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Original Contribution

Glutathione regulates caspase-dependent ceramide production and curcumin-induced apoptosis in human leukemic cells

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ABSTRACT

Depletion of intracellular glutathione (GSH) is the prime hallmark of the progression of apoptosis. Previously, we reported that curcumin induces reactive oxygen species (ROS)-mediated depletion of GSH, which leads to caspase-dependent and independent apoptosis in mouse fibroblast cells (F. Thayyullathil et al., *Free Radic. Biol. Med.* **45,** 1403–1412, 2008). In this study, we investigated the antileukemic potential of curcumin in vitro, and we further examined the molecular mechanisms of curcumin-induced apoptosis in human leukemic cells. Curcumin suppresses the growth of human leukemic cells via ROS-independent GSH depletion, which leads to caspase activation, inhibition of sphingomyelin synthase (SMS) activity, and induction of ceramide (Cer) generation. Pretreatment of leukemic cells with carbobenzoxy-Val-Ala-Asp fluoromethylketone, a universal inhibitor of caspases, abrogates the SMS inhibition and Cer generation, and in turn prevents curcumin-induced cell death. Curcumin treatment of leukemic cells also downregulates the expression of the inhibitor of apoptosis proteins (IAPs), phospho-Akt, c-Myc, and cyclin D1. Extracellular supplementation with GSH attenuates curcumin-induced depletion of GSH, caspase-dependent inhibition of SMS, Cer generation, and downregulation of IAPs, whereas, L-D-buthionine sulfoximine, a widely used inhibitor of GSH synthesis, potentiates GSH depletion, Cer generation, and apoptosis induced by curcumin. Taken together, our findings provide evidence suggesting for the first time that GSH regulates caspase-dependent inhibition of SMS activity, Cer generation, and apoptosis induced by curcumin in human leukemic cells.

Curcumin has been reported to possess anti-inflammatory and antioxidant activities, and it has been reported as a potent inhibitor of mutagenesis and carcinogenesis [1,2]. The anti-cancer property of curcumin has been extensively investigated in various cancer cells and laboratory animal models of cancer. Curcumin was found to inhibit cellular proliferation and enhance apoptosis in a variety of in vitro leukemia cell line models [3–5]. The proposed mechanism of antileukemic action of curcumin in the majority of these studies involves suppression of NF-κB-related gene product expression. Other biological effects of curcumin include cell shrinkage, chromatin condensation, and DNA peroxidation [6]. Curcumin also induces endoplasmic reticulum stress, activation of Bax and caspases, and degradation of PARP in leukemic cells [7–9]. Moreover, curcumin induces inhibition of Akt, c-Myc, inhibitor of apoptosis proteins (IAPs), and specificity protein expression [10–12]. Recently, we reported the modulation of curcumin-induced apoptosis by using a PI3K inhibitor in breast carcinoma cell lines [13].

Ceramide (Cer), a tumor suppressor lipid, has been shown to exert potent growth-suppressive effects in a variety of cell types [14]. A diverse array of stressors, including TNF-α [15], Fas ligation [16], irradiation [17], heat shock [18], and anti-cancer drugs [19], were reported to increase intracellular Cer levels leading to the induction of apoptosis. Generation of Cer, directly or indirectly, through various enzymes, such as sphingomyelinase (SMase) [20], ceramidase (CDase) [21], ceramide synthase [22], sphingomyelin synthase (SMS) [23], and glucosyl ceramide synthase (GCS) [24], has been shown to play an important role in apoptosis. Ceramide synthesis occurs within the endoplasmic reticulum, and it is converted in the Golgi to sphingomyelin by SMS. Sphingomyelin synthase enzymes were identified as important regulators of the kinetics and magnitude of intracellular Cer accumulation, and they are inhibited under various stress conditions, which trigger Cer generation and apoptosis [23–25]. Previously, it was also shown that curcumin induces de novo Cer generation and apoptosis in human colon cells [26].

Abbreviations: ROS, reactive oxygen species; GSH, glutathione; NAC, *N*-acetylcysteine; BSO, L-D-buthionine sulfoximine; Cer, D-*erythro*-ceramide; SMS, sphingomyelin synthase; GCS, glucosyl ceramide synthase; PBS, phosphate-buffered saline; PARP, poly(ADP-ribose) polymerase; IAP, inhibitor of apoptosis protein; PAGE, polyacrylamide gel electrophoresis; z-VAD-fmk, *N*-benzoyloxycarbonyl-Val-Ala-Asp fluoromethylketone.

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Glutathione (GSH) is the most abundant nonprotein thiol in mammalian cells and acts as a reducing agent. Indeed, it is the major antioxidant within cells, maintaining a tight control of cellular redox status. Intracellular GSH depletion is an early hallmark in the progression of apoptosis in response to various cellular stresses [27]. It has been previously reported that depletion of GSH leads to Cer production and apoptosis in MCF-7 cells [28]. Glutathione depletion during apoptosis, induced by cytotoxic agents, has been reported to be mediated by GSH oxidation to GSSG by reactive oxygen species (ROS). Recently, we showed that curcumin induces ROS-dependent depletion of GSH, which leads to caspase-dependent and -independent apoptosis in L929 cells [29]. Moreover, high intracellular GSH levels have been associated with the apoptotic-resistant phenotype in several models of apoptosis [30].

Caspases are a family of cysteine-dependent aspartate-directed proteases that play a critical role in the initiation and execution of apoptosis. Caspase-dependent Cer generation has been proposed in several apoptosis models [23,31–33]. Moreover, it has been reported that cell-permeative Cer induced the cleavage and activation of caspase-3. Therefore, it has been proposed that caspases can act both upstream and downstream of Cer generation. The relationship between caspase activation and Cer production in apoptosis signaling is still not well understood. Caspase-regulated Cer generation has been proposed in Fas-induced apoptosis in human leukemic cells [23,34].

In this study, we investigated the molecular mechanism of the antileukemic potential of curcumin toward human leukemic cell lines. Our data provide the first evidence that curcumin induces apoptosis of human leukemic cells via a mechanism involving GSH depletion, which in turn leads to caspase-dependent Cer generation and apoptosis. We demonstrate that curcumin increases Cer generation via caspase-dependent inhibition of SMS in human leukemic cells. Curcumin-induced Cer generation and apoptosis were inhibited by extracellular GSH or *N*-acetylcysteine (NAC) and the caspase inhibitor z-VAD-fmk. Moreover, L-buthionine *S,R*-sulfoximine (BSO), a widely used inhibitor of GSH synthesis, potentiated GSH depletion, Cer generation, and apoptosis induced by curcumin. In addition, pretreatment with D-609, a putative SMS inhibitor, enhanced curcumin-induced Cer generation and induction of apoptosis. Thus, our results establish that the important enzyme regulating intracellular Cer concentration, namely, SMS, is under the control of curcumin-induced depletion of GSH and activation of caspases. We also examined the involvement of antiapoptotic proteins such as cyclin D1, c-Myc, IAPs, and Akt in the proapoptotic effect of curcumin in these leukemic cells.

Materials and methods

Reagents

Curcumin, glutathione, *N*-acetylcysteine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), glutathione assay kit, bovine liver catalase, superoxide dismutase, BSO, UDP–glucose, phosphatidylcholine, desipramine, phthaldialdehyde, anti-rabbit IgG, and anti-mouse IgG were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Oxidation-sensitive 2′,7′-dichlorofluorescin diacetate (D-399), insensitive 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (C-369), and C_6-NBD–sphingomyelin were purchased from Molecular Probes (Eugene, OR, USA). RPMI 1640 GlutaMAX medium, MEM GlutaMAX medium, and fetal bovine serum (FBS) were bought from GIBCO BRL (Grand Island, NY, USA). Fumonisin B1, myriocin, D-609, C_6-NBD–ceramide, and z-VAD-fmk were from Alexis (San Diego, CA, USA). Anti-actin and donkey anti-goat IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PARP, anti-Akt, anti-phospho-Akt (Ser^{473}), and anti-cyclin D1 were from Cell Signaling Technology (Beverly, MA, USA). Anti-XIAP, anti-cIAP1, and anti-cIAP2 were from BD Biosciences (San Jose, CA, USA).

Leukemic cell lines, cell culture conditions, and drug treatment

The cell lines Jurkat and Molt-4 (ATCC, Rockville, MD, USA) were grown in RPMI 1640 GlutaMAX medium supplemented with 10% (v/v) heat-inactivated FBS and K562 cells (ATCC) were grown in MEM GlutaMAX medium containing 10% FBS. All cell lines were grown without antibiotics in an incubator containing a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Curcumin 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. Before curcumin treatment cells were grown to about 80% confluence and then exposed to curcumin at various concentrations (0–100 μM) and for various periods of time (0–24 h). Cells grown in a medium containing an equivalent amount of DMSO without curcumin served as controls.

Cell viability assay

Cell viability assays were carried out as described previously with slight modifications [29]. Cells were grown in 96-well plates (10,000 cells/well) and they were incubated for 24 h with or without various concentrations of curcumin (0–100 μM). At the required time point 100 μl of medium was removed and 25 μl of MTT (5 mg/ml) was added to each well. The plates were incubated for a further 3 h at 37 °C. After incubation the plates were centrifuged at 1500 rpm for 5 min and the medium was removed from all the wells. The formazan crystals were then solubilized in 200 μl of DMSO. The colored solution was quantified at 570 nm by using a 96-well plate reader (PerkinElmer spectrofluorometer, Victor 3X). The viability was expressed as 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 Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM dithiothreitol and EDTA-free protease inhibitor tablets per 20 ml buffer). The cell lysates were centrifuged at 14,000 rpm for 15 min. Total protein, determined by Bio-Rad protein assay, was mixed with 6× loading buffer and boiled at 100 °C for 3 min. Samples at 40 μg/lane were resolved by SDS–PAGE and the separated proteins were transferred onto a nitrocellulose membrane by wet transfer using a Bio-Rad electrotransfer apparatus. After transfer, the blot was blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 and then incubated with primary antibodies followed by secondary antibody. Proteins were visualized using an enhanced chemiluminescence reagent.

Measurement of PARP cleavage

To determine the activation of caspase-3, we examined the cleavage of PARP as previously reported [29]. Forty micrograms of whole-cell extract was resolved on a 10% polyacrylamide gel, transferred to a nitrocellulose membrane, blocked with 5% nonfat milk protein, and probed with PARP antibodies (1:2000) followed by secondary antibodies, and proteins were detected using an enhanced chemiluminescence reagent.

Determination of total (reduced and oxidized) glutathione

For estimating glutathione, a kinetic assay was used with a continuous glutathione reductase-catalyzed reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to the chromophore, whose rate was