Curcumin Induces Human Colon Cancer Cell death via p62/SQSTM1 Degradation, Phospho-ERK Up-regulation and Ceramide Generation

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Abstract
Curcumin is a natural yellow phenolic compound extracted from the Indian spice, turmeric (Curcuma longa). Several studies demonstrated the ability of curcumin to inhibit events associated with the promotion of cancer. We investigated the effects curcumin on human colon cancer cells in vitro and further examined the molecular mechanisms of curcumin induced cell death. The signaling adapter p62/SQSTM1, a multifunctional protein implicated in autophagy, apoptosis, cell signaling pathways and tumorigenesis, is one of the potential targets for anti-cancer therapy. In this study, we demonstrate a dose and time-dependent down-regulation of p62/SQSTM1 expression by curcumin that correlates with increase in the loss of viability of human colon cancer cells. We also found that curcumin enhanced phospho-ERK expression and ceramide (Cer) generation in human colon cancer cells. However, the present study also shows that, curcumin-induced p62/SQSTM1 degradation, up-regulation of ERK phosphorylation, Cer generation and cell death can be reversed by extracellular anti-oxidants such as glutathione (GSH) and N-acetyl cysteine (NAC). Overall, our results suggest that down regulation of p62/SQSTM1 and up-regulation of phospho-ERK and Cer generation may contribute to the anti-proliferative effects of curcumin against human colon cancer cells.

Keywords: p62/SQSTM1, phospho-ERK, Curcumin, Ceramide, Apoptosis, GSH

Introduction
Curcumin, a well-known chemo-preventive agent, has been shown to possess anti-inflammatory and anti-oxidant activities (1). It also has been reported as a potent inhibitor of mutagenesis and carcinogenesis (2). The anti-cancer property of curcumin has been extensively investigated in various cancer cells and in different laboratory animal models of cancer. Curcumin was found to inhibit cellular proliferation, and enhance apoptosis in a variety of human cancer cell lines in vitro (3). Currently, curcumin is in clinical trials for the treatment of cancers of pancreas, colon and multiple myeloma (3). The proposed mechanism of anti-tumor action of curcumin in majority of these studies involves suppression of NF-kB related gene products expression (4). Previously, we have shown that curcumin induces generation of reactive oxygen species (ROS) which leads to caspase dependent and independent apoptosis (5). We have also reported the modulation of curcumin-induced apoptosis by using PI3K inhibitor in breast carcinoma cell lines (6).

The signaling adapter p62/SQSTM1 is a multifunctional protein implicated in autophagy, apoptosis, cell signaling pathways and

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tumorigenesis (7,8). p62/SQSTM1 was initially believed as interacting partner of atypical protein kinase C (aPKC). Recent studies reveal that p62/SQSTM1 act as a prime signaling molecule through its ability to recruit and oligomerize important signaling proteins in the cytosolic speckles to control cell survival and apoptosis (9,10). Recently, it has also been reported that elimination of p62/SQSTM1 is critical for the autophagy mediated suppression of tumorigenesis. These discoveries shed light on the significant role of p62/SQSTM1 as a central player in the life and death decisions of the cell.

Ceramide (Cer), a tumor suppressor lipid has been shown to exert potent growth suppressive effect on a variety of cell types (11). It has been reported that diverse array of stressors, including TNF-α (12), Fas ligation (13), irradiation (14), heat shock, (15) and anti-cancer drugs (16) were able to increase intracellular Cer level leading to the induction of apoptosis. Ceramide is produced by de novo synthesis in the endoplasmic reticulum or by the hydrolysis of sphingomyelin by acid sphingomyelinases (localized in the acidic compartment) and neutral sphingomyelinases (localized in the plasma membrane and mitochondria). Recently, we have reported curcumin induced caspase dependant Cer generation and apoptosis in human leukemic cells (17).

Extracellular signal-related kinase (ERK) is a family of mitogen-activated protein kinases (MAPKs) that are activated through a sequential phosphorylation cascade that amplifies and transduce signals from the cell membrane to the nucleus. Depending on the cell types and stimulus, ERK activity will mediate different tumor suppressive events, such as apoptosis, autophagy and senescence in vitro and in vivo (18). It has been reported that ERK can promote both intrinsic and extrinsic apoptotic pathways (19). Moreover, it has been established that sustained activation of ERK can induce autophagic cell death (20,21).

In the present study, we investigated the molecular mechanism of the anti-tumor potential of curcumin towards human colon tumor cell lines. The present study demonstrates the effects of curcumin on p62/SQSTM1, ERK and Cer generation in vitro. The results indicate that curcumin is potent regulator of p62/SQSTM1 expression. Curcumin induces degradation of p62/SQSTM1, up-regulation of ERK phosphorylation and Cer generation, which are the prime cellular signaling regulatory molecules. We also showed that curcumin-induced p62/SQSTM1 degradation, up-regulation of ERK phosphorylation, Cer generation and cell death were inhibited by the extracellular administration of glutathione (GSH) and N-acetyl cysteine (NAC).

Materials and Methods

Cell line, cell culture conditions and drug treatment: The HT-29 cells (ATCC, Rockville, MD, USA) were grown in McCoy’s 5A containing GlutaMAX medium supplemented with 10% (V/V) heat inactivated fetal bovine serum (FBS). Cells were grown without antibiotics in an incubator containing humidified atmosphere of 95% air and 5%CO2 at 37°C. Curcumin (Sigma Chemical Co. St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 mM and was stored in a dark colored bottle at -20°C. The stock was diluted to the required concentration with DMSO when needed. Prior to curcumin treatment, cells were grown to about 80% confluence, and then exposed to curcumin at different concentrations (0-100 μM) and for a different period of time (0-24 h). Cells grown in a medium containing an equivalent amount of DMSO served as control. Glutathione, NAC, U0126 and TPA were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell viability Assay: Cell viability assay was carried out as described elsewhere with slight modifications (22). Cells were grown in 96 well microtiter plates (10,000 cells/well) and incubated for 24 h with or without different concentrations of curcumin. At the required time point, 100 μl media were removed and 25 ml of MTT (5 mg/ml) was added to each well. The plates were incubated for further 4 h at 37°C.
the plates were centrifuged at 1500 rpm for 5 min and the media were removed from all the wells. The formazan crystals were then solubilized in a 200 µl of DMSO. The colored solution was quantified at 570 nm by using 96 well plate reader (Perkin Elmer spectrofluorometer, Victor 3X). The viability was expressed as a percentage over control.

**Protein lysate preparation and western blot analysis:** Cells were washed twice with phosphate buffered saline (PBS) and lysed in a RIPA lysis buffer [50 mM Tris HCl (pH 7.4), 1% NP-40, 40 mM NaF, 10 mM NaCl, 10 mM NaVO₄, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM dithiothreitol (DTT) and EDTA-free protease inhibitor tablets per 20 ml buffer]. The cell lysates were centrifuged at 14000 rpm for 15 min. Total protein, determined by Bio-Rad protein assay, were mixed with 6X loading buffer and boiled at 100ºC for 3 min. SDS-PAGE and Western blot analyses were carried out as described previously [23]. The following antibodies were used: Anti-actin, anti-ERK1/2, anti-p62 and donkey anti-goat IgG antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-PARP and anti-phospho ERK1/2 were from Cell Signaling Technology. Anti-rabbit IgG and anti-mouse IgG were purchased from Sigma chemicals Co (St. Louis, MO, USA).

**Intracellular Cer measurement:** Intracellular Cer measured as described previously with little modification (24). After treatment cells were washed in PBS and lysed 50 mM Tris (pH-7.4) containing 0.4% IGEPAL CA 630 by freeze and thaw method. The final concentration of IGEPAL CA 630 in the assay was 0.2%. The lysate were heat at 70°C for 5 min in a water bath and centrifuged at 12000 rpm for 10 min at 4°C. The reaction was started by adding 10 µl of supernatant in the tube containing 20 ng recombinant human neutral ceramidase enzymes (10 µl) for 1 h at 37°C. The reaction was stopped by adding 55 µl of stopping buffer (1:9, 0.07 M potassium hydrogen phosphate buffer: methanol). The released SPH was derivatized with o-phthalaldehyde (OPA) reagent. After stopping the reaction add 25 µl of freshly prepared OPA reagent (12.5 mg OPA dissolved in 250 µl ethanol and 12.5 µl 2-µ-mercaptoethanol and made up to 12.5 ml with 3% (w/v) boric acid) was added. The mixture was allowed to stand for 30 min. An aliquot of 25 µl was injected in the HPLC. HPLC analysis was done using Waters 1525 binary pump system. Waters XTerra C18 column (5 µm, 3 mm x 250 mm) was equilibrated with a mobile phase (20% methanol, 80% 1:9, 0.07 M potassium hydrogen phosphate buffer: methanol) at a flow rate of 0.5 ml/min. The fluorescence detector (Waters 2475) was set at an excitation wavelength of 340 nm and an emission wavelength of 455 nm.

**Results**

**Curcumin induces degradation of p62/SQSTM1, Cer generation and cell death in HT-29 cells:** Recently, it has been reported that p62/SQSTM1 is a multi-domain protein plays a central role in life and death decisions of the cell [7]. Therefore, we first examined the involvement of p62/SQSTM1 in human colon cancer cell line, HT-29 up on curcumin treatment. Curcumin treatment of HT-29 cells resulted in a dose (0-100 µM) dependent suppression of p62/SQSTM1 (Fig 1A). We then examined the optimum time required for the down-regulation of p62/SQSTM1 and found that 6 h incubation with curcumin was sufficient for the maximum suppression p62/SQSTM1 (Fig 1B).

Ceramide has been suggested as a tumor suppressor lipid, and its generation is induced exclusively by apoptotic insult and not during growth stimulation. Therefore, we next examined the involvement of Cer generation in HT-29 cells up on curcumin treatment. Curcumin treatment of HT-29 cells resulted in a dose (0-100 µM) dependent reduction in the expression of p62/SQSTM1 (Fig 1A). We then examined the optimum time required for the down-regulation of p62/SQSTM1 and found that 6 h incubation with curcumin was sufficient for the maximum suppression p62/SQSTM1 (Fig 1B).
Fig. 1. Curcumin induces p62/SQSTM1 degradation, Cer generation and apoptosis in HT-29 cells. (A) HT-29 cells were treated with 0-100 μM concentration of curcumin for 24 h, (B) HT-29 cells were treated with 75 μM curcumin for indicated time period. The expression of p62/SQSTM1 was measured by using Western blot analysis. Actin was used as the loading control. Blots shown here are representative of three independent experiments. (C) HT-29 cells were treated with 0-100 μM concentration of curcumin for 24 h, (D) HT-29 cells were treated with 75 μM curcumin for indicated time period. The Cer levels were measured as described in the “materials and methods”. Data represent the mean ± S. D (n=3). (E) HT-29 cells were treated with 0-100 μM concentration curcumin for 24 h and cell viability was measured using MTT assays as described in the “materials and methods”. Data represent the mean ± S. D (n=3). (F) HT-29 cells were treated with 75 μM curcumin for indicated time period and PARP cleavage was measured by Western blot analysis. Actin was used as the loading control. Blots shown here are representative of three independent experiments.
1h after treatment and sustained up to 24 h (Fig 1D).

In order to check whether p62/SQSTM1 degradation and rapid Cer generation induced by curcumin would lead to loss of cell viability, MTT assay was performed. Curcumin caused a dose dependent reduction in cell viability (Fig 1E). The cytotoxic effect of curcumin was, at least in part, attributable to apoptosis, as evidenced by PARP cleavage in HT-29 cells (Fig 1F).

Curcumin induces sustained ERK activation in HT-29 cell lines: The ERK signaling pathway has been shown to be activated in response to certain cellular stresses (18). Hence, we checked the effect curcumin on the ERK activation. HT-29 cells were exposed to curcumin and activation of ERK was determined by Western blot analysis using antibodies against phosphorylated form of ERK. Curcumin induces both dose and time dependent strong activation ERK (Fig 2A and B). The activation was apparent 1h after curcumin

![Fig. 2](image-url)

Curcumin induces up-regulation of phospho-ERK in HT-29 cells. (A) HT-29 cells were treated with 0-100 μM concentration curcumin for 24 h, (B) HT-29 cells were treated with 75 μM concentration of curcumin for indicated time period and the expression of phospho-ERK was measure using Western blot analysis. Blots shown here are representative of three independent experiments. (C) HT-29 cells were pre-treated with 20 μM concentration of U0126 and (D) 50nM TPA for 1h, followed by incubation with 50 μM curcumin for 24h and cell viability assay was performed as described in the “materials and method”. Role of p62/SQSTM1 in curcumin-induced cell death
treatment and sustained up to 24 h (Fig 2B). The increase in phospho-ERK did not result from the increased ERK expression, as total ERK levels were not altered relative to untreated cells.

In order to check whether ERK activation is required for curcumin induced apoptosis, we used U0126, a selective inhibitor of ERK (25). HT-29 cells were pre-treated with 20 µM concentration of U0126 for 1h prior to the addition of curcumin and viability assay was performed using MTT assay. As shown in the Fig 2C, exposure of curcumin alone to the cell resulted in a significant decrease in the viability and its effect was partially reversed by the U0126. Next, we have used phorbol ester TPA, an agent that capable of stimulating ERK signaling pathway (26, 27). HT-29 cells were pre-treated with 50 nM concentration of TPA for 1h prior to the addition of curcumin and viability assay was performed using MTT assay. As shown in the Fig 2D, TPA pre-treated cells were much more sensitive to curcumin. These results suggest that ERK activation plays an important role in curcumin induced HT-29 cell death.

Glutathione and NAC block curcumin induced down-regulation of p62/SQSTM1 and cell death in HT-29 cells: The antioxidants GSH and NAC play an important role in scavenging reactive oxygen species and the detoxification process (5). The exact molecular mechanism of the protective effects of these antioxidants against curcumin-induced apoptosis is not known. Therefore, we investigated whether GSH and NAC can protect cells against curcumin-induced p62/SQSTM1 down regulation and cell death in HT-29 cells. Pre-treatment of HT-29 cells with 10 mM GSH and NAC strongly suppressed the curcumin-induced degradation of p62/SQSTM1 (Fig. 3A) and cell death (Fig. 3B). These results indicate that the involvement oxidative stress in curcumin-induced HT-29 cells death.

Effects of GSH and NAC on curcumin induced up-regulation of phospho-ERK and Cer generation in HT-29 cells: In order to evaluate the protective mechanism(s) of GSH and NAC on curcumin-induced cell death, the influence of GSH and NAC on curcumin-induced ERK phosphorylation and Cer generation was examined. As shown in the Figure 4A and 4B, pre-treatment of the cells with GSH and NAC inhibited curcumin-induced ERK phosphorylation and Cer generation. These findings indicate that GSH and NAC may, indeed, mediate its anti-apoptotic effects via inhibition of ERK phosphorylation and Cer generation in curcumin-induced HT-29 cell death.

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Curcumin is one of the major anti-cancer drugs widely used in the treatment of wide variety of cancers. However, the signalling pathways triggered by curcumin in human colon cancer cells are not completely understood. The present study was designed to investigate the mechanism by which curcumin mediates its anti-proliferative effects in human colon cancer cells. In this study, we specifically focused on p62/SQSTM1, ERK and Cer, which are some of the prime cellular signalling regulatory molecules. Our data shows that, the ability of curcumin to inhibit the proliferation of human colon cancer cells correlated with the down-regulation of p62/SQSTM1 expression. Curcumin induced down-regulation of p62/SQSTM1 and cell death was reversed by anti-oxidants such as GSH and NAC. We found that curcumin enhanced the expression of phospho-ERK and the generation of tumor suppressor lipid, Cer. In sum, these results suggest that down-regulation of p62/SQSTM1, up-regulation of phospho-ERK and Cer generation contribute to the antiproliferative effect of curcumin in HT-29 human colon cancer cells.

This is first report to show that curcumin can induce the degradation of p62/SQSTM1. p62/SQSTM1 is a ubiquitin-binding multifunctional protein, which promotes survival-critical signals including proliferation, differentiation, and induction of anti-apoptotic genes (28). p62/SQSTM1 interacts with a central component of autophagy of the autophagy machinery, LC3, and transports ubiquitinated proteins to degradation by the autophagosome (29). It has been reported that p62/SQSTM1 is a selective substrate for autophagy. Abnormal expression of p62/SQSTM1 has been documented in various cancers including gastrointestinal, prostate and breast cancers (30,31). Moreover, knock down of p62/SQSTM1 sensitizes SKOV3/DDP ovarian cancer cell to cisplatin (32). Previously, we have shown that anti-oxidants such as NAC and GSH tightly controls curcumin induced cell death in mouse fibroblast L929 cells (5) and human leukemic cells such as Jurkat, Molt-4 and K562 (17). Reversal of the effect of curcumin on p62/SQSTM1 by anti-oxidants suggests the role of oxidative stress in this pathway.

We found that curcumin up-regulated the expression of phospho-ERK in human colon cancer cell. Our data demonstrate that, the activation of ERK plays an important role in

**Fig. 4.** Effects of GSH and NAC on curcumin induced up-regulation of phospho-ERK and Cer generation. HT-29 cells were pre-treated with 10 mM concentration GSH and NAC for 1h, followed by incubation with 75 µM curcumin. (A) Expression of phospho-ERK was measure using Western blot analysis. Blots shown here are representative of three independent experiments. (B) Cellular lipids were extracted and assayed for Cer by HPLC method as described in the “materials and method”.

**Discussion**

Curcumin is one of the major anti-cancer drugs widely used in the treatment of wide variety of cancers. However, the signalling pathways triggered by curcumin in human colon cancer cells are not completely understood. The present study was designed to investigate the mechanism by which curcumin mediates its anti-proliferative effects in human colon cancer cells. In this study, we specifically focused on p62/SQSTM1, ERK and Cer, which are some of the prime cellular signalling regulatory molecules. Our data shows that, the ability of curcumin to inhibit the proliferation of human colon cancer cells correlated with the down-regulation of p62/SQSTM1 expression. Curcumin induced down-regulation of p62/SQSTM1 and cell death was reversed by anti-oxidants such as GSH and NAC. We found that curcumin enhanced the expression of phospho-ERK and the generation of tumor suppressor lipid, Cer. In sum, these results suggest that down-regulation of p62/SQSTM1, up-regulation of phospho-ERK and Cer generation contribute to the antiproliferative effect of curcumin in HT-29 human colon cancer cells.

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We found that curcumin up-regulated the expression of phospho-ERK in human colon cancer cell. Our data demonstrate that, the activation of ERK plays an important role in
curcumin induced cell death in HT-29 cells. Curcumin treatment resulted in high and sustained activation of ERK in these cells. Several other studies also reported that, the activation of ERK leads to increased sensitivity of cancer cells to chemotherapeutic drugs (19,33). Utilizing U0126 and TPA, to modulate ERK activity, we found that down regulation of ERK resulted in partial protection against curcumin-induced cell death, whereas, enhancement ERK activity sensitized it. In our study, the ERK inhibitor did not completely prevent curcumin induced cell death. This may be due to the involvement of other signaling pathways that are independent ERK activation. In the present study, the antioxidants GSH and NAC inhibited curcumin-induced activation ERK, suggesting that, the curcumin-induced activation of ERK is mediated by oxidative stress dependent mechanisms.

In the present work, we demonstrate that Cer production parallels the sensitivity of curcumin, based on the loss of cell viability levels. Consistent with our results, sensitivity of curcumin to various cancer cells including HCT 116 colon cancer cells (34), Jurkat leukemic cells (17), PC3 prostate cancer cells (35) was directly related to the Cer generation. Moreover, recently it has been reported that, C2-Cer sensitizes melanoma calls to curcumin-induced cell death (36). Furthermore, a direct relationship has been observed between resistant to radiation induced apoptosis and defective Cer generation (37). Our results are in agreement with the fact that the kinetics of cell death and Cer generation parallel each other, suggests an involvement of Cer in curcumin-induced cell death in HT-29 cells.

Conclusion

In conclusion, our results provide an additional mechanism through which curcumin may mediate its antiproliferative effects in HT-29 cells, via down-regulation of p62/SQSTM1, up-regulation of phospho-ERK and Cer generation. These results suggest that p62/SQSTM1 could be a novel therapeutic target for cancer treatment using curcumin.

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References


