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Replication-competent Adenovirus-mediated Suicide Gene Therapy with Radiation in a Preclinical Model of Pancreatic Cancer

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In preparation for a Phase I trial, we evaluated the efficacy and toxicity of replication-competent adenovirus-mediated suicide gene therapy in combination with radiation in a preclinical model of pancreatic cancer. Human MiaPaCa-2 and PANC-1 pancreatic adenocarcinoma cells were found to be sensitive to the oncolytic effects of the Ad5-yCD/*mut*TK_{SR39}*rep*-ADP adenovirus and also to the cytotoxic effects of the yeast cytosine deaminase (yCD) and herpes simplex virus thymidine kinase (HSV-1 TK_{SR39}) genes *in vitro*. Combining Ad5-yCD/*mut*TK_{SR39}*rep*-ADP-mediated suicide gene therapy with radiation significantly increased tumor control beyond that of either modality alone. Injection of Ad5-yCD/*mut*TK_{SR39}*rep*-ADP in the dog pancreas at doses (10^{12} virus particle (vp)) to be used in humans resulted in mild pancreatitis but not peritonitis or hepatotoxicity. Following administration of 9-(4-[^{18}F]-fluoro-3-hydroxymethylbutyl)guanine ([^{18}F]-FHBG), a positron-emitting substrate of HSV-1 TK, Ad5-yCD/*mut*TK_{SR39}*rep*-ADP activity could be monitored non-invasively by positron emission tomography (PET). [^{18}F]-FHBG uptake was readily detected in the pancreas but not in other major abdominal organs, indicating that little of the injected adenovirus disseminates to collateral tissues. These results demonstrate that Ad5-yCD/*mut*TK_{SR39}*rep*-ADP-mediated suicide gene therapy has the potential to augment the effectiveness of pancreatic radiotherapy without resulting in excessive toxicity. Hence they provide the scientific basis for an ongoing Phase I trial in pancreatic cancer.

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INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States, accounting for 5% of all cancer deaths. The American Cancer Society estimates that pancreatic cancer will claim 31,800 lives in the United States in 2005. Although these statistics demonstrate the significance of pancreatic cancer as a health problem, no statistic is more chilling than the annual incidence to mortality ratio of almost one. The need for better treatments cannot be overstated.

Replication-competent, oncolytic adenoviruses represent a new investigational therapy that has been evaluated in clinical trials of pancreatic cancer both as a single agent and in combination with chemotherapy. The prototype oncolytic adenovirus, ONYX-015, has been administered to patients with locally advanced pancreatic cancer by computed tomography (CT)-guided injection up to a dose of 2×10^{12} vp/injection.[1] Although the treatment was well tolerated, there were no objective responses. In a follow-up trial, eight weekly cycles of ONYX-015 were administered by endoscopic ultrasound-guided injection concomitant with gemcitabine chemotherapy.[2] Toxicities possibly or probably related to ONYX-015 were mild (grade 1/2) flu-like symptoms, and the vast majority of adverse events could be attributed to either the injection procedure (sepsis, duodenal perforation) or gemcitabine chemotherapy (myelosuppression). It is important to note that, although a minority of subjects developed mild, transient elevations in lipase, none developed pancreatitis. Two (10%) subjects exhibited partial regressions (>50%) of the injected tumor, 8 (38%) had stable disease, and 11 (52%) had progressive disease or had to leave the study because of treatment-related toxicity. The median survival was 7.5 months, and 29% of subjects were alive at 12 months. Although the safety of combining oncolytic adenoviral therapy with chemotherapy was demonstrated, it is clear that further improvements in this technology are needed before it will have applicability in the clinic.

One strategy that has been explored for bolstering the antitumor activity of oncolytic adenoviruses is to "arm" them with therapeutic genes. The first such armed replication-competent adenovirus, Ad5-CD/TK*rep*, contained a bacterial cytosine deaminase/wild-type herpes simplex virus thymidine kinase (HSV-1 TK) fusion gene under the transcriptional control of a strong viral promoter.[3] When administered with the prodrugs 5-fluorocytosine (5-FC) and ganciclovir (GCV), the CD and HSV-1 TK genes contained in Ad5-CD/TK*rep* convert these non-toxic

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agents into toxic metabolites, thereby providing a local chemotherapeutic effect. Not only do the CD/5-FC and HSV-1 TK/GCV suicide gene systems provide a chemotherapeutic effect, they also function as potent sensitizers of radiotherapy.[3–6] The safety of this investigational approach has been evaluated in three Phase I/II trials of prostate cancer, without[7,8] and with[9,10] conformal radiotherapy. The investigational therapy was associated with low toxicity, and signs of efficacy have emerged.

In this report we explore the efficacy and toxicity of combining replication-competent adenovirus-mediated suicide gene therapy with radiation therapy in an experimental model of pancreatic cancer. Using a second-generation adenovirus (Ad5-yCD/*mut*TK_{SR39}*rep*-ADP) that contains two improvements over the parental Ad5-CD/TK*rep* adenovirus, we demonstrate that combining Ad5-yCD/*mut*TK_{SR39}*rep*-ADP-mediated suicide gene therapy with radiation significantly improves tumor control and survival beyond that of either modality alone. Moreover, direct intrapancreatic injection of Ad5-yCD/*mut*TK_{SR39}*rep*-ADP was not associated with excessive toxicity at dose levels to be used in future human trials of pancreatic cancer.

RESULTS

Effects of Ad5-yCD/*mut*TK_{SR39}*rep*-ADP adenovirus and suicide gene systems *in vitro*

A schematic of the second-generation Ad5-yCD/*mut*TK_{SR39}*rep*-ADP adenovirus is shown in **Figure 1**. The parental Ad5-CD/TK*rep* adenovirus,[3] from which Ad5-yCD/*mut*TK_{SR39}*rep*-ADP was derived, is shown for comparison. Ad5-yCD/*mut*TK_{SR39}*rep*-ADP contains a yeast cytosine deaminase (yCD)/mutant$_{SR39}$ herpes simplex virus thymidine kinase (*mut*TK_{SR39}) fusion gene in the E1 region as well as the Ad5 adenoviral death protein (ADP) gene in the E3 region. Both the yCD/*mut*TK_{SR39} and ADP genes are under the transcriptional control of the human cytomegalovirus promoter and produce high constitutive levels of the yCD/*mut*TK_{SR39} fusion and ADP proteins.[6]

To examine the cytopathic effect (CPE) of Ad5-yCD/*mut*TK_{SR39}*rep*-ADP on human pancreatic adenocarcinoma cells, MiaPaCa-2 and PANC-1 cells were infected with increasing amounts of adenovirus and then CPE was examined in the absence of prodrugs. The parental Ad5-CD/TK*rep* adenovirus was included for comparison. At all multiplicities of infection, Ad5-yCD/*mut*TK_{SR39}*rep*-ADP resulted in greater cell kill than Ad5-CD/TK*rep* (**Figure 2**). At day 17, the efficiency of cell kill with Ad5-yCD/*mut*TK_{SR39}*rep*-ADP was more than 100-fold greater than with Ad5-CD/TK*rep* with both cell lines. Because prodrugs were not used and these adenoviruses infect human cells with equal efficiency (data not shown), the greater cytolytic effect of Ad5-yCD/*mut*TK_{SR39}*rep*-ADP can be attributed to the ADP gene.

The replication properties of Ad5-yCD/*mut*TK_{SR39}*rep*-ADP were compared to wild-type (wt) Ad5 following infection of MiaPaCa-2 and PANC-1 cells. As expected for replication-competent adenoviruses, the amount of viral DNA increased with time and peaked 4–6 days after infection (**Figure 3a**). These kinetics of viral DNA replication are somewhat slower than what we observed previously with human prostate adenocarcinoma cells (viral DNA peaks in ~2 days).[3] Although the kinetics of Ad5-yCD/*mut*TK_{SR39}*rep*-ADP and wt Ad5 DNA replication were similar, the amount of Ad5-yCD/*mut*TK_{SR39}*rep*-ADP viral DNA was significantly lower than wt Ad5 at all time points. To measure the viral burst, infected cells were harvested on day 6, rather than day 3,[11] to quantify virus yield before the initiation of CPE (~day 10) but after one complete round of DNA replication. Ad5-yCD/*mut*TK_{SR39}*rep*-ADP yielded a viral burst of ~4,000 (**Figure 3b**), which is twofold to threefold lower than wt Ad5 but consistent with the viral DNA replication assays.

To determine the sensitivity of human pancreatic adenocarcinoma cells to the yCD/5-FC and *mut*TK_{SR39}/GCV suicide gene systems, MiaPaCa-2 and PANC-1 cells were infected with Ad5-yCD/*mut*TK_{SR39}*rep*-ADP and cells were then incubated in increasing concentrations of 5-FC and GCV. To avoid the complications of the Ad5-yCD/*mut*TK_{SR39}*rep*-ADP cytolytic effect, cell kill was examined several days before this effect became apparent (**Figure 4a**, no prodrug wells). Both cell lines were sensitive to the cytotoxic effects of the suicide gene systems as a function

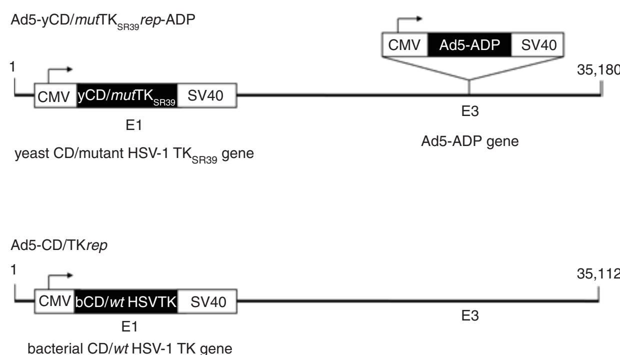

Figure 1 Schematic of Ad5-yCD/*mut*TK_{SR39}*rep*-ADP and Ad5-CD/TK*rep* adenoviruses. CMV, human cytomegalovirus promoter; yCD/*mut*TK_{SR39}, yeast cytosine deaminase (yCD)/mutant$_{SR39}$ herpes simplex virus thymidine kinase (*mut*TK_{SR39}) fusion gene; bCD/HSV-1 TK, bacterial cytosine deaminase (bCD)/wild-type herpes simplex virus thymidine kinase (HSV-1 TK) fusion gene; SV40, simian virus 40 polyadenylation site; and ADP, adenoviral death protein gene. The direction of transcription is indicated.

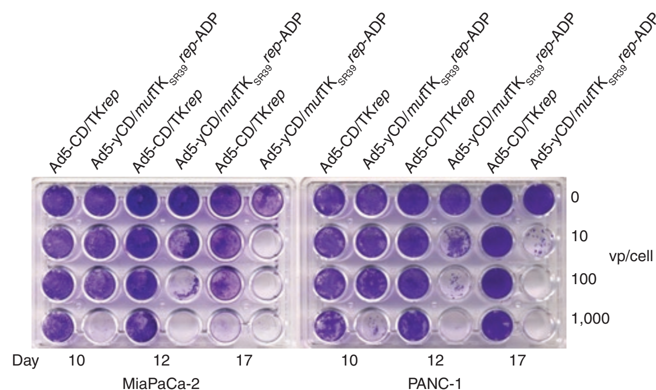

Figure 2 Sensitivity of human pancreatic adenocarcinoma cells to cytolytic effects of replication-competent adenoviruses. MiaPaCa-2 and PANC-1 cells were infected with increasing amounts of the Ad5-yCD/*mut*TK_{SR39}*rep*-ADP and Ad5-CD/TK*rep* adenoviruses as indicated (in virus particle (vp)/cell). Cells were fixed and stained with crystal violet at specific time points thereafter as indicated. ADP, adenoviral death protein; TK, thymidine kinase; yCD, yeast cytosine deaminase.

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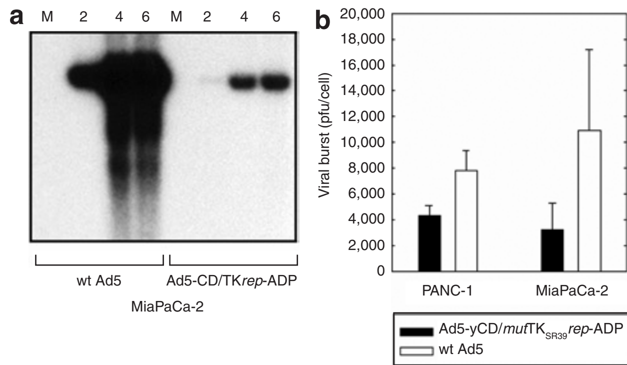

Figure 3 Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP replication. (**a**) MiaPaCa-2 cells were infected with Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP (Ad5-CD/TK*rep*-ADP) and wild-type (wt) Ad5 at a multiplicity of infection (MOI) of 10 plaque-forming units (pfu)/cell. Cells were harvested on the days indicated, and the amount of viral DNA present was examined by Southern blotting. M, Mock-infected cells. (**b**) MiaPaCa-2 and PANC-1 cells were infected with Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP and wt Ad5 at an MOI of 10 pfu/cell. Six days later, when viral DNA was at a peak, the cells were harvested and disrupted; then the yield of virus was quantified. The yield of virus (in pfu) per target cell is plotted. The results represent the mean of three independent infections; bars denote the SD. ADP, adenoviral death protein; TK, thymidine kinase; yCD, yeast cytosine deaminase.

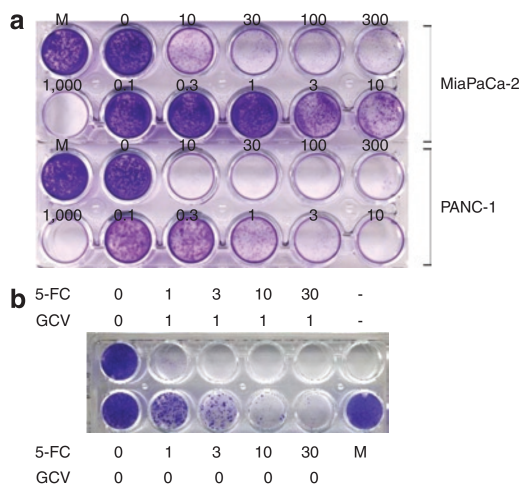

Figure 4 Sensitivity of human pancreatic adenocarcinoma cells to yeast cytosine deaminase (yCD)/5-fluorocytosine (5-FC) and herpes simplex virus thymidine kinase (HSV-1 TK$_{SR39}$)/ganciclovir (GCV) suicide gene systems. (**a**) MiaPaCa-2 and PANC-1 cells were infected with Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP at a virus particle (vp)/cell of 300. The next day, cells were incubated in increasing concentrations of 5-FC and GCV throughout the assay. Cells were fixed and stained with crystal violet 1 week after infection and before the Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP cytolytic effect became evident. M, mock-infection. The number by each well indicates 5-FC (10, 30, 100, 300 and 1,000) and GCV (0.1, 0.3, 1, 3 and 10) concentration in μg/ml. (**b**) MiaPaCa-2 cells were infected with Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP at a vp/cell of 300. The next day, cells were incubated in increasing concentrations of 5-FC (in μg/ml), with or without 1 μg/ml GCV. Cells were fixed and stained with crystal violet 1 week after infection. ADP, adenoviral death protein.

of prodrug concentration (**Figure 4a**). Complete cell kill was observed at >30 μg/ml (0.23 mmol/l) 5-FC (both cell lines) and >3 μg/ml (10 μmol/l) GCV (PANC-1). Although MiaPaCa-2 cells were sensitive to the *mut*TK$_{SR39}$/GCV suicide gene system,

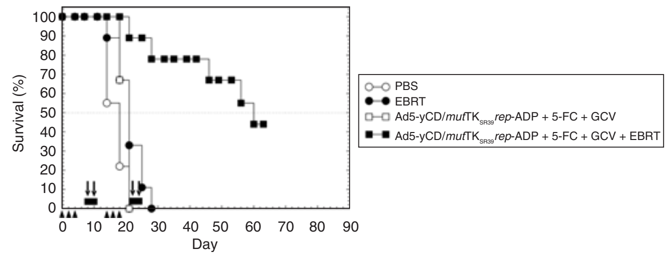

Figure 5 Anti-tumor effects of Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP-mediated suicide gene therapy in combination with radiation. MiaPaCa-2 tumors (100–150 mm^3) were injected with 10^{10} virus particle of Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP on days 0, 2, 4 (cycle 1) and days 14, 16, 18 (cycle 2) (arrowheads). 5-FC (500 mg/kg/day) and ganciclovir (GCV) (30 mg/kg/day) prodrugs were administered on days 7–11 (cycle 1) and days 21–25 (cycle 2) (solid bars). Radiation (2 Gy/dose; 4 Gy/cycle, 8 Gy total) was administered on days 8 and 10 (cycle 1) and on days 22 and 24 (cycle 2) (arrows). Each group contained nine animals. ADP, adenoviral death protein; EBRT, external beam radiation therapy; PBS, phosphate-buffered saline; TK, thymidine kinase; yCD, yeast cytosine deaminase.

they were less sensitive than PANC-1 cells, and complete cell kill was not observed at the highest GCV concentration examined. To examine whether GCV could improve cell kill beyond that observed with 5-FC alone, Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP-infected cells were incubated with increasing concentrations of 5-FC, with or without 1 μg/ml GCV. At all 5-FC concentrations examined, GCV increased cell kill relative to 5-FC alone (**Figure 4b**).

Anti-tumor effect of Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP-mediated suicide gene therapy with radiation

The anti-tumor effect of Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP-mediated suicide gene therapy in combination with radiation (*i.e.*, trimodal therapy) was examined in the MiaPaCa-2 tumor xenograft model. Athymic mice bearing MiaPaCa-2 tumors received intratumoral injections of Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP along with 5-FC + GCV prodrug therapy and fractionated doses of external beam radiation therapy. To mimic treatment schema of future clinical trials, animals were administered multiple cycles (two in this case) of the gene therapy. For comparison, animals received no treatment (controls), radiation only (conventional therapy), or the Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP adenovirus and 5-FC + GCV prodrug therapy only (investigational therapy). Thus, the trimodal therapy represents the combination of the investigational and conventional cancer therapies. Trimodal therapy (Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP + 5-FC + GCV + EBRT) resulted in a median survival of 60 days, which was significantly greater than the control group (18 days) or either single modality (21 days) ($P < 0.001$; Kaplan–Meier Log Rank Test) (**Figure 5**). There were no tumor cures in any group. These results demonstrate that Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP-mediated suicide gene therapy is able to potentiate the effects of radiation in a preclinical model of pancreatic cancer.

Feasibility of monitoring Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP non-invasively *in vivo* by PET imaging

One advantage of therapeutic adenoviruses containing the HSV-1 TK gene is that they can be imaged by positron emission tomography (PET) following administration of positron-emitting,

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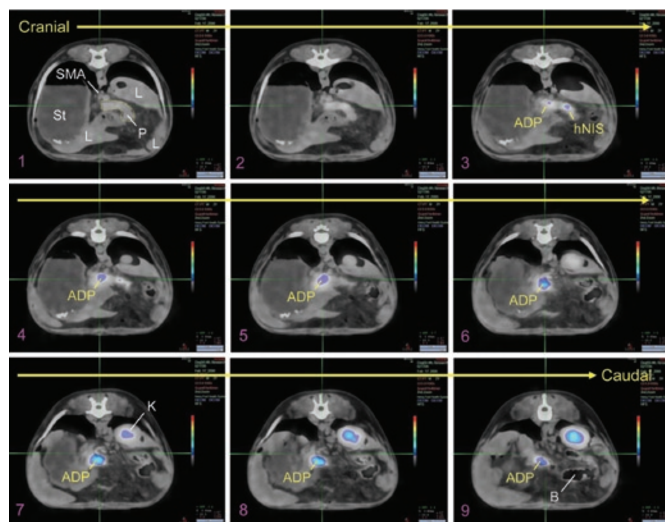

Figure 6 Positron emission tomography (PET)/computed tomography (CT) imaging of Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP in dog pancreas. The Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP and Ad5-yCD/*mut*TK$_{SR39}$*rep*-hNIS adenoviruses (1×10^{12} virus particle each) were injected into the pancreas of a dog. Twenty-four hours later, 1.6 mCi [^{18}F]-FHBG was administered intravenously and the animal was subjected to PET and CT imaging. Consecutive 3.4 mm transverse sections through the abdominal region are shown. B, bowel; K, kidney; L, liver; P, pancreas; SMA, superior mesenteric artery; St, stomach. The dotted yellow line in image 1 denotes the pancreas. [^{18}F]-FHBG uptake in pancreas due to the Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP (ADP) and Ad5-yCD/*mut*TK$_{SR39}$*rep*-hNIS (hNIS) adenoviruses are indicated. ADP, adenoviral death protein; hNIS, human sodium iodide symporter; TK, thymidine kinase; yCD, yeast cytosine deaminase.

HSV-1 TK substrates such as 9-(4-[^{18}F]-fluoro-3-hydroxymethylbutyl)guanine ([^{18}F]-FHBG).[12,13] Thus, the quality of the adenovirus injection and dissemination of adenovirus to collateral tissues can be examined non-invasively.

To determine the feasibility of using PET to monitor Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP non-invasively, adult male dogs (n = 4) were given an intrapancreatic injection of adenovirus at 1×10^{12} vp on day 1. The next day, animals were administered [^{18}F]-FHBG and underwent PET/CT imaging. In the study shown (**Figure 6**), Ad5-yCD/*mut*TK$_{SR39}$*rep*-hNIS[14]—an adenovirus similar to Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP but containing the human sodium iodide symporter (hNIS) gene in place of the ADP gene—was co-injected into the pancreas at a separate site for comparison. Anatomical information provided by CT allowed for precise localization of the [^{18}F]-FHBG signal. Two regions of [^{18}F]-FHBG uptake were detected in pancreas, corresponding to the Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP and Ad5-yCD/*mut*TK$_{SR39}$*rep*-hNIS injection sites. The volume and intensity of the signal obtained with Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP was significantly greater than that obtained with Ad5-yCD/*mut*TK$_{SR39}$*rep*-hNIS. However, the PET imaging was performed 1 day after the adenovirus injection, which is prior to completion of a single viral replication cycle, and so the greater spread observed here is likely due to better distribution of the adenovirus at the time of injection and not to the effects of ADP. As expected, [^{18}F]-FHBG accumulated in kidney (**Figure 6**, panels 7–9) and bladder (not shown in **Figure 6**), as this is the route of [^{18}F]-FHBG excretion. No [^{18}F]-FHBG uptake was detected in other major organs including liver, spleen, stomach, lung, and intestines in any of the four studies. These results demonstrate the feasibility of monitoring Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP non-invasively *in vivo* by PET.

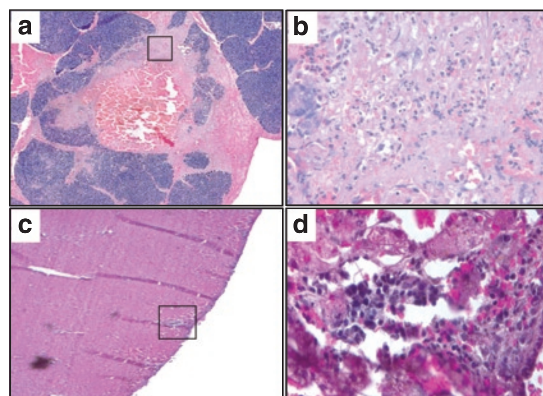

Figure 7 Histopathology of pancreas and liver following intrapancreatic injection of Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP. Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP (1×10^{12} virus particle each) was injected into the dog pancreas. One day later, animals were subjected to positron emission tomography/computed tomography imaging as described in **Figure 6** legend. Shortly after the imaging session, animals were sacrificed and a necropsy was performed. Tissue sections were stained with hematoxylin and eosin. Pancreas at (**a**) low and (**b**) high magnifications, respectively. Liver (**c**) at low and (**d**) high magnifications, respectively. Boxes indicate the region magnified, showing inflammation.

Toxicology studies

The toxicity associated with direct intrapancreatic injection of Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP was examined in the dog. Adult male dogs (n = 3) were injected with 1×10^{12} vp (~8×10^{10} vp/kg) of Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP. On a weight basis, this adenovirus dose is 550 times the starting dose (1×10^{10} vp, 1.4×10^{8} vp/kg) and 5.5 times the highest dose (1×10^{12} vp, 1.4×10^{10} vp/kg) to be administered in an ongoing Phase I trial of pancreatic cancer, assuming a 70 kg (150 lb) patient. Animals were administered [^{18}F]-FHBG and underwent PET/CT imaging as described previously. To maximize the likelihood of detecting inflammation, animals 1 and 2 were sacrificed 1 day after the adenovirus injection and a necropsy was performed. Complete blood cell counts and blood chemistries were obtained at baseline prior to the adenovirus injection and at necropsy. Pancreas, liver, spleen, and lung were removed for histopathological analysis. Animal 3 was sacrificed on day 9 so that additional blood draws could be obtained.

As expected, there was evidence of acute pancreatitis on day 2 as demonstrated by regional inflammation of the pancreas (**Figure 7**). This was accompanied by a minor elevation in amylase (one of three animals) and neutrophilia (three of three animals) (**Table 1**). In one of three animals there was also minor inflammation of the liver (**Figure 7**), which was accompanied by transaminitis (**Table 1**). We believe the latter observation is the result of minor leakage of adenovirus into the abdominal cavity following the injection. However, there was no evidence of overt peritonitis. Spleen and lung were normal in all animals (not shown). The neutrophilia observed on day 2 in animal 3 resolved by day 5, and there was no evidence of inflammation of

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Table 1 Blood chemistries and CBCs[a]

Test	Pre-T[b]	Post-T[c]	Reference range
Animal 1			
GGT	2	19	1–12 IU/l
WBC	8.9	18.6	40–15.5 × 10^3/µl
Neutrophils	6,230	18,042	2,060–10,600 × 10^3/µl
Lymphocytes	2,136	558	690–4,500 × 10^3/µl
Platelets	312	413	170–400 × 10^3/µl
Animal 2			
AST (SGOT)	39	81	15–66 IU/l
Amylase	389	1,631	290–1,125 IU/l
CPK	344	1,057	59–895 IU/l
HGB	13.3	11.6	12.1–20.3 g/dl
Neutrophils	4,018	11,424	2,060–10,600 × 10^3/µl
Lymphocytes	539	357	690–4,500 × 10^3/µl
Animal 3			
CPK (day 2)	205	1,050	59–895 IU/l
CPK (day 5)		152	
CPK (day 7)		164	
CPK (day 9)		155	
Neutrophils (day 2)	7,020	12,760	2,060–10,600 × 10^3/µl
Neutrophils (day 5)		5,940	
Neutrophils (day 7)		6,076	
Neutrophils (day 9)		7,500	

Abbreviations: AST, aspartate aminotransferase; CBC, complete blood cell count; CPK, creatine phosphokinase; GGT, γ-glutamyl transferase; HGB, hemoglobin; SGOT, serum glutamic oxaloacetic transaminase; WBC, white blood cell.
[a]Only values outside the reference range are shown. [b]Pre-T, pre-treatment, prior to the adenovirus injection. [c]Post-T, values are one day post-treatment (day 2) except for animal 3 in which values for days 5, 7 and 9 are also shown.

the pancreas at necropsy (not shown). All other blood chemistries were within normal limits.

DISCUSSION

Here we evaluated the efficacy of combining Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP-mediated suicide gene therapy with radiation in a preclinical model of pancreatic cancer. The combined treatment resulted in significantly greater tumor control and survival relative to radiation or the adenovirus-mediated gene therapy alone. Moreover, direct intrapancreatic injection of Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP at the highest dose (10^{12} vp) to be administered in a Phase I trial of pancreatic cancer resulted in only mild, transient pancreatitis, and little of the injected adenovirus disseminated to collateral tissues including liver. Providing that these preclinical results accurately predict what will occur in the clinic, we believe that the multi-modal, investigational therapy described here will be well tolerated in humans.

One issue that requires careful consideration when developing a new cancer therapeutic is: In what cancer and/or stage should it first be evaluated? Pancreatic cancer is typically categorized into three major classes at diagnosis: potentially resectable, locally advanced, and metastatic. Because early phases of disease progression can occur without symptoms, most pancreatic cancer patients present with metastatic disease and only 15–20% of cases are considered potentially resectable at diagnosis.[15] Although previous gene therapy trials of pancreatic cancer targeted locally advanced disease, we do not believe this is the best setting to examine the safety and potential efficacy of the investigational approach described here. Owing to the low efficiency of gene transduction *in vivo*, most current gene therapy strategies (excluding vaccine approaches) are unlikely to show significant activity against overt, metastatic disease. The vast majority (50–75%) of patients with locally advanced disease have clinically detectable, metastatic disease at diagnosis and so may not benefit from this investigational treatment. In contrast, only ~25% of patients with potentially resectable pancreatic cancer have metastatic liver disease at restaging after neoadjuvant chemoradiation.[15] Thus, many of these latter patients may have solely localized disease at diagnosis and may benefit from better loco-regional therapies. The gene therapy approach described here was designed to improve the effectiveness of radiotherapy, and we believe it is likely to show its greatest activity when targeting loco-regional disease. Thus, in our ongoing Phase I trial, the safety and efficacy of this investigational approach is being evaluated in patients with potentially resectable pancreatic cancer in combination with neoadjuvant chemoradiation.

There are several reasons why the investigational approach described here should be evaluated in pancreatic cancer. First and foremost, it utilizes a biochemical pathway that has demonstrated chemotherapeutic and radiosensitizing activity in human pancreatic cancer. One of the suicide genes contained in Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP, yCD, converts the prodrug 5-FC into 5-fluorouracil. 5-fluorouracil and its prodrug capecitabine have demonstrated chemotherapeutic and radiosensitizing activity in human pancreatic cancer and are now an integral part of standard chemoradiation regimens.[16,17] We believe that, after intratumoral injection of Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP and administration of 5-FC, a high, local concentration of 5-fluorouracil will be produced within the tumor. This may bolster the chemotherapeutic and radiosensitizing effect beyond that of capecitabine alone. Moreover, in addition to the yCD gene, Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP contains a second suicide gene, HSV-1 TK$_{SR39}$, which also has chemotherapeutic and radiosensitizing activity.[18–21] Thus, co-implementation of the HSV-1 TK$_{SR39}$ suicide gene system may increase tumor cell destruction beyond that of the 5-fluoropyrimidine pathway alone, and this in turn may improve local tumor control. An advantage of suicide gene therapy is that the toxic products are produced locally within the tumor, thereby avoiding the systemic toxicity commonly associated with chemotherapy.

Second, the safety of direct intrapancreatic injection of the replication-competent ONYX-015 adenovirus has been demonstrated in humans when administered as a single agent and in combination with gemcitabine chemotherapy.[1,2] Overall, the investigational treatment was well tolerated. The most common ONYX-015-related side effects were mild flu-like symptoms. Despite repeated (up to eight) injections and a maximum dose of 4×10^{12} vp, only 1 of 45 (2%) subjects developed mild pancreatitis. These results, coupled with the well-established safety profile of 5-fluorouracil-based chemoradiation, leads us to believe that the multi-modal approach described here can be applied safely in humans.

Finally, improvements in loco-regional control may translate into an increase in survival and/or a better quality of life in patients with potentially resectable pancreatic cancer. As stated previously, many patients with potentially resectable pancreatic cancer are likely to have solely localized disease at diagnosis and thus may benefit from better loco-regional therapies. If the multi-modal approach could help reduce the frequency of positive margins or

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"down-stage" a patient from being marginally resectable to resectable, then it could have a positive effect on survival. Moreover, better loco-regional control could also translate into improved performance status.

Another important consideration when developing a new cancer therapeutic is treatment-related toxicity. The investigational approach described here utilizes three different cancer modalities: oncolytic adenoviral therapy, suicide gene therapy, and radiation therapy. Based on the results of four Investigational New Drug-directed toxicology studies[5,6,22] and three prostate cancer clinical trials,[7–10] we have found that the toxicities of the individual modalities do not overlap. The most common side effects of the replication-competent adenovirus are local inflammation, mild flu-like symptoms (~30% of patients), and minor (grade 1) transaminitis (33% of patients), the latter likely resulting from viral dissemination to liver. Although hepatotoxicity can be life-threatening and is carefully monitored in our ongoing Phase I trial, we demonstrate here that little of the Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP adenovirus disseminated to liver after direct intrapancreatic injection. Moreover, none of the animals showed any signs of peritonitis. Although the validity of this conclusion rests on the sensitivity of PET imaging, it is supported by blood chemistries that showed only minor evidence of hepatotoxicity. We plan to utilize nuclear imaging to monitor the quality of the adenovirus injection and adenoviral dissemination in all future trials and have successfully imaged adenovirus-mediated gene expression in prostate cancer patients (data not shown). The most common side effects of the 5-FC and GCV prodrugs are hematologic, with lymphopenia (80% of patients) being the most prevalent. The most common side effects of radiation vary with the cancer site but typically include loco-regional toxicity. In pancreatic cancer, the major dose-limiting organs are liver and small bowel. When damage to these organs is severe, the result can be hepatitis, liver failure, or small bowel enteritis and perforation. Fatigue, nausea, vomiting, and diarrhea are also common. Fortunately, none of these treatment-related side effects overlap. However, the side effects of the 5-FC + GCV prodrug therapy and capecitabine chemotherapy do overlap, so hematologic toxicities are being carefully monitored in our ongoing Phase I trial.

The safety of replication-competent adenovirus-mediated suicide gene therapy has been evaluated in three Phase I/II trials of prostate cancer, with and without conformal radiotherapy.[7–10] The first two trials were conducted with the parental Ad5-CD/TK*rep* adenovirus and the third was conducted with Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP, which is described here. When combining the results of these three Phase I/II trials, the investigational therapy was associated with low toxicity and provocative signs of efficacy have emerged. Based on these clinical results and the preclinical studies presented here, we are hopeful that Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP-mediated suicide gene therapy will augment the efficacy of pancreatic chemoradiation without resulting in excessive toxicity.

MATERIALS AND METHODS

Cell lines and adenoviruses. All human pancreatic cell lines used in these studies were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (growth medium) (Invitrogen, Carlsbad, CA). The parental Ad5-CD/TK*rep*[3] and second-generation Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP[6] adenoviruses have been described previously.

In vitro *assays.* CPE and prodrug sensitivity assays were performed as described previously.[3,6] Briefly, MiaPaCa-2 and PANC-1 cells were mock-infected or infected with adenovirus at the vp/cell ratio indicated in the figures. Infection medium was Dulbecco's modified Eagle's medium with 2% fetal bovine serum. One hour later, the medium was changed and cells were maintained in growth medium thereafter. For prodrug sensitivity assays, cells were infected with adenovirus at a multiplicity of infection of 300 vp/cell. The next day, cells were incubated in growth medium containing graded concentrations of 5-FC and GCV as indicated in the figures. Prodrugs were maintained throughout. Wells were fixed and stained with 0.4% crystal violet at specific time points thereafter, as indicated in the figures. To measure viral DNA replication, MiaPaCa-2 cells (12-well plate, 20,000 cells/well) were infected with Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP and wild-type Ad5 (Ad5 Reference Material; American Type Culture Collection, Manassas, VA; catalog # VR-1516) at a multiplicity of infection of 10 plaque-forming units/cell. Cells were harvested at specific times thereafter as indicated in the figures, and low molecular weight (Hirt) DNA was prepared. DNA was digested with HindIII and subjected to Southern blot analysis. The blot was probed with a [^{32}P]-probe that detects a 5.3 kilobase fragment in the E2 region that is common to Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP and wild-type Ad5. To measure the viral burst, MiaPaCa-2 and PANC-1 cells (6-well plate, 250,000 cells/well) were infected in triplicate with Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP and wild-type Ad5 at a multiplicity of infection of 10 plaque-forming units/cell. Six days later, after viral DNA reached a peak and before the appearance of CPE, the cells were harvested and then disrupted by three cycles of freeze-thawing; the amount of infectious adenovirus in the clarified cell lysates were measured by titration on HEK 293 cells using standard procedures.

In vivo *efficacy studies.* MiaPaCa-2 tumor xenografts were established by inoculating 2×10^6 cells prepared in 0.9% NaCl and 50% Matrigel in the right gastrocnemius muscle of female CD-1 athymic mice (20–22 g). Upon reaching 150 mm^3, tumors were injected with phosphate-buffered saline or adenovirus (10^{10} vp, 50 µl) on the days indicated in the figures. Animals receiving prodrugs were administered daily (weekdays only) intraperitoneal injections of 5-FC (500 mg/kg/day) and GCV (30 mg/kg/day) as indicated. Animals receiving radiation received 4×2 Gy (8 Gy total) of γ-irradiation to the tumored leg using a small animal irradiator (CP-160, Faxitron X-ray, Wheeling, VA). Tumor dimensions were measured twice a week, and tumor volume was calculated as previously described.[4] Animals were followed until the tumor volume reached 10% (2 cm^3 or 2 g) of the initial body weight (~20 g), which defined the survival endpoint. These studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the Henry Ford Health System.

PET imaging studies. Sterile surgical procedures were used throughout. Following abdominal incision, the pancreas of adult male dogs (10–12 kg) was exposed and injected with adenovirus (1×10^{12} vp, 0.25 ml) prepared in sterile saline. The next day, animals were administered intravenously 1–5 mCi [^{18}F]-FHBG (Siemens, New York, NY; formerly PETNET). Immediately after administration of [^{18}F]-FHBG, dogs were subjected to PET and CT imaging. Alternating whole body (7–8 beds; 3 minutes acquisition per bed) and abdominal (2 beds; 10 minutes acquisition per bed) emission images were acquired using a PET/CT dual scanner (Biograft Dual Scanner; Siemens, New York, NY). A CT transmission scan was obtained prior to each PET emission scan to generate the attenuation correction. PET/CT fusion images were generated with commercial software (MIM version 3.5; MIMvista, Cleveland OH). These studies were conducted under protocols approved by the Institutional Animal Care and Use Committee and Radiation Safety Committee of the Henry Ford Health System.

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Toxicology studies. Toxicology studies were designed with input from the Food and Drug Administration and conducted under a protocol approved by the Institutional Animal Care and Use Committee of the Henry Ford Health System. The Ad5-yCD/*mut*TK_{SR39}*rep*-ADP adenovirus (1×10^{12} vp, 0.25 ml saline) was injected as described for the PET imaging studies. Blood was taken at baseline prior to the adenovirus injection and at specific times after injection, including necropsy for complete blood cell counts and blood chemistries. Complete blood cell count included absolute counts for white blood cell, red blood cells, lymphocytes, neutrophils, eosinophils, basophils, and platelets. Blood chemistries included determination of hemoglobin, aspartate aminotransferase, alanine aminotransferase, bilirubin, alkaline phosphatase, γ-glutamyl transferase, total protein, albumin (A), globulin (G), A/G ratio, cholesterol, blood urea nitrogen, creatinine, phosphorus, calcium, glucose, amylase, lipase, sodium (Na), potassium (K), Na/K ratio, chloride, creatine phosphokinase, triglyceride, osmolality, and magnesium. Pancreas, liver, spleen, and lung were removed at necropsy for histopathological analysis. Tissues were paraffin-embedded, sectioned into 6 μm sections, and stained with hematoxylin and eosin.

Biostatistics. Statistical analyses were performed by the Department of Biostatistics and Research Epidemiology. Survival among the different treatment groups was compared using Kaplan–Meier analysis.

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