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Prevention of FGF-2-induced angiogenesis by scopoletin, a coumarin compound isolated from *Erycibe obtusifolia* Benth, and its mechanism of action

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ABSTRACT

Previous work in our laboratory has shown that scopoletin, one of the main bioactive constituents of *Erycibe obtusifolia* Benth stems, exerts anti-arthritic activity *in vivo* partly by preventing synovial angiogenesis. The present study was performed to further investigate the anti-angiogenic potential of scopoletin, focusing on the mechanisms of action *in vitro*. In the aortic ring sprouting assay, scopoletin (10, 30 and 100 μM) significantly inhibited the growth of endothelial sprouts in a concentration-dependent manner. As to human umbilical vein endothelial cells (HUVECs), scopoletin could inhibit their proliferation, migration and tubule formation induced by FGF-2, especially the proliferation. It also remarkably decreased the expression of VEGF at mRNA and protein levels, and the phosphorylations of IKKα and IκB but not Akt, as well as the degradation of IκB caused by FGF-2 in HUVECs. These findings suggest that scopoletin is substantially able to attenuate FGF-2-induced angiogenesis, and it might act by directly preventing the stimulation action of FGF-2 and by indirectly decreasing the production of VEGF. Scopoletin down-regulated the VEGF expression through NF-κB rather than PI-3K/Akt signaling pathway.

1. Introduction

Angiogenesis, the formation of capillaries from pre-existing blood vessels, is a physiological and fundamental process, including organogenesis and morphogenesis during embryonic development, wound healing, and regeneration of organs [1–3]. However, persistent angiogenesis plays an important role in the progression of certain pathological conditions, such as diabetic retinopathy, rheumatoid arthritis, psoriasis, and tumor growth and metastasis [4,5]. Anti-angiogenesis has become an important subject in the treatments of these diseases.

A number of cytokines and growth factors participate in the modulation of angiogenesis. Angiogenesis is thought to be controlled by the balance between angiogenic factors and angiogenesis inhibitors. If this equilibrium is broken, serious consequences may arise [6,7]. Among these factors, basic fibroblast growth factor (FGF-2) and vascular endothelial growth factor (VEGF) are recognized as representative angiogenic factors [8,9]. VEGF is considered to be a specific mitogen for endothelial cells, while FGF-2 affects a broad spectrum of cell types including endothelial cells [9,10]. FGF-2 is a potent angiogenic factor that can stimulate quiescent endothelial cells for morphogenesis, proliferation and migration [10]. Moreover, it has synergistic action with VEGF [11], and can facilitate the expression of VEGF in endothelial cells through both autocrine and paracrine mechanisms [12]. Blockade of FGF-2 action is a promising approach for anti-angiogenic therapies in various angiogenesis-related diseases.

Although various potent endogenous angiogenesis inhibitors have been identified and brought into clinical trials in the treatments of angiogenesis-related diseases, their disadvantages are gradually exhibited as most of them are proteins, for instance, difficulties of manufacturing active molecules, high costs, and risk of transmission of microorganism toxins in recombinant proteins [13]. Instead, small molecule inhibitors isolated from natural medicines, such as sinomenine, curcumine and epigallocatechin-3-gallate (EGCG) [14–16], may be more outstanding over protein inhibitors as they can be obtained easily, and confer low cost.

Scopoletin (6-methoxy-7-hydroxycoumarin) is isolated from the stems of *Erycibe obtusifolia* Benth, a traditional Chinese medicine that has been used for the treatment of various rheumatoid diseases for a long history. It possesses various pharmacological properties such as anti-inflammatory, hypouricemic, and anti-oxidant activities [17–19]. Previously, we demonstrated that scopoletin could ameliorate clinical symptoms of rat adjuvant-induced arthritis probably by reducing numbers of new blood vessels in synovium [20]. As an extension, the present study was conducted to address the anti-angiogenic potential of scopoletin in more detail.

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2. Materials and methods

2.1. Materials

Scopoletin (purity>98%) was isolated from the stems of *E. obtusifolia* Benth, which were collected at Guangxi province of China. A stock solution of scopoletin was prepared in dimethyl sulfoxide (DMSO) and diluted to desired concentrations before use. The concentration of DMSO was below 0.1% in treated groups. DMSO 0.1% was used as a vehicle control through the study.

Type II collagenase, heparin, endothelial cell growth supplement (ECGS), 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and FGF-2 were purchased from Sigma (St. Louis, MO). Medium 199 (M199) was purchased from Gibco-BRL (Grand Island, NY). Newborn calf serum was purchased from PAA (Austria). Matrigel was purchased from BD Biosciences. Primary antibodies against p-AKT, p-IKKα, p-IκB, AKT and IκB were purchased from Bioworld Technology (Minneapolis, MN). Primary antibodies against p-ERK1/2, p-p38 MAPK, ERK1/2, p-38 MAPK and GAPDH, as well as secondary antibodies including goat anti-mouse IgG (H&L) [HRP] were purchased from KangChen Bio-Tech (Shanghai, China).

2.2. Rat aortic ring assay

This *ex vivo* angiogenesis assay was performed essentially as described previously [21] with some modifications. Thoracic aortas were excised from 2–3-week-old Sprague–Dawley male rats, and immediately placed into cold M199 free of serum. Clotted blood inside the aorta was flushed with media, and the periadventitial fibroadipose tissue was removed. Aortas were then cut into cross-sectional rings about 1–1.5 mm in length. Aortic rings were placed into wells of a 96-well plate pre-coated with Matrigel, and then incubated at 37 °C until the Matrigel polymerized. The wells were then overlaid with 200 μl of serum free M199 with or without scopoletin, and the rings were maintained at 37 °C for up to 10 days with medium changes every 2 days. Vascular sprouting from each ring was examined on an Olympus IX-70 microscope (100× magnification), and digital images were obtained. Quantitative analysis of endothelial sprouting was performed using Adobe Photoshop software.

2.3. Isolation and culture of endothelial cells

HUVECs were isolated from human umbilical cord veins as described previously [22]. After cannulation and rinsing, the vein was digested with 0.1% type II collagenase in PBS for 20 min at 37 °C. Veins were flushed with warm M199, and the resulting endothelial cell suspension was centrifuged for 10 min at 1000 rpm. Primary cultures of HUVECs were seeded into 25 cm^2 flasks (Costar, Cambridge, MA) precoated with 0.02% (w/v) gelatin. Culture medium consisted of M199 supplemented with 20% (v/v) newborn calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, 10 units/ml heparin and 30 μg/ml ECGS. Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2, and the medium was changed after 24 h and every 2 days thereafter until confluent. Primary cultures of HUVECs were passaged with 0.025% trypsin/0.025% EDTA and collected by centrifugation. Confluent HUVEC monolayers (passages 2–5) were used.

2.4. Cell proliferation assay

HUVECs (4×10^3 cells/well) were seeded to 96-well plates (Costar, Corning, NY) pre-coated with 0.02% (w/v) gelatin. The cells were allowed to attach overnight before beginning the 2-h serum starvation. Then the cultured medium was replaced with medium supplemented with 5% newborn calf serum and containing scopoletin with or without FGF-2 (10 ng/ml) for 48 h before MTT assay, respectively. The medium was renewed every other day. Cells were incubated with MTT at a final concentration of 0.5 mg/ml for 4 h. After incubation, the medium was aspirated, and the cells were lysed and the reduced intracellular formazan product was dissolved by replacing 150 μl of DMSO. Then the absorbance at 570 and 690 nm was measured with a microplate reader. Results were expressed as the percentage of proliferation ratio.

$$\text{Proliferation ratio}\% = \left(OD_{\text{treatment group}} - OD_{\text{control group}}\right)/OD_{\text{control group}} \times 100\%\ (OD = OD_{570} - OD_{690})$$

2.5. Cell migration assay

HUVEC migration assay was performed using a transwell Boyden chamber (Corning Costar, Cambridge, MA, USA) containing a polycarbonate filter with a pore size of 8 μm coated with 0.2% gelatin. The cells were starved for 2 h, then harvested, washed, and suspended in 5% FBS-containing medium with or without scopoletin. The cell suspensions were seeded in Transwell chamber. The chambers were inserted into 24-well plates containing culture medium with (or without) 10 ng/ml FGF-2 and (or) various concentrations of scopoletin. After incubation for 6 h at 37 °C, the nonmigrated cells on the upper surface of the Transwell membrane were removed by cotton swabs. The cells on the filters were fixed with methanol and stained with Wright's stain. Total cell counts per filter were determined by light microscopy.

2.6. Tubule formation assay

The tubule formation assay was performed to determine the effect of scopoletin on angiogenesis in vitro. Matrigel was diluted in serum free medium to 3 mg/ml final concentration and 200 μl of which was added to each well of a 48-well plate and incubated at 37 °C for 1 h to form a gel layer. After gel formation, cells in 5% FBS-containing medium, in the presence of 10 ng/ml FGF-2 with (or without) various concentrations of scopoletin, were applied to each well, and plates were incubated at 37 °C for 18 h. After incubation, the enclosed networks of tubes were photographed randomly under a microscope. Total length of tubes was measured with using Adobe Photoshop software, three images from separate experiments for each data point. Inhibition of tubule formation was calculated as $[1-(\text{tube length}_{\text{treated}}/\text{tube length}_{\text{control}})]\times100\%$.

2.7. Quantification of VEGF levels

HUVECs in logarithmic growth phase were used in this assay. HUVECs were allowed to attach overnight before beginning the 2-h serum starvation. The control group and experimental groups were set. Culture medium in control group was free of FGF-2 and scopoletin. In experimental groups, cells were treated with medium which containing FGF-2 (10 ng/ml) with or without various concentrations of scopoletin for 24 h. Supernatants and lysates at 24 h were collected and VEGF concentrations in the supernatants and cell lysates were measured by ELISA reagent kit, respectively. All analyses and calibrations were carried out in triplicate. VEGF immunoreactivity was determined as compared to recombinant human VEGF standards. Optical densities were determined at 450 nm using a microliter plate spectrophotometer.

2.8. RT-PCR assay

HUVECs in logarithmic growth phase were used in this assay. HUVECs were allowed to attach overnight before beginning the 2-h serum starvation. The control group and experimental groups were set. Culture medium in control group was free of FGF-2 and