Effective Gene Therapy of Biliary Tract Cancers by a Conditionally Replicative Adenovirus Expressing Uracil Phosphoribosyltransferase: Significance of Timing of 5-Fluorouracil Administration

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Abstract

In order to enhance the efficacy of conditionally replicating adenoviruses (CRAd) in the treatment of cancers of the biliary tract, we studied the efficacy in vitro and in vivo of AxE1CAUP, a CRAd vector that carries a gene for uracil phosphoribosyltransferase (UPRT), which converts 5-fluorouracil (5-FU) directly to 5-fluorouridine monophosphate and greatly enhances the cytotoxicity of 5-FU. AxE1CAUP replicated and induced an increased UPRT expression in biliary cancer cells more efficiently than AxCAUP, a non-replicative adenovirus carrying the UPRT gene. Whereas AxCAUP and AxE1AdB, a CRAd without the UPRT gene, modestly increased the sensitivity of BC cells to 5-FU, AxE1CAUP markedly increased the sensitivity, especially when the timing of 5-FU administration was appropriately chosen. AxE1CAUP replicated much less efficiently in normal WI-38 fibroblasts without any change in the sensitivity to 5-FU. In nude mice with s.c. biliary cancer xenografts, i.t. AxE1CAUP/5-FU therapy inhibited tumor growth significantly more strongly than AxCAUP/5-FU or AxE1AdB/5-FU therapy. Furthermore, in mice with peritoneally disseminated biliary cancer, i.p. AxE1CAUP efficiently proliferated in the tumors, decreased the tumor burden, and prolonged the survival of the mice when 5-FU was started 10 or 15 days after the vector inoculation, whereas earlier initiation of 5-FU resulted in early eradication of the vector and no survival benefit. The present study shows that the CRAd expressing UPRT was a more potent sensitizer of BC cells to 5-FU, and that timing of 5-FU administration was a key factor to maximize the efficacy. This gene therapy with appropriately timed administration of 5-FU should be useful in overcoming the resistance of biliary cancers to 5-FU. (Cancer Res 2005; 65(2): 546-52)

Introduction

The prognosis of patients with biliary cancer (BC), cancers of the gallbladder, or bile ducts, is dismal because of the late diagnosis, high incidence of postsurgical local and/or regional recurrence, and frequent distant metastases. Although BC is curable at its early stages, only 10% to 30% of the patients can be considered candidates for curative surgery (1, 2). Many patients with advanced BC receive 5-fluorouracil (5-FU)–based chemotherapy, but the response rate is quite low and the 2-year survival rate is < 8%, with median survival of < 6 months (2–4). New approaches for overcoming resistance to chemotherapy are required to extend the survival. Gene-directed enzyme/prodrug therapy, known as “suicide gene therapy”, such as herpes simplex virus thymidine kinase/ganciclovir (HSVtk/GCV) therapy or cytosine deaminase/5-fluorocytosine therapy, seem to be promising approaches for the treatment of various cancers, but there have been few studies of gene therapy for BC (5).

Conversion of 5-FU to its active and cytotoxic metabolites, 5-fluorodeoxyuridine triphosphate (5-FUTP) and 5-fluorodeoxyuridine monophosphate, requires several steps of enzyme reaction, which are critical in the tumor-killing effect of 5-FU (6). 5-FUTP is incorporated into RNA to yield F-RNA and such F-RNA disrupts the functions of the RNA, whereas 5-fluorodeoxyuridine monophosphate blocks the catalytic activity of thymidylate synthetase by forming a ternary covalent complex with its cosubstrate, 5,10-CH2-FH4, which in turn inhibits DNA synthesis. Uracil phosphoribosyltransferase (UPRT; EC 2.4.2.9) of Escherichia coli is a pyrimidine salvage enzyme that catalyzes the synthesis of fluorouridine monophosphate from uracil and phosphoribosylpyrophosphate. In microorganisms, UPRT converts 5-FU directly to 5-fluorouridine monophosphate as the first step in the activation of 5-FU pathway. By contrast, in mammalian cells, which lack UPRT, 5-FU is converted to 5-fluorouridine monophosphate via a two-step route that is activated only when 5-FU is present at high concentrations. Adenovirus-mediated transduction of UPRT gene markedly sensitzes cancer cells to a low concentration of 5-FU (7–9) and such gene therapy might be an effective approach to overcoming resistance to 5-FU. However, gene therapy trials using nonreplicative viruses as vectors have not been successful, primarily because of the limited efficiency of gene delivery (10, 11).

Conditionally replicating adenoviruses (CRAds) have recently emerged as potentially ideal tools for the treatments of solid tumors (15–17, reviewed in refs. 11–14). Indeed, clinical trials with ONYX-015 (dl1520) in combination with chemotherapy have yielded remarkably good results (14, 17). ONYX-015 is a mutant adenovirus lacking a p53-binding protein of M, 55,000 that is encoded by E1B and it selectively replicate in and subsequently lyse cancer cells that have abnormalities in p53 signaling pathways (14–17). We recently described the experimental efficacy of two CRAds, i.e. AxE1AdB, which encodes a defective E1B-55 kDa protein, and AxdAdB-3, an E1A, E1B double-mutant adenovirus, in the treatment of BC (18).
These approaches seem to be effective in BCs, which have abnormalities in both retinoblastoma protein–related pathways and p53-related pathways at high rates (19–23).

Several groups of investigators have explored the usefulness of a combination of a suicide gene approach with CRAds to enhance antitumor effects, but results have been inconsistent (24–31). In the present study, we aimed to clarify the efficacy in vitro and in vivo against BC of AxE1CAUP gene, an E1B-55 kDa defective CRAD that is armed with the UPRT gene. Our results suggest that gene therapy with the CRAD expressing UPRT is a potentially useful approach for the treatment of advanced BCs and, moreover, that the timing of administration of 5-FU is a critical factor to maximize its efficacy.

Materials and Methods

Cell Lines and Culture. TGBC-ITKB, a line established from a gall-bladder adenocarcinoma, and TGBC-44TKB, a line of poorly differentiated adenocarcinoid gallbladder cancer cells, have a deficient p53 (data not shown) and were established by T. Todoroki (32). The Sk-ChA-1 line, which was established from a bile duct cancer and provided by Dr. A. Knuth (Johannes-Gutenberg University, Mainz, Germany; ref. 33) has a mutated p53. Human embryonic kidney 293 cells were purchased from the American Type Culture Collection (Manassas, VA). A line of human fibroblasts, WI-38 (RCB 702), was purchased from RIKEN Cell Bank (Tsukuba, Ibaraki, Japan). All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum.

Recombinant Adenoviruses. Construction of AxCAUP (7), a recombinant adenovirus that encoded a gene for UPRT driven by the CAG promoter; AxCALacZ (34), a recombinant adenovirus containing a LacZ gene, and AxE1AdB (35), a recombinant adenovirus lacking the gene for E1B-55 kDa was described previously. AxE1CAUP was constructed essentially by the COS-TPC method (36) as follows: the 2,832-bp Sal/HindIII fragment from pCaupp, which contains cDNA for UPRT (649 bp) under the control of the CAG promoter, was inserted at the Sal/HindIII site of the E1AdB cassette in pSkAdB/E1AdB to yield pCaupp-E1AdB. The blunted fragment that contained both the Caupp and E1AdB expression cassettes was ligated to the SwoI site of pAdex1cw to yield pAxE1CAUP. AxE1CAUP was obtained by cotransfecting 293 cells with pAxE1CAUP cosmide DNA together with the adenovirus genomic DNA-terminal protein complex. The TGA stop codon at the third codon of E1B55K in AxE1CAUP was confirmed by DNA sequencing. Adenovirus vectors were propagated in 293 cells, purified by CsCl gradient centrifugation, and preparations were titrated thrice with 293 cells by the standard plaque-forming assay. The two E1-mutant adenoviruses, AxE1AdB and AxE1CAUP, had a deletion in the gene for E1B-55 kDa, which binds to p53 and inhibits its function.

Transduction Efficiency of Adenoviruses. Cells were seeded in six-well plates at a density of 5 × 10^5 cells/well. After incubation for 24 hours, cells were infected with AxCALacZ at a multiplicity of infection (MOI) rate of 0.1 to 100 and were incubated at 37°C for 24 hours. To evaluate the expression of LacZ, the cells were fixed with 0.25% glutaraldehyde and stained by X-gal. Blue stained cells were counted in five independent microscopic fields (×400).

Viral Replication. Cells were seeded in 12-well plates in the appropriate medium at a density of 1 × 10^5 cells/well and were infected with various adenoviruses at a MOI of 10. Fresh medium was replaced 6 hours after infection. The cells and culture supernatant were harvested 2, 4, and 6 days after infection. The cells were sonicated and virus particles in lysates were quantitated. The titer of each adenovirus was evaluated by the standard plaque-forming assay with 293 cells.

Western Blotting of Adenovirus E1A and UPRT. The replication of adenovirus vectors and the transgene expression of UPRT were examined on Western blot. Cells were infected with different adenovirus vectors at a MOI of 10 followed with or without 5-FU administration at different doses (0.01, 0.1, 1, or 10 μmol/L) and then incubated for 1 to 120 hours. The cells were then harvested and lysed with radioimmunoprecipitation assay buffer [150 mmol NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mmol EDTA (pH 7.5), and 50 mmol Tris-HCl (pH 7.4)]. The cell lysate was separated on SDS-PAGE and transferred to an acrylamide membrane. To detect adenovirus E1A, the membrane was incubated with anti-Ads-E1A mouse monoclonal antibody (Ab-1; NeoMarkers, Fremont, CA) and to examine the expression of UPRT protein, the membrane was incubated with anti-UPRT rabbit polyclonal antibody (Cayla, Toulouse, France), for 120 minutes at room temperature. Signals were visualized with the enhanced chemiluminescence-plus system (Amersham, Buckinghamshire, England).

Assay of F-RNA in Biological Cancer Cells. The concentration of F-RNA was determined as described previously (37). TGBC-ITKB cells (1 × 10^7) were infected with different adenovirus vectors at a MOI of 1 or 10. Cells were incubated at 37°C for 72 hours and then exposed to medium that contained 1 μmol/L 5-FU. After 48 hours, the cells were collected and levels of F-RNA were measured by gas chromatography-mass spectrometry.

Sensitivity of Cells to 5-FU in vitro. A cytotoxic effect assay was done to determine whether transgene expression of UPRT increased the sensitivity of cells to 5-FU. BC cells and WI-38 cells were seeded in 96-well plates at a density of 10^5 cells/well with DMEM that contained 10% fetal bovine serum. Cells were infected with different adenoviruses at a MOI of 1, 10, or 100. Then, 24, 72, or 120 hours after infection, cells were exposed to various concentrations of 5-FU (0.001-1,000 μmol/L) and incubated at 37°C for another 5 days. Viable cells were quantitated by a colorimetric WST-1 assay as described previously (18). The 50% inhibitory concentration (IC50) of 5-FU was calculated for each set of conditions.

Animal Studies. Four-week-old female BALB/c nu/nu athymic mice (CLEA Japan, Tokyo, Japan) were quarantined for 1 week. A s.c. BC xenograft model was prepared by injecting TGBC-44TKB (1 × 10^7) cells in 100 μL of DMEM without serum into the left flank of each mouse. Tumors were measured with calipers and the volume of each tumor was calculated as 0.41 × longest diameter × width^2. Fourteen days after inoculation of tumor cells, the mice were randomly divided into eight groups (n = 6) and received i.t. injection of 100 μL of PBS or various adenovirus vectors [2 × 10^7 plaque-forming unit (pfu)/day] on days 1 to 3 followed with or without i.p. 5-FU (10 mg/kg/day) on days 4 to 9 or 7 to 12. The eight groups were as follows: (a) PBS group, (b) 5-FU group (i.p. 5-FU on days 4-9), (c) AxCAUP/5-FU group (i.t. AxCAUP, followed by i.p. 5-FU on days 4-9), (d) AxCAUP group (i.v. AxCAUP alone), (e) AxCAUP/5-FU-d4 group (i.v. AxCAUP followed by i.p. 5-FU on days 4-9), (f) AxCAUP/5-FU-d7 group (i.v. AxCAUP followed by i.p. 5-FU on days 7-12), (g) AxE1AdB/5-FU-d4 group (i.v. AxE1AdB followed by i.p. 5-FU on days 4-9), and (h) AxE1AdB/5-FU-d7 group (i.v. AxE1AdB followed by i.p. 5-FU on days 7-12).

An i.p. dissemination model was prepared by i.p. injection of TGBC-44TKB (1 × 10^7) cells in DMEM (200 μL) without serum with subsequent growth of tumors for 14 days to allow i.p. dissemination of cells. Then, mice were randomly divided into six groups (n = 10) and received i.p. injection of 200 μL of PBS or AxE1CAUP (2 × 10^7 pfu/day) on days 1 to 3 followed with or without i.p. 5-FU (10 mg/kg/day) for 10 consecutive days beginning from days 9, 14, or 19. The six groups were as follows: (a) PBS group, (b) 5-FU group (i.p. 5-FU on days 9-18), (c) AxCAUP group (i.p. AxCAUP alone), (d) AxCAUP/5-FU-d4 group (i.v. AxCAUP followed by i.p. 5-FU on days 9-18), (e) AxCAUP/5-FU-d7 group (i.v. AxCAUP followed by i.p. 5-FU on days 9-18), (f) AxE1AdB/5-FU-d4 group (i.v. AxE1AdB followed by i.p. 5-FU on days 9-18), and (g) AxE1AdB/5-FU-d7 group (i.v. AxE1AdB followed by i.p. 5-FU on days 7-12).

Gene Therapy With CRAD To Overcome Resistance To 5-FU
tissues were fixed in PBS that contained 2% glutaraldehyde and ultrathin sections were stained with uranium acetate and lead nitrate before examination with an electron microscope (H7000; Hitachi, Co., Tokyo, Japan). Effects of the various treatments on survival were compared using the rest of the mice.

**Statistical Analysis.** The significance of differences between groups was assessed by Student’s unpaired two-tailed t test. Survival was analyzed by the Kaplan-Meier method and differences were analyzed by the log-rank test.

**Results**

**Transduction Efficiency and Replication of Adenoviruses in Biliary Cancer Cells and WI-38 Cells.** We examined the transduction efficiency of adenoviruses in three lines of BC cells by infecting cells with AxCA LacZ at various doses. For transduction of Lacz in 50% of TGBC-44TKB cells, Sk-ChA-1 cells, and TGBC-1TKB cells, we had to use MOIs of 10.7, 3.2, and 189.5, respectively.

We examined the replication of various adenovirus vectors in BC cells (Fig. 1A). The two E1B-55 KDa defective CRAds, AxE1CAUP, and AxE1AdB, proliferated effectively (150-1,000 times the initially given doses within 4 days) in TGBC-44TKB cells. These two CRA ds also proliferated effectively in TGBC-1TKB cells, although with slower speed. By contrast, proliferation of these adenoviruses was markedly inhibited in WI-38 cells, a line of normal fibroblasts. We confirmed the replication of AxE1CAUP in BC cells by monitoring expression of adenovirus E1A (Fig. 1B), which increased in a time-dependent manner. After exposure to 5-FU at all tested doses (0.01, 0.1, 1, and 10 μmol/L) immediately after vector inoculation, we were unable to detect any increase in E1A in the cells (Fig. 1B; only data obtained with 0.01 and 0.1 μmol/L 5-FU are shown).

**Expression of UPRT and Production of F-RNA from 5-FU in Biliary Cancer Cells After Infection with Replicative and Nonreplicative Adenoviruses.** We examined the expression of UPRT in BC cells and in WI-38 cells after infection with various adenovirus vectors by Western blotting (Fig. 1C). We detected UPRT only in BC cells that had been infected with AxCAUP or AxE1CAUP, and none were detected in cells that had been infected with AxE1AdB or AxCA LacZ. The expression of UPRT was detected 72 hours after infection with nonreplicative AxCAUP and the level remained constant thereafter. By contrast, UPRT was detected at 48 hours in TGBC-44TKB cells and at 72 hours in TGBC-1TKB after infection with AxE1CAUP and there were time-dependent increases in the level of the expression. The expression of UPRT did not show any time-dependent increase when 5-FU was given immediately after infection at all tested concentrations of 5-FU (results not shown). In WI-38 cells, only slight expression of UPRT was detected at 120 hours after infection with AxE1CAUP. To examine the activity of UPRT in TGBC-44TKB cells, we measured the concentration of F-RNA (Fig. 1D). Cells that expressed UPRT encoded by AxCAUP (10 MOI) or by AxE1CAUP (1 MOI) produced significantly higher concentrations of F-RNA after administration of 5-FU than in the cells infected with a control vector, AxCA LacZ (10 MOI). Moreover, significantly higher levels of F-RNA concentration were generated after administration of 5-FU when the cells had been infected with AxE1CAUP, even at a low MOI of 1, than with AxCAUP (10 MOI).

**Sensitivity to 5-FU of Biliary Cancer Cells and WI-38 Cells After Transduction of UPRT Encoded by Replicative and Nonreplicative Adenoviruses.** We compared the ability of different adenovirus vectors to alter the sensitivity of BC cells to 5-FU, of which administration was started 72 hours after the vector infection (Fig. 2). In TGBC-44TKB cells, a nonreplicative AxCAUP vector increased the sensitivity of the cells to 5-FU 12-fold (IC50 0.5 μmol/L at 10 MOI versus 6 μmol/L 5-FU in the absence of virus), whereas AxE1AdB increased the sensitivity 3.3-fold (IC50 1.8 μmol/L) at 1 MOI. By contrast, AxE1CAUP increased the sensitivity of TGBC-44TKB cells to 5-FU 120-fold (IC50 0.05 μmol/L) at 1 MOI (Fig. 2A and Table 1). In Sk-ChA-1 cells, AxE1CAUP also increased the sensitivity to 5-FU 82.5-fold, even at the low MOI of 0.1, whereas AxC AUP at 1 MOI merely increased the sensitivity 2.5-fold (Fig. 2E and Table 1). The sensitivity to 5-FU did not increase in these BC cell lines when 5-FU was started earlier (24 hours) following infection with AxE1CAUP (Fig. 2B). In TGBC-1TKB cells which had lower adenovirus transduction efficiency,
the sensitivity to 5-FU was moderately increased when 5-FU was started 72 hours after AxE1CAUP infection (Fig. 2C), but it was further enhanced when exposure to 5-FU was delayed (120 hours after the infection; Fig. 2D). In normal WI-38 cells, AxE1CAUP, AxCAUP, and AxE1AdB, each at a MOI of 10, failed to increase the sensitivity to 5-FU (Fig. 2F and Table 1). Therefore, AxE1CAUP, the CRAd vector expressing UPRT, was the most effective in enhancing the sensitivity of BC cells to 5-FU, provided that the interval between inoculation of the vector and administration of 5-FU was appropriately chosen.

**In vivo UPRT/5-FU Gene Therapy Mediated by Replicative and Nonreplicative Adenoviruses for s.c. Biliary Cancer Xenografts.** We compared the antitumor effects of AxE1CAUP/5-FU in mice with peritoneally disseminated BC. We paid special attention to the interval between inoculation of the vector and administration of 5-FU. Four mice in each group were sacrificed on day 30. Large, multiple disseminated tumors were observed in the peritoneum of mice in the PBS group (total weight, 518 ± 195 mg; Fig. 4A) and the 5-FU group (total weight, 462 ± 153 mg), and the tumors were composed of viable tumor cells (Fig. 4D). In mice treated with i.p. AxE1CAUP alone, disseminated tumors of smaller size (total weight, 378 ± 102 mg) were observed, and histologically some tumor cells (approximately one third) had undergone necrosis (Fig. 4E). Adenovirus E1A was detected immunohistochemically (Fig. 4H) and abundant viral particles were seen under the electron microscope (Fig. 4K) in the viable tumor cells that remained around necrotic areas, suggesting that the virus was still spreading within the tumors. No immunostaining of E1A protein was detected in normal tissues, such as liver, peritoneum, small intestine, and colon (results not shown). In the AxE1CAUP/5-FU-d9 group, there were a few, small tumors in the abdomen (total weight, 135 ± 78 mg; Fig. 4B), and histologically approximately two-thirds of tumor cells had undergone necrosis (Fig. 4F). However, in the remaining viable tumor cells in these mice, very little staining of E1A was detected immunohistochemically (Fig. 4I) and very few viral particles were detected by electron microscopy (Fig. 4L), suggesting that the majority of adenovirus vectors had been eliminated as a result of the toxicity of 5-FU. In the mice of AxE1CAUP/5-FU-d14 group or AxE1CAUP/5-FU-d19 group, there were no visible tumors in the abdomen (Fig. 4C).

We compared the survival of mice that were not sacrificed on day 30. In the PBS group, the 5-FU group and the AxE1CAUP/5-FU-d9 group, BC cells continued to spread diffusely, causing extensive abdominal distension with bloody ascites and the cause of death was considered to be peritoneal dissemination. By contrast, all mice of the AxE1CAUP/5-FU-d14 group and of the AxE1CAUP/5-FU-d19 group did not show such abdominal dissemination and multiple metastasis to the liver was considered to be the cause of death. The survival of the 5-FU group (median, <1.5-fold in the AxE1CAUP/5-FU-d7 in 30 days (Fig. 3). Tumor growth was significantly (P <0.05) more suppressed in the AxE1CAUP/5-FU-d7 group than in PBS group, 5-FU group, AxCaUP/5-FU group, or AxE1AdB/5-FU groups.

**In vivo AxE1CAUP/5-FU Gene Therapy for Peritoneally Disseminated Biliary Cancer.** We also examined the antitumor effects of AxE1CAUP/5-FU in mice with peritoneally disseminated BC. We paid special attention to the interval between inoculation of the vector and administration of 5-FU. Four mice in each group were sacrificed on day 30. Large, multiple disseminated tumors were observed in the peritoneum of mice in the PBS group (total weight, 518 ± 195 mg; Fig. 4A) and the 5-FU group (total weight, 462 ± 153 mg), and the tumors were composed of viable tumor cells (Fig. 4D). In mice treated with i.p. AxE1CAUP alone, disseminated tumors of smaller size (total weight, 378 ± 102 mg) were observed, and histologically some tumor cells (approximately one third) had undergone necrosis (Fig. 4E). Adenovirus E1A was detected immunohistochemically (Fig. 4H) and abundant viral particles were seen under the electron microscope (Fig. 4K) in the viable tumor cells that remained around necrotic areas, suggesting that the virus was still spreading within the tumors. No immunostaining of E1A protein was detected in normal tissues, such as liver, peritoneum, small intestine, and colon (results not shown). In the AxE1CAUP/5-FU-d9 group, there were a few, small tumors in the abdomen (total weight, 135 ± 78 mg; Fig. 4B), and histologically approximately two-thirds of tumor cells had undergone necrosis (Fig. 4F). However, in the remaining viable tumor cells in these mice, very little staining of E1A was detected immunohistochemically (Fig. 4I) and very few viral particles were detected by electron microscopy (Fig. 4L), suggesting that the majority of adenovirus vectors had been eliminated as a result of the toxicity of 5-FU. In the mice of AxE1CAUP/5-FU-d14 group or AxE1CAUP/5-FU-d19 group, there were no visible tumors in the abdomen (Fig. 4C).

We compared the survival of mice that were not sacrificed on day 30. In the PBS group, the 5-FU group and the AxE1CAUP/5-FU-d9 group, BC cells continued to spread diffusely, causing extensive abdominal distension with bloody ascites and the cause of death was considered to be peritoneal dissemination. By contrast, all mice of the AxE1CAUP/5-FU-d14 group and of the AxE1CAUP/5-FU-d19 group did not show such abdominal dissemination and multiple metastasis to the liver was considered to be the cause of death. The survival of the 5-FU group (median, 

![Figure 2](image-url). Sensitivity in vitro to 5-FU of BC cells and WI-38 cells after infection with various adenoviral vectors. Three lines of BC cells, TGBC-44TKB (A, and B), TGBC-1TKB (C, and D) and Sk-ChA-1 (E), and normal WI-38 cells (F), were infected with AxE1CAUP (A), AxCAUP (B), AxE1AdB (C), or AxCAULacZ (D) at MOIs from 0.1 to 100 on day 1. 5-FU was supplied to the cells on days 4 to 10 (A, C, E, and F), days 2 to 8 (B), or on days 8 to 12 (D), and cell viability was determined by WST-1 assay. Percentages (mean ± SD) of surviving cells, as compared with control cells without 5-FU, from four individual determinations are shown.

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<tr>
<th>Table 1. Sensitivity (IC$_{50}$) of BC cells to 5-FU exposure 72 hours after infection with different adenoviral vectors</th>
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<tr>
<td><strong>IC$_{50}$ (μg/L) of 5-FU</strong></td>
</tr>
<tr>
<td>TGBC-44TKB</td>
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<tr>
<td>No vector</td>
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<tr>
<td>AxE1AdB</td>
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**NOTE:** MOIs of vectors used are shown in parentheses.
45 days), the AxE1CAUP group (median, 46 days), and of the AxE1CAUP/5-FU-d9 group (median, 49 days) was slightly longer than, but did not significantly differ from that of the PBS group (median, 40 days). By contrast, mice in the AxE1CAUP/5-FU-d14 group (median, 60 days) or the AxE1CAUP/5-FU-d19 group (median, 80.5 days) survived for significantly (P < 0.01) longer periods than the mice in the PBS group, the 5-FU group, or the AxE1CAUP group (Fig. 5). Therefore, it seemed that treatment with AxE1CAUP/5-FU prolonged the survival of mice with peritoneally disseminated BC, but the effect depended on the timing of administration of 5-FU.

Discussion

We found that AxE1CAUP, an E1B-55 kDa–defective CRAd expressing UPRT replicated efficiently in BC cells and had a more potent 5-FU-sensitizing effects than did AxE1AdB, a CRAd without a gene for UPRT, or AxCAUP, a nonreplicative adenovirus expressing UPRT, both in vitro and in vivo, although it had no negative effects on a normal fibroblast line, WI-38.

Gene therapy involving UPRT/5-FU systems has been studied previously using a nonreplicative adenovirus vector (7–9). However, the efficacy of gene therapy using nonreplicative vectors is limited due to the inadequate distribution of the vector and expression of the transgene within tumor masses or multiple lesions (10, 11). CRAds should help to overcome this problem (11). Indeed, we found that AxE1CAUP replicated selectively and efficiently amplified the expression of UPRT and accelerated the production of F-RNA in the BC cells (Fig. 1) and thus enhanced the sensitivity of BC cells to 5-FU in vitro (Fig. 2 and Table 1) and in vivo (Figs. 3–5). These effects were more potent than those of AxCAUP or AxE1AdB (Table 1 and Fig. 3). The ability of the AxE1CAUP/5-FU therapy to successfully prolong the survival of mice with peritoneally disseminated BC suggests the potential clinical efficacy of this approach for advanced BC, a condition that is frequently associated with peritoneal dissemination. The CRAd armed with UPRT gene should also be effective in overcoming the resistance to 5-FU of many other cancers because 5-FU is a widely used chemotherapeutic agent for various cancers.

The dose of AxE1CAUP vector could be reduced to one-tenth of that of AxCAUP vector and yet it was still sufficient to induce stronger 5-FU-sensitizing effects (Fig. 2 and Table 1). A low initial dose of vector should be helpful in avoiding the severe side effects that are caused by acute systemic immune responses to adenovirus vectors. Furthermore, the replication of AxE1CAUP and the expression of UPRT were restricted and the sensitivity to 5-FU did not increase in normal WI-38 cells, which have a normal gene for p53. From these results, we can reasonably expect that use of the replicative AxE1CAUP vector should be safer than use of the nonreplicative AxCAUP vector.

Timing of the administration of 5-FU was clearly a critical factor in the overall efficacy of AxE1CAUP/5-FU gene therapy in vitro (Fig. 2) and in vivo (Figs. 3–5). The observed difference in the antitumor effects of 5-FU given early as compared with late can be explained by the negative effects of 5-FU on viral proliferation (Figs. 1B and 4) and expression of UPRT. Therefore, in order to maximize the efficacy of AxE1CAUP/5-FU gene therapy, it would be reasonable to delay administration of 5-FU until the vector has had time to replicate and to distribute the gene for UPRT and its product extensively within the tumors.

Many recent reports have dealt with the combinations of an oncolytic virus and suicide gene therapy, but results have been inconsistent (24–31, 38–41). The conflicting results might be explained in part by differences in the timing of prodrug administration, but, to our knowledge, only one study (27) addressed this issue previously. Our results are in harmony with the observation by Wildner et al. (27), who studied the efficacy of a CRAd armed with HSVtk in the treatment of s.c. xenografts of melanoma and of cervical cancer cells. They found that GCV given from day 3 prolonged the survival to a greater extent than GCV given from day 1 after the i.t. injection of the CRAd. Investigators who claimed that the HSVtk/GCV system does not enhance the antitumor effects of a CRAd initiated administration of GCV soon after inoculation of the vector (30). Moreover, in the first clinical trial of a CRAd combined with double suicide genes (HSVtk/ cytosine deaminase fusion gene) for locally advanced prostate cancer, the prodrugs were started relatively early (on day 3), and only modest anticancer effects were observed (42). Taken together,
These data support the importance of the timing of prodrug administration in the efficacy of a CRAd that is armed with suicide genes that can potentially eradicate the vector. Other factors that can potentially affect the overall efficacy of the combination include the capacity for replication and oncolysis by the virus, the potency of the suicide gene/prodrug systems and their bystander effects, and the effects of suicide gene/prodrug system on viral replication and eradication (43).

The appropriate timing of prodrug administration might, however, depend on multiple factors, including the number, size, location and adenovirus infectivity of the tumors, and the route of vector administration. Indeed, TGBC-1TKB cells, because of their lower adenovirus infectivity and thus longer time necessary for viral replication and UPRT expression (Fig. 1), required longer interval between the infection of AxE1CAUP and the exposure to 5-FU than TGBC-44TKB cells and Sk-ChA-1 cells (Fig. 2). Furthermore, a 3-day interval between inoculation of the vector and administration of 5-FU was sufficient to show a stronger antitumor effect of the i.t. AxE1CAUP in s.c. BC xenografts (Fig. 3), whereas a 5-day interval did not allow the i.p. AxE1CAUP to prolong survival in the peritoneal dissemination model (Fig. 5). The longer interval required in the latter model was probably related to the longer time required for the i.p. injected vector to infect the disseminated tumors in the peritoneum. The best time for administration of prodrug is probably the time when transgene expression has reached a plateau within target tumors. The critical question in a clinical setting is how to identify the appropriate timing. In considering this important issue, we should pay particular attention to recently developed methods for monitoring and/or visualizing the replication and distribution of viral vectors in vivo (44–46). The role of these methods in deciding adequate timing of 5-FU administration to achieve maximum anticancer effects should be extensively tested in future studies.

Our data also suggest that the UPRT/5-FU systems, just like the HSVtk/GCV system (29) can be used as a fail-safe mechanism to eradicate a CRAd for safety reasons when its unwanted replication in the normal tissues might occur. The inhibitory effect of 5-FU on the expression of adenovirus E1A was observed even at a subtoxic dose (0.01 μmol/L; Fig. 1B) and thus, we were unable to confirm the observations of Bernt et al. (47), who reported that exposure of cells to 5-FU or other prodrugs or drugs at subtoxic concentrations tended to enhance the replication of a CRAd, even though 5-FU did not increase the antitumor effects. The difference between our results and theirs might be due to the difference in the CRAd used or the cancer cells tested.

Recently, Oonuma et al. (48) showed that treatment with AxE1CAUP followed by administration of 5-FU increased the sensitivity of pancreatic cancer cell lines to 5-FU in vitro and caused a dramatic reduction in the tumor burden in mice with i.p. disseminated pancreatic cancer cells. 5-FU was given 3 days after the vector in their in vivo study. However, they neither compared their results to results for AxE1AdB nor did they...
examine whether this treatment prolonged the survival of mice. Our results are basically consistent with their study but we have made three important additional observations. First, we showed that the effects of AxE1ICA/FU-5/FU therapy in vitro and in vivo are superior to those of AxE1AD8/FU-5/FU and of AxCAP/FU-5/FU. Second, we showed that this therapy could significantly prolong the survival of mice with peritoneal dissemination. Third, we showed that the administration of 5-FU inhibits the vector proliferation and thus, the timing of the administration of 5-FU significantly affects the outcome of the therapy.

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Effective Gene Therapy of Biliary Tract Cancers by a Conditionally Replicative Adenovirus Expressing Uracil Phosphoribosyltransferase: Significance of Timing of 5-Fluorouracil Administration

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