Curcumin suppresses constitutive activation of nuclear factor-κB and requires functional Bax to induce apoptosis in Burkitt’s lymphoma cell lines

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Abstract

We provide evidence that curcumin, a natural compound isolated from rhizomes of plant Curcuma longa, induces apoptosis in several Burkitt’s lymphoma cell lines expressing Bax protein (AS283A, KK124, and Pa62PB), whereas it has no effects in cell lines with no Bax expression (BML895 and CA46). Our data show that curcumin treatment results in down-regulation of constitutive activation of nuclear factor-κB (NF-κB) via generation of reactive oxygen species where it causes conformational changes in Bax protein leading to loss of mitochondrial membrane potential and release of cytochrome c to the cytosol. This leads to activation of caspase-9, caspase-3, and poly(ADP)-ribose polymerase cleavage leading to caspase-dependent apoptosis. In addition, curcumin treatment of Burkitt’s lymphoma cell lines also causes up-regulation of DR5; however, this up-regulation does not result in apoptosis. Importantly, cotreatment with curcumin and TRAIL induces apoptosis in Bax-deficient cell lines. Taken together, our findings suggest that curcumin is able to induce apoptosis in Bax-positive cell lines, whereas combinations with TRAIL result in apoptosis in Bax-negative cell lines. These findings also raise the possibility that incorporation of curcumin in treatment regimens may provide a novel approach for the treatment of Burkitt’s lymphomas and provide the molecular basis for such future translational efforts. [Mol Cancer Ther 2008;7(10):3318 – 29]

Introduction

Burkitt’s lymphoma is a high-grade B-cell lymphoma characterized by a translocation involving the c-myc and immunoglobulin gene and has phenotypic features that resemble a germinal center B cell. Mutations in proapoptotic protein Bax has been reported in Burkitt’s lymphoma biopsies as well as cell lines derived from lymphoid tumors (1, 2). The proapoptotic protein Bax (3), which is common to several apoptotic pathways, potentiates death in response to multiple stimuli. Bax mutations have been implicated in resistance to apoptosis after withdrawal of growth factors (4), γ-irradiation (5), dexamethasone (6), and chemotherapeutic agents (7). There are reports that Bax may also participate in activation of caspases (8), and consistent with this, Bax-deficient mice display hyperplasia of both thymocytes and B cells (9), suggesting role of Bax in homeostasis of B cells.

Programmed cell death or apoptosis is a genetically regulated process that plays an essential role in the regulation of homeostasis of higher organisms (10). Defective apoptosis is a mechanism that contributes to the development of different types of malignant phenotypes. Two major pathways that lead to apoptosis exist: (a) the mitochondrion-initiated pathway, also defined as intrinsic pathway, and (b) the cell surface death receptors pathway, also defined as extrinsic pathway (11). In the mitochondrial pathway, cytochrome c is released to cytosol where along with dATP binds to apoptotic protease-activating factor-1 (12). This complex, along with adenine nucleotides promotes pro-caspase-9 autoactivation, which in turn activates caspase-3, ultimately resulting in poly(ADP)-ribose polymerase (PARP) cleavage. In the death receptor-mediated pathway (e.g., TRAIL/TRAIL ligand), caspase-8 is the initiator caspase that activates downstream caspases, including caspase-3. Active caspase-8 also cleaves a proapoptotic Bcl-2 family member, Bid, and truncated Bid induces mitochondrial cytochrome c release (13). There is evidence that cross-talk between the two pathways is mediated by the truncated of Bid (14). In addition, a third pathway has been identified, in which Bid is cleaved downstream of the point of Bcl-2 action (15). This event is catalyzed by caspase-3, upstream of caspase-8 activation, and acts as a potential feedback loop for amplification of apoptosis-associated release of cytochrome c from the mitochondria.
Curcumin (diferuloylmethane) is a naturally occurring product isolated from the rhizomes of the plant *Curcuma longa* (Linn), found in south Asia (16). There has been significant interest on the potential therapeutic value of curcumin and that has previously ignited clinical efforts using curcumin for cancer patients (17). Curcumin has been shown to suppress the activation of nuclear factor-κB (NF-κB) induced by various tumor promoters, including phorbol ester, tumor necrosis factor, and hydrogen peroxide (18). Subsequently, several studies showed that curcumin-induced down-regulation of NF-κB is mediated through suppression of IκB kinase (IKK) activation (19, 20). Another study showed that curcumin suppresses constitutively active NF-κB in multiple myeloma and mantle cell lymphoma (21, 22). Curcumin has also been shown to arrest cell growth at the G2-M phase and induce apoptosis in human melanoma cells by inhibiting NF-κB activation (23). The role of curcumin in Burkitt’s lymphoma has not been investigated. Therefore, in this study, we explore the mechanism of chemopreventive effects of curcumin. We have tested whether curcumin inhibits cell growth and induces apoptosis in a panel of Burkitt’s lymphoma cell lines that express or do not express Bax.

**Materials and Methods**

**Materials**

Curcumin, N-acetylcysteine (NAC), and Bax 6A7 monoclonal antibody were purchased from Sigma. Caspase-9 antibody and zVAD-fmk were purchased from Calbiochem. Antibody against cleaved caspase-3 was purchased from Cell Signaling Technologies. Cytochrome c, β-actin, caspase-3, IKKα, IKKβ, and PARP antibodies were purchased from Santa Cruz Biotechnology. DR5 antibody was purchased from Cayman Lab. TUNEL assay kit was obtained from MBL. Annexin V was purchased from Molecular Probes. Apoptotic DNA-ladder kit was obtained from Roche.

**Cell Culture**

The human Burkitt’s lymphoma cell lines, AS283A, KK124, Pa682PB, BML895, and CA46, were a kind gift from Dr. Ian Magrath (International Network for Cancer Treatment and Research) and Dr. Kishor Bhatia from NIH. The Burkitt’s lymphoma cell lines were cultured in similar condition as described previously (24). Cells were grown in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 units/mL streptomycin at 37°C in an humidified atmosphere containing 5% CO₂.

**Annexin V Staining, Cell Cycle Analysis, and TUNEL Assay**

Burkitt’s lymphoma cell lines were treated with different concentrations of curcumin as described in the legends. Cells were harvested and percentage apoptosis was measured by flow cytometry after staining with fluorescein-conjugated Annexin V and propidium iodide (PI; Molecular Probes) as described previously (25). For cell cycle analysis, cells were washed once with PBS and resuspended in 500 μL hypotonic staining buffer and analyzed by flow cytometry as described previously (26, 27).

**Preparation of Nuclear Extracts for NF-κB**

Nuclear extracts were prepared according to earlier study (28). Briefly, 2 × 10⁶ cells were washed with cold PBS and suspended in 0.4 mL hypotonic lysis buffer containing protease inhibitors for 30 min. The cells were then lysed with 10% NP-40. The homogenate was centrifuged, and supernatant containing the cytoplasmic extracts was stored frozen at 80°C. The nuclear pellet was resuspended in 25 μL ice-cold nuclear extraction buffer. After 30 min of intermittent mixing, the extract was centrifuged, and supernatants containing nuclear extracts were secured. The protein content was measured by the Bradford method. If the nuclear extracts were not used immediately, they were stored at 80°C.

**Electrophoretic Mobility Shift Assay for NF-κB**

The single-stranded 3′-end biotin-labeled probe containing the NF-κB consensus site 5′-AGTGTAGGGGACTTTCCCCAGGC-3′ and 3′-TCAACTCCCCCTGAAAGGTCCG5′ were purchased from Metabion. The biotinylated oligonucleotides were annealed by denaturing at 90°C for 1 min and cooled to room temperature for 1 h. The electrophoretic mobility shift assay (EMSA) binding reactions were done by using a LightShift chemiluminescent EMSA kit (Pierce). Specifically, 3 μg nuclear extract was incubated in 1× binding buffer containing 2.5% glycerol, 0.05% NP-40, 50 mmol/L KCl, 5 mmol/L MgCl₂, 50 ng poly(dl-dC), and biotinylated probe with or without protein extract for 30 min at room temperature. The complexes were separated on a 6% polyacrylamide-0.5% Tris-borate-EDTA gel and transferred to a positive charge nylon membrane. After the transfer was completed, the membrane was crosslinked and biotin-labeled DNA was detected by using a chemiluminescence detection kit (Pierce).

**Luciferase Reporter Assays**

Burkitt’s lymphoma cells were transfected with a β-galactosidase and the plasmid pNF-κB-LUC containing five copies of consensus NF-κB sequences linked to a minimal E1B promoter-luciferase gene using the LipofectAMINE transfection reagent as per the manufacturer’s recommen
dation procedure (Qiagen). Forty-eight hours after transfection, triplicate cultures were either left untreated or treated with curcumin as indicated (29). The cells were washed twice with cold PBS, and after cell lysis, luciferase activity was measured using the protocol of the manufacturer (Promega). The measured luciferase activities were normalized for β-galactosidase activity for each sample.

**Cell Lysis and Immunoblotting**

Cells were treated with curcumin as described in the figure legends and lysed as described previously (30). Briefly, cell pellets were resuspended in phosphorylation lysis buffer on ice for 1 h and spun at 14,000 rpm for 15 min and supernatant was collected. Protein concentrations were assessed by Bradford assay before loading the samples. Proteins (15-20 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore). Immunoblotting was done with
different antibodies and visualized by an enhanced chemiluminescence (Amersham) method.

**InB Kinase Assay**

IB kinase assay was done by a modified method as described earlier (31). Briefly, Burkitt's lymphoma cells were treated with curcumin, cell lysates were immunoprecipitated with 1 µg anti-IKKα and anti-IKKβ antibodies each, and the immune complexes so formed were precipitated with protein G-Sepharose beads for 2 h. The beads were washed first with lysis buffer and then with the kinase assay buffer [50 mmol/L HEPES (pH 7.4), 20 mmol/L MgCl₂, and 2 mmol/L DTT]. The immune complex was then assayed for the kinase activity with the use of kinase assay buffer containing 20 µCi (0.74 MBq) [γ-32P]ATP, 10 µmol/L unlabeled ATP, and 2 µg glutathione S-transferase-IB per sample (1-54). After incubation at 30°C for 30 min, the reaction was stopped by boiling the solution in 6× SDS sample buffer. Then, the reaction mixture was resolved on 12% SDS-PAGE. The radioactive bands of the dried gel were visualized and quantified by autoradiography.

**Detection of Bax Conformational Changes**

Cells were treated with indicated doses of curcumin and lysed with CHAPS lysis buffer [10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, and 1% CHAPS] and immunoprecipitated with anti-Bax 6A7 monoclonal antibody and Bax conformation was detected as described earlier (32).

**Cross-linking of Bax Protein**

Following treatment with 20 µmol/L curcumin for indicated periods, cells were washed twice with PBS and once with conjugating buffer containing 150 mmol/L NaCl, 20 mmol/L HEPES (pH 7.4), 1.5 mmol/L MgCl₂, and 10 mmol/L glucose. DSS in DMSO was added at a final concentration of 2 mmol/L and incubated at room temperature for 30 min. Cross-linked and oligomerized proteins were detected as described earlier. Cross-linking experiments were done as described earlier (32).

**Assay for Cytochrome c Release**

The release of cytochrome c from mitochondria was assayed as described earlier (33). Briefly, cells were treated with and without curcumin as described in figure legends, harvested, and resuspended in hypotonic buffer. Cells were homogenized and cytosolic fractions were isolated by differential centrifugation. Protein (15-20 µg) from cytosolic fraction of each sample was analyzed by immunoblotting using an anti-cytochrome c antibody.

**Measurement of Mitochondrial Potential Using the 5,5',6,6'-Tetrachloro-1,1',3,3'-Tetraethylbenzimidazolylcarbocyanine Iodide Assay Kit**

Cells (1 × 10⁶) were treated with curcumin for 24 h. Cells were washed twice with PBS and suspended in mitochondrial incubation buffer from Alexis. 5,5',6,6'-Tetrachloro-1,1',3,3'-tetracyhanizimidazolylcarbocyanine was added to a final concentration of 10 µmol/L and cells were incubated at 37°C in dark for 15 min. Cells were washed twice with PBS and resuspended in 500 µL mitochondrial incubation buffer and mitochondrial membrane potential (percentage of green and red aggregates) was determined by flow cytometry as described previously (34).

**Reverse Transcription-PCR Assays**

Total RNA was extracted following treatment with 20 µmol/L curcumin for the indicated periods using Trizol and reverse transcribed with random hexamers. Reverse transcription-PCR amplifications were done using the following primers: DR5-forward: GGAGCCGCTCATGAG-GAATTTG and DR5-reverse: GGCAGTCTTCCTCC-CAGGCTCTC for 35 cycles (60°C annealing temperature) to yield an 181-bp product. Amplification of glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Quantification analysis of DR5 using real-time reverse-transcription PCR was done by Pfaffle methodology as described earlier (32).

**Statistical Analysis**

Data are presented as mean ± SD. Comparisons between groups were made with the paired Student’s t test. P values < 0.05 were considered statistically significant.

**Results**

**Curcumin Induces Cell Death of Burkitt's Lymphoma Cell Lines in a Dose-Dependent Manner**

We sought to determine whether curcumin treatment leads to cell death of Burkitt’s lymphoma cell lines. A panel of cell lines either expressing Bax (AS283A, KK124, and PA682PB) or deficient in Bax expression (BML895 and CA46) were cultured in presence of 10 or 20 µmol/L curcumin for 24 h and cell death was determined by trypan exclusion dye assays. Figure 1A shows that significant loss of cell viability was seen at final concentrations of 10 or 20 µmol/L curcumin treatment in AS283A, KK124, and PAPB cell lines, whereas the BML895 and CA46 cell lines were refractory to curcumin-induced cell death.

We next directly determined whether curcumin induces apoptosis and whether it has effects on cell cycle progression in Burkitt’s lymphoma cells. Burkitt’s lymphoma cell lines were treated with curcumin for 24 h, and cells were stained and cell cycle fractions were determined by flow cytometry. As shown in Fig. 1B, the sub-G₁ population of cells increased from 2.77% in control to 24.94% and 32.11% with 10 and 20 µmol/L curcumin, respectively, in AS283A. Similar results were obtained in KK124, 4.34% to 20.59% and 30.68%, and in PA682PB, 5.97% to 20.59% and 37.62%. There were no appreciable sub-G₁ populated cells in response to curcumin in BML895 and CA46 Burkitt’s lymphoma cells. The increase in sub-G₁ population in curcumin-sensitive cells was accompanied by loss in G0-G1, 37.62%. There were no appreciable sub-G₁ populated cells in response to curcumin in BML895 and CA46 Burkitt’s lymphoma cells.

**Gene Silencing Using Small Interfering RNA**

DR5 small interfering RNA (siRNA) and scrambled control siRNA were purchased from Qiagen. For transient expression, cell lines were transfected by using Lipofect-AMINE 2000 reagent (Invitrogen) according to the manufacturer’s instructions. After incubating the cells for 6 h, the lipid and siRNA complex was removed and fresh growth medium was added. Cells were treated 48 h after transfection for 24 h and specific protein levels were determined by Western blot analysis with specific antibodies against the targeted proteins and actin as a loading control.
S, and G2-M phase. It has been reported that cells with these feature are those dying of apoptosis (32–34). To further confirm apoptosis, we also used Annexin V/PI dual staining, TUNEL assay, and DNA laddering detection methods. Burkitt’s lymphoma cells were treated with 10 and 20 μmol/L curcumin and apoptotic cells were assayed by Annexin V/PI dual staining. All the Burkitt’s lymphoma cell lines were treated with 10 and 20 μmol/L curcumin for 24 h and cells were subsequently stained with fluorescein-conjugated Annexin V and PI and analyzed by flow cytometry. Representative of five independent experiments. **Curcumin-induced apoptosis detected by Annexin V/PI dual staining.**

![Figure 1](https://mct.aacrjournals.org/)

**Figure 1.** A, curcumin induces cell death in Burkitt’s lymphoma cell lines. AS283A, KK124, PA682PB, BML895, and CA46 cells were treated with 10 and 20 μmol/L curcumin for 24 h. Percentage cell death was scored using trypan blue exclusion dye. Mean ± SD of three independent experiments. *, P < 0.001, statistically significant (Student’s t test). B, curcumin increases sub-G1 (Apo) population in Bax-expressing Burkitt’s lymphoma cell lines. AS283A, KK124, PA682PB, BML895, and CA46 were treated with 10 and 20 μmol/L curcumin for 24 h. Thereafter, the cells were washed and stained with PI and analyzed for DNA content by flow cytometry as described in Materials and Methods. Representative of five independent experiments. C, curcumin-induced apoptosis detected by Annexin V/PI dual staining. All the Burkitt’s lymphoma cell lines were treated with 10 and 20 μmol/L curcumin for 24 h and cells were subsequently stained with fluorescein-conjugated Annexin V and PI and analyzed by flow cytometry. Representative of five independent experiments. D, PA682PB and CA46 cells were treated with 10 and 20 μmol/L curcumin for 24 h and DNA was extracted and separated by electrophoresis on 1.5% agarose gel.

**Curcumin Suppresses Constitutive NF-κB in Burkitt’s Lymphoma**

Constitutive activation of NF-κB has been reported in a wide variety of cancers including lymphoid malignancies...
We therefore studied Burkitt’s lymphoma cell lines to determine the constitutive activation status of NF-κB using EMSA assays. As shown in Fig. 2A, all the five cell lines used in this study expressed constitutive activation of NF-κB. Next, we sought to determine whether curcumin suppressed such NF-κB activation in Burkitt’s lymphoma cell lines. Bax-expressing PA682PB and Bax-deficient CA46 cell lines were treated with curcumin, as indicated, and EMSA was done. As shown in Fig. 2B, curcumin treatment suppressed NF-κB activity in a dose-dependent manner in both lines.

Curcumin Inhibits IκBα Phosphorylation and IκB Kinase Activity

The degradation of IκBα and subsequently release of NF-κB requires prior phosphorylation at Ser32 and Ser36 residues. We therefore investigated whether the inhibitory effects of curcumin are mediated through alteration of phosphorylation of IκBα. PA682PB cells were treated with curcumin for different periods and 15 μg protein was separated on SDS-PAGE and immunoblotted with antibodies against phospho-IκBα and β-actin. As shown in Fig. 2C, untreated samples showed constitutive phosphorylation of IκBα.

Figure 2. Effect of curcumin on constitutive nuclear NF-κB in Burkitt’s lymphoma cells. A, constitutive expression of NF-κB in Burkitt’s lymphoma cells. Nuclear extracts were prepared and EMSA was done as described in Materials and Methods. The densitometric analysis of NF-κB for each cell line bands was done using Alphalmager Software. Data were plotted as a bar graph. B, curcumin inhibits constitutive nuclear NF-κB in Burkitt’s lymphoma cells. PA682PB and CA46 cells were treated with indicated doses of curcumin for 24 h. Nuclear extracts were prepared and EMSA was done. The densitometric analysis of NF-κB for each cell line after treatment with indicated doses of curcumin and compared with control untreated sample was done using Alphalmager Software. Data were plotted as a bar graph. C, effect of curcumin on IκBα phosphorylation in Burkitt’s lymphoma cells. PA682PB cells were treated with 20 μmol/L curcumin for indicated period. Cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with antibodies against phospho-IκBα and actin as indicated. D, effect of curcumin on IκB kinase activity in Burkitt’s lymphoma cells. Following 24 h treatment with various doses of curcumin, cells were lysed and equal amounts of proteins were immunoprecipitated with IKK antibodies and kinase assay was done using -P32-ATP and glutathione S-transferase-IκBα as described in Materials and Methods. Proteins were separated by SDS-PAGE, transferred to PVDF membrane, and radioactive bands were visualized by autoradiography and membrane was immunoblotted with antibody against IKKa and IKKβ for equal loading.
expression of Ser32-phosphorylated IκBα and treatment of curcumin dephosphorylated IκBα in a time-dependent manner. IKK activation is required for phosphorylation of IκBα. To determine whether curcumin inhibits kinase activity of IKK, in vitro kinase assays were done on immunoprecipitates of curcumin-treated and untreated lysates using glutathione S-transferase-IκBα as an exogenous substrate. As shown in Fig. 2D, treatment of curcumin resulted in inhibition of IKK activity; however, no change was seen on the expression of IKK proteins level, suggesting that curcumin inhibits the kinase activity of IKK.

Curcumin-Induced Reactive Oxygen Species Generation Inhibits Constitutive Activation of NF-κB in Burkitt’s Lymphoma Cells

Several compounds used in chemotherapy induce cell death through the generation of reactive oxygen species (ROS; ref. 37). We examined whether ROS were also generated in Burkitt’s lymphoma cells treated with curcumin for various periods and, if so, whether this is a mechanism for the induction of curcumin-induced apoptosis. H$_2$DCFDA-based fluorescence-activated cell sorting detection of PA682PB cell line revealed that intracellular ROS levels increased in a time-dependent manner, starting as early as 2 h after treatment, peaking at 6 h, and diminishing after 8 h treatment with curcumin (Supplementary Fig. S2A). NAC is a widely used thiol-containing antioxidant that scavenges ROS in cells by interacting with OH and H$_2$O$_2$, thus affecting ROS-mediated signaling pathways. The curcumin-induced increase in ROS levels was blocked by pretreatment with NAC (data not shown). We then examined whether curcumin can repress constitutive active NF-κB gene expression in reporter assays. PA682PB and CA46 cells were transiently transfected with a NF-κB reporter construct, and after treatment with curcumin, luciferase activity was determined. As shown in Supplementary Fig. S2B, constitutive luciferase activity was seen in untreated control cells, whereas curcumin treatment abrogated the constitutive NF-κB reporter activity. We next sought to determine whether curcumin-induced ROS expression inhibits the constitutive activity of NF-κB in Burkitt’s lymphoma cells. PA682PB and AS283A cells were pretreated with NAC and subsequently treated with curcumin as indicated and EMSA was done. As shown in Supplementary Fig. S2C, pretreatment of cells with NAC abrogated curcumin-induced inhibition of constitutively active NF-κB, suggesting that ROS play a role in curcumin-mediated suppression of NF-κB activity. These data were further confirmed using luciferase reporter assays as shown by the inhibition of curcumin-induced NF-κB luciferase activity by NAC NF-κB (Supplementary Fig. S2C).

Curcumin Induces Bax Conformational Changes and Oligomerization

To obtain information on the potential role of Bax in curcumin-induced apoptosis, we initially sought the expression of Bax protein in all the five cell lines used in the study. As shown in Fig. 3A, AS283A, KK124, and PA682PB cell lines expressed Bax as detected by immunoblotting, whereas there was no expression of Bax in BML895 and CA46 cell lines. Next, PA682PB cells were treated with 20 μmol/L curcumin for 4, 8, and 24 h and lysed with 1.0% CHAPS lysis buffer; lysates were immunoprecipitated with an anti-BAX 6A7 antibody that recognizes only the conformationally changed Bax protein. The detergent CHAPS has shown to retain the Bax protein in its native conformation. As shown in Fig. 3B, conformationally changed Bax was detected after 4 and 8 h of treatment with curcumin, whereas the signal decreased after 24 h treatment. To examine whether conformational changes in Bax protein were due to release of ROS or caspase activation, PA682PB cells were pretreated with either NAC or zVAD-fmk for 1 h followed by treatment with curcumin. As seen in Fig. 3C, NAC pretreatment blocked conformational changes of Bax protein, whereas zVAD-fmk pretreatment was unable to inhibit such changes, indicating a role for ROS release but not caspase activation in these changes. The oligomerization of Bax has been shown previously to only occur in apoptotic cells, possibly playing a role in mediating cytochrome c release (32, 38). To test this possibility, we determined whether curcumin treatment of Burkitt’s lymphoma cells could trigger Bax oligomerization. PA682PB cells were treated with curcumin; the cross-linked proteins were separated on SDS-PAGE and immunoblotted for the analysis of Bax oligomerization as described (32). As shown in Fig. 3D, an immunoreactive band of 42 to 46 kDa, reported previously as Bax homodimer (39), could be detected from the curcumin-treated PA682PB cell lysate. In addition, 88- to 90-kDa bands that could be Bax tetramer or oligomer were also detectable. These results suggest that curcumin treatment of Burkitt’s lymphoma cells involve Bax dimerization/oligomerization and play an important role in curcumin-induced apoptosis.

Curcumin Induces Loss of Mitochondrial Membrane Potential and Subsequent Release of Cytochrome c Induced by Curcumin in Burkitt’s Lymphoma Cells

We further sought to determine the effects of curcumin on mitochondrial membrane potential. Burkitt’s lymphoma cells were treated with different concentrations of curcumin for 24 h, cells were labeled with 5,5’,6,6’-tetramethyl-1,1’3,3’-tetraethylbenzimidazolylcarbocyanine dye, and mitochondrial membrane potential was measured by flow cytometry. As shown in Supplementary Fig. S3A, curcumin treatment resulted in loss of mitochondrial membrane potential in AS283A, KK124, PA682PB as measured by 5,5’,6,6’-tetramethyl-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine-stained green fluorescence depicting apoptotic cells. Interestingly, there was no change in the mitochondrial membrane potential in Bax-deficient cells, clearly indicating the importance of functional Bax in curcumin-induced apoptosis. Cytochrome c release, secondary to changes in mitochondrial membrane potential, was also detected in Bax-expressing cell line only (Supplementary Fig. S3B).
Curcumin-Induced Signaling Activates Caspase-9/3 and PARP Cleavage in Burkitt’s Lymphoma Cells

We also investigated whether curcumin treatment of Burkitt’s lymphoma cells activates caspase-9 and -3, further downstream in the apoptotic pathway, and promotes cleavage of PARP. Figure 4A shows that curcumin treatment results in activation of caspase-9 and -3 as well as cleavage of PARP in Bax-positive PA682PB and KK124 cell lines, whereas the Bax-deficient CA46 cell line did not show activation of caspase-9, caspase-3, or PARP cleavage. To address whether ROS release plays a role in curcumin-induced apoptosis, we pretreated the PA682PB and KK124 cell lines with 10 mmol/L NAC and 80 μmol/L zVAD-fmk for 1 h and subsequently treated with 20 μmol/L curcumin for 24 h. Cells were lysed with 1% CHAPS lysis buffer and the lysates were immunoprecipitated with anti-Bax 6A7 antibody and proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with Bax rabbit polyclonal antibody. D, curcumin induced Bax oligomerization in Burkitt’s lymphoma cells. PA682PB cells were treated with 20 μmol/L curcumin for indicated periods. The oligomerization of Bax was assessed by cross-linking with DSS followed by immunoblot analysis using Bax rabbit polyclonal antibody. DMSO was used as the vehicle control.

Curcumin-Induced ROS Generation Regulates Up-regulation of DR5

Recent studies have shown that DR5 expression is up-regulated by ROS generated by several compounds including curcumin (32, 40). In view of these findings, we sought to determine whether curcumin-generated free radicals modulate the expression of DR5 in Burkitt’s lymphoma cells. First, we carried out Western blotting to investigate the induction DR4 and DR5 proteins by curcumin. PA682PB and CA46 cells were treated with curcumin for various periods and cell lysates were separated by SDS-PAGE and immunoblotted with an antibody against DR5. As shown in Fig. 5A, curcumin treatment of Burkitt’s lymphoma cells selectively up-regulated DR5 in a time-dependent manner. When the blots were probed with an antibody against DR4, no up-regulation of this protein was detectable (data not shown). We then examined whether curcumin treatment of Burkitt’s lymphoma cells regulates DR5 expression at the transcriptional level. Reverse transcription-PCR analysis showed that curcumin enhanced DR5 up-regulation at mRNA level in a time-dependent manner (data not shown), indicating a transcriptional regulatory mechanism. To investigate whether ROS generation is directly associated with curcumin-induced DR5 up-regulation, we assessed DR5 expression in PA682PB and CA46 cells pretreated with NAC for 1 h followed by treatment with 20 μmol/L curcumin. As shown in Fig. 5B, treatment with curcumin significantly increased DR5 protein
levels, whereas pretreatment with 10 mmol/L NAC markedly inhibited curcumin-induced DR5 up-regulation. In addition, pretreatment with 80 μmol/L pan-caspase inhibitor, zVAD-fmk, followed by curcumin treatment did not alter the expression level of DR5, suggesting no active role of caspases in the regulation of DR5 expression. Taken together, these data clearly indicate that ROS generation is critical for curcumin-induced DR5 up-regulation.

Figure 4. Activation of caspase-9, caspase-3, and cleavage of PARP induced by curcumin treatment in Burkitt’s lymphoma cells. A, PA682PB, KK124, and CA46 cells were treated with and without 10 and 20 μmol/L curcumin for 24 h. Cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with antibodies against pro-caspase-9, pro-caspase-3, cleaved caspase-3, and β-actin. B, PA682PB and KK124 cells were pretreated with 10 mmol/L NAC for 1 h and subsequently treated with 20 μmol/L curcumin for 24 h. Cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with antibodies against pro-caspase-3 and PARP or live and dead cells were scored using trypan blue exclusion dye (C). Mean ± SD of three independent experiments.
**Up-Regulation of DR5 Does Not Play a Role in Curcumin-Induced Apoptosis**

DR5 up-regulation is important in activating the extrinsic pathway of apoptosis; however, its role in curcumin-induced apoptosis of Burkitt’s lymphoma cells has not been elucidated. To address this issue, PA682PB and AS283A cells were transfected with siRNA specific for DR5 and after treatment with curcumin; apoptosis was evaluated by Annexin V/PI dual staining. Surprisingly, silencing of DR5 by siRNA did not prevent apoptotic effect of curcumin on Bax-expressing PA682PB and AS283A cells (Fig. 5C). Down-regulation of curcumin-induced DR5 by siRNA transfection also did not prevent the activation of caspase-3 as well as cleavage of PARP, strongly suggesting that although curcumin causes up-regulation of DR5, DR5 does not play an active role in curcumin-induced apoptosis in Burkitt’s lymphoma cell lines (Fig. 5D).

**Curcumin Sensitizes TRAIL-Mediated Apoptosis in Bax-Deficient Cell Lines**

Curcumin treatment has a potent apoptotic effect on Bax-expressing Burkitt’s lymphoma cell lines; however, curcumin failed to induce apoptosis in Bax-deficient cell lines despite DR-5 up-regulation. To examine whether up-regulation of DR5 in Bax-deficient cell lines results in induction of apoptosis, CA46 cells were treated with curcumin in combination with TRAIL for 24 h and apoptosis was assessed by flow cytometry and Western blot analysis. Figure 6A and B show that neither curcumin treatment nor TRAIL treatment alone induced apoptosis in Bax-deficient CA46 cells. However, combination treatments of curcumin and TRAIL resulted in strong induction of apoptosis (2.3-27.1% at concentrations of 5 μmol/L curcumin and 50 ng TRAIL and 39.5% at concentrations of 5 μmol/L curcumin and 100 ng TRAIL). We further sought to determine the expression of caspases by Western blot analysis and found that procaspase-8 and -3 was only activated in Bax-deficient CA46 cells following combination treatment with curcumin and TRAIL, but unlike PA682PB where activation of caspase-8 and -3 was evident in all the treatment modalities (Fig. 6C). This suggests a vital role for curcumin in combination with TRAIL in Bax-deficient Burkitt’s lymphoma cell lines.

**Discussion**

Induction of apoptosis in malignant cells is a very important mechanism of action of some chemopreventive
agents (10). Curcumin has well-documented proapoptotic properties in a variety of cell types, including cells of hematopoietic origin (10, 14). Previous studies have shown that curcumin induces apoptosis in various cancer cell types (10, 14) but not in a variety of normal cells including primary hepatocytes, normal lymphocytes, and rat skin fibroblasts (41). We now provide evidence that curcumin induces cell death and apoptosis of Burkitt’s lymphoma cell lines expressing Bax in a dose-dependent manner. On the other hand, Burkitt’s lymphoma cell lines that do not express Bax are resistant to curcumin-induced apoptosis. Our data showed that all Burkitt’s lymphoma cell lines expressed constitutive NF-\(\kappa\)B regardless of Bax status. Curcumin treatment of Burkitt’s lymphoma cell lines suppressed NF-\(\kappa\)B in all cell lines via generation of ROS, such as NAC; a scavenger of ROS abrogated the curcumin-induced suppression of NF-\(\kappa\)B. However, curcumin-mediated ROS induced apoptosis only in those cell lines that expressed functional Bax, suggesting that Bax plays a critical role in curcumin-induced apoptosis.

Curcumin treatment of Burkitt’s lymphoma cells containing functional Bax causes Bax conformational changes and oligomerization that lead Bax to translocate to mitochondrial membrane and causing loss of mitochondrial membrane potential. Our data also show that pretreatment of Burkitt’s lymphoma cells with a universal caspase inhibitor, zVAD-fmk, did not block curcumin-dependent conformational changes of the Bax protein in Burkitt’s lymphoma cell lines. These results suggest that curcumin-mediated Bax conformational change and oligomerization occur upstream of caspase activation. However, pretreatment of NAC blocked Bax conformational changes following curcumin treatment in Burkitt’s lymphoma cell lines, suggesting that Bax conformational change and oligomerization is mediated by ROS. Altogether, these results indicate that curcumin-induced apoptosis requires release of ROS.

There are reports indicating that overexpression of Bax enhances cytochrome c release from mitochondria to the cytosol (42), whereas direct addition of recombinant Bax protein to isolated mitochondria has been shown previously to induce cytochrome c release (43). Our results establish that curcumin induces the loss of mitochondrial potential in all Bax-expressing Burkitt’s lymphoma cell. Loss of

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Figure 6. A and B, combination of curcumin and TRAIL induced apoptosis in Bax-deficient cell lines. CA46 cells were treated with either 5 \(\mu\)mol/L curcumin in the presence and absence of 50 and 100 ng TRAIL for 24 h. Cells were stained with fluorescent-conjugated Annexin V/PI and analyzed by flow cytometry. Average of three independent experiments. *, \(P < 0.001\), statistically significant (Student’s t test). C, PA682PB and CA46 cells were treated with 5 \(\mu\)mol/L curcumin in the presence and absence of 50 and 100 ng TRAIL for 24 h. Cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with antibodies against pro-caspase-8, pro-caspase-3, and \(\beta\)-actin.
mitochondrial membrane potential is one of the main mechanisms responsible for cytochrome c release in response to different cytotoxic stimuli. More recently, ample evidence suggests (44, 45) that some apoptogenic agents induce Bax translocation to the mitochondrial membrane followed by cytochrome c release. In cytosol, cytochrome c plays a key role in the formation of the apoptosome complex by activating the binding of procaspase-9 and apoptotic protease-activating factor-1 in the presence of ATP. The formation of the apoptosome then causes cleavage of caspase-9 that propagates the death signal by activating caspase-3 and causing cleavage of PARP. Activation and cleavage of PARP is the hallmark of apoptosis that in turn causes DNA fragmentation and cell death.

There are studies that have shown that cells lacking Bax in colon cancer cells and mouse embryonic fibroblasts lacking Bax/Bak showed resistance to curcumin-induced apoptosis (46, 47). In both these studies, resistance to curcumin treatment was overcome by overexpression of second mitochondrial derived activator of caspase. Our study also emphasizes the important role of Bax in curcumin-induced apoptosis in Burkitt’s lymphomas. Despite that curcumin treatment causes up-regulation of DR5 in all Burkitt’s lymphoma cell lines, curcumin treatment failed to activate caspase-3 and cleave PARP in Bax-deficient cell lines, suggesting that up-regulation of DR5 is not sufficient to induce apoptosis in Bax-deficient cell lines. There are also studies that have shown that curcumin treatment sensitizes cancer cells to TRAIL-induced apoptosis in other tumors including prostate and ovary (48, 49). In concordance with these studies, our data show that curcumin treatment sensitizes Bax-deficient Burkitt’s lymphoma cells to TRAIL-induced apoptosis. Using this strategy, Bax-deficient cell lines can also be coax into apoptosis by activating the extrinsic apoptotic pathway as well as use subtoxic doses of curcumin and TRAIL to induce apoptosis in Bax-expressing cell lines. This combination strategy using curcumin and TRAIL becomes even more important, as there are reports in which analysis of primary Burkitt’s lymphoma biopsies indicates that loss of Bax expression is not confined to cell lines and also occurs in primary tumors (2).

Clinically, resistance to apoptosis by chemotherapeutic agents is a frequent problem that emerges during the management of malignancies. The cytotoxic inducing ability of curcumin in cancer cells in conjunction with its nontoxic nature to normal cells could make it a potentially effective chemopreventive and/or therapeutic agent for the treatment of different tumors. An increasing number of studies have shown curcumin anticancer efficacy in preclinical and clinical settings (17). Several phase I clinical studies have also shown that curcumin was well tolerated up to 10 to 12 g (17, 50). Furthermore, the combination of curcumin and TRAIL resulted in considerable apoptosis in tumors that do not express Bax such as Burkitt’s lymphoma, which confers resistance to many conventional chemotherapeutic agents. Thus, it is possible that combinations of curcumin with chemotherapeutic agents may be an effective approach to enhance apoptosis of malignant cells and/or reverse resistance in vitro and possibly in vivo, and this remains to be directly addressed in future work.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
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Curcumin suppresses constitutive activation of nuclear factor-κB and requires functional Bax to induce apoptosis in Burkitt’s lymphoma cell lines
