Regulation of Wnt signaling activity for growth suppression induced by quercetin in 4T1 murine mammary cancer cells

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Abstract. Quercetin is a promising chemopreventive agent against cancer that inhibits tumor progression by inducing cell cycle arrest and promoting apoptotic cell death. Recently, the Wnt/β-catenin signaling pathway has been implicated in mammary tumorigenesis, where its abnormal activation is associated with the development of breast cancer. Thus, the objective of this study was to examine the biological activities of quercetin against mammary cancer cells, and to determine whether quercetin could regulate the Wnt/β-catenin signaling pathway. Quercetin showed dose-dependent inhibition of cell growth and induced apoptosis in 4T1 cells. Treatment of 20 µM quercetin suppressed ~50% of basal TopFlash luciferase activity. Moreover, the inhibitory effect of quercetin on the Wnt/β-catenin signaling pathway was confirmed by the reduced stabilization of the β-catenin protein. Among various antagonists screened for the Wnt/β-catenin signaling pathway, the expression of DKK1, 2 and 3 was induced after treatment with 20 µM of quercetin. Stimulation with recombinant DKK1 protein, showed suppressive cell growth of mammary cancer cells instead of quercetin. When 4T1 cells were treated with recombinant Wnt3a or LiCl along with quercetin, both stimulators for the Wnt/β-catenin signaling pathway were able to restore the suppressed cell viability by quercetin. Thus, our data suggest that quercetin exerts its anticancer activity through the downregulation of Wnt/β-catenin signaling activity. These results indicate for the first time that quercetin decreases cell viability and induces apoptosis in murine mammary cancer cells, which is possibly mediated by DKK-dependent inhibition of the Wnt/β-catenin signaling pathway. In conclusion, our findings suggest that quercetin has great potential value as chemotherapeutic agent for cancer treatment, especially in breast cancer controlled by Wnt/β-catenin signaling activity.

Introduction

Breast cancer is the most common malignancy among females, accounting for nearly 1 in 3 cancers diagnosed in women worldwide, and it is the second leading cause of cancer death after lung cancer (1). The major treatment procedures for breast cancer patients are surgery, radiotherapy and chemotherapy; however, the cure rates are not satisfactory. New agents acting on novel targets in breast cancer are currently under investigation.

The role of dietary flavonoids in cancer prevention is widely discussed. Convincing data from laboratory studies, epidemiological investigations, and human clinical trials indicate that flavonoids have imperative effects on cancer chemoprevention and chemotherapy (2). Quercetin (3,3’,4’,5,7-pentahydroxyflavone), a member of the flavonoids family, is one of the most prominent dietary antioxidants present in human diet (3,4) and exerts diverse biological activities in a variety of cancer cell model, including ovarian, endometrial, lymphoma, prostate, liver and gastric cancer (5-7). Although precise molecular mechanisms underlying quercetin-mediated cellular responses on breast cancer remain poorly defined, prior research has shown that it can modulate diverse proteins involved in signal transduction pathways associated with cell survival, apoptosis and proliferation (8-10).

Current studies have shown the importance of several signaling pathways in breast carcinogenesis and progression (11,12) including the Wnt/β-catenin pathway (13-15). The Wnt/β-catenin pathway plays a vital role in mammary tumorigenesis since overexpression of Wnt1 gene in the mammary epithelium is sufficient for mammary gland hyperplasia and adenocarcinomas (16). The canonical Wnt ligands, exemplified by Wnt1 or Wnt3a, bind to frizzled receptor (Fz) and the
low-density lipoprotein receptor-related protein-5/6 (LRP5/6), and prevent phosphorylation and degradation of β-catenin by the GSK3β/β-catenin destruction complex. Subsequent accumulation of cytosolic and nuclear β-catenin bound to T cell factor (TCF) transcription factors results into activation of downstream signals which are important for proliferation and matrix remodeling (17). The elevated levels of nuclear and/or cytoplasmic β-catenin in breast carcinomas correlated with the expression of its target gene cyclin D1 and poor patient prognosis (18). In a microarray based study, Huang et al (19) showed that inhibition of Wnt/β-catenin pathway leads to apoptosis in HeLa cells by upregulating the expression of several pro-apoptotic genes, involved in apoptotic cell death pathways, such as PTEN-P3K-AKT pathway, NfκB pathway and p53 pathway. Furthermore, studies in mice strongly imply that deregulated β-catenin signaling increases risk of breast cancer by inducing stem cell and early progenitor cell accumulation (20,21). Several studies have accounted for increased cytoplasmic and nuclear β-catenin in primary breast cancers, especially basal-like breast cancers, and correlated with poor prognosis and survival (22,23). Thus, it needs to be investigated whether the activation of Wnt/β-catenin signaling can be a potential target for the chemoprevention and treatment of breast cancer.

To identify molecular mechanism for quercetin-induced apoptosis, we used the murine mammary carcinoma cell line, 4T1, which is highly tumorigenic and invasive (24). Contrary to most tumor models, 4T1 cells injected into the mammary fat pads of syngeneic BALB/c mice grow spontaneously into multiple distant organs such as lymph nodes, liver, lung, brain and bone (25). Therefore, 4T1 is widely used as one of the most aggressive type of breast cancer cell for the study of breast cancer. Although quercetin has been reported to stimulate cell cycle arrest and apoptosis in breast cancer cells (26) the exact molecular mechanism of action still remains unclear. In this study, we show that quercetin induces apoptosis of mammary cancer cells through DKK-dependent inhibition of the Wnt/β-catenin signaling pathway.

Materials and methods

Cell culture and reagents. Murine mammary cancer cell line 4T1 (ATCC, CRL-2539) was cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin (Lonza, Basel, Switzerland) at 37°C and 5% CO₂. Quercetin was purchased from Sigma (St. Louis, MO, USA). A stock solution (25 mM) of quercetin was prepared in dimethyl-sulfoxide (DMSO). Recombinant proteins, Wnt3a and DKK1, were purchased from R&D Systems (Minneapolis, MN).

MTT assay. MTT assay was carried out to evaluate the viabilities of 4T1 cells, and performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma). 4T1 cells (1.5x10⁴ cells/well) were plated in 96-well plates and allowed to attach overnight. Cells were then treated with quercetin at various concentrations (5, 10, 20 and 40 µM) or the vehicle (0.16% of DMSO) and incubated at 37°C with 5% CO₂ for 48 h. Fresh MTT (5 mg/ml) was added to growing cells in 96-well plate and incubated at 37°C for 2 h. Following the removal of supernatant, the insoluble formazan crystals were dissolved in 200 µl of DMSO and optical density was measured at wavelength of 570 nm.

Assessment of cell apoptosis. 4T1 cells were treated with quercetin (20 µM) for 48 h, and then apoptotic cells were quantified by using an Annexin V-FITC/PI double staining assay kit (BioPrince, Seoul, Korea). Both, early and late apoptotic changes in 4T1 cells were analyzed. Briefly, cells (1x10⁶) were collected and washed twice with phosphate-buffered saline (PBS) and suspended in 400 µl of binding buffer (added with 5 µl of Annexin V-FITC and 5 µl of PI). The combination of Annexin V-FITC and PI staining permitted the simultaneous quantification of vital, apoptotic and necrotic cells. Thereafter, the samples were incubated in the dark for 15 min at room temperature, and then analyzed on the flow cytometer. The number of Annexin V-FITC-positive and PI-positive cells in each field was determined by counting the cells directly. Experiments were performed in triplicate for accuracy.

RNA isolation and real-time RT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen). RNA purity was verified by measuring 260/280 absorbance ratio. The first strand of cDNA was synthesized with 2 µg of total RNA using SuperScript II (Invitrogen), and one-tenth of the cDNA was used for each PCR mixture containing Express SYBR-Green qPCR Supermix (BioPrince, Seoul, Korea). Real-time PCR was performed using a Rotor-Gene Q (Qiagen, Hilden, Germany). The reaction was subjected to 40-cycle amplification at 95°C for 20 sec, at 60°C for 20 sec and at 72°C for 25 sec. Relative mRNA expression of selected genes was normalized to GAPDH and quantified using the DDCT method. The sequences of the PCR primers are listed in Table I.

Luciferase reporter assay. 4T1 cells were plated at a density of 2x10⁴ cells/well in 48-well plates, and transfected using

<table>
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<tr>
<th>Gene</th>
<th>Forward primer (5’→3’)</th>
<th>Reverse primer (3’→5’)</th>
<th>Size (bp)</th>
<th>Accession no.</th>
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<td>DKK4</td>
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<tr>
<td>GAPDH</td>
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<td>ACCCTGTGTTGCTAGCGCTATTCA</td>
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<td>NM_008084.2</td>
</tr>
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</table>

Table I. Primers for real-time RT-PCR.
Gene transfection reagent (Genetrone Biotech Co., Korea) according to the manufacturer's protocol. The TopFlash (Addgene, Cambridge, MA) luciferase reporter (100 ng) and Renilla luciferase thymidine kinase construct (Invitrogen) (50 ng) were used to determine luciferase activity. Luciferase activity was measured by a luminometer (Glomax, Promega, Sunnyvale, CA), using a Dual-Luciferase assay kit (Promega), according to the manufacturer's recommendations. Total value of reporter activity in each sample was normalized to Renilla luciferase activity.

Protein isolation and western blot analysis. Cells were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol) containing protease inhibitor cocktail (Roche). The concentration of protein was measured with a Protein assay kit (Bio-Rad) following the manufacturer's protocol. Total protein was subjected to SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The blot was probed with primary antibody; anti-β-catenin (Cell Signaling Technology). As a loading control, anti-β-actin antibody (Santa Cruz biotech) was used. Subsequently, the blots were washed in TBST (10 mM Tris-HCl, 50 mM NaCl, 0.25% Tween-20) and incubated with a horseradish peroxidase-conjugated secondary antibody. The presence of target proteins was detected using the enhanced chemiluminescence reagents (BioNote Inc., Hwaseong, Korea).

Lactate dehydrogenase (LDH) activity assay. Measurement of LDH activity was performed with cytotoxicity detection kit (Roche) according to the manufacturer's protocol as follows. Cell culture media (10 µl) from each experimental sample was added to a 96-well plate containing 40 µl of PBS. Next, 50 µl of LDH reagent was added to each well and plates were incubated for 45 min at 25°C in dark, and then enzymatic reaction was stopped by adding the stop solution (50 µl). Absorbance was read at wavelength of 492 nm. Total cell lysate served as a positive control of cell death.

Statistical analysis. All the statistical data were analyzed by GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) and evaluated by two-tailed Student's t-test. Value of P<0.05 was considered to indicate statistical significance.

Results

The treatment of quercetin delays the growth and induces apoptosis of 4T1 cells. Initially, we attempted to determine whether quercetin could exert anti-proliferative effect on 4T1 mouse mammary cancer cell line as reported in case of diverse breast cancer cell lines (27-29). After treatment of quercetin at various doses for 48 h, a dose-dependent decrease in cell viability of 4T1 cells was observed (Fig. 1A). Cells taken as control showed increase in confluence from initial 60 to 100% after 48 h of culture. However, the cells treated with 10 and 20 µM of quercetin reached only 70-80% of confluence. Moreover, a dose of 40 µM of quercetin showed 50% decrease in initial cell number. Hence, for further experiments, 20 µM of quercetin was selected as an ideal dose to detect the effect of quercetin. Since cell apoptosis may be one of the consequences of growth arrest, we stained the cells with Annexin V-FITC and PI, and analyzed the apoptotic effect of quercetin on 4T1 cells using flow cytometry. Fig. 1B showed an increased apoptotic percentage of growth arrest, we stained the cells with Annexin V-FITC and PI, and analyzed the apoptotic effect of quercetin on 4T1 cells using flow cytometry. Fig. 1B showed an increased apoptotic effect of quercetin at a dose of 20 µM (37%) as compared to control (18%). The population of early apoptotic cells increased ~3-fold compared to control whereas, significant increase in late apoptotic cells were also observed.

Wnt/β-catenin signaling activity is decreased by quercetin. As Wnt/β-catenin signaling pathway is implicated in regulating tumor growth (30), the effect of quercetin on Wnt/β-catenin signaling pathway of 4T1 cells needed defining. To detect the activity of Wnt/β-catenin signaling pathway, we used the luciferase reporter assay. 4T1 cells were transfected with TopFlash-luc for Wnt/β-catenin signaling pathway. After 48 h of treatment, quercetin suppressed ~50% of basal level of TopFlash luciferase activity, demonstrating inhibitory effect
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of quercetin on Wnt/β-catenin signaling pathway (Fig. 2A). Canonical Wnt signaling acts through stabilization of β-catenin, which translocate into the nucleus to regulate the expression of genes related to tumor growth, such as cyclin D1 and survivin (31). Thus, in order to confirm the inhibitory role of quercetin on Wnt/β-catenin signaling pathway as observed by luciferase assay, the stability of β-catenin was detected by western blot analysis. We observed reduction of β-catenin stabilization in quercetin treated cells after 1 h of treatment, confirming inhibitory effect of quercetin on Wnt/β-catenin signaling pathway (Fig. 2B).

**Quercetin induces the gene expression of DKK1, 2, 3 and 4.** Wnt/β-catenin signaling pathway is regulated by several processes. Secreted proteins such as secreted frizzled-related protein (sFRP) and Dickkopf (DKK) act as extracellular antagonists and are among various mechanisms to regulate Wnt/β-catenin signaling pathway (32,33). To investigate whether various negative regulators of Wnt/β-catenin signaling pathway is being upregulated in 4T1 cells by quercetin, we next examined the expression of known negative regulators by real-time RT-PCR. Among various antagonists screened for Wnt/β-catenin signaling pathway, mRNA expression level of DKK1, 2, 3 and 4 was found to be elevated dose-dependently (Fig. 3), while any altered expression of sFRP family was not observed (data not shown). Expression of DKK1 increased more than 3-fold while increase in DKK2 and 3 was about 2-fold. A non-significant induction of DKK4 was observed when treated
with 20 µM of quercetin. Induced expressions of DKK family by quercetin suggest their involvement in regulating the Wnt/β-catenin signaling pathway in our system as expected.

Exogenous treatment of DKK1 shows inhibitory effect on cell growth. Among DKK1, 2, 3 and 4, expression of DKK1 have been strongly implicated to proliferation ability of breast cancer cells via regulation of Wnt/β-catenin signaling pathway (34,35). Therefore, to confirm involvement of DKK1 as an antagonist for Wnt/β-catenin signaling pathway after treatment with quercetin, 4T1 cells were stimulated with recombinant DKK1 protein instead of quercetin. Stimulation with 100 µg/ml of recombinant DKK1 showed suppressive effect on cell growth as detected by MTT assay (Fig. 4). The inhibition on cell growth by DKK1 was partial (~10%) as compared to the effect of quercetin, suggesting the possibility of involvement of several other regulatory mechanisms by quercetin for the inhibition of cell growth along with DKK1.

Stimulation of Wnt/β-catenin signaling pathway with Wnt3a or LiCl restored the effect of quercetin. From results obtained, we hypothesized that Wnt/β-catenin signaling activity might be reduced by the induction of the secreted antagonists in response to quercetin. To investigate this possibility, we potentiated the activity of Wnt/β-catenin signaling pathway by exogenous stimulation and observed whether activation of Wnt/β-catenin pathway can effectively restore the inhibition of cell viability in 4T1 cells treated with quercetin. 4T1 cells were incubated with quercetin along with recombinant Wnt3a (Fig. 5), or LiCl (Fig. 6). After 48 h, cell viability was measured by MTT assay and cytotoxicity was detected by LDH assay. Both stimulators for Wnt/β-catenin signaling pathway were able to restore the suppressed cell viability by quercetin in a dose-dependent manner, as well as inhibition of the increased cytotoxicity (Figs. 5 and 6). However, Wnt3a or LiCl did not show any individual effect on cell viability and cytotoxicity in absence of quercetin.

Discussion

The aim of the present study was to investigate the inhibitory role of quercetin on tumorigenic potential of murine mammary cancer cell line, 4T1, and to evaluate its ability for modulating Wnt/β-catenin signaling pathway.

Quercetin is one of the major dietary flavonoids enriched in various fruits and vegetables. Recent studies report that quercetin exert a broad range of pharmacological effects and is a promising chemopreventive agent in a variety of cancer models (36,37). Previous experimental studies indicate that quercetin possesses antitumor activity and induces apoptotic cell death of breast cancer cells by the activation of caspase- or p53-dependent pathway (38-40). Consistent with previous reports, our study showed that quercetin reduces viability and induces apoptosis even in murine mammary cancer cell line, 4T1 (Fig. 1). Though quercetin has attracted much attention in relation to its anticancer activities in many cancer cell models, the molecular mechanisms underlying quercetin-mediated cellular responses remain poorly defined.

The Wnt/β-catenin signaling pathway has been demonstrated to play an important role in tumorigenesis (30). Initially, Wnt/β-catenin signaling activation, as defined by β-catenin nuclear expression and overexpression of the cyclin D1, was reported to be associated with a poorer prognosis in breast cancer patients (27). Recent studies have confirmed the Wnt/β-catenin signaling...
A further investigation from their receptors or contend for receptor binding. In a few and sFRP that can competitively dislodge certain Wnt ligands regulated by a variety of secreted proteins, such as WIF, DKK upstream of Wnt/β-catenin activity in breast cancer cells after treatment of quercetin. According to our results of decreased Wnt/β-catenin signaling regulated by DKK family that may serve as a future target for therapeutic strategies to inhibit the development of breast cancer cells.

References


