Gap Junctions Promote the Bystander Effect of Herpes Simplex Virus Thymidine Kinase in Vivo


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ABSTRACT

Transfer of the herpes simplex thymidine kinase gene (HSVtk) into tumor cells followed by the administration of ganciclovir (GCV) provides a potential strategy for the treatment of some malignancies. During GCV treatment, not only the cells that express the HSVtk gene are killed but also frequently neighboring tumor cells that are not genetically altered. This has been called the "bystander effect." Although the mechanism of the bystander effect in vivo remains elusive, our results suggest that gap junction formation between neighboring cells is an important contributing factor.

The C6 rat glioma cell line, which exhibits a low level of intercellular communication by gap junctions and connexin43 (Cx43)-transfected clones of this cell line forming gap junctions from a moderate level (Cx43-12 and Cx43-14) to a high level (Cx43-13), were transduced with HSVtk. Transduced and nontransduced cells were mixed in various concentrations and then cultured in vitro or injected s.c. into C.B-17/SCID-beige mice followed by i.p. injections of GCV. Cx43-transfected clones showed a significant increase of the bystander effect compared with the less coupled C6 parental cell line. In 11 of 12 mice injected with cells of Cx43-transfected clones, no tumors were seen at the inoculation site when a mixture of 50% HSVtk-negative and HSVtk-positive cells was used. Moreover, in mice injected with cells of clone Cx43-13, which exhibits the highest intercellular communication, tumors were frequently undetectable at the inoculation site when using mixtures of 75% HSVtk-negative and 25% HSVtk-positive cells, and even mixtures containing 5% HSVtk-positive cells of Cx43-transfected clones showed tumor size reduction. All animals in control groups (n = 26) developed large tumors at every injection site. These results demonstrate that gap junctions are an important component in mediating the bystander effect in vivo.

INTRODUCTION

Somatic gene therapy is a rapidly developing therapeutic modality for experimental treatment of some cancers (1). One of the approaches in gene therapy of cancers is virus-directed enzyme/prodrug therapy using viral vectors carrying the HSVtk gene and anti-viral drugs GCV or acyclovir (2, 3). GCV is metabolized by HSVtk into a monophosphate form and subsequently to GCV-triphosphate by endogenous mammalian kinases. GCV-triphosphate is incorporated into the DNA of dividing cells, arresting DNA replication and causing cell death (4). Therefore, cells that express HSVtk become sensitive to the toxic effect of GCV and can be killed in vivo. Moreover, during the treatment, not only do cells that express HSVtk succumb but also frequently nonmodified neighboring tumor cells as well. This phenomenon is called "bystander effect" (5, 6).

Such a strategy has been used to treat various experimental tumors (5, 7–11). After some encouraging results from studies in experimental animals, many clinical protocols have been approved worldwide (12). Although clinical trials using this method have been started, the precise mechanism of the bystander effect in vivo has not been fully elucidated. Several hypotheses have been proposed for its mechanism, including involvement of immune responses (13–16), apoptosis (17), endocytosis of toxic cell debris (6), or blood vessel destruction (8).

It has been shown that gap junctions may play important roles in the mechanism of bystander effect in vitro (18–22). Like other nucleotides, phosphorylated GCV cannot pass through the plasma membrane except when traversing to neighboring cells by gap junctions (23). Gap junctions form channels that connect adjacent cells. The channels are permeable to molecules smaller than Mr 1000, such as cyclic AMP, calcium, and inositol triphosphate, but do not allow the transfer of proteins and nucleic acids. Gap junction channels are formed by proteins called connexins. The family of connexin proteins includes at least 13 members in rodents (24, 25).

Although gap junctions probably play a key role in the mechanism of bystander effect in vitro, their role has not been directly tested in vivo. Many factors may affect a bystander effect in vivo. Particularly, it has been claimed that the host immune system contributes to mediating the bystander effect (16). This effect can be investigated by eliminating the immune system, for instance by means of using immune-deficient animals such as SCID (26) or SCID-beige (bg) mice (27).

We have examined the role of gap junctions as a potential mechanism of the bystander effect in vitro and, particularly, in vivo. C6 rat glioma cells (28), which exhibit a low level of intercellular communication by gap junctions as shown by morphological and functional studies (29, 30), and Cx43-transfected clones of this cell line forming gap junctions from a moderate level (Cx43-12 and Cx43-14) to a high level (Cx43-13), were transduced with the HSVtk gene. Mixtures of transduced and nontransduced cells were tested in vitro and in vivo in SCID-bg mice.

MATERIALS AND METHODS

Cell Line. Rat C6 glioma cells (American Type Culture Collection, Rockville, MD) were grown in DMEM (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% (v/v) FCS (Life Technologies, Inc.), 10 µg/ml streptomycin, and 10 units/ml penicillin. Production of C6 glioma cells transfected with Cx43 cDNA has been described previously (31). Briefly, cells were transfected with the plasmid pLTRCx43, an SV40-based vector (pLTR) in which the Cx43 cDNA clone G2 was inserted. The pLTRCX43 construct contains a dominant selectable marker, the Escherichia coli xanthine-guanine phosphoribosyl transferase (gpt) gene, as well. After transfection and selection, various clones were isolated that were grouped into moderate (Cx43-12 and Cx43-14) or high (Cx43-13) expressers of Cx43 mRNA and protein. The level of expression of Cx43 in the clones correlated well with the level of intercel-
lular coupling. The characteristics of these clones have been described previously (30).

**Retroviral Vector.** A replication-deficient retroviral vector, containing HSVtk and the bacterial neomycin resistance (NeoR) fusion gene, was kindly provided as a high-titer PA317 amphotropic vector-producing fibroblast clone by Dr. Michael Blaese (NIH, Bethesda, MD). To produce virus-containing supernatant, the PA317 vector-producing fibroblasts were plated in 175-cm² flasks in DMEM with high glucose and 10% heat-inactivated FCS. The medium was changed when cells were subconfluent and then collected after 24 h, filtered (0.45 μm), and frozen at −70°C. The titer of supernatant was 4 × 10⁶ viral particles/ml as measured on 3T3 cells. Supernatant containing the viral particles was negative for helper virus when target 3T3 cells were tested by PCR for amphotropic envelope sequences as described previously (32).

**Transduction of Parental C6 and Cx43-transfected Clones, Cx43-12, Cx43-13, and Cx43-14.** One million cells of parental C6 or Cx43-transfected clones were incubated in a 75-cm² flask. Twenty-four h later, cells were incubated in a supernatant containing vector (10 virus particles/cell) with 4 μg/ml polybrene (Sigma Chemical Co., St. Louis, MO) for 24 h. The same transduction procedure was repeated daily for 2 more consecutive days. The cells were then exposed to 1 mg/ml G-418 (Life Technologies, Inc.). After 2 weeks of G-418 selection, resistant cells were obtained for consecutive experiments.

**DNA Isolation and PCR.** Isolation of DNA was performed using SDS/proteinase-K lysis of cells, followed by phenol/chloroform extraction (32). PCR reactions were carried out using 100 ng of each primer and 0.2 unit of Taq polymerase (Life Technologies, Inc.) in the total volume of 50 μl. Samples were incubated initially for 5 min at 95°C followed by 30 cycles using primers neo1 (5'-CAA GAT GGA TFG CAC GCA CG-3') and neo5 (5'-CCC OCT CAG CAG AAG AAC TCG TC-3'). The presence of the neoR gene sequence in DNA samples resulted in a 790-bp fragment when a combination of neo1-neo5 primers were used.

**In Vitro GCV Sensitivity Assay.** To determine the optimal cytotoxic effect of GCV, 10⁶ transduced or nontransduced cells of parental C6 or Cx43-transfected clonal origin were seeded in a well of 24-well plates (triplicate). After 24 h incubation, GCV was added to a final concentration of 0.36 to 72 μM (0.1–20 μg/ml). GCV containing medium was changed every other day for 7 days, after which living cells were counted using the trypan blue dye exclusion test.

**In Vitro Analysis of the Bystander Effect.** To determine the effect of HSVtk-positive cells on HSVtk-negative cells in vitro, experiments were performed in which HSVtk-positive and HSVtk-negative cells were mixed at different ratios. Mixtures of cells were incubated at a concentration of 2 × 10⁶ or 10⁷ cells in a well of 24-well plates (triplicate) in basal medium with or without GCV (final concentration, 36 μM). Medium was changed every other day for 7 days; thereafter, viable cells were counted using trypan blue dye exclusion test.

**RESULTS**

**HSVtk Transduction of C6 Parental and Cx43-transfected C6 Clones and Sensitivity to GCV.** The transduction frequency after 2 weeks of G-418 selection was 27, 25, 30, and 17% for C6, Cx43-12, Cx43-14, and Cx43-13 cells, respectively. Neo resistance cells positive for HSVtk were continuously selected by G-418. To optimize the concentration of GCV, transduced and nontransduced cells were grown in medium containing GCV at various concentrations ranging from 0.36 to 72 μM (0.1–20 μg/ml). Cells were evaluated after 7 days. C6, Cx43-12, and Cx43-14 HSVtk-positive cells were sensitive to GCV treatment at concentrations ≥3.6 μM (≥1 μg/ml), whereas Cx43-13 cells were sensitive at ≥18 μM (≥5 μg/ml), but almost complete eradication of cells was obtained at 36 μM (10 μg/ml; Fig. 1). To use the same concentration of GCV for all cells, 36 μM GCV was used in the subsequent experiments. There was no toxic effect of GCV on nontransduced cells.

**In Vivo Bystander Effect.** For in vitro evaluation of bystander effects, two different concentrations of cells (2 × 10⁶ or 10⁷ cells/well) were used. Transduced and nontransduced cells were mixed in different proportions. In the group of low cell concentration (2 × 10⁶), as few as 10% of HSVtk-positive cells were enough to eradicate the majority of HSVtk-negative cells in all Cx43-transfected clones after 7 days of exposure to GCV (Fig. 2a). Furthermore, when the number of cells was increased in each well (10⁷), a bystander effect was observed.

**KINETIC ASSAYS FOR GROWTH OF PARENTAL C6 OR Cx43-TRANSFECTED CLONES.** To see whether transduced or nontransduced clones had similar growth kinetics in vivo, 10⁶ transduced or nontransduced cells were injected s.c. into four separate locations on the back of each mouse. Tumor size was measured using calipers 20 days after transfer of cells.

**In Vivo Analysis of Bystander Effect.** Various percentages of HSVtk-positive and HSVtk-negative cells of parental C6 or Cx43-transfected clones were mixed. Cells (10⁶ per site) were injected s.c. into four separate locations on the back of each mouse: (a) 100% HSVtk-negative cells into the upper left; (b) 100% HSVtk-positive cells into the upper right; (c) 50% each of HSVtk-negative and HSVtk-positive cells into the lower left; and (d) 75% HSVtk-negative and 25% HSVtk-positive cells into the lower right location of the mice. Similar experiments were performed with 10 or 5% HSVtk-positive cells mixed with 90 or 95% HSVtk-negative cells. Simultaneously, to evaluate the direct effect of GCV on nontransduced cells, 10⁷ cells of parental C6 or Cx43-transfected clones per site were injected s.c. into four different locations on the back of each mouse. Five days after s.c. injection of cells, those mice receiving a mixture of transduced and nontransduced cells as well as those only receiving nontransduced cells were given 25 mg/kg GCV in 500 μl twice a day i.p. injections. Mice in the control groups were injected i.p. with a similar volume of saline.

**Statistical Analysis.** The Wilcoxon-Mann-Whitney test was used in the statistical analysis. The levels are expressed as median ± range. Differences were considered significant when P ≤ 0.05.
In Vivo Bystander Effect. To determine whether the levels of gap junctions are important for a bystander effect, $10^7$ HSVtk transduced and nontransduced C6 parental cells or Cx43-transfected clones, which express Cx43 from a moderate to a high level, were injected into SCID-bg mice in various proportions, followed by the administration of GCV. All animals ($n = 66$) had small palpable tumors before initiation of the GCV treatment 5 days after injection of the cells. Tumor diameters before GCV treatment in C6, Cx43-12, Cx43-14, and Cx43-13 cells were 5.2 ± 2.0 mm, 4.9 ± 2.6 mm, 4.7 ± 1.6 mm, and 4.9 ± 1.2 mm (median ± range), respectively.

In Vivo Bystander Effect in Parental C6 Cells. In the group carrying C6 parental cells, all animals receiving saline only developed large tumors at each injection site, regardless of the proportion of HSVtk-negative or HSVtk-positive cells in the injected mixture at the end of the experiments (20 days after cell injection). All GCV-treated animals ($n = 14$) developed tumors at locations inoculated with HSVtk-negative cells only. Five of 14 animals had small tumors when only HSVtk-positive cells were used (Fig. 4, 5). At the inoculation site of a mixture of 50% HSVtk-negative and HSVtk-positive cells, there were still tumors, although tumor sizes were statistically smaller than in control animals injected with $5 \times 10^6$ non-Cx43-transfected cells.

In Vivo Growth Kinetics of Parental C6 and Cx43-transfected Clones. One $\times 10^6$, $5 \times 10^5$, or $10 \times 10^6$ parental C6 or Cx43-transfected clones (Cx43-12, Cx43-13, and Cx43-14) were injected s.c. into SCID-bg mice. All animals developed tumors. In untreated animals, the tumor diameters from connexin high-expressing Cx43-13 cells were smaller than from C6 parental cells, but there was no statistical difference between parental C6 and Cx43-12 or Cx43-14 clones. Tumor diameters 20 days after injection of $1 \times 10^6$, $5 \times 10^5$, and $10 \times 10^6$ cells, respectively, were 17.2 ± 2.6, 20.6 ± 6.0, and 22.6 ± 5.2 mm (median ± range) for C6 parental cells; 16.5 ± 4.9, 18.7 ± 2.6, and 22 ± 0.8 for Cx43-12; 16.3 ± 4.3, 18.0 ± 2.7, and 20.3 ± 2.0 for Cx43-14; and 9.1 ± 2.3, 10.5 ± 3.4, and 13.7 ± 0.7 for Cx43-13 cells (Fig. 3).

Experiments were also performed to evaluate growth kinetics of transduced and nontransduced cells in vivo. There was no statistically significant difference in the size of s.c. tumors when $10^7$ cells of either nontransduced or HSVtk-transduced cells of the individual clone were injected into SCID-bg mice. Twenty days after injection of cells, the following tumor sizes were observed (nontransduced/HSVtk transduced): C6, 19.5 ± 2.8/19.8 ± 2.1 mm; Cx43-12, 18.2 ± 4.0/19.1 ± 3.0 mm; Cx43-14, 17.8 ± 3.3/18.1 ± 2.6 mm; and Cx43-13, 13.0 ± 1.5/12.7 ± 1.3 mm (median ± range).

In Vivo Bystander Effect. To determine whether the levels of connexins are important for a bystander effect, $10^7$ HSVtk-transduced and nontransduced C6 parental cells or Cx43-transfected clones, which express Cx43 from a moderate to a high level, were injected into SCID-bg mice in various proportions, followed by the administration of GCV. All animals ($n = 66$) had small palpable tumors before initiation of the GCV treatment 5 days after injection of the cells. Tumor diameters before GCV treatment in C6, Cx43-12, Cx43-14, and Cx43-13 cells were 5.2 ± 2.0 mm, 4.9 ± 2.6 mm, 4.7 ± 1.6 mm, and 4.9 ± 1.2 mm (median ± range), respectively.

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over, an even greater degree of bystander effect was observed with the same animals, three of four mice did not have tumors at the inoculation site of a mixture of 75% HSVtk-negative and 25% HSVtk-positive cells (Fig. 4). There was also a significant tumor size reduction or inhibition when administering only 10 or 5% HSVtk-positive cells (clones Cx43-12, Cx43-14, and Cx43-13), even when comparing with untreated animals receiving $5 \times 10^6$ nontransduced cells (Fig. 3b, 20 days, 5).

Effet of GCV on Nontransduced Cells. To rule out the effect of GCV treatment per se on tumor formation, $10^7$ nontransduced cells of each clone were injected into four separate locations on the back of each mouse given either saline or GCV. Twenty days after cell injection, no significant difference was found between the size of tumors appearing in the control and in GCV-treated mice.

DISCUSSION

The transfer of suicide genes to tumor cells has become an important tool for somatic gene therapy of malignancy. Because it is impossible to transduce or transfect 100% of tumor cells using available techniques, a bystander effect in the HSVtk/GCV system is an excellent method to eradicate some tumors. Although the mechanisms of a bystander effect has not been fully understood, several clinical trials have been started with the aim of treating patients with end-stage disease.
brain tumors, ovarian cancer, and pleural mesothelioma (12). However, understanding the mechanism of a bystander effect is of general importance in the design of cancer gene therapy. There is evidence that gap junctions between adjacent cells are important to transfer phosphorylated GCV from HSVtk-positive cells to HSVtk-negative cells in vitro (18—22), but the mechanism of the bystander effect in vivo is still controversial.

To test the role of gap junctions for the bystander effect in vivo, we compared the degree of bystander effect between cells from a poorly coupled C6 rat glioma cell line and clones from this cell line stably transfected with a cDNA encoding an important gap junction protein, Cx43. Our results suggest the importance of gap junctions for the bystander effect of the HSVtk/GCV system in vivo as well as in vitro. We found that Cx43-transfected clones showed clear and significant augmentation of the bystander effect, and furthermore, there was a strong correlation between the bystander effect and the level of gap junctions. For example, about 5% of HSVtk-transduced Cx43-13 cells gave a similar reduction in tumor size as compared to 50% of HSVtk-transduced parental C6 cells. Some of the mice developed tumors at locations inoculated with C6 HSVtk-positive cells only. This is probably due to the low concentration of GCV at the tumor site as compared to in vitro. Reversal of HSVtk-positive cells to HSVtk-negative is unlikely, because cells that had been obtained from tumor masses were sensitive in the culture (data not shown). Statistical comparisons were made with both 5 × 10⁶ and 10 × 10⁶ cells because it could be argued that the direct toxic effect on a mixture of 50% HSVtk-positive and 50% HSVtk-negative cells would result in only 5 × 10⁶ surviving cells in the absence of a bystander effect.

C6 glioma cells were initially derived from rat glial tumors induced by N-nitrosomethylurea (28). It has been shown that C6 glioma cells exhibit marked reduction in expression of Cx43 and a low level of intercellular communication (29, 30). However, Cx43-transfected clones of this cell line express Cx43 mRNA and protein from a moderate (Cx43-12 and Cx43-14) to a high level (Cx43-13). The level of expression of this gap junction gene correlates well with the level of intercellular coupling. The amount of Cx43 mRNA was found to be 30-fold greater in clones Cx43-12 and Cx43-14 and 50-fold greater in clone Cx43-13 relative to that in C6 cells. The level of Cx43 protein, relative to C6 cells, was 3-fold greater for clones Cx43-12 and Cx43-14 and 8-fold greater for clone Cx43-13 (30). Cx43-transfected clones (Cx43-12 and Cx43-14), which express moderate levels of gap junction protein, had similar growth kinetics to C6 parental cells, but the transfected clone Cx43-13, which has the highest level of gap junction protein, grew slowly, probably due to a rise of intercellular communication. However, a stronger bystander effect was obtained from all Cx43-transfected clones. Similar to these results, it has been shown that when uncoupled cells were transfected with one of the genes encoding a connexin, a greater bystander effect was observed in vitro (20—22). Actually, the concept of gap junctions was proposed twice a day for only five doses before sacrifice. A bystander effect was not seen, even when the tumor population comprised 50% HSVtk-positive cells. However, as mentioned above, animals were treated for a short time, and such a brief observation period may not be enough to observe the regression of tumor masses. In our experiments, complete eradication of the tumors was observed 10 days after GCV treatment. Furthermore, we have demonstrated that a bystander effect can be obtained in SCID mice receiving mixtures of HSVtk-transduced and nontransduced cells of the ARH-77 myeloma cell line (11).

In conclusion, this study has shown that the expression of gap junction proteins is an important factor in mediating tumor eradication, or tumor cell reduction, by a bystander effect in vivo as well as in vitro.

ACKNOWLEDGMENTS

We thank Dr. M. Blaese for providing the vector-producing PLTN 3.3 cell line. We also thank A. Holmqvist and A. Dyhr for excellent work in taking care of the animals.

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