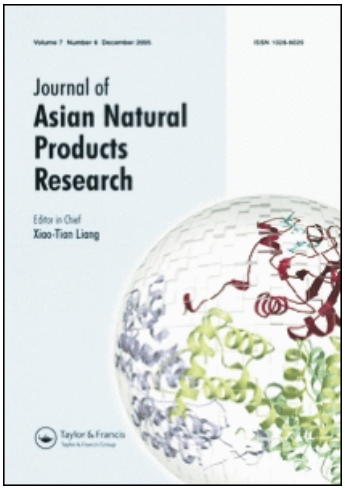

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Autophagic and apoptotic mechanisms of curcumin-induced death in K562 cells

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Curcumin (**1**), a natural polyphenolic compound, has shown strong antioxidant and anticancer activities. Several molecular mechanisms have been attributed to its inhibitory effects on a wide range of tumor cells. In this study, the response of the chronic myeloid leukemia cell line K562 cells to **1** is investigated. Curcumin inhibited the viability of K562 cells in a dose- and time-dependent manner. Furthermore, curcumin-induced cell death was associated with the formation of the apoptosome complex, the collapse of the mitochondrial membrane potential, and caspase-3 activation. Curcumin treatment also induced Bid cleavage and downregulated the expression of Bcl-2 protein. Surprisingly, even with these molecular features of apoptosis, we showed that **1** stimulated autophagy, which was evidenced by microtubule-associated protein light chain 3 (LC3) immunoreactivty. Curcumin also increased the protein levels of beclin 1 and membrane form LC3 (LC3-II). Autophagy inhibitor bafilomycin A1 and the pan-caspase inhibitor Z-VAD-fmk suppressed curcumin-induced K562 cell death. Overall, these results suggest that curcumin induces autophagic and apoptotic death of K562 cells. These findings suggest that both apoptotic and autophagic mechanisms contribute to the curcumin-induced K562 cell death.

Keywords: curcumin; autophagy; K562 cell; mitochondria; apoptosis

1. Introduction

Autophagy is an intracellular degradation system involving sequestration of cytoplasm and organelles into double-membrane vesicles that traffic the contents to lysosomes for degradation [1]. It is a genetically programmed, evolutionarily conserved process, typically observed in all eukaryotic cells from yeast to mammals [2]. There are three primary forms of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Among these forms, macroautophagy is the most prevalent one [3]. Recently, extensive attention has been paid to the role of autophagy in cancer development and therapy [4]. Previous articles suggested that chemotherapeutic agents induce autophagy in many human cancer cell lines [5]. In some cases, activation of autophagy is a cellular survival strategy [6]. On the other hand, persistent activation of autophagy can also lead to programmed cell death [7]. The term 'autophagic cell death' describes a form of programmed cell death morphologically distinct from apoptosis, which is referred to as type I programmed cell death [8]. Recent studies show that an extensive overlap exists between the apoptotic and autophagic cell death. Different forms of the

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cell death are shown to have common aspects and precede each other or even coexist in the same cell [9].

Chronic myelogenous leukemia (CML) is a hematopoietic stem cell cancer cytologically characterized by a translocation that creates a fusion between the *bcr* gene on chromosome 22 and the *c-abl* gene on chromosome 9 to form what is known as the Philadelphia chromosome [10]. CML is originated by the perpetually 'switched on' activity of the tyrosine kinase Bcr-Abl, leading to uncontrolled proliferation and insensitivity to apoptotic stimuli. The genetic phenotype of myeloid leukemic K562 cells expresses the chimeric p210Pbcr/ablP oncoprotein, a protein with a deregulated protein tyrosine kinase activity, which is thought to counteract apoptosis induction and cell death.

In recent years, many compounds that are contained in edible plant products, especially fruits, vegetables, and herbs, have been identified as potential chemopreventive agents [11]. Curcumin (**1**) is one kind of these chemopreventive agents. ($C_{21}H_{20}O_6$, M_w 368.4). **1** is a natural compound present in the spice turmeric, a rhizome of the plant *Curcuma longa* Linn. (Figure 1). It has been shown to prevent and inhibit carcinogen-induced tumorigenesis in different organs in rodent carcinogenesis models, and its cancer chemopreventive effects in these animal models have been extensively reviewed previously [12]. In some countries, **1** was consumed in the diet up to 4 g per adult/day, which appears to lower the incidence rate of colorectal cancer. In addition to its cancer chemopreventive activity, **1** is also well known for its antioxidant and anti-inflammatory properties [13] owing to the inhibition of cyclooxygenase 2 and lipoxygenase [14]. However, the molecular mechanisms of the action of curcumin are not well understood.

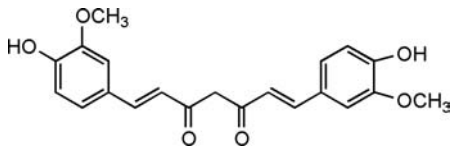

Figure 1. Structure of **1**.

Our pilot studies suggested that K562 cells were particularly sensitive to curcumin-induced cytotoxicity. It has been shown that curcumin can induce non-apoptotic autophagic cell death of human malignant glioma cell lines, U87-MG and U373-MG cells. Furthermore, it inhibits the Akt/mTOR/p70S6K pathway and activates the ERK1/2 pathway as well, which results in the induction of autophagy [15]. The present study sought to evaluate the involvement of autophagy and apoptosis in the curcumin-induced death of K562 leukemia cells.

2. Results and discussion

2.1 Cell viability is inhibited by curcumin

We determined the effects of curcumin on the viability of K562 cells. The cells were treated with various concentrations of curcumin for various periods of time. The cytotoxicity of curcumin was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Curcumin caused a marked growth inhibition and a significant decrease in cell viability in a time- and dose-dependent manner. As shown in Figure 2, after 24 h of treatment, the inhibitory rate of curcumin (20 μM) on the viability of K562 cells had reached 42.17 ± 2.48%. When the incubation time was prolonged to 72 h, the inhibitory rate increased to about 82.49 ± 0.78%. Based on this dose–effect relationship, the curcumin concentration of 20 μM was chosen for subsequent studies.

2.2 Curcumin induces apoptosis of K562 cells

We examined whether curcumin-induced apoptosis contributes, at least in part, to its growth inhibitory effects. K562 cells were

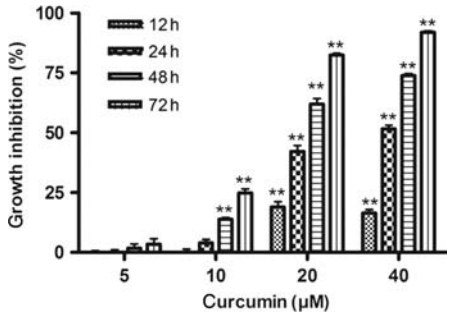

Figure 2. Effects of **1** on viability of K562 cells. K562 cells following exposure to various concentrations of **1** at different lengths of time and cell viability, analyzed with the MTT assay. Values were given as mean ± SD; $^{**}P < 0.05$ vs. control ($n = 6$).

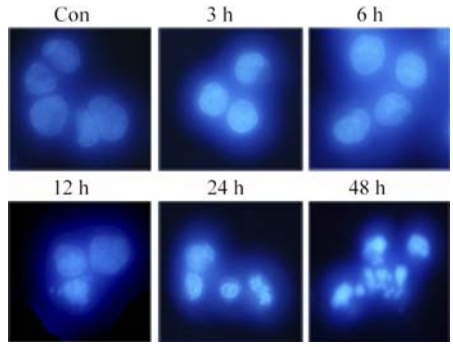

Figure 3. Hoechst 33258 staining. Nuclei of K562 cells were treated with **1** and stained with Hoechst 33258 to detect apoptosis morphologically. Microphotographs were shown as representative results from three independent experiments. Magnification × 400. Bar, 50 μm.

stained with Hoechst 33258, and morphologic changes were examined using a fluorescence microscopy. As shown in Figure 3, typical morphologic features of apoptosis, such as chromatin condensation and nuclear fragmentation, were detected in K562 cells in a time-dependent manner after curcumin treatment.

A few of the Bcl-2 family of proteins are well-characterized regulators of apoptosis. Antiapoptotic protein Bcl-2 protects the organelles from the effects of proapoptotic members such as Bid, Bak, and Bax on mitochondrial membranes. Therefore, we analyzed activation and expression of these proteins to elucidate the initial events of curcumin-induced apoptosis. As shown in Figure 4(a), proapoptotic Bid-cleavage products, t-Bid, were detected to be increased 6 h after curcumin treatment in a time-dependent manner. Meanwhile, the levels of Bcl-2 were found to be reduced 6 h after curcumin treatment. The lowest level of Bcl-2 was observed 24 h after drug treatment (Figure 4(b)).

Curcumin has been reported to induce apoptotic cell death in several cancer cells via mitochondrial disruption [16]. Therefore, to determine whether mitochondrial membrane integrity is damaged in K562 cells induced by curcumin, the mitochondrial membrane potential was measured using JC-1, a sensitive fluorescent probe for $\Delta\Psi_m$. When $\Delta\Psi_m$ was low, JC-1 existed mainly in a monomeric form, which emits green fluorescence. K562 cells were treated with curcumin for different lengths of time. As shown in Figure 5, the levels of FL1 (green) count increased as early as 6 h after curcumin treatment. This alteration reached its peak 24 h after curcumin treatment. These results indicate that a collapse of $\Delta\Psi_m$ is an early event in curcumin-induced cell death.

The loss of $\Delta\Psi_m$ commonly contributes to apoptosis, occurring either as early initiating events before caspase activation or as late downstream consequences of caspase activation [17]. Therefore, the activation of caspase-3 was further investigated. As shown in Figure 6(a), the levels of active caspase-3 (P20 and P17) increased 12 h after curcumin treatment. Maximal activation of caspase-3 was observed 24 h (P20 and P17) after drug treatment.

To confirm an apoptotic mechanism in curcumin-induced death of K562 cells, the effects of pretreatment with the pan-caspase inhibitor Z-VAD-fmk were

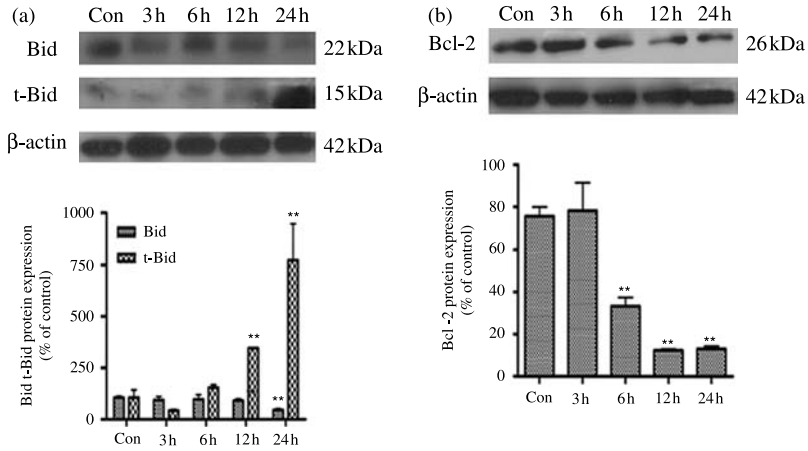

Figure 4. Bid and Bcl-2 protein expressions in K562 cells. (a) Western blot analysis of Bid expression after 3, 6, 12, and 24 h of treatment with **1**. $^{**}P < 0.05$ compared to the control group. (b) Western blot analysis of Bcl-2 expression after 3, 6, 12, and 24 h of treatment with **1**. $^{**}P < 0.05$ compared to the control group.

investigated. K562 cells were pretreated with Z-VAD-fmk 1 h before curcumin treatment, and cell viability was determined with the MTT assay. As shown in Figure 6(b), Z-VAD-fmk significantly inhibited the cytotoxicity of curcumin.

2.3 Curcumin activates autophagy in K562 cells

We next assessed whether curcumin also induces autophagy in K562 cells. Light chain 3 (LC3) is necessary for the formation of intermediate membrane structures which form the autophagosome. LC3 is localized in autophagosome membranes during amino acid starvation-induced autophagy [18]. To assess whether LC3 is altered in curcumin-treated K562 cells, we examined the localization of LC3 immunoreactivity. As shown in Figure 7, punctate patterns of LC3 immunoreactivity in many K562 cells were observed after curcumin treatment in a time-dependent manner, representing increased formation of autophagic vacuoles. In contrast, the control cells showed diffuse distribution of LC3 immunoreactivity only.

Recent investigations suggest that there are two forms of LC3 proteins in various cells: LC3-I and LC3-II [18]. LC3-I is the cytoplasmic form and is processed into LC3-II, which is autophagosome membrane bound. Therefore, the amount of LC3-II is correlated with the extent of autophagosome formation. Using Western blot analysis with anti-LC3 antibody, we examined the expressions of LC3-I (18 kDa) and LC3-II (16 kDa) in K562 cells after treatment with curcumin. As shown in Figure 8(a), an apparent increase in the levels of LC3-II protein was detected in K562 cells 12 h after treatment with curcumin.

Autophagy is controlled by a group of evolutionarily conserved genes (*ATG* genes) [2]. More than 30 *ATG* genes have been identified in yeast and at least 11 have orthologs in mammals; Atg6 is known as beclin 1. Beclin 1/Atg6 is a part of type III PI3 kinase complex, which is required for the formation of autophagic vesicles. Therefore, beclin 1 plays considerable roles in the processes of autophagy. The Western blot analysis revealed that beclin 1 levels were markedly increased 12 h after curcumin treatment (Figure 8(b)).

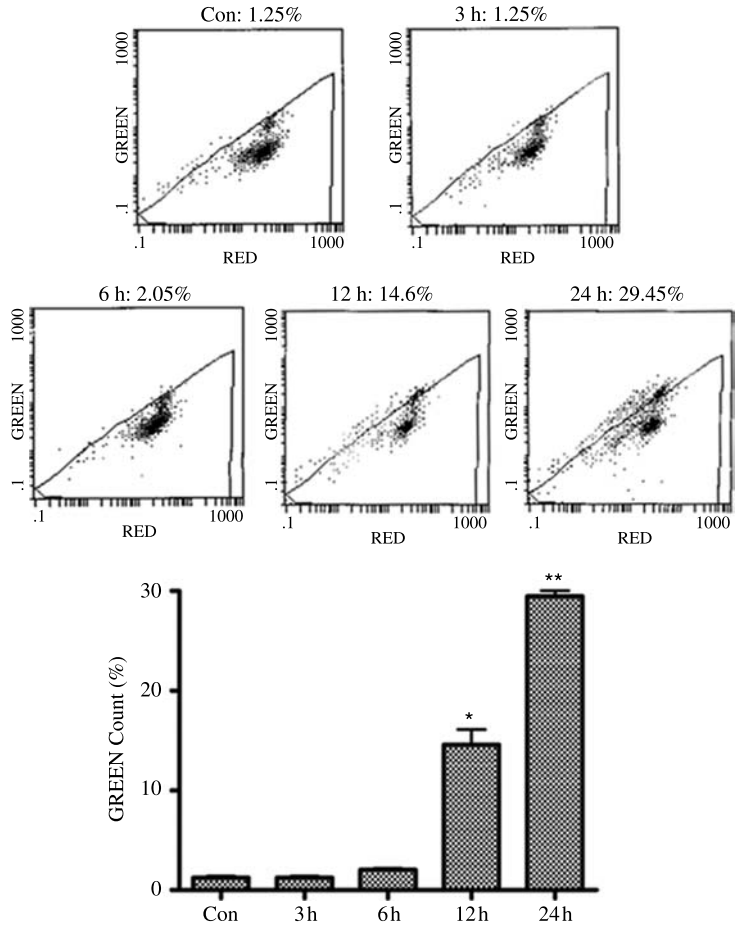

Figure 5. Effects of **1** on alterations in mitochondria membrane potential. K562 cells were treated with **1** for various lengths of time. Mean percentage of K562 cells stained green (FL 1-H) of all total cells was determined by flow cytometry. Results (mean ± SD) from three independent experiments were quantitatively analyzed. $^{*}P < 0.05$ compared to the control group; $^{**}P < 0.01$.

2.4 Autophagy increases curcumin-induced death of K562 cells

Bafilomycin A1, a specific inhibitor of vacuolar H^+-ATPase, prevents autophagy at a late stage by inhibiting fusion between autophagosomes and lysosomes. To explore the inhibition of autophagy at a late stage, bafilomycin A1 was added before curcumin. The results showed that after 24 h of treatment, the rate of inhibition of curcumin (20 μM) on K562 cells reduced. This suggests that autophagy can increase the cytotoxicity of curcumin in K562 cells (Figure 8(c)).

2.5 Discussion

It is of great interest in phytochemicals to search new chemopreventive and antitumor agents, which are more effective and less toxic. Curcumin, a compound known for its antioxidant and anticarcinogenic properties [19], is one of such kind of compounds. It has been used as a dietary spice and herbal medicine for centuries in several southeastern countries. It has also been shown to have an inhibitory effect on a wide variety of tumor cells. Therefore, curcumin has a potent anticancer effect and is a promising new therapeutic strategy.

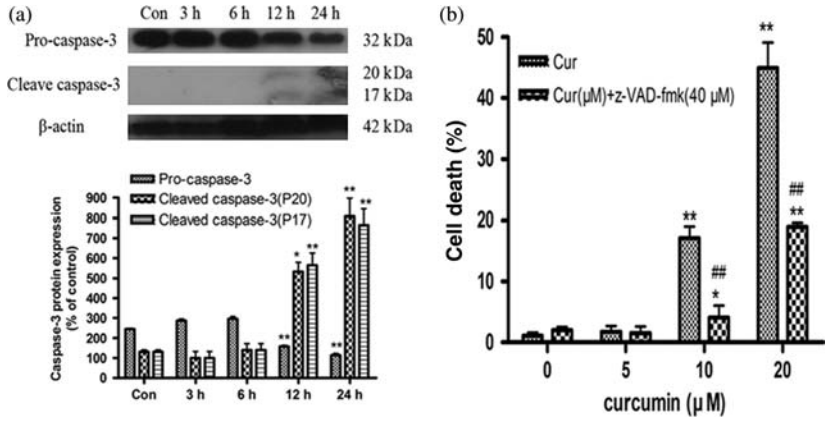

Figure 6. Caspase-3 protein expression in K562 cells and effects of caspase inhibitors on curcumin-induced death of K562 cells. (a) Western blot analysis of caspase-3 expression after 3, 6, 12, and 24 h of treatment with **1**. $^{**}P < 0.05$ compared to the control group. (b) K562 cells were pretreated with Z-VAD-FMK 1 h before curcumin. Cell viability was determined by the MTT assay. Data are presented as means; bars, ± SD ($n = 3$). $^{*}P < 0.05$ compared to the control group; $\#\#P < 0.05$ compared to curcumin alone within the treated group.

Our present study provides experimental evidence to show, for the first time, the activation of an autophagic program in the chronic myeloid leukemia cell line K562 cells following treatment with curcumin. Autophagy is a genetically programmed, evolutionarily conserved process that begins with the sequestering of cytosolic components, often including intracellular organelles within double-membrane structures. The vacuoles (also called autophagosomes) undergo acidification after maturation. Autophagosomes then fuse with lysosomes, where their materials

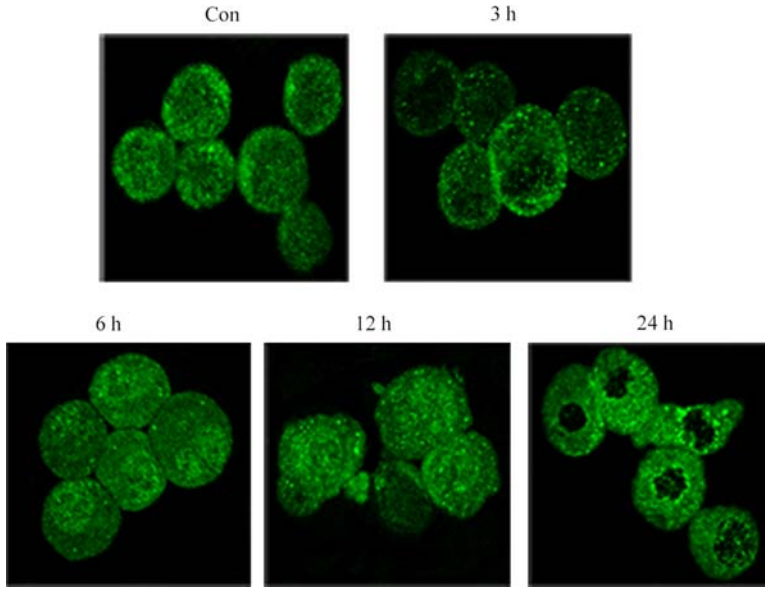

Figure 7. LC3 immunofluorescence in K562 cells after **1** treatment. K562 cells were incubated with **1** for the indicated time and stained with anti-LC3 antibody. Cells were examined by fluorescence confocal microscopy. Magnification × 400. Bar, 50 μm.

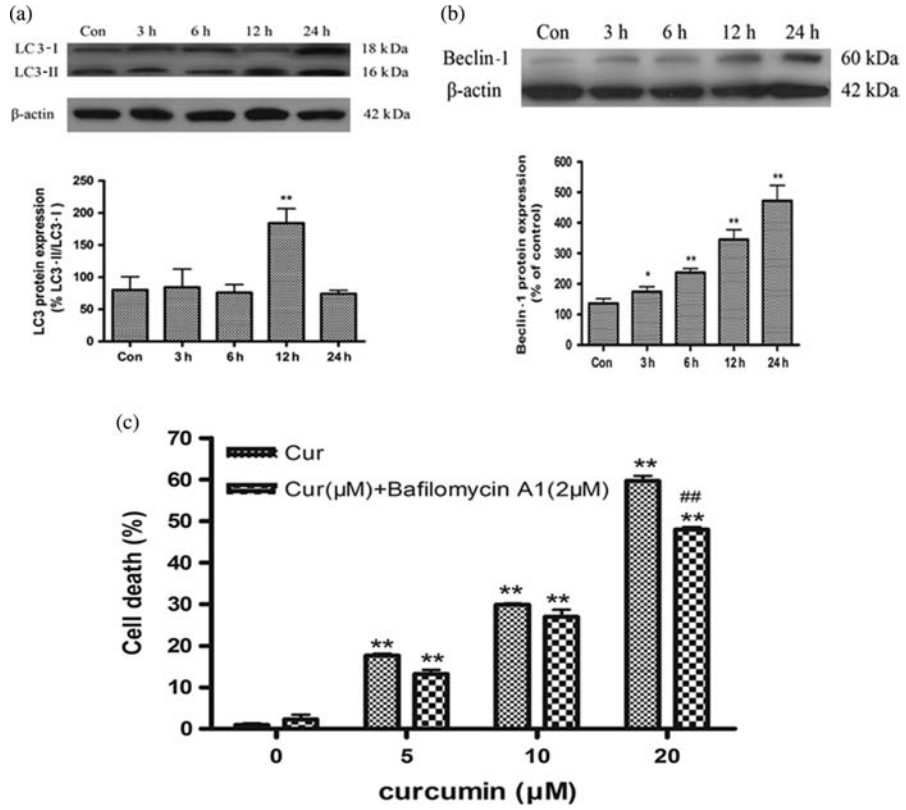

Figure 8. LC3 and beclin-1 protein expressions in K562 cells and effects of bafilomycin A1 on curcumin-induced death of K562 cells. (a) Western blot analysis of LC3 expression after 3, 6, 12, and 24 h of treatment with **1**. $^{**}P < 0.05$ compared to the control group. (b) Western blot analysis of beclin-1 expression after 3, 6, 12, and 24 h of treatment with **1**. $^{**}P < 0.05$ compared to the control group. (c) K562 cells were pretreated with bafilomycin A1 1 h before curcumin. Cell viability was evaluated by the MTT assay. Data are presented as means ± SD ($n = 3$). $^{**}P < 0.05$ compared to the control group; $\#\#P < 0.05$ compared to the curcumin alone group.

inside are degraded and recycled [5]. Our results show that curcumin-mediated autophagy in K562 cells is characterized by green punctate immunostaining of LC3 scattered in the cytoplasm. It has recently been demonstrated that LC3 is the first mammalian protein that is specifically recruited to autophagosome membranes. LC3 is an autophagosomal ortholog of yeast Atg8 and the levels of LC3 might also reflect the levels of autophagy. Our results showed that after incubation with curcumin, the LC3 protein expression was significantly increased after 12 h. Beclin 1 is the first identified tumor-suppressor protein that functions in the lysosomal degradation pathway of autophagy [20]. Furthermore, it is a mammalian Atg6/Vps30 (vacuolar protein sorting 30) ortholog and a subunit of the class III PI3-kinase complex. During tamoxifen-induced death of MCF7 cells (a cell type that contains a mutation in caspase-3), there is a marked upregulation of beclin 1 protein expression [21]. In our study, we also found that beclin 1 protein levels were markedly increased 12 h after curcumin treatment. These findings are similar to those showing autophagy activation after amino acid deprivation.

Whether autophagy promotes cell death or inhibits cell death is circumstantial. Some studies suggest that autophagy serves as a key mechanism of cell survival [22]. This function of autophagy is an evolutionarily ancient process, conserved from yeast to mammals, and best characterized in nutrient deficiency. Autophagy can protect cells by inhibiting them from undergoing apoptosis [23]. On the other hand, autophagy is a cell death mechanism. Autophagic cell death is referred to as programmed cell death type II, while apoptosis is well known as programmed cell death type I [24]. To determine the contribution of autophagy in the curcumin-induced death of K562 cells, a specific inhibitor of the lysosomal protein pump ATP, bafilomycin A1, was used. Bafilomycin A1 is a macrolide antibiotic, which prevents autophagy at a late stage by inhibiting fusion between autophagosomes and lysosomes [25]. In our study, 2 μM bafilomycin A1 was shown to decrease curcumin-induced cytotoxicity. These findings suggest that autophagy plays an important role in curcumin-induced cell death and that inhibition of autophagy significantly affects the anti-tumor effects of curcumin. Therefore, autophagy may serve as a cell death mechanism in K562 cells after treatment with curcumin.

Curcumin has been reported to induce the apoptotic cell death in numerous cancer cell lines. However, the effect of curcumin in K562 cells is still not well understood. Our study intends to examine the apoptotic effect of curcumin in the chronic myeloid leukemia cell line K562 cells. In the search for novel mechanisms underlying curcumin's anti-tumor acions, we have attempted to identify the molecular mechanisms involved in curcumin-induced apoptosis of the chronic myeloid leukemia cell line K562 cells. Our results show that curcumin can induce both dose- and time-dependent apoptosis in K562 cells. Apoptosis is characterized by caspase activation [26]. Curcumin-induced apoptosis requires the activation of caspase-3 and cleavage of Bid, the downregulation of Bcl-2 expression. Curcumin has been shown to induce apoptosis in human androgen-dependent LNCaP as well as androgen-independent DU145 human prostate cancer cell lines by downregulating the expression of Bcl-2 and the activation of pro-caspase-3 [27]. Our results are consistent with other published reports on the mechanisms of curcumin-induced apoptotic cell death in other model systems, suggesting that curcumin may employ common apoptotic signaling pathways in different cancer cell lines. Blockage of apoptosis by the pan-caspase inhibitor z-VAD-fmk, which partially inhibited curcumin-induced cell death, suggests that apoptosis plays an important, but not exclusive, role in curcumin-induced death of K562 cells. The results also suggest that other mechanisms, in addition to activation of caspases, are also involved in the curcumin-induced death of K562 cells.

Mitochondria may act as an orchestrator, integrating apoptotic and autophagic activation [28]. Cell death is often associated with the collapse of $\Delta\Psi_m$. Diverse autophagic or apoptotic signals may converge at mitochondria, provoking a mitochondrial permeability transition (MPT) and thus inducing apoptogenic proteins such as cytochrome *c* release from mitochondria into the cytosol, which trigger caspase-dependent apoptosis or promote autophagy. Previous studies have suggested that apoptosis and autophagic cell death are interconnected through MPT [29]. We have shown that the mitochondrial $\Delta\Psi$ collapsed and cytochrome *c* localization was altered by curcumin treatment. Time-course analysis showed that mitochondrial changes occurred as early as 6 h after curcumin treatment. As a result, the $\Delta\Psi$ collapse appears to be an intracellular signal that may be responsible for the induction of autophagy and apoptosis. How mitochondria

integrate upstream signals and regulate downstream cross-talk between autophagy and apoptosis is still unknown. Thus, the relationship between MPT, apoptosis, and autophagy remains unclear, which requires further investigation.

In summary, this study suggests that autophagy and apoptosis could be triggered by curcumin in K562 cells. Both autophagy and apoptosis make important contributions to curcumin-induced death of K562 cells. Apoptosis is a leading cause of death of K562 cells, while autophagy may promote apoptosis after curcumin treatment. These findings add a novel concept to curcumin-induced cell death pathways. Further studies of upstream signal regulation of autophagy and apoptosis are required to provide new insights on molecular mechanisms of curcumin-induced death of K562 cells and suggest new strategies for tumor chemotherapy.

3. Experimental

3.1 Cell culture and reagents

K562 leukemia cells were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in RPMI-1640 medium (Gibco, Rockville, MD, USA) containing 10% heat-inactivated newborn calf serum (Hangzhou Sijiqing Biological Engineering Material Co. Ltd, Hangzhou, China), 0.03% L-glutamine (Sigma, St Louis, MO, USA), 0.1% Hepes, 0.2% $NaHCO_3$, and incubated in a humidified 5% CO_2 incubator at 37°C. The cells in the mid-log phase were used in the experiments. Curcumin (typical purity: 65–70%) was purchased from Sigma (St Louis). Curcumin was prepared as a 10 mM stock solution in dimethyl sulfoxide (DMSO, Sigma) and stored at −20°C. For each experiment, curcumin was diluted with cell culture medium to the concentrations indicated with a final DMSO concentration of 1% (vol/vol).

3.2 Cell viability assay

Cell viability was assessed by the MTT assay as described previously. K562 cells were planted in 96-well microplates (7×10^4 cells/well). MTT solution was added to the culture medium (500 μg/ml final concentration) 4 h before the end of treatments and the reaction was stopped by addition of 10% acidified SDS (100 μl) to the cell culture. The absorbance value (A) at 570 nm was read using an automatic multiwell spectrophotometer (Bio-Rad, Richmond, CA, USA). The percentage of cell death was calculated as follows: growth inhibition (%) = (1 − A of the experiment well/A of the positive control well) × 100%.

3.3 Apoptosis assay

Nuclei were stained with Hoechst 33258 to detect chromatin condensation or nuclear fragmentation, which were characteristics of apoptosis. We treated K562 cells with curcumin (20 μM) for various times, fixed them with pre-cooled methanol at −20°C, and stained them with Hoechst 33258 (0.5 μg/ml) for 15 min. The cells were washed twice with PBS and analyzed with fluorescence microscopy (Nikon Eclipse TE 300, Tokyo, Japan).

3.4 Assessment of mitochondrial membrane potential

To measure the mitochondrial membrane potential ($\Delta\Psi_m$), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), a sensitive fluorescent probe for $\Delta\Psi_m$, was used. Cells were incubated with curcumin in six-well plates for the indicated time and rinsed with ice-cooled PBS twice, and the incubated cells were treated with 1 μM JC-1 in 10% FBS/RPMI-1640 for 30 min in the dark at 37°C and instantly assessed for red and green fluorescence with flow cytometry (EPICS-XL; Beckman, Ramsey, MN, USA) [30]. A 488 nm filter was used for the excitation of JC-1. Emission filters of

535 and 595 nm were used to quantify the population of mitochondria with green (JC-1 monomers) and red (JC-1 aggregates) fluorescence, respectively. Frequency plots were prepared for FL1 (green) and FL2 (red) to determine the percentage of the mitochondria stained green (low membrane potential) and red (normal membrane potential).

3.5 Immunofluorescence of microtubule-associated protein LC3

LC3, a mammalian homolog of Apg8p/Aut7p essential for amino acid starvation-induced autophagy in yeast, was recruited to the autophagosome membranes in an Apg5-dependent manner [18]. Therefore, autophagosome membrane association with LC3 is a specific marker for autophagy. After being incubated with 20 μM curcumin for 3–24 h, the cells were pelleted and then fixed in methanol at −20°C for 15 min. After rinsing in PBS, the cells were blocked with 0.1% Triton X-100 containing 1% bovine serum albumin in PBS for 1 h. This was followed by incubation in goat polyclonal antibody against microtubule-associated protein 1 LC3 (1:100, sc-16756; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 24 h at 4°C in a humidified chamber. After three washes in PBS, the cells were incubated in donkey anti-goat immunoglobulin G-fluoresceinisothiocyanate (1:400, sc-2024; Santa Cruz) for 1 h at 4°C. Finally, the cells were rinsed in PBS. The coverslips were mounted on the microscopy slides using an anti-fade mounting medium (Vectashield, Co#: H-1000, Vector Lab, Burlingame, CA, USA) to permit examination. Images were captured with a fluorescence microscopy (LEICA TCS SP2, Bensheim, Germany).

3.6 Western blot analysis

Whole cell lysates were prepared from treated cells for Western blot analysis as described previously. Before immunoblotting, the protein concentrations were determined with a BCA detection kit (Pierce, Rockford, IL, USA) and adjusted to equal concentrations across different samples. The proteins were separated by 10% SDS-PAGE gel, transferred to nitrocellulose membrane, and immunoblotted with antibodies against MAP LC3 (1:1000), beclin 1 (1:750), Bid (1:200), caspase-3 (1:200), or Bcl-2 (1:200) at 4°C overnight. The reaction of primary antibodies was detected using horseradish peroxidase-conjugated anti-mouse, goat, or rabbit antibody used at a 1:5000 dilution in blocking solution for 1 h at room temperature. The immunoreactivity was detected using enhanced chemiluminescence (ECL kit; Amersham Pharmacia Biotech, Piscataway, NJ, USA) and visualized by autoradiography. Protein β-actin (1:5000; A5441; Sigma) was used as the loading control.

3.7 Statistical analysis

All experiments were repeated at least three times. The data were presented as means ± SD. Statistical analysis was performed using Student's *t*-test (two-tailed). The criterion for statistical significance was $P < 0.05$.

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