

Curcumin, an antioxidant and anti-tumor promoter, induces apoptosis in human leukemia cells

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Abstract

Curcumin, widely used as a spice and coloring agent in food, possesses potent antioxidant, anti-inflammatory and anti-tumor promoting activities. In the present study, curcumin was found to induce apoptotic cell death in promyelocytic leukemia HL-60 cells at concentrations as low as 3.5 $\mu\text{g}/\text{ml}$. The apoptosis-inducing activity of curcumin appeared in a dose- and time-dependent manner. Flow cytometric analysis showed that the hypodiploid DNA peak of propidium iodide-stained nuclei appeared at 4 h after 7 $\mu\text{g}/\text{ml}$ curcumin treatment. The apoptosis-inducing activity of curcumin was not affected by cycloheximide, actinomycin D, EGTA, W_7 (calmodulin inhibitor), sodium orthovanadate, or genistein. By contrast, an endonuclease inhibitor ZnSO_4 and proteinase inhibitor *N*-tosyl-L-lysine chloro-methyl ketone (TLCK) could markedly abrogate apoptosis induced by curcumin, whereas 12-*O*-tetradecanoylphorbol-13-acetate (TPA) had a partial effect. The antioxidants, *N*-acetyl-L-cysteine (NAC), L-ascorbic acid, alpha-tocopherol, catalase and superoxide dismutase, all effectively prevented curcumin-induced apoptosis. This result suggested that curcumin-induced cell death was mediated by reactive oxygen species. Immunoblot analysis showed that the level of the antiapoptotic protein Bcl-2 was decreased to 30% after 6 h treatment with curcumin, and was subsequently reduced to 20% by a further 6 h treatment. Furthermore, overexpression of *bcl-2* in HL-60 cells resulted in a delay of curcumin-treated cells entering into apoptosis, suggesting that *bcl-2* plays a crucial role in the early stage of curcumin-triggered apoptotic cell death.

Keywords: Antioxidant; Antitumor drug; Apoptosis; Leukemia; (Human)

1. Introduction

Curcumin (diferuloylmethane) is a major active component of the food flavor, turmeric (*Curcuma longa*). Several studies in recent years have shown that curcumin is a potent inhibitor of the initiation and promotion of chemical carcinogen-induced tumor formation in animals [1–3]. Besides its anti-carcinogenic effects, curcumin exhibits remarkable anti-inflammatory and anti-oxidant properties in vivo [4–6]. The pharmacological safety of curcumin is demonstrated by its consumption for centuries at levels of up to 100 mg/day by people in certain countries [4]. However, the mechanism underlying the diverse effects of curcumin is not fully understood. One possible molecular

mechanism that has been suggested is that curcumin can suppress the phorbol ester-induced transcriptional factor *c-jun/AP-1* [7]. Recently, Korutla and Kumar [8] showed that 10 μM curcumin is capable of inhibiting the intrinsic kinase activity of epidermal growth factor (EGF) receptor, leading to growth inhibition of A431 cells. This indicates that curcumin is probably a potent anti-tumor agent for some cancer cells.

Apoptosis, a mode of cell death, plays a crucial role in embryonic development, metamorphosis, hormone-dependent atrophy, and tumor growth as a physiological event, regulating cell number and eliminating damaged cells [9,10]. Several studies have demonstrated that apoptosis may be involved in cell death induced by chemotherapeutic agents including cisplatin, camptothecin, amifostine, etoposide, and teniposide [11–13]. There is accumulating evidence that the efficiency of anti-tumor agents is related to the intrinsic propensity of the target tumor cells to respond to these agents by apoptosis [13]. The present

Abbreviations: TLCK = *N*-tosyl-L-lysine chloro-methyl ketone; TPA = 12-*O*-tetradecanoylphorbol-13-acetate; PKC = protein tyrosine kinase; PKC = protein kinase C; NAC = *N*-acetyl-L-cysteine

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study was designed to investigate the mechanism by which curcumin exerts apoptosis-induced activity in tumor cells. In addition, the role Bcl-2 plays in curcumin-induced apoptosis is also investigated.

2. Materials and methods

2.1. Cell culture and chemicals

Curcumin, cycloheximide, *N*-acetylcysteine, zinc sulfate and TLCK all obtained from Sigma Chemical Co. (Santa Ana, CA, USA). Superoxide dismutase and catalase were purchased from Boehringer Mannheim Co., Germany. Human promyelocytic leukemia (HL-60), human chronic myeloid leukemia (K562) and human colon adenocarcinoma (SW620) were obtained from American Type Culture Collection (Rockville, MD). These cells were cultured in RPMI 1640 medium supplemented with 10% FCS; Hep3B (human hepatocellular carcinoma) was maintained in DMEM medium supplemented with 10% FCS.

2.2. Electron microscopy

Curcumin-treated and untreated cells (0.1% DMSO) were fixed with fixative containing 2% glutaraldehyde and 2% paraformaldehyde in PBS for 15 min. Following several rinses in PBS, cells were postfixed in 1% osmium tetroxide, dehydrated in alcohol and embedded in Epon-Araldite mixture. The sections were cut and doubly stained with uranyl acetate and lead citrate before being examined in a Joel 2000 EXII electron microscope at 100 KV.

2.3. DNA extraction and electrophoresis

Cells (1×10^6) were treated with different concentrations of curcumin for 16 h or fixed concentration for varying exposures of time, then harvested and washed twice in ice-cold PBS, resuspended in 500 μ l TE (10 mM Tris-HCl pH 7.6, 1 mM EDTA pH 8.0), and lysed in 500 μ l lysis buffer (3% SDS, 50 mM Tris, pH 12.6) at room temperature for 10 min. DNA was extracted with phenol and chloroform before precipitation with 95% ethanol. DNA pellets were finally solubilized in TE buffer, and treated with RNase A for 40 min prior to 1.2% agarose gel electrophoresis.

2.4. Flow cytometry analysis

At an indicated time (0, 4, 8 and 24 h), curcumin-treated or untreated cells were harvested and fixed in 75% ethanol at -20°C for at least 1 h. After centrifugation at 800 rpm for 5 min at 4°C , cell pellets were resuspended in 0.5 ml of a buffer (0.5% Triton X-100/PBS and 0.05% RNase A), and incubated for 30 min. Finally, 0.5 ml of propidium iodide (PI) solution (50 μ g/ml) was added, and cells were

allowed to stand on ice for 15–30 min. Fluorescence emitted from the P.I-DNA complex was quantified after laser excitation of the fluorescent dye on a FACSSor flow cytometer (Becton-Dickinson). The effect of various modulators on curcumin-triggered apoptosis was also examined and quantified by FACS analysis.

2.5. Establishment of HL60-Bcl-2 cells

HL-60 cells constitutively expressing human *bcl-2* (HL60-Bcl-2 cells) were created by electroporation (model T800; BTX, San Diego, CA, USA) of HL-60 cells with *bcl-2* expression vector, pC Δ j-bcl2 (a gift from Dr. S.-F., Yang of the Institute of Molecular Biology, Academic Sinica, Taiwan). The expression vector pC Δ j-bcl-2 that carrying the human Bcl-2 cDNA under control of the SV40 promoter/enhancer sequence was developed by Tsujimoto [14] and recently has been used in study on the drug resistance in ovarian cancer [15]. Briefly, cells were suspended in 1 ml Hepes-buffered saline containing plasmid DNA, then received electric treatment with different conditions as follows, electric amplitude, 300–350 V; pulse width, 99 μ s, and subsequently each population of cells was cultured in medium containing G418 (100 μ g/ml). The levels of Bcl-2 expression of each population of cells were examined by immunoblotting. Subsequently, a cell line HL-60-Bcl-2-350, which expressed the most abundant Bcl-2 protein, was selected to perform the apoptosis assay.

2.6. Immunoblotting

Cellular lysates were prepared as described previously [16]. A 50 μ g sample of each lysate was subjected to electrophoresis on 10% SDS-polyacrylamide gels for detection of Bcl-2. The samples were then electroblotted on nitrocellulose paper. After blocking, blots were incubated with anti-Bcl-2 (Santa Cruz, California, USA) antibody in PBST for 1 hour followed by two washes (15 min each) in PBST, and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham, USA) for 30 min. After washing, blots were incubated for 1 minute with Western blotting reagent ECL (Amersham, USA), and chemiluminescence was detected by exposure of the filters to Kodak-X-Omat films for 30 s to 30 min.

3. Results

Curcumin exhibited a remarkable cytotoxic effect on various cancer cells, including HeLa (cervical carcinoma), SW620 (colon cancer), MCF-7 (breast cancer), Hep 3B (hepatocarcinoma) K562 and HL-60 (leukemia) (Fig. 1). Among them, HL-60 cells seemed to be the most susceptible to curcumin treatment, with an LC₅₀ of approx. 5 μ g/ml. To determine the mode of cell death induced by

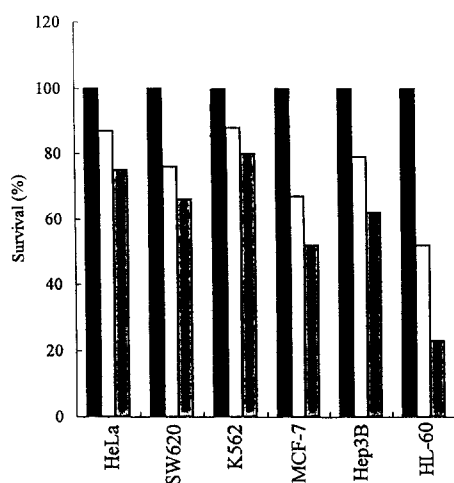


Fig. 1. The relative cell survival of various kinds of cancer cells after curcumin treatment. Each type of cancer cells were seeded at 5×10^5 and viable cells numbers were determined 48 h after culturing in media containing without (0.1% DMSO, black bar) or with 5 µg/ml (white bar) or 10 µg/ml (grey bar) of curcumin. Cell viability was determined by staining cells with trypan blue followed by counting the number of live cells. These are representative data of three separate experiments.

curcumin, HL-60 cells were treated with 5 µg/ml curcumin and morphological alterations were subsequently examined via electron microscopy. Morphological changes characteristic of apoptosis were disappearance of microvilli, cell shrinkage, chromatin condensation and appearance of blebbing and many apoptotic bodies (Fig. 2). Agarose gel electrophoresis of curcumin-treated chromosomal DNA showed a ladder-like pattern of DNA fragments consisting of multiples of approx. 180–200 base pairs (Fig. 3A and B). The apoptosis-inducing activity of curcumin

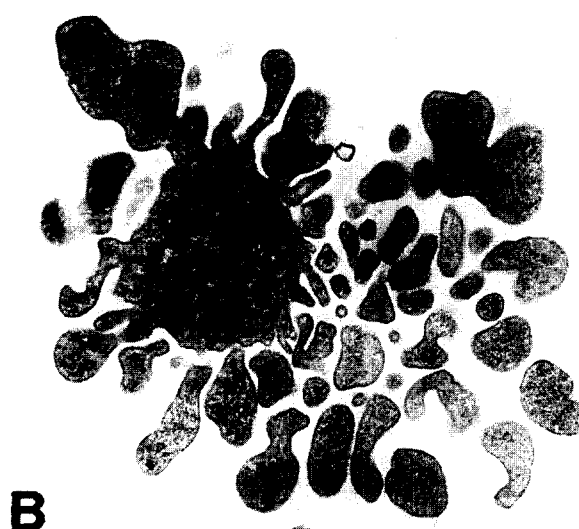
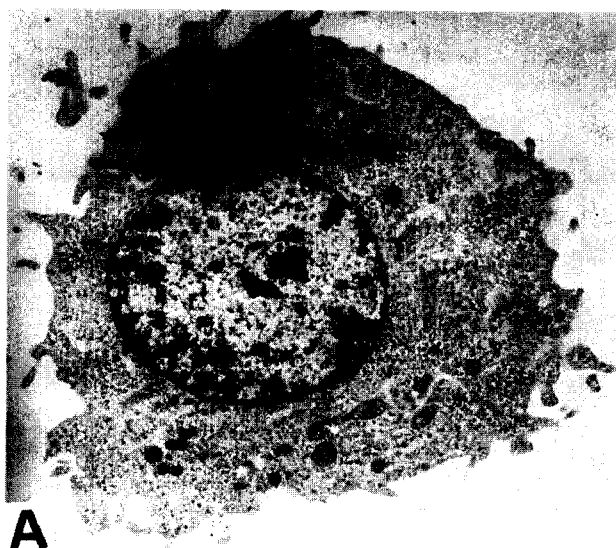


Fig. 2. Morphology of curcumin-treated HL-60 leukemia cells. Cells treated with 7 µg/ml curcumin for 24 h were fixed with 2% glutaraldehyde for 15 min. Following several rinses in PBS, cells were postfixed and dehydrated as described in Section 2. (A) Control HL-60 cells exposed to 0.1% DMSO ($\times 2800$). (B) HL-60 cells exposed to curcumin (7 µg/ml) ($\times 2800$).

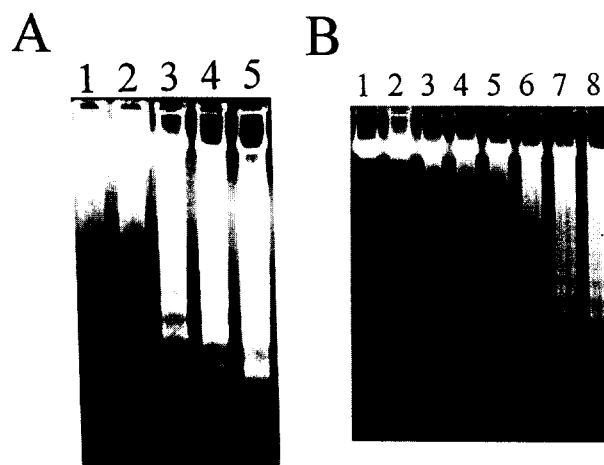


Fig. 3. Agarose gel electrophoresis of DNA extracted from HL-60 cells exposed with various concentrations or fixed concentration for varying periods of time of curcumin. (A) HL-60 cells (1×10^6) were incubated with 0.1% DMSO (lane 1) or with 1.75 µg/ml (lane 2), 3.5 µg/ml (lane 3), 7 µg/ml (lane 4), and 14 µg/ml (lane 5) curcumin for 16 h; (B) HL-60 cells were incubated with 3.5 µg/ml curcumin for 0 h (lane 1), 1 h (lane 2), 2 h (lane 3), 3 h (lane 4), 4 h (lane 5), 8 h (lane 6), 12 h (lane 7) and 16 h (lane 8). DNA was isolated and subjected to electrophoresis on 1.2% agarose gel as described Section 2.

was dose- and time-dependent, being observed at concentration as low as 3.5 µg/ml (Fig. 3A). However, if the concentration was increased up to 14 µg/ml, chromosomal DNA appeared smearing rather than laddering, indicating that the cells underwent necrotic cell death. An early DNA fragmentation was seen at 2–4 h after exposure to 3.5 µg/ml curcumin (Fig. 3B). To further determine the degree of apoptosis, we employed flow cytometry to quan-

Table 1
Effects of various modulators on the curcumin-induced apoptotic cell death

Treatment	Dose	% Apoptosis
DMSO	0.1%	5
Curcumin	7 $\mu\text{g}/\text{ml}$	62
+ Cycloheximide	1 $\mu\text{g}/\text{ml}$	68
+ Actinomycin D	10 ng/ml	66
+ EGTA	1 mmol/ml	71
+ W_7	20 ng/ml	60
+ Na_3VO_4	20 $\mu\text{mol}/\text{ml}$	65
+ Genistein	20 $\mu\text{mol}/\text{ml}$	68
+ ZnSO_4	2 mmol/ml	15
+ TLCK	10 $\mu\text{mol}/\text{ml}$	23
+ TPA	50 ng/ml	50
+ <i>N</i> -acetyl-L-cysteine	5 mmol/ml	12
+ L-ascorbic acid	100 $\mu\text{mol}/\text{ml}$	26
+ Alpha-tocopherol	100 $\mu\text{mol}/\text{ml}$	25
+ Catalase	200 $\mu\text{g}/\text{ml}$	18
+ SOD	100 $\mu\text{g}/\text{ml}$	20

HL-60 cells were pretreated for 16 h with previously determined noncytotoxic doses of the agents and for a further 24 hrs with 7 $\mu\text{g}/\text{ml}$ curcumin. Apoptotic cells were quantitated by flow cytometry analysis of propidium iodide-stained samples, as described in Section 2. Data are averages of two independent experiments.

tify the sub- G_1 peak (apoptotic peak). Fig. 4 shows that when HL-60 cells were exposed to 7 $\mu\text{g}/\text{ml}$ curcumin for 4, 8 and 24 h resulted in 35, 42 and 68% apoptosis, respectively.

To determine the events involved in curcumin-induced apoptosis, we examined a number of agents of known biochemical activity for their capacity to interfere with the cell death process. For this purpose, HL-60 cells were pretreated for 16 h with previously determined noncytotoxic doses of the specified agents and for a further 24 h with 7 $\mu\text{g}/\text{ml}$ curcumin. Cells with apoptotic DNA were quantified in suspension by propidium-iodide staining and flow cytofluorimetry analysis. Table 1 shows that cycloheximide or actinomycin D (inhibitors of protein synthesis

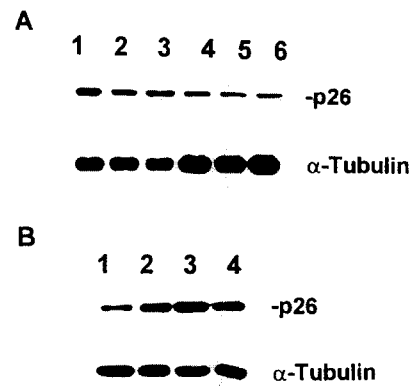


Fig. 5. A. Effects of curcumin on the levels of Bcl-2 protein determined by immunoblotting. Cells were exposed to 7 $\mu\text{g}/\text{mL}$ curcumin, and harvested at different time periods as follows: (1) untreated cells; (2) 1 h of exposure; (3) 2 h of exposure; (4) 4 h of exposure; (5) 6 h of exposure; (6) 12 h of exposure. After treatment, equal amount (50 $\mu\text{g}/\text{lane}$) of each cell lysate was electrophoresed on SDS-PAGE under reducing conditions, and blotted. B. Stable transfection of HL-60 cells with a *bcl-2* expression vector. Cells received electric treatment with different conditions as described in Section 2. The levels of Bcl-2 protein of each population of cells, as indicated as follows: HL-60 NEO (lane 1), HL-60-Bcl-2-300 (lane 2), HL-60-Bcl-2-350 (lane 3) and HL-60-Bcl-2-400 (lane 4) were determined by Western blotting. HL-60 NEO cells were established by electroporation with a control *neo* gene containing plasmid.

and RNA transcription, respectively) could not affect the extent of apoptotic cells induced by curcumin. Deprivation of intracellular calcium by EGTA or addition of calmodulin inhibitor W_7 did not influence curcumin-induced apoptosis. Deregulation of phosphotyrosine (P-tyr) status by the protein tyrosine phosphatase inhibitor, sodium orthovanadium, or the protein tyrosine kinase (PTK) inhibitor, genistein, did not interfere with curcumin-mediated cell death. In contrast, an endonuclease inhibitor ZnSO_4 and proteinase inhibitor TLCK could markedly abrogate apoptosis induced by curcumin, whereas TPA (activator of PKC) had a partial effect. The antioxidants, *N*-acetyl-L-cysteine, L-ascorbic acid, alpha-tocopherol, catalase and su-

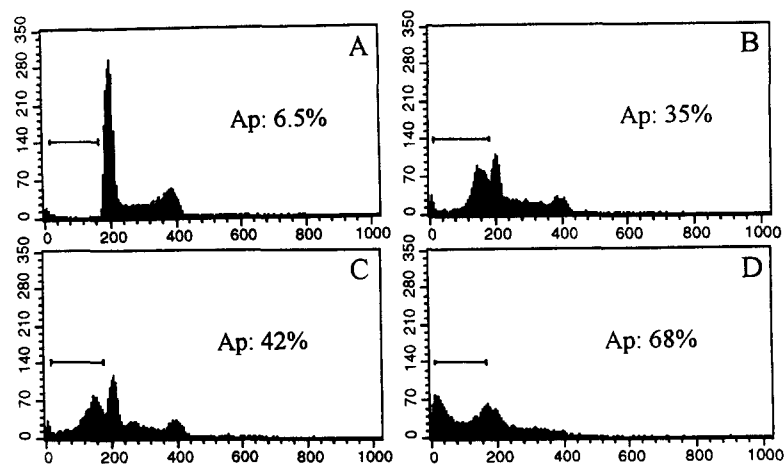


Fig. 4. DNA fluorescence histograms of PI-stained HL-60 cells in FL2-H. Cells were treated with 7 $\mu\text{g}/\text{ml}$ of curcumin for varying periods of time. A, 0 h; B, 4 h; C, 8 h; D, 24 h. The values indicated in figure represent the percentage of apoptosis. Ap, apoptosis.

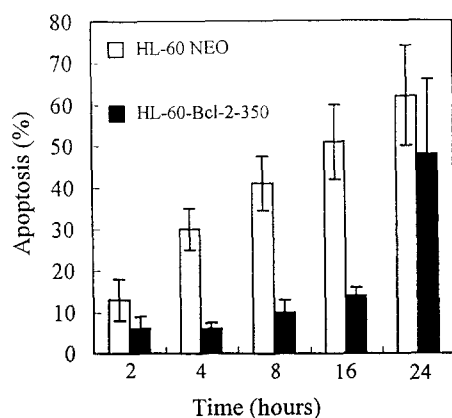


Fig. 6. Overexpression of Bcl-2 confers resistance to apoptosis mediated by curcumin. Cells transfected with *bcl-2* expressing vector (HL-60-Bcl-2-350) or with control *neo* gene plasmid (HL-60 NEO), were exposed to 7 $\mu\text{g}/\text{ml}$ curcumin for indicated time. Cells with apoptotic DNA were quantitated by propidium-iodide staining and flow cytometry analysis. Values shown are means from three separate experiments; the standard errors of mean values are marked by bars.

peroxide dismutase, all effectively prevented curcumin-induced apoptosis.

In an attempt to explore the role of Bcl-2 protein in curcumin-induced apoptosis, we examined the level of Bcl-2 protein in HL-60 cells after treatment with curcumin. Immunoblot analysis of Bcl-2 showed that the antiapoptotic protein was decreased to 30% after 6 h treatment of curcumin, and subsequently was reduced to 20% by further 6 h treatment (Fig. 5A), as compared to control. To determine if deregulated expression of Bcl-2 modifies the susceptibility to death after curcumin exposure, a FACS analysis was performed on the HL-60-Bcl-2-350, a cell line that had been transduced with a *bcl-2* expression plasmid, contained elevated levels of Bcl-2 protein (Fig. 5B). Flow cytometric analysis of hypodiploid cells clearly demonstrated that, while *bcl-2* overexpression delayed entry into apoptosis, e.g., exposing HL-60 cells to 7 $\mu\text{g}/\text{ml}$ curcumin for 8 and 16 h resulted in 41% and 50% apoptotic cells, respectively. In contrast, greater than 85% of HL60-Bcl-2-350 cells were still viable after 16 h treatment with the same concentration of curcumin (Fig. 6). Moreover, Bcl-2 did not influence the cell-cycle distribution of the residual viable cells (data not shown). However, there was no significant difference in apoptosis between HL-60 and HL-60-Bcl-2-350 cells after 24 h exposure to curcumin. These results indicated that the Bcl-2 protein could prevent curcumin-induced apoptotic cell death during early exposure time.

4. Discussion

In the present study we demonstrated that the dietary component curcumin induce apoptosis in human leukemia HL-60 cells at the very low concentration of 3.5 $\mu\text{g}/\text{ml}$.

In addition, the cell death did not occur at a specific phase of cell cycle and as early as 4 h exposure to curcumin resulted in easily detectable chromatin DNA fragmentation. Neither cycloheximide nor actinomycin D could effect curcumin-induced cell death, suggesting that the cell death process did not require new gene products. Curcumin has been shown to inhibit protein kinase C (PKC) [17] and protein tyrosine kinase (PTK) [8] activity in various cell systems. This led us to propose that apoptosis by curcumin may be through means of modulating both kinases. However, neither increasing p-tyr level by orthovanadate nor activation of PKC by TPA prevented the apoptotic cell death, thereby suggesting that PKC and PTK-associated pathways are not involved in curcumin-induced apoptosis. Although calcium- or calmodulin-dependent pathway has been reported to be related to antioxidant gallic acid-mediated apoptosis [18], we did not find the same mechanism was involved in curcumin induced cell death.

Several observations indicate that oxidative stress is an important mediator of apoptosis. For instance, ionizing and ultraviolet radiation, low concentration of H_2O_2 , and certain cytokines stimulate apoptosis and generate reactive oxygen intermediates (ROIs) [19]. On the other hand, antioxidants, such as gallic acid and all-trans retinoic acid, also induced apoptosis in different cell lines by generation of oxidative stress [18,20]. We show here that the antioxidant curcumin-induced cell death can be suppressed by some well-known scavengers of ROIs. This strongly implies that oxidative injury may play a pivotal role in curcumin-elicited apoptotic cell death. Considering some important cellular enzymes, e.g., PTK and PKC, were effectively inhibited by curcumin, it is therefore relevant that antioxidant enzymes such as, superoxide dismutase or catalase, might also be interfered by curcumin, and this interference may subsequently result in an elevation of ROIs within target cells. However, the exact mechanism by which curcumin induce ROIs is completely unknown and remains to be investigated.

Gene transfer studies in several types of mammalian cells have shown that elevations in Bcl-2 protein levels can protect cells from death induced by a wide variety of diverse insults and stimuli, suggesting that Bcl-2 controls a distal step in what may represent a final common pathway for apoptotic cell death [21]. Moreover, recent data support a role for Bcl-2 in an antioxidant pathway, whereby this protein prevents cell death by decreasing the formation of ROIs and lipid peroxidation. Here we found that Bcl-2 proteins can be decreased dramatically by 6 h treatment with curcumin. But, the levels of mRNA did not change during the treatment of curcumin (data not shown). This suggests that curcumin regulates the Bcl-2 expression at a posttranslational level. An interesting result presented here demonstrates that TLCK, a protease inhibitor, significantly abolished curcumin-triggered cell death. This raises a possibility that the protection by TLCK may be through

stabilizing the Bcl-2 proteins via inhibiting some novel proteases. Furthermore, gene transfer study provided convincing evidences that elevation of Bcl-2 protein indeed delayed apoptosis in curcumin-treated HL-60 cells. Taken together, these findings suggest that Bcl-2 proteins play a prominent role in curcumin-induced apoptotic cell death.

The chemoprevention of cancer with curcumin has been intensively investigated [1–3]. In the present study we first demonstrated that curcumin exhibited pronounced anti-tumor activity by triggering apoptosis in human tumor cells. Recently, it becomes clearly that suppression of apoptosis by tumor promoting agents in preneoplastic cells is thought as an important mechanism for tumor promotion [22]. Supportive of this notion, a considerable incidence of apoptosis associated with remodeling has been observed in nodular lesions during their disappearance induced by administration of chemopreventive agent, *S*-adenosyl-L-methionine [23]. Thus, we suggest that the anti-tumor promoting effects of curcumin may be, at least in part, contributed by its apoptosis-inducing activity. Finally, the experiments reported here provide another evidence that curcumin may prove to be of therapeutic value in the treatment of human myeloid leukemia.

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