RESEARCH ARTICLE

Enhanced Anti-tumor Efficacy of Aspirin Combined with Triptolide in Cervical Cancer Cells

Rong-Hui Chen, Yong-Jie Tian*

Abstract

Background: The non-steroidal anti-inflammatory drug (NSAID) aspirin (acetylsalicylic acid) is an inhibitor of cyclooxygenase enzymes. Recent studies have shown that aspirin could be used as an anti-tumor drug. Triptolide, the major compound extracted from the Chinese herb *Tripteryglum wilfordii* Hook.f, has now been shown that it can inhibit tumor growth. The aim of this study was to analyze the anti-tumor efficiency of aspirin and triptolide in cervical cancer cells. Methods: Viability of cervical cancer cell lines was assessed by the MTT method at various concentrations of aspirin and triptolide. Siha and HeLa cell apoptotic analysis was performed by flow cytometry. Real time-PCR and Western Blotting were used to analyze the expression of Bcl-2/Bax, Cyclin D1 and p16. Results: Viability in the combination group was significantly decreased as compared with either drug used alone. Expression change of Bcl-2/Bax, CyclinD1 and p16 appeared to play an important role in the synergistic killing effect on cervical cancer cell apoptosis. Conclusion: Aspirin and triptolide combination treatment may have synergistic anti-tumor effects on cervical cancer cells.

Keywords: Aspirin - triptolide - cervical cancer cells - combined treatment - synergy

Asian Pacific J Cancer Prev, **14 (5)**, 3041-3044

Introduction

Cervical cancer is the second most common cause of cancer and the fourth leading cause of cancer-related death in female all over the world. In majority of the cases, it is associated with the presence of human papilloma virus (HPV) infection, especially in high-risk HPV16 or 18 type infections, being regarded as leading factor for more than 70% of cases of cervical cancer (Green et al., 2001; Pectasides et al., 2008; Jemal et al., 2011; Lea et al., 2012). However, the mechanism of cervical cancer formation is still unstated (Marrazzo et al., 2001; Paavonen, 2007; Gadducci et al., 2011). In present, different therapeutic strategies such as surgery, chemo-radiotherapy and HPV vaccines (applications only for HPV types 16 and 18 but the other high-risk subtypes) related biologic therapy are the main modalities for the treatment of cervical cancer patients, but all have their own limitations. Recently, many researches shown that traditional Chinese medicine (TCM) could be used in cancer therapy (Messina et al., 2011; El Kebir et al., 2013).

Non-steroidal anti-inflammatory drug (NSAID) aspirin (acetylsalicylic acid), is an inhibitor of the cyclooxygenase enzymes, has been widely used in anti-oxidative, anti-microbial and anti-inflammatory (Hsieh et al.,2010; De Luna-Bertos et al., 2012; Dhillon et al., 2012; Pazhang et al., 2012; Kodela et al., 2013; Yan et al., 2013). Studies shown that aspirin could be used as an anti-tumor drug in several cancer cell lines (Xiaoxin et al., 2009; Ou et al., 2010; Park et al., 2010; Singh et al., 2010), but these anti-tumor efficiency is still low. Another widely used drug, triptolide, the major composition extracted from the Chinese herb Tripteryglum wilfordii Hook.f, has now been shown that it can inhibit tumor growth (Clawson et al., 2010; Zhao et al., 2010; Lu et al., 2011; Li et al., 2012; Zhu et al., 2012; Huang et al., 2013; Tan et al., 2013). However, triptolide still have side effects such as immunosuppressive and antifertility (Carter et al., 2008; Westfall et al., 2008; Yang et al., 2008; Chen et al., 2009; Zhu et al., 2009; Borja-Cacho et al., 2010).

In order to enhance the anti-tumor efficiency and reduce the side effects of these two drugs, the present study attempted to analyze the combined efficacy of aspirin and triptolide on cervical cancer cell lines.

Materials and Methods

Cell culture and reagents

Human cervial cancer lines (HeLa, Siha and Caski) were obtained from American Type Culture Collection (ATCC, USA). Cells were cultured in RPMI 1640 culture medium (Hyclone, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, USA), 100 U/mL penicillin and 100 U/mL streptomycin. Cells were incubated in 5% CO2 incubator at 37 °C. Aspirin were obtained from Sigma Chemical (St Louis, MO, USA),

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and kept as a stock solution of 100 mM in PBS, then diluted to the working concentration. Triptolide (purity > 99.0%, Chinese Academy of Medical Sciences, Nanjing, China) was diluted in dimethyl sulfoxide (DMSO) to obtain 1 μM stock solution and then diluted to the working concentration.

Cell viability assay

Cell viability was analyzed by the MTT method. The viability of HeLa, Siha and Caski cells treated by aspirin, triptolide and their combinations were determined respectively. Cells were seeded into 96-well plates at a density of 5000 cells in a well, after 24 h incubation, the culture medium discarded and changed as RPMI 1640 medium supplemented in 2% FBS. Then, cells were treated with different drugs. After treatments over, discard medium, wash with PBS and then add MTT (5 mg/mL), incubated for another 4 h. Discard the medium, 200 μL DMSO was added to dissolve MTT formazan crystals and absorbance at 570 nm was measured by plate reader (BioTek, USA). Cells in RPMI1640 were used as negative control (NC). Cell viability was calculated as percentage of viable cells in total population. Each experiment was performed three replicates.

Flow cytometry

Apoptosis were determinated by flow cytometry. Annexin V-FITC/PI staining followed by flow cytometric analysis was performed for quantitative determination of apoptosis in cervical cancer cells. After the treatments, cells were harvested by trypsin, washed with cold PBS, and stained with Annexin V-FITC/PI staining kit (BD iosciences) according to manufacturer's instructions. Cells were analyzed using a Flow Cytometer (Beckman Coulter).

Mitochondrial membrane potential analysis

Apoptosis also could be evaluated by the loss of mitochondrial membrane potential. MitoTracker Red CMXRos (Invitrogen, USA) accumulated in mitochondria with time dependent manner, and the fluorescence emission will be changed from green to red. After the treatments, cells were harvested, and then centrifuged at 400 x g for 5 min; the cell pellet was resuspended in 0.1 μmol/L final concentration of MitoTracker Red CMXRos (dissolved in dimethyl sulfoxide) for 20 min. Washed and resuspended in PBS, fixed with 4% paraformaldehyde. After the final wash with PBS, the cells on the microplate were read by a spectrophotometer.

Clonogenic survival experiments

Cells were seeded into 6-cm dishes. After treatment of different drugs, culture in CO_2 incubator, 7 days later, colonies were fixed with methanol and stained with 1.25% Giemsa and 0.125% crystal violet for counting. Cell survival was expressed in relation to the untreated control.

Real-time PCR

After the treatments, cells were harvested and total RNA was isolated using Trizol (TianGen, Beijing). RNA was reverse transcribed using a first-strand cDNA synthesis kit from Tiangen Corp (Beijing, China). RNase-free DNase in a column digestion (Qiagen). First-strand cDNA synthesis was performed using 0.5 mg total RNA and the iScript cDNA Synthesis (Bio-Rad) with an oligo-dT primer (Invitrogen). For real-time PCR, the iQ SYBR Green Supermix Kit (BioRad) was used. Primers were p21-F (5'-TGA GCC GCG ACT GTG ATG-3') and p21-R (5'- GTC TCG GTG ACA AAG TCG AAG TT-3'); Cyclin E-F (5'-ATA CAG ACC CAC AGA GAC AG-3') and Cyclin E-R (5'-TGC CAT CCA CAG AAA TAC TT-3'); Bcl-2 forward (5'-CAT GTG TGT GGA GAG CGT CAA-3') and Bcl-2 reverse (5'-GCC GGT TCA GGT ACT CAG TCA-3'); Bax-F (5'-GCC CTT TTG CTT CAG GGT TT-3') and Bax-R (5'-TCC AAT GTC CAG CCC ATG AT-3'); β-actin-F (5'-AGC GCA AGT ACT CCG TGT G-3') and β-actin-R (5'-AAG CAA TGC TAT CAC CTC CC-3').

Western blot analysis

After treatments, cells were harvested, the cell pellets were washed with ice-cold PBS, centrifuged at 10,000 rpm at 4 °C for 10 min. Supernatants were mixed with concentrated sample buffer and they were used as the cytoplasmic fractions for Western blot analysis. The nuclear pellets were washed once with lysis buffer, suspended in sample buffer, sonicated and used as the nuclear fractions for Western blot analysis.

Whole cell lysates or cell fractions prepared as described above were boiled at 95 °C for 5 min and subjected to SDS–PAGE. The resolved proteins were transferred to PVDF membrane using semi-dry transfer apparatus (Bio-Rad). After transfer, the membranes were blocked with 5% non-fat dry milk in TBS for 1 h at room temperature, and then incubated with primary antibodies overnight at 4 °C followed by secondary horseradish peroxidase-labeled antibody (1:2000). The bound antibodies were visualized using the ECL blotting detection system.

Statistical analysis

Statistical analysis was assessed using Student's t-test. $P < 0.05$ was considered statistically significant.

Results

Combination of aspirin and triptolide suppress the proliferation of cervical cancer cell lines

Before the analysis of the synergistic effect of aspirin and triptolide, we tested the cytotoxic effect of aspirin (5–12.5 μM) and triptolide (50–125 nM) separately. After 24 h, 48 h and 72 h of treatment, cell viability was measured with an MTT assay. As shown in Figure 1, both of aspirin (Figure 1A) and triptolide (Figure 1B) suppress the proliferation with a dose-dependent manner in cervical cancer cell line. Combined treatment with aspirin (5-10 μM) and VPA (50-100 nM) was then assessed after 24h and 48 h, the combination of both agents exhibited a significantly synergistic anti-proliferation effect compared with drugs used separately ($P < 0.01$) (Figure 1C). Moreover, this synergistic effect also exists in anther cervical cancer cell HeLa and Caski lines (Figure

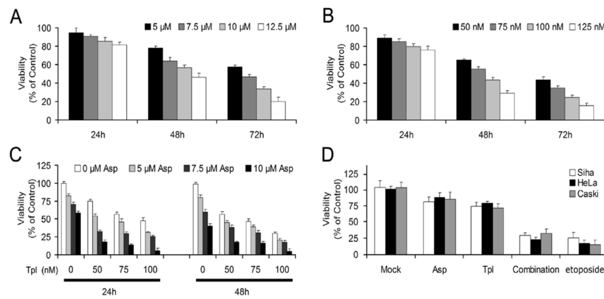

Figure 1. Inhibitory Effect Induced by Combination of Aspirin and Triptolide on Growth. (A) The cells were treated with aspirin with various concentrations (5, 7.5, 10 and 12.5 μM) for 24 h, 48 h and 72 h. (B) The cells were treated with triptolide with various concentrations (50, 75, 100 and 125 nM) for 24 h, 48 h and 72 h. (C) The cells were treated with aspirin (5, 7.5 and 10 μM) and triptolide combination with various concentrations (50, 75 and 100 nM) for 24 h and 48 h. (D) Different cervical cancer cell lines (HeLa and Caski) were used for drug combination. All data represent the mean values ± SD of at least three independent experiments. Abbreviation: Asp as aspirin, Tpl as triptolide

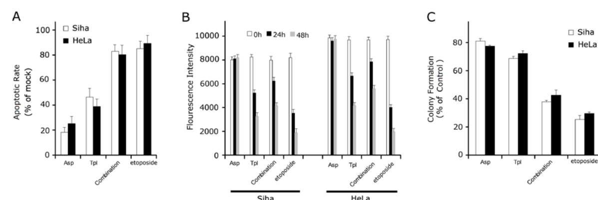

Figure 2. Apoptotic Rate, Mitochondrial Membrane Potential and Colony Formation Assay for Drug Combination. (A) Apoptotic rate of Siha and HeLa cells treated with drug combination or used alone. (B) Mitochondrial membrane potential assay in Siha and HeLa cells after 24 h and 48 h treated with drug alone or combination. (C) Colony formation ability of Siha and HeLa cells were analyzed by colony formation assay. All data represent the mean values ± SD of at least three independent experiments. Abbreviation: Asp as aspirin, Tpl as triptolide

1D). These data indicated that aspirin and triptolide combination have a synergistic anti-proliferation effect in cervical cancer cell line growth.

Cervical cancer cell lines apoptosis, mitochondrial membrane potential colony formation decrease could be induced by the aspirin and triptolide combination

We examined apoptosis induced by aspirin and triptolide, alone and in combination, in the cervical cancer cell line by using flow cytometry, mitochondrial membrane potential assay and colony formation analysis. First, flow cytometry using propidium iodide staining showed that the percentage of apoptotic cells induced by aspirin (10 μM) and triptolide (100 nM) combination was significantly higher than that in drug used alone ($P<0.05$) (Figure 2A). Mitochondrial membrane potential decreased during the process of apoptosis, in combination treatment, mitochondrial membrane potential significantly decreased in the fluorescence intensity of CMXRos (Figure 2B). In addition, colony formation ability of cervical cancer cell lines is the key feature of malignant cells; in Figure 2C, the combination treatment decreased the number of the colonies significantly ($P<0.05$). From these three assays, we found that aspirin and triptolide combination could

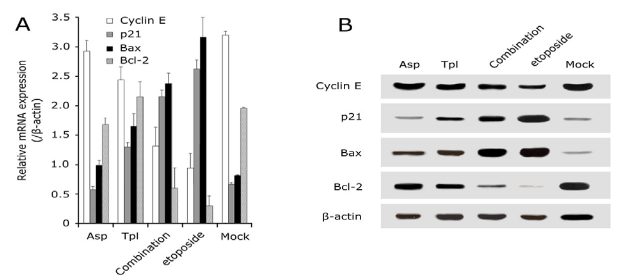

Figure 3. mRNA and Protein Level of Combination of Aspirin and Triptolide in Siha Cells. (A) mRNA expression of cell cycle and apoptosis related genes' expression by qRT-PCR. (B) Immuno blotting of cell lysates of cells treated with drug used alone or combination

induce apoptosis and decrease the colony formation ability in cervical cancer cells.

Cervical cancer cells undergo Bcl-2/Bax and cell cycle related protein-mediated apoptosis

We further examined whether Bcl-2/Bax and cell cycle related gene could be affected by the 48 h of treatment with aspirin and triptolide, alone and in combination, in cervical cancer cell Siha line. Treatment with aspirin and triptolide combination significantly induced the Bax and p21 expression, and decrease the Bcl-2 and Cyclin E expression in the mRNA (Figure 3A) level by qRT-PCR assay and protein (Figure 3B) level by Western blot assay. That means that combination treatment of aspirin and triptolide will synergistically cause the cell cycle arrest, and then induce the apoptosis of cervical cancer cell.

Discussion

Nonsteroidal anti-inflammatory drugs (NSAID) such as aspirin and other NSAIDs were introduced as an anti-inflammatory and analgesic drug (Dhillon et al., 2012; El Kebir et al., 2013; Kodela et al., 2013; Yan et al., 2013), while, recent advance shown that it can reduce the risk for colorectal cancer in clinical observations (De Luna-Bertos et al., 2012; Hsieh et al., 2010; Pazhang et al., 2012; Singh et al., 2010), and induce apoptosis in several cancer (Xiaoxin et al., 2009; Ou et al., 2010; Park et al., 2010). Triptolide is a diterpenoid triepoxide and the major active ingredient of Tripterygium wilfordii Hook. f. that has been used as a natural medicine in China for a long time, which was used for treating autoimmune diseases such as nephritis and rheumatoid arthritis (Li et al., 2012; Zhu et al., 2012; Huang et al., 2013; Tan et al., 2013). Newly report shown that triptolide have the potential to suppress the proliferation of human tumors in vitro (Zhu et al., 2009; Borja-Cacho et al., 2010; Clawson et al., 2010; Zhao et al., 2010; Lu et al., 2011). It is worth noting that triptolide possess side effects in both immunosuppressive and antifertility activities (Carter et al., 2008; Westfall et al., 2008; Yang et al., 2008; Chen et al., 2009). In this, the role of triptolide in cancer treatment needs to be reconsidered. However, in traditional Chinese medicine prescription, we know that different types of Chinese medicine will be made with a mixture for clinical use, and this strategy will decrease the side effects from the drugs used alone with a large amount. Therefore, combination

therapeutic in further research is an alternative strategy compared with conventional.

In this study, the proliferation of cervical cancer cell line was significantly decreased by aspirin and triptolide combination. This combination will lower the dose of drugs respectively but the anti-tumor effects will not be affected. Moreover, this combination not only highly blocked the cervical cancer cell lines' proliferation, but also apoptosis was induced. Cell cycle related gene such as Cyclin E and p21 may play the important role in growth suppression in aspirin and triptolide combination.

In summary, aspirin and triptolide have a synergistic killing effect of cervical cancer cells in vitro, and these data will provide a base for further clinical cancer therapy.

Acknowledgements

The authors have no commercial or financial interest in the products or companies described in this article.

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