Carcinogenesis vol.23 no.1 pp.143–150, 2002

Curcumin (diferuloylmethane) induces apoptosis through activation of caspase-8, BID cleavage and cytochrome c release: its suppression by ectopic expression of Bcl-2 and Bcl-xl

Ruby John Anto, Asok Mukhopadhyay, Kate Denning and Bharat B.Aggarwal[1]

Cytokine Research Section, Department of Bioimmunotherapy, Box 143, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA

[1]To whom correspondence should be addressed
Email: aggarwal@utmdacc.mda.uth.tmc.edu

Pharmacologically safe compounds that can inhibit the proliferation of tumor cells have potential as anticancer agents. Curcumin, a diferuloylmethane, is a major active component of the food flavor turmeric (*Curcuma longa*) that has been shown to inhibit the proliferation of a wide variety of tumor cells. The apoptotic intermediates through which curcumin exhibits its cytotoxic effects against tumor cells are not known, and the participation of antiapoptotic proteins Bcl-2 or Bcl-xl in the curcumin-induced apoptosis pathway is not established. In the present report we investigated the effect of curcumin on the activation of the apoptotic pathway in human acute myelogenous leukemia HL-60 cells and in established stable cell lines expressing Bcl-2 and Bcl-xl. Curcumin inhibited the growth of HL-60 cells (neo) in a dose- and time-dependent manner, whereas Bcl-2 and Bcl-xl-transfected cells were relatively resistant. Curcumin activated caspase-8 and caspase-3 in HL-60 neo cells but not in Bcl-2 and Bcl-xl-transfected cells. Similarly, time-dependent poly(ADP)ribose polymerase (PARP) cleavage by curcumin was observed in neo cells but not in Bcl-2 and Bcl-xl-transfected cells. Curcumin treatment also induced BID cleavage and mitochondrial cytochrome *c* release in neo cells but not in Bcl-2 and Bcl-xl-transfected cells. In neo HL-60 cells, curcumin also downregulated the expression of cyclooxygenase-2. Because DN-FLICE blocked curcumin-induced apoptosis, caspase-8 must play a critical role. Overall, our results indicate that curcumin induces apoptosis through mitochondrial pathway involving caspase-8, BID cleavage, cytochrome *c* release, and caspase-3 activation. Our results also suggest that Bcl-2 and Bcl-xl are critical negative regulators of curcumin-induced apoptosis.

Introduction

Due to pharmacological safety, there has been an increased interest in phytochemicals that may exhibit anticancer activity. Curcumin (diferuloylmethane), a non-nutritive food chemical present in turmeric (*Curcuma longa*), is pharmacologically safe, given that curcumin has been consumed as a dietary spice at the rate of up to 100 mg/day by people in certain countries for centuries (1). Curcumin blocks tumor initiation induced by benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene (2), and suppresses the phorbol ester-induced tumor promotion (3,4). *In vivo,* curcumin suppresses carcinogenesis of the skin (4–8), the forestomach (9,10), the colon (11–13), the breast (14–16) and the liver (17) in mice. Additionally curcumin exhibits antimetastatic activity (18). How curcumin exhibits its anticarcinogenic and antimetastatic effects is not fully understood, but its ability to inhibit the growth of endothelial cells (19), suppresses angiogenesis *in vivo* (20), abrogate FGF-2-induced angiogeneic response and matrix metalloprotease (MMP)-9 expression (21,22), block expression of adhesion molecules (23) and cycloxygenase-2 (24,25) may play important roles. The downregulation of genes involved in carcinogenesis and those in tumor initiation, promotion and metastasis by curcumin could be mediated through the suppression of transcription factors NF-κB (25–27), and c-jun N-terminal kinase (28).

Compounds that block or suppress the proliferation of tumor cells have potential as anticancer agents. *In vitro* curcumin was found to induce apoptosis of a wide variety of tumor cells, including both B-cell and T-cell leukemia (29–32), colon carcinoma (33), breast carcinoma (34–36) and other tumor cell types (37,38). Additionally, curcumin has been shown to block the growth of certain normal cell types including thymocytes, osteoclasts, vascular smooth muscle cells, and endothelial cells (19,39–41). How exactly curcumin induces apoptosis of various cell types is not well understood.

Typically two different pathways leading to apoptosis have been identified, namely receptor-mediated and chemical-induced apoptosis (see Figure 1) (42,43). Most cytokines (usually members of the TNF superfamily) induce apoptosis by interaction of the ligand with its death receptor, which sequentially recruits TNF receptor-associated death domain; Fas-associated death domain (FADD); FADD-like interleukin-1 converting enzyme (FLICE) (also called caspase-8), and caspase-3; the last then cleaves various substrates leading to apoptosis. In contrast, chemical (most chemotherapeutic agents)-induced apoptosis involves cleavage of BID by caspase-8, which causes the release of cytochrome *c* from the mitochondria, and cytochrome *c* together with APAF1 activates caspase-9, and the latter then activates caspase-3, resulting in apoptosis. The chemical-induced apoptotic pathway involving mitochondria has been shown to be negatively regulated by antiapoptotic proteins such as Bcl-2 or Bcl-xl through suppression of cytochrome *c* release (44,45).

Through which pathway curcumin induces apoptosis and whether an antiapoptotic protein plays any role in curcumin-induced apoptosis is not known. Using the acute myelogenous leukemia cell line HL-60, we investigated the mechanism by which curcumin induces apoptotic effects and how these effects are modulated by Bcl-2 and Bcl-xl proteins.

Abbreviations: DN-FADD, dominant negative-FADD; EMSA, electrophoretic mobility shift assay; FADD, Fas-associated death domain; FBS, fetal bovine serum; FLICE, FADD-like ICE; ICE, interleukin-1 converting enzyme; IκB, inhibitory subunit of NF-κB; NF-κB, nuclear transcription factor-κB; PARP, poly(ADP) ribose polymerase; TNF, tumor necrosis factor.

Materials and methods

Materials

RPMI-1640, and fetal bovine serum (FBS) were procured from Life Technologies (Grand Island, NY). Curcumin and MTT (3-(4,5-dimethylthiazol-

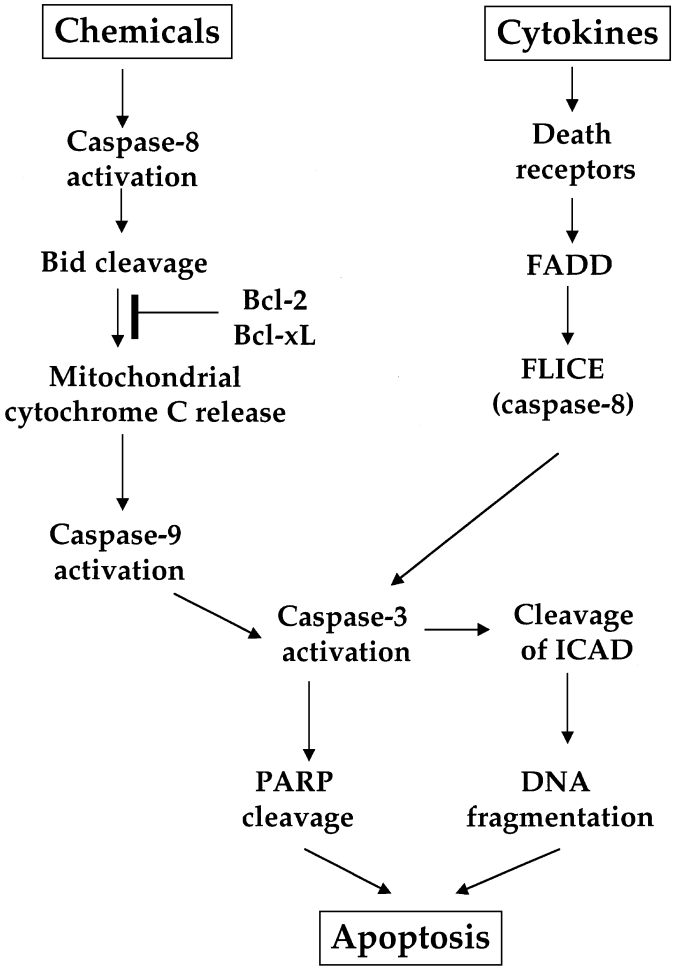

Fig. 1. Two distinct pathways leading to apoptosis activated by chemicals (on the left) and by cytokines (on the right). FADD is Fas-associated death domain, FLICE is FADD-like ICE; ICE is interleukin-1 converting enzyme, and ICAD is an inhibitor of caspase-activated deoxyribonuclease.

2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma Chemicals (St Louis, MO). The rabbit polyclonals, anti-Bcl-2 and anti-cytochrome *c*, the mouse monoclonal anti-Bcl-xL, and the goat polyclonal anti-BID were procured from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-PARP rabbit polyclonal antibodies were purchased from New England BioLab (Beverly, MA). Curcumin was dissolved in dimethylsulfoxide to prepare a stock solution of 20 mM concentration.

Cells

Human myeloid leukemic cell lines (HL-60/neo, HL-60/Bcl-xl, and HL-60/Bcl-2 cells) were originally obtained from Dr Kapil Bhalla, Lee Moffitt Cancer Center & Research Inst., Tampa, FL. The characteristics of these cells have been previously described (46). The cells were maintained in RPMI-1640 containing 10% FBS and a 1× antibiotic-antimycotic. Each cell line was split regularly before attaining 70–80% confluence. The cells were cultured in a humidified incubator in 5% CO_2 in air and were maintained in continuous exponential growth by twice a week passage.

Cytotoxicity assays

Cell growth assays were carried out essentially according to the procedure described (47). Briefly, the HL-60/neo, HL-60/Bcl-xl, and HL-60/Bcl-2 cells (5×10^3/well) were plated in 0.2 ml of the medium (RPMI-1640 with 10% FBS) in 96-well Corning plates in the presence or absence of indicated concentrations of curcumin for different times. At the end of the incubation, cytotoxicity was measured by the modified tetrazolium salt MTT assay. For this, 0.1 ml of cell medium was removed, and 0.025 ml of MTT solution (5 mg/ml in PBS) was added to each well. After a 2 h incubation at 37°C, 0.1 ml of the extraction buffer (20% sodium dodecyl sulfate, 50% dimethylformamide) was added. After an overnight incubation at 37°C, the optical densities at 570 nm were measured using a 96-well multiscanner autoreader (Dynatech MR 5000), with the extraction buffer as a blank.

$$\% \text{ relative cell viability} \frac{\text{A570 of treated samples}}{\text{A570 of untreated samples}} \times 100$$

For [^{3}H]TdR incorporation, cells were cultured and treated with curcumin as indicated above. During the last 6 h, [^{3}H]TdR (6.7 Ci/mmol; New England Nuclear, Boston, MA) was added to each well (0.5 μCi/well). Thereafter, the culture medium was removed, the wells were washed twice with phosphate-buffered saline (PBS), and the cells were detached by the addition of a solution of trypsin (0.5%) with EDTA (5.3 mM). The cell suspension was then harvested with the aid of a Filtermate 196 cell harvester (Packard Instruments, Canberra, Australia) and lysed by washing with distilled water. Radioactivity bound to the filter was measured directly by Direct Beta Counter Matrix 9600 (Model 1600 TR; Packard, Meriden, CT). All determinations were made in triplicate.

Cleavage of PARP and BID

To determine the activation of caspase-3, we also examined the cleavage of poly(ADP-ribose) polymerase (PARP) (49). Forty micrograms whole-cell extracts were resolved on 7.5% polyacrylamide gel, transferred to a nitrocellulose membrane, blocked with 5% non-fat milk protein, probed with PARP antibodies (1:3000), and detected by ECL reagent.

To determine the cleavage of BID, 30 μg of whole-cell extracts were resolved on 12% polyacrylamide gel, transferred to a nitrocellulose membrane, blocked with 5% non-fat milk protein, probed with BID antibodies (1:1000), and detected by ECL reagent.

Measurement of cytochrome c release

Cells were treated with 25 μM curcumin for various time intervals and the cytosolic extracts were prepared as described (45). Briefly the cells were washed first with PBS and then with Buffer A (0.25 M sucrose, 30 mM Tris–HCl (pH 7.9), and 1 mM EDTA). The pellets were then resuspended in Buffer B (Buffer A plus the protease inhibitors: 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml aprotinin) and homogenized gently with a glass Dounce homogenizer and a B pestle (40 strokes). The homogenates were centrifuged at 14 000 r.p.m. for 30 min to remove mitochondria and other insoluble fragments. The supernatants were again centrifuged as above to ensure complete removal of mitochondria. Fifty micrograms of protein was electrophoresed through a 15% SDS-polyarylamide gel and transferred to a nitrocellulose membrane by electroblotting. Cytochrome *c* was detected by Western blotting using mouse monoclonal anti-cytochrome *c* antibody (1:3000) for 2 h. The blot was washed, exposed to HRP-conjugated secondary antibodies for 1 h, and finally detected by chemiluminescence (ECL, Amersham Pharmacia Biotech., Arlington Heights, IL).

Assay of caspase-3 and -8

The enzymatic activity of caspases induced by curcumin was also assayed essentially based on the manufacturer's protocol (R and D Systems, Minneapolis, MN). Briefly, cells were lysed in lysis buffer for 30 min on an ice bath. The lysed cells were centrifuged at 14 000 r.p.m for 10 min, and 100 μg protein was incubated with 50 μl of reaction buffer and 5 μl of either caspase-3 (DEVD-pNA) or caspase-8 calorimetric substrate (IETD-pNA) at 37°C for 2 h. The optical density of the reaction mixture was quantitated spectrophotometrically at a wavelength of 405 nm.

Activation of procaspase-3, -7 and -8 causes the cleavage of these proteins, thus releasing the proteolytically active domain of various caspases. We examined the appearance of active caspase proteins also by western blot analysis.

Expression of Bcl-xl and Bcl-2

The effect of curcumin on the expression of Bcl-xl and Bcl-2 in HL-60 cells was determined by western blot analysis. Briefly, the concentration was measured in each cell lysate using Bradford method. Equal amounts of total protein were loaded for each blot. After electrophoresis, the proteins were electrotransferred to nitrocellulose filters, probed with the specific antibodies, and detected by chemiluminescence (ECL, Amersham–Pharmacia Biotech).

Results

In this report we investigated the pathway through which curcumin induces apoptosis and examined the effect of ectopic expression of Bcl-2 and Bcl-xl on this pathway (see Figure 1). For this, HL-60 cells stably transfected with either the empty vector (neo), or *bcl-2 or bcl-xl* plasmid were used. As shown in Figure 2A, HL-60 cells expressed very little Bcl-2 or Bcl-xl protein; however, Bcl-2 and Bcl-xl transfected cells expressed higher levels of Bcl-2 and Bcl-xl proteins, respectively. Throughout these experiments, the three cell types

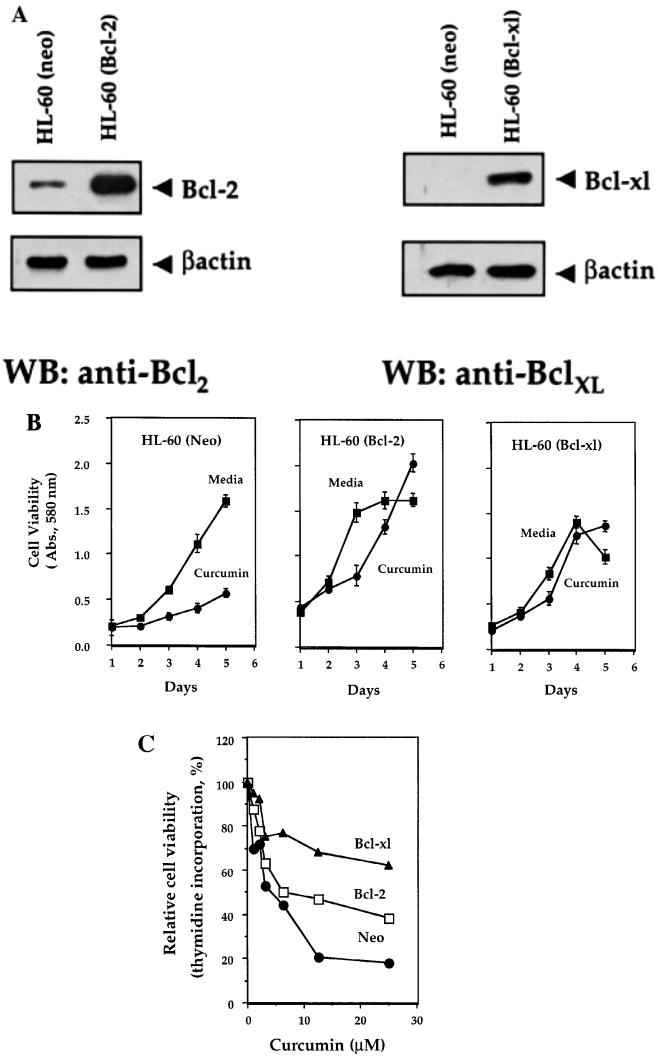

Fig. 2. (**A**) Expression of antiapoptotic proteins in HL-60-neo, HL-60-Bcl-2, HL-60-Bcl-xl cells. Cells were plated in 35 mm dishes and harvested, and whole-cell extracts prepared. Forty micrograms of cell lysate were subjected to SDS–PAGE, and Bcl-2 and Bcl-xl were detected using antibodies specific against each protein as described under Materials and methods. (**B**) Time course of the effect of curcumin on the growth of human acute myelogenous leukemia HL-60 cells (neo) and Bcl-2-and Bcl-xl-transfected cells. Cells (5×10^3 cells/0.2 ml/well) were incubated with either medium or with curcumin (12.5 μM) for different times, and then cell viability was examined by the MTT method. All determinations were made in triplicate. Abs. is absorbance units. (**C**) Dose-dependent growth inhibition of human acute myelogenous leukemia HL-60 cells (neo) and Bcl-2- and Bcl-xl-transfected cells. Cells (5×10^3 cells/0.2 ml/well) were incubated with different concentrations of curcumin for 24 h, and then cell viability was examined by thymidine incorporation method as described in Materials and methods. All determinations were made in triplicate. The results are expressed as a percentage of the control (untreated cells). The variation between triplicates was $<10\%$.

were compared for activation of various signaling intermediates involved in the curcumin-activated apoptosis pathway.

Curcumin has cytotoxic effect against HL-60 (neo) but not against Bcl-2 or Bcl-xl transfected cells

Cells were exposed to 12.5 μM curcumin and then examined for cell growth for different days. Curcumin inhibited the growth of HL-60 cells, but the growth of Bcl-2- or Bcl-xl-transfected cells was minimally affected by curcumin (Figure 2B), suggesting that curcumin is more cytotoxic to HL-60 (neo) than to the transfected cells. Thus expression of Bcl-2 or Bcl-xl appears to protect the cells from the growth-inhibitory effects of curcumin.

We also examined the effect of curcumin on DNA synthesis capability by the thymidine incorporation method. Cells were exposed to different concentrations of curcumin for 24 h and during the last 6 h were pulsed with thymidine. As shown in Figure 2C, curcumin inhibited the thymidine incorporation in a dose-dependent manner and again Bcl-2- or Bcl-xl-transfected cells were protected from the inhibitory effects of curcumin. By the thymidine incorporation method, curcumin (12.5 μM for 24 h) inhibited the incorporation by almost 50% in Bcl-2-transfected cells, whereas the same conditions by the MTT method had no significant effect on cell growth (Figure 2B). The latter is known to measure mitochondrial activity. The difference in results obtained by the two methods may suggest that DNA synthesis is more sensitive to curcumin than its effect on the mitochondria. These results further confirm that curcumin is cytotoxic against HL-60 (neo) but much less so against Bcl-2- or Bcl-xl-transfected cells.

Curcumin activates caspase-8, and ectopic expression of Bcl-2 and Bcl-xl suppresses the activation

Previously it was demonstrated that apoptosis by most agents activates an upstream protease called FLICE, or caspase-8 (42). To determine whether curcumin can also induce the activation of this caspase, we treated the cells with 25 μM curcumin for 6, 12 and 24 h, prepared the cell extracts, and examined the protease activity using a fluorogenic substrate specific for caspase-8. Whereas curcumin activated caspase-8 (Figure 3, upper panel), in a time-dependent manner in the HL-60 neo cells, Bcl-2 and Bcl-xl completely suppressed the curcumin-induced activation of caspase-8 (Figure 3 lower panel). Thus these results for the first time demonstrate that curcumin can activate caspase-8 and ectopic expression of Bcl-2 and Bcl-xl suppresses the activation.

Curcumin induces BID cleavage, and ectopic expression of Bcl-2 and Bcl-xl suppresses the cleavage

When apoptosis is induced by chemotherapeutic agents, it causes the caspase-8 mediated cleavage of BID, a proapoptotic member of the bcl-2 family (48). Western blot analysis demonstrated that curcumin likewise induced BID cleavage (upper panel), in this case in a time-dependent manner in HL-60 (neo) cell (Figure 4). The ectopic expression of Bcl-2 or Bcl-xl in these cells suppressed this cleavage (Figure 4, middle and lower panel).

Curcumin induces cytochrome c release, and ectopic expression of Bcl-2 and Bcl-xl suppresses the release

Caspase-8-mediated cleavage of BID leads to release of cytochrome *c* from the mitochondria, which is an essential step in the apoptosis pathway activated by chemotherapeutic agents (48,49). To determine whether curcumin induced the release of cytochrome *c* from the mitochondria, we treated the cells for 6, 12 and 24 h, prepared the cell extracts, and examined the cytochrome *c* release by western blot analysis. As Figure 5 shows, curcumin induced the cytochrome *c* release in a time-dependent manner in the HL-60 neo cells but the release was minimal in the Bcl-2- or Bcl-xl-transfected cells.

Curcumin activates caspase-3, and ectopic expression of Bcl-2 and Bcl-xl suppresses the activation

Cytochrome *c*, in presence of apaf-1, activates caspase-9, which in turn results in the activation of downstream caspase-3 (42). We investigated whether curcumin also induces the

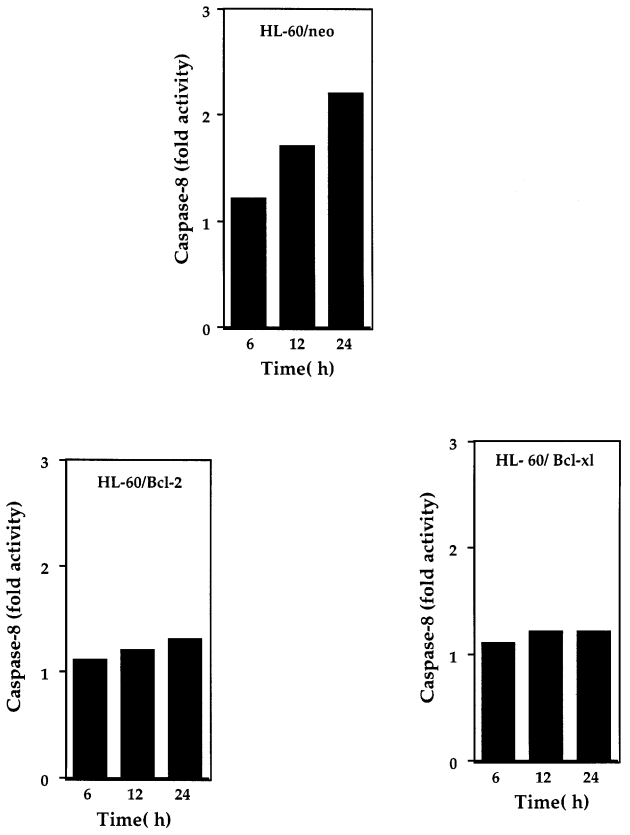

Fig. 3. Effect of curcumin on caspase-8 activation in HL-60 (Neo), HL-60 (Bcl-2), and HL-60 (Bcl-xl) cells. Two million cells were incubated with 25 μM curcumin for the indicated times. The caspase activation induced by curcumin was assayed as described under Materials and methods. The variation between replicates was <10%.

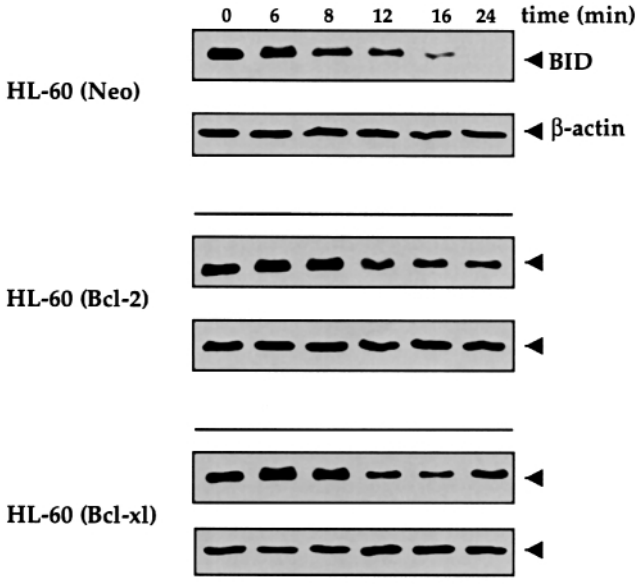

Fig. 4. Effect of curcumin on BID cleavage in HL-60 (neo), HL-60 (Bcl-2), and HL-60 (Bcl-xl) cells. Cells were plated in 35 mm dishes and incubated with 25 μM curcumin for the indicated times. Cells were harvested, and cell lysates prepared. 40 μg of cell lysate was subjected to SDS–PAGE, and PARP was detected with anti-PARP antibodies as described under Materials and methods.

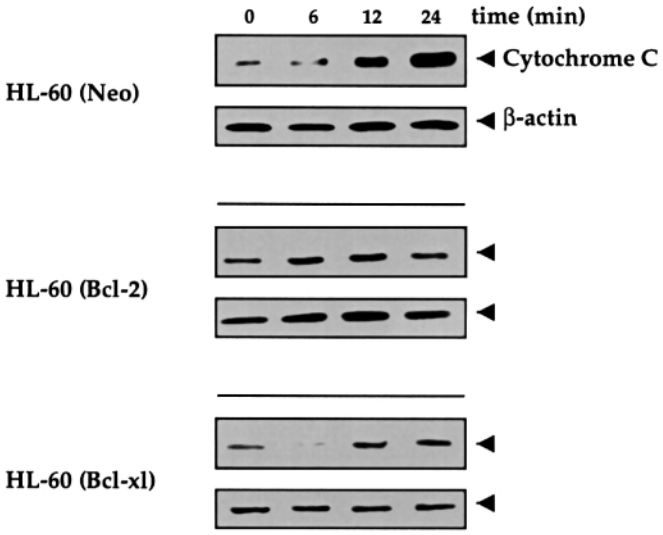

Fig. 5. Effect of curcumin on cytochrome *c* release in HL-60 (neo), HL-60 (Bcl-2), HL-60 (Bcl-xl) cells. Cells were plated in 35 mm dishes and incubated with 25 μM curcumin for the indicated times. Cells were harvested and analyzed for cytochrome *c* release by western blot using anti-cytochrome *c* antibodies as described under Materials and methods.

activation of this caspase, by treating the cells for 6, 12 and 24 h, preparing the cell extracts, and examining protease activity using a fluorogenic substrate specific for caspase-3. As Figure 6 shows, curcumin activated caspase-3 (Figure 6, upper panel) in a time-dependent manner in the HL-60 (neo) cells, but not in Bcl-2-transfected cells. The inhibition was not, however as pronounced with Bcl-xl-transfected cells (Figure 6, lower right panel).

Curcumin induces PARP cleavage, and ectopic expression of Bcl-2 and Bcl-xl suppresses the cleavage

The activation of downstream caspase-3 by most agents causes the cleavage of the PARP protein (50). We treated the cells for 6, 12 and 24 h curcumin, prepared the cell extracts, and examined PARP cleavage by western blot analysis. As Figure 7 indicates, curcumin activated PARP cleavage (upper panel) in a time-dependent manner in the HL-60 (neo) cells but not in the Bcl-2- or Bcl-xl-transfected cells.

DN-FLICE suppresses curcumin-induced apoptosis

Apoptosis mediated by most death receptors requires the recruitment of the adaptor protein FADD, which recruits the upstream protease FLICE, or caspase-8 (42). The dominant-negative form of either FADD or FLICE have been shown to block apoptosis induced by the ligands for the death receptors (42). We investigated whether curcumin-induced apoptosis is also affected by the ectopic expression of DN-FADD or DN-FLICE. Human glioblastoma H4 cells were stably transfected with plasmids for Neo, DN-FADD, and DN-FLICE (Darnay *et al.*, unpublished results) and then examined for sensitivity to curcumin by the MTT and thymidine incorporation assays for cell viability. As shown in Figure 8, H4 (neo) cells were sensitive to curcumin-induced cytotoxicity, and the expression of DN-FLICE suppressed the effect of curcumin. DN-FADD, however, had little effect on curcumin-induced cytotoxicity.

Curcumin downregulates the expression of cyclooxygenase 2 and ectopic expression of Bcl-2 and Bcl-xl prevents down-regulation

We explored alternate mechanisms through which curcumin might mediate apoptosis. Previously it has been demonstrated

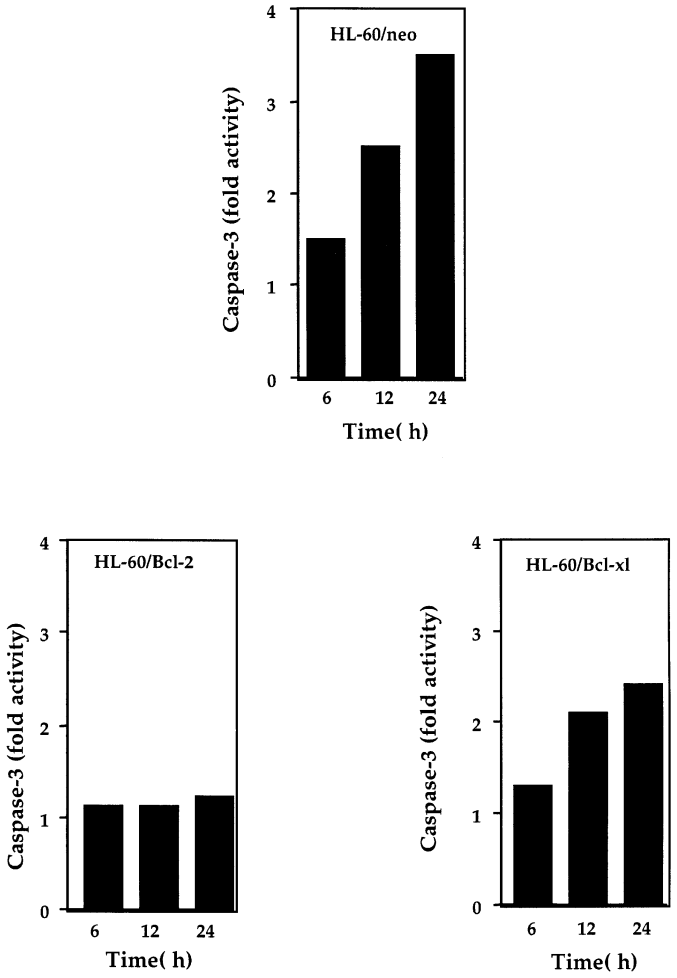

Fig. 6. Effect of curcumin on caspase-3 activation in HL-60 (neo), HL-60 (Bcl-2), HL-60 (Bcl-xl) cells. Two million cells were incubated with 25 μM curcumin for the indicated times. The caspase activation induced by curcumin was assayed as described under Materials and methods. The variation between replicates was <10%.

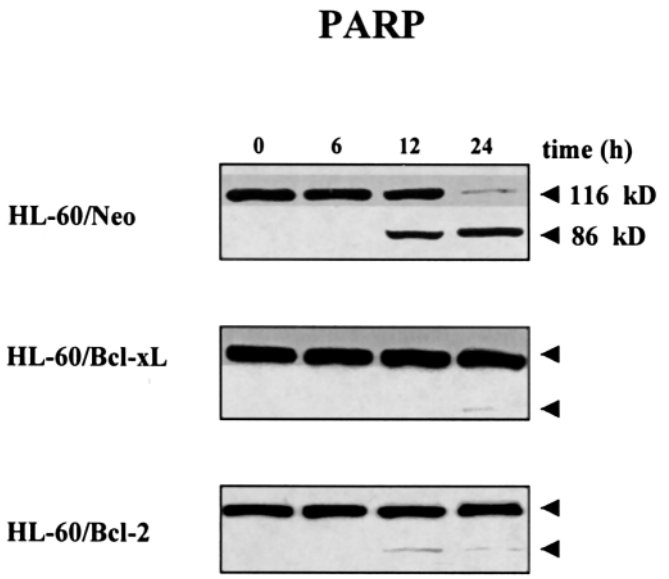

Fig. 7. Effect of curcumin on PARP cleavage in HL-60 (neo), HL-60 (Bcl-2), HL-60 (Bcl-xl) cells. Cells (2×10^6 cells in 4 ml) were plated in 35 mm dishes and incubated with 25 μM curcumin for the indicated times. Cells were harvested and cell lysates prepared. 40 μg of cell lysate was subjected to SDS–PAGE, and PARP was detected with anti-PARP antibodies as described under Materials and methods.

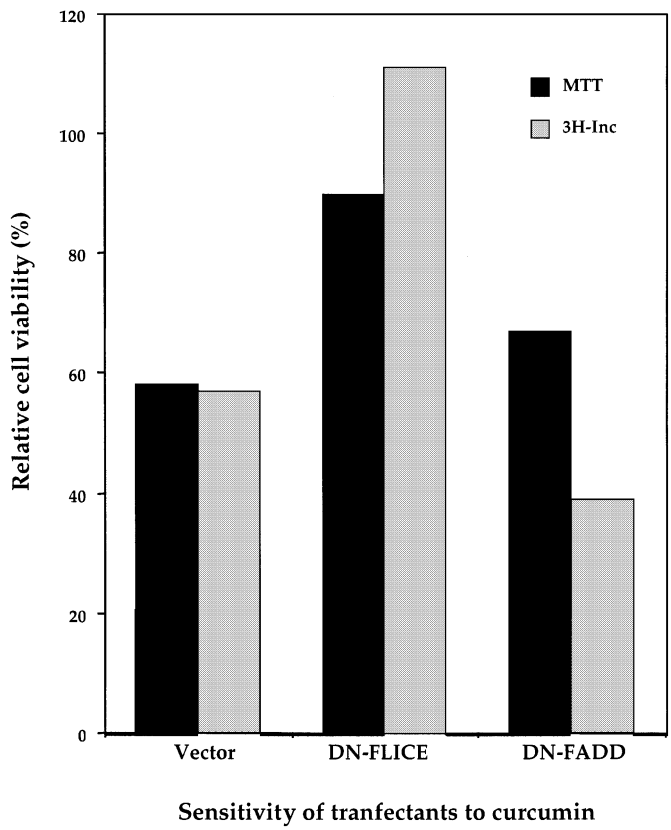

Fig. 8. Effect of curcumin on the viability of H9 cells stably transfected with neo, DN-FADD and DN-FLICE plasmids. Cells (5×10^3 in 0.2 ml) were plated in 96 well plates and incubated with 25 μM curcumin for 24 h. Cells were examined for cell viability by the MTT method (dark bars) and by the thymidine incorporation method as described in Materials and methods. All determinations were made in triplicate. The results are expressed as percentage of the control (untreated cells). The variation between triplicates was <10%.

that the curcumin can downregulate the expression of cyclooxygenase (COX)-2 (24,25). The agents that inhibit COX-2 have been shown to induce apoptosis (51). Therefore, we explored the possibility that curcumin can downregulate COX-2 expression in HL-60 cells, and that ectopic expression of Bcl-2 or Bcl-xl would prevent the effect of curcumin. We treated the cells with 25 μM curcumin for 6, 12, and 24 h, prepared the cell extracts, and examined the COX-2 expression by western blot analysis. Figure 9 indicates that COX-2 was expressed in the HL-60 (neo) cells and that curcumin dowregulated its expression (Figure 9, upper panel) in a time-dependent manner. The ectopic expression of Bcl-2 or Bcl-xl completely suppressed this downregulation (Figure 9, middle and lower panel).

Tumor cell sensitivity to curcumin correlates with the expression of Bcl-xl

Our observations so far indicate that overexpression of anti-apoptotic proteins Bcl-2 or Bcl-xl induces resistance to curcumin-induced apoptosis in HL-60 cells. Whether these results are valid with cell lines that naturally overexpress Bcl-x/Bcl-xl was examined. We found that human T-cell line Jurkat expresses no Bcl-xl, whereas Hut-78, another T-cell line, overexpresses Bcl-xl (Figure 10A). These two cell lines were

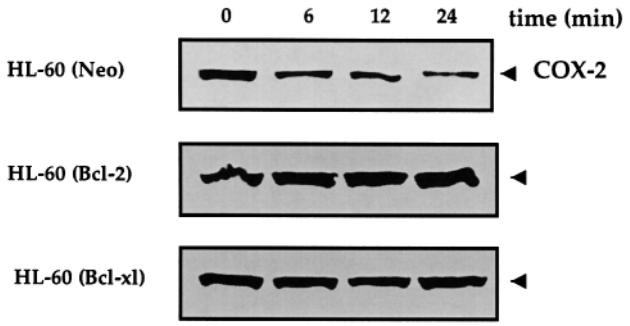

Fig. 9. Effect of curcumin on COX-2 expression in HL-60 (neo), HL-60 (Bcl-2), HL-60 (Bcl-xl) cells. Cells were plated in 35 mm dishes and incubated with 25 μM curcumin for the indicated times. Cells were harvested, and cell lysates prepared. 60 μg of cell lysate was subjected to SDS–PAGE, and COX-2 was detected with anti-COX-2 antibodies as described under Materials and methods.

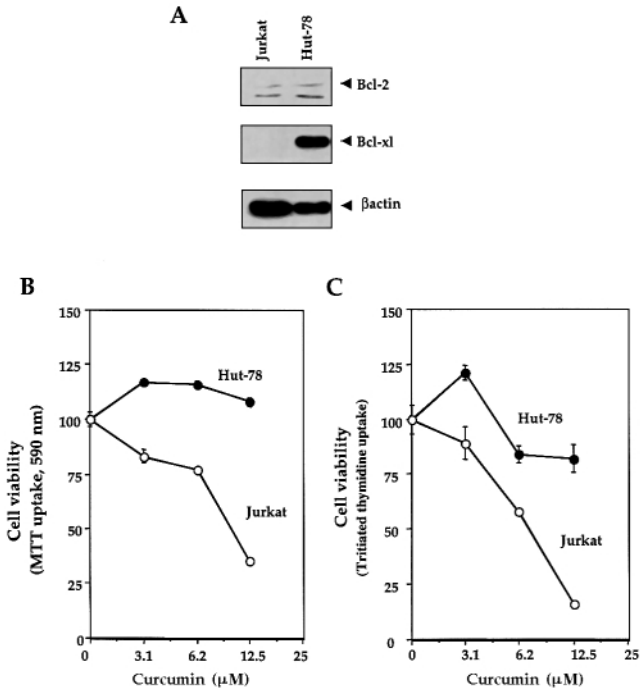

Fig. 10. Effect of curcumin on the proliferation of Jurkat and Hut-78 cells. (**A**) Expression of antiapoptotic proteins in Jurkat and Hut-78 cells. Cells were plated in 35 mm dishes and harvested, and whole-cell extracts prepared. Forty micrograms of cell lysate were subjected to SDS–PAGE, and Bcl-2 and Bcl-xl were detected using antibodies specific against each protein as described under Materials and methods. (**B** and **C**) Cells (5×10^3 cells/0.2 ml/well) were incubated with different concentrations of curcumin for 24 h, and then cell viability was examined by either the MTT method (B) or by thymidine incorporation method (C) as described in Materials and methods. All determinations were made in triplicate. The results are expressed as percentage of the control (untreated cells).

examined for sensitivity to curcumin by the MTT method and by the thymidine incorporation method. The results in Figure 10B and 10C clearly show that Jurkat cells were quite sensitive to the cytotoxic effects of curcumin whereas Hut-78 cells are resistant. These results are consistent with the thesis that Bcl-xl plays an antiapoptotic role in curcumin-induced apoptosis.

Overall, we found that curcumin induced apoptosis, and this activity correlated with the activation of an upstream caspase-8, cleavage of BID, release of cytochrome *c*, activation of a downstream caspase-3, and PARP cleavage. Our results also show that ectopic expression of the antiapoptotic proteins Bcl-2 and Bcl-xl blocked curcumin induced apoptosis, suppressing activation of caspases, cytochrome *c* release, and cleavage of BID and PARP.

Discussion

The search for new chemopreventive and antitumor agents that are more effective and less toxic has kindled great interest in phytochemicals. Curcumin, derived from the root of the plant *Curcuma longa*, is one such compound. It has been used as a dietary factor and as a herbal medicine for centuries in several south-eastern countries and has been shown to inhibit the growth of a wide variety of tumor cells. In the present report, we describe the mechanism through which curcumin induces apoptosis of human acute myelogenous leukemia HL-60 cells. Curcumin activated caspase-8, induced BID cleavage, caused mitochondrial cytochrome *c* release, and induced caspase-3 activation and PARP cleavage in HL-60 (neo) cells but not in Bcl-2 and Bcl-xl-transfected cells. We found that DN-FLICE blocked the curcumin-induced apoptosis, indicating caspase-8 is required. Curcumin also downregulated the endogenous expression of COX-2. Furthermore, we found that the ectopic expression of Bcl-2 and Bcl-xl inhibited both upstream and downstream steps involved in curcumin-induced apoptosis.

This is the first report to suggest that curcumin activates caspase-8 and that it is required for apoptosis induced by curcumin. How curcumin activates caspase-8 is not clear. Because autoactivation induced by oligomerization can activate caspase-8 (52,53), it suggests that curcumin may induce oligomerization of caspase-8. During apoptosis induced by ligands that interact with death receptors, caspase-8 is recruited by FADD and DN-FADD blocks death receptor ligand-induced apoptosis (42). Our results show that while DN-FLICE blocked curcumin-induced apoptosis, DN-FADD had no effect. These results suggest that FADD is most likely not involved in curcumin-induced apoptosis. Certain chemotherapeutic agents are known to induce apoptosis through induction of death receptors (54). Because death receptor-induced apoptosis is mediated through FADD, it suggests that curcumin most likely does not induce apoptosis through induction of death receptors. Curcumin has however, been shown to induce changes in mitochondrial membrane potential (MMP) (55). Therefore, it is possible one of the first steps by which curcumin affects apoptosis is through changes in MMP, which would lead to the release of cytochrome *c* from the mitochondria, thus leading to sequential activation of caspase-9 and caspase-3. We have indeed demonstrated the release of cytochrome *c* and the activation of caspase-3 by curcumin.

Our results indicate that curcumin can induce cleavage of the proapoptotic protein BID. Both caspase-8 and granzyme B are known to cleave BID protein (56,57). Thus it is quite likely that curcumin first activates caspase-8, which leads to cleavage of BID. Cleaved BID has been reported to translocate from the cytoplasm to the mitochondria and induce cytochrome *c* release (49). Thus it is possible that curcumin-induced cytochrome *c* release observed in our studies is mediated through BID cleavage.

Our results also indicate that ectopic expression of Bcl-2 and Bcl-xl proteins blocks curcumin-induced apoptosis and this accompanies suppression of caspase-8 activation, BID

cleavage, mitochondrial cytochrome *c* release, caspase-3 activation and PARP cleavage. How Bcl-2 and Bcl-xl mediate their effects is not fully understood. These proteins are known to inhibit apoptosis by regulating mitochondrial membrane potential (58) and cytochrome c release needed for the activation of caspase-9 (45). Both Bcl-2 and Bcl-xl are also known to form heterodimers with another pro-apoptotic member of the Bcl-2 family of protein, Bad, and thus suppress apoptosis (59). The mechanism by which Bcl-2 or Bcl-xl could block caspase-8 activation is unclear. Both Bcl-2 and Bcl-xl reside on the outer mitochondrial membrane (60), whereas caspase-8 is on the plasma membrane as a component of the death receptor complex. Bcl-xl is known to function downstream of caspase-8 to inhibit Fas and TNFR 1-induced apoptosis of MCF7 breast carcinoma cells (61). It is possible that mitochondrial Bcl-2 and Bcl-xl directly bind to procaspase-8, thus preventing its activation by curcumin.

Though both Bcl-2 and Bcl-xl are antiapoptotic (for references see (44)), the two may not be functionally redundant, as Bcl-xl was found to be a superior inhibitor of apoptosis in some cases (62,63). For instance, immunosuppressant-induced apoptosis in a murine B-cell line is prevented by Bcl-xl but not Bcl-2 (63). In our studies the suppression of apoptotic effects of curcumin were more pronounced with Bcl-xl than Bcl-2 (see Figure 2C). In most other studies, however, we found no significant difference in the activity between Bcl-2 or Bcl-xl for suppression of curcumin-induced caspases or cleavage of various substrates.

Whether all the apoptotic effects of curcumin are mediated through the mechanism as described here is not clear. Curcumin has been shown to downregulate NF-κB and AP-1 activation (23–26). The activation of NF-kB has been demonstrated to play an antiapoptotic role (64). Thus it is possible that downregulation of NF-κB may contribute to the apoptotic effects of curcumin. In fact, we have shown that ectopic expression of rel-A blocks curcumin-induced cytotoxicity (65). Additionally, NF-κB regulates the expression of COX-2 gene transcription (24,25), and ectopic expression of COX-2 has been shown to block apoptosis whereas suppression of COX-2 enhances apoptosis (51). We found that curcumin downregulates the expression of COX-2. These results are in agreement with previous reports (24,25). The suppression of COX-2 by curcumin may also contribute to the apoptotic effects of curcumin. Overall, our results suggest that curcumin can induce apoptosis by multiple mechanisms and that these mechanisms are negatively regulated by antiapoptotic proteins Bcl-2 and Bcl-xl. Besides chemopreventive (1–18), and anti-angiogeneic effects (19–21), curcumin has been shown to downregulate the expression of growth factor receptors (e.g. HER2 and EGFR) (66–68). These activities combined with its ability to induce apoptosis of tumor cells through a mechanism as described here, warrants further investigation into the chemotherapeutic effects of curcumin. It is known that consumption of spices lowers the incidence of certain type of cancers and cardiovascular diseases. For instance, epidemiological studies have revealed that the incidence of large and small bowel adenomas and cancers are extremely low in East Indians and this was attributed to the high intake of natural antioxidants such as curcumin (69).

Acknowledgements

The authors would like to thank Mr Walter Pagel for editorial assistance. This research was supported, in part, by The Clayton Foundation for Research.

References

1. Ammon,H.P. and Wahl,M.A. (1991) Pharmacology of *Curcuma longa*. *Planta Med*., **57**, 1–7.
2. Huang,M.T., Wang,Z.Y., Georgiadis,C.A. *et al.* (1992) Inhibitory effects of curcumin on tumor initiation by benzo[a]pyrene and 7,12-dimethylbenz[*a*]anthracene. *Carcinogenesis*, **13**, 2183–2186.
3. Huang,M.T., Smart,R.C., Wong,C.Q. *et al.* (1988) Inhibitory effect of curcumin, chlorogenic acid, caffeic acid and ferulic acid on tumor promotion in mouse skin by 12-*O*-tetradecanoylphorbol-13-acetate. *Cancer Res*., **48**, 5941–5946.
4. Conney,A.H., Lysz,T., Ferraro,T. *et al.* (1991) Inhibitory effect of curcumin and some related dietary compounds on tumor promotion and arachidonic acid metabolism in mouse skin. *Adv. Enzyme Regul.*, **31**, 385–396.
5. Lu,Y.P., Chang,R.L., Lou,Y.R. *et al.* (1994) Effect of curcumin on 12-*O*-tetradecanoylphorbol-13-acetate- and ultraviolet B light-induced expression of c-Jun and c-Fos in JB6 cells and in mouse epidermis. *Carcinogenesis,* **15**, 2363–2370.
6. Limtrakul,P., Lipigorngoson,S., Namwong,O. *et al.* (1997) Inhibitory effect of dietary curcumin on skin carcinogenesis in mice. *Cancer Lett.*, **116**,197–203.
7. Huang,M.T., Ma,W., Yen,P. *et al.* (1997) Inhibitory effects of topical application of low doses of curcumin on 12-*O*-tetradecanoylphorbol-13-acetate-induced tumor promotion and oxidized DNA bases in mouse epidermis. *Carcinogenesis*, **18**, 83–88.
8. Huang,M.T., Lysz,T., Ferraro,T. *et al.* (1991) Inhibitory effects of curcumin on *in vitro* lipoxygenase and cyclooxygenase activities in mouse epidermis. *Cancer Res*., **51**, 813–819.
9. Huang,M.T., Lou,Y.R., Ma,W. *et al.* (1994) Inhibitory effects of dietary curcumin on forestomach, duodenal and colon carcinogenesis in mice. *Cancer Res.*, **54**, 5841–5847.
10. Singh,S.V., Hu,X., Srivastava,S.K. *et al.* (1998) Mechanism of inhibition of benzo[*a*]pyrene-induced forestomach cancer in mice by dietary curcumin. *Carcinogenesis,* **19**, 1357–1360.
11. Kim,J.M., Araki,S., Kim,D.J. *et al.* (1998) Chemopreventive effects of carotenoids and curcumins on mouse colon carcinogenesis after 1,2-dimethylhydrazine initiation. *Carcinogenesis*, **19**, 81–85.
12. Rao,C.V., Rivenson,A., Simi,B. *et al.* (1995) Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. *Cancer Res.*, **55**, 259–266.
13. Kawamori,T., Lubet,R., Steele,V.E. *et al.* (1999) Chemopreventive effect of curcumin, a naturally occurring anti- inflammatory agent, during the promotion/progression stages of colon cancer. *Cancer Res.*, **59**, 597–601.
14. Singletary,K., MacDonald,C., Wallig,M. *et al* (1996). Inhibition of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary tumorigenesis and DMBA-DNA adduct formation by curcumin. *Cancer Lett*., **103**, 137–141.
15. Huang,M.T., Lou,Y.R., Xie,J.G. *et al.* (1998) Effect of dietary curcumin and dibenzoylmethane on formation of 7,12- dimethylbenz[*a*]anthracene-induced mammary tumors and lymphomas/leukemias in Sencar mice. *Carcinogenesis,* **19**, 1697–1700.
16. Inano,H., Onoda,M., Inafuku,N. *et al.* (1999) Chemoprevention by curcumin during the promotion stage of tumorigenesis of mammary gland in rats irradiated with gamma-rays. *Carcinogenesis,* **20**, 1011–1018.
17. Chuang,S.E., Kuo,M.L., Hsu,C.H. *et al.* (2000) Curcumin-containing diet inhibits diethylnitrosamine-induced murine hepatocarcinogenesis. *Carcinogenesis,* 21, 331–335.
18. Menon,L.G., Kuttan,R. and Kuttan,G. (1999) Anti-metastatic activity of curcumin and catechin. *Cancer Lett*.,**141**, 159–165.
19. Singh,A.K., Sidhu,G.S., Deepa,T. *et al.* (1996) Curcumin inhibits the proliferation and cell cycle progression of human umbilical vein endothelial cell. *Cancer Lett.*, **107**, 109–115.
20. Arbiser,J.L., Klauber,N., Rohan,R. *et al.* (1998) Curcumin is an *in vivo* inhibitor of angiogenesis. *Mol Med*., **4**, 376–383.
21. Mohan,R., Sivak,J., Ashton,P. *et al.* (2000) Curcuminoids inhibit the angiogenic response stimulated by fibroblast growth factor-2, including expression of matrix metalloproteinase gelatinase B. *J. Biol. Chem.*, **275**, 10405–10412.
22. Lin,L.I., Ke,Y.F., Ko,Y.C. *et al.* (1998) Curcumin inhibits SK-Hep-1 hepatocellular carcinoma cell invasion *in vitro* and suppresses matrix metalloproteinase-9 secretion. *Oncology*, **55**, 349–353.
23. Kumar,A., Dhawan,S., Hardegen,N.J. *et al.* (1998) Curcumin (Diferuloylmethane) inhibition of tumor necrosis factor (TNF)-mediated adhesion of monocytes to endothelial cells by suppression of cell surface expression of adhesion molecules and of nuclear factor-kappaB activation. *Biochem. Pharmacol.*, **55**, 775–783.

24. Zhang,F., Altorki,N.K., Mestre,J.R. *et al.* (1999) Curcumin inhibits cyclooxygenase-2 transcription in bile acid- and phorbol ester-treated human gastrointestinal epithelial cells. *Carcinogenesis*, **20**, 445–451.
25. Plummer,S.M., Holloway,K.A., Manson,M.M. *et al.* (1999) Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-kappaB activation via the NIK/IKK signalling complex. *Oncogene*, **18**, 6013–6020.
26. Singh,S. and Aggarwal,B.B. (1995) Activation of transcription factor NF-kappa B is suppressed by curcumin (diferuloylmethane) (corrected). *J. Biol. Chem.*, **270**, 24995–25000.
27. Huang,T.S., Lee,S.C. and Lin,J.K. (1991) Suppression of c-Jun/AP-1 activation by an inhibitor of tumor promotion in mouse fibroblast cells. *Proc Natl Acad. Sci. USA*, **88**, 5292–5296.
28. Chen,Y.R. and Tan,T.H. (1998) Inhibition of the c-Jun N-terminal kinase (JNK) signaling pathway by curcumin. *Oncogene*, **17**, 173–178.
29. Kuo,M.L., Huang,T.S. and Lin,J.K. (1996) Curcumin, an antioxidant and anti-tumor promoter, induces apoptosis in human leukemia cells. *Biochim. Biophys. Acta*, **1317**, 95–100.
30. Ranjan,D., Johnston,T.D., Reddy,K.S. *et al.* (1999) Enhanced apoptosis mediates inhibition of EBV-transformed lymphoblastoid cell line proliferation by curcumin. *J. Surg. Res*., **87**, 1–5.
31. Piwocka,K., Zablocki,K., Wieckowski,M.R. *et al.* (1999) A novel apoptosis-like pathway, independent of mitochondria and caspases, induced by curcumin in human lymphoblastoid T (Jurkat) cells. *Exp. Cell Res*., **249**, 299–307.
32. Han,S.S., Chung,S.T., Robertson,D.A. *et al.* (1999) Curcumin causes the growth arrest and apoptosis of B cell lymphoma by downregulation of egr-1, c-myc, bcl-XL, NF-kappa B and p53. *Clin. Immunol.*, **93**, 152–161.
33. Chen,H., Zhang,Z.S., Zhang,Y.L. *et al.* (1999) Curcumin inhibits cell proliferation by interfering with the cell cycle and inducing apoptosis in colon carcinoma cells. *Anticancer Res.*, **19**, 3675–3680.
34. Mehta,K., Pantazis,P., McQueen,T. *et al.* (1997) Antiproliferative effect of curcumin (diferuloylmethane) against human breast tumor cell lines. *Anticancer Drugs*, **8**, 470–481.
35. Simon,A., Allais,D.P., Duroux,J.L. *et al.* (1998) Inhibitory effect of curcuminoids on MCF-7 cell proliferation and structure–activity relationships. *Cancer Lett.*, **129**, 111–116.
36. Ramachandran,C. and You,W. (1999) Differential sensitivity of human mammary epithelial and breast carcinoma cell lines to curcumin. *Breast Cancer Res. Treat.*, **54**, 269–278.
37. Jiang,M.C., Yang-Yen,H.F., Yen,J.J. *et al.* (1996) Curcumin induces apoptosis in immortalized NIH 3T3 and malignant cancer cell lines. *Nutr. Cancer*, **26**, 111–120.
38. Bhaumik,S., Anjum,R., Rangaraj,N. *et al.* (1999) Curcumin mediated apoptosis in AK-5 tumor cells involves the production of reactive oxygen intermediates. *FEBS Lett.*, **456**, 311–314.
39. Chen,H.W. and Huang,H.C. (1998) Effect of curcumin on cell cycle progression and apoptosis in vascular smooth muscle cells. *Br. J. Pharmacol.*, **124**, 1029–1040.
40. Ozaki,K., Kawata,Y., Amano,S. *et al* (2000) Stimulatory effect of curcumin on osteoclast apoptosis. *Biochem. Pharmacol*., **59**, 1577–1581.
41. Jaruga,E., Bielak-Zmijewska,A., Sikora,E. *et al.* (1998) Glutathione-independent mechanism of apoptosis inhibition by curcumin in rat thymocytes. *Biochem. Pharmacol*., **56**, 961–965.
42. Ashkenazi,A. and Dixit,V.M. (1999) Apoptosis control by death and decoy receptors. *Curr. Opin. Cell Biol.*, **11**, 255–260.
43. Sun,X.M., MacFarlane,M., Zhuang,J. *et al.* (1999) Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. *J. Biol. Chem*., **274**, 5053–5060.
44. Minn,A.J., Swain,R.E., Ma,A. *et al.* (1998) Recent progress on the regulation of apoptosis by Bcl-2 family members. *Adv. Immunol.*, **70**, 245–279.
45. Yang,J., Liu,X., Bhalla,K. *et al.* (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science*, **275**, 1129–1132.
46. Perkins,C., Kim,C.N., Fang,G. *et al.* (2000) Arsenic induces apoptosis of multidrug-resistant human myeloid leukemia cells that express Bcr-Abl or overexpress MDR, MRP, Bcl-2, or Bcl-x (L). *Blood*, **95**, 1014–1022.
47. Haridas,V., Darnay,B.G., Natarajan,K. *et al.* (1998) Overexpression of the p80 TNF receptor leads to TNF-dependent apoptosis, nuclear factor-kappa B activation and c-Jun kinase activation. *J. Immunol.*, **160**, 3152–3162.
48. Luo,X., Budihardjo,I., Zou,H. *et al.* (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell*, **94**, 481–490.
49. Gross,A., Yin,X.M., Wang,K. *et al.* (1999) Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *J. Biol. Chem.*, **274**, 1156–1163.
50. Tewari,M., Quan,L.T., O'Rourke,K. *et al.* (1995) 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.
51. Williams,C.S,. Tsujii,M., Reese,J. *et al.* (2000) Host cyclooxygenase-2 modulates carcinoma growth. *J. Clin. Invest.*, **105**,1589–1594.
52. Muzio,M., Stockwell,B.R., Stennicke,H.R. *et al.* (1998) An induced proximity model for caspase-8 activation. *J. Biol. Chem.*, **273**, 2926–2930.
53. Srinivasula,S.M, Ahmad,M., Fernandes-Alnemri,T. *et al.* (1998) Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol. Cell*, **1**, 949–957.
54. Muller,M., Strand,S., Hug,H. *et al.* (1997) Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. *J. Clin. Invest*., **99**, 403–413.
55. Jaruga,E., Sokal,A., Chrul,S. *et al.* (1998) Apoptosis-independent alterations in membrane dynamics induced by curcumin. *Exp. Cell Res.*, **245**, 303–312.
56. Sutton,V.R., Davis,J.E., Cancilla,M. *et al.* (2000) Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation. *J. Exp. Med.*, **192**,1403–1414.
57. Desagher,S., Osen-Sand,A., Nichols,A. *et al.* (1999) Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J. Cell Biol*., **144**, 891–901.
58. Vander Heiden,M.G., Chandel,N.S., Williamson,E.K. *et al.* (1997) Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. *Cell*, **91**, 627–637.
59. Yang,E., Zha,J., Jockel,J. *et al.* (1995) Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell*, **80**, 285–291.
60. Vander Heiden,M.G., Chandel,N.S., Schumacker,P.T. *et al.* (1999) Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. *Mol. Cell*, **3**,159–167.
61. Srinivasan,A., Li,F., Wong,A. *et al.* (1998) Bcl-xL functions downstream of caspase-8 to inhibit Fas- and tumor necrosis factor receptor 1-induced apoptosis of MCF7 breast carcinoma cells. *J. Biol. Chem.*, **273**, 4523–4529.
62. Boise,L.H., Gonzalez-Garcia,M., Postema,C.E. *et al.* (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell*, **74**, 597–608.
63. Gottschalk,A.R., Boise,L.H., Thompson,C.B. *et al.* (1994) Identification of immunosuppressant-induced apoptosis in a murine B-cell line and its prevention by bcl-x but not bcl-2. *Proc. Natl Acad. Sci. USA*, **91**, 7350–7354.
64. Aggarwal,B.B. (2000) Apoptosis and nuclear factor-kappa B: a tale of association and dissociation. *Biochem. Pharmacol.*, **60**, 1033–1039.
65. Anto,R.J., Maliekal,T.T. and Karunagaran,D. (2000) L-929 cells harboring ectopically expressed RelA resist curcumin-induced apoptosis. *J. Biol. Chem*., **275**, 15601–15604.
66. Korutla,L. and Kumar,R. (1994) Inhibitory effect of curcumin on epidermal growth factor receptor kinase activity in A431 cells. *Biochim. Biophys. Acta*, **1224**, 597–600.
67. Korutla,L., Cheung,J.Y., Mendelsohn,J. *et al.* (1995) Inhibition of ligand-induced activation of epidermal growth factor receptor tyrosine phosphorylation by curcumin. *Carcinogenesis,* **16**, 1741–1745.
68. Hong,R.L., Spohn,W.H. and Hung,M.C. (1999) Curcumin inhibits tyrosine kinase activity of p185neu and also depletes p185neu. *Clin. Cancer Res.*, **5**,1884–1891.
69. Mohandas,K.M. and Desai,D.C. (1999) Epidemiology of digestive tract cancers in India. V. Large and small bowel. *Indian J. Gastroenterol*., **18**, 118–121.

Received July 16, 2001; revised September 21, 2001;
accepted September 28, 2001