CHAPTER ELEVEN

Inhibitory effect of Siwei Xiaoliuyin on glioma angiogenesis in nude mice☆

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☆The authors were supported by Science and Technology Planning Project of Guangdong Province, China (Number: 2014A020212411).

International Review of Neurobiology, Volume 151
ISSN 0074-7742
https://doi.org/10.1016/bs.irn.2020.03.008

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Abstract

Objective: Application of Siwei Xiaoliuyin in glioma mice. Explore the effect of Siwei Xiaoliuyin on angiogenesis of nude mice glioma and its mechanism.

Methods: Establish human glioma cell line U87 tumor model. Mice were randomized to the saline group, the conventional dose of Siwei Xiaoliuyin, high dose group of Siwei Xiaoliuyin, TMZ group, combination therapy group, record the tumor volume. Using the method of Weidner counted the microvessel density. ELISA enzyme-linked adsorption method to detect the content of nude mice serum VEGF and ES. The difference was statistically significant ($P < 0.05$).

Results: The tumor volume and MVD of conventional dose group, large dose group, Siwei Xiaoliuyin combined temozolomide group was smaller than the blank group，the difference was statistically significant ($P < 0.05$). VEGF levels in three groups of nude mice were lower than the blank group and ES content is higher than blank group, the difference was statistically significant ($P < 0.05$).

Conclusion: Siwei Xiaoliuyin can inhibit glioma angiogenesis. Its mechanism of glioma angiogenesis inhibition may be through regulation VEGF and down-regulation of endostatin expression of vascular endothelial growth factor achieved. Down-regulation of endostatin expression of vascular endothelial growth factor achieved.

Glioma is the most common brain tumor with high mobility, invasiveness and proliferation, high recurrence rate, poor prognosis and high mortality, and the 5 year survival rate is less than 5%. In recent years, inhibition of glioma angiogenesis has further explored the occurrence, development and prognosis of glioma (Wen & Reardon, 2016). Tumor vascular microenvironment is carried out under a series of angiogenic factors and inhibition of angiogenesis factors (Hanahan, 2014). Vascular endothelial growth factor (VEGF) and endostatin (ES), as a representative factor for angiogenesis factor and angiogenesis inhibition, play an important role in angiogenesis. Chinese medicine plays an important role in antitumor angiogenesis， Our team found that Curcuma can down regulate the expression of VEGF in nude mice and thus inhibit angiogenesis of glioma (Zhang, Han, et al., 2013). The purpose of this study was to investigate the mechanism of Siwei Xiaoliuyin on inhibiting angiogenesis of glioma in nude mice by dynamically observing the effect of Siwei Xiaoliuyin on glioma.

1. Materials and methods

1.1 Tumor cells

U87 cells are provided by the 629 Laboratory of cancer prevention and treatment center of Sun Yat-sen University. U87 cells in logarithmic growth phase were placed in EME medium. Incubated in incubator at 37 °C and 5% CO_2 and under water saturation conditions. The cytoplasmic retracting was observed under the microscope. Record cell types and date and place them in liquid nitrogen tank.

1.2 Experimental animal

40 healthy nude mice in 6–8 weeks, weight 25–30 g. They are purchased from Guangdong medical experimental animal center and reared by SPF animal experimental center of Sun Yat-sen University.

1.3 Siwei Xiaoliuyin

Siwei Xiaoliuyin: curcuma 15 g (Kangmei Chinese Herbal Pieces, Guangxi, 140303041), Gecko 10 g (Kangmei Chinese Herbal Pieces, Jiangsu, 140302171), *Solanum nigrum* 15 g (Kangmei Chinese Herbal Pieces, Guangdong, 140311081), tuckahoe 60 g (Kangmei Chinese Herbal Pieces, Guangxi, 140401421) purchased by Guangdong Provincial Hospital of Traditional Chinese Medicine. Add 500 mL water, decoct to 100–150 mL, soak for 30 min, boil in water, decoct for 30 min, take juice, two Decoction add water 300 mL, take juice 100 mL, mix twice.

1.4 Temozolomide (TMZ)

Temozolomide was purchased from Jiangsu Tianshili Diyi Pharmaceutical Co., Ltd. TMZ is dissolved in DMSO, and each nude mouse is administered with 50 mg/kg/d.

1.5 Experimental grouping and tumor planting

Forty nude mice were randomly divided into normal saline group, conventional dose Siwei Xiaoliuyin group, high-dose Siwei Xiaoliuyin group, Temozolomide (TMZ) group, Siwei Xiaoliuyin + TMZ group. There was no significant difference in body weight between groups. The cultured U87 cells are made into a cell suspension and injected subcutaneously into the right side of the nude mouse. 200 μL was administered at 10 o'clock every day, group A was given normal saline, group B was given

conventional dose of Siwei Xiaoliuyin, group C was given high dose of Siweixiaoliu drink, group D was intragastrically administered with TMZ, group E was given conventional dose of gavage. Siwei Xiaoliuyin + TMZ, continuous gavage for 28 days.

1.6 Measurement of tumor growth

The tumor tissue was inoculated to observe the tumor formation in nude mice. The long diameter (a) and the broad diameter (b) of the tumor were measured after the third day of tumor formation. The measurement was performed once every 6 days with a vernier caliper. The tumor volume was calculated as follows: Tumor volume $= 1/6 \times \pi \times b2 \times a$.

2. Determination of serum VEGF and ES

2.1 Specimen acquisition

After 28 days of gavage, the eyeballs were taken for blood collection. After centrifugation, the upper serum was taken and stored in a −80 °C refrigerator.

2.2 Detection of serum VEGF and ES in nude mice by ELISA

ELISA kits were used to detect VEGF and ES in nude mice.

3. Tumor tissue MVD determination

The tumor-bearing mice were sacrificed by necking, and the tumor tissues were completely excised. The tumor specimens were sectioned into 5 μm thick sections, dewaxed, repaired with citrate antigen repair buffer, and 5% goat serum was added to the incubator at 37 °C for 10 min. The CD34 primary antibody was added dropwise, incubated at 37 °C for 60 min, and then placed in a refrigerator at 4 °C overnight. After washing PBS three times × 5 min, the reagent biotinylated secondary antibody working solution was added dropwise, placed in a wet box, and incubated at 37 °C for 30 min. Rinse in PBS for 5 min × three times. After DAB color development, the color was satisfied, and the reaction was terminated by tap water washing. The nuclei were counterstained with hematoxylin, rinsed with tap water, dried and sealed with neutral gum, and MVD was counted by Meidner method.

4. Result

4.1 Effect of Siwei Xiaoliuyin on tumor volume in nude mice

The tumor volume of nude mice gradually increased, and there was a difference between the groups at the third week. Siwei Xiaoliuyin could inhibit the tumor growth of nude mice, and the high-dose Siwei Xiaoliuyin had a strong inhibitory effect on tumors in nude mice. In conventional doses, the combination of Siwei Xiaoliu Decoction and TMZ can enhance the inhibition of tumor growth in nude mice (Fig. 1).

4.2 Effect of Siwei Xiaoliuyin on microvessel density in tumor tissue of nude mice

Weidner method for counting microvessel density in tumor tissues of nude mice. As shown in Table 1, The MVD of tumor tissues in the conventional Siwei Xiaoliuyin Group, High-dose Group, TMZ group and combination group were lower than those in the blank group, the difference was statistically significant ($P < 0.05$), indicating that Siwei Xiaoliuyin could reduce the nakedness. Murine tumor tissue MVD inhibits tumor angiogenesis, and the combination of Siwei Xiaoliuyin and TMZ can enhance its inhibition of tumor angiogenesis (Fig. 2).

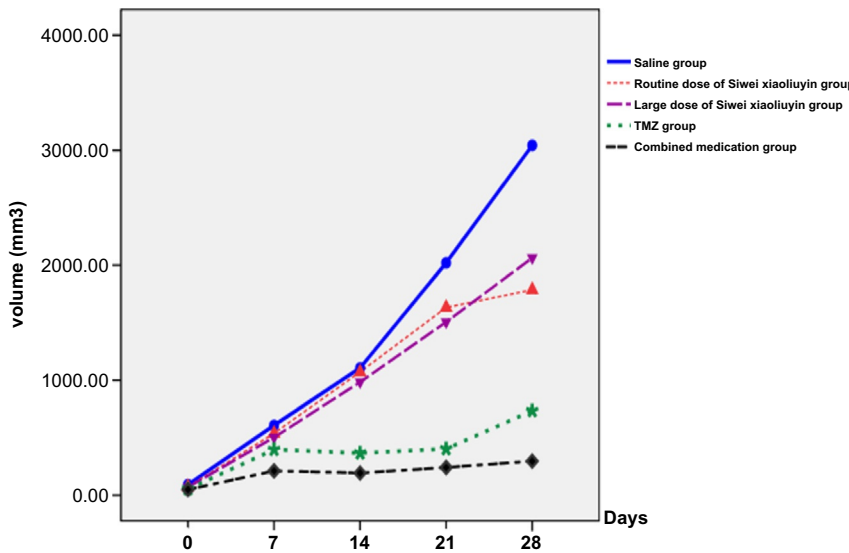

Fig. 1 Changes in body weight curve of nude mice at each time point in each group.

Table 1 MVD count of tumor tissues in nude mice.

Group	$-\bar{x} \pm s$
Saline group	25.1 ± 2.7
Routine dose of Siwei Xiaoliuyin group	20.7 ± 3.6
Large dose of Siwei Xiaoliuyin group	17.2 ± 3.9
TMZ group	24.7 ± 3.4
Combined medication group	15.4 ± 3.5

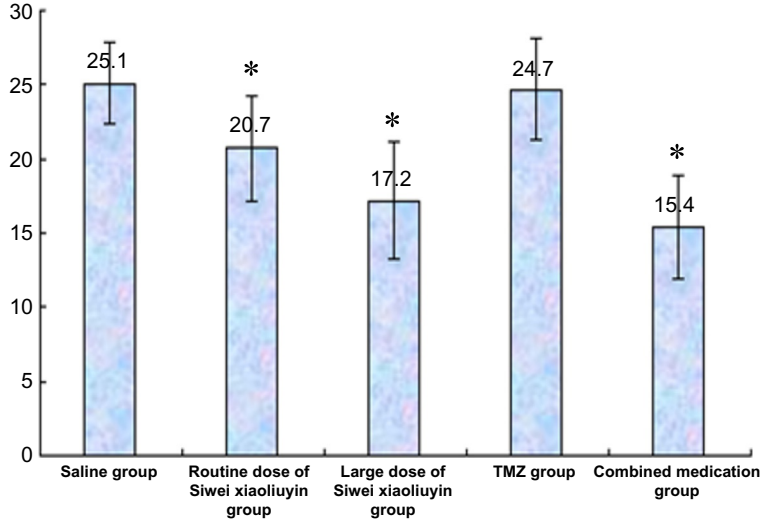

Fig. 2 MVD content in tumor tissues of nude mice.

4.3 Effect of Siwei Xiaoliuyin on the expression of VEGF in serum of nude mice

The serum VEGF content of nude mice was detected by ELISA method, and the serum VEGF content of nude mice was compared. The normal dose group, the large dose group and the combination group VEGF were less than the blank group. The difference was statistically significant ($P < 0.05$). It suggested that the Siwei Xiaoliu yin could reduce the serum VEGF content in nude mice, and had a certain dose effect relationship and Siwei Xiaoliuyin. The combination of temozolomide and drinking has synergistic effect on reducing serum VEGF level in nude mice (Table 2, Fig. 3).

Table 2 Serum VEGF content in nude mice (pg/mL).

Group	$-\bar{x} \pm s$	Max	Min	*M*
Saline group	253.5 ± 63.2	354.5	146.3	258.4
Routine dose of Siwei Xiaoliuyin group	176.2 ± 33.3	232.6	124.6	168.9
Large dose of Siwei Xiaoliuyin group	134.1 ± 35.0	194.5	98.9	126.4
TMZ group	258.1 ± 29.7	297.0	224.5	255.6
Combined medication group	103.4 ± 22.7	147.9	71.5	103.8

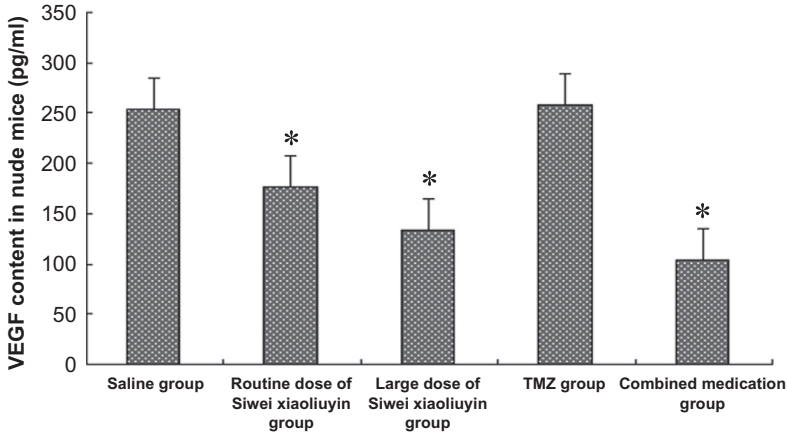

Fig. 3 Serum VEGF levels in nude mice.

4.4 Effect of Siwei Xiaoliuyin on the expression of ES in serum of nude mice

Determination of serum ES content in nude mice by ELISA. As shown in Table 3. The content of ES in the serum of the Siwei Xiaoliuyin group, the large dose group and the combination group was higher than that of the blank group, and the difference was statistically significant ($P < 0.05$). It showed that the Siwei Xiaoliuyin could increase the serum ES content in nude mice and had a certain dose effect relationship. The Siwei Xiaoliuyin combined with temozolomide could further improve the serum ES content in nude mice (Fig. 4).

5. Discussion

The growth and metastasis of tumors depend on the blood vessels. The tumor vessels provide oxygen and nutrients for the growth of the tumor.

Table 3 Serum ES content in nude mice (pg/mL).

Group	$-\bar{x} \pm s$	Max	Min	*M*
Saline group	114.3 ± 19.3	144.6	92.7	105.7
Routine dose of Siwei Xiaoliuyin group	152.1 ± 39.4	197.8	100.8	138.3
Large dose of Siwei Xiaoliuyin group	192.4 ± 31.4	236.9	152.5	180.1
TMZ group	119.8 ± 28.5	168.8	77.9	115.2
Combined medication group	218.4 ± 56.1	295.0	164.8	199.9

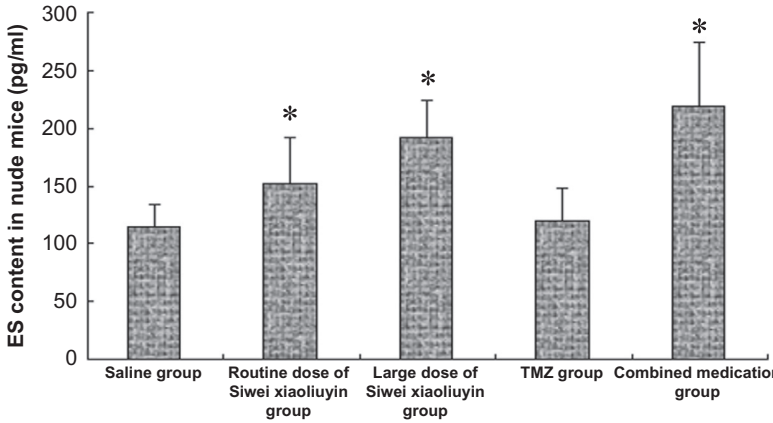

Fig. 4 Serum ES levels in nude mice.

They inhibit the angiogenesis of tumor, cut off the feeding of the tumor, and finally achieve the purpose of restraining the growth and metastasis of the tumor (Jhaveri, Chen, & Hofman, 2014). Therefore, it is important to explore the mechanism of angiogenesis and the influencing factors in the process of tumor formation. The treatment of tumor with the most important starting point of anti-tumor angiogenesis will be an important way to control the growth and migration of tumor, and it is of strategic significance for the treatment of tumor (Borhani & Bamdad, 2017; Sun, Guo, Su, et al., 2014).

Microvessel density (MVD) can directly respond to the angiogenesis of tumor. With the development of tumor angiogenesis, MVD has gradually become an independent prognostic indicator of solid tumors, and is also an important indicator for the detection and evaluation of the role of anti-tumor angiogenesis drugs. CD34 can be used to label vascular endothelial cells and is widely used in the detection of MVD. In this study, the CD34

antibody was used to detect the MVD count in the tumor tissues of nude mice by immunohistochemical method. The results of statistical analysis showed that the MVD of the tumor tissue of the Siwei Xiaoliuyin, the large dose group, the TMZ group and the combined group of nude mice was less than that of the blank group, and the difference was statistically significant ($P < 0.05$), which showed that the Siwei Xiaoliuyin was found. It can effectively reduce the MVD count in the tumor tissue and have a certain dose effect relationship. The combination of the Siwei Xiaoliuyin and TMZ can plays a synergistic role in the angiogenesis of tumor.

The process of neovascularization is very complex, and the basic steps are the balance between the angiogenesis factor and the inhibition of angiogenesis factor, the formation of angiogenesis, the degradation of the vascular endothelial basement membrane, the migration and proliferation of vascular endothelial cells, the endothelization of endothelial cells, and the branching of the endothelial cells. A vascular ring; forming a new basement membrane. Therefore, it is an important way to control the development and metastasis of tumor and to control the development and metastasis of tumor.

The formation of tumor vessels is regulated by a series of factors, including angiogenesis factors and angiogenesis factors, which play an important role in the development and development of tumor. It is mutually antagonistic to promote angiogenic factors and inhibit angiogenesis factors. Under normal conditions, these two kinds of factors maintain a relatively balanced state. When the tumor occurs, this equilibrium state is broken, making the tumor cells producing more angiogenesis substances, starting and promoting the formation of tumor vessels. VEGF is the most powerful angiogenic factor known at present. VEGF can specifically promote mitosis and increase vascular permeability factors in vascular endothelial cells, and exudate a large number of fibrin out of the blood vessels to promote the migration and proliferation of vascular endothelial cells, and then lead to tumor angiogenesis and primary swelling. Tumorigenesis, development and metastasis play an important role (Bhattacharya et al., 2016; Krcek, Matschke, Theis, et al., 2017; MeiYu, Zhong, et al., 2018; Xiang, Deng, Bao, et al., 2013). Endostatin (ES) can inhibit the proliferation of vascular endothelial cells, thereby slowing down the growth and metastasis of tumor. Our study confirmed that the Siwei Xiaoliuyin could reduce the VEGF and the expression of endostatin in the nude mice, which showed that it could inhibit the angiogenesis of glioma.

Glioma is a highly malignant tumor. Glioma cells have strong ability of proliferation, invasion and migration. There are many factors that promote

the progression and recurrence of glioma, which limits the treatment of glioma. It is of great significance to explore the role of traditional Chinese medicine in the treatment of glioma and to explore the role and mechanism of antiglioma angiogenesis in the treatment of glioma with antiglioma angiogenesis as a starting point.

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