**Abstract.** Curcumin, the major phytochemical in turmeric, exerts anti-proliferative, anticancer and anti-inflammatory activities in various types of cancer cells. Curcumin has been demonstrated to induce apoptosis through multiple signaling pathways; however, its association with survival pathways, including the Wnt signaling pathway, is not fully understood. The Wnt signaling pathway is involved in diverse functions, including cell development, growth and proliferation. This pathway is important for cancer cell survival and metastasis. ß-catenin and GSK3β play a key role in the Wnt signaling pathway and therefore, various members of the Wnt signaling pathway have been hypothesized to represent potential targets for anticancer therapy. In the present study, the effect of curcumin on the suppression of migration and proliferation of Hep3B hepatocarcinoma cells was investigated via suppression of Wnt signaling in vitro and in vivo. 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced cell migration was observed to be suppressed by curcumin treatment. In addition, curcumin suppressed TPA-induced activation of Wnt signaling. These results indicate that curcumin induces anti-migratory activity, which functions via the Wnt signaling pathway.

**Introduction**

Curcumin, the major phytochemical in turmeric, has a diverse pharmacological role, including anticancer, anti-inflammatory, antioxidant and anti-proliferative activities (1). In a previous study, curcumin was identified to exhibit anticancer effects by interfering with signaling pathways associated with the initiation, promotion and progression of multistage carcinogenesis (2). In addition, curcumin has been found to induce apoptosis in various types of cancer cells, including colon, breast, lung and ovarian (3-6), and suppresses tumor growth in various cancer xenograft models (7,8). Curcumin inhibits metastasis and angiogenesis by suppression of matrix metalloproteinase-9, vascular endothelial growth factor and hypoxia-inducible factor 1α expression in hepatocarcinoma (9).

The Wnt signaling pathway is important in cell growth, proliferation, differentiation and development. Several studies have reported that overactivation of ß-catenin in the cytosol is associated with cancer metastasis (10-13). Under normal conditions, ß-catenin is phosphorylated at the Ser33/37 residue by GSK3β in the GSK3β/Axin/Ck1 complex, triggering subsequent proteosomal degradation. Phosphorylated ß-catenin translocates into the nucleus, where it activates target genes coding for proteins, including cyclin D and c-Myc in the Wnt pathway, by binding with the transcription factor, T cell factor (TCF) (14). The Akt/mTOR pathway, in addition to overactivated Wnt signaling, inhibits GSK3β activity. Phosphorylation of GSK3β by Akt has been demonstrated in a number of cell lines to promote angiogenesis, metastasis and cell survival by activation of the NF-κB signaling pathway (15-18).

Although several anticancer effects of curcumin in cancer cells have been reported, studies on the molecular mechanisms of these effects via the survival pathways in hepatocarcinoma have not been performed. Curcumin possesses the ability to suppress Wnt signaling and may be important for the development of anti-proliferative and anti-metastatic drugs.

In the current study, curcumin was observed to induce apoptosis by cell proliferation assay and cell cycle analysis. Curcumin induced expression of apoptotic proteins and suppressed Wnt signaling by the reduction of ß-catenin and phospho-GSK3β in vitro. In addition, in vivo suppression of the Wnt signaling pathway by curcumin was identified. Curcumin significantly suppressed 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced cell migration by blocking the Wnt signaling pathway in Hep3B hepatocarcinoma cells.
Materials and methods

Cell culture and reagents. Hep3B cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in DMEM containing 10% fetal bovine serum and 1% antibiotics at 37°C in a 5% CO₂ incubator. Curcumin, TPA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) and BIO were obtained from Sigma-Aldrich (St. Louis, MO, USA). LY294002 was purchased from Tocris Bioscience (Bristol, UK).

MTT assay. Cells seeded in 12-well plates at 1x10⁵ cells/ml were incubated with curcumin for indicated times and concentrations. Respective medium was removed and then incubated with 20 µl MTT solution (5 mg/ml MTT in PBS) for 1 h. Converted purple formazan dye from MTT was solubilized in DMSO and optical densities were measured at 595 nm.

Cell cycle analysis. Cells treated with curcumin at various concentrations (10, 20 and 40 µg/ml for 24 h) were harvested, washed with phosphate-buffered saline (PBS) and fixed in 70% cold ethanol. Following washing with PBS, cells were resuspended in PBS and incubated with RNase A and PI. Cells were analyzed with flow cytometry.

Wound healing assay. Hep3B cells were grown to 90% confluence in a 6-well plate at 37°C in a 5% CO₂ incubator. A wound was created by scratching cells with a sterile 200 µl pipette tip. Cells were washed with PBS to remove floating cells and then added to a medium. The distance between wound edges was measured at a fixed region. Images of the wound were captured under a microscope (magnification, x100).

Western blot analysis. Cells were washed with PBS and lysed with RIPA lysis buffer [50 mM Tris-HCl (pH 8.0), 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl and 1 mM PMSF]. Protein concentrations were determined using the Bradford assay. All samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. The membrane was incubated overnight with primary antibodies. Secondary antibodies, conjugated to horseradish peroxidase, against IgG were used. Proteins were visualized by enhanced chemiluminescence (Intron Biotechnology, Seong nam, Korea) and detected using the LAS4000 chemiluminescence detection system (Fujifilm, Tokyo, Japan).

Xenograft and immunohistochemistry. Hep3B cells (2x10⁶) were inoculated subcutaneously in 4-week nu/nu mice at the left flank. After 1 week, mice were administered curcumin or PBS (control) by injection. Tumor size was measured in two perpendicular diameters by using a caliper every 3 days and tumor volume was calculated using the following formula: Volume = 1/2 x (length x width)².

Tumor specimens from mice were fixed in 10% formaldehyde, embedded in paraffin and sectioned into 5-µm thick slices. Sections were deparaffinized with xylene and dehydrated with 98% ethanol. Serial sections were stained using standard immunoperoxidase techniques with primary antibodies against CD31. For epitope retrieval, specimens were microwave treated for 25 min prior to incubation with primary antibody. Pre-immune serum was used as a negative control for immunostaining and positive staining was visualized with diaminobenzidine, followed by a light counterstaining with hematoxylin. Sections were evaluated by a pathologist, determining stain intensity and percentage of reactive cells. Representative images were captured. All animal experiments were approved by the Ethics Committee for Animal Experimentation of the Hannam University (Daejeon, South Korea).

Statistical analysis. Cell viability data were statistically analyzed using unpaired t-tests (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of curcumin on cell viability and apoptosis in Hep3B cells. To examine the effects of curcumin on Hep3B cell proliferation, cells were seeded in 12-well plates at a density of 1x10⁵ cells/well. The effect of curcumin on cell viability was determined by MTT assay. Fig. 1A demonstrates significant inhibition of cell proliferation in curcumin-treated cells in a dose- and time-dependent manner, compared with control cells. Cell cycle analysis revealed that curcumin induced dose-dependent apoptosis. The percentage of apoptotic cells (sub-G₁ peak) increased from 19.5% (control) to 35.53% (curcumin 20 µM) and 32.15% (curcumin 40 µM) in the Hep3B hepatocarcinoma cells (Fig. 1B). To determine the mechanism of curcumin-induced apoptosis, the expression levels of apoptotic proteins, including procaspase-3, PARP, Bax and Bcl-2, were detected by western blot analysis. Activation of caspase-3 plays a critical role in apoptosis, as it proteolytically cleaves PARP. In Hep3B cells treated with curcumin, the levels of procaspase-3, PARP and Bcl-2 decreased and Bax increased.

Effect of curcumin on the Wnt signaling pathway in vitro and in vivo. The Wnt signaling pathway plays a critical role in tumor cell growth and survival in hepatocarcinoma. Therefore, the inhibitory effect of curcumin on Wnt signaling in Hep3B cells was determined by analyzing the levels of β-catenin and GSK3β, which play key roles in the pathway. Previous studies have demonstrated that GSK3β is inactivated by Akt and regulated by mTOR (25). In addition, Akt and mTOR are associated with cell survival and growth. Therefore, levels of β-catenin, p-GSK3β, p-Akt and p-mTOR were assessed by western blot analysis. As demonstrated in Fig. 2A, curcumin increased phosphorylation of GSK3β, Akt and mTOR and thus, the level of β-catenin, in a dose-dependent manner. To explore the therapeutic effects of curcumin, hepatocarcinoma tumors were established in nude mice and tumors were treated by injecting curcumin. As revealed in Fig. 2B, tumor volume of the curcumin-treated group was significantly reduced compared with that of the control group. No significant differences in the body weight of mice were observed between control and curcumin-treated groups. Furthermore, curcumin was observed to induce antitumor activity by suppression of Wnt signaling molecules. Fig. 2C demonstrates that β-catenin, p-mTOR, p-Akt and p-GSK3β expression was significantly suppressed.
decreased in the curcumin-treated group compared with the control. To further correlate these observations, in vivo tumor therapeutic effects were assessed in vitro. Thus, the expression levels of biomarkers were assessed in tumor tissues by immunohistochemical analysis. The results indicated that CD31-positive microvessels were significantly suppressed following curcumin treatment (Fig. 2D).

**Effect of curcumin on TPA-induced Wnt signaling pathway activation and cell migration in Hep3B cells.** To investigate the effect of curcumin on TPA-induced Wnt signaling activation and cell migration, wound healing assays and western blot analysis were performed in Hep3B cells. Western blot analysis demonstrated that TPA increased β-catenin and phosphorylation of mTOR, Akt and GSK3β, and that curcumin inhibited TPA-induced Wnt signaling pathway activation at 6 h in a concentration-dependent manner (Fig. 3A and B). As demonstrated in Fig. 3C, cell migration was increased in cells treated with TPA. However, the number of migrated cells was significantly decreased in the group treated with curcumin alone and the group pre-treated with TPA and then followed with curcumin for 48 h (for 12 and 24 h; data not shown).

**Effect of curcumin with LY294002 or BIO on Wnt signaling.** Western blot analysis was used to clarify the mechanism of curcumin-mediated suppression of the Wnt signaling pathway in Hep3B cells by the suppression of specific molecules. The results indicate that curcumin significantly suppressed
KIM et al.: CURCUMIN SUPPRESSES MIGRATION AND PROLIFERATION BY INHIBITING WNT

The expression of p-mTOR, p-Akt, β-catenin and p-GSK3β. Pre-treatment using LY294002 and BIO, specific PI3K and GSK3 inhibitors, respectively, followed by curcumin treatment, effectively reduced β-catenin and phosphorylation of mTOR, Akt and GSK3β (Fig. 4).

Discussion

Liver cancer is the leading cause of mortality from cancer and is more prevalent in Asia and Africa. To date, a number of signaling pathways, including Ras/Raf/MEK/ERK, PI3K/Akt/mTOR and NF-κB have been hypothesized to represent potential targets for hepatocarcinoma therapy. Thus, the Wnt signaling pathway is markedly involved in the development of normal cells as well as tumorigenesis. In addition, mutation and aberrant activation of the Wnt signaling pathway has been identified in various types of cancer and is frequently activated in hepatocarcinoma (19). Activation of Wnt signaling results in phosphorylation of GSK3β (inactive form), which in turn leads to transcription of oncogenes by β-catenin translocation and binding with TCF in the nucleus. Following this, the complex activates the transcription of growth-promoting genes, including cyclin D and c-Myc. Previous studies have reported that curcumin represents a potent anticancer agent. In addition, studies have demonstrated that Bcl-2, Bax, procaspase-3, PARP, Noxa and PUMA may serve as predictive markers for the evaluation of apoptosis in various cancer cells (20,21). Thus, in the present study, curcumin was hypothesized to suppress cell migration and proliferation by inhibiting Wnt signaling. Curcumin was found to suppress cell proliferation in vitro by blocking Wnt signaling and inhibition of the pathway, which correlated with the suppression of cell migration. In addition, analysis of the cell cycle and expression of apoptotic proteins was performed, confirming that curcumin induced apoptosis. Curcumin was also identified to suppress the expression of p-mTOR, p-Akt, β-catenin and p-GSK3β regardless of treatment with inhibitors (LY294002 and BIO).

TPA, a tumor-promoting phorbol ester, enhances cellular signaling pathways, including PI3K/Akt, PKC and MAPK (22). TPA also activates the PI3K pathway and downstream Akt, leading to inhibition of GSK3β. In addition, TPA-induced isoforms of PKC has been observed to inactivate GSK3β. This inactivation leads to β-catenin accumulation and Wnt target gene expression, which are involved in cell proliferation (23,24). In the present study, migration triggered by TPA was found to be suppressed by curcumin, confirming that curcumin inhibits TPA-induced Wnt signaling activation. In addition, curcumin was observed to suppress tumor growth and microvessel density by blocking the Wnt signaling pathway in vivo.

In conclusion, results of the present study indicate that curcumin induces apoptosis, as confirmed by cell proliferation assays, analysis of the cell cycle and expression of apoptotic proteins. Curcumin was also found to inhibit TPA-induced migration in vitro as well as tumor growth in vivo through inhibition of the Wnt signaling pathway. Thus, curcumin treatment may be developed as a novel strategy for the suppression of cell proliferation and survival in hepatocarcinoma.

Acknowledgements

The present study was supported by a grant from the National Research Foundation of Korea (KRF-2012-0021402).

References


