The angiosuppressive effects of 20(R)-ginsenoside Rg3

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1. Introduction

Ginseng, referring to the root of Panax ginseng and its related species, has been used as folk medicine in the treatment of various diseases for thousands of years in China. The effects of ginseng include general ‘tonic’, anti-fatigue, anti-stress, immunomodulatory, and anti-cancers [1,2]. Ginsenosides, the major active components of ginseng, are a diverse group of steroidal saponins with multiple pharmacological activities.

Among the ginsenosides, Rg3 (Fig. 1) has been reported to exhibit in vitro and in vivo anti-carcinogenic and anti-metastatic effects. These include the inhibition of growth of LNCaP prostate carcinoma cells, metastasis of B16-BL6 melanoma, and colon 26-M3.1 carcinoma, invasion of human lung carcinoma (OC10) and pancreatic adenocarcinoma (PSN-1) cells [3–7]. In 2000, Rg3 appeared in the market as a new anti-cancer drug called “Shen-Yi Capsule” in China. Its pharmacological mechanisms are prevention of invasion, and metastasis of tumor cells [6].
Angiogenesis is a multistep process which includes degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs), endothelial cell proliferation, migration, and capillary tube formation. The whole process is tightly regulated by the balance between counteracting angiogenic stimulators and inhibitors [8–10]. Apart from the high activity required for placentation, embryogenesis, wound healing, and endometrial repair after menstruation, angiogenesis is a prominent pathological feature of many diseases such as rheumatoid arthritis and psoriasis [11–13]. Moreover, angiogenesis is an obligatory event for the survival, growth, and metastasis of tumors beyond the size limit (1–2 mm diameter) imposed by adequate supply of nutrients, and oxygen [14].

In the early 1970s, Folkman and co-workers hypothesized that tumor growth is angiogenesis-dependent and an anti-angiogenic strategy might constitute a new therapeutic approach for the treatment of solid tumors [14,15]. Nowadays, much effort has been directed toward discovering of new anti-angiogenic agents. A variety of these agents such as Avastin (bevacizumab), thalidomide, TNP-470, endostatin, and angiosatine have been currently undergoing clinical evaluation for their efficacy in anti-angiogenic therapy [16–18]. Moreover, Macugen (pegaptanib sodium), a VEGF165-blocker, is the first anti-angiogenic drug approved by FDA in 2005 [19].

The present study aims to evaluate the anti-angiogenic effect of ginsenoside-Rg3. We have previously demonstrated the counteracting effects of different types of ginsenosides on angiogenesis [20]. Together with the anti-carcinogenic effect of Rg3, it would be a challenge that Rg3 may be a much more powerful drug for the treatment of cancers by “shutting-down” the nutrient supply and blocking the metastatic channel rather than directly targeting on the tumor cells. We have investigated the angiosuppressive properties of Rg3 using various biological assays including the in vitro human umbilical vein endothelial cells (HUVEC) proliferation assay, tube formation of EC in three-dimensional Matrigel, vascular endothelial growth factor (VEGF)-mediated chemoinvasion, microvascular sprouting assay ex vivo, and in vivo basic fibroblast growth factor (bFGF)-mediated Matrigel plug model. The gelatinolytic activity of MMPs was also examined by the zymography. We demonstrated that Rg3 could significantly inhibit angiogenesis in our in vitro and in vivo assay systems.

2. Materials and methods

2.1. Materials

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (TCS Biologicals, UK). Phosphate buffered saline (PBS), fetal calf serum (FCS), trypsin-EDTA, and serum-free endothelial growth medium were supplied by Invitrogen (Carlsbad, CA, USA). Penicillin–streptomycin (PS), dimethyl sulphoxide (DMSO), endothelial cell growth supplement (ECGS), heparin, amphotericin-B and M199 medium were obtained from Sigma (Saint Louis MO, USA). Growth factor-reduced Matrigel (GFR-Matrigel) was provided by BD Bioscience (Palo Alto, CA, USA). Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were purchased from Promega (San Luis Obispo, CA, USA) and R&D system (Minneapolis, USA), respectively.

2.2. Chemicals

Ginsenoside Rg3 is a reference compound (purity >98%) purchased from the Division of Chinese Material Medica and Natural Products, National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), Ministry of Public Health, China. A stock solution of Rg3 (50 mM) was freshly prepared in DMSO.

2.3. Cell culture

HUVEC were cultured in M199 medium supplemented with ECGS (20 µg/ml), 20% heat-inactivated FCS, 1% PS, amphotericin-B (50 ng/ml) and heparin (90 µg/ml) on 0.1% gelatin-coated culture flasks. The cells were grown at 37 °C in humidified air with 5% CO2 incubator. All experiments were conducted with HUVEC from passage 2 to 7.

Fig. 1 – The chemical structure of ginsenoside-Rg3.

Fig. 2 – Anti-proliferative effect of Rg3. HUVEC (2 × 10^4 cells/well) were plated in 96-well plate and grown in medium supplemented with 20% FCS and 20 µg/ml ECGS for 24 h. The cells were incubated with various concentrations of Rg3 for further 48 h. The viable cells were counted using the Trypan blue exclusion method. Values are presented as mean ± S.E.M. obtained from triplicate experiments. *p < 0.05.
2.4. **In vitro proliferation assay**

Briefly, HUVEC were plated at a density of $2 \times 10^4$ cells/well in 24-well plate. Cells were incubated in growth medium supplemented with 20% FCS and ECGS (20 μg/ml) and allowed to attach for 24 h. Then cells were incubated with various concentrations of Rg3 (1–10³ nM) in medium containing 20% FCS and ECGS (20 μg/ml) for 48 h. The cells were then washed in ice-cold PBS and trypsinized. Trypan blue exclusion method was used to distinguish viable cells. Results were expressed as number of cells per culture. Each sample was tested in quadruplicates and the experiment was repeated in triplicates.

2.5. **In vitro tube formation assay**

GFR-Matrigel (500 μl diluted in PBS, 1:3, v/v) was pipetted into wells of 24-well plate. The gel was allowed to solidify at 37 °C for 1 h. HUVEC were plated at a density of $8 \times 10^4$ cells/well and incubated with medium containing 10% FCS, ECGS (20 μg/ml) and various concentrations of Rg3 (1–10³ nM) in the presence or absence of VEGF (20 ng/ml) at 37 °C for 16 h. The images of tube formation were captured by an inverted microscope (Nikon TMS, Japan) using a 10× objective. Images from a total of five microscopic fields per well were analyzed by Motic Image Plus 2.0 software (Motic Instruments Inc., Richmond, Canada). The antiangiogenic activities were determined by counting the branch points of the formed tubes and the average numbers of branch points were calculated. Experiment was repeated in triplicates.

2.6. **Chemoinvasion assay**

Chemotactic motility of HUVEC was studied using Transwell chamber with 6.5 mm diameter polycarbonate filter (8 μm pore size, NUNC). Briefly, the upper and lower surfaces of the filter were coated with GFR-Matrigel (diluted in PBS, 1:30 and 1:100, v/v), respectively. HUVEC (5 × 10⁴ cells/well) were loaded onto the upper wells with culture medium containing 1% serum only or medium containing various concentrations of Rg3 (1–10³ nM). The Transwell chambers were sequentially inserted into 24-well plates containing medium alone or medium with VEGF (25 ng/ml) and the set up was incubated at 37 °C for 5 h. The cells that had migrated to the lower side of the membrane were fixed with methanol and stained with

![Image of tube formation assay](Image)

**Fig. 3** – Inhibition of tube formation by Rg3. HUVEC (8 × 10⁴ cell/well) were seeded into 24-well plate which had been precoated with GFR-Matrigel in medium containing 10% FCS and ECGS (20 μg/ml). The cells were treated with various concentrations of Rg3 (1–10³ nM) in the absence (A, top panel) or presence of VEGF (25 ng/ml) (A, bottom panel) for 16 h. Photomicrographs depict the alignment of HUVEC under defined treatment conditions. (B) Five microscopic fields were counted for each treatment. The data represented mean ± S.E.M. from triplicate experiments. *p < 0.05; **p < 0.01; ***p < 0.001 vs. medium control; #p < 0.05; ##p < 0.01; ###p < 0.001 vs. VEGF control.
DAPI stain (1 μg/ml) (Boehringer Mannheim); those on the top side of the membrane were wiped off using a cotton swab. The cells were visualized using a fluorescent microscope (Axioskop 2, Zeiss) equipped with a 10× objective. Five microscopic fields were counted for each membrane and the images were analyzed using Metamorph software. Each sample was assayed in duplicate, and the assay was repeated in quadruplicates.

2.7. In vivo Matrigel plug assay

Matrigel plug assay was performed as described previously [21]. Briefly, GFR-Matrigel (500 μl) containing bFGF (125 ng/ml) and heparin (32 U) with or without Rg3 (150 and 600 nM) were injected subcutaneously into the left and right abdomen of C57/BL female mice. After injection, the Matrigel rapidly formed a single, solid gel plug. Mice were sacrificed 5 days after injection. Plugs were then removed and the extent of neovascularization was assessed by measuring the hemoglobin content using the Drabkin’s Reagent Kit (Sigma). A control was performed in parallel with another set of mice by injecting GFR-Matrigel alone. Six mice were used for each treatment group and the experiment was done at least twice.

2.8. Aortic ring sprouting assay

Rat aortic rings were prepared as described previously [21]. Briefly, the aortic fragments were placed in GFR-Matrigel precoated 96-well plate. Additional GFR-Matrigel (40 μl) was added to overlay the aortic fragments and the gel was allowed to solidify. The cultures were incubated in 200 μl of serum-free endothelial growth medium containing ECGS (200 μg/ml) with or without Rg3 (1–10^4 nM). Microvascular outgrowths were distinguished from the fibroblasts based on their unique morphology (greater thickness, uniform cohesive pattern of growth and dichotomous branching of one sprout in generating two new sprouts). Culture medium was replaced with fresh medium on day 4 and the aortic fragments were visualized on day 8 with an Olympus SZX9 stereomicroscope using a 10× objective. The images were captured using the Nikon digital sight DS-L1 software. The microvascular sprouting area was calculated by the Image J software (http://rsb.info.nih.gov). The experiment was repeated three times and each culture was performed in duplicate.

2.9. Gelatin zymography

Culture supernatants from Rg3-treated ex vivo rat aortic ring culture were collected on day 8. Gelatinase activity in the culture supernatant was determined by in-gel gelatin zymography. Samples were mixed with 4× Zymograph sample buffer (10% SDS, 40% glycerol, 0.25 M Tris–HCl, pH 6–8, 0.02% bromophenol blue) without heat denaturation. Electrophoresis was performed on 8% (for MMP-9) and 10% (for MMP-2) polyacrylamide gels containing gelatin (1 mg/ml) at 20 mA for 2 h. After electrophoresis, the gels were washed twice in renaturing solution (50 mM Tris–HCl, pH 7.4, 2% Triton X-100). Gels were incubated in substrate buffer (50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 5 mM CaCl₂, 0.02% Tween 20) at 37 °C overnight. Gels were stained with 0.5% Coomassie Blue R-350 and destained with 10% acetic acid in 40% methanol. Gelatinolytic activity was visualized by negative staining. Pre-stained SDS-PAGE protein standards (Bio-Rad) were used for estimation of apparent molecular weights of the protein samples. The intensity of the bands on zymogram was quantified using Kodak 1D software.

2.10. Data and statistics analysis

Data are presented as mean ± S.E.M. of control. Statistical comparisons between groups were performed using the Student’s t test.

3. Results

3.1. Rg3 inhibits the proliferation of HUVEC

We examined the effect of Rg3 on HUVEC proliferation by direct cell counting. As shown in Fig. 2, Rg3 dose-dependently inhibited the proliferation of HUVEC. The 50% of growth inhibition (IC₅₀) of Rg3 on HUVEC was 10 nM. At the concentration of 10^3 nM, the level of inhibition of HUVEC by Rg3 was found to be comparable to the well-known anti-angiogenic agent angiostatin.
3.2. Rg3 attenuates in vitro capillary tube formation of HUVEC

The effect of Rg3 on the capillary tube formation of HUVEC on GFR-Matrigel was examined. In the absence of VEGF (Fig. 3A, top panel), there was a clear inhibition of capillary tube formation by Rg3. The number of branch point was reduced from 52±3 in the control group to 18±4 in the group treatment with Rg3 (10 nM) (p < 0.001). The effect of Rg3 on VEGF induced capillary tube formation was also examined. VEGF alone was found to stimulate the branch point formation from 52±3 (medium control) up to 78±5 branch point per well. Addition of Rg3 resulted in the reduction of VEGF-induced capillary tube formation from 78±5 (VEGF control) to 40±5 (10³ nM Rg3) (p < 0.001) (Fig. 3B, black bars).

3.3. Rg3 inhibits VEGF-induced chemoinvasion

One of the earliest steps in angiogenesis is the invasion of basement membrane and migration of endothelial cells toward the angiogenic stimulation. The effect of Rg3 on the migration of HUVEC was then examined using migration chamber as shown in Fig. 4A. HUVEC were exposed to VEGF (angiogenic chemoattractant) through a Matrigel-coated membrane. Results in Fig. 4B showed that VEGF (25 ng/ml) could significantly induce migration of HUVEC from the upper chamber to the lower chamber (p < 0.001). In the presence of Rg3 (1–10⁵ nM), the ability of HUVEC to migrate through the membrane was reduced by more than 50% (p < 0.05 for 1–10² nM Rg3; p < 0.01 for 10³ nM Rg3). 

3.4. Suppression of in vivo angiogenesis by Rg3

In the Matrigel plug model, GFR-Matrigel containing various compounds were injected s.c. into mice abdomen. After 5 days, the gel was removed for gross morphological examination (Fig. 5A–D), and hemoglobin content determination (Fig. 5E). As shown in Fig. 5E, co-injection of bFGF and heparin significantly induce neovessels formation as judged from the increased hemoglobin content in the gel. However, the content of hemoglobin in Rg3-containing gel was significantly lower than the positive control gel containing bFGF/heparin.

3.5. Rg3 reduces ex vivo microvascular sprouting

In the aortic ring sprouting assay, cultured aortic explants were embedded in a cube of GFR-Matrigel and the explants were then cultured in 96-well plate with serum-free medium containing ECGS or various concentrations of Rg3 (Fig. 6A). ECGS (200 μg/ml) alone, significantly induced microvascular sprouting when compared with the untreated control group. Moreover, it was found that Rg3 ranging from 10–10² nM could effectively inhibit such sprouting in a dose-dependent manner.

3.6. Rg3 inhibits the production of MMP-2 and MMP-9 from aortic explants

To determine the effect of Rg3 on the production of proteinases by HUVEC, culture supernatants collected from aortic ring sprouting assay were subjected to gelatin zymography. As shown in Fig. 7A, the presence of proteinases (MMPs) digested...
the gelatin-containing gel and resulted in a series of clear bands. In order to distinguish the various MMPs, 8% and 10% polyacrylamide gels were used for MMP-2 and MMP-9 analysis, respectively. Different MMPs were assigned according to their molecular weights, clear bands at 86, 72, and 66 kDa bands were assigned to MMP9, proMMP-2, and MMP-2 respectively. Quantified results showed that Rg3 reduced the gelatinolytic activities of secreted MMP-9, proMMP-2 and MMP-2 in a dose-dependent manner which corresponded to the inhibition of aortic ring sprouting activity. Interestingly, data indicated that effective inhibition of MMPs activities was found at the Rg3 concentration higher than 1 nM.

4. Discussion

20(R)-ginsenoside Rg3 (Rg3) has been shown to exhibit anti-cancer activity in many in vivo models. The anti-tumor effect
has been attributed to the actions of anti-invasion and anti-metastasis of tumor cells [3,4,22]. However, the in vivo anti-cancer activities could also result from the inhibition of neovascularization [23]. The angiosuppressive properties of Rg3 have not been studied in detail. In the present study, we used different angiogenesis assays that are related to proliferation, morphological differentiation, invasion, and migration of EC during angiogenic process to assess the angiosuppressive activity of Rg3.

Results from the present study demonstrated that Rg3 exerted inhibitory effect on proliferation, capillary tube formation and invasion of HUVEC in a dose-dependent manner. Interestingly, the effect of Rg3 became more obvious while EC were activated by angiogenic factors such as VEGF. This implied that EC might become more sensitive when they are activated. In fact, under normal condition, EC remain quiescent. They are only activated when angiogenesis is called for, such as during tumor progression. Thus, Rg3 may be useful in this case by acting as a specific and effective angiosuppressive agent. Similar cases were also observed in the ex vivo organotypic cultures of rat aortic rings and in vivo Matrigel plug model; Rg3 was found to effectively suppress the ECGS- and bFGF-mediated angiogenesis. Furthermore, since tube formation of HUVEC involves EC attachment, migration, and production of ECM degrading enzymes, data indicated that Rg3 could possibly interfere all these steps and resulted in the attenuation of angiogenesis in vitro and in vivo.

During the progression of solid tumor, malignant tumor cells and activated EC secret angiogenic factors to initiate the formation of neovessels [8–10]. In this study, angiogenic factors (bFGF, VEGF or ECGS)-mediated angiogenesis models mimicked the microenvironment of endothelium in tumor that the “activated EC” acted as the primary target for the Rg3.
These noteworthy results indicated that Rg3 could be an effective agent for the suppression of neovessels formation. Activated ECs produce many types of enzymes such as matrix metalloproteinases (MMPs) that break down the stroma and ECM proteins, and allow the EC to invade the matrix, to migrate, and to grow in response to growth factors [24,25]. It has been reported that MMPs play a major regulatory role in the ECM re-organization and the initiation of neovascularization [26,27]. In this study, Rg3 was found to reduce the gelatinases activities of the organotypic cultures. Meanwhile, this indication elucidated that the inhibition of EC invasiveness and tube formation of Rg3 could possibly due to the reduction of MMPs activities. Taken together, the angiosuppressive effect of Rg3 would also be related to the differentially regulation of proteinases activities.

Tumor angiogenesis is a complex and obligatory process for the growth and progression of solid tumors beyond the size limit (~2 mm diameter) imposed by simple diffusion for the nutrient supply [14] and that limiting nutrient supply by the blockage of neovessels formation in tumor might be used in cancer therapy [28–30]. To date, this hypothesis has been feasibly culminated in the clinical trials of anti-angiogenic drugs and the first antiangiogenic drug has been marketed in 2005. Moreover, more than 20 anti-angiogenic drugs including TNP-470, thalidomide, and endostatin are subjected to different phases of clinical trials. In addition, phytochemicals such as curcumin, genistein, and ginseng saponins were developed as angiosuppressive drugs in controlling tumor growth and metastasis. In addition, combination of Rg3 with other anti-cancer drugs may be beneficial to treatment of other drug-resistant cancers.

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