

Research on the relationship between artesunate and Raji cell autophagy and apoptosis of Burkitt's lymphoma and its mechanism

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Abstract. – OBJECTIVE: To investigate the effects of Artesunate (ART) on the Raji cell proliferation, apoptosis and autophagy with Raji cells of Burkitt's lymphoma as the objects of study, and to investigate the anti-tumor mechanism of ART.

MATERIALS AND METHODS: Morphological examinations of apoptotic and autophagy were performed. MTT assay was used to detect the cell proliferation after ART treatment. The changes in cell apoptosis and cell cycle were detected by flow cytometer. The expression of Beclin-1 mRNA was detected by qRT-PCR. The expressions of Beclin-1, LC3-I/II and Caspase-3 were detected by Western blotting.

RESULTS: After Raji cells had been treated with ART, typical morphological changes of cell apoptosis were observed, and the proliferation activity of Raji cells was significantly inhibited in a time- and concentration-dependent manner. Compared with that in control group, the apoptotic rate in the treatment group was significantly increased and the expression of the apoptosis-related protein was significantly different. ART induced Raji cells to produce autophagosome, and the expressions of relevant autophagy proteins were significantly different, inducing the autophagy.

CONCLUSIONS: ART can significantly inhibit the proliferation of Raji cells of Burkitt's lymphoma and induce the apoptosis and autophagy excitation of Raji cells with the co-existence of autophagy and apoptosis.

Key Words:

Artesunate (ART), Raji cell, Apoptosis, Autophagy.

nal center and manifested as acute leukemia; it is composed of medium-sized B cells. The occurrence of Burkitt's lymphoma may be associated with malaria, EBV and HIV, etc., and it can invade the central nervous system and bone marrow easily with rapid clinical progression^{1,2}. Over the years, people have tried to understand the pathology of Burkitt's lymphoma and develop new and effective treatment methods, and the most important thing is to understand the mechanism of chemotherapy drug-induced lymphoma cell death^{3,4}.

Artesunate (ART) is one of the derivatives of artemisinin (a sesquiterpene lactone antimalarial drug), which has a high blood brain barrier (BBB) permeability, cerebrospinal fluid (CSF) accumulation and other characteristics⁵. It has been successfully used in the emergency treatment of cerebral malaria and falciparum malaria. Many studies have confirmed that ART can inhibit the cell proliferation of solid tumors, such as colon cancer⁶⁻⁸, and has a higher sensitivity to human erythroleukemia cell line K562^{9,10}. Drugs cannot reach and treat central nervous system (CNS) infiltration and CNS leukemia/lymphoma due to the BBB block, which has always been the difficulty of clinical treatment and is an important factor affecting the long-term survival of patients with lymphocytic leukemia/lymphoma¹¹. Whether the good BBB permeability and CSF accumulation of ART can play roles in the treatment of lymphocytic leukemia/lymphoma deserves the in-depth study.

Introduction

Burkitt's lymphoma, a kind of highly-invasive non-Hodgkin lymphoma (NHL), was reported and named by Donniss Burkitt for the first time in 1958, which is originated from the follicle germi-

Materials and Methods

Cell Morphological Changes DAPI Staining

Raji cells were treated for 24 hours and 48 hours, collected, and smeared. The cells were

then fixed with methanol for 10 minutes and stained with DAPI for 15 minutes. Thereafter, the cells were observed under fluorescence microscope (B×51 EpiFluorescence Microscope; Olympus, Tokyo, Japan).

Detection of Cell Viability via MTT Assay

Cell concentration was adjusted to 5×10^4 /mL and inoculated onto a 96-well plate, and ART in different concentrations (1.5625 μ g/mL, 3.125 μ g/mL, 6.25 μ g/mL and 12.5 μ g/mL) was added with 6 control wells for each group. The cells were incubated in the cell incubator for 24 h, 48 h and 72 h and 5 mg/mL methyl thiazolyl tetrazolium (MTT) solution was added into each well at 4h before the termination of incubation. After cells were mixed and incubated for 4 h, 10% SDS solution (100 μ L) was added to each well to terminate the incubation. Cells were placed in the incubator overnight, and the absorbance (A) value of each well at the wavelength of 570 nm was detected using full-automatic microplate reader. The cell growth inhibition rate was calculated according to the following formula and IC_{50} (50% inhibitory concentration) values of ART in different action time were calculated. Cell growth inhibition rate = (A value in the control group - A value in the experimental group)/A value in the control group $\times 100\%$.

Detection of Apoptosis via Annexin-V/PI Double-Labeled Staining

Raji cells in logarithmic phase in control group treated with 12.5 μ g/mL ART for 24 h and 48 h were taken and washed twice with phosphate buffered saline (PBS). The cells were resuspended in 500 μ L binding buffer, and 5 μ L FITC-labeled Annexin and 5 μ L PI were added and mixed, followed by staining for 30 min at room temperature in a dark place. Then, the cell apoptosis rate was measured by flow cytometer (FCM).

Observation of Autophagosome via Transmission Electron Microscope

Raji cells were treated for 24 hours and 48 hours, collected, and smeared. Ultrastructural changes were examined by transmission electron microscope.

Detection of Protein Content via Western Blotting

The protein expressions of Beclin-1, LC3-I/II, P62 and Caspase-3 were detected via Western Blotting. Raji cells in logarithmic phase in con-

trol group treated with 12.5 μ g/mL ART for 24 h and 48 h were taken and washed with PBS. The total protein was extracted with RIPA lysate, and the protein concentration was measured by bicinchoninic acid (BCA) kit. The extracted protein solution was mixed with 5 \times loading buffer in a volume ratio of 4:1 and boiled in boiling water for 5-8 min. According to the protein concentration measured, the total protein of each sample was adjusted to 50 μ g and added to the loading hole of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel for electrophoretic separation. Then, the protein on the gel was transferred to the polyvinylidene difluoride (PVDF) membrane using the electro-transfer instrument, and the protein was sealed with 5% skimmed milk powder at 4°C overnight or sealed at room temperature for 1 h. The confining liquid was sucked dry, and the rabbit-derived anti-Beclin-1 antibody, anti-LC3-I/II antibody, anti-Caspase-3 antibody and anti- β -actin antibody were added to incubate the protein at room temperature for 2 h or 4°C overnight and, then, the protein was washed with PBS-T for 3 \times 10 min. The secondary antibody was added to incubate the protein at room temperature for 2 h in a dark place and, then, the protein was washed with PBS-T for 3 \times 10 min and finally washed with PBS for 10 min, followed by scan via infrared fluorescent scanning system.

Detection of Beclin-1 mRNA Expression via Quantitative RT-PCR

Primer sequence Beclin-1: upstream primer: 5'-ACCTCAGCCGAAGACTGAAG-3', downstream primer: 5'-AACAGCGTTTGTAGTTCTGAC-3'. β -actin: upstream primer: 5'-TGCTCCTCCTGAGC-GCAAGTA-3', downstream primer: 5'-CCACA-TCTGCTGGAAGGTGGA-3'.

Raji cells in logarithmic phase in control group treated with 12.5 μ g/mL ART for 24 h and 48 h were taken and washed using PBS. 1mL TRIZOL reagent was added and mixed repeatedly. Then, 1/3 volume of iced chloroform was added and mixed. After 5 min at room temperature, it was centrifuged at high speed and low temperature for 15 min (4°C, 12000 rpm). The upper aqueous phase was transferred to another centrifuge tube, and not less than 2/3 volume of isopropyl alcohol was added, followed by precipitation at -20°C for 2 h and centrifugation for 15 min (4°C, 12000 rpm). After the supernatant was discarded, 1 mL 75% ethyl alcohol was added slowly along the tube wall and mixed, followed by centrifugation for 15 min (4°C, 12000 rpm). Then, the super-

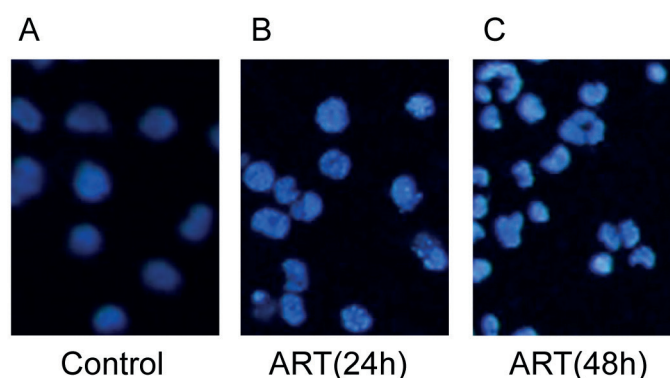


Figure 1. Morphological changes of Raji cells after ART treatment.

nantant was discarded and RNA enzyme-free ddH₂O was added to dissolve RNA, and RNA concentration and purity of each sample were detected. 1 μ L AM Reverse Transcriptase XL, 4 μ L 5 \times AMV Reverse Transcriptase Buffer, 0.5 μ L RNase Inhibitor, 1 μ L 10 mmol/L dNTP, 1 μ L random primer and extracted RNA were added into the reverse transcription tube, and the reaction system was made up to 20 μ L with RNase-free ddH₂O. Reaction conditions: 70°C for 30 min, 42°C for 60 min and 95°C for 5 min, and finally stored at 4°C.

Real-time fluorescence quantitative PCR: 1 μ L upstream primer and 1 μ L downstream primer, 12.5 μ L 2 \times SYBR Green Buffer, 2 μ L cDNA and 8.5 μ L ddH₂O were added into the amplification tube, respectively. Reaction conditions: 95°C for 5 sec, 60°C

for 30 sec, a total of 40 cycles. After the amplification, Rotor-Gene 6.0 software was used for analysis. The relative expression of target gene $F = 2^{-\Delta\Delta CT}$.

Statistical Analysis

All measurement data were expressed as mean \pm standard deviation ($\bar{x} \pm s$). SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for data processing and statistical analysis. Independent *t*-test was used to compare the measurement data between groups. Paired *t*-test was used to compare data within each group, and the χ^2 -test was used to test the attribute data. $p < 0.05$ was considered statistically significant.

Results

ART Induced Raji Cell Apoptosis

After Raji cells had been treated with 12.5 μ g/mL ART for 24 h and 48 h, DAPI staining showed the typical apoptotic morphological changes, and the change was more evident at 48 h (Figure 1). After Raji cells were treated with ART in different concentrations (1.5625 μ g/mL, 3.125 μ g/mL, 6.25 μ g/mL and 12.5 μ g/mL) for different time (24 h, 48 h and 72 h), it was found that ART could significantly inhibit the Raji cell viability in a time- and concentration-dependent manner (Figure 2). Results of flow cytometry also revealed remarkable apoptosis in Raji cells after treatment with 12.5 μ g/mL ART (Figure 3). At the same time, Western Blotting also showed that the expression level of apoptosis-specific protein Caspase-3 was significantly increased compared with that in control group (Figure 4). All the above results indicated that ART induces significant apoptosis in Raji cells.

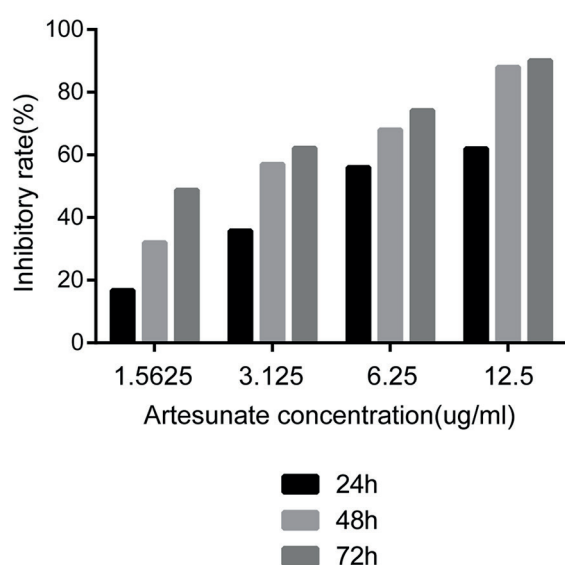


Figure 2. The anti-proliferative effects of ART on Raji cells.

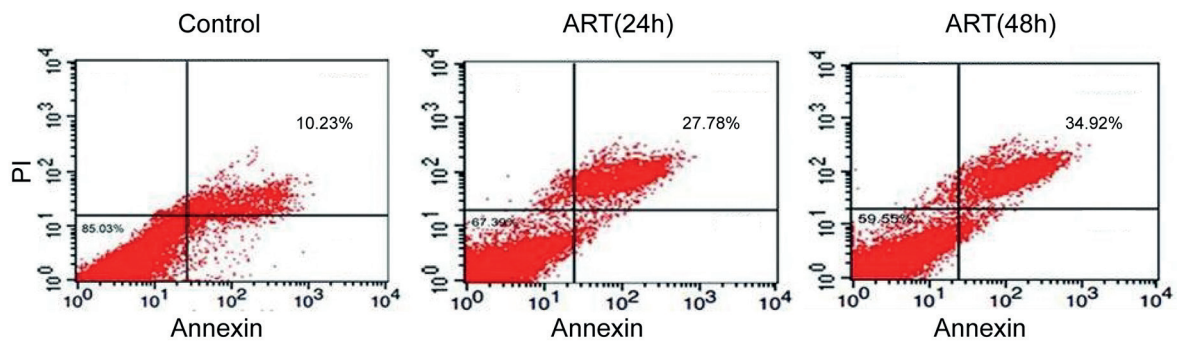


Figure 3. ART induced apoptosis in Raji cells.

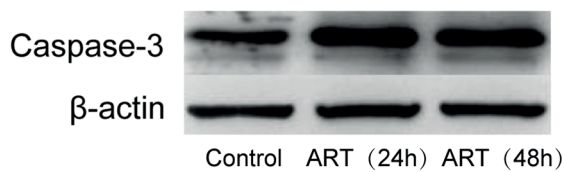


Figure 4. The enhanced expression of Caspase-3 in Raji cells induced by ART.

ART Induced Autophagy in Raji Cells

After Raji cells had been treated with 12.5 $\mu\text{g}/\text{mL}$ ART for 24 h and 48 h, autophagosomes appeared in cells, which was more obvious at 48 h (Figure 5). The protein expressions of autophagy-specific proteins Beclin-1 and LC3-I/II were significantly increased, and results of qPCR also showed that the relative expression quantity of Beclin-1 mRNA was also increased (Figure 6). These findings suggested that ART can induce autophagy in Raji cells.

Discussion

Since the 1940s, chemotherapy has been an important means of clinical treatment of malignant tumors¹², and a large number of studies have confirmed that the main mechanism of chemotherapy drugs of killing tumor cells is to induce cell apoptosis^{13,14}. In mammals, the mechanism of apoptosis can timely remove the aged, damaged, useless or malignant cells in normal cells, so as to limit the tissue elements within the scope of physiological needs, maintain the normal physiological functions of tissue and organs and the stable internal environment and prevent the occurrence of cancer¹⁵. It can be seen that apoptosis is the autonomic and ordered manner of death controlled by the gene, which is an active process¹⁶. Cell apoptosis is like the natural falling of flowers and trees, and people use the term “Apoptosis” to express this biological phenomenon, namely the cell apopto-

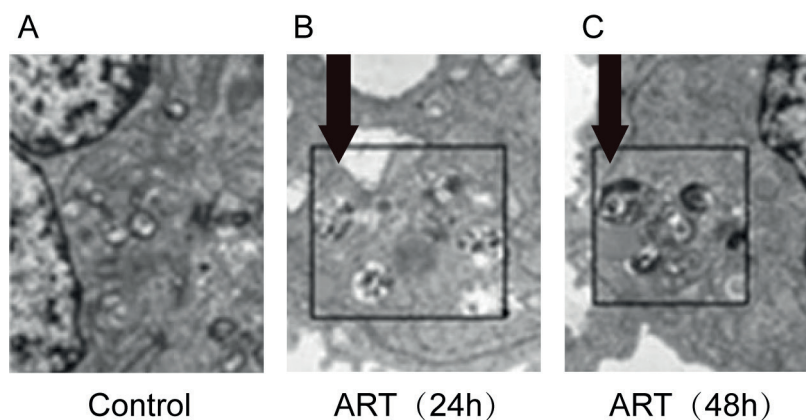


Figure 5. The autophagy activity in Raji cells induced by ART. Arrows indicate the autophagic vacuoles in the treatment groups.

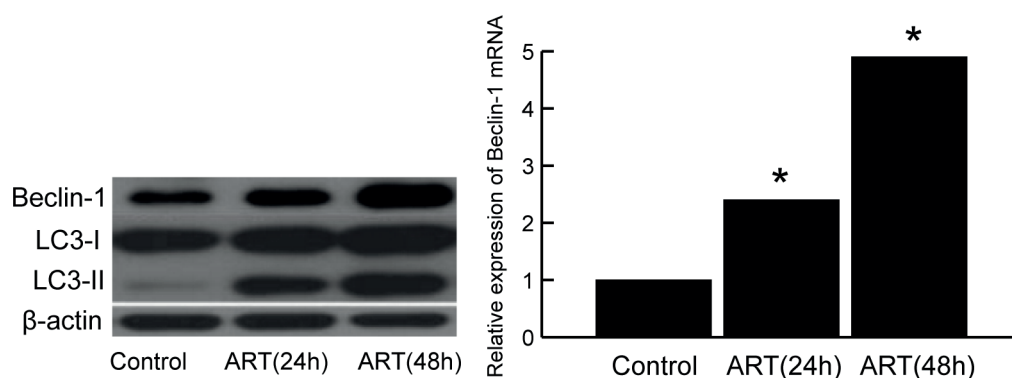


Figure 6. The increased transformation of LC3-I to LC3-II and the enhanced expression of Beclin-1 in ART induced Raji cells. (*Compared with control group, $p < 0.05$).

sis. For decades, it has been thought that apoptosis is the only mechanism of programmed cell death. However, recent studies have found that chemotherapy can not only cause apoptosis but also cause other non-apoptotic forms of death¹⁷, such as necrosis and autophagy. Autophagy, as a new way of cell death, has been widely concerned in recent years.

ART can inhibit the proliferation of solid tumor cells, but the mechanism of anti-tumor effect of ART remains unclear. Some scholars believe that it may be related to the iron ion-mediated cytotoxic effect, and some also believe that artemisinin-based drugs have the effect of inducing tumor cell apoptosis. In 1996, Efferth et al¹⁸ found that ART can induce the tumor cell apoptosis for the first time. Previous studies also reported in succession that artemisinin can induce the K562 cell apoptosis and HL-60 cell apoptosis^{19,20}, decrease the mitochondrial transmembrane potential and activate Caspase pathway. This work confirmed that ART, in addition to inducing Raji cell apoptosis, can also induce the cell autophagy activation, indicating that autophagy may also be one of the important mechanisms of ART of inhibiting tumor cell proliferation.

Conclusions

ART can significantly inhibit the proliferation of Raji cells of Burkitt's lymphoma and induce the apoptosis and autophagy excitation of Raji cells with the co-existence of autophagy and apoptosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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