

Low concentration of quercetin antagonizes the invasion and angiogenesis of human glioblastoma U251 cells

Yue Liu¹
Zhen-Gang Tang¹
Jian-Quan Yang¹
Yi Zhou¹
Ling-Hu Meng¹
Heng Wang¹
Cai-Li Li²

¹Department of Neurosurgery,

²Institute of Esophageal Tumor,
Renmin Hospital, Hubei University
of Medicine, Shiyan, Hubei, People's
Republic of China

Abstract: Glioblastoma is the most aggressive type of brain tumor with a very poor prognosis. Therefore, it is always of great importance to explore and develop new potential treatment for glioblastoma. Quercetin, a flavonoid present in a variety of human foods, has been shown to inhibit various tumor cell proliferation. In this study, we found that treating human glioblastoma U251 cells with 10 µg/mL quercetin for 24 hours, a concentration that was far below the IC₅₀ (113.65 µg/mL) and at which quercetin failed to inhibit cell proliferation, inhibited cell migration (30%) and cell invasion as examined by wound scratch assay and transwell assay, respectively. We further showed that 10 µg/mL quercetin inhibited cell migration and tube formation of human umbilical vein endothelial cells induced by the conditioned medium derived from U251 cell culture. The inhibitory effect of quercetin on migration and angiogenesis is possibly mediated through the downregulation of protein levels of VEGFA, MMP9, and MMP2 as detected by Western blot. Our findings demonstrated that low concentration of quercetin antagonized glioblastoma cell invasion and angiogenesis in vitro.

Keywords: glioblastoma, quercetin, angiogenesis, invasion, metastasis

Introduction

Glioblastoma, also called glioblastoma multiforme (GBM), is the most invasive type of glioma. The incidence of GBM is two to three per 100,000 adults per year, and it accounts for about 17% of all the brain tumors.^{1,2} GBM is composed of several different cell types, and most of these tumors occur in the cerebral hemisphere. Glioblastoma usually grows aggressively and spreads rapidly into the nearby brain tissue.³ The median survival time for adults with glioblastoma treated with concurrent temozolomide and radiation therapy is only around 14.6 months.⁴ The most common treatment for GBM is surgery, followed by radiation and chemotherapy. However, it is impossible to remove the tumor entirely by surgery since GBM usually migrates and invades the surrounding normal brain tissue. The major problem in chemotherapy is the difficulty to deliver drugs into the brain.⁵ So far, no significant improvement has been achieved to increase the survival rates;^{6,7} therefore, researchers are urged to develop new treatments for GBM.

Quercetin (3,3',4',5,7-pentahydroxy-flavone) is one of the most abundant flavonoids found in a variety of human foods such as apples, cherries, grapes, spinach, and onions.^{8,9} Quercetin has the ability to scavenge free radicals and bind transition metal ions, thus functioning as a powerful antioxidant.^{10,11} For instance, the inhibition of peroxidation of low-density lipoprotein by quercetin potentially reduces the risk of

Correspondence: Cai-Li Li
Institute of Esophageal Tumor,
Renmin Hospital, Hubei University
of Medicine, No 39 Middle Chaoyang
Road, Shiyan 442000, Hubei,
People's Republic of China
Tel +86 135 9788 3520
Email licailili@163.com

heart disease and atherosclerosis.^{12,13} Quercetin has also been reported to have anti-inflammatory, antimicrobial, antiviral, antiplatelet, and anti-proliferative activities.^{14–17} Interestingly, quercetin inhibits the proliferation of a wide range of cancers such as lung, breast, liver, colon, and prostate,^{18–22} via inducing cell cycle arrest and/or promoting cell apoptosis.^{23–25} Recent studies also showed that quercetin is able to inhibit DNA topoisomerases I and II²⁶ and modulate the PI3K/AKT and NF- κ B signaling pathways,^{27,28} indicating that quercetin has various anticancer mechanisms.

It is interesting to note that quercetin can also efficiently inhibit the growth of glioblastoma cells and induce apoptosis by suppressing the NF- κ B, Ras/MAPK/ERK, and PI3K/AKT signaling pathways.^{29–31} The minimal inhibitory concentration of quercetin on various glioma cells ranges from 20 μ g/mL to 50 μ g/mL. Our previous work showed that quercetin inhibits the growth of glioblastoma U251 cells in a dose-dependent manner with an IC₅₀ of 113.65 μ g/mL. At 10 μ g/mL, quercetin failed to inhibit the cell growth (Liu et al, unpublished data, 2017). In this study, we investigated the effect of 10 μ g/mL quercetin on cell migration and invasion and found that low concentration of quercetin inhibited glioblastoma cell invasion and angiogenesis in vitro.

Materials and methods

Reagent

Quercetin was purchased from Sigma-Aldrich Co. (St Louis, MO, USA), and Matrigel was obtained from BD Biosciences (San Jose, CA, USA). Transwell with 8.0 μ m pore polycarbonate membrane insert was obtained from Corning Incorporated (Corning, NY, USA). Anti-VEGFA (Cat No sc-7269) was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Anti-MMP2 (Cat No 10373-2-AP) and anti-MMP9 antibodies (Cat No 10375-2-AP) were purchased from Proteintech, Chicago, China. Anti- α -tubulin antibody was obtained from Sigma-Aldrich Co. Radioimmunoprecipitation assay (RIPA) lysis buffer was purchased from Beyotime (Nantong, China). This study was approved by the Institutional Review Board of Ethics Committee of Renmin Hospital, Hubei University of Medicine.

Cell culture

Human glioblastoma cell line U251 and human umbilical vein endothelial cell line CRL-1730 were provided by the Cell Center of Institute of Neurosurgery, Shiyan Renmin Hospital at Hubei University of Medicine. Both cell lines were cultured in DMEM, high glucose medium, supplemented with 10% fetal bovine serum (FBS). No antibiotics were added to

the medium. The cells were grown in 5% CO₂ and humidity saturation at 37°C in culture flasks. Cells were trypsinized with 0.25% trypsin and split at 80% confluence.

Cell migration and invasion assay

The capacity of cell migration was measured by the scratch wound healing assay. Briefly, U251 cells were plated and allowed to grow to the mid-log phase. Cell monolayer was gently scratched with a new 200 μ L pipette tip across the center of the dish. The wells were then washed twice with PBS, replenished with serum-free DMEM containing 10 μ g/mL quercetin, and cultured for 24 hours or 48 hours. Cells were then fixed with 3.7% paraformaldehyde, stained with 1% crystal violet, and examined by microscope. The gap distance was quantitatively evaluated using IPP 6.0 software.

Transwell cell invasion and migration assays were also conducted. Briefly, transwell inserts were coated with matrigel which were 1:6 diluted with serum-free medium. U251 cells were trypsinized, washed, and resuspended in medium containing 1% bovine serum albumin with or without 10 μ g/mL quercetin (1 \times 10⁵/mL). Then, 100 μ L of cell suspensions was added to the upper transwell compartment, and 600 μ L of DMEM with 10% FBS was added to the lower compartment. Cells in the transwell plate were cultured at 37°C with 5% CO₂ for 24 hours. Cells that remained on the upper side of the filter membrane were gently removed with a cotton swab, and cells on the lower side of the insert filter were fixed with 10% methanol and stained with 0.1% crystal violet. Images of the cells on the lower side of the insert filter were taken under a microscope. A total of 16 different 200 \times fields were randomly chosen, and the number of cells was counted.

Preparation of tumor-conditioned medium

U251 cells were cultured in DMEM, high glucose medium, supplemented with 10% FBS. When the cell density reached 60%–70% confluency, cells were washed with PBS for three times after removing the old culture medium and cultured in serum-free medium for 24 hours. The medium was harvested, centrifuged to remove cell debris, filtered with 0.22 μ m membrane, and stored in freezer for later use.

U251 cell-induced migration of endothelial cells

CRL-1730 cells were resuspended in serum-free DMEM with or without 10 μ g/mL quercetin at a density of 1 \times 10⁵/mL. Then, 100 μ L of cells was added to the upper compartment

of the transwell, and 600 μL of U251-conditioned medium was added to the lower compartment. After culturing for 8 hours, cells on the lower side of the insert filter were fixed with 10% methanol, stained with 1% crystal violet, and examined by microscope.

Endothelial cell tube formation assay

Matrigel was thawed at 4°C overnight and then added to a 96-well plate (50 μL /well). The plate was incubated for 30 minutes at 37°C to solidify the gel. CRL-1730 cells were resuspended in U251-conditioned medium with or without 10 $\mu\text{g}/\text{mL}$ quercetin and were gently added at $2 \times 10^5/\text{mL}$ to the gel-coated wells. Cells were cultured for 6 hours and examined by microscope for tubing formation.

Western blot

U251 cells were harvested, washed with cold PBS twice, and lysed with the lysis buffer containing the protease inhibitor for 30 minutes at 4°C. After centrifugation, the supernatant was fractionated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (20 μg total protein/lane). Proteins were transferred to polyvinylidene difluoride (PVDF) membrane, blocked with 5% fat-free milk for 1 hour, and incubated with primary antibodies

(anti-VEGFA: 1:500; anti-MMP9: 1:500; anti-MMP2: 1:500; anti- α -tubulin: 1:1,000) at 4°C overnight. Membranes were washed with PBS containing 0.1% Tween for three times and then incubated with goat anti-rabbit secondary antibodies (1:10,000) for 2 hours. Western blots were developed by using the enhanced chemiluminescence (ECL) kit, and the images were scanned and analyzed by using Quantity One software.

Statistical analysis

All the experiments were repeated three times, and the data were presented as mean \pm standard deviation ($\bar{x} \pm s$). Statistical analysis was conducted by using SPSS16.0 software (SPSS Inc., Chicago, IL, USA). Student's *t*-test was used for two sample comparisons. *P*-value < 0.05 was considered statistically significant.

Results

Effects of quercetin on migration and invasion of U251 cells

To study the effect of quercetin on cell migration, we conducted in vitro scratch assay. Control and quercetin-treated glioblastoma U251 cells were scratched and allowed to migrate for 24 hours or 48 hours (Figure 1A). The migration distance of cells treated with 10 $\mu\text{g}/\text{mL}$ quercetin for 24 hours

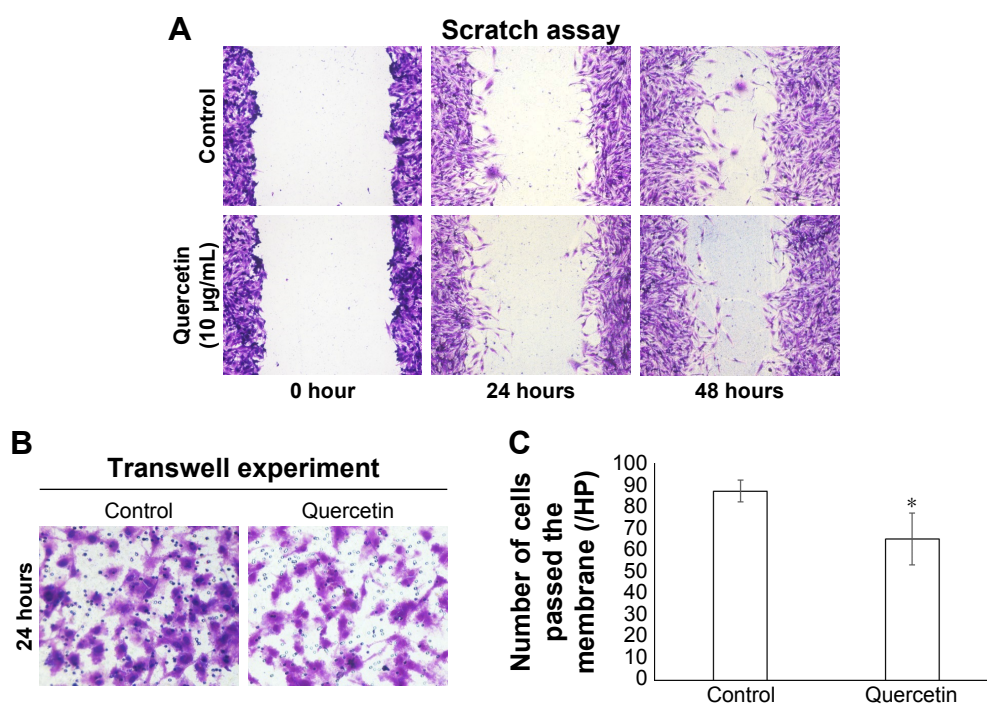


Figure 1 Quercetin inhibits U251 cell migration and invasion.

Notes: (A) Control and quercetin-treated confluent monolayer of U251 cells were scratched (0 hour) and allowed to migrate for 24 hours and 48 hours, respectively. (B) Transwell experiments showing control and quercetin-treated cells passing the membrane. (C) Number of cells passed the membrane for the control and quercetin-treated groups. **P* < 0.05. Magnification (A) $\times 100$, (B) $\times 200$.

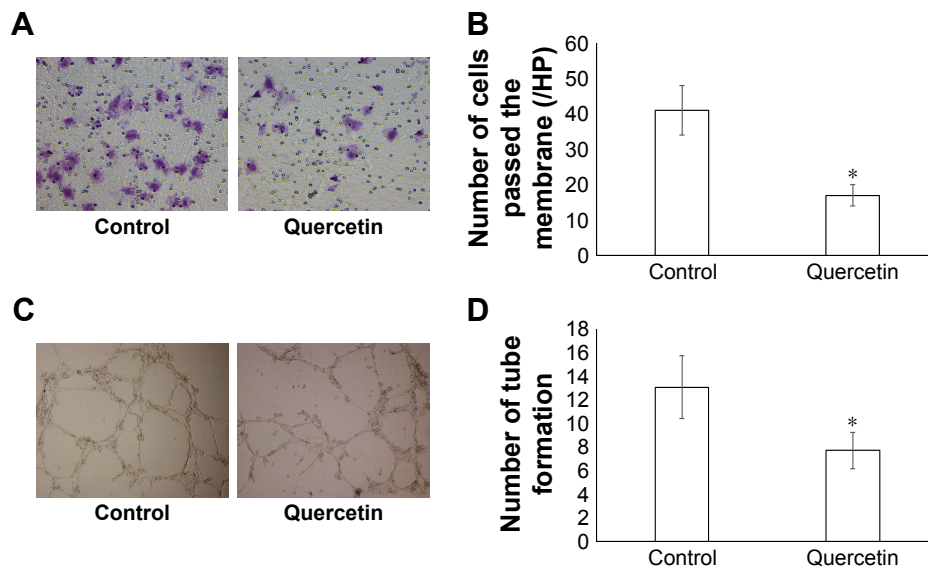


Figure 2 Quercetin inhibits U251-induced endothelial cell invasion and tube formation.

Notes: (A) Transwell experiments showing the control and quercetin-treated cells passing to the bottom wells, where U251-conditioned medium was applied. (B) Quantification of cell numbers for the control and quercetin-treated groups. (C) Endothelial cell tube formation in the presence of control and 10 $\mu\text{g}/\text{mL}$ quercetin. (D) Quantification of tube formation for the control and quercetin-treated groups. * $P < 0.05$.

and 48 hours was $70.10 \pm 1.18\%$ and $67.38 \pm 0.48\%$ of those of the control cells, respectively ($P < 0.05$). Moreover, transwell cell migration assay showed that the cell number passing the membrane for quercetin-treated cells ($65 \pm 12/\text{field}$) was significantly lower than that of the control group ($87 \pm 5/\text{field}$; $P < 0.05$, Figure 1B and C), indicating that quercetin treatment significantly inhibited U251 cell migration and invasion.

The effect of quercetin on U251-induced endothelial cell tube formation

We next used human umbilical vein endothelial cell line CRL-1730 to examine the effect of quercetin on U251-induced cell migration and angiogenesis. CRL-1730 cells, treated with control or quercetin in the transwell experiments, were allowed to invade into the bottom well for 8 hours, where U251-conditioned medium was present. In contrast to control cells, of which 41 ± 7 cells/field passed the membrane, there were only $17 \pm 3/\text{field}$ quercetin-treated cells in the bottom well ($P < 0.05$, Figure 2A and B). The data strongly indicated that quercetin inhibited U251 cell-induced cell invasion. Endothelial cell tube formation was also performed with U251-conditioned medium to examine the effects of quercetin on tumor cell-induced angiogenesis. Consistently, we found an inhibitory effect of quercetin on endothelial cell tube formation (7.67 ± 1.53 in the quercetin group vs 13.00 ± 2.65 in the control group, $P < 0.05$, Figure 2C and D).

Quercetin-regulated proteins involved in cell migration and angiogenesis

Proangiogenic signals such as VEGF are involved in activating endothelial cells to degrade the basement membrane, and proliferate, migrate, and form tubes in angiogenesis.³² Matrix metalloproteinases, MMP9 and MMP2, have been shown to be involved in cell migration by degrading the matrix.³³ Western blot shows that quercetin treatment significantly downregulated the levels of VEGF, MMP9, and MMP2 (Figure 3), suggesting that the inhibitory effects of quercetin on cell migration and angiogenesis are partially due to the deregulation of proangiogenic signals and matrix metalloproteinases.

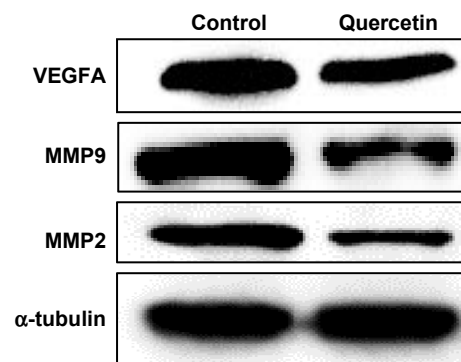


Figure 3 Quercetin inhibits VEGFA, MMP9, and MMP2 expression.

Note: Immunoblot showing protein levels of VEGFA, MMP9, and MMP2 in U251 cells in response to 0 $\mu\text{g}/\text{mL}$ (control) and 10 $\mu\text{g}/\text{mL}$ quercetin.

Discussion

In this study, we have found that quercetin inhibited glioblastoma cell migration and angiogenesis by downregulating the expression of VEGFA, MMP2, and MMP9. Tumor invasion and angiogenesis are very complex and dynamic processes that are made of multistep cascades.³⁴ A number of molecules such as growth factors, cell adhesion molecules, and extracellular matrix have been demonstrated to be involved in these processes. For example, VEGF is a key mediator of cancer angiogenesis.^{35,36} It stimulates the growth of new blood vessels and allows tumors to get nutrients and oxygen for growing. MMPs can degrade and remodel the extracellular matrix, facilitating the invasion and metastasis of tumor cells.^{33,37} MMP2 and MMP9, two major MMPs that proteolyze IV-type collagen, have been recognized as prognostic markers for malignant glioma.^{38,39} Quercetin has been reported to inhibit human prostate tumor angiogenesis by targeting VEGFR2-regulated AKT/mTOR/P70S6K signaling pathways.⁴⁰ Quercetin also inhibits migration and invasion of human oral cancer cells through suppressing MMP2/9 expression.⁴¹ Moreover, Santos et al²⁹ demonstrated that quercetin delayed cell migration in human GL-15 glioblastoma cells, possibly by reducing the expression of metalloproteinase, MMP2, as well as increasing the expression of fibronectin and laminin. In this study, we found that quercetin inhibited glioblastoma cell invasion and angiogenesis at 10 µg/mL, which was the lowest concentration reported so far for quercetin-mediated anti-invasion effects in vitro. In the future, we will examine the in vivo anticancer effects of quercetin using animal models and test any potential synergistic effects of quercetin with other chemotherapy drugs on cell migration and angiogenesis.

Conclusion

Our findings demonstrated that low concentration of quercetin antagonized glioblastoma cell invasion and angiogenesis in vitro.

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Disclosure

The authors report no conflicts of interest in this work.

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