African Journal of Biotechnology Vol. 8 (20), pp. 5527-5533, 19 October, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Curcumin induces cleavage of β-catenin by activation of capases and downregulates the β-catenin/Tcf signaling pathway in HT-29 cells

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Accepted 8 September, 2009

β-Catenin/Tcf-4 signaling pathway plays important roles in colorectal tumorigenesis. RT-PCR, western blotting and immunoprecipitation were used to study the effects of curcumin on β-catenin/Tcf-4 signaling pathway in HT-29 cells. Treatment of curcumin could induce cleavage of β-catenin and the cleavage could be inhibited by caspase inhibitors. The association of β-catenin with Tcf-4 in nucleus could be inhibited by curcumin. The expression of c-myc and cyclinD1 was downregulated by curcumin, which could not be blocked by Z-DEVD-FMK. The results showed curcumin could induce the cleavage of β-catenin by activition of caspases and downregulate the activity of β-catenin/Tcf signaling pathway independent of the caspases in HT-29 cells.

Key words: Curcumin, β-catenin/Tcf signaling pathway, HT-29 cells.

INTRODUCTION

Colorectal cancer is one of the most common and potentially fatal diseases in the world. Dysregulation of the Wnt signaling pathway is believed to play important roles in the pathogenesis of colorectal cancer (Polakis, 2000). In the absence of Wnt signal, β-catenin can be phosphorrylated in the cytoplasm by axin-adenomatous polyposis coli (APC)-glycogen synthase kinase 3β (GSK3β) complex and be degraded by ubiquitin-proteasome pathway (Rubinfeld et al., 1996). When the Wnt pathway is activated, GSK3β is inhibited and β-catenin is not phosphorylated any more and the level of cellular β-catenin increases. Free β-catenin can translocate to the nucleus, forming a complex with T-cell factor 4 (Tcf-4). This complex then binds DNA and induces the expression of genes responsible for cell growth and proliferation such as c-myc, cyclin D1, c-Jun and VEGF (Staal et al., 2002; He et al., 1998; Tetsu and McCormick, 1999; Mann et al., 1999). Mutations of the APC gene or serine-threonine phosphorylation sites for the GSK-3β within exon 3 of the β-catenin gene are associated with the earliest stages of colorectal tumorigenesis (Morin et al., 1996). Humans who inherit mutant APC or β-catenin develop thousands of benign tumors (adenomas) in colon, some of which progress to cancer. Recently, mutations in APC have been identified in up to 80% of 'classic' sporadic colorectal cancers (Bright-Thomas and Hargest, 2003). This means that the dysregulation of β-catenin plays a crucial role in colorectal cancer cells. Therefore if the transactivation ability of β-catenin/Tcf is significantly reduced, tumor growth may be suppressed.

Curcumin is an important polyphenol extracted from the rhizomes of *Curcuma longa* L. Several studies have shown curcumin exerts antioxidant, anti-inflammatory, anti-carcinogenic and chemopreventive activities on many tumor cells (Shishodia et al., 2005). Curcumin can induce the apoptosis of many cancer cell lines such as SW480, HT-116, HL-60, K562, H520 and HeLa, which is mediated by caspase family (Bhaumik et al., 2000; Sen et al., 2005). Accumulating evidence suggests that curcumin has a diverse range of molecular targets, which supports the notion that curcumin influences numerous biochemical and molecular cascades (Goel et al., 2008). Curcumin has been proved to be a potent inhibitor of the activation of various transcription factors including NF-κB, AP-1, STAT, and PPAR-γ (Aggarwal et al., 2006). Cur-

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cumin can also down-regulate the activities of multiple kinases such as PKC, ERK1/ERK2, and JNK (Shishodia et al., 2007). The dysregulation of β-catenin plays crucial roles in colon cancer cells. Several studies have showed that curcumin could inhibit the activity of β-catenin/Tcf signaling pathway (Jaiswal et al., 2002; Park et al., 2005). Another study suggests that curcumin cannot affect the β-catenin/Tcf signaling pathway (Bordonaro et al., 1999). It has been indicated that curcumin induces the cleavage of β-catenin by activation of caspases in HCT-116 cells, but the reduction of transactivation ability induced by curcumin is independent of caspases (Jaiswal et al., 2002). However, another study suggests that curcumin has no significant effects on the levels of β-catenin in HCT-116 and SW480 cells (Park et al., 2005). We hypothesize the mechanisms that curcumin reduces the β-catenin/Tcf transcriptional activity may be various in different cell lines. In the present study, we investigated whether curcumin could affect β-catenin/Tcf signaling in HT-29 cells and further studied the mechanism for the cleavage of β-catenin induced by curcumin. Our data suggests that curcumin can induce cleavage of β-catenin by activation of caspases in HT-29 cells, inhibit the association of β-catenin with Tcf-4 and down-regulate the expression of its downstream genes.

MATERIALS AND METHODS

Reagents

RPMI-1640, fetal bovine serum (FBS), penicillin, streptomycin and trypsin were purchased from GIBCO. Curcumin, sodium dodecyl-sulfate (SDS), dithiothreitol (DTT), phenylmethyl-sulfonylfluoride (PMSF) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Anti-β-catenin, anti-Tcf-4, anti-c-Myc, anti-cyclin D1 andhorseradish peroxidase-conjugated goat anti-rabbit antibodies were obtained from Epitomics. The caspase inhibitors CBZ-Val-Ala-Asp-fluoromethylketone (ZVAD-FMK), CBZ-Asp-Glu-Val-Asp-fluoromethylketone (ZDEVD-FMK), Boc-Asp-fluoro- methylketone (BD-FMK) and the cathepsin inhibitor CBZ-Phe-Ala-fluoromethylketone (ZFA-FMK) were purchased from Biovision (Palo Alto, USA). Nitrocellulose membrane and the enhanced chemiluminescence (ECL) detection system were purchased from Amersham (Pittsburgh, USA). Nuclear and Cytoplasmic Protein Extraction Kits were purchased from Beyotime Biotech, China. Reverse Transcription Kit was from Takara, Japan. Other reagents were of analytical grade and procured locally.

Cell culture and treatment

The human colon cancer cell line HT-29 was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640, supplemented with 10% fetal bovine serum, 100U/ml penicillin and 100 µg/ml streptomycin at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air. Upon reaching 70 - 80% confluence, the cells were exposed to 0 - 80 µM curcumin for 24 h.

Isolation of whole cell extracts

The cells treated with curcumin for 24 h were washed three times with ice-cold PBS. The total proteins were solubilized and extracted with 100 µl lysis buffer (Tris 20 mM, pH 7.5, NaCl 150 mM, Triton X-100 1%, sodium orthovanadate 1 mM, β-glycerophosphate 100 µM, EDTA 5 mM, sodium orthovanadate 0.2 mM, leupeptin 10 µg/ml and PMSF 2 mM). The lysates were centrifugated at 15000 g for 10 min and the supernatants were saved. The extracts were used to estimate their protein content using a BCA Protein Assay kit (Beyotime Institute of Biotechnology, China).

Isolation of nuclear proteins

Nuclear proteins were extracted as described elsewhere with minor modifications (Park et al., 2005). After treatment for 24 h, the cells were washed three times with ice-cold PBS and suspended in 100 µl lysis buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$ and 0.1 mM EDTA). The cells were placed on ice for 15 min and then vortexed after the addition of 5 µl of 10% Nonidet NP-40. After a 5 min centrifugation (16,000 g, 4℃), the pelleted nuclei were resuspended in 100 µl of extraction buffer (20 mM Hepes, pH 7.9, 50 mM KCl, 400 mM NaCl, 1 mML EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF). After vigorously shaking at 4℃ for 30 min, nuclear extracts were aliquoted and stored at - 70℃ until use. Nuclear extracts were quantified with BCA protein assay.

Immunoprecipitation

The immunoprecipitation was done as previously described with minor modifications (Park et al., 2005). The nuclear lysates containing 500µg of protein were incubated with 5 µg primary antibody overnight at 4℃. 50 µl of protein A/G plus-agarose (Santa Cruz, USA) were added and the complexes were incubated at 4℃ overnight. The beads were washed three times with high salt buffer (1 M Tris-HCl, pH 7.4, 0.5 M NaCl and1% Nonidet P-40) and twice with lysis buffer to eliminate nonspecific binding. The immunoprecipitated complexes were released with 2× sample buffer for western blotting.

Western blotting

Western blotting was performed as previously described with minor modifications to detect the expressions of β-catenin, c-myc and cyclin D1 protein (Yan et al., 2009). 50 µg of protein from each sample were subjected to electrophoresis on a 12% SDS-polyacrylamide gel. After electrophoresis, proteins were electroblotted to a Hybond-C Extra nitrocellulose membrane. The membranes were blocked at room temperature with 5% nonfat dry milk in TBS containing 0.3% Tween (TBS-T). The membranes were washed three times with TBS-T and incubated overnight at 4℃ with rabbit monoclonal anti-β-catenin antibody (1:5,000), anti-c-myc antibody (1:10,000), or anti-cyclin D1 antibody (1:1,000) followed by 1 h incubation with the horseradish peroxidase-conjugated secondary antibody (1:5000 dilution). After incubation, the membranes were washed with TBS-T for three times, the antigen-antibody complexes were visualized by enhanced chemiluminescence.

RNA extraction and reverse transcription (RT-PCR)

RNA was extracted with Trizol Isolation Reagent (Invitrogen, USA). Sample RNA content was quantified by measuring absorbance at 260 nm. RT-PCR was performed with PrimeScript RT-PCR Kit (Takara, Japan) according to the manufacturer's instructions. Table 1 showed the primer sequences used for RT-PCR and the product sizes. β-Actin served as an internal control. A total reaction volume of 20 µl contained 4 µl of reverse transcription product, 2.5 U Taq

Table 1. The synthetic primers used for RT-PCR.

Genes	Sense primer (5'→3')	Antisense primer (5'→3')	Size (bp)
c-myc	CAGCTGGAGATGGTGACCGAG	TCTTGCGAGGCGCAGGACTTG	343
Cyclin D1	ATAGGTGTAGGAAATAGCGCT	TCCGGTGTGAAACATCTAAGA	345
B-actin	CATTACAACTCTCCACAACC	CAGATAGCACCTTCAGCAC	450

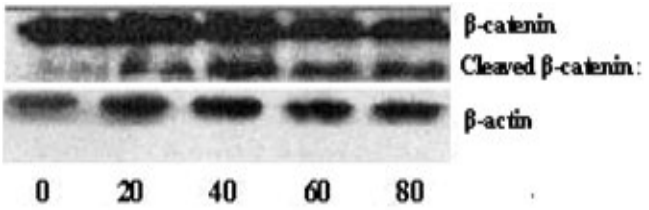

Figure 1. Curcumin induced cleavage of β-catenin in HT-29 cells. The whole cell extracts were immunoblotted with β-catenin antibody. To demonstrate equivalent loading of the lines, β-actin was used as internal control.

DNA polymerase, 20 μM dNTP, 0.1 μM primer and 1× Taq DNA polymerase buffer (Takara, Japan). The reaction mixture was incubated in a thermocycler (Perkin-Elmer, CA, USA) programmed to pre-denature at 94°C for 10 min, denature at 94°C for 30 s, anneal at 55°C for 30 s and extend at 72°C for 1 min. The last cycle was followed by incubation at 72°C for 10 min and cooling to 4°C. The optimum cycle number that fell within the exponential range of response for c-myc (25 cycles), cyclin D1 (28 cycles), or β-actin (22 cycles) was used. The PCR products were analyzed in 1% agarose gels.

Statistical analysis

The student's t-test was used to determine significant differences between the groups.

RESULTS

β-catenin was cleaved by curcumin and caspase family inhibitors blocked the cleavage of β-catenin in HT-29 cells

We performed western blotting to investigate the effects of curcumin on the level of β-catenin in HT-29 cells. The whole cell extracts derived from HT-29 cells were used to western blotting with β-catenin antibody. One truncated β-catenin fragments were detected in the curcumin treated cells (Figure 1). This meant that curcumin could activate the cleavage of β-catenin.

The caspase family play important roles in curcumin-mediated apoptosis. There are also studies indicated that caspases could cleave the β-catenin to several fragments (Steinhusen et al., 2000). To determine whether caspases are involved in the curcumin-mediated cleavage of β-catenin, we studied the effects of various caspase family inhibitors. The most effective nhibiting concentrations of the inhibitors were determined for our assay. In this study, all the inhibitors were used at the concentration of 20 μM. Curcumin induced β-catenin cleavage was inhibited by the caspase inhibitors such as ZDEVD-FMK, ZVAD-FMK and BD-FMK (Figure 2). The cathepsin inhibitor ZFA-FMK was used as a control for the FMK group and found not to block β-catenin cleavage (Figure 2). This result indicated curcumin induced β-catenin cleavage was mediated by the caspase family.

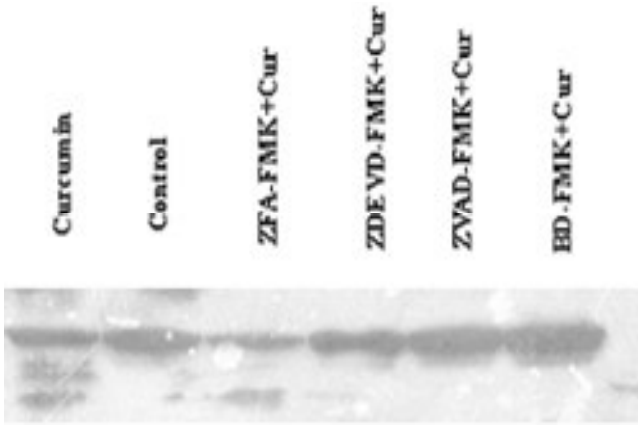

Figure 2. Curcumin induced β-catenin cleavage was inhibited by caspase inhibitors.

HT-29 cells were pre-incubated for 30 min with 20 μM of ZVAD-FMK, ZDEVD-FMK or BD-FMK and then treated with 40 μM of curcumin for an additional 12 h. The cathepsin inhibitor ZFA-FMK (20 μM) was used as a negative control reagent for peptide-FMKs.

Curcumin decreased the level of β-catenin and inhibited the association of β-catenin with Tcf-4 in the nucleus of HT-29 cells

The level of β-catenin and the association of β-catenin with Tcf-4 in the nucleus are very important for the activation of β-catenin/Tcf signaling pathway (Polakis, 2000). We treated HT-29 cells with curcumin (0 - 80 μM) and then determined the level of β-catenin protein in nucleus by western blotting. Our results showed that the amounts of β-catenin in the nucleus of HT-29 cells were markedly reduced by curcumin in a dose-dependent manner (Figure 3a). The association of β-catenin with Tcf-4 is required for activation of Tcf signaling, so we studied

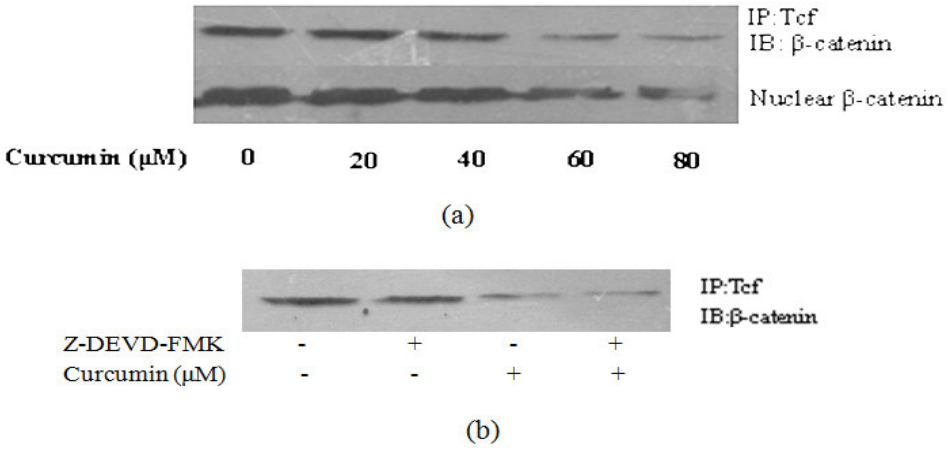

Figure 3. Curcumin inhibited the association of β-catenin with Tcf-4 in nucleus. (a) 50 μg protein extracted from nucleus were used to determine the level of β-catenin by western blotting. β-catenin/Tcf-4 complexes were immunoprecipitated from nuclear extracts with Tcf-4 antibody followed by immunoblotting with β-catenin monoclonal antibody. (b) HT-29 cells were pretreated with or without 20 μM Z-DEVD-FMK for 30 min and then were incubated with or without 60 μM curcumin for 24 h. IP and IB were performed to determine the level of β-catenin/Tcf-4 complex.

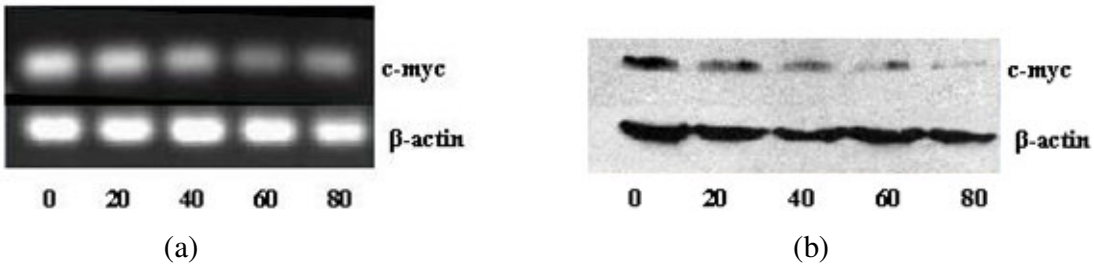

Figure 4. Curcumin downregulated the expression of c-myc. (a) The total RNA was extracted and RT-PCR was performed. β-actin was used as an internal control. (b) The HT-29 cell lysates were immunoblotted with an anti-c-myc antibody.

whether curcumin could affect the formation of the complex β-catenin with Tcf-4. We used anti-Tcf-4 antibody to coimmunoprecipitate the β-catenin/Tcf-4 complex from nuclear extracts and then we determined the amount of β-catenin by immunobloting. Figure 3a showed the level of Tcf-4/β-catenin complex in nucleus was significantly decreased by the incubation of 20 - 80 μM curcumin. The decreased level of β-catenin/Tcf-4 complex was not been changed by Z-DEVD-FMK (Figure 3b). From this result, we can conclude that curcumin inhibits the formation of β-catenin/Tcf-4 complex, which is independent of caspase-3 activation. IP and IB were performed to determine the level of β-catenin/Tcf-4 complex.

Curcumin down-regulated the β-catenin/Tcf signaling pathway

β-Catenin can translocate into the nucleus (Molenaar et al., 1996), where it associates with Tcf-4 and regulates the expression of downstream targets such as c-myc and cyclin D1. These proteins are very important for the growth and proliferation of cancer cells. Oncogene c-myc plays important roles in many cellular events and the over expression of c-myc is related to the increase of cell proliferation in a variety of malignant tumors. c-myc is a target of β-catenin/Tcf-4 signaling pathway, so we hypothesized that the expression of c-myc might be down-regulated by treatment of curcumin. The present results showed the level of c-myc transcript was reduced by treatment of curcumin (Figure 4a). Western blotting results also indicated that the amount of c-myc was markedly decreased in the curcumin treated cells (Figure 4b).

Cyclin Dl, another well known target of β-catenin/Tcf-4, plays important roles in regulating the cellular progression through the G1 phase of the cell cycle (Arber et al., 1997). In this study, we investigated the effects of curcu-

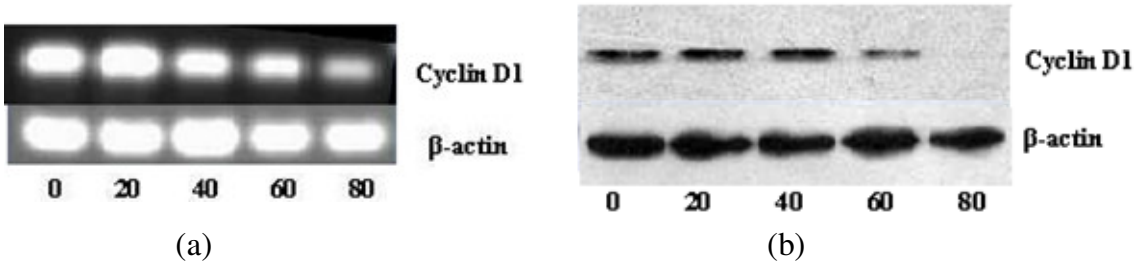

Figure 5. Curcumin reduced the expression of cyclin D1. (a) Semi-quantative RT-PCR was performed to determine the level of cyclinD1 mRNA. (b) Western blotting was performed to study the level of cyclinD1 protein.

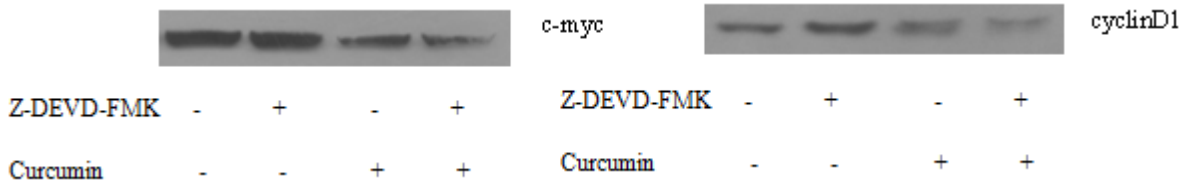

Figure 6. Pretreatment with Z-DEVD-FMK had no effects on the levels of c-myc and cyclinD1.

min on the expression of cyclin D1. Our results indicated that the levels of cyclin D1 transcript were markedly decreased when the cells were treated with 40 - 80 μM curcumin (Figure 5a). Western blotting results showed treatment of curcumin dose-dependently reduced the amount of cyclin D1 in HT-29 cells (Figure 5b).

Z-DEVD-FMK could not inhibited down-regulation of c-myc and cyclin D1 induced by curcumin in HT-29 cells

On the basis of the results that Z-DEVD-FMK pretreatment could not block the decrease of β-catenin/Tcf-4 complex induced by curcumin, we hypothesized the transactivation ability of β-catenin/Tcf complex would not be affected by Z-DEVD-FMK pretreatment. To test this idea, we pretreated HT-29 cells with 20 μM Z-DEVD-FMK and then incubated the cells with 60 μM curcumin for 24 h. The western blotting results showed that the decrease of c-myc and Cyclin D1 induced by curcumin were not affected by caspase-3 inhibitor (Figure 6).

DISCUSSON

Curcumin can strongly inhibit growth of various colon cancer cell lines, but the underlying molecular mechanisms remain unknown. Our work had two interesting findings. First, curcumin induced the cleavage of β-catenin in HT-29 cellular extracts, which was mediated by caspases. Second, curcumin markedly down-regulated the activity of β-catenin/TCF-4 signaling pathway and decreased the expression of the downstream targets such as c-myc and cyclin D1 in HT-29 cell, which was not been affected by the caspase-3 inhibitor, Z-DEVD-FMK.

β-Catenin plays important roles in the apoptosis or survival of cancer cells. Jaiswal et al. (2002) suggested that curcumin treatment induced cleavage of β-catenin in HCT-116 cells. However, Park et al. (2005) reported that the amount of β-catenin had not been affected by curcumin and its derivative in HCT-116 and SW480 cells. The discrepancy in their reports is perhaps due to following two reasons: First, the time period of curcumin treatment was the important difference in HCT-116 cells. The time of treatment was restricted to 24 h in the study of Park et al. (2005); 30 h was used in the study of Jaiswal et al. (2002). Second, there might be different influences between HCT-116 and SW480 cell lines when the cells were treated with curcumin. In previous studies, it had been suggested that the cleavage of β-catenin induced by curcumin may be mediated by caspase-3 in HCT-116 (Jaiswal et al., 2002). In this study, we found treatment with 20-80 μM of curcumin for 24 h could induce β-catenin cleaved into two fragments in HT-29 cells, which was consistent with the results obtained in HCT-116 cell line (Jaiswal et al., 2002). It had been indicated that curcumin can activate caspase family in many cancer cell lines (Bhaumik et al., 2000; Sen et al., 2005). We had found curcumin could activate the caspases in HT-29 cells (Wang et al., 2009). In the present study, we used caspase inhibitors to study whether curcumin induced the cleavage of β-catenin was mediated by caspases. In congruence with the previous

findings (Jaiswal et al., 2002), we found caspase inhibitors blocked curcumin induced cleavage of β-catenin in HT-29 cells, which suggested that the cleavage of β-catenin induced by curcumin was mediated by caspases in HT-29 cells.

It is very important that β-catenin translocates into nucleus and associates with Tcf-4 for growth and proliferation of many cancer cells. So we determined the effects of curcumin on the levels of β-catenin and β-catenin/Tcf-4 complex in HT-29 cell nucleus. Consistent with previous findings of Park et al. (2005), our data showed curcumin decreased the level of the β-catenin/Tcf-4 complex in HT-29 cell nucleus. We next studied the effects of Z-DEVD-FMK on the level of β-catenin in nucleus. Our results indicated pretreatment of HT-29 cells with Z-DEVD-FMK blocked the reduction of the level of β-catenin/Tcf-4 complex in nucleus.

In several colorectal cancer cell lines, the binding of β-catenin/Tcf-4 complex to DNA activates the expression of many genes relevant to colon carcinogenesis, such as c-myc and cyclin D1. c-myc is a well-known oncogene which promotes cell growth and transformation as well as vascular and hematopoietic development (He et al., 2008). Downregulation of c-myc can suppress the growth and proliferation of many cancer cells (Schorl and Sedivy, 2003). Increased nuclear expression of cyclin D1 occurs in around one third of colonic tumors as an early event during multistage process of colon carcinogenesis (Arber et al., 1996). Overexpression of anti-sense cyclin D1 cDNA inhibits the growth of SW480 colon cancer cells in nude mice, indicating a critical role for cyclin D1 in tumorigenesis (Arber et al., 1997). To further clarify the effects of curcumin on β-catenin/Tcf-4 signaling pathway, we studied the expression changes of these downstream genes in curcumin treated HT-29 cells. Our results showed curcumin down-regulated the expression of c-myc and cyclinD1. The decrease in the levels of c-myc and cyclinD1 was not blocked by the pretreatment with Z-DEVD-FMK, which indicated the down-regulation of β-catenin/Tcf-4 transactivation ability was independent of caspases activation.

It is interesting to note that although curcumin induced cleavage of β-catenin was blocked by pretreatment with Z-DEVD-FMK in HT-29 cells; the level of the β-catenin/Tcf-4 complex in nucleus was not reduced. We believe there are two possible reasons. First, either β-catenin or Tcf-4 or both could be directly modified by curcumin in a way and such modification inhibits their association. A second possible explanation is that curcumin may induce a protein(s) that somehow inhibits the formation of β-catenin/Tcf-4 complex.

In summary, the present study shows curcumin can induce cleavage of β-catenin in HT-29 cells by activation of caspases. The association of β-catenin with Tcf-4 was inhibited by curcumin independent of caspases. The main targets of β-catenin/Tcf-4 signaling pathway in HT-29 cells have also been downregulated by curcumin treatments. These targets are important for the growth, proliferation, differentiation and neovascularization of colorectal cancer cells. Based on these findings, we suggest that curcumin may play anti-tumor role by repression of β-catenin/Tcf-4 signaling pathway. In the future, curcumin may prove an important chemopreven-tive agent for colorectal cancer.

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