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Quercetin decreases the expression of ErbB2 and ErbB3 proteins in HT-29 human colon cancer cells☆

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Abstract

Quercetin has chemoprotective properties in experimental colon cancer models, and in vitro studies have demonstrated that quercetin inhibits HT-29 colon cancer cell growth. ErbB2 and ErbB3 receptor tyrosine kinases have been associated with the development of human colon cancer, and the expressions of both receptors are high in HT-29 cells. In this study, we assessed quercetin regulation of HT-29 and SW480 cell apoptosis and the influence of quercetin on the protein expression of ErbB2, ErbB3, Akt, Bax and Bcl-2. We cultured HT-29 cells in the presence of various concentrations (0, 25, 50, or 100 μmol/L) of quercetin or rutin. Quercetin inhibited HT-29 cell growth in a dose-dependent manner, whereas rutin had no effect on the cell growth. DNA that was isolated from cells treated with 50 μmol/L of quercetin exhibited an oliogonucleosomal laddering pattern characteristic of apoptotic cell death. Western blot analysis of cell lysates revealed that Bcl-2 levels decreased dose-dependently in cells treated with quercetin, but Bax remained unchanged. Quercetin increased levels of cleaved caspase-3 and the 89-kDa fragment of poly (ADP-ribose) polymerase. In addition, phosphorylated Akt levels were markedly lower in cells treated with 25 μmol/L quercetin, but total Akt levels decreased only at 100 μmol/L quercetin. Furthermore, a dose-dependent decrease in ErbB2 and ErbB3 levels was detected in quercetin-treated cells. The results obtained using SW480 cells were similar to those obtained with HT-29 cells. In conclusion, we have shown that quercetin inhibits cell growth and induces apoptosis in colon cancer cells, and that this may be mediated by its ability to down-regulate ErbB2/ErbB3 signaling and the Akt pathway.

Keywords: Akt; Bcl-2; Bax; Apoptosis

1. Introduction

Colorectal cancer is a significant public health problem in the western world. Therefore, to prevent and treat this disease, it is necessary to identify and use effective chemopreventive agents. Epidemiological studies suggest that ingesting a lot of dietary flavonoids with fruits and vegetables may help prevent colon cancer in humans [1]. Quercetin, the major constituent of the flavonol subclass of flavonoids, is a major flavonoid present in the human diet [2]. Quercetin is present typically in the form of glycosides, such as rutin in fruits, vegetables, tea and wine (Fig. 1). Because rutin is hydrolyzed to its aglycone quercetin by the β-glycosidase enzymes of the colonic microorganisms [3,4], both quercetin and rutin have been shown to inhibit tumor development in animal colon cancer models [5–8]. In contrast to these observations, other studies reported results that dietary treatment with quercetin resulted in a dose-dependent increase in colonic tumor incidence in azoxymethane (AOM)-treated rats [9]. It was also reported that quercetin induced the focal area of dysplasia in normal mice who were fed the standard AIN-76A diet [7]. However, quercetin used in clinical trials showed antitumor activity in cancer patients [10]. The reason for these conflicting results can be resolved if we understand the mechanisms by which quercetin exhibits anticarcinogenic activities.

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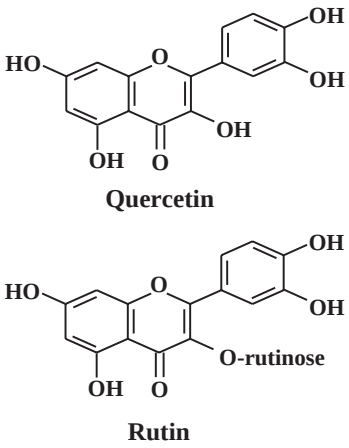

Fig. 1. Chemical structures of quercetin and rutin.

The ErbB family of type I receptor tyrosine kinases (RTKs) has four members, epidermal growth factor (EGF) receptor/ErbB1, ErbB2, ErbB3 and ErbB4. Recent studies have provided evidence for the aberrant overexpression of the ErbB receptor family or their downstream signaling molecules in human colon cancer and colon cancer cell lines and have implicated their involvement in malignant progression [11]. The serine/threonine kinase, protein kinase B or Akt (PKB/Akt), is a downstream target for phosphoinositide 3-kinase (PI3K). PI3K is activated as a result of the ligand-dependent activation of RTKs, such as the ErbB receptor family. Akt has surfaced as an important regulator of widely divergent cellular processes including apoptosis, proliferation, differentiation and metabolism [12]. Since Akt promotes cell survival and enhances cell growth, the Akt signaling pathway has become a promising target for novel anticancer therapy [13].

Research indicates that dysregulation of apoptosis may be involved in the initiation and progression of cancer [14]. Therefore, finding and developing effective agents to enhance the extent of apoptosis might be a good way to prevent and treat cancer. It has been reported that quercetin induces apoptosis in SW480 and T84 colon carcinoma cells [15]. However, the mechanisms by which quercetin induces apoptosis is not currently well known. The Bcl-2 protein family plays an important role in the regulation of apoptosis [16]. Among members of the Bcl-2 family, Bcl-X_L and Bcl-2 provide protection against apoptosis induced by insults, whereas Bax induces apoptosis by inhibiting the death-repressor activity of Bcl-2 [17]. However, one has yet to study the effects of quercetin on the Bcl-2 family proteins in human colon cancer cells.

Previous in vitro studies have reported that quercetin inhibits the growth of HT-29 cells, the human colon adenocarcinoma cell line [18,19]. We performed the present study to learn more about the mechanism of growth inhibition and apoptosis by quercetin in colon cancer cells utilizing HT-29 and SW480 cells. We determined whether quercetin down-regulates the expression of ErbB2, ErbB3, Akt and the phosphorylation of Akt. In addition, we examined whether quercetin is able to alter the expression of Bcl-2, Bax, poly (ADP-ribose) polymerase (PARP) and caspase-3.

2. Materials and methods

2.1. Reagents

We purchased the following reagents from the indicated suppliers: quercetin, rutin (Fig. 1), monoclonal anti-β-actin, RIA grade, bovine serum albumin (BSA), transferrin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO); Dulbecco's modified Eagle medium/Ham's F-12 nutrient mixture (DMEM/F12), fetal bovine serum (FBS) and selenium (Life Technologies, Gaithersburg, MD); horseradish peroxidase (HRP)-conjugated antirabbit and antimouse Ig (Amersham, Piscataway, NJ); anti-caspase-3, anti-cleaved caspase-3 (Asp 175), anti-cleaved PARP (Asp 214), anti-Akt (29752) and anti-phospho-Akt (p-Akt, 473) (New England Biolabs, Beverly, MA); anti-phosphotyrosine-RC20 antibody linked to HRP (PY20) (BD Transduction Laboratories, Palo Alto, CA); and antibodies against ErbB2 (C-18) and ErbB3 (C-17) (Santa Cruz Biotechnology).

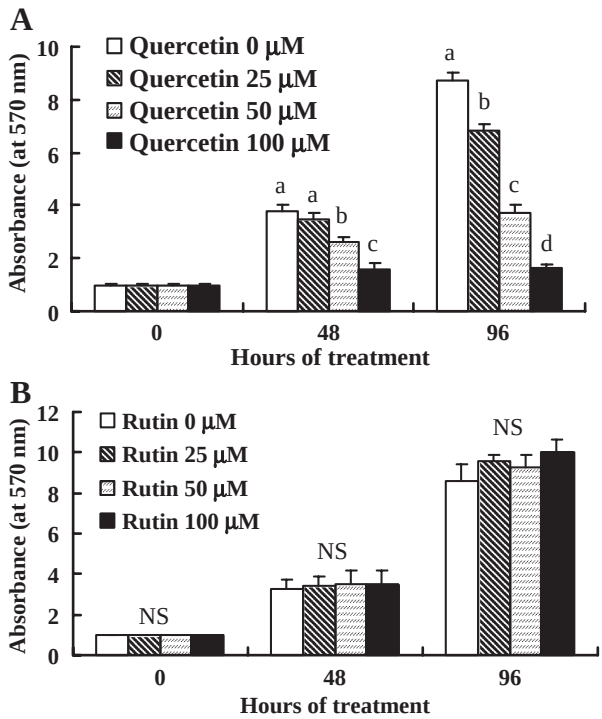

Fig. 2. Effects of quercetin (A) and rutin (B) on HT-29 cell growth. The cells were plated, cultured and serum-starved as described in Materials and Methods. After serum starvation, the cells were cultured in serum-free medium with 0, 25, 50 or 100 μmol/L quercetin or rutin for 48 or 96 h and viable cell numbers were estimated by a MTT assay. Each bar represents the mean±S.E.M. The means without a common letter differ, $P<.05$. NS, not significant, $P>.05$.

2.2. Cell culture

SW480 and HT-29 cells from the American Type Culture Collection (Manassas, VA) were maintained in DMEM/F12 containing 100 ml/L of FBS, 100,000 U/L penicillin and 100 mg/L of streptomycin (regular medium) as described previously [20]. To examine the effect of quercetin, cells were seeded in 24-well plates at 50,000 cells/well with regular medium and allowed to adhere for 24 h. Prior to quercetin treatment, the cell monolayers were rinsed and serum-starved for 24 h with DMEM/F12 supplemented with 5 μg/ml transferrin, 1 mg/ml BSA and 5 ng/ml selenium (serum-free medium). After serum starvation, fresh serum-free medium containing the indicated concentrations of quercetin or rutin was replaced. Quercetin and rutin were dissolved in dimethyl sulfoxide (DMSO), and the concentration of DMSO was kept at 1 ml/L in flavonoid-treated groups. Dimethyl sulfoxide was used as a vehicle control throughout the study. Media were changed every 2 days. Viable cell numbers were estimated by the MTT assay as described previously [21].

2.3. DNA laddering

HT-29 cells were cultured and treated as described above and genomic DNA was extracted as described previously [22]. Equal amounts of DNA samples (20 μg) were electrophoresed on a 2% agarose gel in Tris-borate EDTA buffer and visualized by ethidium bromide staining.

2.4. Immunoblotting analyses

Cell lysates were prepared as previously described [22]. The total cell lysates were resolved on a sodium dodecyl sulfate — 40–200 g/L polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). The blots were blocked for 1 h in 10 g/L BSA in TBS-T (20 mmol/L Tris-Cl, pH 7.5, 150 mmol/L NaCl, 1 g/L Tween 20) or 50 g/L milk TBS-T and incubated for 1 h with either anti-Bcl-2 (1:1,000), anti-Bax (1:1,000), anti-ErbB2 (1:500), anti-ErbB3 (1:1,000), anti-phospho-Tyr (PY20-HRP, 1:5,000), anti-Akt (1:1,000), anti-p-Akt (1:1,000), anti-caspase-3 (1:1,000), anti-cleaved caspase-3 (1:1,000), anti-cleaved PARP (1:1,000) or anti-β-actin (1:2,000) antibody. The blots were then incubated with antimouse or antirabbit HRP-conjugated antibody. Signals were detected by the enhanced chemiluminescence method using Super-Signal West Dura Extended Duration Substrate (Pierce, Rockford, IL). The relative abundance of each protein band was analyzed by scanning the exposed films densitometrically using the Digital Densitro DMV-33C (Tokyo, Japan).

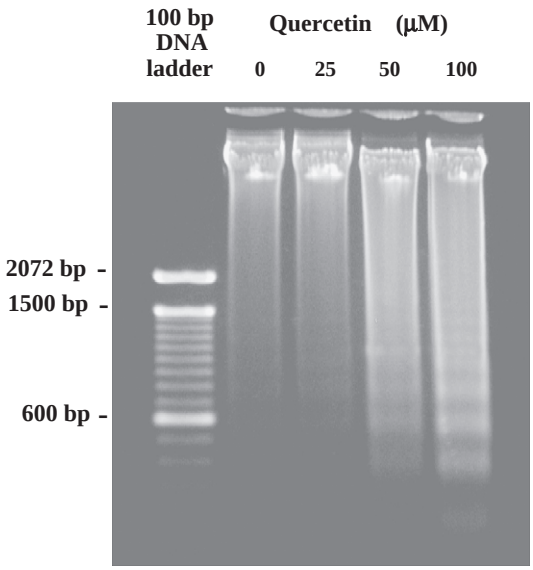

Fig. 3. Effects of quercetin on DNA fragmentation in HT-29 cells. HT-29 cells were cultured and treated with quercetin for 72 h as described in Fig. 2. Genomic DNA samples were prepared and analyzed by agarose gel electrophoresis (20 μg/lane). A photograph of the ethidium bromide-stained gel, which is representative of three independent experiments, is shown.

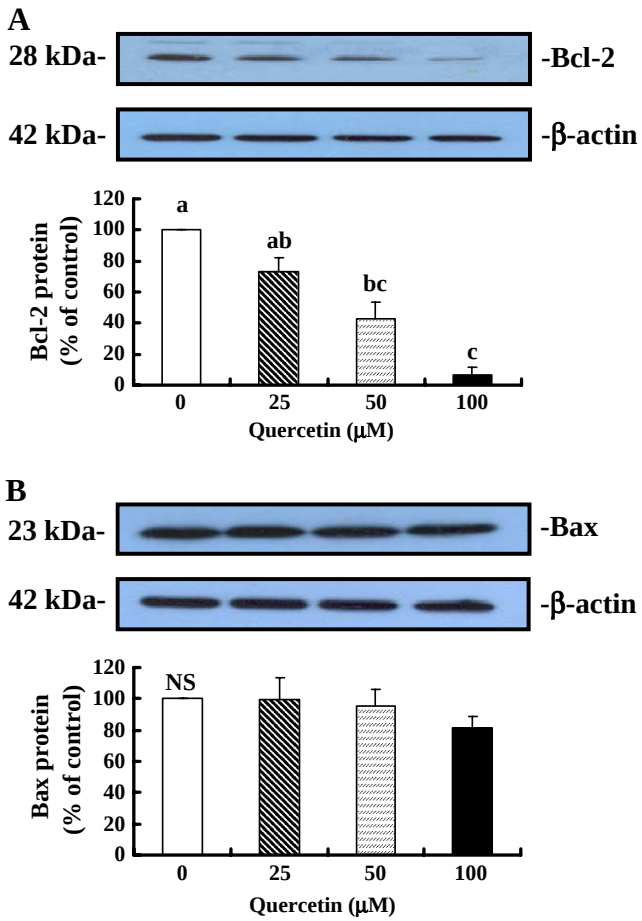

Fig. 4. Effects of quercetin on the expression of Bcl-2 (A) and Bax (B) in HT-29 cells. HT-29 cells were treated with quercetin for 72 h as described in Fig. 2. Cell lysates were subjected to immunoblotting with an antibody against Bcl-2, Bax or β-actin. Photographs of chemiluminescent detection of the blots, which were representative of six independent experiments, are shown. The relative abundance of each band to its own control β-actin band was estimated by densitometric scanning of the exposed films. Each bar represents the mean±S.E.M. (n=6). The means without a common letter differ, $P<.05$. NS, not significant, $P>.05$.

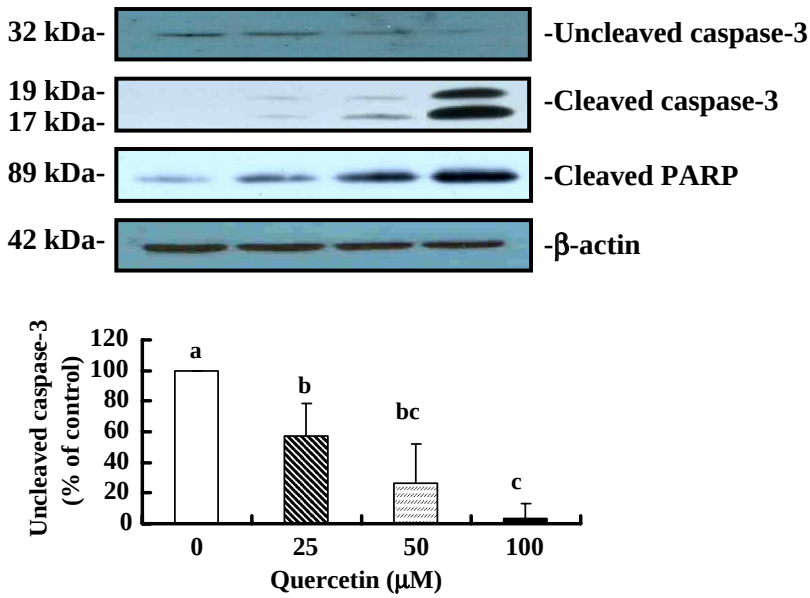

Fig. 5. Effects of quercetin on the levels of caspase-3, cleaved caspase-3 and cleaved PARP in HT-29 cells. HT-29 cells were treated with quercetin for 72 h as described in Fig. 2. Cell lysates were subjected to immunoblotting with an antibody against caspase-3, cleaved caspase-3, and cleaved PARP or β-actin. Photographs of chemiluminescent detection of the blots, which were representative of three independent experiments, are shown. The relative abundance of uncleaved caspase-3 band to its own control β-actin band was estimated by densitometric scanning of the exposed films. Each bar represents the mean±S.E.M. (n=3). The means without a common letter differ, $P<.05$.

Immunoblots were probed with an antibody for β-actin as a control for protein loading.

2.5. Statistical analyses

Data were expressed as the mean±S.E.M. and analyzed by one-way, or two-factor repeated measurements of analysis of variance. Differences between the treatment groups were analyzed by Duncan's multiple-range test.

3. Results

To examine the effect of quercetin on HT-29 cell growth, cells in the monolayer culture were treated with quercetin (0–100 μmol/L) for 48 or 96 h in a serum-free medium, and then the viable cell number was estimated. We have previously shown that these cells grow in serum-free medium as well as in medium containing 10% FBS [23]. As illustrated in Fig. 2A, quercetin decreased the viable HT-29 cell numbers in a dose-dependent manner with an 81±3% decrease in cell number 4 days after 100 μmol/L of quercetin was added. However, the same concentrations of rutin, which is the glycoside of quercetin, had no apparent effect (Fig. 2B). To examine whether quercetin induces apoptosis, HT-29 cells were similarly treated with quercetin for 72 h, and then genomic DNA was prepared. DNA that was isolated from cells treated with 50 μmol/L quercetin displayed an oligonucleosomal laddering pattern that is characteristic of apoptotic cell death (Fig. 3). The intensity of oligonucleosomal laddering was increased, to a greater degree, in cells treated with 100 μmol/L quercetin.

The effect of quercetin on the protein expression of Bcl-2 and Bax was examined by Western blot analysis. The Bcl-2 levels decreased in a dose-dependent manner in cells treated with quercetin for 72 h (Fig. 4A), whereas the Bax levels did not change (Fig. 4B). Western blot analysis utilizing anti-caspase-3 antibodies revealed that treatment of HT-29 cells with increasing concentrations of quercetin led to an increase in the 19- and 17-kDa fragments of cleaved caspase-3 and a decrease in the 32-kDa uncleaved caspase-3. Quercetin induced the proteolytic cleavage of PARP showed by revelation of the 89-kDa fragment of PARP (Fig. 5).

Because Akt plays an important role in cell survival, we next investigated whether the pro-apoptotic effects by quercetin were related to a decrease in the phosphorylation of Akt. P-Akt was significantly lower in cells treated with 25 μmol/L quercetin and was almost undetectable at the 100 μmol/L concentration (Fig. 6A). However, the total Akt levels did not change in cells treated with 50 μmol/L quercetin, but decreased significantly in cells treated with 100 μmol/L of quercetin (Fig. 6B). It is well documented that the activation of PI3K via ErbB3 activation leads to the activation of Akt. Treating HT-29 cells with increasing concentrations of quercetin for 72 h led to a dose-dependent decrease in ErbB2 and ErbB3 levels (Fig. 7). To determine whether quercetin affects the tyrosine phosphorylation of cellular proteins, cells were treated with various concentrations of quercetin for only 24 h and lysates were prepared. Immunoblot analysis of cell lysates revealed that tyrosine phosphorylation of high-molecular-mass proteins (~180 kDa) with the expected mobility of the ErbB family receptors was significantly reduced in cells treated with 50 μmol/L quercetin. The tyrosine phosphorylation was almost undetectable in cells treated with 100 μmol/L (Fig. 8).

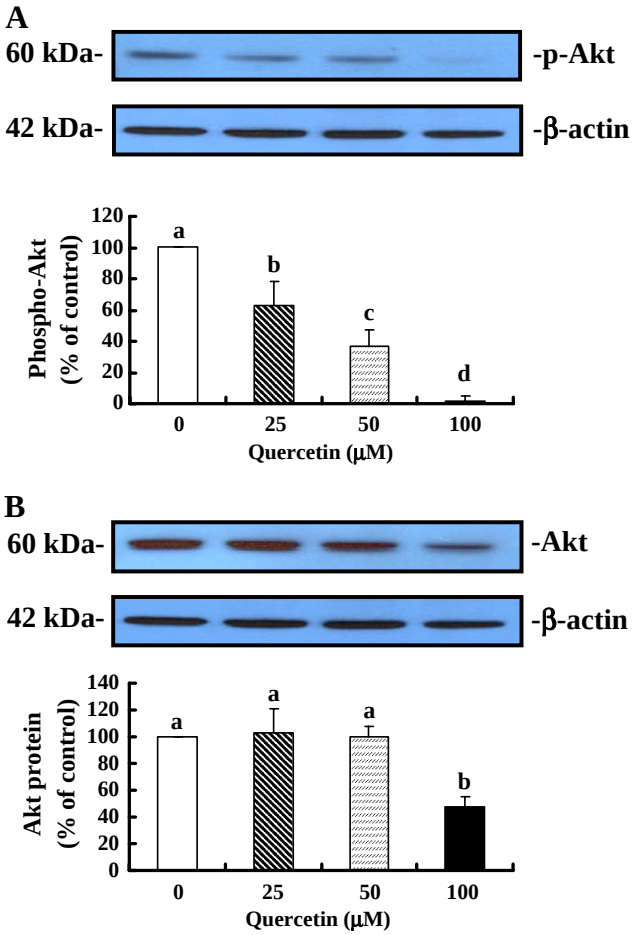

Fig. 6. Effects of quercetin on the levels of phospho-Akt (A) and Akt (B) in HT-29 cells. Protein samples similar to those in Fig. 4 were analyzed by immunoblotting with anti-phospho-Ser473 Akt (p-Akt) or whole Akt protein antibodies. Photographs of chemiluminescent detection of the blots, which were representative of six independent experiments, are shown. The relative fold change in p-Akt or Akt to its own control β-actin band on Western blots was quantitated by densitometric analysis. Each bar represents the mean±S.E.M. (n=6). The means without a common letter differ, $P<.05$.

However, ErbB protein levels remained unchanged in cells treated with quercetin for 24 h (data not shown). We performed similar experiments utilizing SW480 cells and the results obtained were basically the same as those obtained with HT-29 cells (data not shown).

4. Discussion

A diet containing either 2% quercetin or 4% rutin was reported to suppress tumor multiplicity, that is, fewer tumors/animal in mice treated with AOM [6]. Since quercetin is consumed predominantly in the form of the water-soluble glycoside, rutin, we have examined whether rutin or quercetin is effective in inhibiting HT-29 cell growth. Only quercetin is effective in inhibiting HT-29 cell growth, but the glycoside rutin did not inhibit HT-29 cell growth in the present study. This observation indicates that the polyphenols typically found in food as glycosides must be hydrolyzed in vivo to display anticolon cancer activity. Because only free flavonoids without a sugar molecule were considered to pass the gut wall [24], it is reasonable to observe that rutin has no effect on HT-29 cell growth. However, even quercetin was effective in inhibiting HT-29 cell growth at relatively high concentrations (~25 μmol/L). In our subsequent studies, we observed that there was a small (8±1%) but significant decrease in cell growth when HT-29 cells were treated with 15 μmol/L quercetin. These concentrations are much higher than the serum concentrations of quercetin in normal subjects, which can be achieved with usual consumption of dietary flavonoids [25,26]. However, one may achieve the concentrations effective in inhibiting cancer cell growth when quercetin is used as a supplement or chemotherapeutic agent to treat cancer patients. In a phase I clinical trial of quercetin, researchers safely administered quercetin into

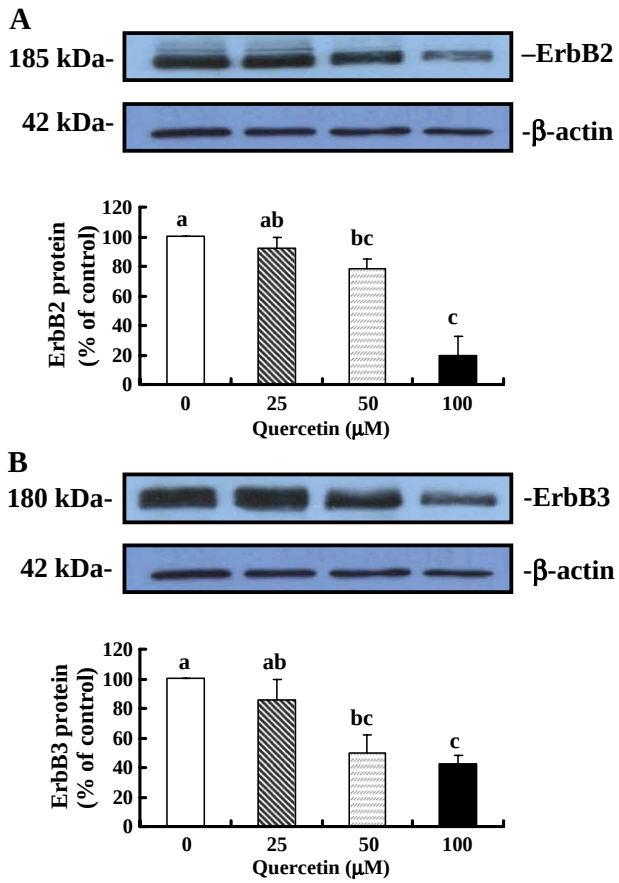

Fig. 7. Effects of quercetin on ErbB2 (A) and ErbB3 (B) levels in HT-29 cells. Protein samples similar to those in Fig. 4 were analyzed by immunoblotting with an antibody against ErbB2, ErbB3 or β-actin. Photographs of chemiluminescent detection of the blots, which were representative of six independent experiments, are shown. The relative abundance of each band to its own control β-actin band was estimated by densitometric scanning of the exposed films. Each bar represents the mean±S.E.M. (n=6). The means without a common letter differ, $P<.05$.

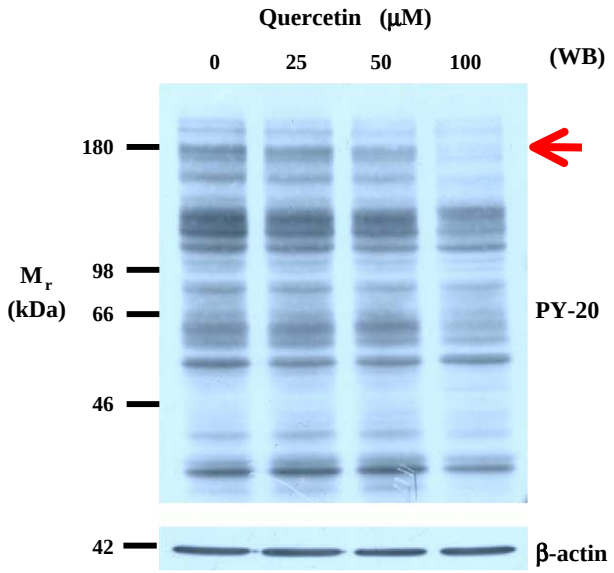

Fig. 8. Effects of quercetin on phosphotyrosine levels in HT-29 cells. Cells were treated in the absence or presence of various concentrations of quercetin for 24 h as described in Fig. 2, and cell lysates were analyzed by immunoblotting with an anti-phosphotyrosine antibody (PY20). A photograph of chemiluminescent detection of the blot, which was representative of three independent experiments, is shown.

subjects by intravenous bolus at a dose of 1400 mg/m^2 [10]. The future studies are needed to investigate the safety and bioavailability of quercetin in supplemental forms to increase serum concentration.

Cancer arises as a result of an increased rate of cell-cycle progression (cell division) and cell growth (cell mass) and/or a decreased rate of programmed cell death (apoptosis) [14]. Apoptosis can be thought of as a 'default' process, intrinsic to all cells, which is stopped by the provision of survival signals [27]. Apoptosis inhibition may be an important mechanism by which gastrointestinal cells containing damaged DNA escape normal clearance mechanisms and grow to become invasive tumors. Previously, Richter et al. [15] reported that quercetin induced apoptosis in SW480 and T84 colon carcinoma cells. The present data confirm that the decreased cell growth by quercetin is, at least in part, due to increased apoptosis in human colon cancer cells.

Apoptosis is controlled by a complicated interaction between regulatory proteins [28]. Bcl-2, a 28-kDa integral membrane oncoprotein, was the first anti-apoptosis gene product discovered [29]. Bax, a 23-kDa protein, is considered as a tumor suppressor, which sensitizes malignant cells to anticancer agents. A popular model of Bcl-2 anti-apoptotic function suggests that Bcl-2 actively forms heterodimers with Bax to deactivate the latter's pro-apoptotic activity [29] and phosphorylation of Bcl-2 functionally stabilizes the Bcl-2-Bax heterodimerization [30]. Therefore, the Bax/Bcl-2 ratio can function as a regulator to modulate cellular fate [31]. In the present study, treating HT-29 cells with quercetin led to a decrease in Bcl-2 levels without change in Bax levels. This finding suggests that the pro-apoptotic effects of quercetin may be mediated by its ability to lower Bcl-2 protein levels. Evidence indicates that Bcl-2 acts to stabilize mitochondrial membrane integrity by inhibiting cytochrome *c* release and subsequent caspase-3 activation [32]. Caspase-3 is one of the key executioners of apoptosis, being responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP) [33]. PARP seems to be involved in DNA repair mainly in response to stress [34] and is one of the main cleavage targets of caspase-3 in vivo [33]. PARP is important for cells to maintain their viability; cleavage of PARP helps cellular disassembly and serves as an indicator of cells going through apoptosis [35]. In the present study, decreased Bcl-2 may have contributed to caspase-3 activation and subsequent PARP cleavage, and resultant induction of apoptosis in quercetin-treated cells.

PKB/Akt is a key mediator of cell survival and suppression of apoptosis in a wide variety of cell types [36–39]. Inappropriate activation of the PI3K/Akt pathway has been associated with the development of diseases, such as cancer [40]. In particular, inhibiting Akt activity may be an effective way to treat cancer and increase the efficacy of chemotherapy [41]. The present data suggest that inhibiting the Akt pathway may be an important mechanism by which quercetin inhibits growth of HT-29 cells.

This study provides the first evidence that quercetin reduces ErbB2 and ErbB3 protein expression and tyrosine phosphorylation of 180-kDa proteins. We believe that these 180-kDa proteins are ErbB2 and ErbB3 because our previous immunoprecipitation studies have shown that the 180-kDa proteins are phosphorylated by heregulin, a ligand for ErbB3, and can be immunoprecipitated by either an antibody raised against ErbB2 or ErbB3 [22]. In addition, ErbB3 receptors are expressed in relatively high levels in HT-29 cells [22]. The present study suggests that inhibiting the expression and signaling of ErbB2 and ErbB3 may be a mechanism by which quercetin inhibits HT-29 cell growth. Overexpression of ErbB genes, particularly ErbB2, has been observed in several types of human cancer [42–44]. In colon cancer, the expression of mRNA for ErbB2 and ErbB3, as well as the corresponding proteins, was increased when compared to the normal mucosa [11,45,46]. However, no difference in the EGFR protein levels was evident between normal colon and cancer [46]. In addition, heregulin is co-expressed with the ErbB2 proteins in human colon cancer specimens, and autocrine activation of ErbB2 occurs through heterodimerization with ErbB3 in GEO colon cancer cells [47]. These results suggest that regulating the heregulin/ErbB2/ErbB3 pathway may be an important modulator of aberrant growth in colon cancer [48,49]. Currently, researchers are performing clinical trials on several agents that target one or more members of the ErbB family of RTKs [50,51]. Quercetin could be used as an anticancer agent targeting ErbB2/ErbB3 RTKs or it could be used in combined therapy to enhance a response to other cytotoxic drugs.

In conclusion, we have confirmed that the mechanism of quercetin inhibition of colon cancer cell growth involves induction of apoptosis. We have also demonstrated that quercetin decreases the expression of Bcl-2, a protein that acts as an inhibitor of programmed cell death. Our results suggest that the decreased Bcl-2 may be one of reasons for the activation of caspase-3 and subsequent cleavage of PARP. Furthermore, we report that quercetin down-regulates ErbB2, ErbB3, Akt and phosphorylation of Akt. The disruption of normal Akt signaling commonly occurs in a number of human cancers, and this disruption contributes to the development of many types of cancer [40]. Therefore, down-regulation of these pathways by quercetin could have important preventive and therapeutic benefits.

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