RESEARCH ARTICLE

Curcumin Induces Apoptosis in Pre-B Acute Lymphoblastic Leukemia Cell Lines Via PARP-1 Cleavage

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Abstract

Curcumin, a polyphenolic compound isolated from the rhizomes of an herbaceous perennial plant, *Curcuma longa*, is known to possess anticancerous activity. However, the mechanism of apoptosis induction in cancers differs. In this study, we have (1) investigated the anticancerous activity of curcumin on REH and RS4;11 leukemia cells and (2) studied the chemo-sensitizing potential of curcumin for doxorubicin, a drug presently used for leukemia treatment. It was found that curcumin induced a dose dependent decrease in cell viability because of apoptosis induction as visualized by annexin V-FITC/ PI staining. Curcumin-induced apoptosis of leukemia cells was mediated by PARP-1 cleavage. An increased level of caspase-3, apoptosis inducing factor (AIF), cleaved PARP-1 and decreased level of Bcl2 was observed in leukemia cells after 24h of curcumin treatment. In addition, curcumin at doses lower than the IC_{50} value significantly enhanced doxorubicin induced cell death. Therefore, we conclude that curcumin induces apoptosis in leukemia cells via PARP-1 mediated caspase-3 dependent pathway and further may act as a potential chemo-sensitizing agent for doxorubicin. Our study highlights the chemo-preventive and chemo-sensitizing role of curcumin.

Keywords: Curcumin - leukemia - PARP-1 - REH - RS4;11

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Introduction

Natural phytochemicals, as supplements or alternative to some chemotherapeutic drugs, show synergistic effect and thereby result in decline in drug related toxicity. Curcumin (diferuloylmethane) is a yellow coloured polyphenol derived from the rhizome of perennial plant Curcuma longa, growing commonly in India. It is one of the extensively investigated compounds for its medicinal values in various diseases. Curcumin is known to possess a significant anti cancerous activity and is reported to induce apoptosis in different cancers like gastric carcinoma (Xue et al., 2014; Ji et al., 2014), skin squamous cell carcinoma (Wu et al., 2015), colon cancer (Dehghan et al., 2015), lung cancer (Badrzadeh et al., 2014; Xia et al., 2014), renal cell carcinoma (Pei et al., 2014), ovarian cancer (Zhao et al., 2014), liver cancer (Li et al., 2014; Dai et al., 2013) leukemia (Banjerdpongchai et al., 2005; Gopal et al., 2014) and cervical carcinoma (Basu et al., 2013) by multiple mechanisms like reactive oxygen species (ROS) generation, autophagy, activation of NF-kB pathway, mitochondrial membrane potential (MMP) dissipation with the release of cytochrome c in cytoplasm, down-regulation of anti-apoptotic marker Bcl-2, up-regulation of pro-apoptotic marker Bax and production of increased cleaved PARP. Apart from having protective effect on normal human cells, curcumin is reported to effectively sensitize cancer cells to a broad range of chemotherapeutic drugs like bortezomib, doxorubicin, 5-FU, paclitaxel, celecoxib, vinorelbine, vincristine, butyrate, cisplatin, melphalan, gemcitabine, etoposide, oxaliplatin, sulfinosine, and thalidomide (Goel, 2010). It enhances apoptotic potential of drugs like etoposide through the generation of reactive oxygen species in myeloid leukemia cells (Papiez et al., 2016), imatinib through disrupting redox potential by generated reactive oxygen species in chronic myeloid leukemia cells (Acharya, 2016), Busulfan via downregulating survivin expression in KG1a cells having stem cell property (Weng et al., 2015), cytarabine via down regulating MDR genes in acute myeloid leukemia cells (Shah et al., 2015), bortezomib in leukemia cells and xenograft models (Nagy et al., 2015), arsenic trioxide via downregulating BCL-2 and PARP in leukemia cells (Wang et al., 2013), imatinib in acute leukemia via downregulating AKT/ mTOR pathway (Guo et al., 2015), ATRA (all-trans retinoid acid) via activating PI3K/AKT pathway in acute promyelocytic leukemia cells (Chen et al., 2013), methotrexate via overexpression of folate receptor in leukemia cells (Dhanasekaran et al., 2013), valproic acid via p38-dependent pathway (Chen et al., 2010) and rapamycin via caspases dependent pathway (Hayun et al., 2009).

Leukemia is a heterogeneous disease affecting

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different types of blood cells. Acute lymphoblastic leukaemia, with prevalence in children of the age group 2 to 5 years, is a malignancy affecting lymphoid progenitor cells (Pui et al., 2008). Revolutionising advances in treatment regimens has resulted in achieving cure rate higher than 80% in children, however using innovative approaches to make chemotherapy more effective, would help in acquiring higher percentage of leukaemia-free survival and reduced treatment related toxic side-effects (Pui et al., 2004; Pui et al., 2006; Pui et al., 2008). Much is known about the anti cancerous role of curcumin, however the accurate mechanism underlying cell apoptosis in pre-B acute lymphoblastic leukemia is still unclear. In the present study, we have investigated anti leukemic effect of curcumin and associated apoptotic pathway. Further, we have also explored chemo-sensitizing role of curcumin and the underlying mechanism of apoptosis involved. We have attempted to define the chemotherapeutic and chemosensitizing potential of curcumin.

Materials and Methods

Cell culture and chemicals

Two pre-B acute lymphoblastic leukemia cell lines RS4;11 and REH were grown in RPMI-1640 medium (Gibco, Life Technologies, USA), supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Life Technologies, USA), 1% antibiotics (Cellclone, Genetix Biotech Asia Pvt Ltd, India) in a 5% CO_2 incubator at 37ºC. Curcumin (Sigma, St. Louis, MO, USA) and doxorubicin hydrochloride (Sigma, St. Louis, MO, USA) main stocks were prepared in pure dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA). All chemicals used were of highest purity grade available. For MTT assay, EZcount MTT cell assay kit (Himedia, India) was purchased. Antibodies anti-Caspase-3, anti-poly-adenosine diphosphate ribose polymerase-1 (anti-PARP-1) and anti-Bcl2 (Cell Signalling Technology, MA, USA), anti-AIF (santa cruz biotechnology, USA) and anti-GAPDH (Imgenex, India) antibodies were used for western blotting.

Cell viability assay

Five thousand cells per well (5×10^3 cells/well) were seeded in 96-well plates. After 24h incubation, cells were treated dose dependently with 10 to 100μM curcumin for 24h or DMSO (control group) and 5nM doxorubicin hydrochloride. Cell cytotoxicity was assessed by MTT cell assay kit as per manufacturer's protocol. Yellow colored water soluble tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduced to formazan crystals was dissolved in DMSO. Absorbance was recorded at 570nm on a microplate reader (BioRad, USA). The percentage of cell viability was calculated using the formula: Cell viability (%) = OD of treated / OD of contro l×100.

Cell cycle assay

To check the proportion of REH and RS;411 cells in different phases of cell cycle, the DNA content was detected by propidium iodide staining (Sigma, St. Louis, MO, USA) by flow cytometry. Cells seeded in 6-well plates were grown for 24h without drug, after 24h both the cell lines were incubated with curcumin (10 and 20 μM) or DMSO (control) for 24h and then collected, centrifuged at 5000 RPM for 5 minutes. Pelleted cells were washed with PBS, and fixed with 70% ethanol. Again after centrifugation, cells were resuspended in PBS containing 0.1 mg/ml RNase A (Amresco, USA) and incubated for 2h at room temperature. Finally just before analysis 2 μg/ml propidium iodide was added and cell cycle distribution was determined by flow cytometry. Stained cell were read for DNA content by BD-FACS calibur instrument (BD-Biosciences, USA), analysis was done by CellQuest pro (BD-Biosciences, USA).

Apoptosis assay

AnnexinV-FITC/ propidium iodide assay was performed to calculate apoptotic cells as per manufacturer's protocol (Invitrogen, Molecular Probes, USA). Cells were seeded in 5ml media in 6-well plates, cells at semi-confluent stage were treated with curcumin (10μM and 20μM) for 24h. Apoptotic events were acquired and analyzed by BD-FACS calibur instrument (BD-Biosciences, USA).

Western blotting

Cells treated with curcumin alone or in combination with doxorubicin were collected after 24h treatment and washed twice with PBS. Cell lysis was done with ice-cold RIPA lysis buffer (Sigma Aldrich, USA). Cell lysate was kept on ice for 30 min followed by gentle vortexing in between, and then centrifugation was done at 12,000 rpm for 10 min at 4°C. The supernatant was collected and transferred in fresh microfuge tube and stored at –80°C for further use. Equal quantity of protein (50μg) was used for western blot analysis. Equal amounts of protein were loaded in each lane of 10% SDS-PAGE gel. Resolved protein was later electro-transferred onto PVDF membranes (Millipore, USA). PVDF membranes were blocked with 5% non-fat milk in TBST containing 0.5% Tween-20. After blocking, PVDF membranes were incubated with anti-PARP-1, anti-AIF, anti-Bcl2 and anti-GAPDH antibodies for overnight at 4ºC. Membranes were further incubated with their respective secondary antibodies conjugated to ALP for 2h. After this membranes were exposed to BCIP/NBT solution (Amersco, USA).

Chemo-sensitization

To study the effect of curcumin as a sensitizing agent to enhance the apoptotic potential of chemotherapeutic drug doxorubicin, cells were seeded in 5ml media in 6-well plates, after 24h incubation, cells at semi confluent stage were treated with curcumin (10μM) alone or doxorubicin (5nM) alone or combined dose of curcumin (10μM) with doxorubicin (2.5nM) and curcumin (10μM) with doxorubicin (5nM) for 24h.

Results

Curcumin decreases leukemia cell viability

Curcumin treatment resulted in cell death in leukemia

cell lines with a half maximal inhibitory concentration (IC_{50}) ranging from 10μM to 20μM. Curcumin induced a dose- dependent decrease in cell viability after 24h treatment (Figure 1b and 1c) suggesting a cytotoxic role.

Curcumin arrests pre-B leukemia cells in G2-M phase and increases sub G1 population

To understand the effect of curcumin treatment on cell cycle distribution it was found that curcumin treatment arrested REH and RS4;11 cells in G2-M phase (Figure 2). Further, a significant increase in sub G1 population was also found after 24h curcumin treatment which signifies rise in apoptotic population.

Curcumin induces apoptosis in leukemia cell lines

To confirm the cell death by apoptosis, annexin V-FITC/PI staining was done on curcumin treated cells.

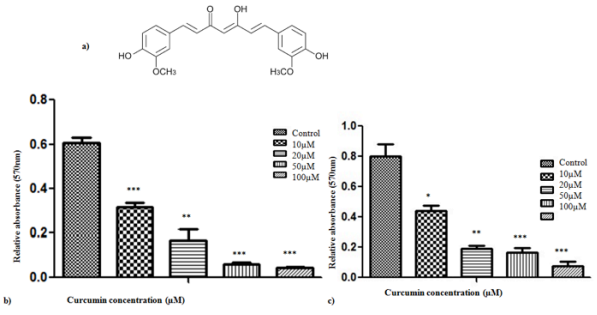

Figure 1. a) Curcumin Structure; Curcumin Decreases b) REH and c) RS4;11 cell Viability when Treated with Varying Concentrations of Curcumin from 10μM to 100μM. IC_{50} was determined by MTT assay after 24h. Two-tailed student t-test was applied

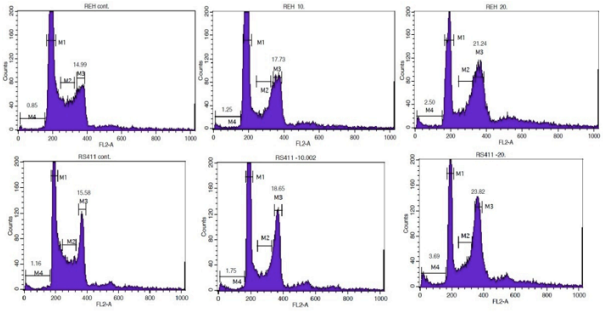

Figure 2. Curcumin Induces G2-M arrest in both REH (Upper Panel) and RS4;11 (Lower Panel) Cells with a Increase in sub G1 Cell Population after 24h after 10μM and 20μM Curcumin Treatment

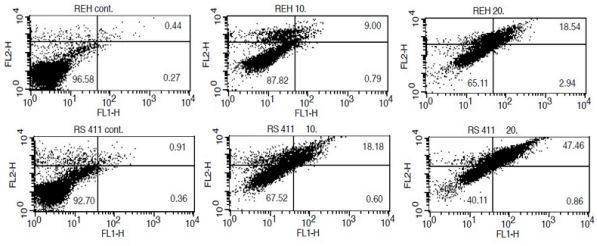

Figure 3. Curcumin Induces Apoptosis in REH (upper panel) and RS4;11 (Lower Panel) Cells Treated with Different Concentrations of Curcumin 10μM and 20μM for 24h, Followed by Annexin V/PI Staining

Q2 quadrant (FITC+/PI+) stands for late apoptotic cells; Q3 quadrant (FITC-/PI-) shows live cells and Q4 quadrant (FITC+/PI-) represents early apoptotic cells. Flow cytometry results suggest a distinct dose-dependent increase in late apoptotic cells after curcumin treatment as compared to control cells (Figure 3). The annexin V–/PI–, annexin V+/PI–, and annexin V+/PI+ quadrants indicate the percentage cell population of normal cells, early apoptosis and late apoptosis respectively. Percentage of early apoptotic REH cells increased dose dependently from 0.27 in control to 0.79 and 2.94 subsequently. Percentage of cells in late apoptosis increased from 0.44 in control to 2.39 and 18.54 in REH cells. Similar pattern was observed in RS4;11 cells, early apoptotic cells increased from 0.36 in control to 0.60 and 0.86 dose dependently. Late apoptotic cells increased from 0.91 to 18.18 and 47.46 dose dependently. Total apoptotic cells (Q2 and Q4 quadrants) increased dose dependently in both cell lines after curcumin treatment.

Curcumin induces apoptosis by cleavage of PARP-1

PARP-1 is the downstream substrate of caspase family and is a hallmark mediator of the apoptotis. Curcumin treatment resulted in significant dose dependent increase in apoptotic cells as indicated from MTT and apoptosis assay in REH and RS4;11 cells, suggesting cell death via apoptotic pathway. This result was further confirmed by western blotting of PARP-1; dose dependent cleavage of full length PARP-1 was found after curcumin treatment. We validated apoptosis by doing western blotting for PARP-1 after 24h treatment. Curcumin cleaves PARP-1 forming 116 and 89kD bands suggesting caspase pathway mediated apoptosis after 24h treatment (10 and 20μM) (Figure 4). PARP-1 cleavage thereby confirms apoptosis. Band densitometric analysis shows increased expression of full length caspase-3 and apoptosis inducing factor

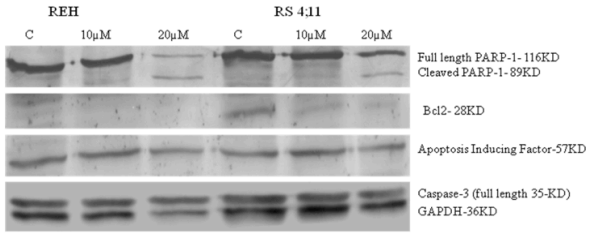

Figure 4. Curcumin Induces Apoptosis in REH and RS4;11 Cells Treated with 10μM and 20μM Curcumin for 24h through Caspase Dependent PARP-1 cleavage

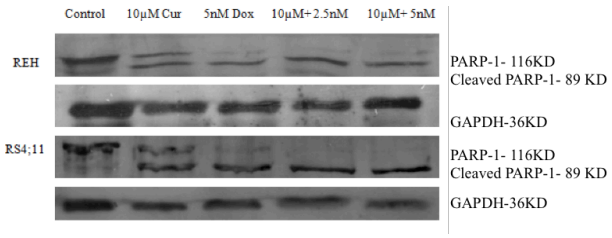

Figure 5. Curcumin Induces Apoptosis in REH and RS4;11 cells treated with Alone and in Combination with Doxorubicin Hydrochloride for 24h, through PARP-1 cleavage

(AIF) with a dose dependent down regulation of Bcl2 (Figure 4).

Curcumin potentiates apoptosis induced by doxorubicin hydrochloride

Doxorubicin is an age old chemotherapeutic drug known to induce apoptosis in various malignancies. IC_{50} dose was calculated using MTT assay. IC_{50} dose 5nM and a dose lower than IC_{50} value 2.5nM was chosen. It was found that both 10μM curcumin and 5nM doxorubicin induced apoptosis as evident by PARP-1 cleavage. However, when given simultaneously, a 2.5nM doxorubicin and 10μM curcumin induced a higher level of apoptosis. With a gradual increase in 89kD fragment of PARP-1, increased level of apoptosis was also observed dose dependently which suggests enhanced level of apoptosis when curcumin was supplemented with doxorubicin in cell culture (Figure 5).

Discussion

Multiple studies have shown chemotherapeutic role of curcumin on variety of tumor cells. Curcumin targets the genes involved in critical pathways of cancer including invasion, migration, and proliferation by inhibiting constitutively active NF-κB (Han et al., 2002), DNA damage to mitochondrial and nuclear genomes (Kong et al., 2009), inhibition of PI3K-AKT activation (Hussain et al., 2006), and regulating cell cycle (Park et al., 2002). In the present study, we evaluated the effect of curcumin on leukemia cell lines REH and RS4;11. Our experimental results showed that curcumin has chemotherapeutic potential as it dose dependently exhibited cytotoxicity to human pre-B acute lymphoblastic leukemia cells, with an IC_{50} ranging in between 10 to 20μM. Annexin V-FITC/PI apoptosis assay suggested that percentage of total apoptotic cells increased after curcumin treatment. Our immunoblotting results validated the curcumin induced caspase-3 dependent PARP-1 mediated apoptosis in these leukemia cells. Further, this study also demonstrates that curcumin has the potential to chemo-sensitize cells for doxorubicin to work at dose lower than IC_{50} value.

In conclusion, this study gives mechanistic insights about curcumin anti leukemia effect. Further it demonstrates that curcumin induces PARP-1 mediated cell apoptosis in leukemia cells and curcumin can behave as a potential sensitizing agent for doxorubicin, enhancing its apoptosis inducing potential.

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