhIFN-β resulted in significant tumor regression with complete elimination of three tumors ($P < 0.05$).

To evaluate the serum and liver pharmacokinetics of IFN after this combination treatment, we measured serum and hepatic levels of hIFN-β in nontumor-bearing mice treated with the same 5-FU and/or AdhIFN-β dose schedule. Measurable serum hIFN-β levels were observed 3 and 7 days after AdhIFN-β administration with or without 5-FU treatment. Approximately 3-fold higher hepatic levels were observed 3 days after AdhIFN-β administration (Table 1).

An independent experiment to correlate serum IFN-β levels with tumor response was performed. Fig. 4 demonstrates the correlation between serum IFN-β levels 3 days after vector administration and tumor size 14 days after treatment with AdhIFN-β and 5-FU. A trend toward decreased tumor size with increasing serum IFN-β levels was observed.

**Recombinant hIFN-β Protein Therapy in Combination with 5-FU Fails to Produce a Therapeutic Response.** We sought to evaluate if recombinant IFN-β protein therapy could engender a response in this model. Therefore, we evaluated the ability of recombinant hIFN-β protein therapy to engender a therapeutic response in combination with 5-FU in this gross disease liver metastases model. Recombinant hIFN-β protein was delivered daily by i.p. injection to nude mice 14 days (gross disease) after tumor cell inoculation (intrahepatic). The addition
of high-dose recombinant IFN-β protein therapy (10,000 units/daily) to 5-FU therapy failed to demonstrate any therapeutic benefit over 5-FU alone (Fig. 5).

**Adenovirus-Mediated IFN-β Gene Therapy in Combination with 5-FU Induces Apoptosis in Established Tumors.** To investigate the potential mechanisms of combination 5-FU and AdhIFN-β therapy on the human colorectal tumors in vivo we evaluated tumors after this therapy. In situ TUNEL was performed on tumor specimens harvested 48 h after the final treatment. Although treatment with 5-FU or AdhIFN-β alone resulted in an apoptotic response similar to or slightly greater than baseline within the tumors, a profound apoptotic response was observed within the tumors of animals treated with the combination of 5-FU and AdhIFN-β (Fig. 6).

**Adenovirus-Mediated IFN-β Gene Therapy in Combination with 5-FU Results in Prolonged Survival in a Gross Disease Liver Metastases Model.** We evaluated if this observed tumor regression with combination 5-FU and AdhIFN-β therapy could be translated into a survival benefit. An intrasplenic model, which results in multiple liver metastases via access from the portal system, was used for these experiments. Mice were treated 14 days after tumor inoculation with the combination of 5-FU and AdhIFN-β. Animals were monitored and euthanized according to predetermined criteria. All mortalities were secondary to tumor progression. As noted in Fig. 7, treatment with PBS, 5-FU, Adβ-gal (control vector) alone, and Adβ-gal/5-FU had little effect on overall survival, with a median survival in the range of 50 days for all these groups. A significant improvement in survival was observed in animals treated with AdhIFN-β compared with PBS and vector controls, with a median survival of 86 days, and 1 animal experiencing long-term survival ($P < 0.05$). However, combination therapy with 5-FU and AdhIFN-β resulted in prolonged survival compared with 5-FU or AdhIFN-β alone with a median survival of 131 days and 4 animals surviving long term without evidence of disease ($P < 0.05$).

**Combination 5-FU/Adenovirus-Mediated IFN-β Gene Therapy Results in Limited Hepatotoxicity.** We evaluated the toxicity to the liver after treatment in an independent experiment. Nontumor-bearing nude mice were evaluated after treatment. Animals were evaluated 7 days after vector administration (3 animals/group). The 7-day time point was chosen based on our previous studies evaluating adenovirus toxicity (24). Serum ALT levels were measured, and histopathological analysis of the liver was performed blinded to treatment. Elevations in ALT were observed after treatment with the adenoviral vectors (Fig. 8). Elevations were greatest in the control vector (Adβgal). The changes observed in ALT after vector administration were less prominent in animals treated with AdIFN-β. The addition of 5-FU to treatments did not significantly alter ALT levels.

Histopathological changes in the liver 7 days after treatment were evaluated. The administration of 5-FU without vector resulted in minimal observable histopathological changes compared with PBS controls. The addition of 5-FU treatment to the control vector or AdIFN-β vector treatments resulted in minimal

### Table 1  Serum and liver IFN levels after systemic delivery of H5.001x1AthIFN-β (AdhIFN-β) at $2 \times 10^{10}$ particles in combination with 5-fluorouracil (5-FU; 40 mg/kg i.p.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>&lt;600</td>
<td>&lt;600</td>
<td>&lt;600</td>
</tr>
<tr>
<td>AdhIFN-β</td>
<td>2080 (1180)</td>
<td>1260 (560)</td>
<td>&lt;600</td>
</tr>
<tr>
<td>AdhIFN-β/5-FU</td>
<td>1940 (820)</td>
<td>1520 (740)</td>
<td>&lt;600</td>
</tr>
</tbody>
</table>

Note: Level of detection was 600 units/ml for serum samples. Liver homogenates were diluted 3:1; therefore, the minimum detectable level was 1800 units/ml.
changes of the observed vector-related histopathological findings. Treatment with control vector (Ad\textit{\#}gal) with or without 5-FU resulted in vascular congestion in most central veins and pericentral sinusoids, hydropic degeneration, and portal inflammatory infiltration of monocytes within the liver. Treatment with Ad\textit{\#}IFN-\textit{\#} resulted in vascular congestion in most lobules within the central vein and pericentral sinusoid and hydropic degeneration. Necrosis was not observed in any of the treatment groups. These results are summarized in Table 2.

DISCUSSION

Colorectal cancer affects >130,000 patients annually and is the second leading cause of cancer deaths in the United States, accounting for 56,300 deaths (37). Many patients present with metastatic disease, and even greater numbers subsequently develop metastases. More than 70% of patients who develop metastasis will have liver involvement, and autopsy studies indicate that this is the sole site of disease in \textasciitilde40% of these patients. Additionally, in many cases, liver metastases are a predominant cause of morbidity and mortality. Systemic chemotherapy offers potential palliation but little to no hope for cure (38). Because of the high incidence of liver metastatic disease only, regional strategies directed toward the liver have been developed in an attempt to improve patient survival.

5-FU-based chemotherapy regimens remain the mainstay of treatment for metastatic colorectal cancer (38). These regimens have measurable but limited activity in colorectal cancers. The combination of 5-FU with type I and II IFNs has been reported to result in improvements in therapeutic effects in preclinical \textit{in vitro} and \textit{in vivo} models (7–18). However, Phase III clinical trials with metastatic colorectal cancer have failed to identify any benefit of IFN/5-FU combination therapy (19–21).

This therapy has been limited by the systemic toxicity and the short half-life of recombinant IFN protein. Parentally administered protein results in peak serum concentrations within minutes to 1 h that fall to below levels of detection within a few hours (22). Adenoviral-mediated IFN-\textit{\#} gene therapy resulted in sustained local concentrations of IFN-\textit{\#} for \textasciitilde7 days and a significant antitumor effect in a model in which recombinant administration of protein resulted in no observable effect (24).

This adenoviral-mediated IFN-\textit{\#} expression resulted in a dose-dependent toxicity. As highlighted by recent studies of toxicity with systemically administered recombinant IFN protein, there is also a dose-dependent nonlinear increase in the acute inflammatory response to systemically administered adenovirus vector (27, 28). This inflammatory response to vector and concurrent toxicity emphasizes the need for strategies that will reduce vector loads.

The gross disease liver metastases model we present is a challenging model, which correlates with clinical stage IV colorectal cancer. As in patients with documented colorectal liver metastases, animals were treated after the establishment of or-

Fig. 6 Adenovirus-mediated IFN-\textit{\#} gene therapy in combination with 5-fluorouracil (5-FU) results in induction of apoptosis in established liver metastases. KM12L4 cells (1 \times 10^6) were directly injected in the livers of athymic nude mice on day 1. Treatment was initiated 14 days after tumor inoculation. Forty-eight h after the final treatment, animals were euthanized, and liver tumors were bisected in the horizontal plane and fixed. Apoptotic cells were detected using the \textit{in situ} death detection kit Fluorescein (Boehringer Mannheim, Indianapolis, IN). T, KM12L4 hepatic metastatic tumor; L, normal liver parenchyma.
Adenovirus-mediated IFN-β gene therapy in combination with 5-fluorouracil (5-FU) results in prolonged survival in a gross disease liver metastases model. KM12L4A cells (1 x 10^6) were injected in the spleens of athymic nude mice followed by splenectomy on day 1. Treatment was initiated 14 days after tumor inoculation. Mice were euthanized when they became moribund as determined by preestablished criteria, and their Kaplan-Meyer survival curves were plotted; n = 9/treatment group.

Liver function tests and histology after AdhIFN-β treatment. Nontumor-bearing nude mice were treated with i.v. administration of vector or vehicle control (PBS) in combination with 5-fluorouracil (5-FU). Animals were evaluated 7 days after vector administration (3 animals/group). Alanine aminotransferase (ALT; units/liter) from treated animals.
results in a short peak in serum IFN levels followed by a fall to baseline. AdhIFN-β therapy results in a continuous level of IFN expression both locally and systemically (22, 24). This continuous local expression of IFN in vivo may better mimic the in vitro environment (where cells are exposed to sustained levels of IFN protein) in which we and others have observed synergy with the 5-FU/IFN combination.

The observed tumor response after Ad IFN/5-FU treatment resulted in a prolonged survival in these animals despite no additional treatment being given. This would indicate that the tumor response to the initial treatment was sufficient to change the natural history of these tumors in these animals. The transient expression of transgenes by adenoviral vectors represents a potential limitation of this vector technology. It is possible that a more prolonged expression of IFN may have been preferable and may have resulted in a more profound tumor response and improvement in animal survival. The evaluation of therapeutic strategies and animal models has many limitations. To evaluate cells derived from human tumors, a nude mouse model was used in these experiments. The use of nude mice in these experiments likely limited immunological responses to the adenoviral vector administration.

This synergy may be secondary to an enhanced apoptotic response. Induction of apoptosis is a fundamental and organized cellular process crucial to development and is an important mechanism by which chemotherapeutics and radiation therapy provide therapeutic benefit. Our in vitro and in vivo data indicates that the interaction between IFN-β and 5-FU is associated with a profound increase in induction of apoptosis.

Colorectal cancer liver metastases remain a significant clinical problem with limited therapeutic options. The application of an adenovirus mediated IFN-β cancer gene therapy strategy resulted in sustained local expression of IFN-β with limited systemic exposure. However, the acute inflammatory response to systemically administered adenovirus may limit our ability to apply this clinically. The combination of 5-FU therapy with adenovirus-mediated IFN-β cancer gene therapy allowed for a significant reduction in vector dose while maintaining a therapeutic response. The systemic delivery of an adenovirus vector secreting IFN-β and the resultant liver transduction generated persistent local peritumoral (hepatic) concentrations of IFN-β at levels where synergistic interactions were observed without significant systemic levels. This strategy may circumvent the potential deficiencies in vector technology and recombinant IFN-β therapy. These responses in an aggressive and established orthotopic animal model are encouraging and warrant additional investigation.

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