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Herpes Simplex Virus Thymidine Kinase/Ganciclovir-mediated Apoptotic Death of Bystander Cells[1]

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Abstract

An emerging strategy for cancer gene therapy involves the transfer of the herpes simplex virus thymidine kinase (*HSV-tk*) gene into tumor cells, rendering them susceptible to the cytotoxic effects of ganciclovir. The observation that HSV-tk-expressing cells can also induce cell death in neighboring cells, which do not express HSV-tk, has been called the bystander effect. Gap junction-mediated transfer of cytotoxic molecules to bystander cells may be an important mechanism of bystander cell death, although others have suggested a role for phagocytosis. In this study, we evaluated the mode of cell death in bystander cells. We detected apoptosis in bystander cells and found that bystander cell death could be inhibited by BCL2 expression. We determined that ganciclovir incubations for 10 h were sufficient to induce cell death in most bystander cells cocultured with HSV-tk-expressing cells. During this period, no phagocytosis was detected, although it was obvious at later stages.

Introduction

A novel approach to the treatment of cancer is based on the transfer into tumor cells of nonmammalian genes encoding enzymes that selectively convert nontoxic prodrugs to highly toxic metabolites (1–3). For example, expression of the *HSV-tk*[3] gene in mammalian cells renders them susceptible to the cytotoxic effects of innocuous nucleotide analogues after their phosphorylation by the viral enzyme (4, 5). One widely used such analogue is GCV. Treatment with GCV also leads to the death of cells that do not express the *HSV-tk* gene that are in the immediate vicinity of HSV-tk-expressing cells. This has been called the "bystander effect" (5–8). The bystander effect greatly enhances the efficacy of HSV-tk-mediated cytotoxicity, and a better understanding of bystander cell death may lead to improvements in this approach to gene therapy.

Apoptosis has been suggested as a mechanism by which bystander cell death is mediated (7, 9), although this is controversial (10), and direct evidence of apoptosis in bystander cells has not been demonstrated. Recent evidence indicating that bystander cell death is mediated by gap-junctional intercellular communication (11–13) suggests that GCV, which is phosphorylated in HSV-tk-expressing cells, may be transferred through gap junctions to HSV-tk-negative cells. This mechanism of bystander cell killing is consistent with an apoptotic cell death, which is known to occur after the GCV treatment of HSV-tk-expressing cells. Phagocytosis of material from dying HSV-tk-expressing cells by bystander cells has also been suggested as a mechanism for the bystander effect, although no functional evidence has been presented to date (7, 14). We sought to examine, therefore, the role of apoptosis in bystander cell killing and to evaluate the possibility of phagocytosis mediating this effect.

Materials and Methods

Cell Culture and Cytotoxic Treatment. Cells were cultured as described (11). PA317 cells (15) and C6 rat glioma cells were obtained from the American Type Culture Collection (Rockville, MD). Human glioma cell lines SF126 and SF188 and the rat gliosarcoma cell line 9L were obtained from the Brain Tumor Research Center, University of California, San Francisco. NIH-3T3 cells engineered to express high levels of BCL2 and NIH-3T3 cells transfected with the gene encoding neo resistance have been described (16). PA317 cells express the *HSV-tk* gene (15), rendering them susceptible to the cytotoxic effects of GCV (provided kindly by Syntex, Palo Alto, CA). We used GCV in a final concentration of 10 μM in all experiments.

Cytological Evaluation of Cultured Cells. Staining of subconfluent 9L or PA317 cells with 10 μM CMFDA (Ref. 17; obtained from Molecular Probes, Eugene, OR) was performed in culture medium at 37°C for 15 min. PA317 cells were stained with PKH26 (Sigma Chemical Co., St. Louis, MO) as described previously (11). Stained and unstained cells were plated in four-well glass chamber slides (Nunc, Naperville, IL) at a density of 5×10^4 cells/well, and the cocultures were treated with 10 μM GCV or left untreated. After treatment for 4–72 h, monolayers were fixed with 10% neutral buffered formalin at room temperature for 10 min. The nuclei were stained with 2 μg/ml DAPI (Molecular Probes) at room temperature for 15 min. The slides were mounted with Antifade (Oncor, Gaithersburg, MD) and viewed with a fluorescence microscope (Zeiss Axioplan, Oberkochen, Germany).

Cytological Analysis of FACS-sorted Cocultures. PA317 cells (10^6) were cocultured with 9L cells (10^6) in 10-cm dishes. Either PA317 or 9L cells had been prestained with CMFDA, and the cocultures were treated for 10 h with 10 μM GCV or left untreated. Cells were detached with enzyme-free Cell Dissociation Buffer (Life Technologies, Inc., Gaithersburg, MD) at room temperature and washed with culture medium. Cells (10^6) were resuspended in 1 ml ice-cold PBS containing 2% FBS, 3% Cell Dissociation Buffer, and 5 μg/ml PI (Boehringer Mannheim, Mannheim, Germany) and analyzed on a Dual Laser FACStar Plus (Becton Dickinson, San Jose, CA) equipped with two argon lasers. PI and CMFDA were excited at 488 nm, and emissions were collected through 630/22- and 530/30-nm band pass filters, respectively. Cell viability was determined by PI exclusion, and cocultured PA317 and 9L cells were separated by sorting on the basis of CMFDA staining. Cells were collected in complete culture medium under sterile conditions. Only the sorted cells that had not been stained with CMFDA were analyzed further for growth and endonucleolytic activity. Cells were plated into four-well glass chamber slides (10^4 cells/well), and the cultures were incubated without GCV. After 60 h, the monolayers were rinsed with PBS and fixed in 10% neutral buffered formalin for 10 min. The fixed samples were washed with PBS and analyzed subsequently using a TdT assay (ApopTag fluorescein kit, Oncor) for free 3′-OH ends, which are characteristic of apoptosis (18). Growth of the sorted cells was assessed after 7 days. Monolayers were washed with PBS and fixed

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[3] The abbreviations used are: HSV-tk, herpes simplex virus-thymidine kinase; GCV, ganciclovir; CMFDA, 5-chloromethylfluorescein diacetate; neo, neomycin phosphotransferase; Gy, Gray; TdT, terminal deoxynucleotidyl transferase; PI, propidium iodide; FBS, fetal bovine serum; FACS, fluorescence-activated cell sorting; DAPI, 4′,6-diamidino-2-phenylindole.

with 0.25% crystal violet in 20% methanol at room temperature for 15 min followed by extensive rinsing with tap water.

Southern Blot Analysis. Mouse PA317 and human SF188 glioma cells (10^6 cells/10-cm dish) were treated with 10 μM GCV or irradiated at a dose rate of 3 Gy/min (total dose, 10 Gy) using a Philips RT 250 X-ray machine, respectively. Untreated PA317 cells were used as controls. In coculture experiments, mouse PA317 cells and human SF126 glioma cells were plated at a ratio of 1:1 (10^6 cells/cell line) in 10-cm dishes and treated with 10 μM GCV or left untreated. Total genomic DNA was isolated 2 and 4 days after irradiation and 4 days after GCV treatment and analyzed by agarose gel electrophoresis. Agarose gels were blotted in 0.4 M NaOH and 1.5 M NaCl onto nylon membranes (Amersham, Arlington Heights, IL) for 12 h. Membranes were hybridized with a human specific COT-1 DNA probe (Life Technologies, Inc.) in 5× SSC (1× SSC = 150 mM NaCl and 15 mM sodium citrate), 1% SDS, 1× Denhardt's solution, and 100 μg/ml salmon sperm DNA at 65°C for 12 h. The filters were washed in 0.05× SSC and 0.1% SDS at 65°C for 45 min and exposed to X-ray films for 48 h.

Analysis of BCL2-expressing Cells. Four NIH-3T3 cell clones transfected with and expressing human BCL2 (nos. 10, 20, 27, and 30) (16) and two pools of NIH-3T3 cells transfected with recombinant plasmid DNA encoding the neo-resistance gene (neo 1 and neo 2) were analyzed for their susceptibility to bystander killing in cocultures with HSV-tk-expressing cells. Each cell line was analyzed in quadruplicate in cocultures, with the HSV-tk-expressing cell line PA317 plated at a ratio of 1:1 in 24-well plates. Cells were counted 1 day after plating (day 0), at which time each of the wells contained about 1×10^5 cells. The mean number of cells from the four wells for an individual cell line on day 0 was defined arbitrarily as 100%. Treatment with 10 μM GCV was initiated on day 0, and quadruplicate samples were evaluated for viable cells by trypan blue exclusion after 2, 4, and 6 days of incubation. The fraction of surviving cells after GCV treatment was calculated by dividing the number of cells after treatment by the number of cells for an individual cell line on day 0, representing 100%. Statistical analysis included one-way ANOVA followed by Student Newman Keuls test (19). Expression levels of human BCL2 protein were determined by Western blot analysis as described (16) using an anti-human BCL2 monoclonal antibody (provided kindly by Dr. D. Delia, Instituto Nazionale dei Tumori, Milan, Italy).

Results

We[4] and others have reported recently that GCV treatment of HSV-tk-expressing cell lines induced apoptosis as determined by oligonucleosomal DNA laddering, nuclear morphological alterations, and endonucleolytic activity (TdT assay; Refs. 7, 14). To determine whether bystander cells, which do not express the *HSV-tk* gene, undergo apoptosis when cocultured with HSV-tk-expressing cells, we examined the morphology of such cells in cocultures after treatment with GCV. In these experiments, 3T3 mouse fibroblasts expressing HSV-tk (PA317 cells) and a rat glioma cell line, 9L, were examined, because these cell lines were critical in the development of this gene therapy strategy for gliomas (e.g., Ref. 6). To differentiate bystander cells (9L) from HSV-tk-expressing PA317 cells, 9L cells were prestained with CMFDA so as to have a characteristic green fluorescence (Fig. 1, *D–F*). In the absence of GCV, no nuclear alterations suggestive of apoptosis could be observed in the nuclei of 9L or PA317 cells (Fig. 1*A*). After incubation of the cocultures in 10 μM GCV for 36 h, highly condensed and partly fragmented nuclei could be observed in close proximity to unaltered nuclei (Fig. 1*B*). Evaluation of the same microscopic field for CMFDA fluorescence (Fig. 1*E*) revealed two important findings: (*a*) few CMFDA-stained 9L cells revealed nuclei with apoptotic changes; and (*b*) unstained cytoplasmic regions that appeared as punched-out sites could be seen in CMFDA-stained cells, and their location corresponded closely to that of apoptotic nuclei identified in the same field by DAPI staining.

The apoptotic nuclei may represent apoptotic bodies derived from PA317 cells that were phagocytosed by 9L cells. The unstained cytoplasmic regions in CMFDA-stained 9L cells were first observed 12 h after treatment with GCV and were most prevalent after 36 h of incubation in GCV (Fig. 1, *B* and *E*). After GCV treatment of the cocultures for more than 36 h, these circular defects were detected less frequently in 9L cells. Concomitant with the apparent decreased frequency of occurrence of these lesions, we observed an increase in the number of structures showing bright green fluorescence (Fig. 1*F*). These circular structures were observed commonly in cells showing green fluorescence of less intensity (Fig. 1*F;* analysis after 72 h of GCV), and some of these structures contained condensed and fragmented nuclei (Fig. 1*C;* analysis after 72 h of GCV). Such structures may represent CMFDA-stained 9L cells that are undergoing apoptosis and that have been phagocytosed by adjacent CMFDA-stained 9L cells.

Control experiments in which PA317 cells were stained with CMFDA revealed the appearance of condensed structures with bright green fluorescence after treatment with GCV for at least 12 h, but no dark punched-out defects similar to those described above could be observed (data not shown). DAPI staining indicated that these bright-green bodies contained apoptotic nuclei that were indistinguishable from the apoptotic nuclei localized in the dark cytoplasmic regions of CMFDA-stained 9L cells (data not shown). These green bodies with apoptotic nuclei were found in CMFDA-unstained cells (9L) but not in CMFDA-stained cells (PA317). Similarly, CMFDA labeling of both PA317 and 9L cells did not reveal such dark, punched-out regions (data not shown). Instead, only small bodies of bright, green fluorescence, containing highly condensed and partly fragmented nuclei, were observed. These data suggest that HSV-tk-expressing cells (PA317), after being killed by GCV, may become phagocytosed by bystander cells (9L). This is followed by the death of some bystander cells, which themselves can be phagocytosed by other bystander cells.

To evaluate further whether the apoptotic nuclei that were observed in close proximity to the unaltered nuclei of bystander cells (Fig. 1, *B* and *C*) resulted from phagocytosis (and do not reflect experimental artifacts, *e.g.,* superimposition), we analyzed cocultures in which each of the two cell lines had been prestained with different fluorescent dyes. We used PKH26 and CMFDA to stain PA317 (HSV-tk$^+$) and 9L cells (HSV-tk$^-$), respectively. PKH26 and CMFDA, which are fluorescent dyes not transferred between adjacent cells, stain the plasma membrane and cytoplasm, respectively. Using such an approach, PKH26-stained PA317 cells (Fig. 1*J*) could be distinguished easily from CMFDA-stained 9L cells (Fig. 1*H*) when these cultures were incubated without GCV. The DAPI-stained nuclei of these cells are shown in Fig. 1*G* and appear normal. After treatment with 10 μM GCV for 36 h, we observed cells containing condensed nuclei (Fig. 1*K, arrows*) adjacent to unaltered nuclei, similar to the pattern observed in Fig. 1*B*. In contrast to untreated cocultures, most cells revealed fluorescence from both PKH26 and CMFDA after GCV treatment (Fig. 1, *L* and *M,* respectively). The PKH26-CMFDA double fluorescence of cells after treatment with GCV suggests that cellular and nuclear material from the PKH26-stained PA317 cells (HSV-tk$^+$), which all go on to die under these conditions, was phagocytosed by CMFDA-stained 9L cells (HSV-tk$^-$). Reverse staining (CMFDA staining of PA317 cells and PKH26 staining of 9L cells) was also characterized by cells exhibiting double fluorescence after incubation with GCV (data not shown). Double staining of cells was not apparent before 12 h of GCV treatment (not shown). Similar results were obtained with C6 rat glioma cells and two different human glioma cell lines (SF126 and SF210) cocultured with PA317 cells in the presence of GCV (data not shown). These data indicate

[4] W. Hamel, P. Dazin, and M. A. Israel. Adaptation of a simple flow cytometric assay to identify different stages during apoptosis, submitted for publication.

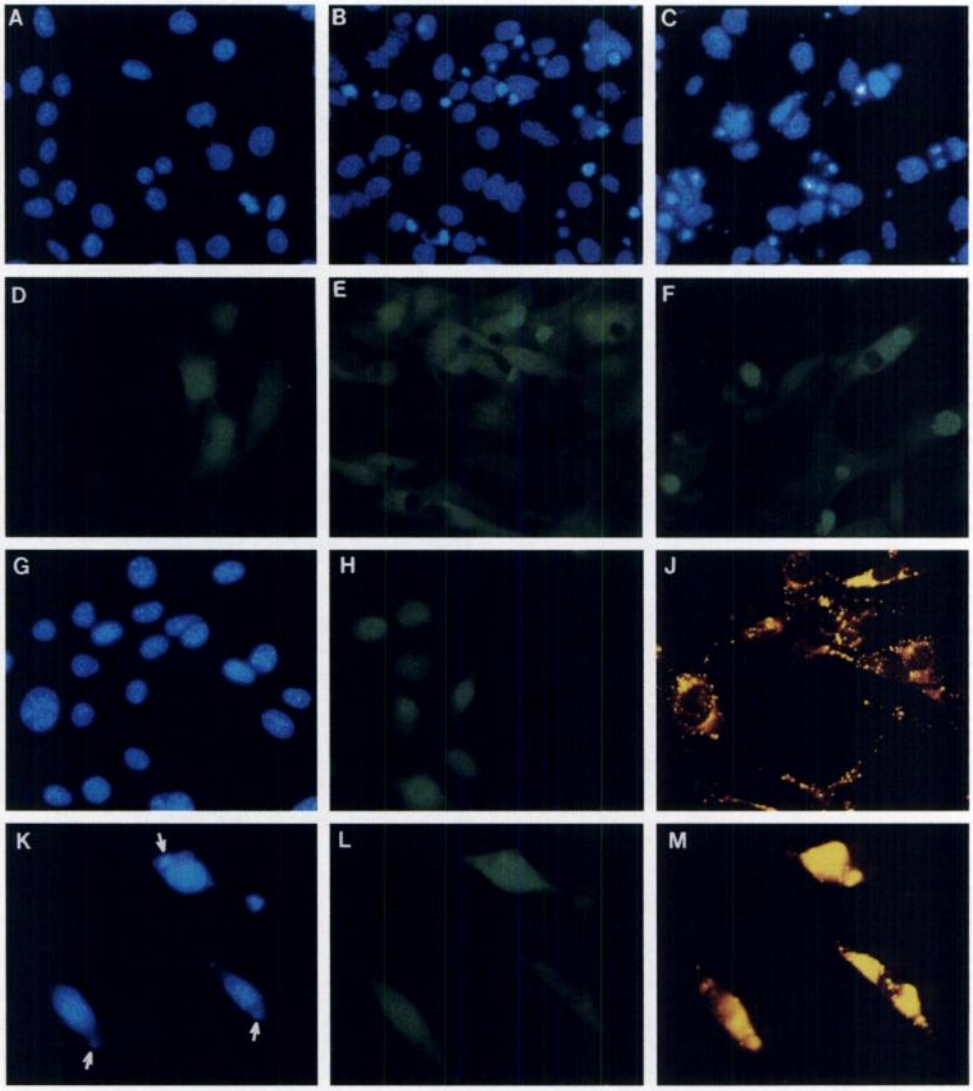

Fig. 1. *In situ* analysis of bystander killing in cocultured 9L and PA317 cells. *A–F*, cocultures of CMFDA-stained 9L cells and unstained PA317 cells. For morphological analysis, the nuclei of untreated (*A*) and GCV-treated cocultures [10 μM GCV for 36 (*B*) or 72 (*C*) h] were stained with DAPI. Green CMFDA fluorescence of untreated (*D*) or GCV-treated (*E* and *F*) cultures is shown below. *D–F*, the same microscopic fields as in *A–C*, respectively. *G–M*, cocultures of CMFDA-stained 9L cells and PKH26-stained PA317 cells. Orange fluorescence (PKH26) of untreated (*J*) and GCV-treated (*M*) cocultures (10 μM GCV for 36 h) and green fluorescence (CMFDA) of the same untreated (*H*) or GCV-treated (*L*) microscopic fields are shown. Nuclear staining of the same cells with DAPI (blue fluorescence) is shown in *G* and *K*, respectively. The *arrows* in *K* indicate extranuclear DNA stained with DAPI. Pictures were taken with ×400 magnification using a 40×/0,75 Zeiss Plan Neofluar objective.

that bystander killing is associated with marked phagocytosis of cellular (cytoplasm and plasma membrane) and nuclear material.

Although our data suggest strongly that bystander cells undergo apoptosis (Fig. 1, *C* and *F*), the nuclear alterations described above cannot be attributed unequivocally to bystander cells. To provide direct evidence that bystander cells undergo apoptosis, we designed an experiment in which the apoptotic response was evaluated in HSV-tk-expressing cells and bystander cells separately. To perform this analysis, we separated PA317 and 9L cells that had been cocultured in the absence or presence of 10 μM GCV for 10 h by FACS on the basis of prestaining with CMFDA, as described in "Materials and Methods." After sorting, the cells were plated in chamber slides containing GCV-free culture media. Because phagocytosis could not be observed until at least 12 h after the initiation of GCV treatment in cocultures of PA317 and 9L cells (see above), these two cell types were separated easily after 10 h of coincubation. Such incubation periods proved to be sufficient for the induction of cell death in HSV-tk-expressing cells as well as bystander cells, and we have routinely observed extensive bystander killing of 9L cells by PA317 cells after treatment with GCV for only 8 h (data not shown). Immediately after the sorted cells had attached, control slides were analyzed by fluorescence microscopy for the purity of the sorted populations and for evidence of phagocytosis. This revealed that the preparation of unstained cells that were used for further analysis contained less than 5% CMFDA-stained cells. Despite extensive morphological evaluation of CMFDA-stained and -unstained cells, no evidence of phagocytosis could be observed.

These sorted cells were cultured for 60 h without GCV, at which time we analyzed 9L cells (HSV-tk$^-$) for the presence of free 3′-OH groups, which are characteristic of apoptosis (18). As shown in Fig. 2*B*, both 9L and PA317 cells exhibited evidence of endonucleolytic activity after coculture in the presence of GCV for 10 h. No detectable activity was observed in cells from untreated cocultures (Fig. 2*B*). Nuclear counterstaining of the same cells with DAPI revealed nuclear condensation and fragmentation in PA317 cells (HSV-tk$^+$) and 9L bystander cells (HSV-tk$^-$) from the GCV-treated cocultures, whereas the nuclei from untreated cocultures appeared normal (data not shown). These data provide evidence that HSV-tk$^-$ cells that are exposed to HSV-tk-expressing cells and GCV for short incubations go on to die by apoptosis.

To confirm that GCV incubation for 10 h had cytotoxic effects on HSV-tk$^+$ (PA317) and HSV-tk$^-$ (9L) cells, we assessed the growth potential of these cells after they were sorted from cocultures that had been treated with GCV for 10 h, as described above. A chamber slide containing sorted cells was cultured for 7 days without GCV and fixed with crystal violet. As shown in Fig. 2*A*, PA317 and 9L cells sorted from GCV-treated cocultures both exhibited drastically reduced growth compared to cells sorted from cocultures of untreated controls.

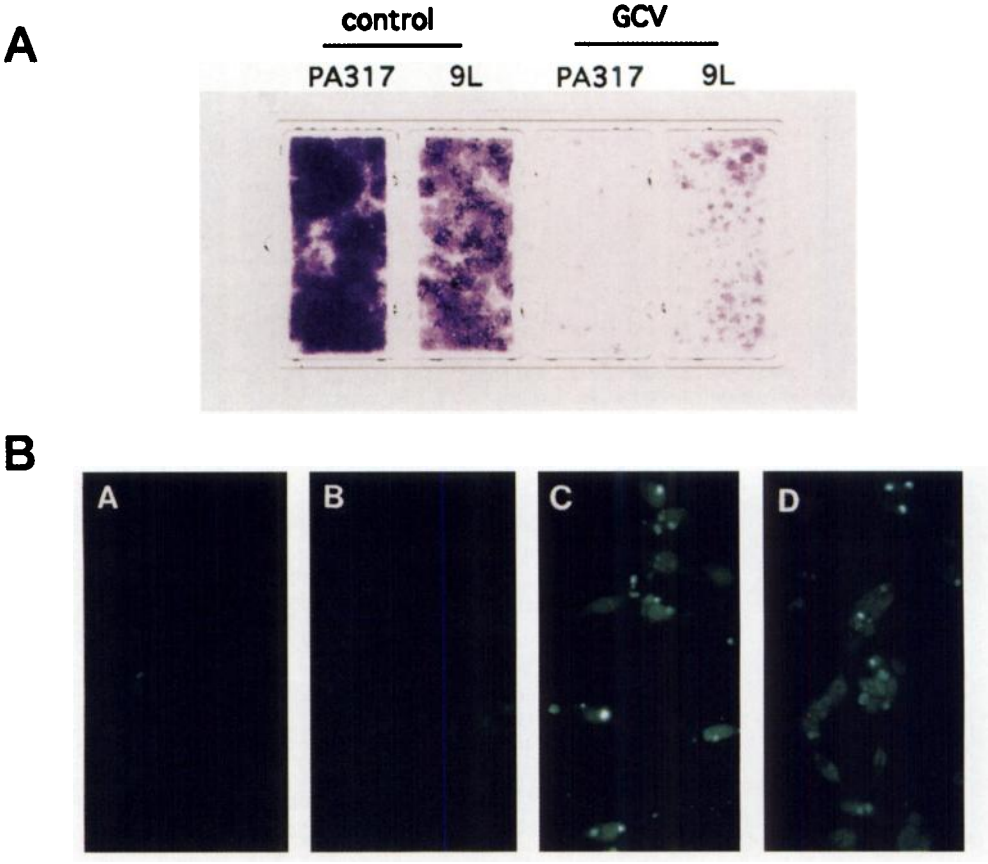

Fig. 2. Induction of apoptosis in bystander cells detected after short incubation with GCV followed by cell sorting. PA317 and 9L cells were cocultured in the absence or presence of 10 μM GCV for 10 h. Both cell lines were separated by flow-cytometric sorting as described in "Materials and Methods." The sorted cells were plated in glass chamber slides (1×10^4 cells/well) without further addition of GCV. After 7 days, the monolayers derived from sorts of untreated (*control*) or GCV-treated (*GCV*) cells were fixed with crystal violet (*A*). Cells were evaluated for free 3'-OH groups with the TdT assay 2.5 days after sorting (*B*).

This indicates that both the HSV-tk-expressing PA317 cells and HSV-tk-negative 9L cells, which served as bystander cells, are susceptible to cell death after GCV treatment for only 10 h. Nevertheless, among the 9L cells cocultured with PA317 cells in the presence of GCV, a number of resistant colonies are obvious (Fig. 2*A*), suggesting that more efficient bystander killing may require longer GCV incubation.

To characterize further the cell death of bystander cells, we sought DNA laddering characteristic of apoptotic cell death in human tumor cells used as bystander cells. Southern blots containing genomic DNA were probed with a human species-specific probe under high stringency conditions. As shown in Fig. 3, genomic DNA from mouse PA317 cells, which served as a negative control, could not be detected with this probe under high stringency conditions. In contrast, we observed DNA laddering in genomic DNA isolated from human SF188 glioma cells 2 and 4 days after irradiation with 10 Gy (Fig. 3), indicating that this probe only detects human DNA under such conditions. Analysis of genomic DNA derived from cocultures of mouse HSV-tk-expressing PA317 cells and the human glioma cell line SF126, used as bystander cells, revealed DNA laddering after GCV treatment, suggesting that the human bystander cells undergo apoptosis (Fig. 3). Only high molecular weight DNA could be detected in untreated control cocultures of PA317 and SF126 cells (Fig. 3). We obtained similar results with two additional human glioma cell lines tested in this manner (data not shown).

To determine whether apoptosis in bystander cells is mediated by a pathway inhibited by BCL2, we analyzed 3T3 cell lines transfected with and constitutively overexpressing BCL2 (Fig. 4*B*) for their susceptibility to bystander killing when cocultured with isogenic PA317 cells (HSV-tk$^+$) in the presence of GCV. Preliminary experiments indicated that, like all other bystander cell killing we examined, 3T3 cells in these cocultures die by apoptosis (data not shown). Therefore, we determined the number of viable cells in such cocultures after 2, 4, and 6 days of GCV treatment by trypan blue exclusion, and viability percentage was calculated based on the number of cells present at the initiation of GCV treatment (Fig. 4*A*). After 6 days of incubation in GCV, all four BCL2-transfected cell clones (clones 10, 20, 27, and 30) revealed significantly ($P < 0.01$) reduced bystander killing compared to two neo-transfected 3T3 cell pools not expressing human BCL2 (Fig. 4*B*). Interestingly, clones 10 and 20, which were the least susceptible to bystander killing (Fig. 4*A*), showed the highest expression of BCL2 protein (Fig. 4*B*).

Discussion

We have demonstrated that cell death in bystander cells cocultured with HSV-tk$^+$ cells in the presence of GCV is mediated by apoptosis. Our data also indicate that cell death in HSV-tk-expressing cells and bystander cells can be induced readily by short incubations with GCV. This is consistent with the observation that the bystander effect is correlated with HSV-tk enzyme activity (20) and gap-junctional intercellular communication (11–13) that may mediate the transfer of metabolites such as phosphorylated GCV from HSV-tk-expressing cells to bystander cells. This is of importance, because systemic side effects have been observed in conjunction with GCV treatment in some animal models (*e.g.*, Refs. 6, 21). Our data suggest that modified regimens (*e.g.*, shorter or fractionated GCV administration schedules) should be evaluated for comparable antitumor efficacy with reduced toxicity.

Phagocytosis of material from dying HSV-tk-expressing cells by bystander cells has been suggested as a mechanism for the bystander effect (7, 14). We observed extensive phagocytosis in conjunction with bystander killing, but phagocytosis occurred after apoptosis had been induced irreversibly in a high proportion of bystander cells. In

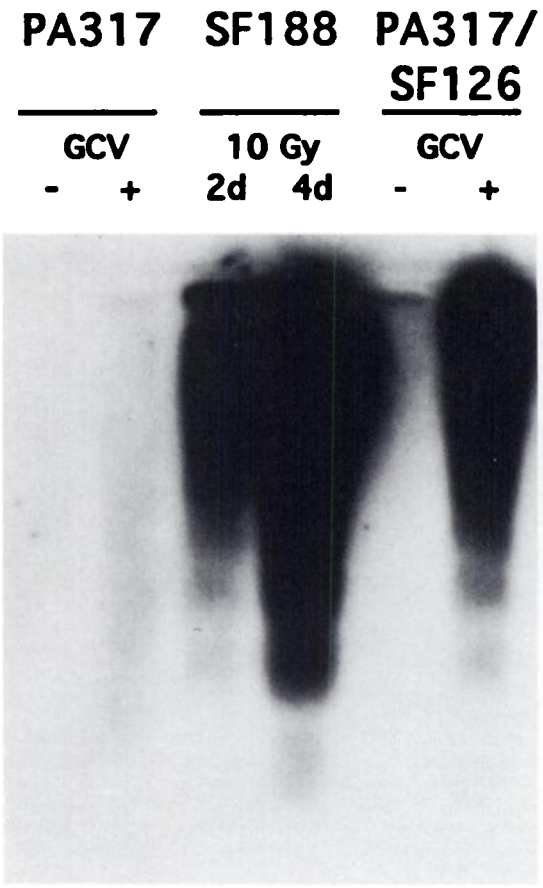

Fig. 3. Southern blot analysis of bystander killing. Southern blots containing undigested genomic DNA were probed with a human species-specific probe (COT-1 DNA) and subsequently washed under high-stringency conditions. Mouse PA317 cells served as a negative control. Human glioma cell line, SF188, which showed DNA laddering when analyzed 2 and 4 days after irradiation with 10 Gy, served as a positive control. Cocultures of mouse PA317 cells and human glioma cell line, SF126, were analyzed for induction of DNA laddering in the human bystander cells after culturing in the absence (−) or presence (+) of 10 μM GCV for 4 days.

addition, mechanical separation of GCV-treated cocultures of HSV-tk-expressing cells and bystander cells before the onset of phagocytosis did not prevent bystander cell death. Other data from our laboratory indicate that PA317 cells (HSV-tk$^+$) preincubated in GCV for 2–12 h had a diminished capacity to induce cell death in subsequently added bystander cells, although phagocytosis still occurred in such experiments.[5] It therefore appears unlikely that phagocytosis contributes significantly to the events leading to bystander cell death, although phagocytosis is found frequently in association with apoptosis (22). These findings, furthermore, suggest that the efficacy of bystander cell killing correlates with the viability of HSV-tk-expressing cells. We found that apoptosis occurring in bystander cells during the course of HSV-tk-mediated GCV cytotoxicity involves a pathway that can be inhibited by BCL2. Thus, efforts to enhance the bystander effect should include modifications in HSV-tk-expressing cells that may exert enhanced bystander cell killing if their own death by GCV is delayed. This might be achieved by the expression of *BCL2* or other antiapoptotic genes, although such strategies could increase the biohazards associated with cellular-based therapies.

The protective role of BCL2 in HSV-tk/GCV-mediated cell death suggests that BCL2 and perhaps other gene products known to inhibit apoptosis, such as BCLX$_L$, influence the efficacy of this treatment strategy as do other biological variables, such as gap-junctional intercellular communication (11–13), HSV-tk enzymatic activity (20), the distribution of HSV-tk-positive cells in the tumor bed, or the specific nucleotide analogue administered. Also, tumors that express high levels of BCL2 might be more resistant to this form of therapy than other tumors in which apoptosis can be induced readily. Optimization of this gene therapy strategy might, therefore, also include the modulation of tumor genes such as *BCL2* that inhibit apoptotic cell death (23). In addition, it is possible that combining HSV-tk-based gene therapy with other antineoplastic therapies that induce apoptosis, such as chemotherapy and irradiation (24), may lead to synergistic antineoplastic activity, because all appear to activate a final common pathway (22, 25).

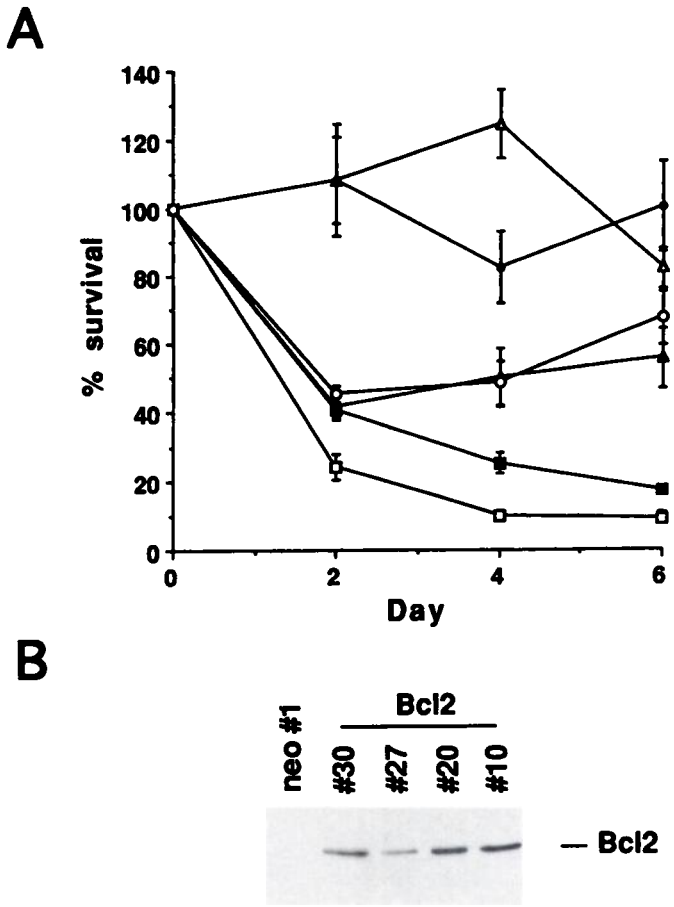

Fig. 4. Analysis of BCL2-transfected 3T3 cells for their susceptibility to bystander killing. *A*, clonal derivatives of NIH-3T3 cells transfected with the human *BCL2* gene, #10 (△), #20 (●), #27 (▲), #30 (○), and two pools of neo-transfected NIH-3T3 cells, neo 1 (□) and neo 2 (■), were analyzed for their susceptibility to bystander killing. Each cell line was analyzed in quadruplicate in cocultures with the HSV-tk-expressing 3T3-derived cell line PA317. All cell lines were seeded at a ratio of 1:1 in 24-well plates as described in "Materials and Methods." Cells were counted 1 day after plating (day 0), at which time each of the wells contained about 1×10^5 cells. The mean number of cells for an individual cell line on day 0 represents 100%. Treatment with 10 μM GCV was initiated on day 0, and the cells were counted in quadruplicates by trypan blue exclusion after 2, 4, and 6 days. The percentage of surviving cells after GCV treatment was calculated and is depicted in the graph. *Bars*, SE. On day 6, all four BCL2-transfected clones (clones 10, 20, 27, and 30) differed significantly from both neo-transfected pools (neo 1 and neo 2; $P < 0.01$). The experiment depicted is representative of three independent experiments. *B*, Western blot analysis of human BCL2-transfected NIH-3T3 cell clones (clones 10, 20, 27, and 30) and a pool of neo-resistance-transfected NIH-3T3 cells (neo 1). Twenty μg of total cell lysate were loaded per *lane*, and BCL2 protein expression was evaluated by immunoblot analysis and detected with an enhanced chemiluminescence system (Amersham, Little Chalfont, United Kingdom) as described in "Materials and Methods."

[5] W. Hamel, and M. A. Israel, unpublished data.

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