RESEARCH ARTICLE

Effects of Valproic Acid on Proliferation, Apoptosis, Angiogenesis and Metastasis of Ovarian Cancer in Vitro and in Vivo

Zhao Shan1*, Rong Feng-Nian2, Geng Jie3, Zhou Ting2

Abstract

Inhibitors of histone deacetylase activity are emerging as a potentially important new class of anticancer agents. In this study, we assessed the anticancer effects of valproic acid (VPA) on ovarian cancer in vitro and in vivo. Cultured SKOV3 cells were treated by VPA with different concentrations and time, then the effects on cell growth, cell cycle, apoptosis, and related events were investigated. A human ovarian cancer model transplanted subcutaneously in nude mice was established, and the efficacy of VPA used alone and in combination with diaminoc dichloroplatinum (DDP) to inhibit the growth of tumors was also assessed. Proliferation of SKOV3 cells was inhibited by VPA in a dose and time dependent fashion. The cell cycle distribution changed one treatment with VPA, with decrease in the number of S-phase cells and increase in G1-phase. VPA could significantly inhibit the growth of the epithelial ovarian cancer SKOV3 cells in vivo without toxic side effects. Treatment with VPA combined with DDP demonstrated enhanced anticancer effects. The result of flow cytometry (FCM) indicated that after VPA in vitro and in vivo, the expression of E-cadherin was increased whereas vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) were decreased. This study suggests that VPA could be a novel attractive agent for treatment of ovarian cancer.

Keywords: Valproic acid - ovarian cancer - apoptosis - DDP

Introduction

Ovarian cancer has the highest mortality rate among gynecologic malignancies, with a 5-year survival rate of 39% in 1990 (Boring et al., 1992). Most neoplastic ovarian tumors produce few symptom until the disease is widely disseminated throughout the abdominal cavity, for this reason, the chemotherapeutic treatment is a attractive therapeutic strategy to improve the efficacy of surgery, reduce the chance of recurrence. But many patients present drug resistance gradually and these common agents maybe associated with some potentially life-threatening toxic side effects. So finding some new agents which has better anticancer effect without toxicities is an area of intensive investigation.

It has been well established that the occurrence and development of cancers involve a substantial change in functions of both oncogenes and tumor suppressor genes. One of the most important mechanisms in chromatin remodeling is the posttranslational modification of the N-terminal tails of histones by acetylation, which are key steps in the regulation of gene expression, consequently affecting proper cell function, differentiation and proliferation. More than thirty years ago, Vincent Allfrey and collaborators reported a strong correlation between increased levels of histone acetylation and gene over expression (Allfrey et al., 1964). The genesis of cancer, malignant dissemination of tumor cells are, at least in part, based on the up-regulation of histone deacetylases (HDACs). So HDACIs are emerging as promising clinical therapeutics for cancer (Bellarosa et al., 2012; Feng et al., 2012; Francisco et al., 2012).

Some HDACIs are of limited therapeutic use, due to poor bioavailability in vivo and toxic side effects at high doses (Marks et al., 2001). The short chain fatty acid valproic acid (VPA), one of the HDACIs, has a good tolerability and safety profile. It emerged in 1997 as an antineoplastic agent as well, when findings indicated that the substance inhibited proliferation and induced differentiation of primitive neuroectodermal tumor cells in vitro and in vivo (Cinatl Jr et al., 1997) The anti cancer characteristics of VPA has meanwhile been investigated in preclinical models of a variety of solid and hematologic tumors. Currently, the drug is in phase II trials (Daud et al., 2009). While these agents show promise, their mechanism(s) of action and selective toxicity against tumor cells have not yet been adequately defined. The current study was designed to define the biological therapeutic effects of VPA in the treatment of ovarian cancer in vitro and in vivo.

1Department of Obstetrics and Gynecology, the First Affiliated Hospital of Guangxi Medical University, Nanning, 2Department of Obstetrics and Gynecology, Shandong Qianfoshan Hospital, Jinan, 3Department of Reproduction, The 174 Hospital of the PLA, Xiamen, China *For correspondence: zhaoshan81641@yahoo.com.cn
Materials and Methods

The human ovarian cancer cell line SKOV3 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). It was routine maintained in RPMI 1640 containing 100 mL/L fetal bovine serum (FBS), 100U/mL penicillin, 100 U/mL streptomycin at 37°C in a humidified atmosphere containing 50 mL/L CO₂. VPA was obtained from Sigma and dissolved in phosphate-buffered saline (PBS) at concentration of 1, 2, 3, 4, 5 mmol/L, PBS was added to culture media as a negative control.

Cell proliferation was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl (MTT) dye reduction assay. Cells were seeded at the density of 5x10⁴ per well in 96-well plates in RPMI-1640 containing 100 μL/L FBS and incubated as described above. After 24 hours, fresh medium was added; containing VPA at concentrations of 0, 1, 2, 3, 4, 5 mmol/L, and every concentration has 10 plates. The plates were allowed to stand 1-3 days at 37°C, 5% CO₂. We observed the cells morphous under light microscope every day. After the termination of the culture, MTT (0.5 mg/mL) was added for an additional 4 hours. Absorbance at a wavelength of 492 nm was determined for each well using a microplate ELISA reader. So we can get optical density (OD) of every well. Inhibition ratio of different concentrations was calculated.

The percentage of G₁, S, and G₂-M phase of the cell cycle was determined by a modified Vindelov propidium iodide DNA staining procedure. Briefly, cells cultured after 48 h and 72 h were isolated from the culture dishes with trypsin, washed once in fresh media, and resuspended at a concentration of 1 x 10⁶ cells/ml. The cell suspension (200 μl) was then incubated with 500 μl of a propidium iodide solution [0.006 g of PI, 70 units of RNase, 0.058 g of NaCl, 0.121 g of Trisma base, and 0.1 ml of NP40; volume to 100 ml (pH 8.0)] overnight at 4°C. The following day, the stained cell nuclei were run on a Coulter Epix XL flow cytometer. The ungated histogram was evaluated using the Phoenix flow DNA modeling system to determine the percentage of cells in G₁, S, and G₂-M phase. On the left side of G₁ summit is SubG₁, it represented the cell apoptotic index.

Cells cultured after 48 h were subsequently dissociated into a single-cell suspension, and resuspended at a concentration of 1 x 10⁶ cells/ml. Cells were initially incubated with rat-monoclonal anti-VEGF, anti-E-cadherin and anti-MMP-9 antibody (1:100, Zymed, USA) respectively at room temperature for 30 min, followed by labeling with anti-rat fluorescein-isothiocyanate (FITC)-conjugated second antibody (1:100, Center Laboratories QiLu Hospital). That was also performed at room temperature for 30 min. Flow cytometry and cell sorting were completed on a Coulter Epix XL flow cytometer. Facility using Expo 32 ADC Analysis Program. Fluorescence index (FI) was used to demonstrate the protein lever of VEGF, E-cadherin and MMP-9 (Morkve et al., 1991).

Twenty four 6-week-old weighing 19-21 g immunodeficient beige/nude/xid nu/nu female mice were purchased from Chinese Academy of Medical Sciences, and maintained under pathogen-free conditions with irradiated chow. The experimental procedures on the use and care of animals had been approved by the Ethics Committee of Shandong University. In our experiment, 5 x 10⁶ SKOV3 cells in 0.2 mL PBS was subcutaneously injected into the trunk of 24 mice, leading to the formation of a tumor per animal. Human ovarian cancer model transplanted subcutaneously in nude mice was established, and randomly divided into 4 groups (6 mice per group): (1) control group, received PBS 0.2ml only; (2) experimental group, including: VPA group, DDP group and VPA+DDP group. Treatment was initiated intraperitoneally when established tumors of 0.2 cm-0.3 cm diameter were detected; VPA group was treated with VPA in doses of 500 mg/kg/day in 0.2 mL for 30 days. DDP group was treated with DDP in doses of 3 mg/kg every 6 days, for 5 times. VPA+DDP group was treated VPA and DDP. Tumors were measured every week with vernier calipers. Tumor volume was calculated using the following formula: volume x length x width x height x 0.5236 respectively. Draw the growth curve of different drugs. At the end of the experiment, animals were sacrificed after which careful resection was performed and tumor weights were measured, and calculated the Tumor growth inhibition (TGI). TGI (%) = (1 - Vt/Vc) x 100%, where Vt is the mean tumor volume of the arsenic-treated group, and Vc the mean tumor volume of the control group. Tumor, brain, liver, spleen, and kidney specimens were fixed and stained for histologic analysis.

Tumors and normal organs were detected apoptosis by flow cytometry, the protein levers of VEGF, E-cadherin and MMP-9 were also assayed by FCM.

All results were presented as mean ± standard error. Date was analyzed by a computer software SPSS10.0, statistical significance was investigated by F test and P<0.05 was considered statistically significant.

Results

We initially examined whether assertion of the VPA to SKOV3 cells cultures produced any effect and cellular morphology. Figure 1 is a photomicrograph composite of SKOV3 cells incubated in the presence and absence of VPA (4 mmol/L 48 h). Under light microscope, cells in the control group grew bloomy and were clustered. The shapes were long, shuttle and polygon. The cell shapes were plump. When the concentration of VPA was less than 2 mmol/L, the growth of cells in the experimental group were not inhibited evidently. As the concentration of VPA rise to 3 mmol/L or more, the growth of the cells was inhibited and the normal cells were dropped off. The residual attached cells were scattered or rickled. The shape of the residual attached cells was round or ellipse. The cytoplasm was crimped. Photomicrographs in the Figure 1 B showed the SKOV3 cells treated with VPA (4 mmol/L, 48 h). As is apparent from the figure, within 48h, VPA had a dramatic effect on the morphology of cells, and the cells density was reduced.

To test whether the reduced cell density after VPA treatment is the result of changes in proliferation, we used MTT to determine the optical density, and then calculated
Valproic Acid Effects on Proliferation, Apoptosis, Angiogenesis and Metastasis of Ovarian Cancer

Valproic Acid Effects on Proliferation, Apoptosis, Angiogenesis and Metastasis of Ovarian Cancer

Figure 1. Inhibition Rate of VPA on SKOV3 Cells Growth. Under light microscope, cells in the control group grew bloomy and were clustered. The shapes were long, shuttle and polygon. The cell shapes were plump (as in A). As the concentration of VPA rise to 3 mmol/L or more, the growth of the cells was inhibited and the normal cells were dropped off. The residual attached cells were scattered or rickled. The shape of the residual attached cells were round or ellipse (as in B). We used MTT to determine the optical density after VPA treatment, and then calculated the inhibition ratio. It shows VPA producing a significant reduction of proliferation in cells. When the concentration of VPA was between 3 to 5 mmol/L, the growth of SKOV3 cells was inhibited evidently in vitro (as in C). The proliferation of SKOV3 cells was inhibited by VPA in a dose and time dependent fashion. *: p<0.05 for the difference in each concentrations and each times. Error bars represent standard deviations.

The inhibition ratio, Figure 2C shows VPA producing a significant reduction of proliferation in cells. When the concentration of VPA was between 3 to 5 mmol/L, the growth of SKOV3 cells was inhibited evidently in vitro. After using VPA for 72 h, the cell inhibition rate was 18.92%, 32.70%, and 48.65% respectively. The proliferation of SKOV3 cells was inhibited by VPA in a dose and time dependent fashion.

We used FCM to determine whether the reduction in the number of cells was the result of increased apoptosis and cell cycle arrest. The cell cycle distribution changed while treated with VPA for 24 h, decrease of S-phase cells and increase of G1-phase cells, but the changes of the G0- and G2-phase were not significant as in Figure 2. The percentage of subG1 was increased in a dose and time dependent fashion and reached differences statistical significance in each group (P<0.05). The subG1, respected the apoptosis rate, as showed in Figure 2C, Apoptotic rate of SKOV3 cells treated with VPA of different concentrations for different times, the differences have statistical significance in each group (P<0.05).

In our study, we tested expression of genes associates with angiogenesis and metastasis, to examine whether VPA can inhibit neoplasms metastasis and angiogenesis. We used FCM to detect VEGF, E-cadherin and MMP-9 protein levels. After treated with VPA at concentration of 3mmol/L for 48 hours ,the expression of E-cadherin was increased but the expression of VEGF and MMP-9 were decreased, as showed in Figure 3, there was a significant difference among each group (P<0.01).

Finally we detected the anticancer effect of VPA and sensitization in combine with DDP on the growth of human ovarian cancer transplanted subcutaneously in nude mice. Under light, in the control group cancer cells growth are active, nucleus were hyperchromasia, with a lot of cellular atyoi and karyokine. But in the tumors of experimental groups, there was zone of necrosis, a number of characteristic apoptotic cells were found, the karyoplasmic ratio was smaller, we also found inflammatory cell infiltrate in tumors of experimental groups. However, in the control groups there were few. In subcutaneous tumor model the dimension of the tumor in experimental group is smaller than that in the control group. The longer VPA being used the smaller the tumors were. The inhibiting rate of VPA group, DDP group and VPA+DDP group were as follows: 40.7%, 45.3%, 58%, as showed in Figure 4. They were significantly higher than control group (P<0.01). The apoptotic rate of experimental groups were as follows: (27.05±1.63)%, (35.93±3.89)%, (42.59±2.55)%, which were significantly higher than control group (16.73±2.82)% (P<0.01). The cell cycle distribution changed in experimental group, the S-phase was decrease. The result (Figure 5) of FCM indicated...
that after using VPA, the expression of E-cadherin was increased but the expression of VEGF and MMP-9 were decreased, there was a significant difference among each group (P<0.05). There were no toxic reactions during the course. The apoptotic rates of the normal organs tissues in nude mice were very low (P>0.05), as showed in Figure 6.

Figure 5. The VEGF, E-cadherin and MMP-9 Protein Level of Human Ovarian Cancer Transplanted Subcutaneously in Nude Mice. The result of FCM indicated that after using VPA, the expression of E-cadherin was increased but the expression of VEGF and MMP-9 were decreased, there was a significant difference among each group (P<0.05)

Figure 6. Apoptotic Rates of Liver and Kidney. There were no toxic reactions during the course. The apoptotic rates of the normal organs tissues (A is liver, B is kidney) in nude mice were very low (P>0.05). Treatment was initiated intraperitoneally when established tumors of 0.2 cm - 0.3 cm diameter were detected. At the end of the experiment, tumor weights were measured, draw the growth curve of different drugs (as in A). In subcutaneous tumor model the dimension of the tumor in experimental group is smaller than that in the control group. The longer VPA being used the smaller the tumors were. After used with VPA combined DDP, the tumors were the smallest, there was a significant difference among each group (P<0.01). The inhibiting rate of VPA group, DDP group and VPA+DDP group were as follows: 40.7%, 45.3%, 58% (as in B), they were significantly higher than control group (P<0.01). The cell cycle distribution changed in experimental group, the S-phase was decrease (as in C). The apoptotic rate of experimental groups were as follows: (27.05±1.63)%,(35.93±3.89)%,(42.59±2.55)% (as in D), which were significantly higher than control group (16.73±2.82)% (P<0.01)

Discussion

Ovarian cancer turned to be the biggest risk of women’s health. Chemotherapy as one of the assisted therapeutic measure once encouraged both patients and gynecologists in the battle against ovarian cancer now facing great challenge due to its serious side effect. With the development of apoptosis and oncology, we began to define neoplasm in a brand new angle.

Valproic acid, a short chain fatty acid, has been used extensively in the clinic for the treatment of a variety of seizure disorders as well as for the treatment of manic depressive bipolar illness. The advantages of VPA include low cost, favorable safety profile, and oral dosing. Recently it has been identified that VPA is an effective inhibitor of HDAC activity at physiologically relevant concentrations. It is also becoming evident that the efficacy of VPA in vivo is in part due to inhibition of cancer cell migration, invasion and angiogenesis (Chou et al., 2011; Wedel et al., 2011; Leiva et al., 2012). This gave us the inspiration to see whether VPA could have the similar effects in ovarian cancer.

In our in vitro experiment, the proliferation of SKOV3 cells was inhibited by VPA in a dose and time dependent fashion. In subcutaneous tumor model the dimension of the tumor in experimental group is smaller than that in the control group. The longer VPA being used the smaller the tumors were. Our study showed VPA can inhibit the proliferation of cancer cells.

The reasons for this effect is completely obscure but might be related to the specific subset of genetic defects affecting regulation of the cell cycle or apoptosis in cancer cells. Researches have showed HDACIs have cytostatic activity characterized by a G1 phase cell cycle arrest that is associated with the increased expression of the cyclin-dependent kinase (cdk) inhibitor p21WAF1/CIP1 (Chen et al., 2011). HDACIs result in diminished proliferation due to cell cycle blocks at the G1 and G2-M checkpoints, consistent with an association between HDAC activity and cell cycle control genes (Ogryzko et al., 1996). Cell cycle arrest maybe the main mechanism of inhibition effects by VPA (Vallo et al., 2011).

Then we use FCM to determine this mechanism. The cell cycle distribution changed while treated with VPA, decreasing of S-phase cells and increasing of G1-phase cells, but the changes of the G2-phase and M-phase were not significant. Diminished proliferation of ovarian cancer cells is the result of a reduction in the S-phase due to cell cycle blocks at the G1 and G2-M checkpoints. The percentage of SubG1 was increased in a dose and time dependent fashion and reached differences statistical significance in each group (P<0.05). We have shown that treatment with VPA dramatically and significantly increase the number of apoptotic cells.
Valproic Acid Effects on Proliferation, Apoptosis, Angiogenesis and Metastasis of Ovarian Cancer

DOI:http://dx.doi.org/10.7314/APJCP.2012.13.8.3977


Chen Y, Tsai YH, Tseng SH, (2011). Combined valproic acid...


