

Inhibition of Curcumin on ZAK α Activity Resultant in Apoptosis and Anchorage-Independent Growth in Cancer Cells

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Abstract

Curcumin, a popular yellow pigment of the dietary spice turmeric, has been reported to inhibit cell growth and to induce apoptosis in a wide variety of cancer cells. Although numerous studies have investigated anticancer effects of curcumin, the precise molecular mechanism of action remains unidentified. Whereas curcumin mediates cell survival and apoptosis through mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF- κ B) signaling cascades, its impact on the upstream regulation of MAPK is unclear. The leucine-zipper and sterile- α motif kinase alpha (ZAK α), a mitogen-activated protein kinase kinase kinase (MAP3K), activates the c-Jun N-terminal kinase (JNK) and NF- κ B pathway. This paper investigated the prospective involvement of ZAK α in curcumin-induced effects on cancer cells. Our results suggest that the antitumor activity of curcumin is mediated *via* a mechanism involving inhibition of ZAK α activity.

Key Words: apoptosis, cell cycle, curcumin, ZAK α

Introduction

Tumorigenesis is a multistep process modulated by complex signal networks. Most cancers are caused by the dysregulation of various genes; agents that target multiple gene products are required for preventing and treating cancer. Molecular markers involved in carcinogenesis and cancer progression have been reported as potential therapeutic targets (14).

A growing number of nutritional compounds have shown applications for their potential beneficial effects in health or therapy (21, 22). Numerous natural compounds have been supposed to be effective anticancer agents, including curcumin (11, 15, 29). Curcumin is a yellow-color polyphenolic pigment isolated from turmeric, which comes from the herbal

root of *Curcuma longa*. Curcumin possesses a wide range of biological functions and cellular activities (20) and exerts multiple anticancer effects by interacting with diverse intracellular targets and altering their expression and activity. The anticancer activities are conducted through the regulation of various biochemical cascades including protein kinases, transcription factors, inflammatory cytokines and other gene products related to the cell cycle and cell survival, proliferation, apoptosis, invasion and angiogenesis (9, 34). The anticancer effects of curcumin include growth inhibition and induction of apoptosis of various tumor cells such as human non-small cell lung cancer HCl-H460 cells, osteosarcoma MG63 cells (4) and oral cancer cells (16). Recent studies have demonstrated that curcumin mediates growth inhib-

itory effects by suppressing protein kinase B (Akt), mitogen-activated protein kinase (MAPK), notch homolog 1 (Notch-1) and nuclear factor-kappa B (NF- κ B) activities, and also NF- κ B-regulated gene expression (26). NF- κ B is a downstream target of MAPK found in all eukaryotes to orchestrate various cellular responses including survival, mitosis, motility and programmed cell death (17). Activation of MAPK cascades plays critical role in different biological responses and initiates many pathological processes (18, 33). Curcumin causes G2/S- or G2/M-phase cell cycle arrest in numerous cancers (8, 24). The leucine-zipper and sterile- α motif kinase (ZAK) is a member of the mixed-lineage kinase (MLK), a group of serine/threonine kinases that function as mitogen-activated protein kinase kinase kinase (MAP3K), and can lead to the activation of the c-Jun N-terminal kinase (JNK) and NF- κ B pathways and induce apoptosis (7, 19). Moreover, ZAK α plays a role in G2 cell cycle arrest, and ZAK activity is essential for cell cycle checkpoint modulation (32).

Extensive *in vitro*, *in vivo* and clinical studies have indicated that curcumin may be a preventative or therapeutic agent for various human diseases and cancers. However, in the absence of reported biological effects of curcumin, further research is necessary to determine the anticancer role of curcumin by elucidating the underlying molecular mechanism. Therefore, we speculated that ZAK α might mediate curcumin-induced growth inhibition and apoptosis. The aims of this study were to determine whether ZAK α was involved in curcumin anticancer effects and to clarify the mechanism of its action.

Materials and Methods

Cell Lines

The human oral epidermoid carcinoma cell line (KB, ATCC CCL-17) and the human osteosarcoma cell line (MG-63, ATCC CRL-1427) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corporation, NY, USA) plus 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μ g/ml) in 5% CO₂ at 37°C in a humidified incubator. Soft agar assays were performed as previous publish (13). In brief, cells were seeding in 0.3% Noble agar in DMEM containing 10% FBS above presolidified 0.5% agar in the same medium. At the end of 2 weeks, colonies were stained with the vital stain 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) for 72 h at 37°C, and colonies were counted under a microscope. Curcumin was purchased from Sigma-Aldrich (St. Louis, MO, USA), and was dissolved in Dimethyl sulfoxide (DMSO) as the stock solution for *in vitro*

study. The vehicle solvent DMSO was used as a negative control.

SiRNA Knockdown

The expression vector pCDNA-HU6, a derivative of pCDNA3.1/Myc-His(-) with a human U6 promoter, was used for expression of short hairpin RNAs (shRNAs). Two oligonucleotides, shRNA-F (36 nucleotides) and shRNA-R (41 nucleotides), were synthesized, each of which consisted of a 19-nucleotide stem sequence. The oligonucleotides used for shRNA were synthesized by QualitySystems (Taipei, Taiwan) and were as follows: ZAK460iF (GATCCGCTCTCGGTTCCATAACCATTTC AAGA-GAA), ZAK460iR (AGCTTAAAAAGCCTCTCGGTTCCATAACCATTCTCTTGAAA), ZAK1712iF (GATCCGCCAGTGGTTAGATACTCTGATTCAA-GAGAT), ZAK1712iR (AGCTTAAAAAGCCAGTGGTTAGATACTCTGATCTCTTGAAT).

Western Blot Analysis

Cell lysates were prepared in IP buffer (40 mM Tris-HCl, pH 7.5, 1% NP40, 150 mM NaCl, 5 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluorid (PMSF), 20 mM NaF, proteinase inhibitors, and 1 mM sodium vanadate). Cell extracts (600 μ g) were incubated with 5 μ g anti-green fluorescent protein (anti-GFP) mAb (Clontech, Palo alto, CA, USA) for 6 h at 4°C, mixed with 20 μ l protein-A sepharose suspension, and incubated for an additional hour. Immunoprecipitates were collected by centrifugation, washed three times with the immunoprecipitation (IP) buffer plus 0.5% deoxycholate, five times with the IP buffer alone, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblot analysis was performed with the anti-FLAG antibody (Sigma, St. Louis, MO, USA). ZAK- or empty vector-expressed cells were harvested in lysis buffer (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1% NP-40, 2 mM ethylenediaminetetraacetic acid [EDTA]) containing 1 mM PMSF, 10 ng/ml leupeptin, 50 mM NaF and 1 mM sodium orthovanadate. Total proteins were separated on SDS-PAGE and specific protein bands visualized with an electrochemiluminescence (ECL) detection system (Amerchanm, Freiburg, Germany).

Cell Cycle Analysis

Cells were grown for 24 h or 72 h in the presence of doxycycline to induce expression of the ectopic genes. The cells were then plated to 40% confluency (2.5×10^6 /plate) on 100-mm plates. Cells were filtered

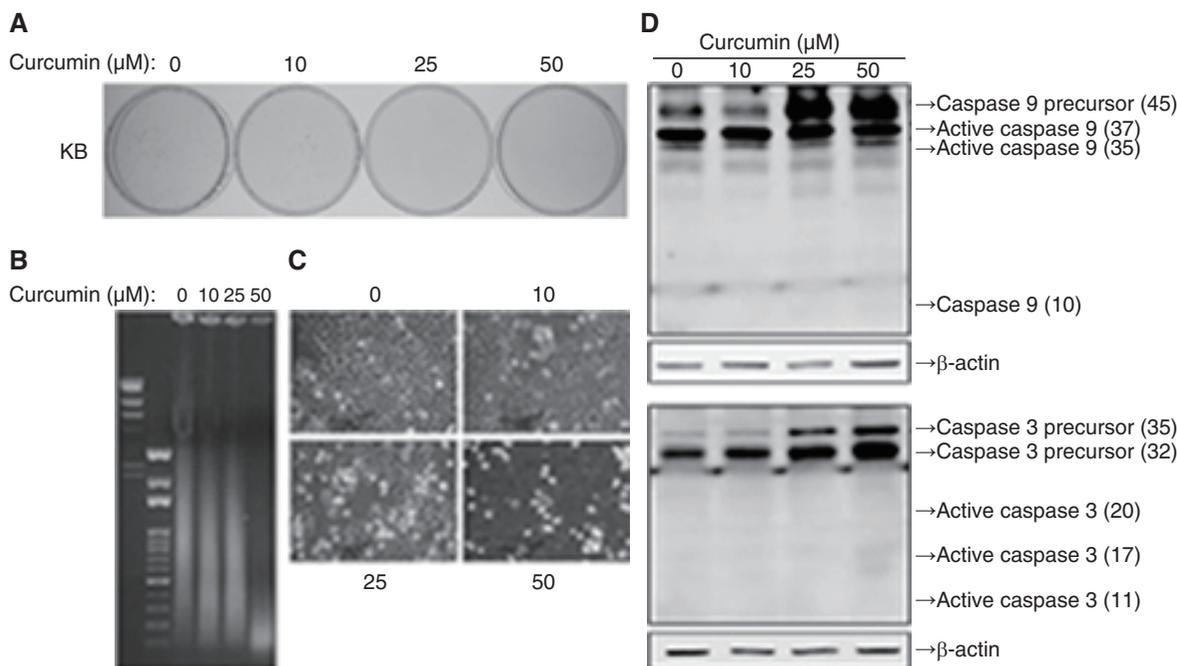


Fig. 1. Curcumin affects cell growth and morphology, and induces apoptosis in the KB cells. (A) Curcumin has dose-dependent inhibition on KB cell colony formation. KB cells were incubated with or without curcumin for 7 days and macroscopically visible colonies were fixed and stained with Giemsa. (B) Curcumin induces apoptosis in KB cells. KB cells were treated with the indicated concentrations of curcumin for 24 h. The cells were harvested and lysed. DNA fragmentation was examined by agarose gel electrophoresis. (C) Morphological changes of KB cells after exposure to curcumin. KB cells were treated with indicated concentrations of curcumin for 24 h. (D) Expression of apoptosis-related proteins pro-caspase 3 and pro-caspase 9 in KB cells after treatment with curcumin.

to remove cell aggregates in Falcon filter-top tubes, and analyzed for DNA content by fluorescence-activated cell sorting (FACS) analysis with a FACSCalibur flow cytometer (BD PharMingen, San Diego, CA, USA). Data were analyzed using the Cell Quest (BD PharMingen) and ModFit (Verity, Software House) analysis software to determine cell cycle fractions.

Results

Curcumin Treatment Leads to Significant Decrease Colony Formation on a Cancer Cell Line

To determine the effectiveness of curcumin on a human oral epidermoid carcinoma cell line, KB, cells were seeded onto 6 cm culture plates at a density of 5,000 cells per plate without or with curcumin treatment at 10, 25, or 50 μ M, and the cells were incubated for 7 days for colony formation. The lowest dose of curcumin at 10 μ M could significantly decrease colony formation (Fig. 1A). At the higher doses of curcumin of 25 and 50 μ M, the KB cells formed much fewer colonies (Fig. 1A). These results showed that curcumin inhibited the growth of KB cells in a dose-dependent manner.

Curcumin Induces Apoptosis in KB Cells

To test whether the effect of curcumin on colony formation in KB cells was caused by apoptosis, KB cells were pretreated with different doses of curcumin and apoptosis was determined by DNA fragmentation assay or Western blot analysis. Agarose gel electrophoresis revealed that exposure to higher doses of curcumin resulted in more DNA fragments (Fig. 1B). Microscopic observation revealed that the fewer KB cells survived higher concentrations of curcumin treatment (Fig. 1C). Furthermore, the higher doses of curcumin altered the cell morphology. Most of the cells were killed or wizened by exposure to curcumin of up to 50 μ M. In order to determine that these cancer cells entered apoptosis when they were exposed to curcumin, Western blot assay was subsequently performed to assess changes of apoptosis-related proteins. The data showed that curcumin could trigger the cleavage of pro-caspase 3 and pro-caspase 9 to form the respective active forms, caspase 3 and caspase 9, in KB cells in a dose-dependent manner (Fig. 1D). However, caspase 8 was not involved in curcumin-induced apoptosis in KB cells. The data suggest that curcumin might

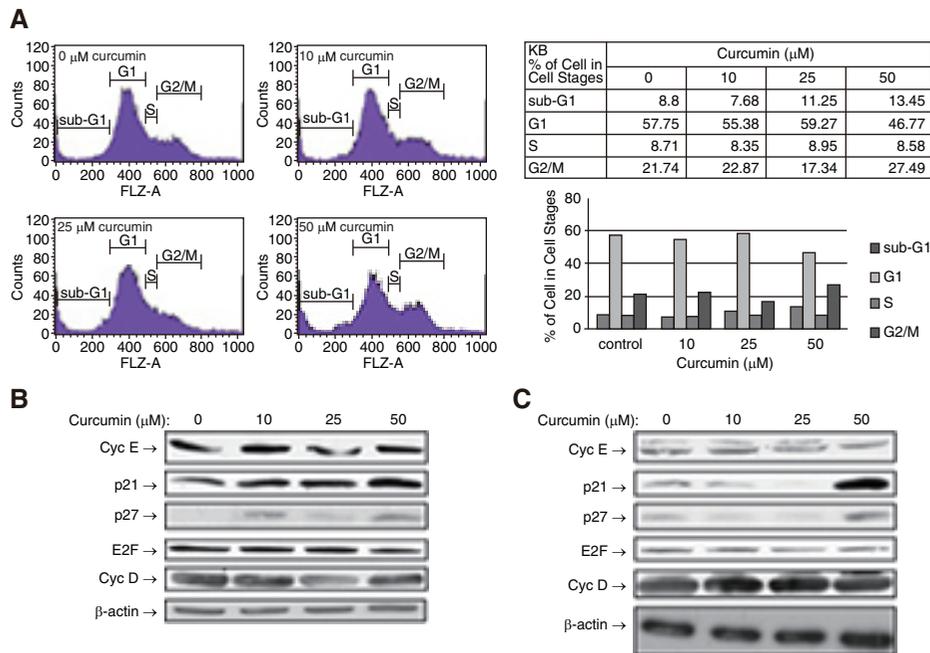


Fig. 2. Curcumin elicits G2/M phase arrest. (A) KB cells were treated with curcumin for 24 h and the cell cycle profiles were analyzed by flow cytometry following propidium iodide staining. Expression of the cell cycle proteins cyclin D, cyclin E, p21, p27, and E2F in KB (B) and MG-63 (C) cells after treatment with curcumin. Data shown are representatives of three independent experiments.

induce apoptosis through intrinsic apoptotic pathways.

Curcumin Inhibits Cell Cycle Progression by Upregulating p21 and p27 Levels

Since curcumin suppressed colony formation and activated apoptotic proteins *via* the caspase pathway, the effect of curcumin on the cell cycle distribution in KB cells was further analyzed by flow cytometry (FACS). When the sub-G1 population, which represents the population of apoptosis cells, were examined, the sub-G1 population was found to be increased proportionately with the escalated dose of curcumin. Moreover, the cells exposed to curcumin tended to induce cell cycle arrest in the G2/M phase also in a dose-dependent manner. To further investigate the effect of curcumin on cell cycle progression, the expression levels of several cell cycle regulatory proteins were analyzed by Western blot analysis after curcumin treatment for 24 h. Notably in the curcumin-treated cells, the levels of cyclin-dependent kinase inhibitors (CDKIs), p21 and p27 were upregulated, but the expression levels of cyclin E, cyclin D and E2F in the KB cells remained unchanged (Fig. 2B). Similar results were found in an osteosarcoma cell line, MG-63 (Fig. 2C). Thus, the results showed that curcumin induced the expression of p21 and p27 proteins in two cancer cell lines, leading to cell cycle arrest.

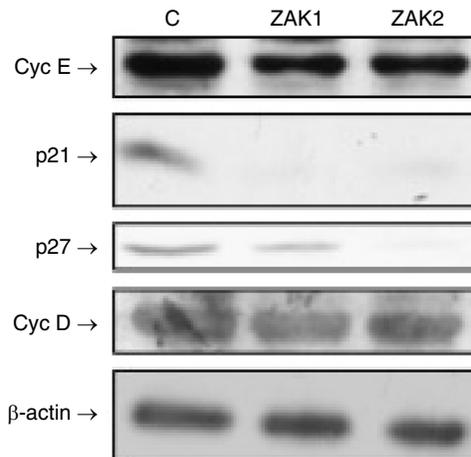


Fig. 3. $ZAK\alpha$ regulates the expression of the cell cycle-regulatory proteins in $ZAK\alpha$ over-expressed KB cells. The proteins analyzed were cyclin D, cyclin E, p21, and p27. Data shown are representatives of three independent experiments.

ZAK α Overexpression Decreases p21 and p27 Levels

Our previous studies reported that $ZAK\alpha$ expression might induce apoptosis and regulate G2 arrest (19, 32). However, data in this study showed that $ZAK\alpha$ induced anchorage-independent growth in cultured KB cells most likely due to the suppression of p21 and p27 levels in $ZAK\alpha$ -overexpressed KB

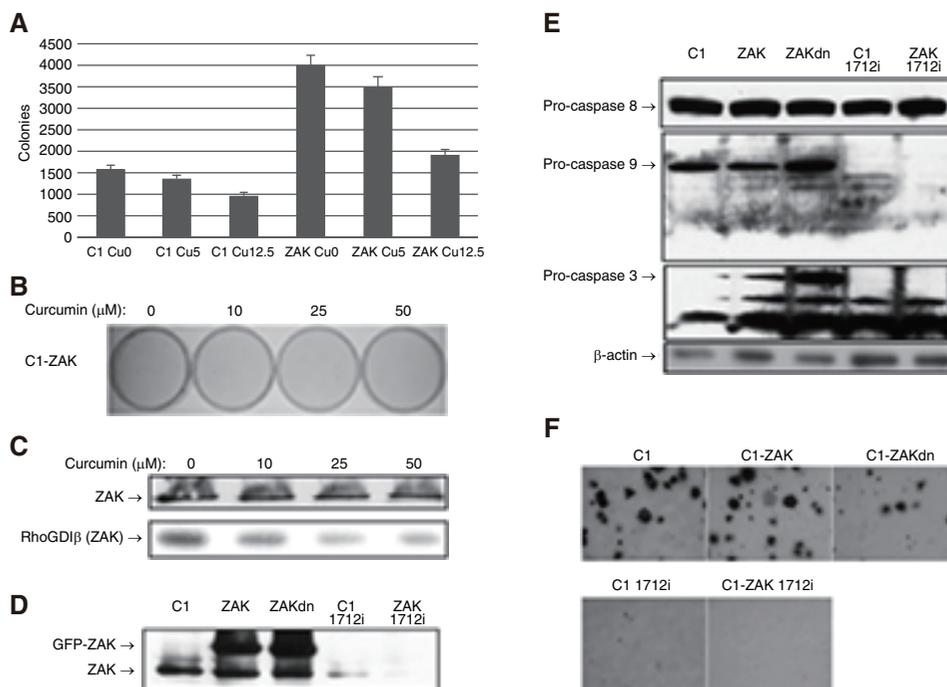


Fig. 4. Curcumin specifically inhibits ZAK α kinase activities. (A) Curcumin showed dose-dependent inhibition of the anchorage-independent growth on ZAK α overexpressed-KB and the control KB cells. The cells were incubated with or without curcumin for 14 days and stained with INT. (B) Curcumin showed dose-dependent inhibition on colony formation by ZAK α over-expressed-KB cells. The cells were incubated without curcumin for 7 days and macroscopically visible colonies were fixed and stained with Giemsa. (C) ZAK α kinase activities were inhibited by curcumin. ZAK α kinase activities were detected by *in vitro* kinase assay using GST-RhoGDI β as a ZAK α 's substrate in the presence of [γ - 32 P] ATP. (D, E) ZAK α regulated the expression of the apoptosis-related proteins pro-caspase 3, 8 and 9 in KB cells. (F) ZAK α was necessary for KB cells to grow in soft agar. The indicated cells were seeded in soft agar for 14 days and stained with INT. Data shown are representatives of three independent experiments.

cells (Fig. 3). Curcumin was shown previously (Fig. 2B) to induce KB cell cycle arrest and apoptosis through upregulation of p21 and p27. Therefore, these data suggest that curcumin-induced growth inhibition is mediated through the ZAK α protein activities. To test this hypothesis, KB cells expressing the control vector pEGFPC1 (C1) or pEGFPC1-ZAK α (C1-ZAK α) were treated with different doses of curcumin to test their responses to curcumin biologically and biochemically. These cells were seeded in soft agar at a density of 2,500 cells/mL with or without different doses of curcumin (5 or 12.5 μ M) and incubated for 2 weeks to determine anchorage-independent property of the cells. The data revealed that ZAK α -expressed cells significantly increased colony formation. Curcumin treatment was able to reduce colony formation either in the control or in ZAK α -expressed cells in a dose-dependent manner (Fig. 4A). Furthermore, curcumin inhibits the growth of ZAK α -expressed KB cells through colony formation assay (Fig. 4B). Therefore, we speculated that curcumin might play as an agent to inhibit ZAK α activities resulting in the inhibition of anchorage-independent growth.

To elucidate how ZAK α participates in curcumin-

induced growth inhibition, the ZAK α protein expression levels after treatment with curcumin were determined. No significant change in the ZAK α protein levels in KB cells was observed after treatment with different doses of curcumin (Fig. 4C), indicating that curcumin treatment has no effect on ZAK α protein levels. In addition, ZAK α kinase activity has been suggested to regulate cell cycle progression (32). Therefore, we investigated ZAK α kinase activities after curcumin treatment. In previous studies, we identified that RhoGDI β was not only a specific ZAK α kinase substrate but also bound to ZAK α (10). Therefore, an *in vitro* kinase assay was conducted using RhoGDI β as a substrate and ZAK α kinase activities were found to be inhibited by curcumin in a dose-dependent manner (Fig. 4C). Therefore, curcumin attenuates ZAK kinase activity without changing ZAK protein levels.

The above data suggest that curcumin induces tumor cell arrest or apoptosis through the inhibition of ZAK α kinase. To further confirm that ZAK α is required for tumor cell cycle progression or survival, we selected tumor cells with ectopic expression of wild-type ZAK α , kinase-dead ZAK α (ZAK α dn), or

short-hairpin RNA (1712i) specific to knock-down either endogenous ZAK α or ectopic expressed ZAK α ; the ZAK α protein expression profiles were shown in Figure 4D. Cell cycle or apoptotic regulatory protein expression profiles in these ZAK α expression variant cells were then determined. Our results indicated that the cleavage of pro-caspase 9 and pro-caspase 3 was inhibited in the presence of ZAK α protein and the ZAK α dn cells had more caspase 9 active forms. However, there was no significant change in pro-caspase 8 levels (Fig. 4E). These results demonstrated that ZAK α was required for the survival of tumor cells and that inhibition of ZAK α triggered the intrinsic apoptotic pathways. These results might suggest that curcumin-treated tumor cells enter the intrinsic apoptotic pathway through the inhibition of ZAK α kinase activities. To determine anchorage-independent growth in ZAK α expressed variants, we found that ZAK α induced colony formation and tumor cells with shRNA knocked-down ZAK α lost their ability to grow in soft agar (Fig. 4F). We suggest that ZAK α is required for tumor cells survive and curcumin exerts an unidentified mechanism to suppress ZAK α kinase activity in tumor cells resulting in cell growth arrest or apoptosis.

Discussion

Numerous studies indicated that curcumin targets cancer cells by regulating various cellular signaling pathways resulting in inhibiting cell proliferation and inducing apoptosis in a variety of tumor cells. Recent studies have indicated that curcumin interferes with the activities of many cellular proteins such as protein kinases or transcription factors NF- κ B or activator protein-1 (AP-1), and the secretion of various inflammatory cytokines (15).

In this study, we found that curcumin inhibited tumor cell growth and induced apoptosis through its ability to suppress ZAK α kinase activities. Tumor cells treated with low doses of curcumin were sufficient to reduce anchorage-independent growth in a dose-dependent manner (Fig. 1A). Cell cycle progression was also inhibited by curcumin in a dose-dependent manner. Curcumin treatment at higher concentrations led to the tumor cells entering apoptotic pathways through the observation of DNA fragmentation, morphologic changes, apoptotic regulating proteins, and the sub-G1 population (Figs. 1, B, C and D, and Fig. 2A). Our results indicated that higher dose of curcumin treatment activated intrinsic apoptotic pathways, as reported by other studies of curcumin treatment in various tumors in activating caspase 9 in prostate (23) and ovarian cancer cells (31).

The cell cycle distribution results revealed that curcumin induced G2/M arrest in KB cells has also

been reported by others in oral carcinoma and non-small cell lung cancer (26, 30). We demonstrated here that curcumin induced p21 and p27 expression in KB and MG63 cells; however, the expression levels of cyclin D and cyclin E remained unchanged (Figs. 2, B and C). Our results are different in some way from other studies in which curcumin is a potent agent to downregulate cyclin E and upregulate CDKI p21 and p27 expression in tumor cells (1). Curcumin was found in this study to upregulate p21 and p27 expression without changing the cyclin D and E expression, suggesting that p21 and p27 play major roles in suppressing cell cycle progression in this system. Cip/Kip family proteins, p21 and p27, participate in cell cycle regulation by binding and inactivating cyclin-CDK complexes (28). The upregulation of p21 is regulated by p53 after DNA damage for maintaining the G2 checkpoint and prolonging G2/M arrest following DNA damage in human cells (3). The p27 protein acts as both a cell cycle regulator and a tumor suppressor, and p27 expression is frequently associated with human cancers (6, 25), suggesting that the upregulation of p21 and p27 is a crucial event in curcumin-induced antiproliferative and apoptotic effects. Hence, curcumin-induced p21 and p27 expression might play a critical role in linking to the cell cycle arrest and apoptosis.

Curcumin activates numerous signal transduction pathways through the interference of MAPKs and NF- κ B signaling cascades, which are thought to regulate apoptosis and cell survival. In a previously study, we demonstrated that ZAK α activated the JNK and NF- κ B pathway (19). We showed here that ZAK α overexpression inhibited the p21 and p27 protein levels in KB cells (Fig. 3); moreover, curcumin could inhibit the growth of ZAK α -overexpressed cells (Figs. 4, A and B) through the inhibition on ZAK α kinase activity. Therefore, we suggest here that curcumin can specifically inhibit ZAK α activities. The decline of ZAK α kinase activity in KB cells was shown to be correlated with increasing doses of curcumin without affecting the total levels of ZAK α protein (Fig. 4C). This finding is similar to other studies, which have shown that curcumin suppresses the activity of several protein kinases linked to tumorigenesis. Curcumin has also been reported to inhibit the JNK pathway (5, 12) and JNK kinase activities are regulated by ZAK α in a kinase-dependent manner (32). Whether curcumin inhibits cancer cell growth through JNK kinase activity will be further examined. Albeit curcumin elicits various biological activities *in vivo*, its undesirable physiochemical properties, such as low water solubility and poor bioavailability, are still problems (2). For applications in *in vivo* studies and clinical trials, many approaches have been undertaken like utilization of nano-base

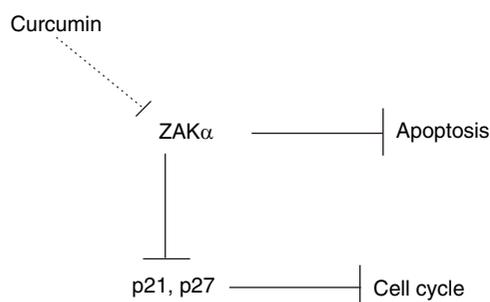


Fig. 5. Schematic showing curcumin induces cell cycle arrest and apoptosis through inhibiting ZAK α activity.

delivery systems and analogs (27).

In this study, ZAK α was required for the anchorage-independent growth in KB cells with kinase-dependent manner, and we found here that curcumin inhibits cancer cells growth through the suppression ZAK α kinase activity (Figs. 4, A and F). Notably, the knock-down of ZAK α proteins by shRNA triggered the apoptotic pathway in KB cells through the activation of caspase 9 and caspase 3. ZAK α kinase plays a critical role to suppress apoptosis or support cell cycle progression in cancer cells. We found here that curcumin specifically suppressed ZAK α kinase activity (Fig. 4C) and resulted in the induction of apoptosis in cancer cells. In summary, curcumin might serve as a ZAK α inhibitor through an unidentified mechanism and it was significant that ZAK α exerts inhibition on the expression of p21 and p27 and the suppression of apoptotic pathway (Fig. 5).

Conflict of Interests

The authors declare that there are no conflicts of interests.

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