

Curcumin Inhibits Cell Migration of Human Colon Cancer Colo 205 Cells through the Inhibition of Nuclear Factor kappa B /p65 and Down-regulates Cyclooxygenase-2 and Matrix Metalloproteinase-2 Expressions

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Abstract. Curcumin (diferuloylmethane) is a chemical derived from several *Curcuma* species (turmeric), possessing anti-inflammatory and antioxidant properties and which, thus, may be a potential anticancer drug. However, its mechanism of action is not fully understood. Our previous studies had shown that curcumin induced cytotoxicity, cell cycle arrest and apoptosis in human colon cancer colo 205 cells. In this study, curcumin affected the levels of NF- κ B/ p65 in a time-dependent manner but did not affect NF- κ B/ p50, based on Western blotting methods. *In vitro* experiments revealed that curcumin inhibited Cox-2 levels, but promoted those of Cox-1 in colo 205 cells. Curcumin also inhibited MMP-2 levels and promoted MMP-9 levels, but did not affect MMP-7 levels, based on Western blotting assays. These effects were also confirmed by cDNA microarray. Remarkably, curcumin not only exerted its effect on the protein levels of NF- κ B, Cox-1 and -2, MMP-2 and -7, but also directly inhibited their mRNA levels. Curcumin was also found to significantly repress the *in vitro* invasion of colo 205 cells.

Colorectal cancer is a major health concern worldwide with approximately 17.42 persons per 100 thousand dying, while in Taiwan, 32.9 persons per 100 thousand die annually based on reports from the "People's Health Bureau of Taiwan". The standard therapies for patients in Taiwan include surgery, chemotherapy and radiotherapy. Over the past

40 years, 5-fluorouracil-based therapies have been routinely used first-line chemotherapy regimens for metastatic colorectal cancer (1). New agents acting on novel targets of colon cancer are currently under development. Bevacizumab combined with fluorouracil-based chemotherapy has now become the standard of care for the first-line treatment of metastatic colorectal cancer (2).

Approximately 15% of all colon cancer patients have genetic syndromes (familial adenomatous polyposis and hereditary nonpolyposis) that increase the risk of colon cancer (3). Adenomatous polyps and subsequent carcinoma then lead to the development of sequential genetic alterations (4). The genes which are deleted in colon cancer include p53 (5) and matrix metalloproteinases (MMPs) (6). MMPs generally function to degrade proteoglycans and matrix glycoproteins. The unregulated degradation of the extracellular matrix, which occurs in the process of carcinogenesis, may lead to an advantage for the cancer cell. It was reported that the loss of basement membrane integrity may correlate with an increased probability of distant metastasis and poor prognosis (7). The overexpression of MMPs may be one part of the multi-step process that leads to neoplastic cell proliferation and metastasis. To date, 3 MMPs (MMP-2, -7 and -9) have been most associated with colorectal adenomas and carcinoma. The overexpression of MMP-2 was reported in colorectal cancer (8). MMP-7 was found at higher levels in colorectal adenomas compared with normal mucosa (6), as well as in mouse models of intestinal neoplastic cells (9). It was reported that increased levels of MMP-9 mRNA in colorectal cancer compared with normal mucosa were associated with significantly shorter disease-free and overall survival (10).

Curcumin (diferuloylmethane) is a natural polyphenol derived from several *Curcuma* species, commonly known as

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turmeric, and has been widely used in the human diet for centuries (11). It was reported that curcumin induces antiproliferative and pro-apoptotic effects against many tumors *in vitro* (12-14). It was also demonstrated that curcumin exerts its anticancer, antioxidant and anti-inflammatory effects *via* the down-regulation of the transcription factors NF- κ B, AP-1 and Egr-1 (15) and through the repression of the genes for cell adhesion molecules (chemokines, TNF, Cox-2 and MMP-9) (16, 17). Animal studies have shown that the cancer chemopreventive activities of curcumin *via* the inhibition of tumorigenesis, during both the initiation and promotion stages (18, 19), may be carried out by inhibition of cyclooxygenase and lipoxygenase (20). In this study, the roles of Cox-1 and -2 and MMPs on the cell migration of the human colon cancer cell line, colo 205, were investigated.

Materials and Methods

Chemicals and reagents. Curcumin, aprotinin, antipain, sodium deoxycholate, leupeptin, propidium iodide (PI), sodium orthovanadate, triton X-100, Tris-HCl, 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).

Human colon cancer cell line (colo 205). The colo 205 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were placed into 75-cm³ tissue culture flasks and grown at 37°C in a humidified 5% CO₂ and 95% air atmosphere in RPMI 1640 medium, containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% glutamine.

The effects of curcumin on cell viability as determined by flow cytometry. The colo 205 cells were plated in 12-well plates at a density of 5x10³ cells/well and grown for 24 h. Curcumin (50 µM) was added and the cells were grown for 6, 12 and 24 h. DMSO (solvent) was used for the control regimen. To determine cell viability, the flow cytometric protocol was used, as previously described (21).

Protein preparation of colo 205 cells after treatment with curcumin. Approximately 3x10⁶ cells/well in 6-well plates were incubated with 50 µM curcumin for 6, 12 and 24 h before the cells were harvested by centrifugation. Protein was extracted as previously described (22).

Western blotting analysis of the effect of curcumin on NF- κ B, Cox-1 and -2, MMP-2, -7 and -9 in 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 (20, 21). Briefly, 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

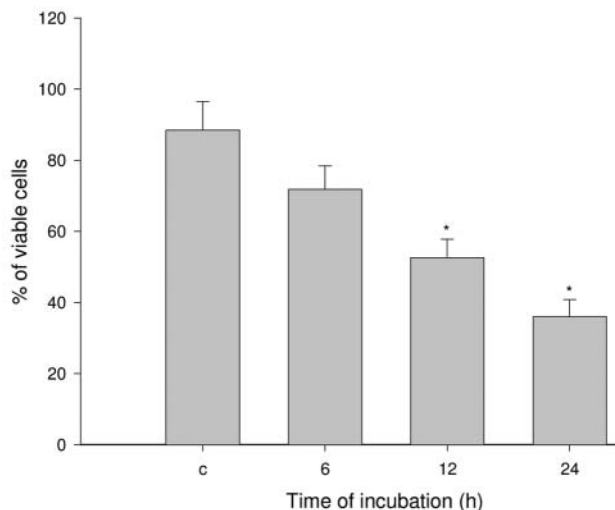


Figure 1. The percentage of viable colo 205 cells after curcumin treatment for various time-periods. The colo 205 cells (2×10^5 cells/well; 12-well plates) were plated in RPMI 1640 medium + 10% FBS with 50 µM curcumin for 6, 12 and 24 h. Then the cells were collected by centrifugation and the viable cells were determined by trypan blue exclusion and flow cytometry, as described in Materials and Methods. Each point is mean \pm S.D. of x3 experiments. * $P < 0.05$

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 NF- κ B, Cox-1 and -2, MMP-2, -7 and -9 (Upstate, Lake Placid, NY, USA) at 4°C overnight. After incubation 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, 22).

Gene expression of colo 205 cells as examined by cDNA microarray hybridization. The human colon cancer colo 205 cells were treated with or without 50 µM curcumin and were incubated in an incubator. cDNA microarray evaluations were then made, as described previously (21).

The effects of curcumin on colo 205 cell migration. The chemotactic migration of colo 205 cells was assayed using a modified Boyden chamber (23). Briefly, the cells were suspended in serum-free RPMI 1640 and were then added to the upper wells (48-multiwell Boyden micro chambers) at 2×10^4 cells per well in the presence or absence of curcumin, with fibronectin (10 mg/mL) present in the lower chamber. Migrating cells will traverse a polycarbonate filter from the upper chamber to the lower chamber. After 24 h at 37°C in 5% CO₂, the cells that had traversed and spread over the lower membrane surface were fixed with methanol and stained with Giemsa stain (Sigma). Utilizing a microscope with a 40x objective, the number of migratory cells per membrane was evaluated. Each experiment was performed in triplicate and migration was expressed as the mean \pm SD of total cells counted per field (24).

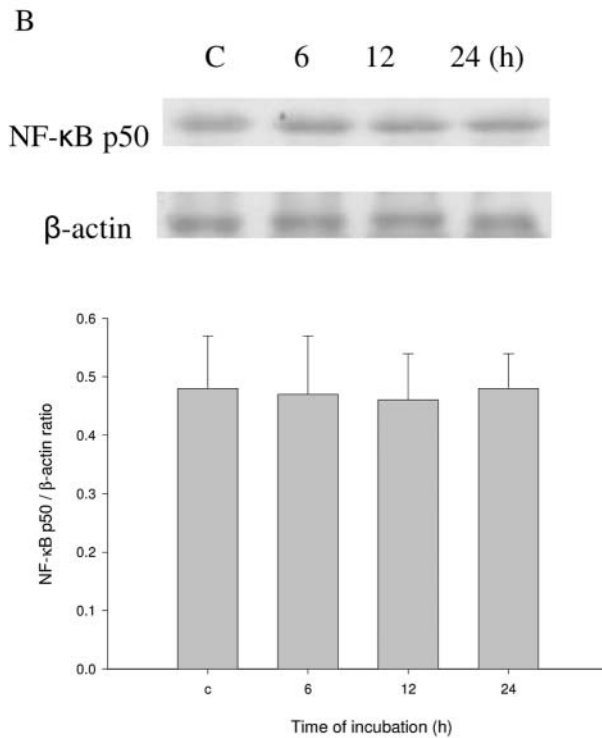
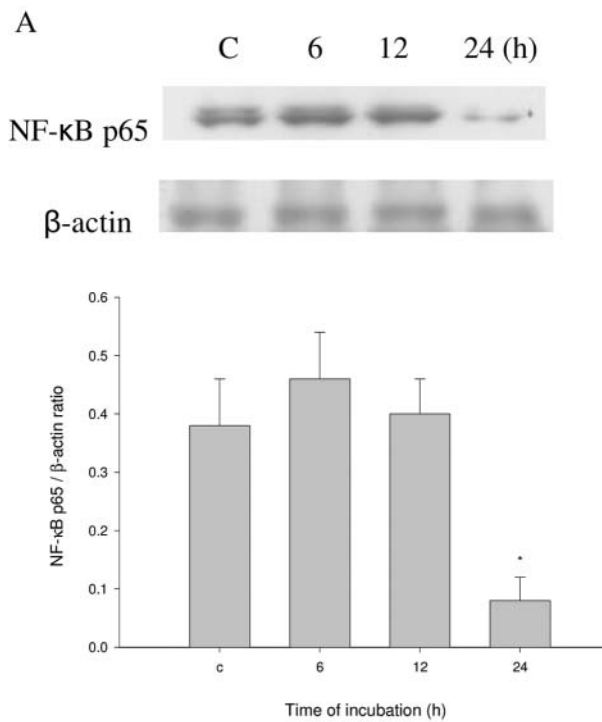


Figure 2. Representative Western blots showing the changes in NF-κB levels in colo 205 cells after exposure to curcumin. The cells ($5 \times 10^6/ml$) were treated with $50 \mu M$ curcumin for 6, 12 and 24 h. The cytosolic fraction and total protein were prepared and determined as described in Materials and Methods. The evaluation of the levels of NF-κB p65 (panel A) and NF-κB p50 (panel B) expressions were estimated by Western blotting, as described in Materials and Methods.

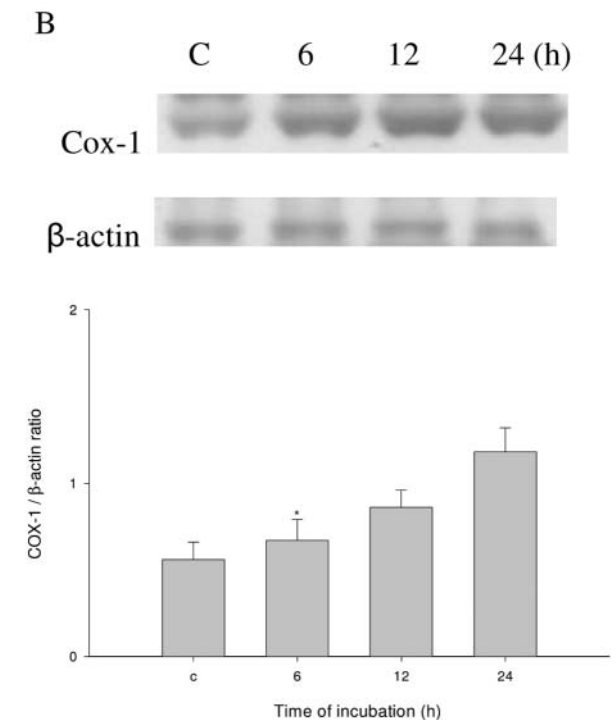
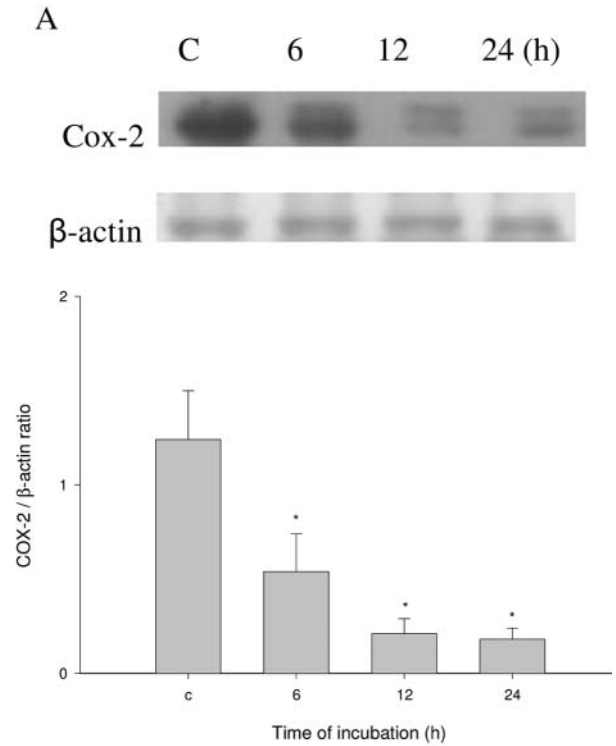


Figure 3. Representative Western blots showing the changes in Cox-1 and Cox-2 levels in colo 205 cells after exposure to curcumin. The cells ($5 \times 10^6/ml$) were treated with $50 \mu M$ curcumin for 6, 12 and 24 h. The cytosolic fraction and total protein were prepared and determined as described in Materials and Methods. The levels of Cox-2 (panel A) and Cox-1 (panel B) expressions were estimated by Western blotting as described in Materials and Methods.

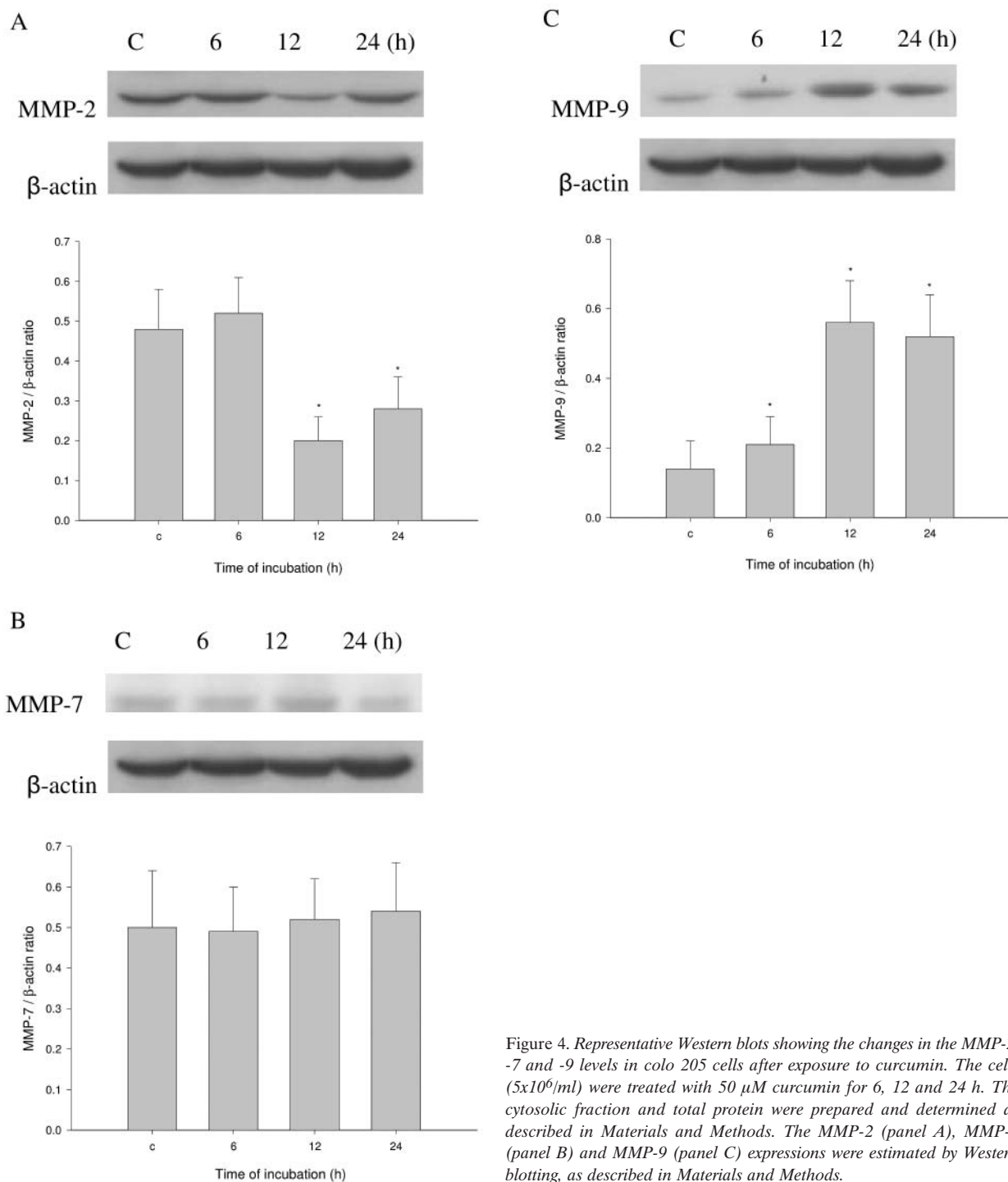


Figure 4. Representative Western blots showing the changes in the MMP-2, -7 and -9 levels in colo 205 cells after exposure to curcumin. The cells ($5 \times 10^6/ml$) were treated with $50 \mu M$ curcumin for 6, 12 and 24 h. The cytosolic fraction and total protein were prepared and determined as described in Materials and Methods. The MMP-2 (panel A), MMP-7 (panel B) and MMP-9 (panel C) expressions were estimated by Western blotting, as described in Materials and Methods.

Statistical analysis. The values are presented as the percentage \pm S.D. of the 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

The effects of curcumin on viability as determined by flow cytometry. In the presence of $50 \mu M$ curcumin, the cells were collected, propidium iodide (PI) stained and analyzed by

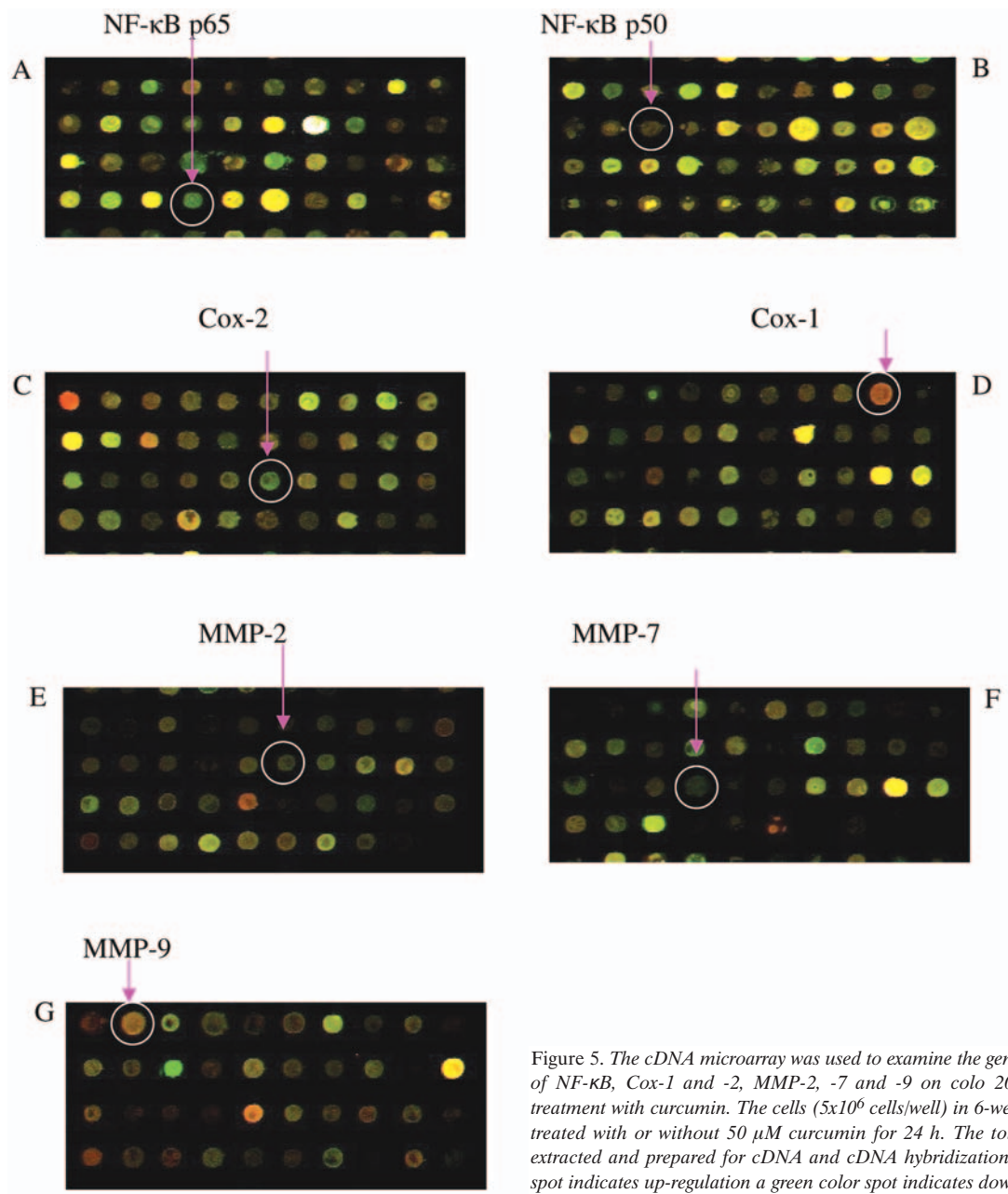


Figure 5. The cDNA microarray was used to examine the gene expressions of NF-κB, Cox-1 and -2, MMP-2, -7 and -9 on colo 205 cells after treatment with curcumin. The cells (5×10^6 cells/well) in 6-well plates were treated with or without 50 μ M curcumin for 24 h. The total RNA was extracted and prepared for cDNA and cDNA hybridization. A red color spot indicates up-regulation a green color spot indicates down-regulation.

flow cytometry. The results indicated that the cells became increasingly PI-stained as time increased, suggesting that curcumin induced colo 205 cell death (Figure 1).

Western blot analysis of the effect of curcumin on NF-κB, Cox-1 and -2, MMP-2, -7 and -9 in colo 205 cells. In the presence of curcumin, Western blotting examinations indicated that curcumin inhibited the levels of NF-κB p65 (Figure 2A), but not those of NF-κB p50 (Figure 2B). In addition, curcumin decreased the levels of Cox-2 (Figure 3A) and increased

those of Cox-1 (Figure 3B). Moreover, curcumin decreased the levels of MMP-2 (Figure 4A), did not affect the levels of MMP-7 (Figure 4B) and increased the levels of MMP-9 (Figure 4C).

Effects of curcumin on NF-κB, Cox-1 and -2, MMP-2, -7 and -9 mRNA expressions in intact colo 205 cells as examined by cDNA microarray. The results of the cDNA microarray are presented in Figure 5. The circle marked in Figure 5A indicates down-regulation of the NF-κB p65 gene (green

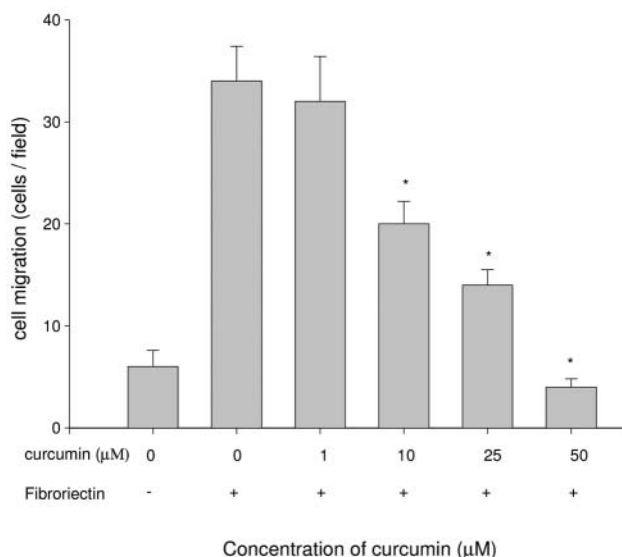


Figure 6. Cell invasion assay in colo 205 cells after treatment with curcumin for 24 h. The cells were treated with 0, 1, 10, 25 and 50 µM curcumin for 24 h and then their cell invasion through the Matrigel basement membrane was analyzed with a modified Boyden chamber method, as described in Materials and Methods. Mean cell counts from at least 10 fields and data represent means ± SD of at least 3 independent experiments.

color spot). Neither NF-κB p50 (panel B, black color spot) nor MMP-7 (panel F, black spot) were affected, while Cox-2 (panel C, green spot) was down-regulated. The MMP-2 gene (panel E: green spot) was down-regulated, and the Cox-1 (panel D: orange spot) and MMP-9 genes were up-regulated (panel G: orange spot) in the curcumin-treated colo 205 cells.

The effects of curcumin on cell migration of colo 205 cells. The effect of curcumin on the invasion of the colo 205 cells through Matrigel was examined. As shown in Figure 6, the treatments with 0, 1, 10, 25 and 50 µM curcumin for 24 h inhibited cell invasiveness in a dose-dependent manner. The strong inhibitory effect of curcumin on *in vitro* invasion appeared to be related to its inhibition of MMP gene expression (Figure 5).

Discussion

Our results demonstrated that curcumin decreased the percentage of viable cells in a time-dependent manner. Western blotting methods were used to show that curcumin affected the levels of NF-κB, decreasing the levels of NF-κB p65, but not affecting the levels of NF-κB p50 in human colon cancer colo 205 cells (Figure 7). This is in agreement with another report, which demonstrated that curcumin inhibited interferon-α-induced NF-κB expression in human lung cancer A549 cells (25). These investigators also suggested that curcumin suppressed the IFN-α-

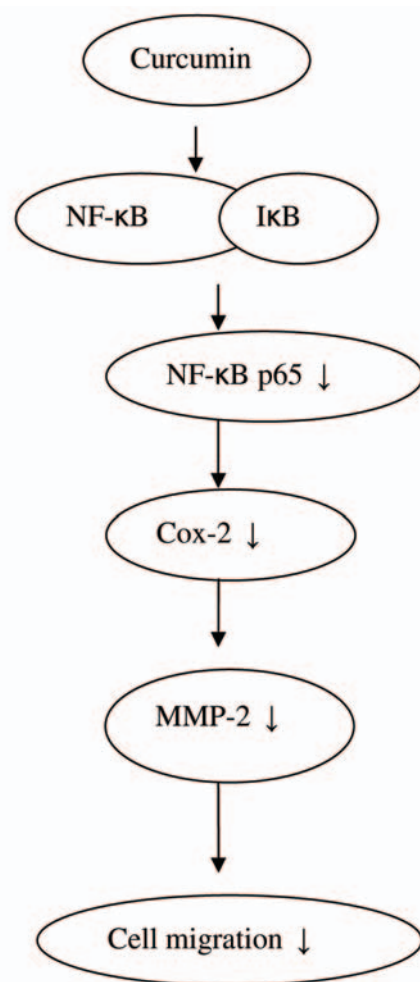


Figure 7. The proposed signal pathways of curcumin-induced NF-κB, Cox-2, MMP-2 in human colon cancer colo 205 cells.

dependent NF-κB activation in their experiment. They speculated that curcumin may potentiate the antitumor effects of IFN-α by selectively targeting the NF-κB pathway (25). NF-κB lies dormant in the cytoplasm through the binding of IκB inhibitory proteins. After the stimulating cytokines had promoted the dissociation of inactive NF-κB/IκB complexes, NF-κB entered the nucleus and bound *cis*-acting κB sites in the promoters and enhancers of key cellular genes (25). It is well known that NF-κB is a complex that contains NF-κB p50 and NF-κB p65. These effects were also confirmed by cDNA microarray, which indicated that curcumin inhibited NF-κB p65 gene expression but it did not affect NF-κB p50 gene expression in the examined cells.

Our results also demonstrated that curcumin inhibited Cox-2 levels and promoted Cox-1 levels, as determined by Western blotting methods and confirmed by cDNA microarray. Our observations are in agreement with the

results of other investigators who reported that curcumin inhibited the IFN- α -dependent activation of NF- κ B, subsequently leading to the down-regulation of Cox-2 expression (25, 26).

It is well known that the abnormal expression of MMPs plays an important role in the invasion of malignant colon cancer cells into the surrounding and distant normal tissue, causing problems with chemotherapy. In brain tumor cells, curcumin was found to significantly repress the *in vitro* invasion of glioma cells. Thus, the broad-spectrum inhibition of MMP gene expression by curcumin might provide a novel therapeutic strategy for treating glioma (27). It is also well known that MMP activity is regulated by several mechanisms, such as gene transcription, pro-enzyme activation and inhibition by various inhibitors (TIMPs) (28, 29). The inhibition of MMP transcription may be a useful strategy for controlling MMP activity, even in early tumor stages. Our results demonstrated that curcumin inhibited MMP-2 expression, did not affect MMP-7 but promoted MMP-9 expression, effects which were confirmed by cDNA microarray. Although it was demonstrated that curcumin affected many genes of apoptosis in human breast cancer cells (MCF-7) (30), there are no reports about whether curcumin affects MMP-2 gene expression in MCF-7 cells. Our findings were the first to show that curcumin inhibited MMP-2 gene expression, as examined by cDNA microarray.

Heslin *et al.* (31) demonstrated that the overexpression of MMP-7 was an early event in adenomas but not in carcinomas. MMP-2 and MMP-9 appeared to be primarily overexpressed in carcinomas. Based on these observations, there may be one mechanism by which adenoma cells gain the ability to invade and carcinoma cells to metastasize. Recently, a phase I clinical trial of oral curcumin (32) in colon cancer patients showed the high tolerability of the drug, suggesting the possibility of developing curcumin as an oral cancer preventive or therapeutic agent.

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