Original Article

Effects of artemether on the proliferation, apoptosis, and differentiation of keratinocytes: potential application for psoriasis treatment

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Abstract: Artemether exhibits diverse pharmacological effects and has multiple applications. This study aimed to investigate its antiproliferative and apoptogenic effects on HaCaT cells and keratinocyte differentiation-inducing activity in vivo. WST-8 analysis demonstrated that Artemether can inhibit the proliferation of cultured HaCaT cells in a time- and dose-dependent manner. Annexin V/PI dual staining and JC-1 staining further revealed that Artemether can dose-dependently augment HaCaT apoptosis. To investigate the keratinocyte differentiation-inducing activity of Artemether, it was prepared as topical creams at concentrations of 1%, 3%, and 5%. During the 4 weeks of topical treatment, no evidence of irritation was observed in the mouse tail test. Artemether cream dose-dependently increased the degree of orthokeratosis and the relative epidermal thickness of mouse tail skin, indicative of the keratinocyte differentiation-inducing activity. Taking the in vitro and in vivo findings together, the present study suggests that Artemether may be a promising antipsoriatic agent worthy of further investigation.

Keywords: Artemether, psoriasis, antipsoriatic, keratinocyte, HaCaT cells, proliferation, apoptosis, differentiation, mouse tail test

Introduction

Psoriasis is a chronic, remitting, and immune-mediated inflammatory skin disease with a worldwide incidence of approximately 2% to 3% [1]. Aside from the infiltration of immune cells, expression of inflammatory cytokines, hyperproliferation and altered differentiation of epidermal keratinocytes, and subepidermal angiogenesis are also observed in psoriatic lesions [2]. Patients seldom die from psoriasis, but the itchy skin, slightly raised erythema, silvery white scales, and comorbidities, such as cardiovascular disease and metabolic syndrome, seriously impair their quality of life [3, 4]. Currently, drug treatment remains the main option for most psoriasis patients. However, the long-term therapeutic efficacy of existing drugs is not ideal because of side effects and the development of pharmacoresistance [5]. Therefore, identification of new effective antipsoriatic agents with less adverse effects remains a hotspot in dermatology to date.

Artemisia annua L., also known as Qinghao in Chinese, has been used in China for more than two thousand years. Artemether (ART) is a semi-synthetic derivative of artemisinin isolated from A. annua. Artemisinin and its derivatives are known for their antimalarial effects, but they can also exert antiproliferative, apoptotic, anti-angiogenic, anti-inflammatory, and immunomodulatory activities [6]. In particular, they can inhibit the activation and proliferation of T cells [7], and they can reduce the production of tumor necrosis factor-alpha [8], a key cytokine that is substantially increased in psoriatic lesions [9].

Considering the pharmacological effects of artemisinin and its derivatives, we speculated that these substances can block the pathological changes brought about by psoriasis in various aspects. In this study, we determined whether or not artemisinin and its derivatives exhibit antipsoriatic activity in an experimental model. This study is the first to evaluate the in
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vitro and in vivo pharmacodynamic effects of ART on the proliferation, apoptosis, and differentiation of keratinocytes.

Materials and methods

Materials for in vitro experiment

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), 0.25% trypsin-EDTA (1×), phosphate-buffered saline (PBS, pH 7.4), and a Dead Cell Apoptosis Kit with Annexin V Alexa Fluor® 488 and propidium iodide (PI) for flow cytometry were purchased from Invitrogen (Carlsbad, USA). A WST-8 assay Kit was purchased from Dojindo Molecular Technologies, Inc. (Tokyo, Japan). ART (purity ≥ 99.3%) was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China) and was dissolved in dimethylsulfoxide (final concentration < 0.25% v/v).

General cell culture

HaCaT cells, an immortalized line of human epidermal keratinocytes extensively used in psoriasis research [10], were obtained from the Experimental Research Center of Changhai Hospital (Shanghai, China). Cells were routinely maintained in DMEM culture medium with 10% FBS, 100 U/mL penicillin, and 100 μg/mL of streptomycin, and then maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Apoptogenic effects of ART on HaCaT cells

HaCaT cells were cultured in six-well plates at a density of 5 x 10⁶ cells/well in 2 mL of DMEM culture medium. After 24 h of culture, the unattached cells were removed by washing twice with PBS. Subsequently, they were incubated with or without ART (0, 14.5, 29, 58, and 116 μg/mL) for an additional 48 h. At the end of the treatment, both floating and adherent cells were harvested, pooled together, and then washed twice with PBS. Subsequently, cell apoptosis was assayed by Annexin V/PI dual staining and JC-1 (5,5”,6,6”-tetrachloro-1,1”, 3,3”-tetrathylbenzimidazolycarbocyanineiodide). The results of these two assays were sufficient to establish the occurrence of ART-induced apoptosis.

Annexin V/PI dual staining. Approximately 1 x 10⁶ cells were collected, suspended in 100 μL of binding buffer, and then stained with 5 μL of Annexin V staining solution and 1 μL of PI working solution (100 μg/mL). After incubation at room temperature for 15 min, an additional 400 μL of binding buffer was introduced into the cell suspension and gently mixed. The cells were then analyzed on a flow cytometer (FACSCalibur, BD), measuring the fluorescence emission at 530 nm and 575 nm using 488 nm excitation.

JC-1 staining. Approximately 1 x 10⁶ cells were incubated with 200 μL of diluted JC-1 (2 μM final concentration) for 20 min at 37°C in the dark and then washed with warm PBS. The cells were pelleted by centrifugation, resuspended in 500 μL of PBS, and then measured with a flow cytometer (FACSCalibur, BD). Green fluorescence (480 nm to 530 nm) was measured in the FL-1 channel, and red fluorescence (580 nm to 630 nm) was measured in the FL-2 channel. The mitochondrial membrane potential (MMP) in HaCaT cells was represented by their red/green fluorescence intensity ratio. Meanwhile, their fluorescence images were observed and obtained under ultraviolet illumination with a BX51 fluorescence microscope (Olympus, Japan).

Preparation of ART cream

All experimental reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. Hyaluronic acid was added to an adequate vol-
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ume of water and heated at 85°C until complete dissolution. Ultrez 10 was soaked in the above solution to achieve complete swelling, and the mixture changed into a transparent non-mass gel after adding triethanolamine. Glycerol, propylene glycol, and tween 80 were gradually added and mixed well. The gel was weighed to calculate water loss. Sodium sulfite was dissolved by water with the volume equivalent to the water loss and slowly added to the gel. After complete mixing, the blank hydrogel was obtained, and its aqueous phase was prepared by heating to 85°C. Monoglyceride, hexadecanol, gerol GTCC, silicone oil, vitamin E, solid paraffin, albolene, nipagin, and laurocapram were heated in 85°C water and melted into the oil phase. The two phases were completely mixed, and then the vehicle cream matrix was prepared. An adequate amount of ART was added to dimethylsulfoxide and heated in an 85°C water bath to dissolve. The mixture was added to the vehicle cream matrix and weighed to replenish the lost water. After stirring, the ART cream was prepared. The prepared vehicle cream and ART cream (1%, 3%, and 5%) were stored separately at 4°C.

All these formulated topical preparations, together with the negative control of normal saline solution and positive control of 0.1% tazarotene cream (Huapont Pharm, Chongqing, China. Lot 2011005), were later evaluated for their keratinocyte differentiation-modulating activities in mouse tail test.

Animals

Male ICR mice weighing 22 g to 25 g were purchased from the Shanghai Lab Animal Research Center. All mice were kept in a specific pathogen-free animal room and had free access to typical mouse food and water. An artificial light cycle (12 h light and 12 h dark) and controlled temperature (23 ± 2°C) were maintained. The mice were allowed to acclimatize for 1 week prior to the experiment. Animal care and treatment were conducted in accordance with the guidelines of the Animal Care and Use Ethical Committee of Zhongshan Hospital, Fudan University.

Experimental protocol of mouse tail test

Sixty mice were divided into six groups composed of 10 animals each. The animals received either normal saline solution (negative control) or 0.1% tazarotene cream (positive control). The remaining four groups were treated with the test samples, namely, vehicle cream, 1% ART cream, 3% ART cream, and 5% ART cream.

In a typical procedure, approximately 0.1 g of topical preparation was thinly coated on the dorsal surface of the mouse tail. To prevent the topical agents from rapidly rubbing off, the mice were individually housed for 2 h and then returned to their animal cages. The treatment was administered twice a day (8:00 and 16:00) for four consecutive weeks. Sixteen hours after the last treatment, the mice were sacrificed by cervical dislocation. Subsequently, the dorsal surface of the mice tails, measuring approximately 2 cm in length starting 1 cm from the anus, were removed from the underlying cartilage and stained with hematoxylin-eosin.

Histological measurement of mouse tail skin

The histological sections were inspected under a BX60 light microscope (Olympus, Japan) and analyzed as described by Bosman et al [12]:

(A) The horizontal length of the fully developed granular layer within an individual scale lying the turning point of the beginning and ending of two adjacent hair follicles (n = 10 scales per animal, n = 10 animals per group; a total of 100 measurements per group).

(B) The whole horizontal length of an individual scale lying the turning point of the beginning and ending of two adjacent hair follicles (n = 10 scales per animal, n = 10 animals per group; a total of 100 measurements per group).

(C) The vertical epidermal thickness between the dermoepidermal borderline and the beginning of the horny layer (n = 5 measurements per scale, n = 10 scales per animal, n = 10 animals per group; a total of 500 measurements per group).

Evaluation of antipsoriatic activity

With the raw data from (A)-(C) mentioned above, the antipsoriatic activities of topical preparations were further evaluated as described by Bosman et al [12]:

(1) The degree of orthokeratosis (OK) per scale was calculated as OK (%) = (A/B) × 100.
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(2) The drug activity was calculated as Drug activity = (OK of the treated group - mean OK of the negative control group)/(100 - mean OK of the control group) \times 100.

(3) Out of the raw data of (C), the mean epidermal thickness of the negative control was set to 100% and served as the baseline data. The relative epidermal thickness of the other treated animals was defined as the percentage ratio of their mean epidermal thickness to that of the baseline data.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical significance was determined with ANOVA, and subsequent significant difference was employed using LSD for multiple comparisons. All analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Values of \( P < 0.05 \) were considered significant.

Results

Effects of ART on HaCaT cell proliferation

Treatment with ART at different concentrations (10, 20, 40, 80, 160, and 320 \( \mu \)g/mL) for 24, 48, and 72 h decreased the proliferation rate of HaCaT cells compared with that of untreated cells (\( P < 0.05 \)). WST-8 analysis showed that the antiproliferative effects of ART on HaCaT cells were dose- and time-dependent (Figure 1).

Quantification of apoptosis by Annexin-V/PI dual staining

ART-induced apoptosis in HaCaT cells was analyzed by Annexin V/PI dual staining. As shown in Figure 2A, early apoptosis in the HaCaT cells treated with 14.5 \( \mu \)g/mL ART was similar to that in the vehicle control. When the concentration of ART was increased to 29, 58, and 116 \( \mu \)g/mL, the percentage of early apoptotic cells continued to substantially increase. Late apoptotic cells also revealed the same trend. These results revealed that ART can increase the apoptosis ratio in a dose-dependent manner as compared with the vehicle control. The typical images of flow cytometric analysis are presented in Figure 2B.

Assessment of apoptosis by JC-1 staining

The loss of MMP is a hallmark of apoptosis. JC-1 is primarily used to detect mitochondrial depolarization in the early stages of apoptosis [13]. It accumulates as aggregates in healthy cells and exists as monomers in apoptotic cells. With the mitochondrial membrane becomes more polarized, the fluorescence of the JC-1 dye changes from red to green. Hence, cells that shifted in fluorescence were classified as apoptotic, and their MMP was represented by the red/green fluorescence intensity ratio.

As shown in Figure 3A, the red/green fluorescence intensity ratio in the untreated HaCaT cells was 1.70, whereas in the cells treated with 14.5 \( \mu \)g/mL ART was 1.61 (\( P > 0.05 \)). As the concentration of ART was increased from 29 \( \mu \)g/mL to 116 \( \mu \)g/mL, the fluorescence intensity ratios in the treated cells significantly decreased from 1.24 to 0.31 (\( P < 0.05 \)). These results not only revealed the ART-induced depolarization in HaCaT cells but also confirmed its dose-dependent apoptogenic effect.

Similar reductions in red fluorescence were also observed by fluorescence microscopy. The typical images are presented in Figure 3B. ART reduced the number of red JC-1 aggregates and increased the number of green JC-1 monomers, suggesting loss of MMP in HaCaT cells. As the concentration of ART was increased, this...
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Figure 2. ART-induced apoptosis in HaCaT cells analyzed by Annexin-V/PI dual staining. HaCaT cells were treated with or without ART for 48 h. Cell apoptosis was analyzed using Annexin V/PI dual staining and expressed as the percentage of total cell populations. A. ART increased the apoptosis ratio of HaCaT cells in a dose-dependent manner as compared with the vehicle control (P < 0.05). B. Cells in the upper right portion are late apoptotic cells, whereas those in the lower right portion are early apoptotic cells. Results are representative of one of three independent experiments.
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Figure 3. ART decreased mitochondrial membrane potential (MMP) in HaCaT cells as measured by JC-1 staining. HaCaT cells were treated with or without ART for 48 h. After staining with JC-1 dye, the MMP in HaCaT cells was analyzed by flow cytometry and observed by fluorescence microscopy. A. As the concentration of ART was increased, decreased ratios of red/green fluorescence intensities were observed, suggesting loss of MMP in HaCaT cells. Data shown are the means ± SD of three independent experiments. * P < 0.05 compared with the vehicle control. B. A similar reduction in red fluorescence was also observed in HaCaT cells by fluorescence microscopy. Cells treated with high concentrations of ART showed a marked reduction in red fluorescence (seen in healthy cells) and a reciprocal increase in green fluorescence (sign for MMP collapse) compared with that of the vehicle control. Images are representative of one of three independent experiments.
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phenomenon became prominent. These results revealed the occurrence of mitochondrial perturbation in the ART-treated HaCaT cells.

*Keratinocyte differentiation-modulating activity in mouse tail test*

No visible skin irritations, such as erythema or edema, were found in any mouse during the 4 weeks of topical treatment. The keratinocyte differentiation-modulating action in mouse tail tests are shown in Figure 4. Among the topical preparations, 0.1% tazarotene cream produced the most evident and striking increase in the degree of OK and the relative epidermal thickness. Compared with the negative control, vehicle cream increased the degree of OK but did not change the relative epidermal thickness. ART cream dose-dependently increased both the degree of OK and the relative epidermal thickness in the mouse tail skin.

Consistent with the measurement results mentioned above, histomorphological changes in the mouse tail also revealed the same trend. The typical pathology images of mouse tail skin are shown in Figure 5. Parakeratotic skin without an apparent granular layer was observed in the mice treated with normal saline solution. By contrast, a well-developed granular layer was clearly observed in the tazarotene-treated mouse tail skin. The OK of the epidermal granular layer slightly increased in the vehicle cream group compared with that in the negative control group. When treated with ART cream at different concentrations, a mild to moderate differentiation of the epidermal granular layer in the mouse tail skin were observed. Their degree of OK increased in an ART dose-dependent manner.

**Discussion**

ART is widely known as an anti-malarial agent [14]. It also has multiple applications because of diverse pharmacological effects [15]. This study is the first to explore the antipsoriatic activity of ART. Results revealed that ART can restore homeostasis to the epidermis and may be a promising agent for psoriasis treatment.

Whereas psoriasis is primarily a T-cell-mediated disease, intrinsic alterations in epidermal keratinocytes also play an important role to induce
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psoriasis [16, 17]. Exposure of altered autoantigens by keratinocytes may be directly responsible for the activation and expansion of certain T-cell subpopulations in psoriatic skin [18]. Furthermore, intrinsic multigenic altered keratinocytes can express inflammatory cytokines and growth factors that modify the microenvironment in psoriatic lesions [19]. Therefore, keratinocytes are closely associated with the development of psoriasis by amplifying the inflammatory response and contributing to angiogenesis.

As mentioned above, regulation of the pathological changes initiated or perpetuated by keratinocytes may be effective in psoriasis management [20]. Given their capability to attenuate inflammatory responses and bring homeostasis to the epidermis, drugs such as dithranol and vitamin D analogs are currently used in clinical settings to treat psoriasis with satisfactory results [21, 22]. Laboratory studies have demonstrated the immunosuppressive effects of ART on T cells both in vitro and in vivo [7]. The present study further revealed that this drug can inhibit keratinocyte proliferation and induce keratinocyte apoptosis. Considering the effects of ART against inflammation and keratinocytes, we speculate that ART may be a potential antipsoriatic agent.

Figure 5. Histological sections of mouse tail skin treated topically for four consecutive weeks (HE, ×200). Note: A. The granular layer is missing in most parts of the tail specimen. B. Tazarotene-induced OK is clearly observed over the whole horizontal length of the scale as a black layer. C. The granular layer is less developed in most parts of the mouse tail skin treated with vehicle cream. D-F. The degrees of OK increased in an ART dose-dependent manner. A well developed granular layer is detected in the mouse treated with 5% ART cream.

Altered keratinocyte differentiation, including parakeratosis and absence of the granular layer, play important roles in the clinical appearance of psoriatic lesions [23]. Such pathological changes can be partially mimicked by the mouse tail model because parakeratosis occurs in the normal adult mouse tail [24]. This model also has the advantages of easy access to animals and experimental procedure. Similar to most pharmacodynamic studies on antipsoriatic drugs [25, 26], our study also evaluated the inducing effect of ART on epidermal differentiation. As expected, 0.1% tazarotene cream significantly enhanced the degree of OK in mouse tail skin. These results not only indicated the rationality of using 0.1% tazarotene cream as a positive control but also further confirmed the reliability of the mouse tail model.

Repeated scratching as a result of itchiness can impair the skin barrier function and increase the transepidermal water loss in psoriatic lesions [27]. A moisturizer can reduce the transepidermal water loss, attenuate their epidermal hyperproliferation, and induce differentiation in psoriasis [28, 29]. Therefore, we added moisturizing ingredients, including silicone oil, solid paraffin, and albolene, into the vehicle cream matrix. Similar to our findings in
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another study [30], the vehicle cream also increased the degree of OK as compared with the negative control. Considering the absence of any anti-psoriatic drug in the vehicle cream matrix, we hypothesized that OK is related to these moisturizing ingredients. The detailed mechanism of such activity will be investigated in another project in the future.

After 4 weeks of treatment with ART cream, the degree of OK and relative epidermal thickness were significantly increased compared with that of the vehicle cream. The keratinocyte differentiation-modulating activity of ART cream increased in a dose-dependent manner (i.e., from 1% to 5% ART cream). In the histological sections, a continuous granular cell layer was clearly observed in the mice treated with 5% ART cream. These results indicated that the ART cream promoted the differentiation of mouse tail epidermis and exerted antipsoriatic activity in a dose-dependent manner.

Psoriasis is an autoimmune disease with multiple disorders, and keratinocyte abnormality is only one of its various pathological changes [31]. Hence, despite the weaker keratinocyte differentiation-modulating activity of ART cream than 0.1% tazarotene cream, ART cream is still a promising antipsoriatic drug because of its immunomodulatory, anti-inflammatory, and anti-angiogenic effects, among others [32, 33]. These effects might be synergistic in psoriasis treatment, and further studies on these areas are warranted.

Conclusions

This study revealed that ART possessed antiproliferative action and apoptogenic effects on HaCaT cells and exhibited keratinocyte differentiation-modulating activity in the mouse tail test. Therefore, ART may be a promising agent for psoriasis treatment and is worthy of further development. To better understand the potential antipsoriatic effects of ART, future studies must develop new experimental methods and animal models.

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Disclosure of conflict of interest

None.

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