

Herpes simplex virus thymidine kinase and granulocyte macrophage colony-stimulating factor combination gene therapy in a murine CT26 cell colon cancer model

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We evaluated the antitumor effects of combination gene therapy on CT26 mouse colon cancer cells, using the genes for herpes simplex virus thymidine kinase gene *HSV-TK* combined with granulocyte macrophage colony-stimulating factor (*GM-CSF*) compared with *HSV-TK* alone. Cells, unmodified or retrovirally transduced with *HSV-TK* or *GM-CSF*, were inoculated subcutaneously into syngeneic BALB/c mice in various combinations. *HSV-TK* and *GM-CSF* were also delivered using different routes (in separate cells vs doubly transfected single cells). Both *HSV-TK* (with i.p. ganciclovir — GCV — treatment) and *GM-CSF* genes had independent antitumor effects, and given together they caused significant reduction in tumor volumes compared with the *HSV-TK* gene alone ($P < 0.001$). Following GCV treatment, however, the treated/control ratios for tumor volumes were not different between tumors containing either *HSV-TK* alone or both genes (0.27 vs 0.25, respectively). Thus, the presence of *GM-CSF* did not increase the bystander effect of *HSV-TK*. Tumors receiving genes transferred in separate cells tended to be more consistently suppressed after GCV treatment than when both genes were transferred in the same cells, although this was not statistically significant. Thus, combination *GM-CSF* and *HSV-TK* gene therapy produced greater therapeutic efficacy than *HSV-TK* alone, but the bystander effect was not enhanced by *GM-CSF*.

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Gene therapy is the optimal strategy for cancer treatment in the sense that cancers can develop because of the activation or suppression of tumor-related genes. However, it is doubtful that any cancer could be eradicated with a manipulation of any single gene, because cancer development requires multi-step processes involving multiple tumor-related genes.¹ Thus, combination gene therapy seems to be inevitable for the treatment of cancer. Furthermore, neither tumor cell-specific nor highly efficient vectors that can convey the therapeutic gene into all target cells are currently available. Given these limitations, the mainstay of current gene therapy protocols remains the use of nonspecific cytotoxic genes such as that for herpes simplex virus thymidine kinase (*HSV-TK*)^{2,3} or immune system-related genes to augment antitumor immunity.^{4,5} Combination *HSV-TK*/ganciclovir (GCV) therapy has the advantage that tumor

eradication may be possible without the need to transfect *HSV-TK* into every tumor cell. GCV is a prodrug that competitively inhibits deoxyguanosine 5'-triphosphate (dGTP) after conversion into its triphosphate form by the action of the enzyme *HSV-TK*. With such alteration of GCV, neighboring cells that do not express the *HSV-TK* gene are killed in addition to the tumor cells expressing the gene. As the phosphorylated GCV is unable to diffuse freely across the plasma membrane, several mechanisms have been suggested for this bystander-killing effect.

The connexin gap junction-mediated intercellular transfer of toxic phosphorylated GCV molecules has been demonstrated in many cell lines.^{6,7} However, this route varies between different tumor types.⁸ Especially in colon tumors, the magnitude of the *HSV-TK*/GCV bystander effect has proven to be modest.⁹

Connexin-independent bystander effects in human colon cancer cell lines have been suggested,¹⁰ and there is evidence that cellular immunity might play a role in mediating bystander effects in mouse colon cancer cell lines.¹¹ *GM-CSF* is one of the best-characterized of the cytokines that induce antitumor immunity. It promotes tumor regression and has long been used as a potent

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cancer vaccine.^{12–14} If an immunological component could contribute to a bystander-killing effect in the colon cancer system, combination gene therapy using *GM-CSF* transfection could synergistically improve the efficacy of the *HSV-TK/GCV* gene therapy system. However, in combination gene therapy, the route of delivery for each gene may have implications for efficacy, especially if a cell suicide gene such as *HSV-TK* is employed. If such a gene were introduced into the same cell with other therapeutic genes, theoretically it could turn off the expression of the other genes and thus reduce the overall efficacy.

In this study, we tested whether combined *HSV-TK* and *GM-CSF* gene therapy could enhance antitumor activities compared with the use of *HSV-TK* alone, and whether the addition of the *GM-CSF* gene could enhance the bystander effect of *HSV-TK*. We also explored the best route of delivery in combination gene therapy by delivering the same genes via different routes.

Materials and methods

Cell line

CT26 cells, an N-nitroso-N-methylurethane (NNMU)-induced undifferentiated colon carcinoma line from BALB/c mice, were obtained from the Korean Cell Line Bank (Seoul, Korea). The cell line was routinely maintained in RPMI medium containing 10% fetal bovine serum under a 5% CO₂-humidified air atmosphere at 37°C.

Vector preparation and transduction

The *HSV-TK*-expressing *LTKSP* vector was constructed by cloning the coding sequence of *HSV-TK*, amplified with a 5'-*Bam*HI and 3'-*Eco*RI primer set, into the multicloning site of *pBabePuro*, a kind gift of Professor Peter J Stambrook (Department of Cell Biology, Neurobiology and Anatomy, University of Cincinnati, Cincinnati, OH, USA). The *GM-CSF*-expressing *LGMSN* vector was constructed by cloning the *GM-CSF* gene into the *Bam*HI site of *LxSN*, kindly provided by Professor William RA Osborne (Department of Pediatrics, University of Washington, Seattle, WA, USA).

The methods used to generate retroviral packaging cell lines minimizing the formation of replication-competent retroviruses, and to produce high-titer amphotropic vectors involving selection, dilution cloning, and screening, were as previously described.¹⁵

The *LTKSP* and *LGMSN* vectors were transduced into parental CT26 cells by retroviral transduction using amphotropic retroviral producer cells (NIH3T3) as previously described.¹⁶ Retroviral-infected cells were selected by the addition of 1 µg/mL puromycin for *LTKSP*, and 400 µg/mL active G418 for *LGMSN*, establishing CT26/TK and CT26/CSF cell lines, respectively. Procedures for the transduction of *LGMSN* were repeated using CT26/TK cells as a parental line, establishing doubly-infected CT26/TK/CSF. Cells were subcul-

tured and grown thereafter in appropriate selection antibiotic-containing media.

In vitro sensitivity to GCV and bystander effect

Cells transduced with the *HSV-TK* gene were tested for their sensitivity to GCV (Cymevene, Roche Products, Basel, Switzerland) and for bystander antitumor effects. CT26/TK and CT26/TK/CSF cells were plated in 10-cm dishes (10⁶ cells per dish) and cultured in RPMI medium with and without (control) GCV, at a concentration of 20 µM. Unmodified CT26 and CT26/CSF cells were used as *HSV-TK*-negative controls. For the analysis of bystander effects, CT26/TK and CT26/TK/CSF cells were plated admixed with unmodified CT26 cells at a ratio of 25:75 and cultured as above. Each cell line or admixtures of cell lines were plated in four sets and the viable cell numbers estimated by Trypan blue exclusion daily for four consecutive days.

In vivo antitumor activity and bystander effects

Syngeneic male BALB/c mice were obtained at age 8 weeks from the Japan SLC (Hamamatsu), and were maintained according to established guidelines. Five groups of mice each consisting of GCV treatment (*n*=6) and control (*n*=6) arm were subcutaneously implanted with tumor cells (6 × 10⁵ cells in 100 µL of phosphate-buffered saline) on the back. The proportions of each type of cells implanted are listed in Table 1. Groups 1 and 2 were used for comparison of direct antitumor activities, and groups 3–5 were used for quantitation and comparison of bystander effect. The proportions of *HSV-TK* gene-expressing cells were maintained at 50% in groups 3–5 for the quantitation and comparisons of bystander effects.

The tumors were left to develop for 7 days before the mice in the treatment arms of each group were intraperitoneally injected twice daily with GCV at a dose of 25 mg/kg. The size of each tumor was measured using calipers and tumor volumes were calculated as: (longest diameter) × (shortest diameter)²/2.¹⁷ All mice were killed on day 18 and the tumors were removed for histological and immunohistochemical studies.

Immunohistochemistry

Immunohistochemical studies were performed with sections of tumor tissue snap frozen and sectioned (4 µm) on a cryostat, fixed in cold 100% acetone for 5 minutes,

Table 1 *In vivo* experimental groups according to the composition of tumor inoculum

Experimental groups	Composition of inoculum
Group 1	100% CT26/TK
Group 2	100% CT26/TK/CSF
Group 3	50% CT26+50%CT26/TK
Group 4	50% CT26+50%CT26/TK/CSF
Group 5	25% CT26+50%CT26/TK+25% CT26/CSF

washed three times in PBS, and incubated in blocking sera for 30 minutes. Sections were incubated with monoclonal rat anti-murine GM-CSF antibody (BMS, Austria). After two washes with PBS, sections were stained according to the standard avidin–biotin complex immunoperoxidase method (ABCComplex/HRP; Dako, Glostrup, Denmark).

Results

In vitro sensitivity to GCV and bystander effects

Figure 1 shows the *in vitro* susceptibilities to GCV for each cell line and bystander effects for *HSV-TK*-expressing cell lines. CT26 and CT26/CSF cells were resistant to GCV treatment as expected. CT26/TK and CT26/TK/CSF cells were completely inhibited by the addition of GCV, implying stable transduction of the *HSV-TK* gene. This effect of GCV was consistent from the beginning of culture. *GM-CSF* had no effect on cell growths or viability compared with the unmodified CT26 cells (data not shown).

When *HSV-TK*-expressing cells were mixed with unmodified CT26 cells at a ratio of 25:75, the cell growths were significantly but not completely inhibited. The treated/control (T/C) ratios for cell viabilities on day 4 were 0.23 and 0.25 for CT26/TK and CT26/TK/CSF cells, respectively (Fig 2). This means that each *HSV-TK*-expressing cell could inhibit approximately two unmodified neighboring cells.

In vivo antitumor efficacy and bystander effects

The sequential effects of GCV treatment on tumors in mice inoculated with CT26/TK and CT26/TK/CSF cells are shown in Figure 3. There was a significant reduction in mean tumor volume after treatment with GCV in the mice inoculated with CT26/TK cells. Similarly, tumors in treated mice inoculated with CT26/CSF cells were significantly smaller than those in the control arm, but tumors were not completely eradicated in both groups until day 18 when all mice were killed. The mean tumor volumes (\pm SD) on day 18 of mice in both the GCV-treated arm ($78 \pm 70 \text{ mm}^3$) and control ($1075 \pm 631 \text{ mm}^3$) of the CT26/TK/CSF group were markedly reduced compared with those in corresponding arms (251 ± 216 and $1830 \pm 646 \text{ mm}^3$, respectively) of the CT26/TK group. This indicated the additional antitumor effect of the *GM-CSF* gene, although the differences were not statistically significant ($P=0.068$ for controls and $P=0.096$ for the treated arms).

Mice in groups 3–5 were inoculated with mixtures of various cell lines, but to quantify the bystander effects, the proportions of *HSV-TK*-expressing cells were fixed at 50% in all three groups. Figure 4 shows the significant inhibition of tumor growths in mice in the GCV-treated arm compared to the controls in all three groups. With regard to the antitumor efficacy of combined *HSV-TK* plus *GM-CSF* vs only *HSV-TK* gene therapy, the mean volumes (\pm SD) of tumors that expressed both *HSV-TK* and *GM-CSF* ($148 \pm 122 \text{ mm}^3$) were significantly smaller

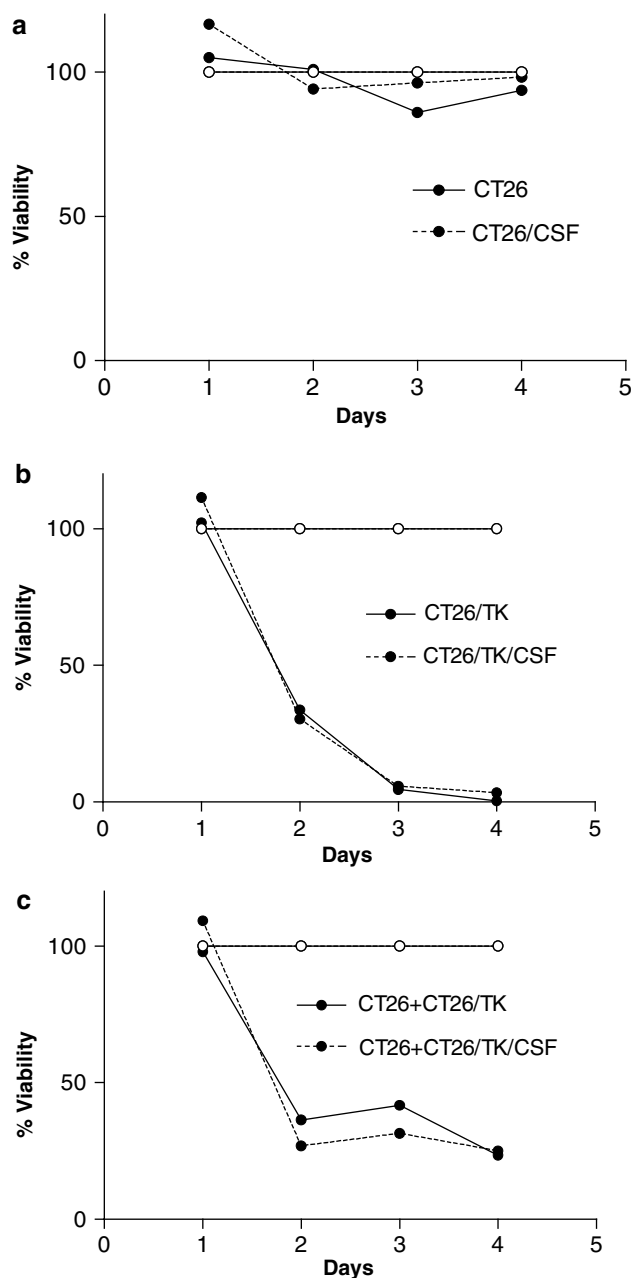


Figure 1 Assessment of GCV sensitivity of various cell lines *in vitro*. Cells or cell mixtures were cultured in media with (●) or without (○) addition of GCV (20 μ M). (a) Unmodified CT26 cells and CT26 cells containing only the *GM-CSF* gene (CT26/CSF) showed no sensitivity to GCV. (b) *HSV-TK*-expressing cells were almost completely suppressed. (c) When co-cultured with unmodified cells (75%), there was no difference in bystander effects between cells transduced with *HSV-TK* alone or with both the *HSV-TK* and *GM-CSF* genes.

than those of tumors that expressed only *HSV-TK* ($455 \pm 386 \text{ mm}^3$) after GCV treatment ($P=0.021$). For the bystander effects, however, there was no difference between the T/C ratios for tumors that expressed only *HSV-TK* (0.27) and those expressing both *HSV-TK* and

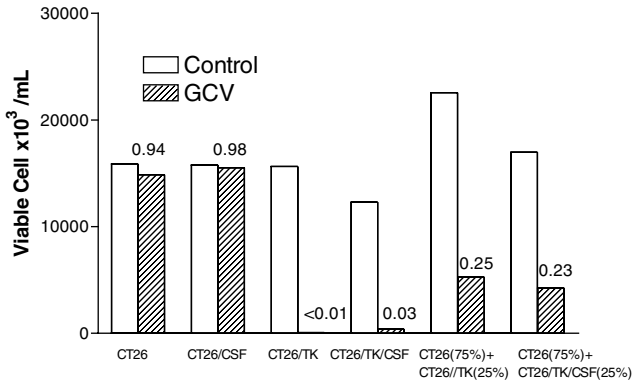


Figure 2 Viable cell numbers and treated/control (*T/C*) ratios on day 4 of treatment with GCV (20 μ M). *T/C* ratios for each cell line show that the addition of GM-CSF had no effect on GCV toxicity or bystander effects in various cell lines.

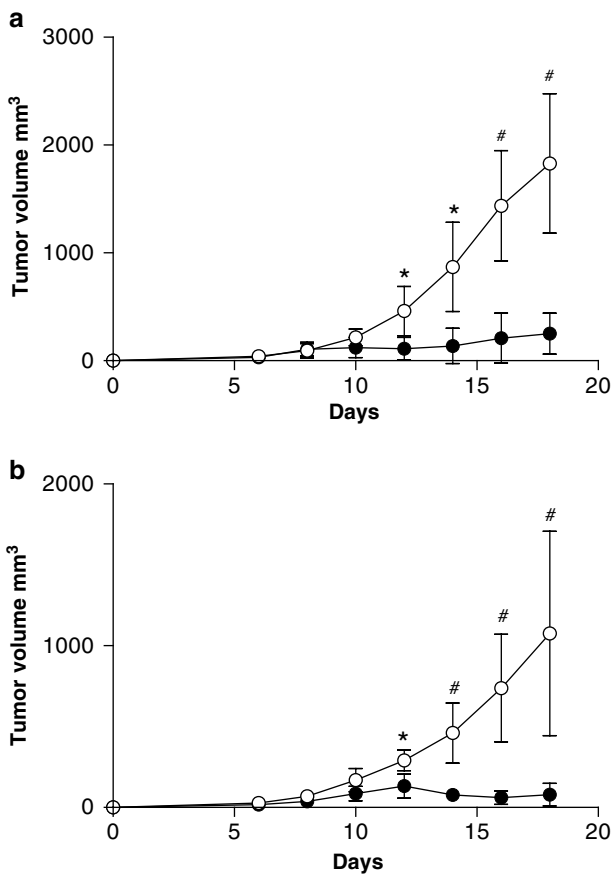


Figure 3 *In vivo* tumor growth curves for (a) CT26/TK and (b) CT26/TK/CSF cells. For the mice in treatment arm in both groups, GCV (25 mg/kg twice daily) was injected intraperitoneally from day 7. Tumor growth was significantly suppressed in GCV-treated mice compared with control mice in both groups, but there was no significant difference in the *T/C* ratio between the groups. * $P < 0.05$; # $P < 0.01$. ●, GCV treated; ○, untreated control.

GM-CSF (0.25) (Fig 5). As shown in Figure 6, the mean *T/C* ratios for both *GM-CSF*-positive and -negative tumors were getting smaller as GCV treatment continued,

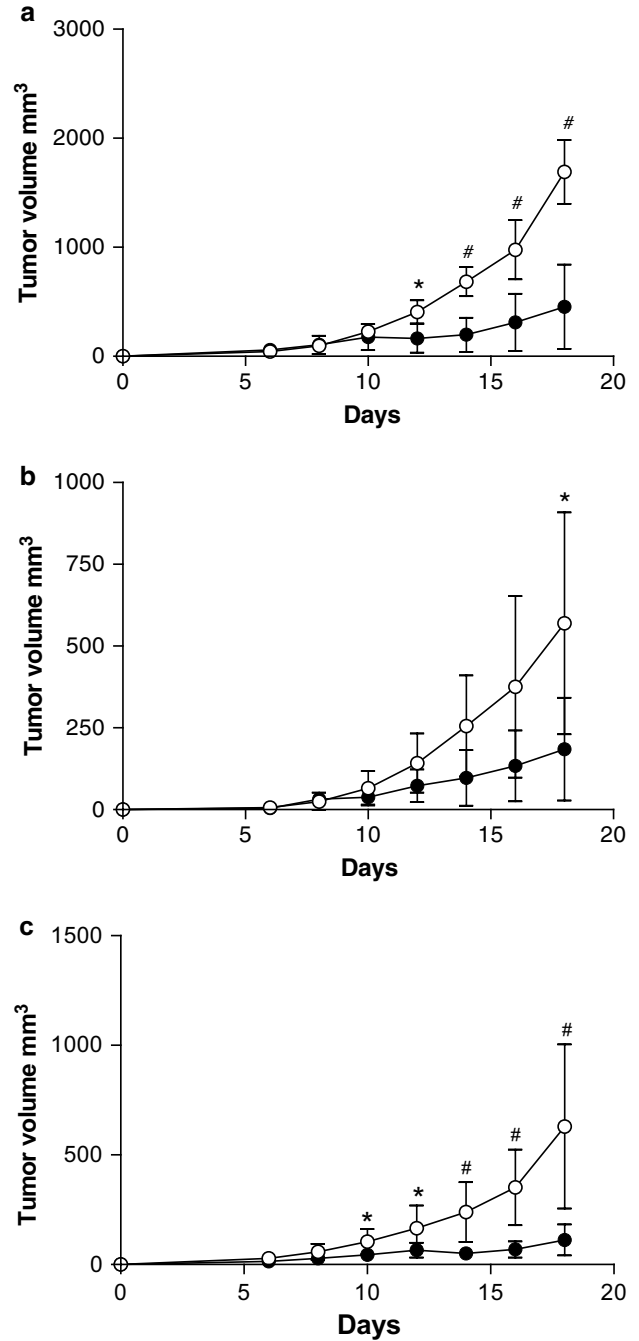


Figure 4 *In vivo* bystander effects for tumors composed of (a) CT26 (50%) + CT26/TK (50%) cells; (b) CT26 (50%) + CT26/TK/CSF (50%) cells, and (c) CT26 (25%) + CT26/TK (50%) + CT26/CSF (25%) cells. Although the tumors secreting GM-CSF tended to be more suppressed, there were no significant differences in *T/C* ratios between groups. Statistics and symbols are as in Figure 3.

and they eventually nearly converged. Thus, the greater tumor-suppressive effect of combined *HSV-TK* and *GM-CSF* gene therapy could be attributed to the additional antitumor effects of *GM-CSF*, but not to the changes in bystander effects caused by *GM-CSF*. The antitumor

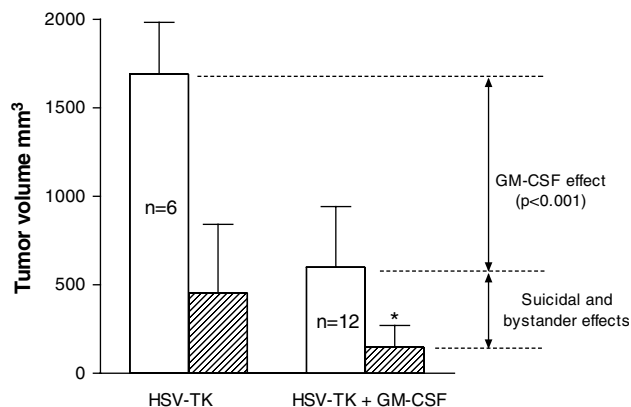


Figure 5 The therapeutic effects of *HSV-TK* and *GM-CSF* expressions on tumor growth. Each decrease in the volume of GCV-treated tumors (hatched box) consists of the growth-suppressive effect produced by *GM-CSF* and the suicide-inducing/bystander effect of the combined *HSV-TK*/GCV treatment. Although the mean final volume of the tumors carrying both genes was significantly smaller than that for the tumors carrying the *HSV-TK* gene alone ($*P=0.021$), there was no significant difference in *T/C* ratios between the experimental groups.

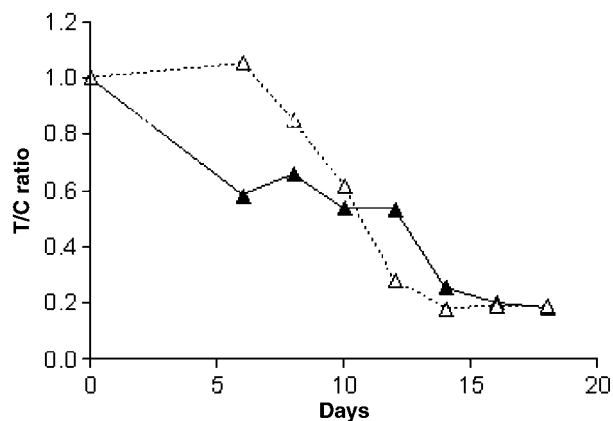


Figure 6 Time-dependent curves of *T/C* ratios for tumors composed of mixed cell lines with (—▲—, $n=24$) and without (—△—, $n=12$) *GM-CSF*-expressing cells. As the GCV treatments continued, the curves converged, indicating that there was no effect of *GM-CSF* expression on the bystander effect.

activities of *GM-CSF* were most evident between control arms in each group because there were no effects from the *HSV-TK*/GCV combined treatment (Fig 7). The tumors containing the *GM-CSF*-expressing cells were significantly more suppressed than those without *GM-CSF* were from the time of inoculation, and this effect of *GM-CSF* was consistently observed during the whole study. On day 18, the mean (\pm SD) tumor volume of tumors carrying the *GM-CSF* gene was $758 \pm 496 \text{ mm}^3$, compared with $1760 \pm 483 \text{ mm}^3$ for tumors that did not carry the gene ($P < 0.001$).

Expression of *GM-CSF* within tumors was not quantitatively determined, but immunohistochemical

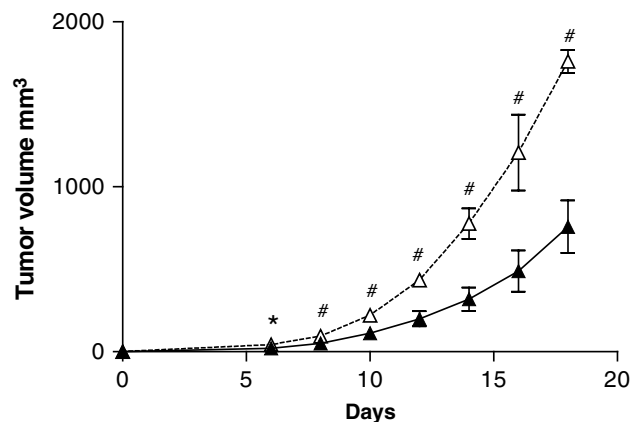


Figure 7 Tumor volumes for control mice in all treatment groups. The formation of tumors with the *GM-CSF* gene ($n=18$) was consistently and significantly suppressed than those without it ($n=12$) over the study period. $*P < 0.05$; $\#P < 0.01$. Symbols are as in Figure 6.

staining for *GM-CSF* protein showed the most intense expression in tumors consisting of CT26/TK/CSF cells, and then in tumors consisting of CT26 + CT26/TK/CSF cells and CT26 + CT26/TK + CT26/CSF cells, in order of decreasing expression (data not shown).

In vivo effects of delivery routes for each gene

Among the mice implanted with tumors expressing both *HSV-TK* and *GM-CSF*, those in group 4 were inoculated with cells that expressed both genes (CT26/TK/CSF) as compared with those in group 5 which were inoculated with a 50:50 mixture of two cell lines (CT26/TK and CT26/CSF), each of which expressed only one of the therapeutic genes. We found that the *HSV-TK* gene in tumors of group 4 appeared to suppress the production of *GM-CSF*. As shown in Figure 4, the mean tumor volume of the GCV-treated arm in group 4 showed a tendency to increase with time, whereas that in group 5 remained suppressed. Although the differences were not statistically significant, the final mean tumor volumes (184 ± 170 vs $112 \pm 70 \text{ mm}^3$) and *T/C* ratio (0.18 vs 0.32) also tended to be smaller in group 4 than in group 5.

Discussion

Many studies have demonstrated the enhanced antitumor efficacy of therapy using a combination of cytokine and suicide-gene (*HSV-TK*) therapy compared with the efficacy of either therapy alone.^{18–20} However, whether the enhanced efficacy results from synergistic effects, such as the enhanced bystander effect of *HSV-TK*, or from the simple additive effects of each therapeutic gene has not been clarified.

We studied the direct suppressive effects of combined *HSV-TK* and *GM-CSF* gene therapy on tumor growth and explored the relative contribution of each gene to the

inhibitory effects. We also assumed that the combined *HSV-TK* and *GM-CSF* efficacy could be resolved into each gene's individual effect, as depicted in Figure 5.

Our results demonstrate that the combined *HSV-TK* and *GM-CSF* gene therapy had a greater antitumor activity compared with therapy using the *HSV-TK* gene alone, but the additional effect was attributable to the independent antitumor activity of *GM-CSF*, and not to the enhanced bystander effect.

Furthermore, our results suggest the possibility that the method of transfer of each therapeutic gene may affect the overall efficacy of combination gene therapy when a suicidal gene such as *HSV-TK* is used.

The mechanism underlying the bystander-killing effect remains to be fully understood, but the theories based on gap junction transmission are getting more persuasive. However, the expression levels of connexin 43 proteins and the extents of the bystander effect are known to be modest in colon cancer cells.⁹ Furthermore, connexin 43 protein was reported to be frequently and specifically mutated in advanced human colon cancer cells.¹⁰ Bystander effects in colon cancer cell lines may therefore be mediated by pathways other than by gap junction intercellular communication, as reported by other groups.²¹ Several studies have suggested the contribution of immunologic mechanisms in mediating the bystander-killing effects in colon cancer cells.^{22,23} We hypothesized that the bystander effect could be enhanced by inducing immunogenicity against the tumor. The *GM-CSF* gene was used because it is known to encode for a cytokine that is highly potent in inducing antitumor immunity. However, we failed to obtain any evidence that the *GM-CSF* enhanced the bystander effect of *HSV-TK* gene expression.

The reported tumor-regressing effects of *GM-CSF* expression vary according to the expression levels and cell lines used.²⁴ Several studies have reported that the antitumor effects or immunity induced by *GM-CSF* are mediated by recruitment of various inflammatory cells.^{25,26} However, the inflammatory cell infiltrate induced by *GM-CSF* expression is known to be dependent on time as well as on the production content of the *GM-CSF* protein: it peaks on days 6–10 and then declines.²⁵ Although we did not quantitatively analyze the recruitment of inflammatory cells histologically, there was no apparent significant difference between *GM-CSF*-producing and non-producing tumors (data not shown). In our study, regardless of the cellular infiltration, the antitumor effect of *GM-CSF* expression independent of *HSV-TK* expression was evident in control mice which were not treated with GCV.

Another potent antitumor cytokine — interferon $\alpha 2a$ — when administered to cells carrying the *HSV-TK* gene, was reported to be able to enhance the bystander effect, but the mechanism of action was also not related to immunological factors.²⁷

In this study, the bystander effects were more potent *in vitro*, in which one tumor cell expressing the *HSV-TK* gene destroyed approximately two neighboring cells, compared with *in vivo*, in which each of two transduced

cells killed only one bystander cell. Taken together with the findings on *T/C* ratios, this makes immunological mechanisms in the mediation of the bystander-killing effect less likely.

In combination gene therapy, the strategies for delivering each gene are speculative, at least if a suicide-inducing gene is included in the combination. The expression of a therapeutic gene contained in the same cell carrying the suicide-inducing gene would also cease when the cell dies following prodrug therapy. In fact, the *HSV-TK* gene has been used as a regulating gene that ablates the production of cytokine or hormones.^{28,29} In our study, mice in group 4 were inoculated with tumors in which both *HSV-TK* and *GM-CSF* genes were expressed in the same cells, as compared with mice in group 5, in which each gene was expressed by different cells. The growth-suppressive effect tended to be more consistent in group 5 than in group 4 (Fig 4). Considering the proportion of *GM-CSF*-expressing cells in group 5 was only 25% compared with 50% in group 4, this tendency was of particular interest. The final tumor volume after treatment also tended to be smaller in group 5 than in group 4, suggesting the persistent antitumor effects of *GM-CSF* expression. Although there was no statistical significance here, these findings suggest that the route of gene delivery might have implications in designing successful combination gene therapy.

In summary, combined *GM-CSF* and *HSV-TK* gene therapy had a greater antitumor efficacy than *HSV-TK* expression alone, but the additive effect was not related to the enhancement of bystander effects. In combination gene therapy employing a suicide-inducing gene, delivering the gene in cells separately from other therapeutic genes might thus help to guarantee the continued activity of the other genes.

Acknowledgments

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