Quercetin inhibits human breast cancer cell proliferation and induces apoptosis via Bcl-2 and Bax regulation

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Abstract. Breast cancer is a disease in which cancer cells form in the tissues of the breast. The present study aimed to explore the effect of the flavonoid compound quercetin on the growth and apoptosis of human breast cancer cells. Varying concentrations (12.5, 25, 50, 100, 200 μM) of quercetin were applied to cultured MCF-7 human breast cancer cells for defined lengths of time. At 50 to 200 μM doses, quercetin significantly inhibited the proliferation of MCF-7 cells assessed by MTT colorimetry, in both dose- and time-dependent manners ($P<0.05$). The compound also increased apoptosis after 48 h of exposure ($P<0.05$). Furthermore, following quercetin treatment Bcl-2 expression decreased significantly while Bax expression increased significantly ($P<0.05$). In brief, quercetin inhibits cell growth and induces apoptosis in MCF-7 human breast cancer cells. The mechanisms behind these effects may stem from the downregulation of Bcl-2 protein expression and upregulation of Bax expression.

Introduction

Breast cancer is one of the most common malignancies in humans, accounting for 7-10% of all malignant tumors; its incidence increases at an annual rate of 1-2% (1). According to World Health Organization Statistics, about 1.3 million women are diagnosed with breast cancer each year. In North America, Europe, and other developed countries, the incidence of breast cancer ranks first among all malignant tumors in women, and its mortality ranks second only to lung cancer among deaths caused by malignant tumors in women (2,3). In recent years, the incidence of female breast cancer in China has risen. In fact, this incidence increased from 19.9/100,000 in 2000 to 24.8/100,000 in 2005, making breast cancer the most common malignancy in Chinese women. Furthermore, the number of deaths from breast cancer in women increased from 34,927 to 48,764 during the same period, with the mortality rate increasing from 5.5/100,000 to 6.7/100,000 (4).

Current treatment of breast cancer follows an integrated therapeutic model, which includes surgery, radiation therapy, chemotherapy, endocrine therapy, and biological therapy; this approach has made breast cancer one of the most successfully treatable solid tumors (5). Chemotherapy is irreplaceable in this treatment plan. In the last 30 years, more than 100 clinical trials from many countries have confirmed that postoperative adjuvant chemotherapy for breast cancer kills systematic subclinical micrometastases, thereby reducing postoperative recurrence and mortality rates of breast cancer (6). Although current chemotherapeutics are able to inhibit or kill tumors, there remain issues of toxicity and severe side effects, restricting the clinical application of these drugs in antitumor treatment. Thus, the exploration and development of new antitumor chemotherapeutics is critical to improving the integrated treatment plan.

One potential antitumor compound is quercetin. Quercetin and its derivatives, which are easily extracted, isolated, and detected, are widely distributed in the natural world; quercetin is a flavonoid compound found in many fruits, vegetables and herbs. This compound is biologically active, able to expand coronary arteries, reduce capillary permeability and fragility, and prevent platelet aggregation, as well as possessing other antioxidant, anti-virus, anti-allergic, analgesic, and other pharmacological effects (7). Recent studies (8-10) have found that quercetin is also able to prevent and treat tumors, by not only inhibiting many carcinogens and tumor promoters, but also preventing growth of tumor cells. To determine the ability of quercetin to treat breast cancer, we investigated the effects

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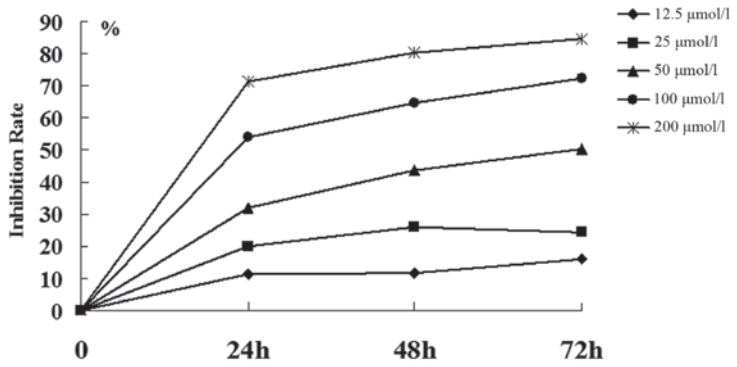

Figure 1. Relationship between time and inhibition rates of MCF-7 cells after treatment with different concentrations of quercetin.

of quercetin on *in vitro* proliferation and apoptosis of human breast cancer MCF-7 cells using MTT colorimetry and flow cytometry. Furthermore, we assessed the induction of apoptosis in MCF-7 cells following quercetin treatment by observing changes in apoptosis-related Bcl-2 and Bax protein expression.

Materials and methods

Materials and reagents. Human breast cancer MCF-7 cells were supplied by the School of Life Sciences, Xiamen University. Quercetin (Sigma, USA; lot no. 100081-200406) was suspended in dimethyl sulfoxide (DMSO) and stored at -20˚C. Prior to use, quercetin was diluted with Dulbecco's modified Eagle's medium (DMEM) to a final concentration of 0.8%. Fetal bovine serum (FBS), DMEM, DMSO, trypsin, and MTT were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. Mouse anti-human Bcl-2 and Bax monoclonal antibodies and the SP immunohistochemistry kit were purchased from Wuhan Boster Bio-Engineering Limited Company. The flow cytometer was purchased from US Gene Company.

Experimental methods. MCF-7 cell growth was assessed using the MTT method. MCF-7 cells were cultured in 10% DMEM (containing 10% FBS and 100 U/ml streptomycin) at 37˚C in 5% CO_2, under saturated humidity conditions. Medium was replaced every two days. MCF-7 cells were harvested during logarithmic growth phase, digested with 2.5 g/l trypsin, and resuspended to a cell concentration of $1x10^5$/ml. Two hundred microliters of suspension was transferred to each well of a 96-well culture plate and cultured for 24 h. Quercetin was then added to a subset of wells at concentrations of 12.5, 25, 50, 100 and 200 μM. Control wells contained only MCF-7 cells without quercetin, and blank wells contained only culture medium. Three replicates were established for each group, and three plates were added at a time. After 24, 48 and 72 h in culture, 20 μl MTT (5 mg/ml) was added to each well, and plates were incubated at 37˚C for 4 h. Supernatants were extracted and discarded, and 200 μl DMSO were added to each well with shaking to completely dissolve the blue-purple precipitate from MTT. A microplate reader was used to test the absorbance (A) of each well at 490 nm, and average values were obtained. Time (x) and A value (y) were used to create a growth curve and determine inhibition rates of quercetin on MCF-7 cells at different time points and concentrations {inhibition rate (IR) = [(A490 in control group - A490 in experimental group)/A490 in control group] x 100%}.

Cell cycle detection by flow cytometry. Following incubation with quercetin, cells were digested with trypsin and dispersed. Samples were centrifuged at 1500 rpm for 5 min, supernatants were discarded, and 200 μl 1X buffer was added. Annexin V (4 μl) and propidium iodide (PI) (5 μl) were added to each sample, which was brought to a final volume of 250 μl with 1X buffer before placing in an ice bath. Flow cytometry was used to determine the cell cycle and apoptosis rate. MCF-7 cells cultured for 72 h without quercetin were used as the negative control.

Bcl-2 and Bax detection by immunohistochemistry. Four bottles of MCF-7 cells in logarithmic growth phase ($1x10^5$ cells/bottle) were randomized to the control and experimental groups, 2 bottles/group. Those in the experimental group were cultured with medium containing 200 μM quercetin; those in the control group were cultured with conventional methods. After 48 h, one bottle was randomly selected from each group and digested with 0.2% trypsin. Cells were then harvested for smears and fixed for 20 min at 4˚C with acetone, then washed with 1X PBS three times for 5 min each and air-dried. Bcl-2 and Bax immunohistochemistry was performed according to instructions supplied in the PV-9000 kits. Bcl-2- and Bax-positive cells displayed yellow or brown cytoplasmic staining. Staining was analyzed with automatic image analysis software using identical capture conditions for each field. Five fields (10x40) were randomly selected for analysis of the average optical density (AOD) of 50 cells to determine staining intensity of Bcl-2 and Bax proteins.

Statistical methods. SPSS13.0 software was used for data analysis. MTT and flow cytometry results were analyzed by single factor analysis of variance and SNK method; comparisons of Bcl-2 and Bax protein expression were analyzed with independent samples t-test. Tests were 2-sided, with α level of 0.05, and $P<0.05$ was considered to denote statistical significance.

Results

Quercetin inhibits MCF-7 cell proliferation. Using MTT colorimetry to measure cell proliferation, we observed that 12.5

Table I. Effects of quercetin on proliferation of MCF-7 human breast cancer cells.

			24 h		48 h		72 h	
Group	Dose (μmol/l)	n	A490	IR (%)	A490	IR (%)	A490	IR (%)
Control	-	3	0.570±0.032	-	0.718±0.028	-	0.818±0.031	-
Quercetin	12.5	3	0.505±0.018[a]	11.4	0.634±0.016[a]	11.6	0.686±0.026[a]	16.1
	25	3	0.457±0.036[a]	20.0	0.532±0.039[a]	26.0	0.620±0.012[a]	24.2
	50	3	0.388±0.016[a]	32.0	0.404±0.033[a]	43.8	0.405±0.025[a]	50.4
	100	3	0.262±0.025[a]	54.0	0.255±0.039[a]	64.6	0.225±0.020[a]	72.5
	200	3	0.164±0.022[a]	71.4	0.140±0.021[a]	80.5	0.124±0.019[a]	84.8
	F	-	108.479	-	158.828	-	457.232	-
	P-value	-	0.001	-	0.001	-	0.001	-

IR, inhibition rate. [a]P<0.05 vs. control group.

Table II. Effects of quercetin on the cell cycle and apoptosis rate of MCF-7 human breast cancer cells.

		Cell cycle			
Group	Dose (μmol/l)	G_0/G_1	S	G_2/M	Apoptosis rate
Control	-	62.41±1.94	26.74±2.75	10.85±0.82	0.42±0.04
Quercetin	12.5	59.92±1.94	27.61±2.76	12.47±0.91	0.68±0.07
	25	51.43±1.56[a]	31.68±2.38	16.89±0.83	1.26±0.09
	50	42.04±1.74[a]	32.74±3.07	25.23±1.36[a]	8.53±0.55[a]
	100	30.70±1.93[a]	37.47±2.81[a]	31.83±0.89[a]	11.54±0.75[a]
	200	29.74±1.24[a]	58.00±1.94[a]	12.26±0.73[a]	23.15±1.91[a]
	F	199.022	57.618	241.377	316.291
	P-value	0.001	0.001	0.001	0.001

[a]P<0.05 vs. control group. Values are expressed as percentages.

Table III. Levels of Bcl-2 and Bax in MCF-7 cells.

Group	n	Bcl-2	Bax
Control group	50	0.372±0.160	0.251±0.092
Test group	50	0.260±0.100	0.336±0.127
t		4.226	3.828
P-value		0.001	0.001

Values for average optical density (AOD) are expressed as the mean ± SD.

and 25 μM doses of quercetin did not inhibit proliferation of human breast cancer MCF-7 cells; however, higher doses (50, 100 and 200 μM) significantly inhibited proliferation of these cells (Fig. 1). Furthermore, inhibition of proliferation displayed both dose- and time-dependent relationships with quercetin treatment. At a quercetin concentration of 50 μM, the inhibition rate ranged between 32.0 and 50.4%; at a concentration of 100 μM, the inhibition rate fell between 54.0 and 72.5%; and when quercetin concentration was 200 μM, the inhibition rate ranged between 71.4 and 84.8%. Compared with control samples, these differences in proliferation were statistically significant (P<0.05; Table I).

Quercetin induces MCF-7 cell apoptosis. Using flow cytometry and comparing with untreated MCF-7 cells, the cell cycles of quercetin-treated MCF-7 cells were skewed (Table II). Specifically, the proportions of G_0/G_1 phase cells decreased significantly with all concentrations of quercetin (P<0.05). In contrast, the proportions of S phase cells increased significantly with quercetin concentrations of 100 and 200 μM (P<0.05), and the proportions of G_2/M phase cells increased significantly with quercetin concentrations of 50, 100 and 200 μM (P<0.05). Additionally, PI staining enabled quantification of apoptosis. Apoptosis rates increased with increasing concentrations of quercetin; the apoptosis rates of MCF-7 cells treated with 50, 100 and 200 μM quercetin were all significantly higher than those of the control MCF-7 cells (P<0.05).

Bcl-2 and Bax protein expression. To further confirm increased rates of apoptosis in quercetin-treated MCF-7 cells, we assessed Bcl-2 and Bax protein expression by immunohistochemistry (Table III). Bcl-2 protein expression was significantly lower in quercetin-treated cells than in control cells (t=4.226, P=0.001), while Bax protein expression was significantly higher following quercetin treatment (t=3.828, P=0.001).

Discussion

Previous *in vivo* and *in vitro* experiments demonstrated that quercetin inhibits the growth of breast, ovarian, colon, liver and stomach cancers as well as leukemia and other malignant tumors, likely by blocking cell cycles, inhibiting tumor cell signaling, and regulating growth factors to prevent further cell proliferation and to induce tumor cell apoptosis (11). Our study of the effects of quercetin on MCF-7 breast cancer cells demonstrates its ability to inhibit proliferation of these cells in a dose- and time-dependent manner.

Proliferation of tumor cells depends on completed cell cycles (12), which are regulated and controlled by the G_1/S and G_2/M checkpoints. These checkpoints trigger mitotic spindle assembly and ensure that downstream activities are initiated when upstream activities are properly completed (13). Cell cycle checkpoint regulation also removes damaged cells by inducing apoptosis and blocking cell cycle progression (causing apoptosis); indeed, apoptosis is often accompanied by cell-growth retardation (14). Using flow cytometry we showed that, after treatment of human breast cancer cells with quercetin for 72 h, the proportion of cells in the G_0/G_1 phase decreased, while the proportion of cells in S phase increased, suggesting that quercetin blocked transition from S phase to G_2/M phase. Notably, the proportion of cells in G_2/M phase also increased, indicating cell cycle retardation at this checkpoint; however, at a quercetin concentration of 200 μM, the proportion of cells in G_2/M phase decreased. The reason for this change remains unclear. However, we used PI staining to verify that more cells underwent apoptosis following treatment with quercetin, and that rates of apoptosis increased as quercetin dose increased.

Bcl-2, or B-cell lymphoma/leukemia-2, is a proto-oncogene that inhibits apoptosis through antioxidant activity and inhibition of transmembrane flow of calcium ions. *Bax* is a related gene with the ability to antagonize Bcl-2 protein and promote apoptosis (15). Bax protein expression is closely related to development of various malignant tumors and is significantly reduced in some of them (16,17). In this study, immunohistochemistry for Bcl-2 and Bax following treatment of MCF-7 cells with 200 μM of quercetin indicated that cells showed decreased expression of Bcl-2 but increased expression of Bax. This finding demonstrates that reduced expression of an anti-apoptotic protein and increased expression of a pro-apoptotic protein resulted in higher rates of apoptosis following quercetin treatment.

In conclusion, quercetin treatment decreases proliferation and increases apoptosis in MCF-7 human breast cancer cells. Apoptosis is induced via upregulation of Bax protein and downregulation of Bcl-2 protein. The mechanisms by which quercetin regulates Bcl-2 and Bax expression require further study, but this compound shows promise as a potential chemotherapeutic.

Acknowledgements

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