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Inhibitory effects of quercetin on angiogenesis in larval zebrafish and human umbilical vein endothelial cells ☆

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ABSTRACT

Angiogenesis plays an essential role in many physiological and pathological processes. Quercetin, a plant pigment and traditional Chinese medicinal herb, is an important flavonoid that has anti-cancer activity. However, the function of quercetin in blood vessel development in vivo and in vitro is still unclear. In this study, we investigated the anti-angiogenic activity of quercetin in zebrafish embryos and in human umbilical vein endothelial cells (HUVECs). Our results showed that quercetin disrupted the formation of intersegmental vessels, the dorsal aorta and the posterior cardinal vein in transgenic zebrafish embryos. In HUVECs, quercetin inhibited cell viability, the expression of vascular endothelial growth factor receptor 2 and tube formation in a dose-dependent manner. In inhibiting angiogenesis, quercetin was found to be involved in suppressing the extracellular signal-regulated kinase signaling pathway in vivo and in vitro. This study has shown that quercetin has potent anti-angiogenic activity and may be a candidate anti-cancer agent for future research.

1. Introduction

The circulatory system in vertebrates is a network of arteries, veins and capillaries, and the formation of the vascular system includes vasculogenesis and angiogenesis (Larrivee et al., 2009; Risau, 1997). Angiogenesis, the emergence of new blood vessels via branching from an existing vascular system, plays an important role in embryonic vascular formation and development (Potente et al., 2011; Risau, 1997). Studies have shown that tumors promote the angiogenic process, including the proliferation and migration of endothelial cells. One novel strategy to suppress tumor development is the inhibition of angiogenesis (McMahon, 2000; Pratheeshkumar et al., 2012), and there is increasing evidence demonstrating that angiogenesis is involved in increased cellular infiltration and proliferation (Jackson et al., 1997). Vascular endothelial growth factor (VEGF) is the most important angiogenic factor that increases mitogenic activity and the survival of vascular endothelial cells (Roberts and Palade, 1997; Yu et al., 2010). VEGF exerts its activity on endothelial cells through two types of receptor tyrosine kinases (RTKs): vascular endothelial growth factor receptor 1 (VEGFR-1) and vascular endothelial growth factor receptor 2 (VEGFR-2). VEGFR-2 plays a critical role in mediating the mitogenesis and proliferation of endothelial cells (Pratheeshkumar et al., 2012), and activation of VEGFR-2 enhances proliferation, migration and tube formation of endothelial cells by activating the phosphorylation of multiple signaling pathways, including the extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), phosphatidylinositide 3-kinase (PI3K), protein kinase B (AKT) and p38 mitogen-activated protein kinase (p38MAPK) pathways (Ferrara et al., 2003).

Quercetin, a bioactive flavonoid with a molecular weight of 302.24 g mol^{-1} is a Chinese herbal medicine found in various edible plants, such as red onions, apples, tea, broccoli, red grapes and a number of berries (Bischoff, 2008). It exhibits a broad range of pharmacological activities, and is considered to be an anti-inflammatory, anti-oxidant, anti-tumor and anti-ulcer agent, as well as exerting immunomodulatory and vasodilatory effects (Ajay et al., 2006; Alvarez et al., 1999; Shoskes and Nickel, 2011). In our study, we chose transgenic zebrafish (*Danio rerio*; fli1: EGFP), which are a useful model for the high-throughput screening of drugs and compounds (Cheng et al., 2001; Peterson et al., 2001), to investigate the effect of quercetin on angiogenesis. We

Abbreviations: HUVECs, human umbilical vein endothelial cells; ISV, intersegmental vessel; DA, dorsa aorta; PCV, posterior cardinal vein; VEGF, vascular endothelial growth factor; RTKs, receptor tyrosine kinases; DMSO, dimethylsulfoxide; hpf, hours postfertilization; PVDF, polyvinylidene difluoride; ERK, extracellular signal-regulated kinase

☆Chemical compounds studied in this article. Quercetin (PubChem CID: 5280343).

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additionally studied the in vitro activity of quercetin in human umbilical vein endothelial cells (HUVECs). We found that quercetin exerts anti-angiogenic activity in zebrafish, and that it significantly inhibits endothelial cell proliferation, migration and tube formation in vitro. Our results show that the molecular mechanism for quercetin-mediated inhibition of angiogenesis involves the ERK signaling pathway and the expression of VEGFR-2.

2. Materials and methods

2.1. Cell lines and chemicals

HUVECs were obtained from ATCC. Quercetin was purchased from Sinopharm Chemical Reagent Co. Ltd. (SCRC, Shanghai, China). HUVECs were cultured in Kaighn's modification of Ham's F-12 medium (F-12K) with 10% heat-inactivated fetal bovine serum (FBS) and 100 U ml^{-1} penicillin–streptomycin (Gibco). Cells were incubated at 37 °C in 5% CO_2 (v/v). Quercetin was dissolved in dimethylsulfoxide (DMSO; Amersco) to give a 200 mM stock solution. The stock solution was diluted with cell culture medium in different concentrations for use.

2.2. Maintenance of zebrafish and treatment of embryos

Transgenic zebrafish Tg(fli1: EGFP) expressing enhanced green fluorescent protein (EGFP) in the endothelial cells were kindly provided by ZFIN (Oregon) for use as the in vivo model. Zebrafish were maintained as described in Westerfield (1993). In brief, zebrafish were maintained at 28.5 °C in 14 h:10 h light/dark cycles. Zebrafish were fed twice daily with dry food in the morning and afternoon. Embryos were collected in the morning and cultured at 28.5 °C in distilled water. At 6 h after fertilization, embryos were distributed into a six-well cell culture plate with 4 ml distilled water containing different concentrations of quercetin. Embryos receiving DMSO (0.1%) only served as a control. All of the experiments were repeated at least three times with 20–30 embryos per group.

2.3. Cell proliferation assay

HUVECs were seeded into 96-well plates at a density of 5×10^3 cells per well. In order to achieve a quiescent state, the complete medium was replaced after 24 h incubation with low serum (0.5% FBS) medium. After this, the medium was replaced with low serum (0.5% FBS) medium containing various concentrations of quercetin. Cells receiving DMSO (0.1%) only served as a vehicle control. Plates were incubated for an additional 48 h, and cell proliferation was assessed using Cell Proliferation Kit II (MTS, Promega) in accordance with the manufacturer's protocol. MTS test solution (20 μl) was added to each well, and the cells incubated for an additional 4 h at 37 °C. The spectrophotometric absorbance of each well was measured using a multilabel-counter fluorescent plate reader (Infinite® M 1000, Tecan, Switzerland). The wavelength used to measure absorbance was 490 nm. The results are expressed as the percentage of proliferating cells.

2.4. Morphological observation of zebrafish

After drug treatment, embryos were anesthetized using 0.01% tricaine (Sigma-Aldrich) and observed for any morphological changes using an Olympus spinning disk confocal microscope system (IX81-ZDC motorized inverted microscope). Images were analyzed with ImageJ and Adobe Photoshop 7.0 software.

2.5. Embryo collection, drug treatment and measurement of the toxic effects of quercetin on zebrafish

Tg(fli1: EGFP) transgenic zebrafish embryos were generated by natural pair-wise mating and were raised at 28.5 °C in distilled water. Quercetin was diluted in DMSO as needed, and then transferred to the embryo water. Healthy, hatched zebrafish embryos were picked out 6 h postfertilization (6 hpf), and treated with DMSO (0.1%) or different concentrations of quercetin (50, 100, 200, 300, 500, 700 or 1000 μM). They were then incubated in six-well plates (20–30 embryos per well) at 28.5 °C from 6 to 72 hpf. Embryos treated with DMSO (0.1%) alone served as a vehicle control. During the experiment, the embryos were observed for survival and morphology under an inverted microscope (Nikon, Japan). Data were analyzed using the statistical package SPSS 17.0 (SPSS Inc., Chicago, IL, USA) for non-linear regression, and the minimum lethal concentration was defined as 0% mortality of zebrafish treated with quercetin. The assay was repeated three times independently with 20–30 embryos per group.

2.6. Assessment of vascular changes in zebrafish embryos by microscopy

Tg(fli1: EGFP) transgenic zebrafish embryos were treated with DMSO (0.1%) or various concentrations of quercetin (50, 100 or 200 μM), and incubated in six-well plates (20–30 embryos per well) at 28.5 °C from 6 to 72 hpf. At 72 hpf, zebrafish were removed from the six-well plates, and anesthetized with a standard solution of 0.02% MS-222 for 10 s until the tail fins stopped moving. Then, the fish were transferred to slides, and observed for viability and morphological changes in blood vessels under a fluorescence microscope (Axio Imager Z1, Zeiss, Oberkochen, Germany). Images were taken with an epifluorescence microscope (Zeiss, Germany). The section of the zebrafish just below the yolk sac was chosen for the measurement of the number of complete intersegmental vessels (ISVs) and angiogenic sprouts by manual counting. Embryos receiving DMSO (0.1%) alone were used as vehicle controls. The assay was repeated three times independently with 20–30 embryos per group.

2.7. Endothelial cell capillary-like tube formation assay

BD Matrigel™ Basement Membrane Matrix (growth factor reduced; BD Biosciences, San Jose, CA, USA) was thawed at 4 °C, pipetted into pre-chilled 24-well plates and incubated at 37 °C for 45 min. HUVECs were first incubated in endothelial cell growth medium (ECGM) supplemented with 0.5% FBS for 6 h and then treated with DMSO (0.1%) or various concentrations of quercetin (50, 100 or 200 μM). Cells were collected and placed onto a layer of Matrigel™ (4×10^4 cells per well) in 1 ml ECGM supplemented with 0.5% FBS. After 6 h of incubation at 37 °C in a 95%:5% (v/v) mixture of air and CO_2, the network-like structures of the endothelial cells were examined under an inverted microscope (Olympus, Center Valley, PA, USA). The tube-like structures were defined as endothelial cord formations that were connected at both ends. Branching points in three random fields per well were quantified by manual counting. Cells receiving only DMSO (0.1%) served as a vehicle control. The inhibition percentage is expressed as the percentage of the vehicle control (100%). The assay was repeated three times independently.

2.8. Quantitative real-time PCR

The effects of quercetin on certain genes were determined by quantitative real-time polymerase chain reaction (qRT-PCR). HUVECs (5×10^5 cells per well) were seeded in 24-well plates,