Molecular Chemotherapy Combined with Radiation Therapy Enhances Killing of Cholangiocarcinoma Cells \textit{in Vitro} and \textit{in Vivo}

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ABSTRACT

Cholangiocarcinoma is a virtually incurable tumor, resistant to current surgical, chemotherapy, and radiotherapy interventions. We applied the gene therapy strategy of toxin gene conversion of nontoxic prodrug to chemotherapeutic drug in combination with radiation therapy to the treatment of cholangiocarcinoma. In this regard, 5-fluorouracil (5-FU) is an accepted radiosensitizing and chemotherapeutic agent presently used in cancer therapy. The \textit{Escherichia coli} enzyme cytosine deaminase (CD) converts the prodrug 5-fluorocytosine (5-FC) to 5-FU. Therefore, our goal was to express the \textit{CD} gene in the human cholangiocarcinoma cell line, SK-ChA-1, assess the cytotoxicity of intracellular production of 5-FU, and determine any enhanced cell killing by the addition of external beam radiation.

The susceptibility of SK-ChA-1 cells to recombinant adenoviral infection was determined by fluorescence-activated cell sorting analysis. We used the recombinant adenoviral vector AdCMVLacZ, encoding the \textit{E. coli} \textit{β}-galactosidase reporter gene under control of the human cytomegalovirus (CMV) promoter, to infect SK-ChA-1 and HeLa cells at 10 and 100 plaque forming units (pfu)/cell, followed by FACS analysis. To evaluate CD-mediated conversion of 5-FC to 5-FU and subsequent cytotoxicity, SK-ChA-1 cells were infected with the recombinant adenovirus AdCMVCD, which encodes CD. Cells were then plated in 96-well microtiter plates and exposed to varying concentrations of 5-FC. Cell proliferation assays (tetrazolium salt conversion to formazan colorimetric assay) were performed 5 days after infection. We evaluated the effects of external beam radiation using a single 8 Gy \textit{60Co} dose to AdCMVCD infected cells, with prior exposure to 5-FC for 2–3 days. MTS assays were performed following radiation treatment. Radiation dose-response analysis, via clongen assay, was used as a more sensitive assay to confirm the interaction of the treatment conditions. s.c. SK-ChA-1 tumors in athymic nude mice were established, which then received three intratumoral injections of 1 × 10⁶ pfu AdCMVCD. Mice received i.p. injections of 400 mg/kg of 5-FC twice daily for 7 days beginning the day of initial AdCMVCD injection (day -2). The radiation treatment group received 10 Gy of \textit{60Co} exposure to their tumor on day 0.

SK-ChA-1 cells were efficiently transduced (48.7 and 99.2%) by 10 and 100 pfu/cell of AdCMVLacZ, respectively. From 37.9 to 84.4% of SK-ChA-1 cells were killed following infection with 10 pfu/cell AdCMVCD and 8 days of exposure to various concentrations of 5-FC (5, 10, 30, 50, and 100 \mu g/ml). Higher 5-FC concentrations and longer duration of exposure resulted in greater cell killing. Radiation treatment (8 Gy) enhanced cell killing by greater than 70% when combined with 10 or 20 \mu g/ml of 5-FC. Radiation dose-response analysis with clongen assay confirmed enhanced SK-ChA-1 cell cytotoxicity as a result of radiation treatment following AdCMVCD infection and 5-FC exposure, with radiobiological parameters \textit{α = 0.44 and D}₅₀ = 0.96. Combined treatment of SK-ChA-1 tumors with AdCMVCD, 5-FC, and radiation in animals resulted in significantly greater survival, time to tumor regrowth, and doubling time compared to the nonradiation treatment group (\textit{P = 0.03, 0.015, and 0.002, respectively}). Significantly greater change in tumor size, smaller ratio of final tumor size to original tumor size, and smaller final tumor size were observed in the radiation treatment group compared to the no radiation treatment group (\textit{P = 0.02, 0.03, and 0.03, respectively}).

Human cholangiocarcinoma cells were transduced with a recombinant adenovirus \textit{in vivo} at high efficiency and were susceptible to CD-mediated intracellular 5-FU production. Radiobiological survival curve parameters confirmed an interactive cytotoxic effect when viral infection and prodrug therapy were combined with external beam radiation exposure. Preliminary \textit{in vivo} studies demonstrated effective tumor growth inhibition following AdCMVCD injection, 5-FC administration, and radiation treatment. These findings suggest the potential for integrating molecular chemotherapy strategies with radiation therapy for the treatment of cholangiocarcinoma.

INTRODUCTION

Cholangiocarcinoma, or carcinoma of the human biliary epithelium, continues to carry a poor long-term prognosis. In this regard, curative therapeutic intervention is limited by the advanced disease stage of most patients at initial presentation (1–4). At diagnosis, approximately 30% of patients are candidates for attempted curative surgical resection. Of these patients, 70% are found to have occult metastatic or advanced local disease, precluding curative resection. Surgical cures do occur; however, the majority of patients undergoing attempted curative resection develop recurrent disease at the anastomotic site or within the intrahepatic biliary tree and succumb due to progression of disease, hepatic failure, or cholangitis (5). Furthermore, strictly nonoperative attempts at palliation of obstructive jaundice include either percutaneous or endoscopically placed stents across the obstructing mass, and survival varies from 3 to 6 months (6–8). The overall survival following diagnosis of cholangiocarcinoma varies from 6 months to 5 years (9, 10).

Chemotherapy with 5-FU, \textsuperscript{2} a well-described radiosensitizing chemotherapeutic drug, has historically been ineffective for therapy of cholangiocarcinoma (11). Clinically effective antineoplastic treatment with 5-FU is generally limited by dose-related toxicity (12). Radiotherapy following attempted surgical resection for cholangiocarcinoma yields minimal prolongation in survival, on the order of 6–24 months (1, 3, 4, 12). There are, however, conflicting reports regarding the efficacy of radiation therapy for cholangiocarcinoma. The combination of treatment modalities (first attempted curative resection, then chemotherapy or radiotherapy) have shown minimal increased survival, although trials are small, not randomized, and retrospective (4, 11). As advances have been seen in combined modality treatment for other refractory malignancies (pancreatic, colon, esophageal, and breast; Ref. 13), application to cholangiocarcinoma has not shown consistent clinical benefits (11, 14–19).

From these results, it is clear that novel treatment strategies for cholangiocarcinoma are required. To this end, gene therapy offers an innovative means to treat this malignancy. An approach to gene therapy, toxin gene/prodrug treatment, involves insertion and expression of a novel gene
in a target cell, and subsequent treatment with a prodrug, which acts as a substrate for the novel gene product. The novel gene product mediates conversion of the nontoxic prodrug into a toxic product (20). For this broad gene therapy approach, most work has been with HSV-TK with GCV and Escherichia coli CD with 5-FC (21–32).

In this regard, CD is a nonmammalian enzyme that normally catalyzes the formation of uracil by the deamination of cytosine. When 5-FC is the substrate, this enzyme will produce 5-FU, a potent radiosensitizing cancer chemotherapeutic agent (33). The introduction of broad gene therapy approach, most work has been with HSV-TK with GCV and approximately 500-fold increased sensitivity to 5-FC. Huber et al. (22) reported antitumor effects of CD insertion (via a retroviral vector) and 5-FC exposure. They noted that even with a low percentage of transduced cells, there was significant antitumor effect, suggesting a “bystander effect” in this system (22). The human genome does not normally encode CD; therefore, humans do not normally deaminate 5-FC, and this prodrug has little systemic toxicity (29, 33). Several investigators have shown in vitro and in vivo efficacy for toxicity/prodrug systems in other gastrointestinal malignancies (colon, pancreatic, and hepatic; Refs. 31–39). Additionally, different vector systems (plasmids, retrovirus, and adenovirus) have been used to deliver CD for pancreatic, colon, and hepatic cancer models (31–39).

Recent studies involving the combination of radiation therapy with toxicity/prodrug therapy have shown efficacy in tumor cell control. Sougawa et al. (40) showed adenoviral introduction of HSV-TK with high dose acyclovir enhanced radiation toxicity in an in vivo tumor model. Kim et al. (41, 42) showed increased glioma and gliosarcoma cell toxicity to radiation therapy following retroviral introduction of HSV-TK and exposure to the substrate 5-[2-bromovinyl]2-deoxyuridine. Recently, Rogulski et al. (43) created a fusion gene of HSV-TK and CD, transduced glioma cells with this fusion gene via a retroviral vector, and demonstrated enhanced radiation cytotoxicity with cellular exposure to 5-FC and GCV.

Our hypothesis for the advantages of combined genetic modification, molecular chemotherapy with subsequent radiotherapy to approach treatment of cholangiocarcinoma include: (a) specific vector delivery of CD to the inherently compartmentalized biliary tree; (b) intracellular production of the active chemotherapeutic agent, 5-FC from 5-FC; and (c) additive, or possibly synergistic tumor cytotoxicity by combination of the known radiosensitizing chemotherapeutic agent, 5-FC, with radiotherapy. Therefore, our goal was to infect cholangiocarcinoma cells with an adenoviral vector encoding CD, assess the cytotoxicity of cellular production of 5-FC, and evaluate the effects of superimposed external beam radiation on cell survival. Furthermore, we evaluated the efficacy of this treatment combination in an animal model.

**MATERIALS AND METHODS**

**Cell Lines.** The human cholangiocarcinoma cell line SK-ChA-1 was the gift of A. Knuth (Ludwig Institute for Cancer Research, London, United Kingdom). SK-ChA-1 cells were maintained in RPMI 1640 supplemented with l-glutamine (200 μM), 10,000 IU/ml penicillin, 10 mg/ml streptomycin, and 10% heat-inactivated FBS (Summit Biotechnology, Ft. Collins, CO) at 37°C in a humidified 5% CO₂ atmosphere. HeLa cells (American Type Culture Collection, Rockville, MD) were used for control purposes. Cells infected with AdCMVlacZ and uninfected controls were harvested 48 h following infection and resuspended at 10⁵ cells/ml in FACS staining media [PBS (Life Technologies, Inc.; 0.2 g/liter KCl, 0.2 g/liter KH₂PO₄, 8 g/liter NaCl, and 1.15 g/liter Na₂HPO₄) containing 10 mM HEPES and 4% FBS]. One hundred μl aliquots were placed into 6-ml tubes and incubated at 37°C for 10 min. One hundred μl of 1% paraformaldehyde were added to the tubes and incubated for 1 min. FACS loading was stopped by adding 1 ml of ice-cold staining media. Cells were washed in FACS buffer, harvested, and resuspended in 1% paraformaldehyde solution for 1 min. Flow cytometry was performed on a FACScan (Becton Dickinson, San Jose, CA).

**Construction of Recombinant Adenovirus Encoding Reporter Gene.** To analyze gene transfer efficiency, a recombinant adenoviral vector containing the LacZ reporter gene encoding β-galactosidase was used. AdCMVLacZ encodes the E. coli LacZ gene, under the control of the human CMV promoter/enhancer. AdCMVLacZ is an E1A/E5-deficient, replication-incompetent adenoviral vector described previously (44).

**Construction of Recombinant Adenovirus Encoding CD.** CD cDNA, contained in the plasmid PTH-CD500, was provided by J. Harris (Imperial Cancer Research Fund, London, United Kingdom) and has been described (27). The CD gene (1.54 kb) was excised using EcoRI and BamHI restriction enzymes (Promega Corp., Madison, WI) and cloned into an adenoviral shuttle vector, pACCMVlpARS(+). The resulting shuttle plasmid, encoding the CMV early promoter, pACCMVCpLpARS(+) was used to construct a replication-deficient recombinant adenoviral vector, AdCMVCD (a recombinant adenoviral vector encoding E. coli CD gene under control of the CMV promoter), following standard homologous recombination techniques (45). Briefly, the shuttle plasmid pACCMVCpLpARS(+) and adenoviral packaging vector pJM17 (provided by F. Graham, McMaster University, Hamilton, Ontario, Canada) were cotransfected into low-passage 293 cells using cationic liposome-mediated transfection with N-[1-(2,3-dioleoyloxy)propyl]-N,N,N,N-tetramethylammonium methylsulfate (Boehringer Mannheim, Indianapolis, IN). Individual plaques produced from recombinant events were isolated and validated for the presence of the CD gene and absence of adenoviral E1 gene (wild-type) by PCR. Appropriately validated plaques were purified by two subsequent passages through 293 cells. The final viral isolate was again verified by PCR and also by restriction enzyme analysis for presence of the CD gene. Quantities of AdCMVCD suitable for in vitro studies were produced by infecting 293 cells with validated viral stock as described above, purified by cesium chloride gradient ultracentrifugation, and dialysis. The preparation of AdCMVCD was titered by plaque assay using 293 cells.

**Recombinant Adenoviral Infections.** Procedures regarding infection of tumor cells in vitro with recombinant adenovirus have been described previously (44). Briefly, 1 × 10⁶ cells were plated in 6-well culture dishes (Costar, Cambridge, MA) and infected with recombinant adenovirus (AdCMVLacZ or AdCMVCD) 24 h later, at a multiplicity of 0.01 to 0.95. Cellular infections were carried out in a minimal volume of OptiMEM (Life Technologies, Inc., Grand Island, NY) at 37°C. Infection was stopped after 1.5–2 h with the addition of an equivalent volume of RPMI 1640 supplemented with 20% FBS. Cells were infected with 10 or 100 pfu/cell.

**Reporter Gene Expression.** Forty-eight h after infection with AdCMVLacZ, SK-ChA-1 and HeLa cells were analyzed by FACS analysis. HeLa cells were used for control purposes. Cells infected with AdCMVLacZ and uninfected controls were harvested 48 h following infection and resuspended at 10⁵ cells/ml in FACS staining media [PBS (Life Technologies, Inc.; 0.2 g/liter KCl, 0.2 g/liter KH₂PO₄, 8 g/liter NaCl, and 1.15 g/liter Na₂HPO₄) containing 10 mM HEPES and 4% FBS]. One hundred μl aliquots were placed into 6-ml tubes and incubated at 37°C for 10 min. One hundred μl of 1% paraformaldehyde were added to the tubes and incubated for 1 min. FACS loading was stopped by adding 1 ml of ice-cold staining media. Cells were washed in FACS buffer, harvested, and resuspended in 1% paraformaldehyde solution for 1 min. Flow cytometry was performed on a FACScan (Becton Dickinson, San Jose, CA).

**Functional Validation of the AdCMVCD Recombinant Adenovirus.** To verify the effectiveness of the AdCMVCD vector to induce expression of CD within the SK-ChA-1 cells, the viral preparation was functionally validated to confirm its ability to convert [6-3H]-5-FC to [6-3H]-5-FU. In a modification of the procedure used by Haberkorn et al. (46) to measure 5-FU production as a result of CD expression in eukaryotic cells, SK-ChA-1 cells were infected with 100 pfu/cell of AdCMVCD or AdCMVLacZ as a control and then harvested after 48 h incubation at 37°C. The cells were lysed by four freeze-thaw cycles in 100 mM Tris-HCl, 1 mM EDTA/DDT (Sigma), pH 7.8. Cellular debris was pelleted by centrifugation at 14,000 rpm for 2 min. The cytosolic fraction was separated, and 10 μl of each cell lysate were incubated with (0.5 μCi)
described above. Twenty-four h later, medium was aspirated and replaced with expressing cells following a single dose of external beam radiation, SK-ChA-1. C-9 80 cm isocenter @°Co clinical irradiator (Cleveland, OH). Following exposure, and AdCMVCD viral preparation was used for all subsequent experiments.

Sensitivity to Cellular Production of 5-FC. SK-ChA-1 cells were infected with 10 pfu/cell of AdCMVCD as described above and with appropriate controls (no viral infection with and without 5-FC exposure, and AdCMVlacZ with and without 5-FC exposure, as an irrelevant virus control). Twenty-four h later, cells were trypsinized, counted, and plated (5000 cells/well) in 96-well microtiter plates (Costar) in 12 replicates. Media were supplemented with 0, 5, 10, 30, 50, and 100 μg/ml 5-FC (Sigma), respectively. Cell proliferation was determined by colorimetric assay (CellTiter 96 AQueous nonradioactive cell proliferation assay kit; Promega Corp.) after 2, 5, and 8 days incubation. This assay measures the conversion of MTS to formazan by viable cells. Briefly, on the day of plate development, medium from each well was aspirated and replaced with 100 μl of fresh RPMI 1640 without 5-FC. To calculate cell number/well from absorbance, known numbers of uninfected cells were plated on the day of assay to generate a standard curve for comparison. According to the manufacturer’s recommendations, 20 μl of assay mixture were added to each well and incubated at 37°C for 1–2 h. The absorbance at 490 nm was then measured in a 96-well plate reader (Molecular Devices, Menlo Park, CA). Data collected by the plate reader was analyzed by the SOFTmax software package (Emax Molecular Devices, Menlo, CA).

Cytotoxicity of Combined Molecular Chemotherapy and Single Dose of External Beam Radiation. To determine an increased cytotoxic effect of CD expressing cells following a single dose of external beam radiation, SK-ChA-1 cells were infected with AdCMVCD at 10 pfu/cell and appropriate controls, as described above. Twenty-four h later, medium was aspirated and replaced with fresh RPMI 1640 supplemented with 10 or 20 μg/ml of 5-FC. After 48–72 h of exposure to 5-FC, cells were irradiated on ice at a dose of 8 Gy with a Picker C-9 80 cm isocenter @°Co clinical irradiator (Cleveland, OH). Following radiation treatment, cells and controls were trypsinized, counted, and plated in 96-well microtiter plates (5000 cells/well) in media free of 5-FC. MTS colorimetric cell proliferation assays were performed as described above, 7–11 days following radiation treatment.

Combined Molecular Chemotherapy and External Beam Radiation Dose Response. To confirm and define the nature of an enhanced cytotoxic interaction between AdCMVCD infection, 5-FC treatment, and radiation therapy, cells were infected with 10 pfu/cell of AdCMVCD, or AdCMVlacZ, treated with 20 μg/ml 5-FC for 72 h and irradiated with 0, 2, 4, or 8 Gy. The cells were irradiated on ice, then trypsinized, counted, and plated in triplicate in 25-cm² tissue culture dishes (Costar) in media free of 5-FC. Approximately 14 days later, the plates were fixed with 3:1 methanol:acetic acid and stained with 1:95 crystal violetethanol. Individual colonies (greater than 50 cells/ group) per plate were counted. Plating efficiencies (number of colonies counted per number of cells plated) for nonirradiated, virally infected/5-FC exposed groups were determined and used to correct for the survival of the corresponding groups exposed to radiation. The surviving fraction was calculated as colonies counted/cells plated × plating efficiency/100. Survival data were analyzed by the FIT version 2.4 software program (kindly provided by Dr. Norman Albright, University of California, San Francisco, CA) to generate the radiobiological descriptive parameters based on the LQ and the SHTM models. The radiation response curves were also generated by the FIT version 2.4 software. In addition, the MTD, which represents the area under the survival curve, was calculated using a modification of Fertil’s equation (Ref. 47; kindly performed by Dr. Brian Davis, Mayo Clinic, Rochester, MN).

Human Cholangiocarcinoma Tumor Therapy Model in Nude Mice. Female athymic nude mice (National Cancer Institute-Frederick Research Laboratory, Frederick, MD) were injected i.c. with 2 × 10⁶ SK-ChA-1 cells in 50 μl of PBS in the right flank. Tumors with diameters of 5–10 mm developed 7 days after injection. Animals then received intratumoral injections of AdCMVCD, at 1 × 10⁶ pfu in a 50 μl volume, using a 25-gauge needle, on days −2, 0, and +2 relative to radiation treatment. Animals were administered 5-FC in doses of 400 mg/kg twice daily by i.p. injection beginning on day −2 and continuing for 7 days. Animals in the radiation treatment group (n = 10) were anesthetized with 2 mg of ketamine (Phoenix Scientific, Inc., St. Joseph, MD) by i.p. injection and received a 10-Gy dose of 60Co radiation to their tumor on day 0, 2 days after initial AdCMVCD injection and 5-FC administration. The nonirradiated animals (n = 10) served as controls. Tumor diameters were measured with a Vernier caliper three times weekly, and the surface area (product of length x width) was calculated. Animals were fed sterilized mouse diet and sterilized tap water.

Statistical Analysis. The reporter gene expression in SK-ChA-1 cells represents the mean of three independent experiments. Logistic regression was used to verify an interaction between pfu/cell, cell type, and to estimate the likelihood of cellular infection.

To determine an interaction between viral infection (AdCMVCD, AdCMVLacZ, or no virus), dose of 5-FC, and duration of 5-FC exposure, a three-factor ANOVA was used. In addition, pair-wise comparisons between specific groups was performed to verify significant differences. These experiments were repeated three times, for confirmation of experimental findings, and the data presented are a representative result.

A four-factor analysis of viral infection (AdCMVCD, AdCMVLacZ, or no virus), dose of 5-FC, radiation exposure, and time following radiation exposure was used to compare the effects of a single radiation exposure on toxicity to SK-ChA-1 cells. Pair-wise comparisons were also performed between specific groups. These experiments were repeated at least three times for confirmation of experimental findings, and the data presented are a representative result.

Radiation dose-response data presented represent the mean of three independent experimental replicates. Logistic regression was used to determine the effect of viral infection (AdCMVCD, AdCMVLacZ, or no virus), 5-FC dose, and 60Co exposure. A two-sample test for proportions was also used to compare the effects of viral infection and radiation, with specific doses of radiation alone.

For analysis of tumor response in the animal model, the log-rank test was used to compare overall animal survival, time to tumor regrowth, and time for tumor doubling in size for the two groups of animals administered AdCMVCD and 5-FC and treated with 10 Gy radiation or no radiation. Comparisons of final tumor sizes between the two groups were performed using analysis of covariance, adjusting for the original tumor size as a covariate. ANOVA was used to compare the changes in tumor size (final tumor size minus original tumor size) and also to determine the ratio of the final tumor size to the original tumor size between the 10-Gy and no radiation groups. Another experiment using an AdCMVLacZ infection and AdCMVCD infection was performed, and due to the small sample size, Wilcoxon’s Rank Sum test was used to compare final tumor size, change in tumor size, and ratio of final tumor size to the original tumor size between these two groups.

RESULTS

FACS analysis indicated that SK-ChA-1 cells were efficiently transduced to a level of 48.7 and 99.2% by the recombinant adenovirus AdCMVLacZ at 10 and 100 pfu/cell, respectively. Infectivity of this cholangiocarcinoma cell line was compared to HeLa cells, which were 96.5 and 98.6% transduced by 10 and 100 pfu/cell of AdCMVLacZ, respectively. Thus, this cholangiocarcinoma cell line was less transducible with the reporter virus at 10 pfu/cell compared with the HeLa cells (P > 0.001) but transduced to a similar level with 100 pfu/cell.

Functional validation of the AdCMVCD viral preparation revealed 61 versus 1.7% conversion of [6-³H]5-FC to [6-³H]5-FU in SK-ChA-1 cells infected with 100 pfu/cell AdCMVCD or AdCMVLacZ, respectively.

In regard to adenoviral vector-mediated delivery of the CD gene inducing cytotoxicity after conversion of 5-FC to 5-FU, Fig. 1 shows the survival of AdCMVCD-infected SK-ChA-1 cells exposed to incrementally higher concentrations of 5-FC with 2, 5, and 8 days of...
prodrug exposure. There were no differences between uninfected/5-FC controls, AdCMVCD/5-FC, or AdCMVLacZ/5-FC-treated groups with 2 days of 5-FC exposure. With 5 days of 5-FC exposure, control groups (uninfected cells with and without 5-FC exposure, AdCMVCD infected cells without 5-FC exposure, and AdCMVLacZ infected cells with and without 5-FC exposure) approximately doubled in cell number, indicating a growing population of cells. However, with 5 days of 5-FC exposure, AdCMVCD/5-FC-treated groups (10, 30, 50, and 100 µg/ml) showed significantly lower cell numbers than the control groups (P < 0.001), which represented 32.1, 47.9, 52.4, and 56.5% cell killing relative to AdCMVCD/0 µg/ml 5-FC, respectively. AdCMVCD infection and exposure to 5, 10, 30, 50, and 100 µg/ml of 5-FC induced 37.9, 55.0, 77.0, 81.1, and 84.4% cytotoxicity compared to no viral infection groups at 8 days of 5-FC exposure, respectively (P < 0.001). The cytotoxicity of 5-FC conversion in AdCMVCD-infected cells appeared to be progressive with time and a function of concentration. The prodrug, 5-FC, alone was nontoxic to the cells in concentrations ranging from 5—100 µg/ml, even in the presence of nonspecific viral (AdCMVLacZ) infection. However, there appeared to be a nonspecific viral toxicity compared to no viral infection. This effect was observed in cells infected with AdCMVLacZ as well as AdCMVCD/0 µg/ml of 5-FC at day 2, accounting for 16.3—22.8% toxicity. A similar toxicity of 17—25% was observed at day 5 in the AdCMVLacZ-infected cells and AdCMVCD/0 µg/ml 5-FC groups.

Intracellular production of the radiosensitizing drug 5-FU and subsequent radiation therapy represents a novel treatment combination for cholangiocarcinoma. To this end, Fig. 2 shows the cytotoxic effects of a single 8-Gy radiation exposure superimposed on AdCMVCD infection and 5-FC treatment. There was significant cytotoxicity observed with AdCMVCD infection and exposure to 10 or 20 µg/ml of 5-FC exposure compared to noninfected cells at the three time points in the nonirradiated controls (P < 0.01; Fig. 2A). This difference in cell numbers represents 51 and 54.7%, 53.1 and 57.5%, and 34 and 34.2% cell killing for 10 or 20 µg/ml 5-FC at days 7, 9, and 11, respectively. There was significant killing of uninfected SK-ChA-1 cells by a single 8-Gy radiation exposure of 57.1% at 7 days, 83.1% at 9 days, and 87.8% at 11 days (Fig. 2B). Despite this baseline toxicity, an increased cytotoxic effect was noted at 11 days in AdCMVCD-infected cells exposed to 10 or 20 µg/ml 5-FC with a single 8-Gy dose of radiation compared to uninfected irradiated cells (P < 0.01), representing 76.8 and 71.8% cell killing, respectively (Fig. 2B).

To further define the interaction between intracellular 5-FU production and radiation-induced cytotoxicity, we performed a radiation dose-response analysis. In this regard, Fig. 3 illustrates the effects of combined modality (AdCMVCD, 20 µg/ml 5-FC with varying radiation exposures) treatment on SK-ChA-1 cells. Plating efficiencies varied from 17.6—22.7% in the control groups, and in the AdCMVCD/20 µg/ml 5-FC group, the plating efficiency was 6%. The radiobiological parameters calculated from the LQ and SHMT models are listed in Table 1. The AdCMVCD/20 µg/ml 5-FC treatment group had the largest α parameter of 0.44, and AdCMVCD/0 µg/ml 5-FC followed with 0.32. The α:β ratio was of greater magnitude in the AdCMVCD groups compared to controls. The β values were similar for all groups. In regard to the SHMT model, the D0 value for AdCMVCD/20 µg/ml 5-FC was the smallest value obtained from the experimental groups at 0.96, whereas the other values ranged from 1.26—1.48. Additionally, D10s for the AdCMVCD groups (1.36 and 1.33) were smaller than for the control groups (1.7—2.7). Another value used to describe the radioresponsiveness of mammalian cells in vitro is the MID. The AdCMVCD/20 µg/ml 5-FC treatment group had a value of 1.04, whereas the value for AdCMVCD/0 µg/ml 5-FC was 2.29, indicating
AdCMVCD and 5-FC with or without 10 Gy radiation treatment is shown in Fig. 4. The combined treatment modalities of intratumoral CD delivery, systemic 5-FC administration, and radiation resulted in significant differences in tumor growth compared to AdCMVCD- and 5-FC-treated animals without radiation. Overall survival, time to tumor regrowth, and doubling time were significantly longer in the radiation treatment group ($P = 0.03, 0.015, \text{ and } 0.002$, respectively). ANOVA was adjusted for the original tumor size to allow determination of treatment-induced tumor response. This analysis indicated significantly greater change in tumor size, smaller ratio of final tumor

over a 2-fold increase in the cytotoxicity due to the 5-FC exposure. Other control groups had a MID in the range of 2.5–3.2.

Stringent mathematical modeling to verify a synergistic interaction between CD-mediated production of 5-FU and radiation therapy was also performed. Synergistic cytotoxicity is defined as cytotoxicity of greater magnitude with simultaneously delivered treatments than the summed cytotoxic effects of single treatments. Strict synergistic interaction between AdCMVCD infection, 20 μg/ml 5-FC treatment, and radiation exposure was not observed in this experimental model. However, a significant positive cytotoxic interaction of these treatments was indicated by the results described above.

The growth of SK-ChA-1 tumors in animals injected with

Fig. 2. Evaluation of cytotoxicity by combination of AdCMVCD-mediated conversion of 5-FC to 5-FU and external beam radiation. A, nonirradiated SK-ChA-1 cells, infected 24 h after plating with AdCMVCD, AdCMVLaCZ, or no virus, treated with 5-FC on days 3–5 after plating, and replated 24 h later; the cell number was counted 7–11 days later. B, SK-ChA-1 cells, infected 24 h after plating with AdCMVCD, AdCMVLaCZ, or no virus, were treated with 5-FC on days 3–5 after plating. Twenty-four h later, SK-ChA-1 cells were exposed to 8 Gy 60Co and replated, and the cell number was counted 7–11 days later. The data points are the mean cell counts of six replicates. Bars, SD. •, AdCMVCD untreated; ◆, AdCMVCD treated with 10 μg/ml 5-FC; △, AdCMVCD treated with 20 μg/ml 5-FC; +, AdCMVLaCZ treated with 20 μg/ml 5-FC; ○, no viral infection.

Fig. 3. Radiation cell survival curves for the human cholangiocarcinoma cell line SK-ChA-1 infected with AdCMVCD treated with 20 μg/ml 5-FC (◆), AdCMVCD without 5-FC (○), AdCMVLaCZ treated with 20 μg/ml 5-FC (△), or no virus without 5-FC (□) prior to 60Co exposure. Clonogenic assays were then performed. The surviving fractions for each treatment condition are presented. Data points represent the mean of three experiments, each performed in triplicate. Bars, SD. A, SHMT model. B, LQ model.
size to original tumor size, and smaller final tumor size in the radiation treatment group compared to the no-radiation treatment group ($P = 0.02, 0.03,$ and $0.03$, respectively). There were 0 of 20 treatment-related deaths at 60 days from initial viral injection. There was a 10% tumor regression rate in the nonirradiated group and a 20% regression rate in the radiated group, but given the small sample size, this difference was not statistically significant. In another experiment, AdCMVLacZ or AdCMVCD was administered in a single intratumoral injection, followed by 5-FC administration and radiation treatment. Although differences in the time to tumor regrowth, time to doubling of tumor size, and overall survival were observed, because of the small sample size ($n = 4$ and 5, respectively), these differences did not reach statistical differences (data not shown).

**DISCUSSION**

Since Gerald Klatskin’s description of adenocarcinoma arising at the hepatic duct bifurcation, the difficulties regarding the diagnosis and treatment of patients with this malignancy continue (48). As advances in therapeutics conferred improved survival to patients with other malignancies, the application of these modalities to cholangiocarcinoma has had little impact on the outcome of biliary malignancy (3–5). Due to minimal therapeutic efficacy observed with traditional multimodality treatment, molecular chemotherapy represents a new and potentially useful therapeutic option for a multidisciplinary approach to cholangiocarcinoma treatment.

The underlying biological principle basic to any gene therapeutic treatment strategy depends on efficient transfer of novel genetic material to the target cell. Specific infectivity of human tissues and tumor cells by adenoviral vectors varies; therefore, accurate determination of the percentage of infected cells is pertinent. Target cell susceptibility to recombinant adenoviral infection is a basic requirement for subsequent attempts to use these vectors for human gene therapy strategies. Also of critical importance is to verify expression of newly introduced genetic material in the target cells.

The high percentage of reporter gene expression ($β$-galactosidase) in the SK-ChA-1 cell line confirms delivery to and expression of the LacZ gene. This observation confirms our group’s previous report of introduction and expression of other novel genes (HSV-TK, LacZ, and firefly luciferase) in cholangiocarcinoma cell lines via adenoviral vectors. The same recombinant adenoviral backbone was used to construct the AdCMVCD vector and to verify the effectiveness of this vector to induce expression of CD within the SK-ChA-1 cells; the viral preparation was functionally validated for its ability to convert [6–3H]5-FC to [6–3H]5-FU. This analysis revealed a high rate of conversion in cells infected with AdCMVCD but not in cells infected with AdCMVLacZ. Demonstration of this 5-FC to 5-FU conversion in AdCMVCD-infected SK-ChA-1 cells confirms the presence of functional CD enzyme.

Another concern regarding human gene therapeutics involves vector delivery to the anatomical compartment of disease. In this regard, cholangiocarcinoma is an attractive target for cancer gene therapy due to the inherent compartmentalization of the biliary system. The anatomically privileged site of the biliary tree has the advantage of limiting dispersal of the vector, in situ cellular transduction is mandatory for solid tumor treatment. To this end, Vickers et al. (49) have shown the ability to infect human biliary epithelium in an explanted human liver with AdCMVLacZ. In addition, they were able to selectively cannulate the left hepatic duct and achieve infection of a segment of the biliary tree, while the excluded right ductal system was not infected.

Traditional applications of chemotherapy to cholangiocarcinoma are ineffective and limited by dose-related systemic toxicity. Harvey et al. (50) reported a 31% response rate to three-agent therapy (5-FU, mitomycin, and doxorubicin) in a Phase II trial for cholangiocarcinoma. Hsue et al. (12) recently published the results of a Phase I trial of combined 5-FU and folinic acid in patients with pancreatico-biliary carcinoma. They concluded that a maximum dose for 5-Hi was 350 mg/m²/day, above which patients experienced intolerable side effects (12). Other in vivo studies of cholangiocarcinoma cell lines indicate

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**Table 1 Radiobiological parameters of in vitro survival curves for human cholangiocarcinoma cell line SK-ChA-1 infected with AdCMVCD, AdCMVLacZ, or no viral infection, treated with 5-FC and exposed to 60Co radiation**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Linear quadratic parameters</th>
<th>SHMT parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha$</td>
<td>$\beta$</td>
</tr>
<tr>
<td>No viral infection, 0 $\mu$g/ml 5-FC</td>
<td>0.03</td>
<td>0.062</td>
</tr>
<tr>
<td>No viral infection, 20 $\mu$g/ml 5-FC</td>
<td>0.131</td>
<td>0.051</td>
</tr>
<tr>
<td>AdCMVLacZ, 0 $\mu$g/ml 5-FC</td>
<td>0.239</td>
<td>0.037</td>
</tr>
<tr>
<td>AdCMVCD, 0 $\mu$g/ml 5-FC</td>
<td>0.188</td>
<td>0.045</td>
</tr>
<tr>
<td>AdCMVCD, 20 $\mu$g/ml 5-FC</td>
<td>0.323</td>
<td>0.030</td>
</tr>
<tr>
<td>AdCMVCD, 20 $\mu$g/ml 5-FC</td>
<td>0.444</td>
<td>0.054</td>
</tr>
</tbody>
</table>

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that high-dose regimes have cytotoxic effects, but equivalent doses of chemotherapeutics in humans are not tolerated (51).

Molecular chemotherapy has been well described in reference to the toxin gene/prodrug system, CD-5-FC. This therapeutic strategy has the advantage of intracellular production of chemotherapeutic drug as an alternative to systemic administration, therefore potentially limiting systemic toxicities (29). Once infection and reporter gene expression was confirmed, sensitivity of this cholangiocarcinoma cell line to cellular production of 5-FU was determined. Because the mechanism for delivery of cytotoxic drug is via intracellular enzymatic production, 5-FC was added to the culture media; subsequent cellular conversion to 5-FU resulting in cell death was measured. We observed that AdCMVCD-mediated conversion of 5-FC to 5-FU was cytotoxic to SK-ChA-1 cells. The effect was most dramatic in the higher 5-FC concentration range but was present to a lesser extent with lower doses of 5-FC. Lack of cytotoxic effects in control cells (uninfected cells with and without 5-FC exposure, AdCMVLacZ-infected cells with and without 5-FC exposure, and AdCMVCD-infected cells without 5-FC exposure) and observed toxicity in the experimental treatment groups (AdCMVCD-infected cells exposed to various 5-FC concentrations) suggest AdCMVCD infection, and treatment with 5-FC was toxic to cells otherwise growing rapidly. Ancoban (5-FC) is used clinically for antifungal therapy and in this regard is typically administered daily for 6 weeks. Nontoxic systemic serum levels of 50–100 μg/ml are achievable with oral administration of 50–150 mg/kg/day in four divided doses (52). Our findings of effective cell killing with tolerable equivalent human doses of 5-FC support this approach for molecular chemotherapy.

The roles for radiation therapy in the treatment of malignancy have been both as a single or multiple agent, but radiation therapy is more commonly used in an adjuvant or neo-adjuvant manner. To date, radiation therapy for cholangiocarcinoma is a variety of clinical settings (external beam, intraoperative, iridium wire brachotherapy, and radiolabeled anti-CEA monoclonal antibodies) has had minimal effect on patient survival, although 6–12 months survival advantage has been reported (1, 2).

The combination of treatments (AdCMVCD infection, 5-FC administration, and radiation) was highly toxic to the SK-ChA-1 cells. The low cell numbers shown in Fig. 2B reflect this toxicity, because equivalent numbers of cells were plated in each group for both panels A (no radiation control) and B. The MTS assay was not sensitive enough to distinguish small differences in survival of these toxic treatments. However, these studies using the MTS assay indicated the significant toxicity of the combined treatments, and also that a low dose of 5-FC induced a cytotoxic effect with the addition of a high dose of radiation. These preliminary studies were performed to determine if an interactive treatment effect occurred. Because these experiments were suggestive of an enhanced cytotoxic effect, a more detailed radiation dose-response analysis was performed to elucidate the nature of the interaction.

The clonogenic survival assay is the standard method to measure radiation effects on mammalian cells and can accurately demonstrate treatment interactions, even in highly toxic treatment combinations. The plating efficiency describes the ability of a particular cell type, when placed into a flask as a single-cell suspension, to adhere and form a visible colony in 2–3 weeks. This ability varies per cell line, and as we observed, was low for SK-ChA-1 cells, although suitable to generate a radiation dose-response analysis. The radiobiological parameters derived from the clonogenic assay were indicative of a significant radiosensitization effect on SK-ChA-1 cells induced by the combined treatments. The AdCMVCD/20 μg/ml 5-FC group had the largest α value and a large α/β ratio, indicating significant cytotoxicity in the low (2 Gy) radiation dose region. This is an important observation with respect to clinical considerations, because most radiation therapy is administered in multiple low-dose fractions. In addition, the small $D_0$ for the AdCMVCD/20 μg/ml 5-FC group suggests there is also significant radiation-induced killing at the higher radiation doses. An interesting effect noted in the AdCMVCD/0 μg/ml 5-FC group, and to a lesser extent in the AdCMVLCaZ groups, was an intermediate range of α values. These intermediate values suggest radiosensitization of the cells as a result of adenosinergic infection alone. Other investigators’ studies with toxin gene/prodrug therapy systems have not described this phenomenon. Kim et al. (42) transduced gliosarcoma cells with a retrovirus encoding HSV-TK, added GCV, and exposed the cells to radiation. Their control groups did not reveal any change in radiosensitivity compared to the experimental groups (42). Gallardo et al. (53) explored the alteration of radiosensitivity in ovarian carcinoma cells following insertion of the p53 gene via adenosinergic infection. Their control virus, encoding the luciferase gene, did not alter the radiosensitivity of SK-OV-3 cells. In addition to viral vectors, insertion of control plasmid vectors by Yamagishi et al. (54) did not confer nonspecific radiosensitivity to melanoma or fibroblast cells. The significance of our experimental observation is uncertain but could be pertinent in human cancer gene therapy applications.

Radiobiological survival curve parameters in Table 1 were derived from mathematical models LQ and SHMT, describing mammalian cell survival following radiation treatment in vitro. The MID is another value to describe radiosensitivity of mammalian cells in vitro and which represents the area under the survival curves in Fig. 3 (47, 55, 56). This value provides a measure of the radiosensitivity of the cells in each treatment group and determination of whether treatment interaction occurs resulting in lower cell survival. The lower MID for the combined modality group (1.04) and a 2-fold higher value for the next closest control group indicate at least an additive effect occurred with molecular chemotherapy and radiation treatment. Using the MID to compare tumor cell types has an advantage over the standard radiobiological parameters α, β and $D_0$ in that an overall radiation effect may be described, rather than only the effect at high or low radiation doses (47).

Our results suggest that although not strictly synergistic, there was a significant positive cytotoxic interaction between radiation therapy and AdCMVCD-mediated conversion of 5-FC to 5-FU, resulting in enhanced cholangiocarcinoma cell killing. One limitation in the mathematical proof of synergism relates to the low plating efficiency of the AdCMVCD-infected, 20 μg/ml 5-FC-treated cells. Specifically, because each treatment (AdCMVCD infection with 5-FC treatment and external beam radiotherapy) was highly toxic to the cells, the combination of the two induced near total cell death, making measurement of a greater than additive cellular response difficult to quantitate.

Combined treatment (AdCMVCD infection, 5-FC, and radiation) to SK-ChA-1 tumors in animals significantly inhibited tumor growth compared to animals receiving AdCMVCD infection and 5-FC treatment without radiation. Despite the inability to identify a synergistic interaction in vitro, the tumor response in the animal model demonstrated an enhanced antitumor effect with the combination of molecular chemotherapy and radiation.

We intend to explore a clinical model for the utility of molecular chemotherapy in combination with external beam radiation therapy for cholangiocarcinoma treatment. The rationale for this approach is based on the dose-limiting toxicity of currently used chemotherapeutics and their ineffectiveness in disease control. Future experimental efforts will include optimization in an animal model and ultimately a human clinical trial.
REFERENCES


Molecular Chemotherapy Combined with Radiation Therapy Enhances Killing of Cholangiocarcinoma Cells in Vitro and in Vivo

Lee C. Pederson, Donald J. Buchsbaum, Selwyn M. Vickers, et al.