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CELL DEATH OF HUMAN ORAL SQUAMOUS CELL CARCINOMA CELL LINE INDUCED BY HERPES SIMPLEX VIRUS THYMIDINE KINASE GENE AND GANCICLOVIR

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ABSTRACT

Suicide gene therapy combining herpes simplex virus thymidine kinase gene (HSVtk) and ganciclovir (GCV) is one strategy for the treatment of head and neck squamous cell carcinoma (HNSCC). The purpose of this study is to determine the mechanism of cell death that occurs in suicide gene therapy using HSVtk and GCV and to assess the safety of that therapy.

The human oral squamous cell carcinoma cell line SAS was treated with adenovirus vector containing HSVtk gene (AdHSVtk) and GCV *in vitro*. Morphological changes including chromatin condensation, cell shrinkage, blebbing of cell membrane, and ballooning formations were observed. Changes in the localization of phospholipids in the cell membrane were also observed. The results of flow cytometry showed a maximum of about 65% of cells in the early phase of apoptosis. In addition, DNA fragmentation was investigated using the TUNEL method *in vivo*. Nude mice (BALB/c AJD^{-nu-}, aged 4 weeks) were implanted with SAS and treated with AdHSVtk and GCV. Tumor sections were then observed. The treatment group was confirmed to have DNA fragmentation-positive cells.

These results suggest that suicide gene therapy using AdHSVtk and GCV led to apoptosis of the oral squamous cell carcinoma cell line.

Key Words: Adenovirus, HSVtk, Suicide gene therapy, Head and neck squamous cell carcinoma, Apoptosis, Safety

INTRODUCTION

Since clinical trials of gene therapy began at the National Institutes of Health (NIH), the research and development of gene therapy for various diseases has been ongoing. Because cancer is such an intractable disease, it is the most widely studied.

With regard to head and neck squamous cell carcinoma (HNSCC), NIH has proceeded with gene therapy research using various strategies. These approaches have included tumor suppressor gene therapy using p53 gene,[1,2] suicide gene therapy using herpes simplex virus thymidine

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kinase gene (HSVtk) and ganciclovir (GCV), cytokine gene therapy using interleukin-2, interleukin-12 and interferon, and the E1B 55-kDa gene-defective adenovirus ONYX-015.[3,4] Clinical trials have already been started in these areas. The earliest clinical protocol was introduced in 1995. The clinical trial of gene therapy for HNSCC using p53 gene is a phase III study at present. (Source: Journal of Gene Medicine website)

In this strategy of suicide gene therapy, 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. Several toxic products have been identified and are available; including HSVtk. Thymidine kinase is an enzyme that acts in the salvage cycle of DNA synthesis. This enzyme alone is not harmful to cells and, in the case of mammalian thymidine kinase negative cells, provides a means for applying a selective pressure for cell survival. Nucleoside analogs such as GCV are phosphorylated by HSVtk to nucleoside monophosphates. The GCV nucleoside monophosphates are phosphorylated by cellular kinase to diphosphates and finally to triphosphates that are incorporated into cellular DNA where they inhibit cellular DNA synthesis and lead to cell death.[5] By these mechanisms, target cells expressing the HSVtk enzyme become susceptible to the toxic effects of GCV.

Both *in vitro* and *in vivo*, the efficacy of suicide gene therapy using HSVtk and GCV for HNSCC at the early stage[6-8] has also been confirmed in some facilities. Moreover, clinical trials have now been started, a strategy designed to produce clinical applications in the near future. However, there is a dearth of research examining the safety factor in suicide gene therapy using HSVtk and GCV.

In this study, the mechanism of cell death in suicide gene therapy using adenovirus vector containing HSVtk gene for the HNSCC cell line was confirmed. The further purpose of this study is to assess the safety of that therapy.

MATERIALS AND METHODS

Cell culture

The human oral squamous cell carcinoma cell line SAS obtained from the Cancer Cell Repository at Tohoku University was used. This cell line was established from a poorly differentiated squamous cell carcinoma of the tongue. SAS was maintained in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum in a 5% CO_2 incubator at 37°C and passaged when the culture plate was 75–90% confluent.

Construction of recombinant adenovirus vector

A recombinant replication-deficient adenovirus vector containing HSVtk gene under transcriptional control of the CAG promoter (AdHSVtk) was constructed. This vector was propagated with 293 cells, human embryonic kidney cells transformed by E1A and E1B genes and purified by double cesium gradient ultracentrifugation. The concentrated virus was dialyzed, aliquoted, and stored at 80°C. The virus titer was determined by plaque assay.

Analysis of morphological changes and changes in localization of phospholipids of cell membrane *in vitro*

For this analysis, 2×10^4 cells/ml of SAS cells were plated in a glass-bottom culture dish (Mat Tec Corporation, USA) and incubated in a 5% CO_2 incubator at 37°C for 24 hours. Twenty-four hours later, the medium was changed, the cells were infected with AdHSVtk vectors at a multiplicity of infection (MOI) of 10, and incubation was continued for another 24

hours, after which 10 μg/ml of GCV was then added to the dish. After 3, 6, 12, 24 and 48 hours, the cells were stained with Annexin V-EGFP and Propidium Iodide (PI) (MBL, Japan). Changes in the localization of the phospholipids of cell membrane were observed under fluorescence microscopy, while morphological changes were observed by microscope.

Analysis of the ratio of apoptosis cells *in vitro*

Apoptosis was analyzed by FACS using the Annexin V-EGFP and PI. In brief, 1×10^5 cells/well of SAS cells were plated in a 6-well tissue culture plate (Becton Dickinson, USA), and incubated in a 5% CO_2 incubator at 37°C for 24 hours. The medium was then changed, the cells were infected with AdHSVtk at a MOI of 10, and incubation was resumed for another 24 hours. Next, 10 μg/ml of GCV was added to the dish. PBS containing no viral constructs and no GCV was used as a control. The cells (including those floating in the medium) were harvested after 3, 6, 12 and 24 hours, treated with 0.02% trypsin EDTA and washed once with PBS. Then, 1 ml of PBS and 200 μl of binding buffer were added to the cells. In the postperiod, 1 μl of Annexin V-EGFP solution and 1 μl of PI solution were also added. The cells at each time point were subjected to flow cytometric analysis using a FACS caliber (Becton Dickinson, USA). They were then analyzed for green FITC fluorescence through a 520-nm BP filter, and for red PI fluorescence through a 620-nm BP filter.

The ratio of the early phase of apoptotic cells or the post-apoptotic secondary necrosis cells was observed at each time point for all cells. Such rates were calculated by deducting the number of green or red fluorescence positive cells in the control group from the number of positive cells in the treatment group, as shown in the following formulas.

% early phase of apoptotic cells = (A-B)/C×100
A = number of green fluorescence positive cells in the treatment group
B = number of green fluorescence positive cells in the control group
C = number of all of treated cells
% post-apoptotic secondary necrotic cells= (D-E)/F×100
D = number of red fluorescence positive cells in the treatment group
E = number of red fluorescence positive cells in the control group
F = number of all treated cells

Analysis of DNA fragmentation *in vivo*

Nude mice (BALB/c AJD^{-nu-}, aged 4 weeks) were anesthetized with an intraperitoneal injection of pentobarbital at 60-70 mg/kg-body weight (Nembutal; Abbott Laboratories, North Chicago, IL, USA). Using a 1-ml syringe and a 26-gauge needle, a 200 μl solution containing 5×10^6 human SAS cells was slowly injected subcutaneously into the right flank of the nude mice, and the needle was then removed with no apparent leakage. The animals were maintained under standard housing conditions for 7 days.

In this experiment, 20 mice were divided into four groups: group 1, no treatment; group 2, injected only with AdHSVtk; group 3, injected only with GCV; and group 4, injected with AdHSVtk and GCV. Seven days later, the established tumor size was about 10 mm; the nude mice in groups 2 and 4 were anesthetized as before for the adenovirus injection. AdHSVtk was injected into the tumor mass by a Hamilton syringe using a 26-gauge needle. After 24 hours, the mice in groups 3 and 4 were injected with GCV intraperitoneally. After 3, 6, 12, 24 and 48 hours, the mice in all groups were sacrificed. The tumors were excised and stored at –80°C until the preparation of frozen sections. The sections were fixed in 4% PFA, stained by the TUNEL method using a MEBSTAIN Apoptosis Kit Direct (MBL, Japan). In this method, dUTP

was labeled by FITC. The sections were then stained with PI for comparison and observed under fluorescence microscopy.

RESULTS

Morphological changes and changes in the localization of phospholipids of cell membrane of human oral squamous cells treated with AdHSVtk and GCV *in vitro*

To study the morphological changes, the living human oral squamous cell carcinoma cell line SAS was treated using HSVtk/GCV and observed by microscope. Chromatin condensation, cell shrinkage, blebbing of cell membrane, and ballooning formations peculiar to apoptosis were observed (Fig. 1).

Changes in the localization of phospholipids of cell membrane were observed under fluorescence microscopy. Green fluorescence indicates the early phase of apoptosis, and red fluorescence indicates the post-apoptotic secondary necrosis. The cells were transduced at a MOI of 10 with AdHSVtk followed by GCV. At each time point after GCV administration, the cultured cells were observed by FITC in developed green fluorescence. Annexin V bound specifically with phosphatidylserine, one of the phospholipids of cell membrane. Thus, the change in the localization of the phospholipids of cell membrane was observed.[9,10] In short, we observed the change in membrane potential based on apoptosis.

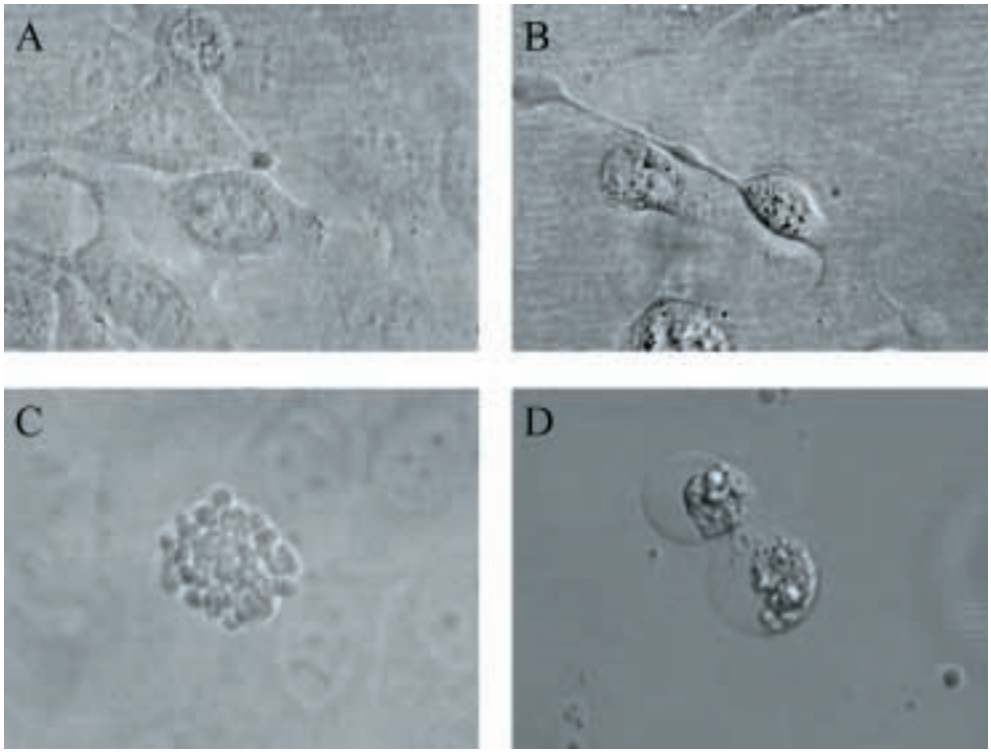

Fig. 1 To observe morphological changes, the living human oral squamous cell carcinoma cell line SAS was treated using HSVtk/GCV. (A) chromatin condensation. (B) cell shrinkage. (C) blebbing of cell membrane. (D) ballooning formation

From 6 to 48 hours after GCV administration, the cultured cells were observed by PI in developed red fluorescence, showing post-apoptotic secondary necrosis (Fig. 2).

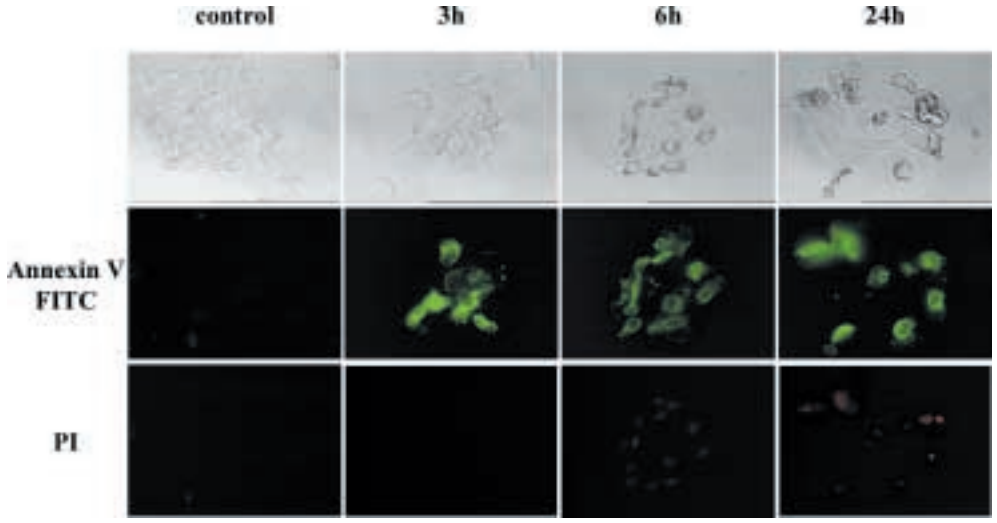

Fig. 2 Changes in the localization of the phospholipid of cell membrane were observed under fluorescence microscopy. Green fluorescence indicates the early phase of apoptosis, and red fluorescence indicates the post-apoptotic secondary necrosis. The cells were transduced at a MOI of 10 with AdHSVtk followed by GCV.

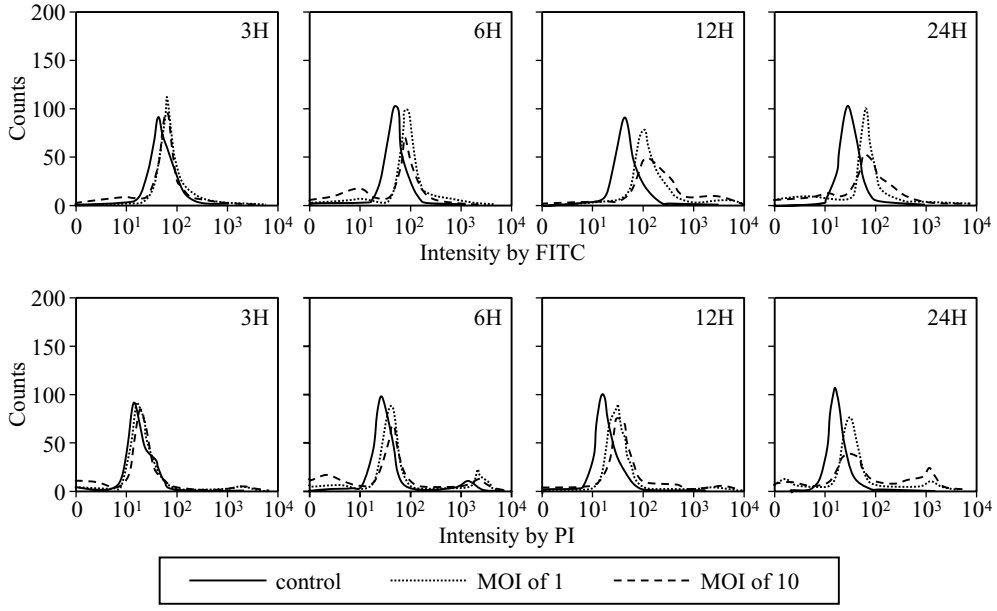

Fig. 3 Treated cells were stained with Annexin-V and PI, and fluorescence was assessed by flow cytometer. The observed reinforcement in fluorescence intensity by FITC at an MOI of both 1 and 10 was compared with that in the control group. Fluorescence intensity was reinforced over time. Similarly, reinforcement in fluorescence intensity by PI was observed.

Flow cytometric analysis of treated SAS cells

To study the ratio of apoptosis in the treated SAS cells, flow cytometric analysis was used. The treated cells were stained with Annexin-V and PI, and fluorescence was assessed by flow cytometer. At each time point, the control group was compared with an MOI of 1 or 10. The observed reinforcement in fluorescence intensity by FITC at an MOI of both 1 and 10 was compared with the control group. The fluorescence intensity was reinforced over time. Similarly, reinforcement in fluorescence intensity by PI was observed (Fig. 3).

With an MOI of 1, the ratio of the early phase of apoptotic cells reached a peak after 12 hours. An MOI of 10 produced the same result (Fig. 4-a).

In the same way, with an MOI of 1 and an MOI of 10, the ratio of post-apoptotic secondary necrosis cells was seen to gradually increase (Fig. 4-b).

Morphological changes in human oral squamous cells treated with AdHSVtk and GCV *in vivo*

Tumor sections were observed by fluorescence microscopy. In group 4, at each time point, fluorescence intensity by FITC was checked, and fluorescence intensity by PI was observed at each time point except at 3 hours. In groups 1, 2 and 3, however, few cells were stained by FITC or PI at each time point (Fig. 5).

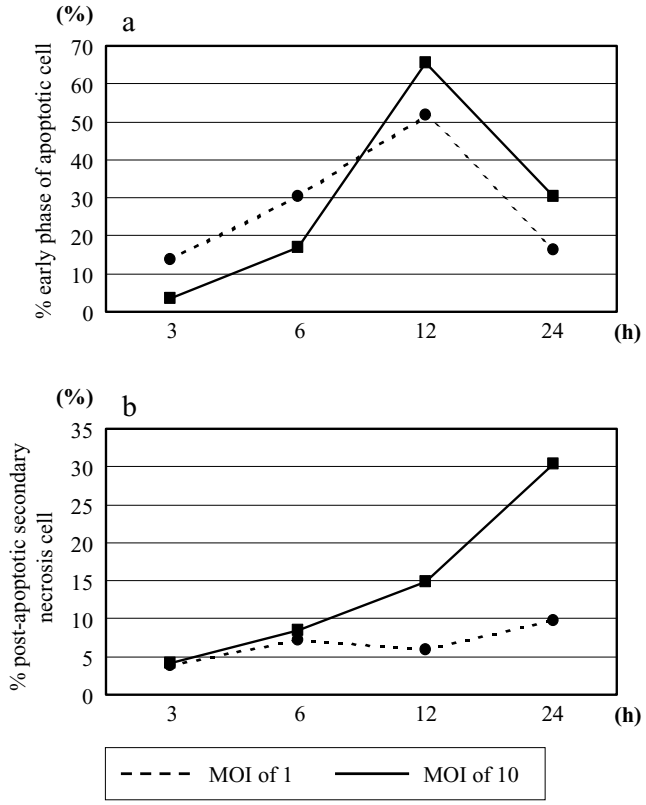

Fig. 4 (a) The ratio of the early phase of apoptotic cells was observed at each time point for all cells.
(b) The ratio of the post-apoptotic secondary necrotic cells was observed at each time point for all cells.

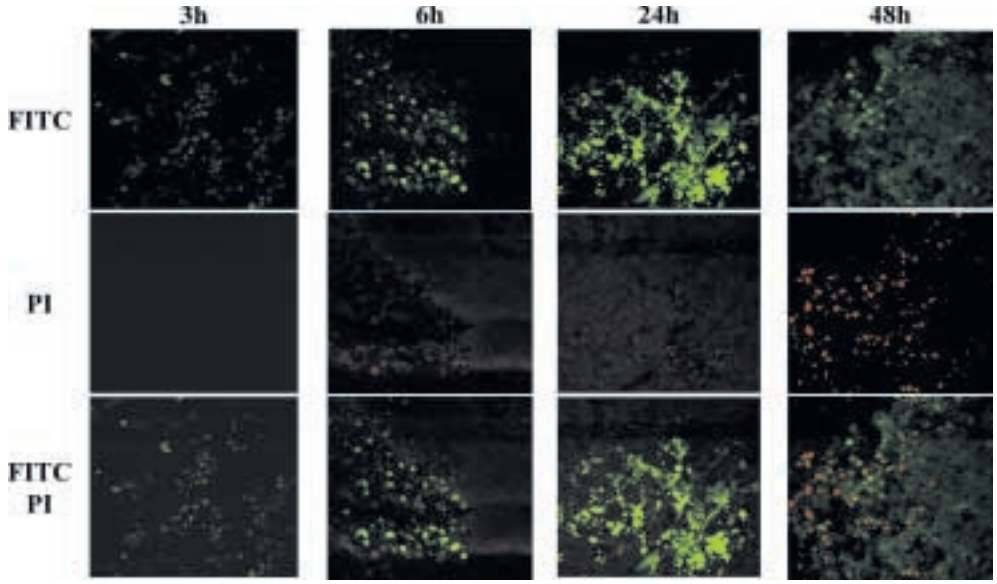

Fig. 5 Tumor sections were stained by the TUNEL method and observed under fluorescence microscopy. At each time point, fluorescence intensity of treated cells was checked by FITC, and fluorescence intensity by PI was observed at each time point except at 3 hours.

Thus, apoptosis was induced in the group treated with HSVtk and GCV. Moreover, the increased intensity of coloring by PI over time was thought to reflect the complete destruction of the cells.

DISCUSSION

Suicide gene therapy using HSVtk gene and GCV is one of the most widely studied along with gene therapy using tumor suppressor p53 gene. In the fields of neurosurgery, urology and gynecology, clinical protocols have already been started.[11,12] For head and neck cancer, expectations for clinical trials are already high.

The purpose of this study is to assess the safety of suicide gene therapy using HSVtk gene and GCV by observing the mechanism of cell death. The mechanism by which cell death is induced in tumor cells by HSVtk gene and GCV is as follows. At first, a viral vector containing the metabolic enzyme gene, HSVtk, infects the target cells. The HSVtk gene produces HSVtk, which is different from mammalian thymidine kinase. The HSVtk enzyme is almost 1000-fold more efficient at monophosphorylating GCV than mammalian cellular thymidine kinase.[13] GCV is a derivative of acyclovir, developed during the 1970s to treat HSV infections. Phosphorylation of GCV by HSVtk into a monophosphate form (GCV-MP) is the rate-limiting step in the generation of a cytotoxic drug. Normal mammalian guanylate kinase, which metabolizes the monophosphate to the diphosphate form (GCV-DP) and then to the triphosphate form, is highly toxic to cells by inhibiting DNA polymerase and by incorporation into DNA with chain termination. Therefore, GCV in the presence of HSVtk becomes a pro-drug that causes cell death. It is important to note that GCV at a therapeutic concentration is nontoxic to cells that lack HSVtk; conversely, the constitutive expression of HSVtk is nontoxic to cells in the

absence of GCV.[14] Now we theoretically consider as apoptosis the cell death due to gene therapy using HSVtk.

Apoptosis, frequently referred to as "programmed cell death," is an active and physiological cell death in which the cell itself designs and executes the program of its own demise and subsequent body disposal.[15] The feature of apoptosis is the fragmentation of cells together with the organelles they contain, at which time apoptotic bodies are formed. They are then encapsulated by macrophages or taken up by surrounding cells and eliminated. Moreover, because the cell membrane is maintained, the cellular contents are not released outside the cells, and no inflammation is ensues.[16]

On the other hand, necrosis generally represents a cell's response to gross injury, and can be induced by an overdose of cytotoxic agents. The early events of necrosis include mitochondrial swelling followed by a rupture of the plasma membrane and a release of cytoplasmic constituents, including proteolytic enzymes. The nuclear chromatin of necrotic cells shows patchy areas of condensation followed by its own slow dissolution. Necrosis triggers an inflammatory reaction in the tissue, often resulting in scar formation[15].

Thus, in gene therapy, during cell death by apoptosis it is important that oncogenes or transduction genes are not introduced from outside or are not allowed to cause an allergic reaction or local inflammation.

Tumor cell death occurred by apoptosis in this study. The mechanisms of the apoptosis involved may be explained as follows. First, when apoptosis is induced, caspase is activated. Some of the processes from apoptosis induction to the activation of caspase are known. Typical of such processes are the activation of the caspase-9 pathway due to the isolation of cytochrome c from the mitochondria,[17] and the signal activation of the caspase-2, 8 pathway through the death receptor, and activation of the caspase-8, 10 pathway by granzyme B[18,19]. After the activation of caspases, changes involving DNA fragmentation and the cell membrane changes, along with changes in phosphatidylserine localization on the cell surface[9,10,20].

We first observed, the histological changes in tumor cells, the results of which are shown in Fig. 1. The chromatin condensation, cell shrinkage, blebbing of cell membrane, and ballooning formations peculiar to apoptosis were then observed. The results are given in Fig. 2; the change in the localization of the phospholipid of cell membrane was confirmed by staining with AnnexinV. Phosphatidylserine existing only inside the normal cell membrane appeared on the surface of the cell membrane in normal cells, thereby indicating apoptosis. Furthermore, the fragmentation of DNA was confirmed *in vivo* using the TUNEL method (Fig. 5). Moreover, apoptosis was induced in more than half the cells, as revealed by flow cytometric analysis (Fig. 3, 4).

From these results, all the phenomena involved in the actual process of apoptosis were observed, thus confirming that apoptosis was induced during suicide gene therapy using the HSVtk/GCV system for human oral squamous cell carcinoma. Such results argue in favor of a clinical trial of suicide gene therapy using the HSVtk/GCV system.

The adenovirus vector was used in this study. When this vector is used in an actual clinical trial, one must consider the possibility that necrosis may be induced by the pathogenic nature of the adenovirus. However, this poses no serious problems since it can be resolved by using a non-virus vector and a non-pathogenic virus vector.

When we actually conduct suicide gene therapy using the non-pathogenic virus vector adeno-associated virus (AAV) containing HSVtk gene it will be shown that the efficacy of suicide gene therapy using AAV is equivalent to that when using the adenovirus vector.[21] This strategy will prove effective for head and neck cancer as long as research on targeting the cancer cells and on developing vector improvements continue.

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