

Cell Cycle Regulation and Induction of Apoptosis by β -carotene in U937 and HL-60 Leukemia Cells

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In this communication, we report the efficacy of β -carotene towards differentiation and apoptosis of leukemia cells. Dose (20 μ M) and time dependence (12 h) tests of β -carotene showed a higher magnitude of decrease (significance $p < 0.05$) in cell numbers and cell viability in HL-60 cells than U937 cells but not normal cell like Peripheral blood mononuclear cell (PBMC). Microscopical observation of β -carotene treated cells showed a distinct pattern of morphological abnormalities with inclusion of apoptotic bodies in both leukemia cell lines. When cells were treated with 20 μ M of β -carotene, total genomic DNA showed a fragmentation pattern and this pattern was clear in HL-60 than U937 cells. Both the cell lines, on treatment with β -carotene, showed a clear shift in G₁ phase of the cell cycle. In addition the study also revealed anti-oxidant properties of β -carotene since there was reduction in relative fluorescent when treated than the control at lower concentration. Collectively this study shows the dual phenomenon of apoptosis and differentiation of leukemia cells on treatment with β -carotene.

Keywords: β -carotene, DNA fragmentation, Cell cycle, Intracellular oxygen tension leukemia cells

Introduction

There is an expanding source of information on the potential application of synthetic non-steroidal, anti-inflammatory drugs (NSAIDs) in cancer prevention but the rate of prevention is

Abbreviations: HL-60, Human leukemia cells; PBMC, peripheral blood mononuclear cells; DMSO, di methyl sulphoxide; DCFA-DA, dichloro dihydrofluorescein diacetate.

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limited to only 40% since the drug's efficacy is associated with side effects to the human body (Schreinemachers and Everson, 1994). Alternate search for different modes of chemoprevention which are more effective in preventing and reducing the risk of cancer growth is consistently gaining immense focus. Nutraceuticals from microorganisms, algae and plants have attained immense importance in cancer prevention (McCarty and Block, 2006). A large scale cohort study showed that intake of major carotenoids prevented ovarian cancer (Genkinger *et al.*, 2006; Koushik *et al.*, 2006). Nutritional factors like carotenoids can also influence the cellular differentiation, apoptosis programme and cellular anti-proliferation potential with different molecules as target points (Neuhouser *et al.*, 2003; Aggarwal and Shishodia, 2006). Recent studies showed that antioxidants, vitamins and related nutrients are able to influence the carcinogenic process by governing the cellular process in the pathogenesis of cancer (Altucci *et al.*, 2001; Paolini *et al.*, 2003; Maillard *et al.*, 2006). Among total carotenoids there is experimental evidence that β -carotene can modulate molecular pathways involved in the cell cycle progression and enhance apoptosis in undifferentiated leukemia cells (Palozza *et al.*, 2002; Aggarwal and Shishodia, 2006). Dietary role of β -carotene as an antioxidative agent is discussed very well in cancer biology and oxidative stress (Jarrett *et al.*, 2006). All trans retinoic acid (ATRA) is one of the major physiologic stimulators able to regulate proliferation and differentiation of haematopoietic cells receptor (Collins, 2002) and the activity depends on the structure of the carotenes (Konishi, *et al.*, 2006). Apoptosis plays a crucial role in normal development, homeostasis and in the defense response against pathogens. This kind of cell death is thought to be an important response to most chemotherapeutic agents in leukemia cells. However the biochemical mechanism of β -carotene's action in well-differentiated leukemia cells remains unclear. This study elucidates some aspects of the β -carotene action in acute and differentiated human leukemia cells like HL-60 and U937 and also in normal cells and its possible mechanism of action causing cell death.

Materials and Methods

Chemicals. Commercially available β -carotene, agarose, DMSO, DCFA-DA and propidium iodide were purchased from Sigma (Sigma-Aldrich). All other chemicals, reagents and solvents were purchased from SD fine chemicals and Qualigen and Wako pure chemicals. Phosphate buffered saline (PBS), RPMI medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from GIBCO (GIBCO Bioscience). Culture vessels were purchased from Nunc, Denmark.

Cell culture and treatment. Human chronic monocytic leukemia U937 and myeloid leukemia, HL-60 (promyelocytic cells) obtained from American Type Culture Collection and normal PBMC cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine. Human peripheral mononuclear cells (PBMC) were isolated from heparin-anticoagulated blood of healthy persons by centrifugation with Ficoll-Paque Plus (Amersham Biosciences). Cell stock culture was maintained in liquid nitrogen and experiments were conducted with periodic changes of culture. Experimental sets were maintained in a humidified atmosphere with 5% CO₂ and 37°C. The initial culture density for experiments was 1×10^5 cell/ml. β -carotene was dissolved in di-methyl sulphoxide (DMSO) and concentration was adjusted according to the dose applied. The final concentrations of di-methyl sulfoxide (DMSO) in the culture medium was <0.03%. The concentrations used were 0, 5, 10, 20, 50 and 100 μ M. 0 μ M β -carotene means addition of DMSO alone. Initially, HL-60 and U937 cells were co-incubated with various concentrations of β -carotene for 24 h and the dose dependency test was studied. After selection of specified dose, time duration study was conducted. Cell viability test was carried out using the Beckman-Coulter automatic cell counter (VI-cell analyzer, Beckman Coulter, Inc).

MTT reduction assay. Cell proliferation assay was determined using MTT assay (Mosmann, 1983). U937, HL-60 and PBMC cells (1×10^5 cells/ml) were incubated in 96 well plates with difference doses of β -carotene (0, 5, 10, 20, 50 and 100 μ M) for 12 h. Ten μ l of MTT (10 mg/ml) was added to each well and incubated further at 37°C for 4 h. After incubation, MTT-formazan precipitate was dissolved in 100 μ l of DMSO and absorbance was recorded at 570 nm in automatic plate reader (BioRAD instrument). Three independent experiments were conducted to quantify the data.

2.4 Cytotoxicity assay. Cytotoxicity parameters of β -carotene on U937 and HL-60 cells were carried out using LDH assay kits. (CytoTox 96^R Non-radioactive cytotoxicity assay, Promega, Madison, USA). Red formazon - a product of conversion of tetrazolium salt (INT) was quantified using multi plate reader (BioRAD instrument). Data are presented as percentage of cytotoxicity of treated VS untreated cells.

DNA ladder assay. DNA ladder assay was carried out as per standard method (Herrmann *et al.*, 1994). This method prevents the contamination of entire genomic DNA with fragmented DNA. Briefly, after treatment with β -carotene, cells were harvested, washed twice with cold PBS and lysed for 30 min at 4°C in lysis

buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.2% Triton X-100) using zirconium beads and automatic cell lyser (MS-100 R, Tomy, Medico Ltd.). After centrifugation at $15,000 \times g$ for 20 min, the supernatant was treated with protease inhibitor cocktail and 0.5% SDS for 1 h at 37°C. DNA was extracted twice with phenol and precipitated with 150 mM NaCl and two volumes of ethanol at -20°C. DNA precipitate was washed twice with 70% ethanol, dissolved in TE buffer and treated for 1 h with Rnase at 37°C. Finally, DNA precipitates were stained with propidium iodide, electrophoresed on 2% agarose gel and visualized in an automatic gel documentation system (BIO-RAD systems).

Confocal microscopy. β -carotene-treated as well as non-treated U937 and HL-60 cells were fixed and stained with wright-giemsa stain on cover-glass slides and observed under light microscope with $400 \times$ magnification (Nikon Optihot model # 88, Osaka, Japan) for apoptotic body accumulation. Photos were taken with a digital camera (Canon).

Cell cycle analysis by Flow cytometer. Cell cycle analysis was done as described previously (Noguchi and Browne, 1978; Jing *et al.*, 1994). Briefly, treated and untreated cells were harvested by centrifugation and washed once with cold PBS. The cells were fixed with ice-cold 70% ethanol at a cell density of 1×10^6 and then treated with 1 mg/ml RNase for 30 min at 37°C. Propidium iodide was added to the cell solution at a final concentration of 50 μ g/ml. The rate of cell cycle were analysed by quantification of DNA content in a Coulter[®] Epics[®] XL[™] Flow Cytometer (Beckman Coulter, Inc).

Cellular redox potential assay. Cells were treated with different concentrations of β -carotene and incubated for prescribed period. After incubation period, cells were harvested and washed with cold PBS. Washed cells were further incubated with 2',7'-dichlorofluorescein-di-acetate (DCFH-DA) at 37°C for 1 h in dark. Intracellular ROS generation was investigated by quantifying H₂O₂ by Coulter[®] Epics[®] XL[™] Flow Cytometer (Beckman Coulter, Inc).

Statistical analysis. Each experiment was carried out in three independent sets. Mean values and standard deviation were calculated. Student 't' test for each set of experiments was calculated in all groups. Statistically significant values was set at the level of $p < 0.05$.

Results

Effect of β -carotene on cells. First, we analysed the uptake of the β -carotene in the U937, HL-60 and normal PBMC cells after lysing treated cells and analyzing cellular β -carotene content by spectrophotometer. Results showed that accumulated β -carotene was in the range of 60-250 pmol level (results not shown). This cellular uptake study analysis confirmed the optimal uptake by the cells and formed the basis for further treatment. A dose-dependent decrease of cell numbers in both cell lines was noticed during 12 h treatment (Fig. 1), with maximum and significant ($p < 0.05$) decrease at 20 μ M concentration. Among the tested cell lines, a differential

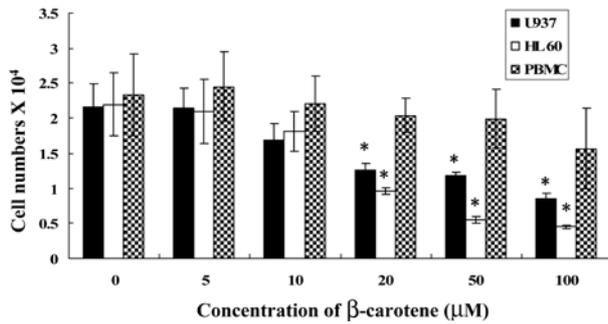


Fig. 1. Cell proliferation assay. U937, HL-60 cells and normal cells (PBMC) were cultured in RPM 1648 medium with various concentrations of β -carotene for 24 h. Cell numbers were counted by trypan dye exclusion method. Values are expressed as mean \pm S.D. of three independent experiments. Significant levels were set as $p < 0.05$. 0 μ M indicates DMSO alone. P -values ≤ 0.05 with * mark were considered significant.

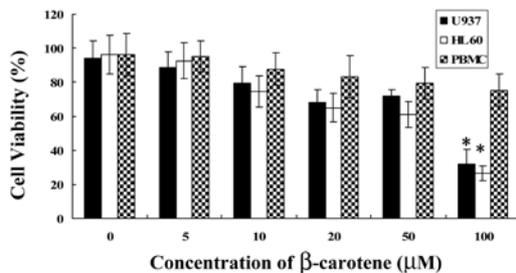


Fig. 2. Cell viability test. Dose-dependent growth inhibition and cytotoxic effect of various concentrations of β -carotene were tested on U937, HL-60 cells and normal cells (PBMC). The cells were treated for 24 h. Total viable cells were counted with automatic cell counting unit. Data presented are the means of three independent experiments. 0 μ M indicates DMSO alone. P -values ≤ 0.05 with * mark were considered significant.

behavior was observed in the cytotoxic effect of the β -carotene, where a higher susceptibility was found in cell viability in HL-60 cells up to 50 μ M in trypan blue dye exclusion study (Fig. 2). The decrease in cell numbers and cell viability show that β -carotene did kill the cells at concentrations higher than 20 μ M. Based on these results, the molecular mechanisms involved in the generation of the antitumor action of these derivatives are now being explored

Cell proliferation/cytotoxicity study. Once we confirmed the degree of β -carotene effect to U937, HL-60 and normal PBMC cells, we confined our goal of cytotoxicity study towards leukemia cells lines. The cytotoxic property of β -carotene was evaluated by the aid of LDH release assay and MTT dye exclusion assay. The limit of the activity was defined as IC_{50} value. Fig. 3 explains the LDH release during the treatment with different concentrations of β -carotene in U937 and HL-60 cells. LDH release to the medium during the drug treatment is the hallmark of cytotoxicity of the compound

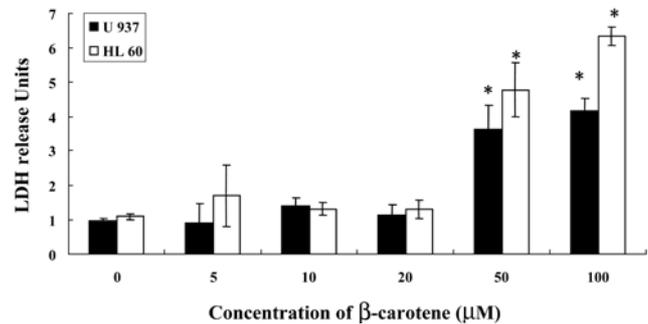


Fig. 3. LDH release assay. U937 and HL-60 cells were seeded in 6 well plates with various concentrations of β -carotene. Percentage of LDH was measured by CytoTox Non-radioactive cytotoxicity assay Kit (Promega Corp. Madison, WI, USA). Significant levels were set as $p < 0.05$. 0 μ M indicates DMSO alone. P -values ≤ 0.05 with * mark were considered significant.

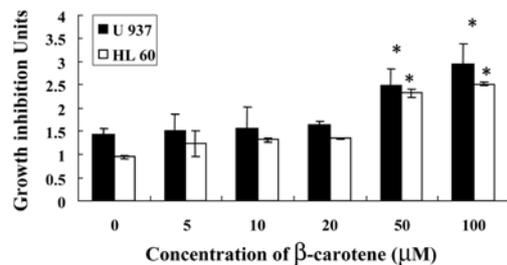


Fig. 4. MTT dye reduction assay. U937 and HL-60 cells were treated with various concentrations of β -carotene for 24 h and MTT dye reduction assay was carried out as per manufacturer's instructions (Promega Corp. Madison, WI, USA). Values are expressed as three independent observations. Significant levels were set as $p < 0.05$. 0 μ M indicates DMSO alone. P -values ≤ 0.05 with * mark were considered significant.

because of membrane lysis and oxidation reaction of lactate to pyruvate and subsequent reaction of pyruvate with INT tetrazolium to form formazan. A significant accumulation of formazan was noticed in doses higher than 20 μ M, clearly showing the degree of toxicity of β -carotene in both cell types studied. This observation was further confirmed by MTT dye reduction assay and results are depicted in Fig.4. There was a stronger and significant ($p < 0.01$) growth inhibition effect of β -carotene at doses higher than 20 μ M. However, there was no strong difference in reduction between 50 μ M and 100 μ M probably due to apoptosis and/or necrosis in both cell lines. In both cases more than 80% of dead cells were observed (Figs. 1 and 2).

Cell morphology and DNA fragmentation study. In order to identify the cause for cell number decrease, we studied the cell morphology to test whether cell death was a result of apoptosis during the β -carotene treatment. Fig. 5 shows a characteristic accumulation of apoptotic bodies in both cells after treatment as denoted by the arrow mark at a concentration

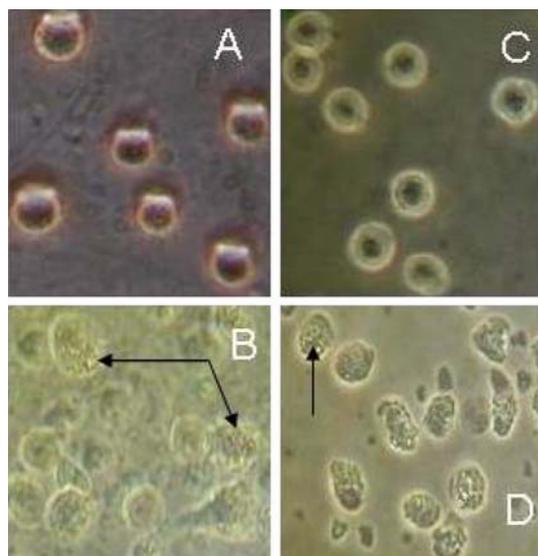


Fig. 5. Morphological appearance of apoptotic cells by confocal microscopy. U937 and HL-60 cells were treated with 20 μM β -carotene for 24 h. Cells were harvested, fixed and stained with Wright-Giemsa stain. Observations were made under phase contrast microscope. Arrow indicates the apoptotic cells. A. U937 control cells; B. U937 β -carotene treated cells; C. HL-60 control cells; D. HL-60 β -carotene treated cells.

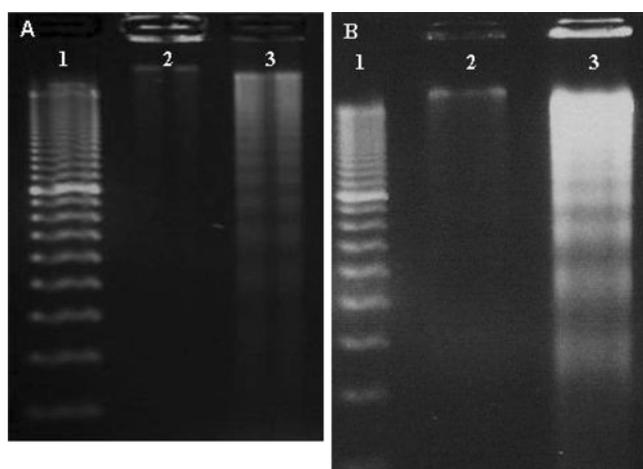


Fig. 6. DNA ladder assay. Agarose gel electrophoresis of DNA from U937 and HL-60 cells treated with 20 μM β -carotene. The gels were photographed under UV illumination. A U937 cells; B HL-60 cells; Lane 1. Molecular marker; Lane 2. Control cell; Lane 3 Treated cells. 0 μM indicates DMSO alone.

of IC-50. The number of apoptotic bodies also increased with increasing concentration of β -carotene (results not shown). The morphological changes of the cells were further ascertained by a strong biochemical confirmatory assay-DNA fragmentation study and results are depicted in Fig. 6. Inter nucleosomal cleavage of cellular DNA by endonuclease to size less than 180 bp could be detected by agarose gel electrophoresis and

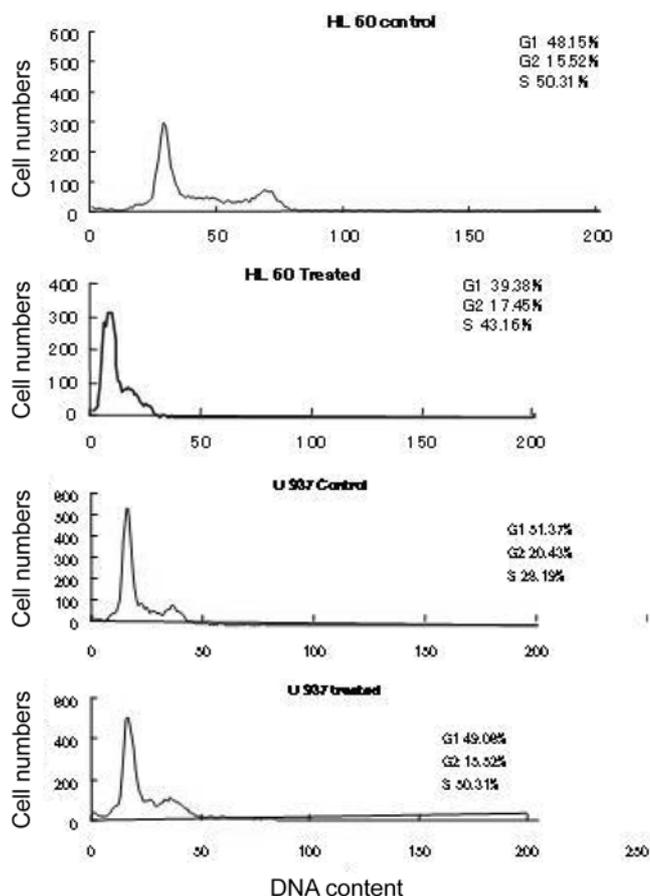


Fig. 7. Flow cytometer analysis of cell cycle. After β -carotene treatment, cells were harvested, washed with PBS and DNA content was analyzed by flow cytometer. The results are expressed as DNA content vs. cell number. 0 μM indicates DMSO alone.

PI Staining. As illustrated in Figs. 6a and 6b, the rate of fragmentation was clear in HL-60 cells than U937, reason being unknown at present. Control cells did not show any DNA fragmentation (Figs. 6 a & b lane 1). This type of nucleosomal cleavage reflects the activity of endonuclease which might have been induced by β -carotene.

Cell cycle study by flow cytometer. Having confirmed the nature of cell death, we further directed our study towards the cell cycle pattern during the cell death. The degree of apoptosis in β -carotene treated cells was further evaluated by DNA content through flow cytometric analysis. Fig. 7 illustrates the different phases of cell cycle in treated and control cells. The control group showed a prominent G_1 phase of 49% and 51% in HL-60 and U937 cells respectively; whereas, in treated cells a significant reduction of G_1 phase to 39.4% in HL-60 and a small reduction of G_1 in U837 cells was observed. This result suggests a possibility that β -carotene induces apoptosis by arresting G_1 phase. In addition to these changes, the presence of distinct sub G_1 peak (sub diploid

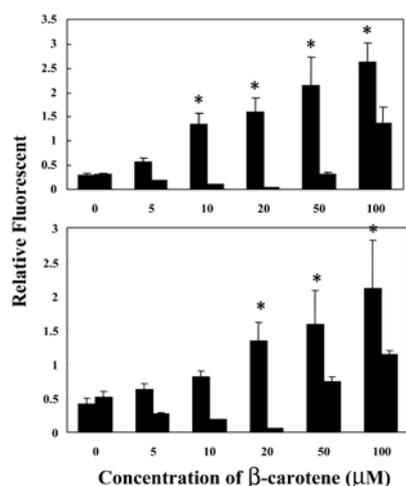


Fig. 8. Quantification of intracellular ROS generation Cells (10^4 cells/ml) treated with varying concentrations of β -carotene for 24 h were harvested to evaluate cellular peroxide content by DCFH-DA labeling. Before addition of fluorescent probe, culture media was removed to eliminate the amount of β -carotene and/or antioxidants that were not cell associated. Untreated cells, treated cells. 0 μ M indicates DMSO alone.

content), a characteristic of apoptotic cells was also noticed (result not shown).

Intra cellular ROS generation assay by flow cytometer.

Oxidative stress is a central action for the anticancer effects, since oxidative stress is coupled with many signals inducing apoptosis (Phalen 2006). The radical scavenging activity of the tested compounds was influenced by the number and location of hydroxyl groups, glycosylation, and other substitutions. To investigate the possible mechanism by which β -carotene could interfere with intra cellular ROS generation in leukemia cells due to oxidative stress, we evaluated the cellular ROS content by flow cytometer with the aid of DCFH-DA labeling of. Fig. 8 depicts the pattern of fluorescence spectra in two experimental groups of treated and non treated groups. β -carotene addition deeply modified intracellular ROS levels. At lower concentrations, β -carotene acted as antioxidant but at higher level it showed pro-oxidant nature. The maximum effect of antioxidant phenomenon was noticed up to 20 μ M in both cells. On the other hand fluorescent in untreated group increased gradually. This clearly indicates pro-oxidants and antioxidant nature of β -carotene.

Discussion

The present study demonstrated that β -carotene can act as both apoptotic inducer as well as antioxidant compound in human leukemia cell lines. β -carotene is one of the major water insoluble but solvent soluble natural colorants widely

present in majority of fruits and vegetables and used as a source of chemopreventive agents. First, we have studied and demonstrated that β -carotene demonstrates antiproliferative and apoptotic inducing properties on human leukemia cell lines. Cytotoxicity can be defined as the cell killing property of a chemical compound independent from the mechanism of death. Our results show that the carotene induced a dose dependent inhibition of cell growth in both cell lines studied but not normal cell (PBMC). The degree of inhibition was severe in HL-60 than the U937 cells, implying that activity is cell dependent. HL-60, a leukemic cell line has been extensively studied as an able cell line because of its nature to differentiate into morphologically mature myeloid cell, a phenomenon similar to neutrophils after certain drug treatment (Covacci *et al.*, 1998). This inhibition phenomenon ultimately leads to the inhibition of cell proliferation followed by cell death via apoptosis (Palozza *et al.*, 2002). When considering the chemotherapy side effects, it is very important to verify whether the compound shows a harmful effect against normal dividing cells such as proliferating mononuclear cells such as PBMC. The present investigation shows a stronger cytotoxic effect of β -carotene as confirmed by LDH release (Fig. 3) and MTT dye reduction assay (Fig. 4) confirming that HL-60 is more prone to apoptosis than U937 cells. In addition to this observation HL-60 cells did not show the significant loss of cell viability at higher concentration of β -carotene (Fig. 2) confirming that cells did differentiate to myeloid type and underwent apoptosis as clearly seen by DNA ladder assay. Moreover, β -carotene did not exhibit any cytotoxic effect towards the normal cells confirming that the effect is cellular specific. Findings from our studies suggest that low doses of β -carotene are dramatically effective in acute promyelocytic leukemia and show considerable promise.

The growth inhibitory effect of β -carotene in HL-60 was accompanied by an arrest in cell cycle. There are some indications that carotene are able to modify the cell cycle pattern. For example, lycopene (Amir *et al.*, 1999) and α -carotene (Murakoshi *et al.*, 1989) were able to block G₀/G₁ phase of the cell cycle. However, β -carotene (>70 μ M) prevents the G₂/M phase of cell cycle in oral cancer cell line SCS-25 (Schwartz, 1993). Our experiments regarding DNA fragmentation (Fig. 6a) leads to the view that β -carotene must have blocked the downstream caspases to degrade the DNA by DNases. However, we have not studied any mechanism of pathway in this experiment. In order to identify the nature of mechanism which governs the rate of apoptosis in β -carotene treated cells, we evaluated the intracellular ROS generation by DCFH-DA shift assay. Clear differences in ROS production was noticed between treated and control cells (Fig. 8). This result is similar to that found in colon cancer cells (Bortuzzo *et al.*, 1996) and leukemia cells (Palozza *et al.*, 2002). Cytochrome C is an electron carrier in oxidative phosphorylation mechanism in mitochondria (Matsuno-Yagi and Hatefi, 1996). It has been recently revealed that cytochrome C is the potent catalyst of DCF oxidation (Burkitt and Wardman, 2001). In

the present investigation, untreated group showed an increase in fluorescence in both cell lines (Fig. 8). This is an indication that cytochrome C is released into cytoplasm. However, in cells treated with up to 20 μ M β -carotene, the degree of fluorescence is decreased, confirming ROS scavenging. Alternatively, in higher concentrations, the rate of ROS production was increased.

Reactive oxygen species (ROS), such as superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radicals (OH^{\cdot}) and peroxy radicals (ROO^{\cdot}), are produced as a part of normal metabolic processes. It has been reported that an elevation in ROS production does induce accumulation of inhibitors of cyclin dependent kinases, mainly p21^{WAF-1} (Esposito *et al.*, 2000). It is possible that β -carotene cleaves into retinoic acid and acts as pro-oxidant in cancer cells like small lung cancer cells (Prakash *et al.*, 1999) and colon adenocarcinoma cells (Quick and Ong, 1990). Results accumulated here show a decrease in cell viability and cell numbers even though oxidation state of the cell increased at higher concentrations. This particular observation needs to be tested since we have not done any experiments on the relationship between apoptosis and pro-oxidation nature of β -carotene.

The significance of the present study lies in the observation that β -carotene induces apoptosis through cell cycle arrest by the mechanism of ROS scavenging system. However, *in vivo* study warrants further confirmation that β -carotene acts as apoptosis agent in cancer cells particularly leukemia cells but not normal cells.

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