Protective role of curcumin in oxidative stress of breast cells

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Abstract. Curcumin (diferuloylmethane) is a well known antioxidant that exerts anti-proliferative and apoptotic effects. The effects of curcumin were evaluated in a breast cancer model that was developed with the immortalized breast epithelial cell line, MCF-10F after exposure to low doses of high LET (linear energy transfer) α particles (150 keV/μm) of radiation, and subsequently cultured in the presence of 17β-estradiol (estrogen). This model consisted of human breast epithelial cells in different stages of transformation: i) a control cell line, MCF-10F, ii) an estrogen-treated cell line, named Estrogen, iii) a malignant cell line, named Alpha3 and iv) a malignant and tumorigenic, cell line named Alpha5. Curcumin decreased the formation of hydrogen peroxide in the control MCF-10F, Estrogen and Alpha5 cell lines in comparison to their counterparts. Curcumin had little effect on NFκB (50 kDa) but decreased the protein expression in the Estrogen cell line in comparison to their counterparts. Curcumin enhanced manganese superoxide dismutase (MnSOD) protein expression in the MCF-10F and Alpha3 cell lines. Results indicated that catalase protein expression increased in curcumin treated-Alpha3 and Alpha5 cell lines. Curcumin slightly decreased lipid peroxidation in the MCF-10F cell lines, but significantly ($P<0.05$) decreased it in the Alpha5 cell line treated with curcumin in comparison to their counterparts as demonstrated by the 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) levels. It can be concluded that curcumin acted upon oxidative stress in human breast epithelial cells transformed by the effect of radiation in the presence of estrogen.

Introduction

Oxidative stress is one of the important pathogenic factors of cancer development. Among the antioxidants, curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione; diferuloylmethane) is a well-known major dietary natural yellow pigment derived from the rhizome of the herb *Curcuma longa* (Zingiberaceae). It is also named turmeric and is a perennial herb belonging to the ginger family, native to India and Southeast Asia. It has been shown to be a potent anti-inflammatory, antioxidant, anticarcinogenic and chemopreventive agent (1,2). This phytochemical has also been shown to suppress the proliferation of numerous types of tumor cells (3-6). Curcumin has been previously shown to prevent the formation of many chemically-induced cancers including mammary cancer in mice (7-9).

Under oxidative stress conditions, superoxide anions are produced which are converted to hydrogen peroxide through a specific antioxidant system, and then to water to complete the detoxification pathway (10,11). The hydrogen peroxide formation favors others reactive oxygen species (ROS) formation and an increase of this molecules may play an important role in carcinogenesis (12).

It is known that curcumin interferes with the transcription activation induced by transcription factors, such as nuclear factor-κB (NFκB) resulting in the negative regulation of various cell cycle control genes and oncogenes (13-20). The nuclear NFκB complex containing p65 (Rel A) and p50 consists of closely related proteins that act as a multifunctional nuclear transcription factor (13) which regulates the expression of multiple genes that promote carcinogenesis. Upstream activators of nuclear NFκB include various cellular stressors such as carcinogens, tumor promoters, apoptosis inducers, cytokines and ROS (13-20).

Abnormal levels of manganese superoxide dismutase (MnSOD) in cancer have been documented to play a critical role in the survival of aerobic life (21). MnSOD is a nuclear encoded mitochondrial antioxidant enzyme, catalyzing the conversion of superoxide radicals into molecular oxygen and hydrogen peroxide, which are further reduced into water by peroxide metabolizing enzyme systems, mainly catalase. This enzyme is an endogenous antioxidant that neutralizes hydrogen peroxide by converting it into water and oxygen. Catalase together with MnSOD, are the principal defense against intracellular oxidative stress (22).

An imbalance of nutrients and generation of ROS can alter antioxidant activity of cells and apoptosis (23). It is also

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known to be an important factor in the pathophysiological functions of numerous diseases (24-26). It has been generally accepted that active oxygen produced under stress is a detrimental factor, which causes lipid peroxidation as well as oxidative damage to DNA (27-29). Specific biomarkers can recognize oxidative cell damage *in vitro*. The best biomarker of lipid peroxidation are the isoprostanes, such as the 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), malondialdehyde that show lipid damage (30-33) and are initially generated *in situ* from esterified arachidonic acid in phospholipids that are released in the free form into the circulation. This end product provides useful tool to monitor oxidative stress in human organisms. It has been shown that there is concurrent damage not only to lipids but also DNA during lipid peroxidation (33). The measurement and correlations between the extent of oxidative DNA damage and lipid peroxidation in different tissues are important indicators of the individual oxidative stress levels in different physiological systems (34).

To gain insight into the effects of curcumin on oxidative stress an established *in vitro* experimental breast cancer model (Alpha model) (35) was used. It was developed with the immortalized human breast epithelial cell line, MCF-10F (36) that was exposed to low doses of high LET (linear energy transfer) α particles (150 keV/μm) of radiation, values comparable to α particles emitted by radon progeny, and subsequently cultured in presence or absence of 17β-estradiol (estrogen). The aim of this study was to evaluate whether curcumin had any effect on oxidative stress in human breast epithelial cells transformed by the effect of radiation in the presence of estrogen.

Materials and methods

Cell culture. The spontaneously immortalized breast epithelial cell line, MCF-10F (ATCC, Manassas, VA), was used as a control. This cell line retains all the characteristics of the normal epithelium *in vitro*, including anchorage dependency, non-invasiveness and non-tumorigenicity in nude mice (36). To analyze the oxidative stress we used the Alpha model (35) that consisted of human breast epithelial cells in different stages of transformation: i) normal cells, MCF-10F; ii) the MCF-10F cell line treated with estrogen (10^{-8} M), called the Estrogen cell line; iii) a malignant non-tumorigenic cell line, named Alpha3; iv) a malignant and tumorigenic cell line, named Alpha5. This cell line was anchorage-independent, invasive and tumorigenic in nude mice. The cell lines were cultured with Dulbecco's modified Eagle's media (DMEM)/F-12 (1:1) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2.5 mg/ml amphotericin B (all from Life Technologies, Grand Island, NY), 10 mg/ml insulin (Sigma-Aldrich, St. Louis, MO), 5% equine serum (Biofluids, Rockville, MD), 0.5 mg/ml hydrocortisone (Sigma-Aldrich) and 0.02 mg/ml epidermal growth factor (Collaborative Research, Bedford, MA). The cells were incubated at 37˚C with 5% CO_2 up to 70% of confluence.

Hydrogen peroxide determination. The Amplex™ Red Hydrogen Peroxide assay was used in these studies (Molecular Probes, Eugene, OR), that is a fluorescent microassay of hydrogen peroxide based on the use of peroxide N-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent). The enzyme-catalyzed oxidation of this reagent is a colorless and non-fluorescent, derivative of dihydroresorufin, which produces highly fluorescent resorufin, with an excitation maximum at 563 nm and an emission maximum at 587 nm. The release of hydrogen peroxide was measured by horseradish peroxidase (HRP)-dependent oxidation of Amplex Red reagent in 96-well cell culture plates. Briefly, 100 μl Amplex Red reagent solution (144.4 mM NaCl; 5.7 mM Na_3PO_4; 4.86 mM KCl; 0.54 mM $CaCl_2$; 1.22 mM $MgSO_4$; 75 μM Amplex Red reagent, pH 7.35) containing 0.5 U/ml HRP (Molecular Probes). Cells were plated onto Matrigel-coated 96-well cell culture plates until 75% confluence was achieved. Then, cells were treated with control cell media and incubated at 37˚C for 4 h. Cells were rinsed in phosphate-buffered saline (pH 7.4) and hydrogen peroxide production was measured per protocol. The fluorescence intensity of each well was measured using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) with an excitation wavelength in the range of 530-560 nm. The concentration of hydrogen peroxide was based on a standard curve.

Western blot analysis. Cells were treated with 1 ml lysis buffer (pH 7.2) (50 mM Tris Base; 1 mM EDTA; 100 mM NaCl; 1 mM PMSF; 1 mM orthovanadate; 0.1% Triton X-100) and centrifuged (10,000 rpm x 10 min). The supernatant with cellular proteins was dissolved in SDS-PAGE sample solution (60 mM Tris, pH 6.5; 10% (w/v) glycerol; 5% (w/v) β-mercaptoethanol; 20% (w/v) SDS; 0.025% (w/v) bromophenol blue) and denatured by boiling (2x5 min), and vortex mixing (2x30 sec). The total amount of protein was 20 μg in each lane with standard protein markers (Bio-Rad Laboratories, Hercules, CA). After fractionation by SDS-PAGE gels (7x14 cm), proteins were electroblotted onto a nitrocellulose membrane (Amersham, Biosciences, UK) using a blotting apparatus (Bio-Rad Laboratories). Blots with prestained SDS-PAGE standard were blocked for 2 h in 10% defatted dry milk-TBS-0.1% Tween and then incubated for 2 h at room temperature with the corresponding primary antibodies (1:200), NFκB (sc-53744), MnSOD (sc-133134) and catalase (sc-58332) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then followed by incubation with secondary peroxidase-conjugated antibodies (1:5,000) (all from Santa Cruz Biotechnology) in 5% defatted dry milk-TBS-0.1% Tween. All steps were performed at room temperature, and blots were rinsed between incubation steps with TBS-0.1% Tween. Cell blots were probed with mouse anti-actin antibody as control. Immunoreactive bands were visualized by using the ECL™ Western Blotting Detection Reagent detection method (Amersham, Dübendorf, Switzerland) and exposure of the membrane to X-ray film. Protein determination was performed using the bicinchoninic acid method (Bio-Rad Laboratories) and bovine serum albumin as a standard.

Immunocytochemical detection. Protein expression was evaluated by peroxidase immunochemical staining (37,38). The cells were plated on a 4-well glass chamber slide (Nunc, Inc., Naperville, IL, USA). Cells were allowed to grow at a density of $1x10^4$ cells in 1 ml of medium for 2-3 days until they reached 70% confluence. The cells were incubated with 1% (v/v) hydrogen peroxide in methanol for 30 min, in order

to block endogenous peroxidase, washed twice with a buffer solution, and fixed with buffered paraformaldehyde in PBS, pH 7.4, at room temperature. Subsequently, cell cultures were then covered with normal horse serum for 30 min at room temperature. Cultures were then washed once and incubated with the corresponding antibodies at a 1:500 dilution overnight at 4˚C as NFκB (sc-53744), SOD2 (sc-133134), catalase (sc-58332) (all from Santa Cruz Biotechnology). The protein expression in the different cell lines was determined by using the avidin-biotin-horseradish immunoperoxidase peroxidase complex (Standard ABC kit; Vector, Burlingame, CA, USA). 3,3'-Diaminobenzidine (DAB) (Sigma-Aldrich) was used as a chromogen. For negative controls, duplicate samples were immunostained without exposure to the primary antibody or substituted with pre-immune serum.

Lipid peroxidation determination. The 8-iso-$PGF_{2\alpha}$ enzyme levels were measured by the immunoassay kit (Assay Designs, Ann Arbor, MI). Oxidative damage was analyzed in cells as described (39). Cells of 50-100 mg was homogenized in cold PBS (pH 7.4) containing 0.005% butylated hydroxytoluene. Cell homogenates (10% w/v) were prepared by using a PRO200 homogenizer with a 5x75 mm generator (PRO Scientific, Oxford, CT) in 2-ml tubes. Homogenization was carried out from 0 to 5 (0-30,000 rpm). The 8-iso-$PGF_{2\alpha}$ esters in 100 μl of the total cell homogenate were hydrolyzed by incubation with 25 μl of 10 N NaOH at 45˚C for 2 h. The reaction mixture was neutralized with 25 μl of 12 N HCl and centrifuged for 5 min. The supernatant was transferred into a new tube and 50 μl of the neutralized sample was used for 8-iso-$PGF_{2\alpha}$ assays. Samples were incubated with 8-iso-$PGF_{2\alpha}$ antibody for 18 h at 4˚C in a 96-well format. After incubation color was developed by incubation with 200 μl of p-nitrophenyl phosphate for 45 min at room temperature. The reaction was terminated by the addition of 50 μl of stop solution, and the plate was read at 405 nm. A standard curve was generated by measuring the optical density of 160-100,000 pg/ml of 8-iso-$PGF_{2\alpha}$ standards that were processed simultaneously with unknown samples on the same plate. The protein concentration was determined by using a Pierce protein assay kit. 8-iso-$PGF_{2\alpha}$ levels were analyzed, and the data are expressed as the mean 8-iso-$PGF_{2\alpha}$ pg/mg protein ± SEM (40).

Results

Effect of curcumin on oxidative damage measured by an agent that recognizes cell damage. An observed excess of hydrogen peroxide can be an indication of the imbalance in redox control that causes oxidative cell damage. Curcumin decreased the formation of hydrogen peroxide in control MCF-10F, Estrogen and Alpha5 cell lines in comparison to their non-treated counterparts. However, this effect was not significant in the Alpha3 cell line (Fig. 1). There was greater background of hydrogen peroxide in the Alpha5 cell line than in the other cell lines.

Effect of curcumin on a multifunctional nuclear transcription factor. The NFκB complex acts as a transcription factor. We therefore, wanted to know whether NFκB protein expression was altered by curcumin since this compound is directly related with the specific oxidative stress pathway. Western blot

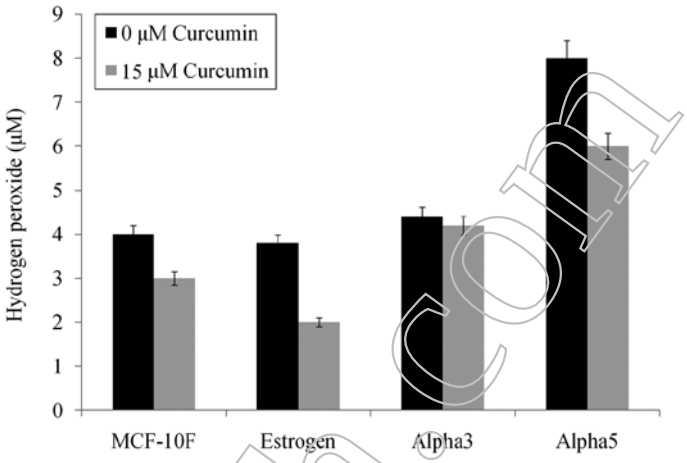

Figure 1. Hydrogen peroxide levels in MCF-10F, Estrogen, Alpha3 and Alpha5 cell lines treated and not treated with curcumin (15 μM) and measured by the Amplex™ Red Hydrogen Peroxide Assay.

analyses indicated that curcumin increased NFκB (50 kDa subunit) protein expression in MCF-10F treated cells, but there was only a slight decrease in the Estrogen and Alpha3 cell lines in comparison to their counterparts without curcumin. However, there was no difference in Alpha5 with or without curcumin (Fig. 2A). The background of NFκB protein expression in the non-treated Alpha5 cell line was higher than the other non-treated cell lines as shown by Western blot analysis. Immunoperoxidase staining corroborated all findings (Fig. 2B).

Effects of curcumin on the antioxidant system. MnSOD is an enzyme that catalyzes the superoxide radical to hydrogen peroxide. Therefore, the effect of curcumin on MnSOD protein expression was analyzed in the 4 cell lines (Fig. 3). The Alpha3 and Alpha5 cell lines had a higher background of MnSOD expression than the other two cell lines as indicated by Western blot analysis. The control cell line was responsive to curcumin effect enhancing it expression in the treated cells. In contrast to these results, the Estrogen cell line did not have any effect in the presence of curcumin. Curcumin increased the MnSOD protein expression in the Alpha3 cell line (Fig. 3A). In the Alpha5 cell line it was not possible to discriminate any changes in the protein expression with curcumin treatment. Peroxidase studies corroborated these results (Fig. 3B).

Effect of curcumin on oxidative stress. Catalase is a peroxisome-specific marker protein that belongs to the catalase family. It is an important regulator of oxidative stress and protector of the cells from hydrogen peroxide. Results indicate that there was no detectable catalase protein expression in the MCF-10F and Estrogen cell lines as shown by Western blot analysis and immunoperoxidase staining (Fig. 4). On the contrary, both techniques indicated that there was a high background of catalase expression in the Alpha5 cell line. Curcumin increased catalase protein expression in the Alpha3 and Alpha5 cell lines in comparison to their counterpart.

Effects of curcumin on oxidative damage. Specific biomarkers can recognize oxidative cell damage *in vitro* such as the isoprostanes. The levels of 8-iso-$PGF_{2\alpha}$ indicated lipid damage by lipid

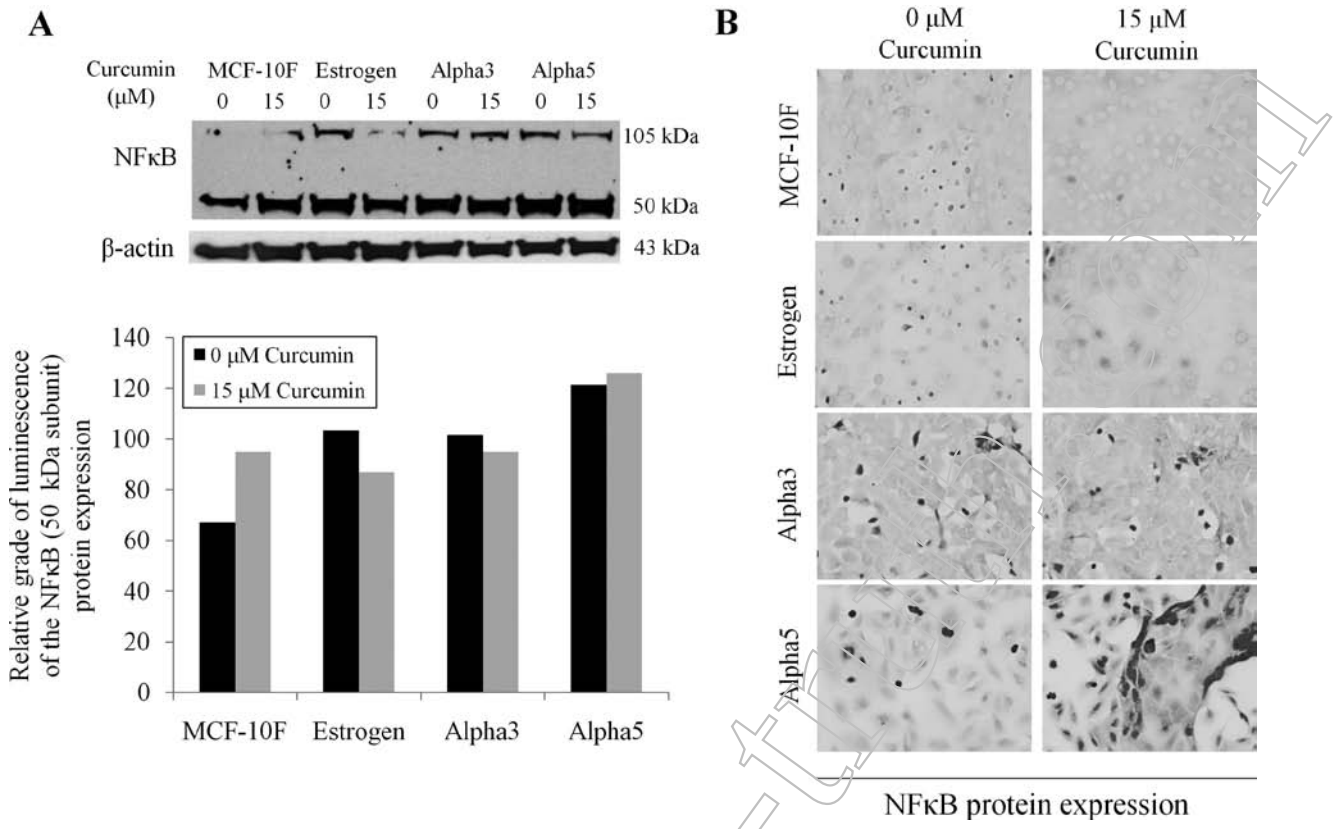

Figure 2. (A) Western blot analysis of NFκB protein expression in MCF-10F, Estrogen, Alpha3 and Alpha5 cell lines treated with 15 μM of curcumin. β-actin was used as a loading control. The graph represents the relative grade of luminescence of the NFκB protein expression level. (B) Representative images of NFκB protein expressions analyzed by the peroxidase technique.

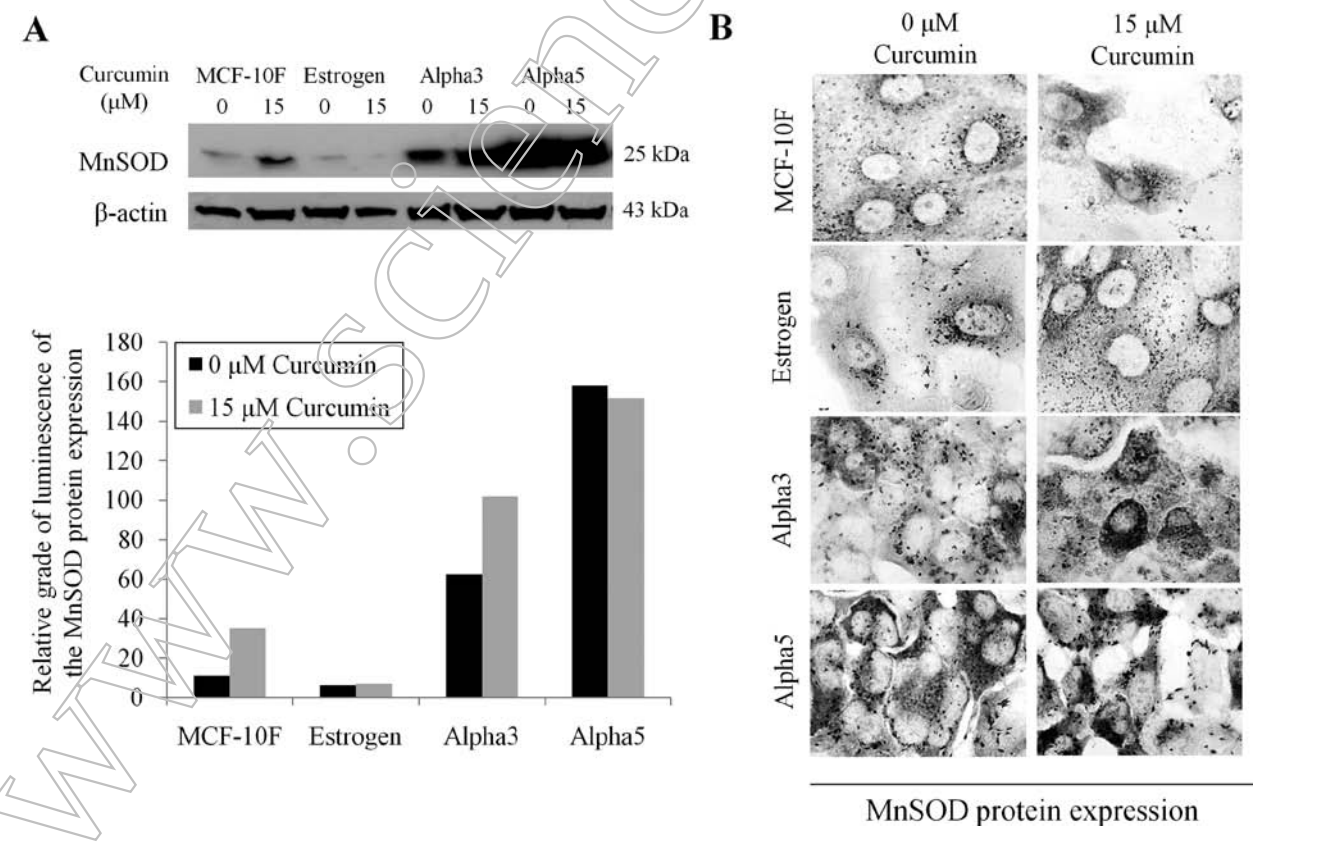

Figure 3. (A) MnSOD protein expression by Western blot analysis in the MCF-10F, Estrogen, Alpha3 and Alpha5 cell lines treated with 15 μM of curcumin. β-actin was used as a loading control. The graph represents the relative grade of luminescence of MnSOD protein expression level. (B) Representative images of MnSOD protein expressions analyzed by the peroxidase technique.

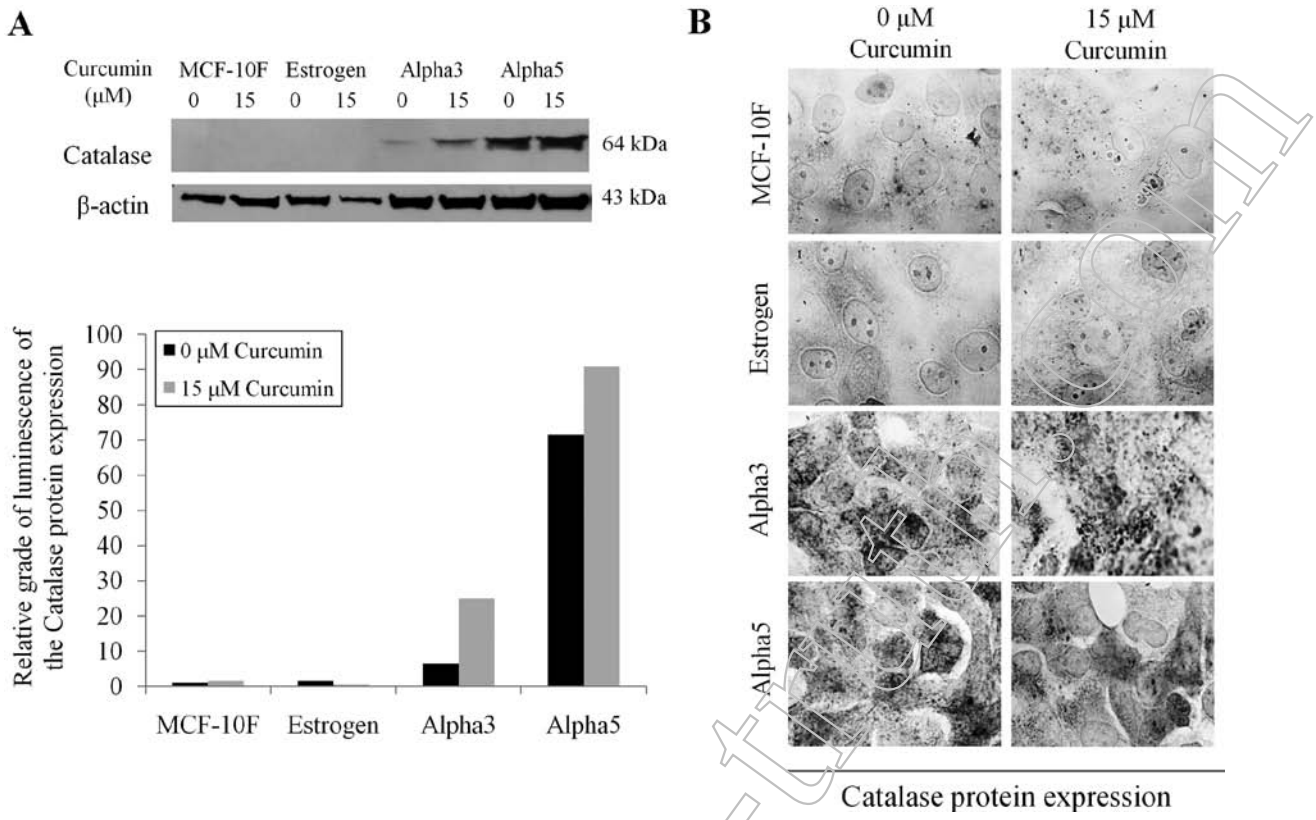

Figure 4. (A) Catalase protein expression by Western blot analysis in the MCF-10F, Estrogen, Alpha3 and Alpha5 cell lines treated with 15 μM of curcumin. β-actin was used as a loading control. The graph represents the relative grade of luminescence of catalase protein expression level. (B) Representative images of catalase protein expression analyzed by the peroxidase technique.

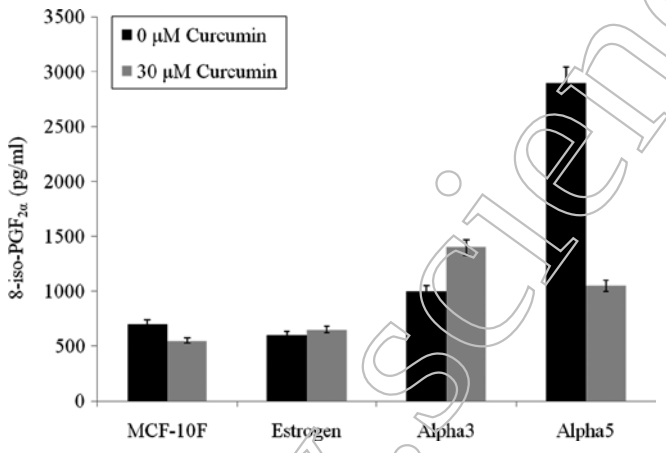

Figure 5. Enzyme levels of 8-iso-$PGF_{2\alpha}$ in MCF-10F, Estrogen, Alpha3 and Alpha5 cell lines treated with curcumin (30 μM) and measured by the immunoassay kit (Assay Designs, Ann Arbor, MI).

peroxidation. It is an end product that provide useful tool to monitor oxidative stress in human organisms. Curcumin resulted a decreased lipid peroxidation in the control MCF-10F cell line. However, curcumin did not have any effect in the Estrogen cell line on such formation. There were higher 8-iso-$PGF_{2\alpha}$ levels in the Alpha3 curcumin-treated cells in comparison to the non-treated cells. However, there was a significant ($P<0.05$) decrease in 8-iso-$PGF_{2\alpha}$ formation in the tumorigenic Alpha5 cell line in comparison to its counterpart. There was also a higher background of lipid formation than in the other cell lines (Fig. 5).

Discussion

Curcumin and its analogues are known to protect against peroxidative damage. In the present study the effect of curcumin as an antioxidant was analyzed by measuring several parameters related to oxidative stress. It is known that hydrogen peroxide can be useful to recognize oxidative cell damage *in vitro*. It was interesting to find that curcumin decreased the formation of such compounds in the control cell line MCF-10F, and in the Estrogen and Alpha5 cell lines. These results also suggest that the Alpha5 cell line had a high level of oxidative damage as demonstrated by a strong background before treatment. Such effects were recognized by the fact that curcumin induced a higher regulation of that compound in the Alpha5 cells than in the other cell lines. Again, there was no effect of curcumin on Alpha3, a malignant and non-tumorigenic cell line and had similar background that the controls. Curcumin is a good scavenger of hydrogen peroxide at high concentrations (over 27 μM), but at low concentrations it activates the Fenton reaction to increase the production of hydrogen peroxide (41).

The present results showed that NFκB, a transcription factor in the cell was altered by curcumin. Curcumin decreased NFκB protein expression in the Estrogen and Alpha3 cell lines studied in comparison to their counterparts. However, there was a higher background in the Alpha5 cell lines than in the other cell lines corroborated by immunoperoxidase staining. Activation of NFκB has been implicated in resistance of cancer cells to radiotherapy and chemotherapy (33). It has also been implicated in growth control and G0/G1 to S-phase transition

(42,43). Curcumin may inhibit NFκB in experimental conditions (44) and may also regulate DNA binding in a gel mobility shift assay in pancreatic cancer cells, inducing apoptosis therefore decreasing cell survival (45,46).

Furthermore, since MnSOD is an enzyme that catalyzes the dismutation of the superoxide radical to hydrogen peroxide we analyzed the effect of curcumin on MnSOD protein expression in the four cell lines. The Alpha3 and Alpha5 cell lines had a higher background of MnSOD expression than the other two cell lines. Curcumin did not reduce MnSOD protein expression in the Alpha5 cell line. It has been reported (47) that MnSOD expression was up-regulated in response to oxidative stress in various types of cells and tissues by toxic stimuli and treatments, such as ionizing radiation and ultraviolet light. Catalase is a regulator of oxidative stress and protector of the cells. There was a higher background of catalase protein expression in the malignant Alpha5 than in the control and Estrogen cell lines. Curcumin had no effect in the MCF-10F and Estrogen cell lines corroborating previous conclusions.

Levels of 8-iso-$PGF_{2\alpha}$ formation were assessed in several cell lines transformed with radiation in the presence of estrogen. Curcumin had a decreased lipid peroxidation in the control MCF-10F and it did not have any effect in the Estrogen cell line on such formation. Peroxidation of lipids is known to be a free radical-mediated reaction leading to cell membrane damage, and the inhibition of peroxidation by curcuminoids is mainly attributed to the scavenging of the reactive free radicals involved in such a process (30-34). DNA damage caused by peroxidation of lipids may be implicated in tumorigenesis. Measurement of isoprostane concentrations is likely to have an important diagnostic potential to assess oxidative stress in several disorders, such as carcinogenesis.

These studies demonstrate that curcumin has a diverse range of molecular targets, confirming the concept that it acts upon numerous biochemical and molecular cascades. In the present study, curcumin acted upon oxidative stress in human breast epithelial cells transformed by the effect of radiation in the presence of estrogen. In summary, curcumin decreased hydrogen peroxide formation in control and malignant cell lines. Results indicate that there was an increase in the scavenger elements in the cell, such as MnSOD and catalase that protect from oxidative stress damage. Among the indicators, 8-iso-$PGF_{2\alpha}$ was a good marker to clarify the effects of an antioxidant, such as curcumin. It can be concluded that curcumin acted upon oxidative stress in human breast epithelial cells transformed by the effect of radiation in the presence of estrogen.

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