RESEARCH ARTICLE

Tanshinone IIA Reverses the Malignant Phenotype of SGC7901 Gastric Cancer Cells

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Abstract

Backgrounds: Tanshinone IIA (TIIA), a phenanthrenequinone derivative extracted from *Salvia miltiorrhiza* BUNGE, has been reported to be a natural anti-cancer agent in a variety of tumor cells. However, the effect of TIIA on gastric cancer cells remains unknown. In the present study, we investigated the influence of TIIA on the malignant phenotype of SGC7901 gastric cancer cells. Methods: Cells cultured *in vitro* were treated with TIIA (0, 1, 5, 10 μg/ml) and after incubation for different periods, cell proliferation was measured by MTT method and cell apoptosis and cell cycling were assessed by flow cytometry (FCM). The sensitivity of SGC7901 gastric cancer cells to anticancer chemotherapy was investigated with the MTT method, while cell migration and invasion were examined by wound-healing and transwell assays, respectively. Results: TIIA (1, 5, 10 μg/ml) exerted powerful inhibitory effects on cell proliferation ($P < 0.05$, and $P < 0.01$), and this effect was time- and dose-dependent. FCM results showed that TIIA induced apoptosis of SGC7901 cells, reduced the number of cells in S phase and increased those in G0/G1 phase. TIIA also significantly increased the sensitivity of SGC7901 gastric cancer cells to ADR and Fu. Moreover, wound-healing and transwell assays showed that TIIA markedly decreased migratory and invasive abilities of SGC7901 cells. Conclusions: TIIA can reverse the malignant phenotype of SGC7901 gastric cancer cells, indicating that it may be a promising therapeutic agent.

Keywords: Tanshinone IIA - gastric cancer - growth - chemo-sensitivity - migration - invasion

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Introduction

Gastric cancer, one of the most aggressive tumors, is the second leading cause of cancer-related deaths throughout the world (Parkin et al., 2001; Parkin, 2004; Li et al., 2012), especially in East Asian countries such as China, Japan and Korea (Alberts et al., 2003; Parkin et al., 2005). The development of gastric cancer is a multi-step process from normal mucosa to chronic gastritis, then to precancerous lesions including gastric atrophy, intestinal metaplasia and dysplasia, and finally to invasive cancer (Correa, 1992). Gastric cancer is frequently associated with lymph node metastasis, peritoneal dissemination, and hematogenous metastasis (IARC, 2004). It is believed that various structural and functional genetic alterations of oncogenes, tumor suppressor genes, DNA repair genes, cell cycle regulators, cell adhesion molecules and growth factors/receptors system are involved in the onset and progression of gastric cancer (Wu et al., 1997; Yasui et al., 2000; Werner et al., 2001) and that the rapid proliferation, multidrug resistance, invasion and metastasis of tumor cells are responsible for poor prognosis (Lochhead and El-Omar, 2008). At present, surgery and chemotherapy (Huang et al., 2012; Shi et al., 2012) are the two main ways to treat gastric cancer. However, the existing drugs are not effective enough and they have many side effects. As a result, it has become a focus to search drugs which are capable of preventing and treating gastric cancer and other malignant tumors.

Tanshinone IIA (TIIA), a phenanthrenequinone derivative extracted from *Salvia miltiorrhiza* BUNGE, is used in Chinese traditional herbal medicine (Danshen) for the treatment of many diseases (Lam et al., 2003; Zhou et al., 2005; Fu et al., 2007; Gao et al., 2008; Yang et al., 2008). Because TIIA inhibits the association of lipid peroxidation products with DNA by breaking chain reactions (Wang et al., 2003; Choi et al., 2004; Yang et al., 2005), TIIA may have biochemical effects as an anti-oxidant with anti-inflammatory properties.

Recent studies indicate that TIIA is also an anti-cancer agent in a variety of tumor cells, including leukemia (Liu et al., 2006; Zhang et al., 2010), breast cancer (Su and Lin, 2008; Lu et al., 2009), colon cancer (Su et al., 2008; Zhou et al., 2012) and hepatocellular carcinoma cells (Yuan et al., 2004; Yuxian et al., 2009). However, the effects of TIIA on gastric cancer cells are not clear yet. In the present study, therefore, we aim to elucidate the effects of TIIA on the malignant phenotype of SGC7901 gastric cancer cells. Our results showed that TIIA efficiently inhibited cell proliferation, induced apoptosis and blocked cell cycle at G1/S phase,while increasing the sensitivity to anticancer chemotherapy and reducing migratory and invasive abilities of SGC7901 cells.

Materials and Methods

Cell culture and grouping

Gastric cancer SGC7901 cell line (obtained from

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ATCC, Rockville, MD, USA) was maintained in RPMI1640 medium. The medium were supplemented with 10% fetal calf serum (FCS), 100 U/ml of penicillin and 100 U/ml of streptomycin and all the cells were kept at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Cells were divided into the following groups: (1) control group: cells were treated under normal conditions without TIIA treatment; (2) TIIA group: cells were treated under normal conditions with incubation with different doses of TIIA (1.0, 5.0 and 10.0 μg/ml).

Cell proliferation assay

Cell proliferation assay was determined by the conversion of 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO) into water-insoluble formazan by viable cells. Briefly, cells of same density were seeded into flat-bottom 96-wells, treated with different concentrations of TIIA (0-10.0 μg/ml) and grown under normal conditions. Cultures were assayed at 24, 48 and 72 h. Then, cells were lysed in dimethyl sulfoxide after incubation with MTT for 4 h and the amount of MTT formazan was quantified by determining the absorbance at 490 nm. Relatively inhibitory rate of cell growth by drugs was calculated according to the following formula: R = (A2-A1)/A2×100% in which R is relatively inhibitory rate of cell growth; A1 is absorbance value of cells in the presence of drugs; and A2 is absorbance value of control cells without any drug treatment. Each experiment was performed in triplicate.

Flow cytometry Analysis

Flow cytometry analysis was used to determine the distribution of cells in cell cycle or apoptosis of the cells. Briefly, after adherent SGC7901 cells treated with different doses of TIIA for 48 h, cells were collected by trypsinization, washed twice with ice-cold PBS, suspended in about 0.5 ml of 70% alcohol and kept at 4°C for 30 minutes. The suspension was filtered through a 50-μm nylon mesh, and the DNA content of stained nuclei was analyzed by a flow cytometer (EPICS XL; Coulter, Miami FL). Cell cycle was analyzed using Multicycle-DNA Cell Cycle Analyzed Soft-ware. The proliferous index (PI) was calculated as: PI = (S + G2)/(S + G2 +G1). Each experiment was performed in triplicate. Apoptotic cells were identified as a hypodiploid DNA peak representing cells (sub-G1). Findings from at least 20 000 cells were collected and analyzed with CellQuest software (Becton Dickinson).

In vitro drug sensitivity assay

MTT assay as described previously was used to evaluate the sensitivity of gastric cancer cells to anticancer drugs. In brief, cells were maintained under normal conditions or treated with different concentrations of TIIA, ADR and 5-Fu for 48 h. Relatively inhibitory rate of cell growth by drugs was calculated according to the following formula: R = (A2-A1)/A2×100% in which R is relatively inhibitory rate of cell growth; A1 is absorbance value of cells in the presence of drugs; and A2 is absorbance value of control cells without any drug treatment.

Wound-healing assay

For wound-healing assays, cells were plated into six-well plates and grown under normal conditions. When cell grew into monolayer, cells were pretreated with TIIA (0 - 10.0 μg/ml) and a plastic pipette tip was drawn across the center of the plate to produce a clean 1-mm-wide wound area after the cells reached confluency. Then cell were cultured in medium with 1% FBS for 48 h. The cell movement into the wound area was examined by a phase-contrast microscope.

Invasion assay

A transwell plate (Costar, Corning, NY) precoated with Matrigel (Becton-Dickinson) was used to perform cell invasion assays. Briefly, the transwell plate was placed on a 24-well plate, and 400 μl culture medium (10% FBS) was added to the lower chamber as chemoattractant. Then, 200 μl cells (1×10^5) suspended in culture medium with 1% FBS were added to the upper chamber. Cells in the invasion chambers were incubated in a humidified incubator for 24 h. The cells that traversed membrane pore and spread to the lower surface of the filters were stained with 5% Giemsa solution for visualization. Cell invasive viability is expressed as a percentage of the value of the control group.

Statistic analysis

All results were presented as mean ± S.E.M. One-way ANOVA (Fisher's PLSD test) was used for statistical analyses. A statistical difference was accepted as significant if $P < 0.05$.

Results

TIIA reduces the proliferation of SGC7901 cells in vitro

We first investigated the effect of TIIA on the cell proliferation of SGC7901 cells. As shown in Figure 1, TIIA significantly depressed the growth of SGC7901 cells as compared with the control group, and the inhibitory effect of TIIA was dose- and time-dependent ($P < 0.05$, and $P < 0.01$). Especially when the cells were treated with TIIA (10 μg/ml) for 72 h, the inhibitory rate reached (53.0 ± 6.1) %. These results indicate that TIIA reduces the proliferation of SGC7901 gastric cancer cells.

TIIA induces the apoptosis of SGC7901 cells in vitro

Meanwhile, the effect of TIIA on the apoptosis of SGC7901 cells was also investigated by flow cytometry (FCM). As shown in Figure 2 A, apoptotic cells was few in the control group, but the percentage of apoptotic cells began to increase when TIIA was at the dose of 1 μg/ml, and the increase was much more significant when TIIA was at the dose of 5 and 10 μg/ml. Meanwhile, Quantitative analysis showed a significant increase in the number of apoptotic cells in the TIIA group compared with the control group (Figure 2B; $P < 0.05$, and $P < 0.01$). These data suggest that TIIA results in the apoptosis of SGC7901 cells in a dose-dependent manner.

TIIA redistributes the cell cycle of SGC7901 cells in vitro

Next, to investigate the mechanism by which TIIA

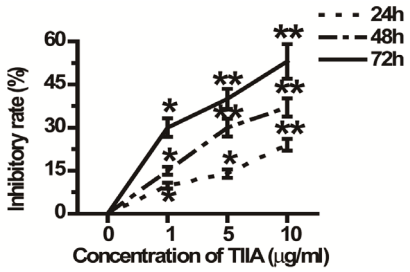

Figure 1. Effects of TIIA on the Cell Growth of SGC7901 Gastric Cancer Cells. Gastric cancer cells were seeded in 96-well microplates and cultured with TIIA (0 - 10 μg/ml) for 24, 48 and 72 h and their numbers were determined by MTT assay. Data are mean ± S.E.M. * P < 0.05, ** P < 0.01 compared with control group

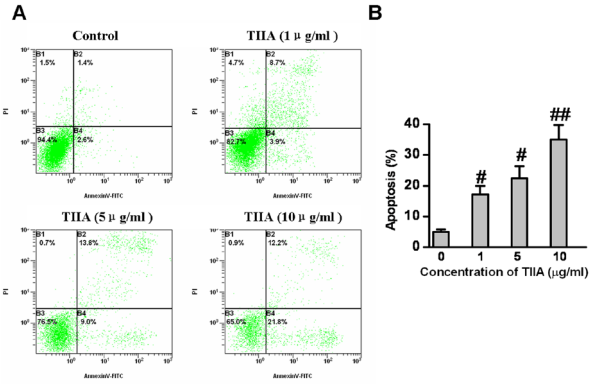

Figure 2. Effects of TIIA of the Apoptosis of SGC7901 Gastric Cancer Cells. (A) For flow cytometric analysis, cells were harvested 48 h after treatment of TIIA (0 - 10 μg/ml), and the percentage of cell apoptosis was determined by flow cytometry. (B) Quantitative analysis of (A). Data are mean ± S.E.M. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with control group

influenced gastric cancer cell growth, we observed the cell cycle of SGC7901 cells (Figure 3) by FCM after the cells were treated with TIIA (0 - 10 μg/ml). Results showed that the percentage of SGC7901 cells at the G0/G1 phase in the TIIA group was much higher than that in the control group (P < 0.05). However, compared with the control group, there was an obvious decrease in the percentage of SGC7901 cells at the S phase in the TIIA group (P < 0.05). These data indicate that TIIA redistributes the cell cycle and exhibits an inhibitory effect on S-phase entry of SGC7901 cells.

TIIA increases the sensitivity of SGC7901 cells to anticancer chemotherapy

We then compared the effect of TIIA on the drug sensitivity of SGC7901 cells with that of control cells using the MTT assay. As shown in Figure 4, TIIA (1 - 10 μg/ml) significantly enhanced the sensitivity of SGC7901 cells to ADR or 5-Fu treatment at three different doses (P < 0.05, and P < 0.01). These results suggest that TIIA may have a beneficial effect on chemosensitivity.

TIIA inhibits the migration and invasion of SGC7901 cells in vitro

To see if TIIA might have an effect on cell movement, we compared the migratory rate of the tumor cells in a wound-healing assay. Figure 5A shows that TIIA significantly decreased cell migration from the edge of

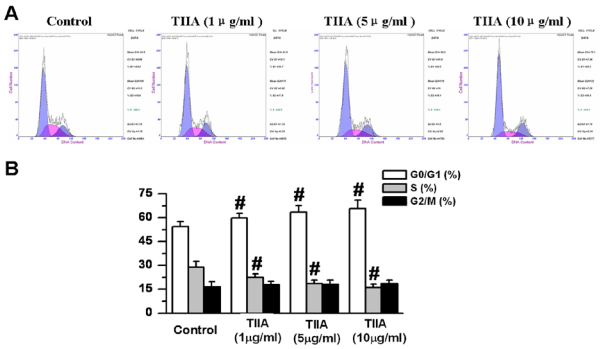

Figure 3. Effects of TIIA of the Cell Cycle of SGC7901 Gastric Cancer Cells. (A) For flow cytometric analysis, cells were harvested 48 h after treatment of TIIA (0 - 10 μg/ml), and the cell cycle was determined by flow cytometry. (B) Quantitative analysis of (A). Data are mean ± S.E.M. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with control group

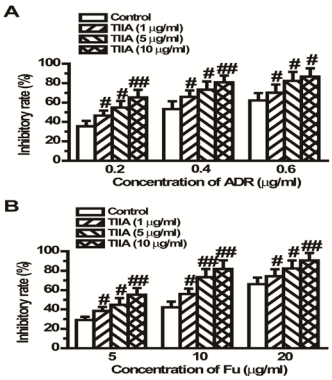

Figure 4. Effects of TIIA on the Sensitivity of SGC7901 Gastric Cancer Cells to ADR or 5-Fu. SGC7901cells were treated with various concentrations of TIIA and ADR or 5-Fu for 48 h. Cell viability was determined by MTT assay. Data are mean ± S.E.M. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with control group

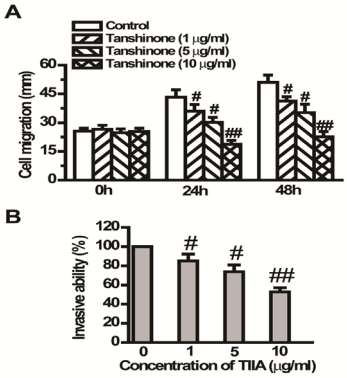

Figure 5. Effects of TIIA of the Migration and Invasion of SGC7901 Gastric Cancer Cells. (A) Cell migration was measured by the wound-healing assay described in Materials and Methods. The cells were incubated for 48 h after cell wounding, and then the photos were taken. (B) The invasive activity of cells was assayed in a Matrigel-coated transwell. The cells that invaded through the filter were quantified 24 h after plating. Data were expressed relative to the invasive ability of control cells. Data are mean ± S.E.M. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with control group

the wound (P < 0.05, and P < 0.01). Similarly, when the cell invasion potential was measured in a matrigel-coated transwell assay (Figure 5B), we found that TIIA markedly inhibited the invasive ability of SGC7901 cells (P <

0.05, and P < 0.01). Therefore, it seems that TIIA has an inhibitory effect on cell migration/ invasion of SGC7901 gastric cancer cells.

Discussion

In the present study, we showed that treatment of TIIA could significantly (i) inhibit the proliferation of SGC7901 cells; (ii) induce the apoptosis of SGC7901 cells, and reduce the number of cells at S phase and increased the number of cells at G0/G1 phase, thus blocking cell cycle at G1/S phase; (iii) increase the sensitivity of SGC7901 gastric cancer cells to anticancer chemotherapy; and (iv) decrease the migratory and invasive abilities of SGC7901 cells.

Danshen, a herbal drug derived from the dried root or rhizome of S. miltiorrhiza Bunge, has been used clinically in China or many Asian countries to manage many diseases, such as angina pectoris, myocardial infarction and stroke. TIIA is one of the key components of Danshen and has been reported to possess the majority of Danshen's properties. Recent researches have shown that TIIA is a natural anti-tumor drug, which has cytotoxic effect on many tumors and inhibits the invasion and migration of cancer cells. For example, TIIA could inhibit the cell growth of reproductive system tumors, such as breast cancer (Su and Lin, 2008; Lu et al., 2009), cervical cancer (Pan et al., 2010), ovarian cancer (Jiao and Wen, 2011) and prostatic carcinoma (Won et al., 2010); TIIA disrupts the membrane potential of mitochondria, induces apoptosis and reduces the adhesion and invasion of cells in acute promyelocytic leukemia. However, few studies have been reported to investigate the effect of TIIA on gastric cancer. As a result, in the present study, we fully studied the influence of TIIA on the malignant phenotype of SGC7901 gastric cancer cells.

Once the balance between the proliferation and apoptosis is disrupted, cells will grow without limits and finally induces the formation of tumor (Liotta and Stetler-Stevenson, 1991). This process is regulated by cell cycle, in which there are some restriction points, such as G1/S point and G2/M point. At present, one of the key points to cure tumor is to interfere with the cell cycle of tumor cells to lower the growth rate of tumor or to induce cell apoptosis. In the present experiment, we first investigated if TIIA could influence the proliferation and apoptosis of SGC7901 cells. Our results showed that, as many other traditional chemotherapeutic drugs, TIIA could inhibit the cell growth of gastric cancer by decreasing the cell proliferation and inducing the apoptosis of SGC7901 cells in a dose-dependent manner. Moreover, TIIA redistributed the cell cycle of SGC7901 gastric cancer cells by blocking the cell cycle at G1/S phase.

Although chemotherapy is one important strategy to treat tumors, the resistance to anticancer agents often occurs and is an important cause of treatment failure and mortality in gastric cancer patients. So, it is meaningful to find new agents which are chemo-sensitizing. Previous reports showed that TIIA could increase the sensitivity of lung cancer cells to anticancer chemotherapy. Here we show for the first time that TIIA could result in the substantial enhancement of sensitivity of SGC7901 cells to two different types of anticancer drugs, ADR or 5-Fu, suggesting that TIIA may be a potential adjuvant of chemo-sensitizing chemotherapy for gastric cancer.

Except for infinite self-duplication, migration and invasion also play an important role in the development of malignant tumor. These two malignant phenotypes not only challenge the treatment of tumors, but also provide new idea to study anti-cancer drugs. The migration of tumor cells involves a complex process, including adhesion, degradation and movement. Once across the basement membrane, tumor cells will grow invasively and quickly, and metastasis will take place. Hence it is beneficial to block any process in the migration and invasion of tumor cells. We then measured the effect of TIIA on the migration and invasion of SGC7901 gastric cancer cells. Results from wound-healing assay and transwell assay indicated that TIIA could reduce the movement, and decrease the migratory and invasive abilities of SGC7901 cells.

In summary, the results of the current study tended to suggest that pharmacological treatment with TIIA can significantly inhibit gastric cell growth by inhibiting cell proliferation, inducing cell apoptosis and redistributing cell cycle, increase chemosensitivity to anticancer drugs and reduce the migration and invasion of SGC7901 gastric cancer cells. At present, although further investigations need to be performed to study the effect of TIIA in vivo, our findings suggest that TIIA might be a promising agent for gastric cancer treatment.

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