Artesunate inhibits proliferation and invasion of mouse hemangioendothelioma cells *in vitro* and of tumor growth *in vivo*

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Abstract. Artesunate has been demonstrated to be a novel potential antitumor agent in numerous studies. However, its efficacy in infantile hemangioma is unknown. The aim of the present study was to investigate the role of artesunate in the control of vascular tumor biological behavior and molecular mechanism using mouse hemangioendothelioma endothelial (EOMA) cells and a nude mouse model. Cell viability, apoptosis and invasion were determined by an MTT assay, flow cytometric analysis and Transwell invasion assay, respectively. Reverse transcription-quantitative polymerase chain reaction and western blotting were utilized to examine the expression of genes and proteins. Inoculated EOMA cells were injected into the subcutaneous tissues of nude mice to observe the effect of artesunate therapy on the vascular tumor, an effect that was similar to that of pingyangmycin (PYM). It was identified that artesunate treatment (0-600 µg/ml) inhibited cell growth in a time- and dose-dependent manner. Artesunate at 300 µg/ml significantly reduced the proliferation and invasion of EOMA cells, and significantly decreased the expression of vascular endothelial growth factor (VEGF)-A, VEGFR-1, VEGFR-2 and hypoxia inducible factor-1α over time; caspase-3 was simultaneously upregulated *in vitro*. Artesunate significantly inhibited tumor growth, and the curative effect was similar to that observed with PYM *in vivo*. It was concluded that artesunate could effectively inhibit the growth of vascular tumors, and thus could be a novel drug candidate for the treatment of infantile hemangioma.

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Introduction

Hemangioma is a type of congenital vascular dysplasia common among infants and young children, with a high incidence rate of ~10-12% (1). The primary pathological features of hemangioma include the excessive proliferation of vascular endothelial cells and the formation of an abnormal vascular cavity (2). Kasabach-Merritt syndrome (K-MS; large hemangioma with thrombocytopenia syndrome) is a serious and life-threatening hemangioma (3-7), which is often associated with comorbid conditions such as hemangioendothelioma (8-10) or plexiform hemangioma (tufted angiomas) (11). In addition to the rapid increase in the size of the tumor, other features of the syndrome include the development of a low platelet count, microvascular disease, anemia and blood coagulation dysfunctions (12). In the present study, it was generally considered that vasculogenesis and angiogenesis are involved in the formation of new blood vessels in the hemangioma (13). The potential underlying molecular mechanisms include vascular endothelial growth factor (VEGF) and receptors (VEGFR) (14), angiogenin and receptors (15), the Notch signaling pathway (16), mammalian target of rapamycin (mTOR) signaling pathway (17), β-adrenergic receptors and the renin-angiotensin system (18), among others.

Artesunate is an important derivative of artemisinin, and its metabolite dihydro-artemisinin has been used to treat malaria (19). In recent years, studies have identified that artesunate has anti-tumor potential (20). Artesunate has a variety of mechanisms as an anti-tumor agent: It can influence tumor cell apoptosis, the iron-mediated formation of free radicals, anti-angiogenesis, block the cell cycle, anti-immunosuppressive (21), reverse drug resistance and restore chemosensitivity (22,23). A large number of studies are underway, evaluating the anti-cancer activity of artemisinin derivatives; however, artesunate has not yet been studied for hemangioma.

The aim of the current experiment was to explore the effect of artesunate on hemangioma. Thus, EOMA cells were used in an established hemangioma nude mouse model (24,25). Subsequently, the mechanisms of the artesunate-mediated inhibition of EOMA cell proliferation and invasion *in vitro*, and of hemangioma growth *in vivo*, were investigated.

Materials and methods

Antibodies and main reagents. Hypoxia-inducible factor (HIF-1α; cat. no. ab82832), caspase-3 (cat. no. ab13847), VEGFR1 (cat. no. ab32152) and β-actin (cat. no. ab8227) were purchased from Abcam (Cambridge, UK). VEGF-A (cat. no. 31274-1) and VEGFR-2 (cat. no. 21079-1) were purchased from Signalway Antibody LLC., (College Park, Maryland, USA). The EOMA cell line was purchased from the American Type Culture Collection, (ATCC, Manassas, VA, USA). Fetal calf serum (FCS) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA); artesunate was purchased from Guilin Pharmaceutical Co. Ltd., (Guilin, China); MTT was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Matrigel-coated Transwell chambers were purchased from Qiagen GmbH (Hilden, Germany). Apoptosis kits, including propidium iodide (PI) and fluorescein isothiocyanate (FITC)-Annexin V work solution, were purchased from Thermo Fisher Scientific, Inc. PrimeScript™ RT reagent kit with gDNA Eraser and SYBR® Premix Ex Taq™ II were purchased from Takara Bio, Inc., (Otsu, Japan). TRIzol Reagent was purchased from Invitrogen; Thermo Fisher Scientific, Inc.

Cell culture. Cells were cultured in DMEM supplemented with 10% FCS and incubated at 37°C with 5% CO_2, prior to being used at the logarithmic growth phase.

Growth inhibition assay. An MTT assay was used to assess cell proliferation. The EOMA cells ($1x10^5$/ml) were seeded onto 96-well plates. Artesunate was diluted in 1 ml 5% sodium bicarbonate solution, shaken for 2-3 min producing 0, 100, 200, 300, 400 and 500 µg/ml concentrations and then 100 µl was added to the EOMA cells in each well. At 0, 24, 48 and 72 h, an equal volume of MTT solution was added to each well and cultured for another 4 h. The incubation conditions in this assay were all 37°C in an atmosphere containing 5% CO_2. MTT-treated cells were fixed with 150 µl dimethyl sulfoxide for 30 min at room temperature and then assayed with an Evolution™ 201/220 UV-Vis spectrophotometer at 490 nm.

Apoptosis assessment. After 24 and 48 h, artesunate-treated (300 µg/ml) cells were collected and washed with cold PBS. Stained cells were assessed with a FACSCalibur™ flow cytometer and analyzed using BD CellQuest™ software (version 5.1; BD Biosciences, Franklin Lakes, NJ, USA). The percentage of early apoptotic cells (stained with Annexin V only) and late apoptotic cells (stained with Annexin V and PI) was recorded. Furthermore, Hoechst 33342 staining solution was used to identify the apoptotic cells, and later detected with fluorescence microscopy. Unstained cells were included as the control.

Transwell invasion assay. A Transwell assay was performed by using Matrigel-coated Transwell chambers (pore size of 8.0 µm). A total of $1x10^5$ cells were re-suspended in 200 µl serum-free medium and seeded into the upper compartment of the chamber. The lower compartment was loaded with 800 µl DMEM containing 10% FCS. After incubation at 37°C for 36 h, the membranes were fixed with formaldehyde at room temperature for 5 min, and counter-stained with DAPI at room temperature for 30 min in the dark. The staining was examined under a vertical fluorescence microscope. Trans-membrane migrated cells from each sample were counted in three random fields at x200 magnification. The assay was performed in triplicate.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. An RT-qPCR assay was used for the quantitative estimation of the mRNA expression of VEGF-A, VEGFR-1, VEGFR-2 and HIF-1α in EOMA cells, in the artesunate and in the control groups. Total RNA from ~$1x10^7$ cells was extracted using TRIzol® reagent, according to the manufacturer's protocol. Total RNA then underwent RT using the PrimeScript™ RT reagent kit with gDNA Eraser. The expression of VEGF-A, VEGFR-1, VEGFR-2 and HIF-1α mRNA was determined using the CFX96 Real-Time Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using SYBR® Premix Ex Taq™ II. β-actin was used as the internal reference. Data were analyzed using the $2^{-\Delta\Delta Cq}$ method (26). Three separate experiments were performed for each clone. Primers used for PCR analysis were as follows: VEGF-A forward, 5'-CAG GCTGCTGTAACGATGAA-3' and reverse, 5'-TTTCTTGCG CTTTCGTTTTT-3'; VEGFR-1 forward, 5'-GAGGAGGAT GAGGGTGTCTATAGGT-3' and reverse, 5'-GTGATCAGC TCCAGGTTTGACTT-3'; VEGFR-2 forward, 5'-TTCTGG ACTCTCCCTGCCTA-3' and reverse, 5'-AAGGACCATCCC ACTGTCTG-3'; HIF-1α forward, 5'-TGAGCTTGCTCATCA GTTGC-3' and reverse, 5'-CCATCTGTGCCTTCATCTCA-3'; β-actin forward, 5'-AAGATGACCCAGATCATGTTTGAG ACC-3' and reverse, 5'-GCCAGGTCCAGACGCAGGAT-3'.

Western blot analysis. EOMA cells were treated with 300 µg/ml artesunate for 24, 48 and 72 h, washed with cold PBS, harvested and extracted using lysis buffer. The protein concentration was detected using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Inc.). A total of 100 µg protein were separated using SDS-PAGE (10% gel) and then transferred to polyvinylidene fluoride membranes. To eliminate background disturbance, membranes were blocked by incubation in blocking solution (5% low fat milk and 0.1% Tween 20 in TBS) at room temperature for 1 h. Cells were then incubated with a rabbit monoclonal primary antibody specific for VEGF-A (dilution, 1:500), VEGFR-1 (dilution, 1:1,000), VEGFR-2 (dilution, 1:500), HIF-1α (dilution, 1:1,000), caspase-3 (dilution, 1:500) and β-actin (dilution, 1:2,000) at 4°C overnight, followed by incubation with a secondary antibody for 1 h at room temperature. Following this, enhanced chemiluminescence protein analysis was performed.

Animals. To observe efficacy of intra-tumor injection of artesunate for mouse K-MS and later a comparison with pingyangmycin (PYM), a total of 20 five-week-old female BALB/c nude mice (weight, between 18 and 20 g) were obtained from Chongqing Medical University (Chongqing, China). The mice were housed under specific pathogen-free conditions in an animal facility with free access to pelleted regular rodent diet (Experimental Animal Centre of the Children's Hospital of Chongqing Medical University, Chongqing, China) and water *ad libitum*. The room temperature was between 22-25°C,
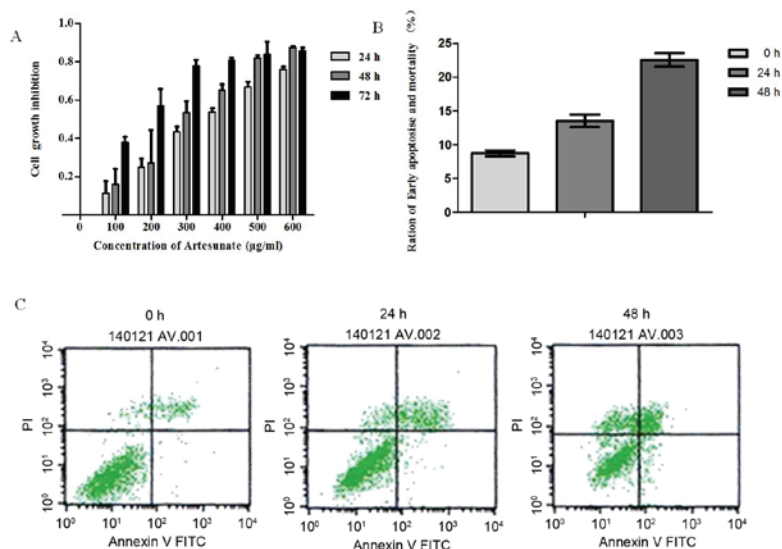
Figure 1. Effect of artesunate on the rate of EOMA cell growth inhibition and the induction of apoptosis in artesunate-treated EOMA cells. (A) Growth inhibition was determined using the MTT assay. Cells were treated with various concentrations of artesunate for the indicated times. The IC_{50} value of artesunate in EOMA cells was 300 μg/ml. The growth of EOMA cells was significantly inhibited by artesunate in a dose- and time-dependent manner. Results presented are the representative data of at least three independent experiments. (B) Annexin V/PI binding assay analysis of apoptosis in artesunate treated EOMA cells lines. The percentages of early apoptosis (Annexin V+/PI-, LR) are depicted. The results are expressed as mean ± standard deviation from three independent experiments (24 h, P<0.05 vs. 0 h; 48 h, P<0.01 vs. 0 h). (C) Representative images of the respective treatments in the Annexin V/PI binding assay. Results presented are representative data of at least three independent experiments. PI, propidium iodide; FITC, fluorescein isothiocyanate.

humidity was between 45-55%, and a 12-h light-dark diurnal cycle (lights on from 7:00 to 19:00) was used. The Experimental Animal Centre of Children's Hospital of Chongqing Medical University approved the experimental protocols. All procedures were carried out according to the Institutional Animal Care and Use Committee Guide in Child Development and Disorders' Laboratories (Chongqing, China; license no. SYXK (YU)2012-0001; sydwzx.cqmu.edu.cn).

An inoculation of 1x10⁷ EOMA cells in 100 μl PBS was injected into the subcutaneous tissues of the right flank of the female nude mice. The female nude mice were randomly divided into the following four groups of five each: Artesunate group, PYM group, PBS group and no treatment group. Tumor-bearing mice were treated with intra-tumoral injections every three days until the tumor was visible to the naked eye. The PBS group (intra-tumoral injection with the same dose of PBS every three days) was used as the negative control group and the no treatment group (tumor growth was not modified) was considered as the blank control group. As PYM is typically used as a treatment modality for hemangioendothelioma, the curative effect of artesunate was compared with that of PYM. The tumors were measured on alternate days using tissue calipers. Tumor volume was determined using the following formula: (Width)²x length x0.52. Mice were sacrificed by cervical dislocation when difficulty with ambulation and lethargy had set in.

Statistical analysis. SPSS 13.0 software was used for all data analysis (SPSS, Inc., Chicago, IL, USA). Numerical data are represented as the mean ± standard deviation. Differences between the control and treated groups were determined using the Student's t-test and one-way analysis of variance. Each experiment was performed in triplicate and P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of artesunate on the proliferation of EOMA cells. Artesunate treatment significantly inhibited the growth of EOMA cells in a time- and dose-dependent manner (Fig. 1A). The inhibition of cell proliferation increased correspondingly with the increase in drug concentration during the same time. Also, on extension of exposure time, there was an increase in cell proliferation inhibition with the same concentration. However, this inhibitory effect became apparent as ≤50% (IC_{50}) at a concentration of 300 μg/ml artesunate; therefore, this concentration was used throughout the study.

Effect of artesunate on the apoptosis of EOMA cells. Based on the results of the MTT assay, 300 μg/ml of artesunate was used for determining apoptosis. To elucidate the mechanism by which artesunate exerts its anti-proliferative effect on EOMA cell lines, an Annexin V/PI binding assay was performed. Results demonstrated an increased percentage of early apoptotic cells (Annexin V/PI) after treatment of EOMA cells with 300 μg/ml artesunate for 24 h (24 h, P<0.05 vs. 0 h), and this percentage of early apoptotic cells increased significantly following incubation for 48 h (48 h, P<0.01 vs. 0 h; Fig. 1B and C). The rate of apoptosis for EOMA cells treated with artesunate increased in a time-dependent manner.

Effect of artesunate on the invasion of EOMA cells. To investigate the inhibitory effect of artesunate on the invasion of EOMA cells *in vitro*. The invasion efficiency of EOMA cells in response to artesunate was tested using a Transwell invasion system. The invasion efficiency was calculated as the number of invaded cells to the control. In the presence of artesunate (300 μg/ml), the number of invaded EOMA cells
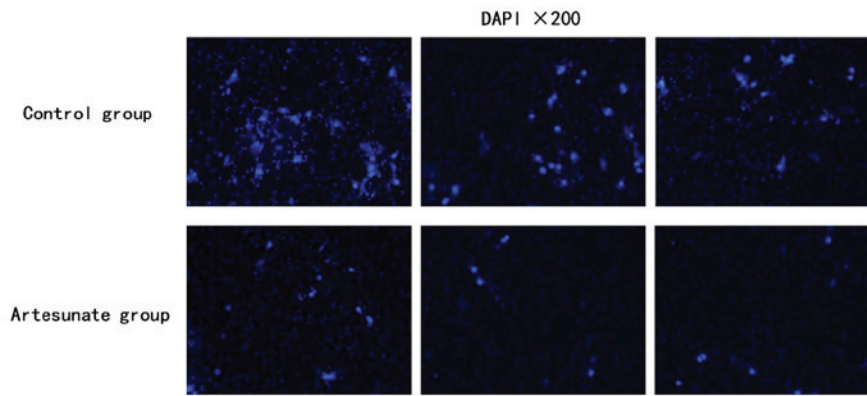
Figure 2. Transwell invasion assay using EOMA cells. The migration efficiency was illustrated as the increase or decrease in the number of invaded cells relative to the control. Cells from each sample were counted in three random fields at x200 magnification (P<0.05). Results presented are representative data of ≥3 independent experiments.

was significantly decreased compared with that of the control group, which has the same number of EOMA cells in the initial stage of this experiment (Fig. 2; P<0.05).

Effect of artesunate on VEGF-A, VEGFR-1, VEGFR-2 and HIF-1α mRNA expression. RT-qPCR was performed to examine the expression of VEGF-A, VEGFR-1, VEGFR-2 and HIF-1α mRNA in EOMA cells treated with artesunate at 300 μg/ml for 24, 48 and 72 h. The results demonstrate that VEGF-A, VEGFR-2 and HIF-1α mRNA expression levels in EOMA cells treated with artesunate were significantly decreased in a time-dependent manner (*P<0.05, $^#$P<0.01; Fig. 3A). There was no statistically significant difference in the VEGFR-1 mRNA expression level among the treatment groups (Fig. 3A).

Effect of artesunate on VEGF-A, VEGFR-1, VEGFR-2, HIF-1 and, caspase-3 protein level. Western blotting was performed to examine the expression of VEGF-A, VEGFR-1, VEGFR-2, HIF-1α and caspase-3 protein in EOMA cells treated with artesunate at 300 μg/ml for 24, 48 and 72 h. The results demonstrated that HIF-1α, VEGF-A and VEGFR-2 protein expression levels in EOMA cells were significantly decreased, but caspase 3 protein expression levels were significantly increased (*P<0.05, $^#$P<0.01; Fig. 3B). There were no statistically significant differences in VEGFR-1 protein expression levels among the treatment groups (Fig. 3C).

Effect of artesunate on the growth of Kasabach-Merritt tumors in nude mice. The outcome of artesunate expression on tumor growth in mice was examined by establishing a subcutaneous K-MS transplantation model. The tumor size was measured every three days (Table I and Fig. 4). The results demonstrated that the volume of the tumor in the artesunate-treated group had significantly reduced, and the effect was approximately equal to that recorded for PYM treatment. Artesunate and PYM can significantly reduce Kasabach-Merritt tumor growth compared with the no treatment and PBS groups (P<0.05). There were no statistically significant differences in tumor size between the artesunate and PYM groups, which was the same between the no treatment and PBS groups (P>0.05).

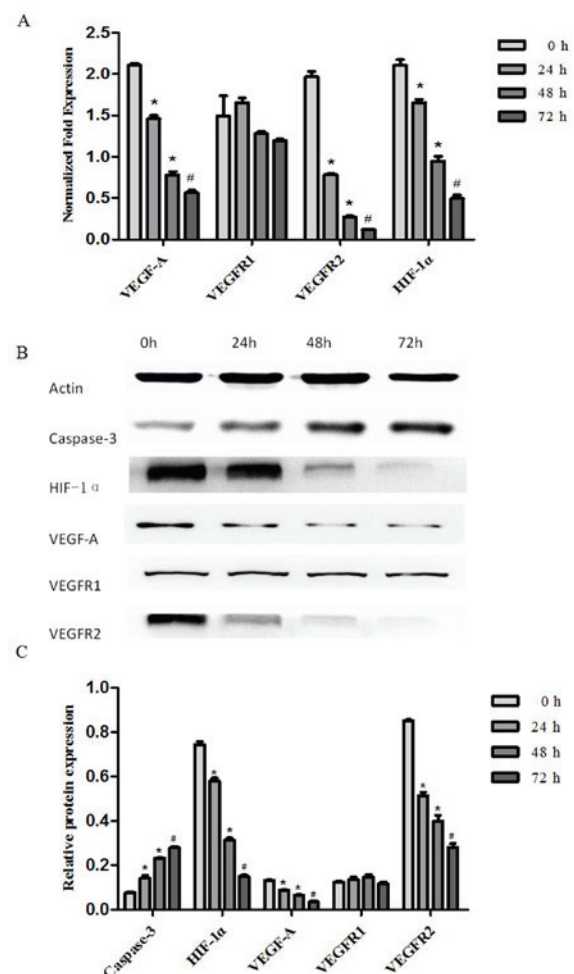Figure 3. Artesunate suppressed the gene and protein expression of VEGF-A, VEGFR-2 and HIF-1α, and increased the protein expression of caspase-3 in mouse EOMA cell lines. (A) The transcript levels of VEGF-A, VEGFR-1, VEGFR-2 and HIF-1α mRNA were analyzed using RT-qPCR; β-actin was included as the loading control. VEGF-A, VEGFR-2 and HIF-1α expression was significantly downregulated by artesunate. The results are expressed as the mean ± standard deviation from three separate experiments (*P<0.05, $^#$P<0.01, vs. control). (B) Western blot analysis of the protein expression in EOMA cells treated with artesunate for 24, 48 and 72 h, to evaluate VEGFA, VEGFR-1, VEGFR2, HIF-1α and caspase 3 expression. (C) Results presented are representative data of at least three independent experiments (*P<0.05, $^#$P<0.01, vs. control). VEGFR, vascular endothelial growth factor receptor; HIF-1α, hypoxia inducible factor-1α.

Table I. The volume of tumor in every group at various times (mm^3; n=5, mean ± standard error of the mean).

| Time	No treatment group	PBS group	PYM group	Artesunate group
0 day	68.9±19.5	80.0±21.2	132.6±23.7	122.4±15.2
3 days	215.3±145.4	161.9±90.4	32.6±79.9	71.4±13.2
6 days	284.9±190.1	241.5±138.4	87.1±213.4	44.4±12.7
9 days	274.3±199.5	492.3±225.1	391.6±161.2	57.4±25.1
12 days	575.8±418.1	751.8±370.9	323.7±135.4	111.1±45.3
15 days	900.3±637.5	1,129.4±319.4	273.9±119.9	139.8±43.0
18 days	1,489.7±1,018.6	1,442.2±604.2	202.2±61.6	167.3±62.9
21 days	1,728.7±975.7	1,559.1±718.2	161.4±58.8	186.8±60.9
24 days	2,165.8±1,041.8	1,819.0±789.3	136.3±58.9	183.4±61.6
27 days	3,163.8±1,688.2	2,033.3±1,021.8	105.6±50.9	184.1±64.3
30 days	3,445.7±1,889.8	2,337.4±1,282.7	84.1±33.3	188.6±61.5
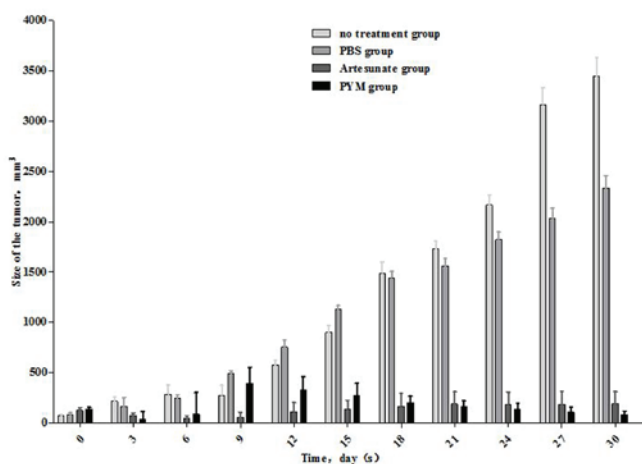
Figure 4. Artesunate, PYM and PBS were used to establish Kasabach-Merritt tumors in nude mice. Tumor size was monitored for 30 days. Artesunate depressed tumor growth, as compared with PYM group (P>0.05), and as compared with the no treatment and PBS groups (P<0.05).

Discussion

K-MS is a life-threatening complication of hemangioma; its most common histological appearance is as Kaposiform hemangioendothelioma (46% cases), followed by plexiform vascular (31%) and capillary tumors (23%) (27). The primary clinical manifestations are a rapid increase in tumor size, progressive decrease in platelet count, microvascular anemia, blood coagulation dysfunction and serious internal bleeding (28). EOMA cells were originally derived from 129/J mice spontaneous hemangioendothelioma (29). *In vitro*, EOMA cells exhibit the typical characteristics of the endothelial cells; these can grow in the matrix with tubular structure rearrangement and their biological actions are similar to those of microvascular endothelial cells (30). Studies have identified that EOMA cells administered to 129/J mice subcutaneously or nude mice can form histological and hematological characteristics similar to hemangioma with a K-MS animal model (24,25). EOMA cells have been demonstrated to be useful in constructing K-MS models with high rates of quick

forming tumors; therefore, EOMA cells are widely used for the study of angiogenesis inhibitors (31).

A number of positive and negative regulators control the angiogenic process, particularly the VEGF family and angiopoietins (32). VEGF-A is one of the most potent angiogenic factors, which induces migration and proliferation in endothelial cells, correlates with vascular density and influences prognosis (33). VEGFR-1 and VEGFR-2 are VEGFA receptors that mediate numerous biological activities, including: Proliferation, migration, tyrosine phosphorylation of downstream targets, Ca^{2+} mobilization, prostacyclin production, extracellular signal regulated kinase activation, nitric oxide production and phosphatidylinositol-3-kinase/protein kinase B activity (34). Hypoxia can induce the expression of HIF-1 and HIF-2, which may then upregulate the transcription factors for VEGF (35). Thus, VEGF secretes under hypoxic conditions and binds to its receptors, which are located on the surface of vascular endothelial cells (36). Hypoxic conditions can upregulate the expression of VEGF, while tissues can increase their oxygenation in order to induce blood vessel growth (37). By contrast, normoxia downregulates the expression of VEGF and can degenerate newly formed blood vessels (38). The vasculature exactly satisfies the metabolic requirements of the tissue through these opposing processes (39). Though hemodynamic forces, such as shear stress, are fundamental for remodeling, tissue hypoxia appears to serve an essential role in the coordinated expression of all angiogenic factors. Once oxygenation has reached normal levels, they are rapidly downregulated and HIF levels decrease (40).

Various treatment modalities are available for K-MS, the commonly used drugs including triamcinolone acetate and PYM (41,42). A disadvantage is that the long-term adverse effects are problematic to assess and the mechanism of action is also unclear. Another study has identified that rapamycin acts by inhibiting the mTOR pathway, thus suppressing HIF-1α and VEGF and subsequently inhibiting vascular endothelial cell proliferation (43). A study also identified that propranolol decreases HIF-1α, reduces downstream VEGF, VEGFR-1 and VEGFR-2 and the expression of monocyte chemoattractant protein-1, thus inhibiting angiomatous proliferation; however,

there were no significant differences in the expression of matrix metalloproteinases (44).

Artesunate, a compound isolated from a traditional Chinese medicine plant, is currently being evaluated for anti-tumor activity (45,46). Chinese medicine workers first identified artemisinin in the early 1970s as an effective antimalarial ingredient, extracted from sweet wormwood (*Artemisia annua*) (47). The history of artemisinin drugs used in the clinical treatment of malaria is decades old, thus it has become a well-established clinical drug and no severe systemic or local adverse drug reactions have been identified to date (48). With the development of scientific technology, the understanding of pharmaceutical ingredients present in traditional Chinese medicine has increased, and the pharmacological effects of the effective components are under further study (49).

Targeting angiogenesis is one of the treatment modalities in cancer management (50-52). Dihydro-artemisinin can decrease the expression of VEGF, inhibit the formation of new blood vessels and promote the apoptosis of tumor cells in leukemia cell line K562 animal models (53). Studies have identified that artemisinin has inhibitory effect on A549 human lung adenocarcinoma cells, and that its mechanism of action may be to inhibit cell proliferation, induce apoptosis and cell cycle arrest (54,55). Thus, the co-administration of artemisinin derivatives with other anti-cancer agents may increase the concentration of those anti-cancer drugs in the cell (56). In the present study, it was identified that artesunate can inhibit the proliferation, apoptosis and invasion of EOMA cells *in vitro* by reducing the expression of HIF-1α, VEGF-A and VEGFR-2. It was also determined that artesunate can inhibit the growth of a vascular tumor *in vivo*, with a curative effect similar to that of PYM.

In conclusion, the traditionally used anti-malarial drug artesunate has a significant inhibitory effect on hemangioma growth. This property should be evaluated further and may become a potential treatment option for hemangioma, with the advantages of fewer toxic side effects and higher cost-efficiency.

Acknowledgements

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