JNK pathway mediates curcumin-induced apoptosis and autophagy in osteosarcoma MG63 cells

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Abstract. Human osteosarcoma is a common primary malignancy of the bone in children and adolescents. It has been reported that curcumin is able to induce apoptosis in osteosarcoma MG63 cells through the mitochondrial pathway. However, whether curcumin is able to induce autophagy and the interaction between apoptosis and autophagy in osteosarcoma cells has yet to be fully elucidated. In the current study, it was determined that curcumin was able to significantly induce apoptosis, and lead to autophagy in MG63 cells. Notably, inhibition of apoptosis enhanced curcumin-induced autophagy due to upregulation of the c-Jun N-terminal kinase (JNK) signaling pathway. This finding was confirmed by the use of JNK-specific inhibitor, SP600125. Furthermore, our data showed that curcumin-induced apoptosis was increased when autophagy was completely inhibited by 3-methyladenine in MG63 cells. These results suggest that autophagy may have an important role in resistance to apoptosis when MG63 cells are incubated with curcumin. Thus, these results provide important insights into the interaction between apoptosis and autophagy in osteosarcoma cells and clinical treatment strategies using curcumin.

Introduction

Osteosarcoma is one of the most common primary malignant neoplasms in children, adolescents, and young adults (1). The introduction of chemotherapy has lead to a significant improvement in the prognosis of patients with localized osteosarcoma, and long-term survival rates of <20% have been observed to improve to >65% after the use of multiagent chemotherapy regimens (2). However, patients with osteosarcoma who present with metastasis continue to have poor prognosis, which is associated with a strong resistance to chemotherapy (3,4). Consequently, it is imperative that novel treatment strategies are developed for such patients.

Curcumin (Fig. 1) is derived from the rhizome of the East Indian plant Curcuma longa. Over the past three decades, curcumin has been widely used as a cancer chemotherapy agent in a wide range of cancer models, including those for colorectal, pancreatic, breast and hematological malignancies, and has been utilized for its ability to alleviate therapy-induced toxicities such as Mitomycin C associated side-effects and chemotherapy-induced mucosal barrier injury (5-10). Curcumin has been found to inhibit the tumorigenesis and progression of various tumors, such as colorectal cancer, lung adenocarcinoma (5). These anti-cancer effects are predominantly mediated through the negative regulation of various oncogenic molecules and pathways, including activator protein 1, nuclear factor κB, peroxisome proliferator-associated receptor gamma, signal transducer and activator of transcription, Wnt/β-catenin, nuclear factor (erythroid-derived 2)-like 2, tumor necrosis factor-α, interleukins, inducible nitric oxide synthase, cyclooxygenase-2, lipoxygenase, p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK1/2), extracellular signal-regulated kinase 1/2, growth factor induced signaling cascades, cyclin D1, p53, in addition to intracellular adhesion molecule-1 (11). Curcumin has also been used in combination with various other anti-cancer agents, such as gemcitabine, docetaxel and acetylcysteine in pre-clinical cancer studies (5,10,12).

Autophagy, which is a process involving self-degradation and the turnover of cellular components, has a complex role in the initiation and progression of cancer. Evidence suggests that autophagy has anti-survival characteristics, and can contribute to tumor suppression in different cancer cell types (13). The predominant function of the JNK signal transduction pathway is to induce defense mechanisms that protect organisms against a number of stressors, including UV irradiation and oxidative stress, which can induce apoptosis or growth inhibition. This pathway has also been confirmed to be associated with the molecular events involved in the regulation of autophagy (14).

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Abbreviations: JNK, c-Jun N-terminal kinase; 3-MA, 3-methyladenine; MDC, monodansylcadaverine; ATG5, autophagy related 5; ERK, extracellular signal-regulated kinase; LC3, microtubule associated protein 1 light chain 3

Key words: osteosarcoma, apoptosis, curcumin, autophagy
The current study aimed to determine whether curcumin was able to induce autophagy in osteosarcoma cells. In addition, the underlying interaction between autophagy and apoptosis was investigated.

Materials and methods

Cell lines and curcumin. The human osteosarcoma cell line MG63 was purchased from the American Type Culture Collection (Manassas, VA, USA). All of the cells were cultured with Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBSand penicillin/streptomycin (both Thermo Fisher Scientific, Inc.).

Proliferation assay. Cell proliferation assays were performed using cell counting kit (CCK)-8 (Dojindo Molecular Technologies, Inc., Rockville, MD, USA) in accordance with a previously described method (15). A total of 2,000 cells were seeded in each well in a 96-well plate. CCK-8 solution (10 µl) was added to 100 µl of culture media, and the optical density was measured at 450 nm. The concentrations of z-VAD-FMK, 3-MA or SP600125 used in the experiments were 5, 5 or 10 µM, respectively. Three independent experiments were performed.

Apoptosis assay. Cell apoptosis assays were performed by flow cytometry (BD FACSCalibur flow cytometer; BD Biosciences, Franklin Lakes, NJ, USA), as previously described (16,17). Cells at a density of 0.5x10^5 cells/dish in 60-mm cell culture dishes were pre-incubated with or without agents for 24 h, collected and stained using the Annexin V-FITC Apoptosis Detection kit (BD Biosciences) according to the manufacturer's instructions. In brief, cells were washed with cold PBS and resuspended in 100 µl Annexin V binding buffer, followed by incubation with fluorescein isothiocyanate-conjugated Annexin V and propidium iodide for 15 min at room temperature in the dark.

Western blot analysis. Total protein (~500 mg) was extracted using lysis reagents (Cell Signaling Technology, Inc., Danvers, MA, USA) and quantified using a bicinchoninic acid assay kit (Pierce; Thermo Fisher Scientific, Inc.). Proteins (50 mg) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with 5% skimmed milk for 2 h at room temperature and incubated overnight at 4°C with mouse anti-actin monoclonal antibody (1:1,000; sc-81178; Santa Cruz Biotechnology, Inc.). Immune complexes were then detected by incubating the PVDF membranes with HRP-conjugated secondary antibody for 2 h at room temperature, followed by exposure of the membrane to enhanced chemiluminescence reagents (Pierce; Thermo Fisher Scientific, Inc.). Quantification of the protein bands was performed using ImageJ 1.48 software (National Institutes of Health, Bethesda, MD, USA).

Autophagy assay. Monodansylcadaverine (MDC; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), a specific marker for autophagic vacuoles was used in order to further confirm whether curcumin was able to induce autophagy. MG63 cells were labeled with 0.05 mM MDC in PBS at 37°C for 10 min, washed three times with PBS and immediately analyzed under an AV300-ASW confocal microscope (Olympus Corp., Tokyo, Japan) with a x60 oil lens. The amount of LC3 puncta per cell was quantified. A minimum of 10 cells in five independent experiments were analyzed at random.

Statistical analysis. Statistical analysis was performed using SPSS statistical software (version 16.0; SPSS, Inc., Chicago, IL, USA). Student's t-test was used to analyze all other data. All tests were two-tailed and P<0.05 was considered to indicate a statistically significant difference.

Results

Curcumin suppresses osteosarcoma cell proliferation. To determine the cytotoxic effects of curcumin (Fig. 1) on osteosarcoma cell lines, a CCK-8 assay was performed to evaluate the proliferation of human osteosarcoma MG63 cells. As shown in Fig. 2A, curcumin treatment markedly decreased the proliferation of MG63 cells in a dose- and time-dependent manner. Furthermore, the exposure of MG63 cells to curcumin at various concentrations for 24 h dose-dependently increased the number of cytolytic cells (Fig. 2B). The IC_{50} value for curcumin was 9.2 µM in MG63 cells.

Curcumin promotes apoptosis in osteosarcoma cells. Flow cytometry was employed to investigate the anti-cancer effects of curcumin on the apoptosis of MG63 cells. Following treatment with different concentrations of curcumin (1, 5 or 10 µM) for 24 h, the apoptosis rate of cells was markedly increased in a dose-dependent manner (Fig. 3A). The Annexin V positive cell ratios (%) for the concentrations of 1, 5 or 10 µM curcumin were 4.6, 23.8 and 41.9%, respectively in MG63 cells. The results showed that 5 or 10 µM curcumin could significantly induce MG63 cell apoptosis compared with the DMSO...
group (Fig. 3B; P<0.05 and P<0.01, respectively). To further investigate the potential mechanisms of curcumin-induced apoptosis in MG63 cells, the expression levels of apoptosis signaling proteins were detected by western blot analysis. The results indicated that the protein expression levels of cleaved caspase-3 and Bax were increased, while the expression of...
anti-apoptosis proteins Bcl-2 and caspase-3 were reduced after treatment (Fig. 3C). These results indicate that the mechanism underlying curcumin-induced apoptosis may involve the activation of the caspase-3 pathway in MG63 cells.

**Inhibition of apoptosis enhances curcumin-induced autophagy through the JNK signaling pathway.** The current study subsequently explored whether curcumin was able to alter the level of autophagy, which would consequently affect cell apoptosis. The auto-fluorescent substance MDC was used as a marker to detect the level of autophagy in MG63 cells. Following incubation with curcumin for 24 h, the percentage of MDC-positive cells significantly increased in a dose-dependent manner, particularly in the curcumin combined with z-VAD-FMK group (Fig. 4A; P<0.05). These results suggest that z-VAD-FMK was able to promote curcumin-induced autophagy in MG63 cells.

Next, we extracted the total protein from MG63 cells incubated with different concentrations of curcumin or with the combination treatment for 24 h. The expression levels of ATG5, LC3-I, LC3-II, p-JNK, p-ERK, and p-p38 were analyzed by western blotting. As shown in Fig. 4B and C, compared with the other groups, there was a significant increase in the expression levels of ATG5 (P<0.01), LC3-II (P<0.05), and p-JNK (P<0.05) in the curcumin combined with z-VAD-FMK group. In addition, there was a significant reduction in the expression levels of LC3-I in the curcumin combined with z-VAD-FMK group compared with the other groups. Notably, JNK inhibitor SP600125 effectively reversed combination treatment-induced autophagy in MG63 cells, suggesting that JNK pathway signaling may have an important role in curcumin-induced autophagy (Fig. 4D).

**3-MA inhibits curcumin-induced hyperactive of p-JNK/autophagy pathway.** To further explore the role of JNK in curcumin-induced autophagy, MG63 cells were used to investigate the effect of 3-MA on autophagic activity. As shown in Fig. 6, curcumin upregulated ATG5, p-JNK, and p53 expression levels in MG63 cells; however, z-VAD-FMK inhibition of autophagy enhances curcumin-induced apoptosis in MG63 cells.

Subsequently, in order to examine whether the inhibition of autophagy sensitizes MG63 cells to curcumin, cells were treated with curcumin, z-VAD-FMK or in combination with 3-MA, which is an autophagy inhibitor. Treatments with these drugs, either alone or in combination, significantly affected the apoptosis of MG63 cells (P<0.05). The results showed that 3-MA alone did not induce apoptosis or inhibit proliferation in MG63 cells (Fig. 5A and B). Furthermore, 3-MA or SP600125 effectively reversed z-VAD-FMK-induced proliferation in curcumin-treated MG63 cells (Fig. 5C).
only upregulated the expression of ATG5 and p-JNK in 3-MA-treated cells. Furthermore, 3-MA effectively prevented the upregulation of ATG5 and p-JNK in MG63 cells induced by the combination of curcumin and z-VAD-FMK. Notably, z-VAD-FMK markedly downregulated p-P53 expression in curcumin-treated MG63 cells, and this phenomenon was inhibited by 3-MA.

Discussion

Given that osteosarcoma is characterized by adjuvant chemotherapy resistance and high rates of recurrence after curative resection (18), it is necessary to develop novel therapeutic agents to achieve improved patient prognoses. Curcumin is a natural compound derived from turmeric (*Curcuma longa*) and exhibits an effective antitumor effect on various cancers, including osteosarcoma (19). In addition, curcumin can reverse chemotherapy resistance in various types of human cancer (7,20,21). Therefore, curcumin may be a promising agent for the treatment of osteosarcoma.

Several studies have confirmed that chemotherapy agents, including tamoxifen, 5-fluorouracil, and rapamycin, are able to induce autophagy (22-24). However, the mechanisms underlying autophagy in cancer therapy are complex and remain controversial. It has been reported that forced autophagy may be an apoptotic enhancer or a survival enhancer, depending on the experimental conditions (22,25). Attempts have been made to elucidate the potential mechanisms involved in autophagy, so that it may be exploited as a target for cancer therapy. The current study showed that the expression levels of the anti-apoptotic protein Bcl-2 were significantly decreased in MG63 cells incubated with curcumin. The results of the present study suggest
that inhibition of the Bcl-2-mediated anti-apoptotic pathway may contribute to curcumin-induced apoptosis in MG63 cells.

Autophagy has been confirmed to have two contrasting roles in cancer, in that it is both a tumor suppressor and a tumor protector (26). Recently, autophagy has been reported as a mechanism by which osteosarcoma cells develop resistance to anti-tumor agents, including cisplatin and doxorubicin (27,28). Dysregulation of the p38 MAPK signaling pathway has an important role in tumor development and progression (29). In addition, p38 MAPK also mediates autophagy in response to anti-cancer agents in the treatment of cancer. Furthermore, p38 MAPK acts both as a positive and negative regulator in the autophagy process (29). JNK is initially activated in response to various stress signals and has been observed to participate in numerous cellular events including apoptosis (30). The current study demonstrated that treatment with curcumin is correlated with increased phosphorylation of JNK in MG63 cells. JNK displays both tumor-promoting and tumor-suppressive functions depending on the genetic context of the tumor cells (31). The JNK pathway has been shown to be important in enforcing autophagy and apoptosis (32). Previous studies have further revealed that elevated JNK signaling is also associated with autophagy induction (32,33). The present study also found that JNK inhibitor SP600125 was able to reverse curcumin-induced autophagy. These results suggest that the JNK pathway has a critical role in curcumin-induced autophagy.

The ERK signaling pathway has an important role in cancer development and progression. In addition, ERK activity has been confirmed to participate in autophagy and autophagic cell death (34). Notably, in human ovarian cancer cells, cytoplasmic sequestration of ERK by PEA-15 has been confirmed to promote autophagy (34). Furthermore, forced ERK activation by overexpression of activated MEK can promote autophagy without any other stimuli (35). However, there was no apparent change in p-ERK expression in osteosarcoma MG63 cells treated with curcumin, suggesting curcumin does not affect the ERK signaling pathway.

Autophagy and apoptosis are well-regulated biological processes that have important roles in tumor development and progression. The role of the anti-apoptotic protein Bcl-2 in autophagy has been explored, and it has been proposed to be a major binding partner of Beclin-1, or to directly inhibit components of the autophagic pathway proteins, Bax and Bak (36,37). As previously demonstrated, p53 also participates in the regulation of autophagy. Nuclear p53 can induce autophagy through transcriptional effects, while cytoplasmic p53 may act as a master repressor of autophagy (38). It has been confirmed that p53 stimulates autophagy by transactivation of damage-regulated autophagy modulator or through the inhibition of the mTOR signaling pathway via activation of the AMP kinase (39,40). Furthermore, the present results revealed that the autophagy inhibitor 3-MA was able to increase curcumin-induced apoptosis in MG63 cells. The present study also confirmed that 3-MA was able to reverse curcumin-induced upregulation of p-JNK and ATG5, a gene that is essential to the process of autophagy. In addition, 3-MA also upregulated curcumin-induced p53 expression. These results also ascertained that the JNK pathway has a critical role in curcumin-induced autophagy.

In conclusion, the present study demonstrated that curcumin inhibited the growth and induced the apoptosis of human osteosarcoma cells. The effects of curcumin-induced apoptosis in osteosarcoma cells were associated with caspase-3 activation and reduced the levels of Bcl-2 expression. In addition, curcumin induces both apoptosis and autophagy in osteosarcoma cells. Furthermore, curcumin-induced autophagy has an anti-apoptotic role in osteosarcoma cells. These results provide important insight into the interaction between apoptosis and autophagy in osteosarcoma cells, and provides further information regarding the clinical treatment strategies using curcumin.

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