ABSTRACT

In vitro experiments from our laboratory and others have suggested that herpes simplex virus thymidine kinase (HSV-TK)/ganciclovir (GCV) gene therapy depends on gap junctional intercellular communication (GJIC) to produce a strong bystander effect. Furthermore, we have shown that cells transduced with HSV-TK can be protected from GCV-mediated toxicity by GJIC with bystander cells. We wished to determine whether GJIC affected either the bystander or protective effect of the cytosine deaminase (CD)/5-flucytosine (5-FC) gene therapy approach, in which CD converts 5-FC to 5-fluorouracil (5-FU). To test this, we designed a coculture system using communication-competent WB rat hepatocytes and a noncommunicating subclone (aBl), which were transduced with CD and with antibiotic resistance genes so that we could independently determine the survival of the CD-containing or bystander cells. We found that, compared to the HSV-TK/GCV strategy, bystander killing resulting from treatment with CD/5-FC does not depend on GJIC. However, our most striking finding was that both communication-competent and -incompetent CD-transduced cells were preferentially killed, by a factor of up to 500, compared to bystander cells. The lesser dependence of the CD/5-FC system on GJIC, combined with the finding that most cancer cells lack the capacity for GJIC, suggest that the CD/5-FC system may be superior to the HSV-TK/GCV approach for gene therapy. However, the premature death of the CD-transduced 5-FU “factory” suggests that other strategies may be necessary to produce a sufficient quantity of 5-FU for a duration long enough to produce permanent tumor regression.

INTRODUCTION

Two of the most widely studied methods of enzyme prodrug gene therapy involve the use of the viral enzyme HSV-TK with the prodrug GCV or the bacterial enzyme CD with the prodrug 5-FC (1–3). In the former approach, GCV is a far better substrate for HSV-TK than for mammalian thymidine kinase, leading to preferential production of GCV monophosphate and, ultimately, cytotoxicity. In the latter strategy, CD can convert the prodrug 5-FC to 5-FU, which has both cytotoxic and radiosensitizing properties (4). A recent comparison of these two approaches in cell culture (5) and implanted tumors (6) suggested that the CD/5-FC system was more effective, although the mechanism underlying the improved efficacy of CD/5-FC is not known.

One possible reason that the CD/5-FC system might be superior to the HSV-TK/GCV approach is that the former may depend less on GJIC for bystander killing. Because gene therapy approaches appear to transduce no more than 1–5% of tumor cells, it is crucial that the transduced cells effectively kill surrounding cells which have not been transduced (the “bystander effect”). Although there exist a number of possible mechanisms for a bystander effect (see “Discussion”), the most direct mechanism involves the transfer of toxic metabolites from one cell to another. In the case of the strategy using HSV-TK and GCV, the toxic metabolite, phosphorylated GCV, cannot transit the membrane. GJIC permits the transfer of molecules up to Mr ~1000 (which includes phosphorylated GCV; Refs. 7–9) directly from one cell to another. This is consistent with the finding that communication-competent cells demonstrate a greater bystander effect than do incompetent cells (10, 11). In the case of the CD/5-FC approach, although the toxic phosphorylated metabolites of 5-FU, such as FdUMP, would, like phosphorylated GCV, require GJIC to affect a bystander cell, 5-FU itself can diffuse across the cell membrane to neighboring cells. Therefore, although the CD/5-FC strategy might have some dependence on GJIC, the influence of communication status should be substantially less than that observed for the HSV-TK/GCV approach. This difference assumes greater importance in light of the fact that the capacity for GJIC has been lost in most cancer cells (12–15) and was not found on ultrastructural examination of the tumors in the study described above (6).

Therefore, we wished to determine the role of GJIC in these two enzyme prodrug strategies. To carry out these studies, we used communication-competent WB rat hepatocytes and a communication-incompetent subclone (aBl). We chose experimental designs that permitted us to quantify separately the effects of drug treatment on both the transduced cell and the bystander cell. Using this approach, we have recently confirmed that the in vitro bystander effect for HSV-TK-induced killing is strongly dependent on GJIC, and we made the novel observation that communication-competent HSV-TK-transduced cells could be protected by communication competent neighbors (the “Good Samaritan” effect; Ref. 11). We next wished to carry out analogous studies to assess the role of GJIC on the efficacy of the CD/5-FC strategy. In confirmation of our hypothesis, we found that treatment of cocultures of CD-transduced cells produced bystander killing that was independent of GJIC. Similarly, although bystander cells protected CD-transduced cells, this protection showed little, if any, dependence on GJIC.

However, we also discovered that the transduced cells were much more effectively killed by 5-FC than were the bystander cells, almost surely because of the high intracellular concentrations of 5-FU that are generated. Although successful gene therapy will require that the transduced cells ultimately be killed, preferential killing of the transduced cells compared to the bystander cells might be disadvantageous because the transduced cell (the 5-FU “factory”) would die before producing sufficient 5-FU to cause tumor regression. This suggests that strategies that produce more uniform killing of the transduced and bystander cells need to be explored.

MATERIALS AND METHODS

Cell Culture. Cells were cultured under standard conditions using DMEM containing 10% bovine calf serum, penicillin-streptomycin, and 2 mM glutamine with a final thymidine concentration of 0.2 μM. All cells were checked for mycoplasma every 3 months.
Cell Lines. Communication-competent WB rat hepatocytes and the communication-incompetent aB1 subclone have been described (16). To construct WB-CD and aB1-CD cells, the CD coding sequence was PCR-amplified using the DNA of Kohara phages 137 and 138 as template (kindly provided by F. Nienhardt, University of Michigan, Ann Arbor, MI; Ref. 17). The 5′ primer (GTCGACGGACAAATGGCTGGTAAACTAGTTA) was designed to introduce a Kozak sequence and an initiator ATG (in place of GTG) for mammalian cell expression, as well as a SaII site. The 3′ primer (TACGACGTTGAAAGCTGGATCTAGTTTA) introduces an EcoRI and an XbaI site after the stop codon. The amplified fragment was cloned into the mammalian expression vector pZ (kindly provided by Genetics Institute, Cambridge, MA) and sequenced to confirm the sequence. We transfected WB and aB1 cells with this plasmid (pZ-CD1) using LipofectAMINE (Life Technologies, Gaithersburg, MD), and 48 h after transfection, cells were split 10-fold in the presence of 600 μg/ml G418. The resulting neomycin-resistant colonies were isolated and screened for the presence of the CD protein by Western blot (Fig. 1) using CD-specific antibodies (described below). Puromycin-resistant WB and aB1 cells were constructed similarly using the pPUR plasmid and selected in 2 μg/ml puromycin. MCF-7 cells were obtained from American Type Culture Collection.

Western Analysis. Logarithmically growing cells were trypsinized, counted, and lysed with NP40 lysis buffer [1% Nonidet –40, 150 mM NaCl, 50 mM Tris (8.0), and 0.05% SDS] containing a cocktail of protease inhibitors (BMB, Indianapolis, IN). Aliquots of the lysates were resolved using standard SDS-PAGE techniques and transferred onto nitrocellulose membrane. CD was detected using a CD-specific rabbit antiserum (kindly provided by Dr. Brian Huber, Glaxo-Wellcome, Raleigh, NC) and processed for horseradish peroxidase-induced chemiluminescense, as recommended by the manufacturer (Pierce, Rockford, IL).

Assessing GJIC by FRAP. The communication status of mixed cultures was determined essentially as described previously (18) using a Meridian ACAS 570 (Meridian Instruments, Inc. Okemos, MI), coupled with a computer workstation. Briefly, one cell type was incubated with fluorescent beads (Fluoresbrite Carboxylate, 1.16-μm diameter; Polysciences, Inc., Warrington, PA), which it phagocytosed, thus permitting it to be distinguished from the other cell types in a coculture. Cells were cocultured for 12–16 h and loaded with carboxyfluorescein. Single bead-loaded cells were then photobleached and observed for recovery of fluorescence by laser scanning. The total fluorescence recovery over 3 min after photobleaching was determined for 12–50 selected cell sites for each coculture condition.

Assessing GJIC by Scrape Loading. Cells were grown to near confluence in 35-mm culture dishes. The medium was removed, and the cultures were rinsed three times with prewarmed calcium magnesium-free PBS. One ml of a 0.05% solution of Lucifer yellow in CMF-PBS was added to each dish. The edge of a razor blade was pressed into the monolayer to form the “scrape line,” along which the dye enters the cells. After 1 min, the dye was removed, and the cultures were rinsed three times with prewarmed PBS. After 15 min at 37°C, the PBS was removed, and the cells were fixed with 3.7% (v/v) formaldehyde in PBS for 15 min, after which the dishes were washed three times with PBS. The culture dishes were examined under blue excitation (450–490 nm) using a Leitz Laborlux D microscope equipped with an epifluorescence unit. Fields were examined for cells beyond those along the scrape line, which had taken up the dye (19).

Cell Survival Assay. Cocultures using different ratios of transduced to bystander cells were constructed so that the total cell density was constant. Separate cultures were plated for each coculture condition to control for the number of CD-transduced cells. The actual ratios of cells at the time of 5-FU addition were 4–7% and 45–60% transduced when the nominal ratios were 5:95 and 50:50, respectively. Cocultures were treated with 5-FU for 48 h. Cell survival of the neomycin-resistant CD-expressing cells (aB1-CD and WB-CD) and the puromycin-resistant bystander cells (aB1-puro and WB-puro) was determined by plating cells into selective medium (Fig. 2) and assessed using a standard clonogenic assay, as described previously (20). The plating efficiencies of WB-puro and aB1-puro cells in puromycin were 0.48 ± 0.08 and 0.49 ± 0.05, respectively. The plating efficiencies of WB-CD and aB1-CD cells in G418 were 0.50 ± 0.03 and 0.69 ± 0.02, respectively. The surviving fractions of the puro clones in G418 and the CD clones in puromycin were all <10%. Cocultures were ~90% confluent at the time of processing for cell survival.

Determination of 5-FU. Conversion from 5-FC to 5-FU by tissue culture cells was measured using a gas chromatographic/mass spectrometric assay, as described previously (20). Briefly, conditioned medium was collected and derivatized, and quantification of the derivatized products was performed using a Hewlett-Packard 5987A gas chromatograph/mass spectograph in selected ion-monitoring mode.

Apoptosis. Cells were trypsinized and washed once with PBS. They were then fixed by incubation in 4% paraformaldehyde at a concentration of 106 cells/ml for 30 min at room temperature and then washed again with PBS. The fixed cells were resuspended in PBS at 106 cells/ml. Thirty-five μl of the cell suspension were mixed with 5 μl of 100 μg/ml acridine orange solution and 10 μl of Vectashield (Vector Laboratories, Burlingame, CA). The mixture was examined using a Leitz Laborlux 5 microscope equipped with a 1-Lambda Pleomonok incident light fluorescence illuminator (450–490-nm excitation wavelength with 520-nm barrier filter). Cells were scored as apoptotic if they exhibited both chromatin condensation and cellular shrinkage. Data are presented as the mean percentages of apoptotic cells.

Statistical Analysis. Data are presented as means ± SE. Student’s t test and the F test were used to compare two means and multiple means, respectively. Statistical significance was defined at the level of P < 0.05 (two-tailed).
RESULTS

To test our hypothesis that bystander cell killing resulting from treatment of cocultures of cells transduced with CD was relatively independent of GJIC, it was first necessary carry out two types of control experiments. First, we needed to confirm the communication status of these cells. It has been previously demonstrated that WB cells are communication competent and that the aB1 clone derived from WB cells does not show detectable GJIC (16). We began with a detailed assessment of WB-CD and aB1-CD cells using FRAP (Fig. 3). As described in "Materials and Methods," we cocultured WB cells (marked by latex beads) with WB-CD cells and photobleached either a WB cell or a WB-CD cell. We found that there was efficient transfer of dye from the surrounding cells into the bleached cell, demonstrating communication competence (Fig. 3, a and c). In contrast, when an aB1-CD cell (marked by beads) was photobleached, dye transfer from surrounding cells did not occur, even when aB1-CD cells were cocultured with WB cells (communication competent; Fig. 3, b and c). Scrape loading, as described in "Materials and Methods," confirmed these results (data not shown) and demonstrated that WB-puro and aB1-puro cells also maintained the communication status of the parental cell (Fig. 4). Second, we needed to determine the 5-FU sensitivity of WB-puro, aB1-puro, WB-CD, and aB1-CD cells. We found that there were no significant differences in 5-FU sensitivity among the WB-puro, aB1-puro, and aB1-CD cells, although WB-CD cells were more resistant to 10 μM 5-FU than the other cell lines (Fig. 5).

We could then determine whether the communication status of cells influenced the bystander effect. For these experiments, WB-CD (communication-competent) cells were cocultured with either (communication-competent) WB-puro cells or (communication-incompetent) aB1-puro cells. Cocultures were constructed using a ratio of transduced cells to bystander cells of either 50:50 or 5:95, exposed to 5-FC, and assessed for survival of the bystander cells according the method described in Fig. 2. Whereas 1 mM 5-FC had no effect on WB-puro cells or aB1-puro cells cultured alone, there was substantial WB-puro and aB1-puro cell killing (a strong bystander effect) at the higher ratio and a weaker one at the lower ratio. However, bystander killing did
PREFERENTIAL CYTOTOXICITY OF CD-TRANSUDED CELLS

Fig. 4. Determination of the communication status of WB-puro and aB1-puro cells by scrape loading. WB-puro and aB1-puro cells were cultured and assessed for GJIC by scrape loading as described in "Materials and Methods." A, WB-puro cells. B, aB1-puro cells. Cells that are capable of GJIC (WB-puro) exhibit penetration of the dye through several cell layers beyond the scrape line. In communication-incompetent cells (aB1-puro), Lucifer yellow is present only in those cells along the scrape line. 

not depend on the communication competence of the cells (Fig. 6A). Similar experiments were carried out using aB1-CD cells cocultured with either WB-puro cells or aB1-puro cells, which confirmed that communication status had no influence on the ability of CD-transduced cells to kill bystander cells (Fig. 6B).

We next wished to determine whether communication status affected the survival of the transduced cells. We conducted these experiments because, as described in the "Introduction," we had found that communication-competent bystander cells protected HSV-TK-transduced cells to a far greater extent than did communication-incompetent bystander cells (11). We found that bystander cells were able to protect CD-transduced cells from 5-FC-mediated cytotoxicity. However, in contrast to our results with HSV-TK-transduced cells treated with GCV, communication-competent WB-puro cells did not consistently provide better protection than communication-incompetent aB1-puro cells (Fig. 7A). Furthermore, communication-incompetent aB1-CD cells were protected from 1 mM 5-FC by coculture with either aB1-puro or WB-puro cells (Fig. 7B). Therefore, although it is possible that GJIC mediates part of the protection, a substantial component seems unrelated to communication status.

Our findings that the capacity for GJIC had little influence on the resulting clonogenicity of cells bystander cells suggested that the secretion of 5-FU was chiefly responsible for bystander killing. Therefore, we measured the 5-FU concentrations resulting from treatment with 5-FC in medium that we had saved at the time the cocultures described above were processed for clonogenic survival. This permitted us to construct a plot of the surviving fraction of both the CD-transduced cells and the bystander cells as a function of the extracellular 5-FU concentration generated by that coculture (Fig. 8). We found that bystander cell cytotoxicity increased only slightly with the extracellular concentration of 5-FU. However, when the ratio of CD-transduced cells to bystander cells was 5:95, both WB-CD and aB1-CD cells were killed to a significantly greater extent than the bystander WB-puro and aB1-puro cells for the same extracellular 5-FU concentration. Likewise, CD-transduced cells were growth arrested to a far greater extent than were bystander cells (data not shown). When the ratio of CD-transduced cells to bystander cells was 50:50, extracellular 5-FU concentrations were in the range of 3–6 μM, and the loss of clonogenicity of the transduced and bystander cells did not consistently differ (data not shown).
Although the chief purpose of this and our previous investigation (11) was to determine the role of GJIC in the bystander effect of gene therapy, it seemed of interest to begin to assess other potential mechanisms. Because it has been proposed that the transfer of apoptotic vesicles could mediate bystander killing (21-23), we decided to determine whether 5-FC produced substantial apoptotic cell killing under the conditions used for our coculture experiments described above. We found that neither WB-CD nor aBI-CD cells showed significant cell death from drug-induced apoptosis, assessed as described in “Materials and Methods,” after a 48-h treatment with 1 mM 5-FC (Table 1). Parallel experiments were carried out with MCF-7 breast cancer cells as a positive control for our ability to detect apoptosis because these cells have been shown to undergo apoptosis after a variety of insults (24). In addition, we found that WB-CD and aBI-CD cells were capable of undergoing apoptosis after etoposide treatment (80 μg/ml for 1 h). These data suggest that, although WB-CD and aBI-CD cells are capable of undergoing apoptosis, they do not do so to a significant extent after exposure to up to 1 mM 5-FC for ≤48 h.

DISCUSSION

In this study, we have used communication-competent cells and a communication-incompetent subclone transduced with CD and antibiotic resistance genes to assess directly the role of GJIC on the killing of both the transduced and bystander cells. We have found that the CD/5-FC gene therapy strategy expresses little or no dependence on GJIC, suggesting that bystander killing is determined chiefly by diffusion of 5-FU into the extracellular space. This result contrasts with our previous findings derived from analogous experiments using the HSV-TK/GCV approach, in which the capacity for GJIC produced both significant bystander cell killing and protection of the transduced cell (11). Because most cancer cells lack functional GJIC, our results suggest a potential advantage of the CD/5-FC system compared to the HSV-TK approach. However, we have also found that, when transduced cells and bystander cells are cocultured at a 5:95 ratio, which is relevant for in vivo gene therapy, the CD-transduced cells are killed to a significantly greater extent than the bystander cells for the same extracellular concentration of 5-FU. This suggests that a potential disadvantage of the current CD/5-FC strategy is that the CD-transduced cell may be preferentially killed before the bystander tumor cell, thus decreasing the bystander effect and permitting tumor regrowth.

Whereas a number of investigators have evaluated the role of GJIC in the HSV-TK/GCV strategy (10-11, 25), we are unaware of other studies of the influence of GJIC on the efficacy of the CD/5-FC approach. Our results are consistent with those of Huber and colleagues (6), who found significant regressions of tumors composed of mixtures of CD-transduced and bystander WiDr cells, which do not contain gap junctions when evaluated by electron microscopy. It is, therefore, possible that at least a component of the superior results they obtained using the CD/5-FC system compared to the HSV-TK/GCV strategy was due to the decreased effectiveness of the latter strategy in tumors that lack the capacity for GJIC.

In this study, we found that increasing concentrations of secreted 5-FU produced greater bystander cytotoxicity. In addition, it is likely that additional factors will influence the bystander effect. For instance, it is possible that the greater diffusion of 5-FU compared to phosphorylated GCV could actually decrease the selectivity of tumor cell killing. In addition, membrane-impermeant toxic metabolites such as phosphorylated GCV or FdUMP (in the case of the HSV-TK/GCV and CD/5-FC strategies, respectively) could be transferred from one cell to another by a mechanism other than GJIC, such as the diffusion
of apoptotic vesicles (21–23). Although neither WB-CD nor aB1-CD cells undergo significant 5-FC-induced apoptosis under the conditions used in this study, it remains possible that transfer of apoptotic vesicles contributes to the bystander effect in other settings. Furthermore, a bystander effect in vivo could be mediated by nonpharmacological means, such as stimulation of an immunological response (21, 26, 27), which were not examined in this cell culture system. Likewise, the finding that CD-transduced cells are partially protected when they are cocultured at a 5:95 ratio regardless of communication status could reflect 5-FU catabolism by neighboring cells. Therefore, it will be important to perform further studies of responses in vivo before drawing conclusions about which enzyme prodrug strategy is superior.

Perhaps the most important finding of this paper is that CD-transduced cells are preferentially killed compared to bystander cells. This result is perhaps even more striking, given that WB-CD cells are relatively resistant to 5-FU compared to the other cell lines used in this study. It is highly likely that preferential cytotoxicity of transduced cells occurs because they generate high intracellular concentrations of 5-FU, which then becomes diluted before reaching bystander cells. This result is consistent with the suggestion that CD/5-FC can be used under some conditions as a negative selection system for selectively removing CD containing 3T3 cells from a mixed culture with minimal killing of bystander cells (28). Thus, although successful gene therapy requires that the transduced cells ultimately die, if this occurs premortally, the great majority of the (nontransduced) tumor cells will not be killed. We have begun to address this potential weakness by designing a CD that is secreted. Our preliminary in vitro data suggest that expression of secreted CD produces more uniform treatment of transduced and bystander cells than the expression of intracellular CD used here. Whether the expression of extracellular CD will prove to be superior in vivo to the current approaches using intracellular CD or HSV-TK remains to be determined.

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 Preferential Cytotoxicity of Cells Transduced with Cytosine Deaminase Compared to Bystander Cells after Treatment with 5-Flucytosine

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