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Original article:

CURCUMIN INDUCED CELL DEATH AND INHIBITION OF TELOMERASE ACTIVITY IN MOUSE LYMPHOMA P388D1 CELLS

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ABSTRACT

Telomerase, a potential marker for tumorigenesis, has been found to be activated in more than 85-90% of human cancer. Curcumin is the major biologically active, yellow phytochemical compound of *Curcuma longa* (Zingiberaceae). The present study is aimed to investigate the capacity of curcumin on the regulation of telomerase activity and induction of apoptosis in P388D1 mouse lymphoma cells. Here, we demonstrate that curcumin at a concentration of 3.5 µM and an incubation period of 48h induces apoptosis and inhibits telomerase activity in the P388D1 cells. Curcumin induced apoptosis and telomerase activity in P388D1 lymphoma cells was confirmed by enumeration of apoptotic cells, % DNA fragmentation and RT-PCR. The culture supernatant of lymphoma cells treated with curcumin showed a higher level of nitric oxide content. RT-PCR analysis revealed over expression of TNF-α and IL-1β and inhibition of the antiapoptotic Bcl-2 and human catalytic subunit hTERT in the curcumin treated lymphoma cells as compared to untreated cells. Taken together the result shows that curcumin could significantly inhibit tumor proliferation and induce apoptosis in lymphoma cells. Thus, curcumin should be further tested as a possible antineoplastic agent.

Keywords: curcumin, apoptosis, RT PCR, lymphoma

INTRODUCTION

Curcumin (diferuloylmethane), a polyphenolic compound (molecular formula $C_{21}H_{20}O_6$) isolated from the plant *Curcuma longa,* has been widely used as a spice and coloring agent (Araÿjo and Leon, 2001; Goel et al., 2008). Curcumin (Figure 1) acts as potent antioxidant, anti-inflammatory and antiproliferative therapeutic agent (Jagetia and Aggarwal, 2007; Alaikov et al., 2007). Commercially, curcumin contains approximately 77 % diferuloylmethane, 17 % demethoxycurcumin, and 6 % bisdemethoxycurcumin (Goel et al., 2008).

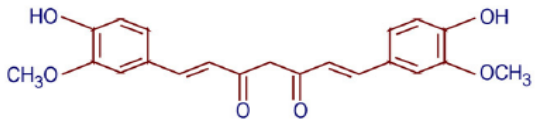

Figure 1: The structure of curcumin

Recently, curcumin has gained special interest due to its curative impact on numerous ailments such as wound healing, diabetes, Alzheimer disease, Parkinson disease, cardiovascular disease, pulmonary disease and arthritis (Goel et al., 2008). Curcumin can act as a potent immunomodulatory agent that can modulate the activation of T cells, B cells, macrophages, neutrophils, natural killer cells, and dendritic cells (Gaurisankar and Das, 2008; Jagetia and Aggarwal, 2007). Several reports have docu-

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mented that curcumin can induce apoptosis in cancer cells from liver, colon, breast, stomach and duodenal tissue (Shi et al., 2006).

Reports suggest apoptosis involves changes in the expression of apoptotic agent's like Bcl-2, TNF-α and IL-1B appears to be a critical determinant of a cells threshold for undergoing apoptosis (Anand et al., 2008). Curcumin has been shown to downregulate the expression of various pro-inflammatory cytokines including TNF-α and interleukin-1, through inactivation of the transcription factor NF-κB (Jagetia and Aggarwal, 2007). Thus, *TNF-α* and *IL-1β* could be identified as the therapeutic targets in cancer therapy. Extensive research work suggest that curcumin acts as a potent scavenger of a variety of reactive oxygen species (ROS) including superoxide anion (Khar et al., 2001), hydroxyl radical, singlet oxygen (Subramanian et al., 1994), and nitric oxide radicals (Sreejayan and Rao, 1997) that leads to apoptosis in several cancer cell lines (Woo et al., 2003; Anand et al., 2008).

Telomere is a repeating (5'-TTAGGG-3'), non-coding DNA sequence located at terminal ends of the chromosomes (Moyzis et al., 1988). Telomere is regulated by an enzyme namely telomerase, a ribonucleoprotein, with the function of a DNA polymerase. The telomerase holoenzyme consists of the catalytic subunit reverse transcriptase protein hTERT (Nakamura et al., 1997), the telomerase RNA template subunit, hTR, that provides a template r-5'-CUAACCCUAAC-3' (Feng et al., 1995) and other associated proteins (Harrington et al., 1997). A strong correlation has been shown between TERT mRNA expression and telomerase activity in a variety of cancer cells such as breast, colon, gallbladder, lung, stomach and oesophagus (Jagetia and Aggarwal, 2007; Alaikov et al., 2007; Goel et al., 2008; Salvioli et al., 2007). Reports suggest that human telomerase subunit hTERT overwhelmingly activated in 80 % human cancer cells, which prevents telomere shortening and activate apoptotic phenomena (Watanabe 2001; Mukherjee et al., 2007). Telomerase is thus proving to be a reliable marker for the proliferating capacity and tumor mass of cancer patients (Watanabe 2001; Gao and Chen, 2007). This makes telomerase a good target for cancer diagnosis. The present work was undertaken to investigate the underlying mechanism involved in induction of apoptosis and chemoprevention by curcumin in P388D1 cells.

MATERIALS AND METHODS

Cell lines and treatment with curcumin

P388D1 mouse lymphoma cells were obtained from the National Centre for Cell Science, Pune, India. Cells were cultured in DMEM culture medium, supplemented with 4 mM L-glutamine containing 10 % fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 mg/ml) at 37 ^{0}C with 5 % CO_2 in air. Before each experiment, cells were passaged three times. Curcumin was dissolved in dimethyl sulfoxide (DMSO) and diluted further in water. P388D1 cells were treated with curcumin at a final concentration of 3.5 μM for 48 h. Cells cultured in media containing an equivalent concentration of DMSO and without curcumin served as a control.

Morphological evaluation of apoptotic cells

Percentage of apoptotic cells were measured using standard protocol of Kerr et al. (1972), where apoptotic cells were stained with Wright's staining. After curcumin treatment, cells were removed from the culture plate using Trypsin/EGTA. A drop of cell suspension after air drying was fixed in methanol and stained with Wright's solution, and then mounted in a permanent medium and analyzed. Apoptotic cells were identified on the basis of morphological features that included, contracted cell bodies, condensed uniformly circumscribed and densely stained chromatin or membrane bound apoptotic bodies containing one or more nuclear fragments.

Agarose gel electrophoresis of DNA

Extraction of DNA from P388D1 lymphoma was performed following the method given by Pringent et al. (1993) with some modifications. Cell suspension was washed in PBS and lysed in 0.5 ml of lysis buffer containing mM Tris HCl, 75 mM NaCl 10 mM EDTA, 0.5 % SDS and 0.15 mg/ml proteinase K and incubated for 4 h at 50 ^{0}C. Lysates were spun down at 10,000xg for 20 min at 4 ^{0}C. The supernatant was collected carefully and a solution of 0.5 M NaCl and 50 % absolute ethanol was added to precipitate DNA. The precipitated DNA was resolubilized in 30 µl TE buffer (10 mM Tris HCl and 1 mM EDTA, pH 8.0) for 1h at 60 ^{0}C and then 10 µl of loading dye (bromophenol blue 0.025 %, xylene cyanol and 30 % glycerol in water) was added. 40 µl of the sample was loaded into the well of 1.5 % agarose gel and electrophoresed in the presence of 0.5 µg/ml ethidium bromide. After the run, DNA was visualized and photographed on a UV transilluminator**.**

DNA fragmentation assay

A quantitative determination of DNA fragmentation was carried out according to the method of Sellins and Cohen (1987), with minor modifications. Cells (1 x 10^6 cells/ml) were lysed in 0.5 ml Tris-EDTA buffer, pH 7.4, containing 0.2 % (v/v) Triton X-100 and the fragmented DNA was separated from intact chromatin in a microfuge tube (labelled as A) by centrifugation at 13,000 g at 4 ^{0}C for 10 min. Supernatant containing fragmented DNA was transferred to second microfuge tube (labelled as T). 25 % TCA (0.5 ml) was added to each T and B tube and vortexed vigorously. DNA was precipitated overnight at 4 ^{0}C and pelleted by centrifugation at 13,000 g at 4 ^{0}C for 10 min. Supernatants were discarded and 80 µl of 5 % TCA was added to each pellet. DNA was hydrolyzed by heating at 90 ^{0}C for 15 min. At this stage, a blank was included containing 80 µl of 5 % TCA. A 160 µl aliquot of freshly prepared DPA reagent [150 mg diphenylamine in 10 ml glacial acetic acid, 150 µl concentrated H_2SO_4 and 50 µl of acetaldehyde solution] were added and the samples were allowed to stand overnight at room temperature to develop colour. 100 µl of the resulting coloured solution was transferred to a 96-well, flat-bottomed ELISA plate and optical density was measured at 600 nm in a microtiter plate reader. The percentage of DNA fragmentation was calculated as: % DNA fragmentation= [T/ (T+B)] x 100. Samples were carried out in duplicate. Blue colour was developed in both treated and untreated samples.

Assay for NO production

The nitrite concentration in medium was measured following according to Srivastava et al. (2004). Briefly, 100 µl of cell-free culture supernatants were collected from each well of the 96-well microplates. An equal volume of Griess reagent (one part 1 % sulfanilamide in 2.5 % H_3PO_4 plus one part 0.1 % naphthylethylenediamine dihydrochloride in distilled water) was added and the mixture incubated for 10 min at room temperature. The absorbance at 540 nm was measured with an automatic microplate reader. Nitrite concentrations were calculated using sodium nitrite as a standard. Data were expressed as µM nitrite/1.5 x10^5 cells originally plated. In all experiments nitrite contents in wells containing medium without cells were also measured and subtracted from the values in the presence of cells. Samples were carried out in triplicate. Pink colour was developed in both treated and untreated samples. The amount produced was expressed as 1x10^{-7} mM.

Isolation of RNA

Total cellular RNA was extracted from P388D1 cells using GeNei Total RNA extraction Kit according to the manufacturer's instructions. Samples were resuspended in RNase-free water. RNA was quantified from the absorbance recorded at an optical density of 260 nm (Jenway, Essex, UK).

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RT-PCR analysis of hTERT, hTR, Bcl-2, IL-1β and TNF-α

The resulting RNA was subjected to single step RT-PCR using the GeNei one step RT-PCR Kit; according to the manufacturer's instructions. Briefly, 3 µg RNase-free RNA template was subjected to RT-PCR amplification in a final volume of 25 µl. The template RNA used for amplification was denatured at 65 ^{0}C for 5 min, followed by heating at 55 ^{0}C for 45 min for initial cDNA preparation in the presence of reverse transcriptase. The primer sets used for each gene of interest were as follows: Bcl-2: 5`-primers: 5`-GAACACCAGAATCAAGTGTTCG-3` and 5`-CAGGTGGACCACAGGTGGC-3`, size of amplicons: 455 bp; *TNF-α*, 5`-primer: 5`-ATGAGCACTGAAAGCATGATCCGG-3` and 5`GCAATGATCCCAAAGTAGACCTGC-3`, 'size of amplicons: 695 bp; *IL-1β*, 5`-primer: 5`-ATGGCAGAAGTACCTAAGCTCGC-3` and 5`-CACAAATTGCATGGTGAAGTCAG-3`, size of amplicon: 802 bp; *β-Actin* 5`- primer: 5`-GGGTCAGAAGGATTCCTATG-3` and 5`-CTAGAAGCATTTGCGGTGGAC-3`, size of amplicons: 1000 bp; hTERT, 5`-primer: 5`-CGGAAGAGTGTCTGGAGCAA-3` and 3`-primer: 5`-GGATGAAGCGGAGTCTGGA-3`, size of amplicons: 145 bp; for hTR, 5`- primers 5`-GGGTTGCGGAGGGTGGGCCT-3` and 3`- primers 5`-ACGGGCCAGCAGCTGACAT-3`, size of the amplicon 185 bp.

The reaction master mix was prepared according to the manufacturer's protocol. The primer (Met. Int. AG, Deutschland, Germany) concentration was 20 pmol for each set. Thermal programme was kept similar for all amplifications. The gene amplifications were performed with an initial incubation step at 94 °C for 3 min, followed by 31 cycles at 94 °C for 60 seconds, 60 ^{0}C for 1 min (*Bcl-2*: 58 ^{0}C for 1 min and hTERT: 57 ^{0}C for 1 min) and 72 °C for 1.5 min with a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis on a 2 % agarose gel containing ethidium bromide and DNA bands were detected in gel documentation unit (Bio-Rad Laboratories, Hercules, CA, USA). The intensity of the bands corresponding to Bcl-2, *TNF-α* and *IL-1B* and β-actin were calculated using Quantiscan software. Due care was taken to prevent PCR carry over contamination, including physical separation of the reaction area from the analysis area. Each RT-PCR was repeated twice and representative results are shown. Housekeeping genes β-actin was used as internal control for equal loading.

Statistical analysis

All statistics were carried out using SPSS software. Values were expressed as mean ± SD. Group means are compared using the unpaired student's t-test. A probability value of 0.05 or less was considered significant.

RESULTS

Curcumin induced apoptosis of P388D1 cells

Cells treated with curcumin and subsequently visualised with Wright's stain and examined under phase contrast microscope showed characteristic apoptotic features, such as chromatin condensation and nuclear fragmentation (Figure 2).

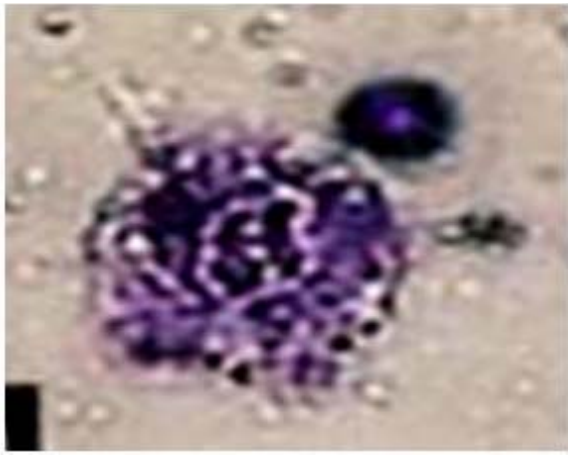

Figure 2: Lymphoma cell undergoes apoptosis after treatment with 3.5 µM of curcumin for 48 h, shows chromatin condensation, nuclear fragmentation, presence of apoptotic bodies and surface blabbing in an apoptotic cell.

Other morphological features of apoptosis such as cell shrinkage and presence of apoptotic bodies were also evident. One hundred cells were scored at randomly and classified into apoptotic and non apoptotic

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cells based on above characteristics. Percentage of apoptotic cells was increased by nearly 2 fold, as shown in Figure 3.

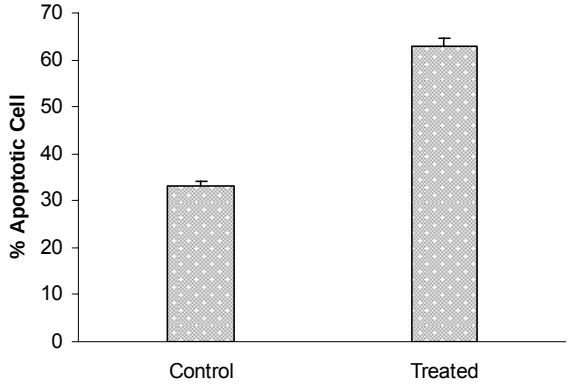

Figure 3: Percent of apoptotic P388D1 cells observed under phase contrast microscope after treatment with 3.5 μM of curcumin for 48 h. Values represented mean ± S.D. (n=100)

% DNA fragmentation assay

To examine further the apoptotic characteristics in curcumin-treated lymphoma cells, the % DNA fragmentation was determined. Induction of apoptosis was evident (Figure 4), whereby the % DNA fragmentation was increased by more than 2-fold in curcumin-treated cells compared to control cells.

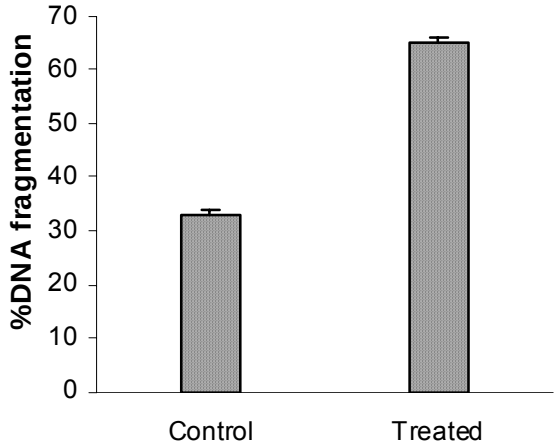

Figure 4: Percent of DNA fragmented P388D1 cells after treatment with 3.5 μM of curcumin for 48 h.

DNA ladder profile

To further examine the effect of curcumin on agarose gel electrophoresis on tumor cells at 3.5 μM concentration for 48 h. Fragmented DNA ladders were observed in curcumin treated tumor P388d1 cells as represented in Figure 5. No significant fragmentation were observed in the untreated tumor cells (reference control).

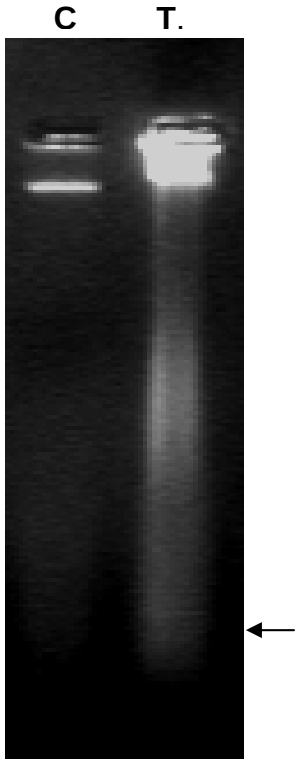

Figure 5: Agarose gel electrophoresis profile of P388D1 cells at 3.5 μM curcumin for 48 h. C indicate untreated and T for treated cells.

NO production analysis

Curcumin treated and untreated culture supernatants of tumor cells, incubated in DMEM medium were analyzed for NO content. Culture supernatant of curcumin treated tumor cells showed higher level of NO content compared to untreated tumor cells. The NO content is shown in Figure 6, which demonstrates stimulation of NO production in the curcumin treated lymphoma cells as compared to untreated cells.

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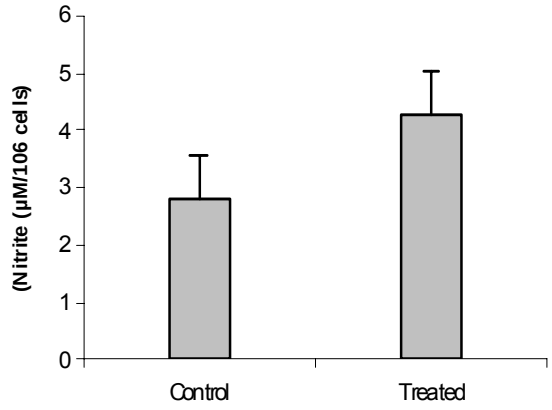
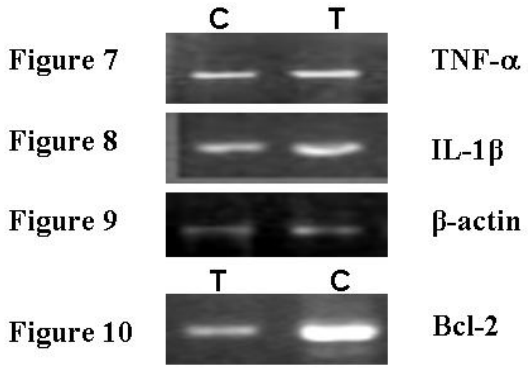


Figure 6: Nitric oxide produced by P388D1 cells after treatment with 3.5 μM of curcumin for 48 h.



Figures 7-10: RT–PCR analysis of TNF-α, IL-1β, β-actin and Bcl-2 gene expression after treatment with 3.5 μM curcumin for 48 h, respectively. Where lane: C represents P388D1 cells without curcumin treatment and T represents with Curcumin treatment.

RT-PCR expression analysis of Bcl-2, IL-1β and TNF-α

To explore the possible mechanism by which curcumin induced apoptosis in P388D1 cells, the expression of both pro- and anti-apoptotic mRNA was analyzed by RT PCR analysis. Changes in the mRNA expression levels of Bcl-2, *TNF-α* and *IL-1B* in P388D1 cells were measured, to examine their possible contribution to the mechanism of curcumin-induced apoptosis. RT-PCR analysis revealed that mRNA levels of *TNF-α* and *IL-1B* (Figures 7 and 8) increased with a concomitant decrease in Bcl-2 mRNA (Figure 10). No significant changes were observed in the levels of β-actin mRNA (reference control) (Figure 9). The expression level of Bcl-2 was decreased to 60 % of control levels at 3.5 μM of curcumin. By contrast, *TNF-α* and *IL-1B* levels were increased to 2 to 3 fold higher than control levels, at the same concentration of curcumin.

RT PCR expression analysis of hTERT and hTR

To assess the effects of curcumin on telomerase activity, P388D1 cells were treated with 3.5 μM of curcumin for 48 h. Telomerase activity was analyzed by RT-PCR profile of human telomerase catalytic subunit (*hTERT*) and RNA subunit of the human telomerase complex (hTR). Results are represented in Figures 11-13. Compared to untreated cells, the telomerase activity of P388D1 cells was significantly suppressed. Percentage inhibition of telomerase was calculated from band intensity. Intensity obtained from untreated cells was considered to have 100 % telomerase activity and accordingly intensities were calculated for the treated cells. Results revealed that the telomerase activity was inhibited to the extent of 25 % and 40 % after 48 h treatment with 3.5 μM curcumin, respectively. Expression levels were calculated from the intensities obtained in two to three independent experiments.

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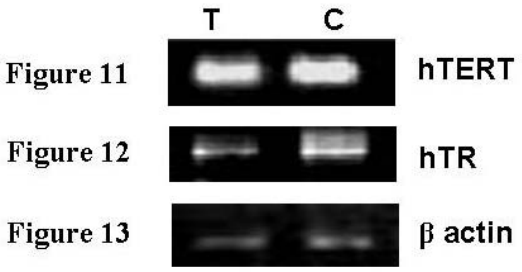

Figures 11-13: RT–PCR analysis of hTERT, hTR and β-actin expression after treatment with 3.5 μM curcumin for 48 h, respectively, where lane C represents P388D1 cells without curcumin treatment and T represents with Curcumin treatment.

DISCUSSION

Over the last decade, a significant research interest has been generated around curcumin, the major constitutes of turmeric, because of its anti-inflammatory and anti-neoplastic properties (D'Incalci et al., 2005). However, hardly any study has been reported to date regarding effect of low dose of curcumin on induction of apoptosis via inhibited of telomerase activity and accumulated reactive oxygen species. In the present study, we focused on the effect of curcumin on telomerase activity to clarify the anti-proliferating effect of curcumin in P388D1 cells derived from mouse lymphoma/leukemia cells. Recent studies have indicated that inhibition of tumor cell growth is associated with the promotion of apoptosis and decrease in telomerase expression (Cui et al., 2006; Chakraborty et al., 2006) in a dose dependent manner (Mukherjee et al., 2007). Moreover, dose dependent cytotoxic effect of curcumin in induction of apoptosis in various cells has been well documented (Alaikov et al., 2007). Curcumin mediated cell death was accompanied by characteristic morphological changes like nuclear condensation and formation of apoptotic bodies, when examined under phase contrast microscope.

The present findings illustrate that curcumin induces the release of cytochrome c to translocate from mitochondria to cytosol at 3.5 μM concentration. Moreover, cytochrome c is involved in the activation of caspases and thus initiates the apoptotic pathway (Khar et al., 2001). It has been suggested that low dose of curcumin treatment leads to increased ROS generation in comparison to higher doses (Chan et al., 2006). Furthermore, the release of cytochrome c leads to inhibition of the mitochondrial respiratory chain, that could be assumed to the overproduction of ROS (Schulze-Osthoff et al., 1992). Studies demonstrated depletion of endogenous antioxidant level or increase of ROS led induction of programmed cell death in various tumor cells (Buttke and Sandstrom, 1994). Therefore, curcumin induced generation of antioxidant associated with apoptosis at 3.5 μM in P388D1 lymphoma/ leukemia cells could not be ignored.

In vitro investigations have demonstrated that Bcl-2 acts as a malignant factor causing various types of carcinoma, includes lung cancer and breast cancer (Ichikawa et al., 2004). On the other hand TNF-α and IL-1β are the key mediator of apoptotic pathway, tumor proliferation and differentiation (Mandal and Kumar, 1997). Studies suggest IL-1β acts synergistically with *TNF-α*, activates proinflammatory responses in a wide range of tumor cells (Goel et al., 2008; Cho et al., 2007). Studies demonstrated the expression of the catalytic subunit TERT correlates with telomerase activity during cellular differentiation and neoplastic transformation. Moreover, curcumin mediated downregulation of telomerase activity is due to the inhibition of translocation of telomerase reverse transcriptase (TERT) from cytosol to nucleus (Chakraborty et al., 2006). The present finding illustrates that suppression of tumor proliferation even in low doses of curcumin is due to the inhibitory effect of the curcumin by inhibition of various cytokines leads to apoptosis and involved in tumor proliferation or inhibiting RNA transcription rather than direct inhibition of the telomerase activity. Therefore, telomerase inhibition by curcumin can be interpreted as an important event that leads to apoptosis.

Thus, inhibition of hTERT could be a possible potential therapeutic agent to induce cell death in tumor cells.

CONCLUSION

In conclusion, our work reveals that curcumin could significantly inhibit telomerase activity and induce apoptosis in P388D1 cells. Although, the modulation of telomerase expression by curcumin in cancer cells is poorly understood. Therefore, curcumin may inhibit cancer cell growth and induce apoptosis via reducing telomerase expression.The present investigation suggests that even low dose of curcumin is sufficient to develop curcumin as possible potential universal antineoplastic agent.

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