Original Article
Effect of apoptosis inducing factors on artesunate-induced astrocytic glioma apoptosis

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Abstract: Objective: To explore the effect of apoptosis inducing factors (AIFs) on artesunate (ART)-induced apoptosis in astrocytic glioma. Methods: CCK-8 Kit was used to detect the viability of U-87 MG cells treated with ART at different concentrations and different processing time. ART-induced cell death was detected by nuclear staining followed by flow cytometry. The effect of ART on mitochondrial membrane potential was determined by Rhodamine123 staining. AIF was then silenced by RNA interference, the effect of ART on cell viability was detected by CCK-8 kit and then its effect on the expression of AIF was measured by Western blot. An ectopic glioma model was established in nude mice and the *in vivo* anti-tumor effect of ART was investigated. Results: ART decreased the viability of cells to various degrees, which was concentration- and time-dependent. ART induced apoptosis and led to the decline of mitochondrial membrane potential. A dose of 40 ug/ml ART induced 58.5 ± 2.9% of apoptosis rate and 61.7% decline of mitochondrial membrane potential. After AIF was silenced, ART-induced cell viability was increased and ART-induced expression of AIF was decreased. *In vivo* anti-tumor experiment indicated that ART could inhibit the growth of glioma effectively. Conclusion: ART can induce mitochondria-mediated apoptosis in U87 cells and AIFs are involved in ART-induced apoptosis.

Keywords: Artesunate (ART), apoptosis inducing factors (AIFs), RNA interference, U-87 MG cells

Introduction

Human brain glioma is one of the malignant tumors which have greatly threatened human health [1]. Despite the development of medical technology, glioma is still hard to be cured. At present, its treatment is mainly based on chemotherapy, supplemented by radiotherapy. The major problem is the strong toxic and side effects produced by chemotherapeutics and radioactive rays. So it is of great clinical significance to find out a new way to treat human brain glioma [2-4]. Traditional Chinese medicine, as bio-regulators, is characterized by its long lasting effect, few side effects, and being easy to be accepted by patients in general. Thus, the application of traditional Chinese medicine may become a new approach for the treatment of tumors [5, 6].

Artesunate (ART), whose chemical name is dihydroartemisinin 1,2-α-succinate, is a derivative of antiperiodic artemisinin with the structure of sesquiterpenes [7, 8]. WHO actively advocates the usage of artemisinin and derivatives in treating serious malaria. In recent years, The anti-tumor effects has been proved in artemisinin and its derivatives [9, 10]. Zheng SQ found that artemisinin drugs had evident inhibitory effect on A549 and Hela cells [11]. Wu FH *et al.* found that ART could inhibit cell proliferation and induce apoptosis in gastric cancer cell line HGC27 [12]. ART could induce expression imbalance of various genes in cancer cells, such as drug resistance genes, DNA damage and repair genes [13, 14]. Reports also found that ART could induce mitochondrial mediated apoptosis containing ROS production and Bcl-2 expression down-regulation [15, 16].

In this study, we firstly investigated the effect of ART on the viability and apoptosis of glioma U-87 cells, and further experiment explored the effect of AIF on ART-induced cell death.

Materials and methods

Investigational drug

ART (CAS: 88495-63-0) was purchased from J&K Scientific (purity ≥ 98%).
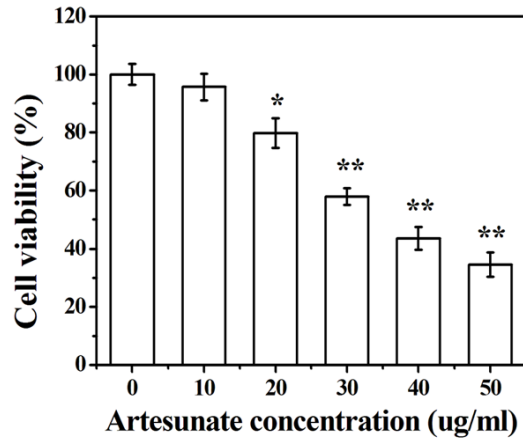
Figure 1. The effect of various concentrations of artesunate on cell viability.
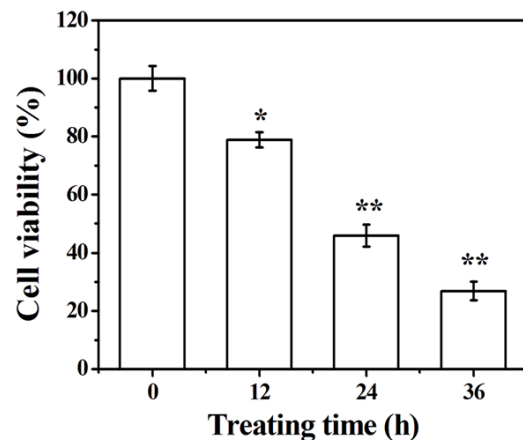

Figure 2. The effect of artesunate on cell viability for various treating time.

Cell line

Human brain astrocytic glioma U-87 MG cells were obtained from the cell resource center of Shanghai Institutes for Biological Sciences, Chinese Academy of Science.

Reagents

MEM medium and trypsin was purchased from Gibco (US); Qualified fetal calf serum from Zhejiang Tianhang Biological Technology Co., Ltd.; Transfection reagent Lipofectamine 2000 from Invitrogen (the US); Cell Counting Kit (CCK-8) from Dojindo; Staurosporine (STS), Rhodamine 123 and Hoechst 33258 from Sigma (the US); And FITC-Annexin V/PI Apoptosis Detection Kit from Beyotime Institute of Biotechnology.

Instruments

Microplate reader (infinite M200, Austria); Laser confocal microscope (LSM510/ConfoCor2, Germany); Flow cytometer (Arla BD, USA); Imaging system (Odyssey, USA); CO_2 incubator (Tempcontrol37-2 digital, Germany).

Methods

Cell culture: U-87 MG cells were cultured in a MEM medium. Major ingredients of MEM complete culture solution included 90% MEM, 10% qualified fetal calf serum and 1% penicillin-streptomycin solution. It was placed into an incubator with constant temperature and humidity and 5% CO_2 at 37°C. Cells were subcultured after trypsinization.

Cell viability assay: Cells during logarithmic phase were inoculated into a 96-well plate with 4000 cellsi/well. Cell adherence was achieved after 24 h, MEM complete medium containing ART at different concentrations was added. Four wells in parallel were used for each concentrations. After culturing for a specific period of time, fresh MEM culture solution with 10% CCK-8 was added in each well and the plate was incubated for 30 min. Then, the absorbance was detected by a microplate reader at 450 nm (OD_{450}), cell viability was in proportion to the value of OD_{450}.

Apoptosis assay: Cells during logarithmic phase were subcultured in a confocal culture dish. After 24 h, ART at different concentrations was added, the dish was then washed by PBS for three times and stained with Hochest 33258 (final concentration 1 µM) for 10-15 min. Cells was washed by PBS for three times. Finally, cell karyotype was observed under confocal fluorescence microscopy.

Cells during logarithmic phase were inoculated into a 6-well plate, ensuring about 1×10^6 cells were inoculated in each well. After cells were treated with ART at different concentrations, 5 µl FITC-Annexin V was added and the mixture was incubated in the dark for 15 min. Then, 5 µl PI was added and the resultant substance was incubated in the dark for another 10 min. flow cytometry was conducted, when cells were filtered with a 300 mesh sieve. A total of 10 000 cells in each specimen were collected.

Mitochondrial membrane potential assay: Rhodamine123 was a specific probe for mitochon-
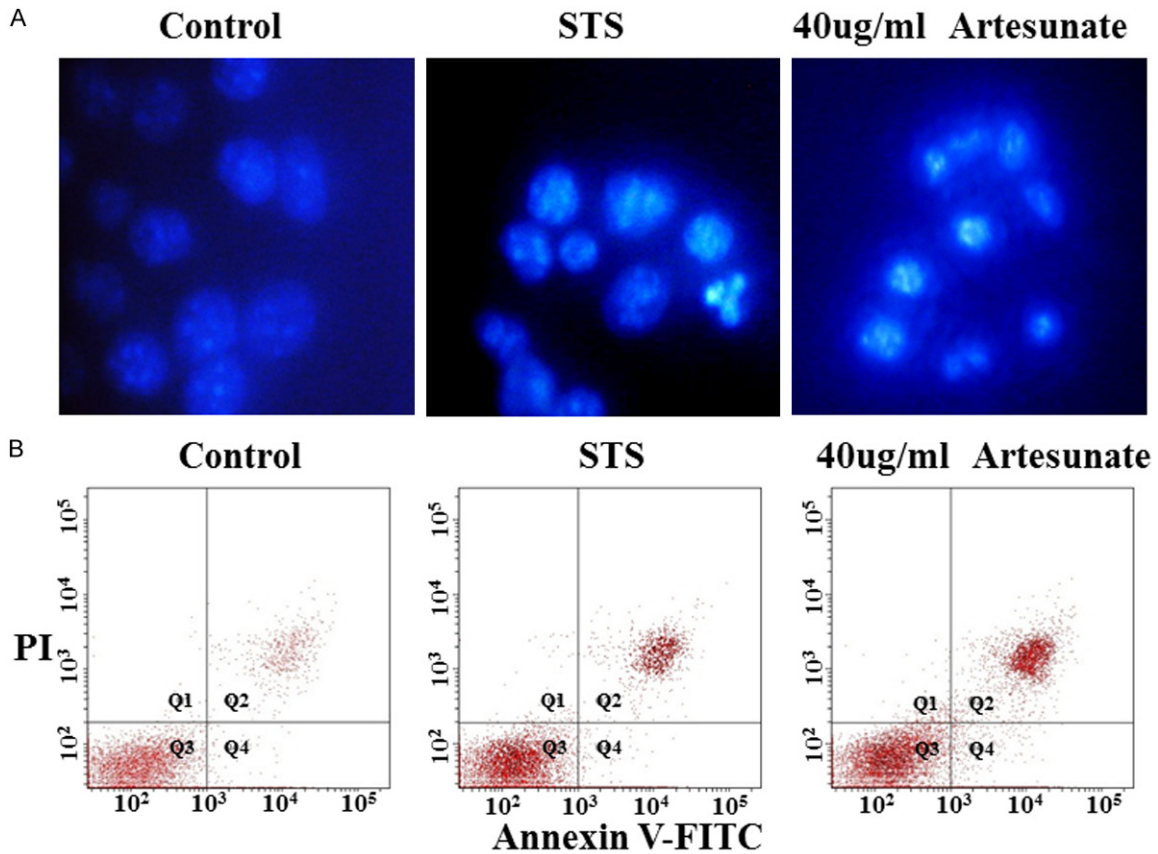
A

B

Figure 3. Artesunate induced cell apoptosis. A. The nucleus change induced by Artesunate; B. Cell apoptosis was detected by flow cytometry.

Table 1. The cell apoptosis rate induced by artesunate

	Cell apoptosis rate (%)		
	Control	STS	40 ug/ml Artesunate
24 h	3.4 ± 2.6	37.9 ± 3.1**	58.5 ± 2.9**

Note: **$P < 0.01$, compared with the control group.

dria. After cells were stained by Rhodamine123, the fluorescence of Rhodamine123 was detected by flow cytometry. It was performed according to the following steps: After being treated by drugs, cells were collected and stained in dark by 10 μM Rhodamine123 for 30 min. Cells were detected by a flow cytometer after being washed by PBS for three times. A total of 10 000 cells in each specimen were collected.

RNA interference

U-87 MG cells were cultured in a 24-well plate. When 60%-70% wells were filled with cells, 0.6 μg interference plasmid shAIF was transfected into cells by siRNA transfection. After transfection for 24 h, different concentrations of ART were added. After 24 h, cells were collected and the expression level of AIF was detected by Western blotting.

Effect of ART on cell apoptosis and mitochondrial membrane potential after AIF silencing

The U-87 MG cells at logarithmic growth period and the plasmid transfected cells were separately inoculated in 96-well plates and treated for 24 hours with ART, to detect the changes in cell apoptosis rate and mitochondrial membrane potential.

In vivo anti-tumor assay

U-87 MG cells during logarithmic phase were collected and 100 ul PBS cell suspension (10^7 cells/ml) was injected subcutaneously in the back of nude mice aging 4-5 weeks. When the
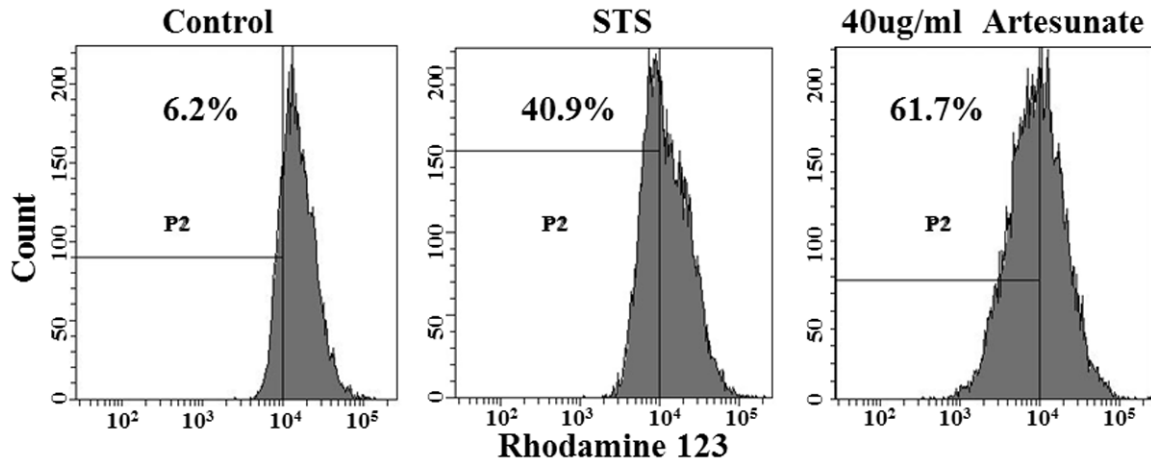
Figure 4. Artesunate induced decrease of cell mitochondrial membrane potential.

Table 2. The effect of artesunate on cell viability after AIF silencing

	Cell viability (%)			
	Control	Artestunate	shAIF	Artestunate + shAIF
24 h	100 ± 3.6	48.9 ± 2.8	98.9 ± 3.1	65.9 ± 3.9**,##

Note: **P < 0.01, compared with the control group; ##P < 0.01, compared with the artestunategroup.

Figure 5. The effect of artesunate on AIF activation after AIF silencing.

size of subcutaneous tumors reached up to 80 mm³, PBS, temozolomide solution and ART were injected through caudal vein. Then, tumor size was observed and measured every three days (tumor volume = length × width × width/2). The body weight of mice was weighed every three days from caudal vein injection until one treatment cycle (1 month) was completed.

Statistical analysis

Data in this study was expressed as mean ± SD and statistical analysis was conducted by an analysis software SPSS 13.0. Comparison between groups was performed by using indepen-dent sample *t-test*. $P \leq 0.05$ indicates significant difference.

Results

Cell viability assay

As shown in **Figure 1**, cells were treated by 10-50 ug/ml ART after 24 h, cell via-bility was decreased with drug concen-tration increasing. When the ART concentra-tion was higher than 20 ug/ml, the decreased proportion was significantly different from the control group ($P < 0.05$). As shown in **Figure 2**, the cell viability was decreased gradually over time ($P < 0.05$) when cells were treated with 40 ug/ml ART during 0-36 h. These findings indicated that ART had killing effect on U-87 MG cells in concentration dependent- and time dependent-manner.
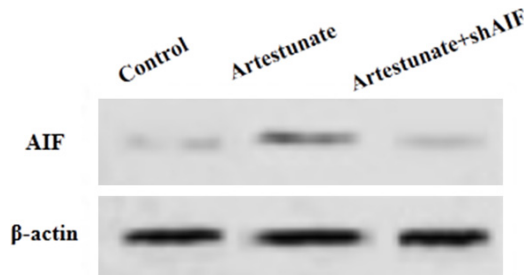
Apoptosis assay

Cells were treated with 40 ug/ml ART for 24 h. Cells treated with STS was used as a positive control. The results demonstrated that cell nu-cleus was shrunk and apoptotic bodies were detected. These results were similar to the find-ing in STS treated cells (**Figure 3A**). ART-induced cell death was primarily judged to be apoptosis. After cells were treated by the same drugs, ART-induced cell death was mainly distributed in the area of apoptosis, which was similar to the effect of STS (**Figure 3B**). Statistical results were shown in **Table 1**. ART (40 ug/ml) induced an apoptosis rate of 58.5 ± 2.9% ($P < 0.01$). These results further confirmed that ART could induce apoptosis.
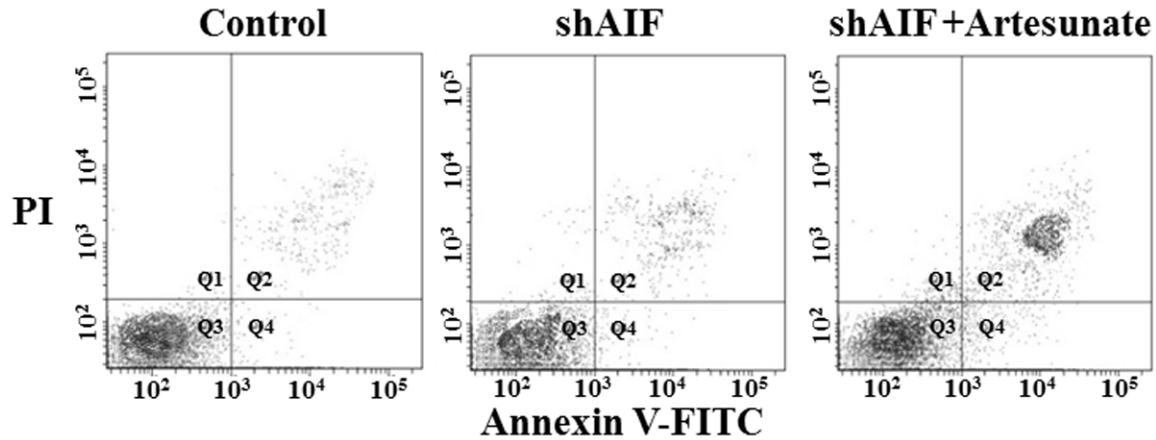
Figure 6. The effect of artesunate on cell apoptosis after AIF silencing.

Table 3. The effect of artesunate on cell apoptosis after AIF silencing

	Cell apoptosis (%)		
	Control	shAIF	Artesunate + shAIF
24 h	6.5	7.9	39.5**

Note: **$P < 0.01$, compared with the control group.

Table 4. The effect of artesunate on cell mitochondrial membrane potential was weakened afterAIF silencing

	Cell apoptosis (%)		
	Control	shAIF	Artesunate + shAIF
24 h	5.2	3.9	42.1**

Note: **$P < 0.01$, compared with the control group.

Mitochondrial membrane potential assay

Rhodamine123 was a specific probe for mitochondrial membrane potential. After drug-treated cells were stained by Rhodamine123, the fluorescence signal of Rhodamine123 was detected by flow cytometry. As shown in **Figure 4**, STS induced 40.9% membrane potential decrease, 40 ug/ml ART induced 61.7% membrane potential decrease. The results showed that mitochondria were involved in ART-induced apoptosis.

The effect of AIF on ART-induced apoptosis

As shown in **Table 2**, after AIF was silenced by RNA interference, ART-induced cell viability was 65.9 ± 3.9%, which was increased significantly compared with cells with unsilenced AIF ($P < 0.01$). Nevertheless, AIF silencing had no evident toxicity for cells. It showed that AIF was involved in ART-induced apoptosis. Results of western blot were shown in **Figure 5**. ART upregulated the expression of AIF evidently. ART-induced expression level of AIF was decreased evidently, when AIF was silenced. After AIF silencing, the cell apoptosis rate induced by ART was decreased significantly (**Figure 6** and **Table 3**), the mitochondrial membrane potential was increased significantly (**Table 4**), thus suggesting that AIF was involved in cell apoptosis induced by ART.

In vivo anti-tumor assay

Results of *in vivo* anti-tumor assay were shown in **Figure 7**. ART obviously inhibited tumor growth under treatment within one month. The body weight of mice showed no evident decrease. The results indicated that drug doses used in this study had no toxic or side effects in mice.

Discussion

Artesunate (ART) is a derivative of artemisinin. Researches showed that the anti-tumor mechanism is similar to the anti-malaria mechanism in artemisinin and its derivatives: The peroxide bridge of artemisinin and its derivatives can react with iron ionin cancer cells and form free radicals, which act on macromolecules and induce apoptosis [17, 18]. Wang Lijuan et al. found that ART could inhibit the proliferation of human endometrial cancer HEC-1B cells and induce apoptosis [19]. In this study, we found

A

B
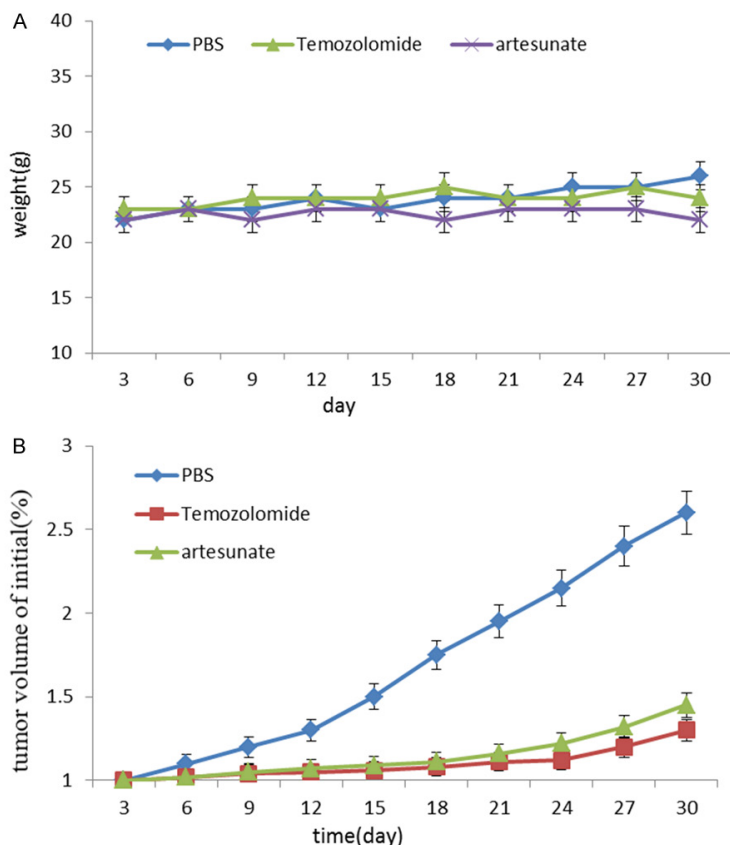
Figure 7. *In vivo* anti-tumor effect of artesunate in tumor-bearing mice.

In this study, U-87 MG cells cultured *in vitro* were treated by ART at different concentrations. Results showed that cell viability was decreased with drug concentration increasing suggesting that the inhibitory effect of ART on tumor cells was concentration- and time-dependent. Typical morphological changes of apoptosis were observed under a transmission electron microscope. Besides, through analyzing apoptotic cells by flow cytometry, ART was found to be able to promote apoptosis.

In conclusion, our results suggested that ART was effective in inducing apoptosis in U-87 MG cells, by morphological observation and flow cytometry analysis. Results of *in vivo* anti-tumor assay also indicated that ART had a good anti-tumor effect in glioma. This study can provide more accurate and strong evidence for the application of artesunate in the treatment of glioma.

that ART could decrease the viability of human brain glioma U-87 MG cells, which was concentration- and time-dependent. Nuclear staining and flow cytometry showed that ART induced cell apoptosis.

Apoptosis is a process of programmed cell death. It is of great importance in biological development and the maintaining of normal physiological activities. Up to now, researches have identified two classical pathways of apoptosis: death receptors pathway; mitochondrial mediated pathway [20, 21]. Therefore, the activation of apoptotic pathways has become a new hotspot in anti-cancer study. It was found that ART could decrease mitochondrial membrane potential. So it could be inferred that ART-induced apoptosis was endogenous, i.e. mitochondrial mediated apoptosis. ART could induce expression imbalance of various genes in cancer cells like drug resistance genes and DNA damage and repair genes. This study indicated that ART activated the expression of AIF and thus induced apoptosis.

Disclosure of conflict of interest

None.

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