Original Article

Artesunate induces apoptosis, inhibits cell proliferation, and decreases growth hormone levels in pituitary tumor

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Abstract: Artesunate (ART) is a kind of drug with an endoperoxide bridge to generate radicals for killing cancer cells and has been shown to act anti-tumor effect in different types of tumors. However, few studies have been reported in pituitary tumors. In this study, we aimed to investigate its effect and mechanisms of action in mouse pituitary adenoma AtT-20 cell line. The MTT assay was used to assess AtT-20 cell proliferation. Flow cytometry was used to analyze AtT-20 cell apoptosis and cell cycle. The expression of growth hormone (GH) protein andsecreted GH were measured by western blot and ELISA assay, respectively. MTT assay showed that ART inhibited AtT-20 cell proliferation in a dose-dependent manner with IC_{50} of (32.58 ± 3.21) μM. ART induced apoptosis and blocked AtT-20 at G2/M phase. The pan-caspase inhibitor V-ZAD-FMK partly attenuated the inhibitory effect of ART. Additionally, ART can inhibit GH synthesis and secretion by western blot and ELISA. Thus, we propose ART as a probably anti-tumor candidate drug in the treatment of pituitary adenoma.

Keywords: Pituitary tumor, growth hormone, artesunate, apoptosis, caspase

Introduction

Pituitary adenomas are abnormal growths that develop in pituitary gland - a small structure plays a major role in regulating metabolic, developmental, and reproductive functions. These tumors are very common, occurring in up to 20% of adults. Although most pituitary adenomas are harmless, some cause serious problems. Typically, a pituitary adenoma develops in the anterior pituitary, and its impact is determined by its size and whether it secretes hormones [1].

Artemisinin is a sesquiterpene lactone compound that is extracted from Artemisia annua L. and used as an antimalaria drug since 1970s [2]. Artesunate (ART) is one of its derivatives, apart from dihydroartemisinin and artemether, which has been proven to be effective in against cerebral malaria [3-5]. Besides of the antimalaria activity, researches indicate that ART is active against several cancer cell lines, such as breast, colon, lung pancreatic cancers, and leukaemias in vitro [6, 7]. Up to now, the most agreement in anticancer mechanism of ART is cell apoptosis inhibiting. It is reported that ART upregulated Wnt/β-catenin pathway in colorectal cancer [8], inhibited proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVEC), inhibited vascular endothelial growth factor (VEGF) binding to surface receptors on HUVEC and reduced expression of VEGF receptors Flt-1 and KDR/flk-1 on HUVECs [9-11]. ART reduces expression of the VEGF receptor KDR/flk-1 in tumor and endothelial cells and slows the growth of human ovarian cancer HO-8910 xenografts in nude mice [12, 13]. HUVEC apoptosis by artesunate is associated with downregulation of Bcl-2 (B-cell leukemia/lymphoma 2) and upregulation of BAX (Bcl-2-associated X protein) [14].

Although intense investigations have been conducted on the antiproliferative and chemotherapeutic effects of ART in several tumor cell lines, its effect on pituitary tumors is unknown. In this study, we examined the effect of ART on mouse pituitary tumor cells (AtT-20 cells) proliferation, apoptosis, and measured its effects on
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hormone levels. The related mechanisms were investigated and discussed in order to identify a promising drug for pituitary tumors.

Materials and methods

Cell lines and cell culture

The mouse pituitary tumor cells (AtT-20 cells) were obtained from the cell bank of Chinese academy of sciences and were cultured in RPMI-1640 (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The primary pituitary cells were purchased from Cell Bioscience Inc (Shanghai, China). The cells were maintained at 37°C in a fully humidified atmosphere of 5% CO₂.

Cell proliferation assay

The anti-proliferation assay of ART on the AtT-20 cells and primary pituitary cells was performed in 96-well plates. The cells were cultured at a density of 5.0 × 10³ cells per well in 100 μL of RPMI-1640 for 24 h, then started incubation with a series of concentrations of ART. Control (without ART) group was also set at the same time. After the cells were incubated for 48 h, 10 μL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, Oakville, ON, Canada) solution (5 mg/mL) was added to each well and incubated for 4 h. Formazan crystals were dissolved in DMSO (100 μL/well). The absorbance was measured at 490 nm with a Multiskan JX microplate reader (Thermo LabSystems, Cheshire, UK). The percentage of inhibited cell growth was calculated as follows: [(OD490control cells-OD490treated cells)/OD490control cells] × 100%. The half maximal inhibitory concentration (IC₅₀) of the drugs was determined as the drug concentration that resulted in 50% cell growth inhibition as compared with the growth of the control cells, following 72 h exposure to the drugs. Six replicate wells were used for each drug concentration and experiments were performed in triplicate.

Cell cycle analysis

Cells in logarithmic phase were plated at a density of 1.0 × 10⁵ cells in a 6-well plate and allowed to adhere to the well walls overnight. After incubation for 24 h, the cells were treated with ART or the procaspase inhibitor V-ZAD-FMK for 48 h at 37°C and 5% CO₂. Thereafter, the cells were collected, washed in PBS and fixed in 70% ice-old ethanol at 4°C. Before the analysis, the cells were treated with 50 μg/ml of propidium iodide (PI) and 200 μg/ml RNase A (Sigma-Aldrich) in the dark at 4°C for 30 min. The stained cells were analyzed using flow cytometry by using a MoFlo cell sorter (Dako Cytomation, Kyoto, Japan).

Cell apoptosis analysis

Cells in logarithmic phase were plated at a density of 1.0 × 10⁵ cells in a 6-well plate and incubated for 24 h prior to treatment with IC₅₀ values of ART for 48 h. Thereafter, the cells were collected and resuspended in 400 μL 1 × buffer. Then, 5 μL Annexin V-FITC was added and the cells were incubated in the dark at room temperature for 15 min, followed by an incubation with 10 μL PI at room temperature in the dark for 5 min. The stained cells were analyzed using a FACScan system equipped with Cell Quest software (BD Biosciences, San Jose, CA, USA) within 30 min. Untreated cells were used as controls.

Western blotting analysis

Cells were collected in 1.5 mL tubes and washed twice with PBS, and then 0.1 ml RIPA lysis buffer (Beyotime Institute of Biotechnology, China) containing 1 mM PMSF (Shanghai Sangon Biotech Co., Ltd, China) were added, then the tubes were placed into ice for 30 min. Supernatant was acquired by centrifugation at 13,000 rpm for 15 min at 4°C. Subsequently, protein concentration was detected by the BCA Protein Quantitative Assay (Shanghai Sangon Biotech Co., Ltd, China). The samples were then boiled with 4X SDS loading buffer for 5 min, and the proteins were separated by SDS-PAGE. After electrophoresis, the proteins were transferred to a PVDF membrane and blocked with 5% nonfat dry milk in TBS-T. The membrane was then incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies for 1 h at room temperature. The bands were visualized using an enhanced chemiluminescence detection kit.
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Biotech Co., Ltd, China). Total 20 μg protein sample was separated on 12% SDS-PAGE gel, and then transferred onto PVDF membranes, which were blocked in 5% nonfat milk for 1 h. The membranes were incubated overnight at 4°C with rabbit anti-mouse β-actin monoclonal antibody (1:1000, Santa Cruz, USA), rabbit anti-mouse GH polyclonal antibody (1:500, Santa Cruz, USA), rabbit anti-mouse cleaved caspase-3 (Asp175) polyclonal antibody (1:1000, Abcam, USA), and washed for three times with PBST, then incubated with Goat anti-rabbit IgG(H+L)-HRP (1:5000, Jackson ImmunoResearch, USA) for 2 h at room temperature, respectively. Ultimately, the proteins were detected with ECL (Millipore, USA).

Measurement of GH level by enzyme-linked immunosorbent assay (ELISA)

The concentrations of GH in AtT-20 cells and primary pituitary cells were determined by ELISA kits in accordance with the manufacturer’s instructions (GH: Millipore, MN, USA).

Statistical analysis

Statistical analysis was performed by SPSS 19.0 software (SPSS, USA). Data were expressed as the mean ± SD and analyzed by one-way analysis of variance. A value of P < 0.05 was considered as significant.

Results

Anti-proliferative effects of ART on AtT-20 cells

The anti-proliferative effects of ART on AtT-20 cells and pituitary cells were determined by MTT assay. The cells were treated with various concentrations (1.5625, 3.125, 6.25, 12.5, 25, 50 and 100 μM) of ART for 48 h. MTT analysis showed that ART treatments caused significant growth inhibition in AtT-20 cells compared to untreated cells. As expected, at 48 h, ART potently inhibited growth in a dose-dependent manner with IC_{50} value of 32.58 ± 3.21 μM. However, there was no significant difference among different dose groups in ART treated primary pituitary cells (Figure 1).

Effect of ART on cell cycle progression

The effects of ART on cell cycle distribution were further determined by flow cytometry. The AtT-20 cells in S phase were significantly reduced while those in G2/M phase were remarkably increased with the increasing concentration of ART (P < 0.05, Figure 2A). Besides, the cells in G1 phase ratio showed no significant difference between untreated cells and ART-treated cells. There was no significant difference between each groups in ART treated pituitary cells (P > 0.05, Figure 2B).

Effect of ART on cell apoptosis

In order to investigate whether ART inhibits proliferation of AtT-20 cells by apoptosis, an apoptosis assay was further performed after treatment with ART. Treatment with ART increased the percentage of cell apoptosis in a dose dependent manner in AtT-20 cells, while the percentage of apoptotic cells was not significantly changed among different dose groups in primary pituitary cells (Figure 3A). The apo-
sis in 25 μM ART treated cells was obviously inhibited after the addition of the pan-caspase inhibitor V-ZAD-FMK (Figure 3B).

Effect of ART on GH and caspase-3 protein

As shown in Figure 4A, the expression of inactive pro-caspase-3 was down-regulated in ART group, while up-regulated in ART adding pan-caspase inhibitor V-ZAD-FMK group. The expression levels of caspase-3 and its substrate, PARP (Poly ADP-Ribose Polymerase), were significantly up-regulated in 25 μM ART treated cells, while were decreased in ART plus V-ZAD-FMK treated cells. Further, the GH expression was detected in ART treated cells of various concentration. From Figure 4B, the expression of GH in AtT-20 cells was decreased in ART treated cells in a dose-dependent manner, while that in primary pituitary cells maintained in a stable level.

ART reduced GH secretion of AtT-20 cells

ELISA results showed that ART inhibited GH secretion in AtT-20 cells in a dose-dependent manner; the levels of inhibition changes (compared with control) were 93.5 ± 0.35%, 87.3 ± 0.27%, 83.9 ± 0.67%, and 68.7 ± 0.28%, respectively (Figure 5A). There was no significant difference of GH secretion in primary pituitary cells among different ART dose groups (Figure 5B).

Discussion

In the present study, we described the anticancer effects of ART on pituitary tumor cells of AtT-20. ART suppressed the growth of AtT-20 cells while promoted the apoptosis in a dose dependent manner. However, these effects were not detected in primary pituitary cells. Furthermore, the expression of inactive pro-cas-
pase-3 was down-regulated in ART group, while up-regulated in ART adding pan-caspase inhibitor V-ZAD-FMK group. The expression levels of caspase-3 and its substrate, PARP, were significantly upregulated in 25 μM ART treated cells, while were decreased in ART plus V-ZAD-FMK treated cells in western blot analysis.

ART was famous of its antimalarial effects from 1970s [15-17]. However, we knew little about the effect of ART on cancer cells up to date, especially in pituitary tumor cells. Our study is the first to investigate ART’s effect on AtT-20 cells and its action mechanism of cell proliferation, migration and apoptosis. In agreement with previous reports in colorectal cancer cells, we revealed that ART significantly blocked cell proliferation and migration, and increased the number of apoptosis cells [8].

Up to date, we knew little about the effect of ART on caspase pathway in AtT-20 cells.

The western blot analysis in this study indicated that the caspase-dependent pathway might participate in ART-induced apoptosis. Caspase activation was proved to execute the apoptosis by induction, amplification and transduction of the intracellular apoptotic signals [18, 19]. Caspase-dependent apoptosis is the most common cell death pathway that involves caspase-3, caspase-7, caspase-9 and caspase-12 pathways. During apoptosis, one of the caspase-3 substrates PARP-1 plays an important role in repairing DNA damage [20]. Once activated, it could mediate cell death in reactive ROS (oxygen species)-induced injury [21]. To our knowledge, there are few published data on the defense mechanism of caspase-3 inhibitor to apoptosis in human AtT-20 cells. Activated caspase-3, after DNA damage by oxidative stress, might either trigger the initiation of DNA repair, which resulted in the completion of the cell cycle, or stop the cell cycle to apoptosis. The activated caspase-3 then decomposes the critical substrate PARP-1 and finally leads to apoptosis [22, 23]. Therefore, the interaction of caspase-3 and its substrate PARP-1 are regarded as key executioners of apoptosis [24]. Thus, the caspase-dependent pathway is likely involved in ART induced AtT-20 apoptotic process.

Functional pituitary adenomas excessively secrete hormones, including prolactin, GH, adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). In western blot and ELISA analysis, we demonstrated the effect of ART on GH synthesis and secretion in AtT-20 cells and primary pituitary cells and found that ART could inhibit GH synthesis and secretion at the translational level. Many compounds, such as curcumin and pyridoxal phosphate, are reported to inhibit GH synthesis and (or) secretion by GH3 cells [25, 26]. We are the first to demonstrate that ART inhibited GH secretion in AtT-20 cells and that this inhibition might be attributed to ART-induced AtT-20 cell apoptosis.

In summary, our results present the molecular mechanism that ART inhibited cell proliferation and migration, and induced cell apoptosis in
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AtT-20 cells. ART reduced the pro-caspase-3 expression, might hereby enhance caspase-3 and PARP expression to block cell proliferation and migration, and to start up apoptosis program to promote cell apoptosis. Future studies are warranted to explore ART’s mechanism of its anti-tumor effect. It may have therapeutic potential to prevent or reduce the development of pituitary adenoma, a hypothesis that merits further investigation in experimental animals and humans.

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Disclosure of conflict of interest

None.

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