Valproic Acid Enhances the Efficacy of Chemotherapy in EBV-Positive Tumors by Increasing Lytic Viral Gene Expression

Wen-hai Feng and Shannon C. Kenney

Abstract

EBV infection in tumor cells is generally restricted to the latent forms of viral infection. Switching the latent form of viral infection into the lytic form may induce tumor cell death. We have previously reported that certain chemotherapy agents can increase the amount of lytic viral gene expression in EBV-positive tumor cells. In this report, we have explored the potential utility of valproic acid (VPA), an anti-seizure drug that also has strong histone deacetylase inhibitory activity, for activating lytic viral gene expression in EBV-positive tumors. Although VPA treatment alone induced only a modest increase in the level of lytic viral gene expression, it strongly enhanced the ability of chemotherapeutic agents to induce lytic EBV gene expression in EBV-positive epithelial and lymphoid cells in vitro. Furthermore, VPA enhanced cell killing in vitro by chemotherapeutic agents in lymphoblastoid cells and gastric cells (AGS) containing wild-type EBV. In contrast, VPA did not enhance the cytotoxicity of chemotherapy in lymphoblastoid cells containing a lytic-defective (BZLF1-knockout) form of EBV or in EBV-negative AGS cells. Finally, we found that the combination of VPA and chemotherapy was significantly more effective in inhibiting EBV-positive tumors than the three forms of latent viral infection (1, 2). In vitro, a variety of different treatments, including phorbol ester, sodium butyrate, transforming growth factor β, and activation of the B-cell receptor, induce the lytic form of EBV infection (1, 2) by activating BZLF1 and/or BRLF1 transcription.

In addition, we previously showed that certain chemotherapy agents induce lytic viral gene expression in EBV-positive epithelial cell tumors [5-fluorouracil (5-FU), cis-platinum, and methotrexate; refs. 6, 7] and/or B-cell tumors (gencitabine, doxorubicin, and methotrexate; refs. 7, 8). The ability of chemotherapy to activate the EBV immediate-early BZLF1 promoter requires cis-acting cyclic AMP-responsive element and MEFD2 binding motifs in the promoter, as well as activated phosphatidylinositol 3-kinase, mitogen-activated protein kinase/extracellular signal–regulated kinase (ERK) kinase (MEK) kinase, and p38 kinase pathways (8). Although chemotherapy treatment alone induces lytic viral gene expression in only a fraction of EBV-positive tumor cells, the combination of chemotherapy and the nucleoside analogue ganciclovir produces potent EBV-dependent killing of EBV-positive tumor cells. This effect is mediated by the ability of lytically infected, but not latently infected, EBV-positive tumor cells to phosphorylate ganciclovir and convert it to the active cytotoxic form.

In the absence of chemotherapy, EBV-associated malignancies generally have only a very low level of lytic EBV gene expression. The EBV genome is highly methylated in a variety of EBV-associated tumors, including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, and a subset of posttransplant lymphomas (9–13). DNA methylation plays a crucial role in modulating expression of cellular and viral genes (14). DNA methylation causes transcriptional repression by multiple different mechanisms, including modification of the histone acetylation state and prevention of transcription factor binding to DNA (15–19). EBV genome methylation likely suppresses the ability of cellular transcription factors to activate expression of the EBV immediate-early gene promoters, thus helping to maintain latent infection. Consistent with this, 5-azacytidine (aza-CR) treatment (an inhibitor of DNA methyltransferase) increases the amount of lytic viral gene expression in some Burkitt's lymphoma cell lines (20–22). However, aza-CR does not significantly increase lytic viral...
protein expression in lymphoblastoid cell lines (8). The recent finding that the BZLF1 protein preferentially binds to, and enhances transcription from, the methylated form of the BRLF1 promoter (23) may explain the variable and inconsistent effects of aza-CR.

Valproic acid (VPA), a short-chain fatty acid that is widely used to treat epilepsy and mood disorders, is also known to be a potent histone deacetylase (HDAC) inhibitor. VPA is unique among HDAC inhibitors by virtue of its ability to preferentially inhibit class I HDACs while also triggering the proteasomal degradation of HDAC2 (24, 25). In addition, VPA can induce the demethylation of exogenous plasmid DNA in mammalian cells (26). VPA has been previously shown to activate lytic viral gene expression in cells infected with human cytomegalovirus or KSHV (27–29). We therefore reasoned that VPA treatment might induce lytic EBV gene expression in latently infected host cells and/or enhance the ability of chemotherapy to activate lytic EBV gene expression in EBV-infected tumor cells.

Here we show that whereas VPA treatment by itself only weakly induces lytic EBV gene expression, it potently enhances the ability of a variety of chemotherapy agents to activate lytic EBV gene expression. In contrast, aza-CR, a demethylating agent, does not significantly enhance the induction of lytic EBV infection by chemotherapy. Furthermore, we show that VPA enhances the cytotoxicity of chemotherapeutic agents in an EBV-dependent manner, and show that this synergistic killing effect does not occur in EBV-negative control cells or cells infected with a lytic-defective form of the virus. Finally, we show that the combination of VPA and gemcitabine is much more effective than either VPA or gemcitabine alone in treatment of EBV-positive tumors in the severe combined immunodeficient (SCID) mouse model. Thus, the addition of VPA may be a valuable approach for enhancing the therapeutic efficacy of chemotherapy in treating EBV-positive tumors.

Materials and Methods

Cell lines. AGS, a gastric carcinoma cell line, was obtained from American Type Culture Collection (Rockville, MD). The green fluorescent protein–containing wild-type EBV and the BZLF1-deleted mutant EBV were constructed using bacterial artificial chromosome technology as previously described (30). The wild-type or BZLF1-deleted (Z-KO) viruses were used to obtain EBV-transformed B-cell lines (lymphoblastoid cell lines) or EBV-positive AGS cells as previously described (7, 8). The cell line cell 666 (a gift from Dolly Huang, Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, China) is an EBV-positive nasopharyngeal carcinoma line that was derived from an undifferentiated NPC biopsy. Cells were cultured in RPMI 1640 (all suspension cells and 666 cells) or Ham’s F-12 medium (AGS and AGS-EBV cell lines) with 10% fetal bovine serum at 37°C with 5% CO2 and 100% humidity. Before plating 666 cells, the flasks were coated with fibronectin (10 μg/mL) in PBS at 4°C overnight.

Lytic induction assays. Cells were treated with either aza-CR (2.5 μmol/L; Sigma Chemical Co., St. Louis, MO), VPA (0.3 or 1 mmol/L; Ben Venue Laboratories, Inc., Bedford, OH), 5-FU (1 μmol/L; Pharmacia Upjohn Co., Schaumberg, IL), cis-platinum (0.25 μg/mL; American Pharmaceutical Partners, Inc., Bedford, OH), doxorubicin (0.05 or 0.2 μmol/L; Sigma Chemical), or gemcitabine (0.25 or 1 μg/mL; Eli Lilly and Company, Indianapolis, IN) for 2 to 4 days. Western blot analysis was done as previously described (31) with anti-BMRF1 (1:100; Capricorn Products, Inc., Bedford, OH), anti-BZLF1 (1:100; Argene, North Massapequa, NY), anti-BRLF1 (1:100; Argene), and β-actin (1:5,000; Sigma Chemical) antibodies and an enhanced chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ).

Immunofluorescence. AGS-EBV cells treated with no drug, VPA (0.3 mmol/L), cis-platinum (0.25 μg/mL), or VPA (0.3 mmol/L) combined with cis-platinum (0.25 μg/mL) for 2 days were fixed in cold 50% acetone/50% methanol for 10 minutes at –20°C. Cells were then stained with anti-BMRF1 antibody (1:500; Argene) or an isotype control antibody for 60 minutes at room temperature. BMRF1 staining was visualized with FITC-conjugated goat anti-mouse immunoglobulin G antibody (1:100; Sigma) by fluorescence microscopy.

Luciferase reporter assays. The BZLF1 promoter sequence from –495 to +27 (relative to the mRNA start site) in the B95-8 virus strain genome was PCR amplified and inserted into the luciferase reporter vector, pGL3 basic (Promega, Madison, WI), between the Xhol and BgII sites to create the Zp-luciferase vector. Following purification of plasmid DNA with Qiagen columns per procedure of the manufacturer, the Zp-luciferase vector (1 μg) was transfected using FuGENE 6 transfection reagent (Roche, Indianapolis, IN) into AGS cells seeded on 60-mm plates. Twelve hours later, cells were treated with VPA (0.3 mmol/L), cis-platinum (0.25 μg/mL), or 5-FU (1 μg/mL) alone, or VPA in combination with cis-platinum or 5-FU, followed by further culture for 48 hours. Luciferase activity was assayed with the luciferase reporter assay system (Promega).

In vitro cell killing studies with chemotherapy and VPA. AGS and AGS-EBV cells were treated with no drug, cis-platinum (0.25 μg/mL), 5-FU (1 μg/mL), or VPA (0.3 mmol/L) alone, or cis-platinum or 5-FU combined with VPA. Lymphoblastoid cell lines (containing wild-type virus or the BZLF1-deleted virus) were treated with no drug, doxorubicin (0.05 μmol/L), gemcitabine (0.4 μg/mL), or VPA (1 mmol/L) alone, or doxorubicin or gemcitabine combined with VPA. Cell killing was determined by trypan blue exclusion at 5 days posttreatment.

In vivo tumor studies. All animal experiments were conducted in accordance with the guidelines of the University of North Carolina Animal Care Committee. LCL-1 cells (5 × 106), which contain wild-type EBV, were implanted s.c. into the flanks of 6-week-old SCID mice. For tumor treatment studies, mice were treated with no drug (six tumors), one dose of gemcitabine alone (160 mg/kg body weight administered i.p. at day 7 following injection of the tumor cells; six tumors), VPA alone (300 mg/kg body weight administered i.p. on days 7, 8, and 11 following injection of the tumor cells; six tumors), or the combination of VPA and gemcitabine administered as above (six tumors). The mice were examined and tumor measurements were obtained thrice per week after drug treatment was initiated. Mice were euthanized when the tumor size exceeded 1 cm3 in size. Statistical analysis was done using the t test.

Results

5-Aza-CR does not enhance the lytic-inducing effect of chemotherapeutic agents in vitro. EBV-positive tumor cells usually express only low levels of lytic viral transcripts. We previously reported (6–8) that certain chemotherapeutic drugs, including gemcitabine, doxorubicin, cis-platinum, 5-FU, and methotrexate, can induce the lytic form of EBV gene expression in latently infected host cells. However, for reasons that are not completely understood, chemotherapeutic agents by themselves generally induce lytic EBV protein expression in only a portion of the treated cells. As the EBV genome is extensively methylated in latently infected cells and methylation may suppress the constitutive activity of lytic viral genes, we determined whether aza-CR, a demethylating agent, can enhance the ability of chemotherapeutic drugs to induce lytic EBV gene expression in LCLs. Cells were treated for 4 days with aza-CR alone, gemcitabine or doxorubicin alone, or the combination of aza-CR and chemotherapy. Following these treatments, the expression of an early lytic viral protein, BMRF1, was quantitated by immunoblot analysis. As previously reported, gemcitabine or doxorubicin alone at the doses given significantly induced BMRF1 expression. However, aza-CR alone did not induce lytic EBV protein expression and did not
significantly enhance the ability of gemcitabine or doxorubicin to activate lytic EBV protein expression in LCLs (Fig. 1A). These results suggest that demethylating agents such as aza-CR are not sufficient to enhance the lytic-inducing effects of chemotherapy in LCLs, at least when given concomitantly with chemotherapy.

Valproic acid enhances the lytic induction effect of chemotherapeutic agents in vitro. Methylated DNA is often associated with an inactive (condensed) form of chromatin that inhibits gene transcription. To determine if an inactive chromatin configuration surrounding the EBV immediate-early promoters prevents chemotherapy from activating EBV immediate-early gene transcription in a portion of latently infected cells, we examined the effect of VPA. Valproic acid is not only a potent inhibitor of class I HDACs but has also been reported to induce demethylation (24, 25).

Two different lymphoblastoid cell lines (Fig. 1B), an EBV-positive gastric cell line (AGS-EBV; Fig. 1C) and a cell line derived from an EBV-positive NPC tumor (666; Fig. 1D), were treated with either no drug, VPA, gemcitabine, doxorubicin, cis-platinum, or 5-FU alone, or the combination of VPA and chemotherapy. The expression level of the viral early lytic protein, BMRF1, or the two viral immediate-early proteins, BZLF1 and BRLF1, was analyzed by immunoblot analysis. Even at a very high dose (1 mmol/L), VPA alone produced only minimal lytic EBV protein expression in lymphoblastoid cells or 666 cells (Fig. 1B and D, and data not shown), although some viral lytic protein expression was induced by VPA alone in the relatively lytic AGS-EBV line (Fig. 2B). However, in all three cell types, VPA substantially enhanced the ability of low-dose chemotherapy to induce lytic EBV protein expression (note that the dose of chemotherapy used to treat lymphoblastoid cells in Fig. 1B is substantially lower than that used to treat cells in Fig. 1A).

Immunofluorescence was also done to assess the number of cells expressing the early lytic viral protein, BMRF1, after 2 days of VPA and cis-platinum treatment (alone or in combination) of AGS-EBV cells (Fig. 2). The combination of VPA and cis-platinum clearly resulted in more cells expressing BMRF1 than the use of either drug alone. In addition, the intensity of BMRF1 staining in cells treated with both drugs was greater than that observed in the cells treated with cis-platinum alone. Although immunoblot analysis suggested that the enhanced lytic-inducing capacity of the VPA/chemotherapy combination did not peak until 4 to 5 days of treatment (Fig. 1 and data not shown), we were unable to quantitate specific immunofluorescent staining of the BMRF1 protein at time points later than 2 days due to nonspecific staining of the chemotherapy-treated cells.

Valproic acid activates the EBV immediate-early BZLF1 promoter. The immunoblot analysis shown in Fig. 1C indicated

Figure 1. VPA, but not aza-CR, enhances the lytic inducing effect of chemotherapeutic agents in vitro. A, LCL cells were treated with either aza-CR (5-AZA; 2.5 μmol/L), doxorubicin (DOXO; 0.2 μmol/L), or gemcitabine (GEM; 1 μg/mL), or the combination of aza-CR and chemotherapy. Immunoblot analysis was done 4 days later to analyze the expression of the early lytic EBV protein, BMRF1, and cellular β-actin. B, LCL lines derived from two different donors were treated as indicated with either VPA (1 mmol/L), doxorubicin (0.05 μmol/L), or gemcitabine (0.4 μg/mL), alone or in combination. Immunoblot analysis was done 4 days later to analyze the expression of the early lytic EBV protein, BMRF1, and cellular β-actin. C, AGS-EBV cells were treated as indicated with either VPA (0.3 mmol/L), 5-FU (1 μg/mL), or cis-platinum (0.25 μg/mL), alone or in combination. Immunoblot analysis was done 2 days later to analyze the expression of three lytic EBV proteins (BMRF1, BZLF1, and BRLF1) and cellular β-actin. D, NPC tumor (666) cells were treated as indicated with either VPA (0.3 mmol/L), 5-FU (1 μg/mL), or cis-platinum (0.25 μg/mL), alone or in combination. Immunoblot analysis was done 2 days later to analyze the expression of the early lytic EBV protein, BMRF1, and cellular β-actin.
that VPA enhances the expression of the two immediate-early proteins, BZLF1 and BRLF1, when given with chemotherapy. To determine whether VPA can directly activate the BZLF1 immediate-early promoter, alone or in combination with chemotherapy, EBV-negative AGS cells were transfected with a promoter gene construct containing the BZLF1 promoter, Zp, linked to the luciferase gene (Zp-luciferase). Interestingly, VPA alone increased the activity of the BZLF1 promoter even in this experiment using "naked" DNA, suggesting that at least part of the VPA effect may be independent of its ability to enhance chromatin acetylation. As previously reported, treatment of cells with chemotherapy alone also increased the luciferase activity derived from the Zp-luciferase construct (Fig. 3). Treatment of AGS cells with VPA and 5-FU together resulted in the greatest level of Zp activity. Nevertheless, in contrast to the dramatic synergistic effect of VPA and 5-FU observed when activating BZLF1 transcription in the context of the intact (chromatinized) viral genome (Fig. 1C), VPA only modestly increased the ability of chemotherapy to activate the naked DNA Zp promoter construct in AGS cells. These results suggest that the ability of VPA to enhance the lytic induction effect of chemotherapy is at least partially related to its HDAC activity.

Valproic acid enhances chemotherapy killing of EBV-positive AGS cells. The finding that VPA enhances the ability of chemotherapeutic agents to induce lytic EBV infection in lymphoblastoid cells and epithelial cells suggests that it could also enhance the killing of these cells by chemotherapy. To test this possibility, EBV-negative or EBV-positive AGS cells were treated with no drug, VPA alone (0.3 mmol/L), cis-platinum alone (0.25 μg/mL), or cis-platinum (0.25 μg/mL) combined with VPA (0.3 mmol/L). Cells were then harvested and expression of the lytic EBV protein, BRLF1, was assessed by immunofluorescence. A, BMRF1 staining. B, combined BMRF1/4',6-diamidino-2-phenylindole staining of the same slides in (A).
Valproic acid enhances the effectiveness of gemcitabine treatment of EBV-positive lymphoblastoid tumors in SCID mice. To determine if VPA can enhance the efficacy of chemotherapy for EBV-positive lymphoblastoid tumors in vivo, SCID mice were inoculated with 5 x 10⁶ LCL cells s.c. in both flanks. Mice were treated with no drug, VPA alone (given in three doses on days 7, 8, and 11 following injection of tumor cells), one dose of gemcitabine alone (given on day 7 following injection of tumor cells), or one dose of gemcitabine and three doses of VPA. As shown in Fig. 6, VPA alone slightly inhibited the growth of EBV-transformed lymphoblastoid cells in SCID mice. The tumors in mice treated with gemcitabine alone, as expected, were also smaller than the tumors in untreated mice. Most importantly, however, the tumors in mice treated with VPA and gemcitabine together were significantly smaller than the tumors in mice treated with gemcitabine alone (P = 0.008, t test). These results suggest that VPA significantly enhances the therapeutic effectiveness of gemcitabine for treating EBV-positive lymphoproliferative disease.

Discussion

EBV is associated with the development of both lymphoid and epithelial cell malignances (1, 2), including Burkitt’s lymphoma, Hodgkin’s disease, natural killer cell lymphoma, transplant-associated lymphoproliferative disease, gastric carcinoma, and nasopharyngeal carcinoma. The consistent presence of the EBV genome within certain tumor types provides an attractive target for the development of the novel strategies to treat such tumors. We previously reported (6–8) that a variety of chemotherapeutic agents (including 5-FU, cis-platinum, gemcitabine, doxorubicin, and methotrexate) increase the level of lytic viral protein expression in EBV-positive tumor cells. In the current study, we have examined VPA on its ability to induce lytic viral gene expression by itself or enhance the lytic-induction effect of chemotherapy. Our results indicate that whereas VPA by itself only weakly activates lytic EBV gene expression (and this effect is limited to a subset of cell lines), it strongly enhances the ability of chemoagents to induce lytic EBV infection in both epithelial and lymphoid tumor cells. Furthermore,

the addition of VPA obviously enhanced the killing effect of cis-platinum and 5-FU in EBV-positive AGS cells, it did not affect the ability of cis-platinum and 5-FU to kill EBV-negative AGS cells. These results indicate that VPA enhances the killing effect of chemotherapy through an EBV-dependent mechanism.

Valproic acid enhancement of chemotherapy killing requires the lytic form of EBV gene expression. To determine if the enhanced killing effect of the VPA/chemotherapy combination that was observed in the EBV-positive (but not EBV-negative) AGS cells requires the lytic form of EBV gene expression, we used LCL lines derived from the same donor, which were immortalized using wild-type versus lytic-defective (BZLF1-deleted) EBV. As previously described, B cells immortalized with the BZLF1-deleted (Z-KO) virus are similar to cells immortalized with the wild-type virus in vitro but cannot enter the lytic form of viral gene expression (30). LCLs containing the wild-type versus Z-KO viruses were treated with VPA alone (1 mmol/L), two different chemotherapy agents previously shown to induce lytic EBV gene expression in LCLs (0.05 μmol/L doxorubicin or 0.4 μg/mL gemcitabine), or the combination of doxorubicin and VPA or gemcitabine and VPA. As shown in Fig. 5, VPA alone was slightly more cytotoxic in LCLs containing the wild-type virus (capable of becoming lytic) versus the LCLs containing the lytic-defective (Z-KO) virus. Likewise, gemcitabine and doxorubicin alone each killed more cells in the LCLs containing wild-type EBV than in the LCLs with lytic-defective virus, suggesting that at least some of the chemotherapy killing could be mediated through the induction of lytic viral protein expression. Most strikingly, whereas VPA clearly enhanced the cytotoxicity of both doxorubicin and gemcitabine in cells containing the wild-type virus, it had no effect on the cytotoxicity of these two drugs in cells from the same donor containing the lytic-defective form of EBV. These results strongly suggest that the ability of VPA to enhance the killing effect of chemotherapy in vitro not only requires that the EBV genome be present but that it is also able to express lytic viral proteins.

Figure 3. VPA activates the EBV immediate-early BZLF1 promoter. EBV-negative AGS cells were transfected with a promoter gene construct containing the BZLF1 promoter, Zp, linked to the luciferase gene (Zp-luciferase; 1 μg). Twelve hours later, cells were treated with VPA (0.3 mmol/L), cis-platinum (0.25 μg/mL), or 5-FU (1 μg/mL) alone, or VPA in combination with cis-platinum or 5-FU, followed by further culture for 48 hours. Luciferase activity was assayed with the luciferase reporter assay system (Promega). The Zp-luciferase construct was also cotransfected with a BZLF1 expression vector (which activates the Zp promoter) as a positive control. Duplicate transfections were done for each condition.

Figure 4. VPA enhances chemotherapy killing of EBV-positive AGS cells. AGS and AGS-EBV cells were treated with no drug, cis-platinum (0.25 μg/mL), 5-FU (1 μg/mL), or VPA (0.3 mmol/L) alone, or cis-platinum or 5-FU combined with VPA. Cell counts in each condition were determined by trypan blue exclusion at 5 days posttreatment. The number of cells obtained in the absence of drugs is set as 100% survival.
the addition of VPA to gemcitabine significantly enhanced the ability of gemcitabine to inhibit the growth of EBV-driven lymphoproliferative disease in SCID mice.

In EBV-associated malignances, the majority of cells contain one of the latent forms of infection and the lytic viral genes are expressed at a very low level. It is clearly advantageous for the growth of EBV-positive tumors \textit{in vivo} that the virus remains primarily in the latent, versus lytic, form of infection. The currently recognized EBV-encoded transforming proteins are all expressed in the latent, rather than lytic, form of infection. In addition, lytic EBV infection may lead to tumor cell death and cause the tumor to express lytic viral proteins (including BZLF1) that are major targets for the anti-EBV CTL response (32, 33).

The key step in the switch from latent to lytic EBV infection (34) is mediated by the viral immediate-early proteins, BZLF1 and BRLF1. BZLF1 and BRLF1 encode transcriptional activators that, in combination, activate the complete cascade of lytic viral gene expression. Thus, whether cells contain the latent versus lytic form of viral infection is largely determined by the activity state of the BZLF1 and BRLF1 promoters (Zp and Rp). A number of cellular transcription factors, including Jun, activating transcription factor-2 (ATF-2), Sp1, EGR-1, ZEB-1, MEF-2D, and YY-1, have been shown to regulate the Zp and Rp promoters \textit{in vitro} (35–40). Interestingly, in reporter gene assays, the Zp and Rp promoters are essentially silent in most B-cell lines but constitutively active in many epithelial lines, correlating with the propensity of the virus to produce latent infection following infection of B cells and lytic infection following infection of normal epithelial cells.

However, even in epithelial cell tumors, EBV infection is largely latent. Thus, a variety of different epigenetic mechanisms, including viral genome DNA methylation and histone modifications of the chromatin, also likely contribute to keeping EBV lytic genes repressed in tumor cells. Viral genomes are highly methylated in a variety of EBV-associated tumors (9–11, 13, 41), and the demethylating agent aza-CR is sufficient to induce lytic viral gene transcription in a subset of Burkitt’s lymphoma lines \textit{in vitro} (11, 21, 22). Recently, it has become clear that modification of histones in chromatin, particularly through acetylation, is also a very widespread mechanism by which the activity of cellular and viral genes is regulated (42–44). Murphy et al. (45) showed that chromatin remodeling of the major immediate-early promoter in human cytomegalovirus by histone acetylation may play a role in reactivation of latent human cytomegalovirus, and Jenkins et al. (46) determined that the acetylation of histones in chromatin around the BZLF1 promoter region is involved in the activation of its expression. The fact that sodium butyrate, a HDAC inhibitor, is widely used to induce lytic EBV gene expression \textit{in vitro} (although its effects are highly cell line dependent) suggests that repressive histone modifications, in addition to viral genome methylation, is important for maintaining EBV latency in tumor cells.

VPA induces both histone acetylation and DNA demethylation (26). Thus, we sought to determine whether VPA would activate EBV immediate-early gene expression in latently infected tumor cell lines. With the exception of the EBV-infected AGS cell line (the line which has the highest level of constitutive lytic EBV gene expression), we did not find that VPA treatment alone is sufficient to substantially increase lytic EBV protein expression. Nevertheless, we found that VPA dramatically increases the ability of low-dose chemotherapy to induce lytic EBV gene expression in three different cell types (gastric cells, lymphoblastoid cells, and NPC

**Figure 5.** VPA enhancement of chemotherapy killing requires the lytic form of EBV gene expression. LCL and LCL-Z-KO cells were treated with no drug, doxorubicin (0.05 μmol/L), gemcitabine (0.4 μg/mL), VPA (1 mmol/L) alone, or doxorubicin or gemcitabine combined with VPA. Cell number was determined by trypan blue exclusion at 5 days posttreatment. The number of cells obtained in the absence of drugs is set as 100% survival.

**Figure 6.** VPA enhances the effectiveness of gemcitabine treatment of EBV-positive lymphoblastoid tumors in SCID mice. EBV-positive lymphoblastoid cells (5 x 10^6 LCL-1 cells) were implanted s.c. into the flanks of SCID mice. Tumors (six tumors in each group) were treated with either no drug, valproic alone (given on days 7, 8 and 11 after injection of tumor cells), gemcitabine alone (given 7 days after injection of tumor cells), or gemcitabine followed by three doses of VPA treatment. Columns, mean tumor volumes at different time points; bars, SE.
cells). Furthermore, VPA enhanced the cell killing activity of chemotherapy in an EBV-dependent manner, and this effect was shown to require the ability of the viral genome to enter the lytic form of viral gene expression.

We previously showed that the ability of chemotherapy to induce transcription of the viral immediate-early genes requires that at least three different signal transduction pathways in the host cell be activated (including phosphatidylinositol 3-kinase, MEK kinase, and p38 kinase) and is mediated through specific cis-acting motifs in the immediate-early promoters that bind to MEF-2D, ATF-2/JUN, and EGR-1 (8, 35). We speculate that whereas chemotherapy induces lytic EBV gene transcription by increasing the availability of cellular transcription factors capable of activating the Zp and/or Rp viral immediate-early gene promoters, VPA enhances the effect of these transcription factors by increasing the acetylation state of the chromatin encompassing the episomal viral genome. As VPA was also recently shown to be able to induce DNA demethylation, it is possible that this effect also contributes to its ability to enhance the lytic-inducing effect of chemotherapy. Nevertheless, as the demethylating agent aza-CR was unable to enhance the lytic-inducing effect of chemotherapy, it seems that demethylation of the viral genome per se is not sufficient to enhance the effect of chemotherapy.

In addition to its ability to regulate epigenetic modifications of the viral genome, which normally serve to repress lytic viral gene transcription, it is also possible that VPA also directly increases the activity of cellular transcription factors that bind to, and activate, the viral immediate-early gene promoters. Consistent with this, our results indicate that VPA significantly enhanced the luciferase activity derived from the Zp-luciferase construct even in the absence of a chromatinized template. Interestingly, one of the most important regulators of Zp transcription is the MEF-2D transcription factor, which is constitutively bound to the BZLF1 promoter but can serve either as a positive or negative regulator of BZLF1 transcription (47). When MEF-2D is complexed with HDAC proteins, it acts as negative regulator of transcription, and thus the ability of VPA to inhibit HDAC activity would be expected to convert MEF-2D bound to the BZLF1 promoter from a negative to a positive regulator of Zp transcription. In addition, a previous report (48) suggested that VPA is also an ERK pathway activator. As phosphorylation of MEF-2D by the ERK5 kinase enhances its ability to positively regulate transcription (49–51), this is another mechanism by which VPA could directly activate Zp transcription even in the absence of a repressive chromatin structure. An unresolved issue is exactly how the combination of chemotherapy and VPA results in more efficient cell killing of EBV-positive cells. Although it is tempting to speculate that this killing is mediated by overwhelming production of infectious viral particles, we have previously shown that, in fact, ganciclovir quite efficiently inhibits the lytic form of EBV genome replication, although it activates the expression of early lytic viral proteins (7). Therefore, it is more likely that an immediate-early or early lytic viral protein (produced before the onset of actual viral replication) in some way potentiates the cytotoxicity of either chemotherapy and/or VPA.

It has previously been suggested that VPA could be used clinically to inhibit the growth of EBV-negative tumors. Among its various effects, VPA has been reported to induce cellular differentiation, produce a G1 cell cycle arrest, reduce vascular endothelial growth factor production, inhibit tumor growth in animal models, and induce apoptosis (52, 53). However, at least at the relatively low doses used in our experiments, we did not find that VPA significantly inhibited tumor cell growth in vitro unless the tumor cell contained an EBV genome capable of converting to the lytic form of viral infection. It is possible that at higher doses, or in different cell types, VPA would also be an effective inhibitor of EBV-negative tumors.

Our results suggest that the addition of short-course VPA therapy might enhance the efficacy of conventional chemotherapy for the treatment of EBV-positive tumors. Based on previous studies, the addition of ganciclovir would likely even further augment the EBV-dependent cytotoxicity of the VPA/chemotherapy combination. VPA may emerge as a particularly appealing candidate for activating lytic EBV gene expression in human tumors because of its long record of widespread and well-tolerated human use. Furthermore, the concentration of VPA necessary to activate enhanced lytic EBV gene expression lies within the physiologically achievable dose level (1 mmol/L) in patient plasma (54). Further studies will be necessary to determine whether this strategy can be clinically useful in patients with EBV-associated malignances.

Acknowledgments

Received 3/17/2006; revised 5/31/2006; accepted 6/14/2006.

Grant support: NIH grants R01-CA66519 and R01-CA58853.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Henri-Jacques Delecluse at the German Cancer Research Center, Heidelberg, Germany, for the BZLF1-knockout virus.

References


Valproic Acid Enhances the Efficacy of Chemotherapy in EBV-Positive Tumors by Increasing Lytic Viral Gene Expression

Wen-hai Feng and Shannon C. Kenney


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/17/8762

Cited articles  This article cites 52 articles, 30 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/17/8762.full.html#ref-list-1

Citing articles  This article has been cited by 25 HighWire-hosted articles. Access the articles at:
/content/66/17/8762.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.