Ganciclovir augments the lytic induction and apoptosis induced by chemotherapeutic agents in an Epstein–Barr virus-infected gastric carcinoma cell line

Eun Ji Jung[a], You Mie Lee[a,c], Byung Lan Lee[a], Mee Soo Chang[b] and Woo Ho Kim[a,b]

Epstein–Barr virus is an oncogenic herpesvirus and has been associated with several human malignancies, including gastric cancer. In Epstein–Barr virus-associated gastric cancer, Epstein–Barr virus is found in virtually all tumor cells, but rarely in normal epithelial cells, thus implying that Epstein–Barr virus-targeting therapies are likely to be an effective treatment strategy. Using the SNU-719 gastric cancer cell line, which is naturally infected with Epstein–Barr virus, we found that the chemotherapeutic agents, such as 5-fluorouracil, *cis*-platinum and taxol, induced the expressions of BMRF1, BZLF1 and BRLF1 proteins that are usually found in the lytic form of the virus. This effect was found to require various signal transduction pathways involving phosphatidylinositol 3′ kinase, mitogen-activated protein/extracellular signal-regulated kinase, protein kinase C δ and p38 mitogen-activated protein kinase. Moreover, the combination of ganciclovir with these agents increased the lytic transformation and induced apoptosis in Epstein–Barr virus-associated gastric carcinoma. We conclude that ganciclovir enhances the therapeutic efficacy of 5-fluorouracil, *cis*-platinum and taxol in Epstein–Barr virus-positive gastric cancer cells. It is hoped that this information will be found useful during the establishment of treatment strategies for Epstein–Barr virus-associated gastric cancer. *Anti-Cancer Drugs* 18:79–85 © 2007 Lippincott Williams & Wilkins.

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[a]Cancer Research Institute and [b]Department of Pathology, Seoul National University College of Medicine, Seoul and [c]Department of Natural Sciences, School of Life Sciences and Biotechnology, Kyungpook National University, Daegu, South Korea.

Correspondence to W.H. Kim, Department of Pathology, Seoul National University College of Medicine, 28 Yongon-dong, Seoul 110-799, South Korea. Tel: +82 2 740 8269; fax: +82 2 765 5600; e-mail: woohokim@snu.ac.kr

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The first two authors contributed equally to this work.

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Introduction

Epstein-Barr virus (EBV) is a large, approximately 172-kb DNA virus of the gammaherpesvirus family and is ubiquitously found in human populations [1,2]. EBV is associated with various malignancies, including Burkitt lymphoma, nasopharyngeal carcinoma, Hodgkin's disease and gastric carcinoma [1]. Recent studies have revealed that EBV is responsible for about 2–16% of gastric cancer cases worldwide. The latency patterns of EBV in gastric carcinoma have been characterized as latency I or II and involve the expression of lytic cycle genes [3]. In Korea, stomach cancer is the most common malignancy and 5.6% of Korean stomach cancers are associated with EBV.

The EBV genome is present in all EBV-associated tumor cells, but not in adjacent normal cells. Therefore, the EBV genome or its products could serve as potential targets for anti-tumor therapies in EBV-associated malignancies [4]. One potential therapeutic strategy that takes advantage of the EBV genome in tumor cells involves the induction of the lytic form of EBV infection in tumor cells [5]. The switch from the latent to the lytic form of EBV infection can be induced by the expressions of one of the EBV immediate-early proteins, i.e. BZLF1 (ZEBRA) or BRLF1. Both BZLF1 and BRLF1 are transcription factors and can induce the entire program of lytic EBV gene expression [6].

SNU-719 is a unique gastric cancer cell line that is naturally infected with EBV. It is one of the several gastric cancer cell lines that were established from Korean patients and, specifically, was derived from a primary gastric tumor that was histopathologically classified as an adenocarcinoma with lymphoid stroma. Moreover, this primary tumor contained EBV in every carcinoma cell and not in normal cells. The expression pattern of EBV genes in this cell line is similar to that of most EBV-associated gastric cancers and, therefore, the SNU-719 cell line is viewed as a useful model for the EBV-associated gastric cancer [7].

Although many randomized chemotherapeutic trials have been reported in gastric cancer, no standard regimens

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have been established. Currently, oncologists agree that *cis*-platinum and 5-fluorouracil (5-FU) are the most effective agents either in combination or as a single agent. In contrast to breast or lung cancers, there is no effective therapeutic regimen to gastric cancer using molecular targeting agents. [8,9].

In this study, we investigated whether the chemotherapeutic agents, 5-FU, *cis*-platinum or taxol, induce the lytic form of EBV *in vitro* and whether ganciclovir (GCV) can enhance the cytotoxic effect of these agents on lytically transformed cancer cells in EBV-positive gastric carcinoma cell lines.

Materials and methods

Cell lines

Two human gastric cancer cell lines, SNU-216 (an EBV-negative gastric cancer cell line) and SNU-719 (an EBV-positive gastric cancer cell line), were obtained from the Korean Cell Line Bank (Seoul, Korea) and were cultured in RPMI-1640 (Hyclone, Logan, Utah, USA), supplemented with 10% heat-inactivated fetal bovine serum (Hyclone).

Chemical agents

The chemotherapeutic agents, 5-FU, taxol and *cis*-platinum, and GCV were obtained from Sigma Aldrich (St Louis, Missouri, USA). Stock solutions of 5-FU (5 mg/ml) and *cis*-platinum (1 mg/ml) were prepared in dimethyl sulfoxide, taxol (1 μmol/l) in ethanol, and GCV (2 mg/ml) in 0.1 N HCl. Signal transduction pathway inhibitors (LY294002, PD98059, Rottlerin and SB202190) were obtained from Calbiochem (Darmstadt, Germany) and 10 mmol/l stock solutions of each were prepared in dimethyl sulfoxide.

Lytic induction assays

To confirm the EBV lytic protein expression, cells were treated with 0.1 μg/ml 5-FU, 0.1 nmol/l taxol or 5 μg/ml *cis*-platinum with/without GCV (10 μg/ml) for 6 days. Proteins were separated on 8% acrylamide gels and transferred to polyvinylidine diflouride membranes. Lytic proteins were detected with anti-BMRF1 (1:100; Capricon, Scarborough, Maine, USA), anti-BZLF1 (1:100; Argene, North Massapequa, New York, USA) and anti-BRLF1 (1:100; Argene). Anti-α-tubulin (1:5000; Sigma) was used as a loading control.

To define the mechanism whereby the chemotherapeutic agents induce the lytic form of EBV infection, cells were grown to 70% confluence and pretreated for 1 h with no agent, LY294002 (15 μmol/l), PD98059 (50 μmol/l), Rottlerin (10 μmol/l) or SB202190 (20 μmol/l). Cells were then treated for 48 h with 5-FU (0.1 μg/ml) in the presence or absence of the inhibitor and then subjected to Western blotting for BMRF1.

Cell viability assays

Cell lines were plated in 96-well cell culture plates and treated with 0.1 μg/ml 5-FU, 0.1 nmol/l taxol or 5 μg/ml *cis*-platinum with/without GCV (10 μg/ml) for the indicated period, or with different concentrations of chemotherapeutic agents with/without GCV (10 μg/ml) for 8 days. Surviving cell numbers were assessed by adding CCK-8 solution [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] (Dojindo, Kumamoto, Japan) to each well and measuring absorbance at 450 nm using a microplate reader. Experiments were performed in triplicate.

Apoptosis assay

To identify apoptotic cells, cells were treated with 0.1 μg/ml of 5-FU with/without 10 μg/ml GCV for 8 days and compared with untreated cells (control). Cells were then harvested, washed and fixed with 4% paraformaldehyde for 30 min at room temperature, and then suspended in phosphate-buffered saline. Cells were then smeared on slides, dried at room temperature and stained with Hoechst 33258 (50 ng/ml; Sigma) for 30 min. Adherent cells were rinsed with phosphate-buffered saline, air dried and mounted with 90% glycerol, and slides were examined by fluorescence microscopy. Apoptotic cells were identified by nuclear condensation and fragmentation.

For cell cycle determinations, DNA contents were measured by propidium iodide (Sigma) staining permeabilized cells. Briefly, cells were harvested, washed, fixed with 70% ethanol and then stained with propidium iodide (20 μg/ml). Cell cycle analysis was performed by measuring cellular DNA contents using a flow cytometer (Beckton Dickinson, San Diego, California, USA).

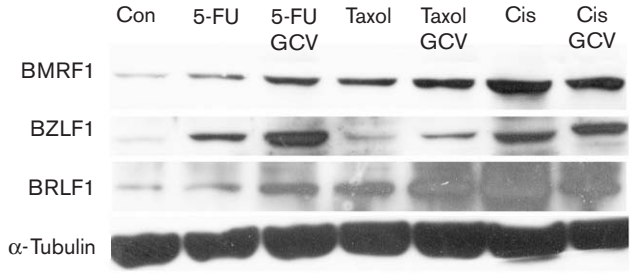
To confirm the involvement of caspase-3, caspase-8 and caspase-9, cells were treated with 0.1 μg/ml 5-FU, 0.1 nmol/l taxol or 5 μg/ml *cis*-platinum with/without GCV (10 μg/ml) for 6 days and then harvested for protein extraction. Caspase expressions were investigated by Western blotting using anti-caspase-3 (1:200; Imgenex, San Diego, California, USA), anti-caspase-8 (1:500; Imgenex) or anti-caspase-9 (1:500; Upstate, Charlottesville, Virginia, USA). Anti-α-tubulin (1:000; Sigma) was used as a loading control.

Results

The induction of lytic Epstein–Barr virus infection *in vitro*

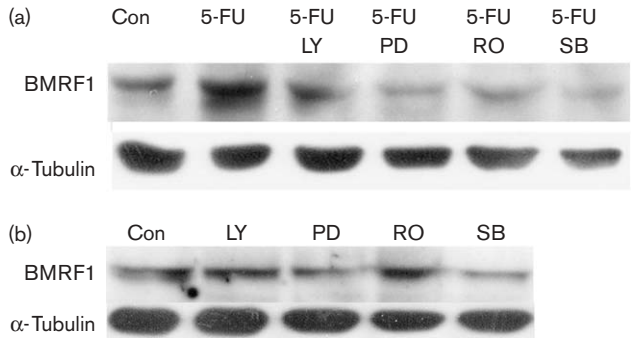
We examined whether 5-FU, *cis*-platinum or taxol induce the lytic form of EBV infection in SNU-719 cell lines. Cells were treated with each of these three agents with/without GCV for 6 days and then the expressions of EBV early lytic proteins, BMRF1, BZLF1 and BRLF1, were examined by Western blotting. All agents induced the expression of lytic EBV proteins (Fig. 1). Moreover, the addition of GCV to the 6-day treatments enhanced these expressions. Expression of these proteins was not

Fig. 1



Induction of lytic cycle proteins by 5-fluorouracil (5-FU), *cis*-platinum (Cis) or taxol and ganciclovir (GCV) in the SNU-719 cell line. Cells were treated with 0.1 μg/ml 5-FU, 0.1 nmol/l taxol or 5 μg/ml *cis*-platinum with/without 10 ig/ml GCV for 6 days, and the expression of lytic proteins, i.e., BMRF1, BZLF1 and BRLF1, was investigated by Western blotting. The control (Con) is the untreated SNU-719 cell.

Fig. 2



The effects of signal pathway inhibitors on immediate-early BMRF1 protein expression. (a) Cells were treated with 5-fluorouracil (5-FU) alone or 5-FU plus phosphatidylinositol 3′ kinase inhibitor (LY), mitogen-activated protein/extracellular signal-regulated kinase inhibitor (PD), protein kinase C δ inhibitor (RO) or p38 MAPK inhibitor (SB) for 2 days and total cell lysates were then subjected to Western blotting. (b) Treatment of cells with the above inhibitors alone did not alter BMRF1 expression. Untreated SNU-719 cell was used as a control (Con).

detected in the SNU-216 gastric cancer cell line irrespective of any treatment (data not shown).

To define the mechanism by which these three agents induce the lytic form of EBV infection, the effects of materials known to inhibit specific signal transduction pathways were examined. Cells were treated with phosphatidylinositol 3′ (PI3) kinase inhibitor (LY294002), mitogen-activated protein (MAP)/extracellular signal-regulated (ERK) kinase inhibitor (PD98049), protein kinase C δ inhibitor (Rottlerin) or p38 mitogen-activated protein kinase (MAPK) inhibitor (SB202190) and expression of the lytic protein, BMRF1, was analyzed. The treatment of cells with inhibitors and 5-FU reduced BMRF1 expression (Fig. 2a), whereas cells treated with an inhibitor alone showed no BMRF1 expressional change (Fig. 2b). These results confirm that various signal transduction pathways (PI3 kinase, MAP/ERK kinase, protein kinase C δ and p38 MAPK) contribute to the induction of lytic EBV forms by chemotherapeutic agents in EBV-positive gastric cancer cells.

Effect of the chemotherapeutic agents and ganciclovir on cell viability

We examined whether GCV enhanced the cytotoxic effects of the three chemotherapeutic agents. SNU-719 cells were treated with 5-FU, *cis*-platinum or taxol with/without GCV at different concentrations for 8 days. The addition of GCV was found to enhance the cytotoxic actions of all three agents, especially of 5-FU (Fig. 3), not in a dose-dependent manner. The same treatment was not effective in killing EBV-negative SNU-216 gastric cancer cells (Fig. 4).

Apoptosis induced by chemotherapeutic agents and ganciclovir

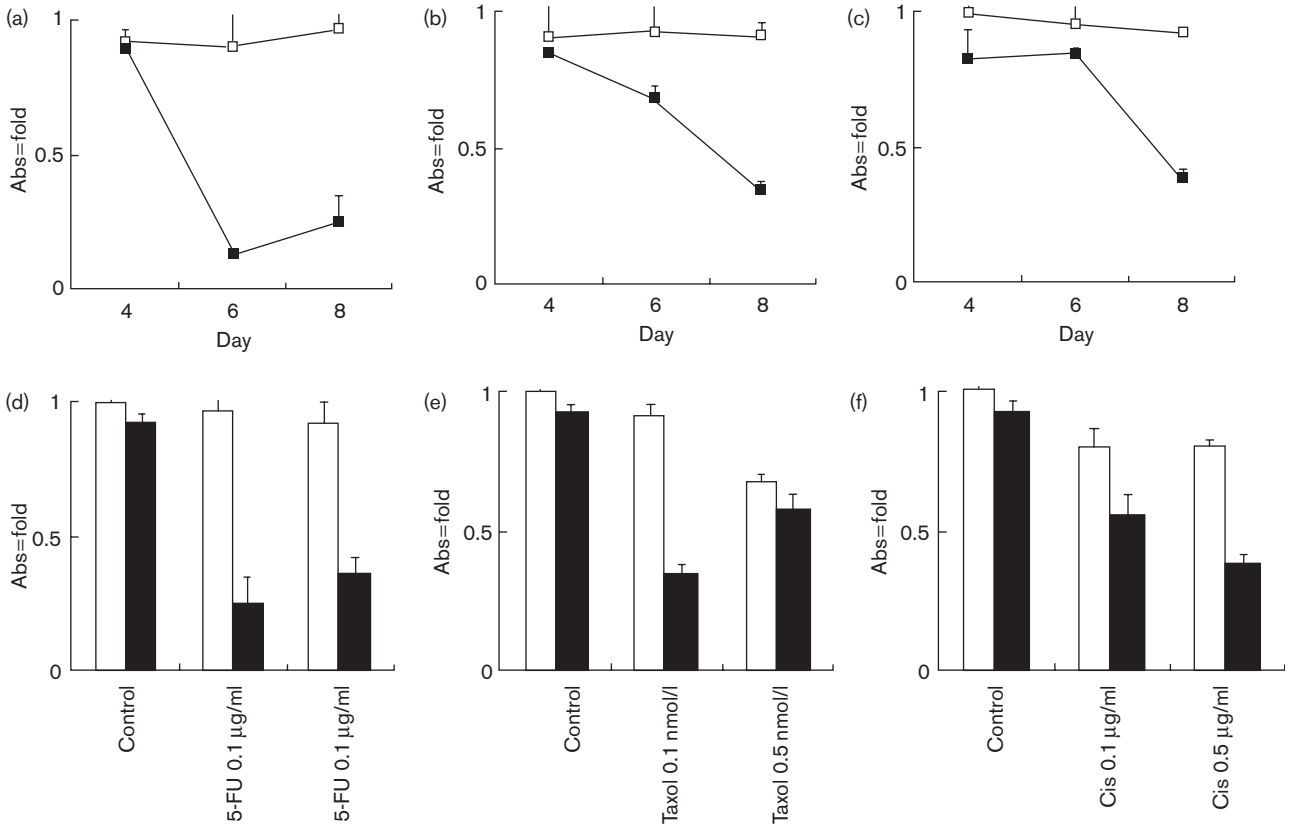
To determine whether the combination of chemotherapeutic agents and GCV leads to apoptotic cell death, SNU-719 cells were treated with 5-FU alone or with 5-FU and GCV, and analyzed by Hoechst 33258 staining and flow cytometry. Cells treated with 5-FU and GCV showed nuclear fragmentation and condensation by Hoechst 33258 staining (Fig. 5a). Apoptosis was also confirmed by the flow cytometric analysis of cellular DNA contents after staining with propidium iodide (Fig. 5b).

To investigate caspase requirements during apoptosis, SNU-719 cells were treated with 5-FU, *cis*-platinum or taxol with/without GCV and the expressions of various caspases were analyzed by Western blotting. The activations of caspases -3, -8 and -9 were suggested by reductions in the intensities of their proenzymes (Fig. 6). Thus, we concluded that in the EBV-positive SNU-719 gastric cancer cell line, the combination of GCV and either of the three chemotherapeutic agents induces apoptosis, which is largely dependent on caspase activation.

Discussion

EBV is found in almost all tumor cells in EBV-positive cancer patients, but not in normal cells. This situation suggests that EBV-targeting strategies could be used to treat such tumors. EBV-based strategies for treating cancer include the prevention of viral oncogene expression, inducing the loss of the EBV episome, purposefully inducing the lytic form of EBV infection and enhancing host immune response to virally encoded antigens [4]. GCV is an anti-viral drug that is used to treat virus-infected cells and can be used to treat cancer if the virus in the tumor cells become lytic. Host cells containing the lytic, but not the latent type of EBV infection, express

Fig. 3



In-vitro cell viabilities of Epstein–Barr virus-positive gastric cancer cell lines treated with each of the three chemotherapeutics with/without ganciclovir (GCV) in comparison with untreated control. SNU-719 cell line was treated with 0.1 μg/ml 5-fluorouracil (5-FU) (a), 0.1 nmol/l taxol (b) or 5 μg/ml *cis*-platinum (Cis) (c) with (solid box) or without 10 μg/ml GCV (open box) and cell viabilities were measured using CCK reagent. SNU-719 cells were treated with different concentrations of 5-FU (d), taxol (e) or *cis*-platinum (f) with (solid box) or without GCV (open box) for 8 days.

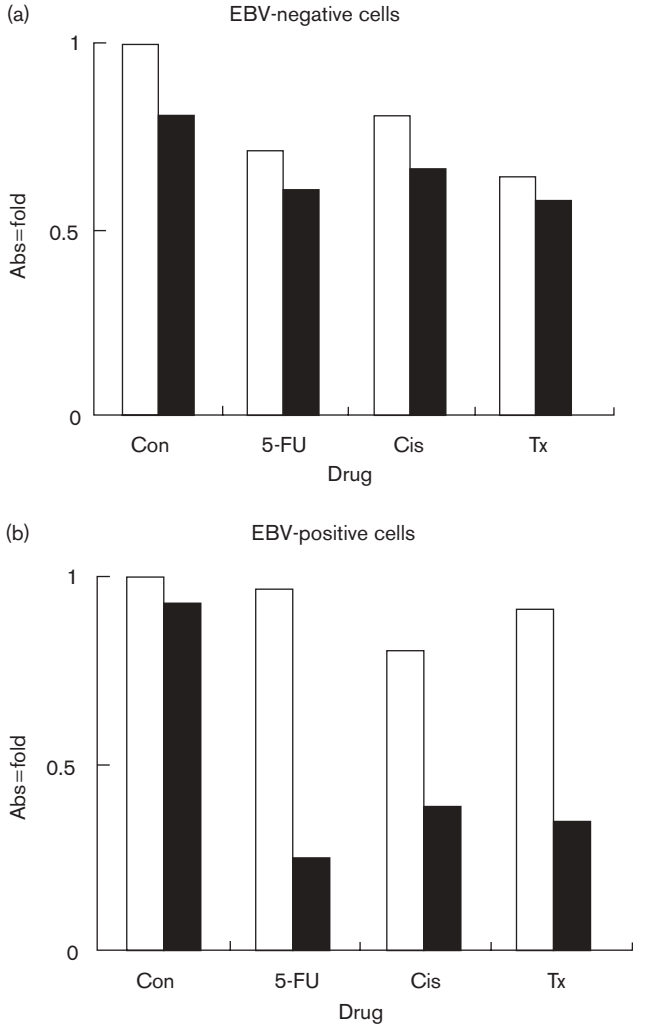
virally encoded kinases that can phosphorylate the prodrug, GCV, and convert it to its active cytotoxic form [10,11]. Furthermore, phosphorylated GCV can be transferred to nearby cancer cells, thus inducing 'by-stander killing' [12]. As EBV-positive tumor cells are primarily infected with latent forms of EBV infection, GCV in itself is not useful for treating EBV-positive tumors until the virus enters into its replicative lytic cycle [4].

The present study confirms that the chemotherapeutic agents, 5-FU, *cis*-platinum and taxol, induce the expressions of immediate-early proteins, BMRF1, BZLF1 and BRLF1. Both BZLF1 and BRLF1 are transcription factors, and activate the transcriptions of other genes required for the lytic conversion of the virus. The overexpression of either of the above proteins in latently infected cells can induce the lytic cycle of EBV [1], but the induction of lytic proteins is not enough to induce the cell death. Also, in the present study, we found that GCV can enhance the lytic conversion induced by these three chemotherapeutic agents.

Three different signal transduction pathways, i.e. p38 stress MAPK, PI3 kinase and the protein kinase C δ pathways, are known to be important for the induction of lytic EBV infection by drugs in epithelial cells [5]. The ability of gemcitabine and doxorubicin to induce lytic EBV gene expression requires the same signal transduction pathways. The present study confirms that PI3 kinase, MAP/ERK kinase, protein kinase C δ and p38 MAPK are involved in the induction of lytic EBV infection by these three agents in an EBV-positive gastric cancer cell line. Although we could not elucidate the mechanism by which these agents induce lytic EBV infection, it appears that the virus recognizes cellular stress in the host cell and that this converts the infection to the lytic form via multiple cellular signal transduction pathways.

Moreover, it was reported that the addition of GCV greatly enhances the ability of both 5-FU and *cis*-platinum to inhibit nasopharyngeal carcinoma growth in nude mice [6], and that the addition of GCV to chemotherapy regimens enhances therapeutic efficacy in EBV-driven

Fig. 4



Effects of 0.1 μg/ml 5-fluorouracil (5-FU), 0.1 nmol/l taxol (Tx) or 5 μg/ml *cis*-platinum (Cis) with (solid bar) or without (open bar) (10 μg/ml) ganciclovir (GCV) for 8 days on the in-vitro cell viabilities. (a) Effect of the addition of GCV was small in Epstein–Barr virus (EBV)-negative gastric cancer cells. (b) GCV effectively blocked the cell proliferation in EBV-positive gastric cancer cells. Con, control.

lymphoproliferative disease patients [5]. Here, we demonstrate that combinations of GCV and the three agents examined were more effective than the agents alone in EBV-positive gastric cancer cells, but not in EBV-negative gastric cancer cells. Previous reports have demonstrated that lytically infected cells are more susceptible to GCV cytotoxicity; these reports involved a non-gastric cancer cell line [5] or were artificially incorporated with viral genome [6]. The present study is the first to show that GCV has a combinatorial effect in a gastric cancer cell line naturally infected with EBV. As the SNU-719 cell line shows the same expression pattern of EBV genes as most EBV-associated gastric cancer cells [3,13], we believe that it serves as a useful model for therapeutic applications concerning EBV-associate gastric cancer.

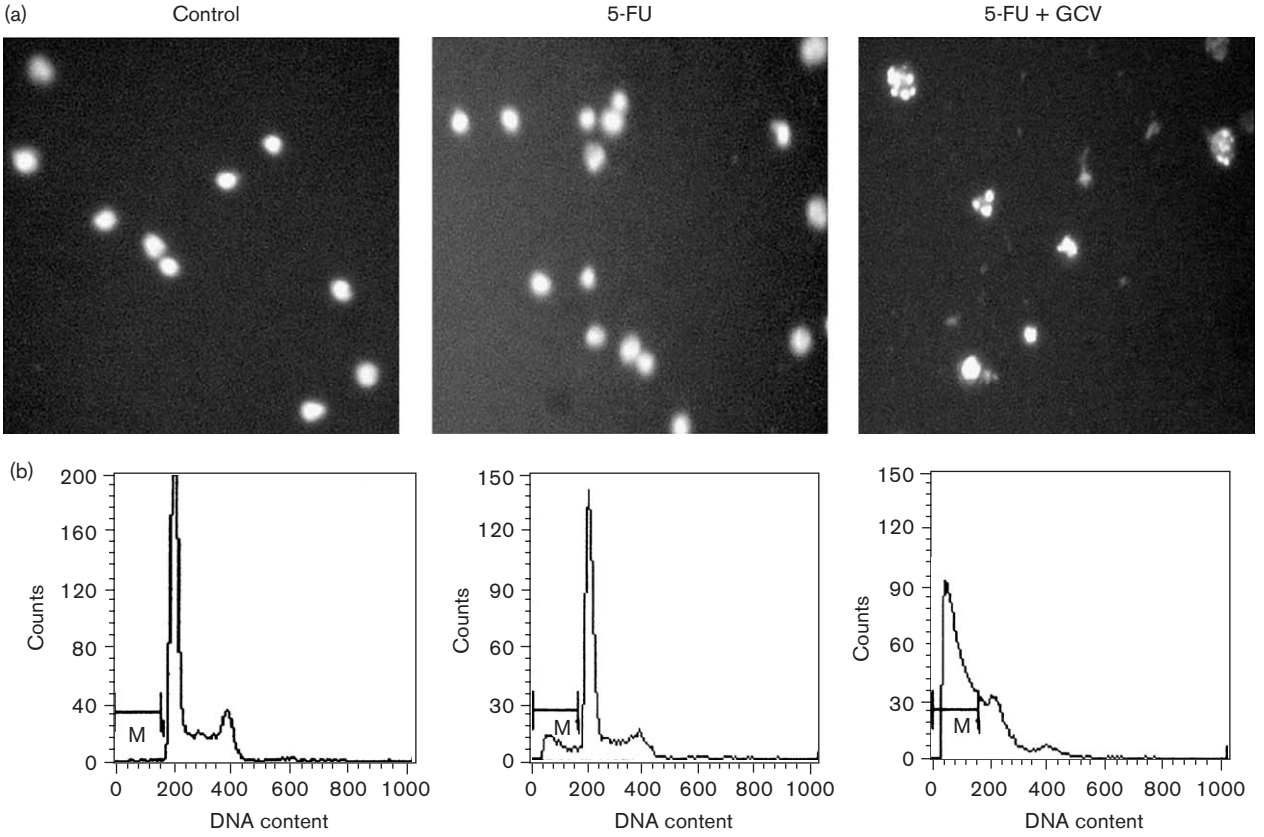
In previous paper, we showed the similar result that epigenetic-modifying agents, 5-aza-2′-deoxycytidine or trichostatin A, induced the expression of BMRF1, BZLF1 and BRLF1 genes in an EBV-positive gastric cancer cell line, and addition of GCV induced apoptosis. In this case, the induction of lytic EBV infection did not involve PI3 kinase, MAP/ERK kinase, protein kinase C δ and p38 MAPK, but probably involved changes in DNA methylation or histone structure [14].

In EBV-positive gastric cancer tissues, some EBV lytic genes are expressed at low level [1]. These are also identifiable in SNU-719 cell line by RT-PCR [13]. If gastric cancers contain sufficient cells with the lytic form of infection, GCV alone is likely to show a significant cytotoxic effect and previous reports have suggested that GCV alone has some clinical benefit in certain tumor containing cells with the lytic form of EBV infection [15]. GCV alone was, however, not found to be effective in treating SNU-719. We believe that only a small proportion of the cell populations contain EBV in the lytic state in gastric cancer and that the great majority of cancer cells are infected with latent EBV.

To induce the lytic form of EBV, we used 5-FU, *cis*-platinum and taxol. Taxol-treated gastric carcinoma cells are known to be arrested in the G_2/M phase and to undergo apoptosis [16]. In the present study, we used a much lower dose (0.1 nmol/l) than the normal growth-inhibiting dose (10 nmol/l) [17]. Moreover, although combination treatment with 5-FU and *cis*-platinum has an anti-tumor action in gastric carcinoma, no significant anti-tumor effect for 5-FU alone was observed at concentrations from 130 to 750 μg/ml *in vitro* and *cis*-platinum showed no significant anti-tumor effect at concentrations from 450 to 900 μg/ml [16]. Even when we treated with low concentrations, i.e., 0.1 μg/ml of 5-FU or 5 μg/ml of *cis*-platinum, GCV enhanced their efficacies in terms of killing cancer cells. We also found that in an EBV-positive gastric cancer cell line, the combination of GCV and any of the three chemotherapeutic agents examined induced more apoptosis than the treatment of chemotherapeutic agent alone, as assessed by Hoechst 33258 staining and flow cytometry. Initiator caspases and effector caspases act together to blow up the death signal, and finally lead to a unilateral process to apoptosis [18,19]. A decrease in procaspases was observed by Western blotting. Thus, it indicated that this apoptosis was largely dependent on caspase activation.

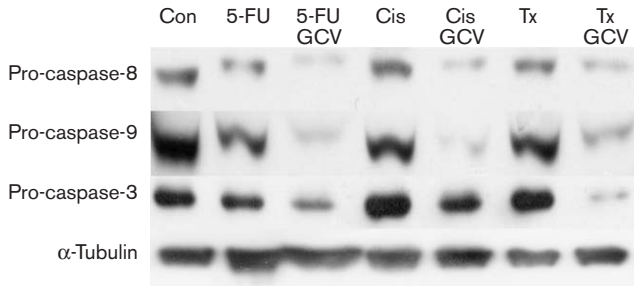
In conclusion, combinations of GCV and 5-FU, *cis*-platinum or taxol were found to show enhanced therapeutic efficacy in EBV-associated gastric cancer.

Fig. 5



Identification of apoptosis in SNU-719 cells induced by 5-fluorouracil (5-FU) with/without ganciclovir (GCV). (a) Morphology of Hoechst 33258-stained cells. Apoptosis was identified by nuclear fragmentation and condensation in cells treated with 5-FU plus GCV. (b) Flow cytometric analysis of DNA contents using propidium iodide revealed that most cells were apoptotic after 5-FU plus GCV treatment. Control is the untreated SNU-719 cells.

Fig. 6



Western blot for caspases after treatment with 5-fluorouracil (5-FU), *cis*-platinum (Cis) or taxol (Tx) plus ganciclovir (GCV). The activations of caspases -3, -8 and -9 were suggested by reductions in the intensities of their proenzymes, especially after treatment with 0.1 μg/ml 5-FU, 0.1 nmol/l taxol, or 5 μg/ml *cis*-platinum and GCV. α-Tubulin was used as a loading control. Untreated SNU-719 served as a control.

We hope that this information will be useful for those trying to establish virus-targeting treatments for EBV-associated gastric cancer and suggest that clinical studies be undertaken to determine whether such strategies are effective in gastric cancer patients.

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