Double Suicide Gene Therapy Augments the Antitumor Activity of a Replication-Competent Lytic Adenovirus through Enhanced Cytotoxicity and Radiosensitization

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ABSTRACT

Replication-competent adenoviruses may provide a highly efficient means of delivering therapeutic genes to tumors. Previously, we evaluated in vitro a replication-competent adenovirus (Ad5-CD/TK_{rep}) containing a cytosine deaminase (CD)/herpes simplex type 1 thymidine kinase (HSV-1 TK) fusion gene that allows lytic viral therapy to be combined with double suicide gene therapy. Both the CD/5-FC and HSV-1 TK/GCV enzyme/prodrug systems enhanced the tumor cell-specific cytopathic effects of the Ad5-CD/TK_{rep} virus in vitro and sensitized cells to radiation. To extend these in vitro findings in vivo, we evaluated the antitumor activity of the Ad5-CD/TK_{rep} virus in combination with double prodrug therapy and radiation therapy. The Ad5-CD/TK_{rep} virus independently demonstrated significant antitumor activity against C33A cervical carcinoma xenografts. Therapeutic outcome was dramatically improved with systemic administration of double, but not single, prodrug (5-FC + GCV) therapy. When used in a neoadjuvant setting, Ad5-CD/TK_{rep}-mediated double suicide gene therapy dramatically potentiated the effectiveness of radiation therapy. The trimodal approach of Ad5-CD/TK_{rep} viral, double suicide gene, and radiotherapies produced significant tumor regression and ultimately 100% tumor cure. The results demonstrate the high therapeutic potential of the trimodal approach and provide a solid foundation for future clinical trials.

OVERVIEW

The ability of double suicide gene therapy to improve the antitumor efficacy of a cytolytic adenovirus was examined utilizing an E1B-attenuated, replication-competent adenovirus (Ad5-CD/TK_{rep}) encoding a CD/HSV-1 TK fusion gene. Despite the inability of the individual CD/5-FC and HSV-1 TK/GCV systems to enhance viral therapy, double suicide gene therapy, employing both 5-FC and GCV prodrugs, markedly enhanced the therapeutic effectiveness of the lytic Ad5-CD/TK_{rep} virus. Consistent with the ability of CD/5-FC and HSV-1 TK/GCV enzyme/prodrug systems to function as effective neoadjuvants to radiotherapy, Ad5-CD/TK_{rep}-mediated double suicide gene therapy markedly sensitized adenovirally infected tumors to \gamma radiation. Concomitant adenoviral, double suicide gene, and radiotherapies resulted in significant tumor regression and tumor cure, demonstrating the therapeutic potential of the trimodal anticancer approach.

INTRODUCTION

A VARIETY OF cancer gene therapies have been developed and proven effective against experimental tumors in vivo. For a given cancer gene therapy to be clinically successful, however, it must selectively destroy malignant cells while minimizing damage to normal tissue. One approach that has proven effective at increasing the differential response between tumor and normal tissue is suicide gene therapy. Suicide gene therapy involves the tumor-targeted delivery of genes encoding metabolic enzymes that convert systemically delivered, innocuous prodrugs into toxic metabolites. This results in a high concentration of toxic product intratumorally, thereby avoiding the systemic toxicity often associated with conventional chemotherapy (e.g., 5-fluorouracil [5-FU]). Two suicide gene systems that have been studied intensely are herpes simplex type 1 thymidine kinase (HSV-1 TK)/ganciclovir (GCV) and Escherichia coli cytosine deaminase (CD)/5-fluorocytosine (5-FC). HSV-1 TK phosphorylates GCV, converting it to a nucleotide analog.
that inhibits DNA synthesis (DeClercq, 1984), while CD converts 5-FC to its highly toxic metabolite, 5-FU (Calabresi and Chabner, 1990).

Effective cancer gene therapy also requires an efficient and reproducible method of in vivo gene delivery. Currently, recombinant adeno-viruses represent the most efficient means of delivering genes to solid tumors. Preclinical studies have demonstrated a high efficiency of gene transfer and effective tumor control after regional delivery of replication-defective, CD- or HSV-1 TK-containing adenoviruses, coupled with 5-FC or GCV therapy (Chen et al., 1994; Hirschowitz et al., 1995; Dong et al., 1996; Eastman et al., 1996; Ohwada et al., 1996; Block et al., 1997; Evoy et al., 1997; Hall et al., 1997; Sutton et al., 1997).

In an attempt to improve the efficacy of these monotherapies, we previously coupled the CD/5-FC and HSV-1 TK/GCV enzyme/prodrug systems and pioneered the concept of using them as a neoadjuvant to radiation therapy (Kim et al., 1994; Hirschowitz et al., 1996; Eastman et al., 1997; Evoy et al., 1997; Hall et al., 1997; Sutton et al., 1997).

As detailed above, C33A cells were implanted into the right gastrocnemius muscle. Intramuscular, rather than subcutaneous, cells (5 \times 10^5/60-mm dish) were either mock infected or infected with Ad5-FGNR at a multiplicity of infection (MOI) of 100 in 1 ml of DMEM with 2% FBS. After 1 hr, cells were replated (1.5 \times 10^4 cells/well, 24-well plate) in DMEM containing 10% FBS (growth medium). Seventy-two hours postinfection, the medium was removed and replaced with fresh medium without or with 5-FC (Sigma, St. Louis, MD) or GCV (Syntex, Palo Alto, CA). Cells were cultured in the presence of prodrugs for 96 hr. Cell viability was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] assay according to the manufacturer protocol.

**Ad5-CD/TKrep viral replication in the presence of prodrugs**

C33A cells (10^6/60-mm dish) were either mock infected or infected with Ad5-CD/TKrep at an MOI of 10 in 0.2 ml of DMEM with 2% FBS. After 1 hr, the medium was removed and cells were immediately replated (2 \times 10^4 cells/well, 24-well plate) in varying concentrations of 5-FC or GCV. Low molecular weight DNA was isolated 48 hr postinfection and analyzed by Southern blotting as previously described (Freytag et al., 1998).

**In vivo adenovirus-mediated suicide gene therapy**

Five- to 6-week-old female athymic mice (*nu/nu* [CD-1]; Charles River Laboratories, Wilmington, MA) were used in all studies. C33A tumors were established by inoculating 2 \times 10^6 cells prepared in 0.9% NaCl and 15% Matrigel (Collaborative Biomedical Products, Bedford, MA), subcutaneously over the abdomen or intramuscularly within the right gastrocnemius muscle. On reaching 100 mm^3 (subcutaneous) or 120 mm^3 (intramuscular), tumors were injected with 10^6 PFU of Ad5-CD/TKrep or phosphate-buffered saline (PBS, 50 \mu l) for five consecutive days (days 0–4). Daily injections were distributed evenly throughout the tumor. At the conclusion of the viral inoculations, mice in the prodrug treatment groups received intraperitoneal injections of 5-FC (500 mg/kg), GCV (60 mg/kg), or 5-FC (500 mg/kg) + GCV (60 mg/kg). Prodrugs were administered daily as indicated in Figs. 1 and 3. Tumors were measured every 2–3 days. Tumor volume was determined by three-dimensional caliper measurements.

**Radiation therapy**

As detailed above, C33A cells were implanted into the right gastrocnemius muscle. Intramuscular, rather than subcutaneous,
tumors are used to minimize stretching of the skin, which can lead to unacceptable ulceration after irradiation. When tumors reached 120 mm³, animals received five daily intratumoral injections of Ad5-CD/TKrep or PBS (days 0–4). After the last viral injection (day 4), Ad5-CD/TKrep-treated mice were divided into two groups (Ad5-CD/TKrep, Ad5-CD/TKrep + 5-FC + GCV; 10 animals per group). Prodrug-treated animals received daily injections of 5-FC (500 mg/kg) and GCV (60 mg/kg) for either 4 or 7 days, as indicated in Figs. 1 and 3, beginning on day 4. On the day of irradiation, the two treatment groups were further subdivided into four groups (five animals per group) based on comparable tumor volumes: Ad5-CD/TKrep, Ad5-CD/TKrep + radiation treatment (RT), Ad5-CD/TKrep + 5-FC + GCV, and Ad5-CD/TKrep + 5-FC + GCV + RT. Radiation was delivered to the tumor leg 2 hr after prodrugs were given for that day. For radiation response controls, five uninjected tumors with volumes comparable to those within the Ad5-CD/TKrep + RT and Ad5-CD/TKrep + 5-FC + GCV + RT groups were also irradiated. Animals in the radiation treatment groups were anesthetized by intraperitoneal injection of Nembutal (60 mg/kg) and a single dose of 56Co γ-radiation (8 or 10 Gy) was delivered to the tumor-bearing leg as described previously (Rogulski et al., 1997b). Volumes of intramuscular leg tumors were determined by the following formula (Alfieri and Hahn, 1978): \( d^3 \times 0.62 = \text{volume (cm}^3\) \), where \( d^3 \) is the average diameter of the tumored leg (cm) and the product (0.62) is the correction factor for normal leg volume. For all tumor studies, animals were followed until death (euthanasia) from tumor burden or for at least 90 days after cessation of treatment. All animal experiments were approved by the Care of Experimental Animal Committee of Henry Ford Hospital (Detroit, MI). An extensive toxicology study has been completed with the Ad5-CD/TKrep virus without and with double prodrug therapy after intraprostatic administration in C57BL/6 mice. The results of these studies will be made available to any investigator on request.

RESULTS

Double suicide gene therapy augments the antitumor activity of the replication-competent Ad5-CD/TKrep adenovirus

The therapeutic efficacy of the CD/5-FC and HSV-1 TK/GCV suicide gene systems in the context of the replication-competent Ad5-CD/TKrep adenovirus was evaluated using the human C33A (cervical carcinoma) tumor xenograft. Subcutaneous abdominal tumors (100 mm³) were injected with either PBS or Ad5-CD/TKrep (10⁸ PFU) for five consecutive days. As both suicide gene systems were previously demonstrated to interfere with Ad5-CD/TKrep replication (Freytag et al., 1998), prodrug therapy commenced shortly after completion of viral inoculation to allow for viral replication and spread. In initial studies, prodrug therapy was continued until tumors stopped regressing (~3 weeks).

Intratumoral inoculation of Ad5-CD/TKrep itself resulted in a significant tumor growth delay of 25 days \( (p = 0.015; \text{ANOVA relative to PBS-injected controls (Fig. 1A)}) \). This was not a nonspecific effect of adenoviral injection, as parallel tumors injected with a replication-defective version of Ad5-CD/TKrep, Ad5-FGMR (Freytag et al., 1998), resulted in a tumor growth delay (7 days) that was not significantly different from the control group (Fig. 1A). Thus, as expected, Ad5-CD/TKrep itself, via its cytolytic activity, provides significant tumor control. The efficacy of Ad5-CD/TKrep viral therapy was markedly enhanced by double suicide gene therapy. On day 23, when Ad5-CD/TKrep-treated tumors averaged ~125 mm³, those treated with Ad5-CD/TKrep viral and double prodrug therapies averaged only ~8 mm³, representing a 94% reduction in volume relative to Ad5-CD/TKrep viral therapy alone. The combination of Ad5-CD/TKrep viral and double prodrug therapies resulted in a tumor growth delay of 66 days relative to PBS-injected controls, which was significantly greater \( (p = 0.011; \text{ANOVA}) \) than Ad5-CD/TKrep therapy alone (Table 1). Importantly, no toxicity (animal death or sustained weight loss) was observed after Ad5-CD/TKrep viral and double prodrug therapies.

To determine whether the dramatic augmentation of Ad5-CD/TKrep viral therapy by double prodrug therapy was attributable predominantly to one of the suicide gene systems, the effects of combining Ad5-CD/TKrep viral therapy with 5-FC or GCV prodrug therapy independently were also examined. As pilot studies demonstrated that extending the prodrug therapy regimen beyond 7 days did not result in significantly better tumor control (our unpublished results, 1999), a 7-day prodrug therapy regimen was adopted in subsequent experiments. As was observed with the 21-day prodrug therapy regimen (Fig. 1A), 7 days of double prodrug therapy (Fig. 1B) resulted in rapid tumor regression, ultimately yielding a tumor growth delay of 67 days, which was more than three times that (20 days) of Ad5-CD/TKrep viral therapy alone (Table 1). These results demonstrate the reproducibility of the double prodrug therapy effect, which was observed in eight independent experiments. In marked contrast to double suicide gene therapy, neither the CD/5-FC nor HSV-1 TK/GCV system significantly \( (p ≥ 0.2; \text{ANOVA}) \) improved tumor control beyond that of Ad5-CD/TKrep viral therapy alone. 5-FC therapy produced a mild, transient enhancement of the Ad5-CD/TKrep viral effect, which, in contrast with double prodrug therapy, was rapidly lost on completion of the prodrug therapy course (Fig. 1B). GCV therapy resulted in tumor growth kinetics that were essentially identical to those of Ad5-CD/TKrep itself (Fig. 1B). These results demonstrate clearly that in the context of a replication-competent adenovirus, double suicide gene therapy is therapeutically superior to single suicide gene therapy and corroborate our previous findings with retrovirotransduced tumor lines (Rogulski et al., 1997b).

The marked enhancement of Ad5-CD/TKrep viral therapy by double prodrug therapy, despite the relative impotence of the individual suicide gene systems, suggests synergistic interactions between the CD/5-FC and HSV-1 TK/GCV systems, which have been documented previously (Rogulski et al., 1997a,b; Aghi et al., 1998; Uckert et al., 1998). The failure of either suicide gene system to independently augment the antitumor effects of the Ad5-CD/TKrep virus could derive from an inherent insensitivity of C33A tumor cells to the individual prodrugs, or a balance between suicide gene-mediated cytotoxicity and inhibition of Ad5-CD/TKrep viral replication (Freytag et al., 1998). To discern between these two possibilities, the suicide gene-mediated effects were examined in vitro. For pro-
drug sensitivity assays, C33A cells were infected with Ad5-FGNR, a replication-defective version of Ad5-CD/TK rep (Freytag et al., 1998), allowing suicide gene-mediated cytotoxicity to be analyzed independently from Ad5-CD/TK rep viral cytopathicity. Ad5-FGNR-infected C33A cells were sensitive to both 5-FC and GCV in a concentration-dependent manner, making it unlikely that the failure of these prodrugs to enhance the Ad5-CD/TK rep viral effect in vivo was due to an inherent insensitivity of C33A tumors to the prodrugs (Fig. 2A).

Analysis of Ad5-CD/TK rep replication in C33A cells revealed that the HSV-1 TK/GCV system was effective at inhibiting viral replication (Fig. 2B). Indeed, the concentration of GCV required to completely inhibit Ad5-CD/TK rep viral replication in vitro (0.2 μg/ml or 0.8 μM) is below that obtainable in humans (2–30 μM) when using lower doses (15 mg/kg/day) of GCV. The CD/5-FC system inhibited Ad5-CD/TK rep viral replication.

### Table 1. Comparison of Therapeutic Effects of Ad5-CD/TK rep Viral Therapy without and with Single and Double Prodrug Therapies

<table>
<thead>
<tr>
<th>Treatment</th>
<th>21 days prodrug therapy</th>
<th>7 days prodrug therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad5-CD/TK rep</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>Ad5-CD/TK rep + 5-FC</td>
<td>ND</td>
<td>27</td>
</tr>
<tr>
<td>Ad5-CD/TK rep + GCV</td>
<td>ND</td>
<td>20</td>
</tr>
<tr>
<td>Ad5-CD/TK rep + 5-FC + GCV</td>
<td>66</td>
<td>67</td>
</tr>
</tbody>
</table>

aNumbers represent average tumor growth delay relative to PBS-injected controls. Each treatment group contained five animals.

bAnimals were given daily intraperitoneal injections of 5-FC (500 mg/kg), GCV (60 mg/kg), or 5-FC (500 mg/kg) + GCV (60 mg/kg) for either 21 or 7 days as indicated.
tion only at the highest concentration examined (100 μg/ml or ~0.8 mM), which is well below the mean serum concentration (3.8 mM) obtainable in mice with this dose (500 mg/kg/day) of 5-FC (Huber et al., 1993). Cumulatively, the in vitro analyses suggest that the failure of either suicide gene system to independently enhance the antitumor effects of the Ad5-CD/TKrep virus likely results from a balance between suicide gene-mediated cytotoxicity and inhibition of viral replication. Nevertheless, it is clear that despite the effects of these suicide gene systems on Ad5-CD/TKrep viral replication, when used concomitantly, they improve dramatically the efficacy of Ad5-CD/TKrep viral therapy in vitro.

**FIG. 2.** Effects of suicide gene systems on Ad5-CD/TKrep replication and cell survival. (A) In vitro sensitivity of C33A cells to the CD/5-FC and HSV-1 TK/GCV enzyme/prodrug systems. Cells were infected with the replication-defective Ad5-FGGR virus at an MOI of 100. Seventy-two hours later, cells were treated with graded concentrations of 5-FC or GCV for 96 hr. Cell viability was determined by the MTT assay. The results represent the mean of triplicate determinations. Error bars represent standard deviations. (B) Ad5-CD/TKrep viral replication in the presence of the 5-FC and GCV. C33A cells were either mock infected (M) or infected with Ad5-CD/TKrep at an MOI of 10. Cells were detached and replated in varying concentrations of prodrugs. Cells were harvested 48 hr later for isolation of viral DNA. DNA was digested with HindIII and subjected to Southern blot analysis. The 32P-labeled probe contains adenoviral type 5 sequences from base 1340 to the right end and detects all Ad5-CD/TKrep viral HindIII fragments.
Potentiation of radiotherapy by Ad5-CD/TKrep-mediated double suicide gene therapy

Using retrovirally transduced, radioresistant tumor lines stably expressing the CD/HSV-1 TK fusion gene, we demonstrated previously that both the CD/5-FC and HSV-1 TK/GCV systems can sensitize tumor cells to the toxic effects of radiation (Kim et al., 1994, 1995, 1997, 1998; Khil et al., 1996; Rogulski et al., 1997a; Freytag et al., 1998; Gable et al., 1998). Even greater radiosensitization is achieved when both enzyme/prodrug systems are used simultaneously (Rogulski et al., 1997a,b; Kim et al., 1998). To assess the full potential of the trimodal (viral, suicide gene, radiation) approach, Ad5-CD/TKrep-mediated double suicide gene therapy was used as a neoadjuvant to radiation therapy. Each single and dual modality was also evaluated to determine the contribution of each therapeutic arm. Because the radiation studies require that the body be shielded during irradiation, C33A cells were inoculated intramuscularly into the leg. After administration of the Ad5-CD/TKrep virus, half of the animals received double prodrug therapy (days 4–10). Animals within the radiation treatment groups received a single dose of γradiation to the tumor-bearing leg midway through the prodrug course.

As observed in the subcutaneous model (Fig. 1A and B), Ad5-CD/TKrep viral therapy alone resulted in a significant \( p = 0.0037 \) (ANOVA) tumor growth delay of 29 days relative to PBS-injected controls and 40% tumor cure (Fig. 3A, Table 2). Notably, with respect to tumor cure rate, Ad5-CD/TKrep viral therapy was equivalent to 10 Gy of radiation, which demonstrates clearly the benefit of using a cytolytic adenovirus as a gene therapy vector (i.e., injection of the replication-defective Ad5-FG NR virus yields no tumor cure; our unpublished results, 1999). As expected, the addition of 7 days of double prodrug therapy markedly enhanced both the tumor growth delay and cure rate of Ad5-CD/TKrep viral therapy in the intramuscular model. Whereas Ad5-CD/TKrep viral therapy itself resulted in an average tumor growth delay of 29 days and 40% cure, Ad5-CD/TKrep viral and double prodrug therapies produced a tumor growth delay of at least 100 days and 80% cure (Fig. 3A, Table 2). The basis for the greater efficacy observed in the intramuscular model (tumor growth delay > 100 days) relative to that achieved in the subcutaneous model (tumor growth delay > 67 days) is unclear, but may be due to better tumor vascularization resulting in more efficient prodrug delivery.

The trimodal Ad5-CD/TKrep viral, double suicide gene, and radiotherapy approach resulted in significant tumor regression, but ultimately produced the same tumor growth delay (> >100 days) and high cure rate (80%) as the combination of Ad5-CD/TKrep viral and double prodrug therapies (Fig. 3A, Table 2). Because the combination of Ad5-CD/TKrep viral and double suicide gene therapies was so effective in this regimen, likely masking any radiosensitization effect, these studies were repeated with an abbreviated prodrug therapy course (4 versus 7 days) and a lower dose of radiation (8 versus 10 Gy).

FIG. 3. Effects of Ad5-CD/TKrep-mediated double suicide gene therapy on the radiation response of C33A leg tumors. (A) Intramuscular C33A tumors (120 mm³) were injected with either PBS or 10⁸ PFU of Ad5-CD/TKrep for five consecutive days (days 0–4, solid bar). Animals receiving double prodrug therapy were administered 5-FC (500 mg/kg) and GCV (60 mg/kg) for 7 days beginning on day 4 (hatched bar). Animals within the radiation treatment groups received a single dose of 10 Gy to their tumor-bearing limb on day 7. The dotted line represents the predetermined end point of 4 mm³. (B) Intramuscular C33A tumors (120 mm³) were injected with either PBS or 10⁸ PFU of Ad5-CD/TKrep for five consecutive days (days 0–4, solid bar). Animals receiving double prodrug therapy were administered 5-FC (500 mg/kg) and GCV (60 mg/kg) for 4 days beginning on day 4 (hatched bar). Animals within the radiation treatment groups received a single dose of 8 Gy to their tumor-bearing limb on day 6. The dotted line represents the predetermined endpoint of 400 mm³.
Independent ly, Ad5-CD/TKrep viral and radiation therapy (8 Gy) each resulted in an average tumor growth delay of 42–45 days and 40 and 20% tumor cure, respectively (Fig. 3B, Table 3). In contrast to the 7-day prodrug therapy regimen (Fig. 3A), the abbreviated 4-day regimen produced only a transient enhancement of the Ad5-CD/TKrep viral effect without improving therapeutic outcome, yielding an average tumor growth delay and cure rate (40%) identical to that of Ad5-CD/TKrep viral therapy alone (Fig. 3B, Table 3). These results demonstrate the critical importance of the length of the prodrug therapy regimen (4 days—same outcome as Ad5-CD/TKrep viral therapy; 7 days—markedly better outcome than Ad5-CD/TKrep viral therapy). A profound antitumor effect resulted when combining Ad5-CD/TKrep viral, double suicide gene, and radiation therapies (trimodal therapy). Rapid tumor regression occurred shortly after radiation therapy (Fig. 3B). On day 18, when tumors within all other treatment groups averaged between 40 to 180% of their pretreatment volumes, those within the trimodal therapy group were apparently eradicated. Trimodal therapy resulted in a tumor growth delay of at least 100 days a high cure rate (80%), which was much greater than that achieved with any other monotherapy or dual therapy. Such results were obtained in two independent experiments. The one animal in the trimodal therapy group that was not cured 90 days posttreatment (60-mm$^3$ tumor) became tumor free at 6 months, ultimately yielding a cure rate of 100%. Thus, whereby shortening the prodrug therapy course to 4 days essentially eliminated the chemotherapeutic effect of double prodrug therapy, the suicide gene-mediated radiosensitization effect remained. The results demonstrate that double suicide gene therapy, in the context of the replication-competent Ad5-CD/TKrep adenovirus, is an effective neoadjuvant to radiation therapy.

DISCUSSION

We demonstrated previously in vitro the potential of the trimodal approach of Ad5-CD/TKrep viral, double suicide gene, and radiation therapies. We now demonstrate in vivo that double suicide gene therapy significantly enhances the antitumor activity of the replication-competent Ad5-CD/TKrep adenovirus and that these modalities, when combined, are an effective neoadjuvant to radiation therapy. Given that 50% of all human cancer patients are treated with radiation, it is possible that Ad5-CD/TKrep viral and double suicide gene therapies will demonstrate value in the clinic.

As demonstrated originally by us (Rogulski et al., 1997a,b; Kim et al., 1998) and confirmed subsequently by others (Aghi et al., 1998; Uckert et al., 1998), double suicide gene therapy is therapeutically superior to single suicide gene therapy. The mechanism underlying the synergistic cytotoxicity of concomitant CD5-FC and HSV-1 TK/GCV therapies is unclear.

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**Table 2. Comparison of Therapeutic Effects of Ad5-CD/TKrep Viral, Double Suicide Gene (7 Day), and Radiation Therapies (10 Gy)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average tumor growth delay (days)</th>
<th>Percentage tumor free at 90 days posttreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>NA</td>
<td>0 (0/5)</td>
</tr>
<tr>
<td>Ad5-CD/TKrep</td>
<td>29</td>
<td>40 (2/5)</td>
</tr>
<tr>
<td>8 Gy</td>
<td>53</td>
<td>40 (2/5)</td>
</tr>
<tr>
<td>Ad5-CD/TKrep + 5-FC + GCV</td>
<td>$&gt;100^a$</td>
<td>80 (4/5)</td>
</tr>
<tr>
<td>Ad5-CD/TKrep + 10 Gy</td>
<td>68</td>
<td>40 (2/5)</td>
</tr>
<tr>
<td>Ad5-CD/TKrep + 5-FC + GCV + 10 Gy</td>
<td>$&gt;100^a$</td>
<td>80 (4/5)</td>
</tr>
</tbody>
</table>

*Note that the mean tumor volume of the Ad5-CD/TKrep + 5-FC + GCV and trimodal therapy groups never reached the predetermined end point of 400 mm$^3$ (even after 6 months). Thus, a statistical analysis of the average tumor growth delays of these groups versus the other treatment groups could not be performed.

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**Table 3. Comparison of Therapeutic Effects of Ad5-CD/TKrep Viral, Double Suicide Gene (4 Day), and Radiation Therapies (8 Gy)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average tumor growth delay (days)</th>
<th>Percentage tumor free at 90 days posttreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>NA</td>
<td>0 (0/5)</td>
</tr>
<tr>
<td>Ad5-CD/TKrep</td>
<td>45</td>
<td>40 (2/5)</td>
</tr>
<tr>
<td>8 Gy</td>
<td>42</td>
<td>20 (1/5)</td>
</tr>
<tr>
<td>Ad5-CD/TKrep + 5-FC + GCV</td>
<td>45</td>
<td>40 (2/5)</td>
</tr>
<tr>
<td>Ad5-CD/TKrep + 8 Gy</td>
<td>58</td>
<td>20 (1/5)</td>
</tr>
<tr>
<td>Ad5-CD/TKrep + 5-FC + GCV + 8 Gy</td>
<td>$&gt;100^a$</td>
<td>80 (4/5)$^b$</td>
</tr>
</tbody>
</table>

*Note that the mean tumor volume of the trimodal therapy group never reached the predetermined end point of 400 mm$^3$ (even after 6 months). Thus, a statistical analysis of the average tumor growth delays comparing the trimodal therapy group with the other treatment groups could not be performed.

$^b$The one animal in this group that was not tumor free 90 days posttreatment had a 60-mm$^3$ tumor and became tumor free at 6 months.
but may stem from the fact that the two suicide gene systems target distinct, yet merging, biochemical pathways (Rogulski et al., 1997a). As was shown here, double, but not single, suicide gene therapy can enhance significantly the antitumor effects of Ad5-CD/TkRep viral therapy. Independently, both the CD/5-FC and HSV-1 TK/GCV systems failed to improve the antitumor effects of the Ad5-CD/TkRep virus, despite their in vitro cytotoxicity. Low efficacy of the CD/5-FC system in vivo, despite in vitro potency, has been observed previously (Huber et al., 1993; Trinh et al., 1995; Kievit et al., 1999) and may be due to either inefficient conversion of the 5-FC substrate (Kievit et al., 1999) or a requirement for an intact host immune system (Consalvo et al., 1995). The failure of the HSV-1 TK/GCV system to enhance the antitumor effects of the Ad5-CD/TkRep virus in the C33A model appears to stem from a balance between suicide gene-mediated cytotoxicity and suppression of viral replication. This antagonism may be tumor specific, as the HSV-1 TK/GCV system has been demonstrated to enhance the antitumor activity of a cytolytic virus in a colon xenograft (Wildner et al., 1999). It should be noted, however, that a much higher dose (200 mg/kg/day) of GCV was used in that study, which is more than 3 times the dose used here and 10 times the dose that can be safely used in humans (15–20 mg/kg/day).

In both the subcutaneous and intramuscular tumor models, CD/5-FC and HSV-1 TK/GCV cotherapy achieved a potent antitumor effect in the context of the replication-com petent Ad5-CD/TkRep adenovirus, suggesting that the combined cytotoxic effects outweigh the suicide gene-mediated antiviral effects (i.e., inhibition of viral replication and its accompanying cytolyis). Hence, despite the apparent ability of the suicide gene systems to interfere with Ad5-CD/TkRep viral replication, the therapeutic benefits of combining double suicide gene therapy with Ad5-CD/TkRep viral therapy are substantial. Because suicide gene therapy requires prodrug administration, maximal viral spread and tumor destruction can be allowed to occur prior to engaging the enzyme/prodrug systems (and radiation), thereby minimizing the deleterious effects of inhibiting viral replication.

Although the therapeutic benefits of coupling the CD/5-FC and HSV-1 TK/GCV enzyme/prodrug systems and their use as a neoadjuvant to radiation therapy are well documented (Rogulski et al., 1997a,b; Aghi et al., 1998; Kim et al., 1998; Uckert et al., 1998; and this report), an important issue not addressed here is the yet unproven benefit of using a replication-competent versus replication-defective adenovirus to deliver the CD/HSV-1 TK fusion gene. Replication-competent adenoviruses have several theoretical advantages over replication-defective viruses, some of which have been demonstrated. As demonstrated here, Ad5-CD/TkRep itself, via its cytolytic activity, results in a profound (20–45 days) tumor growth delay and 40% tumor cure. Similar tumor growth delays have been obtained in several other tumor models (subcutaneous DU145, 45 days; orthotopic LNCAp C4-2, 25 days). In contrast, the replication-defective counterpart of Ad5-CD/TkRep, Ad5-FGNR, has demonstrated either little (C33A, 7 days) or no (Hep3B, SK-OV-3; Xie et al., 1999) tumor growth delay and no tumor cure in three tumor models. Second, as demonstrated previously in vitro, replication-competent adenoviruses can result in markedly higher (up to 2000-fold) transgene expression per cell via their ability to achieve a high copy number (Freytag et al., 1998), which, in turn, should result in greater conversion of prodrugs into their toxic metabolites. Third, replication-competent adenoviruses, via viral spread, have the potential to increase the in vivo gene transduction efficiency (percentage of tumor cells infected) relative to replication-defective viruses. Whether the latter possibility holds true in vivo is presently unknown. Using the DU145 prostate adenocarcinoma model, we have found that Ad5-CD/TkRep viral therapy alone (10⁸ PFU × 5) results in a tumor growth delay (45 days) that is essentially equivalent to that (42 days) of the replication-defective Ad5-FGNR virus (10⁸ PFU × 5) and 14 days of double prodrug therapy (our unpublished results, 1999). Consistent with our observations, Wildner and co-workers demonstrated that a cytolytic HSV-1 TK-expressing adenovirus was as effective as a replication-defective Ad.Tk vector and GCV prodrug therapy (Wildner et al., 1999). The therapeutic efficacy of the cytolytic HSV-1 TK virus could be improved further with high doses of GCV. Cumulatively, these observations indicate that the potency of suicide gene therapy will be greater in the context of a replication-competent adenovirus than in a non-replicating viral background.

Ad5-CD/TkRep is E1B attenuated and was modeled after ONX-015 (Bischoff et al., 1996), a virus originally hypothesized to target only tumors of mutant p53 status by virtue of its inability to express the p53-inactivating 55-kDa E1B protein (Yew and Berk, 1992). Much controversy has arisen regarding the selectivity of the E1B-attenuated adenoviruses (Goodrum and Ornuelles, 1997, 1998; Hall et al., 1998; Rothmann et al., 1998; Harada and Berk, 1999; Turnell et al., 1999). Hall and colleagues demonstrated that such viruses require wild-type p53 function for effective replication and cytolysis (Hall et al., 1998). Our results, and those of others (Harada and Berk, 1999), conflict dramatically with this tenet, as we have found that Ad5-CD/TkRep lysed efficiently tumor cells of both mutant (C33A, DU145, U251, HT-29) and wild-type (LNCAp, RKO, A549, U343) p53 status in vitro (Freytag et al., 1998; and our unpublished results, 1999), and results in significant tumor growth delays with p53 wild-type (orthotopic LNCAp C4-2, 25 days) and mutant (C33A, 20–45 days; DU145, 45 days) tumors. Nonetheless, our in vitro observations regarding the host range specificity of the E1B-attenuated adenoviruses do not agree well with the tenet originally put forth by ONXY (Bischoff et al., 1996). Using isogenic tumor cell lines that express either wild-type (RKO, U343) or a dominant negative mutant (Arg-273→ His) of p53, we have observed reproducibly that the Ad5-CD/TkRep and ONXY-015 viruses replicate about three times faster in mutant p53 cells and that this can lead to slightly (two- to threefold) faster cytolysis in vitro (Rogulski et al., 1999). Our observations agree well with the results of Berk and colleagues, who found, using a cell line expressing a temperature-sensitive mutant of p53, that the d1520 (ONXY-015) virus replicates slower (~3-fold) and yields a markedly lower viral burst when p53 is active (Harada and Berk, 1999). Along these same lines, using the isogenic RKO/ RKOp53.13 tumor model (Slichenmyer et al., 1993), we have found that ONXY-015 demonstrates greater antitumor activity against mutant p53 tumors. Specifically, intratumoral injection of ONXY-015 (10⁸ PFU × 5) resulted in a tumor growth delay with mutant p53 tumors (19 days) that was significantly better (p < 0.001) than with wild-type tumors (1 day) (Rogulski et al., 1999). It is possible that the differential effect observed in vivo is related to the ability of the
virus to replicate and, perhaps, spread faster in mutant p53 tumors. Whether this is universally true will require confirmation in other isogenic tumor models.

Although conventional cancer therapies (surgery, chemotherapy, radiation therapy) are effective at curing early-stage disease, few human cancers are curable with a single modality. Without significant improvements in in vivo gene delivery, suicide gene therapy, as a monotherapy, will have limited value in the clinic. It has been our tenet that adenovirus-medited suicide gene therapy will show the most promise when used as a neoadjuvant to radiation therapy. Previously, we demonstrated that both the CD/5-FC and HSV-1 TK/GCV systems could, independently, potentiate radiotherapy (Kim et al., 1994, 1995, 1997; Khil et al., 1996; Gabie et al., 1998). Even greater radiosensitization is achieved when both suicide gene systems are combined (Rogulski et al., 1997a,b; Kim et al., 1998). We have now extended these observations by demonstrating that double suicide gene therapy, in the context of a replication-competent adenovirus, can potentiate the therapeutic effects of radiation. Combining Ad5-CD/TK<sub>rep</sub> viral and radiotherapies, in the absence of double prodrug therapy, does not significantly improve tumor control beyond that of either monotherapy. In dramatic contrast, therapeutic outcome is markedly improved when the suicide gene systems are engaged (trmodal therapy), even under conditions in which the suicide gene systems themselves produce no apparent chemotherapeutic effect (i.e., 4 days of prodrug therapy). These results demonstrate clearly that the CD/5-FC and HSV-1 TK/GCV enzyme/prodrug systems can function as true radiosensitizers in vivo, improving therapeutic efficacy only through their synergistic interactions with radiation.

Because human adenoviruses do not replicate efficiently in mouse cells, human tumor xenografts in immunodeficient mice had to be used in these studies. It remains to be determined whether the dramatic results observed here can be achieved in an immunocompetent host (i.e., patients). It is presently unclear whether the host immune system will prove to be a “friend” (by generating an effective immune response to virally infected tumor cells) or “foe” (by preventing viral spread) of cancer therapies that utilize replication-competent adenoviruses. The fact that the ONYX-015 virus has demonstrated antitumor activity in the clinic (Kim et al., 1997) indicates that the immune system does not block the cytolytic action of replication-competent adenoviruses, at least with the initial dose. However, repeated doses appear to be less efficacious. Although the immune system may preclude extensive viral spread, it may not necessarily impede viral replication in infected cells. As described above, an advantage of using replication-competent adenoviruses as gene therapy vectors is their ability to achieve a high copy number resulting in significantly greater (up to 2000-fold; Freytag et al., 1998) therapeutic transgene expression per cell. Thus, even if the immune system precludes viral spread, replication-competent vectors should still result in greater therapeutically gene expression per viral dose relative to replication-defective vectors. Moreover, although the immune system may attenuate the Ad5-CD/TK<sub>rep</sub> viral cytolytic effect, there is reason to believe that it will augment the efficacy of CD/5-FC and HSV-1 TK/GCV suicide gene therapies (Vile et al., 1994; Consalvo et al., 1995). Thus, the efficacy of the combined therapy described here may depend on whether the antitumor effects of host immune system (immune response to virally infected cells and augmentation of suicide gene therapies) outweigh its negative effects on viral spread. The merit of this combined approach is best evaluated in the clinic, which will commence in 1999.

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