

Curcumin induces apoptosis through FAS and FADD, in caspase-3-dependent and -independent pathways in the N18 mouse-rat hybrid retina ganglion cells

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Abstract. Curcumin, a naturally occurring yellow pigment isolated from turmeric, is a well known antioxidant with broad spectrum of anti-tumor activities against many human cancer cells. In this study, curcumin-induced cytotoxic effect of mouse-rat hybrid retina ganglion cells (N18) were investigated. For determining cell viability, the trypan blue exclusion and flow cytometric analysis were used. The curcumin-caused cell cycle arrest in N18 cells was examined by flow cytometry. Curcumin affect on the production of reactive oxygen species and Ca²⁺ and on the decrease of the level of mitochondria membrane potential ($\Delta\Psi_m$) were also examined by flow cytometry. Curcumin-induced apoptosis was determined by DAPI staining and Western blotting was used for examining the apoptotic signaling proteins. Cell cycle analysis showed that G2/M phase arrest and sub-G1 occurs in N18 cells following 48 h exposure to curcumin. Curcumin also caused a marked increase in apoptosis, as characterized by DNA fragmentation (sub-G1 phase formation) and DAPI staining, and dysfunction of mitochondria, which was associated with the activation of caspase-8, -9 and -3. Curcumin also promoted the levels of Fas and FADD, Bax, cytochrome c release, but decreased the levels of Bcl-2 causing changes of $\Delta\Psi_m$. Curcumin also induced endoplasmic reticulum stress in N18 cells which was

based on the changes of GADD153 and GRP78 and caused Ca²⁺ release. Curcumin induced apoptosis through the intrinsic pathway and caspase-3-dependent and -independent pathways in N18 cells.

Introduction

Apoptosis is a highly regulated mechanism in which the cells undergo programmed cell death (1) and the two main apoptosis pathways are the extrinsic and the intrinsic pathway (2). The extrinsic pathway is initiated through the interaction of ligands and surface receptors (3) including CD95/Fas/Apo1, tumor necrosis factor receptor 1 (TNFR1), TNF receptor 2 (TNFR2) and death receptors 3-6 (DR3-6) (1), and they deliver a death signal from the extracellular microenvironment to the cytoplasm before leading to apoptosis. The intrinsic pathway is initiated through stimuli such as DNA damage, hypoxia, cell detachment, cellular distress and cytotoxic drugs, which act inside the cell (1). Proteins associated with apoptosis can be divided into the pro-apoptotic such as Bax and Bad anti-apoptotic such as Bcl-2 and Bcl-xl (4,5) and if one of the pro-apoptotic and anti-apoptotic proteins is overexpressed, it will lead to the dysfunction of mitochondria of pro- and anti-apoptotic proteins leading to cytochrome c release and causing the activation of caspase-3 that leads to apoptosis (6).

Curcumin (diferuloylmethane) is an antioxidant and anti-inflammatory substance (7). It was reported that the dietary treatment of 0.5-2% curcumin in mice inhibited the incidence and size of colonic, small intestinal and gastric cancers induced by azoxymethane (8,9). Curcumin inhibited proliferation and caused cell cycle arrest in colon adenocarcinoma cell lines by a prostaglandin-independent pathway (10). Curcumin inhibited the expression of oncogenes *c-jun*, *c-fos*, and *c-myc* in animals and cell lines (11,12) and the

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activity of the AP-1 transcription factor in cells stimulated to proliferate (13). Curcumin was found to be cytotoxic in rat hepatocytes (14) and to stimulate oxidative damage caused by quercetin in rat hepatocytes (15). Investigators also pointed out that curcumin inhibited proteasomal activity (16), caused the accumulation of cytosolic Ca^{2+} (17,18) and disrupt protein disulfide bond formation (19). Curcumin also induced apoptosis in some cells and may be associated with endoplasmic reticulum stress (19,20). However, there is no available information to address the effects of curcumin on mouse-rat hybrid retina ganglion N18 cells. Therefore, in this study, we investigated the induction of cell cycle arrest and apoptosis and the role of reactive oxygen species in mouse-rat hybrid retina ganglion N18 cells and also show the possible signaling pathways.

Materials and methods

Cell culture. Mouse-Rat Hybrid retina ganglion cell line (N18: derived from retina ganglion cells hybrid with lymphoma cells) was purchased from Japanese Collection of Research Bioresources Bank. Cells are cultured in DMEM medium supplemented with 10% FBS, 1% penicillin-streptomycin (100 U/ml penicillin and 100 ng/ml streptomycin) and 1% L-glutamine. All cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells were cultured for several generations, and have been checked for viability as described previously (21).

Trypan blue exclusion assay. N18 cells were trypsinized, isolated and collected by centrifugation. An aliquot of collected cells was mixed with trypan blue dye (Sigma) to final concentration of 0.04% w/v, loaded onto a hemocytometer, and counted under a microscope (21,22).

Viability determination. Cells were analyzed for percentage of viable cells by flow cytometer. Cells at a density of 2×10^5 cells/well were plated onto 12-well plates and incubated at 37°C for 24 h. Different concentrations of curcumin (0, 10, 30, 50, 75 and 100 μM) were added and cells incubated for 24, 48 and 72 h, and the floated cells were collected and adherent cells were washed once with PBS, trypsinized, and collected by centrifugation at 1000 x g for 5 min. The cells (1×10^5 cells per sample) were centrifuged at 1000 x g for 5 min, cell pellets were dissolved with 0.5 ml of PBS containing 100 $\mu\text{g}/\text{ml}$ RNase and 5 $\mu\text{g}/\text{ml}$ propidium iodide and viable cells were determined by using a flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA) (21,22).

Cell cycle analysis by flow cytometry. Cells were analyzed for their cell cycle and sub-G1 (apoptosis) distribution by flow cytometer. Cells at a density of 2×10^5 cells/well were plated onto 12-well plates and incubated at 37°C for 24 h. Different concentrations of curcumin (0, 5, 10, 20, 30 and 50 μM) were added and cells incubated for 48 h, while the adherent cells were washed once with PBS, trypsinized, and collected by centrifugation at 1000 x g for 5 min. The cells (1×10^5 cells per sample) were fixed in 2 ml of cold 70% ethanol at -20°C overnight before cells were centrifuged at 1000 x g for 5 min, and the cell pellets were incubated with

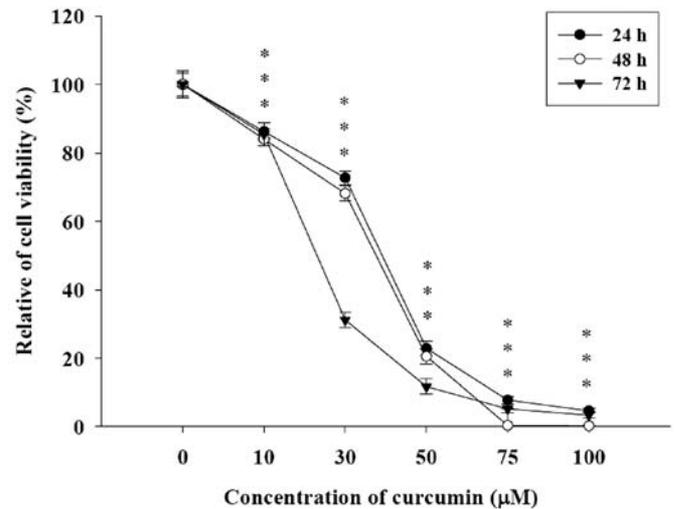


Figure 1. Curcumin effects on cell viability in N18 cells. Cells were cultured with various concentrations of curcumin for 24, 48 and 72 h and were collected by centrifugation. Then for percentage of total viable cells, they were counted by PI-incorporation and flow cytometric analysis as described in Materials and methods. Each point is mean \pm SD of three experiments; *** $P < 0.001$.

0.5 ml of PBS containing 100 $\mu\text{g}/\text{ml}$ RNase and 5 $\mu\text{g}/\text{ml}$ propidium iodide at 37°C for 30 min. Cell cycle and sub-G1 distributions were analyzed by measuring DNA content using a flow cytometer (21,22).

Detection of reactive oxygen species, Ca^{2+} levels and mitochondrial membrane potential. Cells at a density of 2×10^5 cells/well were plated onto 12-well plates and treated with 30 μM of curcumin for 0, 3, 6, 12, 24, 48 and 72 h. Cells were harvested, washed twice with PBS, and re-suspended in the 500 μl of ROS indicator 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA), 500 μl of calcium probe Indo 1/AM and 500 μl of the mitochondrial membrane potential indicator 3, 3'-dihexyloxycarbocyanine iodide (DiOC_6) and incubated at 37°C for 30 min then to detect changes in ROS, Ca^{2+} levels and mitochondrial membrane potential by using flow cytometry as previously described (23).

Western blotting. Cells at a density of 2×10^6 cells/well were plated onto 6-well plates and incubated at 37°C for 24 h. Curcumin (30 μM) was added and cells incubated for 0, 6, 12, 24 and 48 h. The cells were lysed and protein concentration was determined using the BCA assay. Protein samples were separated by SDS-PAGE (12%) and electro-transferred onto PVDF membrane. The membrane was incubated with specific primary antibodies. Mouse anti-human Bax, Bcl-2, cytochrome c, Apaf-1, AIF, GADD153, GRP78, Fas, FADD, caspase-8, -9 and -3 antibodies were from Santa Cruz Bio-technology (Santa Cruz, CA, USA) and β -actin was from Sigma Chemical Co. (St. Louis, MO, USA). Then cells were stained for secondary antibody. The protein levels were normalized by β -actin (22-23).

Statistical analysis. Student's t-test was used to analyze all experimental data, with $P < 0.05$ considered as the level of significance between control and experimental groups.

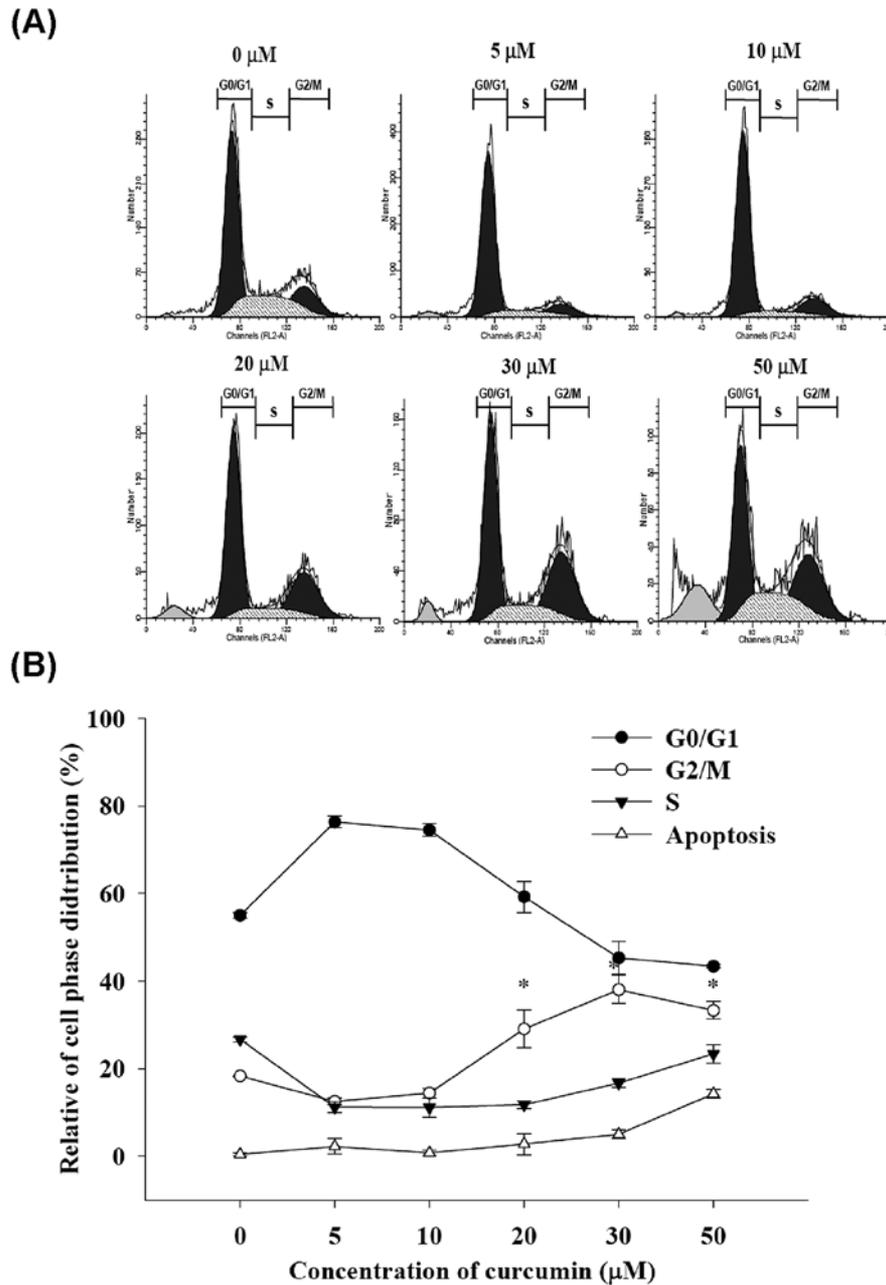


Figure 2. Curcumin effects on the cell cycle arrest and sub-G1 (apoptosis) of N18 cells, and the representative profiles (A) and percentage of each phase and apoptosis (B) on SCC-4 cells. Cells were treated with various concentrations of curcumin for 48 h and the cells were harvested and analyzed for cell cycle and the sub-G1 group (A) and phase distribution (B) by flow cytometry, as described in Materials and methods. Data represent mean ± SD of three experiments; *P<0.05.

Results

Curcumin induces cytotoxicity in N18 mouse-rat hybrid retina ganglion cells. The effects of curcumin on N18 cells were first evaluated by trypan blue exclusion and flow cytometric assay. Both assays indicated that curcumin significantly decreased the percentages of viable N18 cells and these effects are time- and dose-dependent. As shown in Fig. 1, the viable cells at 48 h after the treatment with curcumin were decreased to 74.6 (30 μM), 23.4% (50 μM) and 10.2% (70 μM) of the control. We noted that at a lower concentration, 10 μM, curcumin had little effect on the morphology of the treated cells.

Curcumin treatment alters the distribution of cell cycles and induced apoptosis. To determine whether or not curcumin exerted its cytotoxic effect via the induction of cell cycle arrest, we examined the distribution of cell cycles of N18 cells, by flow cytometry, after their exposure to curcumin. As shown in Fig. 2A and B, curcumin induced an altered cell cycle distribution in a dose-dependent manner. The percentages of cells at G0/G1 phase at 48 h after the treatment with curcumin were increased from low doses of curcumin (5 and 10 μM), the increased doses led to a decrease in the G0/G1 phase. Percentages of cells at S phase were slightly decreased by curcumin treatment. It should be noted that while there was no change in the percentage of cells at G2/M at lower doses

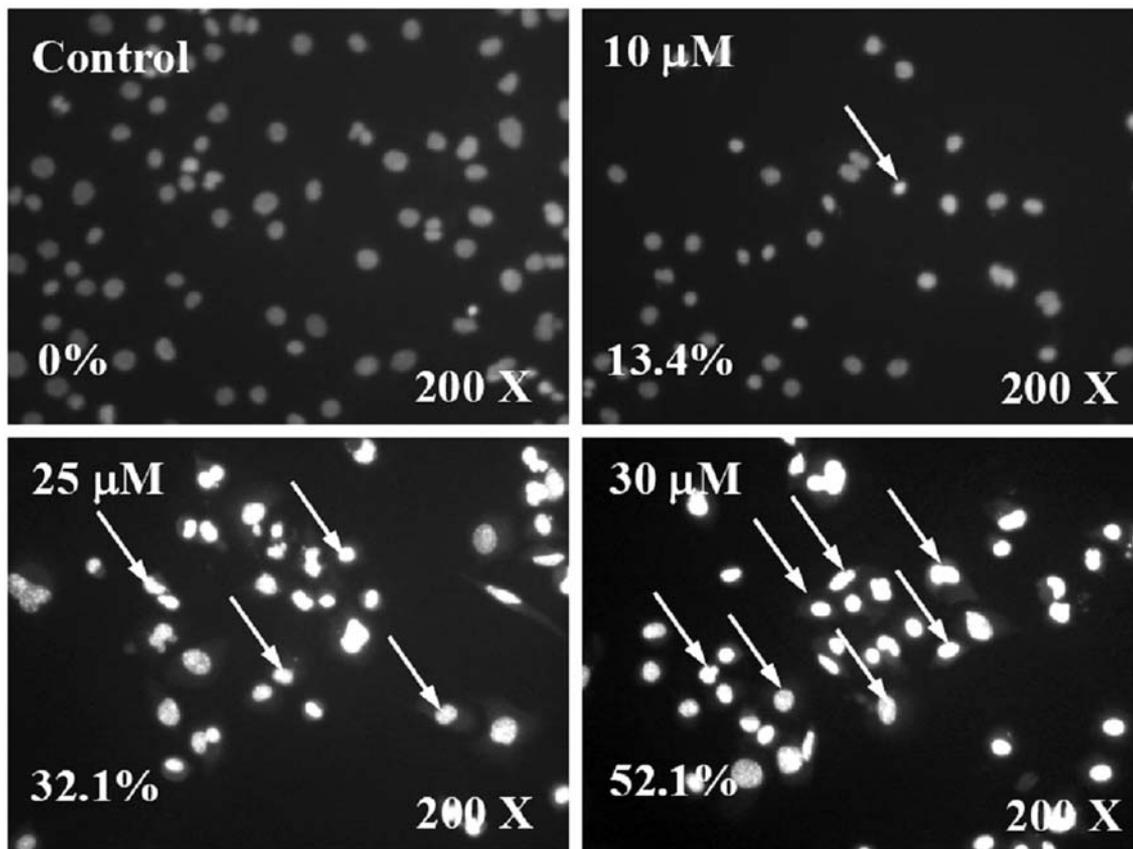


Figure 3. Curcumin-induced apoptosis in N18 cells examined by DAPI staining. N18 cells were treated with various concentrations of curcumin for 48 h and apoptosis was determined by DAPI staining and photographed by fluorescence microscopy (x200) as described in Materials and methods.

(5 and 10 μM), the percentage of cells at G2/M was markedly increased after more than 10 μM of curcumin.

The percentages of cells at G2/M phase at 48 h after the treatment with curcumin were increased, from 20.4% in the control to 40.8%. Sub-G1 was increased after 48 h treatment of curcumin to >20 μM . Thus, curcumin treatment significantly reduced cells in S phase, leading to the accumulation of cells at G2/M and increased sub-G1 phase. These results suggested that curcumin can induce cell cycle arrest at G1 and G2/M phases and produces sub-G1 in a dose-dependent manner in N18 cells. This induction of cell cycle arrest at G2/M is consistent with previous studies on other cancer cells.

Curcumin-induced apoptosis examined by DAPI staining. To determine whether cytotoxic effect of curcumin was due to apoptotic induction, morphological features such as membrane blebbing were observed in N18 cells treated with 10, 25 and 30 μM curcumin for 48 h by fluorescence microscope as shown in Fig. 3. The fluorescence microscopy indicated that DAPI staining assays revealed apoptotic cells in curcumin-treated N18 cells compared with intact control cells and this effect was dose-dependent.

Curcumin induces production of reactive oxygen species and Ca^{2+} and decreases the levels of mitochondria membrane potential ($\Delta\Psi_m$) in N18 cells. In order to examine whether or not curcumin induced apoptosis which is associated with

ROS production, we examined the levels of ROS in N18 cells after exposure to 30 μM curcumin for various time periods. The results indicated that curcumin induced ROS production quite early at 1 h time-dependently (Fig. 4A) up to 24 h of treatment, and the ROS levels were still high when compared to the control. Curcumin increased Ca^{2+} levels time-dependently (Fig. 4B) up to 24 h of treatment. Mitochondrial membrane potential ($\Delta\Psi_m$) was reduced by curcumin in a time-dependent manner (Fig. 4C).

Curcumin affects the levels of associated proteins in apoptosis of N18 cells. To confirm whether the cytotoxic effect of curcumin on N18 cells, as noted in flow cytometric assays, was due to apoptosis, N18 cells were cultured for 0, 6, 12, 24 and 48 h in the presence of the DMSO vehicle alone or with 30 μM curcumin. Cells were harvested from each treatment and cell lysates were prepared for Western blot analysis of Bax, Bcl-2, cytochrome c, Apaf-1, AIF, GADD153, GRP78, Fas, FADD, caspase-8, -9 and -3 proteins expression. The results from Western blotting are shown in Fig. 5A-E. Curcumin promoted the levels of Bax (Fig. 5A), cytochrome c, Apaf-1 and AIF (Fig. 5B), GADD153 and GRP78 (Fig. 5C), Fas and FADD (Fig. 5D), but decreased Bcl-2 (Fig. 5A). However, curcumin promoted the active form of caspase-8, -9 and -3 (Fig. 5E). PARP-1 cleavage and a reduction in the level of pro-caspase-3 or the production of active form of caspase-3, both as a result of caspase activity, are indicators of apoptosis (24,25). As shown in Fig. 5E, a reduction in

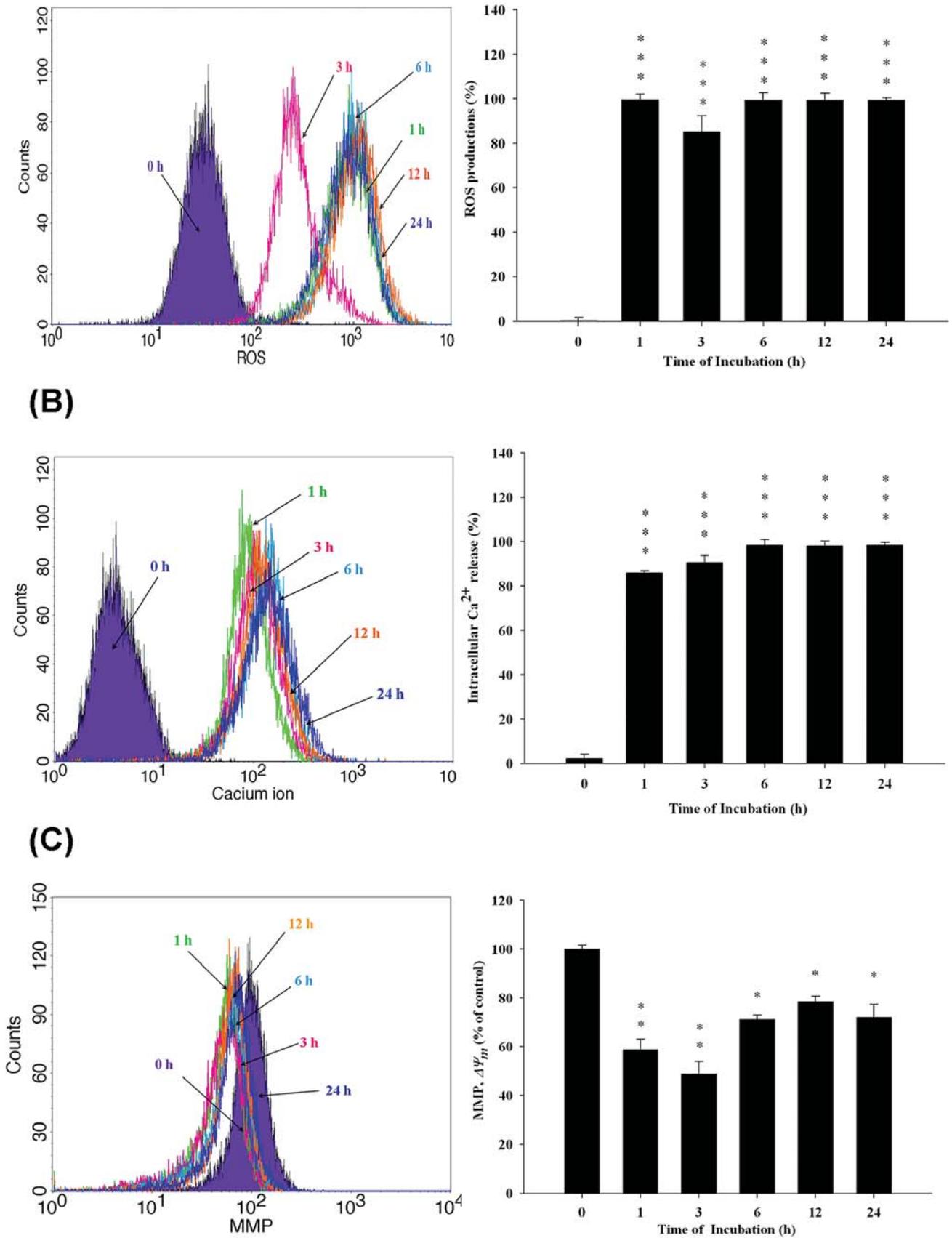


Figure 4. Curcumin induces production of reactive oxygen species (ROS) and Ca²⁺ and decreases the levels of mitochondria membrane potential ($\Delta\Psi_m$) in N18 cells. Cells were treated with 30 μ M curcumin for various time periods before cells were collected, stained by 2,7-dichlorodihydrofluorescein diacetate for ROS levels determined, stained by Indo 1/AM for Ca²⁺ levels determined and stained with DiOC₆ for $\Delta\Psi_m$ levels determined as described in Materials and methods. Data represent mean \pm SD of three experiments; *P<0.05, **P<0.01, ***P<0.001.

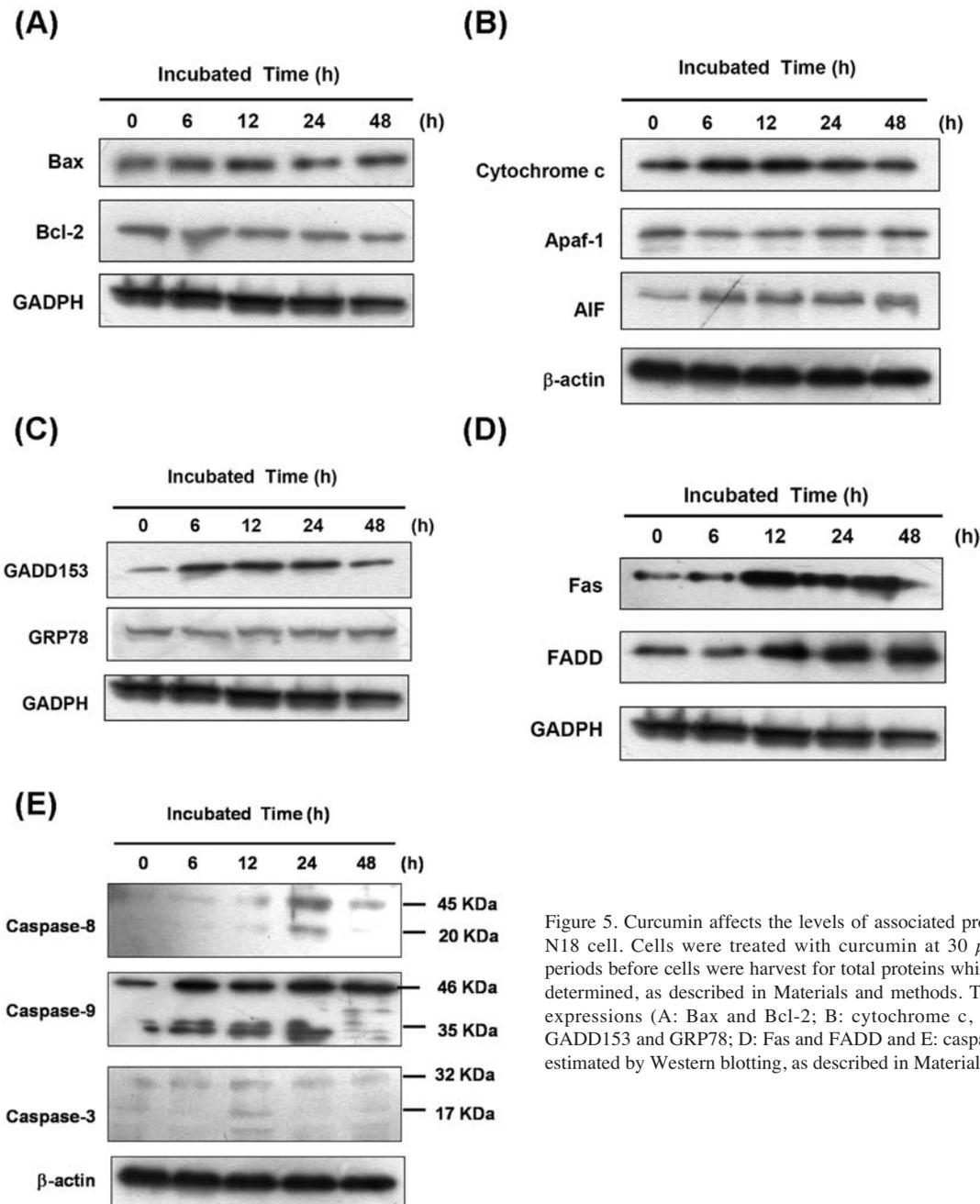


Figure 5. Curcumin affects the levels of associated proteins in apoptosis of N18 cell. Cells were treated with curcumin at 30 μ M for various time periods before cells were harvest for total proteins which were prepared and determined, as described in Materials and methods. The associated protein expressions (A: Bax and Bcl-2; B: cytochrome c, Apaf-1 and AIF; C: GADD153 and GRP78; D: Fas and FADD and E: caspase-8, -9 and -3) were estimated by Western blotting, as described in Materials and methods.

pro-caspase-8, -9 and -3 were seen in N18 cells in response to curcumin treatment. In addition, the PARP-1 cleavage fragment (Fig. 5B) was observed in N18 cells following exposure to curcumin. Collectively, these data are consistent with curcumin-induced apoptosis associated with the activation (via cleavage) of caspase-3.

Discussion

Curcumin has been demonstrated to induce cell cycle arrest and apoptosis in many types of cancer cells (19,20) and it has been suggested as a possible anti-cancer agent. The proposed mechanisms of its anti-cancer properties include direct cytotoxicity (16-18), immune modulatory effects (26) and normalization of carcinogen induced cell proliferation (27,28). The present study showed that curcumin also exerts a strong growth inhibitory activity against the mouse-rat hybrid retina

ganglion N18 cells. Flow cytometric analysis showed that N18 cells treated with different concentrations of curcumin for up to 48 h predominantly accumulated in the G2/M phase of the cell cycle in a dose- and time-dependent manner, and with subsequent accumulation in the sub-G1 phase of cell cycle (Fig. 2A and B), suggesting the sequential events of cell cycle arrest followed by apoptosis. Apoptosis induced by curcumin was also confirmed by the observed changes in nuclear morphology by DAPI staining method (Fig. 3).

Apoptosis is a genetically regulated biological process with two major pathways: the extrinsic pathway involved in death-receptor and the intrinsic pathway involved in mitochondria apoptosome-mediated apoptosis (2). Bcl-2 family proteins such as Bax and Bcl-2 have a central role in controlling the mitochondrial pathway. The pro-apoptotic proteins and anti-apoptotic proteins of the Bcl-2 family may turn on and off apoptosis because of the formation of

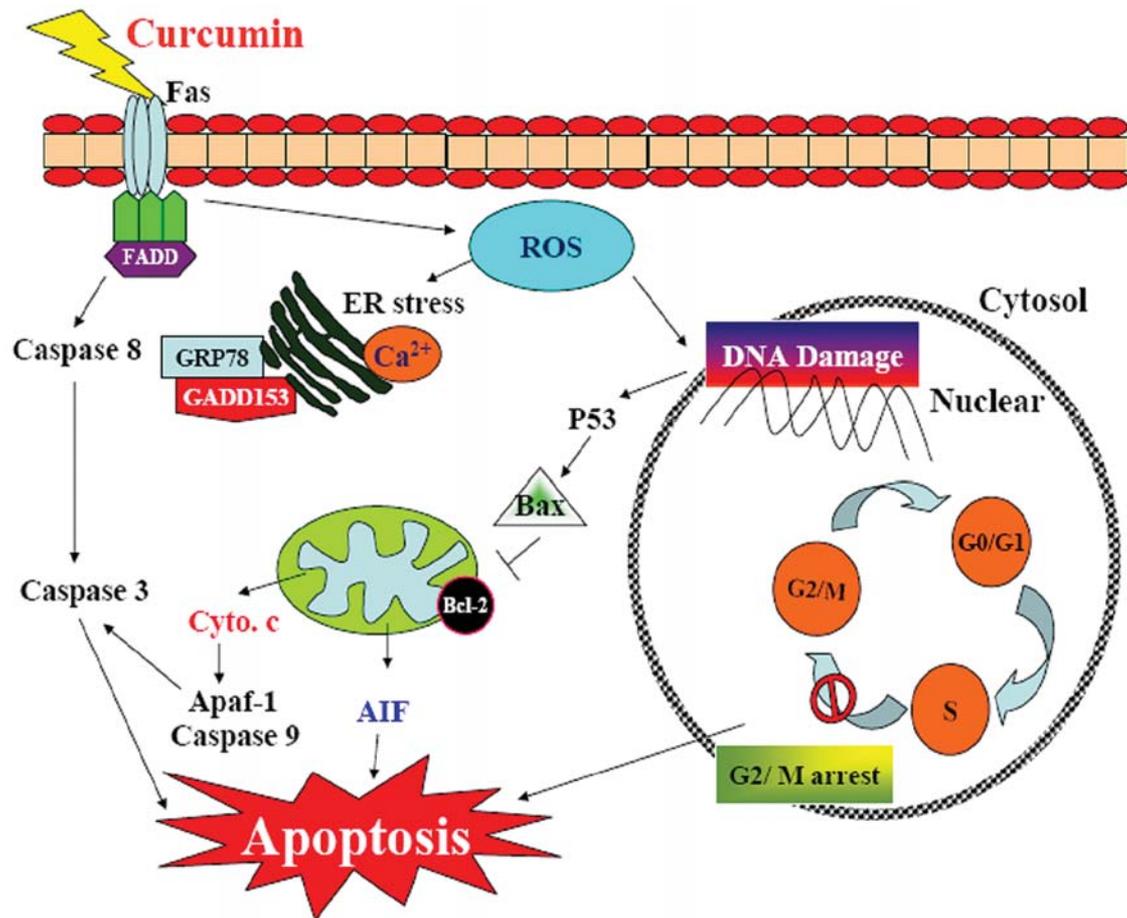


Figure 6. The proposed model of curcumin-mediated cell cycle arrest and apoptosis in mouse-rat hybrid retina ganglion N18 cells. Curcumin promoted the production of ROS and Ca^{2+} and reduced $\Delta\Psi_m$ levels leading to cytochrome c and AIF release, and promoted caspase-8, -9 and -3 activation causing apoptosis in the N18 cells.

heterodimers among these proteins (29-31). The balance between the expression levels of the protein units (e.g. Bcl-2 and Bax) is critical for cell survival or death. In N18 cells treated with curcumin, the level of Bax was increased and Bcl-2 was decreased leading to decrease of the $\Delta\Psi_m$ (Fig. 4C) suggesting that curcumin induced apoptosis through mitochondria-dependent pathway. In addition, human colon cancer cells lacking Bax also resist curcumin-induced apoptosis (32).

Many chemotherapeutic and chemopreventive agents have been shown to cause apoptotic cell death through caspase-dependent pathway (33). Caspase-3 is an executioner caspase, which upon activation can systematically dismantle cells through cleaving PARP then lead to DNA fragmentation. Increased active form of caspase-3 was observed in curcumin-treated N18 cells (Fig. 5E). Caspase-3 plays an effector caspase that can be activated by different stimuli such as a mitochondrial pathway involving caspase-9 or a death receptor pathway involving caspase-8 (34,35). In the present study, curcumin treatment increased the active form of caspase-8 and -9 (Fig. 5E). Fig. 5 also showed that curcumin promoted Fas and FADD, caspase-8, -9 and -3 expression, cytochrome c levels. Fas plays an initial role for extrinsic pathway of apoptosis and it acts as a death-receptor for triggering apoptosis (2). These results suggest involvement of both mitochondrial and death receptor pathways in curcumin-

induced apoptosis. The other important finding is that curcumin promoted the levels of GADD153 and GRP78 which are the hallmark of ER stress (36,37) before leading to Ca^{2+} release. Therefore, we may also suggest that curcumin induced apoptosis in N18 cells also through the ER stress signaling pathway.

In summary, this study shows that curcumin is a potent growth inhibitor of cultured N18 cells. We characterized the molecular mechanisms underlying curcumin-induced apoptosis in N18 cells. The growth inhibition is related to the G2/M phase cell cycle arrest and the induction of apoptosis is associated with up-regulation of Bax and down-regulation of Bcl-2 (Fig. 6). The results provide the molecular basis for curcumin to act as an anti-tumor agent in cancer therapy. These findings should be helpful for the clinical application of curcumin in the future.

Acknowledgements

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