Inhibition effect of triptolide on human epithelial ovarian cancer via adjusting cellular immunity and angiogenesis

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Received August 1, 2017; Accepted December 13, 2017

DOI: 10.3892/or.2017.6158

Abstract. Chemotherapy resistance of advanced ovarian cancers is responsible for death of most cancer patients, so it is necessary to seek safe and effective natural ingredients to lower the chemotherapy resistance of ovarian cancer. In the present study, we studied the anticancer effects of triptolide (TPL) and TPL + cisplatin (DDP) in vitro and in vivo using SKOV3/DDP cell line and a mouse model. In vitro results showed that TPL and TPL + DDP inhibited cellular invasion and migration of SKOV3/DDP cells (P<0.05), and significantly reduced the expression of adhesion-related proteins integrin β1 (ITGβ1) and apoptosis-inhibiting proteins survivin, matrix metalloproteinase 2 (MMP-2) and MMP-9 (P<0.05). Animal results demonstrated that TPL and TPL + DDP had significantly enhanced the inflammatory factor-2 (IL-2) and tumor necrosis factor-α (TNF-α) in serum of mice, and significantly increased the NK cell-related protein levels of CD16 and CD56, while significantly inhibited the production of vascular endothelial growth factor (VEGF) related protein clusters of differentiation 31 (CD31) and CD105. Collectively, the combination of TPL and DDP may produce a synergistic anticancer effect on epithelial ovarian cancer (EOC).

Introduction

Epithelial ovarian cancer (EOC) is regarded as the most common type of ovarian cancers, which is usually diagnosed at an advanced stage due to the ineffective screening strategies, and causes immense morbidity and mortality worldwide (1). Although the surgical resection combined with cisplatin-based chemotherapy has greatly benefited cancer patients, the total survival of patients with advanced disease is <30% due to chemoresistance (2,3). Therefore, it is meaningful to find the sensitizer of EOC to cisplatin and clarify its mechanisms.

Numerous studies indicate that the mechanism of tumor occurrence, development, invasion, metastasis and resistance is complex (4-9). As one of the most well-known natural products, triptolide (TPL) had been used as an anti-inflammatory agent for rheumatoid arthritis for a long time in China, and was also recognized as a potential medicine for various types of cancers (10-16) although some researchers showed its potential toxicity on animal liver, kidney, testes, ovary and heart (17). Recent studies indicated that TPL induced cell apoptosis by inhibiting nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) in a p53-independent pathway, producing reactive oxygen species (ROS) and inactivating the PI3K/Akt signal pathway (9,16,18-21). In previous studies, our group proved that the TPL effectively inhibited cell growth, proliferation, metabolism, survival and cancer genesis by regulating the PI3K/Akt pathway (22). However, scarce study is carried out to evaluate the antitumor effect of TPL on cellular immunity and angiogenesis.

In the present study, we investigated the TPL anticancer effects using SKOV3/DDP cell line and a mouse model, and studied the TPL sensitization effects via inhibiting protein expression of angiogenesis and immunology.

Materials and methods

Cell experiments. Platinum-resistant SKOV3/DDP cell line (purchased from China Center for Type Culture Collection, Wuhan, China), which was derived from human ovarian carcinoma, was cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin. Cells were cultured at 37˚C in a 5% humidified CO₂
atmosphere, and 0.3 μg/ml cisplatin was added to the culture media to maintain the acquired resistance to DDP.

**Cellular migration and invasion assays.** To evaluate the effect of TPL on cell migration, a scratch assay was applied. SKOV3/DDP cells were digested with trypsin and washed twice with cold Hank's solution. Then, SKOV3/DDP cells were suspended in a binding buffer containing Annexin V-FITC and PI. The cell mixture was incubated at room temperature (RT) (in dark) for 15 min, and were then sorted by cell flow cytometry (Becton-Dickinson, San Jose, CA, USA).

**Western blotting.** Cell lysis buffer supplemented with protease inhibitor cocktail and 1 mM phenylmethanesulfonyl fluoride (PMSF) were used to prepare whole-cell lysates, and protein concentrations were measured (10). Then, samples were resolved by polyacrylamide electrophoresis, the polyvinylidene difluoride membranes were blocked with 5% non-fat milk, and then incubated with the appropriate primary antibodies for 3 h at RT, and then incubated with the appropriate HRP-conjugated secondary antibody for another 1 h.

**Mouse model of ovarian cancer.** To establish primary tumor xenografts, five million SKOV3/DDP cells were injected in the BALB/c-nu nude mice in a volume of 20 μl of PBS. When tumors reached 10 mm³, PBS (50 ml/kg/day, every day), DDP reduced the expression of adhesion-related protein integrin β1 (ITGβ1) and apoptosis-inhibiting proteins survivin, matrix metalloproteinase 2 (MMP-2) and MMP-9 (DDP + TPL group > TPL > DDP; P<0.05) (Fig. 2).

**Immunohistochemical staining.** Resected tumors were fixed in the 10% buffered formalin, and then embedded in paraffin and mounted on slides. Tumor sections were paraffinized and stained with hematoxylin and eosin (H&E), and then mounted on glass slides. The stained slides were then analyzed using a light microscope (Leica Microsystems, Wetzlar, Germany).

**ELISA.** The yields of IL-2 and TNF-α in mouse sera were evaluated using the IL-2 ELISA kit and TNF-α ELISA kit (both from eBioscience, San Diego, CA, USA).

**Statistical analysis.** The data are presented as mean ± SD, P<0.05 was considered statistically significant (23-25).

**Results**

**Effects of TPL and DDP + TPL on migration and invasion of SKOV3/DDP cells.** To the best of our knowledge, cellular invasion is an important part of cellular migration. Cells with high migration usually possess high invasion, while cells with high invasion may not possess high migration. So we simultaneously tested the migration and invasion of SKOV3/DDP cells. As shown in Fig. 1, the addition of DDP, TPL and DDP + TPL significantly reduced the cellular invasion and migration of SKOV3/DDP compared with the control group (NC) at 24 h (P<0.05), and the synergistic effect of TPL and DDP had significantly enhanced the inhibition effect on cellular invasion and migration compared with DDP and TPL group (P<0.05).

**TPL + DDP increases the apoptosis rate of SKOV3/DDP cells.** When treated with different concentrations of reagents, the apoptosis rates in the control, DDP, TPL and TPL + DDP group were (4.863±0.930), (8.333±0.965), (19.823±2.358) and (24.733±2.009)% respectively. For the single drug group, DDP and TPL greatly enhanced the apoptosis rate of SKOV3/DDP cells (P<0.05), and the TPL + DDP had the best promoting effect on apoptosis of SKOV3/DDP cells compared with DDP and TPL group (P<0.05) (Fig. 2).

**Effects of TPL and DDP + TPL on protein expression of SKOV3/DDP cells.** As tumor development is a complex process, so cancer-related proteins were tested. As shown in Fig. 3, the treatment of DDP, TPL and DDP + TPL significantly reduced the expression of adhesion-related protein integrin β1 (ITGβ1) and apoptosis-inhibiting proteins survivin, matrix metalloproteinase 2 (MMP-2) and MMP-9 (DDP + TPL group > TPL > DDP; P<0.05), and significantly increased the yields of apoptosis-promoting proteins cleaved caspase-3 and Smac (DDP + TPL group > TPL > DDP; P<0.05).

**Effects of TPL and DDP + TPL on IL-2 and TNF-α expression.** To further study the anticancer effects of TPL and DDP + TPL on tumors, the DDP, TPL and DDP + TPL were intraperitoneally injected or orally administered to mice, and their effects on related cytokines were evaluated. As shown in Fig. 4, the 4 mg/kg/day DDP, 0.15 mg/kg/day TPL,
4 mg/kg/day TPL and 0.15 mg/kg/day TPL greatly enhanced the inflammatory factors IL-2 and TNF-α in serum, and the DDP + TPL possessed the best enhancement effects compared to the other two groups.

**Effects of TPL and DDP + TPL on protein expression of cellular immunity and angiogenesis.** To find the synergistic mechanisms of TPL and DDP, proteins related to tumor immunity and angiogenesis were studied in control, DDP, TPL, and DDP + TPL groups. The results showed that the expression levels of proteins involved in cellular immunity and angiogenesis were significantly different among the groups. Compared with the blank group, *P<0.05; compared with TPL group, ∆P<0.05; compared with DDP group, ▲P<0.05.

Figure 1. Effects of DDP, TPL and DDP + TPL on (A) cancer cell invasion and (B) cancer cell migration. The DDP, TPL and DDP + TPL reduce the cellular invasion and migration of SKOV3/DDP cells, and the DDP + TPL possessed the best inhibition effect. Compared with blank group, *P<0.05; compared with TPL group, ∆P<0.05; compared with DDP group, ▲P<0.05.

Figure 2. Effect of DDP, TPL and DDP + TPL on apoptosis of SKOV3/DDP cells (x±s, n=3). Both the DDP group and TPL group can promote the apoptosis rate of SKOV3/DDP cells, and TPL + DDP had the best apoptosis rate of 24.733% among the groups. Compared with blank group, *P<0.05; compared with TPL group, ∆P<0.05; compared with DDP group, ▲P<0.05.
TPL + DDP groups. As shown in Fig. 5, the NK cell-related protein levels of CD16 and CD56 obviously increased in treatment groups, and the TPL + DDP had the best effect. Moreover, the addition of DDP, TPL and DDP + TPL greatly inhibited the production of vascular endothelial growth factor (VEGF) related proteins CD31 and CD105 (DDP + TPL group > TPL group > DDP group).

Discussion

The platinum-based chemotherapy combined with curative resection was widely used in various cancer treatments, while drug resistance to DDP has emerged as the major hinderence to this method (26), thus naturally-occurring, plant-derived compounds have become a research hotspot in cancer therapies, which have been proven to influence multiple signaling pathways and can enhance the activity of conventional chemotherapy and radiation therapy (27).

As one of the well-known phytochemicals, TPL has been investigated for its pleiotropic anticancer activities by inhibiting cancer cell proliferation and inducing apoptosis of various cancers (11,12,16,28-35), while few studies were carried out on the effect of TPL on the cellular immunity and angiogenesis of human epithelial ovarian cancer (EOC).
In the present study, we first evaluated the combination of DDP and TPL on cell invasion, migration and apoptosis of cisplatin-resistance cell SKOV3/DDP in vitro, and determined the combined effects of DDP and TPL. The expected value of combination effect between DDP and TPL was calculated as: (observed DDP value)/(control value) x (observed TPL value)/(control value) x (control value); and the combination index is calculated as the ratio of (expected value)/(observed DDP + TPL value), and the ratio of >1 indicated a synergistic effect (36). As the ratio of the combination of DDP + TPL was >1, thus, they presented a synergistic effect on the inhibition of human EOC. Our results indicated that the DDP + TPL group significantly inhibited the invasion and migration of SKOV3/DDP cells compared with DDP and TPL group, and the apoptosis rate in DDP + TPL group was as high as (24.733±2.009)%. As is known, caspase-3 belongs to the cysteine protease family, playing a key role in apoptotic pathways via cleaving a series of key cellular proteins, while survivin is a member of the inhibitor of apoptosis (IAP) family to inhibit caspase activation (37), and Smac is a member of promoter for caspase activation via binding to inhibitors of apoptosis-related proteins (38), therefore the negative regulation of survivin and positive regulation of Smac to caspase-3 lead to apoptosis (programmed cell death) of SKOV3/DDP cells.

In addition, the significant reduction of the membrane protein ITGβ1 (39) (a key protein in tumor invasion and metastasis, via mediating the adhesion of cells to the matrix and regulating the adhesion growth, migration, invasion and angiogenesis and chemotherapy resistance of many tumor cells) and MMP-2/MMP-9 (40) (whose altered expression and activity levels had been strongly implicated in the progression and metastasis of many forms of cancers) contributed to tumor apoptosis and tumor suppression. Fig. 4 shows the DDP + TPL greatly enhanced expression of IL-2 (which promotes the differentiation of T cells into effector T cells and into memory T cells to enhance the immunity of host) and TNF-α (whose primary role is to regulate immune cells, and is able to inhibit tumorigenesis), creating a inflammatory environment to promote the death of cancer cells (41,42).

In conclusion, our results indicated that both the TPL and DDP + TPL greatly enhanced cell apoptosis and tumor suppression via adjusting cellular immunity and angiogenesis of human EOC. Therefore, we proposed that TPL can lower the resistance of EOC to cisplatin, and can serve as a promising reagent for the treatment of human ovarian cancer.

Figure 5. Effects of DDP, TPL and DDP + TPL on the expression of CD16, CD56, CD31 and CD105 using immunohistochemical method (at 4°C). The combination of TPL and DDP can greatly increase the NK cell related proteins (CD16 and CD56) and can obviously reduce the vascular endothelial growth factor (VEGF) related proteins (CD31 and CD105) to inhibit tumor growth.
Acknowledgements

The present study was supported by grants from the National Natural Science Foundation of China (nos. 81760729, 81503364 and 31560264), and the Jiangxi Government (2016BBG20718, 2016A078, 20171BBC2B028 and 20175526).

References