ABSTRACT
The efficacy of HSV-1 thymidine kinase (TK) and Escherichia coli cytosine deaminase (CD) suicide gene therapies as cancer treatments are currently being examined in humans. We demonstrated previously that compared to single suicide gene therapy, greater levels of targeted cytotoxicity and radiosensitization can be achieved in vitro by genetically modifying tumor cells to express CD and HSV-1 TK concomitantly, as a fusion protein. In the present study, the efficacy of the combined double suicide gene therapy/radiotherapy approach was examined in vivo. Nude mice were injected either s.c. or i.m. with 9L gliosarcoma cells expressing an E. coli CD/HSV-1 TK fusion gene. Double suicide gene therapy using 5-fluorocytosine (500 mg/kg) and ganciclovir (30 mg/kg) proved to be markedly better at delaying tumor growth and achieving a tumor cure than single suicide gene therapy, which used 5-fluorocytosine or ganciclovir administered independently. Importantly, double suicide gene therapy was highly effective against large experimental tumors (>2 cm³), reducing tumor volume an average of 99% and producing a 40% tumor cure. Moreover, double suicide gene therapy profoundly potentiated the antitumor effects of radiation. The results indicate that double suicide gene therapy, particularly when coupled with radiotherapy, may represent a highly effective means of eradicating tumors.

INTRODUCTION
A major impediment that limits the effectiveness of conventional cancer therapy is the lack of a significant differential response between malignant and normal tissue. Suicide gene therapy provides a means of circumventing this limitation through the selective introduction of genes encoding nonmammalian metabolic enzymes into tumor cells, rendering them susceptible to specific antimetabolites.

Unlike mammalian TK, HSV-1 TK phosphorylates a variety of nucleoside analogues, such as GCV, to their nucleoside monophosphate derivatives (1). Once phosphorylated, these analogues are converted by endogenous cellular kinases into their corresponding nucleoside triphosphates, which inhibit DNA replication by chain termination (2). CD, an enzyme present in a variety of fungi and bacteria, can deaminate the innocuous prodrug 5-FC, forming 5-FU, a widely used chemotherapeutic agent (3). The cytotoxic effect of 5-FU is largely exerted following its conversion to 5-fluoro-dUMP, which irreversibly inhibits thymidylate synthase, resulting in the inhibition of DNA synthesis (3).

The potential of the HSV-1 TK/GCV and Escherichia coli CD/5-FC enzyme/prodrug systems as cancer therapies has been demonstrated extensively in animal models (4–19). In most cases, marked antitumor effects have also been observed following in vivo gene transfer using both recombinant retroviruses and adenoviruses coupled with prodrug (GCV or 5-FC) therapy. These encouraging results have paved the way for the numerous ongoing clinical trials that will determine the efficacy of HSV-1 TK/GCV and CD/5-FC therapies in humans.

Despite the established efficacy of these therapies in animal models, however, limitations have been demonstrated. In many studies, the HSV-1 TK/GCV (8, 17–19) or CD/5-FC (9, 11, 13, 14) enzyme/prodrug combination failed to result in complete tumor regression, and frequently, many tumors recurred once prodrug therapy ceased. Moreover, all of these studies began prodrug therapy when the tumors were relatively small (<2 cm³), and the ability of suicide gene therapy to control large tumors has not been investigated. Thus, it is likely that significant improvements in the conventional suicide gene therapy approach will be needed before this modality will have any value in the clinic.

Toward this end, we have been developing protocols that might improve the efficacy of the conventional suicide gene therapy approach. Because the cytotoxic effects of the CD/5-FC and HSV-1 TK/GCV combinations are mediated through different mechanisms, we hypothesized that combining these two suicide gene systems might result in enhanced cell killing. Indeed, there is a biochemical basis for the fact that these two suicide gene systems, when combined, should result in a synergistic, and not an additive, enhancement of cytotoxicity. As stated previously, the cytotoxic effect of the CD/5-FC combination is mediated predominantly through the inhibition of thymidylate synthase by 5-fluoro-dUMP (3). This results in a

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1 The abbreviations used are: TK, thymidine kinase; GCV, ganciclovir; CD, cytosine deaminase; 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; HSV, herpes simplex virus.
redistribution of cells in early S phase (20), as well as DNA strand breakage due to the disruption of deoxynucleotide pools (21). The cytotoxicity of the HSV-1 TK/GCV system results from GCV-monophosphate inhibition of DNA polymerases, which leads to a disruption of DNA synthesis (2). It was therefore reasoned that when combined, the CD/5-FC and HSV-1 TK/GCV systems may synergize, because CD/HSV-1 TK-expressing cells would accumulate in early S phase, at which point they are most sensitive to GCV. GCV might also inhibit the repair of DNA strand breaks caused by 5-FC treatment. Moreover, because 5-FU (the product of the CD reaction) and certain antiviral agents are proven radiosensitizers (22), we hypothesized that HSV-1 TK/GCV and CD/5-FC suicide gene therapies may potentiate the effect of radiotherapy.

We previously examined these possibilities in vitro by coexpressing CD and HSV-1 TK in 9L gliosarcoma cells as a fusion protein (23). Concomitant treatment of cells with 5-FC and GCV at suboptimal concentrations achieved slightly synergistic cytotoxicity that was approximately 2-fold greater than that expected from solely additive effects. These results raised the possibility that double suicide gene therapy might prove to be an improvement over conventional single suicide gene therapy in vivo, given that plasma prodrug concentrations much higher than those used in the in vitro studies can be achieved. Moreover, our investigations demonstrated that both the CD/5-FC and HSV-1 TK/GCV suicide gene systems radiosensitize tumor cells in vitro (24–27), and that even greater radiosensitization can be achieved when these two suicide gene/prodrug systems are combined (23).

In the present study, we evaluated the in vivo efficacy of double suicide gene therapy, alone and in combination with radiotherapy. An E. coli CD/HSV-1 TK fusion gene (23) provided the means whereby the CD/5-FC and HSV-1 TK/GCV suicide gene systems could function in the same tumor cell. The results demonstrate that dual prodrug therapy generates a significantly greater antitumor effect than conventional single prodrug therapy and is effective against large i.m. tumors. More importantly, double suicide gene therapy was observed to greatly potentiate the therapeutic effects of radiation.

MATERIALS AND METHODS

Cell Culture. The rat 9L gliosarcoma cell line expressing an E. coli CD/HSV-1 TK fusion protein (9L-CDglyTK) was described previously (23). The CDglyTK fusion protein is produced from a stably integrated provirus that encodes a bicistronic mRNA. Cells were maintained in DMEM supplemented with 10% fetal bovine serum and 250 μg/ml G418. No antibiotic or antifungal agents were used.

In Vivo Prodrug Therapy. Female athymic nude mice [nu/nu (CD-1); Charles River Laboratories] were used in all studies. Two × 10⁶ 9L-CDglyTK cells in 0.9% NaCl were implanted into the right hind leg either s.c. (50 μl) above or i.m. (100 μl) within the right gastrocnemius muscle. When tumors reached an average size of 200 mm³ (s.c. model) or 380 mm³ (i.m. model), animals were divided randomly into four treatment groups of four to five mice/group: no treatment, 5-FC (500 mg/kg; Sigma Chemical Co.), GCV (30 mg/kg; Syntex), and 5-FC (500 mg/kg) + GCV (30 mg/kg). Prodrugs were dissolved in 0.9% NaCl and administered i.p. (1 ml) for 14 days beginning on day 0. During prodrug treatment, tumors were measured every other day and intermittently thereafter. Volumes of s.c. and i.m. leg tumors were estimated using the following equation (28):

\[d^3 - (0.6)^2d' = \text{volume (cm}^3\text{)},\]

where \(d\) is the average tumor diameter (cm) and the product \((0.6)^2d'\) is the correction factor for normal leg volume. Tumors were allowed to reach five times their initial volume (start of prodrug), at which time mice were euthanized. For large tumor studies, i.m. 9L-CDglyTK tumors were allowed to attain an average volume of 2300 mm³. Mice subsequently received 21 daily i.p. injections of 5-FC (500 mg/kg) + GCV (30 mg/kg). Animals were euthanized when recurring tumors returned to their original volume (initiation of double prodrug treatment).

Irradiation Procedure. Tumor cells were implanted s.c. over the right gastrocnemius muscle as detailed above. When tumors reached an average volume of 250 mm³, mice were divided into two groups (10 animals/group): no treatment and 5-FC + GCV. Prodrug-treated animals received four daily i.p. injections of 5-FC (500 mg/kg) + GCV (30 mg/kg) beginning on day 0. Shortly after the fourth prodrug injection (day 3), the two groups of animals were subdivided further into four groups (five animals/group) based on comparable tumor volumes: no treatment, 25 Gy of radiation, 5-FC + GCV, and 5-FC + GCV + 25 Gy of radiation. For irradiation, mice were anesthetized by i.p. injection of 60 mg/kg Nembutal. Anesthetized animals were secured in a compartmentalized plastic jig such that only the tumor-bearing legs protruded into the central cavity. A bolus of tissue-equivalent material of 2.0-cm thickness (which allows for a homogeneous dose distribution throughout the treatment volume) was placed over the central cavity, and a lead alloy (cerrobend) donut collimator (three half-value layers) was positioned over the entire jig. This arrangement allowed for delivery of radiation to the tumor itself while simultaneously shielding the body of the animal. A 60Co Theratron 780 irradiator (Atomic Energy of Canada Ltd., Ottawa, Ontario, Canada) was used to deliver 25 Gy at a dose rate of 1.54 Gy/min using a 62-cm source-to-tumor center distance. For 2 days postirradiation, mice within the prodrug treatment groups received i.p. doses of 5-FC + GCV (six doses total). Tumor volumes were determined as described above. Animals were euthanized when tumors reached 1000 mm³ (4 × initial tumor volume).

For all tumor studies, animals were followed until death (euthanasia) from tumor burden or for at least 90 days after cessation of prodrug treatment. All animal procedures were performed with approved protocols and in accordance with published recommendations for the proper use and care of laboratory animals (29).

RESULTS

In Vivo Antitumor Activity of Double Suicide Gene Therapy. In view of the enhanced cytotoxicity attained via combining the CD/5-FC and HSV-1 TK/GCV suicide gene systems in vitro (23), we examined the therapeutic efficacy of double suicide gene therapy in vivo. Rat 9L gliosarcoma cells expressing an E. coli CD/HSV-1 TK fusion protein (9L-CDglyTK) served as our model. A strength of our approach using the CDglyTK fusion protein is that the efficacy of the
nu/nu tumors have been found to be immunogenic (30), we performed administration of tumors expressing the CD/HSV-1 TK fusion gene is equiv-
entially. Importantly, our previous studies demonstrated that the CDglyTK fusion protein functions as well as the mdm-1 suicide gene therapy approach using an i.m. tumor model. 9L-CDglyTK cells were injected into the gastrocnemius muscle of the right hind leg. As expected, the tumors grew rapidly, achieving a volume of \( \sim 380 \text{ mm}^3 \) only 10 days postimplantation. At this point (day 0), prodrug therapy was initiated. The tumors of untreated animals expanded rapidly to \( >2000 \text{ mm}^3 \) (more than five times their initial volume) in approximately 4 days (Fig. 2; Table 2). As observed in the s.c. tumor model, 5-FC treatment caused only a modest slowing of growth, indicative of a weak antitumor effect. Few 5-FC-treated mice experienced any tumor regression, and 100% of these animals eventually succumbed to tumor burden. Collectively, i.m. tumors responded weakly to GCV administration, continuing to grow even during treatment. The resultant GCV-mediated tumor growth delay was \( \sim 6\)-fold relative to untreated animals, and 2-fold longer than that achieved with 5-FC. A fraction (33%) of GCV-treated animals did experience what appeared to be complete tumor regression. However, unlike the s.c. tumors, 20% of which were eliminated completely, all i.m. tumors that had regressed quickly recurred (0% tumor cure).

Double suicide gene therapy proved effective against i.m. tumors. Much like their s.c. counterparts (Fig. 1), 100% of i.m. tumors regressed completely in response to 5-FC + GCV cotreatment (Fig. 2). However, unlike the s.c. tumors, the majority of i.m. tumors rapidly rebounded. This resulted in a reduction in tumor cure, with only 33% of the animals tumor free 90 days after cessation of treatment. Nonetheless, this represented a significant improvement over single suicide gene therapy, which failed to achieve any tumor control. The i.m. results corroborate those of the s.c. studies, demonstrating again that double suicide gene therapy is superior to single suicide gene therapy.

Table 1  Effects of 5-FC, GCV, and 5-FC + GCV therapy on s.c. 9L-CDglyTK tumors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average time to reach five times initial volume (days)</th>
<th>Fold increase relative to untreated group</th>
<th>Percentage tumor-free at day 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>( 7 \pm 1.3 )</td>
<td>0 (0 of 10)</td>
<td></td>
</tr>
<tr>
<td>5-FC</td>
<td>( 15 \pm 2.1 )</td>
<td>2 (0 of 10)</td>
<td></td>
</tr>
<tr>
<td>GCV</td>
<td>( 37 \pm 4.4 )</td>
<td>5 (20 of 10)</td>
<td></td>
</tr>
<tr>
<td>5-FC + GCV</td>
<td>( 54 \pm 3.0 )</td>
<td>8 (80 of 10)</td>
<td></td>
</tr>
</tbody>
</table>

* Animals were given daily i.p. injections of 5-FC (500 mg/kg), GCV (30 mg/kg), or 5-FC (500 mg/kg) + GCV (30 mg/kg) for 14 days. Initial volume refers to tumor size at the start of prodrug treatment.

CD/5-FC and HSV-1 TK/GCV suicide gene systems, when used either alone or combined, can be compared in exactly the same tumor cell line in which both suicide proteins are expressed stoichiometrically. This ideal situation would be difficult to achieve when expressing the two suicide proteins independently. Importantly, our previous in vitro studies demonstrated that the CDglyTK fusion protein functions as well as the individual CD and HSV-1 TK enzymes in conferring prodrug sensitivity (23). Thus, there was no reason to use the individual suicide genes in our analyses, because single prodrug treatment of tumors expressing the CD/HSV-1 TK fusion gene is equivalent to using CD or HSV-1 TK independently. Because 9L tumors have been found to be immunogenic (30), we performed our studies in immunocompromised mice rather than syngenic Fischer rats to minimize any effect from the immune system on the outcome.

Our initial experiments used a s.c. leg model. 9L-CDglyTK cells were implanted on the right hind leg of athymic CD-1 nu/nu mice. Fourteen days after tumor cell implantation, when the average tumor volume was \( \sim 200 \text{ mm}^3 \), administration of 5-FC (500 mg/kg), GCV (30 mg/kg), or 5-FC (500 mg/kg) + GCV (30 mg/kg) began and continued for 14 days (days 0–13). Tumors of untreated animals reached five times their initial size within 7 days (Fig. 1; Table 1). Treatment with 5-FC had a mild antitumor effect, delaying tumor growth by 2-fold relative to untreated animals but failing to result in tumor regression (Fig. 1; Table 1). GCV therapy proved somewhat more effective. Unlike 5-FC therapy, which merely retarded tumor growth, GCV administration delayed tumor growth by approximately 5-fold, induced partial tumor regressions, and resulted in a 20% tumor cure. In marked contrast, 5-FC + GCV cotherapy produced a significant antitumor effect in the s.c. leg tumor model. Double prodrug therapy led to apparently complete tumor regression by day 24 in 100% of these animals. Significantly, 80% of these animals remained tumor free 90 days after cessation of double prodrug therapy. Those tumors that did recur required an average of 54 days (8-fold longer than untreated animals) to attain five times their pretreatment volume (Table 1). Importantly, no drug-related toxicity (animal death or substantial weight loss) was observed following double prodrug therapy. The results demonstrate that double suicide gene therapy is significantly more effective against s.c. tumors than single suicide gene therapy.

Few naturally occurring tumors in humans are s.c. The majority are embedded within a particular organ or tissue. In view of this, we also examined the efficacy of the double suicide gene therapy approach using an i.m. tumor model. 9L-CDglyTK cells were injected into the gastrocnemius muscle of the right hind leg. As expected, the tumors grew rapidly, achieving a volume of \( \sim 380 \text{ mm}^3 \) only 10 days postimplantation. At this point (day 0), prodrug therapy was initiated. The tumors of untreated animals expanded rapidly to \( >2000 \text{ mm}^3 \) (more than five times their initial volume) in approximately 4 days (Fig. 2; Table 2). As observed in the s.c. tumor model, 5-FC treatment caused only a modest slowing of growth, indicative of a weak antitumor effect. Few 5-FC-treated mice experienced any tumor regression, and 100% of these animals eventually succumbed to tumor burden. Collectively, i.m. tumors responded weakly to GCV administration, continuing to grow even during treatment. The resultant GCV-mediated tumor growth delay was \( \sim 6\)-fold relative to untreated animals, and 2-fold longer than that achieved with 5-FC. A fraction (33%) of GCV-treated animals did experience what appeared to be complete tumor regression. However, unlike the s.c. tumors, 20% of which were eliminated completely, all i.m. tumors that had regressed quickly recurred (0% tumor cure).

Double suicide gene therapy proved effective against i.m. tumors. Much like their s.c. counterparts (Fig. 1), 100% of i.m. tumors regressed completely in response to 5-FC + GCV cotreatment (Fig. 2). However, unlike the s.c. tumors, the majority of i.m. tumors rapidly rebounded. This resulted in a reduction in tumor cure, with only 33% of the animals tumor free 90 days after cessation of treatment. Nonetheless, this represented a significant improvement over single suicide gene therapy, which failed to achieve any tumor control. The i.m. results corroborate those of the s.c. studies, demonstrating again that double suicide gene therapy is superior to single suicide gene therapy.
Effects of Double Suicide Gene Therapy against Large Tumors. To date, all suicide gene therapy studies have initiated prodrug therapy when tumors were relatively small (8–200 mm³), and none have investigated the efficacy of this approach against large experimental tumors. To ascertain the effect of double suicide gene therapy on large tumors, i.m. 9L-CDglyTK tumors were allowed to attain an average size of 2300 mm³ (untreated animals; Fig. 2), at which point 5-FC + GCV therapy was initiated. This tumor burden is approximately 10% of total body weight, and if extrapolated to a 70-kg (150-lb) human, would represent a tumor with approximately 24 cm (9 in). An example of such a large tumor is illustrated in Fig. 3 (left). Much like their smaller counterparts, the large i.m. tumors (ranging in size from 1500 to 3160 mm³) responded rapidly to double prodrug therapy, regressing to their initial size of ~380 mm³ 12 days after initiation of treatment (day 18; Fig. 2). Double prodrug treatment was discontinued after 21 days, at which point the average size of these large tumors had been reduced by more than 99% (to ~18 mm³; day 26). The tumors continued to regress after cessation of prodrug therapy, and 100% of the animals were apparently tumor free at day 30. This dramatic reduction in tumor volume is depicted in Fig. 3. As was observed with the small i.m. tumors, the majority of the tumors recurred, achieving their pretreatment volumes within 43 days. 5-FC + GCV therapy produced a tumor cure in approximately one-third (38%) of the animals. Significantly, animals that had tumors as large as 3160 mm³ (Fig. 3) remained tumor free 90 days after prodrug therapy ceased. In sum, these results not only corroborate those of the small tumor investigations, but more importantly, demonstrate that double suicide gene therapy is effective against large i.m. tumors.

Double Suicide Gene Therapy Potentiates Radiotherapy. Previously, we demonstrated that both CD5-FC and HSV-1 TK/GCV systems can sensitize cells to the toxic effects of radiation in vitro (24–27). Even greater radiosensitization is achieved when these two suicide gene/prodrug systems are combined (23). Thus, we examined the degree to which double suicide gene therapy could potentiate the therapeutic effect of radiation in vivo.

For irradiation studies, 9L-CDglyTK cells were implanted s.c. over the right hind leg. When tumors reached an average volume of 250 mm³, half of the animals began receiving 5-FC + GCV therapy. Because 14 days of double prodrug therapy was so effective against s.c. tumors (~80% cure; Table 1), we presumed that it would not be possible to demonstrate any statistically significant increase in the antitumor effect when combining double suicide gene therapy with radiation. Consequently, an abbreviated prodrug dosing schedule (empirically determined) was used. Mice in the double prodrug treatment group received four daily injections of 5-FC + GCV. One to 2 h after administration of the fourth injection, half of the prodrug-treated animals, along with half of the untreated animals, received a single 25-Gy dose of γ-radiation on the tumored leg (Fig. 4). 5-FC + GCV was administered to all animals in the prodrug-treated groups for 2 additional days postirradiation (a total of six doses).

At a dose of 25 Gy, CD-1 nu/nu mice showed moderate and transient evidence of skin damage (i.e., dryness and loss of elasticity). Because s.c. tumor growth would stretch the overlying damaged skin, irradiated mice would sometimes experience skin ulcerations when tumors became large. Consequently, a 4-fold (rather than a 5-fold) increase in tumor volume was used as a biological end point. Reduced radiation doses could not be used, because they had little or no effect on 9L-derived tumors (data not shown).

Tumors of untreated animals reached four times their initial size (1000 mm³) within ~4 days (Fig. 4; Table 3). A single dose of 25 Gy of γ-radiation produced only mild antitumor effects, delaying tumor growth by ~3-fold relative to untreated animals. It should be noted that 9L tumors as small as 1.5 mm³ are incurable using 25 Gy of radiation, demonstrating the radiosensitivity of such cells (data not shown). Tumors of animals treated with 5-FC + GCV or 5-FC + GCV + radiation regressed rapidly in parallel fashion until 11 days after cessation of prodrug treatment (day 16; Fig. 4). As expected, the majority of tumors treated with only six doses of 5-FC + GCV stopped regressing and eventually resumed growth. This abbreviated 6-day regimen of double prodrug therapy produced only a 20% tumor cure and delayed tumor growth by 11-fold relative to untreated controls.

In marked contrast, 100% of the tumors that were subjected to combined double prodrug and radiotherapies regressed completely and resulted in a 60% tumor cure. Those tumors that did recur required 78 days to reach four times their pretreated volume (Table 3). This tumor growth delay exceeded that of 5-FC + GCV therapy and 25-Gy γ-radiation when each was administered independently by 2-fold and 7-fold, respectively. Moreover, combining double prodrug therapy with radiation resulted in a 3-fold increase in tumor cure when compared to 5-FC + GCV therapy alone. The results demonstrate clearly that double suicide gene therapy potentiates the effectiveness of radiotherapy in vivo.
40% tumor cure. Most noteworthy, however, is that double suicide gene therapy potentiates the therapeutic effect of radiation therapy (24). This increase in efficacy is observed following both systemic and local administration of the suicide genes and prodrug (25). For example, Huber et al. (26) showed that systemic administration of suicide gene constructs encoding the CD and HSV-1 TK genes in combination with GCV can result in complete regression of tumors in athymic mice. Similarly, Hildebrandt et al. (27) demonstrated that local administration of suicide gene constructs encoding the CD and HSV-1 TK genes in combination with GCV can result in complete regression of tumors in athymic mice. These results were corroborated directly by enzymic assays (23). These observations indicate that the immunogenicity of the CD and HSV-1 TK-encoding cells remains tumor free 90 days after cessation of double suicide gene therapy.

DISCUSSION

The present study demonstrates that double suicide gene therapy generates a pronounced antitumor effect when targeted against either s.c. or i.m. tumors, under conditions in which single suicide gene therapy produces only partial effects. Moreover, the CDglyTK/5-FC + GCV enzyme/prodrug combination is even effective against large (≥2 cm3) i.m. tumors, resulting in apparently complete tumor regression and an approximately 50% tumor cure. Most noteworthy, however, is that double suicide gene therapy potentiates the therapeutic effect of radiation.

The results of the in vitro analyses demonstrated that CD/HSV-1 TK fusion gene-expressing cells were as sensitive to 5-FC and GCV as their counterparts, which expressed CD or HSV-1 TK independently (23). These observations indicate that the CD and TK enzymic activities of the CDglyTK fusion protein are fully functional, results that were corroborated directly by enzymic assays (23). Moreover, in our system, the immunogenicity of the CD- and HSV-1 TK-expressing cells is also identical, which is not the case when the two proteins are expressed independently in different cell populations. However, the possibility that the efficacy of a given suicide gene/prodrug system is dependent on cell type cannot be excluded, because cellular biochemistry may greatly affect prodrug uptake, sensitivity to drug toxicity, and the efficiency of the bystander effect (32).

Our results using CDglyTK/GCV therapy in the s.c. model are very similar to those obtained by others using the HSV-1 TK/GCV enzyme/prodrug system (4, 17–19), although we are using lower doses of GCV (30 mg/kg versus 50–150 mg/kg in other studies). Consistent with our findings, most studies have observed suppression of tumor growth or partial tumor regression with GCV therapy and tumor regrowth once prodrug therapy stopped.

As stated earlier, treatment of 9L-CDglyTK tumors with 5-FC had only a mild antitumor effect, delaying tumor growth ~2-fold relative to untreated controls. Using the same animal model (female CD-1 nu/nu athymic mice), Huber et al. (9, 10, 31) observed significant tumor regression following 5-FC treatment of a human colon carcinoma cell line (WiDr) transfected with a vector in which expression of the CD gene was driven by the cytomegalovirus promoter. Using transfected cells, we have observed that the level of CD expression driven by the Moloney

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**Table 2** Effects of 5-FC, GCV, and 5-FC + GCV therapy on i.m. 9L-CDglyTK tumors

<table>
<thead>
<tr>
<th>Tumor size</th>
<th>Treatment</th>
<th>Average time to reach predetermined volume (days)</th>
<th>Fold increase relative to untreated group</th>
<th>Percentage tumor-free at day 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small (380 mm³)</td>
<td>None</td>
<td>4 ± 0.2</td>
<td>3</td>
<td>0 (0 of 8)</td>
</tr>
<tr>
<td></td>
<td>5-FC</td>
<td>13 ± 3.2</td>
<td>6</td>
<td>0 (0 of 8)</td>
</tr>
<tr>
<td></td>
<td>GCV</td>
<td>22 ± 6.7</td>
<td>12</td>
<td>33 (3 of 9)</td>
</tr>
<tr>
<td></td>
<td>5-FC + GCV</td>
<td>46 ± 4.6</td>
<td>NA</td>
<td>38 (3 of 8)</td>
</tr>
<tr>
<td>Large (2300 mm³)</td>
<td>5-FC + GCV</td>
<td>43 ± 5.3</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

*For the small tumor studies, animals were given daily i.p. injections of 5-FC (500 mg/kg), GCV (30 mg/kg), or 5-FC (500 mg/kg) + GCV (30 mg/kg) for 14 days. For the large tumor studies, animals were given daily i.p. injections of 5-FC + GCV (500 mg/kg, 30 mg/kg) for 21 days.

**Fig. 3** Effect of double suicide gene therapy on large i.m. 9L-CDglyTK leg tumors. Left, animal with a 3160-mm³ i.m. leg tumor at the initiation of double prodrug therapy (day 6; see Fig. 2). Right, the same animal 3 days after cessation of 5-FC + GCV treatment (day 30; see Fig. 2). This animal (and other similar animals) remained tumor free 90 days after cessation of double prodrug therapy.
Antitumor Effects of Double Suicide Gene Therapy

Fig. 4 Effects of double suicide gene therapy on the radiation response of s.c. 9L-CDg1yTK leg tumors. Daily administration of 5-FC + GCV began on day 0 and was continued through day 5 (stippled bar). Animals received four prodrug doses prior to radiation and two prodrug doses postirradiation. Tumored limbs were exposed to a single radiation dose of 25 Gy on the 4th day of double prodrug therapy as indicated. The average tumor volume of five animals is plotted (bars, SE). · · · · ·, point at which animals were considered tumor free.

murine leukemia virus long terminal repeat (the identical promoter used to drive expression of the CDg1yTK gene) is equivalent to if not better than that driven by the cytomegalovirus promoter. Thus, it is unlikely that the difference between our results and those of Huber et al. (9, 10, 31) can be attributed simply to promoter strength. However, Mullen et al. (13), using mouse fibrosarcomas and adenosarcomas transduced with a retroviral vector encoding CD, observed not tumor regression, but as we had observed, retarded tumor growth following 5-FC treatment. Hence, our inability to achieve significant 5-FC-mediated tumor regression may lie in our choice of vector, more specifically, how the cells were genetically modified. Retrovirus-mediated gene transduction typically introduces a single gene copy per cell in contrast to lipofection or electroporation [both used by Huber et al. (9, 10, 31)], which often results in multiple vector copies per cell. A greater copy number of CD-expressing vectors would result in higher CD enzyme levels and consequently greater sensitivity to 5-FC than that observed with cells expressing only one copy of the CD gene. Finally, pharmacokinetic differences between WiDr and 9L tumors cannot be excluded as a possible explanation for the difference between our findings and those of Huber et al. (9, 10, 31).

Regardless of the existing discrepancies between our single suicide gene/prodrug results and those of others, the results presented here demonstrate clearly that double suicide gene therapy is superior to single suicide gene therapy in its antitumor effects. As stated earlier, we theorized that because the cytotoxic effects of the CD/5-FC and HSV-1 TK/GCV systems are mediated through different mechanisms, these systems may produce enhanced cytotoxicity when combined. Following double prodrug treatment, CDHSV-1 TK-expressing cells may accumulate in early S phase and suffer increased DNA strand breakage (due to the CD/5-FC system) and concomitantly be unable to replicate and repair DNA (due to the HSV-1 TK/GCV system). Our results obtained in vitro support this hypothesis, because concurrent 5-FC and GCV treatment produced mild (2-fold) synergistic cytotoxicity (23). We suspect this modest level of synergy may be attributable to the fact that suboptimal prodrug concentrations had to be used in the in vitro studies when the two prodrugs were combined. 5-FC + GCV cotherapy of s.c. 9L-CDg1yTK tumors, in marked contrast to independent 5-FC or GCV treatment, produced significant and permanent tumor regressions in 80% of the animals. For those tumors that did recur, their growth was retarded significantly (8-fold) relative to untreated animals. This tumor growth delay is significantly greater than that achieved with 5-FC and slightly longer than that achieved with GCV, which produced a 4-fold lower tumor cure rate (20%).

We are very encouraged by our results with the large i.m. tumors, because double suicide gene therapy was able to reduce the volume of such tumors by more than 99%, resulting in a ~40% tumor cure. Although this cure rate was less than that achieved with s.c. tumors, it was markedly better than either 5-FC or GCV therapy, which failed to produce any cure of i.m. tumors. These tumors tend to be better vascularized than s.c. tumors, which may facilitate the removal of antimetabolites and may provide a richer environment for tumor growth. To our knowledge, no other investigations have initiated prodrug therapy when tumors were so advanced. These results may be significant, because most cancer patients enrolled in clinical trials will possess late-stage tumors of appreciable size.

As effective as CDg1yTK/5-FC + GCV therapy was against both large and small tumors, a 100% tumor cure was not achieved. Twenty % of the s.c. tumors (2 of 10) and 65% (11 of 17; results of small and large tumors combined) of the i.m. tumors recurred subsequent to cessation of double prodrug treatment, albeit with major growth delays. Interestingly, 40% of these recurring 5-FC + GCV-treated tumors remained positive for CD (and presumably HSV-1 TK as well), having an activity equal to that of the injected cells as determined by CD assays of tumor cell extracts (unpublished results). Others have also noted that most of the recurring tumors still possessed functional suicide enzymes (17, 18). Because we had already

Table 3 Effects of 5-FC + GCV therapy and radiotherapy on s.c. 9L-CDg1yTK tumors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average time to reach four times initial volume (days)</th>
<th>Fold increase relative to untreated group</th>
<th>Percentage tumor-free at day 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4 ± 0.4</td>
<td>0 (0 of 8)</td>
<td>20 ± 3 (2 of 10)</td>
</tr>
<tr>
<td>Radiation</td>
<td>11 ± 1.6</td>
<td>3 (0 of 9)</td>
<td>60 ± 10 (6 of 10)</td>
</tr>
<tr>
<td>5-FC + GCV</td>
<td>43 ± 4.0</td>
<td>11 (2 of 10)</td>
<td>20 ± 2 (2 of 10)</td>
</tr>
<tr>
<td>5-FC + GCV+ radiation</td>
<td>78.1 ± 12.3</td>
<td>19 (6 of 10)</td>
<td>60 ± 10 (6 of 10)</td>
</tr>
</tbody>
</table>

* For irradiated tumors, a single dose of 25 Gy was delivered shortly after the fourth prodrug injection (day 3). 5-FC (500 mg/kg) + GCV (30 mg/kg) was given i.p. for 6 days beginning on day 0.

* Initial volume refers to tumor size at the start of treatment.

4 Unpublished results.
used the maximum tolerable dose of 5-FC (500 mg/kg) and 60% of the maximum nontoxic dose of GCV (50 mg/kg; Ref. 31), it is unlikely that increasing prodrug concentrations would result in a greater antitumor effect without concomitantly increasing systemic toxicity. Prolongation of prodrug therapy might enhance therapeutic efficacy, providing that the cells responsible for the relapsing tumors were not pharmacologically sequestered. The majority (60%) of the recurring 5-FC + GCV-treated tumors were devoid of CD activity (and presumably HSV-1 TK activity as well). Such tumors may have resulted from the outgrowth of cells that had lost the CD/HSV-1 TK fusion gene, or alternatively, may have originated from a subset of G418-resistant 9L-CDglyTK cells that expressed the neomycin resistance gene product but failed to express the CDglyTK fusion protein. On the basis of immunofluorescence studies, we have observed that only 70% of G418-resistant 9L-CDglyTK cells actually express the fusion protein, although they have been maintained under G418 selection for more than 1 year, and both gene products are translated from the same bicistronic mRNA (23). Thus, the suicide gene status of the recurring tumors was mixed, and the reason(s) for tumor recurrence was unclear. However, it is likely that those cells that had escaped initial treatment could be eliminated by other modalities. Indeed, we have demonstrated that the therapeutic efficacy can be improved further by combining double suicide gene therapy with radiotherapy.

Cancer radiotherapy attains a therapeutic advantage by exploiting differences between malignant and normal tissue with respect to repair, cell-cycle distribution, repopulation, reoxygenation, and limiting the dose to normal tissues (33). In attempts to enhance the therapeutic index of radiation, numerous clinical trials have focused on combining radiotherapy with conventional cytotoxic drugs, such as doxorubicin, methotrexate, 5-FU, and cisplatin. However, injury to normal tissues by the combined regimen remains a major limiting factor. Because drug-radiation interactions lack tumor specificity, altering the genetic makeup of tumor cells to render them more radiosensitive could improve the therapeutic index. As such, we hypothesized that suicide gene therapy might be an effective adjunct to radiotherapy, because the prodrugs used (5-FC and GCV) are relatively nontoxic and well tolerated in humans. Indeed, our in vitro studies have demonstrated that both the CD/5-FC and HSV-1 TK/GCV systems can radiosensitize tumor cells (24–27), and even greater radiosensitization is achieved when these two systems are combined (23). We have now extended these in vitro observations by demonstrating that double suicide gene therapy can potentiate the therapeutic effect of radiation in vivo. Using an abbreviated 5-FC + GCV dosing schedule, an enhanced tumor growth delay resulted from combining double suicide gene therapy with 25 Gy of γ-radiation, exceeding that achieved with independent radiotherapy and double suicide gene therapy. More importantly, combining double suicide gene therapy with radiation resulted in a 200% greater tumor cure than that observed with double suicide gene therapy alone. As stated previously, because a period of 14 days of 5-FC + GCV therapy was so effective at eliminating s.c. leg tumors (80% cure), a suboptimal dosing schedule had to be used in the radiation studies to facilitate the measurement of combined antitumor effects. The possibility that 100% tumor control can be achieved when combining radiotherapy with the “optimal” 14-day 5-FC + GCV regimen is currently being investigated.

The mechanisms underlying radiosensitization are poorly understood. However, studies have suggested that for 5-FU (5-FC), radiosensitization results from the redistribution of cells in early S phase, a radiosensitive phase of the cell cycle (20, 21, 34). The radiosensitization effect of the HSV-1 TK/GCV combination may be due to the inhibition of potentially lethal damage repair, inasmuch as other antiviral agents that inhibit DNA polymerases, such as fludarabine phosphate, are potent radiosensitizers (35). Combining radiotherapy with CDglyTK suicide gene therapy, which on its own is highly efficacious at eradicating tumors, allows both mechanisms of radiosensitization to be used. On the basis of the results presented, double suicide gene therapy in combination with radiotherapy should markedly increase the therapeutic index, greatly increasing the differential response between malignant and normal tissues, and has the potential to achieve a cure of advanced tumors. The observed in vivo efficacy of combining radiotherapy with double suicide gene therapy provides the scientific basis for combining these modalities clinically to treat cancer.

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