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Pharmacological Modulation of the Bystander Effect in the Herpes Simplex Virus Thymidine Kinase/Ganciclovir Gene Therapy System

EFFECTS OF DIBUTYRYL ADENOSINE 3′,5′-CYCLIC MONOPHOSPHATE, α-GLYCYRRHETINIC ACID, AND CYTOSINE ARABINOSIDE

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ABSTRACT. The herpes simplex virus type 1 thymidine kinase (HSV1-*tk*) suicide gene/ganciclovir system was first applied to the treatment of glioblastoma tumors, but was hampered by the low gene transfection yield. Fortunately, the gap junction-dependent diffusion of phosphorylated ganciclovir metabolites from transfected cells to their neighbors proved to enhance the overall benefit of this strategy. However, as tumor cells are often gap junction-deficient, we sought to restore this property pharmacologically and hence to improve the efficacy of the treatment. We demonstrated that this approach was feasible in glioblastoma cells using dibutyryl adenosine 3′,5′-cyclic monophosphate (cAMP) (100 μM) as a pharmacological inducer of gap junctions. α-Glycyrrhetinic acid (25 μM), on the other hand, strongly inhibited both gap junction-mediated intercellular communication and the bystander effect, thus confirming the role of gap junctions in HSV-*tk*-mediated bystander killing. Using cytosine arabinoside as a growth inhibitor, we underlined the role of tumor cell proliferation in the sensitivity of HSV-*tk*-transfected cells to ganciclovir and demonstrated its correlation with the importance of the bystander effect. BIOCHEM PHARMACOL **60**;2:241–249, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. thymidine kinase; gap junctions; bystander effect; cyclic AMP

The HSV¶-*tk* suicide gene/GCV system was first described by Culver *et al.* [1] for the treatment of experimental brain tumors. Following transfection with the HSV-*tk* gene, tumor cells are able to metabolize GCV into a monophosphate derivative, which then is phosphorylated further by cellular kinases into GCV triphosphate. This metabolite is incorporated into replicating DNA strands and acts as both a DNA synthesis inhibitor and cell cycle blocker [2, 3], eventually leading to apoptotic cell death [3, 4]. One major limitation of this strategy is the low *in vivo* efficiency of current gene delivery systems. Interestingly, however, HSV-*tk* gene-transfected cells induce the death of untransfected neighboring cells, a phenomenon called the bystander effect [1, 5]. Different mechanisms have been proposed to explain this effect: (i) the overall immunogenicity of transfected tumor cells increases [6–9]; (ii) the accidental HSV-*tk* transfection of endothelial cells can lead to thrombosis of tumor vessels and hence to ischemic tumoral damage [10]; and (iii) dying cells could release diffusible factors transmitting a cell death signal [11–13]. Another mechanism that has received much experimental support is the diffusion of phosphorylated GCV from HSV-*tk*-positive cells to HSV-*tk*-negative adjacent cells through GJIC [8, 11, 14–16]. This phenomenon could explain the eradication of tumors wherein only 10% of the cells expressed the HSV-*tk* gene [5, 17]. Unfortunately, tumor cells frequently exhibit a reduced level of GJIC [18].

Gap junctions are made up of six protein subunits known as connexins, which are organized in a ring-like structure or connexon [19]. Normal astrocytes express two types of connexins, named, according to their molecular weight, Cx43 and the recently described and poorly characterized Cx30 [20]. Attempts have been made to increase GJIC by acting on connexin gene expression. Dilber *et al.* [15] transfected C6 glioblastoma cells with the Cx43 gene and increased both the GJIC capacity of the cells and the bystander effect following HSV-*tk*/GCV treatment. Although these experiments supported the implication of

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¶ *Abbreviations*: HSV, herpes simplex virus; GCV, ganciclovir; tk, thymidine kinase; AGA, α-glycyrrhetininc acid; Ara-C, cytosine arabinoside; cAMP, adenosine 3′,5′-cyclic monophosphate; GJIC, gap junctional intercellular communication; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; and MTT, dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide.

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connexins in the bystander effect, the application of such a strategy to enhance the therapeutic effect *in vivo* faced two limitations: the low *in vivo* gene transfection rate and the difficulty of transfecting tumoral cells located away from the center of the tumor and/or within the surrounding normal brain tissue. Several authors have considered the possibility of pharmacologically modifying the GJIC capacity of both normal and tumor cells (for a review, see Trosko *et al.* [18]). Few studies have actually addressed the specific issue of pharmacological modulation of the bystander effect [21–23]. We, therefore, sought to determine whether the pharmacological enhancement of the GJIC could lead to an increased bystander effect in glioblastoma cells with the HSV-*tk*/GCV model of gene therapy.

In this study, we used dibutyryl-cAMP as a gap junction inducer and demonstrated that such an approach was effective in glioblastoma cells. We also showed that dibutyryl-cAMP acted on Cx43 protein expression. We further confirmed the implication of gap junctions in the bystander effect by preventing it with AGA, a gap junction blocker. Finally, we showed that Ara-C, a cell growth inhibitor, diminished tumor cell sensitivity to phosphorylated GCV and simultaneously inhibited the bystander effect without influencing the GJIC of the cells.

MATERIALS AND METHODS

Transfection of C6 Cells and Selection of the C6-TK5 Clone

The HSV-*tk* gene was inserted downstream of the cytomegalovirus (CMV) promoter in a pcDNA3 plasmid (In Vitrogen) containing the Neo®ORF (open reading frame) neomycin resistance gene driven by a simian virus (SV40) promoter (pCMV-*tk*). Rat C6 glioma cells were transfected with a *Bgl*II-linearized pCMV-*tk* plasmid, using 1,2-dioleoyloxy-3(trimethylammonium) propane as recommended by the manufacturer (Boehringer Mannheim). Transfected cells were selected with 0.5 mg/mL of G418 in DMEM supplemented with 3% FBS, and grown for 3 weeks. Then clones were isolated and grown in the same medium (DMEM–FBS–G418). GCV sensitivity tests were performed twice on aliquots of each cell clone, and the C6-TK5 clone was selected for further experiments.

Assessment of C6-TK5 Sensitivity to GCV

C6-TK5 cells were grown in 24-well plates at a density of 30,000 cells/well in DMEM–FBS (3%) containing various concentrations of GCV. The media were changed every 2 days for 5 days, and cells were counted in a Thomas cytometer.

Cell Culture and Assessment of the Bystander Effect

Various proportions of C6 and C6-TK5 cells were seeded at a total density of 10,000 cells/well in 96-well plates (Nunc). Three wells of each cell mix were subjected to each of the following conditions: control; 2 μM GCV; assessed drug; assessed drug supplemented with 2 μM GCV. Media were changed every 2 days for 5 days. Then the plates were processed with the Abacus® acid phosphatase cell proliferation assay according to the manufacturer's instructions. Due to a discontinuation of this product by the manufacturer at the end of this study, in some experiments, i.e. *n*-butyrate control tests, we used the MTT cell survival assay [24], which yielded results similar to those of the Abacus® test. Briefly, 5 μL of fresh MTT solution was added to the culture medium for 2 hr. Then the MTT precipitates were dissolved in ethanol, and the absorbance values were read on a Bio-Tek EL309 microplate autoreader at a 570 nm wavelength (reference: 650 nm). All experiments were run at least in triplicate before statistical analysis.

Millicel® Co-cultures

Thirty thousand C6-TK5 cells were seeded in a 24-well plate and allowed to attach for 1 hr. Laminin-coated (10 μg/mL) Millicel® culture inserts then were laid in these wells, and an equal number of C6 cells was seeded on these inserts. Thus, the two cell types were grown in two physically distinct compartments but in the same medium. Control and GCV (2 μM)-containing media were changed every 2 days for 5 days. C6 cells then were rinsed in PBS, trypsinized, and counted in a Thomas cytometer. The experiments were run twice in duplicate.

Communication Assay

GJIC was assessed by microinjection of Lucifer Yellow according to Stewart [25], with slight modifications. Briefly, cells were harvested in culture medium and seeded at a low density on polyornithine-coated glass coverslips. Twenty-four hours later, the coverslips were flooded in test or control medium, and the cells were allowed to grow to confluence for 5–6 days. Media were changed every other day. Then the coverslips were placed in the perfusion chamber of a Zeiss fluorescence microscope and perfused with EA01 buffer (137 mM NaCl, 5.7 mM KCl, 1.8 mM $CaCl_2$, 22.2 mM d-glucose, 10 mM HEPES). Cells in confluent areas were injected with Lucifer Yellow dye [5% (w/v) in 0.1 M LiCl] bypassing hyperpolarizing current pulses (0.5 Hz, 500 msec, 1 mA) for 30 sec through the electrode. Impalement was considered effective when the membrane potential remained negative throughout the injection period and if a single cell was stained by passive dye diffusion before current application. The total number of neighboring cells marked with Lucifer Yellow was counted 60 sec after the end of the injection and served as a measure of GJIC [26]. Photographs were taken on Kodak T-Max CN 400 films with a Minolta 9000 camera.

Northern Blot Analysis

Cells (C6 and C6-TK5) were grown to subconfluence in Falcon T75 flasks for 3 days and then were treated for 48 hr with the different drugs in DMEM–FBS (3%). Cells were harvested in PBS, centrifuged, resuspended in Tri-Pure® isolation reagent (Boehringer Mannheim), and processed as recommended by the manufacturer. Total RNA was dissolved in diethyl pyrocarbonate-treated water and kept at −20° until used for northern blotting. Total RNA (5 μg for Cx43 analysis and 20 μg for TK analysis) was separated by electrophoresis on denaturing formaldehyde–1% agarose gels. Gels were capillary-blotted onto Qiabran Nylon plus membrane (Qiagen). [^{32}P]dATP-radiolabeled cDNA probes to rat Cx43 and to HSV-*tk* were prepared with the random-primed DNA-labeling kit (Boehringer Mannheim), and a [^{32}P]dATP-radiolabeled oligonucleotide probe to human ribosomal RNA 28S subunit was prepared as recommended by the manufacturer (Pharmacia Biotech). Hybridizations were carried out at 68° for 1 hr with Express Hybridization (Clontech) solution as recommended by the manufacturer. After washing, the blots were exposed to Fuji Medical x-ray film at −80°, scanned on a Sharp JX-330 scanner, and analyzed with ImageMaster1D® software (Pharmacia).

Protein Extraction and Western Blot Analysis

Protein extracts were prepared as described by Hayashi *et al.* [27], with slight modifications. Cells were scraped and homogenized in extraction solution (1% SDS, 2 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetoamide, 10 μg/mL of ovomucoid, 1 mM leupeptin, 0.1 mM sodium orthovanadate, 5 mM NaF), heat-denatured, and passed several times through a 27 G needle to disrupt DNA clots. Protein concentrations were measured according to the method of Lowry [28]. The extracts were loaded on 12.5% polyacrylamide gels (PhastGel®, Pharmacia), separated by electrophoresis, electrically transferred onto polyvinylidene difluoride membranes (Boehringer Mannheim), and processed for immunolabeling. Aspecific binding was blocked with Tris-buffered saline (TBS) supplemented with 0.05% Tween 20 and 3% milk powder for 45 min (blocking buffer). A rabbit polyclonal antibody to Cx43 (Zymed, 2 μg/mL in blocking buffer) [29] was then applied for 2 hr at 37°. After several washes, membranes were exposed to a biotinylated goat secondary antibody to rabbit immunoglobulin (Dakopats, 1:500 in blocking buffer), rinsed, and incubated with a streptavidin–peroxidase complex (Boehringer Mannheim, 1:40,000 in TBS–0.05% Tween 20). Revelation of the immunoreactive bands was performed with a mixture of dioctylsulfosuccinate and tetramethylbenzidine in acetate (50 mM)–perborate (0.02%, w:v) buffer, pH 6.0. After drying, the membranes were scanned on a Sharp JX-330 scanner and analyzed with the ImageMaster1D® software (Pharmacia). Western blots were also performed on 10% polyacrylamide gels, incubated with the same polyclonal anti-Cx43 rabbit antibody, and processed with a secondary antibody coupled to horseradish peroxidase (Santa Cruz), and revelation was carried out with a chemoluminescence system (Santa Cruz) according to the manufacturer's instructions.

Statistical Analysis

All statistical analyses were performed with the commercial GraphPad Prism® and GraphPad InStat® programs. The bystander curves were compared with two-way ANOVA. Dye microinjection experiments were compared with one-way ANOVA and Tukey's post tests, except for those experiments concerning cells injected after 48 hr of culture, which were compared with Student's *t*-test, as they concerned only two sets of data at a time. The effect of the different drugs on C6 cell sensitivity to GCV and the gel band densities (western and northern blots) were also analyzed with Student's *t*-test.

RESULTS

Characterization of the HSV-tk-Expressing Cell Clone (C6-TK5)

The HSV-*tk* gene was stably transfected in C6 glioma cells as described in Materials and Methods. Several neomycin-resistant clones were obtained, and the most GCV-sensitive one (C6-TK5) was selected for further experiments. The IC_{50} (GCV) of the C6-TK5 cells was in the nanomolar range, i.e. 2×10^4 times lower than that of the parental C6 cells (Fig. 1a). Expression of the HSV-*tk* gene was confirmed in the C6-TK5 clone by northern blot analysis (Fig. 1b). Whereas no signal was observed in untransfected cells, a strong spot was observed in the C6-TK5 extract at 1.3 kbp, the expected HSV-*tk* mRNA length. The C6-TK5 growth rate was assessed 3 and 5 days after seeding and found to be similar to that of the native C6 cells (Fig. 1c). No difference was found between the GJIC capacity of C6 and C6-TK5 cells, as assessed by Lucifer Yellow dye transfer (Fig. 1d).

Modulation of GJIC

According to the literature, C6 cells are characterized by a low, yet detectable level of GJIC [30, 31]. Our results on short-term cultures (i.e. 5 days) confirmed this finding. The injection of Lucifer Yellow into one C6 cell led to the diffusion of the dye into a mean of 10 neighboring cells (1:10) in 1 min. In contrast, dye injected into normal astrocytes diffused into a mean of 52 adjacent cells under similar conditions (data not shown). Thus, C6 cells displayed a reduced GJIC capability. Hence, the study of both positive and negative GJIC modulators could theoretically be performed on these cells (Figs. 2 and 3, upper left panel). Dibutyryl-cAMP at a concentration of 100 μM significantly increased ($P < 0.01$, Figs. 2 and 3, upper right panel) the GJIC of C6 cells after 5 days of treatment, this effect

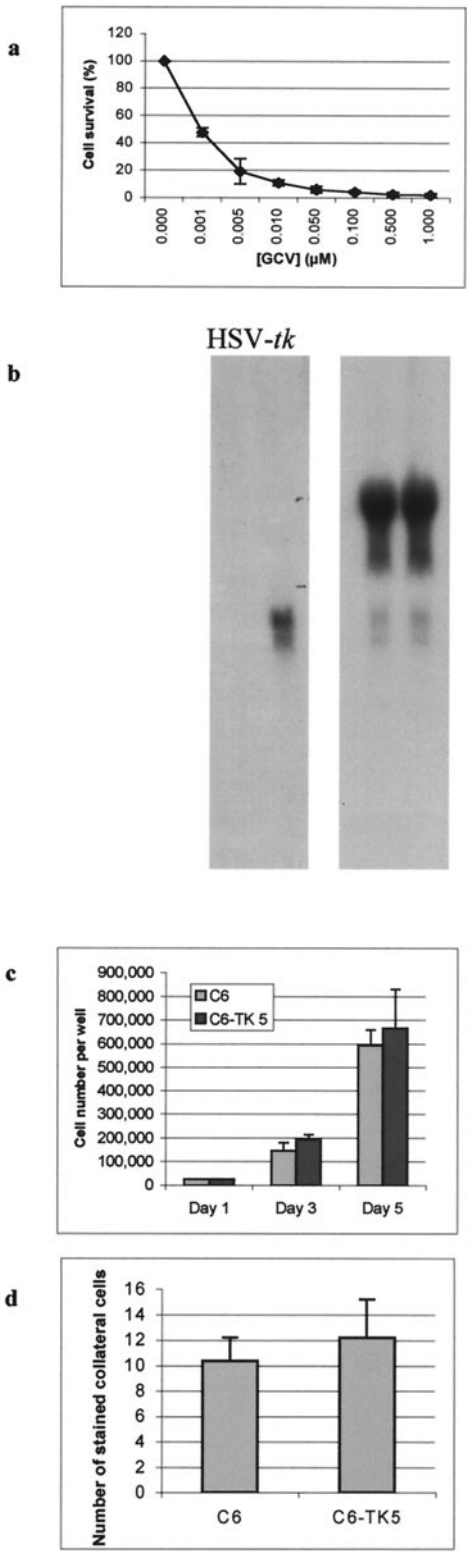

FIG. 1. Characterization of the C6-TK5 clone. (a) C6-TK5 cell survival was assessed after 5 days of treatment with increasing concentrations of GCV. Cell survival is expressed as the percentage (± SD, N = 3) of counted surviving cells in treated wells as compared with that in control wells. (b) Northern blot with a HSV-*tk* probe (left) confirmed the expression of the HSV-*tk* mRNA in C6-TK5 cells (right lane) and its absence in parental C6 cells (left lane); a 28S RNA probe (right) was used to control the deposition of RNA on both lanes. (c) The growth rates of C6-TK5 cells were compared with those of parental C6 cells after 3 and 5 days of culture. Results are expressed as the absolute numbers of cells in the culture wells (± SEM, N = 2 with both experiments performed independently twice in triplicate). (d) Iontophoresis of Lucifer Yellow in confluent C6-TK5 cells led to a diffusion of the dye to 12 adjacent cells (1:12.2, N = 20) similar to that observed in the parental cell line (1:10.36, N = 33). Data are shown ± SEM.

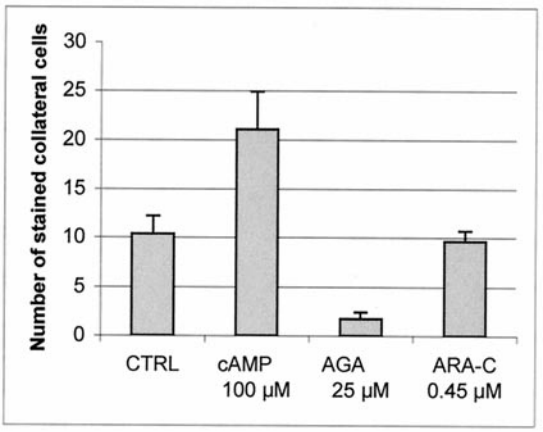

FIG. 2. GJIC in C6 cells. Iontophoresis of Lucifer Yellow was used to assess the GJIC of confluent C6 cells. Means (± SEM; N = 33 for control, 13 for dibutyryl-cAMP, 12 for Ara-C, and 15 for AGA) of the number of stained collateral cells per injected cells are shown for the different drug treatments [control; dibutyryl-cAMP (100 μM); Ara-C (0.45 μM); AGA (25 μM)].

being already detectable after 2 days of treatment. AGA (25 μM), a potent and rapidly acting GJIC blocker [32, 33], strongly decreased the GJIC level in C6 cells after 5 days of treatment. Indeed, in AGA-treated cells, the dye diffused into an average of 1.7 cells ($P < 0.05$, Figs. 2 and 3, lower left panel). Finally, the cytostatic drug Ara-C (0.45 μM) did not modify the GJIC of C6 cells after 5 days of treatment (Figs. 2 and 3, lower right panel).

Cx43 mRNA Expression

Treatment of C6 cells for 48 hr with dibutyryl-cAMP slightly but reproducibly increased their Cx43 mRNA content with respect to their 28S RNA content, as assessed by northern blotting (× 1.3, $P < 0.05$, data not shown). Neither AGA nor Ara-C affected Cx43 mRNA expression significantly.

CX43 Protein Expression and Phosphorylation

Western blots performed on C6 cell extracts using a commercially available polyclonal antibody to Cx43 identified at least three immunoreactive proteins migrating around 43 kDa, named P_0, P_1, and P_2 according to their migration in the gels. On image analysis of the gels, the total Cx43 protein content was slightly but reproducibly increased after treatment with 100 μM dibutyryl-cAMP

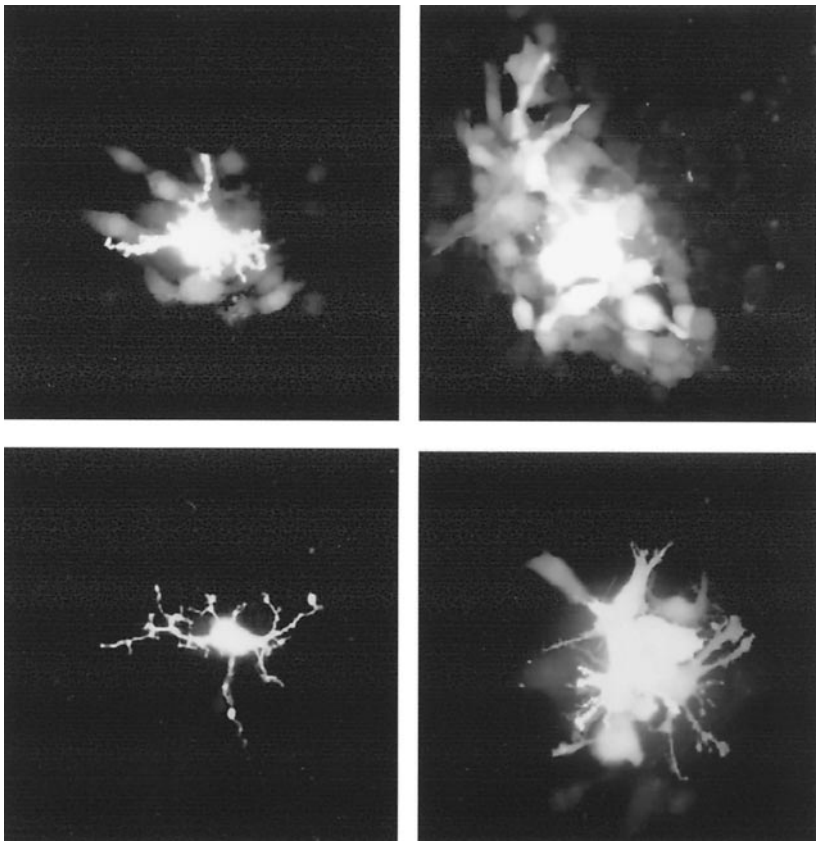

FIG. 3. Dye diffusion in C6 cells. Photomicrographs showing the diffusion of Lucifer Yellow dye in confluent C6 cells after iontophoresis. Upper left, control cells; upper right, dibutyryl-cAMP; lower left, AGA; lower right, Ara-C.

($P < 0.05$, Fig. 4), whereas neither Ara-C nor AGA altered it. The relative intensity of the different phosphorylated bands was not modified by any of the drugs tested, including dibutyryl-cAMP.

Modulation of the Sensitivity of C6-TK5 Cells to GCV Toxicity

The inhibition of C6 cell proliferation by the various drugs was assessed with the Abacus® acid-phosphatase assay. AGA (25 μM), dibutyryl-cAMP (100 μM), and Ara-C (0.45 μM) decreased cell growth by approximately 50% in our culture conditions. To assess the influence of this growth inhibition on the IC_{50} (GCV) of C6-TK5 cells, their survival was measured after 5 days of treatment with various concentrations of GCV, alone or in the presence of each of the three drugs tested (Fig. 5). Dibutyryl-cAMP (100 μM) decreased the toxic response to low concentrations of GCV. The IC_{50} (GCV) was increased significantly ($P < 0.05$) but remained in the nanomolar range. Ara-C (0.45 μM) diminished the sensitivity of C6-TK5 cells to GCV at all concentrations tested ($P < 0.0001$). Although the IC_{50} (GCV) in the presence of Ara-C remained in the high nanomolar range, the fraction of cell survival was much increased at higher GCV concentrations. Ara-C thus somewhat protected the HSV-*tk*-transfected cells from GCV toxicity. However, one must remember that Ara-C by itself has an effect on C6-TK5 cell survival that is not taken into account in these concentration–response curves, which were expressed in percent of control, i.e. cells treated with Ara-C alone. AGA (25 μM) also efficiently protected these cells from the lethal action of GCV ($P < 0.001$). In this case, the survival curve lay somewhere between those of dibutyryl-cAMP and Ara-C. The ic_{50} (GCV) again remained in the higher nanomolar range.

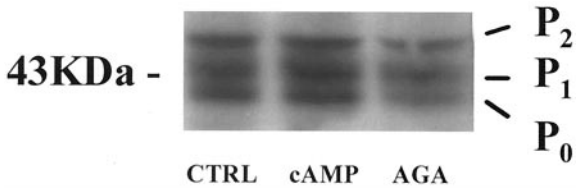

FIG. 4. Cx43 expression in C6 cells. Western blot analysis of Cx43 expression in C6 cell protein extracts (4 μg/lane) in the absence or presence of 100 μM dibutyryl-cAMP ($P < 0.05$) or 25 μM AGA ($P > 0.05$, NS) for 5 days.

Modulation of the In Vitro *Bystander Effect*

The *in vitro* bystander effect in C6/C6-TK5 co-culture was highly significant, the presence of only 5% of C6-TK5 cells leading to an overall 78% decrease in cell survival after 5 days of GCV treatment (mean of all control experiments). We found no evidence of soluble factor involvement in the

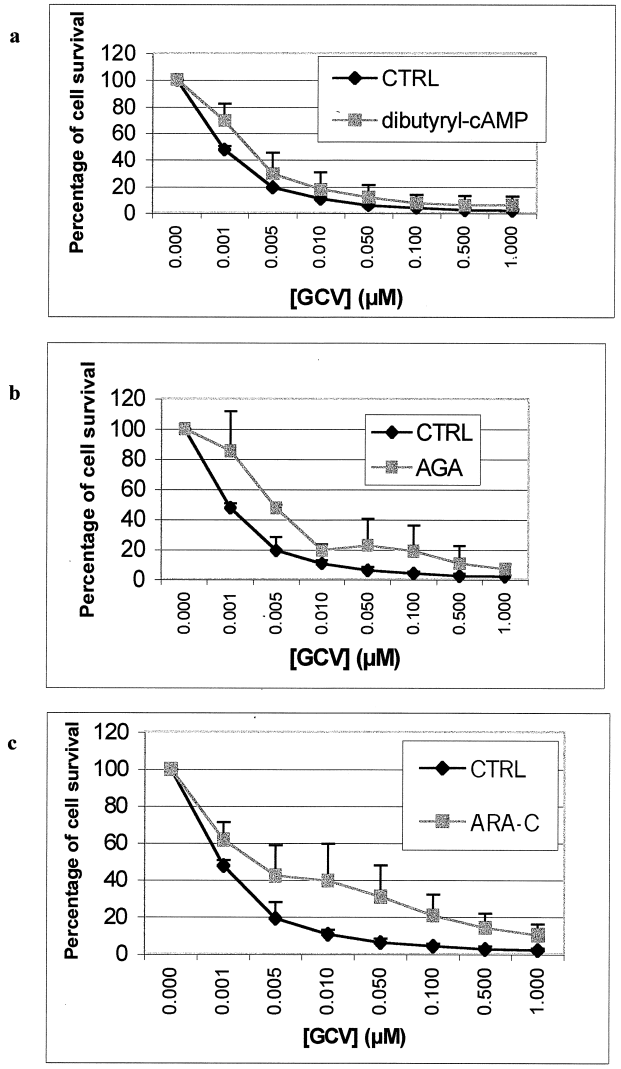

FIG. 5. Modulation of the survival curve of C6-TK5 cells in response to GCV treatment in the presence of 10^{-4} M dibutyryl-cAMP (a), 25 μM AGA (b), or 4.5×10^{-7} M Ara-C (c). Cell survival is expressed as the percentage (± SD, N = 2 with both experiments performed independently twice in triplicate) of optical density in treated wells as compared with that of untreated wells after 5 days of culture and processing with an acid phosphatase assay. Control curves (CTRL) represent the survival of cells treated with GCV alone.

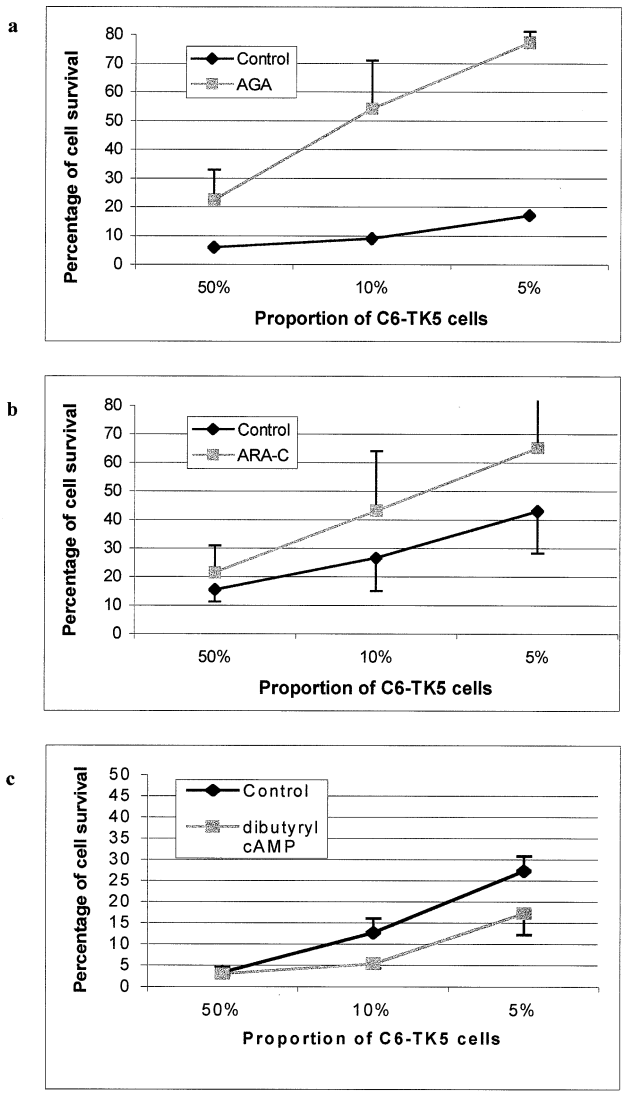

FIG. 6. Pharmacological modulation of the bystander effect. Decreasing proportions of C6-TK5 cells were co-cultured with C6 cells, at a total density of 10,000 cells/well, for 5 days. Cell survival was assessed with an acid phosphatase assay after treatment with GCV. Results are expressed as the percentages of optical density of the GCV-treated wells as compared with GCV-free wells. (a) AGA (25 μM, N = 3); (b) Ara-C (0.45 μM, N = 6); (c) dibutyryl-cAMP (100 μM, N = 3) (results are means ± SD; see text for details). Control curves represent cell survival after treatment with GCV alone.

generation of this bystander effect, as separated cultures of C6 and C6-TK5 cells in two-compartmentalized devices did not result in C6 cell death after GCV treatment (data not shown). As could be expected from its effect on both GJIC and C6-TK5 sensitivity to GCV, AGA (25 μM) strongly inhibited the bystander effect in C6/C6-TK5 co-cultures ($P < 0.0001$, Fig. 6a). This effect was, however, less than total: in the presence of 10% of C6-TK5 cells, cell survival was only reduced to 54% as compared with 10% in the absence of AGA. Ara-C (0.45 μM) also decreased the *in vitro* bystander effect in C6 glioma cells, but to a lesser extent than AGA ($P < 0.01$, Fig. 6b). In the presence of 10 and 5% of C6-TK5 cells, total cell survival increased from 26 to 42% and from 43 to 65%, respectively. Dibutyryl-cAMP (100 μM) increased the *in vitro* bystander effect ($P < 0.001$, Fig. 6c). In the presence of 10 and 5% of C6-TK5 cells, the overall cell survival decreased from 12.7 to 5.3% and from 27 to 17%, respectively, as compared with the controls (i.e. similar cell mixtures treated with GCV only). Interestingly, this effect was only seen when C6 and C6-TK5 cells were pretreated with dibutyryl-cAMP before GCV treatment; it did not occur when dibutyryl-cAMP was added at the time of GCV treatment (data not shown). Finally, none of the three drugs (i.e. dibutyryl-cAMP, Ara-C, and AGA) affected the toxicity of GCV (2 μM) on C6 cells alone, hence confirming that their modulation of

the bystander effect was indeed specifically mediated by the C6-TK5 cells.

DISCUSSION

Multiple studies have underlined the importance of the bystander effect in achieving high rates of tumor cell killing by the HSV-*tk*/GCV gene therapy system [1, 34, 35]. Multiple mechanisms are suspected of playing a role in the bystander effect. The transfer of phosphorylated GCV metabolites from HSV-*tk*-expressing cells to their untransfected neighbors is thought to be the most relevant mechanism. This hypothesis is strongly supported by the fact that the bystander effect is absent in cells that lack functional gap junctions, but can be restored or enhanced by connexin gene transfection [8, 15, 16, 22, 36–38]. Phosphorylated GCV metabolites could also be transferred via the release of apoptotic vesicles by the dying HSV-*tk*-positive cells [21], and their subsequent phagocytosis by neighboring cells, although other data contradict this observation [16]. Phosphorylated GCV could also leak from HSV-*tk*-positive cells in the medium through free hemiconnexons and diffuse into nearby HSV-*tk*-negative cells through similar hemichannels. The release of a soluble death signal by dying HSV-*tk*-positive cells is another mechanism suspected of playing a role in C6 cells [12] as well as in the DHD/K12 cell line [13]. However, we did not observe any bystander killing when C6 and C6-TK5 cells were separated by a porous membrane, confirming, as reported by Samejima and Meeruelo [21], that bystander killing in C6 cells requires at least a close proximity and most likely a direct contact between TK-positive and TK-negative cells.

The implication of gap junctions in the bystander effect was confirmed in our model by assessing the influence of AGA, a known gap junction blocker [33]. This compound indeed strongly inhibited both dye transfer and the bystander effect in our cell cultures. The amplitude of this inhibition was, however, less than total under our experimental conditions, due most likely to the incomplete inhibition of GJIC in C6 cells by 25 μM AGA. A complete block of C6 cell GJIC would have required a higher AGA concentration that was toxic for this cell line (data not shown). As AGA diminished the proliferation rate of C6 cells and GCV acted at least in part through its incorporation into DNA during cell division, the question was raised as to whether the effect of AGA could be related to its dual effect on GJIC and on C6-TK5 cell sensitivity to GCV. However, Ara-C, which strongly reduced GCV sensitivity without affecting GJIC, decreased the bystander effect to a lesser degree than AGA. These results confirmed the role of GJIC in the bystander effect of the HSV-*tk*/GCV gene therapy system in C6 cells.

Most tumor cells exhibit a reduced level of GJIC [39]. Thus, an elegant way to increase the bystander effect in the HSV-*tk*/GCV system would be to increase the GJIC level in the tumor cells. Such an approach was initiated by Naus *et al.* [30] and Dilber *et al.* [15], who transfected C6 glioma cells with the Cx43 gene and increased the HSV-*tk*-related bystander effect. Several authors have demonstrated the possibility of modulating the GJIC level of various tumor cells *in vitro* and the bystander effect [18, 19, 22, 23]. Very little is known about the application of this strategy to glioblastoma cells, and no attempt has been made thus far to characterize the level of action of the tested drugs on the multiple steps of connexin expression. cAMP analogs have been reported to enhance GJIC in a variety of cell lines [18, 19]. Indeed, dibutyryl-cAMP (100 μM) doubled the GJIC level in C6 cells but decreased their growth rate, leading to a statistically significant diminution of C6-TK5 sensitivity to GCV. These seemingly opposite effects of dibutyryl-cAMP resulted in increased bystander killing that was present only if the cells had been pretreated with dibutyryl-cAMP before GCV addition. This observation supports the findings of Denning and Pitts [16], who emphasized the role of the early events of apoptosis in the bystander effect, and could explain the discrepancy between our results and those of Touraine *et al.* [22] and Samejima and Meeruelo [21], who failed to detect an increased bystander effect with cAMP analogs in glioblastoma cells. Finally, the metabolism of dibutyryl-cAMP yields butyrate, a known cell cycle blocker and gene-regulating agent [40–45]. Butyrate (200 μM), however, did not affect the bystander killing (data not shown). Thus, the effect of dibutyryl-cAMP was likely to result from a cAMP-mediated action.

We studied the mechanism of action of dibutyryl-cAMP on the regulation of gap junction expression further. After 48 hr of treatment, this compound led to a reproducible increase in Cx43 mRNA that could be linked to the presence of a cAMP response element on the Cx43 gene promoter sequence [46]. Western blots were then performed and showed three Cx43 immunoreactive bands, as previously described [47]. We found no evidence of phosphorylation-related posttranscriptional alteration of Cx43 in response to this long-lasting dibutyryl-cAMP treatment, but the total Cx43 protein content of C6 cells was increased slightly after 48 hr of treatment. Stagg and Fletcher [48] as well as Azarnia and Russel [49] suspected a rapid and late mechanism of GJIC enhancement by cAMP analogs. In view of our results, if the rapid effect of cAMP on glioblastoma cell GJIC is linked to a difference in Cx43 phosphorylation, it must be transient or would require methods other than western blotting to be demonstrated. The absence of such an early modulation of GJIC, however, could also explain the absence of any dibutyryl-cAMP effect on bystander killing when this drug was added simultaneously with GCV.

In conclusion, our results show the feasibility of a pharmacological enhancement of the bystander effect in HSV-*tk*-based gene therapy through the modulation of the level of GJIC in glioblastoma cells. We also demonstrated the existence of another limiting factor of the bystander effect *in vitro*, i.e. the sensitivity of cells to the action of GCV, which can be altered by drug treatment. Finally, an increased bystander effect was only obtained when tumor

cells were exposed to dibutyryl-cAMP prior to GCV. This observation presumably reflects a delay in the induction of GJIC in glioma cells and stresses the importance of the very first hours of GCV treatment in the induction of the bystander effect. These findings are likely to be relevant for the *in vivo* application of this suicide gene strategy, especially for the design of treatment protocols.

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