Abstract. Curcumin has been documented to exert anticancer effects by interacting with altered proliferative and apoptotic pathways in cancer models. In this study, we evaluated the potential of curcumin to reverse promoter methylation of the p15 gene in Raji cells and its ability to induce apoptosis and genomic instability. Anti-neoplastic action of curcumin showed an augmentation in reactive oxygen species (ROS) and cell cycle arrest in G1 phase. Subsequently, curcumin-exposed Raji cells showed structural abnormalities in chromosomes. These observations suggest that curcumin also causes ROS-mediated apoptosis and genomic instability. The treatment of Raji cell line with 10 μM curcumin caused hypomethylation of the p15 promoter after six days. Hypomethylation of p15 was further found to be favoured by down-regulation of DNA methyltransferase 1 after 10 μM curcumin treatment for six days. Methylation-specific PCR suggested demethylation of the p15 promoter. Demethylation was further validated by DNA sequencing. Reverse-transcription PCR demonstrated that treatment with curcumin (10 μM) for six days led to the up-regulation of p15 and down-regulation of DNA methyltransferase 1. Furthermore, curcumin-mediated reversal of p15 promoter methylation might be potentiated by down-regulation of DNA methyltransferase 1 expression, which was supported by cell cycle analysis. Furthermore, curcumin acts as a double-pronged agent, as it caused apoptosis and promoter hypomethylation in Raji cells.

Introduction

Cancer is a multi-factor, multi-gene-related complex process and DNA methylation plays a major role in tumorigenesis (Wajed et al., 2001). DNA methylation is catalysed by DNMTs (DNA methyltransferases). Among different DNMTs, DNMT1 is responsible for maintaining the methylation pattern during cell division from one generation to the next generation. Overexpression of DNMTs in previous studies with leukaemia samples suggested their role in molecular pathogenesis (Mizuno et al., 2001). The frequent gene silencing by promoter hypermethylation has been implicated in the development of malignancies including lymphoma (Wong et al., 2000; Chim et al., 2003; Zhang et al., 2007). Since epigenetic alterations do not change DNA sequences (Chen et al., 2009), it is proposed that epigenetic changes are dynamic identities and can be reversed. Presently, several synthetic drugs that can reduce DNA methylation are under trial. Therapy to restore silenced gene expression is being tested for anticancer potential (Andreoli et al., 2013). However, these synthetic chemicals are very toxic. Therefore, several natural plant products are being tested for epigenetic reversal (Reuter et al., 2011). One of the natural products, curcumin, has been shown to possess a variety of biological functions including anti-tumour activity and may target all steps of cancer development, including tumour initiation to progression. It has been investigated for its anti-neoplastic action against a variety of malignancies (Aggarwal et al., 2003). However, the mechanism by which curcumin inhibits cancer cell proliferation needed additional clarifications. Only a few studies have reported the effect of curcumin on DNA methylation. We reported reversal of promoter hypermethylation and reactivation of the RARβ gene in a cervical cancer cell line by curcumin (Jha et al., 2010). Recently it has been shown that curcumin can modulate DNA methylation in acute myeloid leukaemia (Yu et al., 2013).

The p15 gene, a target of cyclin-dependent kinase inhibitor, is aberrantly methylated in several human neoplasms, especially among hematopoietic malignancies.
p15 has been postulated to be a tumour suppressor gene modulating pRb phosphorylation (Stone et al., 1995). Among solid and soft tissue tumours, p15 promoter hypermethylation has occasionally been found in plasmacytoma, brain lymphoma, non-Hodgkin’s lymphoma, Burkitt’s lymphoma, and mantle cell lymphoma (Gronbaek et al., 1998; Wong et al., 1998; Zhang et al., 1998). In an attempt to study the methylation pattern of promoters of tumour suppressor genes in acute lymphoid leukaemia (ALL) patients of North India, we observed hypermethylation of the p15/INK4b promoter in 39 out of 125 biopsies (31.2%). The difference in methylation of p15/INK4b (P = 0.000) was statistically highly significant when compared with control samples (data communicated). Therefore, the p15 gene may provide an appropriate model for reversal of hypermethylation studies in ALL. To this aim, the present study was undertaken to examine some of the so far unexplored consequences of curcumin effects on the reversal of promoter methylation and reactivation of the p15 gene in ALL.

Material and Methods

The Raji cell line (ALL) was procured from the National Centre for Cell Sciences (Pune, India). Tissue culture medium RPMI-1640 was obtained from Sigma-Aldrich Chemicals (Pvt) Ltd. (St. Louis, MO) and foetal bovine serum (FBS) was obtained from HiMedia (Mumbai, India). Curcumin was purchased from Acros Organics (Fair Lawn, Nj). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from HiMedia. 5-aza-2'-deoxycytidine, trizol, propidium iodide (PI), and 2,7'-dichlorofluorescein diacetate (DCFH-DA) reagents were obtained from Sigma-Aldrich.

Cell culture and treatment

Raji cells were cultured according to the protocol optimized earlier in our lab. Briefly, the Raji cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS in 5% CO2 at 37 °C. The cells were re-supplemented with fresh medium and test compounds every 48 h. All the treatments for reversal of promoter methylation analysis were carried out for six days since the DNA methylation pattern was transferred from one generation to the next generation and maintained by DNMTs, and therefore it was necessary to analyse its effect in a time-dependent manner. Treatment concentrations were kept low, i.e. 5 μM and 10 μM, in order to find the minimum effective concentrations and to make cells survive for longer duration. All other treatments were carried out using standard concentration at IC50 (20 μM) and below IC50 (10 μM).

Cytotoxicity of chemopreventive agent

The cytotoxic effect of curcumin was studied on Raji cells by the MTT method (Heckenkamp et al., 1999). Briefly, the cells were cultured in 96-well plates at a density of 1 × 104 cells per well with or without different concentrations of curcumin (0–35 μM). After incubation for 48 h, MTT dissolved in PBS was added to each well at a final concentration of 5 mg/ml and then incubated at 37 °C and 5% CO2 for 2 h. The water insoluble dark blue formazan was dissolved in DMSO. The optical density was measured by a microplate reader (Bio-Rad, Berkeley, CA) at a wavelength of 570 nm.

Morphological study

To determine the dose-dependent morphological changes in Raji cells, the cells were treated with different concentrations of curcumin and observed through a phase-contrast microscope (Nikon, Tokyo, Japan) after 48 h of treatment with curcumin at 10 μM or 20 μM. Untreated Raji cells were taken as a control.

Acridine orange/ethidium bromide staining

To study the effect of curcumin on apoptosis induction, Raji cells were grown in the presence of 10 μM or 20 μM of curcumin for 48 h and counted using a haemocytometer and then observed under a fluorescence microscope (Nikon) after staining with acridine orange/ethidium bromide (10 μg/ml).

DNA fragmentation assay

The amount of 1 × 106 cells/ml was treated with curcumin at 10 μM or 20 μM for 48 h along with a proper control (without treatment). Cellular DNA of the treated cells was extracted from the cells according to the Gong’s modified method (Gong et al., 1994). Briefly, curcumin-treated and untreated cells were washed twice in cold PBS (10 mM) and re-suspended in hypotonic lysis buffer (5 mM Tris, 20 mM EDTA, pH 7.4 ) containing 0.5% Triton X-100 for 30 min at 4 °C. The lysate was centrifuged at 13,000 g for 15 min at 4 °C. DNA was extracted from the supernatant with equal volume of phenol-chloroform-isoamylalcohol, precipitated by addition of two volumes of absolute ethanol, 0.1 volume of 3 mM sodium acetate and treated with RNAse A (500 U/ml) at 37 °C for 3 h. The pattern of DNA fragmentation was analysed in 1.5% agarose gel.

Chromosome preparations

Raji cells were seeded at 1 × 105 cells/ml and treated with curcumin at different concentrations (10 μM, 20 μM) for 48 h. Cells without treatment were taken as a control. Chromosomes were prepared after 48 h following 2 h colcemid treatment (0.1 μg/ml). G banding was done using 0.005% trypsin and chromosomes were analysed under a light microscope. Comparative mitotic indices of the slides were calculated by considering the average values of the ratio of metaphase plate vs. the number of cells in five different areas of slides at 10× magnification.

Reactive oxygen species (ROS) estimation

Intracellular ROS accumulation in Raji cells was measured and quantified with DCFH-DA dye. Raji cells
(1 × 10^6 cells/ml) were treated with curcumin at a concentration of 10 μM and 20 μM for 48 h and 72 h, respectively. Harvested cells were incubated with DCFH-DA dye followed and investigated by flow cytometric analysis. DCFH-DA crosses cell membranes and its hydrolysis with nonspecific esterases generates non-fluorescent DCFH. This DCFH in the presence of ROS is further oxidized to DCF, which is readily detected by flow cytometry. The fluorescence is equal to the amount of intracellular ROS generated. Cells without treatment were secured as a control.

**Cell cycle analysis**

Cell cycle analysis was carried out using propidium iodide as described previously (Krishan, 1975). Briefly, 1 × 10^6 cells/ml were treated with or without curcumin at a concentration of 10 μM or 20 μM and then washed with chilled PBS twice. Further, cells were fixed with 70% ethanol for 40 min. After that, cells were washed again with PBS and resuspended in 500 μl of staining solution containing propidium iodide, detergent and RNase as described previously.

**Methylation-specific PCR**

The effect of curcumin on the promoter methylation of p15 gene in the Raji cell line was assessed by methylation-specific PCR (MSP) (Lee et al., 2005). The Raji cell line was exposed to 5 μM or 10 μM curcumin for six days before conducting MSP for the tested gene. DNA extracted from the cell line was modified with sodium bisulphite and MSP was carried out using specific primers for methylation and non-methylation of the tested gene (Herman et al., 1996).

**RT-PCR**

RNA was isolated from the treated and untreated cells after 72 h and 6 days using the trizol reagent (Sigma-Aldrich). An equal amount of RNA was used to synthesize cDNA after DNase treatment using the Revert Aid first-strand cDNA synthesis kit (Fermentas, Glen Burnie, MD). RT-PCR was carried out to check the alteration in the level of mRNA expression after the treatment with curcumin and 5-aza-2’-deoxycytidine using primers as given in Table 1.

**Results and Discussion**

Anticancer drugs have demonstrated distinct mechanisms of action/effects on different types of cancer cells. Zwaan et al. (2000) demonstrated that several drugs showed different cellular resistance in the case of AML and ALL patients. The cellular drug resistance has also been shown to correlate with both the cell lineage and the type of genetic rearrangement (Palle et al., 2005). Since different types of cancer and different cells within a tumour can behave very differently to cancer therapy, and cancer drugs are supposed to be tissue specific and cell specific, it is relevant to explore the curcumin behaviour in the acute lymphoid leukaemia model. Raji cells (ALL) were chosen for the study as the p15 gene promoter is methylated in this cell line. The effect of curcumin was studied extensively in terms of genomic instability, apoptosis, cell cycle, ROS generation, and its association with the reversal of promoter methylation and reactivation of the p15 gene.

**Curcumin exhibits an apoptotic and growth inhibitory effect**

The dose kinetics of curcumin was evaluated by MTT assay (Fig. 1A). The cytotoxicity of curcumin was significantly increased in a dose-dependent manner, whereas the IC50 dose of curcumin was 20 μM ± 2.0. It was also observed that curcumin caused apoptosis as observed by acridine orange/ethidium bromide (AO/EB) staining, which showed nuclear changes and apoptotic body formation (Fig. 1C). Subsequently, DNA fragmentation was observed after treatment with 20 μM curcumin (Fig. 1B).

**Curcumin caused ROS-mediated apoptosis and genomic instability**

The ROS content inside the cells was estimated by flow cytometry as described in Material and Methods. The level of fluorescence is indicative of the presence of oxidative stress inside the cell. ROS have been shown to be involved in cell proliferation and apoptosis. In order to measure the capacity of curcumin to induce ROS, we used DCFH-DA (Fig. 2A, B). Exposure of Raji cells

| Table 1. |
|-----------------|---------------|-------------|----------|
| **Sequence (5'→3')** | **Anneal. temperature (°C)** | **Extent (bp)** | **Reference** |
| p15 | MF: GCCGTTCGATTTTTCGGTT | 62 °C | 148 | Herman et al., 1996 |
| p15 | MR: CCGTACAAATACCGAAGCAA | | | |
| p15 | UF: TTGGATGCGTTGATTTTTGTTT | 59 °C | 154 | Herman et al., 1996 |
| p15 | UR: CCATACAAATACCGAAGCAA | | | |
| p15 | F: GTGGGGCCGGCAGGGATGAG | 63 °C | 451 | Cameron et al., 1999 |
| p15 | R: AGGTTGGGTGAGGGTGGGAAT | | | |
| β-actin | F: GTGGGGCTGCTTCAGGCAACA | 56 °C | 245 | Heyer et al., 2002 |
| | R: GTGGGGCTGCTTCAGGCAACA | | | |
| DNMT1 | F: ACCGGTTCCTCCTCTCCTCCT | 56 °C | 335 | Minami et al., 2010 |
| | R: GTTGCAGTCTCCTCTGGAACACTGTGG | | | |
to curcumin at 10 μM and 20 μM for 48 h and 72 h, respectively, caused significant ROS formation, whose amounts increased in a dose- and time-dependent manner. Culture of Raji cells without curcumin for 72 h was depicted by fluorescence of 2.97. As compared to the control experiment, when the cells were cultured with curcumin at 10 μM and 20 μM (Fig. 2Bb,c), the level of ROS increased to 18.63 and 30.19 after 72 h, respectively. Treatment at 10 μM and 20 μM showed 6.42-fold and 10.4-fold increase in the ROS content after 72 h, respectively. The shift in the peak corresponds to concentration-dependent ROS generation after the treatment with curcumin (Fig. 2). The significant extent of ROS production corresponds to ROS-mediated apoptosis in Raji cells. This report is in agreement with earlier investigation showing ROS production by curcumin in vitro (Sanchez et al., 2010).

After that, we analysed the effect of curcumin on genomic stability in order to assess the ROS contribution to the genomic instability. Chromosome analysis was successfully achieved after G banding. The results were compared between treated and non-treated cells (Fig. 3). Curcumin (20 μM) treatment resulted in breakage of chromosome arms. The comparative mitotic index of control slides was found to be 0.35, while for curcumin (20 μM) 0.19 was observed. Subsequently, the frequency of aberrant metaphases (N = 20) was found for the control: 0.0 (N = 25) and for curcumin: 0.24 (N = 25). It appears that curcumin caused genomic instability in Raji cells. Since ROS was supposed to have a destructive effect on DNA and protein (Simon et al., 2000), ROS generation after curcumin treatment appeared to be associated with chromosomal abnormality or genomic instability in the Raji cells. Previously, curcumin has been reported to cause structural abnormality in chromosomes of the CHO cell line (Antunes et al., 1999).
Curcumin caused cell-cycle arrest

Raji cells were treated with 10 μM or 20 μM curcumin for 48 h and 72 h. Beside induction of apoptosis, curcumin also caused cell-cycle arrest, suggesting curcumin as a dual agent. Curcumin treatment resulted in cell-cycle arrest in G1 phase and depletion in S-phase fraction (Fig. 4). These results are in agreement with earlier studies on Raji cells with curcumin (Sun et al., 2005).

Fig. 2. ROS estimation in Raji cells after 48 h (A), 72 h (B) with (a) control, (b) 10 μM curcumin, (c) 20 μM curcumin. The treatment shows an augmentation in the ROS content in a concentration- and time-dependent manner.

Fig. 3. Representative metaphase plates showing chromosome breaks following curcumin treatment at 10 μM and 20 μM.

Fig. 4. Effect of curcumin on cell-cycle distribution in Raji cells. Cells were treated with curcumin (0, 10 μM, 20 μM) for 72 h. Cell-cycle distribution was determined by flow cytometry. Representative sets of histograms for Raji cells (A) untreated, (B) 10 μM and (C) 20 μM curcumin-treated cells. Percentages of cells in Sub G1, G0/G1, S, and G2/M phase are shown as insets for each experiment. Curcumin caused G1 phase arrest.
Curcumin caused up-regulation of p15 and down-regulation of DNMT1

In order to elucidate curcumin anti-neoplastic action, we checked the expression of p15 after treatment with curcumin at 10 μM concentration for 72 h. We observed up-regulation in expression of the p15 gene (Fig. 5). Since the p15 gene has been reported to be methylated in the Raji cell line, to find out whether these changes are accompanied by promoter demethylation we extended our treatment for six days. For further experiments we found 5 μM and 10 μM as minimum effective concentrations in comparison to 10 μM and 20 μM previously reported in AML. These studies suggested higher sensitivity of ALL toward curcumin as compared to AML (Yu et al., 2013). Despite previous reports regarding the molecular mechanism of curcumin in epigenetic regulations, there are few reports demonstrating that it might display an inhibitory action on DNMT1. To further correlate curcumin-mediated reversal of promoter methylation of the p15 gene and DNMT1 inhibition, gene expression analysis was carried out after treatment of Raji cells with curcumin (5 μM, 10 μM) for six days. The mRNA levels of p15 and DNMT1 genes in Raji cells were examined by reverse transcription PCR with and without exposure to curcumin. Treatment with curcumin at 10 μM resulted in nearly 15-fold up-regulation of the p15 mRNA. Further, to examine whether curcumin-mediated reversal of promoter methylation had any correlation with DNMT1 expression, its expression level was examined in the same samples.

The expression of DNMT1 was found to be down-regulated in a time- and concentration-dependent manner (Fig. 6), while no change in expression was observed with 5-aza-2'-deoxycytidine. Current demethylating drugs, such as 5-aza-2'-deoxycytidine, exert their inhibitory effect by covalent binding to DNMT1, which leads to irreversible inhibition of the DNMT1 activity, causing hypomethylation of the genomic DNA (Christman, 2002). As previously described in the literature, demethylation may be favoured via binding with the catalytic domain of DNMT1 or depletion of DNMT1 protein levels (Loriot et al., 2006). Molecular docking analysis revealed that curcumin covalently blocks the catalytic site of DNMT1 to exert its inhibitory effect (Zhongfa et al., 2009). During our investigation we observed that curcumin also led to the down-regulation of the DNMT1 mRNA level. Melki et al. (1998) reported that leukaemia patients show 4.4-fold higher expression than normal control. This higher activity of DNMT1 could be responsible for hypermethylation of the p15 gene. This suggests that in addition to direct inhibition, down-regulation of DNMT1 expression by curcumin could be an additional factor favouring reversal of promoter methylation and up-regulation of the p15 gene. Cell-cycle analysis revealed G1 arrest. This arrest might be associated with early hypomethylation of the p15 gene promoter. Such reversal of promoter methylation may potentiate curcumin-associated anticancer properties. Generation of ROS is accompanied by a significant decrease in the DNMT1 mRNA level. This suggested that there might be an interplay between ROS generation and DNMT1 down-regulation. These observations also emphasized the role of ROS in epigenetic changes, and further study is needed to elucidate the exact interplay between ROS and DNA methylation.

Reversal of p15 methylation by curcumin

p15 is hypermethylated in cancer cell lines and has been considered as a methylation marker for tumorigenesis (Cameron et al., 1999). The p15 promoter was found to be methylated in the Raji cell line. Methylation-specific analysis showed that curcumin decreased the methylation of the p15 gene. Both the appearance and enhanced intensity of non-methylation-specific amplified product and correspondingly decreased intensity of the methylation-specific amplified product in the gel were observed. The decrease in methylation-specific amplified product intensity after six days confirmed reversal of p15 gene promoter methylation (Fig. 7). Similar results after 5-aza-2'-deoxycytidine (a positive con-
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Conclusion

Based on this study we suggest that curcumin acts as a dual agent causing both apoptosis and reversal of promoter methylation of the \( p15 \) gene in an ALL cell line. Taken together, the results of the present study would be an innovative quest to understand the molecular mechanism of curcumin as an epigenetic modifying anticancer therapeutic agent. Based on these observations, we could draw a possible conclusion that curcumin is a potent demethylating agent that restores the epigenetically silenced \( p15 \) gene expression by down-regulating DNMT1 and could be a novel therapeutic strategy against ALL patients. Demethylation of the \( p15 \) gene by curcumin would contribute to an effective regimen for cancer therapy.

Declaration of interest

None.

References


