ORIGINAL ARTICLES

Erianin Inhibits the Growth of Uveal Melanoma Cells by Regulating VEGF Secretion

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Background: *To investigate the effect of erianin on uveal melanoma cells and its underlying mechanism. Erianin is a broad-spectrum antitumor compound, and whether it is effective in uveal melanoma cells is unclear.*

Methods: *B16F10 cells (murine melanoma cells) were divided into 4 experimental groups and 1 control group. The control group was not treated with drugs, and the experimental groups were treated with 3.125 μmol • L-1, 6.25 μmol • L-1, 12.5 μmol • L-1, and 25 μmol • L-1 erianin. A real-time cell electronic analysis system (RT-CES) and CCK-8 assay were used to investigate the effects of different doses of erianin on the growth of uveal melanoma cells. The VEGF-A mRNA expression in uveal melanoma cells was detected by RT–PCR. ELISAs were used to study the effect of erianin on VEGF-A secretion in uveal melanoma cells. Flow cytometry was used to investigate the effect of erianin on the cell cycle and apoptosis of uveal melanoma cells and its possible mechanism.*

Results: *B16F10 cells (murine melanoma cells) were divided into 4 experimental groups and 1 control group. The control group was not treated with drugs, and the experimental groups were treated with 3.125 μmol • L-1, 6.25 μmol • L-1, 12.5 μmol • L-1, and 25 μmol • L-1 erianin. A real-time cell electronic analysis system (RT-CES) and CCK-8 assay were used to investigate the effects of different doses of erianin on the growth of uveal melanoma cells. The VEGF-A mRNA expression in uveal melanoma cells was detected by RT–PCR. ELISAs were used to study the effect of erianin on VEGF-A secretion in uveal melanoma cells. Flow cytometry was used to investigate the effect of erianin on the cell cycle and apoptosis of uveal melanoma cells and its possible mechanism.*

Conclusion: *Erianin has a significant inhibitory effect on uveal melanoma cells, and its mechanism is to inhibit the level of VEGF and induce apoptosis and S-phase arrest of cells in a concentration-dependent manner.*

Background

Uveal melanoma (UM) is the most common primary intraocular malignancy in adults. Compared with other melanoma subtypes, UM has a very high incidence of liver metastases, and once metastases occur, the survival rate of patients decreases sharply.[1,2] If diagnosis and treatment occur before systemic metastasis, the 5-year survival rate can be improved to 90%.[3] The aim of current ocular treatment for uveal melanoma is to preserve the eyeball and useful vision and prevent metastasis as much as possible.

Previous studies have found that tumor growth, development and metastasis depend on blood vessels to provide essential nutrients and transport metabolic waste. Tumor neovascularization not only provides nutrients for the daily metabolism of tissues but also provides a way for tumor cells to enter the circulatory system and metastasize to distant areas.[4] Vascular endothelial growth factor (VEGF) is an important therapeutic target for fundus neovascular diseases. Uveal melanoma cells can express more VEGF during development and

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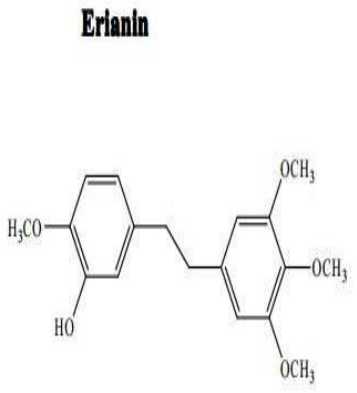
Financial Disclosures: This work was supported by the Shandong Provincial Medical Science and Technology Development Project (2018ws194) and the General Project of Natural Science Foundation of Shandong Province (ZR2020MH393).

metastasis, and the growth of tumor cells is also dependent on VEGF.[5] At present, blocking VEGF production to inhibit tumor neovascularization is an effective antitumor therapy.

Erianin (C18H22O5) is a natural small molecule compound extracted from Dendrobium officinale and D. drumbium. The chemical name is 2-methoxy-5-[2-(3,4,5-trimethoxy-phenyl)-ethyl] -phenol (Figure 1), with a relative molecular weight of 318.36.[6] Erianin can not only relieve heat and pain in traditional Chinese medicine but also plays an important role in antioxidation, antineovascularization and antitumor activity. Yu's et al.7 team demonstrated that erianin could downregulate VEGF expression in retinal endothelial cells and microglia through ERK1/2-mediated HIF-1α activation. Furthermore, the inhibition of retinal neovascularization endothelial cells has potential application in the treatment of diabetic retinopathy.[7] The aim of this study was to investigate the effect of erianin on the growth of B16F10 melanoma cells and its potential mechanism to provide an experimental basis for the application of erianin in the treatment of uveal melanoma (Figure 1).

Figure 1. Molecular formula of erianin.



MATERIALS AND METHODS

Materials

The B16F10 cell line (Shanghai Cell Bank, Chinese Academy of Sciences), erianin (Beijing Yihua Tongbiao Technology Co., Ltd.), HPLC≥98%, and DMEM containing 0.1% DMSO configured into different concentrations were used. Fetal bovine serum (FBS) was purchased from GICO (Australia), and high-sugar DMEM culture medium was purchased from HyClone (USA). PBS reagent, CCK-8 kit and PCR kit were purchased from Shandong Sikejie Biotechnology Co., Ltd.; PCR primers were synthesized by Shanghai Shenggong Biological Co., Ltd. ELISA kits were purchased from Shanghai Future Industrial Co., Ltd. Flow cytometry kits were purchased from Shanghai Biyuntian Biotechnology Co., Ltd. A slit lamp microscope (Zeiss, Germany), high-power microscope (Leica-M841, USA), high-speed refrigerated centrifuge (Heal Force, USA);flow cytometry (BD FACSVerse, USA), rapid temperature gradient PCR instrument (Bio-Rad, USA), fluorescence quantitative PCR machine (Roche 480, Germany); enzyme-linked immunoassay (BioTek, USA), and 37°C CO2 incubator (Xinmiao, Shanghai, China) were used.

Methods

Cell culture

The B16F10 cell line was cultured in DMEM high glucose medium with 10% FBS, 100 mg·L-[1] streptomycin, and 100×103 U·L-[1] penicillin at 37 °C and 5% CO2 by volume. When the cells reached 80-90% confluence, they were digested with 0.05% trypsin. The cells needed for the experiment were at the 3rd to 6th passages.

RT-CES assay

B16F10 cells (10×103 cells) were planted in 16-well plates equipped with an RT-CES machine and cultured in a cell incubator (37 °C, containing a volume fraction of 5% CO2) for 20 h. The culture medium was removed. The experimental group was incubated with different concentrations of erianin (3.125 μmol·L-[1], 6.25 μmol·L-[1], 12.5 μmol·L-[1], 25 μmol·L-[1]) mixed with DMEM, while the control group (0 μmol·L-[1]) was incubated with the same amount of DMEM. The growth trend of melanoma cells treated with different concentrations of erianin was recorded and analyzed by an RT-CES analyzer.

CCK-8 assay

Melanoma cells (10×103) were plated in 96-well plates and cultured in a cell incubator (37 ℃, containing 5% CO2) for 24 h. The experimental group was treated with culture medium containing different concentrations of erianin. The final concentrations of erianin were 3.125 μmol·L-1, 6.25 μmol·L-1, 12.5 μmol·L-1, and 25 μmol·L-1. The control group (0 μmol·L-1) was added to the same amount of DMEM, and each well had three replicates. The culture was continued for 24 h, and 200 μL of CCK-8 detection reagent was added to each well. After 4 h of culture, the test was performed on the machine. Survival rate (%) = (A erianin/A control) ×100. A is the absorbance value of relevant samples at 490 nm.

ELISA

B16F10 cells in the experimental group were treated with mixed culture medium of erianin (3.125 μmol·L-1, 6.25 μmol·L-1, 12.5 μmol·L-1, 25 μmol·L-1) and DMEM, and the control group (0 μmol·L-1) was treated with the same amount of DMEM. After 24 h, 200 μL of supernatant was collected from each well and analyzed by a VEGF-A ELISA kit. The specific operation was carried out in strict accordance with the kit instructions.

RT–PCR assay

Real-time PCR was used to detect the expression of VEGF-A mRNA in uveal melanoma cells after erianin intervention. RNA was extracted according to the method provided by the Sikejet kit instructions, and the concentration of RNA was quantified with a microUV–visible spectrophotometer. Then, cDNA was synthesized by reverse transcription using a Novizan reverse transcription kit. The primer sequences for VEGF-A were as follows: forward: GCCCTTGCCTTGCTGCTCTACC (5–3′) and reverse: GTGATGATTCTGCCTCCTCCTTC (5–3′). PCR amplification was performed with β-actin as an internal reference. The reaction conditions were as follows: predenaturation at 95 °C for 3 min; 40 cycles of 95 °C for 30 s and 60 °C for 30 s, followed by 95 °C for 60 s and 60 °C for 60 s.

Flow cytometry assay

B16F10 cells with a good growth state and in the logarithmic phase of growth were washed with PBS, digested with trypsin and counted. The cells were seeded into 6-well plates at 3×105 cells per well and cultured for 24 h at 37 °C in an incubator with 5% CO2 saturated humidity. After removal of the supernatant and grouping treatment, the cells were cultured for 48 h and centrifuged at 1000 r·min-1 for 4 min to precipitate. Cells were resuspended by adding 1 mL of precooled PBS in an ice bath and transferred to a 1.5 mL centrifuge tube. Then, 1 mL of precooled 70% ethanol (volume fraction) was added in an ice bath, and the samples were gently blown, mixed and fixed at 4 °C for 12 h. The fixed cells were centrifuged to remove ethanol, gently washed twice with precooled PBS, and then resuspended in 0.5 mL of precooled PBS. Then, 0.5 mL of propidium iodide staining solution according to the instructions of the cell cycle detection kit was added to each tube of the cell sample. The cell precipitate was slowly and fully resuspended, and the cell cycle was detected by flow cytometry in a warm bath at 37 ℃ for 30 min.

Detection of apoptosis

B16F10 cells were washed with PBS, digested with trypsin and counted, and 3×105 cells per well were seeded on a 6-well plate. The supernatant was removed after 24 h of culture in the incubator, and the culture was continued for 48 h after group treatment. The supernatant was washed with PBS, and an appropriate amount of trypsin digestion solution was added for digestion. After 1000 r·min -1 for 4 min, the supernatant was discarded, the cells were collected, gently resuspended in PBS and counted. Then, (5-10) x104 resuspended cells were centrifuged at 1000r·min-1 for 5 min, the supernatant was discarded, and the cells were gently resuspended by adding 195 μL of annexin V-FITC binding solution, followed by 5 μL of annexin V-FITC, and finally 10 μL of propidium iodide staining solution The cells were incubated for 15 min at room temperature (20-25℃) in the dark and then placed in an ice bath. The apoptosis of cells in each group was detected by flow cytometry.

Statistical analysis

SPSS 21.0 statistical software was used for data analysis, and the experimental results are

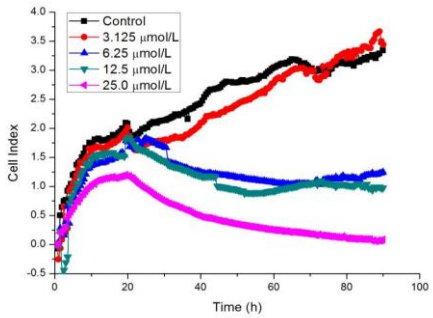
expressed as the mean ± s. An independent sample t test was used to compare the differences between the erianin group and the control group, and one-way analysis of variance was used to compare the differences between the groups treated with different concentrations of erianin. The test level was α=0.05.

RESULTS

RT-CES was used to monitor the cell growth status in each group

RT-CES continuously monitored B16F10 melanoma cells for 90 h after erianin intervention. The results of the dynamic growth curve showed that compared with that of the control group (0 μmol·L-1), the growth of the B16F10 melanoma cells in the experimental group (25 μmol·L-1) was the most significantly inhibited. The cell growth of the experimental groups (12.5 μmol·L-1, 6.25 μmol·L-1) was significantly inhibited. The growth of another experimental group (3.125 μmol·L-1) was not significantly inhibited (Figure 2).

Figure 2. RT-CES dynamic detection of uveal melanoma cell growth curves after erianin intervention.



CCK-8 assays were used to detect the proliferation of cells in each group after erianin intervention

To observe the effect of erianin on the biological behavior of B16F10 cells, we used different concentrations of erianin for intervention, and CCK-8 assays were used to detect cell proliferation. Over time, B16F10 cells were treated with different concentrations of erianin (3.125 μmol·L-1, 6.25 μmol·L-1, 12.5 μmol·L-1, 25 μmol·L-1) for 24 h and compared with the control cells (0 μmol·L-1). The experimental groups showed inhibited cell proliferation, and one experimental group (25 μmol·L-1) had the lowest cell survival rate (35.06±1.40%). In the experimental groups (6.25 μmol·L-1 and 12.5 μmol·L-1), the cell survival rates were 89.46±2.50% and 64.30±2.45%, respectively, while in another experimental group (3.125 μmol· L-1), the cell survival rate was 93.93±0.26%.

RT–PCR was used to detect the relative VEGF-A mRNA expression in cells

B16F10 uveal melanoma cells treated with erianin for 24 h were significantly downregulated in the experimental group (25 μmol·L-1) compared with the control group (0 μmol·L-1), and the relative expression was 0.53±0.01. Compared with that of the control group (0 μmol·L-1), the expression of the experimental group (6.25 μmol·L-1, 12.5 μmol·L-1) was downregulated, and the relative expression levels of the experimental group (3.125 μmol·L-1) were 0.78±0.01 and 0.65±0.01. The relative expression level was 0.85±0.01, and the differences were statistically significant (P<0.05).

ELISAs were used to detect the level of VEGF-A secretion

ELISAs showed that the secretion level of VEGF-A in the control group (0 μmol·L-1) was 99.91±0.8 ng·L-1 after 24 hours of erianin intervention in B16F10 uveal melanoma cells. In the experimental groups (3.125 μmol·L-1, 6.25 μmol·L-1, 12.5 μmol·L-1, 25 μmol·L-1), the level of VEGF-A in B16F10 melanoma cells decreased to 92.06±0.88 ng·L-1, 78.05±0.79 ng·L-1, 60.69±1.20 ng·L-1, and 34.57±0.76 ng·L-1 compared with that of the control group (0 g·L-1), and the differences were statistically significant (P < 0.05).

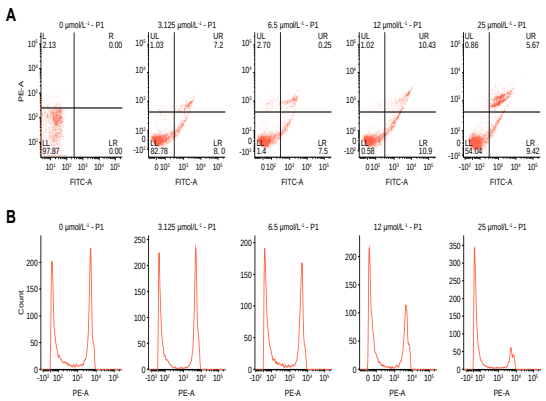
Flow cytometry assay

Cell apoptosis was detected by flow cytometry

The proportion of normal uveal melanoma cells in the control group (0 μmol·L-1) was 97.87% after 48 h of erianin intervention. The percentage of broken and necrotic cells was 0%. The percentage of apoptotic cells was 0%. After treatment with 3.125 μmol·L-1, 6.25 μmol·L-1, 12.5 μmol·L-1, or 25

for 24 h, the ratio of normal cells decreased significantly to 82.78%, 81.48%, 77.58%, and 54.04%, respectively, and the ratio of apoptotic cells increased significantly to 7.29%, 8.25%, 10.04%, and 35.67%, respectively. The results showed that erianin could promote apoptosis of B16F10 uveal melanoma cells in a concentration-dependent manner (Figure 3A).

Figure 3. A Apoptosis results of B16F10 cells treated with erianin by flow cytometry. B Cell cycle results of B16F10 cells treated with erianin.



Cell cycle detection by flow cytometry

After 48 hours of erianin intervention in uveal melanoma cells, the proportion of cells in G1 phase was 38.31% in the control group (0 μmol·L-1), and the proportion of cells in G2 phase was 57.11% in the experimental group (25 μmol·L-1). The inhibitory effect of erianin on uveal melanoma cells was the most obvious, and the proportion of cells in G2 phase was 58.53%. There was no significant difference in the proportion of S-phase cells among the groups. These results suggested that erianin could induce S-phase arrest of cells, thereby inhibiting periodic cell proliferation (Figure 3B).

DISCUSSIONS

Uveal melanoma (UM) is the most common primary intraocular tumor in adults and the second most common type of melanoma after skin melanoma.[8,9] UM is usually asymptomatic in its early stages and is often detected during routine eye examinations.[10] At present, the treatment of uveal melanoma is mainly divided into various forms and combinations of radiotherapy, phototherapy, local excision, and enucleation for advanced cases.[11]

Despite effective treatment of the primary tumor, approximately 50% of UM patients develop metastatic disease, usually liver metastases, and the median survival rate after metastasis is only 4-15 months, which is an important reason for the high mortality of this disease.[12] To date, there are very few drugs specifically used to treat uveal melanoma, and there are currently no effective adjuvant therapies to reduce the metastasis of UM.[13] Ting Wu's team found that the tumor microenvironment plays a decisive role in tumor initiation, progression and metastasis and has profound implications for treatment. The maintenance of tumor microenvironment homeostasis requires tumor neovascularization to provide essential nutrients and carry away metabolic wastes. Antitumor neovascularization therapy[14,15] suggests that if it can successfully block or inhibit the production of tumor blood vessels, cut off their nutrient sources and disturb the homeostasis of the microenvironment of tumor cells, tumor cells will eventually stop growing and die and indirectly achieve antitumor effects.

For decades, many lead compounds isolated from natural resources have influenced modern drug development in an attempt to obtain more therapeutic antitumor drugs. However, erianin is a small molecule compound extracted from Dendrobium that has a wide range of pharmacological activities, such as antitumor, antibacterial and antiviral activities.[16,17] With the deepening of international research on maulanin, it has been found that erianin can activate autophagy of tumor cells, inhibit the invasion and migration of tumor cells, inhibit tumor angiogenesis, and have different inhibitory effects on liver cancer, cervical cancer, breast cancer, rectal cancer and other malignant tumors.[18] However, to date, the mechanism of action of erianin on uveal melanoma cells is still unclear, which hampers the development and utilization of erianin in the treatment of uveal melanoma.

Previous studies have found that neovascularization plays a crucial role in the process of tumor growth and metastasis and is considered to be an important marker of malignant tumors.[19]

Vascular endothelial growth factor (VEGF), a typical angiogenic molecule in malignant tumors, also plays an important role in tumor angiogenesis.[20]

In this study, we investigated the mechanism of action of erianin on uveal melanoma cells by cell biological techniques. The real-time cell detection system (RT-CES) dynamically recorded the whole process of endogenous growth of uveal melanoma cells treated with erianin for 90 h, and the cell growth curve was drawn. The images showed that the cells in the experimental group (25 μmol·L-1) displayed the greatest inhibition. The CCK-8 results suggested that erianin could significantly inhibit the activity of uveal melanocytes. The results of ELISAs and RT–PCR showed that the experimental group (25 μmol·L-1) had the most significant effect in downregulating the expression of VEGF-A mRNA and inhibiting the secretion of VEGF-A. The results of the flow cytometry assay showed that erianin induced apoptosis and S-phase cell arrest in uveal melanoma cells in a dose-dependent manner.

CONCLUSIONS

Combined with the above experimental data, we speculated that erianin could inhibit the secretion of VEGF-A by downregulating the expression of VEGF-A mRNA, thereby antagonizing the generation of VEGF, inducing apoptosis and S-phase arrest of uveal melanoma cells, reducing cell activity and preventing cell growth. The results of this study provide an experimental basis for erianin to become a candidate drug for the treatment of uveal melanoma in the future. However, the safety of high concentrations of erianin on the retina remains to be further studied.

DECLARATIONS

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest and Source of Funding

The authors have declared no conflicts of interest.

Funding

This work was supported by the Shandong Provincial Medical Science and Technology Development Project (2018ws194) and the General Project of Natural Science Foundation of Shandong Province (ZR2020MH393).

Authors' contributions

SH and LJ conceived and designed the study. SH and LJ performed the experiments. ZX and LN analyzed the data. SH, LJ and GB wrote the manuscript. WXR confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Acknowledgments

The authors wish to acknowledge the help provided by the Laboratory of Eye Hospital Affiliated to Shandong University of Traditional Chinese Medicine.

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