Curcumin exerts antitumor effects in retinoblastoma cells by regulating the JNK and p38 MAPK pathways

XIAOMING YU[1], JINGTAO ZHONG[2], LI YAN[1], JIE LI[2], HUI WANG[1], YAN WEN[1] and YU ZHAO[3]

[1]Department of Ophthalmology, Shandong Jiaotong Hospital, Jinan, Shandong 250031; [2]Department of General Surgery, Qianfoshan Hospital, Shandong University, Jinan, Shandong 250014; [3]Department of Otolaryngology, Shandong Jiaotong Hospital, Jinan, Shandong 250031, P.R. China

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Abstract. Curcumin, a naturally occurring polyphenolic compound present in turmeric (*Curcuma longa*), exerts antitumor effects in various types of malignancy. However, the precise mechanisms responsible for the effects of curcumin on retinoblastoma (RB) cells have not been fully explored. In the present study, the molecular mechanisms by which curcumin exerts its anticancer effects in RB Y79 cells were investigated. The results showed that curcumin reduced cell viability in Y79 cells. Curcumin induced G1 phase arrest through downregulating the expression of cyclin D3 and cyclin-dependent kinase (CDK)2/6 and upregulating the expression of CDK inhibitor proteins p21 and p27. Curcumin-induced apoptosis of Y79 cells occurred through the activation of caspases-9/-3. Moreover, flow cytometric analysis showed that curcumin induced mitochondrial membrane potential ($\Delta\Psi m$) collapse in Y79 cells. We also found that curcumin induced the phosphorylation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK). JNK and p38 MAPK inhibitors significantly suppressed curcumin-induced activation of caspases-9/-3 and inhibited the apoptosis of Y79 cells. Taken together, our results suggest that curcumin induced the apoptosis of Y79 cells through the activation of JNK and p38 MAPK pathways. These findings provide a novel treatment strategy for human RB.

Introduction

Retinoblastoma (RB) is the most common intraocular malignancy in children with an incidence of 1 in 15,000 to 1 in 20,000 births, accounting for approximately 4% of all pediatric malignancies (1,2). Most cases of unilateral RB are caused by sporadic somatic mutations in the RB1 gene, representing approximately 60% of all RB cases whereas about 40% of cases occur in infants with germline mutations (3). Treatment strategies for RB include intravenous chemoreduction, enucleation, transpupillary thermotherapy, cryotherapy, thermotherapy, laser photocoagulation, brachytherapy, plaque radiotherapy, orbital exenteration, external beam radiotherapy, and chemotherapy, depending on the stage of tumor development and the location and size of the primary tumor (4,5). Despite progress in the treatment of RB, a number of these treatments have possible side effects, such as blindness, infection, fever, gastrointestinal toxicity and neurotoxicity (6). Therefore, there is an urgent need for the development of novel therapeutic agents for use in the management of RB.

Curcumin [also known as diferuloylmethane and 1,7-bis-(4-hydroxy 3-methoxyphenyl)-1,6-heptadiene-3,5-dione], is a naturally occurring polyphenolic compound present in turmeric (*Curcuma longa*) which has been employed to treat a number of diseases including asthma, bronchial hyperactivity, allergy, anorexia, coryza, cough, sinusitis and hepatic disease in Asian countries for thousands of years (7). Recently, accumulating evidence has demonstrated that curcumin exerts antitumor effects on various types of cancer cells as well as being non-cytotoxic to normal cells (8-13). However, the precise mechanisms responsible for the effects of curcumin on RB cells have not been fully explored. Several studies have reported that curcumin exerts antitumor effects through inducing apoptosis in a variety of types of cancer cells which involves the activation of caspases (14), mitochondrial dysfunction triggered by enhanced Bax levels (15), and pro-apoptotic endoplasmic reticulum stress (16). Furthermore, a number of studies have revealed that curcumin exerts antitumor effects through mediating various cellular signaling pathways including nuclear factor κB (NF-κB) (17), signal transducer and activator of transcription 3 (STAT3) (18), protein kinase B (PKB/Akt) (19), mitogen-activated protein kinase (MAPK) (20), and other pathways. In this study, we examined the molecular mechanism responsible for curcumin-induced cytotoxicity in human RB cells.

Materials and methods

Cell culture. The human RB cell line Y79 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 1% penicillin, and streptomycin (Gibco, Grand Island, NY, USA) at 37˚C in 95% air and 5% CO_2.

Correspondence to: Dr Xiaoming Yu, Department of Ophthalmology, Shandong Jiaotong Hospital, 12 Wuyingshan Middle Road, Jinan, Shandong 250031, P.R. China
E-mail: yuxm1987@163.com

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Reagents and antibodies. Curcumin and ZVAD-FMK were purchased from Sigma-Aldrich (San Diego, CA, USA). SP600125 was obtained from AG Scientific, Inc. (San Diego, CA, USA). SB203580 was purchased from Calbiochem (San Diego, CA, USA). Antibodies against cyclin D3 (ab63535), p21 (ab109520), p27 KIP1 (ab32034), cytochrome *c* (ab53056), caspase-3 (ab90437), caspase-9 (ab25758), and GAPDH (ab37168) were purchased from Abcam (Cambridge, UK). Antibodies against cyclin-dependent kinase (CDK)2 (#2546), CDK6 (#13331), c-Jun N-terminal kinase (JNK; #9252), p38 MAPK (#9212), phospho-JNK (Thr183/Tyr185; #9251), and phospho-p38 MAPK (Thr180/Tyr182; #9211) were obtained from Cell Signaling Technology (Danvers, MA, USA).

Cell treatment. Curcumin was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and diluted immediately prior to each experiment. Final curcumin concentrations of 10-80 μM were obtained by dilution in culture media. Controls containing 0.07% DMSO were included in all experiments. In order to inhibit caspase activity, Y79 cells were treated with ZVAD-FMK at a concentration of 50 μM for 1 h prior to curcumin treatment. Y79 cells were treated in the absence or presence of 20 μM JNK inhibitor (SP600125) or p38 MAPK inhibitor (SB203580) for 1 h, then treated with 80 μM curcumin for 24 h before examining the phosphorylation levels of JNK and p38 MAPK.

Measurement of cell viability. The Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Beijing, China) was used to determine cell viability. Briefly, cells ($5x10^3$/well) were incubated with curcumin-containing RPMI-1640 in 96-well plates for 24 h, and then the culture medium was replaced with fresh medium containing 10 ml CCK-8 solution. The cells were further incubated for 2 h at 37˚C, and the optical density (OD) at 450 nm was measured.

Cell cycle analysis by flow cytometry. Y79 cells were seeded at a density of $4x10^5$ in 6-well culture plates, grown overnight in medium containing 10% FBS, and treated with or without various concentrations of curcumin (0-80 μM) for 24 h. Cell cycle analysis was then performed. Briefly, the cells were suspended in 0.5 ml propidium iodide (PI) solution, and incubated for 30 min in the dark according to the manufacturer's instructions. The cell cycle distribution was analyzed by flow cytometry [fluorescence-activated cell sorting (FACS) analysis; BD Biosciences, San Jose, CA, USA].

Analysis of apoptosis by flow cytometry. Apoptosis was examined using the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (BioVision Inc., Mountain View, CA, USA), according to the manufacturer's instructions.

Western blot analysis. Protein concentrations in the cell extracts were determined (Bio-Rad, Richmond, CA, USA). Briefly, equal amounts of each sample were resolved in SDS-PAGE gels and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), and probed with the primary antibodies described above in Reagents and antibodies. Protein band intensities were quantified by densitometric analysis using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

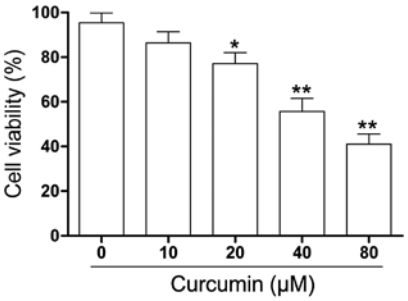

Figure 1. Effect of curcumin on the viability of human retinoblastoma (RB) Y79 cells *in vitro*. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay. Y79 cells were treated with various concentrations (0-80 μM) of curcumin for 24 h. Data are expressed as the percentage of control cells and are the means ± SD of three separate experiments. *P<0.05,**P<0.01 vs. control.

Determination of mitochondrial membrane potential ($\Delta\Psi m$). Briefly, Y79 cells were exposed to various concentrations of curcumin (0-80 μM) for 24 h. The cells were then harvested and incubated with 40 nmol/l $DiOC_6$ (Abcam) at 37˚C in the dark for 20 min. Finally, the mean fluorescence intensity (MFI) was determined by peforming flow cytometric analysis.

Statistical analysis. The data are expressed as the means ± standard deviation (SD). Comparisons were made using a one-way ANOVA followed by Dunnett's test with SPSS software (version 17.0; SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Curcumin significantly inhibits cell viability in Y79 cells. To explore the effect of curcumin on the cell viability of RB cells *in vitro*, human RB (Y79) cells were treated with varying concentrations of curcumin (0-80 μM) for 24 h and changes in cell viability were assessed by the CCK-8 assay. As shown in Fig. 1, the viability of the Y79 cells exposed to curcumin was significantly lower compared with that of the control cells. A sharp decrease in cell viability was present at a curcumin concentration of 40 μM.

Curcumin induces cell cycle arrest of Y79 cells. We explored whether curcumin induces cell cycle arrest in human RB cells. The Y79 cells were exposed to various concentrations of curcumin for 24 h and then analyzed for alterations in the cell cycle by flow cytometry. As shown in Fig. 2A, curcumin-treated Y79 cells were inhibited in the G1 phase after 24 h of treatment. To further examine the molecular mechanisms underlying curcumin-induced G1 phase arrest, the cells were treated with various concentrations of curcumin (0-80 μM) for 24 h, and harvested for protein extraction and western blot analysis. As shown in Fig. 2B and C, the protein expression of cyclin D3, CDK2 and CDK6 was markedly reduced in the curcumin-treated Y79 cells. Moreover, the levels of the CDK inhibitor proteins p21 and p27 were significantly upregulated following the exposure of Y79 cells to curcumin. These results suggest that

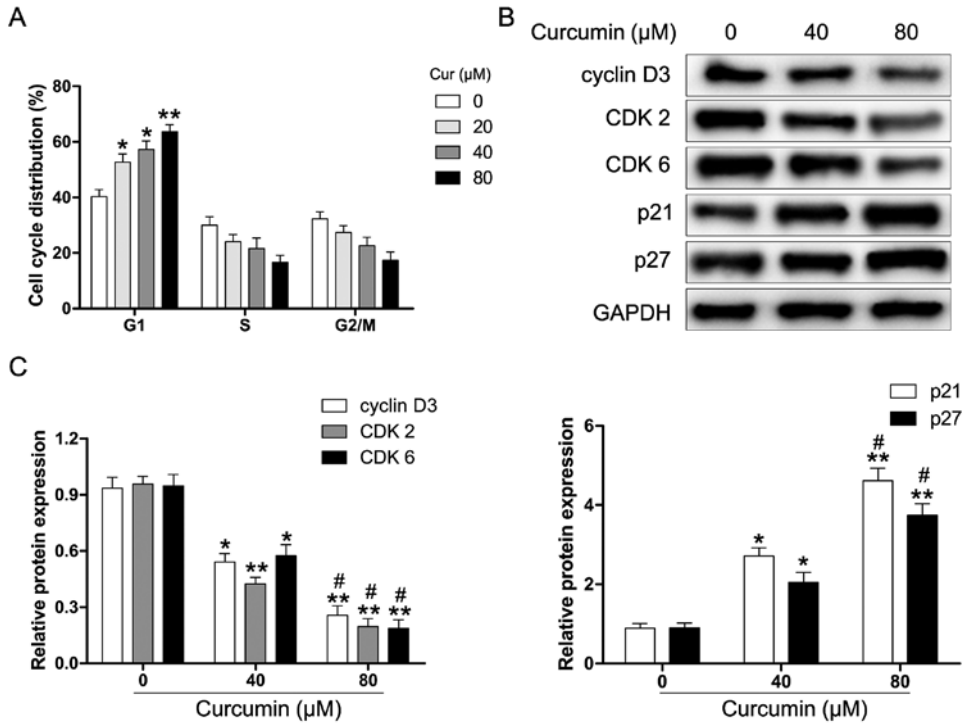

Figure 2. Curcumin induces cell cycle arrest in Y79 cells *in vitro*. (A) Cell cycle distribution of Y79 cells was determined 24 h after treatment with various concentrations of curcumin (0-80 μM). Data are expressed as the means ± SD of three independents experiments. *P<0.05, **P<0.01 vs. control. (B and C) Cells were treated with curcumin (0-80 μM). The cell lysates were harvested at 24 h and evaluated by western blot analysis using specific antibodies against cyclin D3, cyclin-dependent kinase (CDK)2, CDK6, p21, and p27. Levels of GAPDH served as a loading control. The results shown are representative of at least three independent experiments. *P<0.05, **P<0.01 vs. control. $^{\#}$P<0.05 vs. curcumin (40 μM) group.

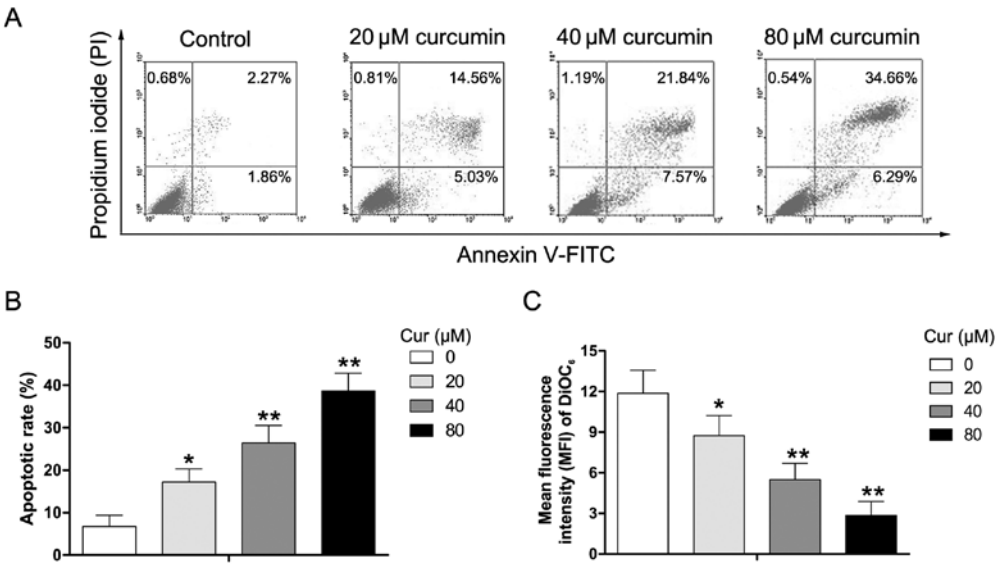

Figure 3. Curcumin induces the apoptosis of Y79 cells i*n vitro*. (A) Y79 cells were untreated (control) or exposed to curcumin (20-80 μM) for 24 h. Apoptotic cells were analyzed by flow cytometry (FACS analysis) with propidium iodide (PI) and Annexin V-fluorescein isothiocyanate (FITC) staining. (B) Quantitative analysis of the total apoptotic (early and late) population. Data are presented as the means ± SD of three independents experiments. *P<0.05, **P<0.01 vs. control. (C) Curcumin induced ΔΨm collapse in Y79 cells. Y79 cells were untreated (control) or treated with curcumin (20-80 μM) for 24 h. The mean fluorescence intensity (MFI) of $DiOC_6$ was obtained by flow cytometric analysis. Data are presented as the means ± SD of three independents experiments. *P<0.05, **P<0.01 vs. control. Cur, curcumin.

curcumin-induced G1 phase cell cycle arrest in human RB cells may be regulated through the cyclin-CDK checkpoint.

Curcumin induces the apoptosis of Y79 cells. To determine whether the effect of curcumin on cell viability was caused by apoptotic cell death, Y79 cells were exposed to various concentrations of curcumin (0-80 μM) for 24 h, and the extent of apoptosis was evaulated using the Annexin V/PI assay. As shown in Fig. 3A and B, the percentage of apoptotic cells (PI-negative/Annexin V-positive and

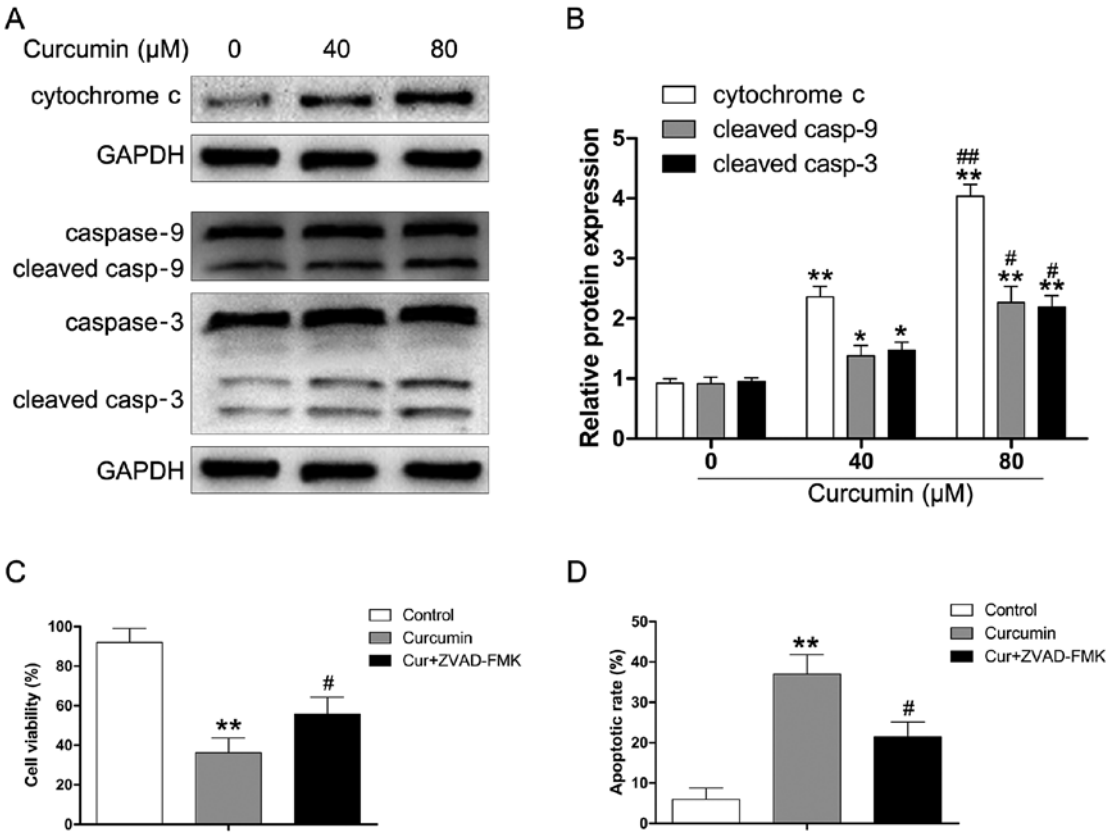

Figure 4. Curcumin induces the apoptosis of Y79 cells through intrinsic pathways. (A and B) Cells were treated with curcumin (0-80 μM). The cell lysates were harvested at 24 h and evaluated by western blot analysis with specific antibodies against cytochrome *c*, caspase-9 and caspase-3. Levels of GAPDH served as a loading control. The results shown are representative of at least three independent experiments. *P<0.05, **P<0.01 vs. control. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. curcumin (40 μM) group. (C) Cells were treated with 80 μM curcumin for 24 h in the presence or absence of 50 μM ZVAD-FMK. Cell viability was determined by the Cell Counting Kit-8 (CCK-8) assay. Data are expressed as the percentage of control cells and are the means ± SD of three separate experiments. **P<0.01 vs. control. $^{\#}$P<0.05 vs. curcumin group. (D) Curcumin-induced apoptosis was inhibited by 50 μM ZVAD-FMK treatment. Data are presented as the means ± SD of three independents experiments. **P<0.01 vs. control. $^{\#}$P<0.05 vs. curcumin group. Cur, curcumin.

PI-positive/Annexin V-positive) increased from 4.13 to 19.59, 29.41, and 40.95%, respectively, after the Y79 cells were either untreated (control) or treated with 20, 40, and 80 μM curcumin. $DiOC_6$, a lipophilic cationic dye, has been reported to specifically accumulate in the mitochondrial matrix depending on the $\Delta\Psi$m which is decreased in apoptotic cells (21,22). To further ascertain the effects of curcumin on $\Delta\Psi$m in human RB cells, Y79 cells were exposed to various concentrations of curcumin (0-80 μM) for 24 h, and then analyzed in order to determine the MFI of $DiOC_6$ by flow cytometry. We found that treatment with curcumin significantly decreased the MFI of $DiOC_6$ (Fig. 3C). The data suggest that curcumin-induced apoptosis may occur through $\Delta\Psi$m dissipation in Y79 cells.

Curcumin induces the apoptosis of Y79 cells through intrinsic pathways. Accumulating evidence has revealed that $\Delta\Psi$m plays an important role in regulating cellular functions. Disturbances of $\Delta\Psi$m may change the membrane dynamics of mitochondria and result in the release of cytochrome *c,* which triggers the formation of the apoptosome complex, and the subsequent activation of caspase-9. To determine whether curcumin induces apoptosis through the release of cytochrome *c* and the activation of caspase-9 in human RB, Y79 cells were exposed to various concentrations of curcumin (0-80 μM) for 24 h. As shown in Fig. 4A and B, curcumin significantly enhanced cytochrome *c* levels in a dose-dependent manner. We also observed that the released cytochrome *c* triggered the activation of caspase-9 and caspase-3 (Fig. 4B). Moreover, we used the pan-caspase inhibitor (ZVAD-FMK) to evaluate the effect of curcumin on apoptotic cell death in Y79 cells. As depicted in Fig. 4C, pre-treatment with ZVAD-FMK attenuated the curcumin-induced reduction of viability in the Y79 cells. We also found that ZVAD-FMK attenuated the apoptotic effect of curcumin in the Y79 cells, which suggested that the activation of caspases is involved in curcumin-regulated apoptosis of human RB cells (Fig. 4D).

JNK and p38 MAPK signaling play essential roles in caspase-9/-3 activation induced by curcumin. Previous studies have demonstrated that JNK and p38 MAPK are involved in the effects exerted by curcumin in tumor cells (10,23). However, the role of MAPKs in curcumin-induced apoptosis of RB cells was not examined. In this study, we explored whether JNK and p38 MAPK were activated in curcumin-treated Y79 cells. As shown in Fig. 5, curcumin induced an increase in the phosphorylation of JNK and p38 MAPK in the Y79 cells. To determine whether JNK and p38 MAPK were necessary for curcumin-induced apoptosis, we examined the relationships among caspases-9/-3 and JNK, and p38 MAPK in the presence of curcumin. The Y79 cells were treated in the absence

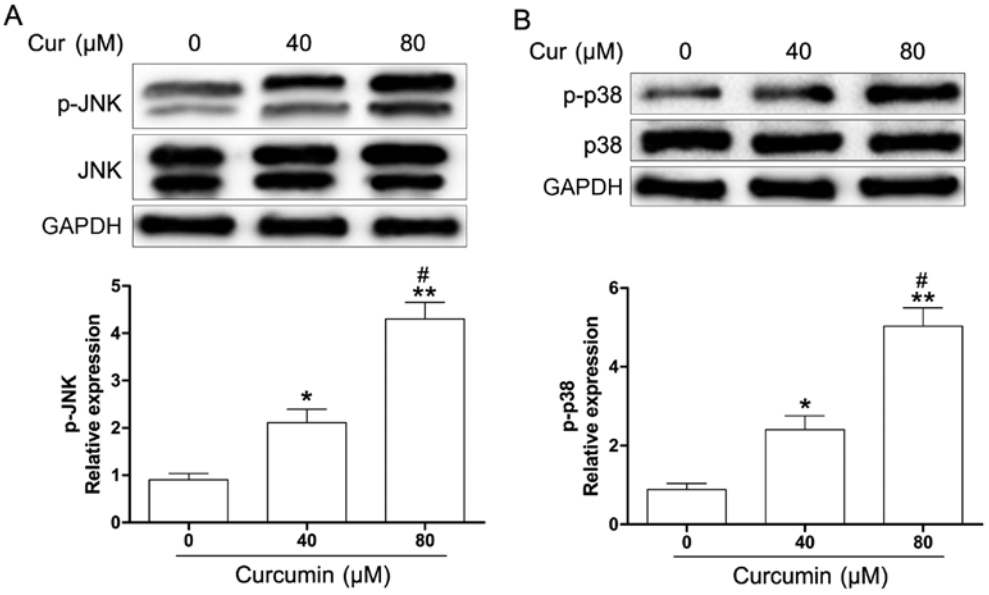

Figure 5. Curcumin induces c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) phosphorylation in Y79 cells. (A and B) Y79 cells were incubated with various concentrations of curcumin (0-80 μM) for 24 h. The levels of both total and phosphorylated (p-)JNK and p38 MAPK were evaluated by western blot analysis. Levels of GAPDH served as a loading control. The results shown are representative of at least three independent experiments. *P<0.05, **P<0.01 vs. control. $^{\#}$P<0.05 vs. curcumin (40 μM) group. Cur, curcumin.

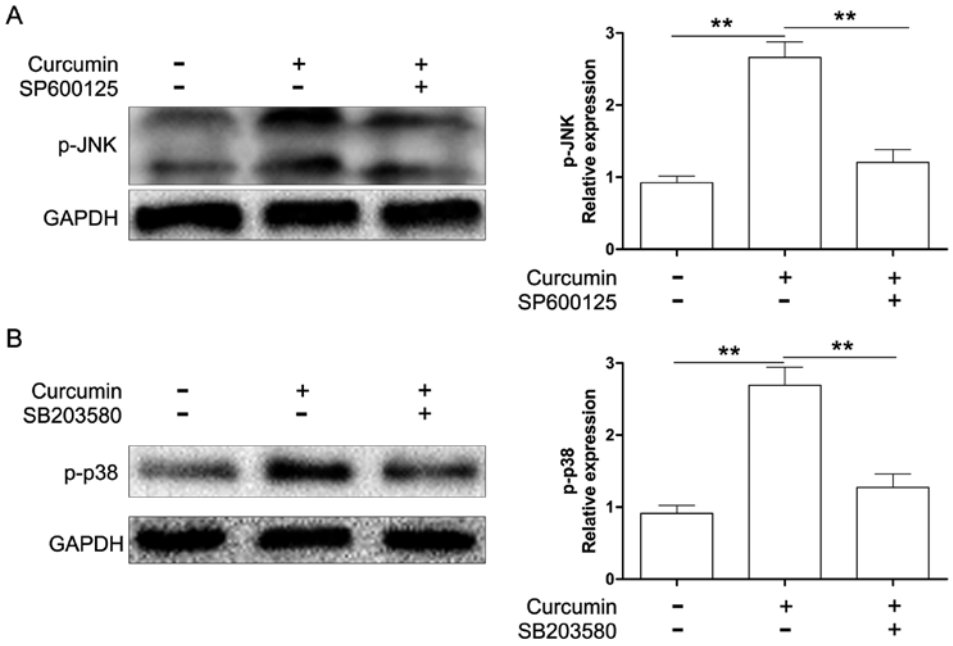

Figure 6. Effects of specific inhibitors of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) on curcumin-induced activation of JNK and p38 MAPK. (A and B) Y79 cells were treated in the absence or presence of 20 μM JNK inhibitor (SP600125) or p38 MAPK inhibitor (SB203580) for 1 h, then treated with 80 μM curcumin for 24 h, and then the phosphorylation levels of JNK and p38 MAPK were assessed by western blot analysis. Levels of GAPDH served as a loading control. The results shown are representative of at least three independent experiments. **P<0.01. p-, phosphorylated.

or presence of 20 μM JNK inhibitor (SP600125) or p38 MAPK inhibitor (SB203580) for 1 h and then treated with 80 μM curcumin for 24 h. Protein expression was evaluated by western blot analysis. As shown in Fig. 6, SP600125 and SB203580 markedly suppressed the activation of JNK and p38 MAPK induced by curcumin. Moreover, we also found that both SP600125 and SB203580 significantly reduced curcumin-induced caspase-9/-3 activation (Fig. 7A and B) and apoptosis (Fig. 7C). These results revealed that the activation of caspase-9/-3 in the presence of curcumin may occur through the activation of JNK and p38 MAPK in Y79 cells.

Discussion

RB is the most common pediatric eye cancer. It is second only to uveal melanoma in terms of the frequency of occurrence of malignant intraocular tumors (24). Although chemotherapy has become an important part of the present management of

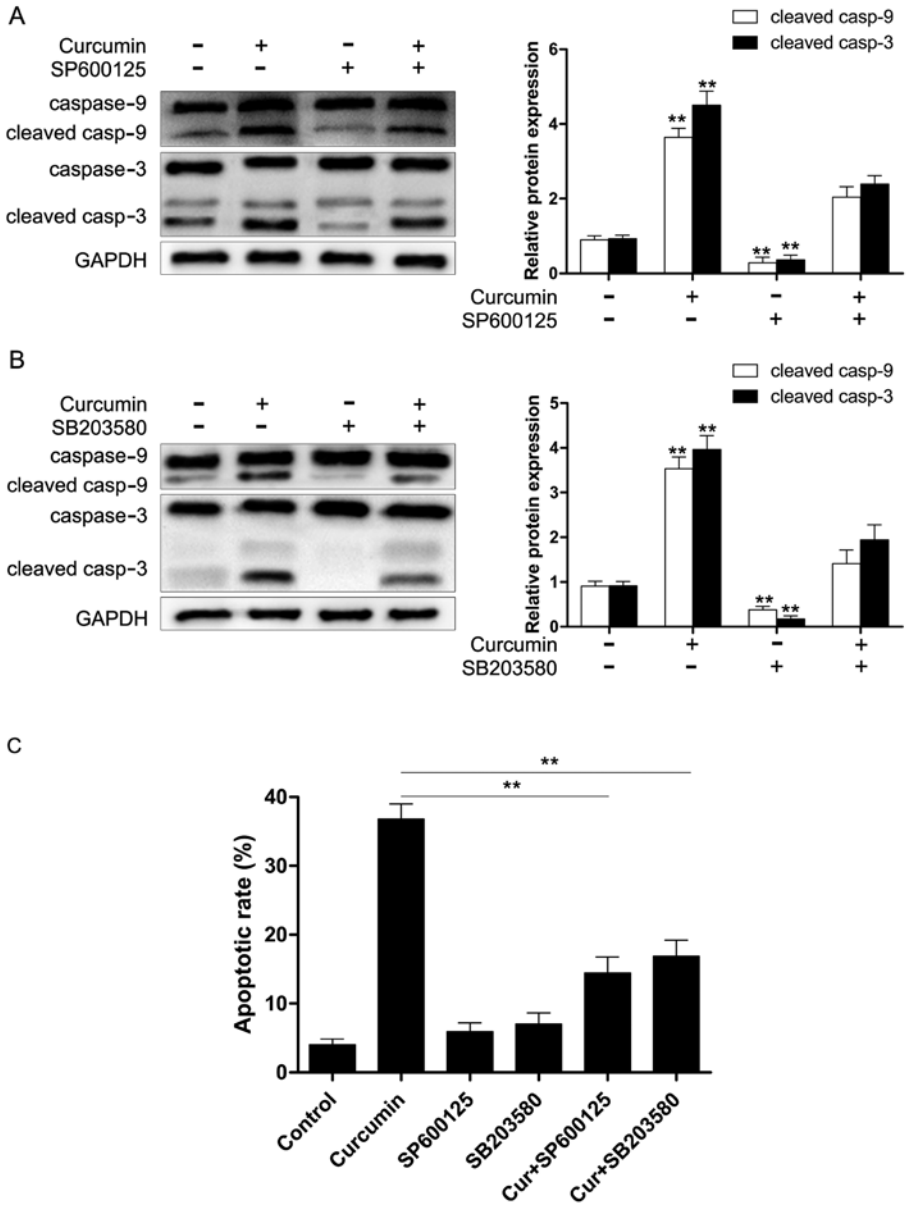

Figure 7. c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) mediate curcumin-induced apoptosis in Y79 cells. (A and B) Y79 cells were treated in the absence or presence of 20 μM SP600125 or SB203580 for 1 h, and then treated with 80 μM curcumin for 24 h. Expression levels of caspase-9 and -3 were determined by western blot analysis. Levels of GAPDH served as a loading control. The results shown are representative of at least three independent experiments. **P<0.01 vs. control. (C) Quantitative analysis of the total apoptotic (early and late) population. Apoptotic cells were analyzed by flow cytometry (FACS analysis) with propidium iodide (PI) and Annexin V-fluorescein isothiocyanate (FITC) staining. Data are presented as the means ± SD of three independent experiments. **P<0.01. Cur, curcumin.

RB, it causes noteworthy complications including secondary malignancies and results in long-term survival rates that remain low in developing countries (25,26). Thus, the search for novel treatment modalities is imperative. Curcumin, a natural polyphenolic compound, exerts powerful growth inhibitory and apoptosis-inducing effects on cancer cells through the regulation of various signaling pathways (27-29). Although the potent anticancer effects of curcumin have been demonstrated in many types of cancer, the precise mechanism responsible for the effects of curcumin in human RB has not been fully explored. In the present study, we examined whether curcumin may potentially be used in the treatment of human RB and explored the potential mechanisms responsible for the anticancer effects of curcumin in Y79 cells.

Our results showed that curcumin reduced cell viability in a dose-dependent manner in Y79 cells. Cell proliferation is regulated by the cell cycle, which is a complex and stepwise process. The activity of CDKs is mediated by cyclin regulatory subunits. These form a complex with the catalytic subunit of CDKs and are controlled at a specific phase of the cell cycle (30,31). In the present study, we found that curcumin treatment induced an accumulation of Y79 cells in the G1 phase of the cell cycle. We also observed that curcumin reduced the protein expression of cyclin D3, CDK2 and CDK6, and enhanced the expression of CDK inhibitor proteins p21 and p27 which suggested that changes in these protein levels appear to make a major contribution to curcumin-induced G1 arrest in Y79 cells.

Apoptosis is a major biological process that leads to specific cell death via an intrinsic 'suicide' mechanism (32). The loss of ΔΨm has been reported to induce cytochrome *c* release which is essential for the activation of caspase-9 (33). A number of studies have demonstrated that caspases-9 and -3 play key roles in the apoptotic cascade (34,35). Our data revealed that curcumin induced ΔΨm dissipation and activated the caspase-dependent apoptotic pathway in mitochondria. Moreover, the pan-caspase inhibitor ZVAD-FMK significantly reduced the curcumin-induced apoptosis of Y79 cells suggesting that activation of caspase-9/-3 is involved in the curcumin-regulated apoptosis of Y79 cells.

MAPKs consists of several subfamilies, such as ERK1/2, JNKs and p38. JNK and p38 MAPK are involved in a variety of cellular responses including cell proliferation, differentiation, and apoptosis (36-39). Our previous study also revealed that advanced glycation end products induce the apoptosis of human corneal epithelial cells through the generation of reactive oxygen species and the activation of JNK and p38 MAPK pathways (40), and it has also been demonstrated that the activation of JNK signaling mediates connective tissue growth factor expression and scar formation in corneal wound healing (41). However, the role of JNK and p38 MAPK signaling pathways in curcumin-induced apoptosis of Y79 cells was not investigated. In this study, we found that curcumin induced the activation of JNK and p38 MAPK in Y79 cells. The JNK-specific inhibitor, SP600125, and the p38 MAPK-specific inhibitor, SB203580, suppressed the activation of caspases-9 and -3, and inhibited the apoptosis of Y79 cells induced by curcumin. These results suggest that the activation of JNK and p38 MAPK signaling pathways plays a crucial role in curcumin-induced apoptosis of Y79 cells by regulating the activity of caspase-9 and -3.

In conclusion, the present study showed that curcumin exerts an antitumor effect on human RB cells by inducing cell cycle arrest and apoptosis. These findings suggest a novel therapeutic strategy for the management of RB which warrants further investigation.

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