Molecular chemotherapy of pancreatic cancer using novel mutant bacterial cytosine deaminase gene

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Abstract
The combination of molecular chemotherapy with radiation therapy has the potential to become a powerful approach for treatment of pancreatic cancer. We have developed an adenoviral vector (AdbCD-D314A) encoding a mutant bacterial cytosine deaminase (bCD) gene, which converts the prodrug 5-fluorocytosine (5-FC) into the active drug 5-fluorouracil. The aim of this study was to investigate AdbCD-D314A/5-FC-mediated cytotoxicity in vitro and therapeutic efficacy in vivo alone and in combination with radiation against human pancreatic cancer cells and xenografts. AdbCD-D314A/5-FC-mediated cytotoxicity alone and in combination with radiation was analyzed using crystal violet inclusion and clonogenic survival assays. CD enzyme activity was determined by measuring conversion of [3H]5-FC to [3H]5-fluorouracil after adenoviral infection of pancreatic cancer cells in vitro and pancreatic tumor xenografts by TLC. S.c. pancreatic tumor xenografts were used to evaluate the therapeutic efficacy of AdbCD-D314A/5-FC molecular chemotherapy in combination with radiation therapy. AdbCD-D314A infection resulted in increased 5-FC-mediated pancreatic cancer cell killing that correlated with significantly enhanced CD enzyme activity compared with AdbCDwt encoding wild-type of bCD. Animal studies showed significant inhibition of growth of human pancreatic tumors treated with AdbCD-D314A/5-FC in comparison with AdbCDwt/5-FC. Also, a significantly greater inhibition of growth of Panc2.03 and MIA PaCa-2 tumor xenografts was produced by the combination of AdbCD-D314A/5-FC with radiation compared with either agent alone. The results indicate that the combination of AdbCD-D314A/5-FC molecular chemotherapy with radiation therapy significantly enhanced cytotoxicity of pancreatic cancer cells in vitro and increased therapeutic efficacy against human pancreatic tumor xenografts. [Mol Cancer Ther 2008;7(9):2845–54]

Introduction
Pancreatic cancer is a common, highly lethal disease that is rising in incidence. Despite advances in the treatment of pancreatic cancer, the prognosis for locally advanced or metastatic disease remains very poor. Thus, the development of more effective alternative treatments will be critical to improve the survival of patients with these tumors (1–3). Among these approaches, molecular chemotherapy has received considerable attention (4).

One of the most widely used molecular chemotherapy systems for cancer uses cytosine deaminase (CD) in combination with the antifungal agent 5-fluorocytosine (5-FC) that has been investigated intensely during the last decade. CD is a bacterial (bCD) or yeast enzyme that can convert 5-FC into the chemotherapy agent 5-fluorouracil (5-FU). 5-FU molecules are able to diffuse across the cell membrane into adjacent cells without passing through the gap junctions to produce a powerful bystander effect (5). It has been shown that 5-FU is a strong radiosensitizer and the increased radiation sensitivity in cells exposed to 5-FU depends on G1-S-phase progression (6).

Molecular chemotherapy using bCD or yeast CD has been studied for pancreatic cancer treatment in vitro and in animal tumor models (7–9). A major problem associated with this molecular chemotherapy approach is the low affinity displayed by the wild-type bCD (bCDwt) gene product toward 5-FC in comparison with cytosine. Recent studies have shown that substitution of an alanine (A) for the aspartic acid (D) at position 314 of bCDwt increased relative specificity of the mutant bCD-D314A enzyme to 5-FC in comparison with bCDwt and may be a superior gene for molecular chemotherapy (10, 11). The utilization of bCD-D314A mutant for pancreatic cancer molecular chemotherapy should increase efficacy as well as decrease possible adverse effects.

The purpose of this study was to evaluate cytotoxicity in vitro and therapeutic efficacy in vivo of the combination of a nonreplicative adenovirus encoding bCD-D314A gene (AdbCD-D314A) and radiation against human pancreatic cancer. Animal model studies using human pancreatic tumor xenografts in nude mice examined the therapeutic efficacy of mutant bCD-D314A/5-FC therapy alone or in combination with radiation therapy. The results show that...
adenovirus-mediated bCD-D314A gene expression and 5-FC significantly enhanced cytotoxicity of pancreatic cancer cells in combination with radiation and increased efficacy against human pancreatic tumor xenografts in vivo compared with AdbCDwt/5-FC and radiation.

Materials and Methods
Recombinant Adenoviruses
A replication-deficient AdbCDwt recombinant adenovirus vector encoding the gene for wild-type CD enzyme from Escherichia coli (bCD) under control of the human cytomegalovirus immediate-early promoter was constructed as described previously (12). The replication-deficient AdbCD-D314A vector encoding the mutant bCD-D314A gene driven by the cytomegalovirus promoter was developed, propagated, and titered as described previously (13).

Cell Culture, Animals, and Chemicals
Human pancreatic cancer cell lines S2O13 and S2VP10 (a kind gift from Dr. Anthony Hollingsworth, University of Nebraska), MIA PaCa-2 and BxPC-3 (American Type Culture Collection), and HEK293 human embryonic kidney cells (Microbix Biosystems) were cultured in DMEM/F-12 (Mediatech) containing 10% fetal bovine serum (Summit Biotechnology). Panc.2.03 human pancreatic cancer cells (a kind gift from Dr. Elizabeth Jaffee, Johns Hopkins University) were cultured in RPMI 1640 (Mediatech) and 10% fetal bovine serum (Summit Biotechnology).

Female athymic nude mice were purchased from the Frederick Cancer Research Facility and housed under aseptic conditions in microisolator cages and experiments were carried out according to the Institutional Animal Care and Use Committee approved protocols.

5-FC and 5-FU were purchased from Sigma and SP Pharmaceuticals, respectively.

In vitro Cytotoxicity Assay
To measure the cytotoxicity of 5-FU and 5-FC, pancreatic cancer cells were plated into 96-well tissue culture plates at 5 × 10^3 per well and allowed to adhere overnight. The next day, serial dilutions of 5-FU or 5-FC were added directly to cells. Cells were incubated for 5 days, and relative cell density was determined using the crystal violet staining assay. Fractional cell survival at each drug concentration was calculated as the ratio of absorbance at 570 nm of cells incubated with drug over that of control. The IC50 values were extrapolated by piecewise linear regression as the concentration of drug producing a 50% reduction in corrected absorbance.

For AdbCD-D314A/5-FC and AdbCDwt/5-FC cytotoxicity experiments, the target cells were plated into 96-well tissue culture plates at 5 × 10^3 per well and allowed to adhere overnight. Twenty-four hours later, cells were inoculated with AdbCD-D314A or AdbCDwt at various multiplicities of infection. The next day, fresh medium supplemented with serial dilutions of 5-FC was added, and 5 days after prodrug treatment, cytotoxicity (IC50) was determined using the crystal violet staining assay.

CD Conversion Assay
Enzyme activity of bCDwt and bCD-D314A in pancreatic cancer cells was determined by measuring the conversion of [3H]5-FC to [3H]5-FU by TLC as described previously (13).

Clonogenic Survival Assay
At 24 h after infection with 50 TCID50/cell of AdbCDwt or AdbCD-D314A, cells were trypsinized and diluted to the appropriate cell density into six-well culture plates and allowed to adhere overnight. 5-FC was added directly to cells the next day, and after 24 h incubation at 37°C, cells were either mock-irradiated or irradiated at 2 Gy using a 60Co γ-iradiator (Picker Unit) and were then returned to the incubator and cultured additionally for 15 days. Cells were then fixed with 70% ethanol and stained with 2% (w/v) crystal violet (Sigma-Aldrich). Colonies comprising at least 50 cells were counted. The plating efficiencies were calculated as the number of colonies divided by the number of test cells plated for each data point. Plating efficiencies were referenced back to the mock-irradiated control plating efficiency to determine the surviving fraction for each dose.

Flow Cytometry
Annexin V staining and propidium iodide (PI) uptake were used for apoptosis evaluation. MIA PaCa-2 and Panc.2.03 cells were infected with 50 TCID50/cell of AdbCDwt or AdbCD-D314A and allowed to incubate overnight. The next day, 5-FC or 5-FU was added to cell culture medium in final concentration of 50 or 15 μg/mL, respectively. Twenty-four hours later, cells were either mock-irradiated or irradiated at 2 Gy. Cells were collected at 24 h after radiation and double stained with FITC-conjugated Annexin V and PI. Annexin V and PI were added according to the manufacturer’s recommendations (BioVision). Samples were immediately analyzed by FACScan. Annexin V and PI emissions were detected in the FL-1 (530/30 nm) and FL-2 (585/40 nm) channels, respectively. Analysis was done with CellQuest software (Becton Dickinson) on cells characterized by forward/side scatter variables. Annexin V-positive cells were considered apoptotic; cells taking only vital dye PI were considered necrotic. The percentages of apoptotic cells were calculated.

Western Blot
MIA PaCa-2 and Panc.2.03 cells were infected with 50 TCID50/cell of AdbCDwt or AdbCD-D314A. The next day, 5-FC or 5-FU was added to cell culture medium in final concentration of 50 or 15 μg/mL, respectively. Twenty-four hours later, cells were either mock-irradiated or irradiated at 2 Gy. Cells were collected at 24 h after radiation, washed in TBS, and homogenized in lysis buffer (Sigma). Each sample was denatured for 5 min at 100°C in loading buffer. Equal amounts of protein (15 μg) were loaded for each sample in all lanes and separated on SDS-PAGE followed by transfer to a polyvinylidene difluoride membrane. The membrane was incubated with anti-Bax, anti-Bcl2 (NeoMarkers), or...
anti-BclXL (Santa Cruz Biotechnology) mouse monoclonal antibodies and then treated with the Western Breeze Chemiluminescent Western Blot Immunodetection Kit (Invitrogen). The membrane was submerged in stripping buffer (Sigma) and incubated for 30 min at 50 °C with occasional agitation, and expression of actin was detected. For immunodetection of actin, the rabbit polyclonal IgG (Santa Cruz Biotechnology) and polyclonal goat anti-rabbit Ig/horseradish peroxidase (DAKO) and the Enhanced Chemiluminescence plus Western Blotting Detection System (Amersham Biosciences) were used.

**CD Conversion in Tumor Xenografts**

For CD conversion assay, 7 × 10⁶ Panc2.03 pancreatic cancer cells were injected s.c. into athymic nude mice. When tumors reached 4 to 6 mm in diameter, they were injected with 1 × 10⁷ TCID₅₀ of AdbCD-D314A or AdbCDwt. Also, 1.6 × 10⁷ MIA PaCa-2 cells were resuspended 1:1 (v/v) in Matrigel (Becton Dickinson) and s.c. injected into athymic nude mice. Our initial in vivo studies showed that employing a reconstituted basement membrane, such as Matrigel, increased tumor take and growth of s.c. MIA PaCa-2 pancreatic tumor xenografts. Thus, Matrigel was used for establishment of MIA PaCa-2 tumor xenografts. MIA PaCa-2 tumors were injected with 5 × 10⁷ TCID₅₀ of AdbCD-D314A or AdbCDwt. Tumors were dissected at different times after adenovirus infection and crushed with a mortar and pestle in protease inhibitor buffer (Roche Diagnostics). Tumor tissues were frozen and thawed three times, and after centrifugation, the supernatant was assayed for protein concentration and subjected to CD conversion assay as described above.

**S.c. Human Pancreatic Tumor Xenograft Studies**

To evaluate in vivo therapeutic efficacy of the combination of 5-FU and radiation treatment, 6 × 10⁶ Panc2.03 cells were injected s.c. into the right flank of athymic nude mice. Treatment was started 11 days post-tumor cell injection at the time of established tumor growth (tumors were 4-6 mm in diameter), noted as day 0. Animals were randomly divided into groups of 9 to 10 mice receiving different treatments: (a) 5-FU alone, (b) 5-FU plus radiation, (c) PBS plus radiation, and (d) PBS alone. Two groups of mice (1 and 2) received 5-FU i.p. at 20 mg/kg on days 1 to 5, 8 to 12, and 15 to 19. Radiation treatment for two groups of animals (2 and 3) was 2 Gy from a ⁶⁰Co γ-iradiator (Picker Unit) on days 1, 9, and 16.

To compare antitumor effects of AdbCD-D314A- and AdbCDwt-mediated molecular chemotherapy on established solid tumors, 7 × 10⁶ Panc2.03 cells were injected s.c. into the flank of athymic nude mice. Treatment was started 11 days post-tumor cell injection noted as day 0. Mice were divided into groups of 9 to 10 animals receiving different treatments: (a) AdbCDwt plus 5-FC, (b) AdbCD-D314A plus 5-FC, (c) PBS plus 5-FC, and (d) AdbCD-D314A alone. Mice were injected i.t. with 1 × 10⁷ TCID₅₀ per tumor AdbCDwt or AdbCD-D314A on days 0, 6, and 12. Three groups of mice (1-3) received 5-FC i.p. at 400 mg/kg on days 1, 5, 8 to 12, and 15 to 19.

For evaluation of therapeutic efficacy of the combination of AdbCD-D314A/5-FC and radiation therapy, 6 × 10⁶ Panc2.03 cells were injected s.c. into the flank of athymic nude mice. Therapy was started 10 days post-tumor cell injection noted as day 0. Animals were divided into groups of 9 mice receiving different treatments: (a) AdbCD-D314A plus 5-FC, (b) AdbCD-D314A plus 5-FC in combination with radiation, (c) AdbCD-D314A plus 5-FC plus 5-FC in combination with radiation, (e) PBS plus 5-FC in combination with radiation, and (f) PBS plus 5-FC. Mice were injected i.t. with 5 × 10⁷ TCID₅₀ AdbCD-D314A or

### Table 1. In vitro CD conversion activity of AdbCD-D314A and AdbCDwt in pancreatic cancer cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>MIA PaCa-2</th>
<th>BxPC-3</th>
<th>Panc2.03</th>
<th>S2VP10</th>
<th>S2O13</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdbCDwt</td>
<td>0.4 ± 0.3</td>
<td>0.6 ± 0.1</td>
<td>0.7 ± 0.04</td>
<td>0.2 ± 0.04</td>
<td>0.2 ± 0.04</td>
</tr>
<tr>
<td>AdbCD-D314A</td>
<td>134.6 ± 6.0</td>
<td>243.9 ± 19.8</td>
<td>112.0 ± 1.6</td>
<td>183.4 ± 6.6</td>
<td>101.4 ± 6.4</td>
</tr>
</tbody>
</table>

**NOTE:** Cells were infected with 25 TCID₅₀/cell of AdbCD-D314A or AdbCDwt and CD conversion activity was determined as pmol/min/mg by measuring the conversion of [³H]5-FC to [³H]5-FU.

### Table 2. Cytotoxicity of 5-FU and adenovirus-mediated molecular chemotherapy in pancreatic cancer cell lines

<table>
<thead>
<tr>
<th></th>
<th>MIA PaCa-2</th>
<th>BxPC-3</th>
<th>Panc2.03</th>
<th>S2VP10</th>
<th>S2O13</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU IC₅₀ (µg/mL)*</td>
<td>1.4 ± 0.3</td>
<td>0.1 ± 0.2</td>
<td>12.4 ± 2.6</td>
<td>2.7 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>5-FC IC₅₀ (µg/mL)†</td>
<td>104.2 ± 19.2</td>
<td>3.7 ± 0.5</td>
<td>397.4 ± 92.4</td>
<td>185.4 ± 74.5</td>
<td>58.7 ± 24.4</td>
</tr>
<tr>
<td>AdbCDwt</td>
<td>21.5 ± 6.8</td>
<td>0.3 ± 0.1</td>
<td>11.3 ± 5.3</td>
<td>25.7 ± 12.1</td>
<td>9.3 ± 0.9</td>
</tr>
<tr>
<td>AdbCD-D314A</td>
<td>94.2 ± 19.2</td>
<td>3.7 ± 0.5</td>
<td>397.4 ± 92.4</td>
<td>185.4 ± 74.5</td>
<td>58.7 ± 24.4</td>
</tr>
</tbody>
</table>

*Cells were incubated with serial dilutions of 5-FU, and IC₅₀ (µg/mL) was determined at 5 days using the crystal violet staining assay.

†Cells were infected with 50 TCID₅₀/cell of AdbCD-D314A or AdbCDwt, and the next day, fresh medium supplemented with serial dilutions of 5-FC was added. 5-FC cytotoxicity was determined on day 5 after adenovirus infection by crystal violet staining assay.
AdbCDwt on days 0, 9, and 16. Three groups of animals (2, 4, and 5) received radiation therapy at 2 Gy on days 2, 10, and 17. All mice were treated i.p. with 400 mg/kg 5-FC on days 1 to 5, 8 to 12, and 15 to 19. To confirm the therapeutic efficacy of the combination of molecular chemotherapy and radiation therapy in a second tumor model, 1.6 x 10^7 MIA PaCa-2 tumor cells were resuspended 1:1 (v/v) in Matrigel (Becton Dickinson) and injected s.c. into the flank of athymic nude mice. Treatment was started 17 days post-tumor cell injection at the time of established tumor growth, noted as day 0. Mice were divided into groups of 10 animals receiving different treatments: (a) AdbCD-D314A plus 5-FC, (b) AdbCD-D314A plus 5-FC in combination with radiation, (c) AdbCDwt plus 5-FC, (d) AdbCDwt plus 5-FC in combination with radiation, (e) PBS plus 5-FC in combination with radiation, and (f) PBS plus 5-FC. Mice were injected i.t. with 5 x 10^5 TCID_{50} AdbCD-D314A or AdbCDwt on days 0, 7, and 14. Radiation treatment for three groups of animals (2, 4, and 5) was 2 Gy on days 2, 9, and 16. All mice were treated i.p. with 400 mg/kg 5-FC on days 1 to 5, 8 to 12, and 15 to 19. Tumor size was monitored twice a week using digital Vernier calipers. Tumor volumes were calculated as 0.5 x length x width^2 (mm^3).

**Statistical Methods**

All error terms are expressed as the SD of the mean. Significance levels for comparison of differences between groups in the *in vitro* experiments were analyzed by Student's *t* test. The differences were considered significant when *P* < 0.05. All reported *P* values are two-sided. In the animal model tumor therapy studies, the treatment groups were compared with respect to tumor size and percent of original tumor size over time. To test for significant differences in tumor volume doubling time between treatment groups, one-way ANOVA test was conducted. When the ANOVA indicated that a significant difference existed (*P* < 0.05), multiple comparison procedures were used to determine where the differences lay.

**Results**

**Enzyme Activity of bCDwt and bCD-D314A In vitro**

To determine the CD enzyme activity, pancreatic cancer cells were infected with 2.5 TCID_{50}/cell of AdbCD-D314A or AdbCDwt and the CD enzyme activity was determined by measuring the conversion of [{}^{3}H]5-FU to [{}^{3}H]5-FU. The results of CD conversion assays using TLC (Table 1) showed that the CD conversion activity was significantly (*P* < 0.001) elevated 336-, 406-, 160-, 917-, and 507-fold for MIA PaCa-2, BxPC-3, Panc2.03, S2VP10, and S2O13 cells, respectively, following infection with AdbCD-D314A, in comparison with the level of conversion activity in AdbCDwt-infected cells. The relative level of CD enzyme activity following infection with AdbCD-D314A was BxPC-3 > S2VP10 > MIA PaCa-2 > Panc2.03 > S2O13.

| Table 3. Adenovirus-mediated molecular chemotherapy and ionizing radiation induced increased pancreatic cancer cell death in a clonogenic survival assay |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | MIA PaCa-2      | BxPC-3          | Panc2.03        | S2VP10          | S2O13          |
| 5-FC            | AdbCDwt         | 89.4 ± 10.0     | 61.3 ± 8.3      | 94.0 ± 9.1      | 90.1 ± 10.2    | 80.5 ± 8.2    |
|                 | AdbCD-D314A     | 51.4 ± 7.2      | 30.1 ± 4.8      | 23.1 ± 4.6      | 49.2 ± 6.0     | 37.4 ± 4.1    |
| 2 Gy            | AdbCDwt         | 45.2 ± 5.5      | 49.2 ± 4.1      | 39.1 ± 5.0      | 46.0 ± 5.1     | 31.1 ± 4.5    |
|                 | AdbCD-D314A     | 39.7 ± 5.2      | 49.7 ± 4.8      | 37.0 ± 4.4      | 51.3 ± 5.7     | 28.9 ± 3.2    |
| 5-FC/2 Gy       | AdbCDwt         | 36.0 ± 4.3      | 20.9 ± 3.1      | 14.5 ± 2.5      | 32.3 ± 3.5     | 15.7 ± 2.2    |
|                 | AdbCD-D314A     | 5.7 ± 1.1       | 1.4 ± 0.7       | 1.1 ± 0.5       | 9.1 ± 2.0      | 2.0 ± 0.9     |

**Note:** Cells were infected with 50 TCID_{50}/cell of AdbCDwt or AdbCD-D314A, treated with 5-FC at 4 μg/mL, and either mock-irradiated or irradiated at 2 Gy using a ^{60}Co γ-iradiator. Cells were fixed and colonies were counted at 15 days after treatment. Data are presented as percent of colonies in comparison with untreated control. Mean ± SD of three independent experiments, each done in six replicates.

| Table 4. Effects of combination adenovirus-mediated molecular chemotherapy with radiation on apoptosis of pancreatic cancer cells |
|-----------------|-----------------|-----------------|
|                 | MIA PaCa-2      | Panc2.03        |
|                  | Mock-irradiated | 2 Gy            | Mock-irradiated | 2 Gy            |
| AdbCD-D314A     | 6.1 ± 3.1       | 8.3 ± 3.4       | 6.0 ± 3.0       | 7.9 ± 3.2       |
| AdbCD-D314A/5-FC| 16.1 ± 5.4      | 25.2 ± 7.5      | 28.5 ± 8.2      | 41.8 ± 9.7      |
| 5-FU            | 18.9 ± 6.1      | 26.8 ± 8.5      | 12.2 ± 5.2      | 20.1 ± 6.6      |
| AdbCDwt         | 5.1 ± 3.0       | 7.9 ± 4.1       | 5.5 ± 3.1       | 9.2 ± 4.0       |
| AdbCDwt/5-FC    | 8.5 ± 3.7       | 11.9 ± 4.1      | 7.5 ± 2.9       | 14.1 ± 4.3      |

**Note:** Cells were infected with 50 TCID_{50}/cell of AdbCDwt or AdbCD-D314A. The next day, 50 μg 5-FC or 15 μg 5-FU was added. Cells were either mock-irradiated or irradiated at 2 Gy using a ^{60}Co γ-iradiator. Data are presented as percentage of Annexin V-positive cells. Mean ± SD of two independent experiments, each done in six replicates.
5-FU Sensitivity of Pancreatic Cancer Cell Lines

In vitro

To determine the sensitivity of pancreatic cancer cell lines to 5-FU, cells were treated with increasing concentrations of 5-FU, and the cytotoxicity of this drug was determined by measuring surviving cells using the crystal violet staining method. The susceptibility to cytotoxic effects of 5-FU was variable in different pancreatic cancer cell lines (Table 2). Cell killing was proportional to the concentration of 5-FU used, and the range of concentration of 5-FU to produce 50% viable cells (IC$_{50}$, 50% inhibitory concentration) was from 0.1 μg/mL for BxPC-3 cells to 12.4 μg/mL for the Panc2.03 cell line (Table 2). The relative sensitivity to 5-FU treatment was BxPC-3 > S2O13 > MIAPaCa-2 > S2VP10 > Panc2.03.

AdbCDwt/5-FC and AdbCD-D314A/5-FC Sensitivity Alone and in Combination with Ionizing Radiation

To evaluate adenovirus infectivity, pancreatic cancer cells were infected with AdLacZ, encoding the β-d-galactosidase gene under control of the cytomegalovirus immediate-early promoter, at different multiplicities of infection. There was a dose-dependent increase of β-d-galactosidase expression in AdLacZ-infected cells. Infection of the five pancreatic cancer cell lines with 50 TCID$_{50}$/cell of AdLacZ produced β-d-galactosidase expression in more than 90% of cells. Thus, to determine the sensitivity of tumor cells to molecular chemotherapy, human pancreatic cancer cells were infected with 50 TCID$_{50}$/cell of AdbCDwt or AdbCD-D314A, treated with increasing concentrations of 5-FC, and the relative cell viability was determined using the crystal violet staining assay. Expression of the mutant bCD-D341A protein in pancreatic cancer cells significantly increased their sensitivity to 5-FC treatment (Table 2). The IC$_{50}$ with 5-FC administration decreased by 4.9-fold for MIA PaCa-2, 12.3-fold for BxPC-3, 35.0-fold for Panc2.03, 7.2-fold for S2VP10, and 6.3-fold for S2O13 in AdbCD-D314A-infected cells in comparison with AdbCDwt-infected cells (P<0.05). The viability of AdbCD-D314A-infected cells incubated with 5-FC decreased in a multiplicities of infection-dependent manner (data not shown). Uninfected cells treated with 5-FC at 764 μg/mL had no detectable cytotoxicity. The relative sensitivity to AdbCD-D314A/5-FC treatment was BxPC-3 > S2O13 > MIA PaCa-2 > S2VP10. The differences in relative sensitivity are a function of variations in infectivity and 5-FU sensitivity as noted above.

The results of a long-term clonogenic survival assay (Table 3) show that the combination of molecular chemotherapy and ionizing radiation produced enhanced cell death in comparison with either treatment alone. The day after infection with 50 TCID$_{50}$/cell of AdbCD-D314A or AdbCDwt, pancreatic cancer cells were treated with 5-FC at 4 μg/mL; the next day, they were exposed to 2 Gy radiation and subjected to clonogenic survival assay (Table 3). Radiation treatment alone caused a dose-dependent reduction in cell survival of uninfected cells (results not shown). AdbCD-D314A/5-FC treatment of pancreatic cancer cell lines produced a reduction in the number of colonies by 1.8-, 2.0-, 4.1-, 1.8-, and 2.2-fold for MIA PaCa-2, BxPC-3, Panc2.03, S2VP10, and S2O13 cells, respectively.
with AdbCDwt/5-FC-treated cells. Also, 2 Gy radiation treatment of AdbCD-D341A-infected cells reduced the number of colonies by 2.5-, 2.0-, 2.7-, 2.0-, and 3.4-fold for MIA PaCa-2, BxPC-3, Panc2.03, S2VP10, and S2O13 cells, respectively, in comparison with mock-irradiated cells (Table 3). There was a significantly greater reduction in the number of colonies following AdbCD-D341A/5-FC plus radiation treatment of MIA PaCa-2, BxPC-3, Panc2.03, S2VP10, and S2O13 cells (8.9-fold, \( P = 0.0002; 21.3\)-fold, \( P = 0.0006; 21.1\)-fold, \( P = 0.0003; 5.4\)-fold, \( P = 0.004; \) and 18.5-fold, \( P = 0.0005, \) respectively) in comparison with AdbCD-D341A/5-FC alone treated cells. Combined treatment using radiation and AdbCD-D341A/5-FC enhanced cell killing, and the cytotoxic effect improved as the multiplicities of infection of AdbCD-D341A or concentration of 5-FC were increased (data not shown). There was greater cytotoxicity with AdbCD-D341A versus AdbCDwt in combination with 5-FC, radiation, or 5-FC plus radiation (Table 3).

To investigate whether AdbCD-D341A/5-FC molecular therapy alone or in combination with radiation produce increased cell death through an apoptotic mechanism, MIA PaCa-2 and Panc2.03 cells were stained with both PI and FITC-labeled Annexin V (Table 4). A significant increase in the percentage of apoptotic cells was observed after AdbCD-D341A/5-FC treatment in comparison with AdbCDwt/5-FC. Also, there was greater apoptosis in MIA PaCa-2 and Panc2.03 cells treated with AdbCD-D341A/5-FC in combination with radiation in comparison with AdbCD-D341A/5-FC alone. The results of sequential 5-FC dose-escalation studies showed a dose-dependent increased induction of apoptosis (data not shown).

Next, the expression of proapoptotic and antiapoptotic members of the Bcl 2 family of proteins was evaluated by Western blot analysis using antibodies against Bax, Bcl2, and BclXL proteins. Data shown in Fig. 1 show increased levels of Bax protein expression in MIA PaCa-2 and

**Figure 2.** Therapy of human pancreatic cancer xenografts using molecular chemotherapy alone or in combination with radiation. A, growth of Panc2.03 xenografts treated with 5-FU in combination with radiation. Treatment was started at the time of established tumor growth (day 0 equal to 11 d after tumor cell injection). 5-FU was administered i.p. at 20 mg/kg qdx5/wk for 3 wk starting on day 0, and tumors were irradiated with 2 Gy on days 1, 9, and 16 using a 60Co \( \gamma \)-iradiator. *, \( P = 0.03, \) 5-FU plus radiation versus 5-FU alone. B, growth of Panc2.03 xenografts treated with AdbCD-D314A or AdbCDwt. Treatment was started at the time of established tumor growth (day 0 equal to 11 d after tumor cell injection). Animals were injected i.t. with PBS or (1 \( \times \) 10\(^7\) TCID\(_{50}\) of AdbCD-D314A or AdbCDwt on days 0, 6, and 12. 5-FC was administered i.p. at 400 mg/kg qdx5/wk for 3 wk starting on day 1. *, \( P = 0.02, \) AdbCD-D314A/5-FC versus AdbCDwt/5-FC. C, growth of Panc2.03 xenografts treated with AdbCD-D314A or AdbCDwt in combination with radiation. Treatment was started at the time of established tumor growth (day 0 equal to 10 d after tumor cell injection). Animals were injected i.t. with PBS or (5 \( \times \) 10\(^7\) TCID\(_{50}\) of AdbCD-D314A or AdbCDwt on days 0, 9, and 16 and irradiated with 2 Gy on days 2, 10, and 17 using a 60Co \( \gamma \)-iradiator. 5-FC was administered i.p. at 400 mg/kg qdx5/wk for 3 wk starting on day 1. *, \( P = 0.04, \) AdbCD-D314A/5-FC + RT versus AdbCDwt/5-FC + RT. D, growth of MIA PaCa-2 xenografts treated with AdbCD-D314A/5-FC or AdbCDwt/5-FC alone and in combination with radiation. Treatment was started at the time of established tumor growth (day 0 equal to 17 d after tumor cell injection). Animals were injected i.t. with PBS or (5 \( \times \) 10\(^7\) TCID\(_{50}\) of AdbCD-D314A or AdbCDwt on days 0, 7, and 14 and then irradiated with 2 Gy on days 1, 8, and 15 using a 60Co \( \gamma \)-iradiator, and 400 mg/kg 5-FC was i.p. administered qdx5/wk for 3 wk starting on day 1. *, \( P < 0.05, \) AdbCD-D314A/5-FC + RT versus AdbCD-D314A/5-FC alone and AdbCDwt/5-FC + RT. Points, mean of tumor volume (mm\(^3\)); bars, SD. The mean time to tumor volume doubling in days for each group of animals is shown in parentheses.
Panc2.03 after AdbCD-D341A/5-FC molecular therapy. Also, there was an increase in Bax expression following exposure to radiation and 5-FU. However, levels of expression of Bcl-xl and Bcl2 remained unchanged (Fig. 1).

**CD Conversion Activity of AdbCDwt and AdbCD-D314A in Human Pancreatic Tumor Xenografts**

Taking into consideration the results of *in vitro* experiments, Panc2.03 and MIA PaCa-2 pancreatic cancer cell lines with different sensitivities to 5-FU were selected for an animal model study to investigate CD conversion activity after i.t. injection with AdbCDwt or AdbCD-D314A. The CD enzyme activity was determined by measuring the conversion of [3H]5-FC to [3H]5-FU in MIA PaCa-2 tumor xenografts after single i.t. injection of 5 x 10⁷ TCID₅₀ AdbCD-D314A or AdbCDwt. The conversion activities on days 2 and 7 post-injection of AdbCD-D314A in MIA PaCa-2 xenografts were 5.7 ± 2.3 and 4.5 ± 2.1 pmol/min/mg, respectively, in comparison with AdbCDwt injection. A similar increase in CD conversion activity was obtained with AdbCD-D314A in Panc2.03 xenografts: 0.4 ± 0.2, 2.4 ± 0.4, and 2.7 ± 0.7 pmol/min/mg on days 1, 4, and 11 after i.t. injection of 1 x 10⁷ TCID₅₀ AdbCD-D314A, respectively, compared with undetectable levels in AdbCDwt-injected tumors.

**In vivo Therapy of Human Pancreatic Cancer Xenografts Using Molecular Chemotherapy Alone or in Combination with Radiation Treatment**

Based on data not shown from an *in vitro* dose-escalation study, the maximum tolerated dose of 5-FU at 20 mg/kg, administered i.p. qdx5/wk for 3 weeks, was chosen to investigate the therapeutic effect of this drug alone versus in combination with radiation treatment. Panc2.03 cells (6 x 10⁶) were s.c. injected into athymic nude mice. *In vivo* tumor therapy was initiated on day 0, which corresponded to 11 days post-tumor cell injection. The baseline mean and SD for tumor volumes on day 0 was 109.8 ± 8.6 mm³. The mean time to tumor doubling for PBS alone, 5-FU alone, PBS plus radiation, and 5-FU plus radiation groups were 34, 42, 56, and 67 days, respectively (Fig. 2A). There were no significant differences in tumor doubling times between groups that received 5-FU alone versus PBS plus radiation or PBS plus radiation versus 5-FU plus radiation (P > 0.05). However, the group treated with 5-FU plus radiation versus 5-FU alone showed significant differences in the time to tumor doubling (P = 0.03).

To compare antitumor effects of AdbCD-D314A- and AdbCDwt-mediated molecular chemotherapy on established solid tumors, 7 x 10⁶ Panc2.03 cells were s.c. injected into athymic nude mice. *In vivo* tumor therapy was initiated on day 0, which corresponded to 11 days post-tumor cell injection. The baseline mean and SD for tumor volumes on day 0 was 118.8 ± 13.7 mm³. The mean time to tumor doubling for PBS/5-FC, AdbCD-D314A/PBS, AdbCDwt/5-FC, and AdbCD-D314A/5-FC groups were 16, 17, 18, and 49 days, respectively (Fig. 2B). There were no significant differences in tumor doubling times between groups that received 1 x 10⁷ TCID₅₀ per tumor of AdbCDwt/5-FC in comparison with PBS/5-FC or AdbCD-D314A/PBS (P > 0.05). Comparison of tumor doubling times of the AdbCD-D314A/5-FC treatment group versus the AdbCDwt/5-FC group showed a significant difference (P = 0.02).

We next determined whether a combination of molecular chemotherapy with radiation therapy increases tumor growth inhibition. Panc2.03 cells (7 x 10⁶) were injected s.c. into athymic nude mice. *In vivo* tumor therapy was started on day 0, which corresponded to 10 days post-tumor cell injection. The baseline mean and SD for tumor volumes on day 0 was 86.0 ± 9.1 mm³. The mean time to tumor doubling for the groups treated with PBS/5-FC, 5-FC plus radiation, AdbCDwt/5-FC, AdbCDwt/5-FC plus radiation, AdbCD-D314A/5-FC, and AdbCD-D314A/5-FC plus radiation were 35, 43, 70, 81, 90, and 105 days, respectively (Fig. 2C). Comparison of the mean time to tumor doubling of the group treated with AdbCD-D314A/5-FC plus radiation versus treated with AdbCDwt/5-FC plus radiation showed a significant difference (P = 0.04). AdbCDwt/5-FC and AdbCDwt/5-FC in combination with radiation increased the mean time to tumor doubling (70 and 81 days, respectively) in comparison with 5-FU alone or 5-FU plus radiation treatment (tumor doubling time was 42 and 67 days, respectively; Fig. 2A). Importantly, employing of mutant CD-D314A instead of CDwt enhanced inhibitory effect of molecular chemotherapy on tumor growth. There was a greater tumor growth delay in mice treated with AdbCD-D314A/5-FC alone or AdbCD-D314A/5-FC plus radiation (tumor doubling time of 90 and 105 days, respectively) in comparison with AdbCDwt/5-FC and AdbCDwt/5-FC plus radiation as well as 5-FU alone or 5-FU plus radiation.

Additionally, we investigated the therapeutic effect of AdbCD-D314A-mediated molecular chemotherapy using the MIA PaCa-2 pancreatic cancer xenograft model. For this study, MIA PaCa-2 cells were resuspended 1:1 (v/v) in Matrigel and s.c. injected into athymic nude mice. To increase the clinical relevance, MIA PaCa-2 tumors were allowed to grow to 205.8 ± 23.7 mm³ on day 0, which corresponded to 17 days post-tumor cell injection. The mean time to tumor doubling for PBS plus 5-FC, 5-FC plus radiation, AdbCDwt/5-FC, AdbCD-D314A/5-FC, AdbCDwt/5-FC plus radiation, and AdbCD-D314A/5-FC plus radiation-treated groups were 17, 27, 29, 42, and 62 days, respectively (Fig. 2D). There were no significant differences in tumor doubling times between groups treated with AdbCD-D314A/5-FC versus AdbCDwt/5-FC (P > 0.05). Comparison of mean time to tumor doubling of the group treated with AdbCD-D314A/5-FC plus radiation versus AdbCD-D314A/5-FC alone or AdbCDwt/5-FC plus radiation showed significant differences between the groups (P < 0.05).

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Discussion

Chemotherapy based on 5-FU administration has been shown to slightly prolong survival of patients with advanced pancreatic cancer (14). Earlier randomized trials showed a significant benefit of combined radiation therapy with 5-FU in comparison with radiation alone (15, 16) or with chemotherapy alone in local pancreatic carcinoma (17). It has been shown that adenovirus-mediated CD therapy, when the vector was directly injected into the tumor sites followed by systemic delivery of 5-FC, resulted in inhibition of pancreatic cancer growth (7). However, results from several studies have shown a limited response to this molecular chemotherapy strategy (18, 19).

In the present study, we developed an adenoviral vector encoding the mutant bCD-D314A gene and investigated the activity of bCD-D314A gene transfer in combination with 5-FC prodrug therapy for treatment of human pancreatic cancer cells in vitro and tumor xenografts in vivo. It was shown previously that the D314A mutation in bCDwt decreased efficiency for endogenous cytosine, which can compete with 5-FC prodrug for the active enzyme site. The increased affinity for 5-FC resulted in a 19-fold relative substrate preference for 5-FC in comparison with bCDwt (10, 11). Thus, the rationale for using the mutant bCD-D314A gene in these experiments was that the bCD mutant D314A would more effectively convert 5-FC to 5-FU and increase the antitumor activity.

The results of AdbCD-D314A- and AdbCDwt-mediated molecular chemotherapy showed that AdbCD-D314A/5-FC treatment produced enhanced cytotoxicity of pancreatic cancer cells in comparison with AdbCDwt/5-FC, which correlated with significantly increased CD conversion activity in AdbCD-D314A-infected cells. Also, these data show that combination AdbCD-D314A-mediated molecular chemotherapy with radiation therapy resulted in increased killing of cancer cells. The induction of apoptosis and the role of proapoptotic and antiapoptotic proteins of the Bcl2 protein family were examined in pancreatic cancer cells after molecular chemotherapy in combination with radiation. This study showed that pancreatic cancer cell toxicity induced by AdbCD-D314A/5-FC alone and in combination with radiation is dependent on the activation of apoptosis and associated with increased Bax expression. Thus, the apoptosis induced by conversion of 5-FC to 5-FU may be a primary mechanism in AdbCD-D314A/5-FC molecular chemotherapy of pancreatic cancer cells. These data agree with our and other investigators observations that induction of apoptosis is linked with CD/5-FC-mediated cytotoxicity (20, 21).

Despite the promise of such therapy, the development of resistance to 5-FU could decrease the efficacy of CD-mediated therapy. The mechanisms for the resistance to 5-FU treatment include rapid degradation of 5-FU to nontoxic derivatives through the action of dihydropyrimidine dehydrogenase (uracil reductase; refs. 22, 23), different expression levels of enzymes involved in 5-FU phosphorylation (24), amplification of the thymidylate synthase gene (25, 26), or reduction in the synthesis of folylpolyglutamine (27). 5-FU is converted into 5-FdUMP via a two-step process that is activated only when 5-FU is present at high intracellular concentrations. Thus, poor efficiency of conversion of 5-FU into its toxic metabolites plays an important role in resistance to 5-FU cancer therapy.

Differences in adenovirus infectivity of cancer cells, variations in levels of CD expression within infected tumor cells, and intrinsic differences in sensitivity to 5-FU and radiation may contribute to overall efficacy of molecular chemotherapy in combination with radiation therapy. The in vitro studies revealed that pancreatic cancer cell lines showed different levels of CD enzyme activity as well as sensitivity to 5-FU and radiation. These factors as well as multiple pathways that are involved in regulation of response to radiation and 5-FU-mediated cytotoxicity resulted in significant variability of effectiveness of the combination of AdbCD-D314A/5-FC and radiation for the various pancreatic cancer cell lines. The precise mechanisms underlying the interaction of radiation and 5-FU are not clear. However, there are several potential pathways by which 5-FU could increase radiation sensitivity at the cellular level. Although the disruption of either RNA or DNA synthesis can produce cytotoxicity, a substantial body of evidence suggests that radiosensitization is a result of thymidylate synthase inhibition (28, 29). It was shown that 5-FU produces tumor cell killing particularly during S phase, when cells are relatively radioresistant (30). Also, the combination of 5-FU and radiation sensitization probably results in cells that are progressing inappropriately through S phase due to misrepair of DNA damage imposed by radiation. This loss of the S-phase checkpoint in cancer cells may provide the molecular basis for selective killing of tumors compared with normal tissues (31, 32).

Higher levels of CD conversion activity in Panc2.03 and MIA PaCa-2 tumor xenografts injected with AdbCD-D314A were associated with increased therapeutic efficacy of AdbCD-D314A/5-FC molecular chemotherapy in comparison with AdbCDwt/5-FC. The animal therapy studies showed that AdbCD-D314A/5-FC in combination with radiation significantly increased the frequency of regression of tumor and the persistence of tumor growth inhibition in comparison with AdbCD-D314A/5-FC alone and radiation therapy alone. However, pancreatic cancer molecular chemotherapy using mutant bCD in combination with radiation did not achieve complete regressions, which resulted in tumor regrowth. It is possible that the progression of tumor after treatment was due to the short duration of treatment used in this particular regimen as well as a low dose of adenovirus and/or 5-FC. Thus, additional experiments that are outside the scope of the present study are required to determine the optimum regimen of pancreatic cancer molecular chemotherapy in combination with radiation therapy.

There are several approaches to increase specificity and improve efficacy of pancreatic cancer molecular chemotherapy using mutant bCD. The rationale behind molecular
chemotherapy with CD and 5-FC is that, after targeted expression of the enzyme into tumor cells, these cells will be rendered sensitive to the cytotoxic action of 5-FU and be more sensitive to radiation treatment. As molecular chemotherapy is essentially a tumor-targeted chemotherapy, the systemic toxicity commonly associated with, and a major limitation of, conventional chemotherapy using 5-FU is avoided. Specifically targeted expression of CD should decrease systemic toxicity and result in a high drug concentration in the tumor mass and an improved therapeutic index compared with systemic 5-FU administration. Thus, tumor-targeted molecular chemotherapy is an attractive approach for locally advanced pancreatic cancer therapy because local gene delivery is feasible. The utilization of replication-competent adenoviruses with selective oncolytic activity may increase therapeutic efficacy of molecular chemotherapy of pancreatic cancer. Oncolytic virotherapy in combination with molecular chemotherapy may produce cytotoxicity by several mechanisms including tumor-targeted CD expression, direct lysis of infected tumor cells as result of viral replication, and induction of inflammatory cytokine expression and T-cell-mediated immune response (33–35). Recently, it was shown that the combination of conditionally replicating adenovirus with 5-FU in subtoxic concentrations can improve virotherapy due to increased adenoviral uptake (36) and blocking cells in the S or G2 phases of the cell cycle (37). In the last decade, several conditionally replicating vectors encoding the CD gene were tested in various preclinical tumor model studies alone and in combination with radiation therapy, including glioma (38), melanoma (39, 40), prostate cancer (41, 42), and pancreatic cancer (43). These studies showed that using conditionally replicating vectors encoding CD in combination with radiation enhanced therapeutic efficacy of molecular chemotherapy.

In summary, a novel mutant bCD-D314A for suicide gene/5-FC prodrug molecular chemotherapy in combination with radiation therapy produced markedly increased cytotoxicity in human pancreatic cancer cells in vitro and xenografts in vivo.

Disclosure of Potential Conflicts of Interest

D.J. Buchsbaum and S.A. Kaliberov, ownership interest in CDEPT, LLC. No other potential conflicts of interest were disclosed.

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