

# Cell Death Induction of Thymidine Kinase Gene Transfer Followed by Ganciclovir Treatment in Oral Squamous Cell Carcinoma Cell Lines

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**The tumoricidal effect of herpes simplex virus thymidine kinase (HSV-tk) gene transfer followed by ganciclovir (GCV) treatment has been demonstrated in relation to the bystander effect. We examined the mode of cell death after GCV treatment in three human oral squamous cell carcinoma (SCC) cell lines that had been infected with adenovirus possessing HSV-tk gene. Oral SCC cell lines displayed high susceptibility to HSV-tk/GCV treatment despite low transduction. Evidence suggests that apoptosis was not found by internucleosomal DNA fragmentation, cell cycle kinetics or gene expressions indicative of apoptosis. However, we observed different levels of expression of annexin V-positive/propidium iodide-negative cells in all the cell lines and occasional TUNEL-positive cells in one cell line. To address these controversial findings, we further confirmed the morphological phenotypes of cell death with semi-thin and ultra-thin sections, which revealed that the cells undergoing death were consistent with necrosis, i.e. swelling of cytoplasm and intracellular organs with membrane disruption. Furthermore, no apoptotic bodies were detected within cytoplasm of apparently intact cells. A nonapoptotic mechanism may play a central role in the HSV-tk/GCV-induced cell death in oral SCC cell lines.**

**Key words:** apoptosis; ganciclovir; necrosis; thymidine kinase

Squamous cell carcinoma (SCC) is the most frequent malignancy in the oral cavity. The prognosis of advanced oral SCC has remained poor over the last few decades despite progress in aggressive approaches using combination therapy. Novel strategies should be explored in the oral cancer field.

One of the potential improvements in current therapeutic strategies is herpes simplex virus thymidine kinase (HSV-tk) gene transfer followed by ganciclovir (GCV) treatment based on

the enzyme/prodrug system concept (Borrelli et al., 1988). HSV-tk-transduced cells selectively convert prodrug GCV into monophosphate GCV. Triphosphate GCV is the final activated form with an ability to kill cells by interfering in DNA synthesis through chain termination and single-strand breaks (Elion, 1982). Another promising aspect of this strategy is the existence of the so-called "bystander effect" (Freeman et al., 1993; Colombo et al., 1995), which mediates cytotoxicity to the adja-

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Abbreviations: Ad, adenovirus; FITC, fluorescein isothiocyanate; GCV, ganciclovir; HSV-tk, herpes simplex virus thymidine kinase; IC<sub>50</sub>, median inhibitory concentration; MOI, multiplicity of infection; PBS, phosphate-buffered saline; pfu, plaque forming unit; PI, propidium iodide; SCC, squamous cell carcinoma; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling

**Table 1. Oral squamous cell carcinoma cell lines and *p53* status**

Cell line	Histology*	<i>p53</i> status	Mutation	Site mutation codon
HSC-3	por	mutant	exon8	TAAG insertion (305 and 306)
HSC-4	well	mutant	exon7	248CGC to CAG
SCCKN	mod	mutant	exon5	178CAC to -AC (frame shift)

\*well, well differentiated type; mod, moderately differentiated type; por, poorly differentiated type.

cent untransduced cells. There have been two hypothesis proposed to explain the bystander effect: (i) transfer of cytotoxic molecules via gap junctions because phosphorylated GCV is unable to diffuse freely across the cytoplasmic membrane (Bi et al., 1993) and (ii) phagocytosis of apoptotic bodies containing GCV-metabolites from HSV-tk-transduced cells by untransduced intact cells (Freeman et al., 1993; Colombo et al., 1995).

HSV-tk/GCV treatment has been successfully applied in both human and animal tumor cell lines of different origins (Moolten et al., 1990; Glaser et al., 2001; Lanuti et al., 1999; Li et al., 1999; Boucher et al., 1998). There have been, however, few studies on oral SCC cell lines (O'Malley et al., 1996; Fukui et al., 2001). While the validity of this approach has been confirmed, the molecular mechanism of HSV-tk/GCV-induced cell death remains to be elucidated. The most commonly described cell death mechanism was related to the induction of apoptosis, not only for the execution pathway but also the bystander effect (Colombo et al., 1995; Samejima and Maruelo, 1995). On the other hand, a few studies have suggested nonapoptotic induction, i.e. necrosis (Kaneko and Tsukamoto, 1995; Vile et al., 1997; Katabi et al., 2002). Recently, activation of caspase-independent apoptotic pathways was also suggested because of the incomplete inhibition of cell death by caspase inhibitors such as z-VAD-fmk (Beltinger et al., 1999; Rivas et al., 2001). Both molecular biological analysis and morphological examinations are indispensable for apoptotic evaluation. Most studies exposing the HSV-tk/GCV-induced cell death mechanism have been performed with apoptosis-related molecular biological analysis, while a few studies using ultra-thin examinations have been performed where apoptosis was originally evaluated from the mor-

phological changes that occurred at ultra-structural levels (Kerr et al., 1972).

The purpose of the present study was to assess the feasibility of adenovirus-mediated HSV-tk gene transfer followed by GCV to oral SCC cell lines, in addition to verifying whether apoptotic responses are associated with HSV-tk/GCV-induced cell death. In addition, we examined the ultra-structural level alterations to identify molecular biological changes.

## Materials and Methods

### Cell lines

Three cell lines derived from human tongue SCCs were used (Table 1). We obtained HSC-3 and HSC-4 from the Health Science Research Resources Bank (Osaka, Japan), and SCCKN from Riken Gene Bank (Tsukuba, Japan). Cell line 293 derived from human embryonic kidney was used for both virus purification and titer determination. All cell lines were grown in Dulbecco's MEM (COSMO BIO, Tokyo, Japan) complemented with 1/100 diluted Gibco solution (29.2 mg/mL L-glutamine 10,000 unit/mL penicillin G, 10,000 µg/mL streptomycin) and 10% inactivated fetal bovine serum and maintained at 37°C in an incubator under 5% CO<sub>2</sub>/95% air atmosphere.

### Adenovirus vectors

Two replication-deficient recombinant adenoviruses containing LacZ marker gene (Ad.β-gal) as a control and luciferase vector, and adenoviruses containing HSV-tk (Ad.tk) as the therapeutic vector under the control of cytomegalovirus promoter

were kindly provided from Professor Kenzo Sato (Division of Molecular Biology, Department of Molecular and Cellular Biology, School of Medicine, Tottori University Faculty of Medicine, Yonago, Japan). The Ad.β-gal and Ad.tk were propagated in 293 cells, which include the Ad5 E1 region in chromosomal DNA. These viruses were purified by sequential centrifugation in CsCl step gradients. Virus titers were determined as plaque forming units (pfu) using a modified end-point cytopathic effect assay. The titers of stock in 5% glycerol and 95% culture medium of Ad.β-gal and Ad.tk were  $2.27 \times 10^{10}$  pfu/mL and  $1.35 \times 10^{11}$  pfu/mL, respectively.

### ***Adenovirus infection efficiency***

Cells were seeded in 24-well culture plates at a density of  $2 \times 10^4$ /well. After overnight incubation, stock Ad.β-gal was diluted in 100 μL of medium and added at varying multiplicities of infection (MOIs) of 0, 6.25, 12.5, 25, 50 or 100. The cells were exposed for 1 h with constant agitation, and then the fresh medium was added up to 500 μL. Plates were incubated for an additional 24, 48 and 72 h at 37°C. After rinsing with phosphate-buffered saline (PBS) twice, cells were fixed with a solution of 2% formaldehyde and 0.2% glutaraldehyde in PBS for 10 min at 4°C. Cells were rinsed with PBS twice and incubated in a solution containing the X-gal (0.2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for 4 h at 37°C. The number of blue cells expressing transgene was counted. Adenovirus infection efficiency was expressed as a mean of the percentage of stained cells to the total cells from three randomly selected microscopic fields at  $\times 200$  magnification.

### ***Cell viability assay***

Cells were seeded in 60 mm dishes at a density of  $3 \times 10^5$ /dish. After overnight incubation, both stock Ad.β-gal and Ad.tk were diluted in 100 μL of medium and adjusted at MOIs of 1.25, 5, 10 and 20. Each dish was exposed for 1 h with viral diluted medium or condition medium, and then fresh medium was added up to 300 μL. After 16 h incuba-

tion at 37°C, cells were trypsinized and replated in 96-well culture plates at a density of  $3 \times 10^3$ /well. After overnight incubation, the medium was replaced with selective 100 μL medium containing different concentrations of GCV (0–1,000 μg/mL). Uninfected cells without GCV treatment were also incubated as controls. Cell viability was assessed at 96 h after GCV treatment using an MTT assay (CellTiter 96 Nonradioactive Cell Proliferation Assay, Promega, Madison, WI). According to the manufacturer's instruction, 15 μL of the dye solution was added to each well and the plates were incubated for 4 h. Subsequently, 100 μL of the solubilizing solution was added. After overnight incubation, the absorbance at 570 nm wavelength was measured using a microplate reader. Cell viability was expressed as the relative percentage to control. The result represents the mean  $\pm$  SE of triplicates.

### ***DNA fragmentation***

Both floating and attached cells were harvested from wells at 0, 12, 24, 48 and 72 h incubation times after HSV-tk/GCV treatment. Cells were incubated for 10 min at 4°C in 100 μL lysis buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM EDTA and 0.5% Triton X-100. After centrifugation for 5 min at 16,000 rpm, the supernatants were decanted and digested with RNase A (100 μg/mL, final) at 37°C for 1 h and then with proteinase K (200 μg/mL, final) for 1 h at 37°C. After these treatments, DNA was extracted by phenol, and isopropanol precipitation. These preparations were subjected to electrophoresis in 2% agarose gels and stained with 0.5 μg/mL ethidium bromide and electrophoretic patterns of the gel under UV light was photographed.

### ***Cell cycle kinetics***

Cells were infected with Ad.tk at MOIs of 5 for HSC-3 or 2.5 for HSC-4 and SCCKN, respectively. After 16 h incubation, the medium was exchanged with GCV medium at a concentration of 1 μg/mL. Both floating and attached cells were harvested from wells at 0, 12, 24, 48 and 72 h incubation times after HSV-tk/GCV treatment. Then,  $1 \times 10^6$ /mL

suspensions were prepared for flow cytometry assay.

To analyze cell cycle kinetics,  $2 \times 10^5$  cells were fixed in 70% ethanol and stored at 4°C until ready for use. Cells were centrifuged and resuspended in 1 mL PBS containing RNaseA (DNase free, 0.1 mg/mL), and stained with 50 µg/mL propidium iodide (PI). Samples were analyzed within 1 h after the staining procedure. Fluorescence intensity was determined by an EPICS XL flow cytometer (Coulter, FL).

### ***Annexin V assay***

To detect the early stage of apoptosis, annexin V-fluorescein isothiocyanate (FITC) and PI double staining were performed according to the manufacturer's instructions for the apoptosis detection kit (Trevigen, MD). Briefly,  $1 \times 10^5$  cells were exposed with 100 µL of labeling reagent containing 1 µL of annexin V-FITC conjugate and 10 µL of PI (50 µg/mL). After 15 min incubation at room temperature in the dark, an additional 400 µL of 1 × binding buffer was added. Samples were analyzed within 1 h after the staining procedure. Sample analysis was also performed using an EPICS XL flow cytometer.

### ***Antibodies***

The following antibodies were used for Western blotting; anti-P53 (BP53-12, diluted 1:500; Novocastra Lab., Claremont Place, United Kingdom), anti-Bax (P-19, 1:250; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bcl-2 (100, 1:250; Santa Cruz Biotechnology, Santa Cruz, CA), anti-caspase-3 (CCP32, 1:250; Transduction Laboratories, Lexington, KY) and anti-β-actin (Ab-1,1:1500; SIGMA, St. Louis, MO).

### ***Expression of apoptosis related protein***

Both floating and attached cells were harvested from wells at 0, 12, 24, 48 and 72 h incubation times after HSV-tk/GCV treatment, and solubilized in lysis buffer with 50 mM Tris-HCl (pH 7.4), 125 mM NaCl, 0.1% NP-40, 1 mM phenyl methyl

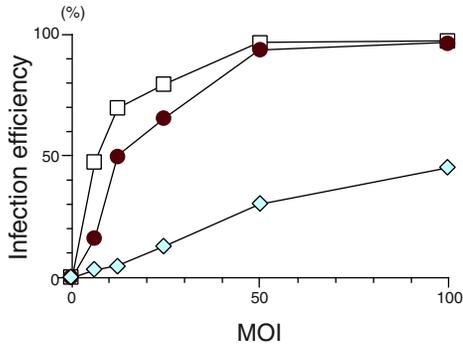
sulfonyl fluoride, 1 ng/mL leupeptin, 10 ng/mL soy bean trypsin inhibitor, 1 ng/mL aprotinin, 10 ng/mL *N*-tosyl-L-phenylalanyl chloromethyl ketone for 1 h on ice. Lysates were centrifuged at 15,000 rpm for 10 min and then supernatants were decanted. Protein concentrations were determined using the Bradford protein assay (Bio-Rad Lab., Richmond, CA). Equal amounts (25 µg) of total protein were loaded onto each lane of 10% SDS polyacrylamide gels, electrotransferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA), and then probed with the respective antibodies. Blots were developed with peroxidase-labeled anti-mouse or anti-rabbit antibodies (1:2000; MBL, Nagoya, Japan) using enhanced chemiluminescence (ECL detection system; Amersham, Bucks, United Kingdom).

### ***TUNEL method***

Cells cultured on cover slips were transduced and incubated for 24, 48 and 72 h with and without GCV (1 µg/mL). After incubation, attached cells were washed with PBS twice. Terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) staining was performed according to kit protocol with minor modification using an Apop Tag Plus peroxidase In Situ Apoptosis Detection Kit (Intergen, Purchase, NY). Briefly, after fixation with 95% ethanol, cells were exposed to terminal deoxynucleotidyl transferase with digoxigenin-11-dUTP and dATP in a moist chamber for 90 min at 37°C. Antidigoxigenin antibody-peroxidase was used for detection of digoxigenin-11-dUTP labeling for 30 min at room temperature, followed by color development with 3,3'-diaminobenzidine containing H<sub>2</sub>O<sub>2</sub> solution. Methyl green was used for counterstaining.

### ***Morphological examinations***

Both floating and attached cells were harvested from wells at 72 h with and without GCV (1 µg/mL) in all cell lines. After centrifugation and washing with PBS twice, cell pellets were fixed in Kalnovsky solution for 4 h at 4°C. After overnight



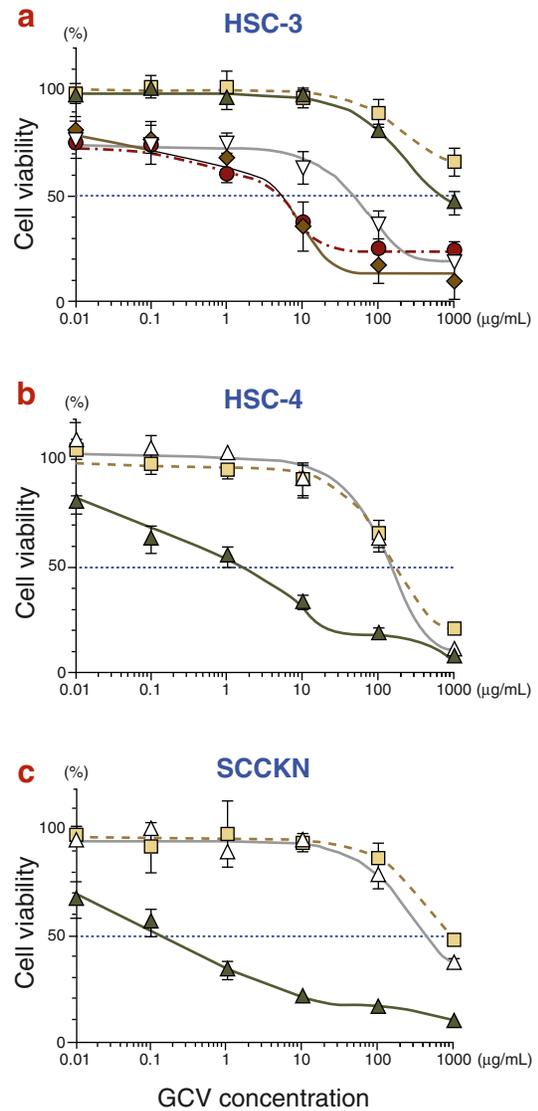
**Fig. 1.** Adenovirus infection efficiency in oral SCC cell lines. Cells were infected with Ad.β-gal at varying MOIs of 0, 6.25, 12.5, 25, 50 or 100 pfu/cell, respectively. At 48 h after the infection, cells were fixed and stained by X-gal. The percentages of cells expressing the transgene were counted. Infection efficiency was expressed by the percent average of blue-staining cells in three randomly selected × 200 microscopic fields. □, HSC-3, ◇, HSC-4 and ●, SCCKN.

washing with 0.1 M phosphate solution buffered at pH 7.4 at 4°C, cell pellets were postfixed in 1% osmium tetroxide solution for 2 h. After washing with 0.1 M phosphate solution buffered at pH 7.4, cell pellets were dehydrated in a graded ethanol series and embedded in Epon 812. After polymerization, 1 μm semi-thin sections were served for light microscopic examinations after toluidine blue staining. Then 60 nm ultra-thin sections were served for electron microscopic examination after uranyl acetate and Reynold’s lead citrate staining. Specimens were examined with an H-800 electron microscope (Hitachi, Tokyo).

## Results

### Adenovirus infection efficiency

Adenovirus infection efficiencies in human oral SCC cell lines were quantified using direct cell counting with X-gal staining. We assessed the infection efficiency at the 48 h time point, when the β-gal expression level was sufficient. For 100% infection, an MOI 50 was needed for HSC-3 and SCCKN cell lines, and impossible for the HSC-4 cell line within an MOI 100 (Fig. 1). However, in-



**Fig. 2.** Cell viabilities after HSV-tk/GCV treatment. Infected cells with either Ad.tk or Ad.β-gal were treated with GCV at concentrations ranging from 0 to 1,000 μg/mL. Uninfected cells without GCV treatment were used as control. Cell viabilities were measured at 96 h after GCV treatment using the MTT assay. Results were calculated as the percentage of viability relative to control. The result represents the mean ± SE of triplicates. The IC<sub>50</sub> were determined. (a) HSC-3, (b) HSC-4 and (c) SCCKN cell lines. □, GCV alone; Δ, Ad.β-gal + GCV; ▲, Ad.tk 1.25 MOI + GCV; ▽, Ad.tk 5 MOI + GCV; ◆, Ad.tk 10 MOI + GCV and ●, Ad.tk 20 MOI + GCV.

fection of Ad.tk at these high titers failed to be cytotoxic to oral SCC cell lines. Then we performed an MOI escalation study to determine the sublethal

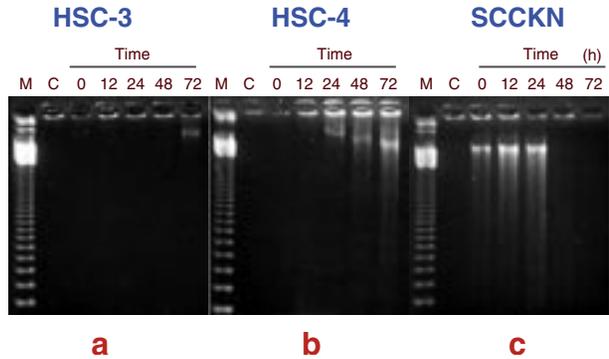
MOI value of Ad.tk infection (data not shown). The results showed MOIs of 20 and less for the HSC-3 cell line and 2.5 and less for HSC-4 and SCCKN cell lines, respectively.

**Cell viability**

Cell viability was determined from the colorimetric quantity of viable cells. HSV-tk gene transfer decreased the median inhibitory concentration (IC<sub>50</sub>) at least 2 logs lower in all cell lines (Fig. 2). Cell viability was dependent on GCV concentration. As the HSC-3 cell line only had resistance to Ad.tk infection, we could examine cell viabilities at several ranges of MOI (Fig. 2a). Though the IC<sub>50</sub> decreased in proportion to the MOI escalation, the results were not different between MOIs of 20 and 10. This fact suggested the upper limit bounds of MOI escalation of this strategy.

**DNA fragmentation**

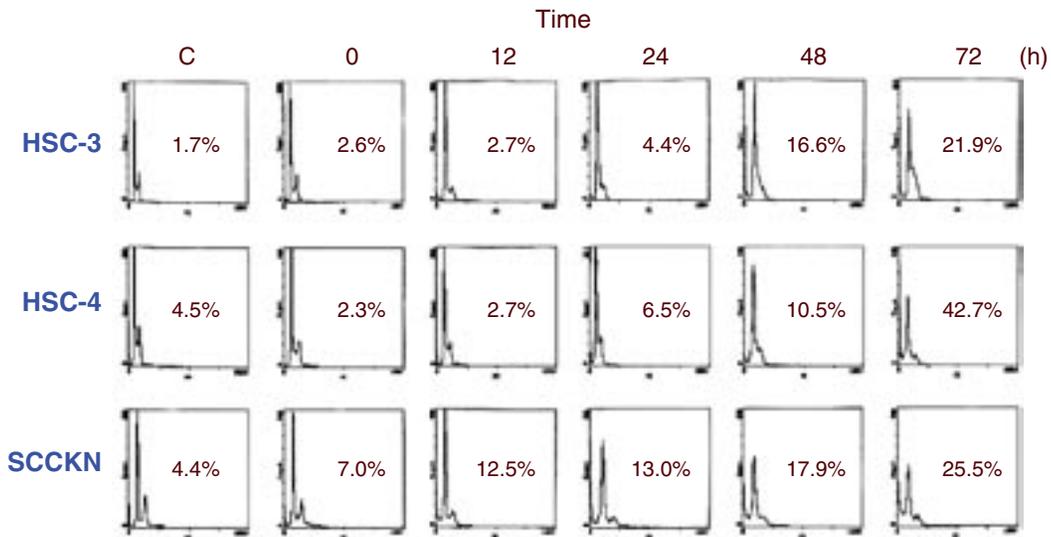
Internucleosomal DNA fragmentation was examined for standard apoptotic hallmarks. They all showed smearing, whose peaks were 72 h, 72 h and 24 h for cell lines HSC-3, HSC-4 and SCCKN, respectively (Fig. 3).



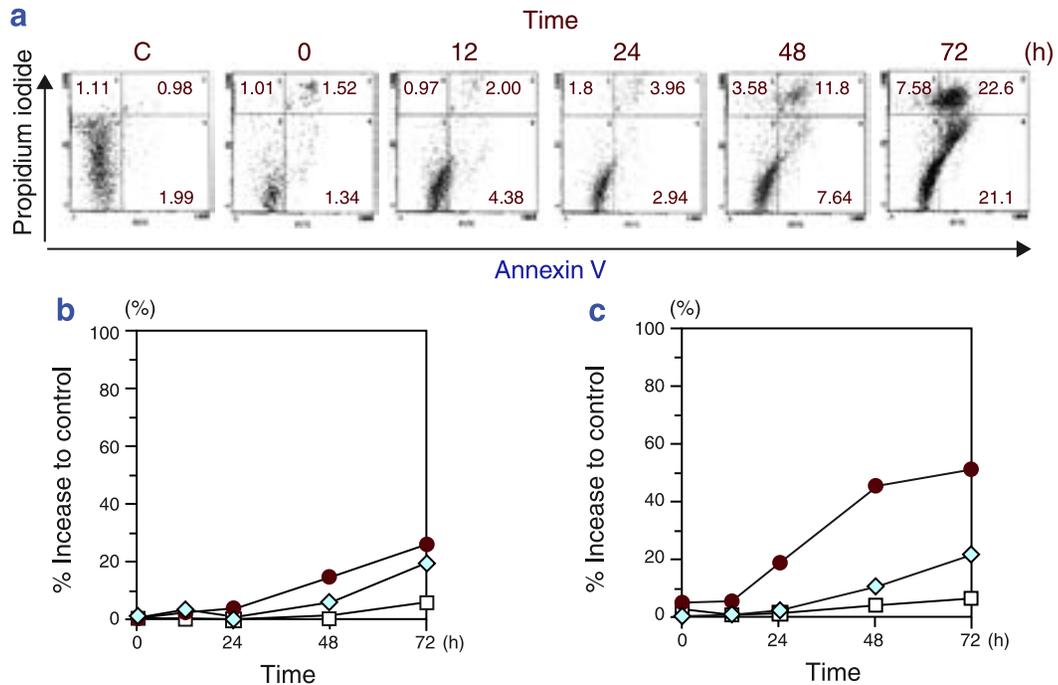
**Fig. 3.** DNA fragmentation after HSV-tk/GCV treatment. Cells were infected with Ad.tk at MOI 5 for HSC-3, 2.5 for HSC-4 and SCCKN, and followed by 1 µg/mL GCV. Cells were harvested in a time-dependent manner. Uninfected cells without GCV treatment were used as control. DNA extracts were loaded on 2% agarose gel by electrophoresis and visualized by staining with 0.5 µg/mL ethidium bromide. (a) HSC-3, (b) HSC-4 and (c) SCCKN cell lines. M, marker; C, control.

**Cell cycle kinetics**

Figure 4 shows the results of cell cycle kinetic analysis. The cells broke down severely showing a time-dependent decrease of G1 and G2/M peaks without cell cycle arrest. Sub-G1 fraction, which means hypodiploid cells correspond to dead cells,



**Fig. 4.** Cell cycle kinetics analysis by flow cytometry. Cells were infected with Ad.tk at MOI 5 for HSC-3, 2.5 for HSC-4 and SCCKN, and followed by 1 µg/mL GCV. Cells were harvested in a time-dependent manner. Uninfected cells without GCV treatment were used as control. The number within each panel shows the percentage of Sub-G1 fractions. C, control.



**Fig. 5.** Annexin V-FITC and PI double staining by flow cytometry. Cells were infected with Ad.tk at MOI 5 for HSC-3, 2.5 for HSC-4 and SCCKN, and followed by 1  $\mu\text{g}/\text{mL}$  GCV. Cells were harvested in a time-dependent manner. Uninfected cells without GCV treatment were used as control.

- a:** Representative results of HSC-4. The number within each panel shows the percentage of each quadrant. C, control.
- b:** Increases in percentage in the lower right quadrant relative to control.  $\square$ , HSC-3,  $\diamond$ , HSC-4 and  $\bullet$ , SCCKN.
- c:** Increases in percentage in the upper right quadrant relative to control.  $\square$ , HSC-3,  $\diamond$ , HSC-4 and  $\bullet$ , SCCKN.

increased in a time-dependent manner in all the cell lines.

### Annexin V assay

Annexin V has an affinity for the phosphatidylserine that is translocated from the inner leaflet to the outer leaflet of the plasma membrane in the early stage of apoptosis (Martin et al., 1995). During the late stage, the plasma membrane showed increased permeability and PI entered the cytoplasm and stained chromatin indicating necrosis. Figure 5a shows representative kinetics of HSC-4 in the cell lines. The proportion of annexin V-positive/PI-negative cells in the lower right quadrant shows the early stage of apoptosis increased in a time-dependent manner (Fig. 5b). The proportion of annexin V-positive/PI-positive in the upper right quadrant shows necrosis also increased in a time-dependent manner (Fig. 5c).

### Expression of apoptosis related protein

Protein expression levels of P53, P21, Bax, Bcl-2 and caspase-3 activation were examined by Western blot analysis (Fig. 6). P53 was stably expressed in HSC-3 and HSC-4 cell lines, and undetectable at all time points in the SCCKN cell line. P21 and Bax was consistently expressed at any time points in all cell lines. On the other hand, Bcl-2 slightly but steadily increased within 24 h after treatment in HSC-3 and SCCKN cell lines. No caspase-3 cleavage or activation was observed throughout the experiment in any cell lines.

### TUNEL method

*In situ* DNA strand breaks were examined by the TUNEL method. TUNEL-positive cells with brown-labeled nuclei were occasionally detected