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CURCUMIN INDUCES APOPTOSIS THROUGH MITOCHONDRIAL-MEDIATED APOPTOTIC PATHWAY IN RETINOBLASTOMA CELLS

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ABSTRACT

Curcumin (diferuloylmethane), the main yellow bioactive component of turmeric has been shown to have wide variety of biological actions like anti-inflammatory, anticancer and antioxidant properties. Its anticancer effect is mainly mediated through induction of apoptosis. In the present study, we have investigated the effects of curcumin on growth and apoptosis in the human retinoblastoma (RB) cell line by MTT assay, fluorescence microscopy, flow cytometry and western blotting. Exposure of human RB cells to curcumin inhibited the growth of cell proliferation and resulted in the induction of apoptotic cell death. We also found loss in the mitochondrial membrane potential on curcumin treated cells in a time dependent manner. A decrease in the expression of anti-apoptotic protein and an increase in pro-apoptotic protein were observed after exposure to curcumin. Curcumin also induced the release of cytochrome c and the activation of caspase 3 and 9 was observed in a dose-dependent manner. Thus, these results suggest that the induction of apoptosis by curcumin involves mitochondrial-mediated cell death pathway in human RB cells.

KEYWORDS: Curcumin, RB cells, Apoptosis, Mitochondria, Caspase

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INTRODUCTION

Retinoblastoma (RB) is the most common intraocular malignancy of childhood, affecting 1 in 15,000 children[1]. The disease is caused by loss of function of both alleles of the RB tumor suppressor gene (RB1) in retinal progenitor cells[2]. Chemotherapy is the treatment of choice following enucleation in patients with optic nerve and choroid invasion and orbital extension[3]. It has been reported that chemotherapy drugs are not well tolerated due to toxicity and undesirable side effects to normal tissue[4]. Therefore, the development of novel chemotherapeutic agent is needed to decrease the toxicity and improve its efficacy in treating cancer. Many medicinal compounds purified from plants, have been identified that can inhibit, retard, or reverse the process of multistage carcinogenesis in various cancers[5]. Curcumin (diferuloylmethane), is the major yellow pigment in turmeric which is derived from the herb *Curcuma longa*[6]. Curcumin has antibacterial, antiviral, anti-cancer, antioxidant activity and anti-inflammatory properties[7]. Furthermore, various studies have been reported that curcumin is known to induce apoptosis in numerous human cancer cell lines established from malignancies like breast, lung, pancreas, bladder, prostate, ovary and skin[8-12]. Curcumin induced apoptosis in human breast carcinoma and hepatoma cell line but failed to do so in normal rat hepatocyte primary cultures[13]. Induction of apoptosis by curcumin involves various mechanisms which include up-regulation of Bax[14], activation of caspases[15], generation of free radicals[16] and impairment of the ubiquitin proteasome pathway[17]. Curcumin also suppresses the activation of nuclear factor kappa B (NF-kB), signal transducer and activator of transcription 3 (STAT3) and the AKT pathway. It has been also shown to down-regulate the expression of various nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) regulated genes, including B-cell lymphoma 2 (Bcl2), cyclooxygenase (COX2), matrix metallopeptidase (MMP-9), tumor necrosis factor (TNF), cyclinD1, and adhesion molecules[18]. In our laboratory, it has been demonstrated that curcumin modulated the expression of drug resistance protein (lung resistance-related protein and multi-drug resistance associated protein 1) and it has also been found to possess anti-proliferative effect in Y79 RB cells[19, 20]. Many of the anti-cancer agents are effective in inducing apoptosis or cell cycle arrest. The effect of curcumin on human RB cells remains unclear. In this study, we investigated the mechanism underlying the induction of apoptosis in human RB cells.

MATERIALS AND METHODS

Curcumin (Sigma) and cell culture materials were purchased from Invitrogen (Carlsbad, CA, USA). Anti-Bax, anti-Bcl-2, anti-Bak and anti-cytochrome c antibodies were from Santa Cruz Biotechnology, Inc. (CA, USA). Other chemicals are from analytical grade.

(i) Cell culture

RB cell lines (Y79 and Weri) were purchased from the Cell Bank (Riken BioResource Center, Ibaraki, Japan). Cells were cultured in RPMI-1640, supplemented with 20% fetal bovine serum and antibiotics in a humidified atmosphere of 95% air/5% CO_2. For individual experiments, the cells were plated down in multi-well plates followed by treatment with various concentrations of curcumin up to 48h.

(ii) Cytotoxicity assay by MTT

RB cells were seeded into 96-well plate at density of $5x10^3$cells/well and incubated overnight. Next day medium containing varying concentration of curcumin from 5-100µM (vehicle control 0.5% DMSO) was added and cells were incubated for 24-72hrs, after which the cell viability was measured using MTT. Thereafter, 10µl MTT (5mg/ml in PBS) was added and incubated for 4hrs. Cells that are viable form formazan crystals by active mitochondria respiration. Crystals were dissolved with 100µl DMSO, after which the

reading was taken spectrophotometrically at 570nm using an ELISA reader. Percentage cell viability was calculated as test absorbance/ control absorbance x 100.

(iii) Examination of apoptosis by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining

RB cells were plated in a 12-well plate at a density of 1x 10^4cells/well. After 24h, cells were treated with or without various concentrations of curcumin (0- 50 µM) for 48h. The cells were collected, washed with ice-cold phosphate buffer saline (PBS) and fixed with methanol: acetic acid (3:1) for 30 min at RT. Cells were stained with DAPI (1µg/ml) for 20 minutes at RT and observed under Nikon TS100 phase-contrast fluorescent microscope mounted with Retiga Exi monochrome cooled CCD camera.

(iv) Annexin V fluos staining by flow cytometry

RB cells (1x10^5cells/cm^2) were plated in 12-well plate overnight at 37^0c. After overnight incubation, the cells were treated with different concentrations of curcumin (0- 50µM) and incubated for 48 h. AnnexinV-fluos staining was performed using an AnnexinV-fluos apoptosis detection kit (Roche, Indianapolis) in accordance with the manufacturer's instructions. In brief, treated cells were centrifuged, re-suspended in 100µl of Annexin-V-Fluos reagent and incubated for 10-15min at RT in the dark. After the incubation period, flow cytometry analysis was immediately performed. Data acquisition and analysis were performed by a FACScalibur flow cytometer using Cell Quest software. Cells that were Annexin V (-) and PI (-) were considered viable cells whereas cells that were Annexin V (+) and PI (-) or Annexin V (+) and PI (+) were considered early stage or late-stage apoptotic cells, respectively.

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(v) Mitochondria membrane potential assay

RB (1x10^5) cells were incubated with 2µM rhodamine 123 for 10 minutes at 37^0C. After 2h, the cells were incubated with 50µM curcumin for different time intervals (30min, 60min, 2h and 4h). After incubation period, the cells were washed twice with PBS and finally re-suspended in 1ml PBS. The fluorescence intensity of the control and curcumin treated RB cells were measured at an excitation wavelength 480nm and an emission wavelength 530nm in a fluorescence spectrophotometer. The fluorescence intensity was used as an arbitrary unit representing the mitochondria transmembrane potential[21].

(vi) Effect of curcumin on apoptosis related proteins by semi-quantitative RT-PCR

RB cells (1x10^5cells/cm^2) were plated in a 12-well plate and after overnight incubation at 37^0C; the cells were treated with different concentrations of curcumin for 48h. Total RNA from each sample was extracted using Trizol reagent (Invitrogen, USA), following the manufacturer's instructions. Turbo DNase treatment was done to remove endogenous DNA contamination. For single-strand cDNA synthesis, 2µg of total RNA from each sample was reverse transcribed (sensiscript, Qiagen) at 37^oC for 1h using sensiscript reverse transcriptase. PCR amplification was carried out in an Eppendorf PCR system using the gene specific primers, Bax, Bak and Bcl-2: 95^oC for 45sec, 69^oC for 1min and 72^oC for 1 min and GAPDH: 94^0C-5 min, 94^0C-45sec, 63^0C-45sec, 72^0C-45sec and final elongation 72^0C-2min. Numbers of cycles were 35 for all the reactions and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used for normalization of the results (Table I). PCR products were fractionated by electrophoresis using 2% agarose gel containing 0.5% Ethidium bromide.

Table I
Primers used for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Gene	Primer sequence	PCR Product size
Bcl-2	FP 5'-AGATGTCCAGCCAGCTGCACCTGAC-3' RP 5'-AGATAGGCACCCAGGGTGATGCAAGCT-3'	366bp
Bax	FP 5'-AAGCTGAGCGAGTGTCTCAAGCGC-3' RP 5'-TCCCGCCACAAAGATGGTCACG-3'	365bp
Bak	FP 5'-TCCAGATGCCGGGAATGCACTGACG-3' RP 5'- TGGTGGGAATGGGCTCTCACAAGG-3'	1191bp
GAPDH	FP 5'-GCCAAGGTCATCCATGACAAC-3' RP 5'-GTCCACCACCCTGTTGCTGTA-3'	498bp

(vii) Effect of curcumin on apoptosis related proteins by western blot

RB cells ($1x10^5$cells/cm^2) were plated in 12-well plate and after overnight incubation at 37^0C the cells were treated with 0- 50µM concentration of curcumin for 48h. The cells were collected and lysated with 100µl RIPA buffer and sonicated at 50-60 pixels/30sec. After sonication, the protein concentration in the lysate was measured by the Lowry method[22]. The lysates (50µg) were run on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (BioRad, Hercules, CA) and then electrophoretically transferred onto the nitrocellulose membranes (Amersham) at 100V for 1hr at 4^0C. Transfer of proteins was checked by staining the membrane with 0.1% Ponceau stain (Sigma) and non-specific sites were blocked with 5% non-fat dry milk for 1hr. The blots were incubated with primary antibody solution (Bak, Bcl-2 and Bax) diluted at 1:1000 for overnight. After washing with TTBS the blots were incubated with the respective HRP-conjugated secondary antibody (Pierce, Italy) diluted 1:2000 for 2hrs at RT. After further washing with TTBS the immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) kit (Amersham Life Sciences). To normalize band intensity, the membranes were probed with β-actin antibodies (Sigma Aldrich, USA).

(viii) Measurement of cytochrome c release

Cytosolic extracts of the cells were prepared as described[23]. RB cells were collected after treatment with curcumin (0-50µM) for 48h and mixed with 100µl of lysis buffer, followed by centrifugation at 12,000rpm for 30 minutes at 4^0C. 100µg of protein was electrophoresed on a 12% SDS-PAGE and transferred onto nitrocellulose membrane. Cytochrome c was detected using polyclonal anti-cytochrome c antibody (1:300 dilutions for overnight). After washing, anti-rabbit horse radish peroxidase (HRP) conjugate antibody was added and incubated for 2hr. Protein bands were visualized using an ECL kit.

(ix) Measurement of caspase 3 and 9 activity

RB cells ($1x10^6$ cells/cm^2) were plated in 12 well plates and treated with different concentrations of curcumin for 48h. Cells were washed with ice-cold PBS and resuspended in a buffer containing 5mmol/L Tris (pH 8), 20mmol/L ethylenediamine tetraacetate (EDTA) and 0.5% Triton-X 100 on ice for 30 minutes. After incubation, lysates were centrifuged for 5 minutes at 13,000rpm and the clear supernatant was taken for caspase activity. Reactions were carried out with 50µg of protein, 20mmol/L4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7), 10% glycerol, 2mmol/L dithiothreitol and 200 µmol/L *N*-acetyl- Asp-Glu-Val-Asp (DEVD)-*p*NA substrate (caspase 3) and Leu-Glu-His-Asp (LEHD)-pNA substrate (caspase 9), in the presence and absence of caspase 3(Ac-DEVD-CHO) and 9(Ac-LEHD-CHO) inhibitor, respectively. Reaction mixtures were placed into a flat-bottomed mitrotiter plate and read with a 405 nm filter using a microtiter plate reader. Caspase activities were detected

by measuring the proteolytic cleavage of the colored substrates.

(x) Statistics

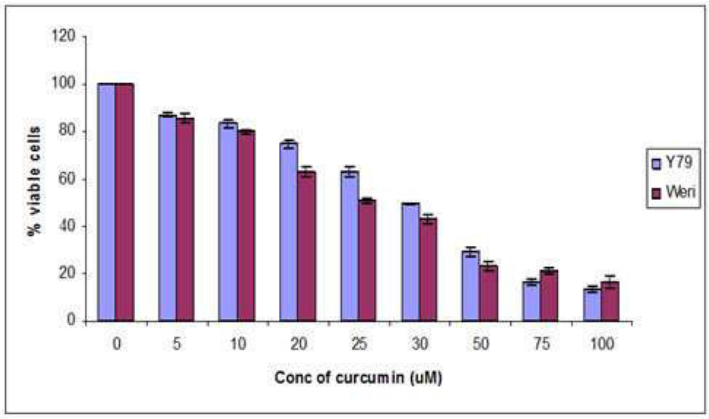
Results are expressed as mean ± standard deviation. Data were analyzed by a one-way ANOVA (SPSS 11.0) and $p<0.05$ were considered to be statistically significant.

RESULTS

1. Cytotoxic effect of curcumin on RB cells

The toxic effect of curcumin on RB cells after 48h was assessed by MTT assay. The IC_{50} of curcumin was 30µM, which cause 50%, cell death in Y79 cells and 25µM in Weri cells. (Fig1**)**.

Figure 1
MTT assay

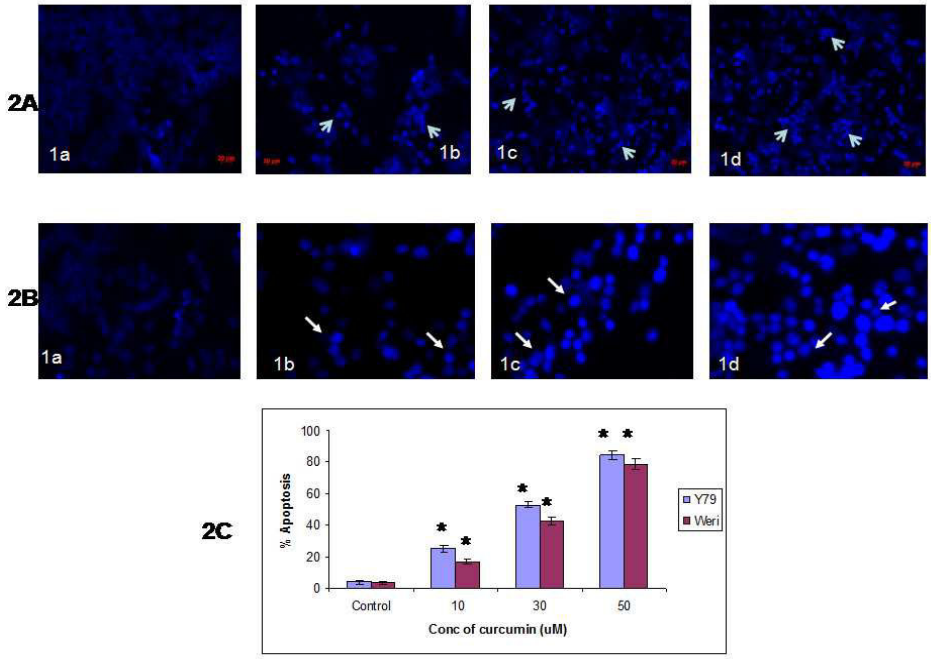


Effect of curcumin on cell viability: RB cells (Weri and Y79) were plated on 96 well plates; cells were treated with different concentrations of curcumin for 48h. The cell viability was then determined by MTT assay. Experiments were done in triplicates. Values represent mean (± SD) cell viability as a percentage of untreated control samples.

2. Curcumin-induced apoptosis examined by DAPI staining

Apoptosis was detected by the DAPI staining method after 48h of exposure to curcumin followed by fluorescence microscopy. As shown in (Fig 2A & B)**,** curcumin induced apoptosis in RB cells compared with control cells. The percentage of apoptotic cells were increased with increase concentration of curcumin in RB cells (Fig 2C).

Figure 2
DAPI staining in RB cell lines

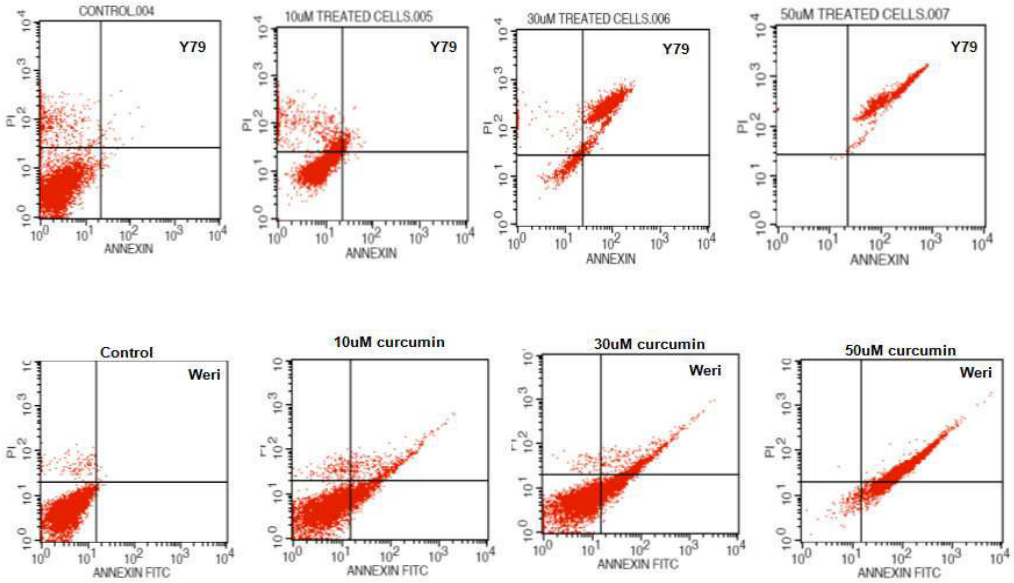


Effect of curcumin on cell nuclear morphology: A) Y79 B) Weri cells were treated with different concentration of curcumin for 48h and untreated cells served as control (1a-control, 1b-10µM curcumin, 1c-30µM curcumin and 1d-50µM curcumin). Arrows indicate the apoptotic cells with condensed and fragmented nuclei. Nuclear staining was examined under a fluorescence microscopy. C) Cells were counted in 5 different fields and the mean values of triplicate samples expressed in percentage are shown.

3. Effect of curcumin treatment on RB cells apoptosis

The effect of a 48h curcumin treatment on RB cells apoptosis was obtained by Annexin V-fluos/PI staining and flow cytometry analysis (Fig3**)**. The number of cells corresponding to the early or late apoptosis was increased at 30 and 50µM concentration of curcumin but was not observed at 10µM concentration. Further, quantitation of apoptotic cells demonstrated that 30 and 50µM curcumin resulted in significantly increased number of apoptotic cells in both Y79 and Weri cells (Table II).

Figure 3
Annexin V staining in RB cell lines



Annexin-V-fluos staining and effect of curcumin on the loss of mitochondrial membrane potential in RB cells. The cells falling in the right upper square in each scatter diagram are considered as apoptotic cells.

Table II
Percent of quadrant analysis of Annexin V Fluos/Propidium iodide flow cytometry of RB cells treated with different concentration of curcumin

Quadrant (%Total) A- Y79 cells	Control	10µM	30µM	50µM
UL	15.45	17.54	3.04	0.20
UR	0.31	2.83	88.64	99.7
LL	83.98	79.24	8.26	0.1
LR	0.26	0.39	0.07	0.0

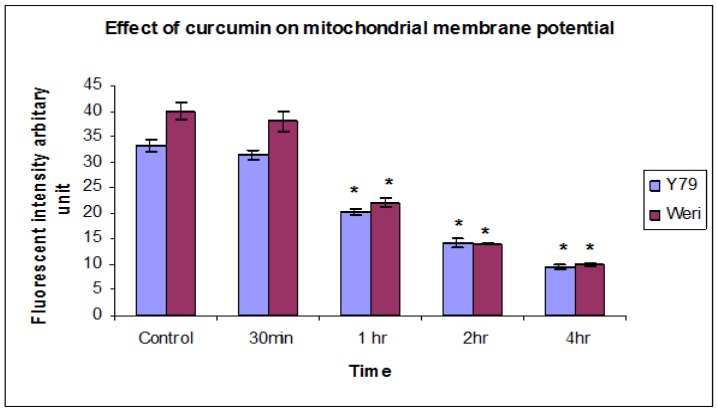
Quadrant (%Total) B-Weri cells	Control	10µM	30µM	50µM
UL	1.86	2.47	1.11	0.02
UR	0.15	8.35	12.95	82.10
LL	97.59	72	58.51	2.64
LR	0.39	16.84	27.44	15.25

Results are expressed as mean ± standard deviation. Experiments were done in triplicates (n=3).

4. Effect of curcumin on loss of mitochondrial membrane potential ($\Delta\Psi_m$) of RB cells

In order to understand the mechanism of curcumin-induced apoptosis in RB cells, we used Rhodamine 123 to acquire the $\Delta\Psi_m$ by examining its fluorescent intensity. As shown in Fig 4 there was a time dependent decrease of Rhodamine 123 fluorescence after treated with curcumin, compared with the control cells. This indicates that curcumin was able to induce $\Delta\Psi_m$ disruption in RB cells.

Figure 4
Mitochondrial membrane potential assay in RB cell lines

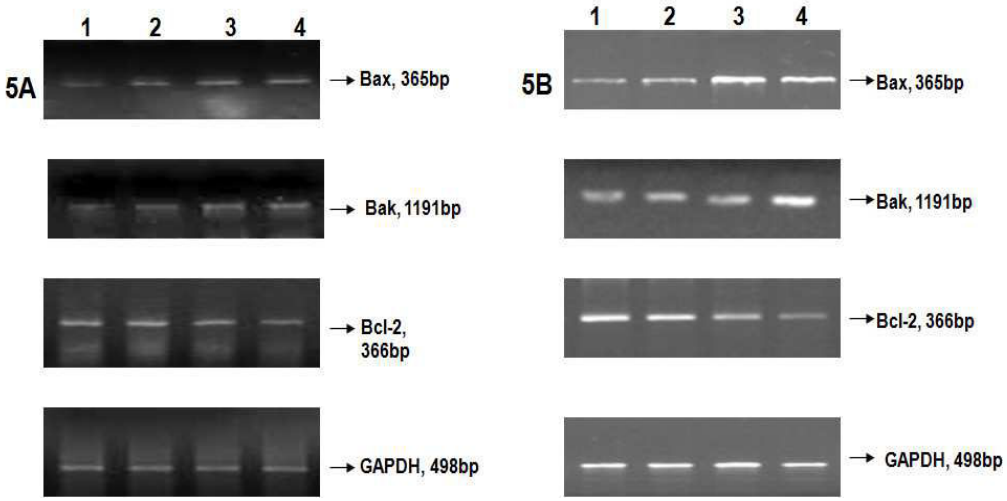


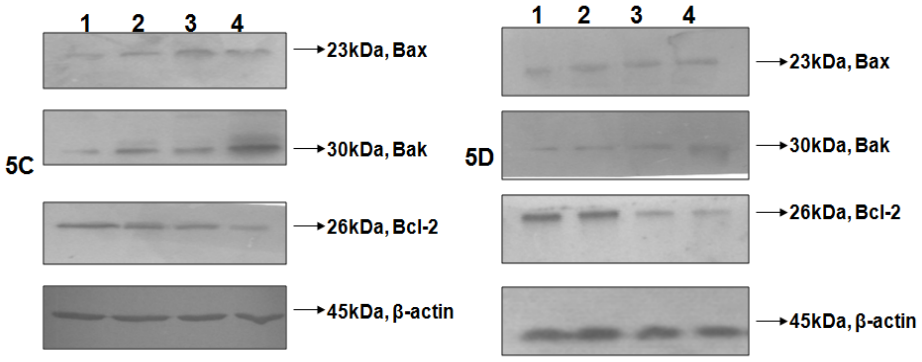
Effect of curcumin on the loss of mitochondrial membrane potential in RB cells: RB cells were treated with and without curcumin at different time intervals, and then incubated with 2µM Rhodamine 123. Experiments were done in triplicates. Asterisks represent significant difference between the controls and the curcumin treated RB cells (*p<0.05, **p<0.01).

5. Effect of curcumin treatment on Bax, Bak and Bcl-2 mRNA and protein expression in RB cells

To investigate the cellular mechanism underlying curcumin-induced apoptosis in RB cells, we analyzed the expression of Bcl-2, Bax and Bak mRNA expression following 48h exposure to various concentrations of curcumin by qRT-PCR. Pro-apoptotic gene Bax and Bak mRNA expression significantly increased whereas Bcl-2 mRNA expression significantly decreased after curcumin exposure (Fig 5A, B). The result of western blotting also showed similar results where Bax and Bak protein expression was increased and Bcl-2 level was decreased in response to curcumin treatment (Fig 5C, D). The Bax/Bcl-2 and Bak/Bcl-2 ratio was significantly increased in cells exposed to 10, 30 and 50µM curcumin (p<0.05).

Figure 5
Effect of curcumin on apoptosis related proteins in RB cell line



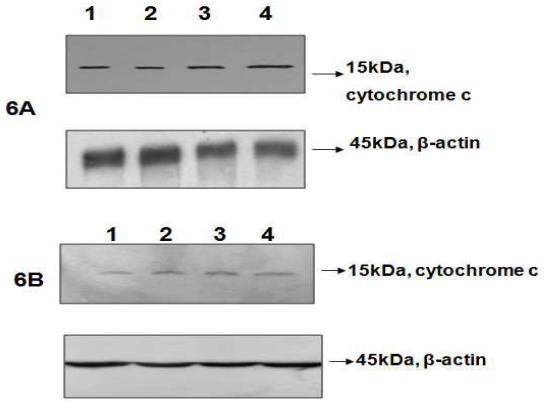


Effect of curcumin treatment on mRNA and protein expression of Bax, Bak and Bcl-2 in RB cells: A&C) Y79 and B&D) Weri cells were treated with different concentration of curcumin for 48h. After curcumin treatment cells were harvested for RNA isolation and protein extraction. RT-PCR was performed as described in methods. A&B) PCR products were resolved on a 2% agarose gel and visualized using ethidium bromide. GAPDH served as an internal control. C&D) Cell lysates were subjected to immunoblot analysis. Equal amounts of total protein (100µg) were resolved on 12% SDS-PAGE and β-actin was used as an internal control.

6. Effect of curcumin on cytochrome c release

The release of cytochrome c from mitochondria is the key event in apoptosis induced by various stimuli. In order to analyze the involvement of mitochondria in the apoptosis induced by curcumin, the cytosolic level of cytochrome c was studied. The release of cytochrome c was significantly increased in RB cells incubated with 10-50µM concentration of curcumin (Fig 6A&B).

Figure 6
Effect of curcumin on cytochrome c expression in RB cell lines

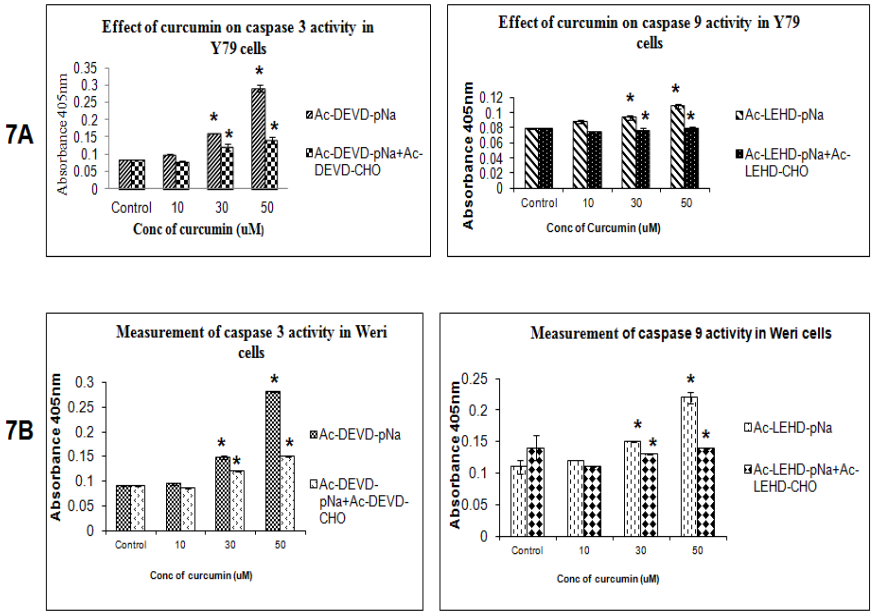


Effect of curcumin on release of cytochrome c: A) Y79 B) Weri cells were treated with indicated concentration of curcumin for 48h. The cytochrome c in cytosolic fraction was assayed by western blot. Lane 1: Control, Lane 2: 10µM curcumin, Lane 3: 30µM curcumin and Lane 4: 50µM curcumin. Data are presented as the means of triplicate experiments.

7. Effect of curcumin on caspase 3 and 9 activities

To examine whether caspase 3 and 9 activity is involved in curcumin induced apoptosis in RB cells. In cells treated with different concentrations of curcumin (10, 30, 50 µM) for 48h, the activity of caspase 3 and 9 was significantly increased, moreover the activity was decreased in the presence of caspase-3 (Ac-DEVD-CHO) and caspase 9 (Ac-LEHD-CHO) inhibitor in RB cells. These results suggest that caspase 3 and 9 is involved in curcumin-induced apoptosis in RB cells (Fig 7A&B).

Figure 7
Caspase 3 &9 activity in RB cell lines



Effect of curcumin on caspase 3 and 9 activity in RB cells: A) Y79 and B) Weri cells were treated with different concentration of curcumin (10, 30 and 50µM) and caspase activity was determined by incubation of 50µg protein with DEVD-pNA substrate (caspase3) and LEHD-pNa substrate (caspase9) and with its respective inhibitor. Data are mean ±SD from three separate experiments. *p<0.05 was considered as statistically significant.

DISCUSSION

Many studies have demonstrated that curcumin, a well-studied cytotoxic compound induces apoptosis in various human cancer cells and that these pathways depend on mitochondrial or receptor mediated activation[24-26]. The apoptotic effect of curcumin is dependent on the cell type. In this study, we investigated the molecular mechanisms where curcumin induces apoptosis in human RB cells. Curcumin-induced apoptosis was observed by the changes in nuclear morphology by DAPI staining in RB cells. The two major signaling pathways for apoptosis are the mitochondria and death receptor pathways[27]. Many studies have shown that the loss of $\Delta\Psi_m$ is a hallmark for apoptosis [28]. Depolarization of the $\Delta\Psi_m$ is observed in some anticancer compounds thereby inducing apoptosis in cancer cell lines[29]. Our data demonstrated reduced mitochondrial membrane potential ($\Delta\Psi_m$) as indicated by Rhodamine 123 (fluorescent dye) following treatment with curcumin at different concentrations. Recent studies suggested that drop in the $\Delta\Psi_m$ is observed in curcumin treated cancer cell lines[30,31]. Our data showed that RB cells treated with curcumin decreased the mitochondrial membrane permeability, which is in agreement with the previous studies. This indicates that

curcumin-induced apoptosis in RB cells is related to the collapse of the $\Delta\Psi_m$. The mitochondrial release of cytochrome c plays an important role in the induction of apoptosis. It is located in the space between the inner and outer mitochondrial membranes. During apoptosis, cytochrome c is released from the mitochondria into cytosol where it binds with apoptotic protease activating factor -1(Apaf-1). The cytochrome c/Apaf-1 complex activates caspase-9 which in turn activates caspase-3 thereby leading to cell death. Curcumin induced cytochrome c release has been observed in various cancer cell lines[32,33,34]. In this study, we also observed that curcumin markedly increased the release of cytochrome c from the mitochondria to cytosol in human RB cells. The Bcl-2 family of proteins has been already identified as regulators of apoptosis. The apoptotic-related activities of Bcl-2 family members are to control mitochondrial integrity. The balance between Bcl-2/Bax and Bcl-X_L/Bax are usually regarded as a criterion in programmed cell death[35]. Mingxin Shi et al. showed that levels of the anti-apoptotic factors Bcl-2 and Bcl-XL, were both decreased after curcumin treatment, while increased levels of the pro-apoptotic factor Bax, were observed in Ho-8910 cells[25]. Our western blot data indicated that curcumin increased the expression of Bax, Bak and decreased the level of Bcl-2 leading to decrease of the $\Delta\Psi_m$. Thus, our data indicates that curcumin-induced apoptosis of RB cells occurs through the mitochondrial pathway. Many anti-cancer agents have been shown to cause apoptotic cell death through caspase dependent pathway. Caspases are a family of cysteine proteases that have been shown to be activated during apoptosis in many cell systems. They play critical roles in both the initiation and the execution of apoptosis[36]. Caspase can be activated by different stimuli such as mitochondrial pathway involving caspase 9 and receptor pathway involving caspase 9[37]. In the present study, we found that curcumin-induced cell death was accompanied by an increase in the activity of caspase 3 and 9 in human RB cells. Thus, curcumin-induced cell death involves the activation of caspase cascade, which then stimulated the molecular cascade of apoptosis (Fig 8).

Mitochondrial mediated pathway in RB cell lines

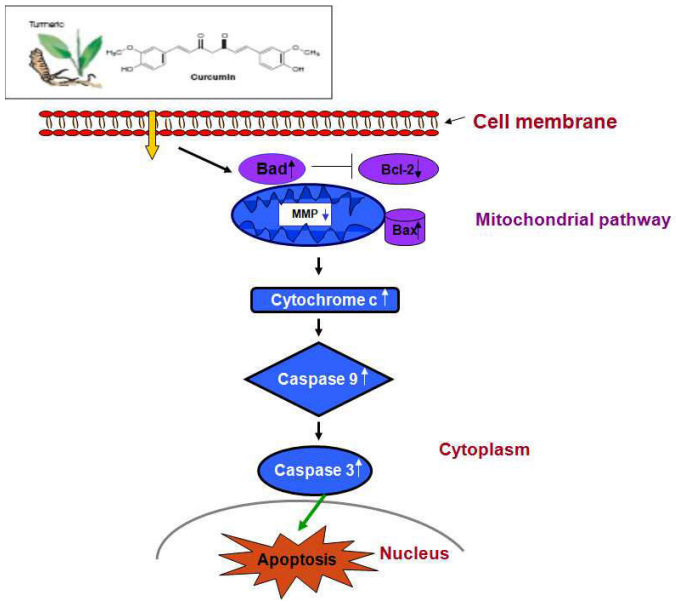


Figure 8
Schematic representation showing induction of apoptosis mediated by curcumin through mitochondrial pathway in human RB cells.

CONCLUSION

Thus, our study shows that curcumin induces apoptosis in human RB cells. Induction of apoptosis by curcumin involves mitochondrial-mediated cell death apoptotic pathway. Curcumin regulates the mitochondrial membrane permeability by down-regulating anti-apoptotic and up-regulating pro-apoptotic proteins, triggering the cytochrome c release into cytosol and thereby activating the caspase 3 apoptotic pathway. Thus curcumin may be used as a potential cancer chemopreventive agent in the treatment of retinoblastoma cancer.

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