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Original Article

Curcumol induced apoptosis of human acute myeloid leukemia KG-1a cells

Feng Li, Qi An, Yubing Zhou, Duolu Li, Chao Han, Xiaojian Zhang, Quancheng Kan

Department of Clinical Pharmacology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, People's Republic of China

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Abstract: Objective: To investigate the effect of curcumol on the apoptosis of human acute myeloid leukemia KG-1a cells and to explore possible mechanism of action. Methods: CCK-8 method was used to detect the cell viability of KG-1a cells treated by 10, 20, 40, 60, 80 uM curcumol, and that of KG-1a cells treated by 40 uM curcumol after different periods of time. Confocal microscopy was used to observe morphologic changes after cells were incubated with 40 uM curcumol for 24 h and 48 h, respectively. Further, morphologic changes of the nucleus were observed after cells were stained by Hochest 33258. Annexin V-FITC/PI staining was also conducted. Then, after Rhodamine 123 staining of mitochondrial membrane potential specific probe, the decreasing ratio of mitochondrial membrane potential was detected with a flow cytometer. Finally, after cells were pretreated by a broad-spectrum caspase inhibitor z-VAD-fmk, the effect of curcumol on cell viability was tested and the expression of caspase 8, 9 and 3 was also detected by Western blot. Results: The inhibitive effect of curcumol on the viability of KG-1a cells showed significant concentration and time-dependence. The viability of cells treated by 10, 20, 40, 60 and 80 uM curcumol were (91.6±3.1)%, (74.1±3.4)%, (57.1±1.9)%, (37.9±2.3)% and (32.6±2.5)%, respectively. For cells treated by 40 uM curcumol for 6, 12, 24 and 48 h, their viability was (75.3±2.102)%, (50.6±1.9)%, (41.6±2.6)%, (20.1±2.1)%, respectively. Compared with blank control, cells treated by 40 uM curcumol for 24 h partially changed to round in shape and some dead ones floated, which was more obvious for cells treated for 48 h. In addition, nuclear staining showed shrinkage of nucleus and the presence of apoptotic body after treatment for 24 h, which was also more significant after treatment for 48 h. Apoptosis was confirmed by flow cytometry. Moreover, it was found that the mitochondrial membrane potential of cells treated by 40 uM curcumol for 24 and 48 h decreased remarkably, which was (43.1±1.9)% and (78.9±2.1)%, respectively ($P<0.01$). At last, before and after pretreatment with broad-spectrum caspases inhibitor z-VAD-fmk, the viability of cells treated for 24 h and 48 h presented no obvious change. Western blot found that caspases 8, 9 and 3 were not involved in the apoptosis induced by curcumol. Conclusion: Curcumol can decrease the viability of KG-1a cells and induce apoptosis via a possible mechanism of caspases-independent cell apoptosis caused by mitochondrial membrane potential dropping.

Keywords: Curcumol, apoptosis, caspases, mitochondrial membrane potential, KG-1a cell, human acute myeloid leukemia

Introduction

The incidence of leukemia accounts for about 3% among cancer patients existing in the whole world. There are about 10 patients with leukemia per 100 000 cases [1, 2]. Acute myeloid leukemia is a blood cancer caused by abnormal proliferation of bone marrow leukocytes (instead of lymphocytic leukocytes). It is characterized by rapid proliferation of abnormal cells within bone marrow, which affects the production of normal blood cells [3]. Currently, the general principle is to eliminate leukemic cell population and control the proliferation of leukemic cells. The major treatment method is chemotherapy. In addition, studies show that berbamine, artesunate, chlorogenic acid and other drugs may kill leukemic cells effectively [4-9].

Curcuma zedoary, a common traditional Chinese medicine in clinical use, can promote the circulation of qi, disintegrating blood stasis, remove food retention and relieve pain [10]. In recent years, the anti-tumor and pharmacological effect of curcuma zedoary has become a research hotspot [11-13]. Curcumol, also called curcumenol, is one of the effective monomers

extracted from curcuma aromatic oil. It has been found that this substance is of good anti-virus and anti-tumor effect [14, 15]. Studies show that curcumol has a good killing effect on gastric cancer SGC-7901 cells. It can reduce the expression of Bcl-2 protein, which promotes apoptosis via mitochondria-dependent Apa-fl/Caspase-9 pathway [16]. In recent years, it is also found that curcumol has a killing effect on leukemic cells as well [17]. However, the mechanism related to curcumol-induced apoptosis is still unknown.

This study mainly investigated the effect of curcumol on human acute myeloid leukemia KG-1a cells. In addition, a preliminary study was conducted to find out the way by which it killed cells and its mechanism. In particular, mitochondrial membrane potential and caspases were detected to make sure whether they were involved in cell apoptosis induced by curcumol. This study preliminarily clarified the mechanism how curcumol fought against acute myeloid leukemia KG-1a cells, which provided a theoretical and experimental basis for the clinical application and experimental research of curcumol.

Experimental materials and methods

Experimental materials and major instruments

Curcumol (purity: 98.9%) was purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd., Iscove's Modiefied Dulbecco's Medium (IMDM), calf serum from Gibco, trypsin, dimethyl sulfoxide (DMSO) and Annexin V-FITC/PI Staining Kit from Aladdin Industrial Corporation in Shanghai, CCK-8 Kit from Dojindo Chemical Institute in Japan, Hoechst 33258, rhodamine 123, broad-spectrum caspases inhibitor z-VAD-fmk as well as primary and secondary antibodies for Western blot from Sigma, confocal laser scanning microscope from Zeiss in German, flow cytometer from BD in the US, carbon dioxide cell culture box from ThermoFisher, automatic microporous microplate reader from TECAN in Australia and human acute myeloid leukemia KG-1a cells from American Type Culture Collection (ATCC).

Experimental methods

Cell culture: KG-1a cell lines were cultured in a IMEM containing 20% calf serum and 10% penicillin-streptomycin, which was incubated at 100% humidity and 37°C with 5% carbon dioxide. These cells were digested with trypsin during logarithmic growth phase and were then subcultured.

Cell viability test: During logarithmic growth phase, cells were digested and inoculated into a 96-well plate with 100 ul suspension per well (10^5 cells/ml). Next, they were cultured in an incubator for 24 h. Then, the old culture solution was removed and new ones with different concentration of drugs (blank control, 10, 20, 40, 60 and 80 uM) were added. The resultant new solution was cultured for another 24 h. After that, cells were treated with a CCK-8 Kit and the OD450 value was measured in a microplate reader. It was found that the cell viability was proportional to OD450 value. In addition, the cell viability at different time points of treatment was detected in the same way.

Morphological observation of cells and their nuclei and apoptosis assay by flow cytometry: Cells in logarithmic phase, after being digested, were inoculated in round cell culture dishes which were 35 mm in diameter (10^5 cells/ml) and cultured for 24 h (the culture dishes were divided into two groups-blank control and treatment group). Afterwards, the culture solution was replaced by 40 uM curcumol. The resultant mixture, after being incubated for 24 h or 48 h, was observed under a microscope for cellular morphological changes.

After cells were incubated with 40 uM curcumol for 24 h or 48 h, they were stained by Hoechst 33258, a dye used specifically for nucleus, according to steps as follows: After the culture solution was sucked out, the remainder was washed by PBS for three times; Then, cells were incubated with PBS solution containing 0.5 ug/ml Hoechst 33258 for 15 min; Next, the mixture was washed by PBS gently for several times until all extracellular Hoechst 33258 was cleaned up; A proper amount of PBS was then added and the resultant substance was observed under confocal microscopy.

Annexin V-FITC/PI staining: Cells, after being incubated with 40 uM curcumol for 24 h or 48 h, were collected and stained according to operating steps shown on Annexin V-FITC/PI Kit; then, they were placed in a flow cytometry to detect the fluorescence signal of FITC and PI; At last, a statistical analysis of apoptosis rate was conducted.

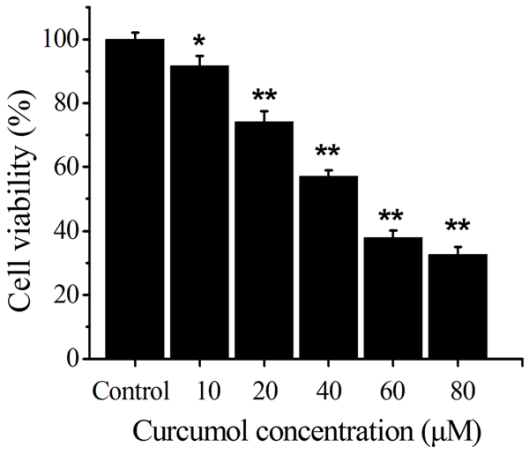

Figure 1. The effect of various concentration of curcumol on cell viability. *P<0.05, **P<0.01, *vs.* the control group.

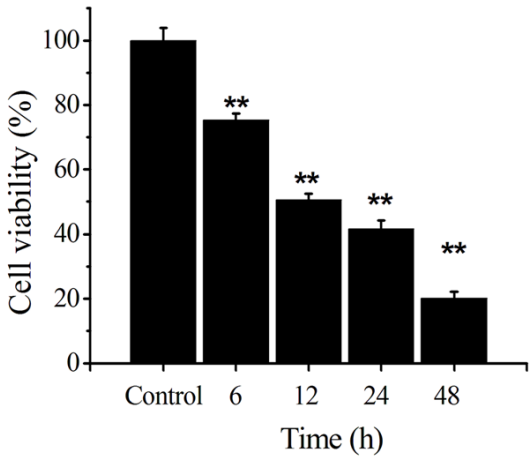

Figure 2. The effect of 40 uM curcumol on cell viability over time. *P<0.05, **P<0.01, *vs.* the control group.

Mitochondrial membrane potential detection: Mitochondrial membrane potential detection was performed by staining with a specific probe Rhodamine 123. The staining method was described as follows: After cells were subcultured for 24 h, they were treated with drugs for different periods of time; After that, the mixture was washed by PBS for three time; Then, the resultant substance was stained for 20 min with Rhodamine 123 whose final concentration was 5 μM; Again, this mixture was washed by PBS for several times until extracellular dyes were cleaned up; Then, cells were digested with trypsin and PBS was dispersed evenly; Finally, the fluorescence imaging of Rhodamine 123 was detected under flow cytometry. The excitation light (wavelength: 488 nm) was emitted by a argon ion laser. For each specimen, 10 000 cells were collected.

The impact of caspases on cell viability: A broad-spectrum caspase inhibitor, z-VAD-fmk, can widely inhibit the activity of the members of the caspase family. After cells were pretreated by z-VAD-fmk for 3 h, they were washed for three times. Then, after these cells were treated with drugs for a different period of time, cell viability was detected by a CCK-8 Kit.

Western blot test: After treatment with single curcumol, broad-spectrum caspase inhibitor z-VAD-fmk and curcumol +z-VAD-fmk, cells were collected and total protein was extracted. Then, they received Western blot test according to the following steps: The concentration of total protein was detected by a microplate reader; Polyacrylamide gel electrophoresis was then conducted by using a constant voltage of 100-120 V; Afterwards, transmembrane was performed and 5% skim milk powder was used for sealing; Finally, corresponding antibody binding and imaging detection was performed.

Statistical analysis of data

For each experiment, there were three replicate samples. Data was expressed as mean ± SD. The difference between two groups was analyzed by t-test of independent samples by using an analysis software SPSS12. When P≤0.05, it was believed that there was significant difference; when P≤0.01, it was believed that there was extremely significant difference.

Results and analysis

The impact of curcumol on the viability of KG-1a cells

Test results by CCK-8 method indicated that curcumol had a concentration and time dependent effect on the viability of KG-1a cells. As shown in **Figure 1**, the viability of KG-1a cells after being treated with 10, 20, 40, 60 and 80 uM curcumol for 24 h was (91.6±3.1)%, (74.1±3.4)%, (57.1±1.9)%, (37.9±2.3)% and (32.6±2.5)%, respectively (P<0.05). It demonstrated that cell viability decreased gradually with increased concentration and the difference was statistically significant compared with blank control. In addition, the viability of

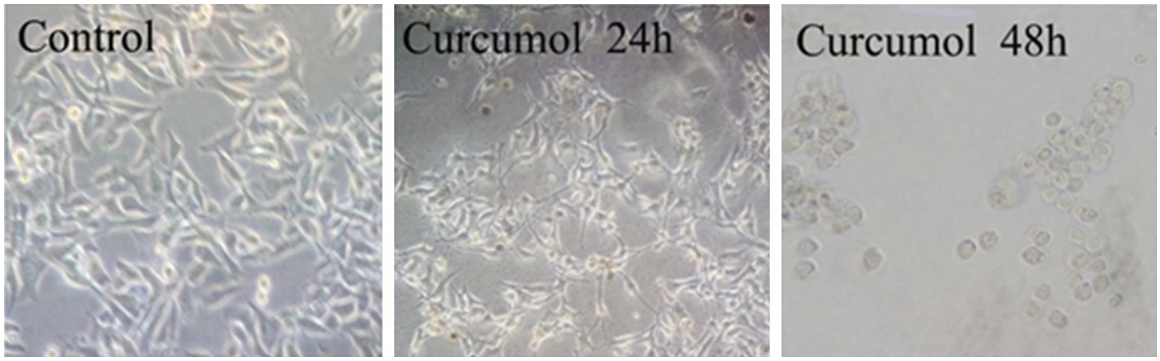

Figure 3. The morphological changes of cells induced by curcumol.

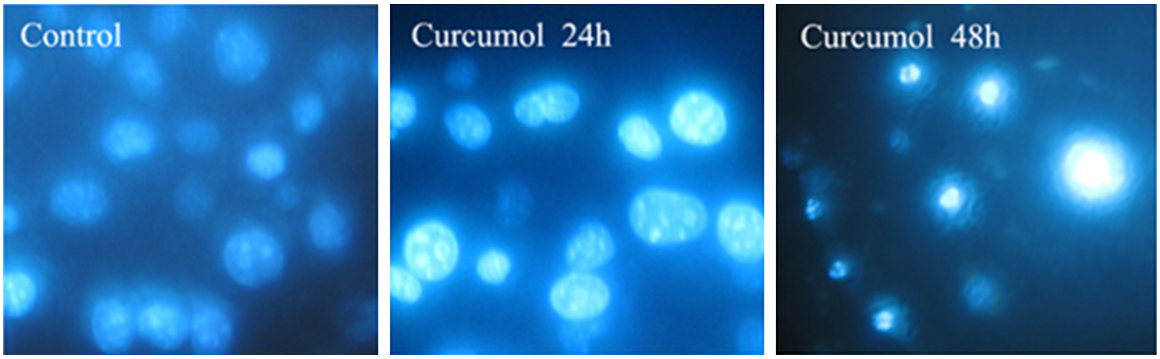

Figure 4. The nucleus changes of cells induced by curcumol.

cells treated with 40 uM curcumol for 6, 12, 24 and 48 h (**Figure 2**) was (75.3±2.102)%, (50.6±1.9)%, (41.6±2.6)% and (20.1±2.1)%, respectively. Likewise, cell viability decreased gradually over time and the difference was statistically significant compared with blank control ($P<0.01$).

Cellular morphological observation and apoptosis assay

As shown in **Figure 3**, after treatment with 40 uM curcumol for 24 h and 48 h, cellular morphology was observed under a microscope. Compared with blank control, cells treated by 40 uM curcumol for 24 h partially changed to round in shape and shrank and some dead ones floated. After 48 h, the majority of cells changed to round in shape and floated. Hence, a preliminary judgment was that curcumol could cause cellular morphological changes and lead to cell death. Then, the abovementioned cells were stained by Hoechst 33258, a dye used specifically for nucleus. As shown in **Figure 4**, nuclei of cells in the control group distributed evenly. By contrast, for cells treated with drugs for 24 h, there was obvious pyknosis and presence of apoptosis bodies. Moreover, pyknosis became more evident after cells being treating for 48 h. As shown in **Figure 5**, the results of Annexin V-FITC/PI staining obtained by flow cytometry demonstrated that the mechanism of cell death induced by curcumol was apoptosis. The apoptosis rate of cells after being treated by curcumol for 24 h and 48 h were (56.9±2.6)% and (78.9±3.1)%, respectively.

Decreased mitochondrial membrane potential induced by curcumol

Rhodamine 123, as a mitochondrial membrane potential probe, could detect mitochondrial membrane potential changes under flow cytometry. As shown in **Figure 6**, statistical analysis of results of flow cytometry suggested that treatment by curcumol for 24 h and 48 h could decrease mitochondrial membrane potential. And the change was of statistically significant difference compared with cells in blank control ($P<0.01$), which preliminarily indicated that curcumol decreased mitochondrial membrane potential and caused cell apoptosis.

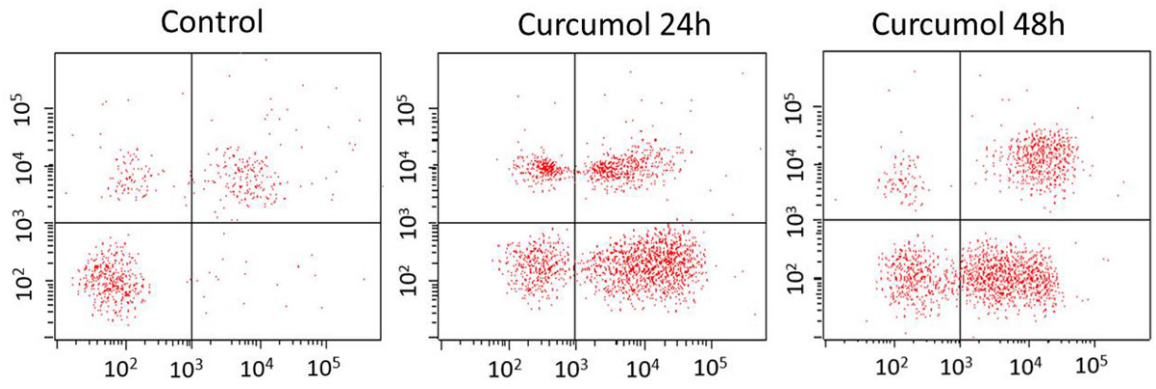

Figure 5. The apoptosis induced by curcumol detected by flow cytometry.

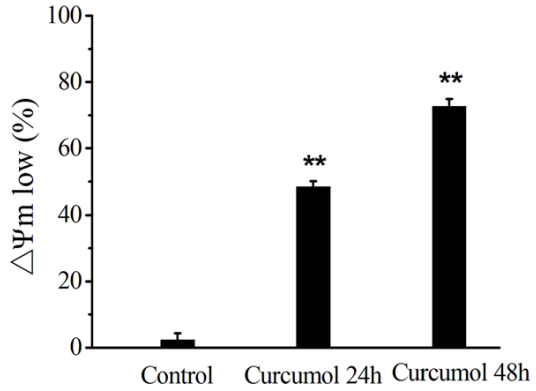

Figure 6. The mitochondrial membrane potential (Δψm) decline induced by curcumol. **P<0.01, *vs.* the control group.

No involvement of caspases in cell apoptosis

Caspases were important factors of apoptosis pathway. A broad-spectrum caspase inhibitor was used to find out whether caspases were involved in cell apoptosis. As shown in **Figure 7**, compared with cells treated with drugs alone, the viability of cells pretreated with caspases showed no significant change. This suggested that the members of the caspase family were not involved in apoptosis induced by curcumol. Besides, the expression of caspase 8, 9 and 3 was detected by Western blot test (**Figure 8**). Results demonstrated that these proteins were not involved in apoptosis induced by curcumol. It further verified the conclusion drawn in **Figure 7**.

Discussions

Acute myeloid leukemia was increasingly becoming a major killer endangering human health. Therefore, it was imperative to find out an effective drug and identify its killing mechanism [18, 19]. As an active extract of curcuma zedoary, a traditional Chinese medicine, curcumol was characterized by low cost and few side effects. Also, it was easy to be obtained. So in recent years, it had been widely used in researches for the treatment of tumor and progress has been made [20]. For instance, studies showed that curcumol had a killing effect on tumor cells in patients with liver, gastric and colon cancer [21, 22].

Through using human acute myeloid leukemia KG-1a cells as our object of study, we investigated the mechanism of cell apoptosis induced by curcumol by fluorescence staining, flow cytometry and confocal microscopy. Results indicated conclusions as follows: 1) The effect of curcumol on cell viability was concentration and time dependent; 2) Curcumol could induce cell apoptosis; 3) Curcumol decreased mitochondrial membrane potential, which indicated that mitochondrial pathway was involved in the apoptosis induced by curcumol; 4) The apoptosis induced by curcumol was independent of caspases.

Apoptosis, as a type of cell death, was closely related to the expression of multiple genes. Many chemical components of traditional Chinese medicine, which were effective in the treatment of cancer, had been proved to be able to act on cancer cells by influencing apoptosis-inducing or regulating factors [23, 24]. This was of great significance for clinical medication and cancer treatment.

Mitochondrion was a very important organelle, which was not only a center for supplying energy but also a major site for the synthesis of pro-

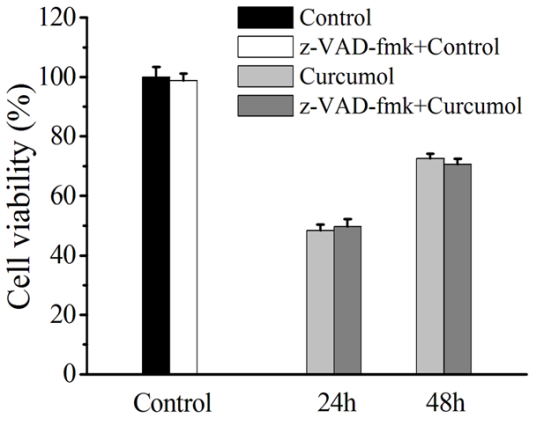

Figure 7. The effect of a broad-spectrum caspase inhibitor on apoptosis induced by curcumol.

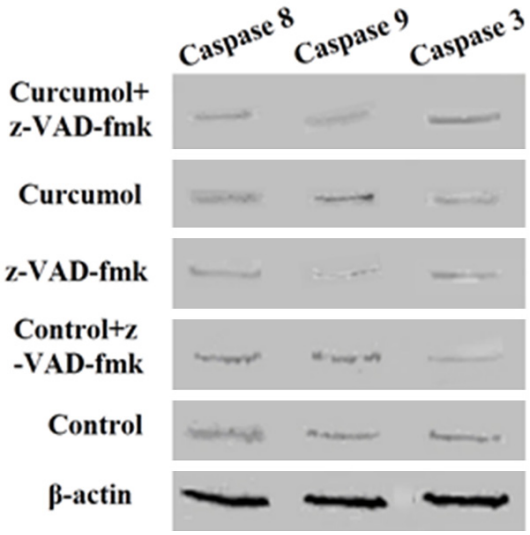

Figure 8. The effect of broad-spectrum caspase inhibitor z-VAD-fmk on the expression of caspase 8, 9 and 3 detected by western blot.

teins. Besides, it was also a target spot for drugs entering into cells. During apoptosis, apoptosis factors like Bax could translocate into mitochondria where they took effect [25]. Researchers found that when lung adenocarcinoma cells were treated with curcumol, Bax translocated into mitochondria and mitochondrial membrane potential decreased. Further, downstream events mediated by mitochondria occurred, which led to apoptosis at last [26]. The results of our study also showed that leukemia cell apoptosis induced by curcumol underwent the same mitochondrial pathway. Furthermore, it was known that the members of caspase family played an important role in apoptosis. They could promote apoptosis. For example, the activation of caspase 8, 9 and 3 could facilitate the process of apoptosis [27]. In this study, we used broad-spectrum caspase inhibitors to inhibit the activation of caspase and discovered that caspases were not involved in the apoptosis induced by curcumol. It indicated that the apoptosis induced by curcumol was independent of caspase. Nevertheless, further studies should be conducted to understand the in-depth mechanism.

To sum up, findings in this study indicate that the mechanism of cell death induced by curcumol is caspases-independent apoptosis in which mitochondrial pathway is involved.

Disclosure of conflict of interest

None.

Address correspondence to: Quancheng Kan, Department of Clinical Pharmacology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, People's Republic of China. E-mail: lf669-12114@163.com

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