

# Combination Electro-gene Therapy Using Herpes Virus Thymidine Kinase and Interleukin-12 Expression Plasmids is Highly Efficient against Murine Carcinomas *in Vivo*

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We report the use of plasmid DNA-mediated combination gene therapy for tumor-bearing mice using *in vivo* electroporation, also called electro-gene therapy (EGT), that resulted in uncomplicated and complete cures in more than 90% of the mice. Subcutaneously inoculated CT26 tumors in syngeneic BALB/c mice were subjected to repeated EGT treatments consisting of intratumoral co-injection of naked plasmids encoding the cytokine interleukin-12 (IL-12) (p35 and p40 subunits) and the suicide gene herpes simplex virus thymidine kinase (HSV-tk), followed by *in vivo* electroporation. The early anti-tumor effect was always stronger, and the rate of cure, as seen in the long-term follow-up, was always greater in the groups treated with combination EGT than in those treated with IL-12 or HSV-tk EGT alone. Systemic levels of IL-12 and IFN- $\gamma$  increased in both combination and IL-12-alone EGT-treated groups. Moreover, combination EGT for established subcutaneous tumors strongly reduced hematogenous lung metastases and increased survival time when live CT26 tumor cells were injected through the tail vein. Limited experiments on C57/B16 mice with murine melanoma also showed very similar trends. These results suggest that this simple and safe method of plasmid-mediated combination EGT may provide a potentially effective gene therapy for cancer.

**Key Words:** electroporation, electro-gene therapy, HSV-tk, ganciclovir, IL-12, colon cancer, melanoma

## INTRODUCTION

Gene transfer methodology using *in vivo* electroporation (EP) is becoming popular, as its advantages, including simplicity and highly efficient transgene expression, have been reported using marker genes in different types of tissues and organs [1–9]. This method has been found to be a powerful and a useful tool for experiments in developmental biology in which region-controlled, rapid, and high transgene expression are essential [10,11]. Recently, gene therapy by this method has been extensively reported in various organs affected by hereditary disease [12–14] or acquired disease such as malignant tumors [15–34].

We have previously reported a method for transfer of expression plasmids for marker as well as functional

genes into experimental brain tumors and subcutaneous tumors using *in vivo* electroporation, a method we designated as electro-gene therapy (EGT) [15,16,19]. We have also reported that (a) EGT using the herpes simplex virus thymidine kinase (HSV-tk) gene against mouse subcutaneous tumors is highly efficient and the transfer is repeatable and reproducible and (b) the effects of this treatment can be enhanced by repeating EGT [18]. In another report, we have also demonstrated that EGT using plasmids containing subunits of IL-12 is also efficient for the suppression of tumor growth and can induce systemic anti-tumor immunity [24,34].

Complete regression and subsequent cures, however, could not be achieved for those experimental tumors using EGT with a single gene. Tumors regressed following

initial EGT but reappeared after a short period of time, presumably by regrowth from a small nest of the tumor cells that either did not get transduced or, more likely, escaped from the gene transfer site.

We, therefore, planned a combination EGT to obtain stronger anti-tumor activity, using expression plasmids for the HSV-tk and interleukin-12 (IL-12) genes. The EGT using (i) HSV-tk, a suicide gene, can induce destruction of tumor cells in a wide area of tumor tissue and (ii) IL-12 can induce infiltration of T cells and systemic anti-tumor immunity. The suicide gene transfer, followed by ganciclovir (GCV) injection, leads to apoptotic cell death of transferred cells [35] and releases tumor antigens in apoptotic bodies [36], which contributes to the establishment of anti-tumor immunity. Therefore, it is conjectured that combination of these two plasmids can efficiently induce stronger anti-tumor immunity than the use of either molecule as a single agent for treatment of tumors.

The purpose of this study is to test whether combination EGT using HSV-tk and IL-12 genes is more efficient than single-gene therapy alone. We performed the combination EGT to treat mouse subcutaneous (sc)

tumors and explored the underlying physiological mechanisms of combination EGT. In this paper we show that the anti-tumor effects of this method were significantly better than using single-gene EGT and complete regression of tumors was frequently obtained by repeating this procedure. Furthermore, we show that we can establish anti-tumoral immunity by such treatment.

## RESULTS

### Response of Mouse Subcutaneous Tumors to Combination EGT Using Plasmids Encoding Therapeutic Genes

We performed several experiments using *in vivo* combination EGT using the HSV-tk and IL-12 genes to treat subcutaneously inoculated CT26 colon cancer and B16 melanoma tumors. Results are summarized in Table 1. In the case of the EGT with the HSV-tk gene alone, the tumor suppression rate on day 20 was ~47%, data similar to what we have previously reported [18]. There was a significant difference in average tumor volumes between the EGT-treated group (HSV-tk alone, treated twice) and the control

**TABLE 1:** Results of EGT using plasmid DNAs for the therapeutic genes

Experiment	Therapeutic genes and No. of treatments	$n^a$	$V_0^b$ (mm <sup>3</sup> )	$V_{20}^c$ (mm <sup>3</sup> )	% suppression	$P$ value
<i>CT26 colon carcinoma</i>						
1	(tk + IL-12) ×2; days 0, 2	5	45.2	355.3 ± 294.0	82.5	<0.001
	(IL-12 alone) ×2	6		488.8 ± 228.9	75.9	<0.001
	(tk alone) ×2	8		1149.6 ± 335.6	46.7	<0.001
	(K-PBS alone) ×2	8		2030.4 ± 222.6	—	
2	(tk + IL-12) ×4; days 0, 2, 8, 10	10	48.2	202.0 ± 217.3	89.0	<0.001
	(IL-12 alone) ×4	8		522.8 ± 785.4	71.5	<0.001
	(K-PBS alone) ×4	10		1831.8 ± 531.8	—	
3	(tk + IL-12) ×4; days 0, 2, 8, 10	10	45.0	92.5 ± 80.9	94.1	<0.001
	(IL-12 alone) ×4	6		172.3 ± 170.6	89.0	<0.001
	(K-PBS alone) ×4	12		1568.3 ± 510.9	—	
4	(tk + IL-12) ×4; days 0, 2, 8, 10	10	49.9	446.3 ± 246.8	88.2	<0.001
	(IL-12 alone) ×4	10		618.8 ± 355.8	83.6	<0.001
	(K-PBS alone) ×4	8		3766.7 ± 693.4	—	
5	(tk + IL-12) ×3; days 0, 2, 4	7	65.1	267.8 ± 285.4	89.8	<0.001
	(tk + IL-12) ×4; days 0, 2, 8, 10	7		182.2 ± 161.5	93.1	<0.001
	(IL-12 alone) ×4	8		268.5 ± 223.3	89.6	<0.001
	(K-PBS alone) ×4	7		2623.2 ± 935.0	—	
6	(tk + IL-12) ×6; days 0, 2, 4, 6, 8, 10	11	64.9	80.9 ± 45.3	97.2	<0.001
	(tk + IL-12) ×4; days 0, 2, 8, 10	8		157.9 ± 91.5	94.5	<0.001
	(IL-12 alone) ×4	9		304.6 ± 244.6	89.5	<0.001
	(K-PBS alone) ×4	10		2892.5 ± 450.2	—	
<i>B16 melanoma</i>						
7	(tk + IL-12) ×4; days 0, 2, 8, 10	9	119.2	406.2 ± 161.5	92.4	<0.01
	(IL-12 alone) ×4	8		610.1 ± 397.1	88.6	<0.01
	(K-PBS alone) ×4	6		5342.0 ± 2423.0	—	

<sup>a</sup> $n$ , number of tumors.

<sup>b</sup> $V_0$ , average volume of all tumors at day 0.

<sup>c</sup> $V_{20}$ , average volume of tumors at day 20.

<sup>d</sup>SD, standard deviation.

group (K-PBS alone, treated twice) at day 20 ( $P < 0.001$ , tk-alone group of experiment 1 in Table 1). The second gene selected for evaluation was the IL-12 gene, due to its well-known ability to enhance anti-tumor immunity. The tumor suppression effects in the IL-12-alone EGT-treated group were much stronger than those of the HSV-tk-alone group. The average tumor suppression rates on day 20 were between 76 and 90% in all six sets of experiments. However, the effect of combination EGT of HSV-tk and IL-12 was better than that of IL-12 alone: average suppression rates for combination EGT on day 20 were 83 and 94%. Average tumor volumes over time in the four groups of experiment 1 are shown in Fig. 1.

Since the differences in therapeutic effects between the combination and IL-12-alone groups and the group receiving HSV-tk alone were significant, we decided to use the conditions of the first two groups in the following series of EGT experiments.

### Effects of Repeating the Combination EGT Treatments

Since we have observed that the effects of single-gene EGT using the HSV-tk gene were enhanced by repeating the treatment [18,24], we set up one experiment in which the EGT was administered sequentially for a total of six times (experiment 6 in Table 1). Average tumor volumes of the four groups in experiment 6 are presented

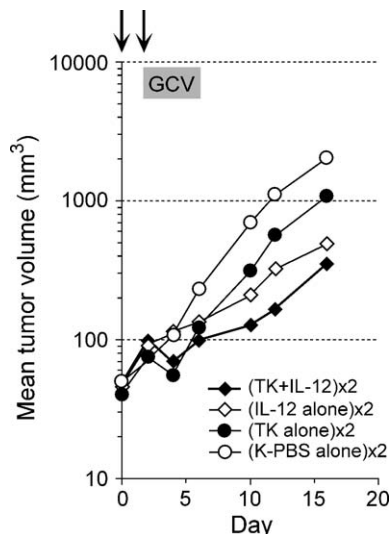


FIG. 1. The average tumor volumes of CT26 subcutaneous tumors following EGT treatment with IL-12 alone or with combination of IL-12 and HSV-tk genes. The symbols represent means. The plasmids pCAGGS/IL12p40 and pCAGGS/IL12p35 (for IL-12 alone) or pCAGGS/IL12p40, pCAGGS/IL12p35, and pCAGGS/TK (for combination) were injected into CT26 tumors by *in vivo* EP on days 0, 2, 8, and 10 in the treatment group, and K-PBS was injected on days 0, 2, 8, and 10 in the control group. GCV injections (3 mg/100 g bw  $\times$ 2/day ip) were administered to mice in all groups from day 2 and continued for 3 days after each gene transfer or each sham procedure. Arrows indicate electro-gene transfer or mock procedures.

in Fig. 2A. The growth curves of treated sc tumors show that the suppression effect of EGT using IL-12 was continuous after treatment, whereas the corresponding effect of HSV-tk EGT lasted for only 3 days after each treatment. The effects of combination EGT were additive for the two genes. Growth curves of each tumor in experiment 6 in Fig. 2B demonstrate that regression of tumors started after some time lag following the end of treatment. Ten of 11 tumors that received combination EGT six times were completely eradicated by day 89. In experiments 4 and 6, all animals were observed at least until day 100. The complete regression rates are presented in Table 2. We observed significant prolongation of survival time in the animals in the combination EGT-treated group in the tumor model we used (Fig. 2C).

### Production of IL-12 and IFN- $\gamma$ in the Mice Treated with Combination EGT

To confirm the transgene expression of plasmids containing IL-12 subunits, we determined the concentration of IL-12 p70 in serum and tumor tissue by the ELISA method. We could not detect the production of IL-12 protein in tumor tissue from the HSV-tk-alone and control groups (K-PBS injection and EP), but in the groups of IL-12 alone and combination EGT, we detected significantly elevated levels of IL-12 p70 on days 5 (data not shown) and 10 (Fig. 3A).

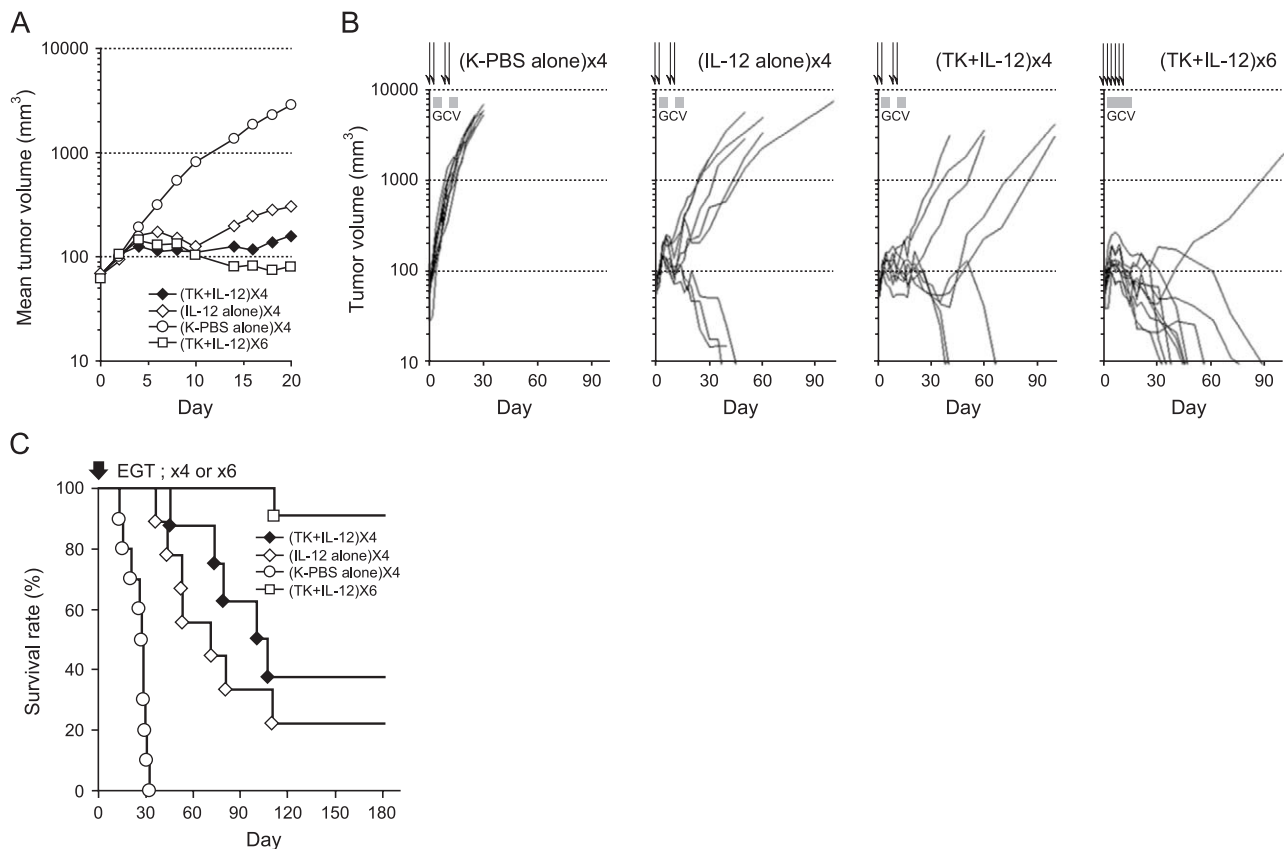
The concentration of serum IL-12 p70 in the HSV-tk group was transiently elevated on day 5 after treatment (data not shown) and then declined on day 10. In the case of IL-12-alone and combination EGT, high levels of IL-12 p70 protein remained on days 10 and 20 (data not shown). We observed the same trend for serum IFN- $\gamma$ .

### Effect on Melanoma Model

To test whether this treatment modality is specific to CT26 tumor, we applied the same method to the B16 melanoma tumor model. With the melanoma model the treatment was just as successful as with the colon tumor (Fig. 4 and Table 1). One notable difference is that the average size of tumors at the initial treatment was significantly larger than that of the colon tumor model. In the previous report, we observed that the success of EGT treatment using the HSV-tk gene in the CT26 tumor model was dependent on the initial size of the tumors. When the initial volume was larger than 80 mm<sup>3</sup>, the effect of EGT was significantly reduced [18]. However, the suppression rate on day 20 for the B16 melanoma was more than 90%, even though the initial volume was over 110 mm<sup>3</sup>. This difference may possibly be attributed to the difference in antigenicity of the tumor cells.

### Establishment of Systemic Anti-tumor Immunity by Combination EGT Using IL-12 and HSV-tk Genes

To test the establishment of systemic anti-tumor immunity by our combination EGT, we rechallenged mice



**FIG. 2.** Effects of repeated EGT treatments on tumor growth. (A) The treatment groups with IL-12 alone and combination of IL-12 and HSV-tk received four EGT procedures (on days 0, 2, 8, and 10), and the control group received four mock procedures of K-PBS injection with *in vivo* EP (days 0, 2, 8, and 10). Another combination group received six EGT procedures (on days 0, 2, 4, 6, 8, and 10). All mice in these four groups received ip injection of GCV (3 mg/100 g bw  $\times$ 2/day) from day 2 and it was continued for 3 days after the gene transfer or the sham procedure. Arrows indicate electro-gene transfer or mock procedures. The symbols represent means. (B) Growth curves of individual tumors in the four treatment groups in A. (C) The survival curves of treated mice in the four groups in A. All mice in the control group were dead by day 32.

that were cured of CT26 tumors by combination EGT in experiment 6 by inoculating  $1 \times 10^5$  CT26 cells into the contralateral flank. Three of the four rechallenged mice completely rejected the inoculated cells, whereas all the naïve mice that received inoculations of the same number of cells, prepared at the same time, rapidly developed subcutaneous tumors (Fig. 5A).

To confirm the establishment of anti-tumor immunity, we performed another set of experiments. We injected CT26 cells into the tail vein of sc tumor-bearing mice and naïve mice and compared the number of metastatic lesions in lung tissue in three groups: the naïve group, the sham-treated group, and the combination-EGT group treated for sc tumors. We observed many metastatic foci in naïve mice on day 10 (Fig. 5B). Fewer foci were observed in sham-treated mice (PBS injection and EP) (Fig. 5B). This may be due to partial establishment of systemic immune response to the tumor cell by the exposure to tumor antigens through the procedures

for inoculation, mock EGT treatment, and excision of residual tumor mass on day 10. We observed no metastatic foci by day 10 in mice that received combination EGT (Fig. 5B). The number of metastatic foci on day 10 in each group is indicated in Fig. 5C. Residual sc tumors on day 10 in sham-treated and EGT-treated groups were resected to compare the survival times for systemic metastasis. All naïve mice with metastatic lesions died by day 28. The mean survival time was  $26 \pm 0.4$  days. Sham-treated mice had longer survival times (mean  $39.0 \pm 2.8$  days) compared to naïve mice. Only 60% of the mice in the combination EGT group died from metastatic lesions by day 100 (Fig. 5D).

## DISCUSSION

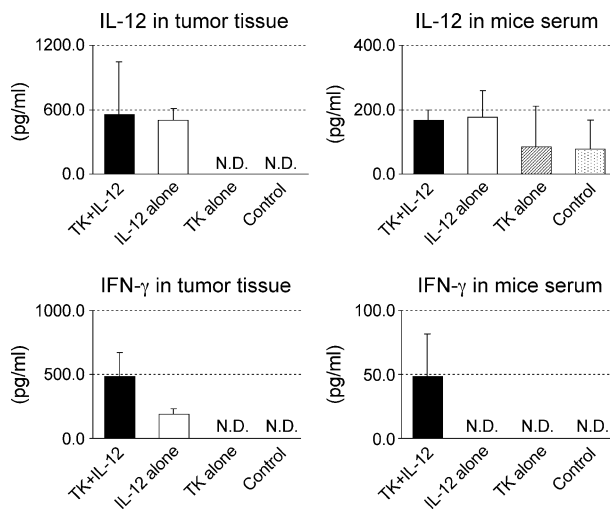
In this study, we have demonstrated that combination EGT using the HSV-tk and IL-12 genes is more efficient for tumor suppression/regression than single-gene EGT

**TABLE 2:** Rates of complete regression of tumors by combination EGT at day 100

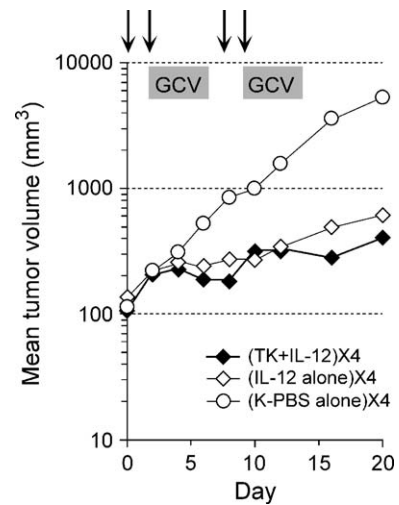
Combination EGT group	Ratio of complete regression at day 100	
(IL-12 alone) ×4; days 0, 2, 8, 10		
Expt 4	20.0%	(2/10)
Expt 6	22.2%	(2/9)
Total	21.1%	(4/19)
(tk + IL-12) ×4; days 0, 2, 8, 10		
Expt 4	40.0%	(4/10)
Expt 6	37.5%	(3/8)
Total	38.9%	(7/18)
(tk + IL-12) ×6; days 0, 2, 4, 6, 8, 10		
Expt 6	90.9%	(10/11)

using either HSV-tk or IL-12 alone. When the combination EGT was administered sequentially six times, complete regression could be obtained in most of the treated animals. The establishment of anti-tumor immunity in those animals with regression was demonstrated.

The efficient anti-tumor action of this method was most probably caused by the combined synergistic effects of the two therapeutic genes. Each gene used in this study has been demonstrated to have strong anti-tumor effects. The HSV-tk-GCV approach is one of the most frequently used methods for anti-tumor gene therapy. By using this method, not only do cells incorporate the HSV-tk gene



**FIG. 3.** Production of IL-12 and IFN- $\gamma$  in treated tumor tissues and serum of treated mice on day 10. Expression levels of IL-12 and IFN- $\gamma$  proteins in sc tumor and serum samples were detected and quantified using mL-12p70 and mIFN- $\gamma$  ELISA kits, respectively (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Tumor lysates were prepared from the sc CT26 tumors and serum was obtained from blood samples on day 10 after two EGT treatments or sham procedures on days 0 and 2 followed by GCV injection for 3 days.



**FIG. 4.** The average tumor volumes of B16 subcutaneous tumors following EGT treatment with IL-12 alone or with combination of IL-12 and HSV-tk genes. The symbols represent means. The plasmids pCAGGS/IL12p40 and pCAGGS/IL12p35 (for IL-12 alone) or pCAGGS/IL12p40, pCAGGS/IL12p35, and pCAGGS/TK (for combination) were injected into B16 tumors by *in vivo* EP on days 0, 2, 8, and 10 in the treatment group, and K-PBS was injected on days 0, 2, 8, and 10 in the control group. GCV injections (3 mg/100 g bw ×2/day ip) were administered to mice in all groups from day 2 and continued for 3 days after the gene transfer or the sham procedure. Arrows indicate electro-gene transfer or mock procedures.

but also other tumor cells in the vicinity of those cells can be killed efficiently by the “bystander effect.”

The major mechanism of this bystander effect is the transfer of phosphorylated GCV via gap junctions [37,38]. IL-12 has also been reported to have strong anti-tumor activity by stimulating the immune system of the host [39,40]. There have been many cancer treatment studies using purified IL-12 and viral vectors producing IL-12 [39–43]. We have also demonstrated the significant anti-tumor activity of single-gene EGT using expression plasmids for IL-12 subunits for the same animal model used in this report [24]. In this model, we showed that we could establish anti-tumor immunity following treatment. Some other groups have reported that the same type of IL-12 EGT was successful in several kinds of tumors such as a hepatocellular carcinoma model [23], 38C13 B cell lymphoma [44], a murine squamous cell carcinoma model [29], and B16.F10 melanoma [26,31]. In the case of the melanoma model, Lucas and Heller demonstrated that administration of a plasmid encoding IL-12 by electroporation has a therapeutic effect on primary tumors as well as on distant tumors and metastases [31].

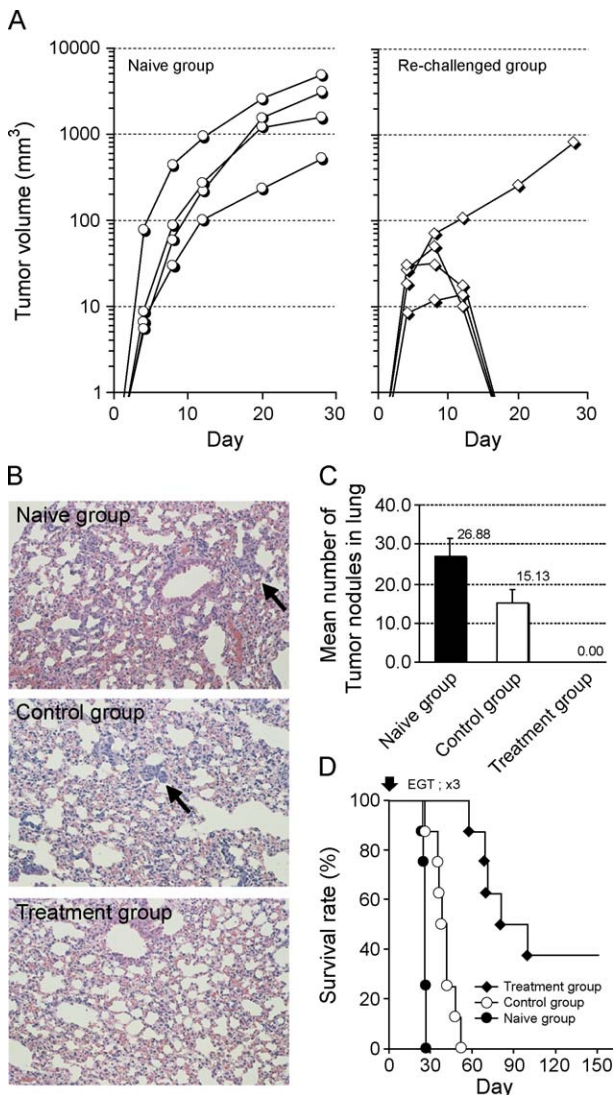
One of the major effects of combining these two genes (HSV-tk and IL-12) is enhancement of the bystander effect of the HSV-tk treatment. Recently, it has been shown that the bystander effect is not limited to cells within the same tumor but, as mentioned earlier, it can

also occur at a distance [45]. Several studies have reported that the immune system participates in the bystander effect following HSV-tk gene therapy [46,47]. For example, several groups highlighted the failure of immunodeficient or sublethally irradiated mice to reject HSV-tk-transfected tumors after GCV treatment [46,48]. Cell death by the HSV-tk-GCV approach is reported to be via an apoptotic process. It has been reported that apoptotic tumor cells can be a source of the specific anti-tumor immune response *in vivo* [36]. Shibata *et al.* also demonstrated the massive apoptotic cell death in chemically induced rat urinary bladder carcinomas following *in situ* HSV-tk electro-gene transfer [35]. As the number of those cells increases, the anti-tumor effect is enhanced [49]. When antigens are released from the tumor that has been destroyed by the HSV-tk gene therapy, simultaneous local expression of appropriate cytokines appears to enhance

the anti-tumor immune response [50,51]. Indeed, a few studies have demonstrated that the efficacy of such gene therapy is improved by simultaneous administration of the IL-2 gene or by a combination of IL-2 and GM-CSF genes [52,53].

In contrast, in the case of anti-tumor gene therapy using the IL-12 gene, antigenicity of targeted tumor cells influences the establishment of anti-tumor immunity through the biological effect of IL-12 [54,55]. Production of a wide variety of proteins, which can be processed as antigens of target tumor cells by HSV-tk gene therapy inside the tumor tissue, enhances the establishment of anti-tumor immunity by IL-12. In this report, we have demonstrated that our method of combining EGT with administration of the two genes can establish anti-tumor immunity in treated animals, using two experimental models of rechallenge and hematogenous metastasis. Therefore, the rationale of using these genes simultaneously and delivered as plasmids with *in vivo* EGT seems justified.

Although this is the first report on plasmid-mediated combination gene therapy using the HSV-tk and IL-12 genes, such a combination using viral vectors has been reported by several groups. Two groups reported such therapy using an adenoviral vector (Av) for an orthotopic model of prostate cancer [56,57]. Although both groups demonstrated an increased efficiency in local tumor control by combination, Nasu *et al.* did not observe suppression of metastasis or increased survival [56]. Interestingly, Hall *et al.* demonstrated the increased expression of Fas and FasL in tumor cells as the mechanism of enhanced apoptosis in tumor site [57]. Similarly, Drozdovik showed that a combination therapy



**FIG. 5.** Establishment of systemic anti-tumor immunity by combination EGT. (A) Growth curves of individual tumors in rechallenge group and naive group. Long-term (>60 days) survivors of combination EGT ( $n = 4$ ) were given a second sc injection of  $1 \times 10^5$  CT26 parental tumor cells into the contralateral flank. Naive mice ( $n = 4$ ) received sc injections of the same number of CT26 tumor cells into the left flank. (B) Histological analysis of hematogenous metastatic model of mouse lungs. Hematogenous metastatic lesions were created by injecting CT26 tumor cells into tail veins of sc tumor-bearing mice and naive mice. On day 0, when the CT26 sc tumors were established and had grown to a mass of approximately 4 to 5 mm in diameter, mice in the treatment group ( $n = 12$ ) and in the control group ( $n = 12$ ) were given tail vein injections of  $1 \times 10^5$  CT26 tumor cells in 100  $\mu$ l saline. At the same time mice in the naive group ( $n = 12$ ) were given injections of CT26 parental tumor cells in the same manner. EGT treatments or sham procedures for established sc tumors were given three times, on days 0, 1, and 2, in the same manner. Four mice in each group were sacrificed on day 10 for histopathological analysis of lung tissue. Lungs were fixed in 10% neutral-buffered formalin overnight, cut into 10- $\mu$ m diameter slices, and stained with hematoxylin and eosin. Arrows indicate microtumor nodules of metastatic tumor. (C) The average number of microtumor nodules in mouse lung tissues. The average total numbers of established microtumor nodules in 20 sections from each lung tissue of four sacrificed mice in the three groups are presented. (D) The survival curves of hematogenous metastasis model animals. Eight mice in each group were kept for observation of survival time.

using an Av system is more effective for the treatment of established hepatocellular carcinoma (HCC) than therapy with a single vector [58]. However, the treatment was not repeated and complete regression in the subcutaneous model of HCC was observed in only very few cases. Using retrovirus-mediated gene therapy, Lechanteur *et al.* were able to obtain a highly significant increase in survival time in their peritoneal dissemination model when IL-12 and HSV-tk or GM-CSF and HSV-tk were combined. By repeating intraperitoneal injections three times, they obtained complete regression in two of the five treated animals [59].

One reason for the fact that our method shows a higher rate of complete regression may be that our EGT is repeated for a total of six times. This repeatability is possible because the procedure is easy to implement and because of the lower antigenicity of the vectors used. Some specific sequences in plasmid DNA have been reported to have antigenic properties [60,61]. However, it is far less than that of viral vectors. In our previous report on EGT using a single expression plasmid for the HSV-tk gene, we demonstrated that each treatment had significant effects even when EGT was repeated after intervals of varying lengths [18].

No major side effects of our EGT have been observed. Other reports using *in vivo* EP, including our previous studies, also support the safety of this method [18,19,23,24,34]. The clinical application step of inserting electrodes into the human body and application of *in vivo* electroporation has been already approved in electrochemotherapy (ECT). The results of phase I/II clinical trials of ECT for head and neck cancer patients using *in vivo* EP in many institutions have shown highly significant clinical responses [62,63]. We feel that the number of repeated treatments could be reduced by improving the efficiency of the gene transfer process. Harrison *et al.* reported that *in vivo* electroporation with their electrical and pulsing conditions was approximately equivalent to an adenovirus dose of  $10^6$  transductions evaluated by the percentage of heart tissue of chick embryos expressing green fluorescent protein [64]. This efficiency can be improved by optimizing the parameters of *in vivo* EP, such as voltage, current, number of pulses, and interval between the pulses for each tumor tissue, and by development of specialized electrodes.

In practical clinical situations of cancer treatment, we frequently experience unresectable large tumors with established multiple distant metastatic lesions. In such cases, it is impossible to deliver genes or anti-tumor agents to all tumor cells using present technology, including viral vectors. Therefore, it is desirable to stimulate and enhance immune responses of the host to obtain complete regression of those lesions. Our method of combining the biological effects of two kinds of genes, providing tumor antigens via destruction of tumor cells by a suicide gene and enhancing establishment of anti-

tumor immunity by simultaneous expression of a cytokine gene, all in a repeatable process, seems to hold promise for application in the clinic.

## MATERIALS AND METHODS

**Plasmids.** The HSV-tk gene was cut from pCEP/TK [18] by *XhoI/NotI* digestion, and murine IL-12 p40 and p35 genes were obtained by PCR using a spleen cDNA library (Clontech, Palo Alto, CA, USA). We constructed plasmids pCAGGS/TK, pCAGGS/IL12p40, and pCAGGS/IL12p35, henceforth to be written as ptk, p40, and p35, respectively, by inserting those fragments of the HSV-tk gene and the murine IL-12 p40 and p35 genes into the unique *XhoI/NotI* sites of the expression plasmid pCAGGSneo containing the CAG promoter. All plasmids were extracted from *Escherichia coli* and purified using the Qiagen Plasmid Mega Kit (Qiagen, Inc., Valencia, CA, USA). The concentration of the plasmids in stock was adjusted to 2.0 mg/ml by dilution with K-PBS (NaCl 30.8 mM, KCl 120.7 mM, Na<sub>2</sub>HPO<sub>4</sub> 8.1 mM, KH<sub>2</sub>PO<sub>4</sub> 1.46 mM, and MgCl<sub>2</sub> 10 mM in distilled water) [18,24,34].

**Animal model and tumor cell line.** We performed mouse studies in accordance with the institutional guidelines of the Laboratory Animal Research Center of Kumamoto University School of Medicine. Male 8-week-old BALB/c mice and C57/B16 mice (Japan SLC, Inc., Sizuoka, Japan), weighing approximately 30 g, were used in this study. The mice were maintained in a specific-pathogen-free environment and fed sterile laboratory pellets and water. We used CT26 murine colon adenocarcinoma [65] and B16 murine melanoma cell lines (Japan Health Sciences Foundation, Osaka, Japan) in this study. The CT26 and B16 cells were maintained in RPMI 1640 and DMEM, respectively, containing penicillin (100 units/ml), streptomycin (100 µg/ml), L-glutamine (4 mg/ml), and 10% FBS (v/v) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. The procedure for making the mouse sc tumor model has been described previously [18,24,34]. When the sc tumors grew to &ndash;5 mm diameter in size, the mice were randomized into experimental groups and EGT treatment was started.

**Procedure for EGT and measurements of tumor growth.** The details of the EGT procedure have been published previously [18]. Briefly, for the transfer of plasmid DNAs, 50 µg of each plasmid DNA in a total of 100 µl of K-PBS was injected into the tumor using a 27-gauge needle. Five minutes after the plasmid injection, the tumor was pulsed using a T830 square-wave electroporator (BTX, a Division of Genetronics, Inc., San Diego, CA, USA) fitted with a 0.5-cm-diameter probe with an array of six needle electrodes. A one-needle clockwise rotation was continued with each pulse until eight pulses were delivered [66]. We set four treatment groups as follows, tk + IL-12, IL-12 alone, tk alone, and PBS alone. In the tk + IL-12 group, a mixture of three plasmids (ptk, p40, and p35) was injected into the tumors. For the IL-12-alone group, only p40 and p35 were injected. For the tk-alone group, only ptk was injected. For the group PBS alone, no plasmid was injected. In each experiment, there were between 5 and 12 mice per group. EGT was administered to tumor-bearing mice twice (on days 0 and 2), or four times (on days 0, 2, 8, and 10), or six times (on days 0, 2, 4, 6, 8, and 10).

Since our previous data indicated that the tumor suppression effect of HSV-tk-EGT does not last more than 5 days after the EGT procedure [18], we administered GCV for 3 days. With one EP (electroporation treatment) on day 0 ( $d = 0$ ), the start day, GCV (3 mg/100 g bw  $\times$  2/day ip) (GCV was a gift from F. Hoffmann Roche Ltd., Basel, Switzerland) was administered on  $d = 2, 3$ , and 4. Similarly, for two EP treatments on  $d = 0$  and  $d = 2$ , GCV was given as before and in addition on  $d = 4, 5$ , and 6. For four treatments, the EP treatment was on  $d = 0, 2, 8$ , and 10, while the corresponding GCV injections were identical to those of  $d = 0$  and  $d = 2$ , as above, and  $d = 10, 11$ , and 12 after EP on  $d = 8$ , and  $d = 12, 13$ , and 14 after  $d = 10$ .

The volume of the tumors was calculated by measuring tumor diameters with a caliper every 2 days, using the formula  $V = a \times b^2/2$ ,

where  $V$  is the volume,  $a$  is the larger diameter, and  $b$  is the smaller diameter. The statistical  $P$  value was determined using the unpaired  $t$  test from data of average tumor volume on day 20 in each of the experimental groups. Animals were maintained for observation after measurement of tumor size. Mouse survival was analyzed using a Kaplan–Meier survival plot followed by a log-rank (Mantel–Cox) test.

**Rechallenge of CT26 cells in EGT-treated mice.** Long-term (>60 days) survivors of combination EGT ( $n = 4$  mice), showing complete regression of the CT26 tumor, were given a second sc injection of  $1 \times 10^5$  CT26 parental tumor cells into the contralateral flank. Naïve mice (same age but no treatment) received sc injections of the same number of CT26 tumor cells into the left flank. These rechallenged mice and naïve mice were kept under observation for sc tumor growth.

**Measurement of IL-12 and IFN- $\gamma$  protein levels.** Expression levels of IL-12 and IFN- $\gamma$  proteins in sc tumor and serum samples were detected and quantified using mIL-12p70 and mIFN- $\gamma$  ELISA kits, respectively (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Tumor lysates were prepared from the sc CT26 tumors and serum was obtained from blood samples 10 days after two EGT treatments or sham procedures on days 0 and 2 following GCV injection.

**Hematogenous lung metastasis model.** We performed another set of experiments to confirm the establishment of systemic anti-tumor immunity. Hematogenous metastatic lesions were created by injecting CT26 tumor cells into tail veins of sc tumor-bearing mice and naïve mice. We set three treatment groups as follows: control group, naïve group, and treatment group. On day 0, when the CT26 sc tumors were established and had grown to a mass of approximately 4 to 5 mm in diameter, mice in the treatment group (receiving combination EGT treatment with HSV-tk and IL-12) and in the control group (receiving K-PBS sham procedure) were given tail vein injections of  $1 \times 10^5$  CT26 tumor cells in 100  $\mu$ l saline. At the same time mice in the naïve group were given injections of CT26 parental tumor cells in same manner as the other two groups. EGT treatment or sham procedures for established sc tumors were given three times, on days 0, 1, and 2, in the same manner. Ten days after the first treatment, all residual sc tumors in both the treatment group and the control group were surgically resected to eliminate the influence of sc tumors on the survival of those mice. Four mice in each group were sacrificed on day 10 for histopathological analysis of lung tissue. Lungs were fixed in 10% neutral-buffered formalin overnight, cut into 10- $\mu$ m slices, and stained with hematoxylin and eosin. We analyzed 20 sections per mice microscopically, looking for evidence of establishment of microtumor nodules, which were counted. Of these, eight mice in each group were maintained for observation of survival time.

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