Effect of curcumin on normal and tumor cells: Role of glutathione and bcl-2

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Abstract

Curcumin, a well-known dietary pigment derived from *Curcuma longa*, inhibited growth of several types of malignant cells both *in vivo* and *in vitro*. However, its mechanism of action still remains unclear. In this study, we have focused primarily on the cytotoxic effects of curcumin on three human tumor cell lines and rat primary hepatocytes. Curcumin induced apoptosis in MCF-7, MDAMB, and HepG2 cells in a dose-dependent and time-dependent manner. Apoptosis was mediated through the generation of reactive oxygen species. Attempts were made to establish the role played by endogenous glutathione on the apoptotic activity of curcumin. Depletion of glutathione by buthionine sulfoximine resulted in the increased generation of reactive oxygen species, thereby further sensitizing the cells to curcumin. Interestingly, curcumin had no effect on normal rat hepatocytes, which showed no superoxide generation and therefore no cell death. These observations suggest that curcumin, a molecule with varied actions, could be developed into an effective chemopreventive and chemotherapeutic agent. [Mol Cancer Ther 2004;3(9):1101–8]

Introduction

Glutathione (GSH), also known as γ-L-glutamyl-L-cysteine-glycine, is an ubiquitous tripeptide that functions as an important intracellular radical scavenger. It protects cells against reactive oxygen species (ROS) as well as against many toxins, mutagens, and drugs. GSH is also essential for the cellular metabolism of various enzymes, hormones, and amino acids. GSH also plays an important role in multidrug resistance either through its spontaneous reactions or through its function as a coenzyme in glutathione *S*-transferases (GST) reacting with the drug (1). Cellular redox potential is largely determined by GSH content, which accounts for >90% of the cellular nonprotein thiols (2). It is important for many biochemical functions including the regulation of gene transcription as well as modulation of apoptosis (3). GSTs belong to a family of enzymes that catalyze the conjugation of GSH to a wide variety of chemical toxins (4) and reactive electrophiles (5). Blockage of the GSH/GST detoxification system enhanced the chemosensitivity of several tumor cell lines (6). Generation of oxidative intermediates has been proposed to be a critical event in the process of programmed cell death induced by various agents. Consequently, depletion of GSH has been found to either precede the onset of apoptosis or render the cells more sensitive to cell death (7).

The ineffectiveness of current chemotherapeutic agents warrants investigations into alternative compounds to improve today's therapy regimens or to act as a means of chemoprevention. Curcumin (diferuloyl methane), a polyphenolic phytochemical, is the primary component of the spice turmeric. The pharmacologic safety of curcumin is well demonstrated by the fact that people in certain countries have been consuming curcumin as a dietary spice for centuries (8). Ample evidence exists to support its use in cancer prevention through its antiproliferative and anticarcinogenic properties or as an adjunct in overall cancer treatment. Curcumin has been shown to possess apoptotic activity against human colon cancer cells (9), stomach and skin tumors (10), breast cancer cells (11), and prostate cancer cells (12). A recent study reported that curcumin inhibited proliferation of colon cancer cell lines (HT-29 and HCT-15) by accumulating cells in G_2-M phase (13). Curcumin also induced apoptosis in NIH3T3 and leukemic cell line HL-60 (14, 15). We have recently shown differential sensitivity of human cancer cell lines to curcumin (16), suggesting that the curcumin effects vary based on the cell type. However, the mechanism of action of curcumin is not well understood. The present study was aimed at understanding the mechanism of action of curcumin on tumor cells.

In the present study, we show that curcumin induced apoptosis in human breast carcinoma cell lines as well as in human hepatoma cells but failed to do so in normal rat hepatocyte primary cultures. Furthermore, our results indicate that GSH plays a vital role in the sensitivity of these cell lines to curcumin. Depletion of GSH further sensitized the cells to curcumin effects, and the cell death is caused by the generation of ROS. Curcumin also down-regulated the expression of bcl-2 in tumor cells, which may be responsible for making them vulnerable to apoptotic death.

Materials and Methods

Cell Culture and Treatments

Human breast tumor cell lines MDAMB and MCF-7 and human hepatocellular carcinoma cell line HepG2 were from the National Cancer Institute (Bethesda, MD)/American Type Culture Collection (Manassas, VA) and were adapted to grow in Iscove's modified Dulbecco's

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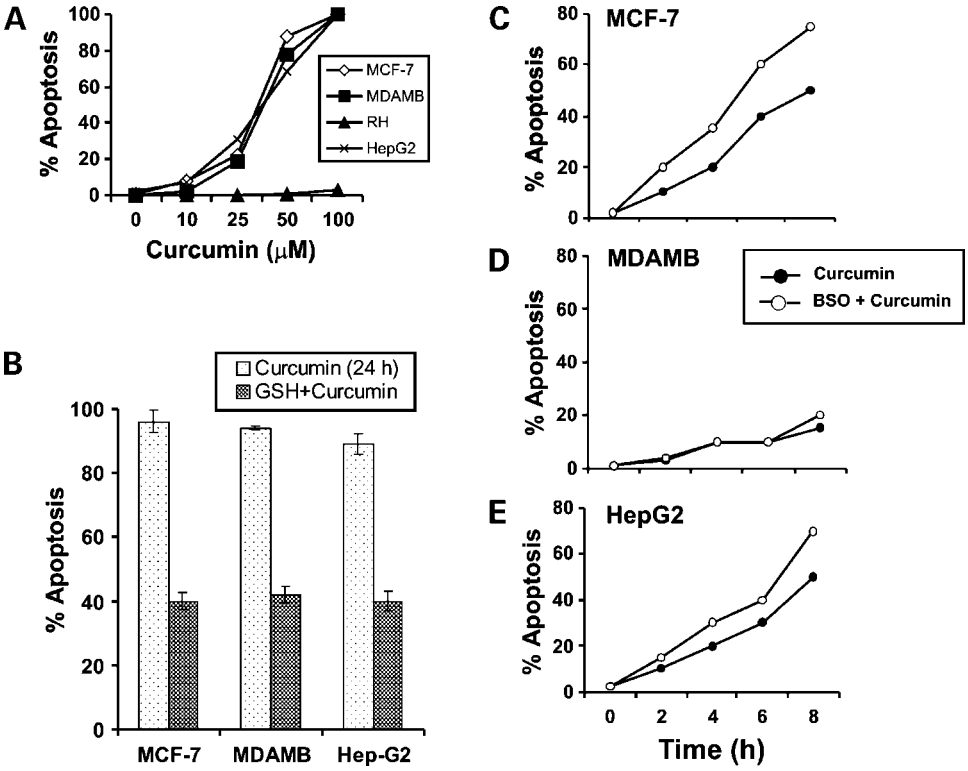

Figure 1. Curcumin induced cell death in different cell lines. **A,** effect of different concentrations of curcumin on MCF-7, MDAMB, and HepG2 cells and rat hepatocytes (*RH*). **B,** effect of GSH (5 mmol/L) on curcumin-induced apoptosis. **C–E,** % apoptotic death induced by curcumin and effect of pretreatment with BSO (1 mmol/L) on curcumin-induced apoptotic death. Percent apoptosis at different time points was evaluated after propidium iodide staining and flow cytometry of 20,000 acquired events. *Columns,* representative of three experiments; *bars,* SD. *, $P \leq 0.001$, with respect to control.

medium containing 10% FCS, 100 units/mL penicillin, and 50 μg/mL streptomycin. Rat hepatocyte primary cultures were prepared after teasing the liver by flushing with PBS with the help of a syringe. Primary cultures were maintained in Iscove's modified Dulbecco's medium-FCS. Cells (1×10^6) were treated with 50 μmol/L curcumin for different time points (0, 2, 4, 6, 8, and 24 hours). This concentration of curcumin was arrived at after a careful titration with different doses of curcumin (Fig. 1A). Stock solution of curcumin (94% purity, Sigma Chemical Co., St. Louis, MO) in ethanol was diluted in culture medium to obtain the required concentration, keeping the final ethanol concentration at <1%. Statistical significance of the data was tested by Student's *t* test.

Cells were depleted of GSH content by 2-hour treatment with 1 mmol/L buthionine sulfoximine (BSO, Sigma Chemical) dissolved in culture medium. Similarly, GSH levels in the cells were enhanced after treatment with GSH (5 mmol/L) for 2 hours. The BSO and GSH concentrations used were optimal and were arrived at after a careful dose-dependent titration.

Detection of Apoptosis

Apoptosis was monitored by flow cytometry and DNA fragmentation assay. Cells were fixed in 80% ethanol and stained with propidium iodide reagent (50 μg/mL propidium iodide in 0.1% sodium citrate containing 0.1% Triton X-100). Ten thousand events were acquired in a flow cytometer and the data were analyzed using CellQuest software (Becton Dickinson, San Jose, CA). For DNA fragmentation analysis, cells fixed in 80% ethanol were suspended in citrate phosphate buffer. Fragmented DNA was electrophoresed on 0.8% agarose gel at 2 V/cm^2 for 16 hours and visualized under UV light after staining with 5 μg/mL ethidium bromide. In addition, curcumin-treated cells after 6 hours were stained with FITC-conjugated Annexin V and the % positive cells were evaluated by flow cytometry.

Cytochrome *c* Reduction

Superoxide-induced reduction of ferricytochrome *c* was monitored spectrophotometrically at 550 nm. Control and curcumin-treated cells were washed and resuspended in complete phenol red–free Iscove's modified Dulbecco's medium and plated in 96-well plates. The time-dependent superoxide anion release was estimated in the presence of 80 μmol/L cytochrome *c* with and without the addition of superoxide dismutase (300 units/mL). The cellular proteins were estimated with Bradford reagent (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard.

Determination of Total GSH

GSH was estimated using a kinetic assay with a continuous GSH reductase catalyzed reduction of 5,5′-dithiobis (2-nitrobenzoic acid) (Sigma Chemical) to the chromophore, with rate monitored spectrophotometrically at 412 nm (17). Cells lysed by alternate freezing and thawing were deproteinized using ice-cold 10% 5-sulfosalicylic acid and the supernatants were used for GSH estimation. To 50 μL sample in 1 mL phosphate buffer (0.1 mol/L, pH 7), 50 μL NADPH (4 mg/mL), 20 μL deoxynucleotide triphosphate (1.5 mg/mL), and GSH reductase (6 units/mL) were added. The contents were mixed and the formation of

5-thio-2-nitrobenzoic acid was monitored for 5 minutes spectrophotometrically at 412 nm. The amount of GSH was calculated from a standard curve generated under similar conditions.

GST Activity

GST activity was measured according to the method of Habig and Jakoby (18) using chlorodinitrobenzene (Sigma Chemical) as substrate. The formation of GSH-chlorodinitrobenzene conjugate was monitored by the change in absorbance at 340 nm. One unit of GST activity is the amount of enzyme catalyzing the conjugation of 1 μmol substrate per minute. Samples were prepared as described above for GSH estimation without any acid treatment.

Reverse Transcription-PCR

Total RNAs were isolated from different cell lines using the guanidium thiocyanate-phenol-chloroform extraction method. RNA was precipitated in cold ethanol washed in 70% ethanol, dried, and resuspended in sterile water. RNA (1 μg) was reverse transcribed into cDNA using the cDNA synthesis kit and oligodeoxythymidylic acid (Promega, Madison, WI). The resulting cDNA was amplified by PCR using the specific primers for bcl-2 (19) and c-*myc* (20). Amplified fragments were electrophoresed on 1% agarose gel, transferred onto nitrocellulose membrane, and hybridized with specific partial cDNA probes for bcl-2 and c-*myc* kindly provided by Dr. S.J. Korsmeyer (Harvard Medical School, Boston, MA) and Dr. A. Kelekar (University of Minnesota, Minneapolis, MN), respectively. The following primers were used in PCR reaction: bcl-2, forward 5′-GAACACCAGAATCAAGTGTTCG-3′ and reverse 5′-TCAGGTGGACCACAGGTGGC-3′; c-*myc*, forward 5′-CCCCTCAGTGGTCTTCCCCTAC-3′ and reverse 5′-TGTTCTCGCCGTTTCCTCAGTA-3′; and glyceraldehyde-3-phosphate dehydrogenase, forward 5′-TGAAGGTCGGTGTGAACGGATTTG-3′ and reverse 5′-TGATGGCATGGACTGTGGTCATGA-3′.

Results

Curcumin Induced Apoptosis

Initially, the sensitivity of cell lines to different concentrations of curcumin was tested. Curcumin at 100 μmol/L was toxic to all the three tumor cell lines MDAMB, MCF-7, and HepG2 and cells became necrotic within 1 hour of treatment. However, at 50 μmol/L curcumin, the cells died mainly by apoptosis; therefore; this concentration was used for all our subsequent experiments (Fig. 1A). Primary rat hepatocytes, on the other hand, did not show any cell death. We also tested the effect of GSH on cell death induced by curcumin. GSH showed a protective effect for all the three tumor cell lines (Fig. 1B).

Because depletion of GSH could either precede the onset of apoptotic cell death or render the cells more sensitive to apoptotic agents, we tested cell viability using propidium iodide staining after treating the cells with BSO (1 mmol/L) for 2 hours prior to curcumin treatment. Viability of cells depleted of GSH was significantly lower showing at least 30% increase in dead cells (Fig. 1C–E). Surprisingly, in MDAMB cells, no significant difference was seen in GSH-depleted or nondepleted cells (Fig. 1D). On the other hand, exogenous GSH protected all the cell lines from undergoing apoptosis (Fig. 1B).

In addition to primary hepatocytes, we have studied the effects of curcumin on normal lymphocytes, rat skin fibroblasts, and a few normal cell lines. Curcumin did not induce any apoptotic death in any of the normal cells tested (Table 1).

To establish that curcumin-induced cell death was through apoptosis, we have studied oligonucleosomal DNA fragmentation in all the cell lines. Curcumin treatment induced cellular DNA fragmentation in all the three tumor cells lines; however, no DNA fragmentation was observed in normal hepatocytes (Fig. 2A). We also confirmed apoptotic death in MCF-7, MDAMB, and HepG2 cells by Annexin V staining (Fig. 2B). Again, normal hepatocytes did not stain with Annexin V.

GSH Levels in Different Cell Types

The increase in GSH levels and the activity of its related enzymes have been characterized as one of the factors, which could render the tumor resistant to either radiotherapy or chemotherapy. We also studied the GSH status of the cells in the present study. There is a significant increase in GSH content in MCF-7 and HepG2 cells after 8-hour incubation (Fig. 3A), whereas there was only 20% increase in GSH in rat hepatocytes. Interestingly, curcumin depleted GSH content in MDAMB cells initially followed by a slight increase. Treatment with BSO (1 mmol/L) depleted GSH content by ~50% and 55% in MCF-7 and MDAMB cells, respectively, but only 21% in HepG2 cells (Table 2). It may be mentioned here that HepG2 cells have higher levels of endogenous GSH content as compared with MCF-7 and MDAMB cells (Fig. 3A; Table 2).

Effect of Curcumin on GST Activity

Increased activity of GST is linked to an enhanced detoxification system. The GST enzymes catalyzed the conjugation of toxic substance to GSH before expelling it out (1). The GST activity increased significantly in MDAMB and HepG2 cells, whereas the increase was negligible in MCF-7 cells and rat hepatocytes. The low

Table 1. Effect of curcumin on primary and nontransformed cells

Cells	% Apoptotic Cells	
	Control	Curcumin
Lymphocytes	1.0	1.6
Hepatocytes	0.5	1.0
Rat skin fibroblasts	0.4	2.6
Chinese hamster ovary	1.4	2.8
Vero	0.6	1.2
F111	0.2	0.8

NOTE: Cells (1×10^6) were incubated with curcumin (50 μmol/L) for 24 hours. Cells were washed and stained with FITC-conjugated Annexin V. % Positive cells were evaluated by flow cytometry.

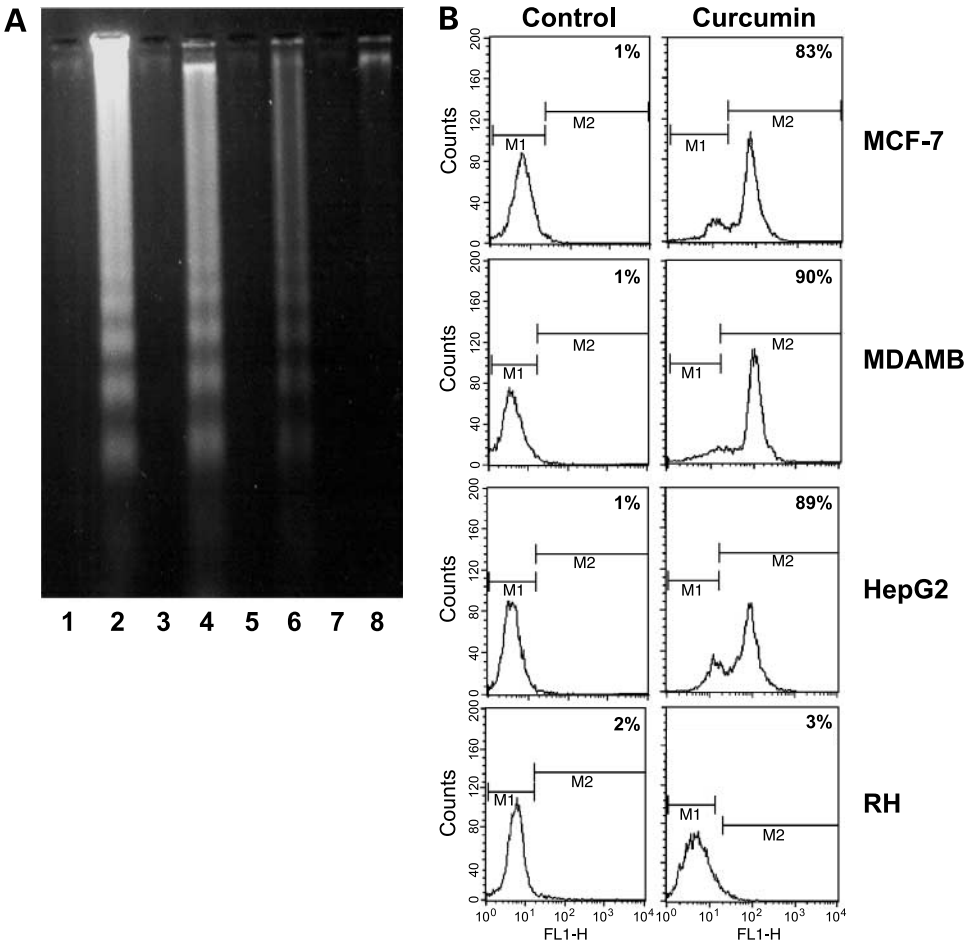

Figure 2. **A,** DNA fragmentation analysis of curcumin-treated human tumor cell lines and rat hepatocytes. *Lanes 1 and 2,* MCF-7 control and treated, respectively; *lanes 3 and 4,* MDAMB control and treated, respectively; *lanes 5 and 6,* HepG2 control and treated, respectively; *lanes 7 and 8,* normal rat hepatocytes control and treated, respectively. The cell lines were treated with curcumin (50 μmol/L) for 24 hours. **B,** Annexin V staining of different cell lines after treatment with curcumin for 6 hours. Percent positive cells were evaluated by flow cytometry.

augmentation of GST activity in MCF-7 cells may be the reason for higher percentage of apoptosis observed at 8 hours after curcumin treatment, implying that the detoxification system is probably not activated (Fig. 3B).

Curcumin Induced Superoxide Generation

To understand the mechanism of action of curcumin-induced apoptosis in tumor cells, we have studied the generation of superoxide anions after curcumin treatment at 0-, 2-, 4-, 6-, and 8-hour incubation. A significant increase

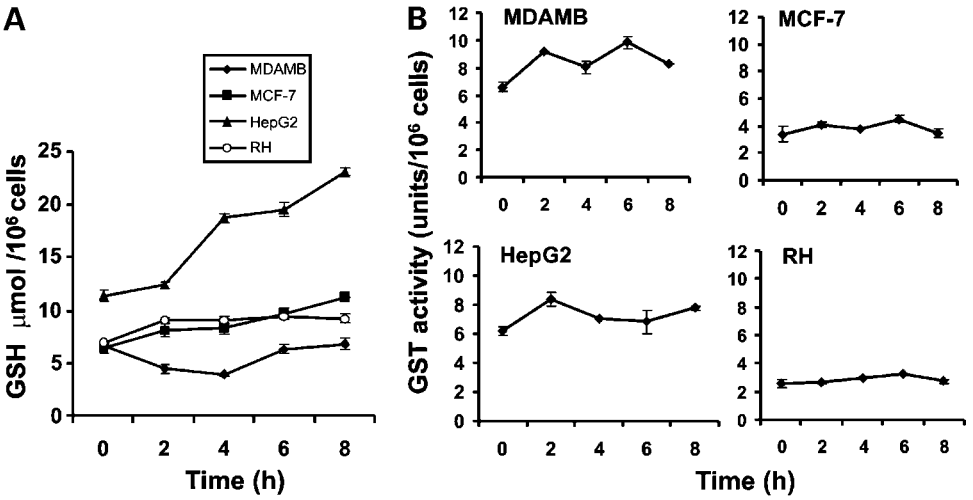

Figure 3. **A,** GSH levels in different tumor cell lines and normal hepatocytes at different time points after curcumin treatment. Basal GSH levels are at time 0 hour. **B,** time-dependent GST activity in MDAMB, MCF-7, and HepG2 cells and rat hepatocytes after treatment with curcumin. Basal GST activity is at time 0 hour. *Columns,* representative of three experiments; *bars,* SD.

Table 2. Effect of BSO on GSH levels in different cell lines after curcumin treatment

Cells	GSH Content (nmol/10^6 Cells)		
	Control	+BSO	% Reduction
MCF-7	6.51 ± 0.60	3.26 ± 0.35*	50
MDAMB	6.27 ± 0.15	2.77 ± 0.07*	55
HepG2	10.80 ± 0.56	8.20 ± 0.42†	21
Hepatocytes	9.20 ± 0.07	10.42 ± 0.85	—

NOTE: GSH levels in different cell lines after treatment with BSO (1 mmol/L) for 2 hours.
*$P \leq 0.001$, significant with respect to controls.
†$P \leq 0.01$, significant with respect to controls.

in the production of superoxide was observed in a time-dependent manner in all the transformed cell lines (Fig. 4). We also measured the amount of superoxide in GSH-depleted cells after curcumin treatment. Higher levels of superoxide were produced when cells were depleted of GSH. Primary rat hepatocytes did not produce significant ROS after curcumin treatment even in the presence of BSO (Fig. 4). GSH therefore seems to play an important role in the apoptotic activity of curcumin.

Effect of Curcumin on bcl-2 Expression

Because bcl-2 has been shown to regulate the generation of superoxide by the cells, we have studied the expression of bcl-2 in all the four cell lines used in this study. Interestingly, curcumin down-regulated the expression of bcl-2 in MCF-7 and MDAMB cells (Fig. 5). We could not detect any bcl-2 expression in HepG2 cells, whereas curcumin had no significant effect on bcl-2 expression in normal hepatocytes (Fig. 5).

Curcumin Induced Down-Regulation of c-*myc* Expression

To understand the mechanism of curcumin action, we investigated the expression of c-*myc* to resolve whether curcumin caused cell cycle arrest before inducing apoptotic death in cells. Our results show a sharp decrease in c-*myc* expression in MCF-7, MDAMB, and HepG2 cells after treatment with curcumin (Fig. 6). Interestingly, c-*myc* expression was partially restored when exogenous GSH was added to MCF-7 cells prior to curcumin treatment (Fig. 6, *lane 3*). These observations suggest a role for ROS in the down-regulation of c-*myc* expression after treatment with curcumin, which may be responsible for the antiproliferative activity of curcumin reported earlier.

Discussion

Therapeutic strategies designed to increase the susceptibility of tumor cells to apoptosis have the potential to significantly augment the efficacy of a variety of cancer treatments. Enhancement of the apoptotic potential of tumor cells increases tumor responses to chemotherapy (21). The increase in GSH content in the cells after curcumin treatment suggests that curcumin interferes with the cell survival systems. Similar observations were also noted with several compounds undergoing anticancer activity tests like organotin compounds (22) and chemotherapeutic

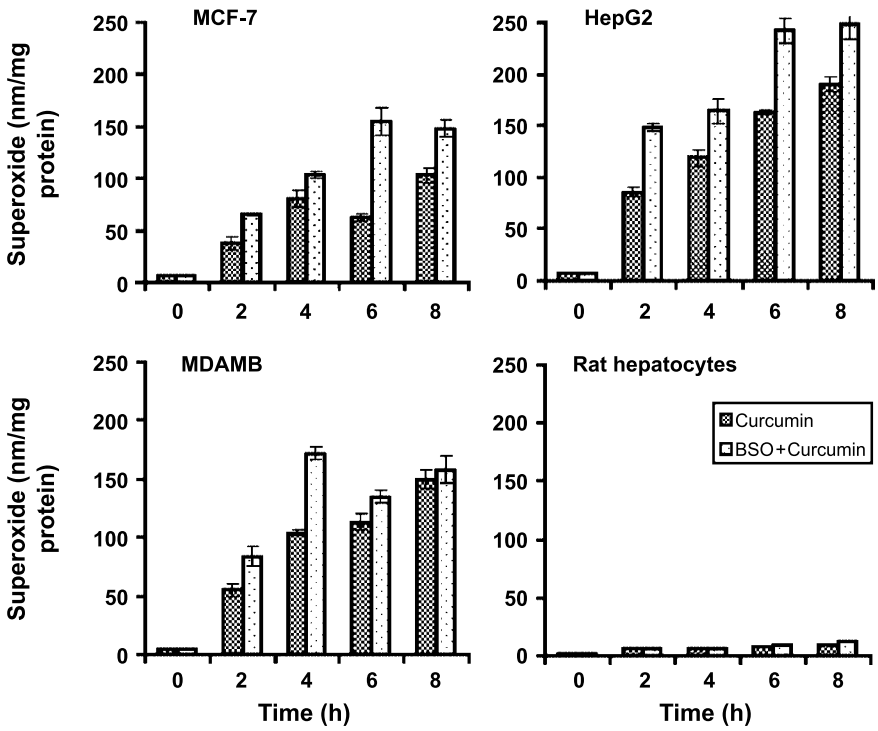

Figure 4. Curcumin induced generation of superoxide anions. Cells were treated with curcumin in the presence and absence of BSO. Superoxide levels were monitored after 0, 2, 4, 6, and 8 hours as described in Materials and Methods. *nm,* nanomoles. *Columns,* representative of three experiments; *bars,* SD.

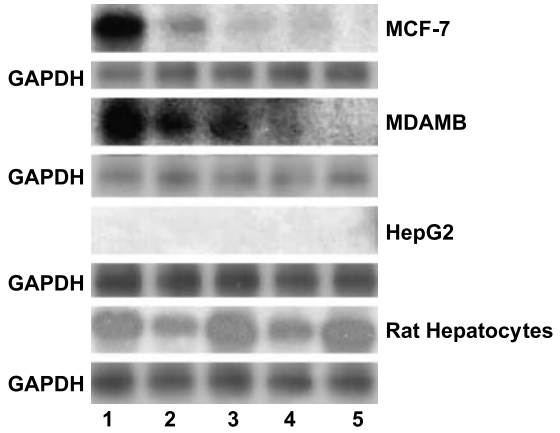

Figure 5. Northern expression of bcl-2 after treatment of cells with curcumin at different time points. *Lane 1,* control; *lanes 2–5,* incubation time of 2, 4, 6, and 8 hours with curcumin (50 μmol/L), respectively. Glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) expression is the loading control.

drugs like cisplatin (23). Curcumin has also been shown to increase the levels of GSH in several other systems like rat hepatocytes (24), kidney cells (25), and human leukemia cells (26). In this study, the depletion of endogenous GSH sensitized the cells to curcumin treatment and thereby increased the percentage of apoptotic cells, thus suggesting that curcumin may be inactivated by the formation of GSH-curcumin conjugate, and GSTs are involved in catalyzing the GSH conjugate formation (27). Therefore, it was interesting to measure GST activity in the present study especially when curcumin elevated the endogenous GSH levels. Recent findings suggest that GSTs play an important role in carcinogenicity and resistance to drugs and their levels are elevated in several tumors (27). Exposure of mammalian cells to a variety of chemical agents results in increased levels of xenobiotic metabolizing enzymes. These enzymes function as an intracellular detoxification system and by decreasing the levels of compounds capable of generating ROS; they are part of the enzymatic antioxidant defense against oxidative stress (28). GSTs are only part of a complex detoxification system because multiple enzymes are involved in the redox cycling of GSH. Therefore, an insight into the kinetics of GSH and GSH-dependent enzymes could yield a more dynamic and reliable representation of the function of this system.

The increase in GSH content in these cells after curcumin treatment prompted us to focus our attention on the production of ROS. Many chemotherapeutic agents have profound effects on the cellular redox status (29) and alteration of redox status plays an important role in the induction of apoptosis (30). ROS have been shown to be involved in cell proliferation and induce apoptosis as well as mediate cell death induced by numerous different factors (31). In several apoptotic models, increased generation of ROS was described as an early event; in addition, enhanced ROS formation and impairment of the cellular antioxidant mechanisms may also lead to cellular apoptosis (32). Decreased antioxidant profiles were found in epithelial cells that undergo apoptosis and antioxidants have been reported to counter apoptotic cell death (33).

Our data show that all the three tumor cell lines are sensitive to curcumin and ~90% cell death were observed after 24 hours, indicating clearly that curcumin acts in a time-dependent manner. In line with our previous report, rat hepatocytes were resistant to curcumin treatment and no cell death was observed (16). Similarly, the normal cell lines and primary cells remained unaffected after curcumin treatment (Table 1). Death in MDAMB cells seems to be delayed initially in spite of the fact that there is a drop in GSH levels on treatment with curcumin (Fig. 3). Our studies show that there is a significant increase in GST activity in this cell line, which could probably be the reason for the delayed induction of apoptosis. Curcumin may be conjugated to GSH catalyzed by GST and is expelled out of the cell. The high production of toxic oxygen metabolites could be the reason for the induction of GST expression observed in the other cell lines (34).

Although there seems to be different pathways by which cells undergo apoptosis, it seems quite clear that the cell death observed is due to the production of ROS. Furthermore, the role of oxidative stress in cell death has been shaped by several independent observations (35). Several groups have suggested that intracellular ROS generation may constitute a conserved apoptotic event and cite ROS production as a critical determinant of toxicity associated with exposure to ionizing radiation and chemotherapeutic drugs (36). Depletion of GSH pool has also been suggested to accompany ROS production during apoptosis in relevant systems (33). The ability of various cellular antioxidants

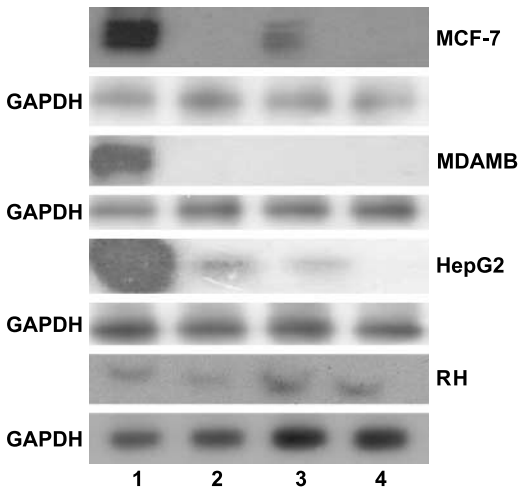

Figure 6. Expression of c-*myc* transcripts by different human tumor cell lines and rat hepatocytes (*RH*). *Lane 1,* control; *lane 2,* curcumin treatment for 8 hours; *lane 3,* pretreatment with GSH (5 mmol/L) for 2 hours; *lane 4,* pretreatment with BSO (1 mmol/L) for 2 hours. Glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) expression is the loading control.

like catalase and *N*-acetylcysteine to block apoptosis induced by diverse agents other than oxidants also argues for a central role for oxidative stress in apoptosis (31). Reciprocally broad-spectrum antiapoptotic proteins like bcl-2 have been ascribed to possess antioxidant function (35), again indicating that ROS generation may be a requisite apoptotic event.

Direct oxidant treatment and dysregulated intracellular production of ROS are equally harmful to the cell and are countered by various antioxidant defenses. Among them, the tripeptide GSH is the most rapid and abundant weapon against ROS and regulates the redox state of many other cellular constituents. Drop in GSH levels and concomitant increase in ROS during the apoptotic process has been reported earlier (36). A significant increase in superoxide levels was observed in curcumin-treated cells in our study. Depletion of GSH by BSO, a potent inhibitor of γ-glutamyl cysteine synthetase, the rate-limiting enzyme in the GSH synthesis pathway, further increased the superoxide levels after curcumin treatment. Treatment with BSO alone does not cause apoptosis in many cell types, indicating that GSH depletion alone may not trigger apoptosis (31).

Involvement of bcl-2, a potent antiapoptotic protein, does not seem to be the only mechanism in the antitumor activity of curcumin in this study. Some reports suggest that curcumin could act on GSH levels via bcl-2, with decreased level connected with several pathways finally leading to cell death (26). There are also studies demonstrating that curcumin in GSH-depleted cells did not alter bcl-2 levels probably because bcl-2 acts upstream of the GSH changes (26). Recently, ROS has been shown to down-regulate bcl-2 expression, thereby sensitizing the cells to apoptotic death (37, 38). Alternatively, curcumin is a known inhibitor of nuclear factor-κB activation, which is essential for the induction of bcl-2 protein (39).

It is known that, before triggering apoptosis, cells are blocked in the G_1 phase of the cell cycle, allowing time to repair its damaged DNA (40). Because the dysregulation of c-*myc* is associated with cellular proliferation, its down-regulation may be linked with growth arrest of cancer cells. Several studies have reported the role of curcumin in the growth arrest of cancer cells in culture and in animal models (41). These studies have implicated curcumin-induced growth arrest, mostly at the G_2-M stage. Expression of c-*myc* is also significant because it acts as a transcription activator of genes required to promote the G_1-S phase transition. Loss of c-*myc* could result in the arrest in G_1-S phase of the cell cycle eventually leading to cell death (42). It was not surprising to note that, in normal rat hepatocytes, which do not divide *in vitro*, c-*myc* expression was not seen; this may be because, in most untransformed cell types, transforming growth factor-β causes the down-regulation of c-*myc*. Because of its short half-life, transforming growth factor-β–dependent down-regulation of c-*myc* results in rapid protein loss and seems to be required for growth inhibition (43).

Thus, the observations made in this study suggest that curcumin does possess antitumor properties but, unlike other known chemotherapeutic compounds, curcumin does not cause any damage to the normal cells. These results further establish its use as a valid chemopreventive and chemotherapeutic agent in malignancy.

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