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Full Length Research Paper

Effect of curcumin on proliferation, cell cycle, and caspases and MCF-7 cells

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We examined the mechanisms by which curcumin inhibits the growth of breast cancer cells. First, we investigated its antiproliferative effects on MCF-7 cells exposed to 2.5 to 40 µM curcumin for 24, 48 or 72 h. Curcumin inhibited cell proliferation in a dose-dependent and time-dependent manner ($p<0.05$) and resulted in significant cell cycle arrest in G1 phase after 72 h of treatment at concentrations of over 10 and 20 µM in MCF-7 cells, respectively ($p<0.05$). In addition, curcumin caused the accumulation of cells in sub-G0 phase in a dose-dependent manner in MCF-7 cells ($p<0.05$). As biomarkers of apoptosis induction, caspase-3 activity and caspase-9 activity were increased by curcumin in MCF-7 cells. To investigate the effects of curcumin on the proteins regulating cell cycle arrest, cells were treated with 20 µM curcumin for 72 h. Similar changes in the expression of regulatory proteins were detected in MCF-7 cells. Curcumin treatment resulted in decreases in cyclin D, cyclin E, CDK2, CDK4 and CDK6. The protein expression of CDK2 related to the G1 phase increased markedly with curcumin treatment. Curcumin treatment increased the expression of the CDK inhibitors $p19^{INK4}$, $p21^{Cip1}$ and $p27^{Kip1}$. Thus, curcumin exerts its anticancer effects in human breast cancer cells via cell cycle arrest at the G1 phase.

Key words: Apoptosis, breast cancer, cell proliferation, cell cycle, caspase, curcumin.

INTRODUCTION

Polyphenols have antioxidant activity and scavenge reactive oxygen species (ROS), blocking the generation of ROS and activating an enzymatic antioxidant defense system *in vitro* (Ying et al., 2004; Guo et al., 2005, 2007). Recent studies have focused on the relationship between antioxidants and cancer (Duthie et al., 2000). In addition, increasing evidence suggests that curcumin can be used to prevent or treat cancer (Verma et al., 1997; Chen et al., 2006; Su et al., 2010).

Curcumin (diferuloylmethane, $C_{21}H_{20}O_6$), an active polyphenolic ingredient isolated from turmeric (the rhizome of the plant *Curcuma longa Linn*.), is traditionally used as a coloring agent and spice in food (Figure 1). Previous studies suggested that curcumin may act as a prooxidant (Yoshino et al., 2004; Ravindran et al., 2010). These prooxidant capacities, such as the pro-apoptotic effect of curcumin, could contribute to cancer chemotherapy and the inhibition of tumor growth (Wu et al., 2011; Semsri et al., 2011; Pandey et al., 2010). Curcumin inhibits the proliferation of cancer-derived cell lines (Elattar and Virji, 2000; Zheng et al., 2004; Giri et al., 2009; Wang et al., 2009). This antiproliferative activity in cancer cells involves the induction of apoptosis (Su et al., 2010). The mechanisms of its anticancer capacity, including cell cycle arrest and the induction of apoptosis, remain poorly understood. Recently, recommendations for the consumption of supplements, such as natural

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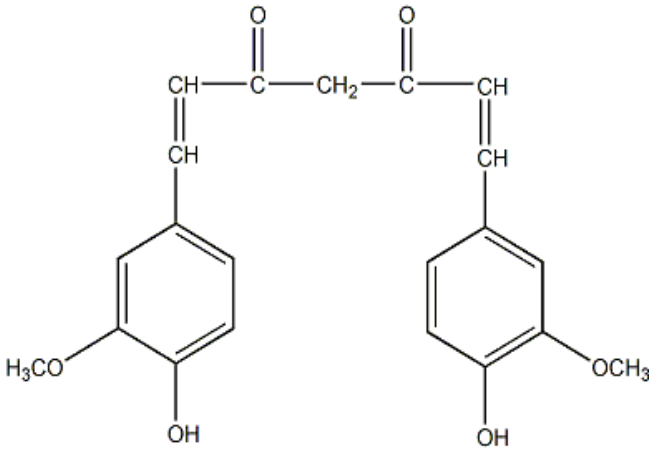

Figure 1. Structure of curcumin.

polyphenols, including curcumin and catechin, are commonly given in grams, although no experimental data support this recommended dose. We therefore, investigated the cellular mechanisms underlying the cell cycle arrest and apoptosis induced by curcumin in human breast cancer MCF-7 cells.

MATERIALS AND METHODS

Cells and cell culture

Human breast carcinoma cells, MCF-7, were cultured in RPMI1640 medium supplemented with 0.22% sodium bicarbonate, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin and incubated at 37°C in 5% CO_2. Curcumin was dissolved in dimethylsulfoxide (DMSO) at a concentration of 5 mM and was diluted to the required concentration with RPM I1640 medium immediately before use. Cells grown in medium containing an equivalent final volume of DMSO (final concentration <0.01%, V:V) served as control.

Cell proliferation

Cell proliferation was determined using the MTT assay. In brief, cells were seeded in 96-well plates (5 to 8×10^3 per well) using RPMI1640 medium with 10% FBS for attachment. At 24, 48 and 72 h point, the cells exposed to equol were added to methyl thiazolyl tetrazolium (MTT). Cells were incubated for 4 h at 37°C and lysed by the addition of 100 µl DMSO, and the optical density (OD) at 595 and 655 nm was then measured on a microplate reader. In parallel, cells were seeded in 24-well plates (5×10^4 per well) and direct cell counting employing trypan blue exclusion analysis was used to confirm significant effects. Each treatment was done in triplicate and repeated three times.

Cell cycle distribution

Cells were then harvested, washed with cold PBS, and processed for cell cycle analysis. Briefly, the cells were fixed in absolute ethanol and stored at -20°C for later analysis. The fixed cells were centrifuged at 1000 rpm and washed with cold PBS twice. RNase A (20 mg/ml final concentration) and propidium iodide staining solution (50 µg/ml final concentration) was added to the cells and incubated for 30 min at 37°C in the dark, and analyzed by flow cytometry.

G1 phase-related protein expression

The expression of G1 phase-related protein CDK2, CDK4, CDK6, cyclin D, cyclin E, $p19^{INK4}$, $p21^{Cip1}$, $p27^{Kip1}$, were determined by assay kits (Ruiqi Biological Technology CO., LTD.).

Apoptosis detection

For caspase-3 and caspase-9 activity analysis, cells were collected by trypsinization and lysed with lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris-HCl, pH 8.0, 2 mM DTT, 1 mM PMSF, 1 µg/ml aprotinin, 1 mg/ml leupeptin). Thereafter, the lysates were transferred to wells in a 96-well flat-bottom plate. A peptide with the caspase-3 and caspase-9 target motif DEVD bound to the chromophore p-nitroanilide was added and incubated at 37°C for 1 h. The intensity of the developed color was read at 405 nm in a microplate reader.

Statistical analyses

Statistical analysis was performed using SPSS 11.5 for Windows. Data were expressed as means±standard deviation (SD) and analyzed by one-way ANOVA with the Student-Neuman-Keuls post-hoc test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Curcumin inhibited the cell proliferation

We examined the effects of curcumin on the viability of MCF-7 cell line using MTT assays (Figure 2). The results showed that curcumin decreased the MCF-7 cells *in vitro* viability in a dose- and time-dependent manner. Compared with control, cell viability inhibition caused by curcumin at 10 µM and above was found to be statistically significant within 24 h. However, curcumin at 5 µM could not cause a growth inhibition until cells were treated for 48 h. Cells exposed to the same concentrations of curcumin under each tested condition were further inhibited as the incubation time periods were extended from 24 to 48 h (data not shown). In parallel, significant effects were confirmed by direct cell counting employing trypan blue exclusion analysis (data not shown). These results indicated that curcumin was actually inhibiting MCF-7 cell growth.

Cell cycle distribution by curcumin

We analyzed cell cycle arrest in MCF-7 cells exposed to curcumin for 24 h (Figure 3). Significant cell cycle arrest at the G1 phase with curcumin treatment was initially

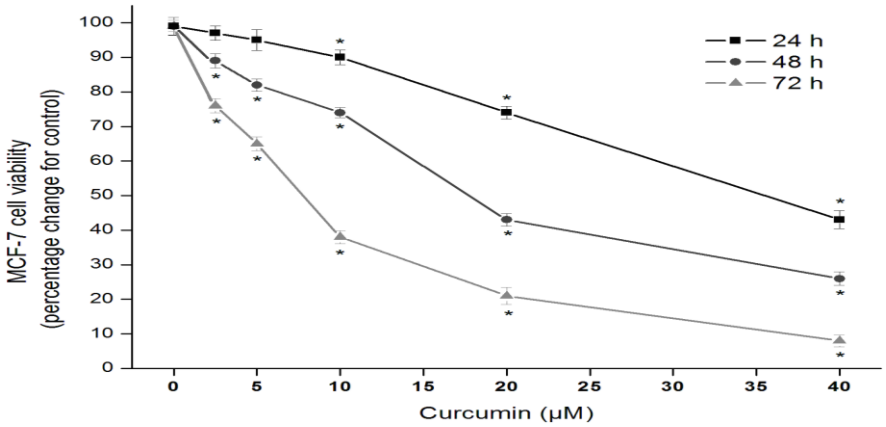

Figure 2. Effect of curcumin on cell proliferation of breast cancers mcf-7 cells. Cells were exposed to curcumin at various concentrations (ranging from 2.5 to 40 μm) and incubated for 24, 48 and 72 h. all data are reported as the percentage change in comparison with the vehicle-only group, which were arbitrarily assigned 100% viability. *, p<0.05, significantly different from the control level (0.1% dmso in medium, that is, curcumin concentration = “0”).

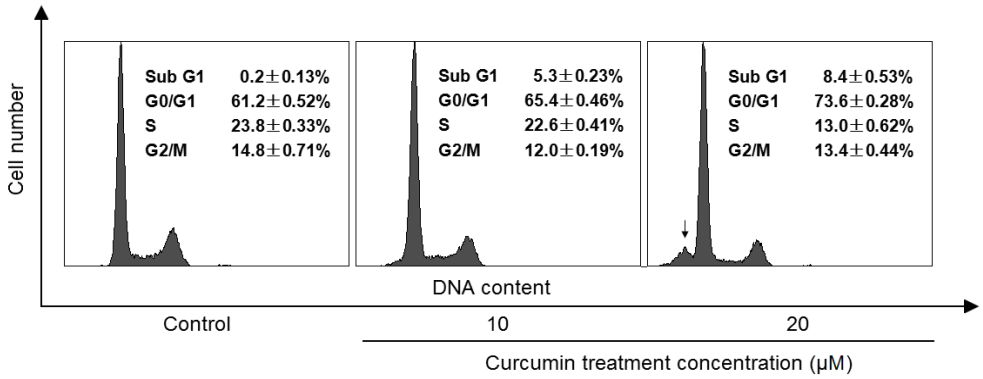

Figure 3. Effect of curcumin on cell cycle distribution of breast cancer MCF-7 cells. Cells were exposed to curcumin at various concentrations (ranging from 10 to 20 μM) and incubated for 24 h. Values are expressed as percentage of the cell population in the G1, S, and G2/M phase of cell cycle. *, p<0.05, significantly different from the control level (curcumin concentration = “0”).

observed using 10 μM curcumin, and a dose-dependent effect was observed in MCF-7 cell line. Curcumin increased the G1-phase population by 4.2 and 12.4% at 10 and 20 μM, concomitant with a decrease in the proportion of G2/M-phase cells (1.2 and 10.8%). In addition, curcumin led to the gradual accumulation of small DNA fragments in the sub-G0 phase, at 5.3 and 8.4% using 10 and 20 μM, respectively (compared to the control of 0.3%; $p < 0.05$), in MCF-7 cells.#

G1 phase-related protein expression by curcumin

When cell cycle arrest was induced in MCF-7 cells using 20 μM curcumin, the modulation of protein expression showed similar patterns. In MCF-7 cells, curcumin decreased cyclin D expression by 30.1%, cyclin E by 27.9%, CDK2 by 28.4%, CDK4 by 45.6%, and CDK6 by 38.2% (Figure 4).

Curcumin targets several components of the cell cycle regulatory apparatus, and it increased the expression of the CDK inhibitory subunits $p19^{INK4}$, $p21^{Cip1}$ and $p27^{Kip1}$ in MCF-7 cells (Figure 5).

Apoptosis induction by curcumin

Caspase-3 activity and caspase-9 activity, biomarkers of apoptosis, were measured in MCF-7 cells after a 72 h

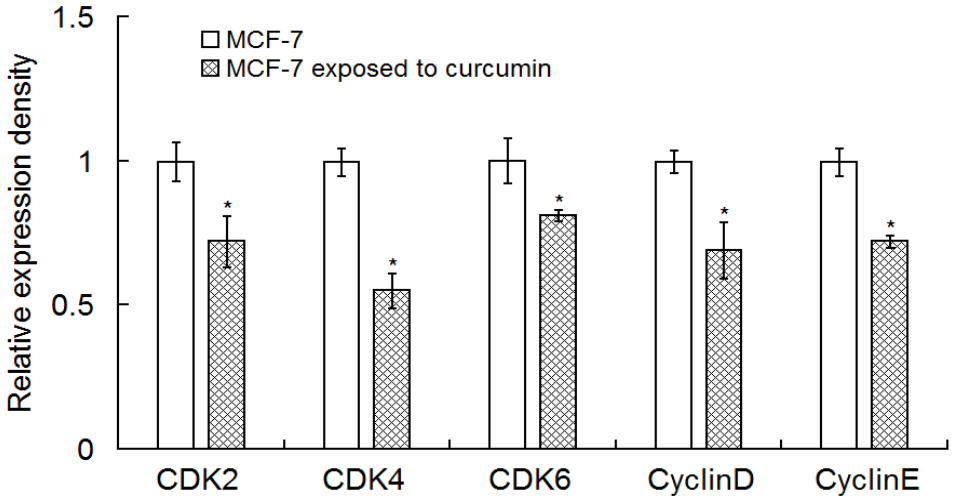

Figure 4. Effect of curcumin on protein expression related cell cycle arrest of breast cancers MCF-7 cells. Cells were exposed to curcumin at 20 μM for 72 h. Control group (vehicle-only group) level was accepted to be "1.0". *, p<0.05, significantly different from the control level (curcumin concentration = "0").

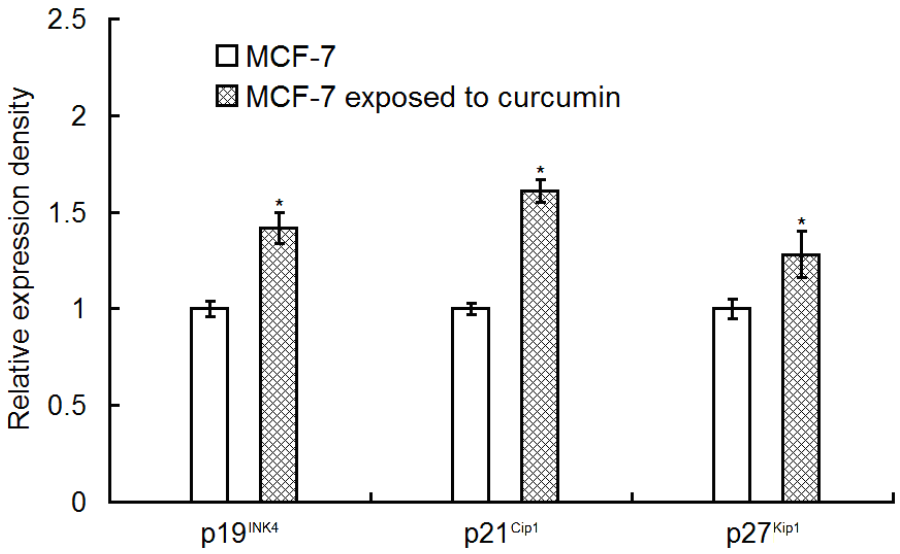

Figure 5. Effect of curcumin on CDK inhibitors expression related cell cycle arrest of breast cancers MCF-7 cells. Cells were exposed to curcumin at 20 μM for 72 h. Control group (vehicle-only group) level was accepted to be "1.0". *,p<0.05, significantly different from the control level (curcumin concentration = "0").

exposure to 20 μM curcumin. Curcumin significantly increased caspase-3 and caspase-9 activity by 73.1 and 18.3% in MCF-7 cells (Figure 6).

DISCUSSION

Recently, a great deal of attention has been focused on how the biological properties of polyphenols, including catechin and curcumin, can be used to develop new cancer chemotherapy strategies. According to previous many reports, catechin as a major polyphenols specifically inhibits cell growth in various types of cancer (Choudhuri et al., 2005; Lee et al., 2009; Sahu et al., 2009; Singh et al., 2010). The anticancer effects of curcumin are not as well understood as those of catechin, although both of them are polyphenols. In the present study, we examined the antiproliferative effect of

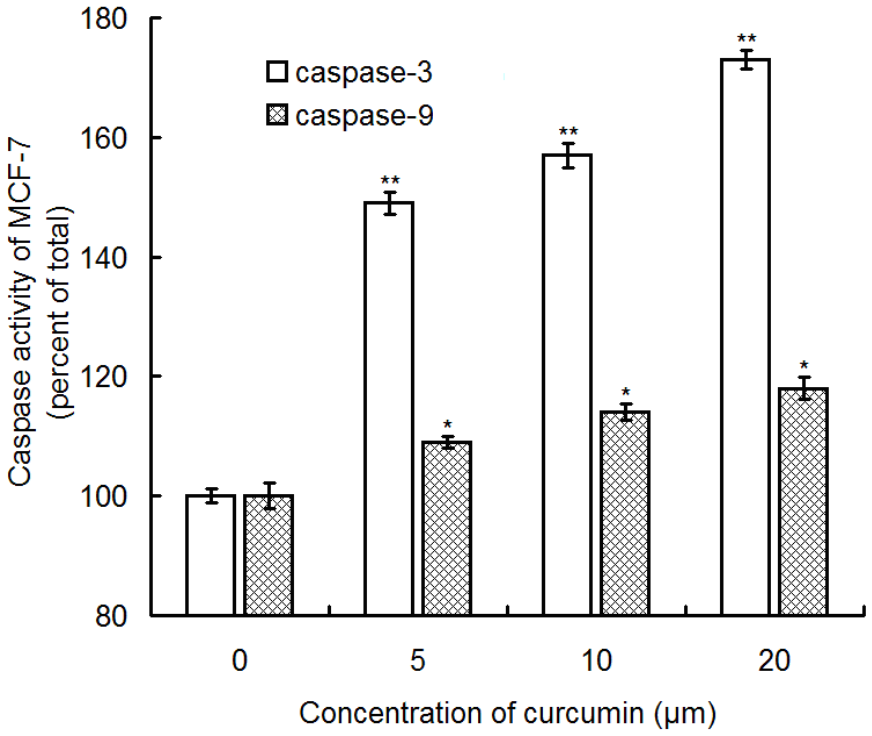

Figure 6. Effect of curcumin on caspase-3 activity and caspase-9 activity of breast cancers MCF-7 cells. Cells were exposed to curcumin at 20 μM for 72 h. All data are reported as the percentage change in comparison with the vehicle-only group, which were arbitrarily assigned 100%. *, $p<0.05$, significantly different from the control level (curcumin concentration = "0").

curcumin on human breast cancer MCF-7 cells at concentrations ranging from 2.5 to 40 μM for 24, 48 and 72 h. Curcumin significantly decreased the proliferation of MCF-7 cell in a dose and time-dependent manner. This was consistent with previous reports that curcumin inhibits the growth of human colon, breast, and ovarian carcinoma cells (Deguchi et al., 2002; Morre et al., 2003; McMillan et al., 2007; Park et al., 2009).

To further scrutinize these results, we analyzed the cell cycle of MCF-7 cells treated with 10 μM curcumin for 24 h. Similar to the results of the cell proliferation experiment, curcumin induced significant G1 phase cell cycle arrest in MCF-7 cell. This was consistent with previous reports that curcumin arrests the cell cycle at G1 phase of MCF-7 cell (Wu et al., 2006, 2008). Many reports indicate that most polyphenols induce G1-phase arrest in human cancer cells whereas some polyphenols inhibit the cell cycle at either the G/S or G2/M phase in various human cancer cells (Chen et al., 2006; Lin et al., 2007; Milacic et al., 2008; Narasimhan and Ammanamanchi, 2008; Nautiyal et al., 2011).

To examine the association between curcumin and the cell cycle, we investigated the modulation of cell cycle regulators in human breast cancer cell lines exposed to high concentrations of curcumin (20 μM). Curcumin caused a remarkable decrease in the expression of cyclin D, cyclin E, CDK2, CDK4, and CDK6. The cell cycle is tightly mediated through a complex network of positive and negative cell-cycle regulatory molecules such as cyclin-dependent kinases (CDKs), CDK inhibitors (CKIs), and cyclins. G1-phase progression and G1/S-phase transition are regulated by CDK2 and CDK4, which assemble with cyclin E and cyclin D. The activated CDK-cyclin complexes are inactivated by binding to CDK inhibitory subunits (CKIs), of which $p19^{INK4}$, $p21^{Cip1}$ and $p27^{Kip1}$ have preferences for CDK4- and CDK2-cyclin complexes (Ahmad et al., 2000; Rafi et al., 2002; Kozikowski et al., 2003; Hakimuddin et al., 2006). Curcumin significantly up-regulated the expression of the CKI inhibitors $p19^{INK4}$, $p21^{Cip1}$ and $p27^{Kip1}$.

The down-regulation of CDK2 by curcumin might be the main cause of the G1 phase arrest. Together with the decrease in CDK2, cyclins E and cyclins D were decreased. CDK2 is a catalytic subunit of the G1-phase promoting factor, which is activated at the S transition (Deng et al., 1995; Sherr and Roberts, 1999). Several investigators have shown that CDK2 in combination with cyclins E is critical in the G1 phase transition (Takizawa and Morgan, 2000). It has been reported that although curcumin and catechin both of which are polyphenols, do not share the mechanism causing cell cycle arrest. Many polyphenols with similar structures have been shown to

target different sites and have different mechanisms in the inhibition of breast cancer cell growth (Porter and Donoghue, 2003).

Note that curcumin caused a significant accumulation of MCF-7 cells in the sub- G0 phase (apoptotic peak). As another measure of apoptosis, we determined the induction of apoptosis by quantifying its effects on caspase-3 and caspase-9 activity. MCF-7 cells were exposed to 20 μM curcumin for 72 h, and curcumin significantly increased the caspase-3 and caspase-9 activity in MCF-7 cells. Apoptosis is induced by multiple pathways, and caspase-9, an early biomarker of apoptosis, is a downstream target of cytochrome c released from mitochondria.

Based on reports that curcumin induces apoptosis directly without altering the cell cycle distribution or that curcumin does not inhibit cell proliferation, curcumin might have different effects depending on the cell type. We have shown that there are multiple pathways by which curcumin causes cell cycle arrest at the G1 phase in human breast cancer cells.

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