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Original Research

Curcumin induce DNA damage and apoptosis through generation of reactive oxygen species and reducing mitochondrial membrane potential in melanoma cancer cells

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Abstract: Melanoma is the most malignant skin cancer. Curcumin has shown to have therapeutic effects when used in the treatment of malignant diseases. However, the precise molecular mechanisms of its action are not fully elucidated. In this research, we hypothesized that reactive oxygen species (ROS) play a key role in curcumin induced DNA damage, apoptosis and cell dead. To test our hypothesis, cytotoxic, genotoxic, apoptotic, ROS generating and mitochondrial membrane potential (MMP) of curcumin on mouse melanoma cancer cells (B16-F10) and fibroblastic normal cells (L-929) were investigated. Our results demonstrated that curcumin decreased cell viability and MMP and, increased DNA damage, apoptosis and ROS levels in both melanoma cancer and normal cells in a dose dependent manner and, these activities were significantly higher in melanoma cells than in normal cells with higher concentrations. There were positive strong relationships between DNA damage, apoptosis, cytotoxicity and ROS generation and MMP levels in curcumin treated melanoma and normal cells. In summary, this *in vitro* study provide clear evidence that curcumin induced DNA damage, apoptosis and cytotoxicity via its pro-oxidant activity in a dose dependent manner in both cancer and normal cells and these activities were higher in cancer cells than those of normal cells.

Key words: Curcumin; DNA damage; Apoptosis; Reactive oxygen species; Melanoma.

Introduction

Malignant melanoma is regarded as the most aggressive and life threatening skin malignancy. In the United States, melanoma is the fifth and sixth most common cancer in men and women, respectively (1, 2). Resistance to chemotherapy indicates that melanoma cells have complex survival mechanisms and therapies also cause severe side effects and kill normal cells of the patients (1). Therefore, the development of alternative and effective therapy is essential. Studies focused on plant-derived compounds or their active components with low toxicity and high selectivity for killing cancer cells.

Curcumin is a polyphenolic compound (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6- heptadiene-3,5-dione) derived from the popular Indian spice turmeric plant. It is a member of the Zingiberaceae (ginger) family, which is native to Southeast Asia (3, 4). It has been extensively studied over the last three to four decades for its anti-inflammatory, anti-angiogenic, antioxidant, wound healing and anti-cancer effects because of its medicinal in Indian and Chinese systems of medicine (5, 6). Moreover, research has shown that curcumin possesses anti-proliferative and anti-carcinogenic properties in a wide variety of cell lines and animals (7-9). Although, many researchers try to explain the anti-cancer effects of curcumin, there is a paucity of data to explain the underlying mechanism of its activity. Recent studies demonstrated that curcumin increases DNA damage, apoptosis and cell death (10-12). However, there is no report about the relationship between ROS and these activities. The aim of the present study is to evaluate association between cytotoxic, apoptotic, DNA damaging and ROS generating effects of curcumin on melanoma cancer cells and to compare normal fibroblastic cells *in vitro*.

Materials and Methods

Materials

Curcumin, fetal calf serum (FCS), Dulbecco's Modified Eagle medium (DMEM), 2', 7'-dichloro-dihydro-fluorescein-diacetate (H2DCF-DA), Penicillin-streptomycin, Annexin-V-FLOUS staining kit and ethidium bromide (EB) were purchased from Sigma-Aldrich (Seelze, Germany). All other reagents used were of analytical grade unless otherwise stated.

Curcumin was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution (40 mM). The stock solution was diluted with DMEM (contains no fetal bovine serum). The final concentration of DMSO in the curcumin solution was <1%. Prior to start of the experiments, we confirmed that this level of DMSO as well as the serum free media did not induce any DNA damages in the cells. Other reagents were prepared freshly for each experiment.

Methods

Cell culture and maintenance

B16-F10 cells (as a standard cell line originated from mouse melanoma cells) and L-929 cells (as a standard cell line originated from mouse fibroblast cells) were obtained from American Type Cell Culture Collection

(ATCC). All cells were cultured in DMEM equilibrated with 5% CO_2 atmosphere at 37 ºC. The medium was supplemented with 10% FCS, 100 U/ml of penicillin and 100 ng/ml of streptomycin. The number of viable cells was estimated by trypan blue exclusion test.

Cytotoxicity assay

Cytotoxic activities of curcumin on B16-F10 and L-929 cells were determined by ATP levels using a luminescence test (Cell-Titer-Glo Luminescent Cell Viability Assay, Promega). Cells were seeded onto 96-well plates at a density of 1.5×10^4 cells per well and incubated overnight at 37 ºC in 5% CO_2. The medium was then replaced with fresh complete medium containing various concentrations of curcumin (2,5 to 100 µM). Control cells were treated with 1 % DMSO. All the cells were incubated in a humidified 5% CO_2 and 95% O_2 at 37 ºC for 24 h. Then, the cells were rinsed with the culture medium and tested for ATP. Each of the samples was supplemented with 100 µL of the prepared reagent (Cell Titer-Glo Luminescent Cell Viability Assay, Promega), mixed for 2 minutes and incubated for 10 minutes at room temperature. The results were read using a luminometer (Varioskan Flash Multimode Reader, Thermo, Waltham, MA). The light emitted in the presence of ATP was quantitated in relative light units (RLU). The intensity of emitted light quants was directly related to ATP content in the tested sample. The cell viability was expressed as the percentage compared with the negative control group designated as 100% (Fig.1). Half maximal growth inhibitory concentration (IC_{50}) values were calculated from the concentration-response curves by non-linear regression analysis. All experiments were repeated three times and standard deviation was within 5%.

Reactive oxygen species generation assay

Generation of ROS was assessed by using a cell-permeable fluorescent signal indicator 2,7-dichlorodihydrofluorescein-diacetate (H2DCF-DA) (13). As described previously, H2DCF-DA is oxidized to a highly green fluorescent 2,7-dichlorofluorescein (DCF) by the generation of ROS. Cancer cell lines were pretreated with various concentrations of curcumin for 24 h. After 24 h. incubation period, the cells were washed with cold phosphate buffere saline (PBS) and incubated with 100 mM H2DCF-DA for another 30 min at 37ºC. DCF fluorescence intensity was measured using the fluorescence plate reader (Varioskan Flash Multimode Reader, Thermo, Waltham, USA) at Ex./Em. = 488/525 nm. The estimations were carried out thrice in triplicate, ensuring each time that the number of cells per treatment group were the same to ensure reproducibility. The values were expressed as % relative fluorescence compared to the control.

Comet assay using B16-F10 and L-929 cells

Genotoxic effects of curcumin on B16-F10 and L-929 cell lines were evaluated by using alkaline single cell gel electrophoresis assay (Comet Assay) according to Singh (14) with slight modification. To determine the genotoxic potential of curcumin, three different cells were seeded into 6-well cell culture plates (approximately $1.5x10^5$ cells per well) with cell culture medium and incubated at 37 ºC in 5% CO_2 for 24 h. for cell establishment. After 24 h., concentrations of curcumin lower than its IC_{50} (2.5 to 50 µM in 1% DMSO) were added to the cells and incubated for another 24 h. at 37 ºC. DMSO (0.1 %) was used as control. After incubation, the cells were washed with phosphate buffered saline (PBS), harvested using trypsin/EDTA and collected for centrifugation at 400 x g for 5 min. at 4 ºC. The supernatant was discharged and the cell density was adjusted to $2x10^5$ cells/ml using cold PBS. Ten µL cell suspension was mixed with 90 µL of 0.6% low melting agarose (LMA) and added to the slides pre-coated with 1% normal melting agarose. After solidification of the agarose, the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM. Tris, 1% Triton X-100 and 10% DMSO, pH 10) for 1 h. at 4°C. Upon removing the slides from lysis solution, they were washed with cold PBS and placed in a horizontal electrophoresis tank side by side. DNA was allowed to unwind for 40 m. in freshly prepared alkaline electrophoresis buffer containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0). Then, electrophoresis was run at 0.72 V/cm (26 V, 300 mA) for 25 min. at 4 °C under minimal illumination to prevent further DNA damage. The slides were washed three times with a neutralization buffer (0.4 M Tris, pH 7.5) for 5 min. at 4 ºC and then treated with ethanol for another 5 min. before staining. Dried microscope slides were stained with ethidium bromide (2 µg/mL in distilled H_2O; 70 µL /slide) covered with a coverslip and analyzed using a fluorescence microscope (Leica DM 1000, Solms, Germany) at a 200 × magnification with epiflourescence equipped with a rhodamine filter (with an excitation wavelength of 546 nm; and a barrier of 580 nm). A computerized image analysis system (Comet Assay IV; Perceptive Instruments) was being used in this study. The DNA percent in tail was used as the primary measure of DNA damage according to Hartmann *et al*. (15).

Acridin orange/ethidium bromide staining test

Morphological changes in cells were studied by acridine orange/ethidium bromide (AO/EB) double staining as described by McGahon et al. (16). In this technique, the cells undergoing apoptosis are distinguished from the viable cells by the morphological changes of apoptotic nuclei. AO and EB are DNA intercalating dyes. AO is taken up by both viable and dead cells and stains double-stranded (ds) and single-stranded (ss) nucleic acids. When AO diffuses into dsDNA, it emits green fluorescence upon excitation at 480-490 nm from viable cells (17). EO is taken up by dead cells and stains DNA orange. Briefly, the cells were cultured in six-well plates (2×10^5 cells/well) and incubated overnight. Then, the cells were treated with curcumin under concentrations of IC_{50} determined by cytotoxicity assay for 24 h. at 37 ºC. DMSO (1%) was used as a negative control. The cells were harvested and washed twice with PBS. Finally, AO/EB solution was added to the cell suspension and the nuclear morphology was evaluated by fluorescence microscopy (Leica DM 1000, Solms, Germany). Multiple photos were taken at randomly-selected areas and a minimum of 100 cells were counted. According to the method, the live cells have normal green nuclei, apoptotic cells have green nuclei with fragmented chromatin,

and dead cells have orange/red nuclei.

Western blotting assay

B16-F10 and L-929 cells (1.5×10^5 cells/well) were seeded onto six well plates for 24 h. and then treated with under concentrations of IC_{50} of curcumin (2.5 to 50 µM). After incubation for 24 h., the cells were harvested and prepared in NP-40 lysis buffer (2 mM Tris–Cl pH 7.5, 150 mM NaCl, 10 % glycerol and 0.2 % NP-40 plus a protease inhibitor cocktail) for 30 min. on ice. Centrifuged at 14.000 rpm for 10 min. at 4 °C (Beckman Coulter, Krefeld, Germany) and the final supernatant was used as the cytosolic fraction. Then, 5 × loading buffer was added to the above obtained supernatant, and the mixture was boiled at 100 °C for 15 min. Protein concentrations were determined using Bradford method (18). Protein were then separated on 8%–12% sodium SDS-PAGE, transferred onto a PVDF membrane, and then incubated with primary antibodies, Caspase 3, Bax; Bcl2 and β-actin (Santa Cruz Biotechnology) and horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology). Protein bands were visualized with Pierce ECL western blotting substrate (Thermo Scientific). Immunoreactive protein bands were detected with an imaging system (Vilber Lourmat Fusion Fx7, French).

Annexin-V and PI staining for apoptosis assessment

Annexin-V-FLUOS staining kit (Roche Life Science) was used for apoptosis detection following the manufacturer's instruction. Briefly, B16-F10 and L-929 cells were seeded (2×10^5 cells/well) onto six-well plates and allowed to adhere overnight to be treated with desired concentrations of curcumin for 24 h. Trypsin-digested cells were centrifuged at 200 x g for 5 min. The cell pellet was re-suspended in 100 µL Annexin-V-FLUOS labeling solution and incubated for 10–15 minutes at 15–20°C and immediately analyzed by flow cytometer (BD, FACS-Canto II) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Determination of Mitochondrial Membrane Potential (MMP)

After different treatments, cancer and normal cells were incubated with 40 nM 3,3 - dihexyloxacarbocyanine iodide (DiOC6(3)) for 15 min at 37^0C then washed with ice-cold PBS, and collected by centrifugation at 500 x g for 10 min. Collected cells were resuspended in 500 ml of PBS containing 40 nM DiOC6(3) (19). In living cells, this cyanine dye can accumulate in the mitochondrial matrix under the influence of the mitochondrial transmembrane potential. Fluorescence intensities of DiOC6(3) were analyzed on a flow cytometer (Becton Dickinson, FACS Canto II) with excitation and emission settings of 484 and 500 nm, respectively. A minimum of ten thousand cells were analyzed by flow cytometer for each data point.

Statistical analysis

The results are presented as the mean ± standard deviation. Data in all experiments were analyzed for statistical significance using analyses of variance (One-Vay ANOVA). IC_{50} values of curcumin over the cell lines were calculated by nonlinear regression analysis. Associations between ROS generation, DNA damage, apoptosis and cell viability parameters were analyzed by Pearson correlation coefficient. The p value <0.05 was considered as statistically significant. All statistical analyses were performed using SPSS package program for Windows (Version 20, Chicago, IL).

Results

Cytotoxicity of curcumin towards cancer and normal cells

Concentration response assays were performed with B16-F10 and L-929 cells for 24 h. to evaluate the effect of curcumin on cell viability. Following incubation, the effects of curcumin was determined by the ATP cell viability assay. Cell viability in B16-F10 and L-929 cell cultures were greater than 95% before all experiments. On the addition of curcumin, cell viability was significantly reduced (p<0.001). 1% DMSO was used as the control. According to the control, the percentage of cell viability progressively increased significantly (p<0.001) in a concentration dependent manner. Higher doses of curcumin resulted in greater cellular death in melanoma cells than fibroblastic cells (Figure 1).

The IC_{50} doses of curcumin for B16-F10 cells and L-929 cells at 24h were calculated concentration-response curve and were found 45 and 60 µM respectively. Further studies were performed assessing apoptosis and genotoxicity of curcumin using under IC_{50} values.

Reactive oxygen species generating activity

We evaluated intracellular ROS generation by using H2DCF-DA as a fluorescence probe. ROS production increased significantly after curcumin exposure on cancer and normal cells (p<0.001), and it could efficiently induce ROS generation in cancer cells than in normal cells in a concentration dependent manner (Figure 2).

Comet assay

To evaluate the ability of curcumin to trigger genotoxic damage in cancer and normal cells were treated with below LD_{50} doses of curcumin, in the range 0–50 µM for 24 h., and the degree of DNA damage was quan-

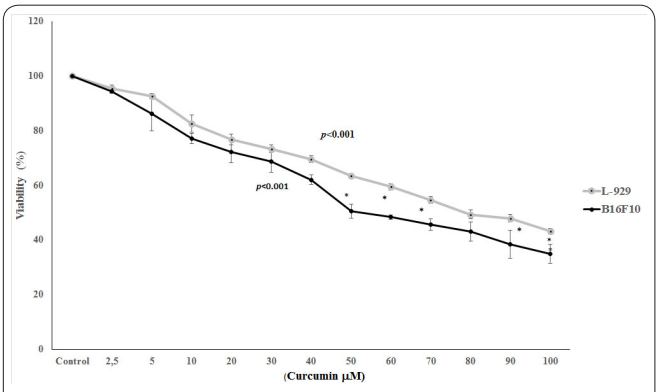

Figure 1. Effect of curcumin on cell viability. B16-F10 and L-929, cells were treated with different concentrations of curcumin or vehicle control DMSO for 24 h. ATP assay was used to determine cell proliferation. The percent cell proliferation was calculated by normalizing with a control panel. Data is representative of three independent trials and are expressed as the mean ± SD. Significant differences between cancer and normal cells were indicated by *, $p < 0.05$.

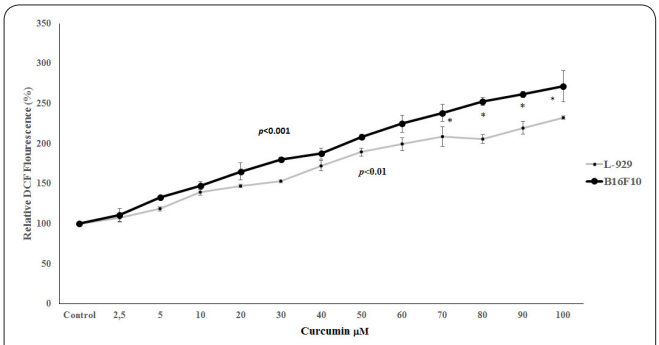

Figure 2. Reactive oxygen species (ROS) generating effect of curcumin on B16-F10 cancer cells and L-929 normal cells. Curcumin induced ROS generation was measured using fluorescent dye H2DCF-DA by fluorimeter. Data is representative of tree independent trials and are expressed as the mean ± SD. Significant differences between cancer and normal cells were indicated by *, $p < 0.05$.

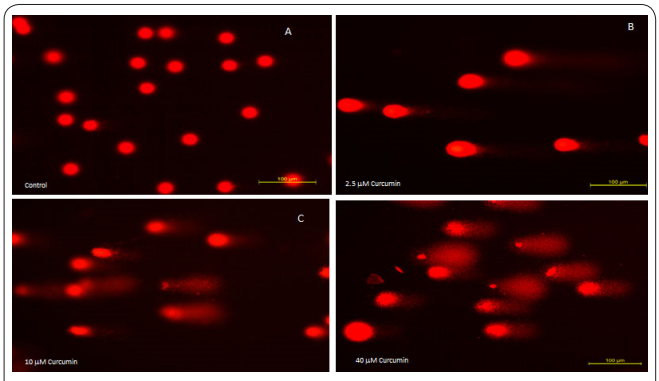

Figure 3. NA damaging effect of different concentrations of curcumin on B16-F10 melanoma cells after 24-h. incubation. Comet formation pattern verify that curcumin induces DNA damage in a dose dependent manner.

tified by the Comet Assay. Nuclei with damaged DNA had a comet feature with a bright head and a tail, whereas nuclei with undamaged DNA appeared to be round without tail. Each figure represents a typical comet tail of the observed cells (at least 100 cells) from two slides in each experiment. Typical micrographs of comet assays are presented in Figure 3.

This Figure showed a significant increase in the percentage of DNA damage and length of comet tail in a concentration-dependent manner in cancer cells exposed to curcumin. After 24 h. curcumin treatment, cancer cells showed different size, fragmentation and comet structures with increased % tail intensity when compared to the normal cells. The results indicated that curcumin induced DNA damage in a concentration-dependent manner ($p<0.001$), and there were significant changes in the tail % of DNA between the normal cells and cancer cells at all doses except the 2.5 µM of curcumin (Figure 4).

Apoptotic effects

Defects in apoptosis are critical in tumorigenesis and resistance to therapy. To clarify whether curcumin induced cell apoptosis in cancer and normal cells, AO/EB double staining, Western blotting and Annexin-V staining methods were performed. To confirm the morphological characteristics of apoptosis, B16-F10 and L-929 cells were exposed to different doses of curcumin for 24 h., stained with AO/EB staining and observed under

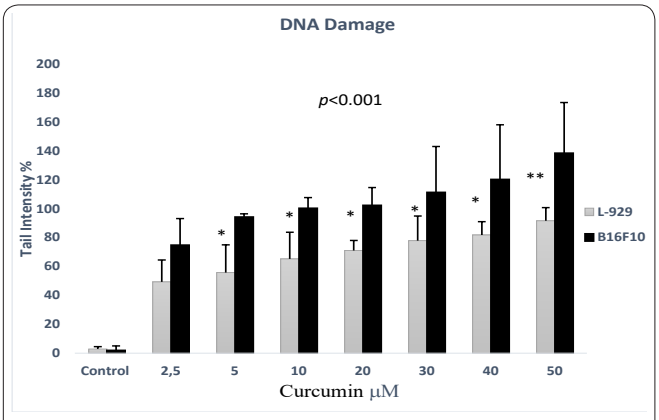

Figure 4. Curcumin induces DNA damage in melanoma and normal cells were treated with different doses of curcumin for 24 h and there were significant changes in the tail % of DNA between the normal and cancer cells. All doses except the 2.5 µM of curcumin. Data presented were Mean ± SD (n = 3). Significant differences indicated by *$p < 0.05$, ** $p < 0.01$.

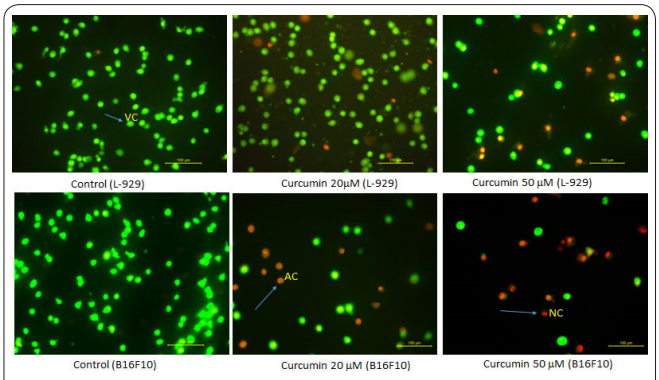

Figure 5. Detection by fluorescent microscopy of AO/EB double-staining with B16-F10 cancer cells (24 h.). Viable cells have uniform green nuclei with organized structure while apoptotic cells would also have bright-red (or yellow-orange) nuclei with condensed or fragmented chromatin.

fluorescence microscopy (Figure 5).

As the concentration of curcumin increased, the number of uniformly green viable cells decreased and yellow-orange apoptotic cells increased after 24 h. in both cancer and normal cells. The higher doses of curcumin resulted in greater apoptosis in cancer than normal cells (Figure 6).

We sought to determine the extent to which apoptosis might govern the anti-proliferative effect of curcumin. Expressional analysis of apoptotic (Caspase-9), pro-apoptotic (Bax) and antiapoptotic (Bcl-2) proteins as well as β-actin in cancer and normal cells have been studied by Western Blotting. The results revealed that 24 h. exposure of curcumin on cancer and normal cells the density of cleaved Caspase-3 and Bax protein increased in a dose dependent manner whereas the density of Bcl-2 decreased in comparison with their levels in control cells. β-actin used as a control (Figure 7).

To quantify curcumin-induced apoptosis, we performed Annexin V/PI double-staining experiments. Flow cytometer was used to detect positive cells after 24 h. of treatment. Annexin-V FLOUS double staining represented early apoptosis and later apoptosis or necrosis (Figure 8).

Higher curcumin concentration resulted in a detection of a higher proportion of Annexin-V staining in the melanoma and normal cell lines. When cancer and

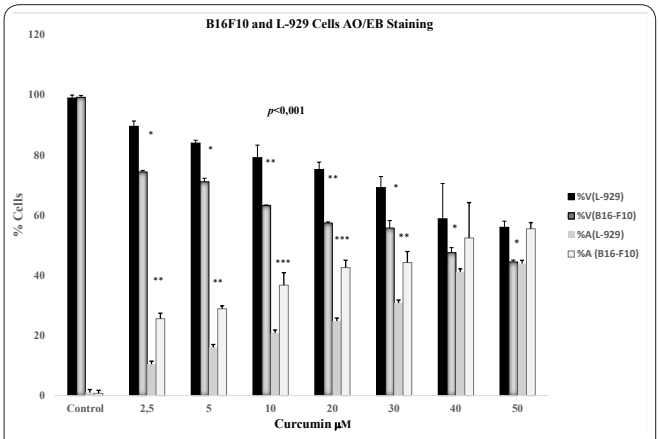

Figure 6. Apoptotic activity of curcumin on B16-F10 and L-929 cell lines. Cells were treated with different concentrations of curcumin (2.5 to 50 µM) for 24 h., AO/PI double staining and measured by fluorescence microscopy. Data presented were Mean ± SD (n = 3). Significant differences according to control indicated by *$p < 0.05$, ** $p < 0.01$.

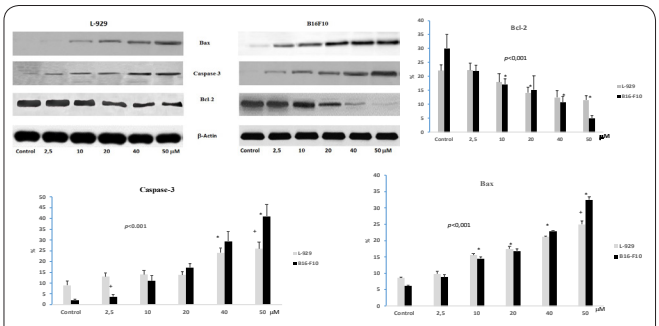

Figure 7. Curcumin treatment regulates intrinsic and extrinsic apoptosis pathways. B16-F10 and L-929 cells were treated with different concentrations of curcumin for 24 h. The vehicle DMSO treatment was used as a negative control. Cell extracts were subjected to western blotting to detect apoptotic protein expression. The β-actin level was used as a loading control.

normal cells were exposed to curcumin (5 to 50 µM) there was a significant increase in both early and late apoptosis in cancer cells when compared with the normal cells in a dose dependent manner. When the curcumin concentration was 50 µM, the proportions of early apoptosis in cancer and normal cell lines were 31.3 and 19.7 %, respectively (Figure 9).

Decreased MMP by curcumin in cancer and normal cells

The mitochondrial apoptotic pathway was investigated to demonstrate mechanisms underlying apoptotic induction in cancer and normal fibroblastic cells. Since loss of DΨ leads to apoptosis via mitochondrial pathways, levels of DΨm were determined. Results from flow cytometric analysis indicated that according to the control curcmin increased loss of DΨm significantly (p<0.05) in a concentrate on dependent manner in cancer cells (Figure10).

However, loss of DΨm was not significantly higher in normal cells. There was also a strong negative relationship between ROS and MMP levels (r=-0,889, p<0.01) in melanoma cancer cells. Results indicate that melatonin increased loss of DΨm and apoptotic induction, supported by linear correlation between cytotoxicity or apoptotic induction and DΨm in cancer cells.

There were statistically significant negative strong

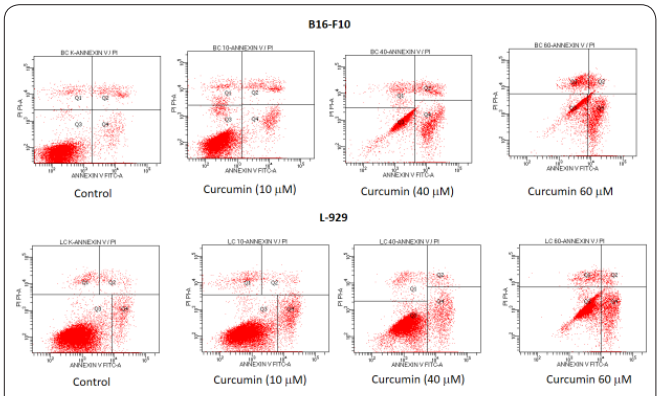

Figure 8. Annexin-V FLOUS double staining to assess apoptosis in melanoma cells. Cells were treated with curcumin for 24 h. After staining with Annexin-V and PI, cells were subjected to flow cytometry analysis.

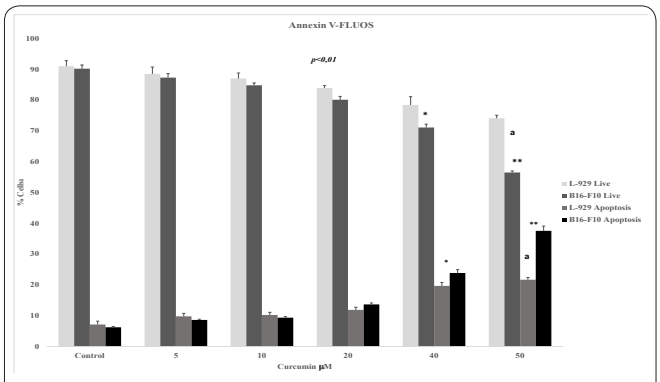

Figure 9. Annexin-V FLUOS double staining to assess apoptotic activity of curcumin on B16-F10 and L-929 cell lines. Cells were treated with different concentrations of curcumin (2.5 to 50 µM) for 24 h., stained with Annexin- V and measured by a fluorimeter. Data presented were Mean ± SD (n = 3). Significant differences according to the control indicated by *$p < 0.05$, ** $p < 0.01$.

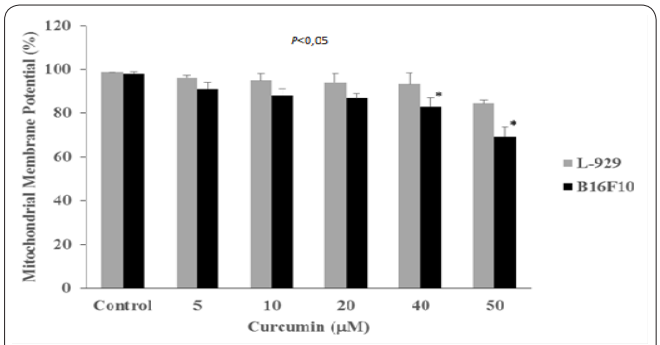

Figure 10. Effects of curcumin on mitochondrial membrane potential (MMP) in B16-F10 and L-929 cell lines. Loss of MMP occurred in response to curcumin treatment of cancer and normal cells. Disruption of MMP was estimated by flow cytometer. The relative signal intensities of DiOC6(3) are shown as a percentage of cancer cells compared with normal cells. Significant differences according to the control indicated by *$p < 0.05$, ** $p < 0.01$.

correlations between melanoma and normal cell viability and ROS (r=-0,994, p<0.001, r=-0,989, p<0.001, respectively) and, MMP and ROS (r=-0,889, p<0.01, r=-0,698, p<0.05, respectively) and, positive correlations between DNA damage and ROS (r=0,920, p<0.001, r=0,879, p<0.05, respectively) and apoptosis and ROS levels (r=0,939, p<0.001, r=0,984, p<0.001, respectively) and DNA damage and apoptosis (r=0,985, p<0.001, r=0,866, p<0.05, respectively in curcumin treated melanoma and normal cells (Figure 11).

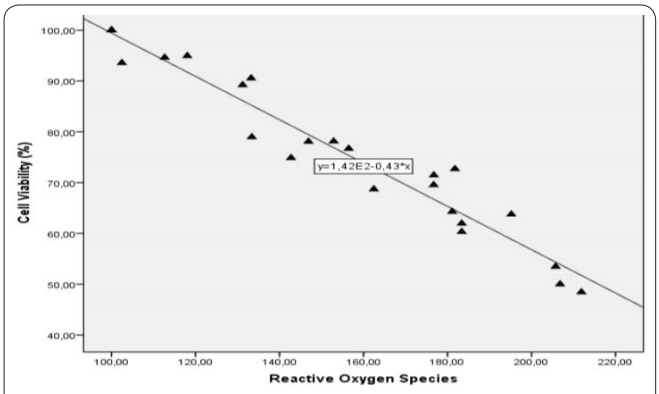

Figure 11. A negative strong correlation between reactive oxygen species and cell viability in curcumin exposed melanoma cells.

Discussion

Various chemotherapy drugs have been used to treat cancer. Unfortunately, all of these drugs affect not only cells, but also normal cells (5, 6). Therefore, the search for new antitumor agents that are more effective but less toxic has become a matter of great interest. Natural products that suppress activation of cell survival pathways while selectively cell death in malignant cell populations are generally regarded to have the greatest therapeutic potential (1). Numerous studies have reported that curcumin shows cytotoxic, genotoxic and apoptotic effects on various cancer cells *(20)*. Here, we also provide evidence that curcumin induce genotoxic, cytotoxic and apoptotic activities in both melanoma cancer and normal fibroblastic cells by their ROS generating activity and these activities were significantly higher in melanoma than normal cells. Additionally, there were strong correlations between cytotoxic, genotoxic, apoptotic and ROS generating activities of curcumin on melanoma and normal cells.

In the present study, we identified effective concentrations for the cell dead activity of curcumin using the most sensitive luminometric ATP cell viability assay and we found that curcumin treatment dramatically decreased the proliferation of B16-F10 cells. The percentage of cell dead activity progressively increased in a dose-dependent manner. IC_{50} dose of curcumin was 45 µM for B16-F10 cells, consistent with the IC_{50} of curcumin previously reported (between 20 and 80 µM) (21). As expected, IC_{50} value of curcumin for L-929 cells was higher than melanoma cells (60 µM).

The potential of curcumin to treat cancer was first evidenced by both *in vitro* cell culture system and *in vivo* mice model in 1985 (21). The other *in vitro* and *in vivo* studies also demonstrated that curcumin has antiproliferative effects in cancer cell lines (22). However, its antiproliferative effect on cancer cells has not been elucidated yet. One of the mechanisms is its ROS generating activity. In fact, curcumin is a phenolic antioxidant and inhibits the generation of ROS including hydroxyl radicals that are responsible for the DNA and membrane damage (23). *In vivo* studies have demonstrated curcumin has also a protective effect against some toxic substances such as perfluorooctane-sulfonate, which cause DNA damage via ROS generation (24, 25). However, it shows cytotoxic effects at high concentrations (26). This dual protective-destructive behavior of flavonoids is not exactly known. There are several mechanisms offered for the cytotoxicity of flavonoids including the inhibition of topoisomerases kinases and pro-oxidant action (27). It is highly possible that the pro-oxidant effect is responsible for the selective antiproliferative activity of these compounds and ROS are key signaling molecules to modulate cell death (28). Beside this, recent studies suggested that cancer cells, compared to normal cells, are under increased oxidative stress due to oncogenic transformation, alterations in metabolic activity, and increased generation of ROS(29). The increased production of ROS in cancer cells was observed in *in vitro* and *in vivo* studies (30, 31). In the present study, it was observed that the cancer cells (B16-F10) generated more ROS than normal cells (L-929). As expected, curcumin also caused significantly high ROS generation in B16-F10 cells in a dose dependent manner, which was consistent with other studies (32, 33). Therefore, cumulative effect of ROS in cancer cells can cause higher cytotoxicity than those of normal cells. We also found in this study that treatment with curcumin for 24 h. in both cancer and normal cells caused dose-dependent increase in DCF-reactive ROS. At the same time, the strong negative correlation was found between cell viability and ROS generating activity in malignant melanoma and normal cells. All these results show that cell dead activity of curcumin might be depending on its pro-oxidant activity. Indeed, under physiological conditions, the maintenance of an appropriate level of intracellular ROS is important in keeping redox balance and cell proliferation (34). Even a modest increase in ROS levels can stimulate cell growth and proliferation (35). However, high levels of ROS can be an important mechanism of cell death (36). Overproduction of ROS lead to serious damage to lipids, proteins, and DNA and regulate the process involved in the initiation of apoptotic signaling (37). Cancer cells with increased oxidative stress are likely to be more vulnerable to damage by further ROS insults induced by exogenous agents (38).

In this study, the alkaline comet assay was employed to assess the genotoxicity of curcumin on cancer and normal cells using under IC_{50} concentrations of this compound. The alkaline comet assay is one of the most important tools for the assessment of DNA damage, in a wide variety of cell lines. (39). We observed that DNA damage increased significantly upon treatment with curcumin in a dose dependent manner and, cancer cells was more sensitive to DNA damage than in normal cells. Recent studies demonstrated that cancer cells prone to curcumin-induced DNA damage, assessed by the comet assay (10, 12, 40, 41).Their results are in accordance with our findings. There are also data that curcumin is not able to induce DNA damage (42). However, it should be noted that DNA damage measurements were performed in a very short period of time (20 min.) in this study. Exposure time may not be enough to cause DNA damage in curcumin exposed cells. We demonstrated that curcumin increases ROS production and correlated with increased DNA damage leading to apoptotic cell death. Consistent with our results, curcumin induces ROS generation, DNA damage and apoptosis in HeLa cervical carcinoma cells (10). Cao (43) also reported that curcumin in high concentrations has induced oxidative stress, with the formation of ROS, induction of lipid

peroxidation, and DNA damages in HepG2 cells; so, the generation of ROS looks to be the main mechanism that results in the clastogenic effect of curcumin. It has been known that the induction of DNA damage in dividing cells results in the activation of cell cycle checkpoints which halt the proliferating cell in its cell cycle progression in order to give time to the DNA damage repair machinery to repair the DNA damage. Eventually, when repair is complete, the cell may proceed into its cell cycle. Alternatively, if the repair process fails, the cell cycle can be blocked permanently, leading to cell senescence or apoptosis.

Induction of apoptosis is the main mechanism for curcumin induced cell dead (44). Apoptosis can be measured by number of methods by taking advantage of the morphological, biochemical and molecular changes undergoing in a cell during this process. We evaluated apoptosis in this study by three different methods: the detection of AO/EB stained condensed nuclei by fluorescent microscopy, the detection of surface expression of phosphatidyl serine by flow cytometer and western blotting. Apoptotic, necrotic and live cells can be distinguished using AO/EB staining. After staining, living cells are distinguished with a normal green nucleus, apoptotic cells show orange-stained nuclei with chromatin condensation or fragmentations, while necrotic cells are clearly observed with uniformly orange-stained cell nuclei with no condensed chromatin. The results of present work revealed that curcumin treated cancer cells clearly exhibit more apoptotic events (chromatin condensation and nuclear fragmentation) with a significant decrease in cell viability than normal cells in a dose dependent manner. These observations are in agreement with the other *in vitro* and *in vivo* studies that showed the treatment of cancer cell with curcumin results in a dose dependent induction of apoptosis (45-47). It has been reported that the anticancer drugs kill the cancer cells by stimulating the apoptotic pathways (48). Phenolic compounds can affect the cellular redox status because of their pro-oxidant properties (49). We also found that there was a strong correlation between ROS and apoptosis in curcumin treated cancer and normal cells. This may result in cytotoxicity through the generation of ROS and apoptosis.

The ongoing development of flow cytometric techniques eventually made it possible to simultaneously identify and quantify apoptotic cells phenotypically defined by the expression of their surface lineage antigens. In this study, apoptotic indexes of the cells were tested by flow cytometer with Annexin-V staining. Apoptotic indexes were increased significantly in cancer cells when compared to the normal cells in a dose dependent manner and, these observations are in agreement with other *in vitro* studies (50, 51).

We also determined apoptotic effect of curcumin on cancer and normal cells by western blotting of Caspase-3, Bax and Bcl-2. Our results demonstrated that Bax expression up-regulated and Bcl-2 down-regulated in melanoma cancer cells after curcumin treatment. There was a dose-dependent increase of Caspase-3 and Bax in curcumin treated melanoma cancer cells and slightly increase normal cells, while the levels of Bcl-2 were lower. These observations show that curcumin-induced apoptosis in melanoma cancer cells triggered by the down regulation of Bcl-2 and the up-regulation of Caspase-3 and Bax. Bcl-2 and Bax both belong to the Bcl-2 family but have the opposite function. Bax can antagonize the biology activity of Bcl-2 and promoting cell apoptosis. The best recognized biochemical hallmark of both early and late stages of apoptosis is the activation of cysteine proteases (caspases) and, detection of active caspase-3 in cells and tissues is an important method for apoptosis induced by a wide variety of apoptotic signals (52). Pharmacological inhibitors of Caspase-3 can prevent the cell death following irradiation significantly indicating that Caspase-3 activation is critical for genotoxic stress-induced apoptosis. Bcl-2, an inhibitor of the mitochondrial apoptosis pathway, exerts its action by blocking pro-apoptotic counterparts, which in turn prevents the release of cytochrome-c and the activation of caspases (53). Curcumin can mediate mitochondrial outer membrane permeability by up-regulating Bax protein expression and down-regulating Bcl-2 expression (54).

Apoptotic induction via the mitochondrial pathway is an important mechanism initiated by chemotherapeutic agents (55). Depolarization of DΨm leads to release of pro-apoptotic proteins, such as cytochrome c, from inner membranes of mitochondria, leading to activation of executioner caspase-3 to induce apoptosis (55). In this study, we demonstrated that curcumin significantly reduced DΨm in melanoma cancer cells in a dose dependent manner and there was negative correlation between ROS and MMP levels. It has been previously reported that curcumin reduced MMP and induce apoptosis activation in human melanoma, MCF-7 and MDA-MB-231 cell lines (56, 57). It has also been demonstrated that 3,3'-OH curcumin anolog causes apoptosis in HepG-2 cells through ROS-mediated mitochondrial pathway (57). These studies support our findings and, ROS generation might be associated with falling apart in the redox buffering system, subsequently induces oxidative stress and collapse of MMP, ultimately leading to cell apoptosis.

In our study it was observed that curcumin can increase the process of planned cell death via inducing the ROS-mediated activation of DNA damage and apoptosis and, these activity is found to be higher in cancer cells than in normal cells especially with higher doses of curcumin. Therefore, targeting ROS generation might be an effective strategy in melanoma cancer treatment.

Disclosure

This study was conducted as an individual project at the Medical Faculty of Medical Biochemistry Research Laboratory of Bezmialem Vakif University.

Competing Interests

The authors declare that they have no competing interests.

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