ABSTRACT

Recent studies have indicated the profound anti-tumor activity of artemisinin's compounds, among which; arteether is an oil-soluble derivative of artemisinin with an endoperoxide bridge that can induce apoptosis in tumor cells but not in the normal cells.

An experiment was carried out on tumor-bearing Balb/c mice to estimate the effects of Arteether on tumor growth and antitumor immune responses. Briefly, 6mg/kg/day of Arteether and diluents were administered to two groups of mice. Tumor sizes were measured using digital vernier callipers. Mice were sacrificed and splenocytes were harvested for lymphocyte proliferation assay, the level of IL-4 and IFN-γ cytokines, and the percentage of splenic T regulatory cells were measured.

According to the findings, there were no statistical differences between the groups with respect to the level of IFN-γ, IL-4 and proliferation assay; while our results showed that Arteether is effective in the reduction of tumor growth rate.

In general, intra-tumoral injection of Arteether as an oil-soluble derivative of artemisinin brings to light some antitumor properties that may aid in development of more effective antitumor agents.

Keywords: Anti-tumor; Arteether; Breast Cancer

INTRODUCTION

Arteether (ARE) is a designation for ethyl-ether derivative of artemisinin (ART) which has been made of dihydroartemisinin "an active metabolite of ART".1

Artemisinin is originally isolated from Artemisia annua, sweet warmwood, and has a history of more than 2000 years in traditional medicine. In 1976 Chinese scientists examined several traditional medicinal herbs and observed the anti-malarial activity of artemisinin, but due to its molecular structure it has low solubility in water and oil, hence much research has focused on modifying its structure in order to enhance its solubility. Many structural
derivatives of artemisinin have been synthesized e.g. arteether.

In recent years, ART and its derivatives such as arteether, not only have been used as anti-malarial drug with low toxicity\(^2\) but also new researches have reported their profound anti-tumor activity \textit{in vitro} and \textit{in vivo}.\(^3\) The selective cytotoxicity of these derivatives is based on their structure. ARE and other derivatives contain an endoperoxide bridge that reacts with iron and produces free radical molecules. On the other hand, most cancer cells such as breast cancer cells, because of their rapid growth, have more iron intake and express a high concentration of transferrin receptor in comparison to normal cells; hence cancer cells are more susceptible to selective cytotoxic effects of ARE.\(^5\)

Although, many factors are involved in failure of tumor control, large bodies of experiments have demonstrated important role of regulatory T cells (Treg) in tumor immune-escape and their increased expansion involved in tumor progression.\(^7\)

In general, inhibition of Treg expansion and tumor metastasis play a critical role in the improvement of anti-tumor outcome.\(^8\) In this study, we investigated the antitumor and immunomodulatory properties of ARE via a loco-regional (intratumoral) injection “as a direct route” \textit{in vivo}.

**MATERIALS AND METHODS**

**Mice**

The groups of inbred female Balb/C mice at the age of 4-6 weeks were purchased from Pasteur Institute of Iran. Each group consisted of five mice which were housed in a standard poly-propylene cage (anti-acid, anti-base cages). The animals were kept under the standard conditions (a cycle of 12/12 h light/dark and a temperature of 20-22\(^\circ\)C) with free access to water and autoclaved standard mouse chow. Animal care and treatment were conducted in conformity with the guideline of Animal Care and Research Committee of Tarbiat Modares University, which is in compliance with the Guide for the Care and Use of Laboratory Animals [DHEW Publication No. (NIH) 85–23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205].

**Arteether**

Arteether powder was purchased from Exim-Pharm International Co., India. It was freshly prepared for each administration by dissolving in absolute ethanol (Merck Co.) and further dilution in PBS (40:60).

**Breast Cancer Model**

Spontaneous mouse mammary tumor (SMMT) is an invasive ductal carcinoma, which develops spontaneously in female Balb/C mouse after transplantation with tumor cells. SMMT is regarded to be similar to the invasive ductal carcinoma observed in human samples.\(^9\)

In our study, mice were subcutaneously transplanted with 0.5mm\(^3\) of tumor tissue that was separated from SMMT-bearing Balb/C mice. Tumors were authorized to reach approximately 500 mm\(^3\). 10 tumor-bearing mice were randomly divided into two groups, each consisting of five mice. The first group was treated with 0.1 mL of arteether at the dose of 6 mg/kg/day via intratumoral (IT) route. In our previous research, this dose had been defined by the DTH test as the optimal immunostimulatory dose of the drug (unpublished data). The other group (negative control) received the ARE diluents (ethanol/PBS) in the same volume and route. The treatments were applied for thirteen consecutive days. The tumor volumes were measured every other day using a digital vernier calliper (Mitutoyo, Japan). The volumes were calculated with the following formula.\(^10\)

\[ V = \frac{1}{2} (LW^2) \]


**Antigen Preparation**

A volume of approximately 3000 mm\(^3\) tumor tissue was extracted from the breast cancer-bearing Balb/C mice. The tumor suspension was prepared using five rounds of freezing/thawing and was passed through a 150 \(\mu\)m stainless steel mesh. The suspension was sonicated in a power of 4W for 30 seconds, followed by a 20-second pause for five times. To inactivate serine proteases, one mM of phenyl methyl sulfonyl fluoride (PMSF) was added to the cell lysate. The extract was filtered through a 0.22 \(\mu\)m filter and its concentration was determined using the Bradford method. Finally, it was stored at -20 \(^\circ\)C until use.

**Separation of Splenic Mononuclear Cells (MNC)**

The control and treated tumor-bearing animals were sacrificed by cervical dislocation on the 13\(^{th}\) day; spleens were removed under sterile conditions and were suspended in PBS. The splenic cell suspension was RBC-lysed with a solution of 0.75% NH\(_4\)Cl and Tris buffer (0.02%) (pH= 7.4). The cells were washed...
and the single-cell suspension was prepared in RPMI 1640 containing stable glutamine (Cytogen) and 10% heat inactivated fetal calf serum (Gibco, England). To define the viability and density of cells in the suspension, Trypan blue dye exclusion method was used. The cells were counted with a homocytometer light microscopy. The viability of splenocytes was generally above 95%. After an additional washing, the suspension was adjusted to 4 ×10^6 cells per milliliters in RPMI 1640 supplemented with 10% FCS, 100 µg/mL streptomycin, and 100 IU/mL penicillin (complete RPMI), and kept at 4°C.

**Lymphocyte Proliferation Assay**

Splenic MNCs were cultured in RPMI 1640 (Gibco) (adjusted to 2 × 10^6 cells/ml in RPMI supplement). Proliferation assay was done using Roche Cell Proliferation ELISA, BrdU kit. Briefly, 100 µl of cell suspensions was dispensed in 96-well-flat bottom micro plates (Nunc, Denmark). Six wells were considered for each sample. Twenty µg/mL of tumor antigen was added to three wells of each sample. Plates were incubated in 37°C (separate wells were cultured with untreated normal splenocytes and incubated with PHA as positive control). After 36 h, cells were labeled with 10 µl/well BrdU. Cells were reincubated for additional 36 h at 37°C. Labeling medium was removed using 10 min centrifuge (Eppendorf) at 300 g and blow dried. Cells were fixed using 200 µl FixDenat solution and incubated for 30 min at room temperature. Removing the FixDenat solution, 100 µl of anti-BrdU-POD was added and incubated for 90 min at room temperature. After the incubation period, microplates were washed three times with PBS buffer and 100 µl substrate solution was added and incubated for 30 min. Stop solution was added (25 µl of 1 M H2SO4) and absorbance of the samples was measured using ELISA reader at 450 nm. Results are presented as stimulation index (SI), using the following formula:

\[ SI = \frac{\text{OD of tumor Ag+splenocytes}}{\text{OD of tumor Ag-splenocytes}} \]

**Splenocyte Cytokine Production**

The isolated spleen MNCs were cultured in 24-well plates (Nunc, Denmark) in a final concentration of 2x10^6 cells/ml. Twenty µg/mL of purified tumor antigen was added to each, in order to stimulate the cells; after 72h incubation at 37°C and 5% CO2, the supernatants were collected and frozen at -70°C until the performing of enzyme-linked immunosorbent assay (ELISA). IFN-γ and IL-4 concentrations were measured using R&D DuoSet ELISA Development kit according to the manufacturers’ protocols. Each sample was analyzed in triplicates.

**Three-Color Immunostaining and Flow Cytometery Analysis**

The MNCs purified from the mice spleens were immunostained with the FITC anti-mouse CD4, PE anti-mouse CD25, and subsequently PE-Cy5 anti-mouse Foxp3, according to the eBioscience mouse regulatory T cell staining kit's instruction. The samples were analyzed by FACS Calibur flow cytometer (BD, USA and the results were analyzed with WinMDI software.

**Statistical Analyses**

In this study each experiment was performed in duplicate or triplicate and one-way analysis of variance (ANOVA) or Mann-Whitney non-parametric test was used to determine the statistical significance (p<0.05) between values of the test and control groups. The data were analyzed using SPSS software version 16 and the results are expressed as the mean ± standard error (mean ± SE).

**RESULTS**

**Effect of Intra-tumoral Injection of Arteether on Lymphocyte Proliferation Index**

In order to estimate the effect of intra-tumoral injection of ARE on lymphocyte proliferation index, the proliferation assay was applied. The data are mentioned as mean value of triplicates after stimulation with specific antigen, as shown in Figure 1. Our results indicated no significant differences between ARE-treated group and control (p>0.05).

**Effect of Intra-tumoral Injection of Arteether on the Splenocyte Cytokine Production**

In order to assess the effect of ARE on concentration of IFN-γ and IL-4 in treated and untreated mice, splenocyte culture of 10 tumor bearing mice was used and ELISA technique was applied. The result demonstrating ARE-treated group in comparison with control showed no significant differences (p>0.05). The results of IFN-γ and IL-4 concentrations are shown in Figure 2.
Effect of Intra-tumoral Injection of Arteether on the Splenic CD4+ CD25+ FoxP3+ T Cells

To define the percentage of splenic CD4+ CD25+ FoxP3+ T reg cells in tumor bearing mice, the flow cytometry technique was used (Figure 3). Although the results showed that the percentage of Treg cells in ARE-treated group is lower than control, this differences is not statically significant (p=0.10).

Effect of Intra-tumoral Injection of Arteether on Tumor Volume

In order to assess the effect of ARE on the tumor volume, 10 mice were included. ARE at a dose of 6mg/kg/day (based on DTH assay) was intratumorally injected for 13 consecutive days. Tumor volume changes during the experiment were shown in Figure 4. Our results indicated that there was a significant difference in the tumor volume between the ARE-treated and control groups, from day 6 of measurement (p=0.01). The results showed that intra-tumoral administration of ARE could decrease tumor growth in the treated group.
DISCUSSION

The anti-malarial activity of artemisinin's compounds such as arteether date back to 1976. Because of their novel cytotoxic mechanisms, these derivatives have been used as cancer therapies. Recent studies have reported the anti-tumor activity of artemisinin and its derivatives. In present investigation; we evaluated the cytotoxicity and immunomodulatory activity of intra-tumoral injection of arteether in vivo. Based on our findings, lymphocyte proliferation assay showed no significant difference in ARE-treated group in comparison with controls. We also reported a similar result about artemisinin and its derivative, suggesting that artemisinin's compounds had no effect on lymphocyte proliferation Index.

As a result intra-tumoral injection of ARE had no effect on the level of IFN-γ and IL-4 cytokines, as typical cytokines for Th1 and Th2 pattern, while previously we showed a significant increase on the level of IFN-γ in artemisinin-treated group; this result may be due to hydrophobic properties of ARE that reduce intra-cellular communication of ARE. Furthermore, ARE and other derivatives showed a dose-dependent manner to interact with immune cells. We have also noticed that arteether, a methyl ether derivative of artemisinin, had no effect on the pattern of cytokine production.

As a positive point, our findings showed that intra-tumoral injection of ARE could have a significant reduction in tumor growth rate. Based on this result and our previous studies, we noticed that the dose-dependent use of oil-soluble derivatives of artemisinin such as ARE was more effective in reducing tumor mass compared to artemisinin. We also demonstrated that however ARE could decrease the total percentage of splenic Treg cells in treated-group, but this difference was not statistically significant. This result could have been due to intra-tumoral injection of ARE, as a local route, that could improve rapid penetration of ARE into tumor cells, hence it could not directly interact with systemic immune cells e.g. splenocytes.

In this investigation, we demonstrated that the specific cytotoxicity of ARE toward tumor cells was distinct from its immunomodulatory properties; on the other hand, intra-tumoral injection of ARE, as a loco-regional administration, may yield higher drug concentration in tumor and may reduce the risk of systemic adverse effects.

REFERENCES