Curcumin induces apoptosis and inhibits prostaglandin E_2 production in synovial fibroblasts of patients with rheumatoid arthritis

CHEOL PARK[1,2*], DONG-OH MOON[3*], IL-WHAN CHOI[4], BYUNG TAE CHOI[5], TAEK-JEONG NAM[6], CHUNG-HO RHU[7], TAEG KYU KWON[8], WON HO LEE[2], GI-YOUNG KIM[3*] and YUNG HYUN CHOI[1,6*]

[1]Department of Biochemistry, Dongeui University College of Oriental Medicine, Busan 614-052; [2]Department of Biology, Pusan National University, Busan 609-735; [3]Faculty of Applied Marine Science, Cheju National University, Jeju 690-756; [4]Department of Microbiology, Inje University College of Medicine, Pusan 614-735; [5]Department of Anatomy, Dongeui University College of Oriental Medicine; [5]Faculty of Food Science and Biotechnology, Pukyong National University, Busan 608-737; [6]Department of Biomaterial Control (BK21 Program), Dongeui University Graduate School, Busan 614-052; [7]Division of Applied Life Science, Gyeongsang National University, Jinju 660-701; [8]Department of Immunology, School of Medicine, Keimyung University, Taegu 700-712, Korea

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Abstract. Rheumatoid arthritis (RA) is a chronic inflammatory disease that is characterized by hyperplasia of the synovial fibroblasts, which is partly the result of decreased apoptosis. This study investigated the mechanisms through which curcumin, a polyphenolic compound from the rhizome of *Curcuma longa*, exerts its anti-proliferative action in the synovial fibroblasts obtained from patients with RA. Exposure of the synovial fibroblasts to curcumin resulted in growth inhibition and the induction of apoptosis, as measured by MTT assay, fluorescent microscopy and Annexin-V-based assay. RT-PCR and immunoblotting showed that treating the cells with curcumin resulted in the down-regulation of anti-apoptotic Bcl-2 and the X-linked inhibitor of the apoptosis protein as well as the up-regulation of pro-apoptotic Bax expression in a concentration-dependent manner. Curcumin-induced apoptosis was also associated with the proteolytic activation of caspase-3 and caspase-9, and the concomitant degradation of poly(ADP-ribose) polymerase protein. Furthermore, curcumin decreased the expression levels of the cyclooxygenase (COX)-2 mRNA and protein without causing significant changes in the COX-1 levels, which was correlated with the inhibition of prostaglandin E_2 synthesis. These results show that curcumin might help identify a new therapeutic pathway against hyperplasia of the synovial fibroblasts in RA.

Correspondence to: Dr Yung Hyun Choi, Department of Biochemistry, Dongeui University College of Oriental Medicine, Busan 614-052, Korea
E-mail: choiyh@deu.ac.kr

Dr Gi-Young Kim, Faculty of Applied Marine Science, Cheju National University, Jeju 690-756, Korea
E-mail: immunkim@cheju.ac.kr

*Contributed equally

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Introduction

Apoptosis is genetically programmed cell death that constitutes a systemic means of cell suicide within an organism during normal morphogenesis and tissue remodelling and as a response to pathogenic infections or other irreparable cell damage. The aberrant regulation of apoptosis has been observed in many human diseases such as inflammation, neuronal disease, autoimmune diseases and cancer. Therefore, understanding the mechanism of apoptosis is important for preventing and treating many of these diseases (1,2). Rheumatoid arthritis (RA) is a chronic inflammatory disease that causes progressive joint destruction, deformity and disability. The initial histological features of RA are characterized by synovial lining hyperplasia, excessive angiogenesis, and the accumulation of mononuclear cells in the synovium (3,4). It should be noted that both macrophage-like synovial fibroblasts residing in the lining layer of the synovium and fibroblast-like synovial fibroblasts located in the sublining layer expand in the synovial tissue of RA patients. T and B lymphocytes and macrophages infiltrate the affected synovial tissues, and promote the release of high concentrations of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-1. In response to these cytokines, the synovial fibroblasts proliferate vigorously and form pannus tissues, which

destroy the cartilage and bone of the joints (5,6). Although the reason for the hyperplasia of the synovial lining in RA is not completely understood, impaired apoptosis or senescence has been proposed as a mechanism to explain their increased numbers. Therefore, the stimulation of apoptosis of the synovial fibroblasts may be useful for treating RA (7-9).

Prostaglandins (PGs) play an important role in synovial inflammation. PG synthesis is initiated by the cyclooxygenase (COX)-catalyzed oxidation of arachidonic acid into PGH_2. From this process, PGE_2, PGF_2, thromboxane A_2, and prostacyclin are produced from PGH_2 in the inflamed rheumatoid synovial tissues (10,11). At least two forms of COX have been identified and their genes cloned (12,13). COX-1 is constitutively expressed in most cells and tissues. On the other hand, COX-2 is highly inducible in certain cell types involved in inflammatory processes. Many clinical studies have indicated that both PGE_2 and COX-2 are up-regulated in inflammation-related pain, and cyclooxygenase inhibitors for RA patients show that inflammation-related pain can be partially reversed by inhibiting PGE_2 (14,15). Recent reports have highlighted the role of COX-2 and PGE_2 in cell apoptosis, particularly in cancer cells. The overexpression of the COX-2 gene protects the cells from apoptosis, and drugs that inhibit COX-2 have been shown to induce programmed death in these cells (16,17). Alterations in the apoptosis of synovial fibroblasts have been observed in resident synoviocytes as well as in inflammatory cells, and are associated with the pathogenesis of RA (8,9). These changes constitute the hallmark of synovial cell activation and contribute to both chronic inflammation and hyperplasia. However, the role of COX-2 and PGs in synoviocyte death is still under investigation. Therefore, the inhibition of COX-2 as well as PGE_2 has a marked impact on the prevention and alleviation of inflammation-related pain.

Curcumin (diferuloylmethane) is a yellow pigment found in the rhizome of turmeric (*Curcuma longa L., Zingiberaceae*) and related species, and has a wide range of pharmacological and biological activity (18,19). The anti-oxidant and anti-inflammatory effects of this compound have been assessed in various *in vitro* and *in vivo* systems. The chemopreventive properties of curcumin are also well documented (19-22). One of the most plausible mechanisms underlying the chemopreventive effects of curcumin involves the suppression of tumor promotion. In addition, this compound has been recognized as a promising anti-cancer drug on account of its ability to induce the efficient arrest cell proliferation and cell death in a variety of tumor cells (19,23,24). Although several studies have reported curcumin to modulate numerous aspects of the cell function relevant to inflammatory arthritis, the molecular mechanisms of curcumin in RA synovial fibroblasts are not completely understood. This study investigated the effects of curcumin on the growth inhibition and apoptosis of synovial fibroblasts obtained from RA patients. Particular attention was paid to the modulation of COX-2 expression and the production of PGE_2 in the curcumin-treated synovial fibroblasts. The results demonstrated that the decreased viability of RA synovial fibroblasts exposed to curcumin was mediated by the induction of apoptosis, which was related to the modulation of the expression of the Bcl-2 family and the activation of caspases. Furthermore, the down-regulation of the COX-2 expression by the curcumin treatment was associated with the inhibition of PGE_2 release.

Materials and methods

Cell culture, curcumin and growth study. The synovial fibroblasts were obtained from RA patients by Dr S-I. Lee at Chonbuk National University Medical School and the Research Institute of Clinical Medicine (Jeonju, Korea) during joint replacement surgery as previously described (25). Briefly, the tissues were minced and incubated with 1 mg/ml collagenase in serum-free DMEM (Life Technologies, Gaithersburg, MD) for 2 h at 37˚C, filtered through a nylon mesh, washed extensively, and cultured in DMEM supplemented with 10% fetal calf serum (FCS), penicillin, streptomycin, gentamicin, and L-glutamine in a humidified atmosphere containing 5% CO_2. After an overnight culture, the nonadherent cells were removed, and the adherent cells were cultivated in DMEM plus 10% FCS. When the cells reached confluency, they were trypsinized, split at a 1:3 ratio, and recultured in medium. Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, diferuloyl methane; Sigma Chemical Co., St Louis, MO] was prepared as a 20-mM solution in dimethyl sulfoxide and further diluted in the cell culture medium. Measurement of cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the conversion of MTT to MTT-formazan by mitochondria. For the morphological study, the synovial fibroblasts were treated with curcumin for 24 h and photographed directly with an inverted microscope (Carl Zeiss, Germany).

Detection of apoptosis by Annexin-V FITC staining. The fibroblasts were washed with PBS and re-suspended in an Annexin-V binding buffer containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$. Aliquots of the cells were incubated with Annexin-V fluorescein isothiocyanate (FITC), mixed, and incubated for 15 min at room temperature in the dark. Propidium iodide (PI) at a concentration of 5 μg/ml was added to distinguish the necrotic cells. The apoptotic cells (V^+/PI^-) were measured by the fluorescence-activated cell sorter analysis in a FACS analyzer (Becton Dickinson, San Jose, CA).

Nuclear staining with DAPI. The cells were washed with PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. The fixed cells were washed with PBS, and stained with a 4,6-diamidino-2-phenylindole (DAPI, Sigma) solution for 10 min at room temperature. The cells were then washed twice with PBS and analyzed by fluorescence microscopy (Carl Zeiss).

RNA extraction and reverse transcription-PCR. The total RNA was prepared using an RNeasy kit (Qiagen, La Jolla, CA) and primed with random hexamers to synthesize the complementary DNA using AMV reverse transcriptase (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions. A polymerase chain reaction

Table I. Oligonucleotides used in RT-PCR.

Name		Sequence of primers
Bcl-2	Sense	5'-CAG-CTG-CAC-CTG-ACG-3'
	Antisense	5'-GCT-GGG-TAG-GTG-CAT-3'
Bax	Sense	5'-ATG-GAC-GGG-TCC-GGG-GAG-3'
	Antisense	5'-TGG-AAG-AAG-ATG-GGC-TGA-3'
XIAP	Sense	5'-GAA-GAC-CCT-TGG-GAA-CAA-CA-3'
	Antisense	5'-CGC-CTT-AGC-TGC-TCT-CTT-CAG-T-3'
cIAP-1	Sense	5'-TGA-GCA-TGC-AGA-CAC-ATG-C-3'
	Antisense	5'-TGA-CGG-ATG-AAC-TCC-TGT-CC-3'
cIAP-2	Sense	5'-CAG-AAT-TGG-CAA-GAG-CTG-G-3'
	Antisense	5'-CAC-TTG-CAA-GCT-GCT-CAG-G-3'
Fas	Sense	5'-TCT-AAC-TTG-GGG-TGG-CTT-TGT-CTT-C-3'
	Antisense	5'-GTG-TCA-TAC-GCT-TTC-TTT-CCA-T-3'
FasL	Sense	5'-GGA-TTG-GGC-CTG-GGG-ATG-TTT-CA-3'
	Antisense	5'-AGC-CCA-GTT-TCA-TTG-ATC-ACA-AGG-3'
COX-1	Sense	5'-TGC-CCA-GCT-CCT-GGC-CCG-CCG-CTT-3'
	Antisense	5'-GTG-CAT-CAA-CAC-AGG-CGC-CTC-TTC-3'
COX-2	Sense	5'-TTC-AAA-TGA-GAT-TGT-GGG-AAA-AT-3'
	Antisense	5'-AGA-TCA-TCT-CTG-CCT-GAG-TAT-CTT-3'
GAPDH	Sense	5'-CGG-AGT-CAA-CGG-ATT-TGG-TCG-TAT-3'
	Antisense	5'-AGC-CTT-CTC-CAT-GGT-GGT-GAA-GAC-3'

(PCR) was carried out using a Mastercycler (Eppendorf, Hamburg, Germany) with the primers shown in Table I. The following conditions were used for the PCR reactions: 1 cycle (94˚C for 3 min); 35 cycles (94˚C for 45 sec; 58˚C for 45 sec and 72˚C for 1 min) and 1 cycle (72˚C for 10 min). The resulting amplification products were separated electrophoretically on 1% agarose gel and visualized by ethidium bromide (EtBr, Sigma) staining.

Gel electrophoresis and Western blot analysis. The cells were harvested, lysed, and the protein concentrations were quantified using a BioRad protein assay (BioRad Lab., Hercules, CA), according to the manufacturer's instructions. For Western blot analysis, an equal amount of the protein was subjected to electrophoresis on an SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) by electroblotting. The blots were probed with the desired antibodies for 1 h, incubated with the diluted enzyme-linked secondary antibody and visualized by enhanced chemiluminescence (ECL) according to the recommended procedure (Amersham). The primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Calbiochem (Cambridge, MA). The peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham.

Assay of caspase-3, caspase-8 and caspase-9 activity. The enzymatic activity of the caspases induced by curcumin was assayed using a colorimetric assay kit according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). Briefly, the cells were lysed in a lysis buffer in an ice bath for 30 min. The lysed cells were centrifuged at 12,000 x g for 10 min, and 100 μg of the protein was incubated with 50 μl of a reaction buffer and 5 μl of the colorimetric tetrapeptides, Asp-Glu-Val-Asp (DEVD)-p-nitroaniline (pNA), Ile-Glu-Thr-Asp (IETD)-pNA and Leu-Glu-His-Asp (LEHD)-pNA for caspase-3, caspase-8 and caspase-9, respectively, at 37˚C for 2 h. The optical density of the reaction mixture was quantified spectrophotometrically at a wavelength of 405 nm.

PGE_2 EIA analysis. After treating the cells with curcumin, the medium was removed and the amount of PGE_2 released by the cells was measured. To measure the level of PGE_2 accumulation, an enzyme immunoassay (EIA) was performed using a commercial kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's protocol. The level of PGE_2 production was normalized with respect to the number of viable cells present in the particular culture.

Results

Inhibition of viability and induction of apoptosis by curcumin in synovial fibroblasts. To determine if curcumin influenced the viability of RA synovial fibroblasts, the cells were cultured in the presence of increasing concentrations of curcumin ranging from 25-100 μM. After 24 h, the cell viability was determined by the MTT assay. The results showed that curcumin led to a reduced viability of synovial fibroblasts in a concentration-dependent manner (Fig. 1A).

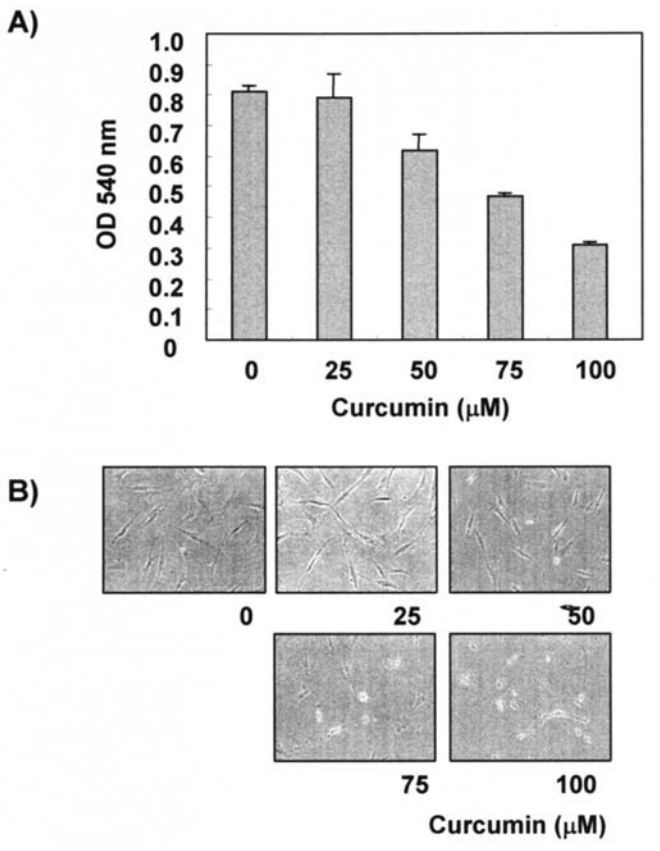

Figure 1. Growth inhibition and morphological changes in the synovial fibroblasts by curcumin. (A) The cells were plated at $1x10^5$ cells per 35-mm dish. The cells were then treated with different concentrations of curcumin (0, 25, 50, 75, and 100 μM) for 24 h, and the level of growth inhibition was measured using a metabolic-dye-based MTT assay. The data is reported as the mean ± S.D. of three independent experiments. (B) After incubation with curcumin for 24 h, the cells were examined by inverted microscopy. These are representative examples for duplicate tests. Magnification, x200.

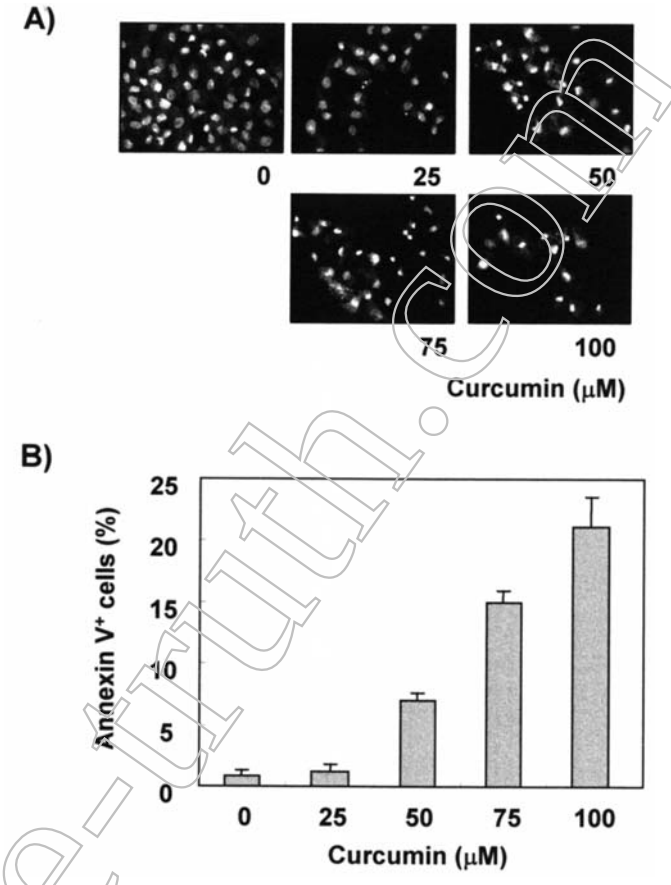

Figure 2. Induction of apoptosis by curcumin in synovial fibroblasts. (A) The cells were incubated with variable concentrations of curcumin for 24 h. The cells were sampled, fixed and stained with DAPI. The stained nuclei were then observed under a fluorescent microscope using a blue filter. Magnification, x400. (B) After curcumin treatment for 24 h, the cells were collected and stained with FITC-conjugated Annexin-V and PI for flow cytometry analysis. The apoptotic cells were determined by counting the percentage of Annexin V^+, PI^- cells and the percentage of Annexin V^+, PI^+ cells. The results are expressed as the mean ± S.E. of three independent experiments.

Direct observation by inverted microscopy showed that the synovial fibroblasts treated with curcumin showed numerous morphological changes from the control cells (Fig. 1B). Cell shrinkage, cytoplasm condensation and the formation of cytoplasmic filaments appeared after a 50-μM curcumin treatment for 24 h. Further experiments were carried out to determine if the inhibitory effect of curcumin on cell viability was the result of apoptotic cell death. Morphological analysis with DAPI staining revealed nuclei with chromatin condensation and the formation of apoptotic bodies in the cells cultured with curcumin in a concentration-dependent manner. However, very few nuclei with chromatin condensation were observed in the control culture (Fig. 2A). Therefore, flow cytometry analysis with Annexin V and PI staining was performed to determine the magnitude of the apoptosis elicited by curcumin. As shown in Fig. 2B, the number of Annexin V^+ cells was increased in the curcumin-treated synovial fibroblasts in a concentration-dependent manner compared with the untreated control cells suggesting that the inhibition of cell viability observed in response to curcumin is associated with the induction of apoptotic cell death.

Modulation of Bcl-2 and IAP family expression in curcumin-treated synovial fibroblasts. The apoptotic cascades caused by curcumin in the synovial fibroblasts were examined by exposing the cells to curcumin and measuring the levels of the Bcl-2 family members. RT-PCR and Western immunoblotting showed that the transcriptional and translational levels of pro-apoptotic Bax expression were induced slightly in the curcumin-treated synovial fibroblasts, whereas the levels of anti-apoptotic Bcl-2 expression were markedly inhibited in response to the curcumin treatment (Fig. 3). The expression levels in the curcumin-treated synovial fibroblasts were also examined in order to determine if curcumin induces synovial fibroblast death through a change in the expression of the inhibitors of the apoptosis protein (IAP) family members. As shown in Fig. 4, curcumin induced a concentration-dependent decrease in the expression levels of the X-linked IAP (XIAP) mRNA and protein but had no effect on the cIAP-1 and cIAP-2 levels. This suggests that curcumin specifically down-regulates the expression of XIAP in synovial fibroblasts.

Activation of caspase-3 and caspase-9 in curcumin-treated synovial fibroblasts. The expression and activity of caspases such as capase-3, caspase-8 and caspase-9 in the curcumin-treated synovial fibroblasts were next examined by Western

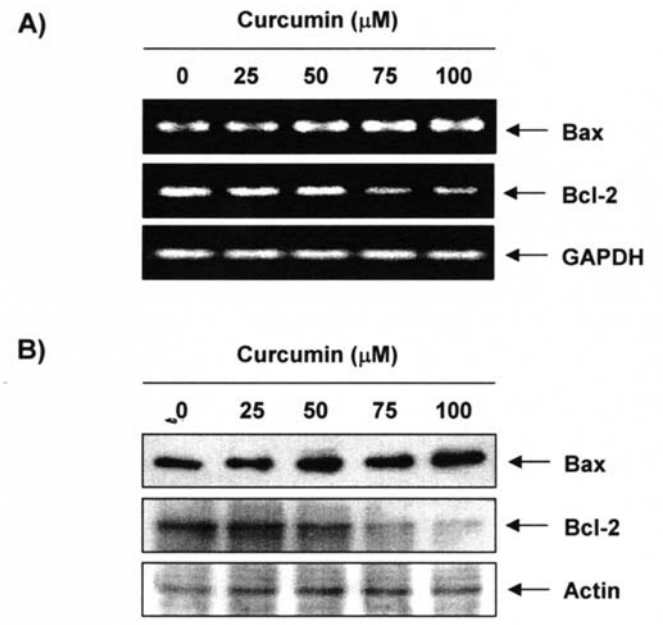

Figure 3. Effects of curcumin on the levels of the Bcl-2 family members in the synovial fibroblasts. (A) After a 24-h incubation with curcumin, the total RNA was isolated and reverse-transcribed. The resulting cDNA was subjected to PCR with the Bcl-2 and Bax primers. The reaction products were subjected to electrophoresis in 1% agarose gel and visualized by EtBr staining. GAPDH was used as the internal control. (B) The cells were lysed and the cellular proteins were then separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the anti-Bcl-2 and anti-Bax antibodies. The proteins were visualized using an ECL detection system. Actin was used as the internal control.

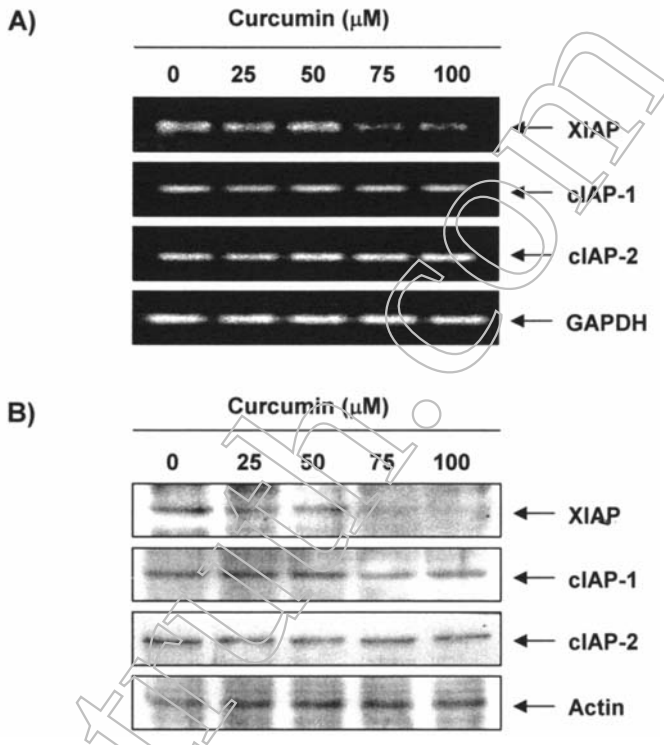

Figure 4. The effects of curcumin on the expression of the IAP family in synovial fibroblasts. (A) The cells were incubated with different curcumin concentrations for 24 h, and the total RNA was isolated and reverse-transcribed. The resulting cDNAs were subjected to PCR with the indicated primers, and the reaction products were subjected to electrophoresis in 1% agarose gel and visualized by EtBr staining. GAPDH was used as the internal control. (B) The cells were lysed and equal amounts of proteins were then separated by SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with the indicated antibodies and detected by ECL. In order to confirm the equal loading, the blot was stripped of the bound antibody and reprobed with the anti-actin antibody.

blot analysis and an *in vitro* activity assay. The results showed that the curcumin treatment down-regulated the levels of the pro-caspase-3 and pro-caspase-9 proteins but had little effect on the pro-caspase-8 levels (Fig. 5A). In order to further quantify the proteolytic activation of the caspases, the lysates equalized for the protein from the fibroblasts treated with curcumin were assayed for their caspase-3, caspase-8 and caspase-9 activity using DEVD-pNA, IETD-pNA and LEHD-pNA as substrates, respectively. As shown in Fig. 5B, the curcumin treatment increased the caspase-3 and caspase-9 activity (~4- to 6-fold after 24-h exposure to 100 μM curcumin) in a concentration-dependent manner; but caspase-8 was not significantly activated in the curcumin-treated cells (Fig. 5B). Subsequent Western blot analysis showed the progressive proteolytic cleavage of the poly(ADP-ribose) polymerase (PARP) protein, which is a downstream target of the activated caspase-3 (26) in the synovial fibroblasts after the curcumin treatment in a concentration-dependent manner (Fig. 4A).

Inhibition of COX-2 activity and PGE_2 production by curcumin. This study next examined whether or not the curcumin-induced anti-proliferative effect of the synovial fibroblasts was connected to the reduced activity of COXs. RT-PCR and Western blot analyses showed a significant decrease in COX-2 mRNA and protein expression after the curcumin treatment, but curcumin had little effect on COX-1 expression (Fig. 6A and B). In order to confirm that PGE_2 production was associated with the catalytic activity of the COX-2 isoform, the synovial fibroblasts were cultured in the presence of curcumin, and the PGE_2 levels were measured. As shown in Fig. 6C, the synthesis of PGE_2 was concentration-dependent, and its production was decreased significantly over time after the curcumin treatment, which was well correlated with the down-regulation of COX-2 expression.

Discussion

RA is an autoimmune disease that is characterized by synovial proliferation, infiltration of lymphocytes and macrophages into the synovial lining, and a paucity of apoptosis (8,9,27). In RA, fibroblasts that reside in the synovial lining markedly increase in number, show a transformed phenotype, and destroy the adjacent cartilage and bone. Protection against synovial fibroblast apoptosis might contribute to the synovial hyperplasia that is associated with RA. Therefore, identifying the agents that induce apoptosis in rheumatoid synovial fibroblasts might be a key step toward a successful treatment of RA (7-9). These results clearly demonstrate that curcumin induces apoptosis in the synovial fibroblasts of RA patients, which appears to account for its anti-proliferating activity. The induction of apoptosis by curcumin was confirmed by the characteristic morphological changes and Annexin-V-based assay (Fig. 2).

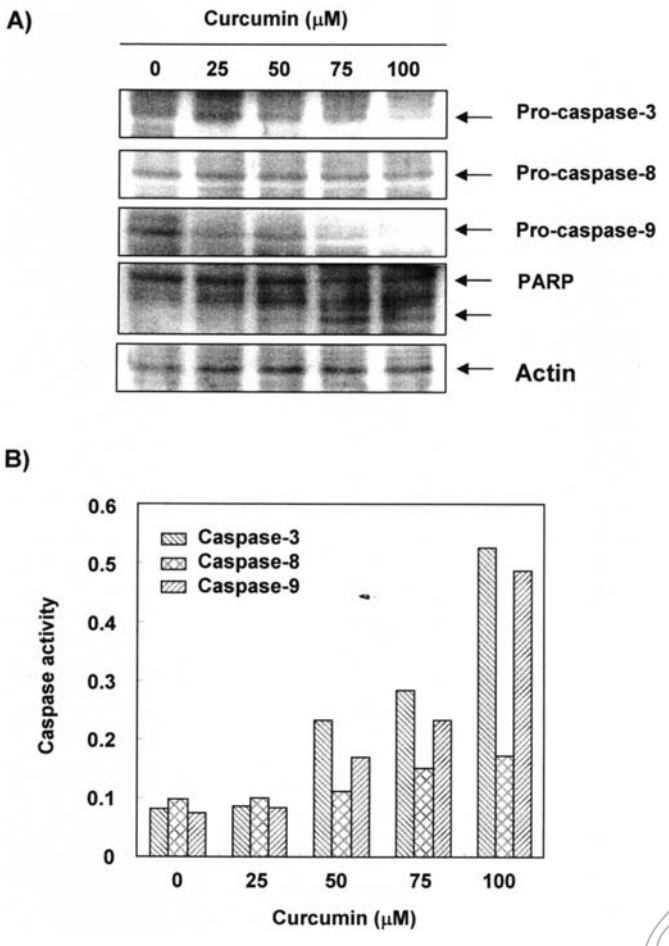

Figure 5. Activation of caspases and degradation of PARP protein by curcumin in synovial fibroblasts. (A) After a 24-h incubation with curcumin, the cells were lysed and the cellular proteins were separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the anti-caspase-3, anti-caspase-8, anti-caspase-9 and anti-PARP antibodies. The proteins were visualized using an ECL detection system. Actin was used as the internal control. (B) The cell lysates from the cells treated with curcumin for 24 h were assayed for *in vitro* caspase-3, caspase-8 and caspase-9 activity using DEVD-pNA, IETD-pNA and LEHD-pNA, respectively, as substrates. The released fluorescent products were measured. The data represents the average of two independent experiments.

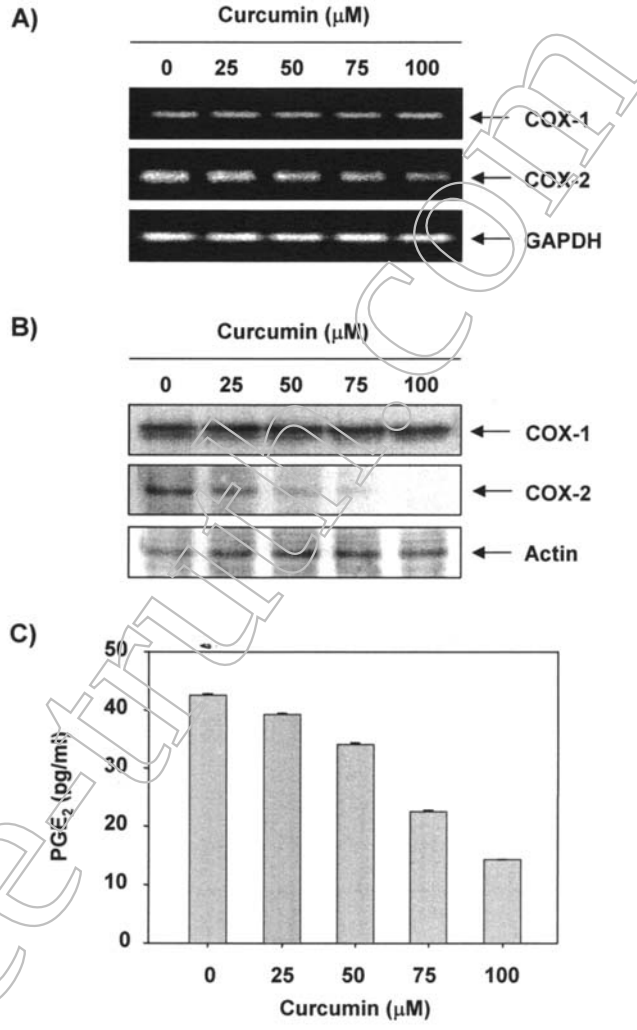

Figure 6. Down-regulation of COX-2 expression and the inhibition of PGE_2 production by curcumin in synovial fibroblasts. (A) After a 24-h incubation with curcumin, the total RNA was isolated and reverse-transcribed. The resulting cDNAs were subjected to PCR with the COX-1 and COX-2 primers and the reaction products were subjected to electrophoresis in 1% agarose gel and visualized by EtBr staining. GAPDH was used as the internal control. (B) The cells were treated with the indicated concentrations of curcumin for 24 h and collected. The cells were then lysed, and the cellular proteins were separated by 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the antibodies against COX-1 and COX-2. The proteins were visualized using an ECL detection system. Actin was used as the internal control. (C) The cells were treated with the indicated concentrations of curcumin for 24 h and collected. The level of PGE_2 accumulation in the medium was determined using an EIA kit as described in Materials and methods. The data is reported as the mean ± S.D. of three independent experiments.

The regulation of apoptosis is a complex process involving a number of gene products including the Bcl-2 family. Bax, a pro-apoptotic factor of the Bcl-2 family, is found in monomeric form in the cytosol or is loosely attached to the membranes under normal conditions. Following a death stimulus, cytosolic and monomeric Bax translocates to the mitochondria where it becomes an integral membrane protein and cross-linkable as a homodimer. After being inserted into the membranes, Bax may form channels or pores allowing for the release of factors from the mitochondria, such as cytochrome *c*, to propagate the apoptotic pathway (28,29). Unlike Bax, which is an anti-apoptotic factor, Bcl-2 is mainly localized as an integral mitochondrial membrane protein and protects the cell from multiple signals that lead to cell death. Bcl-2 forms heterodimers with Bax to prevent mitochondrial changes, thereby blocking the caspase activation step of the apoptotic process (30-32). Therefore, it has been suggested that the ratio between the level of the pro-apoptotic Bax protein and that of the anti-apoptotic factor Bcl-2 protein determines if a cell will respond to an apoptotic signal. In this study, after treating the synovial fibroblasts with different curcumin concentrations, the expression of Bcl-2 decreased significantly, while Bax expression increased in a concentration-dependent manner (Fig. 3). The caspases play a key role in the execution phase of cell death through various apoptotic stimuli (30,32). They are synthesized initially as single polypeptide chains representing the latent precursors that undergo proteolytic processing at specific residues to produce subunits that form an active hetero-tetrameric protease. The activated caspases induce limited proteolysis in a number of cellular proteins including PARP,

which are degraded by the caspase family as a consequence of apoptosis and have been used as a marker of apoptosis (26,33). The processing and activation of caspases can be regulated by molecules such as the Bcl-2 family as well as by IAP family members (34). In the cytosol, cytochrome *c* activates caspase-9, which in turn activates the effector caspases such as caspase-3 and caspase-7. Bcl-2 and its related proteins control the release of cytochrome *c* from the mitochondria, whereas the IAP molecules modulate the activity of caspases through a direct interaction with the active caspases (34). As shown in Figs. 4 and 5, the exposure of synovial fibroblasts to curcumin caused the proteolytic activation of caspase-3 and caspase-9 as well as the selective down-regulation of XIAP, which is associated with the concomitant degradation of the PARP protein and the accumulation of the 85 kDa. Although it is unclear how curcumin decreases the XIAP content in the synovial fibroblasts, curcumin has been shown to activate the downstream caspases. XIAP is a substrate of caspase-3 (34,35). Therefore, it is possible that the observed decrease in the XIAP content is a consequence of caspase-3-mediated processing after the curcumin treatment. In this study, the Bax/Bcl-2 ratio in the synovial fibroblasts increased significantly after the curcumin treatment in a concentration-dependent manner. Likewise, the trend of XIAP expression after the curcumin treatment was consistent with the result of the Annexin-V FITC staining, as well as that of caspase-3 and caspase-9 activation. Therefore, these results strongly suggest that in curcumin-induced apoptosis, an imbalance of Bax/Bcl-2 might be the upstream event in the mitochondrial pathway, and the latter activation of caspase-9 and caspase-3 may lead to apoptosis. However, whether curcumin can induce apoptosis through other pathways, such as the death receptor pathway or endoplasmic reticulum pathway is still unknown. Moreover, the mechanisms for the phosphorylation and activation of multiple apoptosis-related proteins in curcumin-induced apoptosis require further study.

RA is characterized by the proliferation of synoviocytes, which also produce prostanoids. Eicosanoids and prostanoids are important lipid mediators that are produced at elevated levels in inflamed tissues including the rheumatoid synovium as well as in cultured human RA fibroblast-like synoviocytes. COX, which converts arachidonic acid into PG endoperoxides, is the rate-limiting enzyme in prostanoid synthesis (10,11). In normal cells, COX-1 is considered to be the constitutively expressed form and is believed to serve the housekeeping functions. However, COX-2 is expressed at very low basal levels and is rapidly induced by different products such as inflammatory cytokines, tumor promoters or growth factors. Therefore, COX-2 up-regulation is a key step in the initiation of inflammation and carcinogenesis, and the inhibition of COX-2 activity promises to be an effective approach in the prevention and treatment of inflammation and cancer (14,15). Crofford *et al* (36) reported that pro-inflammatory cytokines such as IL-1ß and TNF-α enhanced the *de novo* synthesis of the COX-2 mRNA and protein in rheumatoid synovial explants and cultured rheumatoid synoviocytes, but had no effect on COX-1. This suggests that COX-2 plays an important role in the overproduction of PGE_2 by the rheumatoid synovial fibroblasts. In addition, the newly developed selective COX-2 inhibitors are now being used as anti-inflammatory agents to treat patients with RA. Recently, Lee *et al* (37) reported that curcumin inhibits the interferon (IFN)-α-induced expression of COX-2. The suppression of IFN-α-induced COX-2 activation by curcumin is consistent with a previous study, which showed that curcumin inhibits the TNF-α-induced COX-2 expression in human colon cancer cells via NF-κB activation (38). Zhang *et al* (39) also reported that treating several human gastrointestinal cell lines with curcumin suppressed the expression of the COX-2 protein and mRNA, as well as PGE_2 production by chenodeoxycholate or phorbol ester. This study examined whether or not the curcumin-induced growth inhibitory effect of the synovial fibroblasts was associated with the inhibition of basal COX-2 expression and activity in order to determine the mechanisms by which curcumin inhibits synovial fibroblast proliferation. As shown in Fig. 6A and B, curcumin markedly inhibited the expression of both the COX-2 mRNA and protein in the synovial fibroblasts. However, the levels of COX-1 remained unchanged. In addition, the amount of PGE_2 produced by the synovial fibroblasts decreased with increasing curcumin concentration (Fig. 6C). These results suggest that the inhibition of the COX-2 expression and PGE_2 production is consistent with the results showing that curcumin inhibits synovial fibroblast growth and induces apoptosis.

In conclusion, curcumin inhibits human synovial fibroblast growth through the induction of apoptosis. The apoptotic events of the synovial fibroblasts by curcumin were mediated by an alteration in the Bax/Bcl-2 ratio and the activation of caspase-3 and caspase-9. Curcumin concomitantly causes the loss of COX-2 expression as well as the release of PGE_2. Although further studies are needed, these results have not been previously reported and are expected to provide important new insights into the possible biological effects of curcumin.

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References

1. Murphy FJ, Seery LT and Hayes I: Therapeutic approaches to the modulation of apoptosis. Essays Biochem 39: 131-153, 2003.
2. Fadeel B and Orrenius S: Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. J Intern Med 258: 479-517, 2005.
3. Muller-Ladner U: Molecular and cellular interactions in rheumatoid synovium. Curr Opin Rheumatol 8: 210-220, 1996.
4. Kramer I, Wibulswas A, Croft D and Genot E: Rheumatoid arthritis: targeting the proliferative fibroblasts. Prog Cell Cycle Res 5: 59-70, 2003.
5. Meinecke I, Rutkauskaite E, Gay S and Pap T: The role of synovial fibroblasts in mediating joint destruction in rheumatoid arthritis. Curr Pharm Des 11: 563-568, 2005.
6. Huber LC, Distler O, Tarner I, Gay RE, Gay S and Pap T: Synovial fibroblasts: key players in rheumatoid arthritis. Rheumatology (Oxford) 45: 669-675, 2006.
7. Nishioka K, Hasunuma T, Kato T, Sumida T and Kobata T: Apoptosis in rheumatoid arthritis: a novel pathway in the regulation of synovial tissue. Arthritis Rheum 41: 1-9, 1998.

8. Mountz JD and Zhang HG: Regulation of apoptosis of synovial fibroblasts. Curr Dir Autoimmun 3: 216-239, 2001.
9. Baier A, Meineckel I, Gay S and Pap T: Apoptosis in rheumatoid arthritis. Curr Opin Rheumatol 15: 274-29, 2003.
10. Katori M, Majima M and Harada Y: Possible background mechanisms of the effectiveness of cyclooxygenase-2 inhibitors in the treatment of rheumatoid arthritis. Inflamm Res 47: S107-S111, 1998.
11. Stichtenoth DO, Thoren S, Bian H, Peters-Golden M, Jakobsson PJ and Crofford LJ: Microsomal prostaglandin E synthase is regulated by proinflammatory cytokines and glucocorticoids in primary rheumatoid synovial cells. J Immunol 167: 469-474, 2001.
12. Yokoyama C and Tanabe T: Cloning of human gene encoding prostaglandin endoperoxide synthase and primary structure of the enzyme. Biochem Biophys Res Commun 165: 888-894, 1989.
13. Hla T and Neilson K: Human cyclooxygenase-2 cDNA. Proc Natl Acad Sci USA 89: 7384-7388, 1992.
14. Schuna AA and Megeff C: New drugs for the treatment of rheumatoid arthritis. Am J Health Syst Pharm 57: 225-234, 2000.
15. Kapoor M, Shaw O and Appleton I: Possible anti-inflammatory role of COX-2-derived prostaglandins: implications for inflammation research. Curr Opin Investig Drugs 6: 461-466, 2005.
16. Anderson WF, Umar A, Viner JL and Hawk ET: The role of cyclooxygenase inhibitors in cancer prevention. Curr Pharm Des 8: 1035-1062, 2002.
17. Umar A, Viner JL, Anderson WF and Hawk ET: Development of COX inhibitors in cancer prevention and therapy. Am J Clin Oncol 26: S48-S57, 2003.
18. Ammon HP and Wahl MA: Pharmacology of *Curcuma longa*. Planta Med 57: 1-7, 1991.
19. Aggarwal BB, Kumar A and Bharti AC: Anticancer potential of curcumin: preclinical and clinical studies. Anticancer Res 23: 363-398, 2003.
20. Nagabhushan M and Bhide SV: Curcumin as an inhibitor of cancer. J Am Coll Nutr 11: 192-198, 1992.
21. Conney AH, Lou YR, Xie JG, Osawa T, Newmark HL, Liu Y, Chang RL and Huang MT: Some perspectives on dietary inhibition of carcinogenesis: studies with curcumin and tea. Proc Soc Exp Biol Med 216: 234-245, 1997.
22. Surh Y: Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances. Mutat Res 428: 305-327, 1999.
23. Duvoix A, Blasius R, Delhalle S, Schnekenburger M, Morceau F, Henry E, Dicato M and Diederich M: Chemopreventive and therapeutic effects of curcumin. Cancer Lett 223: 181-190, 2005.
24. Singh S and Khar A: Biological effects of curcumin and its role in cancer chemoprevention and therapy. Anticancer Agents Med Chem 6: 259-270, 2006.
25. Alvaro-Gracia JM, Zvaifler NJ and Firestein GS: Cytokines in chronic inflammatory arthritis. V. Mutual antagonism between interferon-γ and tumor necrosis factor-α on HLA-DR expression, proliferation, collagenase production, and granulocyte macrophage colony-stimulating factor production by rheumatoid arthritis synoviocytes. J Clin Invest 86: 1790-1798, 1990.
26. Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG and Earnshaw WC: Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature 371: 346-347, 1994.
27. Firestein GS: Invasive fibroblast-like synoviocytes in rheumatoid arthritis. Passive responders or transformed aggressors? Arthritis Rheum 39: 1781-1790, 1996.
28. Oltvai ZN, Milliman CL and Korsmeyer SJ: Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74: 609-619, 1993.
29. Reed JC: Bcl-2 family proteins: regulators of apoptosis and chemoresistance in hematologic malignancies. Semin Hematol 34: S9-S19, 1997.
30. Stennicke HR and Salvesen GS: Caspases - controlling intracellular signals by protease zymogen activation. Biochim Biophys Acta 1477: 299-306, 2000.
31. Earnshaw WC, Martins LM and Kaufmann SH: Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 68: 383-424, 1999.
32. Philchenkov A, Zavelevich M, Kroczak TJ and Los M: Caspases and cancer: mechanisms of inactivation and new treatment modalities. Exp Oncol 26: 82-97, 2004.
33. Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS and Dixit VM: Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell 81: 801-809, 1995.
34. Deveraux QL and Reed JC: IAP family proteins - suppressors of apoptosis. Genes Dev 13: 239-252, 1999.
35. Deveraux QL, Leo E, Stennicke HR, Welsh K, Salvesen GS and Reed JC: Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. EMBO J 18: 5242-5251, 1999.
36. Crofford LJ, Wilder RL, Ristimaki AP, Sano H, Remmers EF, Epps HR and Hla T: Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues. Effects of interleukin-1 ß, phorbol ester, and corticosteroids. J Clin Invest 93: 1095-1101, 1994.
37. Lee J, Im YH, Jung HH, Kim JH, Park JO, Kim K, Kim WS, Ahn JS, Jung CW, Park YS, Kang WK and Park K: Curcumin inhibits interferon-alpha-induced NF-κB and COX-2 in human A549 non-small cell lung cancer cells. Biochem Biophys Res Commun 334: 313-318, 2005.
38. Plummer S, Holloway K, Manson M, Munks R, Kaptein A, Farrow S and Howells L: Inhibition of cyclooxygenase-2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-κB activation via the NIK/IKK signaling complex, Oncogene 18: 6013-6020, 1999.
39. Zhang F, Altorki NK, Mestre JR, Subbaramaiah K and Dannenberg AJ: Curcumin inhibits cyclooxygenase-2 transcription in bile acid- and phorbol ester-treated human gastrointestinal epithelial cells. Carcinogenesis 20: 445-451, 1999.