

RESEARCH ARTICLE

Growth Inhibition and Apoptosis Induction of Human Umbilical Vein Endothelial Cells by Apogossypolone

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Abstract

Aims and Background: Prostate cancer is one of the most common malignant tumors in the male reproductive system, which causes the second most cancer deaths of males, and control of angiogenesis in prostate lesions is of obvious importance. This study assessed the effect of apogossypolone (ApoG2) on proliferation and apoptosis of human umbilical vein endothelial cells (HUVECs). **Subjects and Methods:** HUVECs were treated with different concentrations of ApoG2. The survival rate of HUVECs were determined by MTT assay. Ultrastructural changes of HUVECs were assessed with transmission electron microscopy. Apoptosis in HUVECs was analyzed by flow cytometry and cell migration by Boyden chamber assay. Matrigel assays were used to quantify the development of tube-like networks. **Results:** ApoG2 significantly inhibited HUVEC growth even at 24 h ($P < 0.05$). The inhibitory effect of ApoG2 is more obvious as the concentration and the culture time increased ($P < 0.05$). These results indicate that ApoG2 inhibits the proliferation of HUVECs in a time- and concentration-dependent manner with increase of the apoptosis rate. Besides, ApoG2 reduced the formation of total pseudotubule length and network branches of HUVECs. **Conclusions:** The results suggest that ApoG2 inhibits angiogenesis of HUVECs by growth inhibition and apoptosis induction.

Keywords: Apogossypolone - human umbilical vein endothelial cell - cell apoptosis

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Introduction

Prostate cancer, one of the most common malignant tumors in the male reproductive system, causes the second most deaths of males. The mortality rate resulted from this cancer is only lower than that caused by lung cancer. Angiogenesis is an important process in growth, development, and in wound healing and granulation tissues. It is known that tumor expansion with formation of new blood vessels are related to metastasis (Weidner et al., 1991; Weidner et al., 1993; Holash et al., 1999; Hood et al., 2002). Therefore, the use of angiogenic inhibitors in order to reduce tumor metastasis is a promising method for treating tumors (O'Reilly et al., 1994; Konno et al., 1995; Zetter, 1998; Ebos et al., 2009; Pàez-Ribes et al., 2009). Prostate cancer is related to angiogenesis and vascular invasion and thus inhibition of angiogenesis can be used as a method to treat prostate cancers (Weidner et al., 1993; Bagley et al., 2011; Gyftopoulos et al., 2011; Assadian et al., 2012; Lynch et al., 2012; Pande et al., 2012; Pinto et al., 2012). We previously found that ApoG2, a gossypol derivative, inhibits proliferation of prostate cancer cells both in vitro and in vivo and induces their apoptosis and autophagy (Zhang et al., 2010a; Zhang et al., 2010b). Although it is reported that the anti-tumor effect of

ApoG2 in vivo is partially associated with the decrease of the microvessel density (Zhang et al., 2010a), the exact mechanism still remains unknown.

ApoG2 belongs to a new type of semi-synthesized gossypol derivative. Fluorescence polarization analysis indicated that ApoG2 is a small molecule antagonist of Bcl-2, an inhibitor of apoptosis. ApoG2 has stronger affinity and higher anti-tumor activities than gossypol (Shelley et al., 1999). Recent studies revealed that ApoG2 could also block the proliferation of liver cancers (Mi et al., 2008), nasopharyngeal (Hu et al., 2008) and lymphoma (Arnold et al., 2008; Sun et al., 2008) cells. Considering that ApoG2 could inhibit angiogenesis of tumors, which fundamentally depends on the proliferation of vascular endothelial cells, we aim to probe whether ApoG2 inhibits angiogenesis by targeting endothelial cells. In this study, we investigate whether ApoG2 inhibits proliferation of human umbilical vein endothelial cells (HUVECs) to provide experimental evidence for the application of ApoG2 as a new anti-tumor drug.

Materials and Methods

Cell culture

HUVECs were obtained from American Type Culture

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Collection (ATCC, Manassas, VA, USA), and cultured with DMEM medium complemented with 10% fetal bovine serum in a humidified incubator (5% CO₂) at 37°C. ApoG2 was synthesized by modifying gossypol's two aldehyde groups and prepared at a stock concentration of 0.20 mmol/L in DMSO and obtained from Xi'an Jiaotong University (Xi'an, China).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay

Cell viability was determined by using the MTT assay. Briefly, 2×10⁵/ml HUVECs were seeded onto 96-well plates and incubated for 24h at 37°C in a 5% CO₂ culture incubator. Cells were treated with ApoG2 at the final concentration of 0, 0.01, 0.02 and 0.04 mmol/l, respectively, with triple wells for each group. After incubation of 24, 48 or 72h, 20 µl MTT reagent (5 mg/ml) was added to each well and cells were incubated for 4h. Then the formazan precipitate was dissolved in 150 µl DMSO and the absorbance value was measured in a microplate reader at the wavelength of 570 nm. The cell viability rate was calculated as the percent of viable cells relative to the control using the equation 100% × A/A₀, in which A is the mean absorbance value of the sample cells and A₀ is the mean absorbance value of the control samples without ApoG2 treatment.

Ultrastructure observation by transmission electron microscopy

Briefly, 2×10⁵/ml HUVECs were seeded and incubated for 24 h at 37°C in a 5% CO₂ culture incubator. ApoG2 was added to a final concentration of 0.01, 0.02 and 0.04 mmol/l, respectively. After incubation for 48 h, cells were harvested with serum-free DMEM and fixed in 3% glutaraldehyde for 4h. Then the cells were washed twice with PBS, fixed in 1% osmic acid for 1h, dehydrated and embedded. The 70 nm-thick slices were prepared and observed under transmission electron microscope after staining with uranyl acetate and lead citrate.

Flow cytometric analysis of apoptosis

Cells were harvested and washed twice with PBS. 1×10⁶ HUVECs were incubated in PBS with 1 µg/ml Annexin V-FITC and 1 µg/ml propidium iodide at room temperature in dark for 10 min. Apoptotic cell death was assessed by flow cytometric analysis using the CellQuest software (Becton Dickinson, NJ,USA). The tests were performed in triplicate.

Cell migration assay

Boyden Chamber Transwell was used to evaluate cell migration ability. The polycarbonate filter of the inner chamber was coated with 1% gelatin and equilibrated with serum-free DMEM for 1h. HUVECs were harvested and re-suspended in DMEM medium supplemented with 20% FBS and ApoG2 at the indicated concentrations. Then aliquots (1.5×10⁵ cells) of prepared cell suspension were added into the inner chamber and the outer chamber was filled with 0.6 ml of DMEM containing fetal bovine serum. Cells were fixed with 90% methanol for 10 min after they were incubated at 37°C for 6h. After the removal of cells

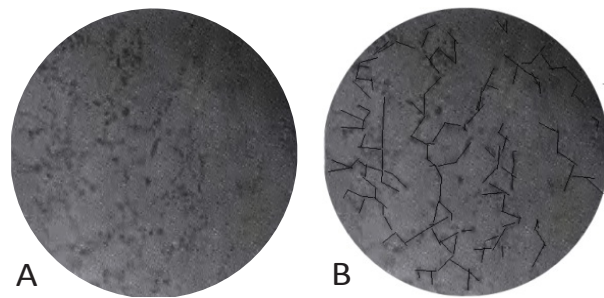


Figure 1. Analysis of Angiogenesis activity. (a) Typical appearance of HUVECs on Matrigel after 24 h. (b) Traces of pseudotubules using Microsoft Palette Manager to facilitate the quantification of the total pseudotubules and the network branches

on the upper side of the membrane by using a cotton swab, the cells were stained with crystal violet for 10 min and washed with PBS. Then the cells were observed using a light microscope at 200× magnification. Finally the cells were treated with 10% ethonal for 10 min, and the absorbance was measured at 600 nm using a microplate reader.

Angiogenesis assessment

Growth factor-free matrigel recovered to 0 °C was added to 96-well microplate and maintained at 37°C for 1 h. HUVECs of 5×10⁵/ml was incubated for 24h with 50µl ApoG2 at the concentration of 0.01mmol/l, 0.02 mmol/l or 0.04 mmol/l, respectively. Then the cells were cultured for 3h after the addition of 50 µl MTT of 1.25 mg/ml, and the graphs were collected using an inverted microscope. The pseudotubules were traced using Microsoft Palette Manager. The total pseudotubules and the network branches were quantified (Figure 1).

Stastical analysis

SPSS (Chicago, IL, USA) version 11.0 for Windows was used for all statistical analyses. All data were presented as mean±SD. ANOVA followed by the Tukey test or Dunnett's test were used to analyze the significance of any difference between different groups. P < 0.05 was considered statistically significant.

Results

ApoG2 inhibits the proliferation of HUVECs

The MTT assay was used to evaluate the effect of ApoG2 on the proliferation of HUVECs. As shown in Figure 2, ApoG2 significantly inhibited cell growth even at 24h (P<0.05). The inhibitory effect of ApoG2 is more obvious as the concentration and the culture time increases (P<0.05). The results show that ApoG2 inhibits the proliferation of HUVECs in a time- and concentration-dependently manner.

ApoG2 alters the ultrastructure of endothelial cells

The transmission electron microscope was used to evaluate the effect of ApoG2 on the ultrastructure of cells. As shown in Figure 3A, normal HUVECs has several microvilli on the surface, and organelles such as Weibel-Palade bodies, endoplasmic reticulum and mitochondria were also visible in the cytoplasm. Besides, it could be

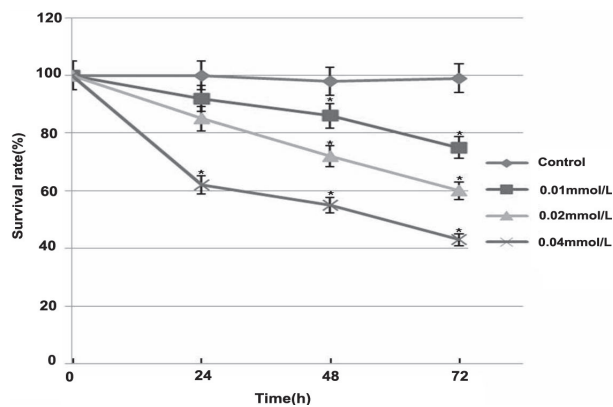


Figure 2. Effect of ApoG2 on Cell Viability of HUVECs. HUVECs were treated without or with different concentrations of ApoG2 for 24, 48, 72 h. Cell viability was evaluated with MTT assay. All results were expressed as mean \pm S.E.M. of three independent experiments. * $P < 0.05$ compared with control

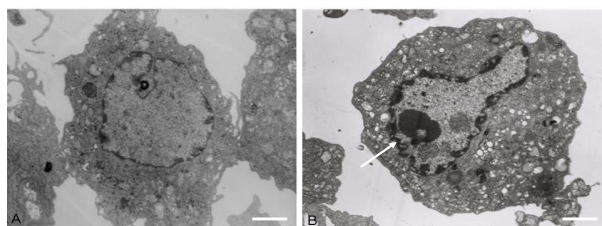


Figure 3. Effect of ApoG2 on the Ultrastructure of HUVECs. A, Ultrastructure of normal HUVECs; B, Ultrastructure of HUVECs after treatment with 0.02 mmol/l ApoG2 for 48h. The arrow indicates a representative apoptotic nucleus. Bar=500 nm

notable that the nucleus displays an irregular shape, the chromatin were mainly euchromatin, and inter-cellular connections were obvious. However, after the treatment of ApoG2, HUVECs displays a phenotype of apoptotic cells (Figure 3B). The intercellular connections and the microvilli on the surface disappeared, and the edge of the cells becomes smooth with some apparent bubble-shaped projection. The nucleic chromatic were aggregated along the margin of the nuclear membrane, and apoptotic bodies were also observed.

ApoG2 increases the apoptotic rate of HUVECs

During apoptosis, the negatively charged phospholipid phosphatidylserine (PS) redistributed from the inner to the outer leaflet of the plasma membrane. To observe the inhibitory mechanism of ApoG2 on HUVECs, we used Annexin V-FITC which binds to PS to mark apoptotic cells, and propidium iodide (PI) was used to stain dead cells. The effect of ApoG2 treatment for 48h on the apoptotic rate of HUVECs was measured by flow cytometry. Compared with the control group, HUVECs treated with ApoG2 at the concentration of 0.01 mmol/l, 0.02 mmol/l, 0.04 mmol/l show apparent apoptosis and necrosis (Table 1). Besides, the apoptotic rate increases with the increase of ApoG2 concentration.

ApoG2 inhibits the migration rate of endothelial cells

To investigate whether ApoG2 inhibits angiogenesis, we examined the effect of ApoG2 on the migration rate of HUVECs considering that the migration of endothelial cells is the basis of angiogenesis. We found that HUVECs

Table 1. Apoptosis Rate of HUVECs

Groups	Average apoptosis rate (%)
Control group	1.4 \pm 0.3
0.0 1mmol/l ApoG2	13.2 \pm 1.5*
0.02 mmol/l ApoG2	25.1 \pm 3.2*
0.04 mmol/l ApoG2	62.0 \pm 5.3*

* $P < 0.01$ vs control group

Table 2. Effect of ApoG2 on Migration of HUVECs ($x \pm s$)

Groups	n	Density (OD)	Inhibition rate (%)
Control group	6	1.5 \pm 0.4	0
0.0 1mmol/l ApoG2	6	0.9 \pm 0.2	40.0 \pm 7.3*
0.0 2mmol/l ApoG2	6	0.7 \pm 0.1	53.3 \pm 3.4* Δ
0.0 4mmol/l ApoG2	6	0.5 \pm 0.1	66.7 \pm 5.4* Δ

* $P < 0.05$ vs control group; $\Delta P < 0.05$ vs 0.0 1mmol/l ApoG2

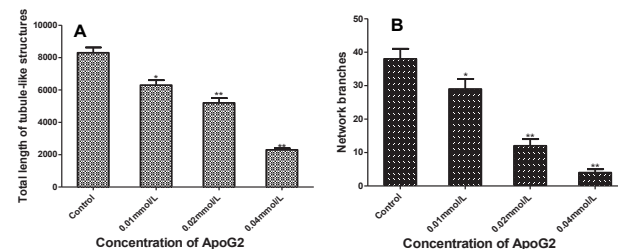


Figure 4. Effect of ApoG2 on the Pseudotubules and Network Branches of HUVECs. The length of pseudotubules (A) and the number of network branches (B) without or with the treatment of ApoG2 at the indicated concentrations were analyzed

migrates through the membrane of the Boyden Chamber Transwell under the stimulation of 20% serum. However, the migration of HUVECs was significantly inhibited by ApoG2, and the inhibitory effect of ApoG2 was concentration-dependent (Table 2). These results suggest that ApoG2 could inhibit the angiogenesis of HUVECs.

ApoG2 inhibits the formation of pseudotubules of endothelial cells

The formation of vessel-like structures in endothelial cells is the basis for angiogenesis. To probe the mechanism of the inhibitory effect of ApoG2, we investigated the ability of HUVECs to form vessel-like structures. The results show that HUVECs could differentiate well and formed capillary-like pseudotubules after 24h. However, after the treatment of ApoG2 for 24h, HUVECs clustered together with the significant decrease of vessel-like structures. The decrease of length and branches of the vessels was more obvious as the concentration of ApoG2 increases (Figure 4). No complete vessels were formed with the treatment of 0.04 mmol/L ApoG2, and the network branches became sparse and even completely disappeared in some areas.

Discussion

Angiogenesis is a complex, dynamic process that involves multiple pathways that converge to affect carcinogenesis, proliferation, and tumor growth. As the basis for tumor growth and metastasis, the angiogenesis of tumors is the fundamental cause of tumor growth, invasion

and metastasis (Fidler and Ellis, 1994; Hanahan, 1997; Risau, 1997). Extensive studies on angiogenesis in prostate cancer to date have revealed that angiogenesis plays a role in the progression of prostate cancer (Abdollahi et al., 2003). And it has been reported that microvessel density, a measurement of prostate cancer angiogenesis, has been shown to be a predictor of metastasis and survival (Weidner et al., 1993). It is shown that the microvessel density in prostate cancer tissues is significantly higher than normal prostate, and the microvessel density in metastatic prostate cancer is higher than that in non-metastatic cancer (Brawer et al., 1994; Mucci et al., 2009; Aragon-Ching et al., 2010). Thus, targeting angiogenesis has been the subject of several clinical investigations. In our previous studies, we have found that Apog2 could inhibit the angiogenesis of microvessels in prostate cancer.

Angiogenesis occurs via the proliferation of vascular endothelial cells. Therefore, screening anti-tumor drugs by targeting endothelial cells has attracted the attention of world-wide pharmacologists in recent years. Based on our previous finding that ApoG2 has an inhibitory effect on angiogenesis, we explored its potential use as an anti-tumor drug by checking its effect on endothelial cells. We found that ApoG2 could inhibit the proliferation of HUVECs and induce their apoptosis. Besides, ApoG2 inhibits the migration of endothelial cells, and both the formation of pseudotubules and network branches were significantly reduced. Our study demonstrate that ApoG2 could inhibit angiogenesis of endothelial cells by inhibiting cell proliferation, inducing apoptosis, reducing cell migration and the formation of pseudotubules.

Various apoptotic signaling pathways are involved in cell apoptosis. However, it remains unclear how ApoG2 induces the apoptosis of endothelial cells. Studies have shown that ApoG2 is a pan-active inhibitor of B-cell lymphoma (Bcl-2) proteins (Carmeliet et al., 1996; Zhang et al., 2009). It is also reported that ApoG2 could induce the production of reactive oxygen species (Ferrara et al., 1996). However, whether these are related with the apoptosis of endothelial cells still awaits further investigation.

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