Total glucosides of *Paeonia lactiflora* Pall inhibit vascular endothelial growth factor-induced angiogenesis

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**ABSTRACT**

Objective: To evaluate the anti-angiogenesis effect of total glucosides of *Paeonia lactiflora* Pall.

Methods: In this study, we determined the effect of TGP on the proliferation of human vascular endothelial cells through 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and fluorescence-activated cell sorting analysis. A migration assay and a tube formation assay were used to investigate the migration properties and tube formation abilities of human vascular endothelial cells after being treated with TGP. Furthermore, the in vivo anti-angiogenic ability of TGP was determined through a chick chorioallantoic membrane assay.

Results: TGP (12.5, 62.5, and 312.5 μg/ml) resulted in a dose-dependent reduction in the proliferation of endothelial cells. This inhibition effect began 6 h after treatment and lasted at least 24 h. Fluorescence-activated cell sorting analysis data showed an accumulation of cells in the G0/G1 phase of the cell cycle, which exhibited apoptotic features indicative of cell death. The migration properties and tube forming abilities of endothelial cells were dramatically inhibited by the TGP extract.

Conclusion: Our results show that TGP can inhibit angiogenesis in vitro and in vivo.

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1. Introduction

Total glucosides of *Paeonia lactiflora* Pall (TGP) is a new drug extracted from the traditional Chinese herb, *Paeonia lactiflora* Pall (Chang et al., 2008; Xu et al., 2007; Zheng and Wei, 2005; Zhu et al., 2005; Wu et al., 2009). While the underlying pharmacological mechanisms of this new drug are still a matter of debate, TGP has been widely used in the treatment of rheumatoid arthritis (RA) and other rheumatoid diseases in China. Previous studies have mainly focused on the anti-inflammatory effects of TGP, but some data have suggested that it also has anti-angiogenesis properties (Chang et al., 2008; Xu et al., 2007; Zheng and Wei, 2005; Zhu et al., 2005; Wu et al., 2009). RA is currently considered as an angiogenic disease because it is associated with active tissue neovascularization. The perpetuation of angiogenesis involves numerous soluble and cell surface-bound mediators, which are also abundantly produced in the arthritic synovium (Szekanecz and Koch, 2007; Lainer-Carr and Brahn, 2007; Costa et al., 2007; Maruotti et al., 2006). Zhu et al. (2005) found that TGP treatment can alleviate synovial hyperplasia and pannus in a rat arthritis model. A more detailed study confirmed that TGP can downregulate the expression of many pro-angiogenic factors in synoviocytes from this rat arthritis model, including vascular endothelial cell growth factor (VEGF), basic fibroblast growth factor (bFGF) and matrix metalloproteinases (MMPs) (Chang et al., 2008). To date, the direct effect of TGP on angiogenesis has not been fully studied. In this research, we first investigated the effect of TGP on the proliferation, apoptosis, migration and tube formation abilities of human vascular endothelial cells (HUVECs) in vitro. We also utilized the chick embryo chorioallantoic membrane (CAM) model to examine the anti-angiogenic effect of TGP in vivo.

2. Materials and methods

2.1. Materials

Sliced dried roots of *Paeonia lactiflora* Pall (10 kg) were extracted three times with 70% aqueous ethanol for 2 h per extraction. Then, the extracts were combined and evaporated to dryness under reduced pressure, which yielded 1.2 kg of dry powder. About 1 kg of...
the residue obtained from the combined extract was dissolved with 8 l of water. After filtration, the aqueous solution was successively extracted three times with 61 ethyl acetate, which yielded 362.4 g of dry powder after being combined and evaporated to dryness under reduced pressure. Then the ethyl acetate extract was chromatographed on polystyrene resin (D101, 0.3–1.25 mm: Shanghai Hualing Resin Corporation, Shanghai) with water and 20% ethanol. Portions of the 20% ethanol were collected and evaporated to dryness under reduced pressure, which yielded 225.5 g of dry powder. The dry powder contained 72.5% TGP by chromatometry. The TGP fraction consisted of paoniflorin, hydroxyponinflorin, paonin, albiflorin, benzoylpaeoniflorin, etc. Paoniflorin, accounted for more than 90% of the constituents as determined by high performance liquid chromatography (HPLC) analysis, and is one of the main therapeutic components of TGP. Before use, TGP was dissolved in a 0.5% sodium carboxymethylcellulose solution (Xu et al., 2007). Subsequently, the suspension was dissolved in phosphate-buffered saline and sterilized for subsequent use (pH 7.2). Human vascular endothelial cells (HUVECs) and endothelial cell growth supplement (EGM-2 Single-Quots) were obtained from Clonetics Co. (San Diego, CA). Human recombinant vascular endothelial growth factor (VEGF) was obtained from Gibcol BRL (Rockville, MD). Matrigel was obtained from Becton Dickinson Co. (Bedford, MA). Transwell was obtained from Corning Science (Acton, MA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture

HUVECs were grown on a 0.2% gelatin-coated flask and supplemented with endothelial cell growth supplement. HUVECs were used between passages 3 and 6. Prior to stimulation, cells were starved for 6 h in serum-free endothelial medium lacking growth factors.

2.3. MTT assay

We performed the MTT assay to determine the effect of TGP on cell viability. Briefly, cells were plated in 96-well tissue culture plates at a density of 3 \( \times 10^5 \) cells per well in a volume of 100 \( \mu l \) and were treated with varying doses of TGP (0, 12.5, 62.5, and 312.5 \( \mu g/ml \)) and VEGF (10 ng/ml) for 6, 12 and 24 h. Then, 10 \( \mu l \) of MTT (5 mg/ml) was added to each well and incubated with the endothelial cells for 3–4 h. Cells were suspended with phosphate-buffered saline three times and the precipitates were dissolved with DMSO, then the plates were read at 570 nm in a SpectraMax 250 ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

2.4. Flow cytometry

After being treated with TGP for 24 h, cells (1 \( \times 10^4 \)) were stained with propidium iodide (PI, 5 mg/l) for 15 min at room temperature. The fluorescence intensity was analyzed by fluorescence-activated cell sorting analysis (FACS) (Becton Dickinson, Mountain View, CA) (Vitale et al., 1991). DNA histograms were subsequently analyzed to determine the cell cycle phase distribution [%] using the Modfit software program.

2.5. Cell migration assay

Cell migration was assessed in Transwell cell culture chambers according to methods reported previously (Lee et al., 2008). Polycarbonate filters of the upper chamber were coated with Matrigel (Becton Dickinson, 4 mg/ml) and the lower chamber was filled with 0.6 ml of endothelial basal medium containing VEGF (10 ng/ml). The filters were dried for 60 min at 37 °C and then washed extensively in phosphate-buffered saline. Cells (5 \( \times 10^4 \) cells/ml) were incubated with TGP in serum-free endothelial basal medium for 24 h. Cells on the upper surface of the filters were removed by wiping with a cotton swab. Cells that had migrated to the lower surface of the filters were photographed and counted.

2.6. Tube formation assay

Anti-angiogenic activity was measured using a method reported previously with slight modifications (Ponce, 2009). Briefly, HUVECs (2 \( \times 10^5 \) cells) were seeded in a 96-well plate that had been pre-coated with 100 \( \mu l \) of Matrigel (10 mg/ml). After incubation with various concentrations of TGP (0, 12.5, 62.5, and 312.5 \( \mu g/ml \)) and VEGF (10 ng/ml) for 24 h at 37 °C in a 5% CO2 humidified atmosphere, four different phase-contrast microscopic fields (magnification, 100 \( \times \)) per well were photographed. The numbers of tubes in every field were counted and recorded to evaluate the tube formation abilities of HUVECs.

2.7. Chick chorioallantoic membrane (CAM) assay

The CAM assay was performed as described previously (Miyazawa et al., 2008). Seven-day-old chick embryos were incubated at 37 °C with 55% relative humidity. A small craft grinding wheel was used to cut a window 1 cm\(^2\) over the dropped CAM. Sterile filter disks adsorbed with TGP (0, 12.5, 62.5, and 312.5 \( \mu g/ml \)) and VEGF (10 ng/ml) were placed onto an a vascular area of the CAM and incubated for 24 h. Angiogenesis was quantified by counting the number of blood vessel branch points in each photograph under the coverslips. At least six viable embryos were tested for each treatment.

2.8. Statistical analysis

Data were expressed as mean ± S.D. ANOVA was used to assess statistical significance between means. \( P < 0.05 \) was considered significant.

3. Results

3.1. MTT assay

To determine if TGP affected the proliferation of HUVECs, cell counts were measured with the MTT assay. The proliferation of HUVECs treated with TGP was significantly inhibited. The inhibitory effect began at 6 h and lasted 24 h. Three doses of TGP were used in the experiment, which produced a dose-dependent inhibition of proliferation in the HUVECs (Fig. 1).

3.2. Induction of G0/G1 arrest and apoptosis by TGP

To determine in which phase of the cell cycle HUVECs undergo arrest, and the effect of TGP on the apoptosis rates of HUVECs, flow cytometry was performed. Results from replicate experiments are summarized in Fig. 2. TGP resulted in a dose-dependent accumulation of cells in the G0/G1 phase of the cell cycle, and a dramatic reduction of cells in the G2/M phase. Apoptosis, as quantified by the sub-G1 fraction, increased after TGP treatment. In the group treated with 312.5 \( \mu g/ml \) of TGP, the percentage of apoptotic cells was 21.25 ± 1.7%.

3.3. Effect of TGP on the migration of HUVECs

Angiogenesis is highly dependent on endothelial cell motility and invasion. Therefore, we determined the effect of TGP on the
migration of HUVECs towards VEGF-containing media. TGP significantly inhibited the migration of HUVECs in a dose-dependent manner (Fig. 3) with maximal inhibition observed at the concentration of 312.5 μg/ml.

3.4. Effect of TGP on the tube formation ability of HUVECs

Tube formation assays were carried out in a three-dimensional gel consisting of diluted Matrigel. When HUVECs were plated on the diluted Matrigel, cells displayed high motility and increased cell–cell communication, and aligned to form an anastomosing capillary-like network within 24 h. After treatment with TGP, HUVECs showed only a few spontaneous tube formations, and most of them were still in a highly proliferative state with a cobblestone shape (Fig. 4).

3.5. Inhibition of CAM angiogenesis by TGP

Since in vitro studies using HUVEC have suggested that TGP can inhibit angiogenesis, we tested the in vivo effect of TGP on angiogenesis using the CAM assay. In the control CAMs, a developed orderly arrangement of the vessels with a ladder-like interdigitation was evident. In contrast, administration of TGP to the CAM led to a significant inhibition of neovascularization. At the
concentration of 312.5 μg/ml TGP, a complete prevention of neovascularization was observed in CAMs (Fig. 5).

4. Discussion

Paeonia lactiflora Pall root is one of the most well-known herbs in China and has been used in the treatment of cramps, pain, giddiness and RA (Zhang et al., 2008; Seo et al., 2007; Lee et al., 2005; Hsu et al., 1997). TGP, the extract of Paeonia lactiflora Pall root, has been confirmed as the main therapeutic component of the herb for the treatment of RA (Chang et al., 2008; Xu et al., 2007; Zheng and Wei, 2005; Zhu et al., 2005; Wu et al., 2009). RA is considered to be an angiogenic diseases, because leukocytes emigrate into the synovium through the vascular endothelium. In addition, the RA synovium is rich in newly formed vessels. Furthermore, overexpression of strong angiogenic inducers, including VEGF and basic fibroblast growth factor (bFGF), have been demonstrated in the synovium of RA patients. Hence, anti-neovascularization therapy has been recognized as a promising therapeutic approach for the treatment of RA (Szekanecz and Koch, 2007; Lainer-Carr and Brahn, 2007; Costa et al., 2007; Maruotti et al., 2006). Recent studies in RA animal models have confirmed that TGP treatment results in a reduction of neovascularization and a reduced expression of angiogenic factors (Zhu et al., 2005; Chang et al., 2008). In the present study, we are the first to demonstrate direct anti-angiogenic effects of TGP on HUVECs and CAM vascularization.

Angiogenesis is a multi-step process involving basement membrane dissolution, endothelial cell proliferation and migration, vessel lumen and branch formation, vessel maturation and new basement membrane formation (Cao, 2008; Kerbel, 2008; Carmeliet, 2005). Endothelial cell proliferation provides the basis for new vascular formation. In this study, TGP inhibited the proliferation of HUVEC in a dose-dependent manner, and this effect lasted at least 48 h. FACS analysis showed G0/G1 arrest and the apoptosis of HUVECs after exposure to TGP. In the cell migration assay, TGP efficiently inhibited the migration of endothelial cells towards VEGF-containing media. In the tube formation assay, cells treated with TGP lost their ability to form tubes and remained primarily as single cells on the Matrigel. These observations suggest that TGP interferes with the early processes of angiogenesis involving cell motility and matrix remodeling. The CAM is an extraembryonic membrane that is commonly used in the study of anti-angiogenesis agents. In our study, TGP reduced vascular density and formation in this angiogenesis model.

The current study evaluated TGP, which is a new angiogenic modulator, in HUVECs and CAM model systems. Our findings confirm the anti-angiogenic effects of TGP and provide useful clues for new therapeutic applications in the study of this traditional drug.

References


