Valproic acid suppresses the self-renewal and proliferation of head and neck cancer stem cells

SANG HYUK LEE1, HYO JUNG NAM1, HYUJN JUNG KANG2, TINA L. SAMUELS3, NIKKI JOHNSTON3 and YOUNG CHANG LIM2

1Department of Otorhinolaryngology-Head and Neck Surgery, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul; 2Department of Otorhinolaryngology-Head and Neck Surgery, Research Institute of Medical Science, Konkuk University School of Medicine, Seoul, Republic of Korea; 3Department of Otolaryngology and Communication Sciences, Medical College of Wisconsin, Milwaukee, WI, USA

Received April 6, 2015; Accepted June 30, 2015

DOI: 10.3892/or.2015.4145

Abstract. Emerging evidence suggests that cancer cells present profound epigenetic alterations in addition to featuring classic genetic mutations. Valproic acid (VPA), a histone deacetylase inhibitor, can potently inhibit tumor growth and induce differentiation. However, the effect and underlying mechanism of VPA on head and neck squamous cell carcinoma (HNSCC) cancer stem cells (CSCs) remain unclear. In the present study we investigated the effects of VPA on the characteristics of HNSCC CSCs in vitro and in vivo. As a result, VPA inhibited the self-renewal abilities of HNSCC CSCs during two serial passages and decreased the expression of stem cell markers, such as Oct4, Sox2 and CD44. VPA also potentiated the cytotoxic effect of cisplatin by suppressing the ABCC2 and ABCC6 transporters as well as by inducing caspase-mediated apoptosis. In addition, the combination of VPA and cisplatin attenuated tumor growth and induced apoptosis in a xenograft model. Our results suggest that VPA might be a potential therapeutic strategy in combination with conventional cisplatin for HNSCC patients by elimination of CSC traits.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, with an annual incidence of more than 500,000 cases (1). Despite recent advances in treatment regimens including surgery, radiotherapy, chemotherapy and introduction of novel therapeutic agents, the 5-year survival rate of these patients has been virtually unchanged in the past three decades, remaining at only 50% (2). Further improvements in curative rates in HNSCC will require significant advances in the development of new drugs and treatment strategies. A deeper understanding of the precise molecular mechanisms responsible for HNSCC tumorigenesis and progression will contribute significantly toward the development of new and improved therapeutic agents.

It has been reported that many solid human cancers, including HNSCC, are maintained by a small subpopulation of cells called cancer stem cells (CSCs) (3-6). These CSCs have the unique features of self-renewal, asymmetrical differentiation into multiple lineages and enhanced tumor-initiating capacity in xenograft models (7-9). Accumulating evidence suggests that CSCs are responsible for tumor metastasis and the acquisition of resistance to treatment with conventional chemotherapeutic agents, which leads to tumor relapse (10,11). Therefore, targeting CSCs with conventional or other targeted therapies may be required to effectively treat cancers (12-15).

Epigenetic regulations are required for normal development and gene expression. Disruption of epigenetic regulations often leads to aberrant gene expression and malignant cellular transformation (16). Epigenetic alterations commonly observed in malignant cells include DNA methylation and changes in histone modification patterns as well as expression profiles of chromatin-modifying enzymes (16,17). Histone modification patterns are dynamically regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), which add and remove specific covalent modifications, respectively (18). An imbalance between HATs and HDACs leads to aberrant gene expression and tumorigenesis (19).

Several epigenetic drugs that effectively reverse aberrant DNA methylations and histone modifications have been discovered. Valproic acid (VPA), a branched short-chain fatty acid, inhibits HDACs causing increased chromatin acetylation (20). HDACs have been found to be overexpressed or mutated in many types of human tumors (21,22). A recent study demonstrated that HNSCCs are primarily hypoacetylated which may account for the accumulation and maintenance of CSCs (23). They also showed that inhibition of HDACs using Trichostatin A reduced the number of HNSCC CSCs and inhibited clonogenic sphere forma-
CSCs was confirmed by functional assays of sphere forma-
tion (23). In contrast, a separate report showed that VPA
promoted the expansion of breast CSCs through reprogram-
mation of differentiated cancer cells into stem-like cells. The
causes of this discrepancy might lie in the different cancer
cell systems as well as the different HDAC inhibitors used.

Unfortunately, previous reports regarding the effect of
VPA on HNSCC CSCs have been extremely scarce. The
objectives of the present study were to evaluate the effect of
VPA on HNSCC CSCs and to delineate the mechanisms by
which VPA inhibits the characteristics of CSCs derived from
human primary HNSCC. Here, we present evidence for the
therapeutic value of VPA in combination with conventional
cisplatin, which suppressed the self-renewal and proliferation
and induced apoptosis of CSCs in HNSCC.

Materials and methods

Isolation and culture of HNSCC stem-like cells. HNSCC
stem-like cells (K3 and K5) were isolated and characterized
from the primary surgical specimens of HNSCC patients,
as previously described (8). The CSC phenotype of HNSCC
CSCs was confirmed by functional assays of sphere forma-
tion, stemness-associated gene expression and xenograft
tumor formation. The cells were grown in serum-free media
composed of DMEM supplemented with B27 (Invitrogen),
N2 supplement (Invitrogen), basic fibroblast growth factor
(bFGF; 20 ng/ml; R&D Systems, Minneapolis, MN, USA) and
epidermal growth factor (EGF; 20 ng/ml; R&D Systems).

Sphere formation assays. To assess the self-renewal capacity
of HNSCC CSCs in vitro, the cells were dissociated into single
cells, seeded in a 24-well plate at a density of 200 cells/well,
and cultured in serum-free medium, with EGF and bFGF
supplementation every other day. Spheres with a diameter
exceeding 10 μm were counted after 14 days.

Western blot analysis. Western blot analysis of electropho-
retically separated proteins from cells was performed as
previously described (8). Specific antibodies against Oct4,
Sox2, Bcl-2, Bax and caspase 3 were purchased from Santa
Cruz Biotechnology (Santa Cruz, CA, USA). Secondary anti-
bodies, anti-rabbit IgG and anti-mouse IgG were purchased
from Jackson ImmunoResearch Laboratories (West Grove,
PA, USA).

Detection of CD44 expression by flow cytometry. HNSCC
CSCs were dissociated into single cells and washed twice in
cold phosphate-buffered saline (PBS). Cells were labeled with
anti-CD44 and fluorescein isothiocyanate (FITC)-labeled
secondary antibodies, then subjected to flow cytometry using
a FACS Calibur machine (BD Biosciences). The percentages of
CD44+ cells in cultures were determined.

Chemosensitivity assay. HNSCC CSCs were dissociated into
single cells and then plated in a 96-well plate at a density of
7x10^3 cells/well under serum-free culture conditions. Cells
were treated with cisplatin at the indicated concentrations
and then cultured at 37°C under a humidified 5% CO2 atmopshere.
Twenty-four hours later, 20 μl of 3-(4,4-dimethylthiazol-2-yl)2,
5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in
PBS) was added to each well, and the plate was placed at room
temperature for 3 h. The absorbance at 570 nm was measured
using a SpectraMax 190 (Molecular Devices) instrument.

Quantitative reverse transcription-polymerase chain reaction
(qRT-PCR). Total cellular RNA was reverse-transcribed using
a reverse transcriptase (RT) kit (Fermentas, Glen Burnie, MD,
USA) according to the manufacturer’s instructions. For semi-
quantitative PCR, cDNA was added to a mixture of specific
primers and 1 U of Taq DNA polymerase (Roche Diagnostics,
Indianapolis, IN, USA), and amplified using an MJ Research
MiniCycler (Bio-Rad Laboratories, Waltham, MA, USA). PCR
products were separated by agarose gel electrophoresis
(1.5% agarose gels) and detected under ultraviolet light
(Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR
was performed on an iCycler IQ real-time detection system
(Bio-Rad Laboratories), using IQ Supermix with SYBR-Green
(Bio-Rad Laboratories). The human sequence-specific primers
used are listed in Table I.

Xenograft tumor formation assay. All animal studies were
approved by the Institutional Animal Care and Use Committee
of Konkuk University. HNSCC CSCs were treated for 48 h with
cisplatin (5 μM) alone, cisplatin (5 μM) plus VPA (400 μM) or
dimethyl sulfoxide (DMSO; vehicle) in vitro. Following this,
10^5 or 10^6 cells were subcutaneously injected into the flank
of 8-week-old female BALB/c nude mice using a 22-gauge
needle. Mice were visually inspected and palpated weekly to
monitor tumor formation. The mice were sacrificed 8 weeks
after transplantation, and subcutaneous tumor tissues were harvested for estimating tumor size, weight and apoptosis.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Tumor tissues collected from mice injected with HNSCC CSCs treated with DmSO, cisplatin (5 µM), or cisplatin (5 µM) and VPA (400 µM) were used for TUNEL assays. Sections (4-µm) from formalin-fixed, paraffin-embedded tumors were deparaffinized and rehydrated using xylene and ethanol, respectively. The slides were rinsed twice with PBS and treated for 15 min at 37˚C with proteinase K (15 µg/ml in 10 mM Tris-HCl, pH 7.4-8.0). Endogenous peroxidases were blocked using 3% hydrogen peroxide in methanol at room temperature for 10 min. The tissue sections were then analyzed with an In Situ Cell Death Detection kit-POD (Roche) following the manufacturer's instructions.

Statistical analysis. The results are presented as mean ± SD. Statistical analyses were performed with the SPSS 10.0 statistical software (SPSS, Inc., Chicago, IL, USA). Statistical significance was determined by one-way ANOVA followed by post hoc tests for multiple comparisons. P<0.05 was considered to indicate a statistically significant result.

Results

VPA suppresses the CSC properties in HNSCC. The sphere formation assay can be effectively used to assess the self-renewal capacity of HNSCC CSCs, and has been reported to correlate closely with tumorigenicity (8). We examined whether VPA had the ability to inhibit the self-renewal capacity of HNSCC CSCs. VPA significantly suppressed sphere formation in two HNSCC CSC cultures during two serial passages in a dose-dependent manner (Fig. 1). CSCs found in HNSCC express typical stem cell markers, including Sox2, Oct4 and CD44. Therefore, we examined whether VPA treatment interfered with the expression of these markers. As evident from the results of the western blot analysis, treatment of HNSCC CSCs with VPA significantly suppressed Sox2 and Oct4 expression (Fig. 2A). Furthermore, VPA treatment significantly reduced the number of CD44+ cells in the HNSCC CSCs (Fig. 2B).

VPA enhances the chemosensitization of cisplatin and apoptosis in HNSCC CSCs. Chemoresistance is one of the most important characteristics of CSCs. We examined whether VPA increases the susceptibility of HNSCC CSCs to cisplatin. A combination of VPA and cisplatin increased the susceptibility of HNSCC CSCs to cisplatin in a dose-dependent manner (Fig. 3A). In order to understand the mechanisms responsible for the increased susceptibility of VPA-treated HNSCC CSCs to cisplatin, we analyzed the changes in expression of ATP-binding cassette (ABC) transporters following VPA treatment. Real-time qPCR analysis revealed that VPA treatment (400 µM) reduced the transcript levels of the ABCC2 and ABCC6 genes in the HNSCC CSCs (Fig. 3B).

To further determine whether the VPA-induced suppression of CSC properties is due to increased apoptosis, we investigated the expression of the apoptosis-related proteins...
Figure 2. Valproic acid (VPA) reduces stemness-associated marker expression in HNSCC CSCs. (A) Protein levels of Sox2 and Oct4 in HNSCC CSCs treated with DMSO or VPA (400 µM) for 48 h. (B) FACS analysis of CD44 expression in HNSCC CSCs treated with DMSO or VPA (400 µM) for 48 h.

Figure 3. Valproic acid (VPA) enhances the chemosensitivity of cisplatin in HNSCC CSCs. (A) MTT assay of the cell viability after VPA treatment at various concentrations in HNSCC CSCs treated with DMSO or cisplatin (5 µM). Data represent mean ± SD. **P<0.01. (B) mRNA expression levels of various ABC transporter genes after VPA (400 µM) treatment in HNSCC CSCs. Data represent mean ± SD. **P<0.01. (C) Protein expression level of Bcl-2 and Bax in HNSCC CSCs treated with DMSO, VPA (400 µM) alone, cisplatin (5 µM) alone, and cisplatin combined with VPA. (D) Protein expression level of cleaved caspase-3 detected by western blotting in HNSCC CSCs treated with VPA (400 µM) alone, cisplatin (5 µM) alone, zVAD (50 µM) alone, and their combinations. zVAD, a caspase inhibitor.
Bax and Bcl2. When compared to the cells treated with VPA (400 µM) or cisplatin (5 µM) alone, the expression of Bax in the CSCs was increased following treatment with cisplatin (5 µM) and VPA (400 µM) (Fig. 3C). To test whether caspase activity was required for VPA-mediated apoptosis, we examined the expression of cleaved caspase-3 in the CSCs before and after VPA treatment. Cleaved caspase-3 protein levels were significantly increased in the cells treated with a combination of cisplatin and VPA. A known caspase inhibitor, zVAD, abrogated the VPA-induced changes in cleaved caspase-3 expression (Fig. 3D). These results suggested that VPA is a potent inducer of apoptosis in HNSCC CSCs.

VPA inhibits the growth of HNSCC CSCs in a xenograft model. To confirm the combined effect of VPA and cisplatin on HNSCC CSCs, the inhibitory effect of VPA on the capacity of HNSCC CSCs to initiate tumor formation in nude mice was examined. Palpable tumor masses developed in 100% (6 of 6) of mice injected with 10^5 DMSO-treated cells and 83.3% (5 of 6) of mice injected with 10^5 cisplatin-treated cells; in contrast, only 16.6% (1 of 6) cells with administration of VPA plus cisplatin formed tumors when 10^5 cells were injected (Table II). In contrast to the large tumors generated by HNSCC CSCs treated with cisplatin or DMSO alone, cells treated with 400 µM VPA and 5 µM cisplatin generated tumors that were small, and the average tumor weight in nude mice (N=5) was significantly lower following treatment with cisplatin and VPA (Fig. 4A). TUNEL assay revealed a significant increase in the number of apoptotic cells in tumors generated from CSCs treated with VPA and cisplatin compared to those generated from cells treated with cisplatin or DMSO alone (Fig. 4B). Taken together, these data suggest that combination treatment of cisplatin and VPA induced apoptosis and reduced the growth rate of CSCs in vivo.

Discussion

Recent advances in the research of epigenetics have shown that human cancer harbors global epigenetic alterations, in addition to numerous genetic alterations (24). These genetic and epigenetic alterations may have critical effects on all stages of cancer development and progression. Unlike genetic alterations, epigenetic alterations are potentially reversible. This reversibility of epigenetic alterations has led to the possibility of developing a new class of therapeutics that restores the normal epigenetic state in malignant cell populations (16,25). Thus, many drugs that target specific enzymes involved in the epigenetic regulation of gene expression have been introduced, and the utilization of these drugs is emerging as an effective and valuable approach to combination treatment with...
conventional chemotherapy (25). Of these, HDAC inhibitors that help in restoring normal histone acetylation patterns have been shown to induce growth arrest, apoptosis, and differentiation by reactivating silenced tumor-suppressor genes (26).

Valproic acid (VPA), a well-known anticonvulsive agent, emerged in 1997 as an antineoplastic agent (27), and has been described to have antiproliferative effects in a variety of human malignancies (28,29). VPA modulates the biology of cancer cells by inducing differentiation, inhibiting proliferation, increasing apoptosis, and decreasing metastatic and angiogenic potential (30,31). A recent report showed that VPA induced differentiation and apoptosis in ETO-positive leukemic cells (32), and now this drug has been tested in differentiation therapy of acute myeloid leukemia (33). VPA also exerted inhibitory effects on the migration and invasion of prostate cancer cells (34). In HNSCC, VPA has been shown to have acute and chronic growth inhibitory effects (35), and causes a 3- to 7-fold increase in cisplatin cytotoxicity (28).

The recent interest in CSC research has emerged from their expected role in the initiation and progression of cancer. Moreover, CSCs are thought to be responsible for resistance to current anticancer treatment and early recurrence in many cancers (36). According to the CSC hypothesis a different treatment strategy focusing mainly on CSCs is required. However, only few attempts have been made to target CSCs epigenetically. VPA, a known HDAC inhibitor, was found to decrease proliferation potential and multilineage differentiation capability of human mesenchymal stem cells (37). Therefore, we hypothesized that inhibition of HDACs by VPA could suppress CSC activity in HNSCC. In the present study, we demonstrated that VPA interfered with the self-renewal of HNSCC CSCs. Accordingly, VPA effectively suppressed the expression of stem cell markers, including Oct4, Sox2 and CD44. A combination of VPA and cisplatin reduced HNSCC CSC chemoresistance, likely by suppressing ABCC2 and ABCC6 expression and increasing caspase-mediated apoptosis. Furthermore, this combination treatment significantly inhibited the tumor growth and induced apoptosis in a xenograft model.

Several studies have shown that these antimut and tumor cell differentiation effects of VPA are primarily mediated through inhibition of HDACs (20,38). Furthermore, inhibition of HDACs appears to interact with various other signaling pathways through complex molecular mechanisms. HDAC inhibition is linked to the modulation of phosphatidylinositol-3-kinase/Akt signaling (39). The Akt signaling pathway has been proved to interact with WNT/β-catenin signaling (40). HDAC inhibition was also found to be increased in inhibition-associated phosphorylation of GSK3β (41). Inactivation of the E-cadherin gene was also demonstrated to be triggered by DNA hypermethylation (42).

The present study further elucidated the VPA-induced antimutator effects in HNSCC CSCs. VPA in combination with cisplatin may disrupt the population of CSCs in HNSCC and thus be a potential curative strategy for the management of HNSCC.

Acknowledgements

The present study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (grant no. 2011-0014237 to S.H.L. and 2012R1A2A2A01046214 to Y.C.L.).


