Inhibitory effect of tetrandrine on angiogenesis

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Background and Objective: Angiogenesis plays an important role in tumorigenesis and metastasis. This study was to investigate the inhibitory effect and mechanism of tetrandrine on angiogenesis in vitro and in vivo. Methods: MTT assay was used to observe the effect of tetrandrine on the proliferation of human umbilical vein endothelial cells (HUVECs) and human colorectal carcinoma LoVo cells. Transwell migration assay and tube formation assay were used to observe the impact of tetrandrine on cell migration and tube forming ability of HUVECs. LoVo cells were transplanted into nude mice. MicrovesSEL density (MVD) was measured in nude mouse xenografts after intraarterial administration of tetrandrine. Results: The inhibition rates of tetrandrine (2–8 μg/mL) on the proliferation of HUVECs and LoVo cells were 24.6%–76.9% and 11.6%–14.0% after 48 h treatment. When HUVECs were incubated with 2.8μg/mL tetrandrine, the number of tubes was reduced and the lumen lost its integrity after 24 h; the number of migrating HUVECs was decreased after 12 h as compared with the control group (p < 0.001). Furthermore, MVD was much less in LoVo cell xenografts treated with 80mg/kg tetrandrine than in those treated with normal saline (p = 0.035). Conclusions: Tetrandrine effectively suppresses angiogenesis in vitro, which may be through inhibiting cell proliferation, migration and tube formation, inducing apoptosis and suppressing DNA synthesis of HUVECs. Tetrandrine also has an antiangiogenic effect on LoVo cell xenografts in nude mice.

Material and Methods

Cell lines. Human colorectal cancer cell line LoVo was preserved and cultured in our Lab. HUVECs were isolated from fresh human umbilical cord from the Department of Gynecology and Obstetrics of Drum Tower Hospital, Nanjing University.

Material and reagents. Tetrandrine (Tet) was provided by Jiangxi Yibo Medical Technology Company Ltd. EGM-2 was bought from Cambrex. PRMI, Fetal Bovine Serum and 0.25% trypsin were purchased from Gibco, USA. New born calf serum (NBS) was the product of Gibco, USA. Annexin V-FITC apoptosis detection kit was the product from Biovision. BALB/c/nu nude mice, male, aged 4–6 weeks with the body weight of 18–22 g were purchased from Shanghai Shilaike Company, and fed in a specific pathogen free (SPF) grade animal lab.

Methods. The primary culture of HUVECs. Under the sterile condition, the umbilical cord of approximately 20 cm long extracted from a newborn was flushed three times using sterile normal saline, followed by digestion with 0.25% trypsin for 7 min, and then centrifuged at 1,000 t/min for 5 min. Isolated HUVECs were collected and cultured in EMG supplemented with 10% FBS in an incubator with an atmosphere of 5% CO₂ at 37°C. The third to sixth passage cells were applied as test subjects in the following experiments. MTT assay. HUVECs were cultured with EMG-2 supplemented with 10% FBS, while LoVo cells were cultured with PRMI-1640 supplemented with 10% NBS. The cells in the logarithm phase of
growth were harvested. The density of the cells was adjusted to 2 x 10^4/mL. Cells were inoculated to a 96-well plate (200 μL/well). The culture medium was then replaced with fresh medium containing 2 μg/mL, 4 μg/mL, 8 μg/mL of Tet, respectively. Control group without Tet treatment and the blank group containing medium only were set up. Experiments were performed in triplicates. All wells were mixed thoroughly, placed in an incubator at 37°C for 48 h. MTT (20 μL/well) was added to each well. After incubation with MTT for 4h, 200 μL of DMSO was added to each well. When the crystal was completely dissolved, the plate was subject to measurement using an enzyme-linked immunoassay analyzer. The absorbance (A value) of each well was measured at 490/630 nm and the inhibition rate was calculated according to the formula: inhibition rate = (A value of the control group-A value of the experimental group) / (A value of control group - A value of the blank group) x 100%.

**Endothelial tube formation assay.** BD Matrigel™ matrix (300 μL) was added to each well of a 24-well plate to polymerize and form gel at 37°C. The suspension of HUVECs with a density of 2 x 10^5/mL was inoculated to a 24-well plate coated with Matrigel gel (500 μL/well). Different concentrations of Tet (2 μg/mL, 4 μg/mL, and 8 μg/mL respectively) were added into the culture medium. No drug was added in the control group. Cells were cultured at 37°C for 24 h. The formed tubes were observed under optic microscopy and recorded with a digital camera in five randomized chosen vision fields of every well.

**Transwell chamber assay.** On the lower surface of the Transwell chamber, 100 μL of fibronectin solution (10 μg/mL) was added and air dried. The surface was flushed with PBS (140 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, and 1.8 mmol/L KH_2PO_4, pH 7.3) to remove the surplus of the binding protein. HUVECs were cultured in EGM-2 supplemented with 0.5% FBS for 12 h, digested and then adjusted to a density of 4 x 10^5/mL. Cell suspension (50 μL) containing 0.5% EGM-2 was added to each inner chamber, and 2 μg/mL, 4 μg/mL, and 8 μg/mL Tet was added, respectively; 600 μL of 10% EGM-2 was added to the lower chamber. All of these chambers were incubated at 37°C for 12 h. Cells on the upper surface of the micro-porous membrane were removed using a swab; while cells on the lower surface were fixed with formalin for 30 min, rinsed with PBS, stained in crystal violet solution (1% of crystal violet, 2% of alcohol, 100 mmol/L of boric acid, pH 9.0) for 20 min and rinsed with PBS again. Subsequently, the micro-porous membrane was cut off, turned up side down and clung to a slide. The number of migrated HUVECs was counted under an optic microscope. Five randomized chosen vision fields were snapshot for each chamber with a digital camera.

**Measurement of apoptosis by flow cytometry.** HUVECs were digested with 0.05% trypsin after being treated with various concentrations of Tet (2 μg/mL, 4 μg/mL, 8 μg/mL) for 48 h. After rinse with PBS and centrifugation at 1000 r/min for 5 min, cells were resuspended in 500 μL buffer solution, incubated with 5 μL Annexin V-FITC and 5 μL PI. The mixture was well shaken and analyzed.

**Cell cycle distribution detected by flow cytometry.** After treatment with various concentrations of Tet for 48 h, HUVECs were digested with 0.05% trypsin, washed with PBS and the buffer solution, centrifuged at 1000 r/min for 5 min. The upper clear liquid was discarded and the solution A, B and C in the reagent kit were added in turn. The cells were incubated for 10 min (The one incubated with solution C should be kept away from light) and assessed by flow cytometry. Results were analyzed by software CellQuest.

**The establishment of the LoVo cell xenograft model in nude mice.** LoVo cells in the logarithm phase of growth were adjusted to a density of 1 x 10^7/mL. Cell suspension of 0.2 mL (approximately 2 x 10^6 cells) was subcutaneously injected into the left axilla of the mice. After approximately five days, tumor nodules started to appear. After 13 days, when the diameter of the tumor was larger than 5 mm, Tet was administered.

**The drug administration regimen for the nude mice bearing human LoVo cell xenografts.** On the 13th day of LoVo cell transplantation, the mice were randomized into five groups with five mice in each group. Tet was intragastrically administered every day. The mice were divided to three groups based on the concentration of Tet: 20 mg/kg group, 40 mg/kg group and 80 mg/kg group, respectively. The mice in the control group were administered with the same amount of distilled water. The mice in the positive control group were treated with 20 mg/kg cyclophosphamide (CTX).

**Measurement of microvessel density (MVD).** After the administration of Tet, the cancerous tissues were extracted from the nude mice, fixed in formalin, embedded with paraffin, cut into slices and stained using the SP immunohistochemistry technique. According to the manufacture’s protocol, anti-CD34 monoclonal antibody was used as the primary antibody. PBS replacing the anti-CD34 was regarded as negative control; while a known positive slide was used as positive control. The CD34 labeled intra-tumor capillaries and microvessels were measured. According to the criteria proposed by Weidner, a single cluster of brown endothelial cells was recorded as one separate microvessel, while vessels with thick muscular layers or vessels whose lumen were greater than the diameter of eight red blood cells were excluded. The whole section slide was firstly scanned under a microscope with 100 magnitudes to choose three fields with most densely distributed microvessels, which was the hot spots of neo-angiogenesis; then the number of microvessels was counted. The average microvessel number of three selected vision fields was taken as the microvessel number of the slide.

**Statistical analysis.** Values are shown as mean ± SD. Data were analyzed using software SPSS 13.0. p < 0.05 was considered as statistically significant.
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Figure 2. The effect of tetrandrine on the tube formation ability of HUVECs after 24h treatment (crystal violet×100). (A) Control group; (B) 8μg/mL tetrandrine group.

Figure 3. The effect of tetrandrine on the migration ability of HUVECs after 12h treatment (crystal violet×100). (A) Control group; (B) 8μg/mL tetrandrine group.

Figure 4. The effect of tetrandrine on microvessel density of LoVo cell xenografts in nude mice after the treatment of tetrandrine (IHC×100). (A) Control group; (B) 80μg/kg tetrandrine group.
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| Table 1 The effect of tetrandrine on apoptosis and cell cycle of HUVEC for 48 h |
|-------------------------|-----------------|
| Group                  | Cell apoptotic rate (%) | Cell proportion (%) |
|                        | G_0-G_1 | S | G_2-M |
| Control                | 6.25    | 74.16 | 17.83 | 8.01 |
| 2 μg/mL tetrandrine    | 12.32   | 92.31 | 4.63  | 3.06 |
| 4 μg/mL tetrandrine    | 30.90   | 93.03 | 4.65  | 2.32 |
| 8 μg/mL tetrandrine    | 35.55   | 97.01 | 1.59  | 1.40 |

Results

Effects of Tet on the cell proliferation of HUVECs and LoVo cells in vitro. As shown in Figure 1, Tet significantly inhibited the proliferation of HUVECs after 48 h treatment at the concentration of 2–8 μg/mL, compared with LoVo Cells. The inhibition effect was in a dose-dependent manner, (p < 0.05).

The inhibition effect of Tet on the tube formation of HUVECs in vitro. Compared with the control group, 2–8 μg/mL Tet inhibited the tube formation of HUVECs after 24 h treatment in a dose-independent manner (r = -0.933, p < 0.01). As shown in Figure 2, the number of tubes was (79.4 ± 5.2) μm, (56.4 ± 3.1) μm, (41.6 ± 1.8) μm, (15.6 ± 2.7) μm in the control, 2 μg/mL, 4 μg/mL and 8 μg/mL Tet group, respectively. Moreover, Tet treatment also affected the incompleteness of the tube.

The inhibition effects of Tet on the migration ability of HUVECs. As shown in Figure 3, compared with the control group (394.8 ± 54.2), the number of cells passing through the microporous membrane was markedly decreased in the 2 μg/mL, 4 μg/mL, and 8 μg/mL Tet-treated groups after 12 h treatment, which were (219.0 ± 10.8), (124.0 ± 13.4) and (33.6 ± 3.9) (p < 0.05). In the mean time, the proliferation inhibition rates of Tet on HUVECs were only 0.5%, 3.7% and 9.1% in 2 μg/mL, 4 μg/mL and 8 μg/mL Tet groups. The inhibition effect of Tet on the migration capacity of HUVECs was in a dose-dependent manner (r = -0.933, p < 0.01).

The effect of Tet on the apoptosis of HUVECs. As shown in Fig 1, the apoptotic rate in the control group was 6.25%, whereas it was 12.32%, 30.90% and 35.55% in the 2 μg/mL, 4 μg/mL and 8 μg/mL Tet groups (all p < 0.05). After the treatment with Tet, the number of HUVECs in G_0–G_1 phase increased, while the number of cells in S and G_2–M phases decreased. The inhibition effect of Tet on G_0–G_1 phase of HUVECs was in a dose-dependent manner (r = -0.922, p < 0.05).

The effects of Tet on the microvessel density of LoVo cell xenografts in nude mice. The number of microvessels was significantly less in the group treated with 80 mg/kg Tet (29.0 ± 6.8) than in the control group (44.0 ± 11.3), as shown in Figure 4 (p = 0.035); whereas the number of microvessels in the 20 mg/kg or 40 mg/kg Tet group was not statistically significant different from that in the control group (40.2 ± 11.1 and 38.6 ± 8.5 vs. 44.0 ± 11.3, p = 0.607 and 0.418, respectively). However, the number of microvessels in the Tet-treated group at the concentration of 80 mg/kg (29.0 ± 6.8) was similar to that in the 20 mg/kg CTX-treated group (25.2 ± 6.7), without significant differences (p = 0.400).

Discussion

Angiogenesis plays an important role in the progression of malignancy, invasion of cancer cells into vascular circulation, and the development and the rupture of metastatic lesions. Studies on the mechanism of anti-angiogenesis, and the research on the development of new drugs to counteract angiogenesis have brought hopes for the treatment of cancers.

There are some effective anti-angiogenic drugs, such as Avastin and Endostatin. However, most cancer patients in China can not afford the high cost of these drugs. Therefore, this study investigated the anti-angiogenic effect and the underlying mechanism of Tet, an ingredient extracted from a Chinese herb.

Numerous studies reveal that Tet could not only inhibit the growth of tumors directly, such as acting as a radio-sensitizer, alleviating the side effect of toxicity of chemotherapy, but also enhance the efficacy of drug therapy. It is also reported that, combined with cortisol, 1.72 mmol/L Tet could inhibit HUVECs to inhibit angiogenesis through inhibiting post-receptor IL-1 and PDGF-BB.

We found that Tet decreased the number of tubes and inhibited the formation of tubes in a dose-dependent manner within the concentration of 2–8 μg/mL, suggesting a marked anti-angiogenic effect of Tet. Moreover, Tet substantially reduced the migration of HUVECs in a dose-dependent manner, without significantly decreasing the proliferation of HUVECs. We suppose that Tet can effectively block tumor angiogenesis through its inhibition on migration of HUVECs. In addition, Tet induced apoptosis, and suppressed the synthesis of DNA in HUVECs in a dose-dependent manner, thus to inhibit angiogenesis and prevent the growth and metastasis of tumors.

Miller et al. proposed four criteria to screen out anti-angiogenic drugs in 2001. (1) Drugs with differential cytotoxicity, which means that the dose of the anti-angiogenic drug killing or inhibiting endothelial cells is lower than its toxic dose against tumor cells; (2) Drugs which could interfere the function of endothelial cell before it causes the death of those cells; (3) Drugs whose working mechanisms are clearly elucidated; (4) Drugs which can inhibit angiogenesis in vivo.

Our study showed that, below the subcytotoxic concentration, Tet apparently inhibited the proliferation and migration of HUVECs as well as the formation of tubes, induced the apoptosis of HUVECs and suppressed the formation of DNA in HUVECs. Tet meets the four requirements listed above to be considered as an anti-angiogenic drug. A small dose of CTX (20 mg/kg), which has been proven to have an anti-angiogenic effect, was used as the positive control. The in vivo study of Tet showed that, at the concentration of 80 mg/kg, Tet significantly decreased the number of microvessels as compared with the control group, though the inhibition effect of Tet was similar to 20 mg/kg CTX. These results suggest that 80 mg/kg Tet can suppress the angiogenesis in LoVo cell xenografts in nude mice.

In summary, in this study, we studied the anti-angiogenic activity of Tet in vitro, and the primary underlying mechanism. Tet probably is a cheap and toxicity free anti-angiogenic agent, which may be developed as an ideal anti-angiogenic drug for cancer therapy. Further studies are needed to verify the anti-angiogenic activity of Tet by a large number of experimental samples to further explore the mechanism of its anti-angiogenesis in vivo.
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References