Apoptotic death in curcumin-treated NPC-TW 076 human nasopharyngeal carcinoma cells is mediated through the ROS, mitochondrial depolarization and caspase-3-dependent signaling responses

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Abstract. Curcumin, a potent candidate anticancer agent, is a dietary pigment (phenolic compound) derived from the food flavoring spice turmeric (Curcuma longa), and it has been shown to have inhibitory effects on tumor cells through anti-proliferative and proapoptotic activities. However, there is no report showing curcumin-induced apoptotic cell death in human nasopharyngeal carcinoma cells in vitro. Thus, this study was performed to elucidate whether mitochondria and caspase cascades are involved in the modulation of apoptosis and cell cycle arrest in curcumin-treated NPC-TW 076 human nasopharyngeal carcinoma cells. The effects of curcumin on cell cycle arrest and apoptosis were measured by flow cytometry, and caspase-3 activity, apoptosis-associated protein levels and its regulated molecules were studied by flow cytometric assay and immunoblots. The results indicated that curcumin-induced G2/M phase arrest was associated with a marked decrease in the protein expression of cyclin A, cyclin B and cyclin-dependent kinase 1 (Cdk1). Curcumin-induced apoptosis was accompanied with upregulation of the protein expression of Bax and downregulation of the protein levels of Bcl-2, resulting in dysfunction of mitochondria and subsequently led to cytochrome c release and sequential activation of caspase-9 and caspase-3 in NPC-TW 076 cells in a time-dependent manner.

These findings revealed that mitochondria, AIF caspase-3-dependent pathways play a vital role in curcumin-induced G2/M phase arrest and apoptosis of NPC-TW 076 cells in vitro.

Introduction

Cancer is one of the major cause of death in human populations. Approximately 90% of all human cancers are carcinomas. Nasopharyngeal carcinoma (NPC) is an endemic disease in southern China and Southeast Asia, and the incidence rates are 15 to 50 per 100,000 (1). In Asian population, especially among Chinese people, NPC exists with high frequency (2,3). NPC has a poor prognosis due to the spread to lymph nodes and metastases to other tissues (4). Currently, the treatments of NPC are still problematic, and thus new therapeutic agents are required. Numerous studies have been tried to identify new naturally occurring chemopreventive or chemotherapy agents for using to inhibit cancer in vitro and in vivo (5-7).

Based on the epidemic studies, it has been demonstrated that among the potential dietary contributors to cancer incidence is turmeric (Curcuma longa), a spice consumed frequently by people from Southeast Asia and India, a continent with low incidence of most cancers. Curcumin (diferuloyl-methane), a yellow-colored polyphenol, is isolated from the plant curcuma longa, and it has been shown to have anticancer activities in many types of human cancer cells in vitro (8) and in vivo (9). Curcumin has been shown to inhibit tumor initiation (10) and promotion (11,12) in animal models.

The anticancer activities of curcumin have been shown to be involved in multiple signaling pathways and on many molecular targets that are related to inhibition of cell proliferation, cell cycle arrest, invasion, metastasis, and angiogenesis of tumor cells (13). Curcumin has been used in clinical trials on cancer patients (9). In a phase I study, the safety of curcumin for oral ingestion of curcumin up to 8,000...
mg/day for 3 months did not produce toxicity (14) neither in a phase II trial on pancreatic cancer patients with curcumin daily (15). Curcumin influences structurally unrelated membrane proteins (16), and then inserts deep into the cellular membrane in a transbilayer orientation inducing negative curvature in the bilayer (17). Moreover, the promotion of negative curvature by curcumin may cause a direct effect on apoptosis by increasing the permeabilizing activity of the apoptotic protein tBid (18). Our previous studies have also shown that curcumin induced cell cycle arrest and apoptosis in human colon cancer cells (19), acute promyelocytic leukemia HL-60 cells (20), murine leukemia WEHI-3 cells in vivo (21), N18 mouse-rat hybrid retina ganglion cells (22), lung carcinoma A549 cells (23), non-small cell lung cancer NCI-H460 cells in vivo (24) and in vitro (25). However, there is no available report addressing the curcumin-induced apoptosis in human nasopharyngeal carcinoma cells. Therefore, in the present study, we investigated the effects of curcumin on cell cycle distribution and induction of apoptosis in human nasopharyngeal carcinoma NPC-TW 076 cells. Results indicated that curcumin induced G2/M phase arrest and induced apoptosis through a mitochondria-dependent pathway.

**Materials and methods**

**Chemicals and reagents.** Curcumin, RNase, propidium iodide (PI), dimethyl sulfoxide (DMSO), trypan blue, and Triton X-100 were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). All primary and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The fluorescent probes DiOC₆ for mitochondrial membrane potential, 2',7'-dichlorofluorescin diacetate (DCFH-DA) for hydrogen peroxide (H₂O₂), and Indo 1/AM for Ca²⁺ staining were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Caspase-3-, 8-, 9-activity assay kits were obtained from OncoImmunin, Inc (Gaithersburg, MD, USA).

**Cell culture.** The human nasopharyngeal carcinoma NPC-TW 076 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 Units/ml penicillin and 100 µg/ml streptomycin in 75 cm² tissue culture flasks at 37°C under a humidified 5% CO₂ and 95% air atmosphere as we have previously reported (26).

**Morphological changes and percentage of viable cell examinations in NPC-TW 076 cells.** NPC-TW 076 cells at density of 2x10⁵ cells/well were placed on 12-well plates and treated with 0, 1, 5, 10 or 25 µM curcumin, or only with vehicle (DMSO, 1% in culture media) then cells were incubated for 24 and 48 h. Then the cells were photographed under a phase-contrast microscope for morphological changes. Cells were harvested for determination of percentage of viable cells by a PI exclusion method and a flow cytometric assay as previously described (22,27). Briefly, an aliquot of the total cell suspension from each well was mixed with PI (5 µg/ml) dye in PBS and immediately determined by using a FACSCalibur instrument (BD, San Jose, CA, USA) equipped with BD Cell Quest Pro software (19).

**Determinations of cell cycle distribution and apoptosis of NPC-TW 076 cells by flow cytometric assay.** NPC-TW 076 cells at density of 2x10⁵ cells/well were placed on 12-well plates and exposed to 10 µM curcumin, or only with vehicle (DMSO, 1% in culture media), and then cells were incubated for 6, 12, 24 and 48 h. After incubation, cells from each treatment were individually harvested by brief trypsinization then washed twice, and then PBS containing 40 µg/ml PI, 0.1 mg/ml RNase and 0.1% Triton X-100 was added for 30 min at 37°C and left in the dark. The PI stained cells were analyzed for quantification, cell cycle distribution and sub-G1 phase (apoptosis) by flow cytometry (28,29). The sub-G1 group was representative of the mean apoptosis.

**DAPI staining and Comet assay.** About 2x10⁵ NPC-TW 076 cells/well on 12-well plates were treated with 0, 1, 5, or 10 µM curcumin, and then all cells were incubated for 24 h under 5% CO₂ and 95% air at 37°C. The treated cells were divided into two parts for DAPI staining and Comet staining by 4'-6-diamidino-2-phenylindole (DAPI) (Invitrogen) for DAPI staining and PI for Comet assay, respectively. Then all samples were examined and photographed using fluorescence microscopy as described elsewhere (29,30).

**Flow cytometric assay for mitochondrial membrane potential (ΔΨₘ), reactive oxygen species (ROS) and cytosolic Ca²⁺ release.** About 2x10⁵ NPC-TW 076 cells/well on 12-well plates were incubated with 10 µM curcumin for different time periods to measure the levels of ΔΨₘ, the productions of ROS and cytosolic Ca²⁺. After cells were incubated for various time periods, all cells from each treatment were harvested, washed twice by PBS, then re-suspended in 500 µl of DiOC₆ (1 µmol/l) for evaluation of the level of ΔΨₘ, 500 µl of DCFH-DA (10 µM) for ROS and in Indo 1/AM (3 µg/ml) for cytosolic Ca²⁺ production in the dark for 30 min at 37°C, then all samples were analyzed immediately by flow cytometry as described previously (27,29,31).

**DNA gel electrophoresis for apoptosis.** Cells (2x10⁵/well) on 12-well plates were cultured with 0, 10, 20, 30 and 40 µM curcumin for 24 h. Chromosomal DNA was isolated (Genomic DNA Purification kit, Genemark technology Co., Ltd. Tainan, Taiwan) and ladder formation assays were performed by DNA gel electrophoresis as described previously (27,28).

**Determination of viability and ROS in NPC-TW 076 cells by pretreatment with N-acetylcysteine (NAC) before curcumin treatment.** Approximately 2x10⁵ cells/well on 12-well plates were pretreated with 20 mM NAC for 2 h, and then all samples except the control were treated with 10 µM curcumin for 24 h. The cells were harvested and examined for percentage of viability and the ROS productions as described above then assayed by flow cytometry (22,27).

**Determination of caspase-3 activity.** NPC-TW 076 cells (2x10⁵) on 12-well plates were incubated with 10 µM curcumin for 0, 24 and 48 h. Cells from each well were harvested and washed twice with PBS, and then substrates were used (PhiPhiLux-G₁D₂ for caspase-3, OncoImmunin, Inc) and the...
activities of caspase-3 was determined by flow cytometry as described previously (22,27).

**Western blot analysis.** Approximately 5x10⁶ NPC-TW 076 cells/well on 6-well plates were incubated with 10 µM curcumin for 0, 3, 6, 12, 24, 48 or 72 h. Then cells from each treatment were harvested by centrifugation and washed twice with PBS, then total protein was determined. The protein levels (Cyclin B, CDK1, cdc25c, Bcl-2, cytochrome c, pro-caspase-9, PARP, XRCC-1, GADD153, SOD and catalase) associated with cell cycle arrest and apoptosis were determined by Western blotting. Lysates of treated cells from each well or isolated nuclei were prepared by using the PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Seongnam, Gyeonggi-Do, Korea) as described previously (22,27). Each sample was incubated with primary antibody (Santa Cruz Biotechnology Inc.) and then washed twice followed by secondary antibody and then detected with an ECL kit (Millipore, Bedford, MA, USA) and autoradiography using X-ray film (22,27). Each membrane was stripped and anti-β-actin antibody was used to ensure equal protein loading.

**Confocal laser scanning microscopy.** NPC-TW 076 cells at density of 5x10⁶ cells/well were cultured on 4-well chamber slides, and then treated without or with 10 µM curcumin for 24 h. At the end of incubation, cells were fixed directly in 4% formaldehyde in PBS for 15 min then permeabilized with 0.3% Triton X-100 in PBS for 1 h. The fixed cells were stained by using the primary antibody to AIF (1:100 dilution) overnight. Then all cells were washed twice with PBS and were stained with secondary antibody (FITC-conjugated goat anti-mouse IgG at 1:100 dilution) (green fluorescence) followed by DNA staining with DAPI (blue fluorescence) as described previously (22,32). Photomicrography was performed using a Leica TCS SP2 Confocal Spectral Microscope (22,32).

**Statistical analysis.** The quantitative data are shown as the mean ± SD and results are representative of three independent experiments. The statistical differences between the curcumin-treated and control samples were calculated by Student’s t-test. A p<0.05 was considered significant.

**Results**

*Curcumin induces morphological changes and decreases percentage of viable NPC-TW 076 cells.* Cells were treated with different concentrations of curcumin for 24 and 48 h, and then cell morphological changes were examined and photographed by a phase-contrast microscope. Results are shown in Fig. 1A, indicating that curcumin induced morphological changes of NPC-TW 076 cells. The results revealed that some cancer cells became round, smaller and blunt in size and cells became detached and suspended in the medium, especially for 48 h exposure with 10 µM curcumin. Then all cells from each treatment were harvested by centrifugation for determining the percentage of viable cells by flow cytometric assay. The results shown in Fig. 1B
Curcumin induces G2/M phase arrest and apoptosis in NPC-TW 076 cells. Cells were treated with 10 μM curcumin for 0, 6, 12, 24 and 48 h. The cells were harvested and analyzed for cell cycle distribution and apoptosis (A) by flow cytometry. Data from flow cytometric profile were quantified (B) as described in Materials and methods. Each point is the mean ± SD of three experiments. *p<0.05, significantly different compared with DMSO-treated control and curcumin-treated groups.
the sub-G1 fraction in curcumin-treated cells was increased in a time-dependent manner, indicative of apoptotic cell death.

Curcumin induces apoptosis and DNA damage in NPC-TW 076 cells. Cells were exposed to different concentrations of curcumin for 24 h, and then all samples were harvested for determining apoptosis by DAPI staining and for DNA damage by Comet assay and for DNA fragmentation occurrence by DNA gel electrophoresis. Fig. 3A demonstrates that curcumin induced apoptosis in a dose-dependent manner based on the higher density of white color on the nuclei from a fluorescence microscope examination (DAPI staining). Comet assay demonstrated that curcumin (1, 5 and 10 µM) for 24 h induced DNA damage (Fig. 3B) and DNA gel electrophoresis indicated that curcumin induced DNA fragmentation in a dose-dependent manner (Fig. 3C).

Figure 3. Curcumin induced apoptosis and DNA damage in NPC-TW 076 cells. Cells were treated with various concentrations of curcumin for 24 h. The cells were harvested and were examined for apoptosis by DAPI staining (A), DNA damage by Comet assay (B) and the DNA fragmentation (C) was performed by DNA gel electrophoresis as described in Materials and methods.

Curcumin changes the levels of mitochondria membrane potential ($\Delta \Psi_m$), reactive oxygen species (ROS) and the production of cytosolic $Ca^{2+}$ in NPC-TW 076 cells. Cells were treated with 10 µM curcumin for different periods of time and then all samples were harvested and the levels of $\Delta \Psi_m$, ROS and cytosolic $Ca^{2+}$ measured by flow cytometric assay. The results are shown in Fig. 4 indicating a significant decrease of $\Delta \Psi_m$ level (Fig. 4A), and the increases of intra-cellular ROS (Fig. 4B) and cytosolic $Ca^{2+}$ level (Fig. 4C) in the curcumin-treated NPC-TW 076 cells. Fig. 4A indicates that curcumin significantly decreased the level of $\Delta \Psi_m$ around 99 to 92% in 1 to 6 h-treatment. Following 0.5 to 3-h treatment of curcumin in NPC-TW 076 cells, it initially significantly increased cytosolic $Ca^{2+}$ level (Fig. 4C). On the contrary, after 3-h treatment, cytosolic $Ca^{2+}$ level did not have significant difference in comparison to that in the control sample (data not shown). However, at the treatment of curcumin at 6 h until 36 h, the
ROS levels were significantly higher than those of the control group (Fig. 4B).

**NAC affects curcumin-treated NPC-TW 076 cells on cell viability and ROS productions.** Cells were pretreated with 20 mM NAC for 2 h, and then exposed to 10 µM curcumin for 24 h. The cells from each treatment were harvested and examined for percentage of viability and the ROS production. The results shown in Fig. 5, indicate that NAC protected against cell viability of curcumin-treated NPC-TW 076 cells, then led to increase the percentage of viable cells (Fig. 5A) and decreased ROS production (Fig. 5B) in curcumin-treated cells. Based on these results, curcumin-triggered apoptosis is involved in the ROS-dependent pathway.

**Curcumin stimulates caspase-3 activity in NPC-TW 076 cells.** Cells were treated with or without 10 µM curcumin for 24 and 48 h, and then cells from each treatment were harvested for determination of the caspase-3 activity. The results shown in Fig. 6 indicate that curcumin induced caspase-3 activity in NPC-TW 076 cells. The results suggest that curcumin-induced apoptosis might be mediated through the caspase-3-dependent signal pathway.

**Curcumin affects the levels of G2/M phase and apoptosis-associated proteins in NPC-TW 076 cells.** Cells were incubated with 10 µM curcumin for 0, 3, 6, 12, 24, 48 and 72 h, and then were harvested for determining the protein levels by Western blotting. The results are shown in Fig. 7, indicating that curcumin decreased Cyclin B, CDK1 and cdd25c (Fig. 7A), Bcl-2, pro-caspase-9 and XRCC1 (Fig. 7B). However, it promoted the levels of cytochrome c, PARP, GADD153 (Fig. 7B), SOD and catalase (Fig. 7C). In the nucleic fraction, cyclin B was decreased and AIF was increased (Fig. 7D). The level of anti-apoptosis protein Bcl-2 (Fig. 7B) was decreased. The p53 and p21 were also increased which could contribute to cell cycle arrest and apoptosis.
Curcumin promotes AIF distribution in NPC-TW 076 cells. Cells were treated with 10 µM curcumin for 24 h, and were harvested for determination of location of AIF. The results...
indicated that curcumin promoted the release of AIF from mitochondria to nuclei followed by apoptosis in NPC-TW 076 cells as seen in Fig. 8.

**Discussion**

The efficacy of treatment with chemotherapeutic agents is largely based on their ability to induce cell death in tumor cells; therefore, the inducer of apoptosis is able to offer a new therapeutic approach design for anticancer agents (33-35). In mammalian cells, the levels of CDKs regulated the cell cycle event (36,37) and among the CDKs such as CDK1 and CDK2 kinases were activated primarily in association with cyclin A and B in the G2/M phase progression (37). In this study, curcumin induced morphological changes and decreased the percentage of viable NPC-TW 076 cells in a dose- and time-
dependent manner. Furthermore, the DNA content indicated that a prominent G2/M phase arrest of NPC-TW 076 cells upon exposure to curcumin (Fig. 2A), and the levels of CDK1, cyclin B and cdc25c proteins were decreased after curcumin treatment in a time-dependent manner (Fig. 7A). Thus, it is plausible that alterations in cell cycle-associated proteins lead to arrest at G2/M phase in curcumin-treated NPC-TW 076 cells.

Our data also demonstrated that curcumin induced apoptosis of NPC-TW 076 cells as verified by condensation of DNA by DAPI staining, sub-G1 phase occurred as shown by flow cytometry. DNA fragmentation from DNA gel electrophoresis indicated a decrease of the level in XRCC1 by Western blotting. It was reported that apoptosis is a tightly regulated process under the control of several different signaling pathways including caspase and mitochondrial pathways (38,39). In the present study, curcumin treatment caused the productions of ROS and Ca\(^{2+}\), but it decreased the level of \(\Delta\Psi_m\) in NPC-TW 076 cells (Fig. 4A-C). Furthermore, curcumin promoted the caspase-3 activity in a time-response manner that is consistent with the DNA fragmentation (Fig. 2C).

Herein, we also found that curcumin stimulated the ROS production in NPC-TW 076 cells. However, cells were pretreated with NAC (an antioxidant scavenger) and then treated with curcumin that led to decrease of the level of ROS and increased the percentage of viable NPC-TW 076 cells (Fig. 5A and B). An increase of cytosolic cytochrome \(c\) was observed in curcumin-treated NPC-TW 076 cells (Fig. 7B). Bcl-2 family proteins including the anti-apoptotic Bcl-2 and the pro-apoptotic Bax proteins have been reported to regulate cytochrome \(c\) release from mitochondria (39). The proto-oncogene Bcl-2, encodes an inner mitochondrial protein that reportedly antagonizes apoptosis in many tumor cells (40). Interestingly, it was reported that a decreased expression of Bcl-2 might contribute to the drug-mediated lethality (41,42).

Our earlier experiments have revealed that curcumin-induced apoptosis in human non-small cell lung cancer NCI-H460 cells and colon cancer colo 205 cells were associated with decreased Bcl-2 expression (19,32). In the present study, consistent with these observations, down-regulation of Bcl-2, a decrease of the mitochondrial membrane potential (\(\Delta\Psi_m\)), and the release of cytochrome \(c\) occurred with curcumin-treated NPC-TW 076 cells. It was reported that the agent disturbed mitochondrial function may be via an increase in the ratio of Bax and anti-apoptotic (Bcl-2) members leading to an increase in mitochondrial membrane permeability, the release of cytochrome \(c\), and activation of caspasas (43,44).

In conclusion, curcumin induces cell cycle arrest at the G2/M phase and apoptosis of NPC-TW 076 cells. Curcumin-induced cell cycle arrest was associated with reduction of CDK1, Cyclin B protein expression. Curcumin-induced apoptosis was accompanied with up-regulation of Bax and down-regulation of Bcl-2 leading to cytochrome \(c\) release, then to activation of caspase-9 and caspase-3, leading to apoptosis of NPC-TW 076 cells.

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References


