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Cell Cycle Regulator Activation Induced by Ganciclovir in Human Non-small Lung Cancer Cells, CL-1, Transfected with Herpes Simplex Virus-*thymidine Kinase*

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ABSTRACT

Human non-small cell lung cancer (NSCLC) cells were transfected with herpes simplex virus-thymidine kinase (HSV-*tk*) cDNA and the cells resistant to G418 were selected. Two clones were investigated based on their difference of the expression level of HSV-tk and investigated for their sensitivity toward exogenous ganciclovir (GCV). The apoptosis from cell susceptibility to GCV and the bystander effect is dependent on the level of HSV-tk followed by activation of cell cycle modulators and tumor suppressor p53. The study provided a model for better understanding of gene therapy in cancer cells that helps the *in vivo* work.

Key words: Human non-small-cell-lung cancer cells, herpes simplex virus-thymidine kinase, gene therapy

Introduction

Lung cancer is one of the most leading malignancies in the overall global population and in Taiwan. It is the second global eminent cause of cancer deaths in men. The major impediment in cancer treatment is that cancer cells do not exhibit unique biochemical properties that distinguish them from normal cells. One promising approach takes advantage of the normal physiological response to suicide/prodrug therapy leading to terminal cellular apoptosis. The approach is gradually becoming a promising alternative to the conventional therapeutic modalities (Deonarain *et al.*, 1995).

One of the mostly promising gene therapeutic studies uses the herpes-simplex-virus-thymidine kinase (HSV-*tk)* gene, which confers sensitivity to exogenous ganciclovir (9-(1,3-dihydroxy- 2-pro-proxy-methyl)-guanine; GCV). An important aspect of this enzyme-prodrug antitumor therapy is the ability to kill cells that do not express the transgene, a course termed the "bystander effect". Expression of HSV- *tk* gene product in fewer as 10% of the tumor cells in animal models accounts for the complete tumor regression after GCV treatment. In this study, we describe the occurrence of apoptosis following ganciclovir treatment after introduction of HSV-*tk* in human non- small-cell-lung cancer (NSCLC) cells. The bystander effect was detected and an endogenous escalation of cell cycle regulator was also shown to accompany cell apoptotic progression.

Materials and Methods

Cell line and tissue culture

Human NSCLC cells, CL-1, kindly given by Dr. Pang-Chr Yang (Department of Internal Medicine, National Taiwan University), were grown in RPMI-1640 medium (Gibco-BRL, Grand Island, NY) supplemented with 5% FBS (HyClone, Logan, UT), 2 mM of L-glutamine, 100 U/ml of penicillin, and 100 mg/ml of streptomycin sulfate (all from Life Technologies Inc., Grand Island, NY). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cell line was maintained free of mycoplasma infection.

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Recombinant pCMV-HSV-*tk* construct and transfection

The pCMV-HSV-*tk* construct was cloned as follows: DNA fragment was amplified from pKO containing HSV-*tk* gene with opening reading frame and the product digested with *Eco*RI. The gel-purified fragment was ligated into *Eco*RI-linearized pcDNA3 mammalian vector (Invitrogen, San Diego, CA) that contains human mammalian cytomegalovirus promoter to form pcDNA-HSV-*tk* construct. The size, sequence and orientation of the inserted fragment in the selected clones were verified through restriction mapping and sequenced from both directions.

A total of 5×10^3 exponentially growing CL-1 cells cultured in 60-cm^2 flasks were transfected with one microgram of either recombinant pcDNA-HSV-*tk* construct or the empty pcDNA3 vector by Lipofectamine (Gibco-BRL, Gaithersburg, MD) in serum-free media following manufacturer's protocols. After three weeks of culturing and selection with 400 μg /ml of G418 (Gibco-BRL, Gaithersburg, MD), twelve colonies were harvested and, after limited dilution, the selected subclones containing HSV-*tk* insert, B9 and C6, were isolated further established as stable cell lines, and propagated in 6% FCS-supplemented RPMI-1640.

Detection of HSV-*tk* genomic DNA incorporation and HSV-*tk* gene expression

To detect the integrated HSV-*tk* gene in the transfected clones, total genomic DNA was isolated for PCR amplification of HSV-*tk*. To determine the expression of HSV-*tk*, cellular RNA isolated with TRIZOLE was extracted four times with phenol-chloroform, ethanol- precipitated, and resuspended in diethyl pyrocarbonate-treated water to give a final concentration of 0.5 mg/ml. The contaminated DNA was removed by incubating RNA mixture with DNase I (50 units/ 0.5 mg RNA) at 37°C for 1h. The reaction was terminated by adding 100 μl of 0.1 M EDTA (pH 8.0), and the RNA was extracted twice with phenol-chloroform. The RNA was ethanol-precipitated, washed once with 80% ethanol, and resuspended in 2 ml of diethyl pyrocarbonate-treated water. The primers for PCR were used at an excess concentration of 500 ng in a 50-μl final reaction volume. The PCR was performed using AmpliTaq DNA polymerase as described in the CLONTECH Advantage RT-for-PCR kit protocol (94°C for 45 s; 60°C for 45 s; 72°C for 2 min; 25 cycles; 7 min final extension at 72°C). The amplified product separated in a 0.7% agarose gel.

Western blot against cell cycle regulator antibodies

Cells (1×10^7 cells) exposed to GCV were washed twice in PBS. Cell pellets were resuspended in lysis buffer (50 mM HEPES, pH 7, 2% SDS, 5 mM EDTA, 10% glycerol and 0.1% NP-40, 0.5 mM DTT, 0.5 mM PMSF, 2μlg/ml aprotinin, 10 mM NaF and 50 mM β-glycerophos-

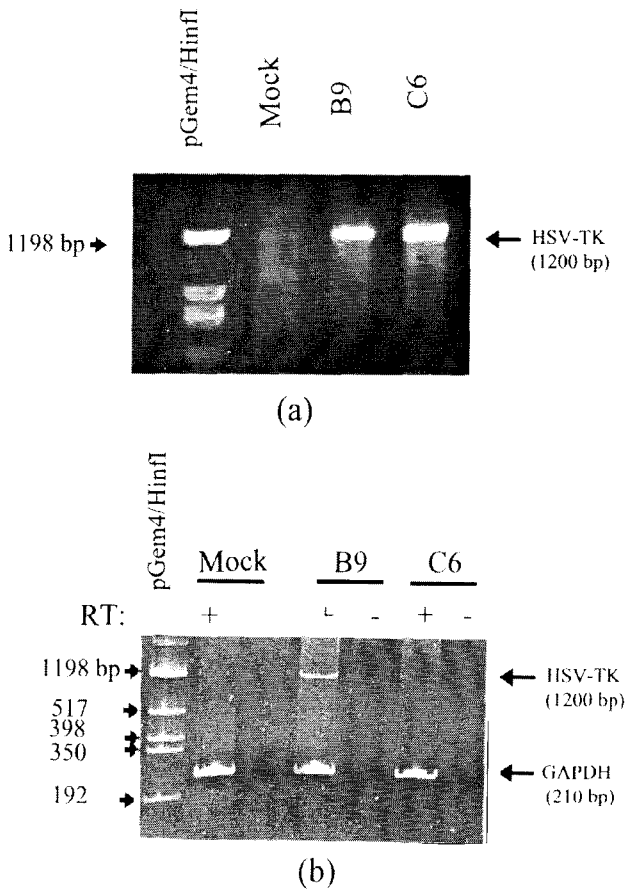

Figure 1. (a) DNA incorporation in the selected clones. Two selected clones, B9 and C6, were shown incorporating HSV-*tk* after G418 selection. The extracted cellular DNA of the two clones along with that of the empty construct-transfected clone were amplified with PCR as described in Materials and Method. The PCR products were separated in a 0.7% agarose gel. (b) Differential expression of HSV-TK in the selected clones. The RNA for selected cultured cells with 70-80% confluency were extracted and 10 μg RNA amplified by RT-PCR for their expressed HSV-*tk* determination and GAPDH (297 bp) to indicate equal RNA loading. Symbols (+) and (-) represent PCR with or without reverse transcriptase reaction on RNA, respectively.

phate). The collected lysates were centrifuged at 12,000 *g* for 5 min, and quantitated with Coomassie protein assay reagent (Pierce, Rockford, IL). Cell extracts with equal amount of protein were separated by 10% SDS-PAGE separating gel and electroblotted to nirtocellulose. The blots are stained in 0.1% amido-black and destained in 5% acetic acid to ensure transfer and equal loading. The blots were blocked in PBS-Tween (PBS-005% T) and non-fat milk for 1 at 22℃. The blots were then incubated in fresh blocking solution and probed for 1 hr with 1:3,000 dilution of cyclin B1, cyclin A and p53 antibodies (Santa Cruz; Lexington, KY). Blots were washed twice for 10 min in PBS-T and then incubated with a 1:4,000 dilution of peroxidase-conjugated secondary antibody (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) in PBS-T for 1 hr at 22℃. Blots were again washed 2 × 10 min in PBS-T and then developed by the ECL detection system (Amersham, Arlington Heights, Il).

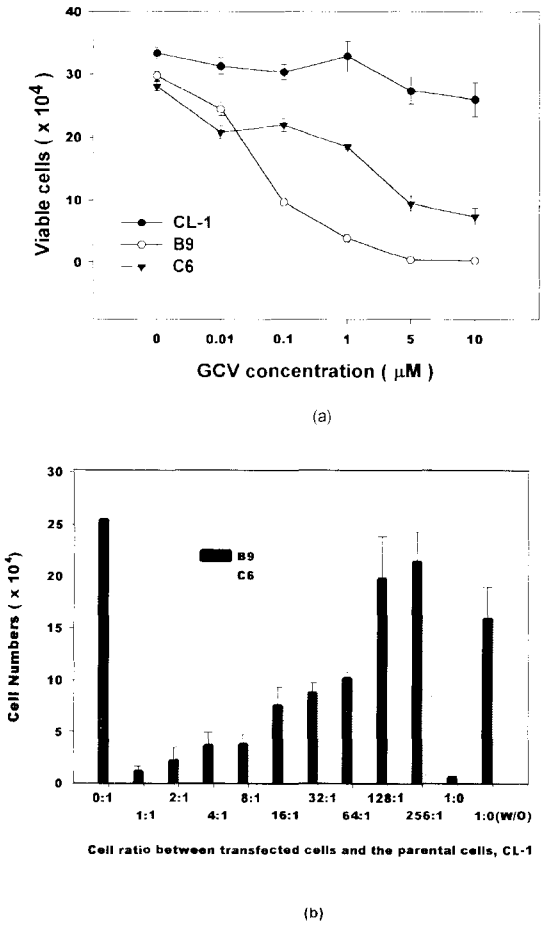

Figure 2. (a) Growth curves of CL-1, B9 and C6 as induced by different concentrations of GCV. A total of 1×10^5 cells of the selected clones were cultured in 6-well plates in 6% serum-supplemented RPMI-1640. Twenty-four hours later, the cells were exposed to different concentrations of GCV. Four days later, the cells were trypsinized at each time point for trypan blue exclusion counting. (b) Differential bystander effect in clones B9 and C6 mixed with the parental cells, CL-1, in different ratios . Both clones were mixed, separately, with the parental cells Cl-1 in different ratios in a total of 1×10^5 cells that were cultured in 6-well plates. The cells were induced by 0.5 μM GCV for four days and counted. The values in both figures represented the average cell numbers counted in three experiments and error bars as standard error.

Result

The growth mediated by HSV-*tk* in human NSCLC tumor cells, CL-1, was abrogated proportional to the level of expressed HSV-*tk*

In this study, an HSV-tk construct that encoded the complete sequence and was motivated by a strong cytomegalovirus promoter was transfected into NSCLC cells, CL-1. The G418-resistant clones were selected, propagated and confirmed incorporating and expressing the HSV-*tk* gene (Figure 1a). Stable clones had identical growth rate compared to cells carrying empty vector alone and the parental cells. The addition of GCV into the culture medium enhanced sensitivity to cells accompanied with the induced morphological change of HSV-*tk* gene-transfected cells. The apoptosis was observed in a significant portion of the target cell population. Two isolated clones, B9 and C6, expressed HSV-*tk* gene differently (Figure 1b). The response toward exogenous GCV differed correspondingly (Figure 2a).

Determination of bystander effect of HSV-tk transfected human NSCLC tumor cells with untransfected cells

Bystander effects were measured by mixing cells with HSV-*tk*-transfected cells, B9 or C6, , and untransfected cells at ratios of 1:0, 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, and 0:1 and plating onto 24-well plates at 1×10^5 cells /well (Figure 2b). Cells were then exposed to GCV at 5 μg/ml at 24 h after plating. Cell viability was measured 4 days after GCV treatment. Transfected cells with highly expressed HSV-*tk* gene, B9, exerted stronger bystander

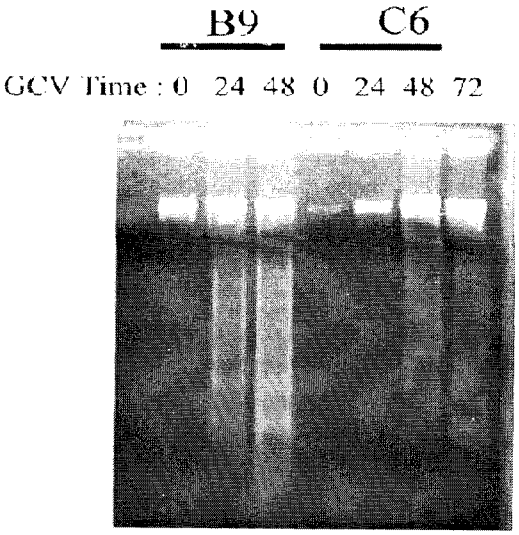

Figure 3. Cellular DNA internucleosomal fragmentation after GCV stimulation. A total of 1×10^5 cells of the selected clones were cultured and exposed to 0.5 μM GCV for four days. The supernatant was collected for DNA extraction. The collected DNA was separated in a 0.7% agarose and stained with ethidium bromide.

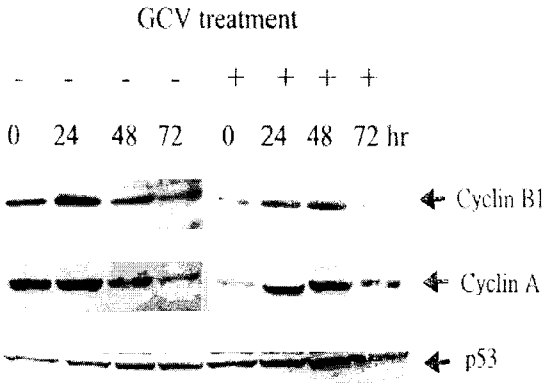

Figure 4. Western blot with cyclin B1, cyclin A and p53 antibodies. A total of 1×10^7 cells of the selected clone B9 was cultured and exposed to 0.1 μM GCV for the time indicated. Cell pellets were resuspended in lysis buffer. Cell extracts with equal amount of protein were separated by 10% SDS-PAGE separating gel and electro-blotted. The blots were then incubated in fresh blocking solution and probed for 1 hr with 1:3,000 dilution of cyclin B, cyclin A or p53 antibodies followed by hybridization with a 1:4000 dilution of peroxidase-conjugated secondary antibody and then developed by the ECL detection system.

effects as compared to C6 clone which had lower HSV-*tk*. Thus GCV significantly inhibited cellular proliferation in a manner dependent on the level of HSV-*tk* gene product.

GCV-induced apoptotic cell death in NSCLC cells transfected with HSV-*tk*

Morphological changes were observed 12 h after treatment with 5 μM of GCV in cells with transfected HSV-*tk*. After 24 h of GCV exposure, characteristics of membrane blebbing, cell detachment, cell shrinkage, vesicle formation, compaction and margination of nuclear chromatin, and convolution of nuclei were conspicuous in HSV-*tk*-transfected cells, while no such effects were observed in the control, the parental cells , or in HSV-*tk*-positive cells without GCV treatment. Further support indicating apoptotic changes was provided by the characteristic pattern of ladder fragmentation (Figure 3).

Elevation of cell cycle regulator in GCV-treated HSV-*tk*-transfected cells

To learn more about the involvement of cell growth in GCV-induced apoptosis, the cell cycle regulators were examined during the time course of apoptosis development (Figure 4). The results clearly indicated a persistent elevation of both cell cycle regulators-cyclin A and cyclin B1 and tumor suppressor p53, 72 h following GCV treatment in HSV-*tk*-transfected cells. The level of cyclin D1, however, remained intact throughout the duration of GCV stimulation. In the transfected cells not subjected to GCV-induced apoptosis, the appearance of both cyclin A and B1 dropped 24 h following cell culture. The finding differed from those stimulated that an escalation of G_2/M cell cycle regulators, cyclin A and cyclin B1 was detected.

Dicsussion

The potential use of suicide gene is to genetically modify a portion of the tumor so that tumor burden can be reduced upon GCV treatment. The key point of the strategy is that GCV is a specific anti-herpes drug which kills infected cells containing HSV-*tk* gene, not the cellular thymidine kinase, which is able to convert monophosphorylated GCV into the DNA precursor. We have described an efficient transfer of HSV-*tk* gene into tumor cells provided a novel and pivotal strategy for cell apoptosis in human NSCLC cells followed by GCV in dose dependent manner. We have clearly demonstrated that the level of cell apoptotic progression is proportional

to the level of HSV-*tk* gene expressed. Thus, the potency of this cytotoxicity can be propagated and amplified. With less than 10% of the cells in culture expressed HSV-TK, significant bystander effect was obsrved.

More data implicating gap junction mediate GCV nucleotide transfer have been accumulated recently. The channel being permeable to small (less than 1 kDa) hydrophilic molecules, gap junctions are formed by the hexameric connexin protein assembly (Cx43) that connect adjacent cells allowing the passage of phosphorylated GCV molecules (Moolten *et al.*, 1990; Su *et al.*, 1997). Studies have indicated the bystander effect involves a transfer of phosphorylated ganciclovir from HSV-TK-containing cells to HSV-TK-negative cells through gap junction-mediated intercellular communication (Mesnil *et al.*, 1996; Rubsam *et al.*, 1999). The protein pores allow molecules of less than 1 kDa to diffuse between cells across a concentration gap and, since GCV nucleotides can be observed in bystander cells within 4 h after coculture, days before significant apoptosis appeared, it is unlikely that apoptotic bodies in HSV-TK-containing cells should contain phosphorylated GCV, and that the debris from phagocytosis appears in the neighboring cells (Freeman *et al.*, 1996).

The appearance of DNA fragmentation agarose electrophoresis indicated internucleosomal cleavages of cellular DNA following integration of phosphorylated GCV. Previously, in melanoma cells with wild type p53 transduced with HSV-*tk*, the continued GCV treatment was followed by p53 elevation and then by S/G_2-phase cell cycle arrest with increases in cyclin B1 level (Wei *et al.*, 1998). In the present work, an escalation of p53 was observed both in the presence of GCV indicating slow turnover of p53 for CL-1 cells. Further characterization of the intrinsic *p53* genotype is needed. With incorporation ganciclovir triphosphate, an extended cell cycle arrest was prolonged to repair the damaged DNA, thus leading to apoptosis progression of p53 escalation. In this work, however, the apoptosis appeared 24 h after drug exposure with no apparent cell cycle arrest. This is distinct from the description in the published work with wild type *p53* genotype, in which cell killing emerged 60 h after sensitization (Wei *et al.*, 1998). An apparent alternative signaling must have triggered apoptosis in human NSCLC cells with transfected HSV-*tk*. Since the concurrent upregulation of two $S/G_2/M$ cell cycle regulators, but not that of G_0/G_1, the repair of damaged DNA must have passed G_1/S checkpoint. In the reported cases of DNA damage, either cyclin A (Knudson *et al.*, 1999) or cyclin B1 (Maity *et al.*,1996; Porter *et al.*, 2000) elevation were needed at the G_2/M phase to allow for DNA repair progression. In this work, an apparent synchronized accumulation of cyclin A and cyclin B1 accompanied with apoptotic development. Thus, it will be of great interesting to determine how variation of different cell cycle modulator associated with mitochondrial control regulators, such as anti-apoptotic gene, Bcl-2, and other apoptotic effectors, such as caspase family and bax, confer cell apoptosis during drug exposure. It will also be worth exploiting to determine how other apoptotic chemotherapeutic agents affect apoptosis efficacy as well as to enhance bystander effect in both *in vitro* and *in vivo* systems.

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人類非小細肺癌細胞攜帶第一型皰疹病毒胸腺嘧啶激素的細胞自戕現象與週期蛋白的活化調控

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摘　要

本文以第一型皰疹病毒胸腺嘧啶激素(HSV-*tk*)基因載體轉殖至人類非小細肺癌腺細胞株(CL-1)之內。由所收集的兩個純品系顯示，它們生長的速率與母細胞沒有任何差異，但是當轉殖細胞以 GCV 處理之後，生長的速率明顯降低，也產生"旁觀者效應"(bystander effect)，生長抑制效應與轉植入基因表現程度相關。實驗也顯示細胞的生長抑制會由含轉殖自殺基因細胞影響至不含基因細胞基因的細胞，主要原因是被磷酸化的 GCV 經由細胞間孔道(gap junction)的流通，影響到不含基因細胞核內 DNA 的合成，致使細胞生長的抑制作用得以蔓延，達到以微量化學藥物產生最大療效的目的。初步實驗結果也顯示，轉殖 HSV-*tk* 的細胞，於 GCV 處理之後，生長機制的抑制，源自細胞的自戕，實驗也可觀察細胞於 DNA 損害後，同時活化週期蛋白，進而引發自戕現象。

關鍵詞：人類非小細肺癌細胞，第一型皰疹病毒胸腺嘧啶激素，基因治療

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