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Biochemical and Biophysical Research Communications 328 (2005) 227–234
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Quercetin, a potent inhibitor against β-catenin/Tcf signaling in SW480 colon cancer cells

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Received 20 December 2004
Available online 6 January 2005

Abstract

Dysregulation of Wnt/β-catenin pathway plays a central role in early events in colorectal carcinogenesis. We examined the effect of quercetin, a famous anti-tumor agent, against β-catenin/Tcf signaling in SW480 cells. Quercetin inhibited the transcriptional activity of β-catenin/Tcf in SW480 and also in HEK293 cells transiently transfected with constitutively active mutant β-catenin gene, whose product is not induced to be degraded by APC–Axin–GSK3β complex, so we concluded that its inhibitory mechanism was related to β-catenin itself or downstream components. To investigate the precise inhibitory mechanism, we performed EMSA showing that binding of the Tcf complexes to its specific DNA-binding sites was strongly suppressed by quercetin. Immunoprecipitation analysis also showed that the binding of β-catenin to Tcf-4 was also disrupted by quercetin. Western blot analysis proved these decreased bindings resulted from decreased level of β-catenin and Tcf-4 product in nucleus caused by quercetin. Together, we suggest that quercetin is an excellent inhibitor of β-catenin/Tcf signaling in SW480 cell lines, and the reduced β-catenin/Tcf transcriptional activity is due to the decreased nuclear β-catenin and Tcf-4 proteins.

Keywords: Quercetin; Colon cancer; β-Catenin/Tcf signaling; Inhibitory mechanism

β-Catenin plays a dual role in a cell. It associates with the cytoplasmic domain of E-cadherin and α-catenin in order to link these proteins to the actin cytoskeleton [1], and is involved in the Wnt signaling pathway to transactivate T cell factor (Tcf)/lymphocyte enhancing factor (Lef) transcription factors in a nucleus [2]. Some of the genes activated by β-catenin/Tcf signaling are *c-jun*, *c-myc*, *fibronectin*, *cyclin D1*, and *fra-1* [3–5]. In normal cells, most β-catenin protein is present in cell–cell junctions with very little in cytoplasm or nucleus. Cytosolic β-catenin is phosphorylated by Axin–adenomatous polyposis coli (APC)–glycogen synthase kinase (GSK)3β complex and recognized by β-TrCP, an F-box component of the E3 ubiquitin ligase complex that promotes ubiquitination of β-catenin and increases its susceptibility to degradation by a ubiquitin–proteasome system [6]. If Wnt signaling is activated, β-catenin degradation is inhibited due to the decreased ability of GSK-3β to phosphorylate β-catenin and β-catenin translocates into the nucleus to transactivate the Tcf/Lef transcription factor leading to upregulation of many genes responsible for cell proliferation.

Activated β-catenin/Tcf signaling by accumulation of β-catenin in the nucleus has been implicated in human carcinogenesis. This accumulation may result from the mutation of either the β-catenin gene itself or the tumor suppressor gene, APC. Actually the APC gene or serine–threonine phosphorylation sites for the GSK3β within exon 3 of the β-catenin gene are mutated in many cancer

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0006-291X/$ - see front matter © 2005 Published by Elsevier Inc.
doi:10.1016/j.bbrc.2004.12.151

cells including colorectal cancer, melanoma, hepatocellular carcinoma, and gastric carcinoma, and the transcriptional activity of β-catenin is upregulated in these cancer cells [7–9]. At least 60% of sporadic colorectal cancers (CRC) contain one APC mutation and almost half of them show abnormalities in both APC alleles [10]. More recently, approximately half of the largest group of sporadic colorectal cancers and CRC cell lines lacking APC mutations were shown to possess somatic mutations in the β-catenin gene [11,12]. This means that the dysregulation of β-catenin plays a crucial role in colon cancer cells. Therefore, we hypothesized that reduced β-catenin/Tcf transcriptional activity may lead to suppressed tumor growth. However, there exist few β-catenin inhibitors. Aspirin, one of the nonsteroidal anti-inflammatory drugs (NSAIDs) was reported to attenuate β-catenin/Tcf signaling in colon cancer cells [13].

Flavonoids are a group of more than 4000 polyphenolic compounds that occur naturally in foods of plant origin. They have been shown to possess a variety of biological activities at nontoxic concentrations in organisms. The role of dietary flavonoids in cancer prevention is widely discussed. Quercetin, the major representative of the flavonol subclass of flavonoids, is an integral part of the human diet and the average human intake has been estimated to be 25 mg/day. In animals developing aberrant crypt foci by the application of a chemical carcinogen, a diet supplemented with 2% quercetin markedly reduced the development of the initial stages of colorectal cancer development [14]. In addition, it exerts an antiproliferative effect in vitro on colonic cancer cell lines of diverse lineages [15–17]. Despite these promising actions of quercetin as significant anti-tumor activities, the molecular mechanisms underlying theses effects are generally unknown. To elucidate why intake of quercetin is disadvantageous to colonic cancer cells, we investigated the effect of quercetin on β-catenin/Tcf signaling, which is particularly important in colon cancer. In this paper, we describe the evidence that quercetin could be an inhibitor of β-catenin/Tcf signaling and its inhibitory mechanism is related to the decreased β-catenin and Tcf-4 proteins in nucleus.

Materials and methods

Cell lines and reagents. SW480 and HEK293 cell lines were derived from Korean Cell Line Bank (KCLB, Korea). Quercetin was purchased from Acros. Stock solutions (50 mM) were made in DMSO. TOPflash was provided by Hans Clevers via Bart O Williams, and wild (pcDNA β-catenin) and mutant β-catenin gene (pcDNA S33Y) were provided by Eric R. Fearon.

Isolation of cellular and nuclear extracts. Cells were trypsinized and whole cell protein was obtained by lysing the cells on ice for 20 min in 700 μl lysis buffer (0.05 M Tris–HCl, pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, 0.5 M PMSF, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate). The lysates were then sonicated for 20 s and spun at 15,000*g* for 10 min, and the supernatant was saved. Protein determinations were made using the method of Bradford. The nuclear pellets were prepared by resuspending cells in 800 μl lysis buffer (10 mM Hepes, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, and 0.1 mM EDTA), placing them on ice for 15 min, and then vigorously mixing after the addition of 50 μl of 10% Nonidet P-40. After a 30-s centrifugation (16,000*g*, 4 °C), the pelleted nuclei were resuspended in 120 μl extraction buffer (50 mM Hepes, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, and 10% glycerol) and incubated on ice for 30 min. Nuclear extracts were stored at −70 °C.

Isolation of cytoplasmic proteins. Cell pellets were suspended in 800 μl lysis buffer (see whole cell protocol) without Tween 20 detergent, pulse sonicated (1 s 30×) on ice, and then spun at 100,000*g* for 1 h. The supernatant (cytoplasmic fraction) was saved at −70 °C.

Immunoprecipitation. Nuclear lysates or cellular lysates were prepared and 1 ml lysates were incubated with 5 μl primary antibody for 90 min at 4 °C. Thirty microliters of protein A/G plus (Santa Cruz, USA) was added and the complex was incubated at 4 °C overnight. The pellet was washed two times with high salt buffer (1 M Tris–HCl, pH 7.4, 0.50 M NaCl, and 1% Nonidet P-40) and twice with lysis buffer. The immunoprecipitated complexes were released with 2× sample buffer for Western analysis.

Western analysis. Western analysis for the presence of β-catenin, Tcf-4, GSK-3β, and c-myc was performed on the whole extract, cytosol, or nuclear proteins from SW480 and HEK293. Whole cell, nuclear extracts, and cytoplasmic proteins were isolated as described above. Protein (20–100 μg) was mixed 1:1 with 2× sample buffer (20% glycerol, 4% SDS, 10% 2-ME, 0.05% bromophenol blue, and 1.25 M Tris–HCl, pH 6.8), loaded onto a 12% SDS–PAGE gel and run at 120 V for 2 h. Cell proteins were transferred to nitrocellulose (ECL; Amersham, Arlington Heights, IL) for 3 h at 250 mA. Equal loading of the protein groups on the blots was evaluated using Bradford assay (nucleus or membrane proteins) or control band (cytosol or whole extracts). The nitrocellulose was then blocked with 5% milk in TBST overnight, washed four times, and then incubated with the primary Ab (anti-β-catenin, c-myc, GSK-3β, and Tcf-4 antibody, Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature. The blots were washed four times with TBST and incubated for 1 h with HRP-conjugated anti-IgG AB (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were developed using a chemiluminescent substrate (WEST-ZOL plus, iNtRON BioTechnology, Korea). An autoradiograph was obtained with exposure times of 2 min to 12 h.

Electrophoretic mobility shift assay (EMSA). Nuclear extract, prepared as described above, was incubated for 30 min at room temperature with inhibitor and ^{32}P-labeled double stranded oligomer probe (5′-CCCTTTGATCTTACC-3′). It was analyzed by nondenaturing 4% polyacrylamide gel electrophoresis in 0.5× TBE buffer at 100 V for 40 min. Gels were visualized by autoradiography.

RT-PCR. Total RNA was isolated from SW480 cells using easy-BLUE RNA Extraction Kit (iNtRON, Korea), according to the manufacturer's instructions. For cDNA synthesis, 4 μg of total RNA was included in a 25 μl reaction volume by using oligo(dT) primers (iNtRON, Korea) and Reverse Transcriptase (iNtRON, Korea) following the manufacturer's instructions. For PCR, 1/25 of the reverse transcription reaction mixture was amplified with 22 cycles for *c-myc* or 17 cycles for β-actin. To amplify *c-myc* and β-actin fragments, 10, 14, 17, and 22 cycles were used to determine whether the DNA amplification was linear. All PCR products were analyzed by electrophoresis on a 1.5% agarose gel and photographed. The sequences of primers (Bionics, Korea) used in the RT-PCR were as follows:

β-Actin: 5′-TCATGAAGTGTGACGTTGACATCCGT-3′ (sense) and 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′ (anti-sense); c-myc: 5′-TGTCAAGAGGCGAACACACAACGTC-3′ (sense) and 5′-ATCTTTCAGTCTCAAGACTCAGCCA (anti-sense).

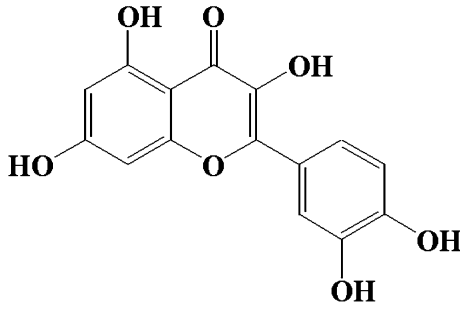

Fig. 1. Structure of quercetin.

Transfection and luciferase assay. Transient transfection was performed using Lipofectamine and plus reagent (Invitrogen, USA). Briefly, 6.5×10^5 cells (HEK293) or 1.5×10^6 cells (SW480) were seeded in the medium onto a 60 mm dish. After 24 h, cells were transfected with 1.5 μg of the luciferase reporter constructs (TOPflash) and 1.5 μg of β-galactosidase gene for normalization. Especially, HEK293 cells were cotransfected with one another gene, 1.5 μg pcDNA β-catenin or pcDNA S33Y gene. After 3 h of posttransfection, inhibitor was added with a medium containing FBS. Cells were incubated for 24 h, lysed in Reporter lysis buffer (Promega, USA), and collected for assays of luciferase and galactosidase activity, respectively Fig. 1.

Results

Quercetin downregulates the β-catenin/Tcf signaling

To investigate whether quercetin modulates β-catenin/Tcf signaling, we used SW480 human colon cancer cells (Fig. 2A) and HEK293 cells transiently transfected with wild β-catenin gene (Fig. 2B). The transcriptional activity of β-catenin/Tcf is constitutively active in SW480 cell line (mutant APC, wild-type CTNNB1, and COX-2 negative). We used reporter gene constructs containing four copies of either an optimized (TOPflash) or mutant (FOPflash) Tcf-binding element. SW480 cells were cotransfected with TOPflash or FOPflash and pCMV-β-galactosidase to normalize for transfection efficiency and sequentially treated with quercetin. Fig. 2A shows that quercetin suppresses the Tcf transcriptional activity in SW480 cell line in a concentration-dependent manner. Within 24 h of treatment, 50 μM concentration of quercetin reduced β-catenin/Tcf transcriptional activity by 55 ± 10% compared with control, whereas the FOPflash activity, a mutant for β-catenin/Tcf binding, remained unchanged after quercetin treatment. This proposes that the functional binding of β-catenin/Tcf may be important for TOPflash. Next, we performed reporter gene assay with HEK293 cells. HEK293 cells have low transcriptional activity of β-catenin/Tcf for the low endogenous amounts of β-catenin protein, so they were cotransfected with wild β-catenin gene (pcDNA β-catenin) and TOPflash or FOPflash. Wild β-catenin gene transfection elevated the Tcf signaling about 30-fold, however, quercetin

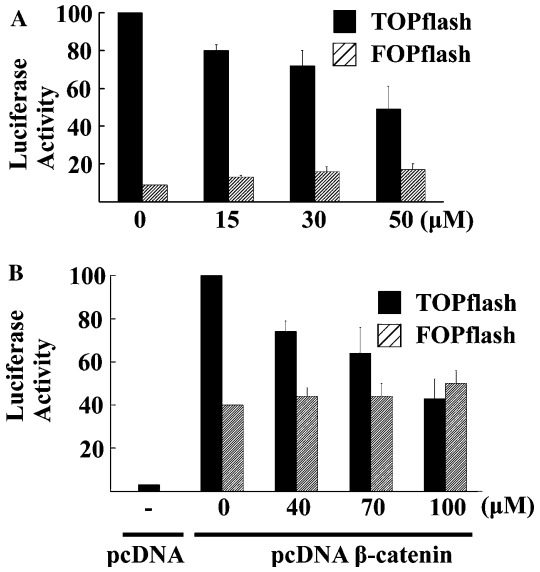

Fig. 2. Quercetin inhibits the transcriptional activity of β-catenin/Tcf in SW480 cells (A) and HEK293 cells (B). SW480 cells were cotransfected with reporter genes harboring Tcf-4 binding sites (TOPflash) or a mutant Tcf-binding site (FOPflash), respectively, and β-galactosidase gene (A). However, HEK293 cells were cotransfected with TOPflash (or FOPflash), β-galactosidase gene, and pcDNA3.1 (mock) or pcDNA β-catenin (B). Three hours posttransfection, increasing amounts of quercetin as indicated were added to the cells. Luciferase activity was determined 24 h posttransfection, normalized against values for the corresponding β-galactosidase activity. An equivalent volume of DMSO substituted for quercetin was used as a vehicle control. Values represent means ± SEM of four independent experiments.

treatment decreased Tcf signaling. One hundred micromolar quercetin reduced Tcf signaling to 42 ± 8% of that in DMSO-treated cells. It is of note that the results presented in Fig. 2 show that quercetin is a good inhibitor of β-catenin/Tcf signaling.

Quercetin acts on β-catenin or its downstream elements

We investigated whether quercetin confers its inhibitory effect upstream or downstream of β-catenin/Tcf signaling. HEK293 cells were cotransfected with a constitutively active mutant of β-catenin (pcDNA S33Y) together with the reporter constructs. S33Y gene products cannot be phosphorylated by GSK3β–APC–Axin complex. Fig. 3A shows 100-fold increase in luciferase activity when 1.5 μg mutant β-catenin (pcDNA S33Y) gene was transfected into HEK293 cells compared with transfection with pcDNA(mock). One hundred micromolar quercetin reduced the increased Tcf activity to about 23 ± 8% of that in DMSO-treated cells. This strongly suggests that quercetin exerts its inhibitory effect on β-catenin/Tcf signaling by acting either on β-catenin itself or on downstream components rather than on upstream regulators. To confirm this

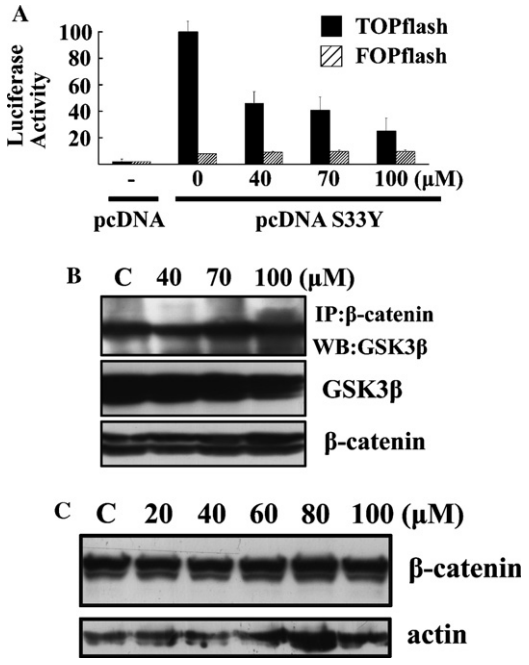

Fig. 3. The inhibition of β-catenin/Tcf signaling is not related to β-catenin degrading machinery, Axin–APC–GSK complex. (A) HEK293 cells were cotransfected with TOPflash (or FOPflash), β-galactosidase gene, and pcDNA3.1 (vehicle) or pcDNA S33Y (mutant β-catenin gene). Three hours posttransfection, increasing amounts of quercetin as indicated were added to the cells. Luciferase activity was determined 24 h posttransfection, normalized against values for the corresponding β-galactosidase activity. An equivalent volume of DMSO substituted for quercetin was used as a vehicle control. Values represent means ± SEM of four independent experiments. (B) SW480 cells were incubated with quercetin for 24 h and the cellular lysates were isolated for Western blot with GSK-3β and β-catenin antibody. GSK-3β and β-catenin were coimmunoprecipitated from cellular lysates using an anti-β-catenin antibody followed by immunoblotting with GSK-3β antibody. (C) SW480 cells were incubated with quercetin for 24 h and cytosolic fraction was isolated for Western blot with β-catenin antibody. To demonstrate equivalent loading of the lines, anti-actin was used.

suggestion, GSK3β, an important upstream regulator of β-catenin in this pathway, and β-catenin were coimmunoprecipitated from cellular lysates (Fig. 3B). SW480 cells were incubated on the 100 mm dishes with quercetin for 24 h and cellular lysates were isolated. Lysates were immunoprecipitated with β-catenin antibody and blotted with GKS3β antibody. The protein levels of GSK3β and β-catenin was unchanged by quercetin treatment. Immunoprecipitation data show that the binding of β-catenin with GSK3β is not affected by quercetin. Moreover, Fig. 3C shows that the level of SW480 cytoplasmic β-catenin is unchanged and no degradation of β-catenin by quercetin occurred. This means that quercetin does not activate the degradation pathway of cytoplasmic β-catenin. Taken together, we conclude that quercetin inhibits β-catenin/Tcf signaling by acting either on β-catenin itself or on downstream components.

Quercetin disrupts the binding of Tcf complexes to its DNA response element

We considered the possibility that the suppressed transcriptional activity of β-catenin/Tcf resulted from the decreased binding of Tcf complexes to DNA by quercetin (Fig. 4). Nuclear extracts derived from 24 h quercetin treated cells were analyzed by EMSA for their ability in association with ^{32}P-labeled oligonucleotide containing Tcf response elements (TRE). SW480 cells have substantial binding activity of Tcf complexes to TRE (lane 1). However, binding of the Tcf complexes to their specific binding element was decreased by quercetin in a concentration-dependent manner (lanes 2–4). This means that quercetin interferes with the binding of Tcf complexes to DNA. The binding of Tcf complexes to TRE was completely eliminated by adding a 100-fold excess of competitive ^{32}P-unlabeled probe (lanes 5–7). In addition, as the amount of unlabeled probe incubated with DMSO-treated nuclear extracts (3 μg) increases, the intensity of the bands weakens (lanes 8–10). These results of lanes 5–10 confirm that the upper bands are specific for Tcf-binding. Therefore, the suppressed β-catenin/Tcf signaling could be ascribed to a reduction of binding activity of Tcf complexes to TRE.

Quercetin interferes with the formation of β-catenin/Tcf complex in the nucleus

The binding of β-catenin to Tcf-4 is inevitably required for the activation of Tcf signaling [18,19], so we examined whether quercetin disrupts the association

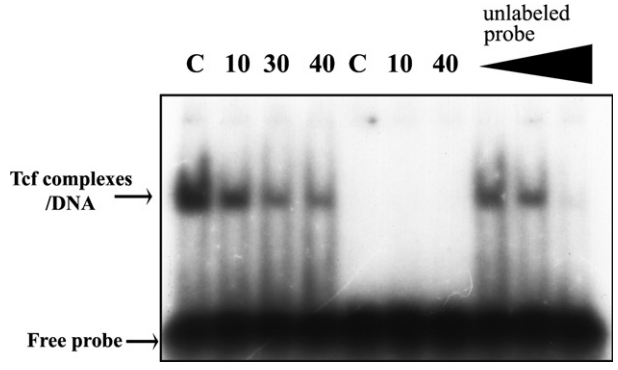

Fig. 4. The binding of Tcf complexes to DNA is decreased by inhibitor. SW480 cells were treated with quercetin for 24 h and nuclear extracts were isolated. Nuclear extracts were isolated from cells treated with DMSO (lanes 1, 5, and 8–10) or quercetin at 10 μM (lanes 2 and 6) or 30 μM (lane 3) or 40 μM (lanes 4 and 7). EMSA was performed with 2 μg nuclear extracts from treated cells. Lanes 1–4 are the results of binding to a ^{32}P-labeled optimal Tcf-binding region. Lanes 5–7 show that 100-fold excess of unlabeled optimal Tcf-binding region used as a competitor prevents binding of the β-catenin/Tcf complexes to the Tcf-binding region. The results of lanes 8–10 were obtained by increasing the amounts of unlabeled oligonucleotide gradually.

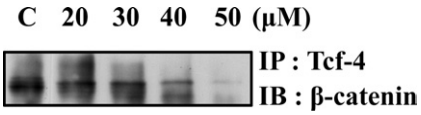

Fig. 5. The association of β-catenin with Tcf-4 is decreased by inhibitor. Quercetin was treated in SW480 cells with increasing doses (0, 20, 30, 40, and 50 μM) for 24 h. Nuclear extracts were prepared and immunoprecipitation was performed with Tcf-4 antibody followed by immunoblotting with β-catenin monoclonal antibody. C, control.

of β-catenin with Tcf-4. SW480 cells were treated with quercetin at the concentration indicated for 24 h, and the nuclear extracts were isolated. β-Catenin and Tcf-4 were coimmunoprecipitated from nuclear extracts using an anti-Tcf-4 antibody and β-catenin levels were analyzed by immunoblotting. Fig. 5 shows that DMSO-treated cells have substantial level of β-catenin/Tcf-4 complex in the nucleus (lane 1). However, quercetin decreased the level of β-catenin/Tcf-4 complex dose dependently (lanes 2–5). Therefore, we can conclude that quercetin inhibited β-catenin/Tcf signaling by decreasing the amount of β-catenin/Tcf complex.

Quercetin reduced the level of nuclear β-catenin and Tcf-4 proteins

We considered the possibility that the reduction in the level of nuclear β-catenin or Tcf-4 proteins by quercetin results in the reduced association of β-catenin with Tcf-4 and reduced binding of Tcf complexes to TRE, leading to the suppressed β-catenin/Tcf signaling. In addition, it is well known that Tcf signaling-activation results from accumulation of nuclear β-catenin [20]. To investigate this possibility, we performed Western blot with the nuclear extracts. SW480 cells were seeded for 1 day and treated with quercetin for 24 h. After 24 h cells were trypsinized and nuclear extracts were prepared for immunoblotting. Fig. 6A shows that substantial levels of nuclear β-catenin and Tcf-4 products in SW480 cells exist. However, 10–50 μM quercetin decreased the amount of nuclear β-catenin and Tcf-4 proteins markedly, leaving β-tubulin unchanged. Unlike SW480 cells, low endogenous amounts of β-catenin protein were detected in HEK293 cells (lane 1 in B and C), so wild β-catenin gene (B) or constitutively active mutant β-catenin gene (C) was transfected into HEK293 cells. As a result, the level of β-catenin products in the nucleus is elevated considerably by gene transfection (lane 2 in B and C). However, like SW480, the level of exogenously expressed nuclear β-catenin products is decreased markedly by quercetin. Taken together, we could conclude that quercetin decreases the level of nuclear β-catenin and Tcf-4, leading to the reduced association of β-catenin with Tcf-4 and reduced binding of Tcf complexes to TRE.

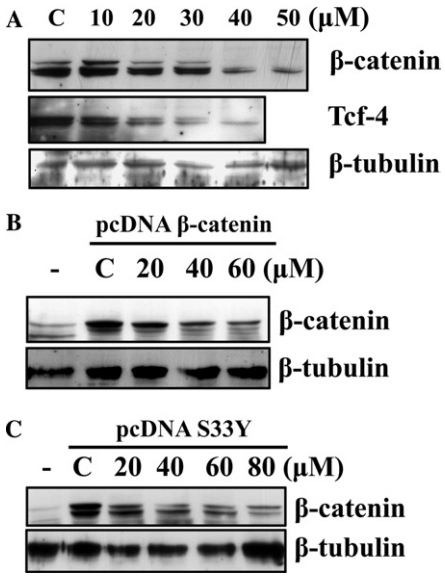

Fig. 6. β-Catenin and Tcf-4 products in the nucleus are decreased by inhibitor. SW480 cells were treated with quercetin for 24 h (A), while HEK293 cells were transiently transfected with WT β-catenin constructs (B) and with constitutively active mutant β-catenin constructs (C) prior to quercetin treatment for 24 h. Lane 1 in (B) and (C) contains the results of mock transfection (pcDNA). Cells were trypsinized for preparing nuclear extracts, which were used for Western blot with anti-β-catenin antibody or with anti-Tcf-4 antibody. To demonstrate equivalent loading of the lines, anti-tubulin was used. All pictures are representative of two independently repeated experiments. C, control.

Quercetin downregulates the expression of β-catenin/Tcf-dependent gene

We investigated whether quercetin affects the expression of β-catenin/Tcf target gene in SW480 cells (Fig. 7). It is well known that c-Myc is a β-catenin target gene [3]. Therefore, we hypothesized that quercetin would downregulate the c-Myc gene expression by inhibiting β-catenin/Tcf signaling. In SW480 cell, as we expected, c-Myc proteins (A) and c-myc gene transcription (B) were downregulated by quercetin. These results indicate that

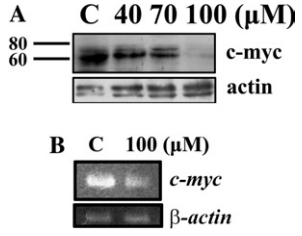

Fig. 7. Quercetin downregulates the c-Myc protein and mRNA. (A) SW480 cells were treated with quercetin (40, 70, and 100 μM) for 24 h and trypsinized to isolate cellular extracts, and we performed Western blot to study the change of c-Myc protein level due to quercetin. To demonstrate equivalent loading of the lines, anti-actin was used. (B) SW480 cells were treated with quercetin (0, 100 μM) for 20 h and trypsinized to isolate RNA, and we performed RT-PCR to study the change of *c-myc* mRNA by quercetin. C, control.

the functioning of β-catenin as a transcription activator is rendered inoperative by inhibitor.

Discussion

Flavonoids constitute one of the largest groups of naturally occurring phenols and are commonly present in plants as glycosides. These compounds possess a common phenylbenzopyrone structure (C6–C3–C6), and they are categorized according to the saturation level and opening of the central pyran ring, mainly into flavones, flavanols, isoflavones, flavonols, flavanones, and flavanonols [21,22]. These dietary anti-oxidants are well known to exert significant anti-tumor effects and have been extensively reviewed [23,24]. The elevated β-catenin/Tcf signaling is an important event in the genesis of a number of malignancies, such as colon cancer. Mutations in the regulatory region of β-catenin or loss of APC function have been identified in human colon cancers [7,25]. Activation of an abnormal APC/β-catenin/Tcf signaling pathway and alterations in cellular adhesion mediated through changes in β-catenin homeostasis within the colonic epithelium are initiating factors in the development of the majority of colorectal cancers. Here, we tested the effect of quercetin, which is the most famous one of various flavonoids, against the transcriptional activity of β-catenin/Tcf. Quercetin has demonstrated chemopreventive activity in a variety of laboratory animal models, including azoxymethane (AOM)-induced colonic tumorigenesis in mice and rats, dimethylbenz[*a*]anthracene (DMBA) and *N*-nitrosomethylurea (NMU)-treated mammary glands of rats [26–29]. Quercetin has also been used in clinical trials in cancer patients [30]. We hypothesized that the anti-tumor effects of quercetin in colon cancer are mediated by its ability to downregulate the β-catenin/Tcf signaling. Studies on the inhibitory agent against β-catenin/Tcf signaling in cancer cell lines have been performed. Dihlmann et al. and Nath et al. reported that NSAID and nitric oxide-donating aspirin, respectively, are good inhibitors of β-catenin/Tcf signaling in colon cancer cell lines [13,31]. Dashwood et al. [32] also reported that EGCG inhibits β-catenin/Tcf activity in HEK293 cells transiently transfected with constitutively active mutant β-catenin gene. In addition, it was revealed by Jaiswal et al. that curcumin inhibits the transcriptional activity of β-catenin/Tcf so as to induce growth arrest and apoptosis in HCT116 colon cancer cells [33]. Recently, Orner et al. [34] suggested that a combination of tea plus sulindac is highly effective at inhibiting intestinal neoplasia in male Apc^{min} mice via direct or indirect effects on the β-catenin/APC pathway. As the importance of β-catenin as a cause of tumorigenesis increases, many more studies on the β-catenin inhibitor and its inhibitory mechanism are being conducted. Our data on luciferase activity show that quercetin inhibits the transcriptional activity of β-catenin/Tcf in SW480 colon cancer cells in a concentration-dependent manner. Similarly, in HEK293 cell, quercetin downregulates the elevated β-catenin/Tcf signaling by wild β-catenin gene transfection (Fig. 2). Next, we focused on the inhibitory mechanism. Quercetin also inhibited the constitutively active mutant β-catenin/Tcf signaling in HEK293 cells, but does not affect the binding of GSK3β to β-catenin in cytosol. In addition, the cytosolic β-catenin proteins in SW480 cells were not degraded by quercetin treatment. Taken together, these results strongly suggest that the inhibitory mechanism of quercetin is not related to the upstream regulators of the β-catenin/Tcf pathway but to β-catenin itself or to the downstream components (Fig. 3). To transcript target genes, transcription factors, including β-catenin/Tcf, must bind to consensus DNA, so we performed EMSA (Fig. 4). EMSA data show that the binding of Tcf complexes to consensus DNA is blocked by quercetin. Furthermore, it is well known that the binding of β-catenin to Tcf-4 induces a significant increase in the Tcf transcriptional activity [35]. However, quercetin suppressed the association of β-catenin with Tcf-4 (Fig. 5). From these data we conclude that the reduced binding of Tcf complexes to DNA and the reduced association of β-catenin with Tcf-4 caused by quercetin lead to the inhibition of β-catenin/Tcf signaling. Here, we hypothesized that quercetin decreased the amount of nuclear β-catenin proteins and that this leads to the reduced binding of β-catenin/Tcf to DNA and of β-catenin to Tcf. As we know, β-catenin is ubiquitous and moves freely in a cell. It contributes to the cell–cell adhesion in the membrane and functions as a transcriptional activator in the nucleus. Dihlmann et al. and Nath et al. reported that subcellular localization of β-catenin protein was not affected by NSAID or NO-donating aspirin, inhibitors against β-catenin/Tcf signaling, that is, the amounts of nuclear β-catenin protein is unchanged by NSAID and NO-donating aspirin [13,31]. We conducted the same experiments with quercetin. Interestingly, the amounts of nuclear β-catenin and nuclear Tcf proteins were decreased significantly by quercetin in SW480 cells and in HEK293 cells transiently transfected with wild β-catenin gene or with constitutively active mutant β-catenin gene (Fig. 6). Here, we could conclude that this reduction of nuclear β-catenin and Tcf-4 proteins results in the decreased binding to DNA containing Tcf-binding element, as demonstrated by EMSA, and results in the decreased association of β-catenin with Tcf-4. Eventually, we could conclude that the reduction of the β-catenin and Tcf-4 level in the nucleus is the root of the suppressed β-catenin/Tcf signaling. Studies in recent years have suggested that β-catenin accumulation in the nucleus has been implicated in tumorigenesis in a wide variety of human cancers [20,36,37]. Conversely, we could suggest from these studies that the blocking of

nuclear accumulation of β-catenin suppresses the tumorigenesis. This paper reveals the molecular mechanism underlying the anti-tumor effect of quercetin by suppressing β-catenin/Tcf signaling via decreasing the nuclear β-catenin proteins. In further studies, we will focus on the way in which nuclear β-catenin and Tcf proteins were decreased.

Acknowledgments

We thank Dr. H. Clevers for the gift of TOPflash. We also thank Dr. Eric R. Fearon for the wild (pcDNA β-catenin) and mutant β-catenin gene (pcDNA S33Y). This research was supported by a grant (KRF 2001-015-DP0344) from the Korean Ministry of Science and Technology. Financial support in part from the Brain Korea 21 program is gratefully acknowledged.

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