Anti-Cancer Effects of Sulfasalazine and Vitamin E Succinate in MDA-MB 231 Triple-Negative Breast Cancer Cells

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Abstract

Aim: Sulfasalazine (SSZ) displayed anti-cancer activities. Vitamin E succinate (VES) could inhibit cell growth in various cancer cells. However, chemical therapies were often not useful for triple-negative breast cancer cells (TNBCs) treatment. Here, this study investigated the anti-cancer effects and the mechanisms on TNBCs under combination treatment with SSZ and VES.

Methods: Cell viability was analyzed by using the MTT assay. The H2O2 levels were determined by using lucigenin-amplified chemiluminescence method. In addition, caspase and MAPs signals were studied by using western blotting.

Results: Low-dose VES antagonized the SSZ-induced cytotoxicity effects while high-dose VES promoted the SSZ-induced cytotoxicity effects on TNBCs. In addition, SSZ alone treatment activated both caspase-3 and ERK signals, however, VES alone treatment only activated JNK signals. On the other hand, activation of caspase-3, JNK, and ERK were found in SSZ plus VES-treated cells.

Conclusion: Combined SSZ and VES has synergistic or antagonistic cytotoxic effects depending on VES concentration. In addition, different cytotoxic signals are induced on SSZ-treated, VES-treated and SSZ plus VES-treated cells.

Key words: vitamin E succinate, sulfasalazine, triple-negative breast cancer cells

Introduction

Triple-negative breast cancer cells (TNBCs) are estrogen receptors-deficient, progesterone receptors-deficient and epidermal growth factor receptor 2-deficient breast cancer, therefore, endocrine and targeted therapies do not applied for clinical TNBCs treatment [1, 2]. Today, to develop a potential therapy for TNBCs is important due to there are not useful clinical treatment for TNBCs [3, 4]. Sulfasalazine (SSZ), an anti-inflammatory drug, is commonly used as a first-line treatment for many rheumatic diseases [5, 6]. On the other hand, many studies has demonstrated that SSZ can inhibit cell proliferation on various cancers including primary brain tumors, lung adenocarcinoma cells, Hepatocellular carcinoma cells
and SSZ/VES-treated TNBC cells. Previous studies also showed SSZ can inhibit cell proliferation of breast cancers including MCF-7 cells (ER-negative breast cancer) and MDA-MB-231 cells (TNBC) though many signal pathways remain to study [12, 13]. However, SSZ can cause adverse effects in human containing mitochondrial dysfunction and acute renal injury [14, 15]. In order to promote anti-cancer activity and decrease SSZ-induced adverse effects, many studies suggested SSZ in combination with other therapies may be a useful treatment for cancer treatment [9, 10].

Vitamin E succinate (VES) is the most useful form of vitamin E derivatives to inhibit cancer proliferation. VES has broad anti-cancer effects to suppress cell growth by inducing mitochondria dysfunction and apoptosis [16-18]. In addition, many studies showed VES can inhibit cell growth on various hormone-dependent breast cancer cells such as MCF-7 and MDA-MB-435 cells [19-21]. However only few study indicated VES can inhibit TNBCs proliferation [22, 23]. The studies showed that VES inhibit cell growth inefficiently on TNBCs, only high-dose VES can suppress TNBCs proliferation. In addition, VES can induce apoptosis and activate Fas signals on TNBCs while lots of mechanisms remained unclear.

The mitogen-activated protein kinase (MAPK) signaling pathways majorly contain three phosphorylation signals: ERK, JNK and p38 phosphorylation [24-26]. Many studies demonstrated the MAPK signaling pathways control cell proliferation, cell death and differentiation [26-28]. SSZ is majorly used as a NF-kB inhibitor in many studies [11, 29]. Only few studies to investigate whether SSZ influences MAPK signals. Previous studies showed that SSZ can activate p38 phosphorylation in cholangiocarcinoma and melanocytes [30, 31]. However, whether SSZ can activate MAPK signals in TNBCs remained unclear.

On the other hand, VES has anti-cancer effects on various cancers [16-18]. Previous studies showed that VES-induced-apoptosis may activate ERK pathway on human gastric cancer cells [32, 33] and VES-induced-apoptosis mediated ERK and JNK pathways on hormone-dependent breast cancer cells [19]. However, whether VES can induce MAPK signals in TNBCs is unclear. In this study, the anti-cancer effects on SSZ-treated, VES-treated and SSZ/VES-treated TNBC cells were studied. Our study firstly showed VES has a synergistic or an antagonistic cytotoxic effect on SSZ-treated cells depending on the concentration of VES. In addition, different signal pathways were induced on SSZ-treated, VES-treated and SSZ/VES-treated TNBC cells.

Materials and methods

Materials

Vitamin E succinate, Luminol and Lucigenin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-tubulin (1:1,000; cat. no. BS1699), anti-p38 (1:2000; cat. no. BS3567), anti-p-p38 (1:2000; cat. no. BS4766), anti-ERK (1:2000; cat. no. BS1112), anti-p-ERK (1:2000; cat. no. BS5016), anti-JNK (1:2000; cat. no. BS1544), and anti-p-JNK (1:2000; cat. no. BS4763) primary rabbit polyclonal antibodies were obtained from Bioworld (Louis Park, MN, USA). Anti-cleaved PARP (1:2000; cat. no. 9544) and anti-caspase-3 (1:1000; cat. no. 9965) primary rabbit polyclonal antibodies and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (1:2000; cat. no. 7074) were obtained from Cell Signaling Technology (Danvers, MA, USA). The MTT assay kit was obtained from BIO-BASIC CANADA INC (Markham, OT, Canada). Fetal bovine serum, Dulbecco’s modified Eagles medium (DMEM), non-essential amino acids, L-glutamine, and penicillin/streptomycin were obtained from Gibco BRL (Invitrogen Life Technologies, Carlsbad, CA, USA). Sulfasalazine was kindly obtained from Dr. Chou PL (Division of Allergy-Immunology-Rheumatology, Department of Internal Medicine, Saint Mary’s Hospital Luodong, Yilan, 265, Taiwan, R.O.C.).

Cell line and cell culture

MDA-MB-231 (Triple-negative breast cancer cell line) was obtained from the Bioresource Collection and Research Center (Shin Chu, Taiwan). MDA-MB-231 cells was cultured in a humidified atmosphere containing 5% CO2 at 37 °C and supplies the cells with DMEM media containing 10% fetal bovine serum, 0.1 mM non-essential amino acids, 2 mM L-glutamine, and 100 IU/ml penicillin/streptomycin.

Cell viability assay

Cell viability was analyzed by using the MTT assay kit described in previous studies [26, 34]. In brief, MDA-MB-231 cells were cultured into 96-well culture dish (1x10^4 cells/well). Every 24 hour, the MTT assay kit was added into the control and experimental groups. After incubation for 3 hours at 37°C, the purple formazan products were measured at 570 nm (A570) using a Multiskan™ FC Microplate Photometer (Molecular Devices, Sunnyvale, CA, USA). The cell viability (%) was indicated as (A570 experimental group)/(A570 control group) × 100%.

Measurements of H2O2 levels

Intracellular H2O2 levels was determined using the lucigenin-amplified chemiluminescence method.
The control and experimental groups (200 μl) were treated with 0.2 mmol/ml of luminol solution (100 μl). Next, all samples were observed and analyzed for 5 minutes using a chemiluminescence analyzing system (CLA-FSI; Tohoko Electronic Industrial Co., Ltd., Sendai, Japan).

**SDS page and western blotting**

Control and experimental cells were lysed in the radio-immunoprecipitation assay buffer (cat. no. 20-188; EMD Millipore, Billerica, MA, USA). Cellular proteins were obtained from the supernatant with centrifugation (16,000 × g; 4 °C) for 20 min. The protein concentration was determined using a protein assay kit (cat. no. 23200; Thermo Fischer Scientific, Inc., Waltham, MA, USA). Equal quantities (40 μg) of protein were separated by SDS-PAGE (13.3% gels, 80 volts) and transferred onto polyvinylidene difluoride membranes (EMD Millipore). The membranes were treated with 5% non-fat milk at room temperature for 2 hours and washed with PBS buffer for 15 minutes (three times). Next membranes were incubated with primary antibodies at room temperature for 4 hours. After membranes were washed with PBS buffer for 15 minutes, the membranes were treated with anti-rabbit HRP-conjugated secondary antibodies at room temperature for 1 hour. Finally, the membranes were treated with Western Lightning® Chemiluminescence Plus reagent (PerkinElmer, Inc., Waltham, MA, USA) and observed with a Luminescence Image Analysis system (LAS-4000, FUJIFILM Electronic Materials Taiwan Co., Ltd., Tainan, Taiwan).

**Statistical Analysis**

All data were analyzed from four independent experiments. The values are presented as the mean ± standard error. Student's t-test was used for the analysis of the data using Microsoft Excel (http://microsoft-excel-2010.updatestar.com/zh-tw). P value < 0.05 was considered statistically significant.

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**Results**

**Low-dose VES antagonizes SSZ-induced cytotoxic effects**

Combination treatments of SSZ with low-dose VES (20 μM) on MDA-MB-231 cells was studied. Our data showed that the percentages of cell viability were about 70%, 93% and 80% with 0.25 mM SSZ, 20 μM VES and 0.25 mM SSZ plus 20 μM VES treatments at 48 hours respectively (Fig. 1A). The data indicated low-dose VES attenuated 0.25 mM SSZ-induced cytotoxicity on MDA-MB-231 cells. In addition, the percentages of cell viability were about 47%, 93% and 60% with 0.5 mM SSZ, 20 μM VES and 0.5 mM SSZ plus 20 μM VES treatments at 48 hours respectively (Fig. 1B). The data also suggested low-dose VES decreased 0.5 mM SSZ-induced cytotoxicity on MDA-MB-231 cells. These results demonstrated that low-dose VES antagonized SSZ-induced cytotoxic effects on MDA-MB-231 cells.

**High-dose VES has synergistic effects on SSZ-induced cytotoxicity**

Combination treatments of SSZ with high-dose VES (80 μM) on MDA-MB-231 cells was further determined. The percentages of cell viability were about 70%, 55% and 38.36% with 0.25 mM SSZ, 80 μM VES and 0.25 mM SSZ plus 80 μM VES treatments at 48 hours respectively (Fig. 2A). The data indicated high-dose VES promotes 0.25 mM SSZ-induced cytotoxicity. In addition, the percentages of cell viability were about 47%, 55% and 38.47% with 0.5 mM SSZ, 80 μM VES and 0.5 mM SSZ plus 80 μM VES treatments at 48 hours respectively (Fig. 2B). The data also indicated high-dose VES increased 0.5 mM SSZ-induced cytotoxicity on MDA-MB-231 cells. These results suggested high-dose VES has synergistic effects on SSZ-induced cytotoxicity on MDA-MB-231 cells. Next, intracellular H2O2 counts were determined and was increased in SSZ plus VES-treated group (Fig. 2C). The data indicated H2O2 might be related to synergistic cytotoxic effects on SSZ plus VES-treated MDA-MB-231 cells.

**Caspase-3 activation is found in SSZ-treated and VES plus SSZ-treated cells**

Whether caspase-3 signals related to SSZ-induced, VES-induced or SSZ plus VES-induced cytotoxicity was determined. Cleaved caspase-3 and caspase-3 were assayed by western blot, the data showed the ratio of cleaved caspase-3/caspase-3...
was increased in SSZ-treated and VES plus SSZ-treated groups (Fig. 3A). PARP is a downstream substrate of caspase-3. PARP can be cleaved by activated caspase-3. Cleaved PARP was determined in SSZ-treated, VES-treated and VES plus SSZ-treated cells. Similar to the result of caspase-3 activity, cleaved PARP was increased in SSZ-treated and VES plus SSZ-treated groups (Fig. 3B). Taken together, these data indicated that SSZ- and VES plus SSZ-induced cell death were associated with caspase-3 signals.

**Different phosphorylated MAPKs are found in SSZ-treated, VES-treated and VES plus SSZ-treated cells**

MAPKs contain JNK, ERK and p38 pathways. Activation of JNK, ERK or p38 was determined by western blot and the ratio of activated (phosphorylated) forms were calculated (Fig. 4). The ratio of p-JNK to JNK was increased in VES-treated and VES plus SSZ-treated cells (Fig. 4A). The data indicated JNK phosphorylation was majorly related to VES treatment. In addition, the ratio of p-ERK to ERK was increased in SSZ-treated and VES plus SSZ-treated cells (Fig. 4B). The data indicated ERK phosphorylation was majorly related to SSZ treatment. However, the ratio of p-p38 to p38 was only increased in VES plus SSZ-treated cells (Fig. 4C). The data indicated p38 phosphorylation was only induced by combination treatments of SSZ with VES. That is different phosphorylation of MAPKs was induced by different treatments (SSZ, VES, or VES plus SSZ) on MDA-MB-231 cells.

**Discussion**

In this study, combination treatment of high-dose VES with SSZ exerted a synergistic anti-cancer activity on MDA-MB-231 cell (Fig. 2). Interestingly, low-dose VES has an antagonistic effect on VES-treated MDA-MB-231 cells (Fig. 1). Recently, a study demonstrated high-dose sodium selenite and VES exerted cytotoxic effects on breast cancers while low-dose sodium selenite could antagonize VES-induced cytotoxic effect[20]. Our study has a similar result to this study. Therefore, these studies indicated synergistic or antagonistic effects might be a dose-dependent manner. In addition, our data showed that the cell viability was about 38% on 0.5 mM SSZ plus 80 μM VES-treated group (Fig. 2B) and the cell viability was also about 38% on 0.25 mM SSZ plus 80 μM VES-treated group (Fig. 2A). The data suggested combination of 0.25 mM SSZ with 80 μM VES might be a good choice for MDA-MB-231 treatment.

Previously, some studies indicated that oxidative stress might be involved in SSZ-
and VES-induced cell cytotoxicity[36-39]. Whether SSZ and VES can induce oxidative stress on MDA-MB-231 cells has remained unclear. Both intracellular H$_2$O$_2$ and O$_2^-$ belonging to ROS family can induce oxidative stress and cytotoxicity[26, 35]. SSZ, VES and SSZ plus VES treatments could induce cell cytotoxicity, however, H$_2$O$_2$ levels increased was only found in SSZ plus VES-treated group (Fig. 2C). We suggested intracellular H$_2$O$_2$ was not major factor in SSZ- and VES-induced cytotoxicity on MDA-MB-231 cells while H$_2$O$_2$ levels increased might play an important role to induce synergistic cytotoxic effects on SSZ/ VES-treated MDA-MB-231 cells. On the other hand, O$_2^-$ levels were not significantly increased in SSZ-, VES- and SSZ plus VES-treated groups (data not show). These results were similar to the acetaminophen-induced and methotrexate-induced cytotoxicity as described in previous studies[26, 40].

Caspases activation can induce cell death has been reported [26, 40]. Previous studies showed SSZ-induced cell death mediated caspase-3 activity [41, 42] as well as VES can induced caspase-3 activity resulting in cell death [21, 43]. Today our data showed that caspase-3 could be activated and PARP could be cleaved in SSV- and SSZ plus VES-treated groups. However, caspase-3 activation and PARP cleavage did not found in VES-treated group. These data suggested caspase-3 signal was involved in SSZ-induced cytotoxicity on MDA-MB-231 cells. However, caspase-3 signal was not activated in VES-induced cytotoxicity on MDA-MB-231 cells. A previous study showed VES-induced cytotoxicity can mediate caspase-3-independent pathways[44]. Similar to this study, our data indicated VES-induced cytotoxicity on MDA-MB-231 cells might mediate caspase-3-independent pathway.

MAPK signaling pathways mediated cellular biologic responses including growth, cell death, differentiation and inflammation have been reported [26-28]. Many studies found the cellular biologic responses might be through different MAPKs activation with distinct treatments. Previous studies showed SSZ could activate p38 signals in cholangiocarcinoma and melanocytes while VES could activate ERK and JNK signals in hormone-dependent breast cancer cells[30-33]. Unlike above studies, today, our data showed that ERK activation but not p38 activation was found in SSZ-treated MDA-MB-231 cells. In addition, only JNK activation was found in VES-treated MDA-MB-231 cells. Taken together, these studies suggested different MAPKs could be activated by using same drugs in different cancers. Furthermore, our data showed p38 activation only found in VES plus SSZ-treated MDA-MB-231 cells but not found in VES- and SSZ-treated groups. Our data also showed H$_2$O$_2$ levels increased was only found in SSZ plus VES-treated group (Fig. 2C). On the other hand, previous studies demonstrated oxidative stress was related to p38 activation [45, 46]. Therefore, we considered the H$_2$O$_2$ levels increased might be association with p38 activation on MDA-MB-231 cells with SSZ plus VES treatment.

Figure 4. MAPKs phosphorylation analysis. (A) JNK phosphorylation (containing JNK1 and JNK2) was assayed by western blot and phosphorylated JNK(1/2)/JNK(1/2) intensity ratio was calculated after 60 minutes treatments. (B) ERK phosphorylation was assayed by western blot and phosphorylated ERK/ERK intensity ratio was calculated after 60 minutes treatments. (C) p38 phosphorylation was assayed by western blot and phosphorylated p38/p38 intensity ratio was calculated after 30 minutes treatments. These proteins were determined in control (CON), 0.5 mM SSZ (S0.5), 80 μM VES (VES80) and 0.5 mM SSZ plus 80 μM VES (VES80+S0.5) groups. The number indicated above the protein band was individual intensity value. Results were obtained from three independent experiments and presented as mean ± SD. *P<0.05, compared to control group.
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Competing Interests

The authors have declared that no competing interest exists.

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