Tetraarsenic oxide and cisplatin induce apoptotic synergism in cervical cancer

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Abstract. Tetraarsenic oxide (As_4O_6, TAO) is a new arsenic compound that inhibits cell growth and induces apoptosis in human cervical cancer cell lines. In the present study, we report that the growth of tumor cells (CaSki) was inhibited by treatment with TAO alone or in combination with cisplatin or paclitaxel *in vitro* and *in vivo*. Proliferation was assessed by WST-1 assay, and apoptosis was assessed by Annexin-V/PI FACS analysis in the CaSki cell line treated with a single agent or with the combinations of two agents. Expression of apoptosis-related proteins was analyzed by western blot analysis. A mouse xenograft model using CaSki cells was used to determine the *in vivo* activity of tetraarsenic oxide alone and in combination with cisplatin or paclitaxel by estimation of tumor size. At the end of the experiment, tumor tissue from each mouse was removed and processed for TUNEL analysis for confirmation of apoptotic cells. TAO was able to inhibit cell proliferation in a time- and dose-dependent manner. A combination of TAO and cisplatin effectively induced apoptosis by activating caspase-3. Using a mouse xenograft model, the sizes of tumors which were treated with a single agent and with a combination of agents decreased in a time-dependent manner. A combination of TAO and cisplatin resulted in a significantly reduced tumor size ($P<0.05$). The data for the histochemical staining of TUNEL-positive cells showed that the number of apoptotic cells was significantly increased by the combination of TAO and cisplatin. Thus, TAO is a good candidate for use in a combined regimen with cisplatin for patients with cervical cancer.

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Introduction

Cervical cancer is the third most common cancer among females worldwide. Approximately 530,232 new cases of cervical cancer were diagnosed and an estimated 275,008 deaths were reported in 2008 (1). Although the overall mortality of patients with cervical cancer has decreased over the past few years due to the widespread availability of effective screening programs, cervical cancer is a major cause of morbidity and mortality in women. Overall, the 5-year survival rate is 73%, yet the prognosis for advanced cervical cancer or recurrent cervical cancer still remains poor (2).

Most women with early stage cervical cancer (stage Ib-IIA) are treated with surgery, radiation or chemoradiation therapy. Since patients with locally metastatic or advanced lesions are at significant risk for recurrence, they require concurrent chemoradiation therapy (3). To eradicate micrometastases and sensitize radiation, concurrent chemotherapy has been added to pelvic radiation, with an apparent improvement in survival rates compared to radiation therapy alone (4-8). Patients with distant metastases are rarely curable, and most of the patients are treated either with chemotherapy or supportive care.

Cisplatin is the most active agent against cervical cancer, with a response rate of 17-21% (9). The most common non-platinum-based agent for cervical cancer is paclitaxel; its response rate is 17% (10). Single-agent chemotherapy plays a limited role in improving survival rates among patients with distant metastasis. Therefore, combination chemotherapy with existing agents is necessary to improve response rates and patient survival. Combination chemotherapy includes drugs that have demonstrated single agent activity (antitumor effects), different toxicity spectra, and synergistic activity with no increase in toxicity to improve response rates and survival of patients. A classic example of a combined regimen is cisplatin-based chemotherapy (11). Cisplatin-based combination chemotherapy with paclitaxel in stage IVb, recurrent, or persistent cervical cancer has a 36% response rate but does not improve the median survival when compared with the use of cisplatin alone (12). A recent phase III trial (GOG 204) evaluated the toxicity and efficacy of four cisplatin-based doublet combinations (ciaplatin/paclitaxel, cisplatin/vinorelbine, cisplatin/gemcitabine, and cisplatin/topotecan) among patients with advanced and recurrent cervical carcinoma (13).

The response rates for cisplatin/paclitaxel, cisplatin/vinorelbine, cisplatin/gemcitabine and cisplatin/topotecan were 29.1, 25.9, 22.3 and 23.4%, respectively. That study was closed early since cisplatin/vinorelbine, cisplatin/gemcitabine and cisplatin/topotecan did not exhibit superior efficacy to cisplatin/paclitaxel. Therefore, other regimens are needed to improve survival in patients with advanced and recurrent cervical cancer.

Arsenic compounds have been used to treat leukemia, particularly chronic myeloid leukemia and Hodgkin's lymphoma, since 1865 (14). Tetraarsenic oxide (As_4O_6; TAO) is a new arsenic compound. TAO is more effective at inhibiting human cervical cancer cell (SiHa cells) growth than arsenic trioxide (As_2O_3) (15). TAO may exert potential anticancer activity via vascular shutdown *in vivo* (16). TAO exhibits a synergistic effect with paclitaxel in gastric, cervix and head and neck cancer cell lines by inducing caspase-3 and poly(ADP-ribose) polymerase-dependent apoptosis (17).

The present study was conducted to investigate the anti-tumor effect of TAO compared with cisplatin and paclitaxel, which are conventional chemotherapy agents. The effects of a combination of TAO and conventional chemotherapeutic agents were evaluated and analyzed *in vivo* and *in vitro* using a cervical cancer cell line.

Materials and methods

Cell lines and chemical reagents. HPV 16, an immortalized human cervical carcinoma cell line, and CaSki cells (CRL-1550; American Type Culture Collection, Manassas, VA, USA), were cultured in RPMI-1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA), 100 U/ml penicillin and streptomycin (Gibco-BRL) at 37˚C in a humidified 5% CO_2-95% air incubator under standard conditions.

Paclitaxel (Genexol®, Samyang Co., Seoul, Korea) and cisplatin (Unistin®, Korea United Pharmaceutical, Seoul, Korea) were purchased and used, and TAO (Tetras®) was provided from Chunjisan (Seoul, Korea).

Cell viability. To evaluate the inhibition of tumor cell viability, a water-soluble tetrazolium salt (WST)-1 assay (EZ-CyTox Enhanced Cell Viability Assay kit; DaeiLab Service, Seoul, Korea) was used according to the manufacturer's instructions. Briefly, $5x10^3$ cells were treated with various concentrations of cisplatin (10-500 μM) and/or paclitaxel (0.001-10 μM) and/or TAO (0.5-25 μM) for 72 h, and the WST-1 solution was added. After 4 h, cell viability was measured at an absorbance of 480 nm using BIO-TEL™ (EL-800). The experiment was repeated three times.

Apoptosis. Apoptosis in the CaSki cell line was measured using the Annexin V-FITC Apoptosis Detection kit (BD Bioscience, San Jose, CA, USA). After a 24-h incubation in 6-well plates at a density of $2x10^5$ cells/well, the cells were treated with a single agent or with a combination of agents at the IC_{50} concentration for each agent. Cells were washed twice with cold PBS, resuspended in 100 μl binding buffer, and incubated with 3 μl Annexin V-FITC (BD Bioscience) and 10 μl propidium iodide (PI; BD Bioscience) at room temperature for 15 min. Fluorescent intensities were determined by flow cytometry (Becton-Dickinson, San Jose, CA, USA). The experiment was repeated three times.

Western blot analysis. CaSki cells were plated in a 6-cm dish at $1x10^6$ cells/dish and incubated for 24 h. After a 24-h incubation with the drugs, the CaSki cells were washed with PBS and lysed with a mammalian tissue lysis/extraction reagent including a protease inhibitor. After the protein was quantified with a BCA protein assay kit, the proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with anti-caspase-3, anti-cleaved caspase-3 and anti-α-tubulin at 4˚C overnight. Goat anti-rabbit or anti-mouse-conjugated alkaline phosphatase secondary antibodies were applied for 1 h at room temperature, and the membrane was developed using an AP-Conjugated Development kit (Bio-Rad Laboratories). The developed protein bands were quantified using the Multi Gauge V2.2 program.

Treatment of human cervical cancer xenografts with cisplatin, paclitaxel and TAO. BALB/c nu/nu female mice (age, 6 weeks; weight, 20-25 g) were obtained from Orient Bio Inc. (Seongnam, Korea). All animal procedures and care were performed under the guidelines approved by the Animal Ethics Committee of the College of Medicine at Inje University. CaSki cells ($2x10^6$) were injected subcutaneously into the backs of mice anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg).

After 24 days, the mice were randomized into the following 7 treatment groups with 6 mice in each group and treated for 35 days. Each agent was administered by intraperitoneal injection: i) control (0.9% sodium chloride injected once per week), ii) cisplatin (4 mg/kg body weight per injection, once per week), iii) paclitaxel (20 mg/kg body weight per injection, twice per week), iv) TAO (8 mg/kg body weight per injection, once per week), and v) cisplatin and paclitaxel and vi) TAO and cisplatin and vii) TAO and paclitaxel at the same doses and schedule.

Tumor sizes were assessed twice per week. Tumor size was calculated using the formula: Tumor size = length x width.

Histological examination. Tumor tissue was removed from each animal at 24, 48 and 72 h following the administration of each agent, and a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was carried out.

Assessment of cell death was carried out via the TUNEL method using an In Situ Cell Death Detection kit conjugated with horseradish peroxidase (Roche Applied Science, Indianapolis, IN, USA), according to the manufacturer's instructions. Five equal-sized tissue section fields were randomly chosen and analyzed under a Leica DMI microscope (Leica, Wetzlar, Germany).

Statistical analysis. Statistical analyses were performed using the MedCalc version 10.0 program (Frank Schoonjans, University of Gent, Belgium). The Mann-Whitney U-test, analysis of variance, and the Kruskal-Wallis tests were used. P-value <0.05 was considered to indicate a statistically significant result.

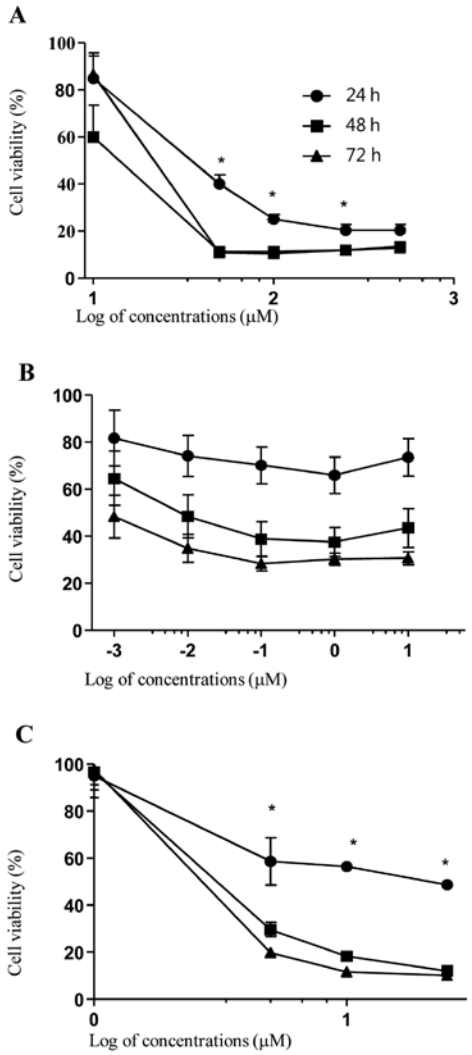

Figure 1. Time- and concentration-dependent inhibition of cell growth in the CaSki cell line by (A) cisplatin, (B) paclitaxel, and (C) tetraarsenic oxide (TAO). Cell viability is presented as a percentage of the control. Inhibition of cell growth in the CaSki cell line by each agent was time- and concentration-dependent (*P<0.05). Vertical bars indicate standard deviations.

Results

TAO induces time- and dose-dependent growth inhibition of CaSki cells. To investigate the effect of cisplatin, paclitaxel, and TAO on CaSki cells, proliferation of CaSki cells was assessed via WST assay using various concentrations of each agent applied over 24-72 h. Cisplatin decreased cell growth slowly at 10 μM but decreased cell growth rapidly at 50 μM (at 24, 48 and 72 h; P<0.001, P=0.001 and P<0.001, respectively). Paclitaxel also tended to decrease cell growth at 0.001 μM, but cell growth did not differ significantly from the control. TAO decreased cell growth at 1 μM and decreased cell growth rapidly at 5 μM (24, 48 and 72 h; P<0.001, P<0.001 and P<0.001, respectively). Each agent decreased cell growth over time. As shown in Fig. 1, time- and concentration-dependent inhibition of cell growth was observed upon incubation with each agent.

The IC_{50} values of cisplatin, paclitaxel, and TAO at 48 h were 29.74, 0.009 and 3.72 μM, respectively.

TAO induces apoptosis and interacts synergistically with cisplatin. To determine whether the inhibited cell growth was a result of apoptosis induced by each agent, an Annexin V/PI double staining-based flow cytometric analysis was performed in the CaSki cells after 12, 24 and 48 h of treatment using the IC_{50} concentrations of each agent alone and in combination. Early apoptotic cell populations were regarded as Annexin V(+)/PI(-) and late apoptotic cell populations were regarded as Annexin V(+)/PI(+) (Fig. 2).

Apoptosis was observed following 12 h of treatment with the IC_{50} concentrations of cisplatin, paclitaxel, and TAO, and early and late apoptosis was increased compared to that in the control group after 24 and 48 h (Fig. 2A). Cisplatin at 29.74 μM induced apoptosis in 20.3 and 36.8% of the cells after 24 and 48 h, respectively (Fig. 2A). Paclitaxel at 0.009 μM induced apoptosis in 19.8 and 30.0% of cells after 24 and 48 h, respectively (Fig. 2A). TAO at 3.72 μM induced apoptosis in 24.8 and 27.5% of cells after 24 and 48 h, respectively (Fig. 2A).

The combinations of cisplatin and paclitaxel, cisplatin and TAO, or paclitaxel and TAO induced apoptosis after 12 h of treatment, and apoptosis was increased to a greater extent in a time-dependent pattern compared to that in the control group after 24 and 48 h (Fig. 2B). The combination of cisplatin and paclitaxel increased the percentages of apoptotic cells to 33.7 and 37.8% after 24 and 48 h of treatment. The combination of paclitaxel and TAO increased the percentages of apoptotic cells to 22.8 and 37.0% after 24 and 48 h of treatment, respectively (Fig. 2B). The combination of cisplatin and TAO increased apoptotic cells to 34.2 and 60.3% after 24 and 48 h of treatment, respectively (Fig. 2B).

The combination of cisplatin and TAO significantly increased apoptosis in the CaSki cell line compared to that of TAO or cisplatin used alone after 24 and 48 h (TAO vs. cisplatin + TAO after 48 h, P=0.0286; cisplatin vs. cisplatin + TAO after 24 and 48 h, P=0.0286 and 0.0286, respectively). The combination of cisplatin and paclitaxel or paclitaxel and TAO tended to increase apoptosis but the difference was not significant when compared to the apoptosis rate with TAO or cisplatin used alone after 24 and 48 h. Apoptosis in CaSki cells treated with a combination of cisplatin and TAO after 48 h was increased to a greater exent when compared with that in the other two-agent combinations (cisplatin + paclitaxel or paclitaxel + TAO; P=0.0231). TAO exhibited more synergism together with cisplatin in inducing apoptosis in CaSki cells when compared with that in combination with paclitaxel (Fig. 2C).

Caspase-3 is highly activated by TAO and cisplatin. Western blot analysis was performed after treatment with each agent alone and in combination to evaluate the expression of apoptosis-related proteins (Fig. 3A). The cisplatin + TAO treatment resulted in prominent caspase-3 activation compared to that in the other treatment groups (Fig. 3B).

TAO in combination with other chemotherapeutic agents inhibits tumor growth in CaSki cell xenografts. Mice bearing CaSki cell tumors were treated with TAO, cisplatin and paclitaxel alone and in combination to test whether TAO synergizes with conventional chemotherapeutic agents (Fig. 5). Tumor size was decreased consistently in the paclitaxel-treated group. Tumor size was decreased significantly on days 32

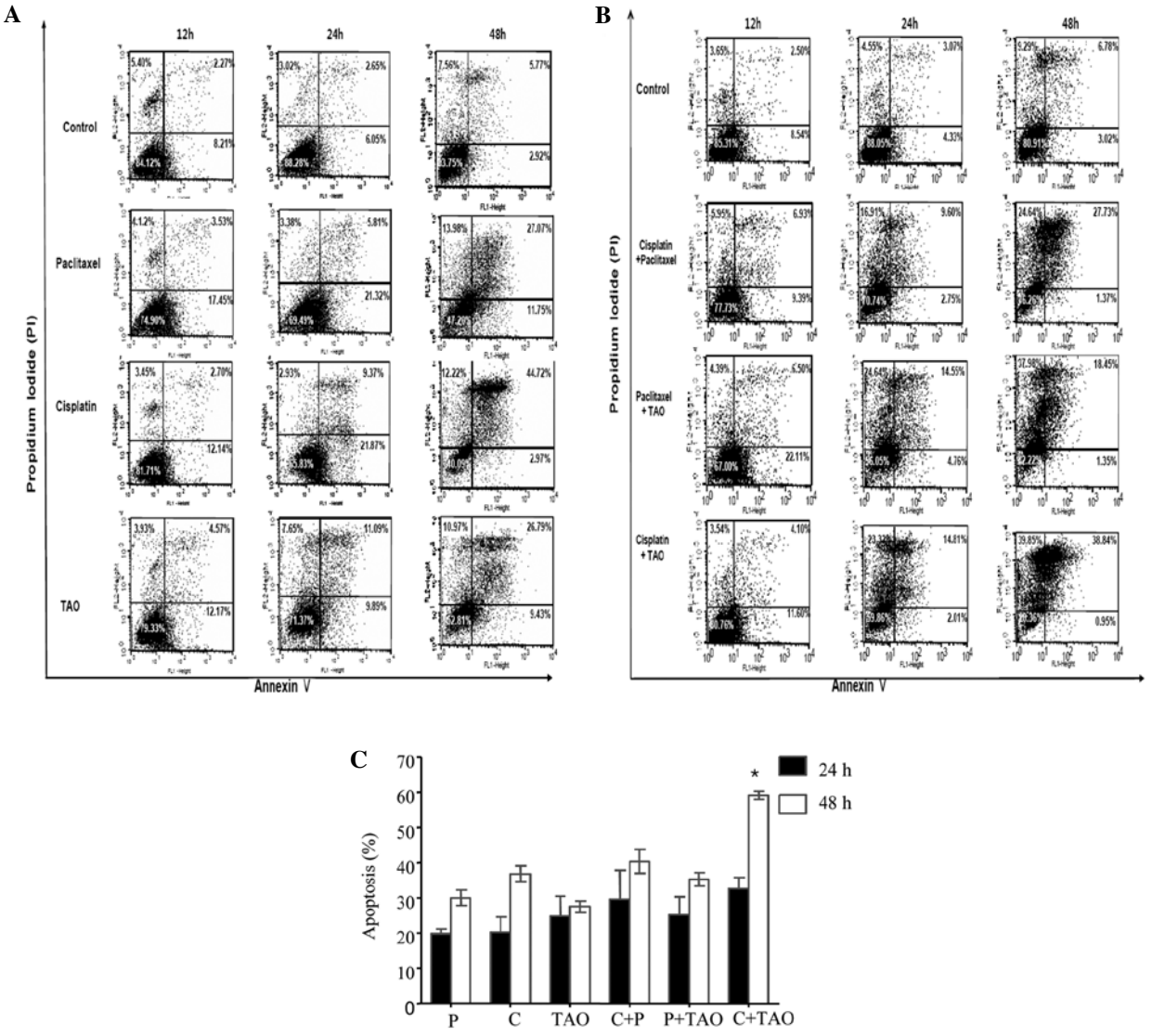

Figure 2. Induction of apoptosis in the CaSki cell line treated with each agent. CaSki cells were double-stained with Annexin V and propidium iodide (PI) followed by flow cytometry analysis. Data are presented as a percentage of the cell population. (A) Induction of apoptosis in the CaSki cell line treated with paclitaxel, cisplatin and tetraarsenic oxide (TAO). Induction of apoptosis in the CaSki cell line treated with each agent increased with time. (B) CaSki cell apoptosis in cells treated with a combination of cisplatin and tetraarsenic oxide (TAO) was increased more significantly when compared with that of the other two-agent combinations (cisplatin + paclitaxel, paclitaxel + TAO, cisplatin + TAO; $P<0.05$). (C) Time-dependent apoptosis was induced by each agent. All values represent the means and standard deviations (bars) of three samples in an independent experiment, which was repeated three times with similar results. TAO, tetraarsenic oxide; P, paclitaxel; C, cisplatin (*$P<0.05$).

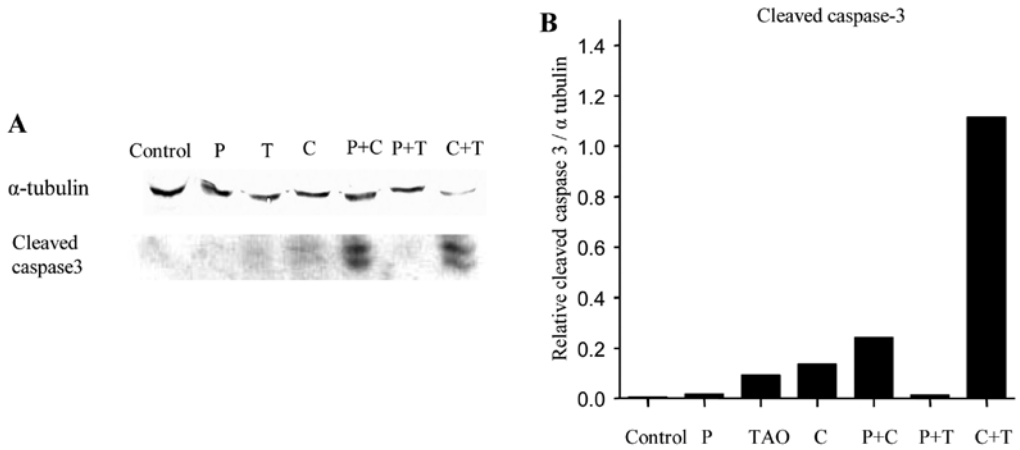

Figure 3. Western blot analysis of CaSki cells exposed to paclitaxel (0.05 μM), tetraarsenic oxide (TAO) (5 μM), cisplatin (30 μM), paclitaxel + cisplatin (P+C), paclitaxel + tetraarsenic oxide (P+T) and cisplatin + tetraarsenic oxide (C+T) for 24 h. (A) Active caspase-3 was detected by western blot analysis. Representative gel image. P, paclitaxel; C, cisplatin; T, tetraarsenic oxide (TAO). (B) Caspase-3 was highly activated by C+T.

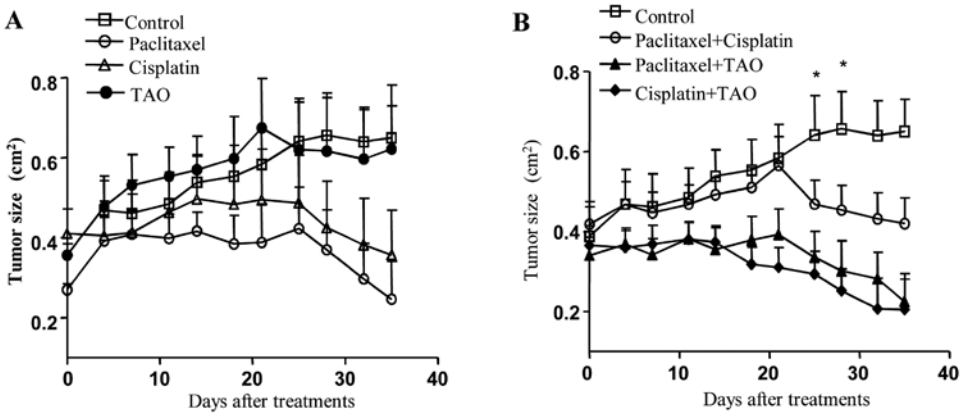

Figure 4. Comparison of tumor size after administration of a single agent and combinations of the agents. Chemotherapy was initiated after inoculation for 24 days. Each point represents the mean value of tumor size. Vertical bars indicate standard deviations. (A) Cisplatin and paclitaxel decreased tumor size to a greater extent when compared with that in the control but the difference was not significant among the three agents. (B) When compared with tumor sizes after administration of the combined agents among the three groups, tumor size in animals treated with the combination of tetraarsenic oxide and cisplatin was decreased significantly when compared to the tumor size in animals treated with a combination of cisplatin and paclitaxel on days 18 and 21 after the injection (P=0.038, 0.05, respectively) (*P<0.05).

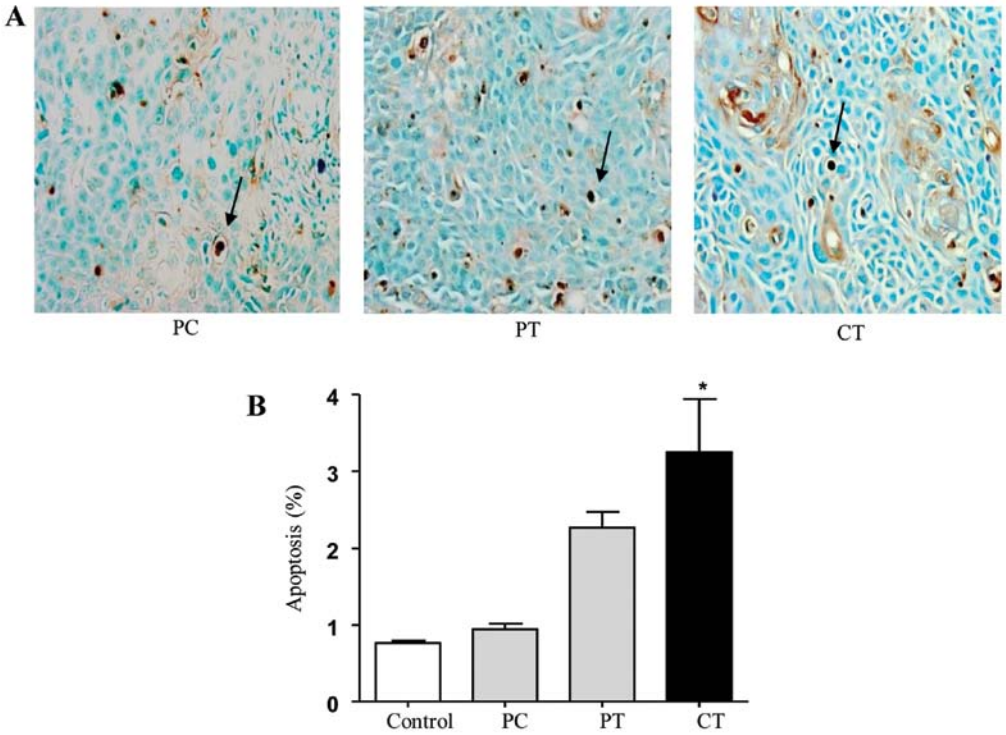

Figure 5. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Tumor samples were removed from mice and TUNEL staining was performed. (A) The nuclei of positive cells (arrows) were stained dark brown at 72 h after treatment (magnification, x400). The apoptosis index was calculated by dividing the number of apoptotic cells by the total number of cells in the field. (B) More TUNEL-positive apoptotic tumor cells were observed in the PT and CT groups compared with the PC group at 72 h after treatment. Vertical bars indicate standard deviations (*P<0.05 vs. paclitaxel + cisplatin). PC, paclitaxel + cisplatin; PT, paclitaxel + tetraarsenic oxide; CT, cisplatin + tetraarsenic oxide.

and 35 after administration of paclitaxel when compared with that in the control group (P=0.00152, 0.0087, respectively). While treatment with cisplatin alone significantly suppressed tumor growth on day 35 of treatment compared with that in the control group (P=0.0411), TAO alone did not inhibit tumor growth (Fig. 4A).

Although the paclitaxel and cisplatin groups exhibited significant differences in tumor size compared with the control group, no significant differences were observed among the paclitaxel, cisplatin and TAO treatments.

The tumor size was markedly decreased following treatment with the cisplatin and TAO combination (Fig. 4B). Tumor size was significantly decreased following treatment with the combination of paclitaxel and cisplatin, cisplatin and TAO, or paclitaxel and TAO when compared with that in the control group. Tumor size was decreased significantly in the paclitaxel

and cisplatin combination group on day 35 of treatment (P=0.0411). Tumor size also decreased significantly from days 21 to 35 after treatment in the TAO and cisplatin combination group (P<0.05). Tumor size was significantly decreased from days 14 to 35 of treatment in the TAO and paclitaxel combination group (P<0.05).

Tumor size in the group treated with a combination of TAO and cisplatin was significantly decreased when compared to that in the group treated with a combination of cisplatin and paclitaxel on days 18-21 of treatment (P=0.0380, 0.050, respectively).

TAO and cisplatin combination induces apoptosis in vivo. A TUNEL assay was conducted to determine whether the drug-induced inhibition of tumor growth was caused by apoptosis. Histochemical staining of TUNEL-positive cells (apoptotic cells) (Fig. 5A) revealed that the number of apoptotic cells increased significantly in the paclitaxel and TAO and cisplatin and TAO combination groups compared with that in the paclitaxel and cisplatin combination group, respectively (P=0.0037, 0.024 respectively). The combination of paclitaxel and TAO tended to increase apoptosis more than the combination of paclitaxel and cisplatin, but the difference was not significant in comparison to the apoptosis noted in the three groups (Fig. 5B). The TUNEL analysis in the three groups revealed a significant increase in apoptosis induced by the combination of cisplatin and TAO when compared with the combination of paclitaxel and cisplatin (P=0.022).

Discussion

The recurrence rate of cervical cancer is 10-20% for FIGO stages Ib-IIa and 50-70% for stages IIb-IVa (18). Recurrent and advanced cervical cancers are associated with high mortality rates and a lack of effective treatment options. To date, management of advanced cases of cervical cancer has included radiotherapy and chemotherapy (19). The efficacy of treatments in patients with recurrent or metastatic disease is palliative, and treatment for recurrent cervical cancer has not improved significantly despite the progress in modern chemotherapy.

Cisplatin binds to and causes cross-linking of DNA and induces DNA damage, which leads either to cell cycle arrest or immediate activation of apoptosis and killing of cancer cells (19). Cisplatin has been the standard cytotoxic agent for treating advanced cervical cancer (20) and has been combined with other chemotherapeutic agents, including 5-fluorouracil (21), bleomycin (22), ifosfamide (23), gemcitabine (24), vinorelbine (25), paclitaxel (26-28) and topotecan (29).

Zhang *et al* (30) reported a synergistic effect of arsenic trioxide in combination with cisplatin in human ovarian cancer cells. Arsenic compounds have been used as a treatment for various hematologic diseases, and arsenic trioxide induces apoptosis in numerous cancer cell lines *in vitro* (14). TAO has the same antitumor effects as arsenic trioxide for inhibiting cell growth, inhibiting angiogenesis, and inducing apoptosis in cancer cells by arresting cells in the G1 or G2/M phases of the cell cycle (31).

In the present study, the antitumor effect of TAO was investigated in the CaSki human cervical cancer cell line, and in nude mice bearing CaSki xenografts. The antitumor activity of TAO was then compared with cisplatin and paclitaxel and the interactions among these agents were tested.

Cisplatin, paclitaxel and TAO induced similar rates of apoptosis *in vitro*. Apoptosis in CaSki cells was also induced by the combinations of the agents; in particular, the combination of cisplatin and TAO after 48 h increased apoptosis more than that of the other two-agent combinations (cisplatin + paclitaxel and paclitaxel + TAO) (P=0.0231).

Western blot analysis revealed higher expression of apoptosis-related proteins in cells treated with a combination of TAO and cisplatin when compared with that in the other combinations.

TAO alone demonstrated less of an antitumor effect than cisplatin or paclitaxel, conventional chemotherapeutic agents. However, TAO had a stronger synergistic antitumor effect in combination with the other chemotherapeutic agents. When combined with cisplatin, the effect of TAO was enhanced to a greater extent than that with paclitaxel on days 18-21 *in vivo* (P=0.038, 0.050). The cytotoxic effect of TAO *in vivo* was similar to that reported *in vitro*. Although we did not investigate the mechanism of the synergistic interaction between TAO and cisplatin in this study, we hypothesize that TAO may improve the apoptotic effect of cisplatin. The TUNEL analysis confirmed that TAO combined with paclitaxel or cisplatin induced cell death, and that TAO enhanced apoptosis in combination with cisplatin more than in combination with paclitaxel *in vivo*. The mechanism of action of cisplatin is DNA damage, which induces apoptosis. Together, these results suggest that TAO enhanced the apoptotic signaling of cisplatin. Although the combination of TAO and paclitaxel induced tumor shrinkage, the effect was not as strong as that of TAO + cisplatin.

Cisplatin is considered the most effective agent for treating cervical cancer and has been placed among the most active drugs in combination with paclitaxel (32). Rowinsky *et al* (33) suggested a possible synergistic interaction between cisplatin and paclitaxel. We demonstrated that the antitumor effect of TAO and cisplatin was superior to that of the cisplatin and paclitaxel combination *in vitro* and *in vivo*. Furthermore, cell growth was inhibited more when TAO was combined with cisplatin than when it was combined with paclitaxel.

Various combination regimens have become a major strategy for overcoming drug resistance and improving response and cure rates (34-36), and these should be able to decrease the adverse effects of the drugs. Therefore, drug interactions may be fully recognized when TAO + cisplatin combination chemotherapy is administered to patients with advanced cervical cancer.

In conclusion, TAO and cisplatin may be a key regimen for cervical cancer chemotherapy. Therefore, a comprehensive examination of the interaction of TAO and cisplatin and the nature of the biochemical mechanisms of the synergistic effect between them is warranted. The combination of TAO and cisplatin merits further investigation.

Acknowledgements

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