Curcumin Sensitizes Acute Promyelocytic Leukemia Cells to Unfolded Protein Response–Induced Apoptosis by Blocking the Loss of Misfolded N-CoR Protein

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Abstract

Acute promyelocytic leukemia (APL) is characterized by accumulation of apoptosis-resistant immature promyelocytic cells in the bone marrow and peripheral blood. We have shown that endoplasmic reticulum (ER)–associated degradation (ERAD) and protease-mediated degradation of misfolded nuclear receptor corepressor (N-CoR) confer resistance to unfolded protein response (UPR)-induced apoptosis in APL. These findings suggest that therapeutic inhibition of N-CoR misfolding or degradation may promote growth arrest in APL cells by sensitizing them to UPR-induced apoptosis. On the basis of this hypothesis, we tested the effects of several known protein conformation–modifying agents on the growth and survival of APL cells and identified curcumin, a natural component of turmeric, as a potent growth inhibitor of APL cells. Curcumin selectively inhibited the growth and promoted apoptosis in both primary and secondary leukemic cells derived from APL. The curcumin-induced apoptosis of APL cells was triggered by an amplification of ER stress, possibly from the accumulation of misfolded N-CoR protein in the ER. Curcumin promoted this net accumulation of aberrantly phosphorylated misfolded N-CoR protein by blocking its ERAD and protease-mediated degradation, which then led to the activation of UPR-induced apoptosis in APL cells. The activation of UPR by curcumin was manifested by phosphorylation of protein kinase RNA-like endoplasmic reticulum kinase (PERK) and eukaryotic translation initiation factor 2 alpha (eIF2α), and upregulation of C/EBP homologous protein (CHOP) and GADD34, the principal mediators of proapoptotic UPR. These findings identify the therapeutic potential of curcumin in APL and further establish the rationale of misfolded N-CoR protein as an attractive molecular target in APL.

Mol Cancer Res; 9(7): 878–88. ©2011 AACR.

Introduction

Acute promyelocytic leukemia (APL), designated as acute myelogenous leukemia (AML-M3) in French-American-British (FAB) classification, is caused by a fusion protein PML-RARα formed by a reciprocal translocation between PML and RARα genes located on chromosome 15 and 17, respectively. APL represents around 10% of all AMLs in adults, with lower incidence reported in children (1, 2). At cellular level, APL is characterized by accumulation of blast cells in the bone marrow and peripheral blood that display features of differentiation arrest and resistance to programmed cell death. Although there is no dispute on the principal role of PML-RARα in the transformation of APL cells, the molecular mechanisms through which PML-RARα promotes transformation are still a matter of intense debate. We have shown that endoplasmic reticulum (ER)–associated degradation (ERAD) and protease-mediated degradation of misfolded nuclear receptor corepressor (N-CoR) plays a significant role in resistance of APL cells to unfolded protein response (UPR)-induced apoptosis (3, 4). N-CoR, a key component of the generic corepressor protein complex involved in transcriptional control mediated by various transcription factors, was first identified as a corepressor of unliganded nuclear hormone receptors (5, 6). N-CoR was later shown to be essential for the transcriptional repression mediated by the tumor suppressor Mad and other sequence-specific transcription factors (7, 8). We identified N-CoR as a Ski-interacting protein in yeast 2-hybrid assay (9) and further shown to be essential for the transcriptional repression mediated by the tumor suppressor Mad and retinoblastoma protein (Rb; refs. 10, 11).

UPR constitutes a coordinated transcriptional activation of a set of genes that encode for ER chaperones and certain...
death signals and is activated when net accumulation of misfolded proteins in the ER threatens the survival of cell (12, 13). Functionally, the UPR comprises 2 branches, the cytoprotective UPR and the cytotoxic UPR, although distinction between these 2 branches is not very clear. The cytoprotective UPR protects cells from the harmful effects of misfolded proteins by promoting chaperone-mediated refolding of misfolded proteins and ERAD of misfolded proteins that could not be refolded (12). In many protein conformation disorders, such as Alzheimer’s and Parkinson diseases, these corrective measures are often impaired, leading to the accumulation of potentially toxic misfolded proteins in the ER and cytosol. This, in turn, activates cytotoxic UPR and eliminates cells harboring the damaged proteins through programmed cell death (12). The cytotoxic UPR is mediated through the activation of c-Jun N-terminal kinase (JNK) by apoptosis signal-regulating kinase (ASK1) and upregulation of C/EBP homologous protein (C/EBP homologous protein (CHOP); ref. 14) by protein kinase RNA-like endoplasmic reticulum kinase (PERK). The final steps of UPR-induced apoptosis involve the activation and processing of caspases 3 and 9 (15).

We have previously shown that cytoprotective UPR can protect APL cells from the cytotoxic effects of misfolded PML-RAR/N-CoR proteins by inducing their degradation (4). This finding not only identified an important role of cytoprotective UPR in the pathogenesis of APL but also suggested that strategic conversion of cytoprotective UPR into cytotoxic UPR is an attractive therapeutic strategy for APL. On the basis of this hypothesis, we screened several known protein conformation-modifying agents to identify suitable compounds that could modulate response of APL cells to UPR and promote growth arrest in APL cells through targeting the degradation of misfolded N-CoR protein. In particular, our goal was to identify agents that would promote selective growth arrest in APL cells either by rescuing the native conformation and function of N-CoR or by aggravating ER stress through inducing further accumulation of misfolded N-CoR in the ER. In our initial screening of selected compounds on APL-derived cells, we identified genisteen and curcumin as potent inhibitors of growth of multiple APL-derived cells. The therapeutic effect of genisteen and its underlying molecular mechanism of action have been documented previously (16). Here, we report that curcumin, a natural component of turmeric that has previously been shown to rescue the native conformation of several misfolded proteins such as the amyloid β (Aβ), mutated cystic fibrosis transmembrane conductance regulator (CFTR), and truncated myelin protein zero (MPZ; refs. 17–19), selectively sensitizes APL cells to UPR-induced apoptosis by promoting the net accumulation of misfolded N-CoR protein in the ER.

**Material and Methods**

**Cell lines, human primary leukemia cells, and reagents**

The retinoic acid (RA)-sensitive APL cell line NB4 and its RA-resistant variant NB4-R1 and UF-1 were from Drs. Homma (Japan), Lanotte (France), and Matsushita (Japan), respectively. Other leukemic cell lines were purchased from American Type Culture Collection (ATCC) and Japan Health Sciences Foundation (Osaka) and were grown in RPMI, supplemented with 10% heat-inactivated FBS, 50 μg/mL penicillin, and 10 μg/mL streptomycin, with the exception of UF-1 cells, which were grown with 15% FBS. The APL cells were validated by their distinct morphology and processing of N-CoR protein. The primary human leukemia samples were collected after informed consent in accordance with the Helsinki Declaration. This study was approved by the Institutional Review Board (IRB) of National University of Singapore and National University Hospital, Singapore.

**Cell proliferation assay**

The cell proliferation assay was carried out using the Cell Proliferation Kit I (MTT; Roche) or CellTititer 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega), as described by the manufacturers. Cells were seeded into 96-well plates at 5 × 10^3/well in 100 μL culture medium containing various concentrations of curcumin at 37°C for the durations stated. MTT or MTS labeling reagent was added and incubated further at 37°C for 5 hours. For MTT, this was followed by suspension in solubilization buffer overnight at 37°C. The spectrophotometric absorbance of the samples was measured using a microplate reader (Ultragold; Bio-Rad) at wavelength 595 nm, with a reference wavelength of 655 nm. Alternatively for MTS, the spectrophotometric absorbance of the samples was measured immediately at wavelength 495 nm. Proliferation index of the cells was calculated as (OD_sh with 20 μmol/L at a particular time point)/OD_sh with 0 μmol/L at the same time point).

**Detection of apoptosis through flow cytometry**

Bone marrow or peripheral blood from APL patients was collected and processed with Ficoll-Paque PLUS (GE Healthcare) for isolation of mononuclear cells (MNC) as described by the manufacturer. The MNCs were cultured with Iscove’s modified Dulbecco’s medium supplemented with 10% FBS, 20 ng/mL FLT3 ligand, 20 ng/mL SCF, 20 ng/mL interleukin-3 (IL-3), 50 ng/mL granulocyte colony-stimulating factor (G-CSF), TPO 50 ng/mL, and 50 μg/mL/10 μg/mL penicillin/streptomycin. Cells were seeded at a density of 10 × 10^4 cells/mL and were treated with either dimethyl sulfoxide (DMSO) or 20 μmol/L of curcumin for 72 hours. They were then harvested, washed, and tested for apoptosis, using the Annexin V apoptosis detection kit (BD Pharmigen). Data were analyzed using the FlowJo program. Apoptosis assays of APL-derived cell lines were conducted similarly.

**Wright-Giemsa staining**

Suspension cells were cytospin onto glass slides, stained in May-Gruwald’s eosin methylene blue solution (Merck) for 5 minutes, followed by staining in Giemsa’s azur eosin methylene blue solution (Merck) diluted at 1:20 in Giemsa buffer (33 mmol/L KH₂PO₄, 33 mmol/L Na₂HPO₄, pH
6.81) for 30 minutes with agitation. Slides were mounted with cover slips and visualized under a Nikon eclipse TE 2000-S microscope.

**Immunostaining and fluorescence microscopy**

Suspension cells were cytopsin onto glass slides, whereas the adherent cells were grown on coverslips in culture medium. Cells were then fixed with 4% freshly prepared paraformaldehyde in PBS for 20 minutes at 37°C and permeabilized with 0.2% Triton X-100 in PBS at 4°C for 5 minutes. They were incubated with the respective primary antibodies at appropriate dilutions in 2.5% bovine serum albumin (BSA) in PBS for 2 hours at room temperature or overnight at 4°C. Alexa Fluor secondary antibodies (Molecular Probes) at a dilution of 1:200 in 2.5% BSA in PBS were incubated with the cells for 1 hour at room temperature. The cells were counterstained with 150 nmol/L 4,6-diamidino-2-phenylindole (DAPI).

**N-CoR solubility assay**

293T cells were seeded at a density of 200 × 10⁶ cells per 10-cm plate and transfected with 3 μg of pACT-N-CoR-FLAG and 3 μg of pACT-PML-RARα-HA expression plasmids. Forty-eight hours after transfection, cells were harvested with NET buffer (20 mmol/L Tris-HCl, pH 8, 300 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP-40, 1 mmol/L NaF, 1 mmol/L Na₃VO₄, 20 mmol/L β-glycerophosphate, 1.5 mg/mL IAA, protease inhibitor cocktail (Roche)) at 4°C with rotation for 1 hour. The lysates were then centrifuged at 15,000 rpm at 4°C for 10 minutes. The supernatant was collected as the soluble fraction, and the pellet, collected as the insoluble fraction, was suspended in SDS sample buffer and sonicated. The samples were then denatured at 50°C for 10 minutes, separated on 6% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane, and blotted with anti-FLAG M2 antibody (Santa Cruz). For solubility assay of endogenous N-CoR protein, around 8 × 10⁷ vehicle or curcumin-treated cells were suspended in 500 μL of NET buffer, centrifuged to separate soluble and insoluble fractions, loaded to 6% SDS-PAGE after brief sonication, and stained with anti-N-CoR antibody (Santa Cruz) after transfer to PVDF membrane.

**Immunoprecipitation and phosphorylation assays**

293T cells transfected with N-CoR-Flag and PML-RARα-HA expression plasmids were treated with vehicle, curcumin, or genistein. After incubation for 48 hours, whole cells extracts were prepared in phosphorylation buffer (20 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA, 10 mmol/L NaCl, 0.1% NP-40, 1 mmol/L NaF, 1 mmol/L Na₃VO₄, 20 mmol/L β-glycerophosphate, 1.5 mg/mL IAA, protease inhibitor cocktail) by mild sonication. Lysates were centrifuged at 15,000 rpm for 15 minutes at 4°C and the supernatants were collected. Protein concentration in the supernatant was determined using the Bio-Rad Protein Assay. N-CoR from the supernatant containing 1 mg of protein was immunoprecipitated with anti-FLAG M2 antibody (Sigma) and level of phosphorylated N-CoR protein was determined in Western blotting with generic phospho-antibody. The blot was then stripped and reprobed with anti-FLAG M2 antibody to quantify the amount of immunoprecipitated N-CoR protein.

**In vitro cleavage assay**

N-CoR substrate was prepared from 293T cells transfected with pACT-N-CoR-FLAG and pACT-PML-RARα-HA expression plasmids. Cell extracts from NB4, HL60, or K562 were prepared by pelleting 1 × 10⁷ cells, lysing them on ice in 200 μL of RSB buffer (10 mmol/L Tris, pH 8.0, 10 mmol/L NaCl, 3 mmol/L MgCl₂, 0.1% NP-40) buffer for 10 minutes. Nuclei were removed from the crude lysates by centrifugation at 3,000 rpm at 4°C for 5 minutes. Cleavage assay was then carried out by mixing 25 μL of N-CoR substrate with 25 μL of cell extracts from leukemic cells or purified fractions through size-exclusion chromatography and incubated at 25°C or 37°C for 60 minutes. The cleavage reaction was stopped by adding 16.7 μL of 4× SDS sample buffer and heating reaction mixture at 50°C for 10 minutes. Proteins were resolved with 6% SDS-PAGE and transferred to PVDF membranes for Western blotting. N-CoR was detected with anti-FLAG M2 antibody.

**Analysis of ER expansion**

NB4 cells seeded at 10 × 10⁴ cells/mL were treated with 0 or 5 μmol/L curcumin for 72 hours. Cells were incubated with 0.2 μg/mL of brefeldin A-BODIPY (BFA-BODIPY) for 45 minutes at 37°C in 5% CO₂. An aliquot of the stained cells was analyzed by a Dako Cytomation Cyan LX flow cytometer, using the SUMMIT software. Another aliquot of stained cells was cytopsin onto glass slide, fixed with paraformaldehyde, and stained with rabbit polyclonal calnexin antibody (SPA-865; Stress Gene) and rabbit Alexa Fluor 594 antibody.

**Statistical analysis**

The statistical significance of differences in values of data was determined using the unpaired Student’s t test. A P value of less than 0.05 was considered statistically significant.

**Results**

**Selective inhibition of growth of APL cells by curcumin**

To investigate whether therapeutic modulation of N-CoR misfolding could sensitize APL cells to UPR-induced apoptosis, we first tested the effect of curcumin on the growth of 3 APL-derived cell lines: the RA-sensitive cell NB4 and the RA-resistant variants NB4-R1 and UF-1. Curcumin, at concentrations ranging from 2.5 to 20 μmol/L, inhibited the growth and viability of all 3 APL-derived cells when analyzed by MTT assay (Fig. 1A) and Wright-Giemsa staining (Supplementary Fig. S1). The effect of curcumin seems to be selective for APL cells, as growth and viability of 6 non-APL cells, namely, K562, U937, HL60, Jurkat, THP-1, and MOLT-4, were not significantly affected after 72 hours of treatment (Fig. 1B and C). Although several non-APL cells also showed varying...
degree of growth inhibition when exposed to curcumin for 96 hours (Fig. 1B), their viability was not affected as much as observed with NB4 cells (Fig. 1C). The curcumin-induced growth inhibition observed in these non-APL cells at 96 hours could be due to its nonapoptotic growth inhibitory effect that has been documented against various cancer cells. All 3 curcumin-treated APL-derived cells displayed identical features of extensive cell death when analyzed by gross morphologic analysis (Supplementary Fig. S1) or fluorescence activated cell-sorting (FACS)-based qualitative apoptosis assay (Fig. 2A). As observed in APL-derived cell lines, curcumin also triggered apoptosis in 2 APL-derived human primary leukemic cells (Fig. 2B).

Because caspases are known to be principal mediators of apoptosis, we decided to identify the caspase(s) selectively involved in curcumin-induced apoptosis of APL cells. A fluorometric caspase assay was employed to identify the caspases and to measure their degree of activation by curcumin in APL cells. In this assay, caspase activation was quantified by measuring the amount of free 7-amino-4-trifluoromethylcoumarin (AFC), which is released when AFC-linked synthetic substrates are cleaved by activated caspase. AFC-linked substrates of all known mammalian caspases were used to measure the effect of curcumin on the activity of individual caspase in APL cells. As showed in Figure 2C, a selective activation of caspases 2, 3/7, and 9 was observed in NB4 cells treated with curcumin for 72 hours at 10 and 20 μmol/L concentrations. In agreement with this finding, curcumin, when used at 10 μmol/L concentration, induced the cleavage of caspases 9 and 3, which was followed by the activation of PARP (Fig. 2D). Comparable levels of caspases 3 and 9 and PARP cleavage by curcumin were also observed in RA-resistant APL cells NB4-R1 and UF-1 (Supplementary Fig. S2A and B). Apart from promoting apoptosis, curcumin also induced cell-cycle arrest at G2-M (M3) phase after 24 hours of treatment (Supplementary Fig. S3), along with a decrease in the percentage of cells at G1 phase (M1). The number of cells at S-phase was not significantly affected.
Curcumin blocks protease-mediated degradation of misfolded N-CoR protein

We previously reported how selective cleavage or degradation of misfolded N-CoR protein was linked to resistance of APL cells and UPR-induced apoptosis (4). To investigate whether curcumin-induced apoptosis of APL cells was mediated through an inhibition of N-CoR cleavage or degradation, effect of curcumin on the cleavage of N-CoR protein was analyzed. Treatment of NB4 cells with curcumin in a dose-dependent manner led to the stabilization of full-length N-CoR protein along with a corresponding reduction in the level of cleaved N-CoR fragment (Fig. 3A). To test whether the observed stabilization of N-CoR protein was a result of the inhibition of proteases activity that cleaved N-CoR protein in NB4 cells, an in vitro N-CoR cleavage assay was conducted. In this assay, Flag-tagged N-CoR protein ectopically expressed in 293T cells was incubated with the extracts of NB4 cells treated with 0 to 20 μmol/L curcumin for 72 hours was calculated on the basis of the fluorescence intensity of their substrate in arbitrary units per microgram of protein. Activation of caspases was represented as fold increase of specific activity of each caspase in cells treated with the respective curcumin concentrations over the specific activity of the same caspase in cells treated with DMSO only. D, activation of caspases by curcumin in APL cells. NB4 cells were treated with various concentrations of curcumin as stated for 72 hours. Cells were then harvested and protein directly extracted with SDS sample buffer. Western blot analyses of the crude lysates were carried out using caspase 9, procaspase 3, cleaved caspases 3, and PARP antibodies.

Curcumin blocks protease-mediated degradation of misfolded N-CoR protein

Figure 2. Curcumin-induced apoptosis of APL cells is mediated by the activation of caspases. A and B, the percentage of Annexin V-positive cells in NB4 (A) and 2 human primary APL samples (B) treated with curcumin for 72 hours in a dose-dependent manner was determined through Annexin V assay. FITC, fluorescein isothiocyanate; PI, propidium iodide. C, curcumin treatment led to the activation of caspases 2, 3/7, and 9. Activity of all the caspases in NB4 cells treated with 0 to 20 μmol/L curcumin for 72 hours was calculated on the basis of the fluorescence intensity of their substrate in arbitrary units per microgram of protein. Activation of caspases was represented as fold increase of specific activity of each caspase in cells treated with the respective curcumin concentrations over the specific activity of the same caspase in cells treated with DMSO only. D, activation of caspases by curcumin in APL cells. NB4 cells were treated with various concentrations of curcumin as stated for 72 hours. Cells were then harvested and protein directly extracted with SDS sample buffer. Western blot analyses of the crude lysates were carried out using caspase 9, procaspase 3, cleaved caspases 3, and PARP antibodies.
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Vehicle or curcumin, as well as with AEBSF, a broad-spectrum protease inhibitor that abrogated N-CoR cleavage in NB4 cells (4). Extract of curcumin-treated NB4 cells did not cleave Flag-tagged N-CoR protein as efficiently as extract of vehicle-treated NB4 cells. This suggests that potency of N-CoR cleaving proteases was significantly reduced after curcumin treatment and the reduction was comparable with that achieved by AEBSF (Fig. 3B). Apparently, the stabilization of N-CoR protein by curcumin was mediated by an inhibition of N-CoR degradation rather than an inhibition of N-CoR misfolding as observed previously with genistein (16). Consistent with this idea, relative level of detergent-insoluble N-CoR protein in NB4 cells or 293T cells overexpressing N-CoR was increased after curcumin treatment while there was hardly any change in the level of detergent-soluble N-CoR protein (Fig. 3C and D). The misfolded N-CoR protein was characterized by aberrant phosphorylation (Fig. 3E), and the level of phosphorylated N-CoR protein was significantly increased after curcumin treatment (Fig. 3F, left panel). On the other hand, genistein treatment resulted in a noticeable reduction in phosphorylated N-CoR level (Fig. 3F, right panel). These findings collectively suggested that curcumin-induced N-CoR stabilization mainly resulted from an inhibition of degradation of misfolded N-CoR protein rather than an inhibition of N-CoR misfolding as observed previously with genistein.

Curcumin blocks proteasomal degradation of misfolded N-CoR protein

In addition to proteolytic processing, misfolded N-CoR was also subjected to ubiquitin-proteasome–mediated degradation, specifically stimulated by the E3 ligase activity of PML-RAR protein (3). As curcumin is also known to be a potent inhibitor of proteasomal activity (20–22), we tested whether curcumin-induced stabilization of N-CoR protein was a result of an inhibition of cellular proteasomal activity. Effect of curcumin on the proteasomal activity of 293T cells was tested using the proteasome sensor pZsProSensor-1, which consists of the mouse ornithine decarboxylase degradation domain (MODC d410) fused to green florescent...
The intensity of the green signal emitted from the pZsProSensor-1 is hence inversely proportional to the level of cellular proteasomal activity in cultured cells. As expected, intensity of the green fluorescence signals in 293T cells transfected with pZsProSensor-1 vector increased with increasing concentrations of curcumin, confirming that curcumin could robustly inhibit the cellular proteasomal activity of 293T cells (Supplementary Fig. S4).

Next, the effect of curcumin on the degradation of misfolded N-CoR protein in 293T cells was determined using GFP-fused N-CoR as a possible substrate of proteasome. When expressed alone, native GFP–N-CoR was confined to the nucleus in the absence of PML–RARα but localized mostly in the cytoplasm in the presence of PML–RARα. Curcumin inhibited the degradation of endogenous N-CoR in NB4 cells, which were treated with 0 to 10 μmol/L of curcumin for 48 hours. Cells were cytospun onto glass slides, fixed, and stained with N-CoR antibody (red). DNA was stained with DAPI (blue).

Figure 4. Curcumin blocked the proteasomal degradation of misfolded N-CoR protein. A, curcumin inhibited the degradation of the GFP–N-CoR in 293T cells transfected with GFP–N-CoR and PML–RARα in a dose-dependent manner. Curcumin (0–10 μmol/L) was added to transfected 293T cells 2 hours after transfection. GFP–N-CoR was visualized 48 hours after curcumin treatment. GFP–N-CoR was localized in the nucleus in the absence of PML–RARα but localized mostly in the cytoplasm in the presence of PML–RARα. B, curcumin inhibited the degradation of endogenous N-CoR in NB4 cells, which were treated with 0 to 10 μmol/L of curcumin for 48 hours. Cells were cytospun onto glass slides, fixed, and stained with N-CoR antibody (red). DNA was stained with DAPI (blue). C, curcumin inhibited the trypsin-, chymotrypsin-, and caspase-like peptidase activities of the proteasome. Crude extracts of NB4 cells treated with 0 to 10 μmol/L curcumin for 48 hours were used to determine the cleavage of proteasome substrates.
Curcumin may have blocked the proteasomal degradation of GFP–N-CoR protein (Fig. 4A, bottom panels). Furthermore, the level of cytosolic N-CoR protein in NB4 cells was also increased after treatment with curcumin in a dose-dependent manner, confirming that curcumin could trigger cytosolic accumulation of misfolded N-CoR protein in APL cells (Fig. 4B). The proteasome, which is a multisubunit and multicatalytic proteinase, possesses multiple trypsin, chymotrypsin, and peptidylglutamyl-like hydrolysing activity (23). To find out which of these 3 catalytic activities was inhibited by curcumin, a fluorometric proteasomal activity assay was carried out using synthetic substrates selectively hydrolysed by each activity. In this assay, crude cellular extract of curcumin-treated NB4 cells was incubated separately with peptides Boc-Leu-Arg-Arg-AMC, Z-Gly-Gly-Leu-AMC, and Boc-Leu-Leu-Glu-AMC, which are the synthetic substrates of trypsin, chymotrypsin, and caspases-like activities, respectively. Curcumin consistently inhibited the activities of all 3 catalytic subunits, though the inhibitory effect was most prominent on both the chymotrypsin and caspases-like peptidase activities (Fig. 4C).

Curcumin activates UPR-induced apoptosis in APL cells

We have previously shown how degradation of misfolded N-CoR protein led to the attenuation of ER stress and protected APL cells from UPR-induced apoptosis (4). On the basis of this finding, we hypothesized that amplification of ER stress due to curcumin-induced accumulation of misfolded N-CoR protein in the ER will eventually resensitize APL cells to UPR-induced apoptosis. To test this hypothesis, effect of curcumin on the level of ER stress in APL cells was first determined by measuring the relative levels of high-molecular-weight (HMW) GRP78 and protein disulfide isomerase (PDI) proteins, which are known to be bona fide ER stress markers in mammalian cells. Curcumin induced the formation of HMW detergent-resistant GRP78 and PDI proteins in NB4 and NB4-R1 cells.
However, level of PERK phosphorylation was augmented of a sustained level of chronic ER stress in APL cells. PERK was already activated in NB4 cells, probably because shown in Figure 6A, a basal level of PERK phosphorylation activation of UPR in APL cells, we first determined the CHOP (14). To assess the effect of curcumin on the activation of JNK and by PERK-induced upregulation of by cells. The cytotoxic UPR is triggered by ASK1-induced mediators of cytotoxic UPR. The initial phase of UPR is neutralized of misfolded proteins accumulated in the ER is neutralized by curcumin treatment (Fig. 5D).

Next, to show that curcumin-induced apoptosis of APL cells was mediated through the activation of cytotoxic UPR, we studied the effect of curcumin on the activation of mediators of cytotoxic UPR. The initial phase of UPR is mediated by the activation of PERK and inositol-requiring enzyme (IRE) through phosphorylation (12, 13), whereas the final outcome of UPR could be either cytoprotective or cytotoxic, depending on how efficiently the cytotoxic effect of misfolded proteins accumulated in the ER is neutralized by cells. The cytotoxic UPR is triggered by ASK1-induced activation of JNK and by PERK-induced upregulation of CHOP (14). To assess the effect of curcumin on the activation of UPR in APL cells, we first determined the phosphorylation status of PERK and IRE in NB4 cells. As shown in Figure 6A, a basal level of PERK phosphorylation was already present in untreated NB4 cells, indicating that PERK was already activated in NB4 cells, probably because of a sustained level of chronic ER stress in APL cells. However, level of PERK phosphorylation was augmented significantly after 16 hours of curcumin treatment, which was followed by phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α) and upregulation of proapoptotic proteins CHOP and GADD34 (Fig. 6A). Moreover, ASK phosphorylation was induced within 4 hour of curcumin treatment, which peaked at 16 hours and was followed by JNK phosphorylation at 24 hours and Bcl-2 phosphorylation at 4 to 24 hours (Fig. 6B). These finding showed a sequential escalation of proapoptotic cascade of UPR after curcumin treatment (Fig. 6B). Consistent with our finding, a recent report also showed misfolded PolyQ-induced JNK phosphorylation at 24 hours, although JNK phosphorylation by the chemical ER stress inducer thapsigargin was evident within 4 hours of treatment (25). Together, these findings provided compelling evidence that curcumin-induced apoptosis of APL cells was mediated through the activation of cytotoxic branch of UPR.

Discussion

Curcumin was selected in our initial screening assay against APL-derived cells for its potent inhibitory effect on the misfolding of Aβ, CFTR, and MPZ proteins (17–19). Although curcumin was as effective as genistein in stabilizing the full-length N-CoR protein, the N-CoR stabilization by curcumin did not result from an inhibition of N-CoR misfolding as observed with genistein. It is likely that molecular mechanism underlying the misfolding of N-CoR protein is substantially different from that involved in the misfolding of Aβ and CFTR proteins. For instance, misfolding of N-CoR is triggered primarily by aberrant posttranslational modification, which could be effectively blocked by genistein, a bona fide inhibitor of tyrosine kinase, but not by curcumin, which is not known to possess any antikinase activity.

Curcumin is the major yellow pigment found in turmeric derived from the roots of the plant Curcuma longa. It

Figure 6. Curcumin activated UPR-induced apoptosis in APL cells. A, curcumin activated the PERK pathway in NB4 cells, which were treated with 10 μmol/L curcumin for 0 to 72 hours. Western blot analysis was carried out using phospho-PERK (pPERK), PERK, phospho-eIF2α (peIF2α), eIF2α, CHOP, and GADD34 antibodies. B, curcumin activated the IRE pathway in NB4 cells, which were treated with 10 μmol/L curcumin for 0 to 72 hours. Western blot analysis was carried out using phospho-ASK (pASK), ASK, phospho-JNK (pJNK), JNK, phospho-Bcl-2 (pBcl-2), and Bcl-2 antibodies.
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possesses strong antioxidant activity and inhibits angiogenesis at pharmacologic concentrations (26–28). The principal anticarcinogenic properties of curcumin, such as inhibition of cellular proliferation, activation of apoptosis, suppression of inflammation, and sensitization of tumor cells to chemotherapy, have been well documented (29–32). Many of these anticarcinogenic effects of curcumin have been attributed to the inhibition of various survival pathways, including the phosphoinositide 3-kinase (PI3K)/Akt1 and nuclear factor kappaB (NF-κB), and upregulation of genes associated with growth arrest and apoptosis. As shown by us in this report, the major anticarcinogenic effect of curcumin in APL cells was mediated through its potent inhibitory effect on the protein quality control mechanism, although involvement of other pathways could not be ruled out. Perhaps, a more thorough understanding of the mechanisms underlying curcumin-induced apoptosis of APL cells could be gained from the findings in proteasome inhibitor–treated multiple myeloma cells. Multiple myeloma cells exhibit a lower threshold for proteasome inhibitor–induced UPR, as they are constitutively under ER stress due to their role as secretory cells. Bortezomib, a proteasome inhibitor, rapidly induced apoptosis or cytotoxic UPR in multiple myeloma cells through the induction of activating transcription factor 4 (ATF-4) and CHOP (33). Likewise, APL cells too experienced a relatively low level of chronic ER stress possibly because of constant production of misfolded N-CoR protein.

Current therapeutic options available for APL in the form of RA, arsenic trioxide (ATO), and chemotherapy have proved to be very effective in inducing clinical remission in the majority of APL patients. However, serious systemic toxicity of RA and a relapse after initial remission followed by resistance to RA limits its scope of therapeutic application in clinical practice. Clinical application of ATO is also fraught with complications, such as leukocyte activation syndrome and arrhythmia. These obstacles underline the need of newer approaches in the treatment and management of APL. Curcumin, being a natural component of food product, could become an adjuvant to currently available therapeutic agents for APL if its therapeutic utility and usefulness could be established through further preclinical and clinical studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Yeo Hui Ling Angjie and Chew Poh Yeong for the analysis of results.

Grant Support

This work was supported by grants SSSC-04-06 and NMRC/1213/2009 to M. Khan from Singapore Stem Cell Consortium and National Medical Research Council of Singapore. A.P.P. Ng was supported by a graduate research scholarship from the Agency of Science and Technology, Singapore.

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Received December 2, 2010; revised May 4, 2011; accepted May 16, 2011; published OnlineFirst May 20, 2011.

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www.aacrjournals.org Mol Cancer Res; 9(7) July 2011 887
Molecular Cancer Research

Curcumin Sensitizes Acute Promyelocytic Leukemia Cells to Unfolded Protein Response–Induced Apoptosis by Blocking the Loss of Misfolded N-CoR Protein

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Mol Cancer Res 2011;9:878-888. Published OnlineFirst May 20, 2011.

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