Protection of Herpes Simplex Virus Thymidine Kinase-transduced Cells from Ganciclovir-mediated Cytotoxicity by Bystander Cells: The Good Samaritan Effect

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ABSTRACT

Although considerable attention has been directed in the field of gene therapy toward elucidating the mechanism by which a transduced cell could kill a bystander cell, little is known about how bystander cells may affect transduced cells. We hypothesized that bystander cells, particularly if they were capable of gap junctional communication, could protect cells transduced with the herpes simplex virus thymidine kinase (HSV-TK) from ganciclovir (GCV)-induced cytotoxicity. To test this hypothesis, we used a rat hepatocyte cell line (WB) that can carry out efficient gap junctional communication, a WB clone transduced with HSV-TK (WB-TK), and a communication-incompetent subclone of WB cells (aBi). We cocultured WB-TK cells with either WB or aBi cells, treated them with GCV, and then plated the cells into selective media that permitted us to quantify independently the surviving fraction of WB-TK cells or bystander cells. We found that WB bystander cells conferred up to a 1000-fold protection on WB-TK cells treated with GCV. aBi cells conferred detectable, but significantly less, protection. These findings demonstrate that herpes simplex virus thymidine kinase-transduced cells can be significantly protected by bystander cells, particularly those that can carry out gap junctional communication. Whether this “Good Samaritan” effect improves the overall efficacy of gene therapy, by prolonging the survival of the source of toxic metabolites, or decreases effectiveness by increasing the survival of transduced cells will need to be determined in vivo.

INTRODUCTION

The HSV-TK and GCV system is the most widely used for enzyme prodrug strategy gene therapy (1—4). GCV, a derivative of the nucleoside analogue Acyclovir, can be phosphorylated by HSV-TK to form GCV triphosphate, which causes DNA chain termination and cell death. The selective killing of HSV-TK transduced cells is based on the fact that the viral TK is almost 1000-fold more efficient than the mammalian TK at phosphorylating GCV.

One of the major obstacles to successful gene therapy for cancer is the difficulty of transcribing a high fraction of a cell population. Typically less than 10% (and often less than 1%) of tumor cells are successfully transduced. Because killing only a small fraction of a tumor would not be clinically meaningful, it is crucial that GCV treatment kill both the transduced cells and nontransduced (bystander) cells.

Substantial evidence suggests that GJIC can play a role in mediating the bystander effect. Gap junctions are specialized structures allowing for direct communication between apposing plasma membranes composed of connexons that act as intercellular channels, permitting the transfer of molecules less than Mr 1000 directly from one cell to another (5—7). Because GCV triphosphate cannot freely diffuse across the cell membrane, it seemed likely that GJIC could mediate transfer of this toxic metabolite from a transduced to a bystander cell. Indeed, this hypothesis has been substantiated by a number of recent investigations (8, 9) demonstrating that bystander killing is increased in communicating cells compared to noncommunicating cells (see “Discussion”).

Although it is now clear that GJIC can facilitate bystander cell killing, we hypothesized that GJIC could also protect the transduced cell, presumably by lowering its concentration of cytotoxic metabolites. If this were the case, it would have important (but potentially complex) implications for the success of HSV-TK gene therapy. On the one hand, protection of the transduced cell could lead to the failure to observe any cell killing. Conversely, protection could permit the transduced “factory” to live longer, which could permit it to kill more bystander cells.

To test the hypothesis that gap junctional communication could protect a transduced cell from GCV-induced cell killing (the “Good Samaritan” effect), we used a rat hepatocyte line (WB), which evidence GJIC (communication competent), and its subclone (aBiI), which does not (communication incompetent). Using retroviral transduction, we constructed a derivative of WB cells (WB-TK) that expresses HSV-TK. This permitted us to coculture WB-TK cells with either communication competent WB or incompetent aBiI cells, treat the coculture with GCV, and then plate the cells into selective media to determine the survival of the transduced cells or the bystander cells. We found that, in addition to the bystander effect reported previously, GJIC mediated a strong protective effect that needs to be taken into consideration when considering the application of the HSV-TK strategy for gene therapy.

MATERIALS AND METHODS

Tissue Culture and Cell Lines. Rat liver epithelial cells that demonstrate GJIC (WB) as well as a communication-incompetent clone (aBiI) were derived as described (10). The cells were cultured in DMEM containing 10% calf serum/penicillin (50 units/ml)/streptomycin (50 μg/ml) at 37°C in 5% CO2.

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 calcium/magnesium-free 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 Labrolux D microscope equipped with an epifluorescence unit. Fields were examined for cells beyond those along the scrape line that had taken up the dye (12).

Assessing GJIC by FRAP. The communication status of mixed cultures was performed essentially as described by Kalani et al. (13) using a Meridian ACAS 570 (Meridian Instruments, Inc., Okemos, MI) coupled with a computer workstation. Briefly, one cell type was incubated with fluorescent beads (Polysciences, Inc.; Fluoresbrite carboxylate; 1.16-μm diameter), which it phagocytosed, thus permitting it to be distinguished from the other cell type 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 rescanning. The total fluorescence recovered over 15 min after photobleaching was determined for approximately 30 selected cell sites.

Determination of Sensitivity to GCV. Log phase cultures were treated for 24 h with varying doses of the drug. Clonogenicity was assessed using a standard assay, as described previously (14).

Assay for the Bystander and Protective Effects. A total of 100,000 cells, comprised of WB-TK (transduced) cells and either WB or aB1 (bystander) cells, were plated at a ratio of transduced to bystander cells of either 50:50 or 5:95 onto either a 100-mm dish (low density) or a 35-mm dish (high density). Two days later, the cells were exposed to GCV (2 or 10 μM) for 24 h trypsinized, and plated at clonal density into either: (a) media containing 1 mg/ml G418 to determine the surviving fraction of G418-resistant WB-TK transduced cells; or (b) media containing 10 μg/ml GCV to determine the surviving fraction of WB or aB1 bystander cells. Under this latter condition, GCV was lethal only to the HSV-TK-transduced cells (data not shown). This scheme is outlined in Fig. 1.

The following controls were included in each experiment: (a) cocultures were plated into nonselective media to ensure that the total cell number equaled the sum of conditions 1 and 2 above; and (b) the plating efficiencies of each coculture condition were determined in the absence of GCV pretreatment. The results of drug-treated samples were corrected for plating efficiency using the appropriate samples exposed to media only.

To quantify the bystander and protective effects, we used the Levenberg-Marquardt algorithm to fit the results of our experiments determining the effect of GCV on the surviving fraction of WB-TK cells cultured alone (see "Results" and Fig. 2) to the following form of the equation of Chou and Talalay (15):

\[
\ln(1/SF - 1) = m \ln([GCV]/[GCV]_{50})
\]

in which SF is the surviving fraction, \([GCV]_{50}\) is the concentration of GCV that produces a surviving fraction of 50%, and m is the slope of the sigmoid curve relating surviving fraction to concentration.

Fig. 1. A diagrammatic representation of the experimental design to measure the bystander effect and the protective effect. WB-TK cells were cocultured either with WB or aB1 at a total of 100,000 cells/dish. After 48 h, the cells are exposed to GCV for 24 h, trypsinized, and plated onto dishes in the presence of selection with G418 or GCV. Small solid circles (○, WB-TK) and open circles (○, WB or aB1) indicate individual cells in the coculture plated at high density. Large solid and open circles indicate colonies that survive GCV or G418 treatment.

Fig. 2. Sensitivity of WB, aB1 and WB-TK to GCV. WB, aB1, or WB-TK cells were plated, treated with GCV for 24 h, and assessed for clonogenic survival as described in "Materials and Methods." Each symbol represents the mean of three independent experiments; bars, SE.

Statistical Analysis. All data are presented as the mean ± SE of at least three experiments. Student's t test was used to compare two means. Statistical significance was defined at the level of P < 0.05 (two-tailed).

RESULTS

Characterization of the Cell Lines. We first needed to confirm the sensitivity of cells transduced with HSV-TK to GCV and the communication status of the individual cells lines and the cocultures. As anticipated, HSV-TK-transduced WB cells were exclusively sensitive to GCV. We found that greater than 99% of the WB-TK cells were killed after a 24-h exposure to 1 μM GCV, and almost 99.99% were killed when exposed to 10 μM GCV. The parental WB cells as well as aB1 cells were not significantly affected, even when treated with 100 μM GCV (Fig. 2). We obtained a good fit of all our data, using the model described in "Materials and Methods," when \( m = 0.20 \) and \([GCV]_{50} = 0.12\) μM (r2 = 0.83).

To confirm the GJIC status of the three cell lines, two types of experiments were carried out. Scrape loading experiments demonstrated that aB1 cells were communication incompetent because Lucifer yellow was found to be present only within the cells along the scrape. In contrast, WB and WB-TK cells were communication competent in that Lucifer yellow was able to penetrate at least 10 cell layers deep (Fig. 3). A second method was used to determine if WB and aB1 cells communicated. In this assay, one cell type was preloaded with fluorescent beads followed by FRAP (fluorescence recovery after photobleaching). We found that the extent of communication between WB and aB1 was no greater than that between aB1 cells, and that both types of cultures demonstrated far less dye transfer than between WB cells (Table 1). These experiments indicate that our model system could be used to determine the role of cellular communication in bystander killing and to detect a protective effect should it exist.

Bystander Killing Is Dependent on GJIC. We found that the communication-competent WB cells tended to show a greater extent of bystander killing than the communication-incompetent aB1 cells. This was most evident when the ratio of transduced to bystanders was 50:50, when the concentration of GCV used was 10 μM, and when the cell density was high (Table 2).

We wished to determine the role of cell density on WB bystander killing. We predicted that the bystander effect would increase with increased cell density. To test this hypothesis, we compared bystander killing of similar numbers of WB cells cocultured with WB-TK cells in 35-mm dishes (high density) versus 100-mm dishes (low density). We found that bystander killing was significantly greater at higher...
compared the surviving fraction of the WB-TK cells exposed to GCV. To examine if such a protective phenomenon existed, we hypothesized that cell-to-cell communication may also play a role. The observation that the bystander effect is potentiated by GJIC led us to hypothesize that cell-to-cell communication may also result in protection of HSV-TK cells from the toxic metabolites of GCV. To try to put this difference in perspective, we compared WB-TK and WB cocultures exposed to either 2 or 10 μM GCV, and plated at clonal density. This was also true when WB-TK and WB cells were plated at a ratio of 5:95 (Table 2).

We then assessed the influence of the ratio of transduced to WB bystander cell on bystander killing. There was less bystander killing when the ratio of transduced to bystander cells was 5:95 compared to 50:50 (Table 2). To try to put this difference in perspective, we calculated the effective concentration of GCV received by the bystander cells based on the relationship between the concentration of GCV and surviving fraction described above. Based on this calculation, bystander WB cells received the equivalent of 1.6 ± 0.1 μM and 0.17 ± 0.02 μM GCV when they were cocultured with WB-TK cells at a ratio of 50:50 or 5:95, respectively. This 10-fold difference in equivalent GCV concentration compares well with an approximately 10-fold difference in the number of WB-TK cells. Bystander killing was also dose dependent, with 10 μM GCV producing significantly more bystander killing than 2 μM GCV (Table 2).

GJIC Protects WB-TK Cells from the Effects of GCV Treatment. The observation that the bystander effect is potentiated by GJIC led us to hypothesize that cell-to-cell communication may also result in protection of HSV-TK cells from the toxic metabolites of GCV. To examine if such a protective phenomenon existed, we compared the surviving fraction of the WB-TK cells exposed to GCV in cocultures with either WB cells or aB1 cells. We found that WB cells provided a significantly stronger protective effect than aB1 cells when WB-TK cells and bystander cells were plated at a ratio of 5:95 at both high and low cell densities (Fig. 4). Although the protection conferred by WB cells was significantly greater than that of aB1 cells, both cell types significantly increased WB-TK survival because, in the absence of bystander cells, the surviving fraction of WB-TK cells after treatment with 10 μM GCV was 0.08 ± 0.03% (Fig. 2).

To determine the concentration dependence of the protective effect, we compared WB-TK and WB cocultures exposed to either 2 or 10 μM GCV and surviving fraction described above. Based on this calculation, bystander WB cells received the equivalent of 1.6 ± 0.1 μM and 0.17 ± 0.02 μM GCV when they were cocultured with WB-TK cells at a ratio of 50:50 or 5:95, respectively. This 10-fold difference in the number of WB-TK cells. Bystander killing was also dose dependent, with 10 μM GCV producing significantly more bystander killing than 2 μM GCV (Table 2).

GJIC-MEDIATED PROTECTION FROM GCV

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cells</th>
<th>Average</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>aB1:aB1 (1:25)</td>
<td>33</td>
<td>1.32</td>
<td>1.09</td>
</tr>
<tr>
<td>WB:aB1 (1:25)</td>
<td>27</td>
<td>1.72</td>
<td>1.69</td>
</tr>
<tr>
<td>WB-WB (1:25)</td>
<td>19.69</td>
<td>4.42</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 The dependence of the bystander effect on GJIC

<table>
<thead>
<tr>
<th>Ratio</th>
<th>GCV (μM)</th>
<th>Density</th>
<th>Surviving fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>50:50</td>
<td>2</td>
<td>High</td>
<td>0.069 ± 0.038</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>0.057 ± 0.057</td>
</tr>
<tr>
<td>5:95</td>
<td>10</td>
<td>High</td>
<td>0.006 ± 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>High</td>
<td>0.077 ± 0.011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>0.55 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>High</td>
<td>0.28 ± 0.06</td>
</tr>
</tbody>
</table>

aB1 surviving fraction is significantly greater than the corresponding WB surviving fraction (P < 0.05).

Fig. 3. Determination of the communication status of WB, aB1, and WB-TK cells using fluorescent-dye scrape loading. WB, aB1, and WB-TK cells were cultured and assessed for GJIC by scrape loading, as described in "Materials and Methods." a, WB cells; b, aB1 cells; c, WB-TK cells.

Fig. 4. WB-TK cells are protected from GCV toxicity by coculture with bystander cells. WB-TK cells and either aB1 (●) or WB cells (0) were cocultured either at high or low density (100-mm dishes) conditions. The surviving fraction of the bystander cells (WB or aB1) was determined as described in "Materials and Methods." a, WB cells; b, aB1 cells; c, WB-TK cells.

Fig. 5. The influence of GCV concentration on the protective effect. WB-TK cells and WB cells (a) or aB1 cells (b) were cocultured in 35-mm dishes, treated with GCV at a concentration of 10 μM (●) or 2 μM (□) in 418-containing media to attest clonogenic survival of the WB-TK cells (see Fig. 1). Each column represents an average of three independent experiments; bars, SE.
μM GCV (Fig. 5). To quantify protection, we calculated the effective concentration of GCV represented by these surviving fractions, using the data from Fig. 2 (see “Methods and Materials”). Compared to the survival of WB-TK cells when cultured alone, this represents an effective concentration of GCV of 0.7 ± 0.15 μM and 5.9 ± 2.9 μM GCV after treatment with 2 and 10 μM GCV, respectively. Note that if protection were mediated by dilution of cytotoxic metabolites throughout the entire cell culture population, the expected effective concentrations would be 1 and 5 μM, respectively. A similar dose dependence of protection was observed when the WB-TK:WB ratio was 5:95. Under these conditions, the surviving fraction after treatment with 2 and 10 μM represented an effective GCV concentration of 0.08 ± 0.035 μM and 0.32 ± 0.01 μM. If protection were mediated by dilution, the expected concentrations would be 0.1 and 0.5 μM, respectively.

WB-TK cells were minimally protected or not protected by an equal number of WB or aB1 cells. The surviving fraction of WB-TK cells when cultured at low density with aB1 cells was 0.08 ± 0.01% and with WB cells was 0.10 ± 0.07%, neither of which differed significantly from WB-TK cells treated alone. WB cells, but not aB1 cells, did confer slight protection at higher density when transduced and bystander cells were plated in equal numbers (Fig. 5). The overall low surviving fractions under 50:50 plating made it difficult to perform an accurate calculation of an equivalent concentration of GCV for WB cells.

DISCUSSION

We have designed a model system that has enabled us to study the potential role of GJIC on the cytotoxicity of GCV against HSV-TK-expressing transduced cells and bystander cells. Using this system, we have made the novel observation that GJIC can protect HSV-TK-transduced cells from GCV-induced cytotoxicity. We found that protection is more effective in communication-competent cells compared to incompetent cells and at a higher ratio of bystander to transduced cells. The extent of protection observed is, overall, consistent with diffusion of the toxic metabolites of GCV throughout the entire population of transduced and bystander WB (communication-competent) cells, although our data do not rule out other mechanisms. These observations are consistent with the hypothesis that protection is mediated chiefly by GJIC.

Our conclusion that GJIC can mediate bystander killing is consistent with a number of recent investigations. First, it has been reported that TK− cells incorporate 3H-labeled GCV only when in contact with TK+ cells (16). This shows that GCV can be transferred from one cell to another by a contact-dependent mechanism, which is a requirement for GJIC-dependent killing. In addition, the bystander effect proved to be highly correlated with the extent of GJIC as quantified by flow cytometry (17). Similarly, Mesnil et al. (18) demonstrated that whereas no bystander effect was observed in cocultures of TK+ and TK− cells among the inherently GJIC-incompetent HeLa cells, a strong bystander effect was seen when these cells were also transfected with a gene encoding the gap junctional protein connexin43. It has also been shown that there is no bystander effect in model systems with lymphocytes, which do not carry out GJIC (19). The fact that our findings confirm those of a number of other investigators (20, 21) suggests that our results concerning the potential protective effect of GJIC on GCV-induced cytotoxicity in HSV-transduced cells will also be widely applicable.

Communication-incompetent aB1 cells also demonstrate both the bystander and Good Samaritan effects, although to a far lesser extent than WB cells. The mechanisms underlying these interactions are not known. It is possible that this represents a small extent of GJIC that is below the level of detection of our assay. It has also been proposed that the cytotoxic metabolites of GCV can be transferred from cell to cell by apoptotic vesicles (22–24), which would not require GJIC, although we have been unable to detect apoptosis in WB-TK cells treated under the conditions used in this study.4

Because aB1 cells were derived from WB cells by mutagenesis and selection against a communication-competent phenotype, it is possible that aB1 cells and WB cells have differences in addition to communication status that contribute to their differing participation in the bystander and protective effects described here. However, the finding that WB and aB1 cells are similar with respect to population doubling times and response to a variety of chemotherapeutic agents and radiation5 suggest that it is unlikely that there are differences other than GJIC status that are important in the context of this study.

It is difficult to predict the consequences of the Good Samaritan effect on the success of HSV-TK-based gene therapy. On one hand, it may be a disadvantage in that it may protect some transduced cells from being killed. However, it is also possible that the prolonged survival of transduced cells may improve overall tumor killing by permitting prolonged synthesis of toxic metabolites, particularly in tumors that are capable of GJIC. It is likely that the outcome of these competing factors will vary as a function of the responsiveness of the individual tumor and of the extent of GJIC evidenced by the tumor. Note, however, that many cancer cells have defects in GJIC (25–28). In addition, mechanisms other than GJIC, such as in situ retroviral infections (26) or immunological influences (29–31), which were not examined in this cell culture system may operate in vivo. It is possible that enzyme prodrug systems such as the cytosine deaminase/5-flucytosine, in which communication status does not influence the bystander effect and which have no Good Samaritan effect,6 will be superior to the HSV-TK GCV strategy, as has been suggested recently (32).

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REFERENCES


4 Unpublished observations.

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