

SUICIDE GENE THERAPY USING ADENOVIRUS VECTOR FOR HUMAN ORAL SQUAMOUS CARCINOMA CELL LINE *IN VITRO*

NORIYUKI YAMAMOTO^a, YASUSHI HAYASHI^a, HIDEAKI KAGAMI^a, TAKAFUMI FUKUI^a, HIROKAZU FUKUHARA^a, IWAI TOHNAI^a, MINORU UEDA^a, MASAAKI MIZUNO^b and JUN YOSHIDA^{c*}

^aDepartment of Oral and Maxillofacial Surgery, Nagoya University Graduate School of Medicine

^bDepartment of Molecular Neurosurgery, Nagoya University Graduate School of Medicine

^cDepartment of Neurosurgery, Nagoya University Graduate School of Medicine
65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan

ABSTRACT

Recently, suicide gene therapy using the herpes simplex virus thymidine kinase (HSV t_k) gene followed by ganciclovir (GCV) administration was evaluated for the treatment of cancer. The purpose of this study was to investigate the effectiveness of suicide gene therapy using the replication-deficient recombinant adenovirus vector for human oral squamous carcinoma cell lines. To evaluate transduction efficiency, each cell line was transduced *in vitro* with an adenovirus vector containing the β -galactosidase gene. By 24 hours after transduction, nearly 100% of the cells were transduced at a multiplicity of infection (MOI) of 10, and from 30 to 10% at an MOI of 1. Next, each cell line was transduced with an adenovirus vector containing the HSV t_k gene, and a subsequent administration of GCV for the assessment of suicide gene therapy. A subsequent administration of GCV resulted in complete tumor cell death. In addition, we conducted a morphological analysis of that cell death using video-enhanced contrast differential interference contrast microscopy, and we observed that it included both apoptosis and necrosis after HSV t_k gene and GCV treatment. These results suggest that adenovirus-mediated suicide gene therapy induced remarkable cytotoxicity with a bystander effect in human oral squamous cell carcinoma thus suggesting an effective treatment strategy for that tumor.

Key Words: Squamous Cell Carcinoma, Adenovirus, Herpes Simplex Virus Thymidine Kinase Gene, Ganciclovir, Bystander effect

INTRODUCTION

Patients with advanced oral squamous cell carcinoma have a poor prognosis, even given the current standard treatment modalities of surgery, radiation, and chemotherapy, either alone or in combination. Surgical resection is often accompanied with functional deficits in speech and swallowing, as well as significant cosmetic deformity. Chemotherapy often causes side effects such as gastrointestinal, bone marrow, or renal toxicity. Radiotherapy results in tissue necrosis, inflammation, pain, and xerostomia. In addition, conventional palliative treatments are often toxic and sometimes ineffective.¹⁾ For these reasons, new strategies are needed both to improve the

Address correspondence to: Noriyuki Yamamoto, Department of Oral and Maxillofacial Surgery, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan.
Phone: +81-52-744-2348 FAX: +81-52-744-2352 E-mail: noriyuki@med.nagoya-u.ac.jp

survival rate and to reduce complications.

Expectations for new treatment strategies have thus focused on gene therapy. Suicide gene therapy is one strategy for the treatment of cancer.²⁾ In this strategy, viral vectors containing metabolic enzyme genes infect the target cells, and enzyme expression converts an inactive pro-drug to a toxic product. Upon viral transduction, the toxic product then selectively kills target cells. One such prodrug system has involved the introduction of herpes simplex virus thymidine kinase gene (HSV \underline{tk}) into tumor cells followed by the administration of ganciclovir (GCV). This system has already entered clinical trials for the treatment of brain tumors.³⁾

Previous studies on suicide gene therapy have concentrated on a gene delivery system with retrovirus^{4,5)} or adenovirus vectors.^{6,7)} Although retrovirus-mediated gene transfer is ideal for many *ex vivo* applications of gene therapy, this delivery system with retrovirus vector poses a number of problems *in vivo*. In using the retrovirus vector involves only actively dividing cells can be introduced, and the viruses are rapidly inactivated in the blood. Compared with the retrovirus vector, the advantages of the adenovirus vector include its ability to transduce both dividing and non-dividing cells, its physical stability and high transduction efficiency, and the existence of efficient protocols for producing clinical-grade material at high concentrations.⁸⁾

In this article, we evaluated the efficacy of suicide gene therapy using adenovirus-mediated transduction of HSV \underline{tk} gene and the subsequent administration of GCV in four oral squamous cell carcinoma cell lines *in vitro*. Additionally, cell death was analyzed using video-enhanced contrast differential interference contrast microscopy.

MATERIALS AND METHODS

Cell culture

We used four human oral squamous carcinoma cell lines, i.e., SAS, HSC-2, HSC-3 and HSC-4, obtained from the Cancer Cell Repository of Tohoku University. All cell lines were maintained in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum in a 5% CO₂-incubator at 37°C and passaged when the culture plates were 75–90% confluent.

Construction of recombinant adenovirus vectors

The replication-deficient recombinant adenovirus (AxCALacZ) was a gift from Izumu Saito (Institute of Medical Science, Tokyo Univ.). The AxCALacZ was constructed with human adenovirus type 5 by replacing the E1A and E1B genes with *Escherichia coli* lacZ gene under transcriptional control of the CAG promoter (the cytomegalovirus immediate early enhancer, modified chicken β -actin promoter, and rabbit β -globin polyadenylation signal) as described elsewhere.^{9,10)} A recombinant replication-deficient adenovirus vector containing the HSV \underline{tk} gene under transcriptional control of the CAG promoter (AxCAHSV \underline{tk}) was similarly constructed. Each recombinant adenovirus vector was propagated with 293 cells, human embryonic kidney cells transformed by the E1A and E1B genes, and purified by double cesium gradient ultracentrifugation. The concentrated virus was dialyzed, aliquoted, and stored at –80°C. The viral titer was determined by plaque assay.

Transduction efficiency

Twenty thousand cells were inoculated into each well of an 8-well glass slide (Nalge Nunc International, USA) with 500 μ l of medium. Twenty-four hours later, the medium was changed, AxCALacZ vectors were added to each well at varying multiplicities of infection (MOI; for the purposes of this study the number of vector genomes per target cell), and incubation was

continued for another 24 hours. The viral suspension was then aspirated, the cells were washed twice with phosphate buffered saline (PBS), and were then fixed with 2.5% glutaraldehyde in PBS for 15 minutes at 5°C. Next, a solution of X-gal (5-bromo-4-chloro-3-indolyl β -D-galactosidase, Wako Pure Chemical Industries, Ltd.) in PBS was added to each well. The cells were inoculated for 24 hours at room temperature. For each well, 500 cells were counted by light microscopy, and the percentage of cells with blue stained nuclei was determined.

Cytotoxicity studies

Experiments for toxicity of the recombinant adenovirus vector were performed in 24-well plates (Becton Dickinson, USA) at a density of 5×10^4 cells per well with 1 ml of medium. Twenty-four hours later, the medium was changed, AxCAHSV \underline{tk} vectors were added to each well at varying multiplicities of infection, and incubation continued for another 24 hours. The virus-containing medium was then aspirated, and replaced with fresh medium. Numbers of living cells were counted 96 hours after AxCAHSV \underline{tk} vector infection, using the trypan blue dye exclusion method. Next, experiments for the toxicity of GCV were performed similar to the experiments with adenovirus vector. Twenty-four hours later, the medium was changed, and incubation continued for another 24 hours. The medium was then removed, and the various concentrations of GCV were added with the fresh medium. Numbers of living cells were counted 72 hours after GCV administration, using the trypan blue dye exclusion method.

Effects on cancer cell death

Fifty thousand cells were inoculated into each well of a 24-well plate (Becton Dickinson, USA) with 1 ml of medium. Twenty-four hours later, the medium was changed, AxCAHSV \underline{tk} vectors were added to each well at varying multiplicities of infection, and incubation continued for another 24 hours. Ten μ g of GCV (Denosine; Roche, Switzerland) was added to each well, and PBS was added to the control plates. Numbers of living cells were counted 72 hours after GCV administration, using the trypan blue dye exclusion method.

Analysis of morphological changes

We plated 2×10^4 cells/ml of the SAS line into a glass bottomed culture dish (Mat Tec Corporation, USA) and incubated a 5% CO₂-incubator at 37°C for 24 hours. Then the medium was changed, the cells were infected with AxCAHSV \underline{tk} vectors at an MOI of 10, and incubation continued for another 24 hours. Ten μ g/ml of GCV was added to the dish. The cells were cultured for 72 hours and observed under video-enhanced contrast differential interference contrast (VEC-DIC) microscopy (AG-7750, Panasonic, Osaka, Japan).

RESULTS

In vitro efficacy of adenovirus vector transduction into human oral squamous carcinoma cell lines

Transduction efficiency in four human oral squamous carcinoma cell lines (SAS, HSC-2, HSC-3 and HSC-4) was determined using an adenovirus vector containing the β -galactosidase gene. The transduced HSC-4 cells were stained blue by X-gal staining (Fig. 1). By 24 hours after transduction, nearly 100% of all cells were transduced at a multiplicity of infection of 10. Transduction efficiencies at an MOI of 1 were $27.7 \pm 2.6\%$ on SAS, $13.0 \pm 2.0\%$ on HSC-2, $12.0 \pm 1.0\%$ on HSC-3, and $14.8 \pm 2.5\%$ on HSC-4 (Fig. 2). No evident toxicity was detected at the AxCA \underline{LacZ} vector concentration mentioned above.

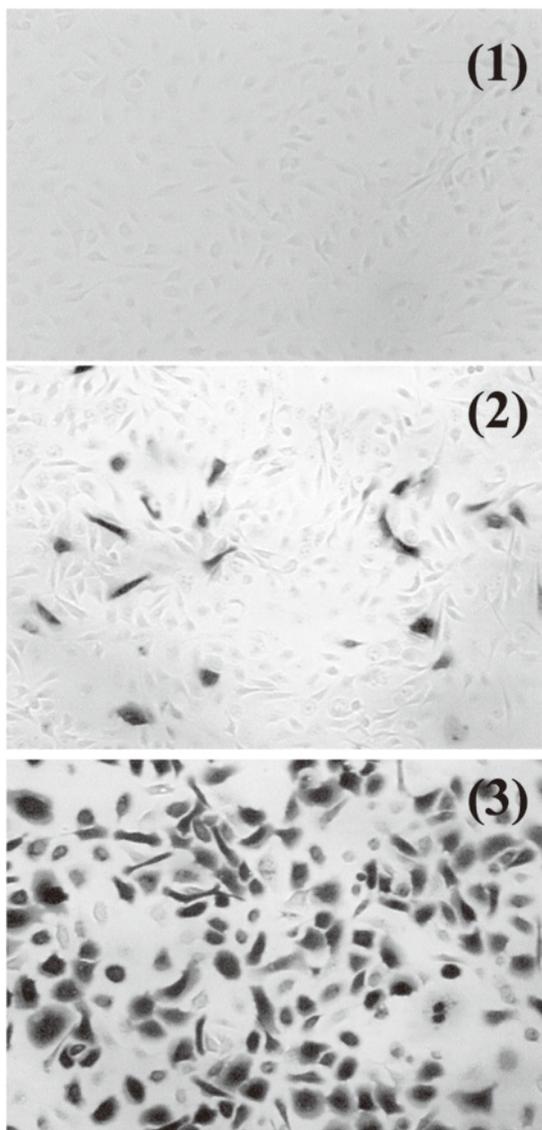


Fig. 1 Light microscopic assay for expression of lacZ gene. Human squamous carcinoma cell line HSC-4 was transduced with AxCALacZ, incubated for 24 hours, then stained for β -galactosidase activity with X-gal. (1) Control cells (MOI: 0) show no positive staining. (2) Transduced cells show 30% positive nuclear staining at MOI of 1. (3) Transduced cells show nearly 100% positive nuclear staining at MOI of 10.

In vitro transduction of human oral squamous carcinoma cell lines with AxCAHSVtk

Four human oral squamous carcinoma cell lines were transduced with adenovirus vector containing the HSVtk gene. Figure 3 shows the cell-survival percentage of each cell line transduced at varying multiplicities of infection with AxCAHSVtk. As compared with control, AxCAHSVtk showed toxicity in all cell lines at an MOI of more than 100 ($p < 0.01$), but there was no evident toxicity at an MOI of 10 or less. Next, the cell toxicity to GCV was evaluated in all cell lines,

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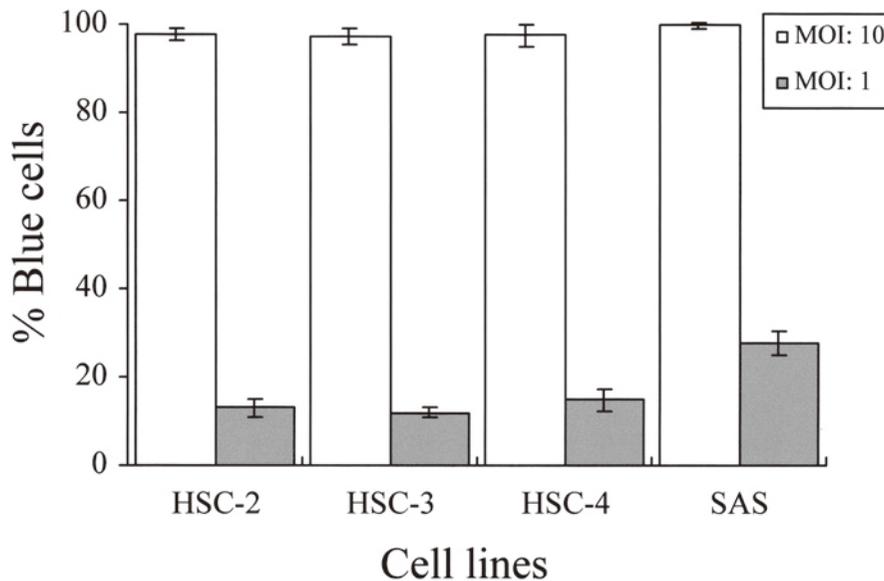


Fig. 2 Transduction efficiency in human oral squamous carcinoma cell lines SAS, HSC-2, HSC-3 and HSC-4 *in vitro* using recombinant replication-deficient adenovirus vector containing lacZ gene (AxCALacZ). By 24 hours after transduction, nearly 100% of all cells were transduced at MOI of 10, and from 10 to 30% at MOI of 1. Each point represents the mean \pm SD.

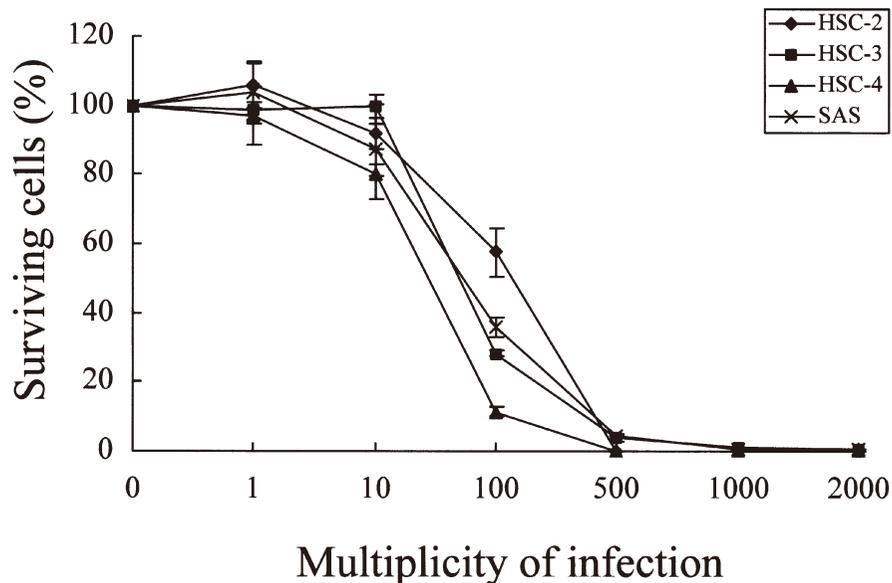


Fig. 3 *In vitro* toxicity study of recombinant adenovirus vector (AxCAHSVtk) in (-♦-) HSC-2, (-■-) HSC-3, (-▲-) HSC-4, and (-×-) SAS human squamous carcinoma cell lines. 5×10^4 cells were inoculated into each well of a 24-well plate with 1 ml of medium. Twenty-four hours later, AxCALacZ vectors were added to each well. Numbers of living cells were counted 96 hours after AxCALacZ infection and compared with nontransduced cells. Results represent the mean of three individual experiments. Each point represents the mean \pm SD.

and found to be 25 $\mu\text{g/ml}$ ($p < 0.01$) or more (Fig. 4).

In order to evaluate the effect on suicide gene therapy using the HSV tk /GCV system, the 4 cell lines were transduced with AxCAHSV tk and then added to either PBS or GCV at 10 $\mu\text{g/ml}$. Figure 5 shows the cell-survival percentage of each cell line transduced at two levels of MOI with AxCAHSV tk followed by GCV. Each experiment was performed in triplicate. The incidence of survival was $17.8 \pm 3.4\%$ on SAS, $31.6 \pm 4.1\%$ on HSC-2, $26.5 \pm 3.5\%$ on HSC-3, and $29.4 \pm 2.9\%$ on HSC-4 at an MOI of 1. Despite less than a 30% transduction efficiency using AxCALacZ, more than 60% cell death was induced at an MOI of 1. In addition, almost 100% cancer cell death was obtained at an MOI of 10.

Morphological changes in human oral squamous cells treated with AxCAHSV tk and GCV.

To study the morphological changes in a living human oral squamous carcinoma cell line SAS using HSV tk /GCV system, we employed VEC-DIC microscopy. The cells were transduced at an MOI of 10 with AxCAHSV tk followed by GCV. At 24 hours after administration of GCV, the cultured cells showed signs apoptosis such as chromatin condensation, cell shrinkage, blebbing of cell membrane, and ballooning formation (Fig. 6).

DISCUSSION

In promising gene therapy strategies to date, highly efficient adenovirus vectors have been employed to transfer a therapeutic gene into HNSCC.^{6,7,11,12} The herpes simplex virus thymidine kinase gene^{6,7} and the tumor suppressor gene $p53$ ^{11,12} have been the most widely studied for gene therapies in HNSCC. The purpose of the present study was to evaluate the efficacy of suicide

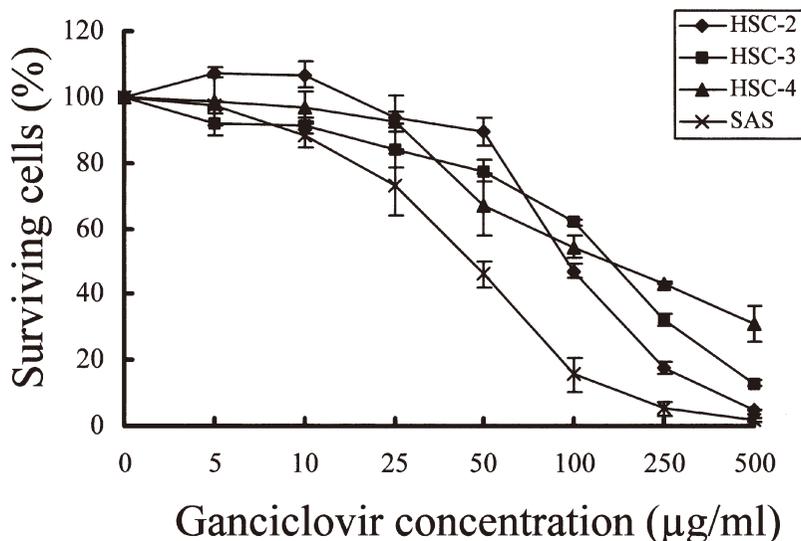


Fig. 4 *In vitro* toxicity study of ganciclovir (GCV) in (-◆-) HSC-2, (-■-) HSC-3, (-▲-) HSC-4, and (-×-) SAS human squamous carcinoma cell lines. 5×10^4 cells were inoculated into each well of a 24-well plate with 1 ml of medium. Forty-eight hours later, GCV was added to each well. Numbers of living cells were counted 72 hours after GCV administration and compared with nontransduced cells. Results represent mean of three individual experiments. Each point represents the means \pm SD.

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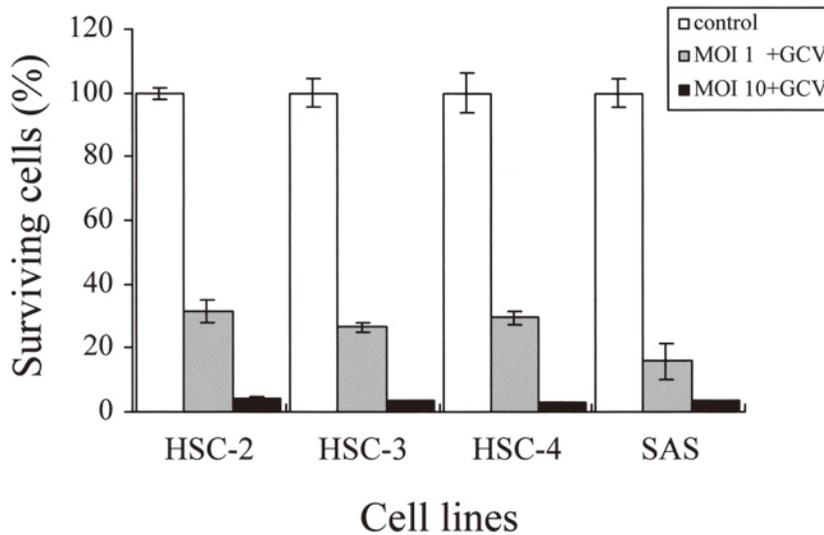


Fig. 5 Rates of cancer cell killing in human squamous cell carcinoma cell lines. Cell-survival percentage of each cell line transduced at two MOI levels with AxCaHSV Δ tk. Cell survival rates ranged from 17 to 32% at MOI of 1 and almost 100% cancer cell death at MOI of 10. PBS controls demonstrated no toxicity. Each point represents the mean \pm SD.

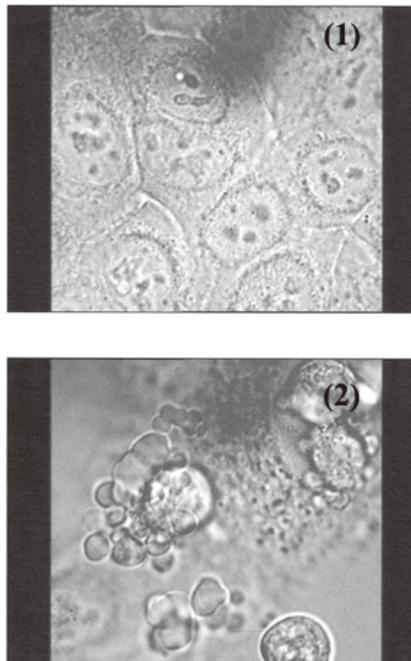


Fig. 6 Morphological analysis of cancer cell death by VEC-DIC microscopy. Human oral squamous carcinoma cell line SAS was infected with AxCaHSV Δ tk vectors at MOI of 10, and incubation continued for another 24 hours. Then 10 μ g/ml of GCV was added to the dish. At 24 hours after administration of GCV, the treated cells were showed apoptosis. (1) Normal cell. Development of apoptosis is associated with chromatin condensation, cell shrinkage, blebbing of cell membrane, and ballooning formation (2).

gene therapy using an adenovirus-mediated transfer of HSV \underline{tk} gene and subsequent administration of GCV in four human oral squamous cell carcinoma cell lines *in vitro*.

The present experiment demonstrated that a replication-deficient recombinant adenovirus vector effectively transduced a foreign gene into four human oral squamous cell carcinoma cell lines (SAS, HSC-2, HSC-3 and HSC-4). In order to evaluate the transduction efficiency, a replication-deficient recombinant adenovirus vector containing the β -galactosidase gene was transduced into each of the cell lines. Resulting in a transduction efficiency close to 100% at an MOI of 10, and from 10 to 30% at the latter an MOI. The transduction efficiency in each cell line showed substantial differences at a MOI of 1. Although the cause of those differences was not clear from this study, it was suggested that they might result from differences in the cell surface receptor for adenovirus or variations in adenovirus receptor density.

In the present study, HSV \underline{tk} gene transduction with an adenovirus vector followed by GCV exposure resulted in the dramatic death of cancer cells. A rate of almost 100% cancer cell death was observed at an MOI of 10 in all cell lines. Moreover, more than a 60% tumor cell death was produced at an MOI of 1, despite the fact that less than 30% of the target cells expressed the β -galactosidase gene as the result of AxCA \underline{LacZ} . This suicide gene therapy using an adenovirus vector killed more tumor cells than the number of actually transduced cells, possibly due to the so-called bystander effect.^{13,14} The mechanism underlying the bystander effect in the HSV \underline{tk} /GCV system has been demonstrated both *in vitro* and *in vivo* in several types of tumor cells. It is hypothesized that two pathways exist in the mechanism *in vitro*. In one pathway, experiments using flow cytometric and electron microscopic analyses have suggested that HSV \underline{tk} -modified cells dying by apoptosis generate apoptotic vesicles containing GCV-metabolites that are phagocytosed by nearby unmodified cells.¹³ In another pathway, cytotoxic molecules are transferred from transduced cells to non-transduced cells via a gap junction.¹⁴

In this study, using VEC-DIC microscopy we evaluated the morphological changes in living cells treated with the HSV \underline{tk} /GCV system. Freeman *et al.* using fluorescence microscopic and electron microscopic analyses, reported that the mechanism of the cell death is apoptosis or programmed cell death (cell shrinkage, cell detachment, vesicle formation, and chromatin condensation).¹³ To better understand the mechanism of cancer cell death using the HSV \underline{tk} /GCV system, we used VEC-DIC microscopy, which is a valuable system for studying the morphological changes in living cells because of its real-time visualization of such phenomend. We demonstrated that suicide gene therapy using the HSV \underline{tk} /GCV system was induced apoptosis within 24 hours after GCV administration.

Adenovirus vectors have numerous advantages such as their capability of efficiently delivering a gene to both dividing and non-dividing cells, high transduction efficiency, and the availability of efficient protocols for producing clinical-grade material at high concentrations. However, because the adenovirus vector carries most of a virus gene, adenoviruses expressing proteins are able to induce direct toxicity and immunogenicity. In the present experiment, AxCAHSV \underline{tk} showed toxicity at an MOI of more than 100 in all cell lines. Although the reason for this remains unclear, there is the possibility that many virus particles themselves bind like the molecules of an integrin cell to send the same kind of signals to the cell. Moreover, the adenovirus vectors carry virtually all of the targeted virus genes, which might block protein synthesis in a transduced cell. Various new-generation adenovirus vectors are now being developed to resolve these problems.¹⁵ However, we consider that the most practical approach is to reduce the effective therapeutic dose through the use of powerful promoters. In the present experiment, we produced cell death with a high transduction efficiency and effectiveness using a CAG promoter.

In conclusion, we demonstrated that all four of the human oral squamous carcinoma cell lines were sensitive to suicide gene therapy using AxCAHSV \underline{tk} and GCV. Based on this and other

reports, this strategy has been well established as highly effective for these cell lines *in vitro*. Our results suggest that the transfer of the HSV θ k gene using adenovirus vector and GCV administration may be an effective treatment strategy for oral squamous cell carcinoma in humans.

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