

Curcumin Induces Cell Cycle Arrest and Apoptosis in Human Osteosarcoma (HOS) Cells

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Abstract. *Background:* Curcumin is a major component of *Curcuma longa* rhizome and has been used as a traditional medicine for centuries. In this study, we showed that curcumin induced cell cycle arrest followed by antiproliferation and apoptosis in human osteosarcoma (HOS) cells. *Materials and Methods:* Antiproliferative activity was measured with the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Nuclear fragmentation was observed with a fluorescence microscope. Flow cytometry was performed to observe cell cycle distribution and apoptotic body appearance. Changes in cell cycle regulatory and apoptosis-related proteins were investigated by Western blot analysis. *Results:* The IC_{50} value of curcumin was ~ 4.0 $\mu\text{g/ml}$. Induction of apoptosis was evidenced by apoptotic body appearance and chromosomal DNA degradation. Flow-cytometric analysis indicated that curcumin induced successive G_1/S and G_2/M phase arrest followed by apoptosis in HOS cells. The G_1/S and G_2/S phase arrest was accompanied by down-regulation of cyclin *D1*, *cdc2* and cyclin *B1*, respectively. Apoptosis was induced by caspase-3 activation and poly(ADP-ribose)polymerase (PARP) cleavage. *Conclusion:* Our results demonstrated that curcumin caused death of HOS cells by blocking cells successively in G_1/S and G_2/M phases and activating the caspase-3 pathway.

Curcumin is a major phenolic component of turmeric *Curcuma longa* Linn (Zingiberaceae) and it has been used for many centuries as a food additives and a traditional medicine in Asian countries. Traditional use of curcumin includes most inflammatory conditions and other diseases (1-2). Recently, the beneficial effects of curcumin have been demonstrated including cancer preventative activity, anti-inflammatory and anticancer activity (3-6). Curcumin exerts its anti-inflammatory effect through inhibition of cyclooxygenase-2

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Key Words: Curcumin, antiproliferative, cell cycle arrest, apoptosis.

(COX-2), lipogenase (LOX), and inducible nitric oxygenase (iNOS) (7-8). Curcumin is capable of inhibiting carcinogen bioactivation through suppression of cytochrome enzymes (9-10) and thus has been suggested as a chemopreventative agent in several cancer models (11-12).

The antiproliferative effect of curcumin against various types of cancer, including gastrointestinal, head and neck, liver, lung and skin, have been reported (13). The molecular targets for curcumin are rather broad and include inflammatory cytokines, transcription factors, gene products related to cell invasion and others (13). The successful completion of phase I clinical trials of curcumin against colon and pancreatic cancer patients have lead to the phase II trials (14-15).

Antiproliferative and apoptosis-inducing mechanism of curcumin has been studied by many researchers and it has been shown that curcumin has multiple targets in the cell. Molecular targets of curcumin include transcription factors such as nuclear factor kappaB (NFkB) and transcription factor activator protein-1, protein kinases such as mitogen-activated protein kinase and anti-apoptotic proteins such as bcl-2 (13). Most studies regarding anticancer activity of curcumin have investigated its action against carcinomas and melanomas (16-20); we attempted to evaluate antiproliferative and apoptosis-inducing activities of curcumin against human osteosarcoma cell (HOS) cells.

Materials and Methods

Chemicals. Curcumin [diferuoylmethane; (E,E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] was purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were of the highest analytical grade and were purchased from common sources.

Cell culture and cytotoxicity test. Human osteogenic sarcoma cell line HOS (ATCC CRL-1543) was cultured in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics. Exponentially growing HOS cells were seeded at 5×10^4 cells/well in a 96-well plate and treated with 0-20 $\mu\text{g/ml}$ of curcumin or vehicle. After various periods of exposure, the general viability of cultured cells was determined by assaying the reduction of MTT to formazan (21). Experiments were performed at least in triplicate.

DNA fragmentation analysis. HOS cells were treated for 72 h with 0-8 $\mu\text{g/ml}$ of curcumin. Cells were then harvested and DNA fragmentation was analyzed as described elsewhere (22).

Nuclear morphology and flow cytometric analysis. HOS cells were treated with 10 $\mu\text{g/ml}$ of curcumin for 24 h. Morphology of cellular nuclei was recorded as described previously (23). For flow-cytometric analysis, cells were incubated with 10 $\mu\text{g/ml}$ of curcumin for 0-24 h or 0-10 $\mu\text{g/ml}$ of curcumin for 24 h, harvested for propidium iodide (PI) staining and used for flow-cytometric analysis as described previously (24).

Western blot analysis. HOS cells were treated with curcumin at 10 $\mu\text{g/ml}$ for 0-48 h or at 0-20 $\mu\text{g/ml}$ for 24 h and subjected to Western blot analysis, as described previously (24). Blots were probed with mouse monoclonal anti-human anti-caspase-3 (Transduction Laboratory, Lexington, KY, USA), and anti-PARP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. For cell cycle-related molecule analysis, anti-cyclin D1, anti-cdc2 p34 and anti-cyclin B1 (Santa Cruz Biotechnology) antibodies were used. Immunoreactivity was detected using either an anti-mouse (Santa Cruz Biotechnology) or anti-rabbit (Amersham Biosciences, Buckinghamshire, UK) peroxidase-conjugated secondary IgG antibody and an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences).

Results

Antiproliferative effect of curcumin on HOS cells. The antiproliferative effect of curcumin on a human osteosarcoma cell line, HOS was evaluated with the MTT assay. When cells were treated for 48 h with 0, 1, 3, 7, 10 and 20 $\mu\text{g/ml}$ of curcumin, the relative cell proliferation progressively decreased in a dose-dependent manner, as shown in Figure 1. The IC_{50} value for curcumin on HOS cells was approximately 4.0 $\mu\text{g/ml}$.

Induction of apoptosis by curcumin. To determine whether curcumin-mediated inhibition of growth and proliferation was associated with apoptosis, curcumin-induced chromosomal DNA degradation in HOS cells was examined. HOS cells were treated with 0, 0.5, 1, 2, 4 and 8 $\mu\text{g/ml}$ of curcumin for 72 h and chromosomal DNA was purified and analyzed by agarose-gel electrophoresis (Figure 2). Analysis of DNA extracted from curcumin-treated HOS cells revealed a progressive dose-dependent increase in chromosomal DNA degradation, which may have been associated with the degradation of chromosomal DNA at the linker regions characteristic of apoptotic cells.

In order to observe the nucleus fragmentation induced by curcumin treatment, which is an important hallmark of apoptosis, we used PI was used to stain nuclei of HOS cells treated with curcumin. As shown in Figure 3, control cells exhibited intact nuclei, but cells treated with curcumin showed significant nuclear fragmentation (Figure 3A and B). *Flow-cytometric analysis.* The induction of apoptotic bodies in curcumin-treated HOS cells was further analyzed by flow-cytometric determination of DNA content (Figure 4).

Histograms of DNA content obtained from PI-stained HOS cells showed that the percentage of cells with reduced DNA content progressively increased over time (Figure 4A). It was interesting to find the gradual accumulation of cells in G_1/S phase at earlier time points and successive accumulation in G_2/M phase at relatively later time points after treatment of curcumin. On longer incubation, curcumin-treated cells went into apoptosis. A similar observation was made regarding the concentration-dependent treatment of curcumin (Figure 4B). When cells were treated with an increasing amount of curcumin, cells accumulated at the G_1/S phase at a relatively lower concentration of curcumin and at the G_2/M phase at a relatively higher concentration of curcumin before the appearance of apoptotic death. The profile for the curcumin-induced increase in hypodiploid DNA content closely correlated with the chromosomal DNA degradation described above.

Effect of curcumin on cell cycle-related proteins. Since cell cycle arrest was observed in curcumin-treated HOS cells by flow-cytometric analysis, it was of interest to test the effect of curcumin on cell cycle regulatory molecules (Figure 5). The level of cyclin D1 decreased upon treatment with curcumin in a time- and dose-dependent manner. The level started to decrease at a relatively early time after curcumin treatment, *i.e.* at 3 h after treatment. The cyclin D1 level began to decrease at 5 $\mu\text{g/ml}$ of curcumin treatment.

The level of cdc2 was significantly reduced in cells treated with curcumin in a time- and dose-dependent manner. In addition, the level of cyclin B1, a regulator molecule of cdc2, decreased after curcumin treatment in time- and dose-dependent manner. However, the decrease of cyclin B1 began somewhat later and at a higher concentration of curcumin, *i.e.* 24-48 h and 10 $\mu\text{g/ml}$ of curcumin treatment. If we consider the findings above that the relative population of cells in G_1/S was increased at slightly earlier incubation time points and at a lower concentration of curcumin treatment compared to the appearance of G_2/M arrest, the results acquired from Western blot analysis correspond well with those of the flow cytometric analysis.

Effect of curcumin on apoptosis-related proteins. In order to investigate the mechanism by which curcumin induces apoptosis, the changes in the level of apoptosis-related proteins were examined. HOS cells were cultured in media containing 10 $\mu\text{g/ml}$ of curcumin for 0, 3, 7, 16, 24 and 48 h. At each time point, total protein was isolated and pro-caspase-3, and PARP immunoreactivity levels were measured by Western blotting (Figure 6). Pro-caspase-3 levels decreased on the treatment with curcumin, implying that the levels of active caspase-3 increased. To investigate the enzymatic activation of caspase-3, the cleavage of PARP, which is a caspase-3 substrate was measured. The formation of the 85 kDa fragment and a

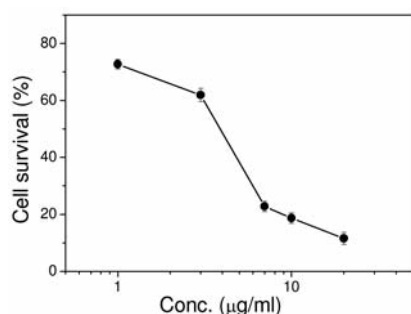


Figure 1. Antiproliferative activity of curcumin in HOS cells. HOS cells were incubated with 0, 3, 7, 10 and 20 µg/ml of curcumin for 48 h. Cell survival rate was measured by the MTT assay. Data are presented as means±SD. The percentage of cell growth in the control group was designated as 100%.

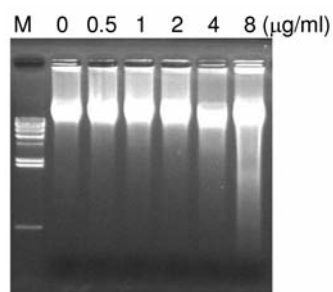


Figure 2. Curcumin induced chromosomal DNA degradation in HOS cells. Cells were treated with curcumin at concentrations of 0, 0.5, 1, 2, 4 and 8 µg/ml for 72 h. Chromosomal DNA was extracted and subjected to electrophoresis on 1.5% agarose gels followed by ethidium bromide staining.

decrease in the 116 kDa PARP were observed (Figure 6A). When cells were treated with 0, 2.5, 5, 10 and 20 µg/ml of curcumin, a dose-dependent decrease in pro-caspase-3 was observed, which again reflected an increase in active caspase-3 (Figure 6B). Accordingly, an increase in the cleavage of PARP to 85 kDa fragment was also apparent.

Discussion

Among many *Curcuma* species, the rhizomes of *C. longa* are used worldwide. The main components in *C. longa* and other *Curcuma* species are mainly curcumin, bis-demethoxycurcumin and demethoxycurcumin (25). Among these components, curcumin is the most studied (3, 5, 26). In order to increase its solubility thus to enhance its activity, curcumin conjugates with amino acids or poly(ethylene glycol), or curcumin analogs have been synthesized and assessed for activity (27-29). Derivatives of curcumin which contain higher efficacy have been developed for therapeutic application (30-31).

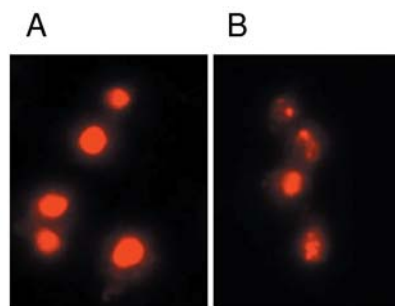


Figure 3. Appearance of apoptotic bodies by curcumin treatment. HOS cells were incubated in the absence (A) or presence of curcumin (10 µg/ml) for 24 h and stained with propidium iodide. Cellular morphological changes were observed using a fluorescence microscope at a magnification of ×600.

The mechanism of action of curcumin has been studied and it is known that curcumin has multiple targets (13). Curcumin interacts with a variety of proteins and modifies their expression and activity, and is thus a good candidate for preventive agents and/or therapeutic agents of cancer. Curcumin inhibited proliferation and induced apoptosis in a several cancer cell types including human ovarian, prostate, pancreatic, human colon, melanoma, and lung carcinoma (14, 18-20, 32-33). The suggested mechanism in these cancer cells was the inhibition of a transcription factor, NFκB, the inhibition of secondary signal molecules such as phosphatidylinositol 3-kinase, the decrease in the expression of anti-apoptotic protein Bcl-2, and activation of an executive enzyme, caspase-3.

In this study, we observed that curcumin inhibited the proliferation of human osteosarcoma (HOS) cells and induced apoptosis through activation of caspase-3. Prior to apoptosis induction, treatment of curcumin caused cell cycle arrest; cells were arrested at the G₁/S phase and successively at the G₂/M phase. Earlier studies reported that curcumin induced G₁/S phase arrest in mantle cell lymphoma, and prostate cancer cells (34-35). In other studies, G₂/M phase block was observed in other human cancer cells including melanoma, bladder cancer and lung carcinoma upon curcumin treatment (20, 33, 36). However, successive cell cycle arrest in G₁/S and then G₂/M phase in curcumin-treated cells was first observed in this study. To further analyze the molecular mechanism by which curcumin caused cell cycle arrest, we evaluated the changes in cell cycle regulatory protein levels. Cyclin-dependent kinases (CDK) are the key regulatory proteins that are activated at specific points of the cell cycle and remain stable during the cell cycle (37). The levels of the CDK-activating proteins, cyclins, change during the cell cycle (38). Cyclin D1 is one of the cyclins required for advance from the G₁ to the phase S. It seems that the reduction of cyclin D1 level is one of the

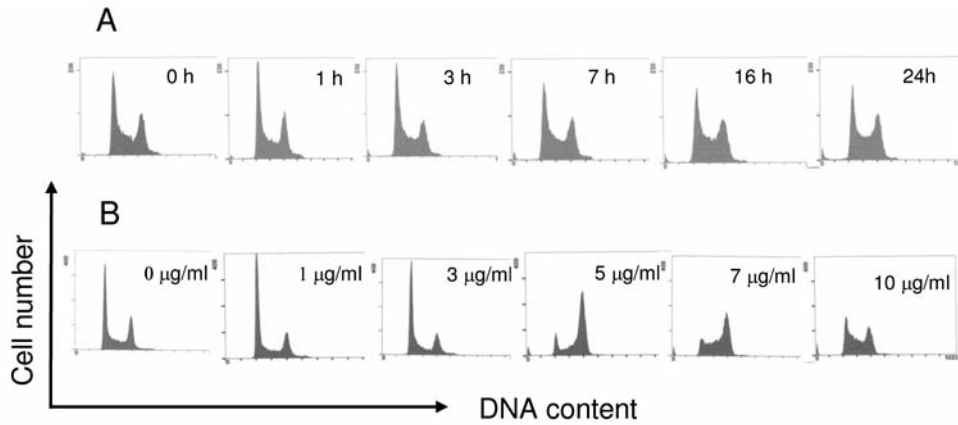


Figure 4. Flow cytometric analysis of curcumin-treated HOS cells. A, HOS cells were treated with curcumin at a concentration of 10 µg/ml and harvested at 0, 1, 3, 7, 16, and 24 h. B, Cells were treated with curcumin at concentrations of 0, 1, 3, 5, 7, and 10 µg/ml for 24 h. Propidium iodide-stained cells were analyzed for DNA content using flow cytometry. Representative histograms are shown.

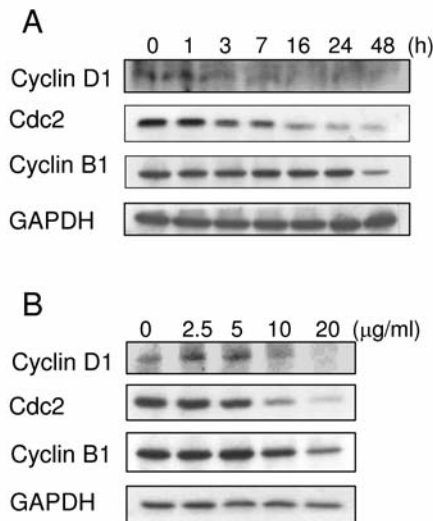


Figure 5. Changes in the expression of cell cycle-regulating proteins in response to treatment with curcumin. HOS cells were treated (A) with 10 µg/ml of curcumin for 0, 1, 3, 7, 16, 24 and 48 h or (B) with 0, 2.5, 5, 10 and 20 µg/ml of curcumin for 24 h. Cell extracts were subjected to Western blotting to determine immunoreactivity levels for cyclin D1, cdc2 and cyclin B1 as described in Materials and Methods. Representative Western blots are shown.

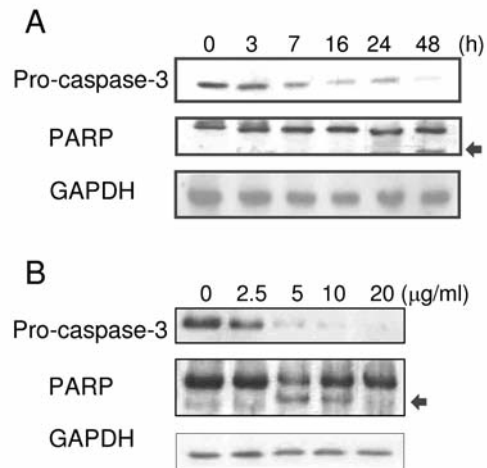


Figure 6. Changes in the expression of apoptosis-related proteins in response to treatment with curcumin. HOS cells were treated (A) with 10 µg/ml of curcumin for 0, 3, 7, 16, 24 and 48 h or (B) with 0, 2.5, 5, 10 and 20 µg/ml of curcumin for 24 h. Cell extracts were subjected to Western blotting to determine immunoreactivity levels for procaspase-3, and PARP as described in Materials and Methods. Arrowheads indicate cleaved PARP. Representative Western blots are shown.

main causes for the G₁/S block that appeared in curcumin-treated HOS cells. The cdc2/cyclin B complex is one of the major regulatory elements governing the G₂ to M progression (39). Cdc2 is activated by both phosphorylation and by binding to cyclin B, which is synthesized during the S and G₂ phases of the cell cycle. Thus, our data suggest that

cell cycle arrest at the G₂/M phase is mediated by reduction of cdc2/cyclin B complex formation, which is an essential step in regulating the cells passage into mitosis.

These results demonstrated that curcumin induced successive cell cycle arrest at G₁/S and G₂/M phase in human osteosarcoma (HOS) cells and through DNA

damage led to a decrease in the level of cell cycle regulatory molecules. Our data showed that the level of cyclin D1, which controls the G₁ to S advance, decreased at earlier time points and at a lower concentration of curcumin treatment. The levels of cdc2 and cyclin B1, G₂ to M entry regulators, decreased at a relatively later time point and at a higher concentration of curcumin treatment. Subsequent curcumin-induced apoptosis was observed through nuclear fragmentation and chromosomal DNA degradation and it involves a caspase-3-mediated mechanism. In conclusion, curcumin induced apoptotic cell death of human osteosarcoma (HOS) cells by blocking cells successively in G₁/S and G₂/M phases and activating the caspase-3 pathway.

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Received July 13, 2009

Revised November 11, 2009

Accepted November 12, 2009