Antibody-Mediated p53 Protein Therapy Prevents Liver Metastasis In vivo


Abstract

To evaluate the clinical efficacy of monoclonal antibody (mAb) 3E10 Fv antibody-mediated p53 protein therapy, an Fv-p53 fusion protein produced in Pichia pastoris was tested on CT26.CL25 colon cancer cells in vitro and in vivo in a mouse model of colon cancer metastasis to the liver. In vitro experiments showed killing of CT26.CL25 cells by Fv-p53 but not Fv or p53 alone, and immunohistochemical staining confirmed that Fv was required for transport of p53 into cells. Prevention of liver metastasis in vivo was tested by splenic injection of 100 nmol/L Fv-p53 10 min and 1 week after injection of CT26.CL25 cancer cells into the portal vein of BALB/c mice. Mice were sacrificed 1 week after the second injection of Fv-p53 and assigned a quantitative metastasis score. Control mice had an average metastasis score of 3.3 ± 1.3, whereas mice treated with Fv-p53 had an average metastasis score of 0.8 ± 0.4 (P = 0.004). These results indicate that Fv-p53 treatment had a profound effect on liver metastasis and represent the first demonstration of effective full-length p33 protein therapy in vivo. mAb 3E10 Fv has significant clinical potential as a mediator of intracellular and intranuclear delivery of p53 for prevention and treatment of cancer metastasis. [Cancer Res 2007;67(4):1769–74]

Introduction

Often referred to as the guardian of the genome, p53 plays a critical role in tumor suppression. Defects in p53 are linked to >50% of human cancers, and numerous studies have shown that restoring p53 function to p53-deficient cancer cells induces growth arrest and apoptosis (1, 2). Developing a method to safely and efficiently restore p53 activity to tumor cells in vivo has become a key goal in cancer research.

Missing or defective cellular proteins, such as p53 in many cancer cells, may be replaced via gene therapy or protein therapy. Gene therapy relies on the capacity of a cell to synthesize protein by using information encoded on exogenously provided DNA. Numerous viral and nonviral DNA delivery vectors have been tested, and p53 gene therapy has met with varying degrees of success both in vitro and in vivo (2). The primary factors limiting gene therapy at present include concerns over potential vector toxicity and immunogenicity, inefficient delivery of genes to cells, and relative instability of the transgene resulting in limited expression (3). As a potential alternative to gene therapy, protein therapy involves direct delivery of protein to the cells.

The discovery and development of protein transduction domains (PTD), peptides or fragments of proteins that carry cargo proteins into cells in an apparently receptor-independent manner, has facilitated a significant expansion in protein therapy. PTDs, such as the HIV Tat peptide, polyarginine peptides, and Fv [the single-chain fragment of autoantibody monoclonal antibody (mAb) 3E10], have delivered functional p53 to cancer cells in vitro (4–6). However, there have been no reports of successful full-length p53 protein therapy in vivo.

Fv possesses molecular characteristics that distinguish it as a unique PTD well suited to p53 protein therapy. First, Fv selectively localizes to cell nuclei. Induction of cell cycle arrest and apoptosis by p53 depends in part on its activation of target genes in the nucleus. Fv-mediated intranuclear delivery of p53 correctly positions p53 within the cell for transactivation of target genes. Second, given that Fv was originally derived from an antibody, Fv should not generate significant inflammation in vivo (7, 8). Inflammation or specific immune responses would hinder the therapeutic efficacy of p53 protein therapy. Whereas highly basic PTDs may generate significant inflammation on administration in vivo (9–12), Fv as a delivery vehicle for p53 protein therapy should bypass this potential obstacle.

We have previously shown that Fv-p53 selectively kills cancer cells and has no effect on nontransformed primary cells in vitro. Moreover, Fv-p53 effectively induces cell death in cancer cells with a variety of defects in p53, including absence of p53, mutations in p53, nuclear exclusion of p53, and overexpression of MDM2 (6). In the present study, we have extended the in vitro experiments to include additional control proteins to verify that Fv-p53 is the factor responsible for cell killing in cancer cell lines. Furthermore, we have tested Fv-p53 protein therapy in vivo and found it strikingly effective in preventing metastasis of colon carcinoma cells to the liver.

Materials and Methods

Plasmids

pPICZA-Fv-p53, cDNA encoding an Fv-p53 fusion protein was ligated into pPICZA as described previously (6).

pPICZA-Fv(R95Q)-p53. The pPICZA-Fv(R95Q)-p53 construct was generated by site-directed mutagenesis of the pPICZA-Fv-p53 construct using the QuikChange kit (Stratagene, La Jolla, CA) with mutagenesis primers 5’-CATGTAT- CATAGTCAGTCTAACCTGGCTTGCACAG-3’ and 5’-CATGTAT- TACGTGCAGGGTTACTACTT-3’.
pPICZA-p53. cDNA encoding wild-type p53 was PCR amplified from the pPICZa-Fv-p53 construct using sense primer 5'-GAATTCCATGATCATCAGTACATCATCATCAGGAGGGCACTGAC-3' and antisense primer 5'-CTCGAGTGTAAAGTCGTAGCAGGGCC-3'. The PCR product was inserted into the pCR2.1 vector with use of the TA Cloning kit (Invitrogen, Carlsbad, CA). The p53 cDNA insert was liberated from pCR2.1-p53 by digestion with EcoRI and XhoI and ligated into EcoRI and XhoI sites in pPICZa.

Recombinant Proteins
Fv-p53, Fv(R95Q)-p53, wild-type p53, Fv, and X-33 control proteins were produced in and purified from Pichia pastoris and analyzed by SDS-PAGE followed by Western blot analysis as described previously (6). Typical yields of Fv-p53 and Fv(R95Q)-p53 were 30 μg from a 500 mL culture. Typical yields of wild-type p53 and Fv were 3 mg from a 500 mL culture. Concentrations of Fv-p53 were determined by an ELISA capture assay with anti-p53 antibodies and comparison with a standard curve.

Cell Lines
Skov-3 ovarian cancer and CT26.CL25 colon cancer cell lines were acquired from the American Type Culture Collection (Rockville, MD).

Nuclear Penetration Assay
Fv-p53, Fv(R95Q)-p53, or wild-type p53 (100 nmol/L) was applied to Skov-3 cells. As a positive control, 100 μmol/L Fv was also applied to the cells. After 1 h of incubation, cells were washed, fixed, and stained with anti-p53 antibodies (Fv contains a myc tag). Ponceau S stain provides an approximation of protein purity.

Microscopic Images
An Olympus IX70 inverted microscope with RC reflected light fluorescent attachment and MagnaFire SP Digital Imaging System (Olympus, Melville, NY) was used to acquire microscopic images of cells as described previously (14).

In vitro Cytotoxicity Assay
Fv-p53, Fv(R95Q)-p53, wild-type p53, or Fv (100 nmol/L) was applied to Skov-3 and CT26.CL25 cells. Control cells were incubated with X-33 yeast protein controls. Twenty-four hours after addition of proteins to the cells, Skov-3 and CT26.CL25 cells. Control cells were incubated with X-33 yeast protein controls. Twenty-four hours after addition of proteins to the cells, proteins were generated and purified from P. pastoris as described previously (6).

In vivo Liver Metastasis Model
A "hemispleen" model, as first described by Schulick et al. (15), was optimized. BALB/c mice at 10 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME). The fur on the left flank was removed using clippers. The animals were anesthetized using halothane, and the surgery. The animal was anesthetized, and the left flank was prepped with povidone iodine. A small portion of the incision was opened, and the surgical area was prepped with povidone iodine. The surgery. The animal was anesthetized, and the left flank was prepped with povidone iodine. A small portion of the incision was opened, and the surgical area was prepped with povidone iodine. A 1.0-cm to 1.5-cm incision was placed across the vascular pedicle and the inferior hemispleen was removed. Ten minutes later, the treatment or control solution was injected into the superior hemispleen in a similar manner. The hemispleen was left in place for a second injection 7 days later. The abdomen was then closed in a single layer using 5-0 Prolene suture. The animals were euthanized 2 weeks later, and the livers were examined. The whole liver was assigned a metastasis score of 0 (no gross metastasis), 1 (<1 cm² area of tumor), 2 (1–2 cm² area of tumor), 3 (>2 cm² area of tumor), or 4 (complete infiltration).

A "portal vein" model was also optimized (16). BALB/c mice at 10 weeks of age were used. The animals were prepped and anesthetized as described previously. An upper midline incision was made, and the peritoneal cavity was entered. The intestines were eviscerated and reflected to the right. A piece of warm saline-soaked gauze measuring 2 × 2 inches was placed over the intestines. A 31-gauge needle was used to inject 4 × 10⁶ CT26.CL25 colon cancer cells in 200 μL HBSS into the portal vein. A small piece of moist Gelfoam (Pharmacia Corp., Kalamazoo, MI) was then pressed over the injection site. Pressure was continued for 2 to 3 min, and the Gelfoam was left in place. The intestines were then returned to the abdomen, which was closed in one layer using 5-0 Prolene suture. The animal was then turned, and a second incision was made over the left flank. A s.c. pocket was dissected, and then, the abdomen was entered. The whole spleen was used for injection of either Fv-p53 treatment or X-33 yeast protein control. After the injection, the whole spleen was placed into the s.c. pocket to facilitate subsequent injections. The spleen was held in position by closing the abdominal wall with 5-0 Prolene suture as described by Kasuya et al. (17). The skin was then closed in a separate layer using the same suture. A second spleen injection was done 7 days later via a minor surgery. The animal was anesthetized, and the left flank was prepped with povidone iodine. A small portion of the incision was opened, and the material was injected into the spleen under direct visualization. Seven days after the second injection, the animals were euthanized and a metastasis score (see above for criteria) was given to the left lobe of the liver that receives drainage from the splenic vein.

Statistics
A P value was determined by using a two-tailed Student’s t test.

Results
The Fv fragment is required for nuclear delivery of p53. Fv-p53, Fv(R95Q)-p53, p53 alone, Fv alone, and X-33 control proteins were generated and purified from P. pastoris as described previously (Fig. 1). Fv(R95Q)-p53, abbreviated as R95Q, contains a 27-gauge needle was used to inject 1 × 10⁶ CT26.CL25 colon cancer cells into the inferior hemispleen. Before this injection, the syringes were preloaded with 250 μL HBSS. During the surgery, 50 μL of cell suspension were aspirated into the syringe, thus providing a saline flush after the cells were injected. Three minutes after the cell injection, a medium vascular clip was placed across the vascular pedicle and the inferior hemispleen was removed. Ten minutes later, the treatment or control solution was injected into the superior hemispleen in a similar manner. The hemispleen was left in place for a second injection 7 days later. The abdomen was then closed in a single layer using 5-0 Prolene suture. The animals were euthanized 2 weeks later, and the livers were examined. The whole liver was assigned a metastasis score of 0 (no gross metastasis), 1 (<1 cm² area of tumor), 2 (1–2 cm² area of tumor), 3 (>2 cm² area of tumor), or 4 (complete infiltration).

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Results
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mutation in Fv that renders the protein incapable of penetrating into the cells (13). X-33 proteins were eluted from Ni-NTA agarose (Qiagen, Valencia, CA) incubated with lysates of X-33 cells free of plasmids. The X-33 control showed the same pattern of proteins found in preparations of Fv-p53 and served as a control for protein impurities that copurify with Fv-p53(Fig. 1). Fv-p53 and control proteins were tested for penetration into Skov-3 cells. Control cells treated with X-33 yeast proteins showed an absence of staining. Cells treated with Fv or Fv-p53 exhibited distinct nuclear staining representing nuclear penetration. As expected, cells treated with R95Q or p53 alone did not show nuclear staining. Because p53 alone failed to penetrate into Skov-3 cells, these results indicate that the Fv fragment is necessary for nuclear delivery of p53 (Fig. 2).

Functional Fv-p53 is required for induction of cell death in cancer cells. Induction of cell death in cancer cells by Fv-p53 was compared with control proteins by applying equimolar amounts of the proteins to Skov-3 and CT26.CL25 cells. Twenty-four hours after application of protein, the cells were analyzed under fluorescence microscopy using propidium iodide staining to quantify cell death. Skov-3 cells (93.8 ± 8.9%) were killed by Fv-p53 compared with 5.2 ± 4.3% by R95Q, 4.0 ± 2.8% by p53, 3.0 ± 1.4% by Fv, and 2.2 ± 1.5% by X-33 proteins (Fig. 3A). Similarly, 74.6 ± 24.1% of CT26.CL25 cells were killed by Fv-p53 compared with 13.8 ± 10.9% by R95Q, 3.0 ± 1.4% by p53, 2.5 ± 0.7% by Fv, and 2.5 ± 0.6% by X-33 proteins (Fig. 3B). Both wild-type p53 and the R95Q mutant fusion protein failed to penetrate into the cells and to kill Skov-3 or CT26.CL25 cells, indicating that transducible p53 is required for cell killing. Taken together, the penetration and killing assays indicate that Fv-p53 is the functional reagent responsible for killing the Skov-3 and CT26.CL25 cells.

To determine the concentration of Fv-p53 required for killing CT26.CL25 cells, the cell death assay was repeated using 12.5, 25, 50, and 100 nmol/L of Fv-p53. Both 50 and 100 nmol/L Fv-p53 were highly effective in killing the CT26.CL25 cells, whereas lower doses

![Figure 2](image-url)  
Figure 2. Fv facilitates nuclear delivery of p53. Fv-p53, R95Q, p53, Fv, or X-33 yeast proteins (control) were tested for nuclear penetration in Skov-3 cells. Cells were washed, fixed, and stained with anti-p53 or anti-myc antibodies after 1 h of incubation with the proteins. Only cells treated with Fv-p53 or Fv exhibited nuclear staining, indicating that Fv facilitates and is required for nuclear delivery of p53. Bar, 5 μm.

![Figure 3](image-url)  
Figure 3. Fv-p53 kills cancer cells in vitro. Equimolar amounts of Fv-p53, R95Q, p53, Fv, or X-33 yeast proteins were applied to Skov-3 and CT26.CL25 cells in vitro, and the percentage of cells killed was determined 24 h later by propidium iodide staining. Columns; mean; bars, SD. A, Skov-3 cells: 93.8 ± 8.9% of Skov-3 cells were killed by Fv-p53 compared with 5.2 ± 4.3% by R95Q, 4.0 ± 2.8% by p53, 3.0 ± 1.4% by Fv, and 2.2 ± 1.5% by X-33 proteins. B, CT26.CL25 cells: 74.6 ± 24.1% of CT26.CL25 cells were killed by Fv-p53 compared with 13.8 ± 10.9% by R95Q, 3.0 ± 1.4% by p53, 2.5 ± 0.7% by Fv, and 2.5 ± 0.6% by X-33 proteins. C, concentrations ranging from 0 to 100 nmol/L Fv-p53 were tested on CT26.CL25 cells. Fv-p53 killing of CT26.CL25 cells was dose dependent.
exhibited significantly less activity (Fig. 3C). This result showed that Fv-p53 has a dose-dependent effect on CT26.CL25 cells and suggested the concentration of Fv-p53 to be tested in vivo.

**Fv-p53 prevents liver metastasis in vivo.** A liver metastasis model, generated by injecting CT26.CL25 colon carcinoma cells into BALB/c mice, was used to test the efficacy of Fv-p53 protein therapy in vivo. The first mouse experiment used the "hemispleen" method to optimize the timing of Fv-p53 delivery after injection of the cancer cells. CT26.CL25 colon carcinoma cells were given to 12 BALB/c mice, which were divided into four groups. Mice in each group received two hemispleen injections of 100 nmol/L. Fv-p53 or control medium. The first injections of Fv-p53 or control medium were made 10 min after administration of the CT26.CL25 cells, whereas the second injections occurred 1 week later. Mice in group 1 received control medium for both injections. Mice in group 2 received Fv-p53 for the first injection and control medium for the second injection. Group 3 mice received control medium for the first injection and Fv-p53 for the second injection. Finally, group 4 mice received Fv-p53 for both the first and second injections. Two weeks after the second injections, the mice were euthanized and the livers were examined to determine the extent of tumor burden. The mice in group 1 had an average metastasis score of 2.7 ± 0.5. In contrast, group 4 had an average metastasis score of 1.0 ± 0.0, indicating a decrease in tumor burden in the treated mice. Mice in groups 2 and 3 had scores of 0.7 ± 0.9 and 2.0 ± 0.8, respectively (Table 1). This shows that Fv-p53 seems to have an effect on the prevention of liver metastasis, and as expected, early treatment was more effective than delayed treatment.

In the first mouse experiment, significant local recurrence of tumor in the left upper quadrant of the abdomen near the spleen site was noted. Therefore, the "portal vein" method was used for the second experiment in an effort to decrease or eliminate the amount of local recurrence of tumor. In this experiment, liver metastases were established by injecting CT26.CL25 cells into the portal vein, and the mice were treated via splenic injection with either Fv-p53 or X-33 yeast protein control at 10 min and again 7 days later. The animals were euthanized 7 days after the second injection, and the livers were examined for tumor burden. Mice treated with Fv-p53 had a significantly lower metastasis score than mice treated with X-33 control (Table 2). Figure 4 shows that a reduction in metastasis score from 3.3 ± 1.3 to 0.8 ± 0.4 represents a clinically significant decrease in tumor burden. Control mice had severe to complete infiltration of livers, whereas mice treated with Fv-p53 had minimal liver infiltration. Taken together, the two mouse experiments show that Fv-p53 inhibits liver metastasis and provides the first experimental evidence of effective full-length p53 protein therapy in vivo.

### Discussion

Developing therapeutic agents that selectively kill cancer cells while sparing healthy cells and tissues will significantly increase the likelihood of achieving cure in cancer patients. Whereas solitary primary tumors may be amenable to surgical removal, metastatic disease is often incurable. Present efforts to treat metastatic disease primarily rely on radiation therapy and chemotherapeutic drugs, which may cause significant side effects. mAbs that bind specific tumor antigens, such as trastuzumab (Herceptin), typically have fewer side effects but usually have the greatest activity when used together with more toxic chemotherapy agents (18). p53 therapy presents a potentially elegant solution to the problem of metastatic disease in that small doses of p53 induce growth arrest and apoptosis in transformed cells but do not seem to adversely affect normal cells (6). It also has the profound advantage of likely being applicable to greater than half of all tumor cells. The obvious question to be answered pertains to the best means of delivering p53 to cells.

Protein therapy could be effective in treating nearly any protein deficiency disease but is particularly well suited to the treatment of cancer. Whereas patients with chronic diseases might require continuous replacement therapy, cancer patients would potentially require only a limited number of doses of p53 to eliminate cancer cells. Furthermore, p53 is functional at a very low intracellular concentration, and the frequency and duration of p53 infusions could be easily modified to minimize side effect profiles.

The Fv fragment of mAb 3E10 is an ideal transport vehicle for p53 protein therapy. Fv specifically delivers most cargo proteins to the nucleus and should promote less inflammation than other PTDs (7, 8). In the present study, no side effects of Fv-p53 therapy were observed in any of the experimental mice. Fv also has a short half-life inside the cells, and previous studies using single-chain Fv fragments to visualize tumors in vivo found that Fv fragments localize into the tumor cells more readily than normal cells and are

### Table 1. Optimization of Fv-p53 delivery

<table>
<thead>
<tr>
<th>Group</th>
<th>No. mice</th>
<th>Treatment at 10 min</th>
<th>Treatment at 1 wk</th>
<th>Metastasis score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>Control</td>
<td>Control</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>Fv-p53</td>
<td>Control</td>
<td>0.7 ± 0.9</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>Control</td>
<td>Fv-p53</td>
<td>2.0 ± 0.8</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>Fv-p53</td>
<td>Fv-p53</td>
<td>1.0 ± 0.0</td>
</tr>
</tbody>
</table>

NOT: The hemispleen mouse model was used to deliver either Fv-p53 or control medium at 10 min and 1 wk after injection of cancer cells. Results are reported as mean ± SD. Fv-p53 seems to decrease the metastatic burden, particularly if given early.

### Table 2. Effect of Fv-p53 on the development of liver metastasis

<table>
<thead>
<tr>
<th>Group</th>
<th>No. mice</th>
<th>Treatment at 10 min</th>
<th>Treatment at 1 wk</th>
<th>Metastasis score*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>Control</td>
<td>Control</td>
<td>3.3 ± 1.3</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>Fv-p53</td>
<td>Fv-p53</td>
<td>0.8 ± 0.4</td>
</tr>
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*P = 0.004.

Fv-p53 had minimal liver infiltration. Taken together, the two mouse experiments show that Fv-p53 inhibits liver metastasis and provides the first experimental evidence of effective full-length p53 protein therapy in vivo.

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6 J.E. Hansen et al., unpublished data.
rapidly cleared from the body (19, 20). The propensity of Fv fragments to localize to tumors would facilitate delivery of p53 to target tissues, and if side effects become a concern, the short half-life of Fv and rapid plasma clearance of Fv fragments would aid in limiting therapy duration.

The timing of Fv-p53 treatment in relationship to the development of metastatic disease seems to be important. In our mouse model, Fv-p53 given 10 min after injection of cancer cells seemed to suppress metastasis to the greatest extent (Table 1). Although the mice receiving Fv-p53 at 7 days seemed to have a larger metastatic burden compared with those receiving an initial dose of Fv-p53 at 10 min, there was still a suggestion that they had a lower metastasis score compared with those that received the control injection at both times. The ability of Fv-p53 to suppress metastasis whether given 10 min or 1 week after administration of cancer cells suggests that Fv-p53 may be able to kill both recently metastasized cells and established tumor cells.

The second in vivo experiment showed that Fv-p53 treatment had a profound effect on liver metastasis. Control mice treated with X-33 yeast proteins had severe to complete tumor infiltration of the liver, whereas mice treated with Fv-p53 had minimal to no liver metastasis. This result was not only statistically significant but also readily apparent on gross observation of the livers from control and Fv-p53–treated mice (Table 2; Fig. 4). This shows that Fv-p53 has activity even in the setting of a very large metastatic burden.

Figure 4. Determination of metastasis scores. Two weeks after the injection of CT26.CL25 cells into BALB/c mice, mice were sacrificed and assigned metastasis scores based on the following criteria: 0, no liver metastasis; 1, minimal infiltration (<1 cm² area of metastasis); 2, mild to moderate infiltration (1–2 cm² area of metastasis); 3, severe infiltration (>2 cm² area of metastasis but the liver was not entirely infiltrated); and 4, complete infiltration. Examples of mice receiving each score in the representative images.
Future studies will focus on specifically quantifying Fv-p53 antitumor activity, doing survival studies, and testing the ability of Fv-p53 to kill established, solid tumors. Technical studies will also be done to increase the yield and purity of Fv-p53. The primary significance of this work is that, for the first time, full-length p53 protein therapy is effective \textit{in vivo}, suggesting that Fv-p53 could potentially be developed into an effective cancer therapy.

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