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Original article

Inhibitory effect of quercetin on colorectal lung metastasis through inducing apoptosis, and suppression of metastatic ability

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ABSTRACT

Background: Quercetin is a major dietary flavonoid found in a various fruits, vegetables, and grains. Although the inhibitory effects of quercetin have previously been observed in several types of cancer cells, the anti-metastatic effect of quercetin on colorectal metastasis has not been determined.
Purpose: This study investigated whether quercetin exhibits inhibitory effect on colorectal lung metastasis.
Study design: The effects of quercetin on cell viability, mitogen-activated protein kinases (MAPKs) activation, migration, invasion, epithelial-mesenchymal transition (EMT) and lung metastasis were investigated.
Methods: We investigated the effect of quercetin on metastatic colon cancer cells using WST assay, Annexin V assay, real-time RT-PCR, western blot analysis and gelatin zymography. The anti-metastatic effect of quercetin *in vivo* was confirmed in a colorectal lung metastasis model.
Results: Quercetin inhibited the cell viability of colon 26 (CT26) and colon 38 (MC38) cells and induced apoptosis through the MAPKs pathway in CT26 cells. Expression of EMT markers, such as E-, N-cadherin, β-catenin, and snail, were regulated by non-toxic concentrations of quercetin. Moreover, the migration and invasion abilities of CT26 cells were inhibited by quercetin through expression of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) regulation. Quercetin markedly decreased lung metastasis of CT26 cells in an experimental *in vivo* metastasis model.
Conclusion: In conclusion, this study demonstrates for the first time that quercetin can inhibit the survival and metastatic ability of CT26 cells, and it can subsequently suppress colorectal lung metastasis in the mouse model. These results indicate that quercetin may be a potent therapeutic agent for the treatment of metastatic colorectal cancer.

Introduction

Colorectal cancer (CRC) is the most commonly diagnosed cancer worldwide, and up to 50% of patients suffer from aggravated metastatic disease (Jemal et al., 2011). Over the past 15 years, the incidence and mortality rates of CRC have also increased in Korea (Jung et al., 2013). In particular, colorectal lung metastasis will be seen in 10–20% of patients with CRC, with studies reporting 5-year survival rates of 20–60% in patients who undergo resection (Cidón 2010).

Apoptosis is the process of programmed cell death and apoptotic cells are eliminated by phagocytes, such as macrophages, without eliciting inflammation (Kerr et al., 1972). The intrinsic apoptotic pathway is regulated by the anti-apoptotic and pro-apoptotic members of the Bcl-2 family, including Bcl-2 and Bcl-xL (Fulda and Debatin 2006). After intrinsic changes, activated caspase-9 induces the activation of caspase-3, which in turn cleaves poly (ADP-ribose) polymerase (PARP), which finally leads to apoptosis (Boulares et al., 1999).

Abbreviations: CRC, colorectal cancer; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; ERK, extracellular-regulated protein kinase; JNK, c-Jun-N-terminal kinase; MAPK, mitogen-activated protein kinases; MMP, matrix metalloproteinases; PARP, poly (ADP-ribose) polymerase; TIMP, tissue inhibitor of metalloproteinases.

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Mitogen-activated protein kinases (MAPKs), including c-Jun-N-terminal kinase (JNK), extracellular-regulated protein kinase (ERK), and p38 kinase, are important mediators of cell membrane-to-nucleus signal transduction and are activated by diverse extracellular stimulation. MAPK pathway is linked to the triggering of apoptosis because they are often activated in response to various cellular stress and growth factors (Gomez-Sarosi et al., 2009; Chang et al., 2001). Thus, many anticancer agents show their effect through the regulation of MAPKs activation in most cancer cell lines (Wada and Penninger 2004).

Tumor metastasis occurs through a multistep process that includes vessel formation, cell attachment, invasion, and abnormal cell growth (van Zijl et al., 2011). This event initiates cytophysiological changes of cancer cells, such as epithelial-mesenchymal transition (EMT), in which primary tumor cells lose cellular adhesion and there is increased cellular motility. EMT is a cellular process during which epithelial cells become mesenchymal-like cells, resulting in the loss of epithelial polarity and intercellular adhesion (Thiery and Sleeman 2006). After this process, metastatic cancer cells migrate and invade the lymph and blood vessels. Then, they adhere and survive in the target organs (Gupta and Massagué 2006). Matrix metalloproteinases (MMPs) are important extracellular proteases, and at least 20 types of MMPs are known. The activation of MMPs enables the degradation of the extracellular matrix (ECM) and provides the opportunity for cancer cells to access the vasculature, migrate, and ultimately invade target organs (Nabeshima et al., 2002; Itoh and Nagase 2002). Among the MMPs, MMP-2 and MMP-9 efficiently degrade collagen, fibronectin, and elastin, which are associated with tumor migration and invasion (Khasigov et al., 2003). Therefore, the inhibition of MMP activity is extremely affective in slowing cancer progression, including suppression glio, aof metastasis.

Quercetin, a flavonoid contained in various foods, such as red onion, cranberry, broccoli, and black or green tea, plays a role in improving several diseases, such as psoriasis, neurodegenerative diseases, diabetes, inflammation, and allergic diseases (Chen et al., 2006; Formica and Regelson 1995; Shen et al., 2012). In particular, quercetin has shown an anti-cancer effect through the regulation of angiogenesis, apoptosis, cell cycle arrest, and inhibition of cell migration and invasion ability in several types of cancer, including breast, prostate, and lung cancer, without any damage to normal cells (Ansó et al., 2010; Lin et al., 2008; Temraz et al., 2013; Hung 2007). It has been reported that quercetin inhibits CRC by inducing cell cycle arrest and apoptosis as well as enhancing the effects of anti-cancer drugs (Zhang at al., 2012; Kim et al., 2010; Atashpour et al., 2015). However, it has not been reported whether quercetin could suppress colorectal metastasis. In this study, we attempted to confirm the effect of quercetin on colorectal metastasis and related molecular mechanisms.

Materials and methods

Materials

Quercetin (Que, purity > 95%) and 5-Fluorouracil (5-FU) were obtained from Sigma (St Louis, MO, USA). Anti-phospho-p38, -ERK, -JNK, PARP, and caspase-3 antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Caspase-9 antibody was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Bcl-2 and α-tubulin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Bcl-xL antibody was purchased from Bioworld Technology (Louis Park, MN, USA).

Cell culture

The mouse colon carcinoma cell line colon 26 (CT26), colon 38 (MC38), and CCD-18Co cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Grand Island, NY), and the human colon adenocarcinoma cell line HT29 was maintained in RPMI 1640 (Gibco BRL, Grand Island, NY). These mediums were supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin in humidified air containing 5% CO_2 in a 37 °C incubator.

Mice

BALB/c female mice (5 weeks, 19–20 g) were purchased from Da-Mool Science (Daejeon, Korea). All mice were housed six per cage in a laminar air-flow room maintained at a temperature of 22 ± 1 °C and a reactive humidity of 55 ± 1% throughout the study. The research was carried out in accordance with the internationally accepted principles for laboratory animal use and care as found in the Wonkwang University guidelines (WKU14-16).

WST assay

Cells were seeded in 96-well microplates (3×10^3 cells/well) and a quercetin-containing medium was added to the wells. After 24–72 h incubation, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt) solution (Enzo Life Sciences, Farmingdale, NY, USA) was added with new medium, and absorbance was measured at 450 nm.

Annexin V assay

Annexin V assay was performed using an FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Diego, CA, USA). Harvested cells were washed twice with cold phosphate-buffered saline (PBS), and the cells were resuspended in 1X Annexin V binding buffer (1×10^6 cells/ml). Then, 100 µl of the solution (1×10^5 cells) was transferred to a 5 ml tube and added with 5 µl titrated FITC Annexin V and Propidium Iodide (PI) Staining Solution. Cells were incubated for 15 min at room temperature in the dark. The volume was made up to 700 µl and analyzed with the FACS Calibur system (BD Biosciences, San Diego, CA, USA).

Western blot analysis

CT26 cells (1×10^6 cells/well) were incubated with various concentrations of quercetin. Stimulated cells were rinsed with PBS and then lysed in lysis buffer (iNtRon Biotech, Seoul, Korea) for 1 h Cell lysates were centrifuged for 10 min, and the quantity of protein in the supernatant was evaluated by using a bicinchoninic acid (BCA) protein assay. The supernatant was mixed with 2X sample buffer for sulfate-polyacrylamide gel electrophoresis and transferred to a membrane. The membranes were blocked with 5% skim milk for 1 h 30 min and incubated over 3 h with primary antibodies. The antibodies were detected using horseradish peroxidase-conjugated anti-rabbit, anti-mouse, and immunoglobulin G (Dako, Glostrup, Denmark), and blots were detected using the ECL system (Santa Cruz, CA, USA).

Wound healing assay

CT26 cells were seeded in a 6-well plate (5×10^5 cells/well) to form a monolayer overnight. Using a 200 µl pipette tip, a scratch of ~1 mm width was made in triplicate. Detached cells were removed, and the scratches were monitored at regular intervals over the course of 0–24 h under serum-free conditions containing