RESEARCH COMMUNICATION

Effects of Water-soluble Antioxidants and MAPKK/MEK Inhibitor on Curcumin-induced Apoptosis in HL-60 Human Leukemic Cells

Ratana Banjerdpongchai[1], Prapon Wilairat [2]

Abstract

Curcumin is the main biologically active phytochemical compound in turmeric. It has been shown to have anticarcinogenic activity. The aims of the study were to identify the mechanism of apoptosis of HL-60 human promyelocytic leukemic cells induced by curcumin and to determine the effects of water-soluble antioxidants, ascorbic acid, Trolox (a water-soluble form of vitamin E), glutathione (GSH) and N-acetylcysteine (NAC) on this process. HL-60 cells were incubated with curcumin for 24 h and apoptotic cells were quantitated by flow cytometry following staining with annexin V-FITC and propidium iodide. Curcumin-treated HL-60 cells produced reactive oxygen species as detected by the dichlorofluorescein fluorescent assay. Apoptosis occurred via the mitochondria pathway as curcumin reduced mitochondrial membrane potential in a dose-dependent manner. In the presence of 10 μM curcumin, vitamin C (56 nM – 5.6 μM) inhibited apoptosis of HL-60 cells; GSH at low concentration (1 μM) reduced apoptosis but had no effect at higher concentrations (10, 100 μM); and Trolox and NAC at 10 and 100 μM, respectively, enhanced apoptosis, but this effect was abolished at higher concentration (1 mM) of NAC. MAPKK/MEK inhibitor PD98059, enhanced curcumin-induced HL-60 apoptotic cell death.

Key words: apoptosis - curcumin - HL-60 cells - MAPKK/MEK - reactive oxygen species - antioxidant

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Introduction

Curcuma longa Linn., belonging to the family *Zingiberaceae*, is a perennial herb cultivated widely in tropical regions of Asia, from whose dried rhizome is isolated the spice turmeric that is used for a variety of medicinal purposes, such as an antiseptic, a cure for poisoning and skin diseases including wound healing, to eliminate body waste products, for treating dyspepsia and respiratory disorders, and as a household remedy for treating sprains and swellings caused by injury (Ammon and Wahl, 1996).

Curcumin, also known as diferuloylmethane (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), is the major yellow pigment extracted from turmeric. Its properties as a coloring and flavoring agent have led to its use as a dietary additive in variety of foods (Ammon and Wahl, 1996; Lin et al., 2000). As a result, dietary intake of curcumin is especially high in Asia, where adults consume up to 200 mg of curcumin/day or up to 7-8 μmol/kg of body weight. Even in France, where curcumin consumption may be more representative of that in non Asian regions, intake of as much as 3.4 μmol/kg/day has been documented (Verger et al., 1998).

Curcumin shows a variety of physiological effects, and several studies indicate that curcumin is anticarcinogenic (Conney et al., 1991) and anti-inflammatory (Huang et al., 1991). Several reports have documented the antiproliferative effects of curcumin on cultured cancer human cells, including prostate, promyelocytic leukemic (HL-60), basal cell carcinoma, melanoma, colon and breast (Mukhopadhyay et al., 2001; Anto et al., 2002; Jee et al., 1998; Bush et al., 2001, Van Erk et al., 2004; Squires et al., 2003). Curcumin-induced apoptotic cell death involves production of reactive oxygen species (ROS) (Bhaumik et al., 1999, Yoshino et al., 2004). Generation of ROS can result in mitochondrial abnormalities that promote p53-dependent apoptosis via activation of caspase-8 and caspase-3, as reported in curcumin-treated human prostate, HL-60, basal cell carcinoma and melanoma cells (Mukhopadhyay et al., 2001; Anto et al., 2002; Jee et al., 1998; Bush et al., 2001).

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In this study, we determined the mechanism of curcumin-induced apoptosis in HL-60 cells by measuring mitochondrial membrane potential. The effects of four water-soluble antioxidants, namely, ascorbic acid, glutathione (GSH), N-acetylcysteine (NAC) and Trolox (water-soluble vitamin E) on curcumin-induced apoptosis of HL-60 cells were evaluated. As induction of apoptosis by curcumin in breast cells involves mitogen activated protein kinase (MAPK) signaling pathway (Squires et al. 2003), to gain further insight into the mechanism of action of curcumin, the effect of PD98059, an inhibitor of the activator of MAPK, MAPKK/MEK, was also studied.

Materials and Methods

RPMI-1640 and fetal bovine serum were obtained from Gibco-BRL, New York, NY, USA, annexin V-FITC kit from Roche, Indianapolis, IN, USA, propidium iodide (PI), curcumin, glutathione, N-acetylcysteine, ascorbic acid, 2',7'-dichlorofluorescein diacetate (DCFH-DA) and 3,3'-dihexyloxacarbocyanine iodide ($DiO_6(3)$) from Sigma, St. Louis, MD, USA, Trolox from Aldrich, Milwaukee, WI, USA, and PD98059 (MAPKK/MEK inhibitor) from Calbiochem, La Jolla, CA, USA.

Cell Culture and Treatment

HL-60 human promyelocytic leukemic cells were cultured in 10% fetal bovine serum in RPMI-1640 medium supplemented with penicillin G (100 units/ml) and streptomycin (100 µg/ml) at 37˚C in a humidified atmosphere containing 5% CO_2. The preconfluent (growth phase) HL-60 cells ($1x10^6$ cells) were treated with curcumin and antioxidants for 24 h and then processed for flow cytometry analysis. Stock curcumin solution was made up in alcohol with final concentration never exceeding 0.5%, which had no effect on cell viability in culture. Antioxidants at the required concentrations were added at the same time as curcumin. The concentrations of antioxidants used were nontoxic to HL-60 under the treatment conditions. HL-60 cells were pretreated with PD98059 for 50 min and then with and without 20 µM of curcumin for 4 h.

For measurement of mitochondrial membrane potential and intracellular ROS, either 40 nM of $DiOC_6(3)$ (for mitochondrial membrane potential) or 5 µM of DCFH-DA (for ROS detection) were added for 15 min at 37˚C and the cells were then subjected to flow cytometry.

Flow Cytometry

Cells were washed once in phosphate-buffer-saline solution, centrifuged at 200 x *g* and the cell pellet was suspended in 100 µl of binding buffer provided by the the annexin V-FITC reagent kit. Annexin V-FITC (2 µl) and PI (2 µl) were added and the cell suspension was left at room temperature for 15 min in the dark. Finally 900 µl of binding buffer were added. Analysis was conducted using FACScan (Becton Dickinson, USA). Cells that were stained with annexin V-FITC and annexin V-FITC together with PI were designated as apoptotic cells.

Statistical Analysis

Duplicate tests were performed in 3 independent experiments and analyzed by Kruskal Wallis analysis. If p value of Kruskal Wallis was less than 0.05 then each value was then compared to control by using the Mann-Whitney U test, with p value <0.05 considered as significant.

Results and Discussion

Curcumin induced HL-60 cells to undergo apoptosis in a dose-dependent manner (5-25 µM) (data not shown) in agreement with our previous report (Banjerdpongchai et al., 2002). Curcumin acted through the production of ROS, as measured by the production of the more fluorescent DCF (oxidized form of dichlorofluorescein) from DCFH (Fig. 1). ROS produced by 10 µM curcumin was nearly twice that seen with hydrogen peroxide at the same concentration.

Curcumin caused the reduction in mitochondrion membrane potential in a dose-dependent manner (Fig. 2), as has been shown in other cells, viz. human prostate cancer cells, acute myelogenous leukemia cells, human basal cell carcinoma cells, and human melanoma cells (Mukhopadhyay et al., 2001; Anto et al., 2002; Jee et al., 1998; Bush et al., 2001). The decrease in mitochondrial membrane potential by the addition of curcumin to mouse neuro2a cells has recently been demonstrated to be mediated by the impairment to ubiquitin-proteasome system (Jana et al., 2004).

The effects of water-soluble antioxidants on apoptosis of HL-60 cells induced by 10 µM curcumin depended on the nature of the antioxidant tested (Fig. 3). Complete protection against curcumin-induced apoptosis was observed with 56 nM ascorbic acid, a phenomenon that remained unchanged even when a hundred fold higher concentration

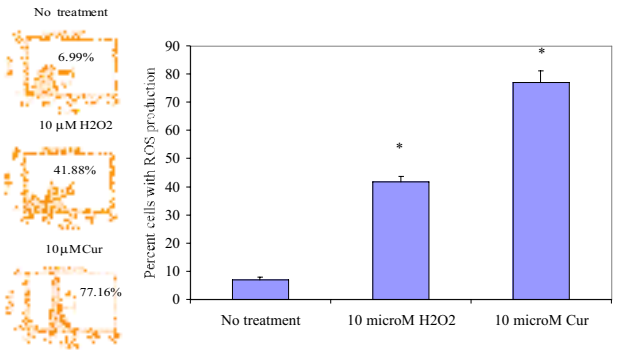

Figure 1. Generation of Reactive Oxygen Species (ROS) in HL-60 Cells by Curcumin. HL-60 cells were treated with 10 µM curcumin or 10 µM H_2O_2 (positive control) for 4 h, incubated with 5 µM of DCFH-DA for 15 min and subjected to flow cytometry. The left panels shows representative histograms from flow cytometry and cells with increased fluorescence, indicating presence of ROS, are located in the region designated M1. Mean and SEM of 3 independent experiments in duplicate are shown. (*) indicates significant difference. Cur: curcumin.

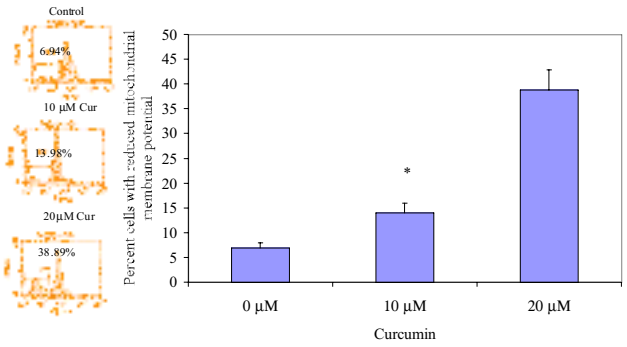

Figure 2. Dose-response of Curcumin on Reduction of Mitochondrial Membrane Potential of HL-60 Cells. HL-60 cells were treated for 24 h with various concentrations of curcumin as indicated, incubated with 40 nM of $DiOC_6(3)$ for 15 min and subjected to flow cytometry. The left panels shows representative histograms from flow cytometry and cells with reduced mitochondrial membrane potential are located in the region designated M1. Percent cells with reduced mitochondrial membrane potential are plotted against curcumin concentration. Mean and SEM of 3 independent experiments in duplicate are shown. (*) indicates significant difference. Cur: curcumin.

of this antioxidant was added (Fig. 3A). Previous study has shown that HL-60 cells undergo apoptosis when treated with high concentrations (0.25-1 mM) of vitamin C alone for 24 hours, due to its prooxidant property (Park et al., 2004). At the much lower concentrations used in this study, vitamin C acts as an antioxidant in protecting HL-60 cells against oxidative damage of curcumin.

Trolox (a water-soluble form of vitamin E) enhanced HL-60 cell apoptosis at 10 and 100 μM as shown in Fig. 3B. The study for Trolox could not be performed at higher concentrations as 1 mM Trolox was toxic to HL-60 cells, possibly due to its prooxidant property. This is an unexpected finding and there is no explanation for this phenomenon.

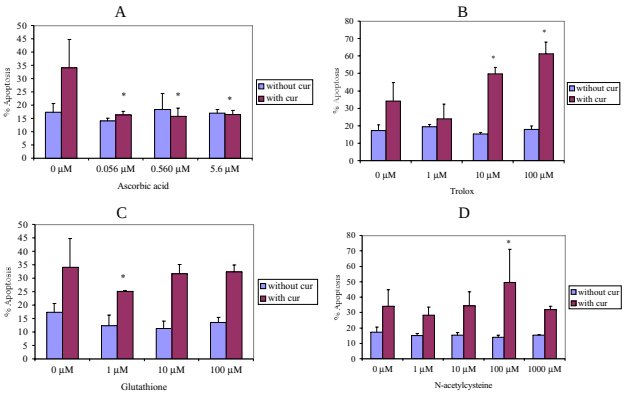

Figure 3. Dose Effects of Water-soluble Antioxidants on Curcumin-induced Apoptosis of HL-60 Cells. HL-60 cells were incubated for 24 h with 10 μM curcumin in the presence of ascorbic acid (A), Trolox (B), glutathione (C) and N-acetylcysteine (D) at concentrations indicated. Percent apoptotic cells were determined by flow cytometry. Mean and SEM of 3 independent experiments in duplicate are shown. (*) indicates significant difference from antioxidant-minus control (curcumin treatment alone).

The addition of 1-100 μM GSH produced significant reduction in curcumin-induced apoptosis only at a dosage of 1 μM (Fig 3C). At this concentration GSH may help to maintain mitochondrial membrane potential as has been shown in TNF-alpha-induced HL-60 cell apoptosis (Liu et al., 2005).

NAC at 100 μM enhanced apoptosis (Fig. 3D), but this effect was abolished at higher concentration (1 mM) of NAC. It was reported that NAC (5 mM) was a potent inhibitor of hydrogen peroxide production and caspase-3 activation in homocysteine thiolactone-treated HL-60 cells (Huang et al., 2002).

Using HL-60 cells, Chen et al (2005) have demonstrated that ascorbic acid, GSH and NAC enhanced both the antioxidant and the anticancer activity at low level of curcumin. Chen et al (2005) measured the antioxidant effects against ROS generation by determining malondialdehyde production, which is less direct than the fluorescence assay used in our studies. Discrepancies between their and our observations need to be investigated further, but, nevertheless, these results indicate the potential role of antioxidants in improving the anticancer property of curcumin.

PD98059 (50 μM), an inhibitor of MAPKK/MEK, enhanced apoptosis of HL-60 cells induced by 20 μM curcumin as indicated by the increase in the number of cells that have reduced mitochondrion membrane potential (Fig. 4). This finding may help to explain why treatment of B16F10 murine melanoma cells with curcumin (15 μM) significantly inhibits focal adhesion kinase, an important downstream component of the intracellular MAPKK/MEK signaling pathway (Banerji et al., 2004). PD98059 may have a pharmacological role in enhancing apoptosis induced by curcumin.

In summary, our studies have shown the potential benefit of some, but not all, antioxidants in augmenting the apoptotic property of curcumin. However, further *in vivo* investigations are needed before any recommendations regarding antioxidant supplementations with curcumin can be made.

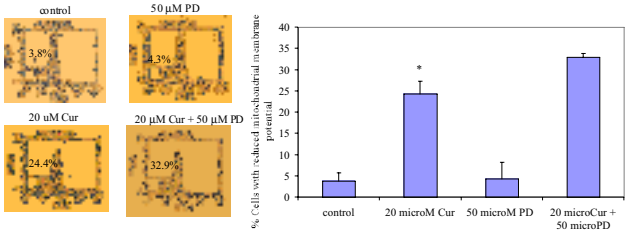

Figure 4. Stimulation of curcumin-induced apoptosis of HL-60 cells by MAPKK/MEK inhibitor PD98059. HL-60 cells were pretreated for 50 min with 50 μM PD98059, followed by incubation for 4 h with 20 μM curcumin and analyzed by flow cytometry for loss in mitochondrial membrane potential according to the procedure described in legend of Fig. 2. Mean and SEM of 3 independent experiments in duplicate are shown. (*) indicates significant difference for 20 microM Cur sample when compared to control, and for 20 microM Cur+50 micro M PD sample when compared to 20 micro PD. Cur: curcumin; PD: PD98059.

Acknowledgement

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