

Second-Generation Replication-Competent Oncolytic Adenovirus Armed with Improved Suicide Genes and ADP Gene Demonstrates Greater Efficacy without Increased Toxicity

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Replication-competent adenovirus-mediated suicide gene therapy has proven to be safe in humans when delivered intraprostatically. Although signs of efficacy are emerging, it is likely that further improvements will be needed before this technology will have widespread applicability in the clinic. Toward this end, we have developed a second-generation, replication-competent adenovirus (Ad5-yCD/*mutTK*_{SR39}*rep*-ADP) containing an improved yeast cytosine deaminase (yCD)/mutant_{SR39} herpes simplex virus thymidine kinase fusion (yCD/*mutTK*_{SR39}) gene and the adenovirus death protein (ADP) gene. Relative to the first-generation Ad5-CD/*TKrep* adenovirus, Ad5-yCD/*mutTK*_{SR39}*rep*-ADP demonstrated greater tumor cell kill *in vitro* and significantly greater tumor control in preclinical models of human cancer. Quantification of transgene volume following direct injection of adenovirus into human tumor xenografts and the naïve canine prostate demonstrated that ADP enhanced adenoviral spread *in vivo*. Toxicology studies were performed to determine whether the improved yCD/*mutTK*_{SR39} fusion and ADP genes increased toxicity. Intraprostatic injection of Ad5-yCD/*mutTK*_{SR39}*rep*-ADP did not result in significantly increased toxicity relative to the parental Ad5-CD/*TKrep* adenovirus, the latter of which has proven to be safe in two Phase I prostate cancer clinical trials. Together, these results provide the scientific basis for evaluating the safety and efficacy of the second-generation Ad5-yCD/*mutTK*_{SR39}*rep*-ADP adenovirus in humans.

Key Words: gene therapy, oncolytic adenovirus, radiation therapy

INTRODUCTION

Replication-competent, oncolytic adenoviruses represent a new investigational therapy that has been evaluated in human trials both as a single agent and in combination with conventional cancer therapies [1–10]. Although the safety of this approach has been demonstrated in multiple settings, it has become clear that oncolytic adenoviruses do not show sufficient activity as single agents to justify their use as a first-line cancer therapy. Nevertheless, anti-tumor activity has been demonstrated and it is possible that further improvements will bring this novel technology to a point at which it will have applicability in the clinic.

One strategy that has been used to increase the anti-tumor activity of replication-competent adenoviruses is to “arm” them with therapeutic genes. The first such armed

replication-competent adenovirus, Ad5-CD/*TKrep*, contained a bacterial cytosine deaminase (bCD)/wild-type herpes simplex virus thymidine kinase (HSV-1 TK) fusion gene in the E1 region in place of the 55-kDa E1B gene [11]. In preclinical models of human cancer, the anti-tumor activity of Ad5-CD/*TKrep* could be significantly enhanced by combining it with 5-fluorocytosine (5-FC) and ganciclovir (GCV) prodrugs, which are converted into toxic agents by the CD and HSV-1 TK genes contained in the virus [12,13]. Not only do the CD/5-FC and HSV-1 TK/GCV enzyme/prodrug systems provide a chemotherapeutic effect, they also function as potent sensitizers of radiotherapy [14–20]. The safety and preliminary efficacy of this combined approach have been evaluated in two Phase I clinical trials of prostate cancer without and with conventional radiotherapy [21,22]. In both studies, the combined

approach was associated with low toxicity with the vast majority (>90%) of adverse events being mild to moderate and self-limiting. In the absence of radiotherapy [21], anti-tumor activity was demonstrated by significant (>50%) declines in serum prostate-specific antigen (PSA) and histopathologic evidence of tumor destruction in post-treatment prostate biopsies. When combined with radiotherapy [22], better than expected declines in serum PSA were observed and the negative biopsy status at 1 year was better than historical controls (67% vs 45%) with the same patients and radiation doses. Although the results of these small Phase I studies must be interpreted cautiously, they are encouraging and raise the possibility that replication-competent, oncolytic adenoviruses armed with therapeutic genes may demonstrate meaningful clinical activity when applied to the right cancer and in the right setting.

Prior to evaluating the efficacy of this investigational approach in a randomized, prospective, two-arm Phase II trial, we have made two improvements in Ad5-CD/TKrep, generating the second-generation, replication-competent adenovirus Ad5-yCD/mutTK_{SR39}rep-ADP. First, Ad5-yCD/mutTK_{SR39}rep-ADP contains an improved yeast cytosine deaminase (yCD)/mutant_{SR39} herpes simplex virus thymidine kinase fusion (yCD/mutTK_{SR39}) gene in place of the bCD/wild-type HSV-1 TK fusion gene. Previous studies have demonstrated that both the yCD and the mutant_{SR39} HSV-1 TK enzymes are more catalytically efficient than their bacterial and wild-type counterparts, respectively [23–26]. Second, Ad5-yCD/mutTK_{SR39}rep-ADP contains the 11.6-kDa adenovirus death protein (ADP) gene that has been shown to enhance the cytolytic activity of replication-competent adenoviruses *in vitro* [27–31]. In this report, we compared the toxicity and efficacy of Ad5-yCD/mutTK_{SR39}rep-ADP versus the parental Ad5-CD/TKrep adenovirus in preclinical models. We demonstrate that Ad5-yCD/mutTK_{SR39}rep-ADP results in greater tumor

cell kill and tumor control without significantly increasing toxicity.

RESULTS

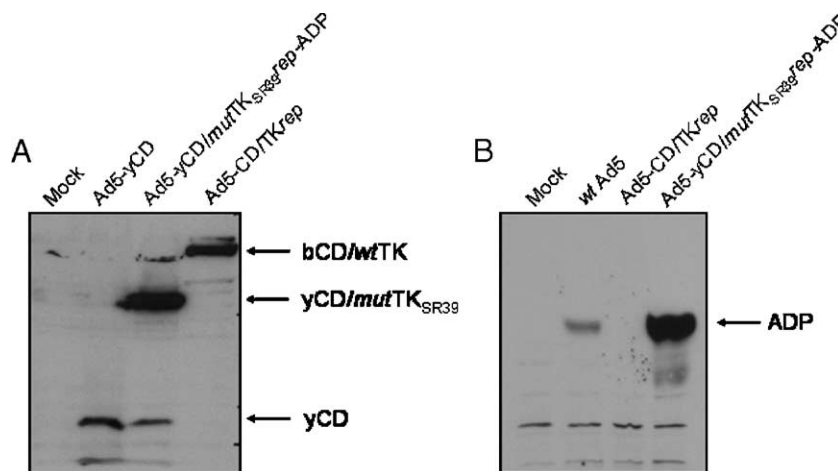
In Vitro Assays Demonstrating Therapeutic Advantage of yCD/mutTK_{SR39} and ADP Genes

We made two improvements in the parental Ad5-CD/TKrep adenovirus, generating the second-generation adenovirus, Ad5-yCD/mutTK_{SR39}rep-ADP. We replaced the prototype bCD/wild-type HSV-1 TK fusion gene located in the E1 region of Ad5-CD/TKrep with a yCD/mutant_{SR39} HSV-1 TK (mutTK_{SR39}) fusion gene. Second, whereas Ad5-CD/TKrep lacks any genes in the E3 region, Ad5-yCD/mutTK_{SR39}rep-ADP contains the Ad5 ADP gene. Both the yCD/mutTK_{SR39} and the ADP genes are under the transcriptional control of the human CMV promoter. Because these identical promoters are separated by approximately 27 kb, no rearrangements in the Ad5-yCD/mutTK_{SR39}rep-ADP genome have been observed in over a dozen laboratory, and one clinical, preparations.

Western blotting demonstrated that Ad5-yCD/mutTK_{SR39}rep-ADP expressed the expected 59-kDa yCD/mutTK_{SR39} fusion and 11.6-kDa ADP proteins (Figs. 1A and 1B). Ad5-yCD/mutTK_{SR39}rep-ADP expressed ADP at a level 5- to 10-fold greater than wild-type Ad5. This is likely attributable to the relative strengths of the promoters driving ADP expression (CMV in Ad5-yCD/mutTK_{SR39}rep-ADP and Ad5 major late promoter in wild-type Ad5).

To demonstrate the therapeutic advantage of the yCD/mutTK_{SR39} fusion gene *in vitro*, we performed 5-FC and GCV prodrug sensitivity assays. To minimize interference due to the ADP cytolytic effect, we infected mouse, rather than human, prostate adenocarcinoma cells with Ad5-CD/TKrep and Ad5-yCD/mutTK_{SR39}rep-

FIG. 1. Western blotting demonstrating expression of yCD/mutTK_{SR39} and ADP proteins. (A) The blot was probed with a mixture of polyclonal antibodies to yCD and bCD. The positions of the 17-kDa yCD, 59-kDa yCD/mutTK_{SR39}, and 88-kDa bCD/wtTK fusion proteins are indicated. The yCD/mutTK_{SR39} fusion protein is susceptible to proteolysis between the yCD and the mutTK_{SR39} moieties and the yCD moiety is detected. Ad5-yCD is a replication-competent adenovirus containing only the yCD gene in the E1 region. (B) The blot was probed with an antibody to ADP. The position of the 11.6-kDa ADP protein is indicated.



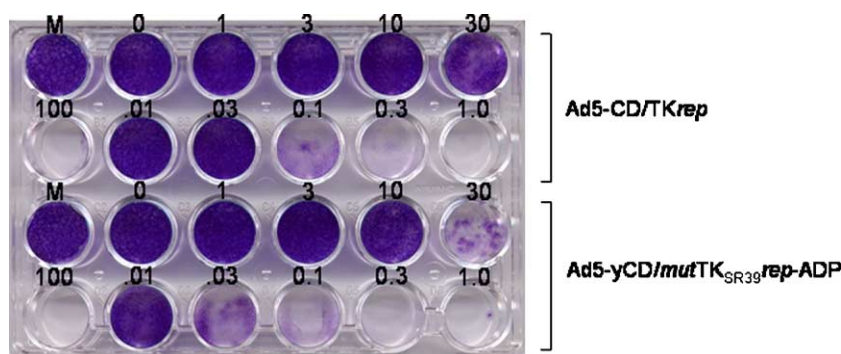


FIG. 2. Prodrug sensitivity assays. Mouse TRAMP-C2 cells were mock-infected (M) or infected with the Ad5-CD/TKrep or Ad5-yCD/mutTK_{SR39}rep-ADP adenovirus as indicated. Following infection, cells were incubated in medium containing varying concentrations of 5-FC (1, 3, 10, 30, 100 µg/ml) or GCV (0.01, 0.03, 0.1, 0.3, 1.0 µg/ml).

ADP and incubated them with increasing concentrations of 5-FC or GCV. Both Ad5-CD/TKrep and Ad5-yCD/mutTK_{SR39}rep-ADP replicate inefficiently in mouse TRAMP-C2 cells and the enhanced cytolytic effect of ADP is not observed during the course of the prodrug sensitivity assay. Both the yCD and the mutTK_{SR39} genes contained in Ad5-yCD/mutTK_{SR39}rep-ADP resulted in greater cell kill than the bCD and wt TK genes contained in Ad5-CD/TKrep (Fig. 2). Specifically, at a concentration of 30 µg/ml (0.23 mM) 5-FC and 0.03 µg/ml (0.1 µM) GCV, Ad5-yCD/mutTK_{SR39}rep-ADP resulted in significantly greater cell kill than Ad5-CD/TKrep. Clonogenic assays demonstrated that the increase in cell kill ranged from two- to fivefold for both the yCD/5-FC and the mutTK_{SR39}/GCV enzyme/prodrug systems (not shown). These results could not be explained by the presence of ADP in Ad5-yCD/mutTK_{SR39}rep-ADP because no cell kill was observed in the absence of prodrugs (Fig. 3, 0 viral particles (vp)/cell). Results of

the prodrug sensitivity assays were supported by *in vitro* enzyme assays demonstrating that the yCD and mutTK_{SR39} enzymes were five- and twofold better at converting 5-FC and GCV into their corresponding products than their bacterial and wild-type counterparts (not shown).

To demonstrate the therapeutic advantage of the ADP gene *in vitro*, we performed cytopathic effect (CPE) assays (see Materials and Methods for discussion regarding basing adenovirus input on vp versus plaque-forming units (pfu)). We infected human LNCaP prostate adenocarcinoma cells with increasing amounts of Ad5-CD/TKrep and Ad5-yCD/mutTK_{SR39}rep-ADP and examined cell kill in the absence of prodrugs. At all time points, one-tenth the amount of Ad5-yCD/mutTK_{SR39}rep-ADP was required to produce the same amount of cell death as Ad5-CD/TKrep (Fig. 3). We obtained similar results with human pancreatic adenocarcinoma cells (PaCa-2 and Panc-1) and human glioma (U251 and U87) cells (not shown).

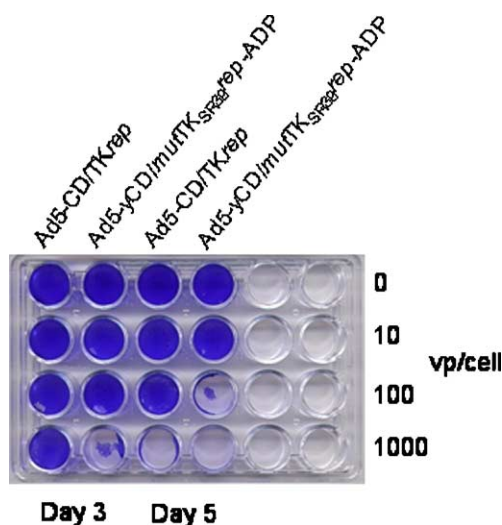


FIG. 3. Cytopathic effect assays. LNCaP cells were mock infected or infected with increasing amounts of the Ad5-CD/TKrep or Ad5-yCD/mutTK_{SR39}rep-ADP adenoviruses. Wells were fixed and stained with crystal violet 3 and 5 days later.

In Vivo Assays Demonstrating Therapeutic Advantage of yCD/mutTK_{SR39} and ADP Genes

To determine whether Ad5-yCD/mutTK_{SR39}rep-ADP was more efficacious than the parental Ad5-CD/TKrep adenovirus *in vivo*, we injected human LNCaP tumor xenografts with adenovirus and administered half the animals in each treatment group a 1-week course of 5-FC and GCV prodrug therapy. Ad5-yCD/mutTK_{SR39}rep-ADP resulted in significantly greater tumor control than Ad5-CD/TKrep both in the absence and in the presence of prodrugs (Fig. 4). In the absence of prodrugs, which reflects the effect of ADP only, Ad5-yCD/mutTK_{SR39}rep-ADP resulted in a tumor growth delay (relative to PBS-injected controls) of 41 days vs 9 days for Ad5-CD/TKrep ($P < 0.05$). In the presence of prodrugs, which reflect the combined effects of yCD/mutTK_{SR39} and ADP, Ad5-yCD/mutTK_{SR39}rep-ADP resulted in a tumor growth delay of 85 days vs 18 days for Ad5-CD/TKrep ($P < 0.05$). Importantly, 5-FC + GCV prodrug therapy significantly enhanced the anti-tumor effects of the Ad5-yCD/mutTK_{SR39}rep-ADP adenovirus alone (85 days vs 41 days,

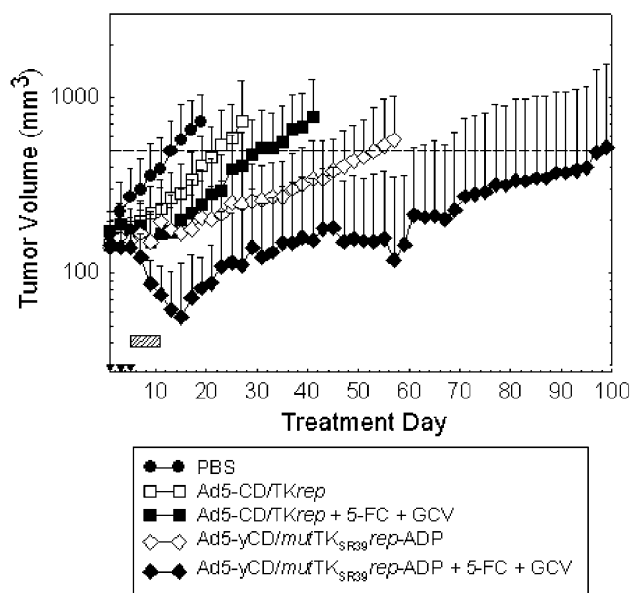


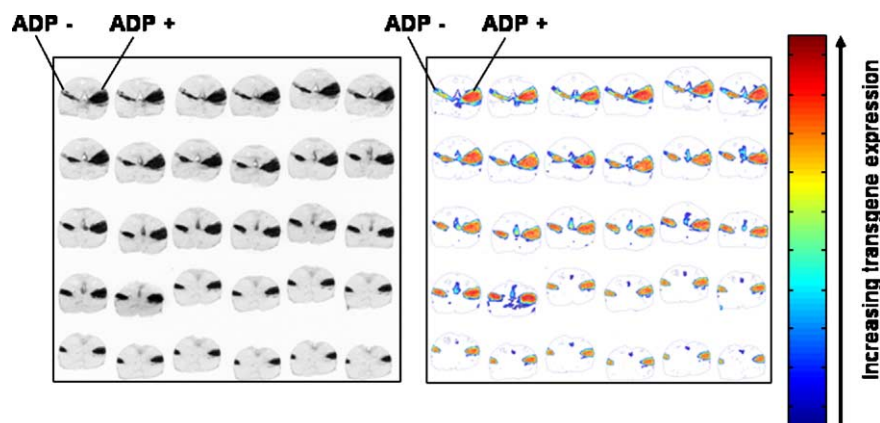
FIG. 4. Comparison of *in vivo* efficacy of Ad5-CD/TKrep versus Ad5-yCD/*mutTK_{SR39}rep*-ADP. LNCaP tumors (~150 mm³) were injected with PBS or the Ad5-CD/TKrep or Ad5-yCD/*mutTK_{SR39}rep*-ADP adenovirus (10^{10} vp per injection) on days 1, 3, and 5 (arrowheads). Half the mice in each treatment group were administered daily injections of 5-FC (500 mg/kg/day) and GCV (30 mg/kg/day) for 1 week (days 5–11, hatched bar). Each data point represents the group mean and the bars the standard deviation. Each group contained 10 animals.

$P < 0.05$). Similar results were obtained with the C33a (cervical) tumor model (not shown).

ADP Improves Local Spread of Replication-Competent Adenoviruses *in Vivo*

Previously, we developed a quantitative method to measure the magnitude and volume of transgene expression *in vivo* with submillimeter resolution [32,33]. This method involves quantification of a reporter gene, the human sodium iodide symporter (hNIS), from two-dimensional tissue sections followed by stacking of sections to create a 3D representation of the magnitude

FIG. 5. Effect of ADP on reporter gene expression volume *in vivo*. Replication-competent hNIS-containing adenoviruses, one lacking (Ad5-hNISrep, ADP-) and the other containing (Ad5-hNIS-ADPprep, ADP+) the Ad5 ADP gene, were injected into contralateral sides of the canine prostate. Representative autoradiograms depicting consecutive tissue sections (left) and computer-generated (i.e., digitized) representations of reporter gene expression (right) are shown. A color scale representing different relative levels of reporter gene expression is shown.



and volume of reporter gene expression *in vivo*. Although it has been demonstrated previously that ADP can enhance adenoviral spread *in vitro* [27–31], to our knowledge, this has never been demonstrated *in vivo*.

We injected a pair of replication-competent hNIS-containing adenoviruses, one lacking (Ad5-hNISrep) and the other containing (Ad5-hNIS-ADPprep) the Ad5 ADP gene, into mice bearing human tumor xenografts and the naïve canine prostate. Although the latter model lacks malignant tissue, the size and structure of the canine prostate resembles that of humans, making it an excellent preclinical model to examine issues such as adenoviral spread *in vivo*. In all studies, the dose, concentration, and vp/pfu ratio of adenovirus; the injection volume; the tumor and prostate volumes; and the injection plan were essentially identical so as not to introduce bias into the results. The next day, we administered Na^{99m}TcO₄ to the animals, removed and sectioned tissue, and quantified the volume of reporter gene expression. Representative autoradiograms of tissue sections depicting the raw data and transgene expression isodose curves depicting computer-generated representation of the raw data are shown in Fig. 5, with the results summarized in Table 1. In the human tumor xenograft model, the presence of ADP increased reporter gene expression volume by an average of 2.5-fold ($n = 5$). Similarly, in the canine prostate model, the presence of ADP increased transgene expression volume by an average of 3.3-fold ($n = 3$). These results demonstrate in two preclinical models that ADP increases the local spread of replication-competent adenoviruses *in vivo* and may provide an explanation for how ADP improves the anti-tumor effects of replication-competent, oncolytic adenoviruses.

Inclusion of yCD/*mutTK_{SR39}* and ADP Genes Does Not Significantly Increase Toxicity

Two toxicology studies were performed in C57BL/6 male mice to evaluate the toxicity of administering Ad5-yCD/*mutTK_{SR39}rep*-ADP intraprostatically (Table 2). The first study compared the toxicity of a single intraprostatic

TABLE 1: Transgene expression volume of ADP-containing versus non-ADP-containing adenoviruses

(A) Mouse studies			
Mouse	Reporter gene expression volume (% of tumor volume)		ADP (+)/ADP (-) ratio
	ADP (-)	ADP (+)	
1	3.1	9.9	3.2
2	6.9	25.7	3.7
3	6.9	7.4	1.1
4	8.8	19.1	2.2
5	3.2	6.9	2.2
Mean ± STD			2.5 ± 1.0

(B) Dog studies			
Dog	Reporter gene expression volume (% of prostate volume)		ADP (+)/ADP (-) ratio
	ADP (-)	ADP (+)	
1	1.3	4.1	3.2
2	1.2	4.3	3.6
3	2.1	6.5	3.1
Mean ± STD			3.3 ± 0.3

injection of Ad5-yCD/*mutTK_{SR39rep}*-ADP versus the parental Ad5-CD/*TKrep* adenovirus followed by 2 weeks of 5-FC + GCV prodrug therapy and pelvic radiation. For Ad5-yCD/*mutTK_{SR39rep}*-ADP treatment groups, we also evaluated every possible mono- and dual-therapy combination for comparison. The second study compared the toxicity of one versus two intraprostatic injections of Ad5-yCD/*mutTK_{SR39rep}*-ADP each followed by a 2.6-week cycle of 5-FC + GCV prodrug therapy and pelvic radiation. We recorded general observations daily and measured body weights weekly. We performed necropsies at three time points for histopathological analysis of major abdominal organs, blood cell counts, and blood chemistries.

Overall, the combination of Ad5-yCD/*mutTK_{SR39rep}*-ADP with 5-FC + GCV prodrug therapy and concomitant pelvic radiation (trimodal therapy) did not demonstrate significantly increased toxicity relative to the parental Ad5-CD/*TKrep* adenovirus. There were no deaths attributable to the investigational therapy in either study. In the first study, the most notable histopathological change was the presence of minimal to mild inflammation in all groups that received the Ad5-yCD/*mutTK_{SR39rep}*-ADP and Ad5-CD/*TKrep* adenoviruses (Table 3). The inflammation was often present along the serosal membranes of several abdominal organs (seminal vesicle, urinary bladder, liver) or within the organ parenchyma as was the case with the prostate gland and liver. Subacute inflammation was characterized primarily by the presence of a mixed inflammatory reaction consisting of polymorphonuclear inflammatory cells and mononuclear cells indicative of a more acute and ongoing active response. For instance, when present in the prostate or on serosal surfaces,

subacute inflammation was noted only at day 5. By day 17, the inflammatory reaction had become more chronic. Chronic inflammation was characterized by more mononuclear cells, fewer polymorphonuclear cells, and subtle fibrosis. We considered this pattern of serosal inflammation to represent a low-grade, and somewhat regional (seminal vesicles and urinary bladder), form of peritonitis related to leakage of adenovirus into the abdominal cavity following the intraprostatic injection. Subacute inflammation and periportal mononuclear cell infiltrate were two lesions commonly observed within the livers of mice. By day 53, many of the inflammatory reactions noted at days 5 and 17 had resolved.

Prodrug-treated groups (Groups 1, 2, and 7) exhibited leukopenia and lymphopenia on day 17 relative to controls (Table 3). These are expected side effects of the 5-FC + GCV prodrugs and resolved by day 53. Animals injected with Ad5-yCD/*mutTK_{SR39rep}*-ADP exhibited statistically higher serum ALT levels on day 17 relative to similarly treated Ad5-CD/*TKrep* animals and the controls. These elevations were minor (Group 1 vs Group 7—44 ± 8 vs 31 ± 6 IU/L, $P < 0.05$; Group 4 vs Group 8—34 ± 15 vs 19 ± 5 IU/L, $P < 0.05$; Group 6—28 ± 8 IU/L); however, because they were observed in the absence of prodrugs and radiation (Groups 4 and 8), they were likely attributable to ADP gene expression. These events resolved by day 53. Animals in both trimodal therapy groups (Groups 1 and 7) developed neutropenia on day 53, which may have been a consequence of the combined myelosuppressive effects of the prodrug and radiation therapies. We noted no other significant differences in the blood chemistries.

The second study demonstrated that two cycles of Ad5-yCD/*mutTK_{SR39rep}*-ADP/5-FC + GCV prodrug therapy concomitant with pelvic radiation did not significantly increase toxicity relative to a single treatment cycle (Table 3). As was observed in the first study, the most notable histopathological change was inflammation along the serosal membranes of several abdominal organs or within the organ parenchyma in both treatment groups (Groups 1 and 2). In addition, there was minimal inflammation of the colon and the presence of apoptotic glandular epithelial cells in the small bowel, which are expected side effects of pelvic radiation. As expected, animals in the treatment groups exhibited myelosuppression during the prodrug therapy and radiation course. However, there was no significant difference between Group 1 (one cycle) and Group 2 (two cycles), and all events, except for slight anemia in Group 2, resolved by the end of the study. Interestingly, animals in both treatment groups demonstrated slight elevations in alkaline phosphatase at the end of the study (Group 1—90 ± 15, Group 2—83 ± 9 vs 60 ± 10 IU/L for controls, $P < 0.05$). Because these events were not accompanied by elevations in hepatic transaminases or bilirubin, indicating damage to the hepatobiliary system,

TABLE 2: Design of toxicology studies

Study 1: Single intraprostatic injection of Ad5-yCD/*mutTK_{SR39}rep*-ADP versus Ad5-CD/*TKrep* followed by 2 weeks of 5-FC + GCV prodrug therapy and 56 Gy radiation to pelvic region

Adenovirus	Treatment plan		EBRT ^b	Endpoints ^c
	Prodrugs ^a			
Gr 1, Ad5-yCD/ <i>mutTK_{SR39}rep</i> -ADP (10 ¹⁰ vp, day 1)	2 weeks (days 3–16)		56 Gy (days 5–51)	Day 5—histopathology of major abdominal organs, CBC, and blood chemistries on 5 animals in adenovirus-only (Gr 4 and 8) and control (Gr 6) groups. Days 17 and 53—same analyses as on day 5 for all groups.
Gr 2, Ad5-yCD/ <i>mutTK_{SR39}rep</i> -ADP (10 ¹⁰ vp, day 1)	2 weeks (days 3–16)		None	
Gr 3, Ad5-yCD/ <i>mutTK_{SR39}rep</i> -ADP (10 ¹⁰ vp, day 1)	Saline		56 Gy (days 5–51)	
Gr 4, Ad5-yCD/ <i>mutTK_{SR39}rep</i> -ADP (10 ¹⁰ vp, day 1)	Saline		None	
Gr 5, vehicle	Saline		56 Gy (days 5–51)	
Gr 6, vehicle	Saline		None	
Gr 7, Ad5-CD/ <i>TKrep</i> (10 ¹⁰ vp, day 1)	2 weeks (days 3–16)		56 Gy (days 5–51)	
Gr 8, Ad5-CD/ <i>TKrep</i> (10 ¹⁰ vp, day 1)	Saline		None	

Study 2: One versus two intraprostatic injections of Ad5-yCD/*mutTK_{SR39}rep*-ADP each followed by a 2.6-week cycle of 5-FC + GCV prodrug therapy and a total of 56 Gy radiation to pelvic region

Adenovirus	Treatment plan		EBRT ^e	Endpoints ^f
	Prodrugs ^d			
Gr 1, Ad5-yCD/ <i>mutTK_{SR39}rep</i> -ADP (10 ¹⁰ vp, day 1)	2.6 weeks (days 4–20)		56 Gy (days 5–50)	Days 21, 55, and 69—histopathology of major abdominal organs, CBC, and blood chemistries on 5 animals in all groups.
Gr 2, Ad5-yCD/ <i>mutTK_{SR39}rep</i> -ADP (10 ¹⁰ vp, day 1)	2.6 weeks (days 4–20)		56 Gy (days 5–50)	
Ad5-yCD/ <i>mutTK_{SR39}rep</i> -ADP (10 ¹⁰ vp, day 22)	2.6 weeks (days 25–41)			
Gr 3, vehicle	Saline		None	

^a 5-FC 500 mg/kg/day, GCV 30 mg/kg/day for 14 consecutive days.

^b 14 × 4 Gy, given twice per week for 7 weeks, for a total of 56 Gy.

^c Day 5 (early time point taken in adenovirus-only and control groups); day 17 (taken in all groups after completion of prodrug therapy), day 53 (taken in all groups after completion of study treatment). At each time point, a necropsy was performed and gross observations were recorded, a blood sample was taken, and the following organs were removed for histopathological analysis: prostate, seminal vesicles, urinary bladder, liver, kidney, spleen, small intestines (3 sections—duodenum, jejunum, ileum), large intestines (3 sections—cecum, colon, rectum). CBC included WBC, RBC, lymphocytes, neutrophils, eosinophils, basophils, and platelets. Blood chemistries included HGB, AST, ALT, bilirubin, alkaline phosphatase, and albumin.

^d 5-FC 500 mg/kg/day, GCV 30 mg/kg/day for 2.6 weeks (weekdays only, 13 days).

^e 14 × 4 Gy, given twice per week for 7 weeks, for a total of 56 Gy.

^f Day 21 (immediately after completion of first cycle of adenovirus and prodrugs), day 55 (shortly after completion of treatment), day 69 (2 weeks after second time point). At each time point, the same analyses were performed as in Study 1 except that prostate and seminal vesicles were not analyzed because the histopathological results were certain in these tissues (i.e., inflammation) based on Study 1.

and the animals did not have evidence of bone disease, we believe this event was likely associated with the observed inflammation of the small bowel.

DISCUSSION

The purpose of these studies was to evaluate in preclinical models the toxicity and efficacy of the second-generation Ad5-yCD/*mutTK_{SR39}rep*-ADP adenovirus to provide the scientific basis for its evaluation in humans. We demonstrate here that relative to the parental Ad5-CD/*TKrep* adenovirus, Ad5-yCD/*mutTK_{SR39}rep*-ADP exhibited greater tumor cell kill and tumor control without significantly increasing toxicity. Moreover, administering two cycles of the Ad5-yCD/*mutTK_{SR39}rep*-ADP adenovirus/5-FC + GCV prodrug therapy combination concomitant with

radiotherapy resulted in little added toxicity relative to a single cycle. Together, the results indicate that Ad5-yCD/*mutTK_{SR39}rep*-ADP is suitable for evaluation in future clinical trials of human cancer in which the safety and efficacy of combining replication-competent adenovirus-mediated suicide gene therapy with radiotherapy will be compared directly to radiotherapy alone.

The safety of replication-competent adenovirus-mediated suicide gene therapy using the parental Ad5-CD/*TKrep* adenovirus has been evaluated in two Phase I clinical trials of prostate cancer without and with conventional radiotherapy [21,22]. Overall, this investigational therapy has been well tolerated. When combining the results of both studies (31 patients), the most frequent side effects attributable to the investigational therapy were mild (grade 1) to moderate (grade 2)

TABLE 3: Results of toxicology studies

Study 1				
Group	Group description	Day 5	Adverse events	
			Day 17	Day 53
1	Ad5-yCD/ <i>mutTK_{SR39}rep</i> -ADP + PD + EBRT	Acute inflamm.; Pr, SV, UB, L	Chronic inflamm.; Pr, SV, UB, L Leukopenia, lymphopenia Slightly elevated ALT	Resolved Resolved Resolved Neutropenia
2	Ad5-yCD/ <i>mutTK_{SR39}rep</i> -ADP + PD	Acute inflamm.; Pr, SV, UB, L	Chronic inflamm.; Pr, SV, UB, L Leukopenia, lymphopenia	Resolved Resolved
3	Ad5-yCD/ <i>mutTK_{SR39}rep</i> -ADP + EBRT	Acute inflamm.; Pr, SV, UB, L	Chronic inflamm.; Pr, SV, UB, L	Resolved
4	Ad5-yCD/ <i>mutTK_{SR39}rep</i> -ADP only	Acute inflamm.; Pr, SV, UB, L	Chronic inflamm.; Pr, SV, UB, L Slightly elevated ALT	Resolved Resolved
5	EBRT only	No AE	No AE	No AE
6	Vehicle	No AE	No AE	No AE
7	Ad5-CD/ <i>TKrep</i> + PD + EBRT	Acute inflamm.; Pr, SV, UB, L	Chronic inflamm.; Pr, SV, UB, L Leukopenia, lymphopenia	Resolved Resolved Neutropenia
8	Ad5-CD/ <i>TKrep</i> only	Acute inflamm.; Pr, SV, UB, L	Chronic inflamm.; Pr, SV, UB, L	Resolved
Study 2				
Group	Group description	Day 21	Adverse events	
			Day 55	Day 69
1	Ad5-yCD/ <i>mutTK_{SR39}rep</i> -ADP + PD + EBRT (one cycle of adenovirus and PD)	Acute inflamm.; UB, L Leukopenia Lymphopenia Neutropenia Anemia Thrombocytopenia	Chronic inflamm.; UB, L Leukopenia Lymphopenia Resolved Anemia Resolved	Resolved Resolved Resolved Resolved Resolved Resolved Elevated ALKP
2	Ad5-yCD/ <i>mutTK_{SR39}rep</i> -ADP + PD + EBRT (two cycles of adenovirus and PD)	Acute inflamm.; UB, L Leukopenia Lymphopenia Neutropenia Anemia Thrombocytopenia	Chronic inflamm.; UB, L Leukopenia Lymphopenia Resolved Anemia Resolved	Resolved Resolved Resolved Resolved Sl. anemia Resolved Elevated ALKP
3	Vehicle	No AE	No AE	No AE

Abbreviations used: PD, prodrugs; EBRT, external beam radiation; inflamm., inflammation; Pr, prostate; SV, seminal vesicles; UB, urinary bladder; L, liver; AE, adverse event; UB, urinary bladder; ALKP, alkaline phosphatase; sl., slight.

myelosuppression (~90% of patients) and mild hepatotoxicity (~33% of patients). These events were self-limiting and resolved shortly after completion of the prodrug therapy course. When combined with radiotherapy, there was no significant increase in acute bladder and bowel toxicities relative to conventional radiotherapy alone [22]. It is comforting that these same toxicities were observed in our preclinical toxicology studies, suggesting that they may accurately forecast what will occur in humans. We are pleased to find that despite having a more catalytically efficient yCD/*mutTK_{SR39}* fusion and the ADP genes, the latter of which is expressed at a level five times that of wild-type Ad5, Ad5-yCD/*mutTK_{SR39}rep*-ADP resulted in little added hepatotoxicity relative to Ad5-CD/*TKrep*, which has a proven safety profile in humans [21,22]. Indeed, Ad5-yCD/*mutTK_{SR39}rep*-ADP has already been administered

intraprostatically to humans (10^{11} , 10^{12} vp) followed by 2.6 weeks of 5-FC + GCV prodrug therapy and 76-Gy intensity-modified radiotherapy, and there have been only minor elevations in liver transaminase levels. These results are consistent with our previous studies in the dog, which demonstrated that following direct intraprostatic injection, little of the injected adenovirus disseminates beyond the prostate gland [32]. Nevertheless, because hepatotoxicity can be life-threatening, it will be monitored closely in all future trials, particularly those in which the injected tumor is in close proximity to the liver.

A major limitation of human cancer gene therapy, however, is not excessive toxicity but rather a lack of significant efficacy. The limited efficacy observed in human studies to date is likely attributable to poor *in vivo* delivery of the therapeutic agent. Both replication-

competent, oncolytic adenoviruses and the CD/HSV-1 TK suicide gene systems are very efficient at killing tumor cells *in vitro* and it is possible that if a high fraction of tumor cells could be transduced *in vivo*, significant outcomes would be observed in humans. In an attempt to improve the local spread of these therapeutic agents *in vivo*, we generated an armed replication-competent adenovirus containing the ADP gene. As demonstrated here for the first time, inclusion of the ADP gene increased reporter gene expression volume approximately threefold *in vivo*. Our results confirm and extend those of Wold and colleagues, who demonstrated that ADP increases adenovirus spread *in vitro* [27–31]. In our studies, an ADP-dependent increase in adenoviral spread was detectable at 24 h based upon evidence obtained in two animal models. It is possible that the observed increase in adenoviral spread at this early time point might not be explained solely on the basis of ADP's known cytolytic effect. Oncolytic adenoviruses expressing ADP might elicit a more robust immune response, which, in turn, might facilitate adenoviral spread through faster cell death. We have observed robust immune responses to replication-competent adenoviruses in the dog prostate as early as 24–48 h postinfection [32]. The immune response is characterized by a massive infiltration of neutrophils and macrophages, which are also present in athymic mice. Thus, the observed increase in adenoviral spread *in vivo* may reflect ADP's cytolytic effect, which can be observed *in vitro*, and the possible effect of ADP on the magnitude of the immune response. If these effects of ADP can be reproduced in humans, it should result in greater tumor destruction via the cytolytic effects of the Ad5-yCD/*mutTK_{SR39}rep*-ADP adenovirus, the chemoradiosensitization effects of the CD/HSV-1 TK suicide gene systems, and the resulting immune response.

Prior to these studies, it could have been argued that a potential drawback of including ADP in an armed replication-competent adenovirus is that it may shorten the duration of therapeutic gene expression. Replication-competent adenoviruses expressing ADP spread much faster, resulting in greater cell death when observed at a fixed time point. Although this effect of ADP clearly enhances the anti-tumor activity of the adenovirus itself ([29–31], this paper), it may weaken the chemoradiosensitization effects of the CD/HSV-1 TK suicide gene systems. Our preclinical studies indicate that ADP does not circumvent the chemoradiosensitization effects of the CD/HSV-1 TK suicide gene systems. As demonstrated here, the addition of 5-FC + GCV prodrug therapy significantly improved tumor control over that of the Ad5-yCD/*mutTK_{SR39}rep*-ADP adenovirus alone. Similar results have been obtained with the C33a (cervical) tumor model (unpublished). Although not examined here in the prostate model, we have observed marked synergy when combining Ad5-yCD/*mutTK_{SR39}*.

rep-ADP + 5-FC + GCV with radiation in a preclinical model of human pancreatic cancer. Moreover, as demonstrated here, significant tumor regression, rather than a slowing of tumor growth, was observed in the Ad5-yCD/*mutTK_{SR39}rep*-ADP-treated groups only when the 5-FC + GCV prodrugs were administered. Such effects were not observed with Ad5-yCD/*mutTK_{SR39}rep*-ADP itself, and after completion of the prodrug therapy course, tumor growth resumed. Taken together, these observations demonstrate that ADP does not circumvent the chemotherapeutic or radiosensitization effects of the CD/HSV-1 TK suicide gene systems and it can provide a therapeutic benefit even in the context of an armed replication-competent, oncolytic adenovirus. Based on these results, we believe that Ad5-yCD/*mutTK_{SR39}rep*-ADP will demonstrate greater efficacy than Ad5-CD/TK*rep* in human cancer trials and therefore is a better agent to test the hypothesis that replication-competent adenovirus-mediated suicide gene therapy can improve the outcome of conformal radiotherapy.

MATERIALS AND METHODS

Cell lines and adenoviruses. LNCaP cells were obtained from Dr. Leland Chung (Emory University, Atlanta, GA, USA). TRAMP C2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were grown in Dulbecco's modified essential medium with 10% fetal bovine serum.

The replication-competent Ad5-CD/TK*rep* adenovirus has been described previously [11–13]. Details regarding the construction of Ad5-yCD/*mutTK_{SR39}rep*-ADP will be provided upon request. The entire DNA sequence (35,180 bases) of Ad5-yCD/*mutTK_{SR39}rep*-ADP has been determined and is in 99.92% agreement with the predicted sequence. There were no alterations in the yCD/*mutTK_{SR39}* fusion or ADP genes.

Comparing the *in vitro* and *in vivo* properties of the Ad5-CD/TK*rep* and Ad5-yCD/*mutTK_{SR39}rep*-ADP adenoviruses, all studies were performed with an equal input of adenovirus based on viral particles. As previously described by Wold and colleagues [27,28], adenoviruses containing ADP develop plaques at a much faster rate than do adenoviruses lacking ADP. Therefore, the determination of plaque-forming units is very time dependent and can be highly variable depending on the condition of the infected cells. When plaque formation is scored 5 days postinfection, Ad5-yCD/*mutTK_{SR39}rep*-ADP typically has a vp/pfu 10 times that of Ad5-CD/TK*rep*. However, because Ad5-CD/TK*rep* (which lacks ADP) plaques develop much more slowly, the final vp/pfu of the two adenoviruses is essentially identical (10–20) when observed over a protracted period of time (i.e., 3 weeks). Thus, an equal input of vp is equivalent to an equal input of final pfu (21 days).

Western blotting. Western blotting was performed as previously described [11]. Rabbit polyclonal antibodies to bCD, yCD, and ADP were obtained from C. Richards (Glaxo-Wellcome, Inc., Research Triangle Park, NC, USA), A. Rehemtulla (University of Michigan, Ann Arbor, MI, USA), and W. Wold (St. Louis University, St. Louis, MO, USA), respectively. ECL sheep anti-rabbit IgG horseradish peroxidase was used as secondary antibody (Amersham Biosciences, Piscataway, NJ, USA).

***In vitro* prodrug sensitivity, cytopathic effect, and enzyme assays.** Prodrug sensitivity and CPE assays were performed as described previously [11]. Briefly, LNCaP cells were mock infected or infected with adenovirus. One hour later, the medium was changed and cells were maintained thereafter in growth medium. For prodrug sensitivity assays, 5-FC and

GCV prodrugs were maintained throughout. Wells were fixed and stained with 0.4% crystal violet at specific time points thereafter.

CD and HSV-1 TK assays were performed as described previously [11]. Briefly, LNCaP cells were mock infected or infected with adenovirus at a vp/cell of 1000. Forty-eight hours later, cells were harvested and a cell extract was prepared by three cycles of freeze–thawing. Clarified cell extracts containing an equal amount of protein were incubated with [¹⁴C]cytosine and 5-[¹⁴C]fluorocytosine for CD assays and [³H]ganciclovir for HSV-1 TK assays at 37°C for 1 h. Substrates and products were resolved by thin-layer chromatography, the chromatogram was exposed to X-ray film, and the amount of radioactivity in each reaction species was quantified using a Bio-Rad FX molecular phosphorimager.

In vivo efficacy and toxicology studies. Animal studies were approved by the Institutional Animal Care and Use Committee of the Henry Ford Health System and Good Laboratory Practices were used throughout. LNCaP tumors were established by inoculating 2×10^6 cells prepared in 0.9% NaCl and 50% Matrigel in the right gastrocnemius muscle (im) of male SCID mice (20–22 g). Upon reaching ~150 mm³, tumors were injected with PBS or adenovirus (10^{10} vp, 50 μl) on days 1, 3, and 5. Half the mice in each treatment group were administered daily intraperitoneal (ip) injections of 5-FC (500 mg/kg/day) and GCV (30 mg/kg/day) for 1 week (days 5–11). Tumor dimensions were measured every 2–3 days.

Toxicology studies were designed with input from the FDA. In the first study, 102 male C57BL/6 mice (20–22 g) were injected intraprostatically with 10^{10} vp (10 μl) Ad5-γCD/*mutTK_{SR39rep}*-ADP (Groups 1–4), 10^{10} vp (10 μl) Ad5-CD/*TKrep* (Groups 7 and 8), or PBS (Groups 5 and 6) on day 1. Animals that were scheduled to receive prodrugs (Groups 1, 2, and 7) were given 14 daily ip injections of 5-FC (500 mg/kg/day) and GCV (30 mg/kg/day) (days 3–16). Other groups received saline in place of prodrugs. Animals that were scheduled to receive radiation (Groups 1, 3, 5, and 7) received 4 Gy of γ-irradiation to their left (2 Gy) and right (2 Gy) pelvic region twice per week for 7 weeks for a total dose of 56 Gy using a ¹³⁷Cs irradiator.

In the second study, 60 male C57BL/6 mice (20–22 g) were injected intraprostatically with 10^{10} vp (10 μl) Ad5-γCD/*mutTK_{SR39rep}*-ADP on day 1 (Groups 1 and 2) and day 22 (Group 2) or injected with PBS (Group 3). Animals in treatment groups received 13 ip injections (weekdays only) of 5-FC (500 mg/kg/day) and GCV (30 mg/kg/day) in one (Group 1) or two (Group 2) 2.6-week cycles following each adenovirus injection. Prodrugs were administered on weekdays only to mimic future clinical trials. Animals in treatment groups (Groups 1 and 2) received 4 Gy of γ-irradiation to their left (2 Gy) and right (2 Gy) pelvic region twice per week for 7 weeks for a total dose of 56 Gy using a small-animal linear accelerator.

In both studies, animals were examined for a number of toxicological parameters: general observations were noted daily; body weights were taken weekly and at each necropsy; gross observations were noted at time of necropsy; and at necropsy either a partial or a full set of major abdominal tissues was taken for histopathological analyses. A blood sample was taken at every necropsy for complete blood cell counts (CBC) and blood chemistries. CBC included absolute counts for white blood cells (WBC), red blood cells (RBC), lymphocytes, neutrophils, eosinophils, basophils, and platelets. Blood chemistries included determination of hemoglobin (HGB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, alkaline phosphatase, and albumin. CBC was performed by Laboratory Corporation of America (Burlington, NC, USA). Histopathology was performed by Experimental Pathology Laboratories, Inc. (Herndon, VA, USA). Statistical analyses were conducted by the Department of Biostatistics and Epidemiology at the Henry Ford Health System.

Quantification of reporter gene expression volume in vivo. In the mouse studies, tumor cells were implanted into both hind legs of CD-1 (*nu/nu*) athymic mice. When tumors reached 150 mm³, 1×10^{10} vp (50 μl) of replication-competent hNIS-containing adenoviruses, one lacking (*Ad5-hNISrep*) and the other containing (*Ad5-hNIS-ADPrep*) the Ad5 ADP gene, was injected into contralateral tumors of each mouse. One day later, animals were administered 0.15 mCi Na^{99m}TcO₄ ip. One hour later,

tumors were excised, frozen, and completely sectioned in 100-μm-thick sections, and sections were exposed to X-ray film for 60 h. In the dog studies, 1×10^{11} vp (100 μl) of the replication-competent hNIS-containing adenoviruses (one containing and one lacking the ADP gene) was injected into contralateral sides of the naïve canine prostate. The next day, animals were administered 20 mCi Na^{99m}TcO₄ ip. One hour later, the prostate was excised, frozen, and completely sectioned in 100-μm-thick sections, and sections were exposed to X-ray film for 60 h. In both mouse and dog studies, autoradiograms were developed and digitized and the volume of reporter gene expression was determined using methods previously described [32,33].

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