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Induction of Apoptosis by Curcumin in Murine Myelomonocytic Leukemia WEHI-3 Cells is Mediated via Endoplasmic Reticulum Stress and Mitochondria-Dependent Pathways

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Received 31 July 2010; revised 14 February 2011; accepted 20 February 2011

ABSTRACT: Curcumin, derived from the food flavoring spice turmeric (*Curcuma longa*), has been shown to exhibit anticancer activities and induce apoptosis in many types of cancer cell lines. In our previous study, curcumin was able to inhibit murine myelomonocytic leukemia WEHI-3 cells *in vivo*. However, there is no report addressing the cytotoxic responses and the mechanisms underlying curcumin-induced apoptotic cell death in WEHI-3 cells. Therefore, we hypothesized that that curcumin affected WEHI-3 cells and triggered cell death through apoptotic signaling pathways. The effects of curcumin on WEHI-3 cells were investigated by using flow cytometric analysis, comet assay, confocal laser microscopy and Western blotting. In this study, we found that curcumin induced apoptosis in WEHI-3 cells in a dose-dependent (5–20 μM) manner. Interestingly, curcumin enhanced the level of the antiapoptotic protein Bcl-2 which might show that curcumin-induced apoptosis is done through the ER stress signaling pathways based on the increase of CIEBP homologous protein (CHOP), activating transcription factor 6 (ATF-6), inositol-requiring

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Contract grant sponsor: Taiwan Department of Health China Medical University Hospital Cancer Research Center of Excellence.

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enzyme 1 (IRE1), and caspase-12 in WEHI-3 cells. Moreover, curcumin increased the reactive oxygen species (ROS) production and cytosolic Ca^{2+} release, and induced DNA damage, but decreased the level of mitochondrial membrane potential ($\Delta\Psi_m$) in WEHI-3 cells. In conclusion, curcumin-induced apoptosis occurs through the ROS-affected, mitochondria-mediated and ER stress-dependent pathways. The evaluation of curcumin as a potential therapeutic agent for treatment of leukemia seems warranted. Environ Toxicol 00: 000–000, 2011.

Keywords: curcumin; murine myelomonocytic leukemia WEHI-3 cells; apoptosis; ER stress; mitochondria

INTRODUCTION

Cancer is a one of the major concerns for health in worldwide populations. This disease has caught the attention of people including medical doctors and scientists and they are all in a race to develop new drugs to treat patients. Numerous works have been focused on identifying new naturally occurring chemopreventive compounds for use to inhibit, retard, or reverse the processes of multistage carcinogenesis. It is well documented that apoptosis plays an important role in normal biological processes and also in cancer (Hengartner, 2000). Apoptotic signaling pathways can be divided into caspase-dependent and -independent or mitochondria-dependent and -independent pathways. Various chemotherapeutic agents-involved in the induction of apoptosis have been recognized to be the best strategy to manage cancer (Hengartner, 2000; Okun et al., 2008).

Curcumin (diferuloylmethane), one of phenolic compounds isolated from *curcuma longa*, has been demonstrated to have anticancer activities of many cancer cell lines, including hematologic malignancies cells *in vitro* (Anand et al., 2008) and *in vivo* (Hatcher et al., 2008). Curcumin reduced the *N-bis*(2-hydroxypropyl) nitrosamine-induced lung tumorigenesis in BALB/c mice *in vivo* (Huang et al., 2008). Many clinical trials on curucmin with cancer patients are actively going on right now (Hatcher et al., 2008). These anticancer activities of curcumin that were reported may associate with its effects on many molecular targets that are involved in cell cycle, apoptosis, transformation, proliferation, angiogenesis, survival, invasion, and metastasis of tumor cells (Lin, 2007). In a phase I study, the safety of curcumin for oral ingestion of curcumin up to 8000 mg/day for 3 months did not produce any treatment-related toxicity (Cheng et al., 2001). In a phase II trial, it has been demonstrated on pancreatic cancer patients taking curcumin daily (Dhillon et al., 2008). Curcumin-triggered apoptosis in B-cell chronic lymphocytic leukemia (B-CLL) cells may relate to the inhibition of constitutively activated NF-κB (Everett et al., 2007). It was reported that curcumin-induced apoptosis through a mechanism of down-regulating ornithine decarboxylase and along a ROS-dependent mitochondria-mediated pathway in human acute promyelocytic leukemia HL-60 cells (Liao et al., 2008). Our previous study also showed that curcumin induced cell cycle arrest and apoptosis in HL-60 cells via changes of mitochondrial membrane potential ($\Delta\Psi_m$) and caspase-3 activation (Tan et al., 2006), and it inhibited WEHI-3 cells in BALB/c mice and prolonged leukemia animal survival rate *in vivo* after the establishment of leukemia mice (Su et al., 2008). However, there is no available information to show that curcumin induced apoptosis in WEHI-3 cells *in vitro*. Therefore, in the present study, we focused on the induction of apoptosis by curcumin and investigated the mechanisms of cell death in the unfolded protein response (ER stress) and mtochondria-dependent apoptotic signaling pathways in the mouse myelomonocytic leukemia WEHI-3 cells *in vitro*.

MATERIALS AND METHODS

Materials and Chemicals

Curcumin, dimethyl sulfoxide (DMSO), propidium iodide (PI), trypan blue and triton X-100 were obtained from Sigma-Aldrich Corp. (St. Louis, MO). All primary and secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The fluorescent probes 2′,7′-dichlorofluorescin diacetate (DCFH-DA), Indo 1/AM and $DiOC_6$ were from Invitrogen Life Technologies (Carlsbad, CA). All primary and secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

WEHI-3 Cell Culture

The mouse myelomonocytic leukemia cell line (WEHI-3) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 100 units/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and 2 mM L-glutamine (Invitrogen) under an atmosphere of humidified 5% CO_2 and 95% air grown at 37°C and 1 atm. in an incubator (Su et al., 2008; Yu et al., 2009).

Morphological Changes and Percentage of Viable Cell Examination in WEHI-3 Cells

About 2×10^5 WEHI-3 cells/well in 24-well plates were treated with 0, 1, 5, 10, 15, and 20 μM curcumin, or only

with vehicle (DMSO, 1% in culture media) and all cells were incubated for 24 or 48 h. For the morphological changes examination, cells in the well with or without curcumin were undertaken and photographed under a phase-contrast microscope. For determining cell viability, trypan blue exclusion (live cells is white color but the dead cells is blue color) was used as previously described (Chiang et al., 2006; Lin et al., 2007a,b). An aliquot of the total cell suspension from each treatment was mixed with an equal volume of trypan blue in PBS and incubated for 5 min at room temperature and viable cells are a white color and dead cells are a light blue color under microscope examination. The total viable cells on the Neubauer chamber were counted under a phase-contrast microscope (Tan et al., 2006; Lu et al., 2010a).

Determination of Cell Cycle Distribution and Apoptosis in WEHI-3 Cells by Flow Cytometric Assay

A total of 2×10^5 WEH-3 cells/well in 12-well plates were treated with or without 10 μM curcumin, or only with vehicle (DMSO, 1% in culture media) and all cells were incubated for 0, 6, 12, 24, and 36 h. After incubation at various time periods, cells from each treatment were individually harvested and fixed gently with 70% ethanol at -20°C overnight. Cells were washed twice with PBS and then incubated with 20 μg/mL PI, 100 μg/mL RNase, and 0.1% Triton X-100 in PBS for 30 min in the dark. The PI stained cells were analyzed for cell cycle distribution and apoptosis (sub-G1 phase) by using a FACSCalibur instrument (BD Biosciences, San Jose, CA) equipped with Cell Quest software as described previously (Tan et al., 2006; Chiu et al., 2009). The sub-G1 group was representative of mean apoptosis.

DAPI Staining and Comet Assay for Apoptosis and DNA Damage in WEHI-3 Cells

About 2×10^5 WEHI-3 cells/well in 12-well plates were treated with 0, 5, 10, 15, and 10 μM curcumin were incubated for 24. Cells in each treatment were individually fixed with 4% formaldehyde for 15 min and stained with 4′-6-diamidino-2-phenylindole (DAPI, Invitrogen) for nucleic acid condensation as described elsewhere (Chiang et al., 2006; Yang et al., 2010). The examined cells were prepared as previously described (Chiang et al., 2006; Chen et al., 2010) for Comet assay and DNA was stained by PI to determine the DNA tail in curcumin-treated WEHI-3 cells. All samples were photographed on a fluorescence microscope.

DNA Gel Electrophoresis for DNA Fragmentation Determination

DNA fragmentation in WEHI-3 cells was exposed to various doses of curcumin and determined by DNA gel electrophoresis. About WEHI-3 cells at 2×10^6 cells/well in 12-well plates were cultured with 0, 5, 10, 15, and 20 μM curcumin for 24 h. DNA was isolated (Genomic DNA purification kit, Genemark Technology Co, Ltd, Tainan, Taiwan) and the ladder formation assay were done as previously described (Lu et al., 2010b; Yang et al., 2010).

Assay for Mitochondrial Membrane Potential ($\Delta\Psi_m$), Reactive Oxygen Species (ROS), Cytosolic Ca^{2+} Release in WEHI-3 Cells

About 2×10^5 WEHI-3 cells/well in 12-well plates were incubated with 10 μM curcumin for 0, 1, 3, 6, 9, 12, or 24 h to determine the level of $\Delta\Psi_m$, and the productions of ROS and cytosolic Ca^{2+}. After cells were incubated for various time periods, all cells in each treatment were harvested, washed twice by PBS, then re-suspended in 500 μL of $DiOC_6$ (1 μmol/L) for the level of $\Delta\Psi_m$, 500 μL of DCFH-DA (10 μM) for ROS and in Indo 1/AM(3 μg/mL) for cytosolic Ca^{2+} production at 37°C in dark room for 30 min. Then all samples were analyzed immediately by flow cytometry as previously described (Lin et al., 2010; Lo et al., 2010; Chiang et al., 2011).

Determinations of Viability, Apoptosis, and Ca^{2+} in WEHI-3 Cells were Pretreated with *N*-Acetylcysteine (NAC) and then Exposed to Curcumin

About 2×10^5 WEHI-3 cells/well in 12-well plates were pretreated with 20 mM NAC (Sigma-Aldrich Corp.) for 2 h then before all samples in well were treated with or without 10 μM curcumin for 24 h. The cells were harvested and examined for percentage of viability. The sub-G1 phase (apoptosis) and the Ca^{2+} release as described above were assayed by flow cytometry (Huang et al., 2010; Wu et al., 2010).

Determinations of Caspase-3, -8, and -9 Activities in WEHI-3 Cells after Exposure to Curcumin

About 2×10^5 WEHI-3 cells/well in 12-well plates were incubated with 10 μM curcumin for 0, 12, 24, and 48 h. The cells from each well were individually harvested and washed twice with PBS for determination of the activities of caspase-3, -8 and -9 by adding specific substrates (PhiPhiLux-G_1D_2 for caspase-3, CaspaLux8-L_1D_2 for caspase-8, CaspaLux9-M_1D_2 for caspase-9, OncoImmunin, Inc., Gaithersburg, MD), and then the activities of caspase-8, -9, and -3 were determined by flow cytometry as previously described (Ji et al., 2009; Lu et al., 2010c).

Western Blotting Analysis

About 5×10^6 WEHI-3 cells/well in 6-well plates were incubated with 10 μM curcumin for 0, 6, 12, 24, and 48 h. The cells from each treatment were harvested and washed twice with PBS for determination of proteins levels

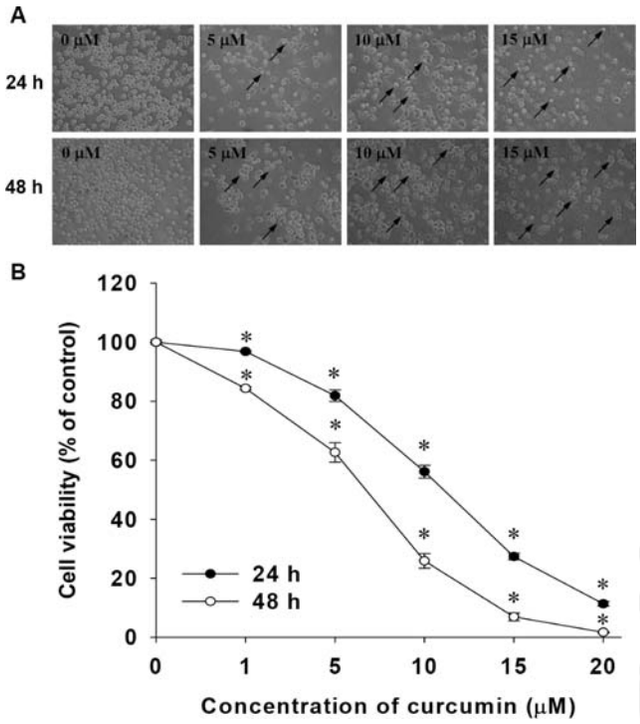

Fig. 1. Curcumin induced cell morphological changes and decreases the percentage of viable WEHI-3 cells. Cells were cultured in RPMI 1640 medium + 10% FBS with 0, 1, 5, 10, 15, and 20 μM curcumin for 24 and 48 h. The cell morphological changes were examined and photographed by phase-contrast microscopy (200×) at 24 and 48 h treatments (A), the percentage of viable WEHI-3 cells (B) were determined as described in Materials and Methods. Each point is mean ± SD of three experiments. *$P < 0.05$, significantly different compared with DMSO-treated control and curcumin treatment.

(caspase-3, AIF, Bcl-2, Bax and p53, CHOP, Bip, ATF-6α, ATF-6β, IRE1α, IRE1β, caspase-12, calpain1, and calpain 2) associated with apoptosis which were determined by Western blotting. Lysates of treated cells from each well were prepared using lysis buffer as described previously (Chung et al., 2007; Ip et al., 2008). Each sample was incubated with primary antibody for secondary antibody, detected by ECL reagent kit (Millipore, Billerica, MA) and then autoradiography using X-ray film (Chiu et al., 2009; Harikumar et al., 2009). Each membrane was re-probed with anti-β actin antibody to ensure that equal protein was loaded. The image is the outcome of protein quantification by NIH ImageJ software (Chiang et al., 2011).

Confocal Laser Scanning Microscopy for the Protein Translocation in WEHI-3 Cells

WEHI-3 cells at density of 5×10^4 cells/well were cultured in 4-well chamber slides and then were treated without or with 10 μM curcumin for 24 h. Cells were fixed in 4% formaldehyde in PBS for 15 min, permeabilized with 0.3% Triton-X 100 in PBS for 1 h. The fixed cells were stained with the AIF antibody (1:100 dilution) (green fluorescence) for overnight before being washed twice with PBS and were stained with FITC-conjugated goat antimouse IgG secondary antibody at 1:100 dilution, and followed by DNA staining with PI (red fluorescence) as previously described (13, 14). All samples were photomicrographed and obtained by using a Leica TCS SP2 Confocal Spectral Microscope (Lo et al., 2010; Wu et al., 2010).

Statistical Analysis

The quantitative data are shown as mean ± SD. The statistical differences between the curcumin-treated and control samples were calculated by Student's t test. A P value of less than 0.05 was considered significant. Results are representative of three independent experiments.

RESULTS

Curcumin Induced Morphological Changes and Decreased Percentage of Viable WEHI-3 Cells

WEHI-3 cells were treated with different concentrations of curcumin for 24 and 48 h, and then the morphological

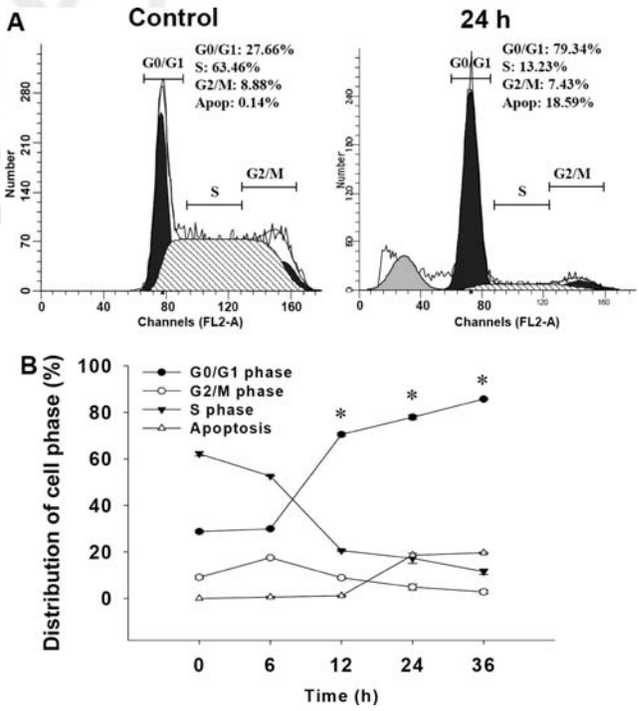

Fig. 2. Curcumin affected the cell cycle distribution and apoptosis in WEHI-3 cells. Cells were cultured with 20 μM curcumin for 0, 6, 12, 24, and 36 h. The cells were examined and analyzed for cell cycle distribution (A) and apoptosis (B) by flow cytometry as described in Materials and Methods. Each point is mean ± SD of three experiments. *$P < 0.05$, significantly different compared with DMSO-treated control and curcumin-treated groups.

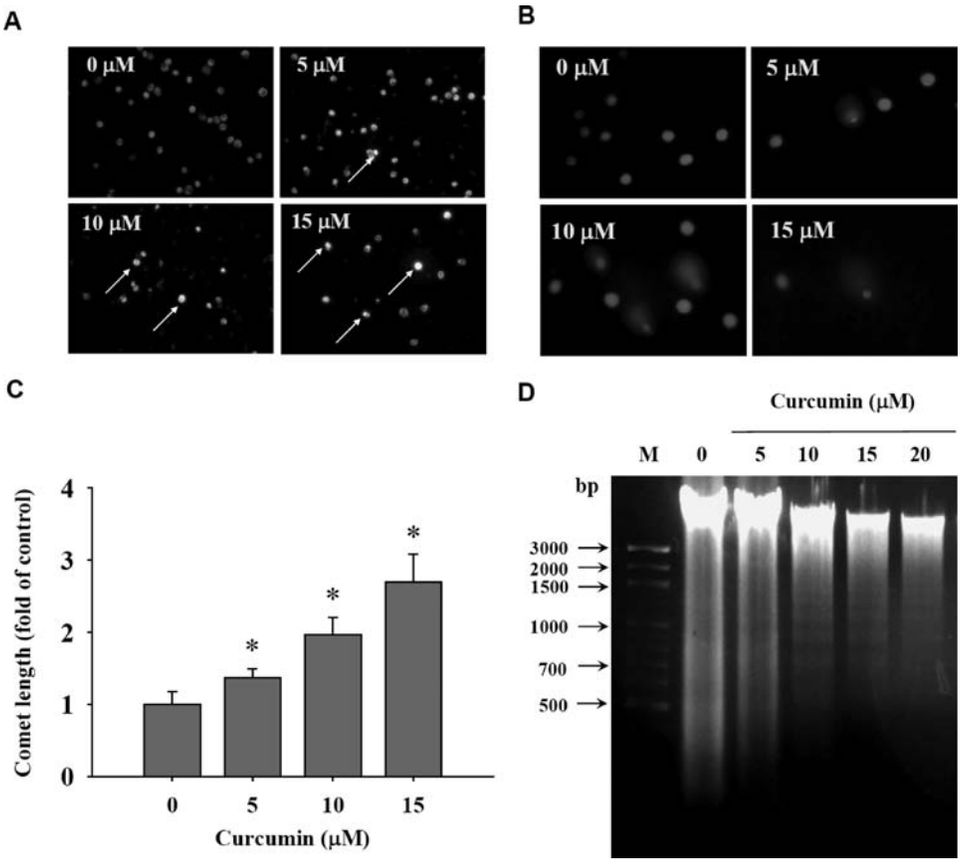

Fig. 3. Curcumin induced apoptosis and DNA damage in WEHI-3 cells. Cells were incubated with various concentrations of curcumin for 24 h. The cells were harvested and were examined for apoptosis by DAPI staining (A) and DNA damage by Comet assay (B) with quantification of fluorescence intensity (fold of difference between control and curcumin treatment) and Comet tail (% of difference between control and curcumin treatment) (C) were determined, the DNA fragmentation (D) is performed by DNA gel electrophoresis as described in Materials and Methods. Each point is mean ± SD of three experiments. *$P < 0.05$, significantly different compared with DMSO-treated control and curcumin-tereated groups. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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changes of cells were examined and photographed by a phase-contrast microscope. The results shown in Figure 1 F1 indicated that curcumin induced morphological changes in a dose-dependent manner. Cells from each treatment were also harvested for determining the percentage of viable cells by the trypan blue exclusion method. Results were shown in Figure 1(B), which showed that curcumin decreased the percentage of viable WEHI-3 cells in a dose-dependent manner.

Curcumin Induced Apoptosis in WEHI-3 Cells

WEHI-3 cells were treated with different concentrations of curcumin for 24 h and then were harvested for determining the cell cycle distribution and sub-G1 phase (apoptosis). The number of cells in each compartment of the cell cycle distribution and sub-G1 phase were expressed as % of the total number of cells and the results are shown in Figure 2(A,B). The results indicated that curcumin induced sub-G1 phase (apoptosis) in a dose-dependent manner and promoted G0/G1 phase arrest after 12 h-exposure [Fig. 2(B)]. F2

Curcumin Induced Chromatin Condensation and DNA Damage in WEHI-3 Cells

WEHI-3 cells were treated with different concentrations of curcumin for 24 h and then were harvested for determining the apoptosis by DAPI staining, for DNA damage by Comet assay and for DNA fragmentation by DNA gel electrophoresis. It can be seen in Figure 3(A–D). DAPI staining assay F3 demonstrated that curcumin induced apoptosis in a dose-dependent manner [Fig. 3(A)]. Comet assay demonstrated that curcumin (5–15 μM) for 24 h-treatment induced DNA damage in a dose-dependent manner [Fig. 3(B,C)]. DNA gel electrophoresis indicated that curcumin induced DNA fragmentation in a dose-dependent manner [Fig. 3(D)].

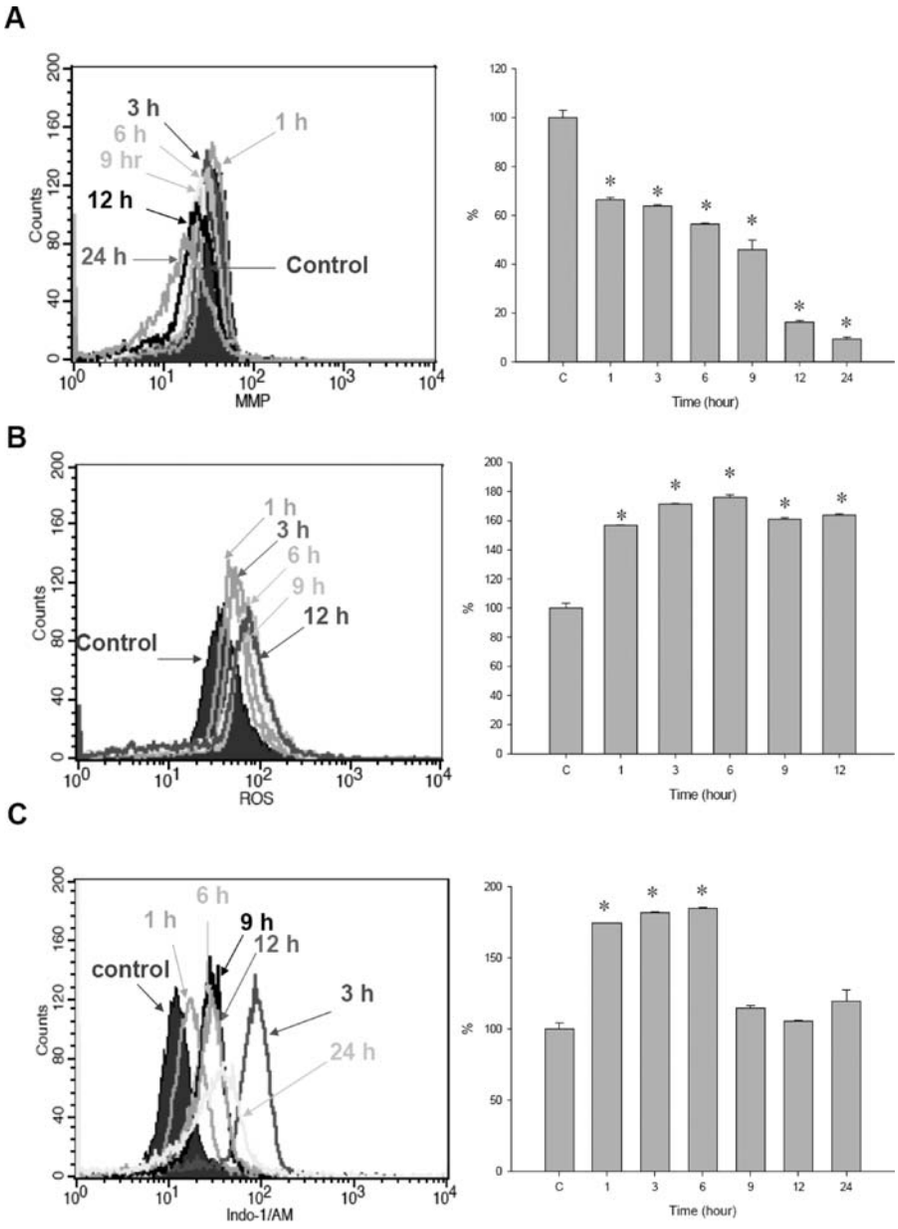

Fig. 4. Curcumin altered the levels of mitochondria membrane potential ($\Delta\Psi_{m)}$, reactive oxygen species (ROS) and the production of cytosolic Ca^{2+} in WEHI-3 cells. Cells were treated with 10 μM curcumin for 0, 1, 3, 6, 9, 12, or 24 h before being collected, and stained with $DiOC_6$ (1 μmol/L) for the level of $\Delta\Psi_m$ (A), DCFH-DA (10 μM) for ROS (B) and Indo 1/AM (3 μg/mL) for cytosolic Ca^{2+} production (C) as described in Materials and Methods. Each experiment was done with triple sets (mean $\pm$ SD): $^*P < 0.05$, significantly different compared with DMSO-treated control and curcumin treated groups.

Curcumin Affected the Levels of Mitochondria Membrane Potential ($\Delta\Psi_m$), Reactive Oxygen Species (ROS), and Cytosolic Ca^{2+} in WEHI-3 Cells

The WEHI-3 cells were treated with 10 μM curcumin for different periods of time and measured the levels of $\Delta\Psi_m$, ROS and cytosolic Ca^{2+} release by flow cytometric assay and the results are shown in Figure 4(A–C). There was a significant decrease in $\Delta\Psi_m$ level [Fig. 4(A)] and an increase in intracellular ROS [Fig. 4(B)] and cytosolic Ca^{2+} level [Fig. 4(C)] was observed in the curcumin-treated cells. Figure 4(A) indicated that curcumin significantly decreased the level of $\Delta\Psi_m$ in WEHI-3 cells in a time-dependent manner [Fig. 4(A)]. After 1 h-treatment of curcumin, there was initially significantly increased cytosolic Ca^{2+} levels, but 6 h-treatment, cytosolic Ca^{2+} levels were not significantly different from that in the control

F4

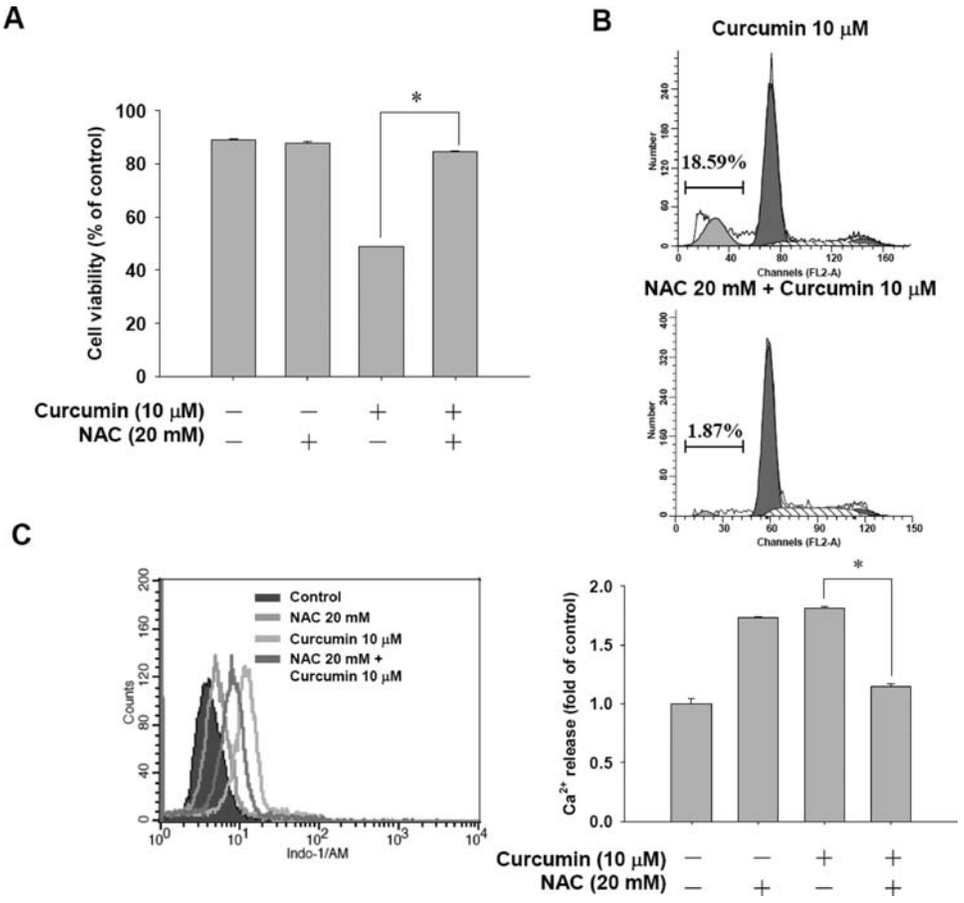

Fig. 5. The effects of viability, apoptosis and Ca^{2+} release in WEHI-3 cells pretreatment with NAC and then treatment with curcumin. Cells were before with 20 mM NAC for 2 h, and then were treated with or without 10 μM curcumin for 24 h. The cells were harvested and examined for percentage of viability (A), the sub-G1 phase (apoptosis) (B) and the production of Ca^{2+} (C) were assayed by flow cytometric assay as described in Materials and Methods. Each point is mean $\pm$ SD of three experiments. $^*P < 0.05$, significantly different compared with DMSO-treated control and curcumin-tereated groups. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

sample [Fig. 4(C)] and 12 h-treatment led to decrease the initially significantly increased cytosolic Ca^{2+} levels. However, the ROS level was significant higher than these of the control [Fig. 4(B)].

NAC Protected the Effects of Curcumin-Affected Cell Viability, Apoptosis, and Ca^{2+} in WEHI-3 Cells

The WEHI-3 cells were pretreated with 20 mM NAC for 2 h, and then were treated with or without 10 μM curcumin for 24 h. The cells were harvested and examined for percentage of viability, the sub-G1 phase (apoptosis) and the production of Ca^{2+}. Results are shown in Figure 5(A–C) and indicated that NAC can decrease the cytotoic Ca^{2+} effects of curcumin, and then lead to increase the percentage of viable cells [Fig. 5(A)] and decrease the percentage of apoptotic cells [Fig. 5(B)] and decrease the level of Ca^{2+} [Fig. 5(C)] in WEHI-3 cells.

F5

Curcumin Increased the Activities of Caspase-8, -9, and -3 in WEHI-3 Cells

The WEHI-3 cells were treated with 10 μM curcumin for 0, 12, 24, and 48 h, and then cells were harvested for the determination of the activities of caspase-8, -9, and -3. Results are shown in Figure 6(A–C) and revealed that curcumin induced caspase-8, -9, and -3 activities in a time-dependent manner. These results suggest that curcumin-induced apoptosis might be done through a caspase-dependent signal pathway.

F6

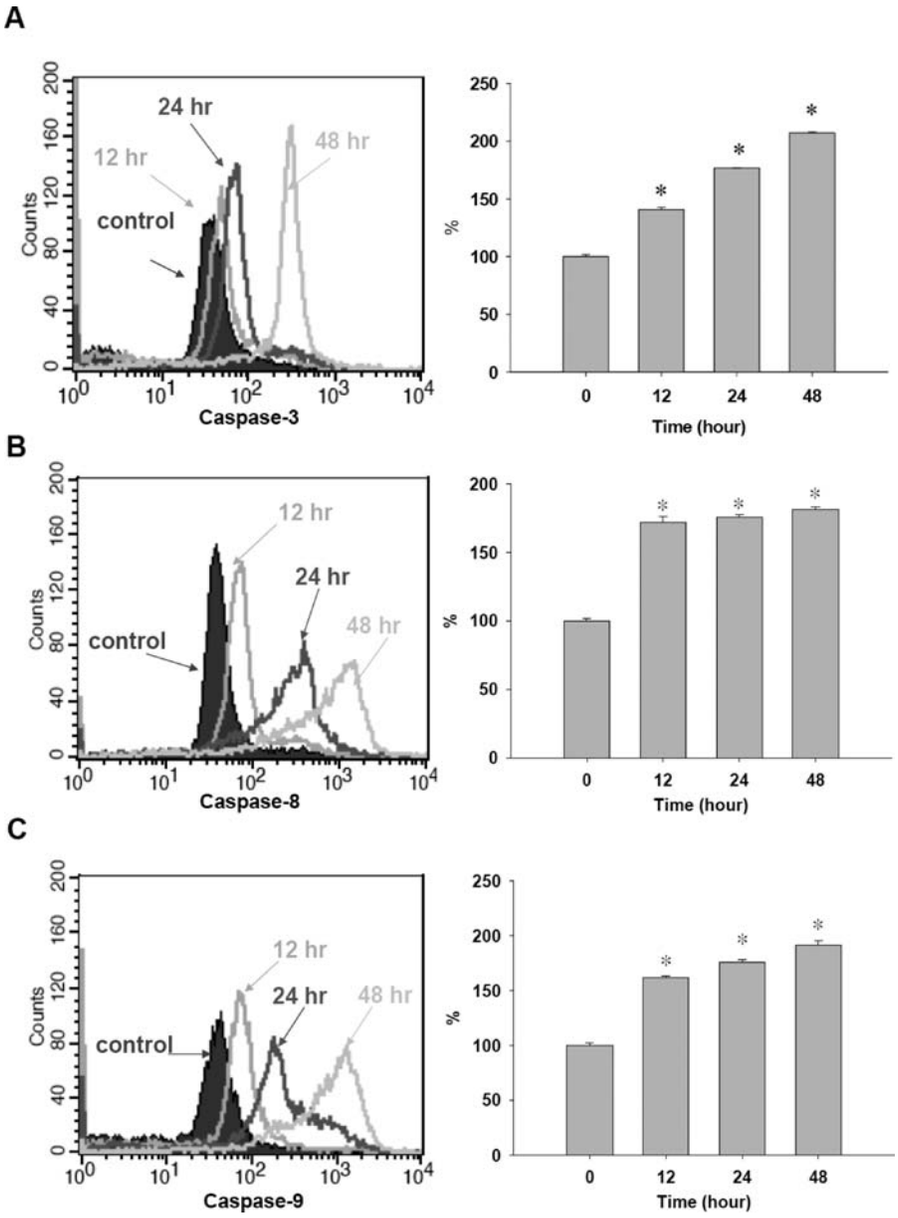

Fig. 6. Curcumin stimulated the activities of caspase-3, -8, and -9 in WEHI-3 cells. Cells were treated with or without 10 μM curcumin for 0, 12, 24, and 48 h. The cells were harvested and washed for measurement of caspase-3 (A), -8 (B), and -9 (C) activity as described in Materials and Methods. Each experiment was done with triple sets (mean $\pm$ SD). *$P < 0.05$, significantly different compared with DMSO-treated control and curcumin treated groups.

Curcumin Altered the Levels of Apoptosis and ER Stress-Associated Proteins in WEHI-3 Cells

The WEHI-3 cells were treated with 10 μM curcumin for 0, 6, 12, 24, and 48 h and then were harvested for determination of apoptosis-associated proteins such as caspase-3, AIF, Bcl-2, Bax, and p53 [Fig. 7(A)], CHOP, Bip, ATF-6α, ATF-6β, IRE1α, IRE1β, caspase-12, calpain 1 and calpain 2 [Fig. 7(B)]. The level of antiapoptotic protein Bcl-2 [Fig. 7(A)] was decreased and the levels of pro-apoptotic protein Bax [Fig. 7(A)] were up-regulated in WEHI-3 cells after exposure to curcumin. ER stress-associated protein levels such as caspase-12, calpain1 and calpain2, CHOP, Bip ATF-6α, and ATF-6β [Fig. 7(B)] were up-regulated, the p53 was also increased which could contribute to apoptotic cell death [Fig. 7(A)] in WEHI-3 cells.

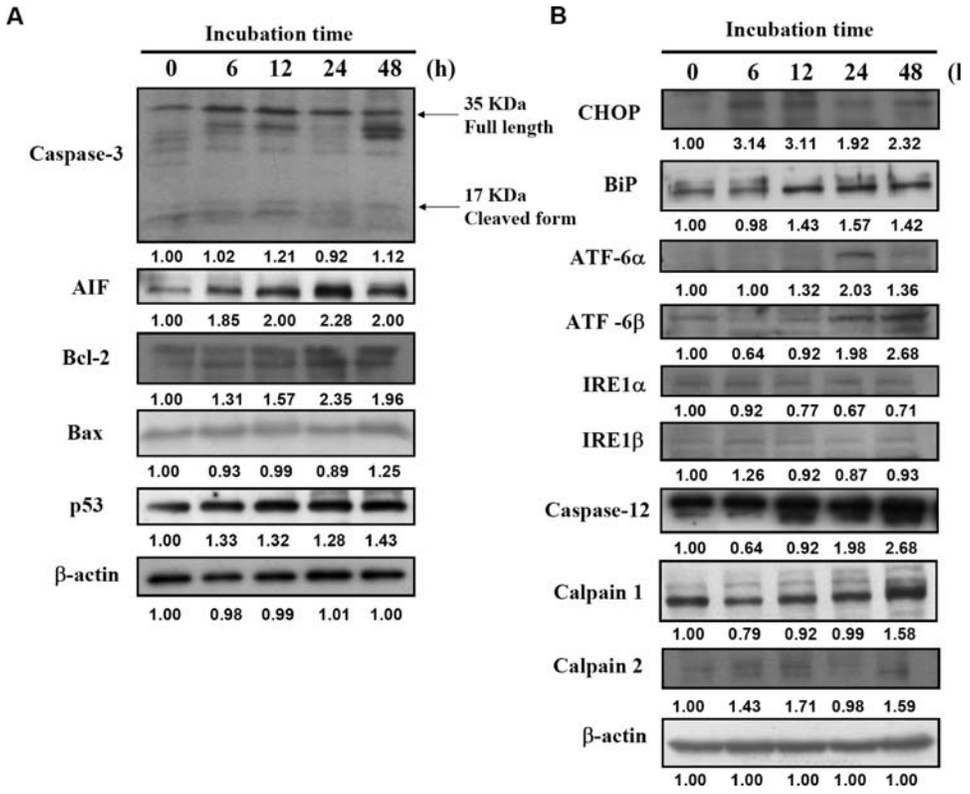

Fig. 7. Representative Western blotting showing changes in the levels of apoptosis and ER stress-associated proteins in WEHI-3 cells after curcumin exposure. Cells were treated with 10 μM curcumin for 0, 6, 12, 24, and 48 h before the total proteins were prepared and determined, as described in Materials and Methods. The levels of apoptosis-related protein expressions (A: caspase-3, AIF, Bcl-2, Bax and p53; B: CHOP, Bip, ATF-6α, ATF-6β, IRE1α, IRE1β, caspase-12, calpain1, and calpain2) were estimated by Western blotting analysis as described in Materials and Methods.

Curcumin Promoted AIF Translocation in WEHI-3 Cells

The WEHI-3 cells were treated with or without 10 μM curcumin for 24 h and then were harvested for the determination of the location of AIF. Results are shown in Figure 8 F8 and indicated that curcumin promoted the level of AIF which release from mitochondria, leading to apoptosis.

DISCUSSION

In this study, we showed that curcumin is an apoptotic induction agent in WEHI-3 cells that go through the ER stress-associated signaling and mitochondria-dependent pathways. Importantly, our results indicated that curcumin differed from most other therapeutic agents currently under the studies in WEHI-3 cells. Based on the observations (1) curcumin-induced apoptosis in WEHI-3 cells is dependent on ER stress activation. (2) Curcumin specifically down-regulated the calpin1 and 2 without discernible effects on level of Bcl-2, a finding that is relatively uncommon among most therapeutics used to treat lymphoid malignancy (Liu et al., 2008). (3) the biological effects of curcumin on WEHI-3 cells seems to be explained, at least in part, through the ER stress, including promotions of CHOP, ATF-6α, ATF-6β, IRE1α, IRE1β, and caspase-12. It is reported that curcumin is well tolerated with minimal toxicity at up to 8 g/d (f115 mg/kg/d) in a phase I clinical trial (Cheng et al., 2001). Collectively, these findings indicate that the use of curcumin in the treatment of leukemia may be worthy of study in the future.

It is well documented that curcumin induced apoptosis in malignant cells with numerous mechanisms. Although reports have demonstrated that curcumin reduces the constitutive phosphorylation level of IκBα, suggesting an inhibition of NF-κB activity in B-CLL cells (Everett et al., 2007), our analyses showed that curcumin induced a significant increase in Bcl-2 expression, an antiapoptotic protein elevated in WEHI-3 cells and also up-regulated AIF and

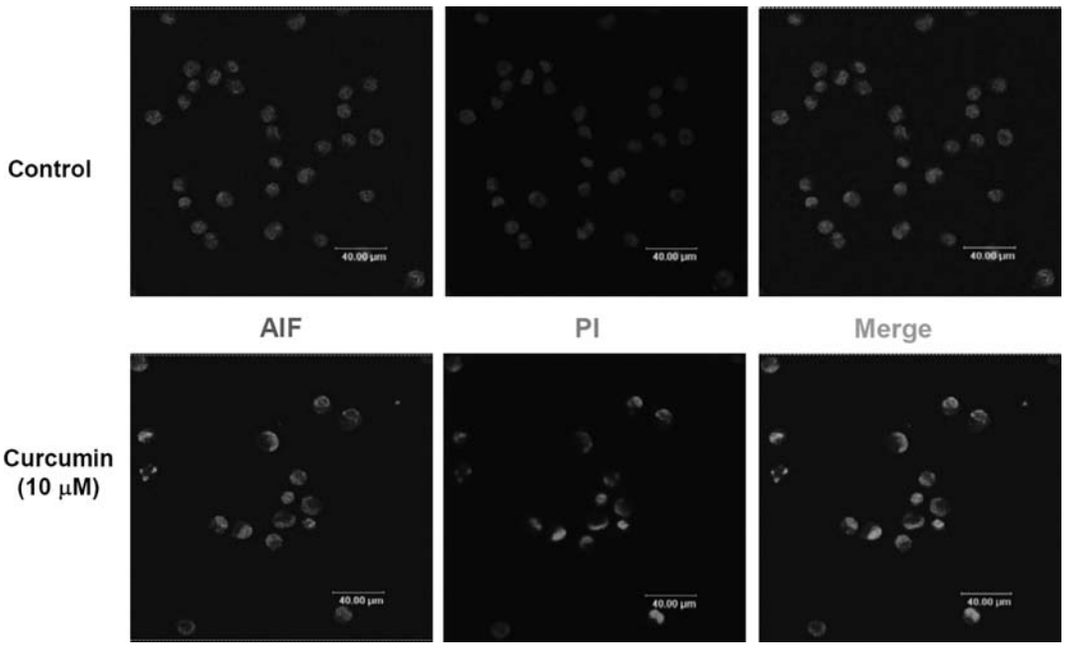

Fig. 8. Curcumin translocated AIF distribution in WEHI-3 cells. Cells were incubated with or without 10 μM curcumin for 24 h, and then fixed and stained with AIF antibody before the FITC-labeled secondary antibody were used (green fluorescence) and the protein was detected by a confocal laser microscope. The nuclei were stained by PI (red fluorescence). Areas of colocalization between AIF expression and nuclei in the merged panels are yellow. Scale bar, 40 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

p53 (data not shown) levels. Other report showed that curcumin treatment inhibited STAT3 activity and decreased the expression of Mcl-1, a downstream target of activated STAT3, in most B-CLL cells (Ghosh et al., 2009). Moreover, curcumin reduced the phosphorylation level of Akt. The serine/threonine kinase, Akt, has been considered an attractive target for cancer therapy and prevention (Datta et al., 1999). Apparently further investigation is needed in the future.

Furthermore, the doses (5–20 μM) of curcumin are able to induce apoptosis in WEHI-3 cells and our earlier study has shown that curcumin can promote the survival rate of leukemia mice after intraperitoneal injection with WEHI-3 cells (Su et al., 2008). These *in vitro* and *in vivo* experiments (Tan et al., 2006; Su et al., 2008) strongly encourage us to use a sequential approach in designing subsequent clinical trials with curcumin. Subsequent assessment of association with clinical outcome will be of great interest.

In conclusion, the present study indicates that curcumin is cytotoxic in murine leukemia WEHI-3 cells. These effects of curcumin are complex and promoted the apoptotic pathway through ER stress and mitochondrial dysfunction-dependent influences. These possible signaling pathways are summarized in Figure 9. Additional evaluation of curcumin as a potential therapeutic agent for the treatment of leukemia seems warranted and further investigation is needed.

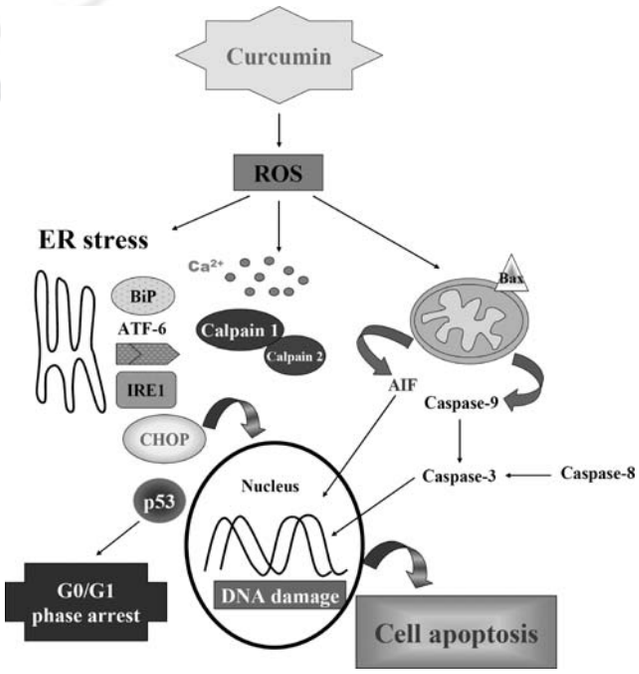

Fig. 9. The proposed model of molecular signaling pathways from murine myelomonocytic leukemia WEHI-3 cells after exposure to curccumin. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

REFERENCES

Anand P, Sundaram C, Jhurani S, Kunnumakkara AB, Aggarwal BB. 2008. Curcumin and cancer: an ''old-age'' disease with an ''age-old'' solution. Cancer Lett 267:133–164.

Chen YY, Chiang SY, Lin JG, Yang JS, Ma YS, Liao CL, Lai TY, Tang NY, Chung JG. 2010. Emodin, aloe-emodin and rhein induced DNA damage and inhibited DNA repair gene expression in SCC-4 human tongue cancer cells. Anticancer Res 30:945–951.

Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, Ko JY, Lin JT, Lin BR, Ming-Shiang W, Yu HS, Jee SH, Chen GS, Chen TM, Chen CA, Lai MK, Pu YS, Pan MH, Wang YJ, Tsai CC, Hsieh CY. 2001. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res 21:2895–2900.

Chiang JH, Yang JS, Ma CY, Yang MD, Huang HY, Hsia TC, Kuo HM, Wu PP, Lee TH, Chung JG. 2011. Danthron, an anthraquinone derivative. Induces DNA damage and caspase cascades-mediated apoptosis in SNU-1 human gastric cancer cells through mitochondrial permeability transition pores and Bax-triggered pathways. Chem Res Toxicol 24:20–29.

Chiang LC, Ng LT, Lin IC, Kuo PL, Lin CC. 2006. Anti-proliferative effect of apigenin and its apoptotic induction in human Hep G2 cells. Cancer Lett 237:207–214.

Chiu TH, Lai WW, Hsia TC, Yang JS, Lai TY, Wu PP, Ma CY, Yeh CC, Ho CC, Lu HF, Wood WG, Chung JG. 2009. Aloe-emodin induces cell death through S-phase arrest and caspase-dependent pathways in human tongue squamous cancer SCC-4 cells. Anticancer Res 29:4503–4511.

Chung JG, Yang JS, Huang LJ, Lee FY, Teng CM, Tsai SC, Lin KL, Wang SF, Kuo SC. 2007. Proteomic approach to studying the cytotoxicity of YC-1 on U937 leukemia cells and antileukemia activity in orthotopic model of leukemia mice. Proteomics 7:3305–3317.

Datta SR, Brunet A, Greenberg ME. 1999. Cellular survival: A play in three Akts. Genes Dev 13:2905–2927.

Dhillon N, Aggarwal BB, Newman RA, Wolff RA, Kunnumakkara AB, Abbruzzese JL, Ng CS, Badmaev V, Kurzrock R. 2008. Phase II trial of curcumin in patients with advanced pancreatic cancer. Clin Cancer Res 14:4491–4499.

Everett PC, Meyers JA, Makkinje A, Rabbi M, Lerner A. 2007. Preclinical assessment of curcumin as a potential therapy for B-CLL. Am J Hematol 82:23–30.

Ghosh AK, Kay NE, Secreto CR, Shanafelt TD. 2009. Curcumin inhibits prosurvival pathways in chronic lymphocytic leukemia B cells and may overcome their stromal protection in combination with EGCG. Clin Cancer Res 15:1250–1258.

Harikumar KB, Kuttan G, Kuttan R. 2009. Phyllanthus amarus inhibits cell growth and induces apoptosis in Dalton's lymphoma ascites cells through activation of caspase-3 and down-regulation of Bcl-2. Integr Cancer Ther 8:190–194.

Hatcher H, Planalp R, Cho J, Torti FM, Torti SV. 2008. Curcumin: from ancient medicine to current clinical trials. Cell Mol Life Sci 65:1631–1652.

Hengartner MO. 2000. The biochemistry of apoptosis. Nature 407:770–776.

Huang AC, Lin SY, Su CC, Lin SS, Ho CC, Hsia TC, Chiu TH, Yu CS, Ip SW, Lin TP, Chung JG. 2008. Effects of curcumin on N-bis(2-hydroxypropyl) nitrosamine (DHPN)-induced lung and liver tumorigenesis in BALB/c mice in vivo. In Vivo 22:781–785.

Huang WW, Chiu YJ, Fan MJ, Lu HF, Yeh HF, Li KH, Chen PY, Chung JG, Yang JS. 2010. Kaempferol induced apoptosis via endoplasmic reticulum stress and mitochondria-dependent pathway in human osteosarcoma U-2 OS cells. Mol Nutr Food Res 54:1585–1595.

Ip SW, Liao SS, Lin SY, Lin JP, Yang JS, Lin ML, Chen GW, Lu HF, Lin MW, Han SM, Chung JG. 2008. The role of mitochondria in bee venom-induced apoptosis in human breast cancer MCF7 cells. In Vivo 22:237–245.

Ji BC, Hsu WH, Yang JS, Hsia TC, Lu CC, Chiang JH, Yang JL, Lin CH, Lin JJ, Suen LJ, Gibson Wood W, Chung JG. 2009. Gallic acid induces apoptosis via caspase-3 and mitochondrion-dependent pathways in vitro and suppresses lung xenograft tumor growth in vivo. J Agric Food Chem 57: 7596–7604.

Liao YF, Hung HC, Hour TC, Hsu PC, Kao MC, Tsay GJ, Liu GY, 2008. Curcumin induces apoptosis through an ornithine decarboxylase-dependent pathway in human promyelocytic leukemia HL-60 cells. Life Sci 82:367–375.

Lin CC, Yang JS, Chen JT, Fan S, Yu FS, Yang JL, Lu CC, Kao MC, Huang AC, Lu HF, Chung JG. 2007a. Berberine induces apoptosis in human HSC-3 oral cancer cells via simultaneous activation of the death receptor-mediated and mitochondrial pathway. Anticancer Res 27:3371–3378.

Lin JK. 2007. Molecular targets of curcumin. Adv Exp Med Biol 595:227–243.

Lin ML, Lu YC, Chung JG, Li YC, Wang SG, N GS, Wu CY, Su HL, Chen SS. 2010. Aloe-emodin induces apoptosis of human nasopharyngeal carcinoma cells via caspase-8-mediated activation of the mitochondrial death pathway. Cancer Lett 291:46–58.

Lin YT, Yang JS, Lin HJ, Tan TW, Tang NY, Chaing JH, Chang YH, Lu HF, Chung JG, 2007b. Baicalein induces apoptosis in SCC-4 human tongue cancer cells via a Ca2+-dependent mitochondrial pathway. In Vivo 21:1053–1058.

Liu Q, Zhao X, Frissora F, Ma Y, Santhanam R, Jarjoura D, Lehman A, Perrotti D, Chen CS, Dalton JT, Muthusamy N, Byrd JC. 2008. FTY720 demonstrates promising preclinical activity for chronic lymphocytic leukemia and lymphoblastic leukemia/lymphoma. Blood 111:275–284.

Lo C, Lai TY, Yang JH, Yang JS, Ma YS, Weng SW, Chen YY, Lin JG, Chung JG. 2010. Gallic acid induces apoptosis in A375.S2 human melanoma cells through caspase-dependent and -independent pathways. Int J Oncol 37:377–385.

Lu CC, Yang JS, Huang AC, Hsia TC, Chou ST, Kuo CL, Lu HF, Lee TH, Wood WG, Chung JG. 2010a. Chrysophanol induces necrosis through the production of ROS and alteration of ATP levels in J5 human liver cancer cells. Mol Nutr Food Res 54:967–976.

Lu HF, Lai TY, Hsia TC, Tang YJ, Yang JS, Chiang JH, Lu CC, Liu CM, Wang HL, Chung JG. 2010b. Danthron induces DNA damage and inhibits DNA repair gene expressions in GBM

8401 human brain glioblastoma multiforms cells. Neurochem Res 35:1105–1110.

Lu HF, Wang HL, Chuang YY, Tang YJ, Yang JS, Ma YS, Chiang JH, Lu CC, Yang JL, Lai TY, Wu CC, Chung JG. 2010c. Danthron induced apoptosis through mitochondria- and caspase-3-dependent pathways in human brain glioblastoma multiforms GBM 8401 cells. Neurochem Res 35:390–398.

Okun I, Balakin KV, Tkachenko SE, Ivachtchenko AV. 2008. Caspase activity modulators as anticancer agents. Anticancer Agents Med Chem 8:322–341.

Su CC, Yang JS, Lin SY, Lu HF, Lin SS, Chang YH, Huang WW, Li YC, Chang SJ, Chung JG. 2008. Curcumin inhibits WEHI-3 leukemia cells in BALB/c mice in vivo. In Vivo 22:63–68.

Tan TW, Tsai HR, Lu HF, Lin HL, Tsou MF, Lin YT, Tsai HY, Chen YF, Chung JG. 2006. Curcumin-induced cell cycle arrest and apoptosis in human acute promyelocytic leukemia HL-60 cells via MMP changes and caspase-3 activation. Anticancer Res 26:4361–4371.

Wu SH, Hang LW, Yang JS, Chen HY, Lin HY, Chiang JH, Lu CC, Yang JL, Lai TY, Ko YC, Chung JG. 2010. Curcumin induces apoptosis in human non-small cell lung cancer NCI-H460 cells through ER stress and caspase cascade- and mitochondria-dependent pathways. Anticancer Res 30:2125–2133.

Yang JS, Hour MJ, Huang WW, Lin KL, Kuo SC, Chung JG. 2010. MJ-29 inhibits tubulin polymerization, induces mitotic arrest and triggers apoptosis via CDK1-mediated Bcl-2 phosphorylation in human leukemia U937 cells. J Pharmacol Exp Ther 334:477–488.

Yu FS, Wu CC, Chen CT, Huang SP, Yang JS, Hsu YM, Wu PP, Ip SW, Lin JP, Lin JG, Chung JG. 2009. Diallyl sulfide inhibits murine WEHI-3 leukemia cells in BALB/c mice in vitro and in vivo. Hum Exp Toxicol 28:785–790.

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