Curcumin sensitizes lung adenocarcinoma cells to apoptosis via intracellular redox status mediated pathway

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The present study demonstrates that curcumin acts as pro-oxidant and sensitizes human lung adenocarcinoma epithelial cells (A549) to apoptosis via intracellular redox status mediated pathway. Results indicated that curcumin induced cell toxicity (light microscopy and MTT assay) and apoptosis (AnnexinV-FITC/PI labeling and caspase-3 activity) in these cells. These events seem to be mediated through generation of reactive oxygen species (ROS) and superoxide radicals (SOR) and enhanced levels of lipid peroxidation. These changes were accompanied by increase in oxidized glutathione (GSSG), reduced glutathione (GSH) and γ-glutamylcysteine synthetase (γ-GCS) activity, but decrease in GSH/GSSG ratio. The induction of apoptosis and decrease in GSH/GSSG ratio was also accompanied by sustained phosphorylation and activation of p38 mitogen activated protein kinase (MAPK). On the other hand, addition of N-acetyl cysteine (NAC), an antioxidant, blocked the curcumin-induced ROS production and rescued malignant cells from curcumin-induced apoptosis through caspase-3 deactivation. However, L-buthionine sulfoximine (BSO), a GSH synthesis blocking agent, further enhanced curcumin-induced ROS production and apoptosis in A549 cells. Decreased GSH/GSSG ratio seems to be a crucial factor for the activation of MAPK signaling cascade by curcumin. The study therefore, provides an insight into the molecular mechanism involved in sensitization of lung adenocarcinoma cells to apoptosis by curcumin.

Keywords: A549 cells, Adenocarcinoma, Apoptosis, Curcumin, MAPK signaling, Reactive oxygen species, Redox status

Lung cancer is one of the most common malignant neoplasms and a cause of cancer related deaths worldwide\(^1\). Due to lack of diagnostic tools for early detection and efficient treatment of the advanced disease, the prognosis of lung cancer is still poor with less than 15% of patients surviving nearly 5 years after its diagnosis\(^2\). Chemotherapy for lung cancer lengthens the survival and improves quality of life in selected patients. Most of the new anticancer therapies can be broadly classified as targeted “cytotoxic agents” or targeted “biological agents”. These agents inflict cell death, inhibit tumor cell proliferation, induce apoptosis, inhibit angiogenesis, or enhance antitumor immune response\(^3\). Still none of these drugs either alone or in combination prove to be of a great clinical advantage. This may be due to development of drug resistance by tumors\(^4\), alterations in biochemical and molecular pathways which lead to escape of tumor from immune surveillance and cell death. Further, anticancer drugs also damage normal tissues, resulting in significant side effects affecting quality of patient’s life, a major clinical limitation in chemotherapy\(^5\). This has prompted a search for natural compounds which are non-toxic to normal cells but have selective tumor cell killing activity.

Curcumin (diferuloylmethane) is a yellow colored spice extract (a component of turmeric) from the rhizome of *Curcuma longa* L. (Zingiberales). Curcumin is widely used as a food flavoring and coloring agent and has a long history of medicinal use in India and Southeast Asia for a wide variety of medical conditions\(^6\). It has been shown in several experimental studies that curcumin has anti-inflammatory, anti-mutagenic, anti-angiogenic, anti-proliferative, anti-migratory and anti-invasive properties\(^7,8\). Interest in curcumin and its promising cancer-preventive potential is growing, owing to its non-toxicity to normal cells and tissues\(^9\). Several possibilities have been raised regarding the potential mechanisms of the observed chemopreventive effects of curcumin, and among these its antioxidant and anti-inflammatory properties have received major attention. Curcumin scavenges ROS including SOR, hydroxyl radical (\(\cdot\)OH), and nitric oxide (\(\cdot\)NO)\(^10\).
It decreases the 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced expression of c-jun, c-fos and c-myc proto-oncogenes\textsuperscript{11} and suppresses activation of NF-kB\textsuperscript{12,13}. Curcumin acts as a ‘dual agent’ by inhibiting pro-carcinogen activating Phase-1 enzymes especially cytochrome P4501A1\textsuperscript{14} and elevating the activities of Phase-2 detoxification enzymes such as glutathione transferases, NAD(P)H: and quinine reductase (QR)\textsuperscript{15}. Moreover, curcumin has also been reported to inhibit the development of chemically induced tumors\textsuperscript{15}.

In addition to their scavenging abilities, phenolic antioxidants like curcumin also regulate the expression of various genes critical to tumorigenesis, increase the binding of transcription factors to promoter regions, thus regulating their activation or deactivation. These effects of phenolic antioxidants are associated with the activation of MAPK, AP-1, and ARE\textsuperscript{16}. Despite extensive analysis of chemopreventive activities of curcumin, its ability to modulate lung cancer growth and the underlying mechanism has not yet been well characterized in various tumors. Therefore, the present study was designed to evaluate the effect of curcumin on cell viability and apoptosis related modulation of genes in lung cancer cells (A549 cells) in an attempt to develop effective pharmacological strategies for the treatment of lung cancer.

Materials and Methods

Chemicals—All rare chemicals were purchased from M/s Sigma Chemical Company, MO, U.S.A. Dichlorodihydrofluorescin diacetate (DCFH-DA) and Dihydroethidium (DHE) were purchased from M/s Molecular Probes, OR, U.S.A. Kits for MAPK activity (p38 and SAPK) were purchased from M/s Cell Signaling Inc., U.S.A. Titanium one step RT-PCR kit (cDNA synthesis kit) was purchased from M/s Clontech, CA, U.S.A. AnnexinV-FITC/PI apoptosis detection kit was purchased from Calbiochem U.S.A. Oligonucleotide primers were designed using the published sequence of desired genes, and were purchased from M/s Biobasics Inc., Canada. All cell culture related products were purchased from M/s GIBCO-BRL, Grand Island, NY, U.S.A. Plastic ware for cell culture was purchased from M/s Falcon-BD Biosciences, U.S.A. All other reagents of highest purity were purchased locally.

Cell culture—Human lung adenocarcinoma epithelial cells (A549) were procured from American Type Culture Collection (ATCC), Rockville, MD, U.S.A. The cells were maintained in continuous culture at 37 °C and 5% CO\textsubscript{2} in Ham’s F-12K medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin and 0.5 µg/ml fungizone. After confluence, cells were trypsinized, deadhered and used for experiments. Cell number for cell plating was counted by trypan blue dye exclusion assay. For curcumin stock preparation curcumin was dissolved in ethanol, in such a way that the final concentration of ethanol in culture media of plates and dishes did not exceed 0.025%. Dilutions of curcumin stocks were prepared in Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-free sterile phosphate buffered saline (PBS).

Light microscopy—To observe changes in cellular morphology, light microscopy experiments were performed. Cells (5 × 10\textsuperscript{4} cells/well) were plated into 48-well flat-bottom culture plates. After 24 h of incubation, cells were replenished with fresh media and treated with different concentrations of curcumin for desired period of time. Thereafter, cells were examined under a light microscope and photographed at 10x magnification by Olympus Inverted microscope (Olympus, Tokyo, Japan).

Cell viability—Effect of curcumin on cell viability was evaluated by reduction of yellow 3-(4, 5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) dye to insoluble purple formazan crystals by dehydrogenase activity in mitochondria of viable cells\textsuperscript{17}. Briefly, 2 × 10\textsuperscript{5} cells were seeded in 96-well plate and after 24 h of priming, cells were treated with different concentrations of curcumin for 24 h. At the end of treatment duration, 20 µL of MTT solution (2.5 mg/mL) was added to each well. After 4 h, resulting formazan crystals were dissolved in 40 µl of lysis buffer (20% SDS dissolved in 50% each of DMF and ddH\textsubscript{2}O). The developed color was read at 540 nm on ELISA reader. Results were expressed as relative cell viability. Each experiment was performed in triplicates and repeated thrice.

Apoptosis assay—Apoptosis in A549 cells after treatment with desired test compounds was quantified with AnnexinV-FITC/PI staining method as described earlier\textsuperscript{18}. Briefly, 1 × 10\textsuperscript{5} cells were plated in 12-well culture plate and cultured for 24 h. Thereafter, cells were replenished with fresh media and treated with various concentrations of desired compounds. The cells were washed, harvested and resuspended in 200 µL of staining solution (supplied with kit) and incubated for 30 min at 40 °C with 2 µl of annexin V-antibody labeled with FITC (provided in kit) and
followed by PI staining as per manufacturer’s instruction. Later, cells were acquired and analyzed by flow cytometer (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, U.S.A). Phosphatidylserine (PS) expressing cells with increased annexinV/PI binding were quantified as the percentage of total cells. Results are expressed in terms of percentage of annexinV/PI positive cells.

Caspase-3 activity—Caspase-3 activity in A549 cells was measured by the methods of Chen et al. In brief, cells were treated with curcumin, BSO (1 mM) (inhibits GSH synthesis by irreversible inhibition of γ-glutamyl cysteine synthase), GSH (1 mM) and NAC (100 µM) (a thiol antioxidant and cysteine source for GSH synthesis) for desired time period and were harvested, collected, washed with PBS and lysed on ice in lysis buffer [25 mM HEPES (pH 7.4), 0.5% NP-40, 2 mM EDTA, and 1 mM DTT], in the presence of a protease inhibitor cocktail. The lysates were centrifuged at 13,000 rpm for 12 min at 4 °C and assayed for protease activity using 40 µg of protein and 100 nM of acetyl-Asp Glu-Val-Asp-μ-nitroanilide (Ac-DEVD-p-nitroanilide) substrate. Samples were incubated at 37 °C for 60 min, after which the enzyme-catalyzed release of p-nitroanilide was quantified by measuring absorbance at 405 nm using a microplate reader. Caspase-3 activity was expressed in terms of nmoles of pNA liberated per mg protein.

Intracellular ROS and SOR estimation—Levels of intracellular ROS and SOR were measured using DCFH-DA and DHE fluorescent dyes respectively by the method of Wan et al. In brief, cells were challenged with different concentrations of curcumin, BSO (1 mM), GSH (1 mg/mL) and NAC (100 µM) for desired period of time in CO₂ incubator at 37 °C. Cells were washed and incubated with 5 µM DCFH-DA or 10 µM DHE for 30 min at 37 °C. After that, cells were washed, harvested, rewashed and transferred to polystyrene tubes for FACS analysis (Beckton Dickinson FACSScan). Shift in fluorescent peak due to oxidation of DCFH and DHE dyes by ROS and SOR was represented in terms of mean fluorescent intensity (MFI).

Lipid peroxidation—Lipid peroxidation (LPO) in the cells was estimated by the method of Ohokawa et al. Briefly, cells were lysed in 50 mM Tris-HCl buffer (pH 7.4). Equal amount of protein for assay was loaded in 15 ml glass tubes and final volume was constituted with reaction buffer. Thereafter, reaction mixture was incubated at 37 °C for 1.5 h after which the reaction was stopped by adding 2 mL of 8% SDS followed with 1.5 mL of 20% acetic acid (pH 3.5). The amount of malondialdehyde (MDA) formed during incubation was estimated by adding 1.5 mL of 0.8% thiobarbituric acid (TBA) and heating the samples at 95 °C for 45 min. After cooling, samples were centrifuged and the TBA-reactive substances (TBARS) were measured in supernatants at 532 nm by using 1.53 x 10⁷ M⁻¹cm⁻¹ as extinction coefficient. Levels of TBARS reactive substances were expressed as nmoles MDA/µg protein.

Intracellular GSH, GSSG and Gamma-glutamylcysteine synthetase (γ-GCS)—Intracellular GSH levels were measured by the modified method of Tietze. In brief, cells after curcumin treatment were rinsed with ice-cold PBS, de-adhered from the culture plate and suspended in 1 mL of ice-chilled PBS containing 0.01% Triton X-100 and 0.6% sulfoalicylic acid. Cells were homogenized and centrifuged at 4 °C for 5 min at 2,500 rpm. 20 µL of supernatant was added to 120 µL of 0.1 M phosphate buffer, 5 mM EDTA (pH 7.5), containing 100 µL of 5 mM DTNB and 0.5 units of glutathione reductase (GR). 60 µL of 2.4 mM NADPH was added and the rate of change in absorbance was measured for 3 min at 412 nm. A standard curve using GSH in the range of 0.33 to 1.35 nmaleoles was prepared prior to measurement of the samples. The results were expressed in terms of nmoles of GSH/µg protein.

Intracellular GSSG levels were estimated by the method of Griffith et al. For the assay, supernatants were first treated with 2-vinylpyridine and triethanolamine. Finally, the processed cell lysate was used for GSSG estimation as described above for the estimation of GSH. Gamma-glutamylcysteine synthetase activity in cell lysate was assayed by the method of Seelig and Meister using a coupled assay with pyruvate kinase and lactate dehydrogenase. Enzyme-specific activity was expressed as nmoles of NADH oxidized/µg protein/min, which is equal to 1 International Unit (I.U.).

mRNA expression study by Reverse transcription-polymerase chain reaction (RT-PCR)—Total RNA from A549 cells was isolated by the acid guanidinium isothiocyanate/phenol/ chloroform method of Chomczynski and Sacchi. After DNase treatment, the quality of the RNA was checked for integrity by 28S/18S ratio, and OD₂₆₀/OD₂₈₀ ratio. Reverse transcription was performed at 58 °C for 60 min. The primer sequences used were as follows: γ-GCS,
5'-GTGGTACTGCTCACCAGAGTGATCCT-3' (forward) and 5'-TGATCCAAGTAACCTCTGGACATTCACA-3' (reverse); and \( \beta \)-2-microglobulin, 5'-GAATTGCTATGTGTCTGGGT-3' (forward) and 5'-CATCTTCAAACCTCCATGATG-3' (reverse). The protocol consisted of 30 cycles at 94 °C for 30 sec and the optimal annealing temperature (with respect to desired gene) for 30 sec, and 72 °C for 30 sec, followed by a final extension at 72 °C for 10 min. The PCR products obtained were run on a 1.8% agarose gel.

MAPK activity—SAPK and p38 MAPK activities in A549 cells were assayed by using the commercially available kits consisting of specific monoclonal antibody-sepharose beads. These beads selectively remove SAPK or p38 kinases from cell lysate. The kinase reaction was carried out in the presence of specific substrate and unlabelled ATP. Briefly, 5×10^5 cells were cultured in 100 mm^2 cell culture dishes and treated with different curcumin concentrations. After 24 h, cells were washed and lysed in lysis buffer supplied with kits. After 5 min of incubation on ice, cells were scraped, transferred to microcentrifuge tubes and then sonicated on ice thrice for 5 sec each. For Western blot analysis, equal amount of protein (10–15 µg) was separated by SDS-PAGE, transferred onto nitrocellulose membrane and immuno-detected according to the manufacturer’s protocol.

Protein estimation—Protein content of cell lysate was measured in all samples by Bradford method. Bovine serum albumin (BSA) was used as a protein standard.

Statistical analysis—All results were expressed as mean±SD. Statistical differences between groups were evaluated by one way analysis of variance (ANOVA). \( P < 0.05 \) was considered to be statistically significant.

Results

Cell growth, viability and apoptosis—Effect of curcumin on cell growth and the associated morphological changes were studied by light microscopy after 24 h of treatment (Fig. 1). It was observed that curcumin concentration above 50 \( \mu \)M induced morphological changes and de-adhered the cells from substratum. Hence, curcumin at higher concentrations was not employed for further studies.

No significant change in cell viability was observed when A549 cells were treated with up to 50 \( \mu \)M concentrations of curcumin (Table 1). However, curcumin at concentrations of 100, 250 and 1000 \( \mu \)M significantly decreased the cell viability of cells by 34.67, 56. and 89.6% respectively. Studies related to apoptosis by using Annexin-V FITC/PI staining method showed significant induction in apoptosis by 20.13 and 44.25% at 50 and 100 \( \mu \)M concentrations of curcumin. Caspase-3 activity was also found to increase concomitantly from 50.00±5.14 (control) to 128.03±10.31 and 176.31±11.02 nmoles of pNA formed/mg protein at 50 \( \mu \)M and 100 \( \mu \)M concentrations of curcumin respectively in these cells (Table 1).

Fig. 1—Effect of treatment with various concentrations of curcumin on growth pattern of A549 cells after 24 h of treatment. [(A)-untreated cells; (B)-10 \( \mu \)M; (C)-50 \( \mu \)M, (D)-100 \( \mu \)M, (E)-250 \( \mu \)M and (F)-1000 \( \mu \)M of curcumin. All images were taken at 10 × magnification with Olympus inverted microscope.]
ROS, SOR and LPO—Treatment of A549 cells with curcumin at 0.1, 1, 10, and 50 µM concentrations increased ROS generation from 3.78% (control) to 6.54, 7.20, 10.2 and 20.1%, and SOR from 5.34% (control) to 8.78, 8.56, 11.10 and 33.8% respectively (Table 1). However, a significant increase was observed only at 50 µM concentration of curcumin. Formation of TBA reactive products, taken as the index of LPO, was not affected up to 10 µM concentrations of curcumin. Like ROS and SOR, significant increase in LPO by 64.4% (from 0.411±0.058 to 0.676±0.064 nmoles TBARS/mg protein), was observed after treatment of cells only at 50 µM concentration of curcumin (Table 1).

Levels of GSH, GSSG and γ-GCS activity—To determine the redox status of the cells GSH, GSSG and their ratios were determined (Table 1). Incubation of cells with 10 and 50 µM curcumin concentrations significantly increased GSH by 55.51 and 29.56% from 732 ± 25.16 (control) to 1138.40 ± 27.00 and 948 ± 42.32 nmoles GSH/mg protein respectively. Like GSH, GSSG level was also induced to an extent of 37% by 50 µM concentration of curcumin as compared to control. The GSH/GSSG ratio thus decreased significantly by 29% from 14.6 ± 0.360 (control) to 10.38 ± 0.652 at 50 µM concentration. Concomitantly, it was found that curcumin treatment increased the γ-GCS activity by 41 and 42% at 10 and 50 µM of its concentrations respectively (Table 1).

ROS, apoptosis and curcumin—To study the mode of action of curcumin and role of ROS in curcumin-induced apoptosis, we further used BSO (a GSH synthesis blocking agent) and antioxidants (GSH and NAC) (Fig. 2A). Treatment of cells with 50 µM concentration of curcumin significantly induced the ROS production which was significantly decreased
(from 100 ± 8.07 to 57.37 ± 3.01 MFI by using 100 µM concentration of NAC. However, BSO at 1mM concentration further increased curcumin–induced ROS production by almost two fold i.e. from 100 ± 8.07 to 201.23 ± 21.01 MFI with respect to curcumin alone. Further, treatment of cells with 1 mM GSH did not change the curcumin induced ROS levels in A549 cells. Interestingly, GSH failed to lower the curcumin induced caspase-3 activity in these cells (Fig. 2B).

**γ-GCS gene expression study and p38 and SAPK MAPK activities**—Curcumin showed significant increase in mRNA expression of γ-GCS at 10 µM and 50 µM concentrations (Fig. 3). Similarly, curcumin treatment at 0.1, 10 and 50 µM concentrations induced the expression of p38 kinase by 75, 96 and 120% respectively. However, the mRNA expression of SAPK was slightly but not significantly elevated at any of the curcumin concentrations.

**Discussion**

Many epidemiological and experimental studies have demonstrated the chemopreventive and anticancer potential of curcumin in various cancers. However, the mechanism of its anticancer action is poorly understood in the lung cancer cells. The present study highlighted the cytotoxic and apoptotic potential of curcumin and the role of GSH and cellular redox status of human lung adenocarcinoma epithelial cells (A549) in sensitizing these cells to undergo apoptosis. To explore the underlying mechanism of curcumin induced apoptosis, we assayed the free radical generation, cellular oxidant-antioxidant homeostasis, down-stream effector molecules such as kinases, activation of γ-GCS and caspase-3 activity in response to curcumin treatment. We found that curcumin-induced apoptosis in A549 cells involved the activation of caspase-3. The present results revealed that decreased GSH/GSSG ratio seems to be a crucial factor for the activation of MAPK signaling cascade by curcumin which leads to apoptosis by activation of caspase-3.

Curcumin-induced ROS generation has been reported in tumor cells such as rat histocytoma (AK-5), human renal carcinoma cell line (Caki cells) and human submandibular gland carcinoma (HSG). It has been reported that oxidative stress is an important mediator of apoptosis in normal as well as cancer cells and may vary in different cell types and in various conditions. Several workers have observed that antioxidants such as gallic acid, all-trans retinoic acid, and α-tocopherol succinate also induce apoptosis in other cancer cell lines by generation of ROS.

The treatment of A549 cells with curcumin led to induction of ROS and SOR production which was concomitantly accompanied by an increase in LPO. Further, curcumin is known to exhibit pro-oxidant activity at high concentrations and anti-oxidant activity at low concentrations. The present results also corroborate with these findings since significant generation of ROS and SOR occurred only when these cells were treated with curcumin at 50 µM concentration.
NAC has been known to quench the generation of SOR in the cellular system. After challenging the A549 cells with curcumin at a concentration of 50 μM, it was observed that the generation of SOR was significantly enhanced as compared to ROS generation (Table 1). Further, in these cells, the increased level of SOR was found to be abrogated significantly after treatment with NAC and not with GSH. This reflects that curcumin primarily causes oxidative stress in these cells through the generation of superoxide radicals. In addition, NAC also significantly neutralized the apoptotic effects of curcumin in these cells which further confirms that generation of oxidative stress could be the underlying cause of cell death. Tan et al. have recently reported that ROS is involved in curcumin-induced cell cycle arrest and apoptosis in Human acute promyelocytic Leukemia HL-60 Cells via MMP changes and caspase-3 activation and caspas have been reported to be activated during apoptosis in many cell systems and are believed to play critical roles in both initiation as well as execution of apoptosis. However, on the contrary, a report from Senft et al. have suggested that anticancer activity of curcumin against malignant gliomas is attributed to its anti-proliferative activity and not its ability to induce apoptosis. From the above studies including the present one, it appears that the anti-cancer property of curcumin is highly cell specific which probably is regulated by its cellular concentration.

Curcumin behaves as a pro-oxidant in the lung malignant cells since it undergoes autoxidation to produce superoxide anions, hydrogen peroxide and hydroxyl radicals via Fenton chemistry involving transition metals which have been found to be increased in tumors. Intracellular phenoxy radicals (redox-cycling phenols) formed endogenously further induce LPO and co-oxidize GSH or nicotinamide adenine dinucleotide (NADH) to form thyl and phenoxyl radicals which result in extensive oxygen uptake and further lead to formation of superoxide radical anion.

Many chemotherapeutic agents including natural polyphenols have been reported to have profound effects on cellular redox status which play an important role in the induction of apoptosis in cancer cells. Moreover, the ratio of reduced and oxidized form of glutathione has been found to serve as a good indicator of cellular redox homeostasis. GSH, a major low molecular weight thiol in cells has been suggested to control intracellular thiol disulfide redox state essential for normal redox signaling and is involved in the protection of normal as well as cancer cells against oxidant-induced injury. In the present study, although curcumin increased both the intracellular GSH and GSSG levels concomitantly in these cells, there was a significant decrease in GSH/GSSG ratio after treatment with curcumin at 50 μM concentration. This was probably due to enhanced GSSG content as compared to GSH levels.

The decrease in GSH/GSSG ratio during oxidative stress appears to activate several signaling pathways including MAPKs which are likely to be responsible for apoptosis induction in response to curcumin treatment. In the present study, curcumin treatment significantly increased p38 MAPK activity in A549 cells, which is also an indication of enhanced oxidative stress. Our results are in conformity with the recent report by Bian and his colleagues. The curcumin-induced apoptosis in A549 cells through ROS-dependent mitochondrial signaling pathway appears to be due to sustained phosphorylation and activation of p38. However, marginal though not significant induction of SAPK in this study suggests that both SAPK and p38 kinases may act differentially on the same cellular system depending upon the nature and magnitude of the stress induced.

In conclusion, curcumin has been found to induce oxidative stress which appears to regulate apoptosis in human lung adenocarcinoma A549 cells. The apoptotic behaviour of curcumin is in part because of its pro-oxidant activity which may be p38 and SAPK dependent and is also regulated by the redox status of the cells.

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References


