RESEARCH ARTICLE

Curcumin Induces Caspase Mediated Apoptosis in JURKAT Cells by Disrupting the Redox Balance

Priya Kalyan Gopal[1], Mausumi Paul[2], Santanu Paul[1]*

Abstract

Background: Curcumin has has been reported to exert anti-inflammatory, anti-oxidation and anti-angiogenic activity in various types of cancer. It has also been shown to induce apoptosis in leukemia cells. We aimed to unravel the role of the redox pathway in Curcumin mediated apoptosis with a panel of human leukemic cells. Materials and Methods: In this study *in vitro* cytotoxicity of Curcumin was measured by MTT assay and apoptotic effects were assessed by annexin V/PI, DAPI staining, cell cycle analysis, measurement of caspase activity and PARP cleavage. Effects of Curcumin on intracellular redox balance were assessed using fluorescent probes like H_2DCFDA, JC1 and an ApoGSH Glutathione Detection Kit respectively. Results: Curcumin showed differential anti-proliferative and apoptotic effects on different human leukemic cell lines in contrast to minimal effects on normal cells. Curcumin induced apoptosis was associated with the generation of intracellular ROS, loss of mitochondrial membrane potential, intracellular GSH depletion, caspase activation. Conclusions: As Curcumin induces programmed cell death specifically in leukemic cells it holds a great promise as a future therapeutic agent in the treatment of leukemia.

Keywords: Leukemia - ROS - GSH - mitochondrial membrane motential - cell cycle

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Introduction

Curcumin, a diferuloylmethane is a naturally occurring hydrophobic polyphenol that can be isolated from the rhizome of a perennial herb *Curcuma longa* L. Colloquially called turmeric, belonging to the family Gingiberaceae indigenous to southern and southeastern tropical Asia (Aggarwal et al., 2005). Besides Curcumin, demethoxyCurcumin, bisdemethoxyCurcumin, and the recently identified CycloCurcumin are other major Curcuminoids present in turmeric (Zhou et al., 2011). It is these Curcuminoids which is liable for the orange-yellow colour of turmeric (Kiuchi et al., 1993). Widespread studies corroborate that Curcumin is the principal active component of turmeric, constituting about 77% of this spice. Research in the latter half of the 20th century identified Curcumin as the agent responsible for most of the biological activity associated to turmeric. As reported pervasively, plant derived polyphenols make the major area of interest for anti-cancer drug development, attributed to their ability of inducing programmed cell death in majority of cancer cells reported so far. Curcumin similarly, is a historically acknowledged component of the Indian Ayurvedic medicine. *In vitro* and *in vivo* investigations accounting to about more than 3000 research publications suggest a wide range of potential therapeutic or preventive effects associated with Curcumin (Aggarwal et al., 2007). Apart from being an established anti-oxidant, the preventive effects of Curcumin encompass antibacterial, antifungal, antiviral, anti-inflammatory, anti-proliferative, pro apoptotic and anti-atherosclerotic effects (Huang et al., 1992; Gupta et al., 2012). Also very well acclaimed medicinal features of Curcumin enlist many diseases like: arthritis, allergy, inflammatory bowel disease, nephrotoxicity, AIDS, psoriasis, diabetes, multiple sclerosis, cardiovascular disease, lung fibrosis, drug-induced myocardial toxicity, prevents pancreatitis, inhibits multi-drug resistance, overall drug-induced cytotoxicity (Vankatesan et al., 1995; James et al., 1996; Piwocka et al., 2002; Aggarwal et al., 2009). The mechanisms underlying various biological effects of Curcumin have been investigated in the past four decades and still hold a major research interest. It is well established with Curcumin that it's potential behold in its ability to interact and affect expression of not one but several signaling molecules significant to cellular growth and progression. Curcumin proficiently induces programmed cell death equally well in both adherent and non-adherent cancer cell types (Huang et al., 1994; Kuoet al., 1996; Ramchandran et al., 2005). The anticancer property of Curcumin has been attributed to the ability of regulating the expression of both pro and anti-apoptotic proteins e.g. Bad, Bim, Caspase-3, p27, Bak, Bcl-2, Bax, Mcl-1, n-PARP (Woo et al., 2003; Goel et al., 2008; Reuter et al., 2008). Despite much work been done regarding the anti-cancer trends of Curcumin,

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the light on its anti-leukemic features is still dim. In the present study we looked at the cytotoxic and apoptotic potential of Curcumin on different human leukemic cell lines. We further intended to also evaluate the mechanism underlying the anti-leukemic phenomenon of Curcumin by studying the cell cycle progression, generation of reactive oxygen species (ROS), depletion of GSH content and loss of mitochondrial membrane potential on the most sensitive leukemic cell line JURKAT.

Materials and Methods

Cell and cell culture reagents

K562 (chronic myelogenous leukemia), REH (acute lymphocytic leukemia), JURKAT (T-leukemic cell line) and MOLT-4 (T-leukemic cell line) were kindly gifted by Dr. Santu Bandyopadhyaya and Dr. Chitra Mandal, IICB, Kolkata, India. Leukemic cell lines were cultured at 1×10^6 cells/ml in RPMI 1640 (Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated FBS (Invitrogen), 2mM L-glutamine,10 U/ml penicillin and streptomycin (Sigma-Aldrich) and maintained in humidified 5% CO2incubator (HF-90). Curcumin and cell viability assay stains Trypan Blue, MTT 3-(4, 5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Propidium Iodide for cell cycle progression study and DAPI for microscopic analysis of nuclear fragmentation were purchased from Sigma-Aldrich. CM-H_2DCFDA for ROS measurement and JC-1 for measuring mitochondrial membrane potential were purchased from Invitrogen (USA). For measuring apoptosis by flow Cytometry FITC Annexin-V/PI apoptosis detection Kit was purchased from BD-Pharmingen, USA.ApoGSH Glutathione detection Kit and Caspase detection Kit was purchased from BioVision. Primary antibodies, secondary antibody for PARP andβ-actin were purchased from Cell Signaling Technologies.

Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood of healthy normal donors by percoll density gradient centrifugation (d=1.082 g/L). PBMC were recovered at the interface and the recovered cells were washed and re-suspended in phosphate-buffered saline (PBS) and then finally put in RPMI 1640 supplemented with 2mM glutamine, gentamicin and 10% heat inactivated FBS.

Cell viability assay

Cells were seeded at 1×10^5 cells/well in 96-well plate with different concentrations of Curcumin and observed at 24 and 48hrs time point by MTT assay (Pal et al., 2004). In brief 10^5 cells were incubated in triplicate in a 96-well plate at different concentrations of Curcumin in a final volume of 200μl for 24 hrs and 48 hrs at 37°C. Three hours before the completion of time points, 20μl of MTT solution (5mg/mL) was added to each well. Formazone crystals formed, representing percentage of cell viability was assessed at 560 nm. Cell apoptosis (100%) was obtained by lysis of cells in 5% SDS lysis buffer. The percentage of cell viability was calculated as mentioned below: % ***cell viability= 100×(O.D. sample-O.D. 100%lysis)/ (O.D. 0%lysis-O.D.100%lysis)***

DAPI staining

To visualize apoptosis, nucleus of cells with or without treatment with Curcumin for 24 hrs were stained with DAPI (Tan et al., 2006). Briefly, after treatment with Curcumin cells were fixed with 4% para-formaldehyde prior to washing with PBS. Washed cells were then stained with 2μg/ml DAPI for 15 min in the dark. Slides were viewed with a fluorescent microscope at 340-380 nm and ×1000 magnification (Olympus BX43 Motorized fluorescence microscope). Cells were evaluated as normal or apoptotic depending on morphological characteristics. Normal nuclei (smooth nuclear structure) and apoptotic nuclei (condensed or fragmented chromatin) were easily distinguished. Thus, analysis of nuclear morphology was observed in three independent experiments.

Measurement of apoptosis by annexinV-PI staining

Apoptosis was determined by AnnexinV-PI double staining of cells with or without Curcumin treatment. In brief, cells were seeded in 96-well plate at 1×10^5cells/well, and treated with or without Curcumin at different concentrations (0-50μM) for different time points. After incubation, cells were washed with cold PBS and re-suspended in 100μl AnnexinV/PI binding buffer and incubated with 5μl of AnnexinV-FITC and 5μl of PI for 15 min at room temperature in the dark and then analyzed in flow cytometer within one hour (Damle et al., 2012). Viable cells were scored as those that were negative for AnnexinV and PI. The stained cells were analyzed by flow cytometry to determine the percentages of $AnnexinV^+/PI^-$(early apoptosis) and $AnnexinV^+/PI^+$ (late apoptosis) cells.

Cell cycle analysis

JURKAT cells (1×10^6/ tube) treated with Curcumin (0-10μM, 18 h) were fixed in chilled ethanol (70%) and kept for overnight at 4°C. Prior to analysis, cells were washed in PBS containing 2% FBS and the resultant pellet re-suspended in DNase free RNase (200μg/ml, 0.5 ml) for 2 h at 37°C; cells were then stained with PI (40μg/ml) and acquired on a flow Cytometer (Damle et al., 2012).The data were analyzed using *Flow Jo* software and expressed as % of cells in each phase of cell cycle.

Measurement of ROS

Measurement of reactive oxygen species using H_2DCFDA fluorescence was done in a 24 well plate format using conditions recommended by Molecular Probes. In brief, cells were seeded in 24-well plate at 5×10^5cells/well, were pretreated with or without Curcumin for different time points. After incubation, cells were washed with cold PBS and incubated with CM-H2DCF-DA (5μM) for another 30 min at 37°C, after which ROS production was analysed by flow Cytometry (Manna et al., 2012).

Measurement of mitochondrial membrane potential

One of the hallmark for apoptosis is the loss of mitochondrial membrane potential ($\Delta\Psi$m). $\Delta\Psi$m was measured using JC-1. In brief, JURKAT cells (1×10^6/ml)

were harvested at different time points (0-6 hr) during Curcumin treatment and were stained with JC-1 (10μM in PBS, 15 minutes, 37°C). Cells were then acquired in a flow cytometer on the basis of quadrant plot to distinguish monomers from J-aggregates and analyzed using Flow Jo software. Cells treated with H_2O_2 (100mM, 37°C, 30 minutes) serves as representative of cells with depolarized mitochondrial membrane potential. Data was expressed as the ratio of J aggregates/monomers which serves as an effective indicator of the cellular mitochondrial trans-membrane potential, allowing apoptotic cells to be easily distinguished from their non-apoptotic counterparts (Manna et al., 2012).

Measurement of intracellular GSH

Intracellular GSH was measured spectrofluorometrically using ApoGSH Glutathione Detection Kit (BioVision). In brief, 1×10^6 cells of both treated and control were collected in 1.5ml microcentrifuge tubes and centrifuged at 700g for 5 min to remove the supernatant. Then the cell pellets were lysed in 100μl of ice-cold cell lysis buffer. They were then incubated on ice for 10 min and centrifuged at high speed for 10 min, and the cell lysate was transferred into new tubes for GSH assay. Assay samples were diluted with cell lysis buffer to total volume of 100μl. 2μl of the 50U/ml GST reagent and 2μl of monochlorobimane dye were added into each sample, and the reaction was incubated at 37°C for 30 min. Then the fluorescence value was measured in a fluorescence plate reader at excitation/emission wavelengths of 380/460nm (Guha et al., 2011). The result was expressed in ng/ml of sample.

Determination of caspase activity

Activity of caspases-3 and 9 was detected in cell lysates (100μg protein in 50μl lysis buffer) as per the manufacturer's instructions. Briefly, JURKAT cells (5×10^5/ml) after incubation with Curcumin (10μM, 18 h at 37°C) was washed twice with ice cold PBS, cell lysates prepared and protein concentration estimated. Lysates were combined with 50 μl of 2× reaction buffer (containing 10 mM DTT), caspase 3 substrate DEVD-pNA (4 mM, 5 ml) or caspase 9 substrate LEHD-pNA (4 mM, 5 ml) following incubation at 37°C for 0-3 h; the release of chromophoreparanitroanilide (pNA) was quantified by measuring absorbances at 405 nm every 30 minutes for 3 h. To establish whether Curcumin induced death was caspase independent or not, JURKAT cells (1×10^4 in 200 μl/well) were pre-incubated with a pan caspase inhibitor Z-VAD-FMK (20 mM, 1 h) followed by 48 h co-incubation with Curcumin and cell viability evaluated by the MTT assay as described above.

Cell lysis and immunoblotting

Cells were treated with Curcumin analogues for 24 hrs and then lysed as previously described (Aggarwal et al., 2005). Briefly, cell pellets were resuspended in lysis buffer (0.5-1.0% TritonX-100, 150mM NaCl, 1mMEDTA, 200μM sodium orthovanadate, 10μM sodium pyrophosphate, 100μM sodium fluoride, 1.5mM magnesium chloride, 1 mmol/L phenylmethylsulfonyl-flouride, 10μg/ml aprotonin). Protein concentrations were assessed by Lowry assay before loading the samples. Equal amount of proteins were separated by SDS-PAGE and transferred to polyvinylidenedifluoride membrane (Sigma). Immunoblotting was performed with different antibodies and visualized by an enhanced chemiluminescence (ECL, Amersham, Illinois, USA) method.

Statistical analysis

All data are expressed as mean±SEM. Student's t-test was used to calculate the significance between treated and untreated cells and a p value <0.05 was considered significant.

Results

Differential cytotoxicity and anti-proliferative activity induced by Curcumin on leukemic cells

We wanted to study whether Curcumin treatment leads to the inhibition of leukemic cell proliferation. For this reason, four different leukemic cell lines i.e. K562, REH, JURKAT and MOLT-4 were cultured in the presence of 10, 20, 30 and 50μM of Curcumin for 24 and 48 hrs time point. Cell viability was measured using MTT assay. As shown in Figure 1A and 1B Curcumin shows a dose dependent induction of anti-proliferative activity in all four leukemic cell lines tested. The growth inhibition, induced by Curcumin treatment was found to be statistically significant ($p<0.05$) in all four leukemic cell lines. JURKAT cells were shown to be most sensitive to Curcumin. IC_{50} of Curcumin on REH,

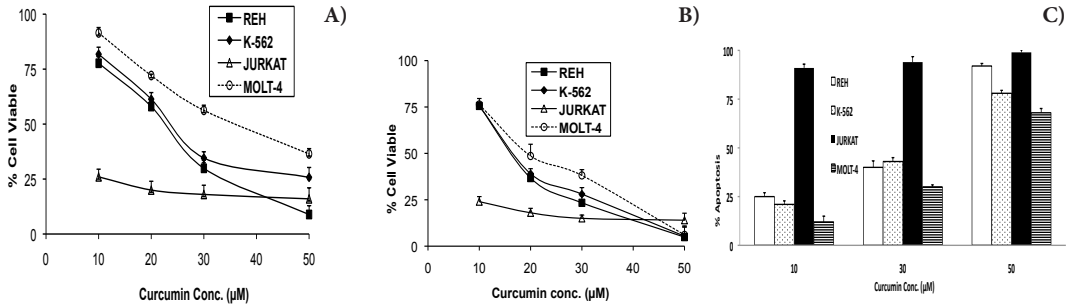

Figure 1. Effect of Curcumin on four Different Leukemic Cell Lines. Effect of Curcumin(10-50 μM) as determined by MTT assay at 24 h time point (A) and at 48 h time point (B). Induction of apoptosis at 24 h of all four leukemic cells by Curcumin at the following concentrations (10-50μM) was measured by annexin V binding and PI staining (C). Data was acquired using BD FACSVerse flow cytometer and data analyzed using flowJo software. Each point represents mean±SD of four independent experiments

K562, JURKAT and MOLT-4 cells is 21.81, 23.14, 4.22 and 37.27μM respectively at 24 hrs time point (Figure 1A). At 48 hrs time point IC_{50} of Curcumin on REH, K562, JURKAT and MOLT-4 cells is 18.62, 18.93, 2.89 and 23.72μM respectively (Figure 1B). The result shows a time dependent increase in growth inhibition of leukemic cell lines (Figure 1). Taken together the data suggests that Curcumin inhibits the cell proliferation of different leukemic cell lines in a dose and time dependent fashion and JURKAT is found to be the most sensitive of all leukemic cell lines tested. As Curcumin induced anti-proliferative activity in different leukemic cell lines, next we performed experiments to look at the apoptotic potential of Curcumin.

Curcumin induces enhanced apoptosis in leukemic cells as compared to minimal effect in normal PBMCs

To confirm whether Curcumin could induce apoptosis on these leukemic cell lines, we performed AnnexinV/PI staining, which detects an early stage of apoptosis and combined staining with propidium iodine, which detects a late stage of apoptosis. All the four human leukemic cell lines were co cultured in the presence of 10, 20, 30 and 50μM of Curcumin for 24 and 48 hrs and apoptosis was measured by flow cytometry. Figure. 1C shows that as the dose of Curcumin increased from 10μM to 50μM, cell apoptosis increased in a dose dependent fashion. Here also we observed JURKAT as the most sensitive cell line followed by K562, REH and MOLT-4 (Figure 1C). IC_{50} values of Curcumin as evidenced by AnnexinV/PI on different human leukemic cell lines are concomitant with that of the MTT assay. In short, Curcumin induces an enhanced but differential pattern of apoptosis in leukemic cell lines.

IC_{50} value of JURKAT was found to much lower as evidenced by MTT and confirmed by AnnexinV/PI data, we conducted experiments with incubating JURKAT cells with Curcumin at concentrations below 10μM. The results indicated that Curcumin induces a robust apoptosis in JURKAT cells with IC_{50} 4.2μM. Curcumin induces 19.8±2.3, 41.2±3.1, 56.1±3.4, 82.3±4.1 and 91±3.6% apoptosis at 1, 2.5, 5, 7.5 and 10μM concentrations respectively (Figure 2A). Fig. 2B depicts a representative profile of induction of apoptosis at different concentration of Curcumin on JURKAT cells as evidenced by AnnexinV-PI staining. Next we tried to observe the effect of Curcumin on PBMC collected from normal healthy donors. PBMCs from normal donors (n=3) showed minimal apoptosis (4±2%) when incubated with Curcumin at 10μM concentration at 24 hrs as compared with JURKAT cells which induces a robust (91±3.6%) apoptosis (Figure 2C). Figure. 2D is a representative profile of apoptosis as evidenced by Curcumin treatment for 24 hrs at a concentration of 10μM on Normal PBMC and JURKAT cells. Taken together, all these results suggest that Curcumin is a potent compound which induces robust apoptosis in all the four leukemic cell lines tested but having minimal effect on normal PBMC. As Curcumin induced apoptosis is specific to leukemic cells and not to normal cells thus it holds a great promise in the treatment of leukemia in future.

Curcumin augments nuclear fragmentation in JURKAT cells

Since Curcumin could effectively lead leukemic cells to undergo apoptosis, we next looked at the nuclear

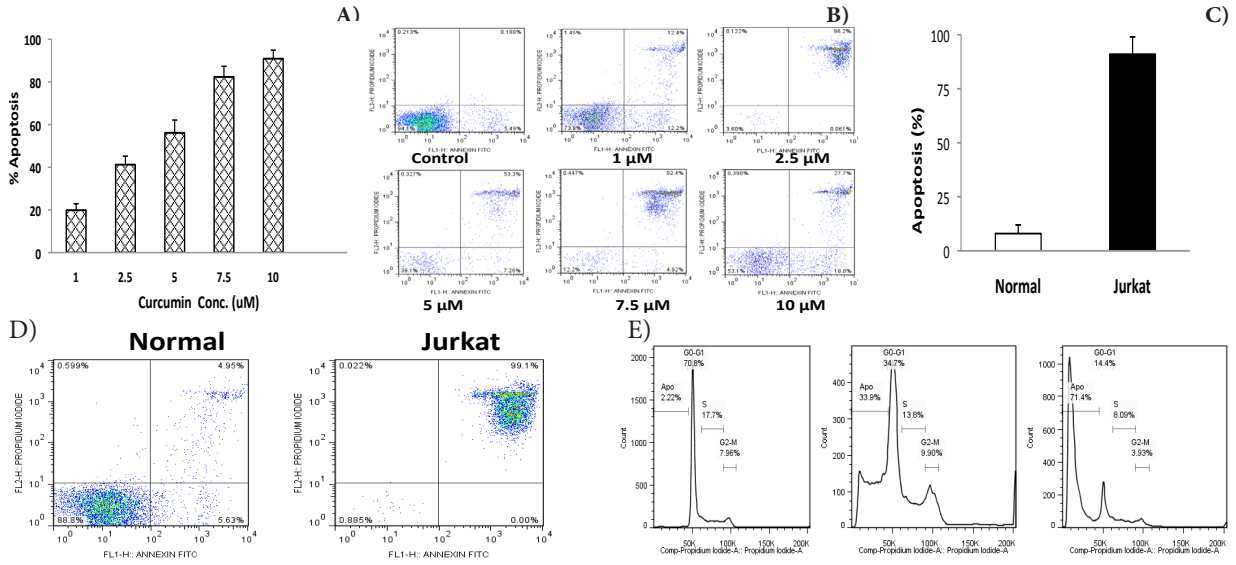

Figure 2. Percentage of Apoptosis of JURKAT Cells Exhibiting Phosphatidylserine Translocation, as Measured by Cell Surface Annexin V binding and PI Uptake. Curcumin was used at indicated concentration (1-10μM) treated for 24 h (A).Each point represents mean±SD of three independent experiments. A representative profile of annexin V/PI staining of JURKAT cells treated with Curcumin (B). Comparative study of the effect of Curcumin on normal PBMC and JURKAT cells. Normal PBMC and JURKAT cells were treated with 10μM Curcumin for 24h and cells were subsequently stained with FITC conjugated Annexin-V/PI and analyzed by BD FACSVerse flow cytometer (C). Each point represents mean±SD of three independent experiments. A representative profile of normal PBMC and JURKAT cells treated with 10μM Curcumin at 24 h time point (D). Effect of Curcumin on cell cycle progression in JURKAT cells. Cells were treated for 24 h with Curcumin at concentrations 5μM & 10μM. Then, cell cycle analysis was performed by PI staining and data acquired using flow cytometry (E). One representative of three separate experiments is shown that yielded similar results

morphology of treated as well as untreated control cells by DAPI staining. Study revealed that the nuclei of control cells were round and smaller as compared with the condensed and larger nuclei of JURKAT cells exposed to 2.5, 5 and 10μM Curcumin for 24 hrs (Supplementary Figure). The nuclear damage induced by Curcumin was in a dose-dependent manner.

Curcumin induced apoptosis by targeting G0/G1 population of JURKAT cells

Because Curcumin could appreciably induce apoptosis in JURKAT cells, we further wanted to check its effect on the cell cycle progression. Treatment with 5 and 10μM of Curcumin for 24 hrs resulted in a significant decrease of the G0/G1 cells followed by the augmentation of sub G0/G1 population (Figure 2E) ($p<0.05$).70% cells representing the G0/G1 stage in untreated control was reduced to 34.2±4% and 14.6±5% following Curcumin treatment at 5 and 10μM respectively. The plot shows cells accumulating in the sub-G0/G1 region indicating apoptosis increased from 2.22% in control cells to 33.6% and 71.8% following Curcumin treatment at 5μM and 10μM respectively. This finding suggests that Curcumin induced anti-leukemic activity in JURKAT cells by targeting the G0/G1 population.

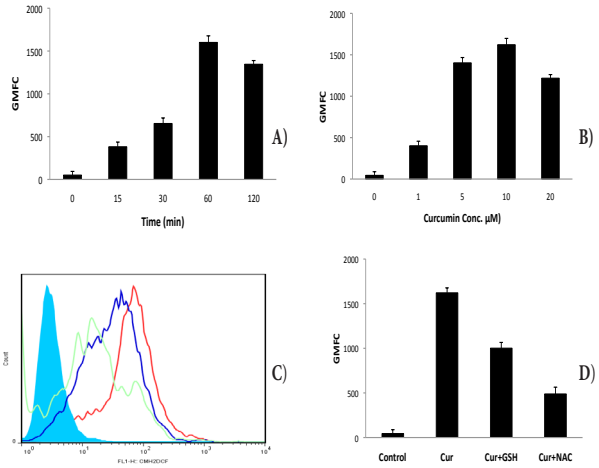

Figure 3. Modulation of Oxidant Status of JURKAT Cells by Curcumin. (A) Time dependent effect of Curcumin on generation of ROS. Cells (5×10^5) were incubated with Curcumin (10 μM) for 0-120 minutes, labeled with CM-H_2DCFDA (0.5 mM) and analyzed for fluorescence of DCF as described in baterials and Methods. Data are expressed as mean GMFC±SEM of three independent experiments. (B) concentration dependent effect of Curcumin on generation of ROS. Cells (5×10^5) were incubated with Curcumin (0-20μM) for 1 h, labeled with CM-H_2DCFDA (0.5 mM) and analyzed for fluorescence of DCF as described in Materials and Methods. Data are expressed as the mean GMFC±SEM of three independent experiments. *p 0.0001 as compared to controls. (C) A representative histogram profile of DCF fluorescence. JURKAT cells (5×10^5, unstained) were incubated in the presence of Curcumin at concentrations of 1 μM, 5 μM and 10 μM for 1hr followed by labeling with CM-2DCFDA (0.5 mM) and GMFC measured as described in Materials and Methods. (D) Effect of Curcumin on intracellular ROS generation by pre-treating cells with ROS scavenger NAC or supplementing medium with GSH. Data are expressed as mean GMFC±SEM of three independent experiments.*p <0.0001 as compared to controls

Curcumin induces ROS in leukemic cell lines

It has been entrusted that different ROS species are produced endogenously in many cell types in diverse cellular processes (Finkel et al., 2000). It has also been proposed that ROS are active cellular signaling molecules (Finkel et al., 1998; Droge et al., 2002) and have unabated role in the induction of apoptosis. In this context to check whether ROS production is associated with Curcumin induced apoptosis in JURKAT cells, we evaluated intracellular ROS generation by flow cytometry using CM-H_2DCFDA as probe. It has been observed that Curcumin could efficiently induce ROS generation in a concentration dependent manner (Figure 3B). The induction of ROS in JURKAT cells was an early event and this was as early as 30 min from the treatment point. The generation of ROS reached its peak at 60 min post Curcumin treatment in JURKAT cells (Figure 3A).The results show that at 10μM there is a maximum intracellular ROS production in Curcumin treated cells as against the untreated control cells (Figure 3C). The association of apoptosis to early ROS generation by Curcumin was further confirmed by pre-treating JURKAT cells with antioxidant N-acetyl cysteine (NAC). The results showed ROS scavenger NAC could effectively lower production of early ROS in Curcumin treated cells. Supplementation of the media with GSH also prevented intracellular ROS generation but not to the extent that with NAC (Figure 3D). Data taken together confirms that generation of early high intracellular ROS is phenomenal to Curcumin treatment of JURKAT cells and there is a probable role of GSH levels.

Generation of ROS was modulated by efflux of GSH and loss of mitochondrial membrane potential

Studies report that one of the phenomenon observed in ROS induced apoptosis is the depletion of GSH. We therefore wanted to measure the GSH levels on the JURKAT cells after Curcumin treatment. Time kinetic

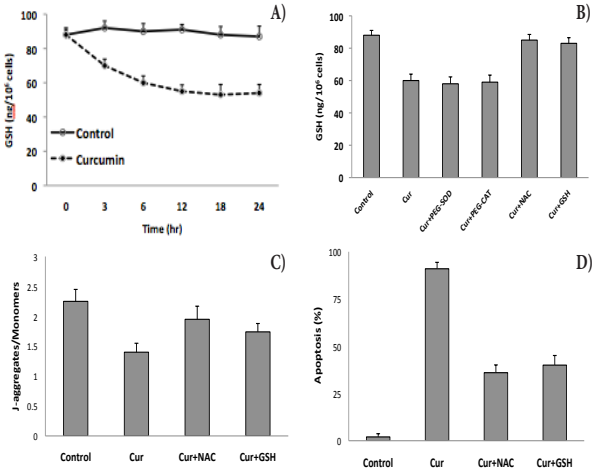

Figure 4. Efflux of Curcumin on GSH Level and Apoptosis in Leukemic Cells. A time-dependent study on intracellular GSH depletion was evaluated after Curcumin treatment (10μM) (A). The role of cell-permeant antioxidants in GSH depletion was explored (B). The role of GSH efflux on the loss of mitochondrial membrane potential (C) and apoptosis (D) was evaluated. The data are represented as mean±SD of five independent experiments. ***p <0.001 versus Curcumin

study reveals that there is a gradual depletion of GSH from 3 hrs onwards and increased over time and maintained up to 24 hrs post Curcumin treatment (Figure 4A). Many reports have suggested GSH depletion during apoptosis is an indicator for ROS formation and oxidative stress. So in the next experiments we used a variety of antioxidants to have a clear idea of the role of ROS on the modulation of GSH. Pretreatment of cells with PEG-SOD and PEG-Cat, potent intracellular scavenger for H_2O_2 did not significantly affect the depletion of GSH. On the other hand pretreatment of cells with NAC or supplementation of the media with GSH prevented intracellular GSH depletion (Figure 4B), thereby confirming that intracellular ROS generation is a phenomenon of Curcumin induced apoptosis in JURKAT cells. Loss of mitochondrial membrane potential ($\Delta\Psi m$) is one of the major consequences in ROS induced apoptosis. Therefore we investigated the loss of mitochondrial membrane potential in Curcumin treated cells. Results indicate a massive loss of $\Delta\Psi m$ which was evident with Curcumin treated JURKAT cells. Pre-treatment of cells with NAC or supplementation of the media with GSH could however prevent the loss of $\Delta\Psi m$ and apoptosis to a greater extent (Figure 4C and 4D) confirming that early ROS production is one of the hallmark events in the induction of apoptosis by Curcumin in JURKAT cells. All data taken together proves that induction of apoptosis by Curcumin on JURKAT cells is through the induction of intracellular ROS followed by disruption of mitochondrial membrane potential and depletion of intracellular GSH.

Curcumin induces apoptosis via caspase dependent pathway

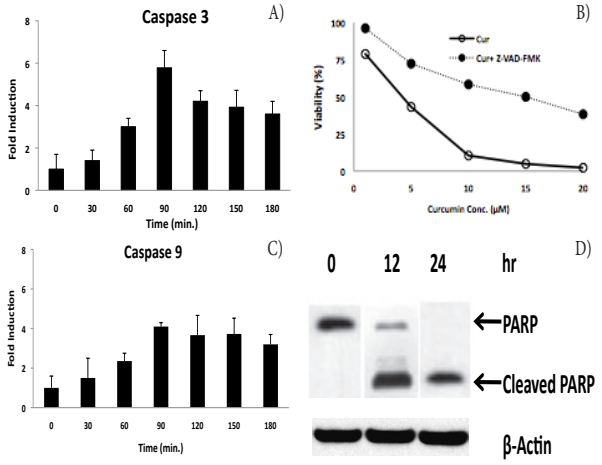

Figure 5. Effect of Curcumin on Caspase Activity and Cleavage of PARP. Lysates of JURKAT cells (o) following treatment with Curcumin (•) were used to study the activity of caspase3and 9 as described in Materials and methods (A). Each point corresponds to the mean±SD of at least three experiments in duplicate. Effect of Z-VAD-FMK on cell viability. JURKAT cells (1×10^4cells/200 μl/well) were incubated with Curcumin (0-20 μM) and Z-VAD-FMK (20mM) for 24 h and cell viability measured by the MTT assay as described in Materials and methods(B). Each point corresponds to the mean±SD of at least three experiments in duplicate. JURKAT cells were treated with Curcumin (10 μM, 12 and 24 h) as described in Materials and methods(C). The figure is a representative profile of at least three experiments

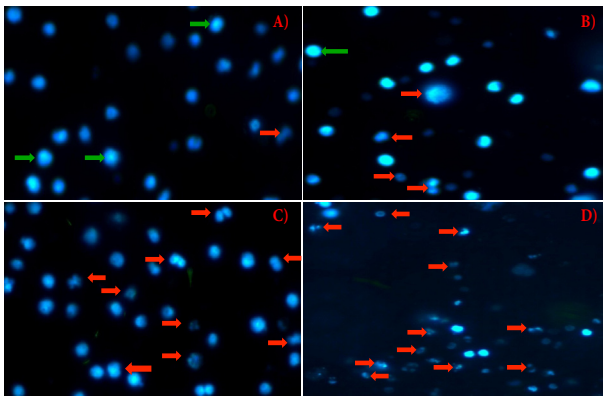

Figure 6. Fluorescence Microscopic Images of JURKAT Cells Treated with or without Curcumin at Different Concentrations. Control JURKAT cells (A), Curcumin treated JURKAT cells at concentrations 1 μM (B), 5 μM (C) and 10μM (D). The control cells were with intact nucleus and gave bright blue fluorescence indicated by green arrows; whereas treated cells showed intense fragments of nucleus indicated by red arrows as signs of apoptosis by DAPI staining

As caspases are effector molecules of the apoptotic pathways, their activity was examined in Curcumin treated cells. Lysates were prepared from cells treated with Curcumin for 24 h based on a dose response pilot study where maximal caspase activity was observed (data not shown). An exponential increase in the activity of the two caspases was observed up to 2 h after which the activity plateaued. The increased activation of caspase3 and 9 (Figure 5A), collectively indicated the potential of Curcumin to induce a marked degree of caspase activation. To confirm the role of caspases in Curcumin induced cytotoxicity, JURKAT cells were co-incubated for 24 h with Curcumin (10 μM), in the absence/presence of a nontoxic concentration of Z-VAD-FMK (20 mM, 1 h), a pan caspase inhibitor and cell viability was measured by MTT assay. The addition of Z-VAD-FMK attenuated Curcumin induced cytotoxicity, as its IC_{50} increased to 15μM from 4.22μM validating that induction of apoptosis is a caspase-dependent phenomenon in JURKAT cells (Figure 5B).

Curcumin induces cleavage of PARP

Poly (ADP) ribose polymerase (PARP), a DNA repair enzyme serves as a substrate for active effector caspase 3 and therefore when cells undergo apoptosis and the caspase cascade is activated, activated effector caspase 3 causes cleavage of PARP, resulting in abrogation of the DNA repair machinery, thereby enhancing cell death. Therefore in the preswent study we checked the effect of Curcumin on PARP cleavage. As a result, our data shows that as Curcumin activated the caspase cascade in JURKAT cells it also effectively cleaved PARP (Figure 5C).

Discussion

In this study, we have taken an effort to present a comparative study on the ability of Curcumin, a long standing polyphenol with profound anticancer effects, for its cytotoxicity and anti-proliferative activity on a panel

of different human leukemic cell lines. Results indicate that IC_{50} value of Curcumin is much lower in case of JURKAT cells as compared to other leukemic cell lines i.e. K562, REH and MOLT-4 tested (Figure 1A and 1B). One of the early events during apoptosis is the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane and that can be determined by utilizing AnnexinV, a Ca^{2+} dependent phospholipid binding protein to phosphatidylserine. So, to confirm whether Curcumin could induce apoptosis in all the four leukemic cell lines we performed AnnexinV/PI staining. Results indicate that Curcumin showed a differential apoptotic pattern in the tested leukemic cells K562, REH, JURKAT and MOLT-4 (Figure 1C). IC_{50} value of Curcumin on the most sensitive cell line JURKAT is 4.2μM (Figure 2A and 2B). To check the apoptotic effect of Curcumin on normal cells, PBMC from normal donors (n=3) were purified and cultured in the presence of Curcumin at various concentrations. Curcumin at concentrations of 10μM showed a minimal apoptosis (4±2%) in normal PBMC as compared to enhanced apoptosis (91±3.6%) on JURKAT cells (Figure 2C and 2D). The results clearly prove that Curcumin is specific for leukemic cells and does not affect normal counterparts. Since from the AnnexinV/PI staining data Curcumin was seen to be most effective on JURKAT cells we further focused our study on JURKAT cells alone. We next looked at the effect of Curcumin on the nuclear morphology. Clear nuclear fragmentation was visualized by DAPI staining after Curcumin treatment of JURKAT cells and it is concentration dependent in contrast to intact nucleus as observed in untreated cells (Figure 6). Cells undergoing apoptosis generally have active endonucleases that preferentially induce double or single stranded breaks in DNA along with chromatin condensation that translate into an increased cell population indicating a sub G0/G1 peak (Sanchez et al., 2008). Curcumin caused apoptotic fragmentation of DNA which was evidenced by increased sub G0/G1 population on a DNA frequency histogram (Figure 2E) Numerous reports suggest (Ray et al., 2012) that enhanced generation of ROS plays an important role in cancer development. It has been recognized to play a "two faced" role displaying both harmful as well as advantageous effects (Pelicano et al., 2004). Basal levels of ROS are higher in cancer cells and are often accompanied by an enhanced anti-oxidant system in respect of their normal counterparts (Storz et al., 2005). However, if an oxidative assault beyond a critical threshold is mounted, it actually leads to an imbalance in the redox homeostasis and can translate into apoptosis (Cadenas et al., 2004). Higher basal levels of ROS have been reported in leukemic cellsand are more sensitive to pro-oxidants as compared to their normal counterparts (Doering et al., 2010).Wetherefore tested the effectiveness of Curcumin to generate ROS. Exposure of JURKAT cells with Curcumin dramatically enhanced generation of intracellular ROS. Intracellular ROS production is an early event and maximal ROS generation was achieved with 10μM Curcumin concentrations within 60min of treatment. To specifically check the involvement of Curcumin in the production of ROS, we pretreated the cells with known ROS scavenger NAC, followed by Curcumin treatment. Results indicate that the intracellular ROS production was drastically lower pointing out that Curcumin was involved in the generation of ROS in JURKAT cells (Figure 3). Oxidants play a vital role to initiate the cellular apoptotic cascade by disturbing the balance between cellular signals for survival and suicide. GSH, the most abundant intracellular low molecular weight thiol is among the many detoxification processes that maintain cellular redox homeostasis (Kizhakkayil et al., 2012). We therefore supplemented the media with GSH followed by Curcumin treatment, which also decreased the generation of intracellular ROS, thereby pointing out the role of GSH in the apoptotic cascade on JURKAT cells (Figure 4). Intracellular loss of GSH has been reported as an early event in the progression of cell death in response to different apoptotic stimuli (Hammond et al., 2004; Franco et al., 2006) and has been associated with activation of a plasma membrane transport mechanism rather than with its oxidation (Van den et al., 1996; He et al., 2003). Several studies have also shown a correlation between cellular depletion of GSH and progression of apoptosis (Ghibelli et al., 1998; Franco et al., 2006). Generation of ROS usually oxidizes GSH to GSSG and ultimately reduces the total GSH level. However, many *in vitro* studies have pointed out that a reduction in the intracellular GSH is necessary for the formation of ROS (Franco et al., 2007). Therefore we measured the levels of GSH on JURKAT cells post Curcumin treatment. Results indicate that there is a drastic depletion of GSH level post Curcumin treatment of JURKAT cells, which could not be reverted back upon treatment with potent intracellular scavenger for H2O2 i.e. PEG-SOD or PEG-Cat. But pretreatment of JURKAT cells with ROS scavenger NAC or supplementation of the media with GSH prevented GSH depletion and apoptosis (Figure 4). Data taken together suggests a depletion of GSH level and augmentation of intracellular ROS generation in Curcumin induced apoptosis on JURKAT cells. Depletion of GSH has been shown to directly modulate both the loss of mitochondrial membrane potential and the activation of executioner caspases (Armstrong et al., 2002; Franco et al., 2007). Hence, we sought to clarify the precise mechanism of GSH loss in Curcumin-induced apoptosis. There is a drastic loss of $\Delta\Psi$m post Curcumin treatment on JURKAT cells and that could be reverted back by pre-treating the cells with ROS scavenger NAC or supplementing the media with GSH (Figure 4C). From this observation it is clear that loss of $\Delta\Psi$m was a ROS dependent phenomenon in Curcumin induced apoptosis in JURKAT cells. One of the feature of apoptosis is the release of cytochrome C in the cytosol which inturn forms complexes with Caspase 9 and Apaf-1 that helps to activate executioner Caspase 3 leading to DNA fragmentation and cell death. Here also we observed increase in caspase 9 and caspase 3 activity (Figure 5A) post Curcumin treatment as compared to untreated control cells.

Activated caspase-3 in turn carries out cleavage of the DNA repair enzyme, PARP culminating in DNA degradation. Executioner caspases are therefore considered critical in the apoptotic cascade and are inducible by different stimuli that include growth-factor deprivation and various environmental stresses, including anti-cancer drugs (Zou et al., 2003). Here also we observed PARP cleavage following Curcumin treatment of JURKAT cells (Figure 5C). Taken together, Curcumin acting as pro-oxidant, effectively raised the cell's oxidative status beyond a threshold limit inducing apoptosis in leukemic cells.

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References

Aggarwal BB, Kumar A, Aggarwal MS, et al (2005). Curcumin derived from turmeric (Curcuma longa): a spice for all seasons. *Phytopharm Cancer Chemo Prev*, 349-387.

Aggarwal BB, Sundaram C, Malani N, Ichikawa H (2007). Curcumin: the Indian solid gold. *AdvExpt Med Biol*, **595**, 1-75.

Aggarwal BB, Harikumar KB (2009). Potential therapeutic effects of Curcumin, the anti inflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases. *Int J Biochem Cell Biol*, **41**, 40-59.

Armstrong JS, Jones DP (2002). Glutathione depletion enforces the mitochondrial permeability transition and causes cell death in Bcl-2 overexpressing HL60 cells. *FASEB J*, **16**, 1263-5.

Cadenas E (2004). Mitochondrial free radical production and cell signaling. *MolAspects Med*, **25**, 17-26.

Damle RN, Temburni S, Banapour T, et al (2012). T-cell independent, B-cell receptor-mediated induction of telomerase activity differs among IGHV mutation-based subgroups of chronic lymphocytic leukemia patients. *Blood*, **120**, 2438-49.

Droge W (2002). Free radicals in the physiological control of cell function. *Physiol Rev*, **82**, 47-95.

Doering M, Ba LA, Lilienthal N, et al (2010). Synthesis and selective anticancer activity of organochalcogen based redox catalysts. *J Med Chem*, **53**, 6954-63.

Finkel T (1998). Oxygen radicals and signaling. *Cur Opinion Cell Biol*, **10**, 248-53.

Finkel TR (2000). Redox-dependent signal transduction. *FEBS Lett*, **476**, 52-4.

Franco R, Cidlowski JA (2006). SLCO/OATP-like transport of glutathione in FasLinduced apoptosis: glutathione efflux is coupled to an organic anion exchange and is necessary for the progression of the execution phase of apoptosis. *J Biol Chem*, **281**, 29542-57.

Franco R, Panayiotidis MI, Cidlowski JA (2007). Glutathione depletion is necessary for apoptosis in lymphoid cells independent of reactive oxygen species formation. *J Biol Chem*, **282**, 30452-65.

Gupta SC, Patchva S, Aggarwal BB (2012). Therapeutic roles of Curcumin: lessons learned from clinical trials. *Am Ass Pharm Sc*, **15**, 195-218.

Goel A, Jhurani S, Aggarwal BB (2008). Multi-targeted therapy by Curcumin: how spicy is it? *Mol Nut Food Res*, **52**, 1010-30.

Ghibelli L, Fanelli C, Rotilio G, et al (1998). Rescue of cells from apoptosis by inhibition of active GSH extrusion. *FASEB J*, **12**, 479-86.

Guha P, Dey A, Sen R, et al (2011). Intracellular GSH depletion triggered mitochondrial Bax translocation to accomplish resveratrol-induced apoptosis in the U937 cell line. *J Pharmacol Expt Ther*, **336** 206-214.

Huang HC, Jan TR, Yeh SF (1992). Inhibitory effect of Curcumin, an anti-inflammatory agent, on vascular smooth muscle cell proliferation. *Eur J Pharmcol*, **221**, 381-4.

Huang MT, Lou YR, Ma W, et al (1994).Inhibitory effects of dietary Curcumin on fore-stomach, duodenal, and colon carcinogenesis in mice. *Cancer Res*, **54**, 5841-7.

Hammond CL, Madejczyk MS, Ballatori N (2004). Activation of plasma membrane reduced glutathione transport in death receptor apoptosis of HepG2 cells. *Toxicol App Pharmacol*, **195**, 12-22.

He YY, Huang JL, Ramirez DC, et al (2003). Role of reduced glutathione efflux in apoptosis of immortalized human keratinocytes induced by UVA. *J Biol Chem*, **278**, 8058-64.

James JS (1996). Curcumin: clinical trial finds no antiviral effect. *AIDS Treat News*, **242**, 1-2.

Kiuchi F, Goto Y, Sugimoto N (1993).Nematocidal activity of turmeric: synergistic action of Curcuminoids. *Chem Pharmacol Bul*, **41**, 1640-3.

Kizhakkayil J, Thayyullathil F, Chathoth S, et al (2012). Glutathione regulates caspase-dependent ceramide production and Curcumin induced apoptosis in human leukemic cells. *Free Rad Biol Med*, **52**, 1854-64.

Manna A, Saha P, Sarkar A, et al (2012).Malabaricone-A induces a redox imbalance that mediates apoptosis in U937 cell line. *PLoS One*, **7**, 1-11.

Piwocka K, Bielak-Mijewska A, Sikora E (2002). Curcumin induces caspase-3independent apoptosis in human multidrug-resistant cells. *Ann New York Aca Sci*, **973**, 250-4.

Pal S, Ghosh S, Bandyopadhyay S, et al (2004). Differential expression of 9-O-acetylated sialoglycoconjugates on leukemic blasts: a potential tool for long-term monitoring of children with acute lymphoblastic leukaemia. *Int J Cancer*, **11**, 270-7.

Pelicano H, Carney D, Huang P (2004). ROS stress in cancer cells and therapeutic implications. *Drug Resis Update*, **7**, 97-110.

Ramachandran C, Rodriguez S, Ramachandran R (2005). Expression profiles of apoptotic genes induced by Curcumin in human breast cancer and mammary epithelial cell lines. *Anti Cancer Res*, **25**, 3293-302.

Reuter S, Eifes S, Dicato M, et al (2008). Modulation of anti apoptotic and survival pathways by Curcumin as a strategy to induce apoptosis in cancer cells. *Biochem Pharmacol*, **76**, 1340-51.

Ray PD, Huang BW, Tsuji Y (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal*, **24**, 981-90.

Sanchez CA, Rodriguez E, Varela E, et al (2008). Stat induced inhibition of MCF-7 breast cancer cell proliferation is related to cell cycle arrest and apoptotic and necrotic cell death mediated by an enhanced oxidative stress. *Cancer Invest*, **26**, 698-707.

Storz P (2005). Reactive oxygen species in tumor progression. *Front Biosc*, **10**, 1881-96.

Tan TW, Tsai HR, Lu HF, et al (2006). Curcumin-induced cell cycle arrest and apoptosis in human acute promyelocyticleukemia HL-60 cells via MMP changes and caspase-3 activation. *Anticancer Res*, **26**, 4361-71.

Venkatesan N, Chandrakasan G (1995). Modulation of cyclophosphamide-induced early lung injury by Curcumin, an anti-inflammatory antioxidant. *Mol Cell Biochem*, **142**, 79-87.

Van den, Dobbelsteen DJ, Nobel CS, et al (1996).Rapid and specific efflux of reduced glutathione during apoptosis induced by anti-Fas/APO-1 antibody. *J Biol Chem*, **271**, 15420-7.

Woo JH, Kim YH, Choi YJ, et al (2003). Molecular mechanisms of Curcumin-induced cytotoxicity: induction of apoptosis through generation of reactive oxygen species, down regulation of Bcl XL and IAP, the release of cytochrome c and inhibition of Akt. *Carcinogenesis*, **24**, 1199-208.

Zou H, Yang R, Hao J, et al (2003).Regulation of the Apaf-1/caspase-9 apoptosome by caspase-3 and XIAP. *J Biol Chem*, **278**, 8091-8.

Zhou H, Beevers SC, Huang S (2011).Targets of Curcumin. *Curr Drug Targets*, **12**, 332-47.