AAV-HGFK1 and Ad-p53 cocktail therapy prolongs survival of mice with colon cancer

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Abstract

This study tried to evaluate the application of a novel cancer gene therapy using recombinant adeno-associated virus (AAV) carrying the kringle 1 domain of human hepatocyte growth factor (AAV-HGFK1) in combination with recombinant adenovirus carrying p53 gene (Ad-p53). BALB/c nude mice models of colon cancer were established and the mice were treated with AAV-HGFK1 alone or in combination with Ad-p53. Combination of AAV-HGFK1 and Ad-p53 significantly prolonged the survival of the mice and also significantly inhibited primary and secondary tumor growth. Histochemical examination of the tumors revealed that AAV-HGFK1 + Ad-p53 combinatorial treatment not only induced necrosis and apoptosis in the tumors but also suppressed tumor angiogenesis. The antiangiogenesis effect could likely be attributed to the ability of AAV-HGFK1 + Ad-p53 viral cocktail to inhibit endothelial cell migration and proliferation. AAV-HGFK1 + Ad-p53 also inhibited tumor cell growth in vitro by inhibiting epidermal growth factor receptor phosphorylation. Therefore, AAV-HGFK1 + Ad-p53 cocktail therapy has a significantly higher therapeutic effect than AAV-HGFK1 or Ad-p53 alone and is a novel promising gene therapy for colon cancer. [Mol Cancer Ther 2008;7(9):2855–65]

Introduction

Colorectal carcinoma is the third leading cause of cancer-related deaths worldwide and the second most frequent cancer in all developed nations. Liver metastasis of colorectal cancer is the major cause of death and occurs in >60% of colon cancer patients (1). Because no therapy at present has significant effect on hepatic metastasis of colorectal cancer, it is necessary to develop alternative treatment strategies for liver metastasis (2).

Because metastasis needs angiogenesis to supply nutrients and oxygen and remove waste products, antiangiogenic therapy is promising for hepatic metastasis of colorectal cancer (3). A growing number of antiangiogenic molecules have been discovered, and some of which are used in clinical treatment of metastasis (4). Antiangiogenic therapy needs constant administration of antiangiogenic proteins to achieve its therapeutic effect (5). Because high therapeutic doses of these recombinant proteins are required and their half-life in vivo is short, their widespread clinical use is limited (6). Furthermore, it was reported that bolus administration of recombinant proteins is insufficient to maintain their therapeutic levels in the tumor mass (7, 8). One potential solution for this is gene therapy. In gene therapy, vectors expressing antiangiogenic genes are used to produce antiangiogenic proteins within the cells themselves (9, 10). Preclinical data are extremely promising and many clinical trials are under way (2).

Hepatocyte growth factor (HGF) is a multifunctional growth factor that regulates both cell growth and cell motility and is often overexpressed in colorectal carcinoma (11). The α-chain of HGF composes of five domains: the N-terminal domain (N-domain) and four kringle domains (12). It has been shown that the hairpin loop and the first kringle domain (K1) are indispensable for receptor binding (13). A recombinant protein comprising the hairpin loop domain and the four kringle domains (HGF/NK4) has been shown not only to be a specific antagonist of HGF but also interferes with other proangiogenic signals, such as basic fibroblast growth factor and vascular endothelial growth factor (14–16). NK2, which has hairpin loop, K1 and K2, is also sufficient for antagonizing HGF and proangiogenic signals (17). Recently, a recombinant protein containing the HGF α-chain kringle 1 domain HGFK1 has been shown to have antiangiogenic activity (18). HGFK1 is the smallest HGF α-chain variant displaying antiangiogenic activity thus far. Therefore, it is very suitable to be carried by adeno-associated virus (AAV)
in a gene therapy system. Recently, we reported that AAV-expressing HGFK1 (AAV-HGFK1) has antiangiogenic and antitumor cell effects on hepatocellular carcinoma (19). In endothelial cells, AAV-HGFK1 inhibits the activation of epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor, and basic fibroblast growth factor receptor but not Met (19).

The tumor suppressor protein p53 is often mutated in cancers. The deletion of the p53 gene leads to increased aggressiveness of cancers, whereas tumor chemoresistance and radioresistance are conferred by the expression of mutant p53 proteins (20). Gene transfer cancer therapy of p53 has shown promising potential in preclinical studies (21). For instance, wild-type p53 gene transfer inhibited cell growth not only in cancer cell lines with deleted p53 gene but also in those with mutated p53 (22). Adenovirus-mediated p53 gene transfer sensitized colorectal cancer cells to ionizing radiation (23). Moreover, Ad-p53 induced apoptosis and inhibited the growth of tumors in a syngeneic rat model of colorectal cancer (24). Meanwhile, Ad-p53 has been approved by the State Food and Drug Administration of China for its clinical use in the treatment of cancer.

We have recently described a simple and feasible strategy using a combined AAV-BMP2 and Ad-BMP2 vector system to increase in vivo bone induction. Our results clearly showed that low-level Ad-BMP2 can greatly enhance AAV-BMP2-mediated osteoinductive activity without provoking an undesired immune response. Here, we tried to adopt a similar strategy to enhance the effectiveness of AAV-HGFK1 by administering the virus together with Ad-p53 and test the virus treatments in colorectal carcinoma models.

Materials and Methods

Cell Culture

CT26 (colon carcinoma cells derived from BALB/c mice), Lovo (human colon carcinoma cells), MILE SVEN 1 (MS1; mouse pancreatic islet endothelial cells transduced with a SV40 large T antigen (tsA-58-3)), and SV10 (SVEC4-10EE2 tumorigenic mouse endothelial cell transformed with SV40) were obtained from the American Type Culture Collection.

Preparation of Viruses

Plasmids pAAV-CAG-sec-EGFP and pAAV-CAG-sec-HGFK1 were constructed by inserting the enhanced green fluorescent protein (EGFP) and human HGFK1 cDNAs into AAV serotype 2 vector. Recombinant AAV (rAAV) particles were produced by a helper virus-free system as previously described (25) with minor modifications. rAAV vectors and helper plasmid pD2 were cotransfected into HEK 293-FT cells (American Type Culture Collection) by calcium phosphate precipitation method. Transfected cells were harvested in Tris-Cl (pH 8.0) buffer 60 h later. After two cycles of freezing/thawing, the cells were centrifuged at 12,000 rpm for 20 min. The supernatant fraction containing rAAV-HGFK1 or rAAV-EGFP particles was collected. The rAAV particles were then purified by HiTrap heparin column chromatography (Sigma). Peak virus fractions were collected and dialyzed against PBS containing 1 mmol/L MgSO4. Samples were then concentrated using a 100K-MicroSep centrifugal concentrator (Life Technologies). Viral titer was quantified by real-time PCR using the Taqman Universal PCR kit (Applied Biosystems), with the forward primer 5′-CGCGTGGTGGCCTGA-3′ and the reverse primer 5′-CCGAGGGGACAAGCA-GAAC-3′. Aliquots of viral stocks [2 × 1012 viral genomes (vg)/mL] were stored at −80°C until ready for use.

For construction of recombinant adenovirus vectors, cDNA encoding wild-type p53 was first cloned into an adenovirus expression vector pAd/CMV/V5-DEST (Invitrogen). The recombinant adenoviruses, Ad-p53 and Adv-null, were produced by transfecting 293A cells with the pAd/CMV/V5-DEST containing p53 or not using Lipofectamine 2000 reagent (Invitrogen). The viruses were then desalted, concentrated through cesium chloride gradients, and purified. The purified viruses were stored at −80°C in Tris solution [10 mmol/L Tris (pH 8.0), 2 mmol/L MgCl2, 4% sucrose]. Viral titer [plaque-forming unit (PFU)] was determined using tissue culture infectious dose 50 method. Viral particles (vp) were assayed by the absorbance of disrupted virions at 260 nm compared with a traceable standard (AdEasy Vector System Application Manual, Qbiogene). The results showed that 1 PFU of Ad-p53 prepared was equal to ~48 vp.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

CT26, Lovo, SV10, or MS1 cells were transfected by the viruses as described above and incubated for 48 h. Colon cancer cells or endothelial cells were incubated for 24 h in 100 μL medium without fetal bovine serum or with 0.2% fetal bovine serum. The cells were seeded at a density of 1 × 103 per well in a 96-well plate for 12 h and incubated in 100 μL medium with or without HGF or EGF for an additional 72 h. To plot a standard curve, the corresponding cells were seeded at different densities in 100 μL of medium per well in a 96-well plate at the end of the incubation period of experimental cells. Finally, 1 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added and the plates were incubated in the dark for 4 h. Water-insoluble MTT formazan crystals were dissolved in 150 μL DMSO, and reduction of MTT was determined at 570 nm with an ELISA reader. Each condition was done in triplicate and the data were from at least three independent experiments.

Western Blot

Cells were seeded at a density of 2 × 105 per well in six-well plates and allowed to adhere overnight. After adhesion, cells were treated with AAV-EGFP+Adv, AAV-EGFP [multiplicity of infection (MOI), 2 × 103] + Ad-p53 (MOI, 10), AAV-HGFK1 (MOI, 2 × 105) + Adv (MOI, 10), or AAV-HGFK1 (MOI, 2 × 103) + Ad-p53 (MOI, 10), incubated for 48 h in fetal bovine serum–free DMEM, and stimulated with different concentrations of HGF or EGF in DMEM for an additional 24 h. At the end of incubation, total proteins were extracted for Western blot analysis. For Western blot, samples were homogenized in a buffer containing 10 mmol/L HEPES, 10 mmol/L KCl, 1 mmol/L MgSO4, Samples were then concentrated using a 100K-MicroSep centrifugal concentrator (Life Technologies). Viral titer was quantified by real-time PCR using the Taqman Universal PCR kit (Applied Biosystems), with the forward primer 5′-CGCGTGGTGGCCTGA-3′ and the reverse primer 5′-CCGAGGGGACAAGCA-GAAC-3′. Aliquots of viral stocks [2 × 1012 viral genomes (vg)/mL] were stored at −80°C until ready for use.

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1.5 mmol/L MgCl₂, 0.5 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin at pH 7.9. Protein extracts were then subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with primary and secondary antibodies, respectively, for 1 h at room temperature. The primary antibodies against EGFR, phosphorylated EGFR, c-Met (HGF receptor), phosphorylated c-Met, and actin were purchased from Santa Cruz Biotechnology. Signals were developed with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Inc.).

Murine Models of Colorectal Carcinomas

Eight-week-old female BALB/c and nude mice, obtained from the Animal Unit of the University of Hong Kong, were acclimated for 1 wk while caged in groups (five each). The mice had free access to a standard animal food and water throughout the experiment. All animal studies were conducted under guidelines approved by the Animal Care and Use Committee of the University of Hong Kong.

CT26 cells were injected into the spleens of 24 BALB/c mice. The mice were randomly assigned to four groups (six each): (a) AAV-EGFP control (1.5 × 10¹¹ vg per mouse), (b) AAV-HGFK1 (1.5 × 10¹¹ vg per mouse), (c) Ad-p53 (2.5 × 10⁹ vp per mouse), and (d) AAV-HGFK1+Ad-p53 (1.5 × 10¹¹ vg + 2.5 × 10⁹ vp per mouse). Similar arrangement was applied to the nude mice model, with Lovo injected into 24 nude mice. Viruses were injected through the tail vein 1 d after injection of CT26 or Lovo cells.

Figure 1. Ad-p53 enhanced the expression of AAV transgenes in vivo and in vitro. A, the indicated viruses were injected into tail veins of BALB/c mice. The mice were sacrificed 20 d after injection and their pancreas and livers were immunohistochemically examined for EGFP expression. Scale bars, 25 μm (in pancreas) and 50 μm (in liver). B, CT26 and Lovo cells were transduced with the indicated viruses and the percentage of cell expressing EGFP was measured at 0-, 4-, and 7-d time points. C and D, Lovo cells were seeded into 96-well plate (1,000 per well) and infected with the indicated viruses in serum-free medium (MOI: AAV, 10⁴ vg per cell; Adv, 10 PFU per cell). The cells were cultured in medium with 2% fetal bovine serum for 5 d before switching to serum-free medium for 8 h. The supernatant was then analyzed by Western blotting. E, BALB/c mice were injected with AAV-HGFK1 alone or with Ad-p53 and their liver vessels were examined immunohistochemically for HGFK1. Scale bar, 100 μm.
into the spleen. The mice were observed daily for 90 d. The survival time of each mouse was recorded.

**Liver Metastasis in CT26-BALB/c Model**

Forty BALB/c mice were randomly assigned to four groups (10 mice each group) as above. One day after injection of CT26 cells into the spleen, viruses were injected through the tail vein. On days 12 and 20 after inoculation, five mice per group were sacrificed with their livers and spleens excised, weighted, and preserved in 10% formaldehyde-PBS for paraffin embedding.

**Immunohistochemistry**

Liver or spleen tissues were fixed in 10% buffered formalin and embedded in paraffin and cut into 5-μm-thick sections for histologic studies. The sections were stained with H&E or an anti-EGFP antibody (1:100) to observe the efficacy of AAV infection in vivo.

For assessing angiogenesis, liver tissue sections were stained with a goat anti-human HGFK1 polyclonal antibody (1:1,000) and a rabbit anti-human CD31 polyclonal antibody (1:100) and counterstained with hematoxylin.

**Detection and Quantification of Apoptotic Cells**

Apoptosis was detected by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay with an in situ cell death detection kit (Roche Molecular Biochemicals) following the manufacturer’s instructions. Quantification of apoptotic cells was expressed as a mean of the ratio of apoptotic cells to the total number of tumor cells in 10 random fields containing distinct TUNEL-positive cells at ×200 magnification. Morphologically necrotic areas were excluded from all analyses.

**Wound-Healing Assay**

Three days after infection of MS1 or SV10 cells with the virus, confluent cells grown in six-well plates were cultured in 2% fetal bovine serum DMEM. The monolayers were mechanically wounded to obtain a denuded area of 1 mm wide and observed under a 100× microscope 20 h after incubation.

**Statistical Analysis**

Comparisons of numbers of endothelial cells and numbers of apoptotic cells were carried out using the two-tailed Student’s t test application of MS Excel. Animal survival was analyzed by log-rank test using the GraphPad Prism software (GraphPad Software, Inc.). A P value of < 0.05 was considered statistically significant. Error bar denotes SE unless specified otherwise.

**Results**

**Adenovirus Enhanced Transduction Efficiency of AAV**

To verify that coadministering low level of adenovirus with AAV enhances the expression of the AAV transgene, we tested the transduction efficiency of AAV-EGFP administered into BALB/c mice through tail vein, with or without a small amount of Ad-p53. Injected mice were sacrificed 20 days after injection and the expression of EGFP was examined in the livers and pancreas of the mice.

As shown in Fig. 1A, control uninfected mice or mice infected with Ad-p53 alone did not express EGFP in their livers and pancreas. Although there was strong EGFP expression in AAV-EGFP–infected group, we observed that Ad-p53 could enhance the expression of EGFP in the pancreas and liver of infected mice (Fig. 1A). We also tested the transduction efficiency of AAV-EGFP in two colon cancer cell lines, CT26 and Lovo. We infected the two cell lines with two dosages of AAV-EGFP (1,000 vp per cell and 10,000 vg per cell) in the presence or absence of a small amount of Ad-null (10 PFU per cell). We found that at both AAV dosage, the addition of Ad-null could enhance EGFP expression in both cell lines (Fig. 1B). The expression enhancement was most obvious when Lovo cells were transduced with 10,000 vg per cell AAV-EGFP; thus, the addition of Ad-p53 increased the EGFP expression by 8- to 10-fold. Similar expression enhancement was also observed when Lovo cells were transduced with AAV-HGFK1+Ad-null (Fig. 1C) or AAV-HGFK1+Ad-p53 (Fig. 1D). Moreover, both Ad-EGFP and Ad-p53 were able to enhance the expression of HGFK1, suggesting that the enhancement was not due to the functioning of p53 but instead depended on the presence of adenovirus. When AAV-HGFK1 was injected into mice, we found that the expression of HGFK1 in the liver concentrated in vascular epithelia (Fig. 1E), and in vivo, HGFK1 expression could be enhanced by Ad-p53 (Fig. 1E).

**AAV-HGFK1+Ad-p53 Treatments Prolonged Survival of Murine Models of Colorectal Carcinoma**

We have shown that AAV-HGFK1 is an effective cancer gene therapy for hepatocellular carcinoma (19). To test the effectiveness of AAV-HGFK1 and AAV-HGFK1+Ad-p53
viral cocktail treatment in colorectal carcinoma, we used two mouse colon cancer models. Murine colorectal carcinoma cells CT26 were injected into the spleens of BALB/c mice. Similarly, Lovo cells (human colon carcinoma cells) were injected into the spleens of nude mice. Lovo was chosen because it is one of the most metastatic human colon carcinoma cell lines, and because adenovirus is immunogenic, we used the CT26-BALB/c model as an immunocompetent model to assess the feasibility of AAV-Ad cocktail. The mice were injected with one of the following virus treatments through tail vein 1 day after inoculation of tumor cells: (a) AAV-EGFP control, (b) AAV-HGFK1, (c) AAV-EGFP+Ad-p53, or (d) AAV-HGFK1+Ad-p53. The virus treatment was administered 1 day after tumor inoculation because the expression of AAV transgenes does not begin until 1 week after infection. At that time, the tumor has already grown and established. Mice inoculated with CT26 or Lovo cells developed primary tumor at spleen and metastasis at liver (Supplementary Fig. S1). A control (AAV-EGFP treated) mice succumbed to tumor burden quickly, with median survival of 21.5 days (CT26) and 33 days (Lovo; Fig. 2A and B; Table 1). AAV-HGFK1 or Ad-p53 treatments could mildly prolong the survival of the mice (Fig. 2A and B; Table 1). The median survival for AAV-HGFK1–treated mice was 27 days (CT26) and 36.5 days (Lovo; both \( P < 0.05 \), compare with AAV-EGFP, log-rank test). The median survival for Ad-p53–treated mice was 27.5 days (CT26) and 39.5 days (Lovo; both \( P < 0.05 \), compare with AAV-EGFP, log-rank test). Interestingly, we found that AAV-HGFK1+Ad-p53 viral cocktail could remarkably prolong survival of the mice (Fig. 2A and B). The median survival of AAV-HGFK1+Ad-p53–treated mice was 63 days (CT26) and 60.5 days (Lovo; both \( P < 0.01 \), compare with AAV-EGFP, log-rank test). Furthermore, one mouse inoculated with CT26 and two mice inoculated with Lovo in the AAV-HGFK1+Ad-p53 group survived through the window of observation (90+ days) and did not develop tumors in their livers and spleens (Table 1). Our data indicated that AAV-HGFK1+Ad-p53 viral cocktail is a potent antitumor agent.

### Table 1. Survival time and tumor growth of mice injected with Lovo or CT26 cells inoculated at their spleens

<table>
<thead>
<tr>
<th>Strain of mice</th>
<th>Cell injected</th>
<th>Treatment</th>
<th>Days of survival</th>
<th>Median survival time (d)</th>
<th>% of mice with liver metastasis</th>
<th>% of mice with spleen tumor</th>
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</thead>
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<tr>
<td>BALB/c</td>
<td>Lovo</td>
<td>AAV-EGFP</td>
<td>21, 30, 33, 34, 37</td>
<td>33</td>
<td>4/6 (66.7%)</td>
<td>6/6 (100%)</td>
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<td></td>
<td></td>
<td>AAV-HGFK1</td>
<td>33, 34, 36, 37, 43, 50</td>
<td>36.5</td>
<td>4/6 (66.7%)</td>
<td>6/6 (100%)</td>
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<td></td>
<td></td>
<td>AAV-EGFP+Ad-p53</td>
<td>31, 35, 36, 43, 46, 51</td>
<td>39.5</td>
<td>3/6 (50%)</td>
<td>6/6 (100%)</td>
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<td></td>
<td></td>
<td>AAV-HGFK1+Ad-p53</td>
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<td>2/6 (33.3%)</td>
<td>4/6 (66.7%)</td>
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<td>Nude mice</td>
<td>CT26</td>
<td>AAV-EGFP</td>
<td>20, 20, 21, 22, 25</td>
<td>21.5</td>
<td>6/6 (100%)</td>
<td>6/6 (100%)</td>
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<td></td>
<td>AAV-HGFK1</td>
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<td>6/6 (100%)</td>
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<td>27.5</td>
<td>6/6 (100%)</td>
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<td>63</td>
<td>5/6 (83.3%)</td>
<td>5/6 (100%)</td>
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</table>

AAV-HGFK1 and Ad-p53 Viral Cocktail Inhibited Primary Tumor and Metastasis Growth and Induced Cell Death

To investigate the effects of the viral treatments on the growth of primary tumor and liver metastasis, we examined the weights of the spleens and livers of CT26-inoculated mice at days 12 and 20 after injection of tumor cells. The weights of spleens and livers of mice from the different treatment groups did not differ significantly at day 12 (Fig. 3A). But at day 20, the weights of spleens and livers from AAV-HGFK1+Ad-p53 viral cocktail treatment group were significantly \( (P < 0.05) \) lower than AAV-EGFP control group (Fig. 3A). This suggested that the growth of tumor at primary and secondary site of AAV-HGFK1+Ad-p53 group was slowed.

When the tumors in CT26-injected mice were examined histochemically, it was found that the AAV-HGFK1+Ad-p53 combinatorial treatment group induced large area of tumor necrosis in primary tumors and liver metastases (Fig. 3B, arrows). Furthermore, TUNEL staining revealed that whereas AAV-HGFK1 or Ad-p53 alone could mildly induce apoptosis in the tumors (Fig. 3C and D), AAV-HGFK1+Ad-p53 combinatorial treatment caused much more apoptosis in the tumors (Fig. 3C and D). Taken together, AAV-HGFK1 and Ad-p53 exhibited mild therapeutic effect on their own, but when administered together, they synergistically prolonged survival, reduced tumor growth, and induced tumor cell death.

AAV-HGFK1 and Ad-p53 Viral Cocktail Inhibited Tumor Angiogenesis and Endothelial Cell Migration

Because HGFK1 has antiangiogenic activity \textit{in vitro} (18), we asked whether AAV-HGFK1 and AAV-HGFK1+Ad-p53 can inhibit tumor angiogenesis \textit{in vivo}. We immunohistochemically stained the liver tumor tissues from day 20 CT26-injected BALB/c mice for CD31, an endothelial cell marker. It was found that tumors in AAV-EGFP, AAV-EGFP+Ad-p53, and AAV-HGFK1 treatment groups were heavily infiltrated with endothelial cells (Fig. 4A and B). On the other hand, AAV-EGFP+Ad-p53–treated tumor contained very limited number of endothelial cells (Fig. 4A and B). To exclude the possibility that the viral cocktail may down-regulate CD31 expression instead of inhibiting angiogenesis, we stained the tissue for another endothelial...
marker vWF. We found that the relative abundance of vWF-positive cells across the treatment groups was similar to CD31-positive cells (Fig. 4A and B). This indicated that AAV-EGFP+Ad-p53 treatment could inhibit tumor angiogenesis.

To shed light on the mechanism of angiogenesis inhibition by AAV-EGFP+Ad-p53, we investigated the effect of AAV-EGFP+Ad-p53 on cell migration in two endothelial cell culture system. MS1 is a mouse pancreatic islet endothelial cell line transduced with a SV40 large T antigen. SV10 is a tumorigenic endothelial cell line. The cells were transduced with (a) AAV-EGFP+Ad-EGFP, (b) AAV-HGFK1+Ad-EGFP, (c) AAV-EGFP+Ad-p53, or (d) AAV-HGFK1+Ad-p53. The relative ability of cell migration was determined by wound-healing assay. In both MS1 and SV10 systems, the wounds in AAV-EGFP+Ad-EGFP group

Figure 3. AAV-HGFK1+Ad-p53 inhibited primary and secondary tumor growth and induced cell death. A, BALB/c mice inoculated with CT26 at their spleens were sacrificed at day 12 or day 20 and the weights of their spleens and livers were recorded. n = 5. *, P < 0.05, ANOVA (and Student-Newman-Keuls) test compared with AAV-EGFP. B, H&E staining of tumors in spleens and livers of CT26-inoculated mice. Scale bar, 50 μm. Arrows, necrotic areas. C, TUNEL staining of liver metastases at 20 d after CT26 inoculation. Arrows, apoptotic cells. D, percentage of apoptotic cells per field in various treatment groups at days 12 and 20 after tumor cell injection. *, P < 0.05; **, P < 0.01, ANOVA (and Student-Newman-Keuls) test compared with AAV-EGFP.
nearly completed healing in 20 h (Fig. 4C). AAV-HGFK1+Ad-p53 cells migrated the slowest (Fig. 4C). The speeds of wound healing of AAV-HGFK1+Ad-EGFP and AAV-EGFP+Ad-p53 groups were intermediate between AAV-EGFP+Ad-EGFP and AAV-HGFK1+Ad-p53 (Fig. 4C). These findings suggested that Ad-p53 or AAV-HGFK1 has an inhibitory effect on endothelial cell migration.

Because proliferation of the endothelial cells could have contributed to the closing of the wounds, we asked whether the virus treatments had any effect on the proliferation of the endothelial cells. We used MTT assay to monitor the growth of SV10 and MS1 cells in culture with or without EGF or HGF, two known growth factors for endothelial cells. In the absence of EGF or HGF, the growth rates of SV10 and MS1 cells were insignificantly affected by the virus treatment (Fig. 5A). So, the extended time for wound closure observed in AAV-HGFK1+Ad-EGFP–treated, AAV-EGFP+Ad-p53–treated, and AAV-HGFK1+Ad-p53–treated SV10 and MS1 cells was not due to a retardation of cell proliferation.

Proliferation of SV10 cells but not MS1 cells increased in response to increasing doses of EGF or HGF (Fig. 5A, AAV-EGFP+Ad-EGFP control). This increase in proliferation was completely negated by treatments of AAV-HGFK1+Ad-EGFP or AAV-HGFK1+Ad-p53 (Fig. 5A). When the activation statuses of HGF receptor c-Met and EGFR in SV10 cells were checked, it was clear that AAV-HGFK1 and AAV-HGFK1+Ad-p53 strongly inhibited the phosphorylation of EGFR but not c-Met (Fig. 5B).

We found that MS1 cells did not show any proliferation response to either EGF or HGF (Fig. 5A). Virus treatments did not alter the response of the cells toward HGF (Fig. 5A),...
but if the cells had been treated with EGF, AAV-HGFK1+Ad-p53 combinatorial treatment significantly reduced the proliferation rate (Fig. 5A). This may be due to the fact that AAV-HGFK1+Ad-p53 could strongly inhibit EGFR phosphorylation in MS1 cells (Fig. 5B). The unresponsiveness of MS1 cells toward HGF could be attributed to the low expression level of HGF receptor in the cells (Fig. 5B).

AAV-HGFK1 and Ad-p53 Treatments Inhibited Tumor Cell Proliferation

Recently, we presented evidence suggesting that AAV-HGFK1 directly suppressed hepatocellular carcinoma cell proliferation in addition to inhibiting tumor angiogenesis (19). Here, we investigated the effect of AAV-HGFK1/Ad-p53 virus treatments on the proliferation of colorectal carcinoma cell lines CT26 and Lovo in response to HGF and EGF. CT26 proliferation was unresponsive to HGF (Fig. 6A). This unresponsiveness may be due to the fact that the expression of Met in CT26 was undetectable (Fig. 6B), and we did not observe any significant effect on CT26 proliferation by AAV-EGFP+Ad-EGFP, AAV-EGFP+Ad-p53, AAV-HGFK1+Ad-EGFP, or AAV-HGFK1+Ad-p53 treatment (Fig. 6A). CT26 is also mildly responsive to EGF; thus, treatment of increasing concentration of EGF led to mild increase of growth ratio (Fig. 6A). AAV-EGFP+Ad-EGFP, AAV-EGFP+Ad-p53, and AAV-HGFK1+Ad-EGFP could all inhibit this increase of proliferation.

Proliferation of Lovo cells, on the other hand, increased remarkably in response to increasing dose of HGF or EGF (Fig. 6A). These increases were inhibited by AAV-EGFP+Ad-p53, AAV-HGFK1+Ad-EGFP, or AAV-HGFK1+Ad-p53 treatment. We observed a mild decrease in phosphorylation of Met and a strong inhibition of phosphorylation of EGFR in Lovo cells treated with AAV-HGFK1+Ad-EGFP or AAV-HGFK1+Ad-p53 (Fig. 6B), suggesting that HGFK1 may inhibit Lovo cell proliferation partially through acting as a HGF antagonist.

Discussion

Antiangiogenic gene therapy for colon cancer has been widely studied with certain exciting results in animal trials. In one study, two groups of mice received intrasplenic injection of CT26 cells expressing apolipoprotein(a) kringle LK68. The median survival period of the mice was 24.5 days (26). In our study, the cocktail treatment with AAV-HGFK1 in combination with Ad-p53 prolonged the median survival to ~60 days. Moreover, a few of our mice were healthy and tumor-free even 110 days after tumor cell inoculation. Therefore, our study represents a significant improvement of AAV tumor gene therapy.

It has been shown that HGFK1 can inhibit proliferation of bovine aortic endothelial cells and HGFK1 is a selective inhibitor for bovine aortic endothelial cell proliferation.

Figure 5. AAV-HGFK1 + Ad-p53 inhibited proliferation of endothelial cells in response to EGF and HGF. A, proliferation of MS1 and SV10 cells was assessed by MTT assay. SV10 or MS1 cells were infected by the indicated viruses and incubated for 48 h. The cells were then serum starved for 24 h before being seeded at a density of $1 \times 10^5$ per well in a 96-well plate and incubated in medium with or without HGF or EGF for an additional 72 h. Growth ratio was calculated as the ratio of the absorbance measured at 12 h after seeding to the absorbance measured after 72 h of HGF or EGF incubation. Columns, mean (n = 6); bars, SE. *, P < 0.05, ANOVA (and Student-Newman-Keuls) test, compared with AAV-EGFP+Ad-EGFP. MOI: AAV, $10^5$ vg per cell + Adv, $10^8$ vp per cell. B, phosphorylation status of Met receptor and EGFR in cell lines treated with AAV-HGFK1, Ad-p53, or AAV-HGFK1+Ad-p53 was monitored by Western blotting.
stimulated by bovine FGF (18). In addition, AAV-HGFK1 could transfect mouse endothelial cell lines and human carcinoma cell lines to express HGFK1 and inhibit cell growth by EGF or HGF stimulation (Fig. 3). One of the possible mechanisms behind this is that HGFK1 binds to the c-Met receptor without activating it, thereby inhibiting the c-Met receptor (15). HGF did not stimulate CT26 cell growth in vitro (Fig. 4), indicating that perhaps c-Met overexpression in cancer cells is not necessary for HGFK1 gene therapy. Similarly, HGF/NK4 may have antitumor activities not only by antagonizing HGF but also by inhibiting HGF amplification via tumor-stromal interactions. Because NK4 induced at a CT26 tumor site by gene transfer shows multiple antitumor activities with potential therapeutic benefit (27), we hypothesized that HGFK1 has multiple antitumor activities as well.

p53 is one of the genes that determine apoptotic incidence in colon carcinoma (28). In our study, although overexpression of wild-type p53 mediated by a low dose of Ad-p53 had no significant effect on the proliferation of wild-type cells in vitro, wild-type p53 gene therapy for malignant carcinomas revealed its in vivo effect. Other than the promotion of apoptosis, a possible mechanism could be that p53 down-regulates the expression of other angiogenesis-promoting growth factors such as vascular endothelial growth factor (29, 30). Dual inhibitory effect of EGFR and vascular endothelial growth factor receptor signaling pathways in tumor cells and tumor-associated endothelial cells has been shown to provide a new approach to colon cancer treatment (31). The effect of AAV-HGFK1+Ad-p53 cocktail on various growth factors and their receptors implicated in angiogenesis warrants further investigations.

Both AAV and Adv have their own advantages over the other. The AAV vector is able to maintain high levels of therapeutic molecules in situ after a few injections, AAV carrying the transgene is expressed for at least 100 days, whereas Adv carrying the transgene is expressed in vivo for 14 days (32). AAV also has a low immunogenicity and toxicity (33), thus causing a mild increase in endothelial VCAM-1 expression and proliferation of vascular cells, whereas Adv causes not only extensive VCAM-1 expression and vascular cell proliferation but also inflammatory cell infiltration and morphologic damages (32). On the other hand, the Adv vector carrying transgenes can be strongly expressed 24 h after treatment, whereas AAV-mediated expression can be delayed for ~4 days. Furthermore, Adv vectors have a broad tropism and high transduction efficiency, transduce both quiescent and dividing cells, and are easy to produce in high-concentration viral stocks. We therefore propose that treatment with AAV plus one injection of Adv can prolong the expression time and immune effect.
It was reported that treatment with AAV and Adv could increase 100- to 1,000-fold AAV transduction efficiency (34). The mechanism behind this dramatic increase has been widely studied. A previous study showed that this enhancement of adenovirus for in vitro AAV transduction is neither dependent on AAV gene cassette nor facilitated by adenovirus-mediated viral uptake but is dependent on adenovirus gene expression (35). Another study reported that the early-region E4 open reading frame 6 is involved in the second-strand synthesis of the AAV life cycle. This expression of adenovirus genes E1 and E4 can sufficiently enhance AAV transduction (34, 36).

Introduction of a low level of adenovirus in vitro can greatly enhance AAV-mediated gene transfer without severe immune responses (37). Therefore, in this study, we used one injection of the therapeutic dose of AAV-HGF1 to treat colon carcinoma of immunocompetent and nude mice. The therapeutic effect of AAV-HGF1 was significantly enhanced due to the fact that Adv increased the efficacy of AAV-HGF1 in vitro. If AAV is used alone in clinic, it is too expensive to treat most patients. The cocktail therapy system can significantly decrease the cost while obtaining the same beneficial therapeutic effect.

In conclusion, adding Adv into AAV can increase the efficacy of treatment with AAV for human and mouse colon cancer cell lines in vitro. More importantly, the therapeutic effect of combined treatment with AAV-HGF1 and Ad-p53 is significantly higher than AAV-HGF1 or Ad-p53 alone treatment. AAV plus Adv cocktail gene therapy is a novel treatment for colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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