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Full Length Research Paper

Inhibition of angiogenesis and metastasis of uveal melanoma cells by astragaloside IV

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Astragaloside IV (AS) has been recently shown to possess pharmacologic activities against cancer. Vascular endothelial growth factor (VEGF) plays a prominent role in the induction of physiological or pathophysiological processes of tumor angiogenesis. The present study focuses on the anti-angiogenesis effects of AS in uveal melanoma cells. In this study, AS was demonstrated to exhibit higher anti-proliferation activity against cultured uveal melanoma cells compared with control. AS was also found to inhibit VEGF-a expression and secretion in human cells; functional assays also indicated inhibition of invasion and migration of the cells. This provides new information on a significant anti-tumor effect of AS. This saponin may be used as a novel therapeutic drug for the inhibition of tumor angiogenesis and metastasis.

Key words: Astragaloside IV, anti-cancer, vascular endothelial growth factor, uveal melanoma.

INTRODUCTION

Radix astragali (Huangqi) is one of the most widely prescribed Chinese herbs (Zhao et al., 2009; Xu et al., 2008). It has been widely used in Chinese medicine since ancient times. It is highly safe and demonstrates efficacy in the improvement of immune disorders and lung diseases (Zhang et al., 2006; Yuan et al., 2008; Jiang et al., 2008; Lv et al., 2010). The major active constituents of *R. astragali* are believed to be the total saponins and the total flavonoids. Astragaloside IV (AS) is a naturally occurring saponin isolated from *R. astragali* and is used for the quality evaluation of the herb, as listed in the 2005 edition of Pharmacopoeia of the People's Republic of China. Astragaloside IV is a major saponin of this herb. It has been recently shown to possess anti-inflammatory activities and pharmacologic activities against cancer, fatigue, and the coxsackie B virus Nalbantsoy et al., 2011; Chen et al., 2011; Shang et al., 2011).

Vascular endothelial growth factor (VEGF) plays a prominent role in the induction of physiological or pathophysiological processes of angiogenesis, vasculogenesis, arteriogenesis, and lymphangiogenesis, collectively termed as vascularization Dome et al., 2007). Although the evidence in the literature supports the idea that VEGF is a positive regulator of tumor growth, more reports indicate that VEGF also acts as a regulator that promotes tumor migration and invasion (Folkman, 1996; Dome et al., 2007; Xue et al., 2008; Siironen et al., 2006). In general, VEGF promotes angiogenesis by induction of the enzymes cyclooxygenase-2 (COX-2) and nitric oxide synthase. Over-expression of VEGF and COX-2 in cancerous tissues has been reported to be associated with poor prognosis. COX-2 is an inducible enzyme produced by many cell types in response to multiple stimuli (Shang et al., 2011). Recently, COX-2 over-expression has been detected in several types of human cancers such as those of the colon, breast, prostate, lung, pancreas, and blood (Toomey et al., 2009). It appears to control many cellular processes. Due to their roles in angiogenesis, carcinogenesis, and apoptosis, VEGF and COX-2 are excellent targets for developing new drugs.

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In this study, AS was demonstrated to exhibit higher anti-proliferation activity against cultured uveal melanoma cells compared with controls. AS was also found to inhibit VEGF-a expression and secretion in human cells; functional assays also indicated inhibition of invasion and migration of the cells. This provides new information on a significant anti-tumor effect of AS. This saponin may be used as a novel therapeutic drug for the inhibition of tumor angiogenesis and metastasis.

MATERIALS AND METHODS

Cell cultures and AS treatment

Human uveal melanoma cell-line OCM-1 was obtained from the American Type Culture Collection (Rochville, MD). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen). All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. AS powder (> 98% assay by high-performance liquid chromatography) was purchased from the RuiQi Chemical Co. (Shanghai, China) and was dissolved in dimethylsulfoxide just before use. For the experiments on the influence of AS on OCM-1 cells, the cells were seeded onto flat-bottomed 96-well or 6-well plates (Costar) with or without AS in the medium at the time of seeding.

Co-culture with HUVEC cells

Indirect co-culture was established using cell culture inserts (0.4 µm pore size, 0.33 cm^2, 24 wells; Transwell, Costar). Inserts were dehydrated and were loaded with OCM-1 melanoma cells while the bottom was inoculated with HUVEC cells. AS was added to the medium and cells were collected at certain times within a 24 h period for Western blot.

Proliferation, migration, and invasion assay

The cell proliferation test involved the MTT assay. Cell invasion assay was performed using Transwell cell culture inserts (Invitrogen). The transfected cells were maintained for 48 h and allowed to migrate for another 24 h. The passed cells were stained with crystal violet solution and their absorbance at 570 nm was determined. Cell motility in the wound healing assays was assessed by measuring the movement of cells into a scrape. The speed of wound closure was monitored after 10 and 24 h by measuring the ratio of the distance of the wound to that at 0 h. Each experiment was done in triplicate.

Western blot

RIPA lysate was used to lyse cells. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the lysed cells were transferred in the wet state onto PVDF film. Skimmed milk powder was used to block the reaction. Information on the primary and secondary antibodies is given in the Supplementary Data. Enhanced chemiluminescence method was used to determine protein expression.

Enzyme-linked immunosorbent assay (ELISA)

The VEGF-a detection ELISA kit was used to detect the concentration of VEGF-a in the medium, according to the manufacturer's instructions. The reaction between POD and ABTS at 405 nm was photometrically determined using a microplate reader.

Statistical analysis

All data in the study were evaluated using SPSS11.5 (SPSS Inc., USA). Differences were considered significant at $p < 0.05$. Significant results are marked with an asterisk (*).

RESULTS

AS inhibits proliferation, migration, and invasion of uveal melanoma cells

As shown in Figure 1, *in vitro* MTT analysis screening of AS demonstrated a strong inhibitory effect on OCM-1 cells. It showed Inhibition was dose-dependent and directly proportional to the AS concentration. At concentrations of 20 mg/L and above, AS killed more than 98% of the cells. The 50% inhibitory concentration (IC_{50}) of AS was 4.028 mg/L. This indicates that AS was cytotoxic to uveal melanoma cells and an $IC_{50} < 5$ mg/L was cytotoxic to the cells.

To determine if AS has a motility inhibitory effect, a wound healing assay was developed. OCM-1 cells were treated at various concentrations of AS for 10 and 24 h after being scratched (Figure 2). In the transwell invasion assay presented in Figure 3, around fourfold decrease in samples with the treated cells (compared the control group) occurred after exposure to 5 mg/L AS, following the Matrigel invasion assay. The results show that the cells proliferation, migration, and invasion were inhibited in a dose-dependent manner.

AS inhibits VEGF-a expression and secretion in uveal melanoma cells

To screen further the functional expression level of VEGF-a, we compared the level in cells treated with AS against those that were not exposed to AS. Western blot and ELISA of VEGF-a were done to analyze levels in the cytoplasm and those secreted into medium. As shown in Figure 4, the VEGF-a expression in the cytoplasm was not detected; this indicates a significant difference between the AS treatment and control groups. However, a significant difference was detected in media of the AS group and control. When treated by 5 to 20 mg/L AS, secretion of VEGF-a was completely inhibited compared with the control.

AS inhibits VEGFR-2 expression in HUVEC cell co-culture with uveal melanoma cells

Tumor cells can secrete VEGF-a to promote proliferation,

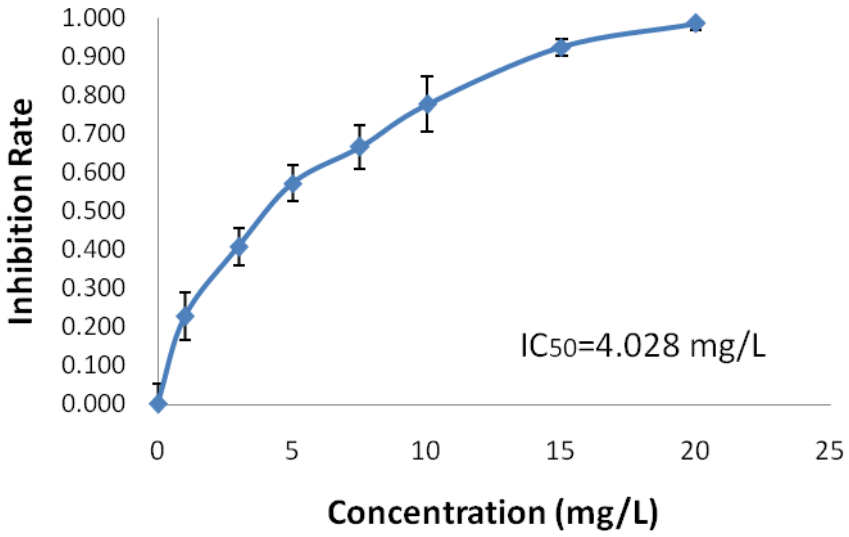

Figure 1. MTT assay analysis inhibitory effect of AS on OCM-1 cells. It showed inhibition was dose-dependent and directly proportional to the AS concentration. The 50% inhibitory concentration (IC_{50}) of AS was 4.028 mg/L. This indicates that AS was cytotoxic to uveal melanoma cells.

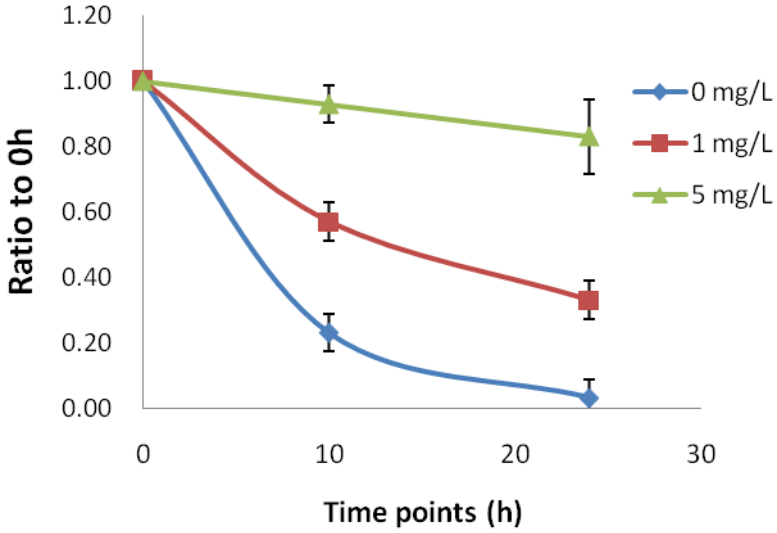

Figure 2. Wound healing assay was developed to analysis the migration of OCM-1 cells were treated at various concentrations of AS for 10 and 24 h after being scratched. This indicates that AS was inhibited uveal melanoma cells migration.

migration, and division of endothelial cells. VEGF presents its function through the VEGF receptor on the cell membrane. In the current study, we developed a co-culture system to detect the influence of AS on VEGFR expression in HUVEC cells co-cultured with OCM-1. The results show that VEGFR-2 expression levels decreased in HUVEC cells treated with AS, compared with the control. The treatment and control groups did not show a significant difference in VEGFR-1 expression levels.

DISCUSSION

The anti-tumor activity of astragaloside IV was confirmed in the *in vitro* experiments by its suppression of VEGF-a secretion in the uveal melanoma cell-line OCM-1. More importantly, this activity was confirmed by inhibition of migration and invasion of the cells.

Tumor metastasis is a multistep process by which a subset of cancer cells or individual cells disseminate from

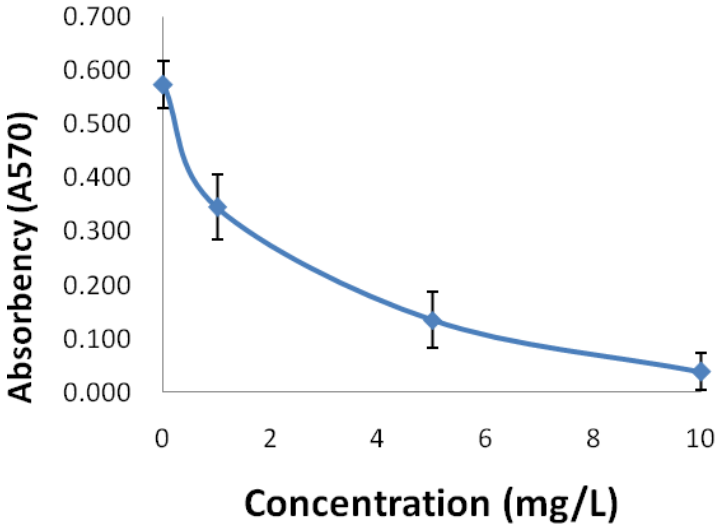

Figure 3. The transwell invasion assay was developed to analysis the invasion of OCM-1 cells were treated at various concentrations of AS. Fourfold decrease in samples with the treated cells (compared the control group) occurred after exposure to 5 mg/L AS. This indicates that AS was inhibited uveal melanoma cells invasion.

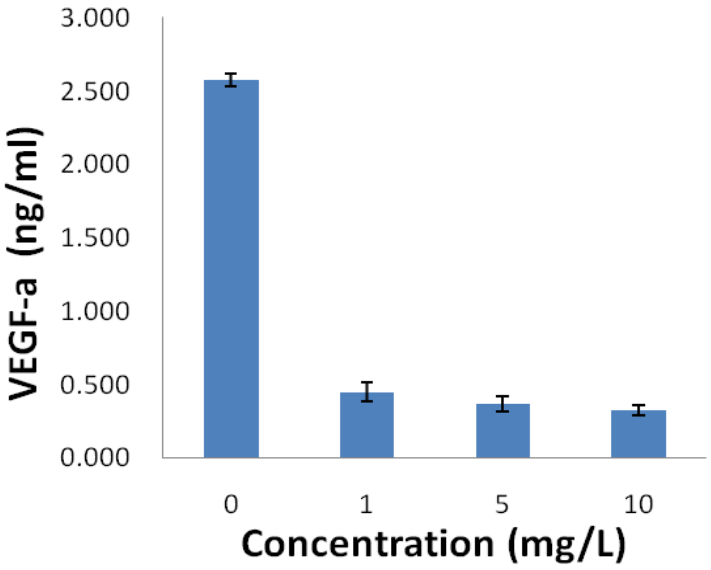

Figure 4. ELISA assay to screen the functional expression level of VEGF-a, we compared the level in cells treated with AS against those that were not exposed to AS. A significant difference was detected in media of the AS group and control. When treated by 5 to 20 mg/L AS, secretion of VEGF-a was completely inhibited compared with the control.

a primary tumor to distant secondary organs or tissues (McCawley and Matrisian, 2001). Tumor cells fulfill their metastatic potential after acquiring advantageous characteristics that allow them to escape from the primary tumor, migrate and invade surrounding tissues, enter the vasculature, circulate and reach secondary sites, extravasate, and establish metastatic foci (Pietras and Ostman, 2010; Josson et al., 2010; Anton and Glod, 2009; Bertinet al., 2010). All these steps of the metastatic cascade require survival of tumor cells and communication among cells. During metastasis, tumor cells are involved in numerous interactions with the

extracellular matrix (ECM). The tumor cells also interact with proteins, growth factors, and cytokines associated with the ECM, basement membranes, endothelial cell lining of the vasculature, blood cells in the circulation, and the microenvironment of the secondary site, where they eventually displace the normal tissue as they grow out and form metastatic foci (Josson et al., 2010; Peng and Wang, 2010). Several regulatory processes either are altered or are aberrant. This gives tumor cells the ability to accomplish all steps of the metastatic process, such as migration and invasion.

Tumor angiogenesis is essential for tumor growth and metastasis. Without active angiogenesis, tumor diameters rarely exceed 2 to 3 mm. Angiogenesis is mediated by the release of angiogenic factors by tumor cells, cells in the tumor stroma and microenvironment, which include endothelial cells (Dome et al., 2007; Folkman, 2006). We report that AS decreases the synthesis and release of angiogenic factors by uveal melanoma cells and inhibits VEGF-a expression and secretion in melanoma cells. Endothelial cell (EC) migration, proliferation, and differentiation are essential to tumor angiogenesis. EC proliferation, *in vitro* tubulogenesis, and survival are known to be stimulated in large part by VEGF. Decreased VEGF levels or inhibition of receptor activation in ECs often correlate with decreased tumor size and metastatic potential. VEGF binds to the extracellular domain of VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1), induces receptor dimerization, and activates tyrosine kinases by autophosphorylation; this leads to angiogenesis, increased vascular permeability, and EC proliferation and survival (Bertin et al., 2010; Karaca et al., 2011). In general, VEGFR-2 is the major mediator of these effects. We found that AS decreases the expression of VEGFR-2 by HUVEC in co-culture with OCM-1. By reducing the VEGF-R2 levels, AS appears to have similar effects on tumor cells. VEGF also induces leakage within tumor vessels, and thereby allows tumor cells to infiltrate blood vessels and migrate into the blood stream. Hence, changes in angiogenic factors even early in tumor formation can affect metastasis. Spreading and inhibiting VEGF production by AS is expected to reduce the metastatic potential of tumor cells. Additionally, increased blood vessel permeability within the tumor may interfere with adequate delivery and retention of chemotherapeutic agents. Indeed, certain anti-angiogenic agents that prevent tumor vessel leakage (a phenomenon called vessel normalization) have been shown to enhance the delivery of chemotherapeutic agents into tumors. Thus, combined treatment with anti-angiogenic factors and conventional chemotherapeutic agents may be superior to using the latter alone. Furthermore, as VEGF is likely required for migration and recruitment of ECs, AS-mediated VEGF reduction may decrease the number of blood vessels in the tumor.

Our present findings suggest that AS may induce potent anti-angiogenic effects, and enhance their potential as a therapeutic option against cancer. Our data demonstrate that astragaloside IV has potent anti-tumor and anti-angiogenesis qualities and deserves to be further evaluated for the treatment of human uveal melanoma. Currently, astragaloside IV is under early development as an anti-tumor candidate.

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