p53 Gene Therapy in a Rat Model of Hepatocellular Carcinoma: Intra-Arterial Delivery of a Recombinant Adenovirus

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ABSTRACT
p53 tumor suppressor gene therapy has been proposed for cancers characterized by inactivation of p53 function, and successful therapy will require efficient strategies for gene delivery. To maximize transgene expression in tumors, a clinical strategy has been proposed to treat neoplasms in the liver via hepatic artery administration of a recombinant adenovirus encoding wild-type p53 (rAd-p53). We have developed a syngeneic rat model using a p53mut hepatocellular carcinoma cell line (McA-RH7777) that results in multifocal liver tumor nodules to provide experimental support for this strategy. Treatment of McA-RH7777 cells with rAd-p53 in vitro resulted in efficient transgene expression, growth suppression, and apoptosis. Intrahepatic artery dosing with rAd-p53 or an adenovirus encoding β-galactosidase (rAd-βgal) increased transgene expression in tumor tissue and decreased systemic exposure when compared with i.v. dosing. Daily hepatic artery dosing of rAd-p53 suppressed tumor growth when compared with untreated rats or animals treated with rAd-βgal. These data demonstrate the potential for arterial gene delivery to tumors using recombinant adenoviruses, and support continued investigation of rAd-p53 gene therapy for liver malignancies.

INTRODUCTION
HCC is perhaps the most common form of lethal cancer, resulting in greater than 1 million deaths/year worldwide (1, 2).

Received 12/2/97; revised 4/16/98; accepted 4/29/98.

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4 The abbreviations used are: HCC, hepatocellular carcinoma; rAd-p53, recombinant adenoviruses encoding wild-type p53; rAd-βgal, rAd encoding β-galactosidase; RT-PCR, reverse transcription-PCR; CMV, cytomegalovirus; MOI, multiplicity of infection; GCV, ganciclovir; FBS, fetal bovine serum; IHA, intrahepatic artery.

Although its incidence in the United States and Europe is modest, the disease is endemic in regions of South Africa and Southeast Asia. Conventional chemotherapy is essentially ineffective in this disease and current treatment of HCC includes surgery, radiation therapy, radioimmunotherapy, and liver transplantation. Alternatively, modified intra-arterial chemotherapy has been investigated to capitalize on the unique physiology of tumor blood flow (2). In contrast to normal hepatocytes that receive blood primarily from the portal vein, the hepatic artery supplies nearly all (80–100%) of the blood to malignancies in the liver (3). Clinical trials have compared i.v. and IHA administration of conventional chemotherapeutics, and improved response rates in both primary and secondary liver tumors have been reported with arterial delivery (4–8). The overall clinical and economic benefit of intra-arterial chemotherapy is under discussion, and effective therapies for hepatocellular carcinoma remain elusive (9).

The p53 tumor suppressor is a 393 amino acid nuclear transcription factor that plays a central role in cell cycle regulation and apoptosis (10). Inactivation of p53 function has been reported in approximately 50% of all human cancers, including primary and secondary malignancies of the liver (11, 12). Reintroduction of wild-type p53 into p53-altered cells can suppress tumors (13), lead to apoptosis (14), increase the sensitivity of tumor cells to chemotherapeutic agents (15), and inhibit angiogenesis (16). Recombinant adenoviruses encoding wild-type p53 (rAd-p53) can suppress growth of a spectrum of human cancer cell lines (17), and direct injection of tumors in nude mice with rAd-p53 can inhibit growth of tumors derived from malignancies of the lung, head and neck, colon, breast, brain, and prostate (17–22).

As an alternative to direct intratumoral administration, we have considered rAd-p53 delivery via the primary tumor blood supply (i.e., hepatic artery). The unique circulation of liver tumors provides an attractive system to test this concept. Because i.v. administered recombinant adenovirus has a natural tropism for the liver (23), regional delivery via the hepatic artery should also limit systemic exposure of rAd-p53. Increased local exposure coupled with decreased systemic exposure provides advantages for evaluating gene therapy in this disease (24). To evaluate the potential for gene delivery to liver malignancies, we have developed a syngeneic rat model of HCC. IHA administration of recombinant adenoviruses resulted in transgene expression in the tumor tissue and p53-specific effects on tumor growth. Data from this model support continued investigation of rAd-p53 gene therapy for hepatocellular carcinoma.
MATERIALS AND METHODS

Tumor Cell Lines. The McA-RH7777 rat hepatoma cell line was originally derived from carcinogenicity studies using female Buffalo rats that were maintained on a diet including N-2-fluorenylphthalamic acid (25). McA-RH7777 cells were obtained from the American Type Culture Collection and propagated in Ham's F12/DME supplemented with 2 mM L-glutamine, 20% horse serum, and 5% FBS at 37°C, 7% CO2. The HLE human hepatocellular carcinoma cell line containing a mutation in p53 at codon 249 was obtained from Dr. Takahashi Morisaki (Kyushu University, Japan). These cell lines were propagated in DME supplemented with 1% L-glutamine, and 10% FBS at 37°C, 7% CO2. A549 (human non-small cell lung cancer), SW480 (human colon carcinoma), and 293 (human embryonic kidney) cells were obtained from American Type Culture Collection.

Sequence Analysis of the McA-RH7777 Cells. Total RNA was isolated from McA-RH7777 cells using a TRI Reagent kit (Molecular Research Center, Inc., Cincinnati, OH). The primers CCGGAGGCAGTCAATCA and AACGGGAAAGGAAAAAAGG were used to amplify the entire rat p53 cDNA by RT-PCR. Sequence analysis of the 1.3 Kb PCR product was carried out using an ABI 373 automated sequencer (Applied Biosystems, Foster City, CA) and the additional sequencing primers: AGTCCAGGTCCCTGTGCT, GTCATCTGCCGTCTCTTC, AGAAGATCCCCACTGGAGTC, and TCCACTACAAGTACATGTC. Sequence analysis identified a bp change at amino acid 280 of the rat p53 cDNA from CGT3AAGTACATGTGC to TACAAGTACATGTGC. and TCCACATGCCATATGC. This mutation corresponds to amino acid 282 of human p53 at Exon 8 (26, 27).

Recombinant Adenoviruses. An El-deleted replication-deficient recombinant adenovirus encoding wild-type human p53 (rAd-p53) under the control of the human CMV immediate early promoter was used. El-deleted control adenoviruses were constructed either containing the bacterial lacZ gene (rAd-3gal) or without a specific gene in the expression cassette (rAd-control; 28). Adenovirus production was carried out using the 293 human embryonic kidney cell line that provides the El-deleted function in trans. CsCl-purified recombinant human adenoviruses were stored in a PBS solution containing 2-3% sucrose, and 2 mM MgCl2 (VPBS). Infectious titers (iu/ml) in adenovirus stocks or serum samples were determined in a limiting dilution bioassay using the 293 cell line (29), and testing for replication competent adenoviruses was performed.

Detection of Transgene Expression in Vitro. β-galactosidase expression was assayed by plating cells in 24-well plates (Costar Corp., Cambridge, MA) at 1 X 10^3 cells/well. After overnight incubation, infections with rAd-3gal were performed using a MOI of 0, 1, 3, 10, 30, and 100 in a volume of 1 ml. After 24 h, cells were fixed with 3.7% formaldehyde/PBS, washed, and stained overnight with 1 mg/ml X-gal solution containing 10% DMF, 1.2 mM MgCl2, 3 mM K4FeCN, and 3 mM K3FeCN in PBS (17).

Detection of cellular p53 protein was accomplished by Western blot in untreated cells or after treatment with rAd-p53. Cells were lysed in buffer containing 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.1% NP-40, 50 mM NaF, 5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. After centrifugation, samples of supernatant were normalized for total protein content and loaded onto a 10% Tris-glycine gel. A549 (Arginine) to CCT (Proline). This mutation corresponds to amino acid 282 of human p53 at Exon 8 (26, 27).

Fig. 1. McA-RH7777 cells were treated with rAd-p53 overnight at a MOI of 3 or 30. Cells were harvested and lysed, and samples of supernatant were loaded onto a 10% Tris-glycine gel. After electrophoresis, proteins were transferred and blotted with an antibody that detects both human and rodent p53 (mAb 240) or an antibody that detects human p53 (PAb 1801). Endogenous p53 was detected by mAb 240, and adenovirus-mediated expression of p53 was detected by both antibodies. Untreated Mc2-RH7777 cell lysates and cell lysates from the SW480 human colon carcinoma cell line that expresses mutant p53 were used as controls.
Fig. 2. Rat (McA-RH7777; A) and human (HLE, p53 mutant; B) hepatocellular carcinoma cell lines were treated with rAd-bgal, and transgene expression was evaluated using an X-gal stain. Dose-dependent transgene expression was detected for both cell lines. Proliferation of HCC cell lines McA-RH7777 (C) and HLE (D) was measured by [3H]thyminidine incorporation 72 h after adenovirus infection and expressed as a percentage of untreated controls (mean ± SD). The solid symbols represent cells treated with rAd-p53; open symbols represent cells treated with rAd-control. Estimates of ED50 were determined from logistic equations fitted to these data.

Table 1  Cell cycle analysis after treatment of McA-RH7777 cells

<table>
<thead>
<tr>
<th>Cell cycle</th>
<th>Untreated (MOI = 30)</th>
<th>rAd-control (MOI = 30)</th>
<th>rAd-p53 (MOI = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 (%)</td>
<td>58</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>S (%)</td>
<td>14</td>
<td>17</td>
<td>12</td>
</tr>
<tr>
<td>G2 (%)</td>
<td>25</td>
<td>58</td>
<td>42</td>
</tr>
<tr>
<td>sub G1 (%)</td>
<td>2</td>
<td>1</td>
<td>21</td>
</tr>
</tbody>
</table>

Detection of Apoptosis in Vitro. McA-RH7777 cells were plated in a volume of 5 ml in T25 flasks (1 × 10⁶ cells/flask), infected with rAd-p53 or rAd-control at MOI 30, and incubated for 42 h. Cells were harvested, fixed with 1% formalin and postfixed with 70% ethanol. After equilibration buffer was added to each sample for 1–5 min, TdT enzyme/reaction buffer solution was added, and the cells were incubated for 30 min at 37°C. The reaction was stopped by applying stop/wash buffer according to the manufacturer’s instructions (ApopTag; Oncor, Gaithersburg, MD). After the wash, antidigoxigenin fluorescein solution was applied and incubated for 30 min at room temperature, and cells were additionally washed in 0.1% Triton X-100. Cells were then counterstained with propidium iodide and analyzed by flow cytometry (Becton Dickinson, San Jose, CA). A minimum of 10,000 events were counted at a 515–565 nm wavelength for propidium iodide staining and TUNEL (TdT-mediated dUTP-x nick end labeling).

Rat Model of Hepatocellular Carcinoma. Experimental protocols conformed with the Guide for the Care and Use of Laboratory Animals published by the NIH (NIH publication No. 85–23, revised 1985). Young adult female Buffalo rats (175–200 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and allowed ad libitum access to food (Pico rodent chow; PMI feeds, St. Louis, MO) and tap water. To establish liver tumors, rats were anesthetized with isoflurane (Isoflor; Abbott Laboratories, North Chicago, IL) administered by a
vaporizer. Animals were prepared for surgery and a 1-cm incision was made along the left side approximately 5 mm distal to the rib cage. The spleen was retracted and exteriorized. McA-RH7777 cells (1 x 10^6 in 1 ml sterile HBSS) were injected directly into the spleen over a 10-15-s interval. Pressure was applied to the injection site for 1 min after injection, and the spleen was removed after ligation of blood vessels (30). The abdominal musculature was sutured and the skin was closed using wound clips (Clay Adams, Parsippany, NJ). After animals recovered on a thermal barrier, they were returned to their cages for routine observation.

**Hepatic Artery Catheterization.** At scheduled times after tumor cell inoculation, animals were anesthetized with isoflurane, prepared for surgery, and a mid-line ventral incision was made. The liver was then retracted and connective tissues were cleared to visualize the hepatic and gastroduodenal arteries (31, 32). A polyethylene catheter (modified PE-50 tubing, Clay Adams) was placed in the gastroduodenal artery, forwarded to the bifurcation of the common hepatic artery and secured. For single administration studies, recombinant adenovirus was delivered in a 1 ml volume via infusion pump (0.33 ml/min.). Following dosing, the catheter was removed, the gastroduodenal artery was ligated, and the animals were sutured as described previously. For studies involving multiple administration, catheters were filled with a heparin saline solution and partially exteriorized to a s.c. pouch to facilitate subsequent dosing.

**Detection of Transgene Expression in Vivo.** Two to three weeks after intrasplenic injection multifocal tumor lesions were established, and Buffalo rats received a single infusion of rAd-3gal either via the hepatic artery or via tail vein. Forty-eight hours after dosing, animals were euthanized and liver samples were frozen in liquid nitrogen. Tissues were quickly thawed, rinsed in RNase free water, minced, and 1 ml of TRI-Reagent (Molecular Research Center) was added to 100 mg of tissue for homogenization. Chloroform (200 µl) was then added to homogenates and pelleted. Pellets were washed with isopropanol, 75% ethanol, air dried, resuspended in water, and incubated at 55°C for 5 min. Samples were then incubated with DNase for 15 min, and EDTA (10 mm) was added and incubated for an additional 15 min at 65°C. Absorbance was determined (260/280 nm), and less than 1 µg of total RNA was used for amplification. Primers were designed to amplify recombinant p53 mRNA expression. Primers used were a 5' from the CMV region (CCACTGCTACTGGGTATCAGAA) and a 3' from the p53 region (AAGCGTGTCACCGTCGTGGAAA-GC'). RT-PCR was carried out by preparing a 50 µl sample containing 0.33 µM of each primer, 1 x of Gene Amp PCR buffer II (Perkin-Elmer Biosystems, Foster City, CA), 1.5 mM MgCl2, 0.5 mM dNTPs, 5 units of 0.5 mM AMV-RT (BM), 2.5 units of a 0.5 mM Ampli Tag DNA Polymerase (Perkin-Elmer, Foster City, CA), 16 units RNase inhibitor (Perkin-Elmer), and RNase free water. Reverse transcription was at 65°C 10 min, 50°C 8 min, and 95°C 5 min, and PCR amplification was for 32 cycles at 94° 30 s, 56°C 30 s, 72°C 1 min, and a 10 min 72°C final extension period in a Perkin-Elmer 9600 thermal cycler (Foster City, CA). PCR products were loaded into a 10% TBE polyacrylamide precast gel (Novex) and visualized with ethidium bromide staining.

**Serum Pharmacokinetics of rAd-p53.** Five young adult female S/A Simonsen Albino rats (Simonsen Laboratories, Gilroy, CA) were anesthetized with isoflurane, and catheters were secured in the carotid artery for serial blood sampling. An additional catheter was placed either in the femoral vein (n = 3) or gastroduodenal artery (n = 2) for i.v. or IHA injection, respectively. A single 3-min infusion of rAd-p53 (2.4 x 10^10 IU) was administered in 1 ml to each animal. Blood samples (0.5 ml) were collected before administration and at scheduled times during the 120 min after infusion. Anesthesia was maintained throughout the experiment. Blood samples were centrifuged, and serum was frozen. Serum concentrations (iu/ml) of recombinant adenovirus were determined using a limiting dilution bioassay (29).
p53 Gene Therapy. For experiments testing the efficacy of rAd-p53 in vivo, six female Buffalo rats received intrasplenic injections of approximately $1 \times 10^6$ McA-RH7777 cells. Seven days later, rats were grouped in pairs to receive either rAd-p53 or rAd-βgal (3 × $10^6$ iu/infusion). Surgery was performed to catheterize the gastroduodenal artery (described above), and each rat received a 1 ml infusion (0.33 ml/min) of rAd-p53 while its matched pair received rAd-βgal. Catheters were partially exteriorized to a s.c. pouch, and daily infusions were administered for 5 consecutive days. At day 31, animals were euthanized, livers were weighed, tumor nodules were counted, and livers were photographed.

RESULTS

Cell Line Characterization. The McA-RH7777 cell line is one of a series of previously characterized Morris hepatoma cells lines derived from female Buffalo rats after exposure to N-2-fluorenylphthalanic acid (25). A Western blot of cell lysates from untreated McA-RH7777 cells probed with an antibody that detects both human and rodent p53 (mAb 240) demonstrated high levels of endogenous protein (Fig. 1, Lane 2), typically associated with the accumulation of mutated p53. No endogenous protein was detected with the PAb-1801 antibody that detects human p53. Sequence analysis confirmed a CGT to CCT mutation at codon 280, corresponding to an arginine to proline mutation.
amino acid substitution in this conserved region of the protein (data not shown).

**Adenovirus-mediated Transgene Expression in Vitro.** Treatment of McA-RH7777 cells with rAd-p53 resulted in an increase of p53 detected by mAb 240. Western blot analysis using an antibody (PAb 1801) that detects human but not rodent p53 further demonstrated the presence of transgene-mediated expression following treatment of cells with rAd-p53 (Fig. 1). Because differential levels of transgene expression have been reported for human tumor cell lines, and human adenoviruses may not efficiently transduce rodent cells, a recombinant adenovirus encoding β-galactosidase was used to compare expression in rat (McA-RH7777) and human (HLE) cells. Expression of β-gal was dose-dependent in cell lines from both species (Fig. 2A and B). Transgene expression was detected in >90% of the McA-RH7777 cells at MOI 3 (3 × 10^9 iu/ml), indicating a level of transduction comparable to human hepatocellular carcinoma cell lines (17). These data demonstrate efficient adenovirus-mediated gene transfer and expression in McA-RH7777 cells at relatively low adenovirus concentrations.

**rAd-p53 Growth Inhibition and Apoptosis in Vitro.** Adenovirus mediated transfer of wild-type p53 has been shown to inhibit the proliferation of a spectrum of human tumor cell lines, and the degree of growth inhibition depends on the extent
of transgene expression (17, 34, 35). A 3-day [3H]thymidine incorporation assay was used to evaluate the effects of rAd-p53.

Table 2  Quantification of reporter gene expression in liver tumors

<table>
<thead>
<tr>
<th>Animal ID No.</th>
<th>Route of administration</th>
<th>Dose (iu)</th>
<th>% Positive in Tumor ± SD</th>
<th>% Positive in Normal ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>730</td>
<td>i.v.</td>
<td>7.4 × 10^9</td>
<td>0.6 ± 0.3</td>
<td>19.4 ± 8.4</td>
</tr>
<tr>
<td>732</td>
<td>i.v.</td>
<td>7.4 × 10^9</td>
<td>0.2 ± 0.3</td>
<td>13.5 ± 1.5</td>
</tr>
<tr>
<td>734</td>
<td>i.v.</td>
<td>7.4 × 10^9</td>
<td>0.2 ± 0.1</td>
<td>20.6 ± 11.6</td>
</tr>
<tr>
<td>738</td>
<td>i.v.</td>
<td>1.5 × 10^10</td>
<td>1.6 ± 1.8</td>
<td>61.9 ± 9.4</td>
</tr>
<tr>
<td>749</td>
<td>i.v.</td>
<td>1.5 × 10^10</td>
<td>0.3 ± 0.4</td>
<td>43.7 ± 13.0</td>
</tr>
<tr>
<td>47</td>
<td>IHA</td>
<td>1 × 10^9</td>
<td>2.4 ± 1.2</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>55</td>
<td>IHA</td>
<td>4.0 × 10^9</td>
<td>63.1 ± 1.6</td>
<td>14.6 ± 0.1</td>
</tr>
<tr>
<td>48</td>
<td>IHA</td>
<td>4.0 × 10^9</td>
<td>16.2 ± 16.8</td>
<td>4.6 ± 3.9</td>
</tr>
<tr>
<td>505</td>
<td>IHA</td>
<td>4.3 × 10^9</td>
<td>8.7 ± 5.0</td>
<td>27.4 ± 25.3</td>
</tr>
<tr>
<td>508</td>
<td>IHA</td>
<td>4.3 × 10^9</td>
<td>13.5 ± 12.0</td>
<td>10.2 ± 5.8</td>
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<tr>
<td>300</td>
<td>IHA</td>
<td>5.5 × 10^9</td>
<td>5.2 ± 3.6</td>
<td>0.9 ± 0.6</td>
</tr>
<tr>
<td>294</td>
<td>IHA</td>
<td>5.5 × 10^9</td>
<td>1.5 ± 1.5</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>296</td>
<td>IHA</td>
<td>5.5 × 10^9</td>
<td>4.2 ± 5.7</td>
<td>0.3 ± 0.2</td>
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<tr>
<td>295</td>
<td>IHA</td>
<td>5.5 × 10^9</td>
<td>15.0 ± 12.9</td>
<td>0.8 ± 0.8</td>
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<tr>
<td>418</td>
<td>IHA</td>
<td>1.0 × 10^10</td>
<td>15.1 ± 19.9</td>
<td>3.9 ± 3.8</td>
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<tr>
<td>103</td>
<td>IHA</td>
<td>1.0 × 10^10</td>
<td>27.5 ± 16.9</td>
<td>4.8 ± 5.0</td>
</tr>
<tr>
<td>10</td>
<td>IHA</td>
<td>2.5 × 10^10</td>
<td>6.2 ± 4.8</td>
<td>3.6 ± 2.9</td>
</tr>
</tbody>
</table>

Tumor cells were introduced to female Buffalo rats by intrasplenic injection, and rats subsequently developed aggressive multifocal tumors localized to the liver. Microscopic examination of liver sections collected 7 days after cell injection demonstrated the presence of tumor, and macroscopic tumors were typically visible by 14 days. Consistent with other rat models of liver malignancy, tumor burden increased with time resulting in death 4–6 weeks after cell injection (Fig. 4).

Detection of Transgene Expression in Tumor and Normal Tissue. To evaluate in vivo gene transfer and expression in normal and tumor tissue, rAd-βgal was injected i.v. or intra-arterially (hepatic) to tumor-bearing rats at doses ranging from 1 × 10^9 to 2.5 × 10^10 iu/dose. When the adenovirus was administered by tail vein (n = 5), hepatocytes stained positive for the transgene product. In contrast, low levels of transgene expression were detected in the tumor tissue (Fig. 5). After intra-arterial delivery of rAd-βgal (n = 12), transgene expression was clearly evident in the tumor tissue, with greater than 50% of tumor cells expressing β-gal in some sections. Representative sections from six animals are shown in Fig. 6. When compared with i.v. dosing, IHA delivery resulted in increased tumor to nontumor ratios (Table 2), consistent with dye-injection studies that demonstrated preferential arterial vascularization of tumor nodules (data not shown). X-gal positive stained tissue was not detected in untreated rats or rats treated with rAd-p53.

To confirm p53 transgene expression in vivo, tumor-bearing rats were treated via the hepatic artery with rAd-βgal or rAd-p53 (approximately 10^9 iu/dose). Two days after injection, liver samples containing both normal and tumor tissue were homogenized and evaluated by RT-PCR using primers specific for exogenous p53. p53 mRNA was detected in samples from rats treated with rAd-p53 but not rAd-βgal (Fig. 7). The PCR reaction was also performed with these samples in the absence of reverse-transcriptase to confirm that measured expression was not a result of DNA contamination (data not shown).

Serum Pharmacokinetics of rAd-p53. To compare systemic exposure after single i.v. or IHA administration, rAd-p53 (2.4 × 10^9 iu) was administered to nontumor bearing female rats. Serial blood samples were collected from a catheter placed in the carotid artery, and serum samples were analyzed for recombinant adenovirus concentration. Infectious adenovirus concentrations (iu/ml) were detected in all serum samples analyzed from 1 min to 30 min after i.v. and IHA administration. No infectious rAd-p53 levels were detected in the samples collected before dosing or 2 h after administration. Peak serum concentrations of rAd-p53 measured in rats dosed i.v. were 10–100-fold greater than those measured after IHA administration (Fig. 8). Data from this study indicate that the first-pass effect after IHA administration can limit the systemic exposure of rAd-p53 in rats. However, detection of infectious rAd-p53 in the circulation after IHA administration demonstrates that the hepatic extraction of recombinant adenovirus was not complete.

In Vivo Effects of rAd-p53. To test efficacy in vivo, multiple injections of rAd-p53 or rAd-βgal were administered to rats via IHA administration beginning 7 days after tumor cell inoculation. Because of interexperiment variations in tumor formation, direct comparisons were made between animals that received intrasplenic injections of McA-RH7777 cells on the
Fig. 7 Animals received an injection of McA-RH7777 cells, and tumors were allowed to grow for 2 weeks. The gastro-duodenal artery was then catheterized, and rats received single infusions (IHA) of rAd-p53 or rAd-βgal (approximately 10^9 iu/dose). Two days after test article administration, livers were harvested and evaluated for mRNA by RT-PCR. Primers specific for recombinant p53 were used. mRNA was extracted from A549 (human NSCLC) cells treated with rAd-p53 as a positive control. p53 mRNA was detected in samples that were treated with rAd-p53 and absent in samples treated with rAd-βgal. No contaminating DNA was detected when homogenates were evaluated in the absence of reverse transcriptase (data not shown).

Fig. 8 Serum samples were analyzed for infectious adenovirus concentration (iu/ml) after a single i.v. or IHA administration of rAd-p53 (2.4 × 10^9 iu/dose) to nontumor bearing rats. Data shown represent samples collected from three animals after i.v. injection (closed symbols) and two animals after IHA injection (open symbols). Pooled pharmacokinetic analysis of area-under the curve suggests first-pass extraction of rAd-p53 exceeds 90%. No infectious virus concentrations were detected in samples drawn 120 min after injection from any animal. Limit of detection was approximately 10^4 iu/ml.

same date. In one experiment, rats received five daily doses of rAd-p53 or rAd-βgal (3 × 10^9 iu/injection). Livers were harvested and photographed 31 days after cell inoculation. Untreated animals were used as a positive control for tumor growth. In a second experiment, paired animals were treated with four daily injections (4 × 10^9 iu/injection) of rAd-p53 or rAd-βgal beginning 7 days after intrasplenic injection of cells. Rats treated with recombinant adenoviruses were harvested 31 days after tumor cell inoculation together with untreated controls. Tumors were present in the livers of all animals, but growth was visibly reduced in rats that received rAd-p53 (Fig. 9, A and B). In the second experiment tumor-bearing livers were weighed wet, and visible tumor nodules on the liver surface were counted to quantify tumor growth (Table 3). Consistent with qualitative observations, the liver weights and number of tumors were reduced in animals that received rAd-p53. In contrast to IHA administration, no reduction in tumor burden was detected after i.v. injection of rAd-p53 or rAd-βgal (n = 4/group) when compared with untreated controls (data not shown).

**DISCUSSION**

Strategies for p53 tumor suppressor gene therapy have been proposed that use retroviral (36), adenoviral (18), and lipid-based delivery (37) systems. Clinical trials are currently underway to evaluate the biological efficacy of intratumoral administration of an adenovirus encoding wild-type p53 to patients with lung or head and neck cancers (38). As an alternative to intralesional injection, we have proposed adenoviral delivery of the p53 gene via the vascular supply to the tumor. Using a syngeneic model of HCC, we have demonstrated transgene expression in tumor tissue and antitumor effects of rAd-p53 after IHA administration. Dosing via the arterial rather than venous circulation increased delivery of recombinant adenovirus to the tumor and reduced exposure to the systemic circulation. The data presented provide preclinical support for adenovirus-mediated p53 gene therapy of hepatocellular carcinoma.

The direct comparison of livers from paired rats treated with rAd-βgal indicates that the effects of rAd-p53 treatment were not due to an immune response or to nonspecific cytotoxic effects of the adenoviral delivery. Although we observed some antitumor effects of rAd-βgal, the in vivo results are consistent with the in vitro data and demonstrate a p53-specific effect of rAd-p53 on the growth of established tumors. Importantly, complete tumor suppression was not observed with the dosing regimen described. Limited delivery of the recombinant adenovirus to all tumor cells provides a likely explanation for tumor growth after rAd-p53 treatment. The variation in gene transfer and expression detected in tumors after a single injec-
Fig. 9 A, animals received an injection of McA-RH7777 cells, and tumors were allowed to grow for 7 days. The gastroduodenal artery was then catheterized, and paired rats received five daily 1-ml infusions (3 × 10^6 IU/dose) of rAd-p53(ACN53) or rAd-βgal(ACBGL). Thirty-one days after cell inoculation, livers were harvested, weighed, and tumor burden was evaluated by counting tumor nodules. B, animals received an injection of McA-RH7777 cells, and tumors were allowed to grow for 7 days. The gastroduodenal artery was then catheterized, and paired rats received four daily 1-ml infusions (10^6 IU/dose) of rAd-p53 or rAd-βgal (4 × 10^6 IU/dose). Thirty-one days after cell injection, livers were harvested, weighed, photographed, and visible tumor nodules were counted. Tumor nodule counts and liver weights are summarized in Table 3.

Table 3 Effects of multiple rAd-p53 treatment on tumor growth

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. tumors</th>
<th>Liver weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>&gt;150</td>
<td>22.90</td>
</tr>
<tr>
<td>Untreated</td>
<td>&gt;150</td>
<td>24.43</td>
</tr>
<tr>
<td>rAd-βgal</td>
<td>92</td>
<td>27.45</td>
</tr>
<tr>
<td>rAd-βgal</td>
<td>111</td>
<td>28.02</td>
</tr>
<tr>
<td>rAd-p53</td>
<td>63</td>
<td>13.58</td>
</tr>
<tr>
<td>rAd-p53</td>
<td>46</td>
<td>16.01</td>
</tr>
</tbody>
</table>

In vivo transfer of the herpes simplex virus-thymidine

treatment of rAd-βgal supports this contention. Furthermore, collateral circulation has been reported for liver neoplasms (39, 40), suggesting that the injected arterial blood vessel may not vascularize each tumor nodule in this animal model. Alternatively, the incomplete tumor suppression in vivo may be attributed to the reduced function of wild-type human p53 in rodent tumor cells. In vitro, rAd-p53 was approximately 10-fold less potent in McA-RH7777 cells than in human hepatocellular carcinoma cells comparably transduced by recombinant adenovirus. Results with this model may, therefore, underestimate the effects of rAd-p53 expected in human malignancies.

I.v. delivery of rAd-p53 did not significantly suppress tumor growth in this model. The lack of efficacy following i.v. dosing corresponded to limited transgene expression detected via this route of administration (Fig. 5). These data are consistent with the limited transgene expression and efficacy of rAd-p53 after portal vein injection to transgenic mice bearing hepatocellular carcinoma (41). When delivered i.v., transgene expression from recombinant adenoviruses is localized primarily to the liver, presumably because the fenestrated nature of the microvasculature facilitates direct interaction between the extravasated adenoviral particles and normal parenchymal cells (23). As primary or secondary malignancies grow in the liver, the nature of the developing tumor vasculature will depend on various factors, including location in the liver, tissue of origin, age, and size of tumor (39). Primary hepatocellular carcinoma in particular may be expected to retain the hypervascular nature of normal parental tissue, where as tumor microcirculation may differ and adenovirus delivery may be less efficient in metastatic tumors of nonliver origin. Additional experimental data are required to determine whether adenovirus-mediated gene transfer will be efficient after arterial delivery to secondary malignancies in the liver, or to other localized tumors with a defined arterial blood supply (e.g., glioblastoma, soft-tissue sarcomas).

Physiological and pharmacological methods may also increase adenovirus targeting to liver malignancies, including obstruction of the hepatic blood supply after administration (42), chemoembolization (43), and use of vasoactive agents to enhance tumor delivery (44). Such aggressive strategies have been evaluated for more conventional therapeutics and may warrant investigation with rAd-p53 in hepatocellular carcinoma because of the serious nature of the disease.
kinase gene that confers sensitivity to the prodrug GCV has been proposed for the treatment of hepatocellular carcinoma (28) and colorectal cancer (45, 46). One theoretical advantage of this strategy is the potential for a bystander effect that can inhibit the growth of neighboring nontransduced tumor cells (47). Adenovirus-mediated delivery of the herpes simplex virus-thymidine kinase gene (rAd-TK) with GCV may, therefore, be efficacious after transduction of only a limited number of cells in the tumor, and tissue-specific promoters may add selectivity to this antitumor approach. Adenovirus transgene expression after intra-arterial delivery supports not only the potential for p53 gene therapy, but also the potential for recombinant adenovirus delivery of enzymes for directed prodrug therapy of HCC. Indeed, a pilot study with this Buffalo rat model demonstrated a reduction in tumor growth after rAd-TK/GCV treatment (data not shown). Antitumor effects have also been reported in a carcinogen-induced model of hepatocellular carcinoma after portal vein administration of rAd-TK/GCV (48). However, these investigators observed deaths in 47% of rats treated with rAd-TK/GCV, and other investigators have recently demonstrated severe liver toxicity associated with the rAd-TK/GCV combination in mice (49). In contrast, rAd-p53 toxicities have not been reported in a regenerating liver model (50). Because p53 overexpression is tolerated in normal cells, an improved combination in mice (49). In contrast, rAd-p53 toxicities have not been reported in a regenerating liver model (50). Because p53 overexpression is tolerated in normal cells, an improved therapy may be expected with tumor suppressor gene therapy. Further experimentation is warranted to compare the effects of these alternative gene therapy strategies for liver malignancies.

A CTL response to recombinant adenoviruses has been reported to limit the duration of transgene expression in vivo and cause an inflammatory response to transduced cells (51). This cellular response may limit the use of adenoviral vectors for diseases requiring prolonged transgene expression. However, in vitro data demonstrate that only short-term expression of p53 (on the order of days) is sufficient to trigger apoptosis in p53-altered cells (21). If delivery of rAd-p53 to tumor cells can be optimized by arterial administration, the CTL response may not compromise the antitumor effects of recombinant p53 expression. In addition, the inflammatory response to positively transduced tumors after rAd-p53 treatment may result in antitumor effects independent of the transgene.

Development of neutralizing antibodies after i.v. administration of recombinant adenovirus has been reported, and this antibody response can be expected to limit transgene expression after subsequent cycles of treatment (52). The extent of antibody neutralization will likely depend on dose and route of delivery. High local concentrations after arterial infusion of rAd-p53 may enable transgene expression even in the presence of serum antibodies. Transient immunosuppression with available pharmaceutical agents has been shown to facilitate gene transfer and expression after multiple administration (53–55). The ability to overcome a neutralizing humoral response may be of critical importance for successful rAd-p53 gene therapy.

ACKNOWLEDGMENTS

We thank Drs. John Park, Robert Warren, Alan Venook, and Jordan Gutterman for advice; Dr. Elaine Sigurdson and Erika Sutanto-Ward for valuable discussions concerning the animal model; and Drs. Monica Zepeda and Robert Bookstein for critical review of the manuscript.

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