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Homoharringtonine induces apoptosis of endothelium and down-regulates VEGF expression of K562 cells

YE Xiu-jin (叶琇锦), LIN Mao-fang (林茂芳)†

(*Department of Hematology, the First Affiliated Hospital, Medical College, Zhejiang University, Hangzhou 310003, China*)

†E-mail: jcai@mail.hz.zj.cn

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Abstract: Homoharringtonine (HHT) has currently been used successfully in the treatment of acute and chronic myeloid leukemias and has been shown to induce apoptosis of different types of leukemic cells in vitro. Emerging evidence suggests that angiogenesis may play an important role in hematological malignancies, such as leukemia. However, whether HHT can relieve leukemia by anti-angiogenesis is still unknown. We investigated the anti-angiogenesis potential of HHT with the human umbilical vein endothelial cell line (ECV304) and leukemic cell line (K562) in vitro. Cellular proliferation was determined by MTT assay and apoptosis was analyzed by flow cytometry. The mRNA expression of vascular endothelial growth factor (VEGF) was assessed by RT-PCR and VEGF protein production was detected by Western blot. Inhibition of cell proliferation and induction of apoptosis by HHT were discovered in ECV304 cells, and appeared in a dose- and time-dependent manner. Also, treatment with HHT caused down-regulation of VEGF mRNA expression in K562 cells in similar dose- and time-dependent manner and inhibition of VEGF protein production in K562 cells in response to the enhancing concentration of HHT. The results demonstrated that HHT could also induce apoptosis in endothelium and down-regulate VEGF expression in K562 cells. In conclusion, we believe HHT has anti-angiogenesis potential and speculate that HHT might exert its anti-leukemia effects via reduction of angiogenesis.

Key words: Homoharringtonine (HHT), Leukemia, Angiogenesis, VEGF
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INTRODUCTION

Homoharringtonine (HHT) is a cephalotoxin alkaloid with anti-leukemic activity and had been used successfully in the treatment of acute and chronic myeloid leukemias (O'Brien *et al*., 1995; 1999; Feldman *et al*., 1992). The principal mechanism of action by HHT is the inhibition of protein synthesis in a dose- and time-dependent manner by binding to ribosome and inhibiting polypeptide chain elongation (Tujebajeva *et al*., 1989; Zhou *et al*., 1995). HHT had been shown to induce apoptosis in different types of leukemic cells in vitro and the HHT-induced apoptotic cascade is characterized cytochrome c release and caspases activation (Cai *et al*., 2001).

It had been established that angiogenesis, the process of new blood vessel formation, is very important for the growth and viability of solid tumors. Recent studies suggested that, as in solid tumors, angiogenesis may play a crucial role in hematological malignancies, such as leukemia (Perez-Atayde *et al*., 1997; Padro *et al*., 2000). Tumor growth requires expansion of existing endothelium. It had been demonstrated that activated endothelial cells can release a variety of cytokines that may stimulate leukemic cell growth. Leukemic cells, in turn, have the capacity to release endothelial growth factors, such as VEGF (Fiedler *et al*., 1997). Therefore, leukemic blasts may be able to support

their own development both by promoting neoangiogenesis and via an autocrine loop (Bellamy *et al*., 1999).

In this study, we investigated the anti-angiogenesis potential of HHT through inducing apoptosis of endothelium and down-regulating VEGF expression of K562 cells.

MATERIALS AND METHODS

Reagents

HHT was obtained from Mingsheng Pharmagen (Zhejiang, China). HHT stock solution was prepared at a concentration of 1 mg/ml in distilled water and kept at 4 ºC. Further dilutions to working concentrations were made before use.

Cell lines and cell culture

Human umbilical vein endothelial cell line (ECV304) was obtained from the American Type Culture Collection, and the leukemia cell line K562 was from the Shanghai Institute of Cell, Chinese Academy of Sciences, Shanghai, China. Both cells were cultured in RPMI 1640 standard medium containing 2 mmol/L L-glutamine and supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 unit/ml penicillin and 100 unit/ml streptomycin in humidified atmosphere 100% air and 5% CO_2 at 37 ºC.

Cell proliferation assay

Cellular proliferation was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide] assay. ECV304 Cells (5×10^3 cells/well) were incubated in sextuplicate in 96-well plates. Different concentrations of HHT were added to each well for 20−68 h, then, 20 μl of MTT (5 mg/ml)(Sigma) was added to each well for an additional 4 h. The reaction was stopped by adding 200 μl of DMSO and the absorbance (A) at 570 nm were determined on a multiwell plate reader (Bio-Rad).

$$\text{Cell inhibitation rate} = 100\% \times \frac{\text{Experimental group } A \text{ values} - \text{control group } A \text{ values}}{\text{control group } A \text{ values}}$$

Flow cytometry analysis

A total 1×10^6 ECV304 cells were harvested after HHT treatment at different time points with a different final concentration. The cells were washed twice with cold PBS and resuspended in 2 ml of 70% ethanol and kept at 4 ºC overnight; and subsequently rinsed with PBS twice, and incubated with 100 μl Rnase (10 mg/ml). Finally, the cells were stained with propidium iodide (PI) 500 μl (0.05 mg/ ml). Distribution of cell cycle and the rate of apoptosis were determined by flow cytometry (Becton Dickinson). All data analysis were performed by using Multicycle 3.0 software (Becton Dickinson).

RNA extraction and RT-PCR for VEGF

Total cellular RNA was extracted using the Trizol RNA extraction kit (Gibco BRL) according to the manufacturer's instructions. cDNA synthesis was performed using the Reverse Transcription System (Gibco BRL) which supplied the random sexamer primers. For PCR reactions the following primers were used: β-actin (sense: 5'-CGC TGC GCT GGT CGT CGA CA-3'; antisense: 5'-GTC ACG CAC GAT TTC CCG CT-3') and VEGF (sense: 5'-TCG GGC CTC CGA AAC CAT GA-3'; antisense: 5'-CCT GGT GAG AGA TCT GGT TC-3'), produce 619 bp products for β-actin and 516 bp, 648 bp, 720 bp, 771 bp for VEGF, respectively. The above primers were designed according to reference (Fiedler *et al*., 1997) and synthesized by the Shanghai Shenggong Biological Engineering Company. RT-PCR for VEGF was performed with initial denaturation at 94 ºC for 4 min, followed by 35 cycles (1 min denaturation at 94 ºC, 1 min annealing at 60 ºC and 90 sec extension at 72 ºC) and with an additional extension at 72 ºC for 10 min in a final volume of 25 μl. The cycle number for β-actin was 25, as VEGF. PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light and photographed with Kodak gel automatic photography. A gel thin-layer scan for semiquantitive assay was done. The relative content of each expected product and β-actin were determined by the ratio of corresponding optic density.

Protein extraction and Western blotting

Total cell extracts from K562 were obtained by lysing the cells in cold RIPA buffer in the presence of protease inhibitors (1 mg/ml aprotinin, and 1 mmol/L PMSF). After centrifugation to remove cell debris, equal amounts of protein (30 μg/well) were resolved by (SDS-PAGE) and transferred to nitrocellulose membrane. The membrane was blocked in 5% nonfat dry milk/PBS with 0.02% sodium azidobenzyl for 1 h at room temperature and incubated with primary rabbit antihuman antibodies, VEGF polyclonal antibody (Santa Cruz), then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz) as the secondary antibody. The membrane was processed and developed as the manufacturer suggested (ECL Western Blot Analysis System, Amersham).

Statistical analysis

All experiments were performed in triplicate unless otherwise noted. Comparison and relationship of means were performed by univariate analysis of variance and bivariate correlation procedure, respectively. $P<0.05$ was considered to indicate statistical significance. All calculations were performed by SPSS for Windows, Version 10.0.

RESULTS

Effect of HHT on proliferation of ECV304 cells

To investigate the effect of HHT on the proliferation of ECV304 cells, the cells were cultured with 0.01−2.00 μg/ml rang concentrations of HHT for 24 to 72 hours and cell proliferation was assessed by MTT. Our data showed that HHT markedly inhibited the ECV304 cells proliferation. Treated with HHT at different concentrations for the same time, the growth inhibition rate of the ECV304 cells increased with increased concentration of HHT (F=202.579, P=0.000). And treated with HHT at the same concentration, the growth inhibition rate increased with prolongation of the time (F=98.363, P=0.000). MTT assay demonstrated that HHT had direct cytotoxic effects on ECV304 cells (Fig.1). The effect was in a time- and dose-dependent manner.

Increased apoptosis of ECV304 treated by HHT

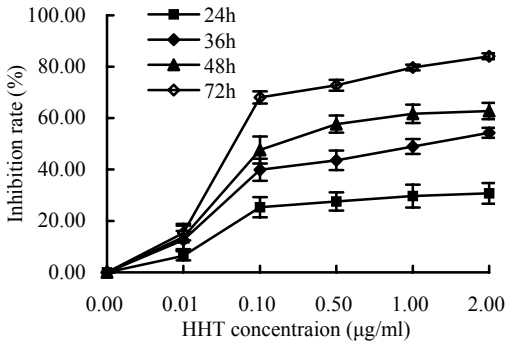

Fig.1 Proliferation inhibition of ECV304 cells by HHT with different concentrations and different time points. After having stopped the reaction, the absorbency at 570 nm was measured by the microplate reader

To demonstrate that the growth inhibition on endothelial cells by HHT was caused by induction of apoptosis, ECV304 cells were incubated with different concentrations of HHT (0.01−2.00 μg/ml), stained with PI and analyzed by flow cytometry. The results indicated that HHT alter the cell cycle of ECV304 cells, showing typical sub-diploid apoptosis peaks before G1 phase and a time- and dose-dependent increase in apoptotic cells. At 0.10 μg/ml concentration tested, nearly 20% of the cells showed apoptosis, and at 1.00 μg/ml concentration approximately 45% of the population underwent apoptosis after 72 hours (Fig.2, see the next page).

Effect of HHT on VEGF mRNA and protein expression of K562 cells

To show whether the HHT on leukemic cells were involved in VEGF mRNA expression, RT-PCR evaluation of VEGF mRNA level was carried out in K562 cells. The treatment with 0.01−2.00 μg/ml concentration HHT for 12 h, down-regulated the transcriptional expression of VEGF splice variant genes (Fig.3). There was correlation between the VEGF mRNA expression (VEGF/β-actin) and HHT concentration (γ=−0.842, P=0.036), and the time of HHT treatment, too (γ=−0.882, P=0.009) (data not shown).

Furthermore, we used Western blot to evaluate the VEGF protein production in the K562 cells. In agreement with the above results, HHT at 0.01−2.00 μg/ml concentration could down-regulate the level of VEGF protein in a dose-dependent manner after 24 h (Fig.4).

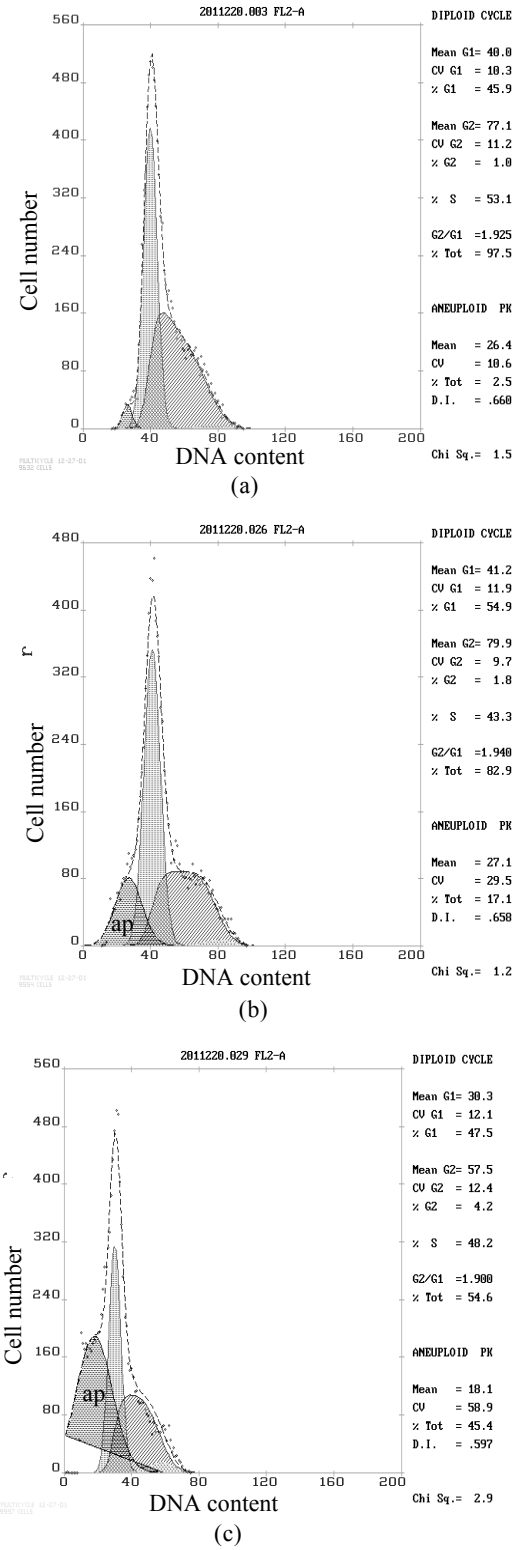

Fig.2 Flow cytometry analysis for apoptotic percentage of ECV304 cells after treatment with different concentration of HHT for 72 h

(a) control; (b) HHT 0.10 μg/ml; (c) HHT 1.00 μg/ml; 'ap' represents apoptotic cells

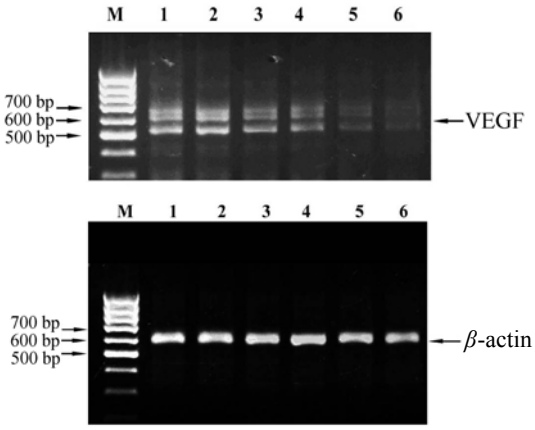

Fig.3 VEGF mRNA expression in K562 cells after treatment with different concentration of HHT for 12h

β-actin used as an internal control. M: marker; lanes 1−6: control, 0.01, 0.10, 0.50, 1.00, 2.00 (μg/ml) HHT, respectively

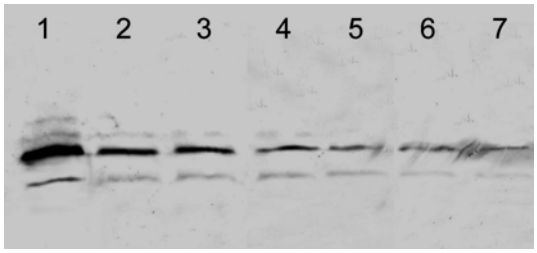

Fig.4 Western blot analysis for VEGF protein in K562 cells after treatment with different concentration of HHT for 24 h

lanes 1−7: control, 0.05, 0.10, 0.25, 0.50, 1.00, 2.00 (μg/ml) HHT, respectively

DISCUSSION

More recently, accumulating evidence linked angiogenesis to the pathophysiology of leukemias, as in the tumors and, therefore, an anti-angiogenic strategy may be effective as antileukemia therapy. Several reports indicated that some conventional chemotherapeutic agents, such as arsenic trioxide (Roboz *et al*., 2000), cyclophosphamide (Browder *et al*., 2000), vinblastine (Vacca *et al*., 1999) had the potential of anti-angiogenic activity, which might be one of their anti-leukemic mechanisms.

In this context, we tried to investigate the anti-angiogenic potential of HHT. The results first demonstrated that HHT could inhibit proliferation and induce apoptosis of ECV304 cells in a dose- and

time-dependent manner. Analysis of cell cycle phase distribution showed that the percentage of cells in subdiploid apoptotic peak increased coincidently with the drug concentration and the time, which suggested that the inhibition effect of HHT on cell proliferation could be attributed mainly to the induction of apoptosis.

Perez-Atayde *et al.*(1997) first noted increased microvessel density in the bone marrow of children with acute lymphoblastic leukemia. A similar phenomenon occured in the bone marrow of adult patients with acute myeloid leukemia (Padro *et al*., 2000). We also found increased vascularity in bone marrow biopsy specimens from adult patients with different types of acute leukemia; and that leukemic blasts could secret VEGF (manuscript in preparation). Release of VEGF by leukemic blasts might be an important mediator of angiogenesis in the bone marrow. In our study, we found HHT could decrease the VEGF protein production and mRNA expression using K562 cells as an *in vitro* model. Therefore we believe one of HHT's anti-leukemic mechanisms might be reduction of the signals stimulating the angiogenesis and induced apoptosis of the vascular endothelium.

Angiogenesis is a possible therapeutic target in hematologic tumors. And now, a lot of anti-angiogenesis agents have been tested in preclinical models and clinical trials alone or in combination with established drug regimens. Anti-angiogenesis therapies for hematologic tumors may be a new strategy and a kind of antitumor agents combining both anti-angiogenic and cytostatic or cytotoxic effect might have bright prospects. The desirable dose of HHT to prevent tumor-associated endothelial cell proliferation in vivo and its molecular mechanism await further study.

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