Down-regulation of Cdc25c, CDK1 and Cyclin B1 and Up-regulation of Wee1 by Curcumin Promotes Human Colon Cancer Colo 205 Cell Entry into G2/M-phase of Cell Cycle

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Abstract. *Background: Curcumin (diferuloylmethane) exhibited potent inhibitory activities against proliferation and induced apoptosis in several tumor cell lines. It was recently reported that curcumin induced cell cycle arrest in several human cancer cell lines. However, the exact mechanisms are unclear. Materials and Methods: Flow cytometry was used to analyze the cell cycle in human colon cancer colo 205 cells treated with various concentrations of curcumin for 48 h. In order to further understand the mechanism of curcumin-induced G2/M arrest, the checkpoint associated with enzymes of the cell cycle were also investigated by Western blotting methods. Results: Curcumin induced G2/M arrest in the examined cells and these effects were dose- and time-dependent. Futhermore, curcumin induced Wee1 expression and decreased the Cdc25c, cyclin B1 and CDK1 expressions, resulting in the induction of G2/M cell cycle arrest in the colo 205 cells. The cDNA microarray assay was also employed to confirm gene expressions (mRNA Wee1, Cdc25c, cyclin B1 and CDK1). Conclusion: The results indicate that curcumin promoted the gene expression of Wee1 and inhibited that of Cdc25c, CDK1 and cyclin B1.*

It is well accepted that defective regulation of the cell cycle is one characteristic of cancer. Many cell types have modulated expression of cell cycle regulatory molecules responsible for cell cycle arrest or apoptosis (1-3), including cyclins and cyclin-dependent protein kinases (CDKs). Distinct pairs of cyclins and CDKs regulate progression through different stages of the cell cycle. CDKs are modulated by their periodic phosphorylation and by interaction with CDK inhibitors (4, 5). During the G1-phase, there is a progressive accumulation of cyclin E and expression above the threshold level leads the cell to enter the S-phase (6). Cyclin B1 was reported to be an essential cell cycle component required for the transition from G2- to M-phases (7-10). NF-κB regulates a number of genes involved in signaling different stress responses (11). It was reported that NF-κB blocked by overexpressed mutant forms of IκB reduces NF-κB translocation to the nucleus and decreases cell survival and cyclin B1 expression (12).

Curcumin (1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione), is a natural compound abundant in several *Curcuma* species. As a component of turmeric (*Curcuma longa*), it has been widely found in the human diet for centuries (13). Curcumin has antiproliferative and pro-apoptotic effects against diverse tumors *in vitro* (14-16). The cancer chemopreventive activities of curcumin, which inhibited tumorigenesis during both the initiation and promotion stages in several animal models (17, 18), may be due to the inhibition of cyclooxygenase and lipoxygenase (19) and by blocking the formation of arachidonic acid metabolites. In this study, the molecular mechanisms of curcumin-induced cell cycle arrest in the human colon cancer cell line colo 205 were investigated.

Materials and Methods

Chemicals and reagents. Curcumin, triton X-100, sodium deoxycholate, propidium iodide (PI), sodium orthovanadate, aprotinin, antipain, Tris-HCl, leupeptin, ribonuclease-A and trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium phosphates and TE buffer were purchased from Merck Co. (Darmstadt, Germany). RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA and glutamine were obtained from Gibco BRL (Grand Island, NY, USA).

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Key Words: Curcumin, cell cycle arrest, Cdc25c, cyclin B1, CDK1, Wee1.

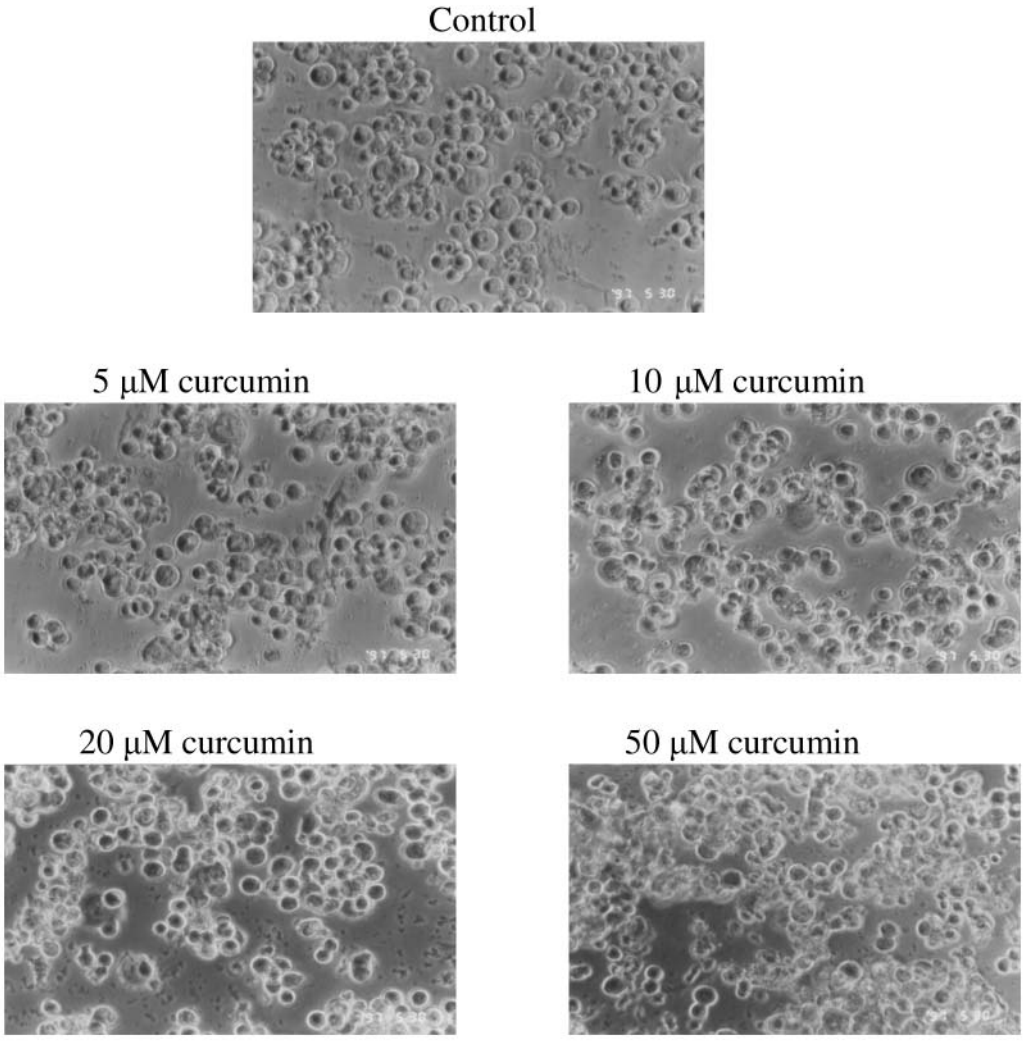

Figure 1. *Morphological changes of human colon cancer colo 205 cells in response to curcumin. The colo 205 cells were treated with varying concentrations of curcumin for 24 h, examined under contrast-phase microscope (200x) and photographed.*

Human colon cancer cell line. The human colon cancer cell line (colo 205: human colon adenocarcinoma) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were placed into 75-cm^3 tissue culture flasks and grown at 37˚C in humidified 5% CO_2 and 95% air atmosphere in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin and 1% glutamine. All data presented in this report are from at least 3 independent experiments.

Cell viability as determined by flow cytometry. The colo 205 cells were plated in 12-well plates at a density of $5x10^3$ cells/well and grown for 24 h. Various concentrations of curcumin (0, 5, 10, 20 and 50 μM) were added and the cells were grown for different periods of time. DMSO (solvent) was used for the control regimen. For cell viability determinations, the flow cytometric protocol was used as previously described (20).

Flow cytometry analysis of DNA content in colo 205 cells co-treated with different concentrations of curcumin. The percentage of cells in G0/G1-, S- and G2/M-phases were determined by flow cytometry as described previously (20). Briefly, $5x10^5$ colo 205 cells/well in 12-well plates were incubated with curcumin at 0, 5, 10, 20 and 50 μM concentrations for different time periods before the cells were harvested by centrifugation. After being harvested, the cells were washed with PBS, fixed gently (drop by drop) in 70% ethanol (in PBS) in ice overnight and then resuspended in PBS containing 40 μg/mL PI, 0.1 mg/mL RNase (Sigma) and 0.1% triton x-100. After 30 min at 37˚C in the dark, the cells were analyzed by a flow cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser at 488 nm. The cell cycle and apoptosis were then determined and analyzed (20). The average of the percentage of each phase in the cell cycle was representative of the 3 independent experiments.

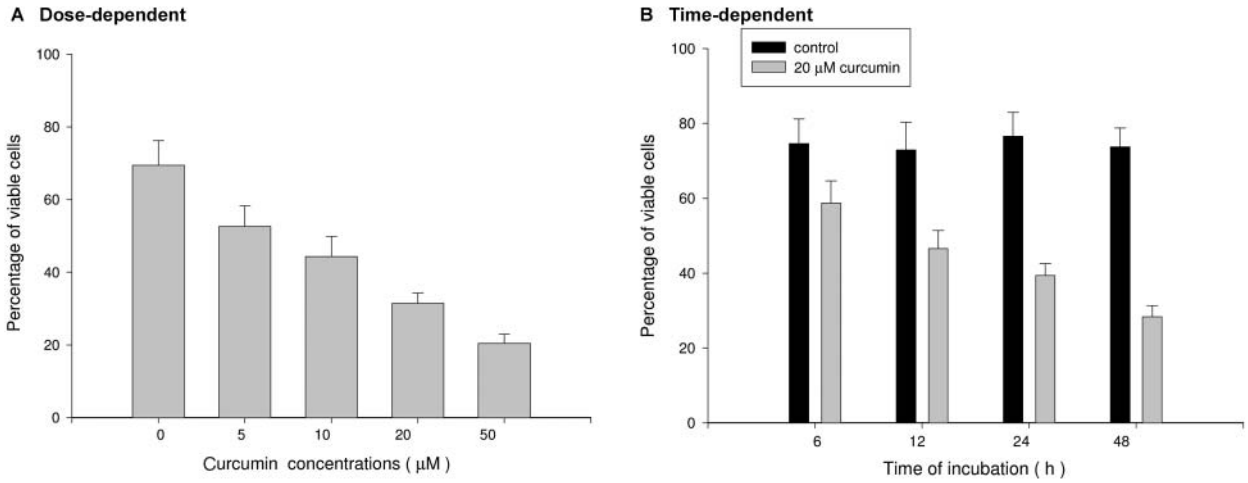

Figure 2. *The percentage of viable colo 205 cells after curcumin treatment and 24-h incubation. The colo 205 cells (2x10^5 cells/well; 12-well plates) were plated in RPMI 1640 medium + 10% FBS with different concentrations of curcumin for 24 h (panel A) or 20 μM curcumin for 6, 12, 24, and 48 h (panel B). Then, the cells were collected by centrifugation and the viable cells were determined by trypan blue exclusion and flow cytometry, as described in Materials and Methods. Each point is the mean±S.D. of 3 experiments. *P<0.05*

Protein preparation. Approximately 3x10^6 cells/well in 6-well plates were incubated with curcumin at 20 μM concentration for different time periods (6, 12, 24 h) before the cells were harvested by centrifugation. The protein was extracted as previously described (21). Briefly, the cell pellets were resuspended in modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet p-40, 0.25% sodium deoxycholate, 1mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 5 μg/ml antipain) for 30 min at 4˚C. The lysates were immediately centrifuged at 13,000 xg for 20 min at 4˚C and the supernatant was collected, aliquotted (50 μg/tube) and stored at –80˚C until assay. The protein concentrations were estimated by the Bradford method (22).

Western blot examination of the effect of curcumin on cyclin B1, CDK1, Cdc25c and Wee1 of colo 205 cells. All samples were separated by sodium dodecylsulfate polyacrylamide (SDS-PAGE) gel electrophoresis (10 and 13%) (Bio-Rad Life Science Products, Hercules, CA, USA) as described previously (21). The SDS-separated proteins were followed by equilibration in transfer buffer [50 mM Tris, pH 9.0, 40 mM glycine, 0.375% SDS, 20% methanol and electrotransferred to Immobilon-P Transfer Membrane (Millipore Corporation, Bedford, MA, USA)]. The blot was then blocked with a solution containing 5% non-fat dry milk in Tris-buffered saline [10 mM Tris, 150 mM NaCl, Sigma Chemical Co.] containing 0.05% Tween 20 for 1 h, washed and incubated with antibodies to cyclin B1, CDK1, Cdc25c, Wee1 and β-actin. (Upstate, Lake Placid, NY, USA) at 4˚C overnight. After incubating with anti-mouse peroxidase-conjugated antibody (Santa Cruz, CA, USA), the signal was visualized by enhanced chemiluminescence (ECL, Amerham Pharmacia Biotech). The detection of β-actin was used as an internal control in all the data for Western blotting (21).

Microarray hybridization. Colo 205 cells were treated with or without 20 μM curcumin and were incubated in an incubator as described above for 24 h before the total RNA was extracted with a Qiagen Rneasy Mini Kit. The cDNA was then synthesized from total RNA before labeling for microarray hybridization. The cDNA was labeled with fluorescence then hybridized to its complement on the chip and the resulting localized concentrations of fluorescent molecules were detected and quantitated, as previously described (Asia BioInnovations Corp., Taipei, Taiwan, ROC).

Statistical analysis. Values are presented as percentage ± S.D. of control. The Student's *t*-test was used to analyze the statistical significance between the curcumin-treated and control groups. A *p* value less than 0.05 was considered significant for all tests.

Results

Effects of various concentrations of curcumin on colo 205 cell morphology and viability. In the presence of curcumin (0-50 μM), the cells were photographed by a phase-light microscope and were then collected and PI stained and analyzed by flow cytometry. The results indicated that the cells were increasingly stained and morphologically changed as time and concentration increased, suggesting that curcumin induced morphological changes and death of the colo 205 cells (Figures 1 and 2A, B).

Curcumin induced cell cycle arrest in colo 205 cells. The flow cytometry results for the cell cycle analysis indicated that, during the 48-h time period, curcumin increased the percentage of cells in G2/M (enhanced G2/M peak) and decreased the percentage of cells in G0/G1. The control cells showed a typical pattern of DNA content that reflected the G0/G1-, S- and G2/M- phases of the cell cycle (Figure 3

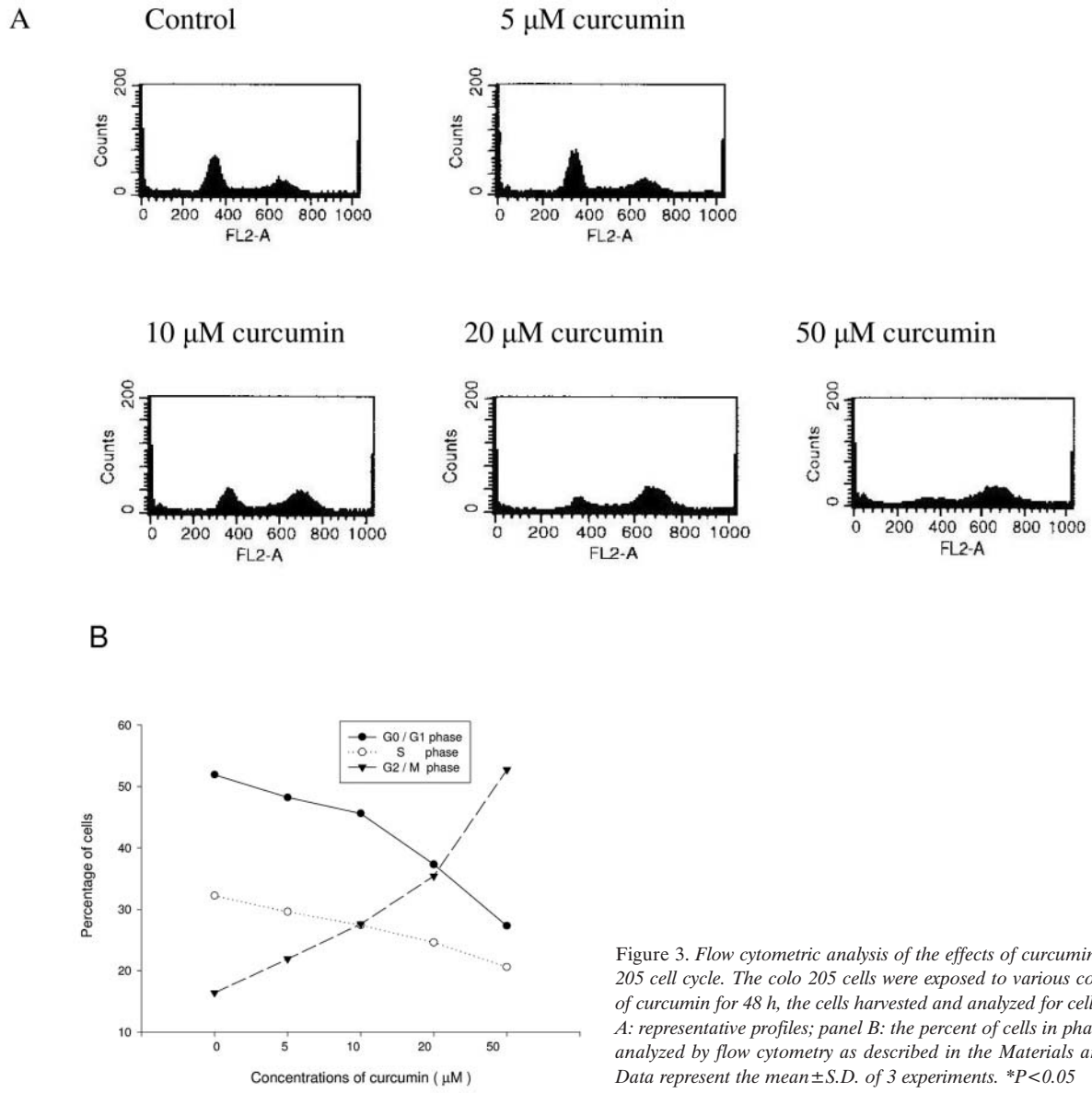

Figure 3. *Flow cytometric analysis of the effects of curcumin on the colo 205 cell cycle. The colo 205 cells were exposed to various concentrations of curcumin for 48 h, the cells harvested and analyzed for cell cycle (panel A: representative profiles; panel B: the percent of cells in phase) and were analyzed by flow cytometry as described in the Materials and Methods. Data represent the mean±S.D. of 3 experiments. *P<0.05*

A, B). An increase in curcumin concentration led to an increase of G2/M-phase in the cells.

Western blot examination of the effect of curcumin on CDK1, cyclin B1, Wee1 and Cdc25c of colo 205 cells. The results indicated that curcumin increased the expression of Wee1 (Figure 4D) but decreased those of cyclin B1 (Figure 4A), CDK1 (Figure 4B) and Cdc25c (Figure 4C).

Effects of curcumin on cyclin B1, Cdc25c, CDK1 and Wee1 mRNA expressions in intact colo 205 cells as examined by cDNA microarray. The results from the cDNA microarray assays are presented in Figure 5. The circle marked on the cDNA microarray of Figure 5A indicates that the Wee1 gene is up-regulated (red spot) and Cdc25c (Figure 5B), cyclin B1 (Figure 5C) and CDK1 (Figure 5D) genes are down-regulated (green spot) in the curcumin-treated colo 205 cells. Curcumin affected the corresponding gene expression of G2/M arrest.

Discussion

Although many studies have demonstrated that curcumin decreased the number of viable cells and induced cell cycle

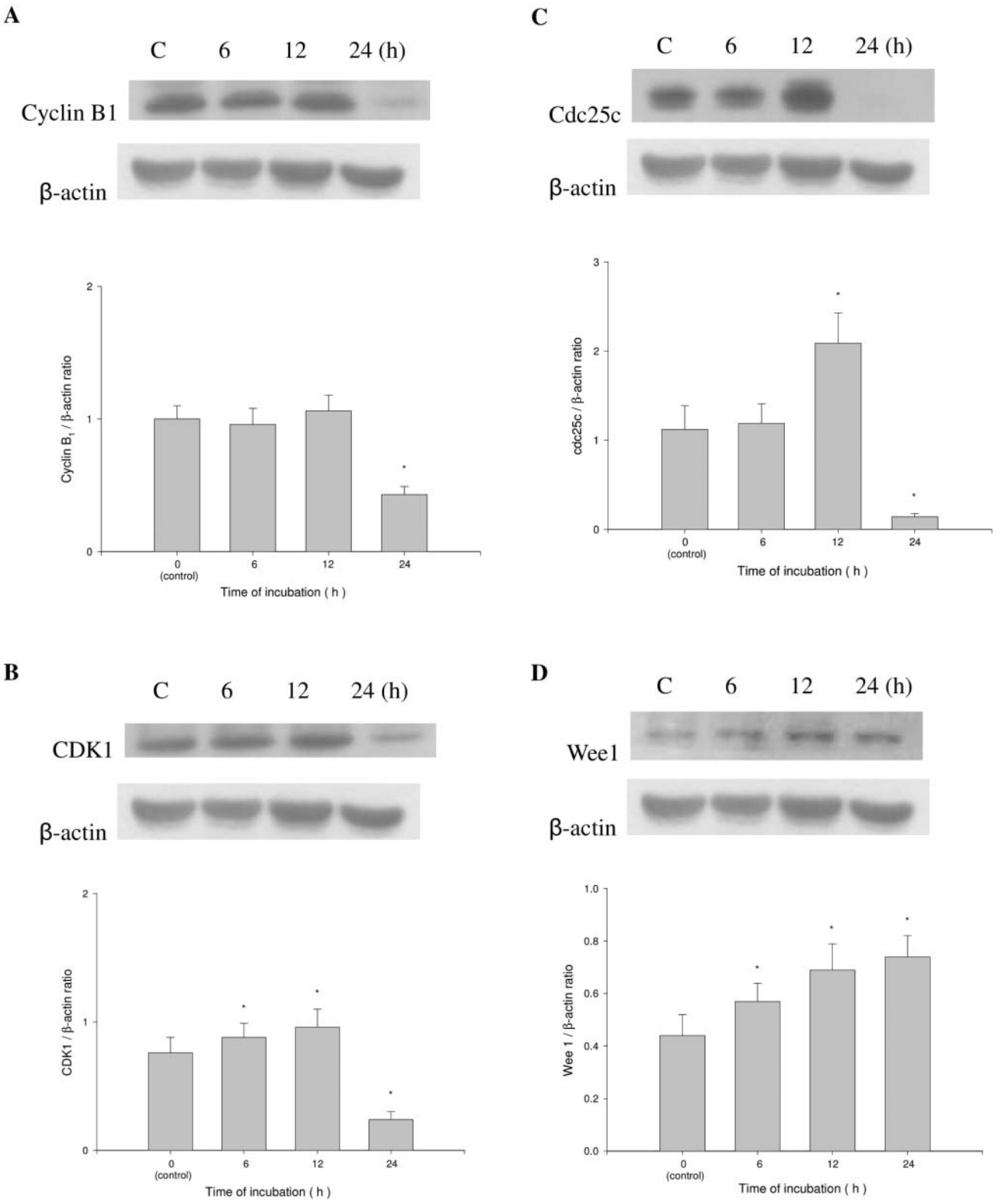

Figure 4. *Representative Western blot showing changes on the levels of CDK1, cyclin B1, Wee1 and Cdc25c, in colo 205 cells after exposure to curcumin. The cells (5x10^6/ml) were treated with 20 μM curcumin for 6, 12, and 24 h and the cytosolic fraction and total protein were prepared and determined as described in Materials and Methods. The evaluation of the levels of Wee1, Cdc25c, CDK1 and cyclin B1 expressions was estimated by Western blotting as described in Materials and Methods.*

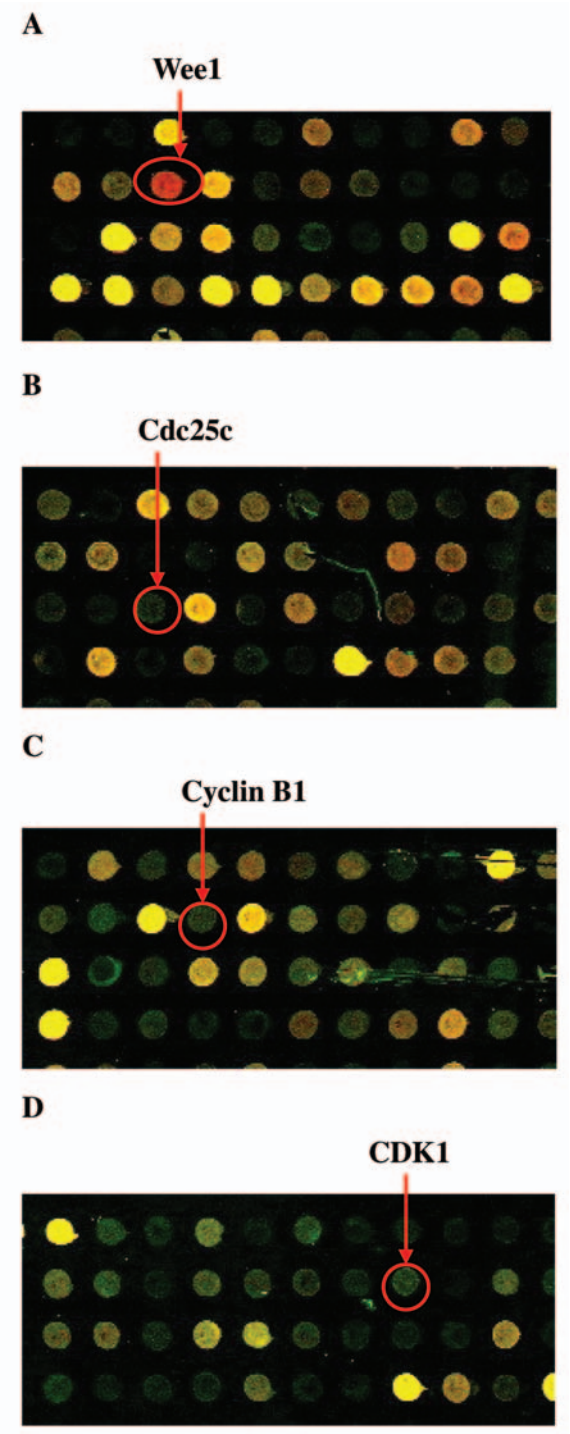

Figure 5. *Cyclin B1, CDK1, Cdc25c and Wee1 gene regulation in colo 205 cells treated with curcumin was assayed by cDNA microarray. The cells ($5x10^6$ cells/well, in 6-well plate) were treated with or without 20 μM curcumin for 24 h. The total RNA was extracted and prepared for cDNA, followed by cDNA hybridization and gene regulation on the chip. The red spot indicates up-regulation and green spot indicates down-regulation. The Wee1 gene was up-regulated (panel A), while the Cdc25c, cyclin B1 and CDK1 genes were down-regulated (panels B, C and D, respectively).*

arrest in many cell lines, the activity of curcumin is still controversial since some data showed that curcumin induced G0/G1 arrest in one cell line while other reports demonstrated that curcumin induced G2/M arrest in other cell lines. Therefore, in order to clarify the action of curcumin, the present studies were focused on the effects of curcumin on cell cycle arrest in a human colon cancer cell line. The choice of human colon cancer colo 205 cells for this study was based on 2 observations: a) that there are no other reports indicating that curcumin affects colo 205 cells and b) that we had cultured this cell line for long periods and had used it for other experiments. We previously showed that curcumin inhibited the gene expression and activity of *N*-acetyltransferase in this cell line (23).

Curcumin (5-50 μM) was found to be cytotoxic and caused G2/M arrest in colo 205 cells in a dose- and time-dependent manner, as examined by flow cytometric methods. This is in agreement with other reports that demonstrated that curcumin induced cytotoxicity and G2/M arrest in human mammary epithelial carcinoma cells (MCF-7, T47-D, MDA-MB-468 and MDA-MB-231) (24). Other investigators also demonstrated that curcumin inhibited cell proliferation and induced G2/M arrest in human colon cancer HCT-116 cells (25). Based on the Western blotting analyses, we also demonstrated that curcumin promoted the levels of Wee1 but decreased those of Cdc25c, CDK1 and cyclin B1. These effects were associated with G2/M arrest in the examined cells.

Cyclin B1 was reported to be an essential cell cycle component required for the transition from G2- to M-phase (1-4). Cyclin B1 and phosphorylated Cdc2 are able to accelerate cyclin B1/Cdc2 translocation into the nucleus and cell cycle regulation (5). Our data also show that curcumin inhibited the amounts of cyclin B1 protein, which may be one of the reasons behind the G2/M arrest in these cells. Other investigators demonstrated that the cyclin-dependent kinases, Cdk2 and Cdc2, changed markedly in response to curcumin (24). It was reported that the ability of Lovo cells (human colon carcinoma cell) treated with curcumin to form colonies was depressed and that curcumin was cytotoxic to the cells. Curcumin was added to Lovo cells that were largely accumulated in the S-, G2/M-phase and prevented the cells from entering the next cell cycle (26). In order to investigate whether or not curcumin affected the gene expressions of Wee1, Cdc25c, cyclin B and CDK1, associated with the cell cycle, especially the G2/M-phase, we used the cDNA microarray assay to examine those expressions in colo 205 cells after treatment with 20 μM curcumin for 24 h. Our results demonstrated that curcumin induced the up-regulation of Wee1 gene expression and the down-regulation of Cdc25c, cyclin B1 and CDK1 expressions in colo 205 cells.

Acknowledgements

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References

1 Farhana L, Dawson M, Rishi AK, Zhang Y, Van Buren E, Trivedi C, Reichert U, Fang G, Kirschner MW and Fontana JA: Cyclin B and E2F-1 expression in prostate carcinoma cells treated with the novel retinoid CD437 are regulated by the ubiquitin-mediated pathway. Cancer Res *62*: 3842-3849, 2002.
2 Freya W, Bora A, Antje C and Jurgen S: Down-regulation of the cyclin D1/cdk4 complex occurs during resveratrol-induced cell cycle arrest in colon cancer cell lines. J Nutr *131*: 2197-2203, 2001.
3 Joe AK, Liu H, Suzui M, Vural M, Xiao D and Weinstein B: Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. Clin Cancer Res *8*: 893-903, 2002.
4 Hartwell LH and Kastan MB: Cell cycle control and cancer. Science *266*: 1821-1823, 1994.
5 Sherr CJ: Cancer cell cycle. Science *274*: 1672-1677, 1996.
6 Gong J, Traganos F and Darzynkiewicz Z: Threshold expression of cyclin E but not D type cyclins characterizes normal and tumor cells entering S phase. Cell Prolif *28*: 337-346, 1995.
7 Muschel RJ, Zhang HB, Iliakis G and McKenna WG: Cyclin B expression in HeLa cells during the G2 block induced by ionizing radiation. Cancer Res *51*: 5113-5137, 1991.
8 Metting NF and Little JB: Transient failure to dephosphorylate the cdc2-cyclin B1 complex accompanies tradition-induced G2-phase arrest in HeLa cells. Radiat Res *143*: 286-292, 1995.
9 Kao GD, McKenna WG, Maity A, Blank K and Muschel RJ: Cyclin B1 availability is a rate-limiting component of the radiation-induced G2 delay in HeLa cells. Cancer Res *57*: 753-758, 1997.
10 Azzam EI, de Toledo SM, Gooding T and Little JB: Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluency of alpha particles. Radiat Res *150*: 497-504, 1998.
11 Baeuerle PA and Baltimore D: NF-kappa B: ten years after. Cell 87: 13-20, 1996.
12 Guo G, Yan-Sanders Y, Lyn-Cook BD, Wang T, Tamae D, Ogi J, Khaletskiy A, Li Z and Li JJ: Manganese super oxide dismutase-mediated gene expression in radiation-induced adaptive responses. Mol Cell Biol *23*: 2362-2378, 2003.
13 Ammon HP and Wahl MA: Pharmacology of *Curcuma longa*. Plant Med *57*: 1-7, 1991.
14 Kuo ML, Huang TS and Lin JK: Curcumin, an antioxidant and anti-tumor promoter, induces apoptosis in human leukemia cells. Biochem Biophys Acta *1317*: 95-100, 1996.
15 Chen H, Zhang ZS, Zhang YL and Zhou DY: Curcumin inhibits cell proliferation by interfering with the cell cycle and inducing apoptosis in colon carcinoma cells. Anticancer Res *19*: 3675-3680, 1999.
16 Mehta K, Pantazis P, McQueen T and Aggarwal BB: Antiproliferative effect of curcumin (diferuloylmethane) against human breast tumor cell lines. Anticancer Drugs *8*: 470-481, 1997.
17 Huang M-T, Ma W, Lu Y-P, Chang RL, Fisher C, Manchand PS, Newmark HL and Conney AH: Effects of curcumin, demethoxycurcumin, bisdemethoxycurcumin and tetrahydrocurcumin on 12-O-tetradecanoylphorbol-13-acetate-induced tumor promotion. Carcinogenesis *16*: 2493-2497, 1995.
18 Rao CV, Rivenson A, Simi B and Reddy BS: Chemoprevention by dietary curcumin, a naturally occurring plant phenolic compound. Cancer Res *55*: 259-266, 1995.
19 Huang M-T, Lysz T, Ferraro T, Abidi TF, Laskin JD and Conney AH: Inhibitory effects of curcumin on *in vitro* lipoxygenase and cyclooxygenase activities in mouse epidermis. Cancer Res *51*: 813-819, 1991.
20 Li TM, Chen GW, Su CC, Lin JG, Yeh CC, Cheng KC and Chung JG: Ellagic acid induced p53/p21 expression, G1 arrest and apoptosis in human bladder cancer T24 cells. Anticancer Res *25*: 971-979, 2005.
21 Chen HC, Hsieh WT, Chang WC and Chung JG: Aloe-emodin induced *in vitro* G2/M arrest of cell cycle in human promyelocytic leukemia HL-60 cells. Food Chem Toxicol *42*: 1251-1257, 2004.
22 Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem *72*: 248-254, 1976.
23 Chen JC, Hwang JM, Chen GW, Hsia TC and Chung JG: Curcumin decreases the DNA adduct formation, arylamines N-acetyltransferase activity and gene expression in human colon tumor cells (colo 205). In Vivo *17*: 301-309, 2003.
24 Tathagata Choudhuri, Suman Pal, Tanya Das and Gaurisankar Sa: Curcumin selectively induces apoptosis in deregulated cyclin D1 expressed cells at G2 phase of cell cycle in a p53-dependent manner. J Biol Chem *280*: 20059-20068, 2005.
25 Moragoda L, Jaszewski R and Majumdar AP: Curcumin induced modulation of cell cycle and apoptosis in gastric and colon cancer cells. Anticancer Res *21*: 873-878, 2001.
26 Chen H, Zhang ZS, Zhang YL and Zhou DY: Curcumin inhibits cell proliferation by interfering with the cell cycle and inducing apoptosis in colon carcinoma cells. Anticancer Res *19*: 3675-3680, 1999.

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