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Vitamin E Protects DNA from Oxidative Damage in Human Hepatocellular Carcinoma Cell Lines

ORNELLA FANTAPPIÈ[a], MAURA LODOVICI[b], PAOLA FABRIZIO[a], SERENA MARCHETTI[a], VALENTINA FABBRONI[a], MICHELA SOLAZZO[a], NADIA LASAGNA[a], PIETRO PANTALEO[a] and ROBERTO MAZZANTI[a,*]

[a]*Department of Internal Medicine, U.A. Oncologia, Azienda Ospedaliero-Universitaria Careggi, Viale G.B. Morgagni 85, I-50134 Florence, Italy;*
[b]*Department of Preclinical and Clinical Pharmacology, Azienda Ospedaliero-Universitaria Careggi, University of Florence, I-50134 Florence, Italy*

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Expression of multiple drug resistant (MDR) phenotype and over-expression of P-glycoprotein (P-gp) in the human hepatocellular carcinoma (HCC) cell clone P1(0.5), derived from the PLC/PRF/5 cell line (P5), are associated with strong resistance to oxidative stress and a significant ($p < 0.01$) increase in intracellular vitamin E content as compared with the parental cell line. This study evaluates the role of vitamin E in conferring resistance to drugs and oxidative stress in P1(0.5) cells. Parental drug-sensitive cells, P5, were incubated in α-tocopherol succinate (α-TS, 5 μM for 24 h) enriched medium to increase intracellular vitamin E content to levels comparable to those observed in P1(0.5) cells at basal conditions. Susceptibility to lipid peroxidation and oxidative DNA damage were assessed by measuring the concentration of thiobarbituric-reactive substances (TBARS) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) at basal and after experimental conditions. Cell capacity to form colonies and resistance to doxorubicin were also studied. P5 cells, treated with α-TS, became resistant to ADP-Fe^{3+} and to ionizing radiation-induced lipid peroxidation as P1(0.5) cells. Exposure to ADP-Fe^{3+} or ionizing radiation increased TBARS and the 8-OHdG content in the P5 cells, while vitamin E enrichment abolished these effects. Irradiation doses at 5 cGy increased TBARS and 8-OHdG. They also inhibited cell capacity to form colonies in the untreated P5 cells. Incubation with α-TS fully reverted this effect and significantly ($p < 0.01$) reduced the inhibitory effect of cell proliferation induced by irradiation doses at >500 cGy. Resistance to doxorubicin was not affected by α-TS. These observations demonstrate the role of vitamin E in conferring protection from lipid peroxidation, ionizing radiation and oxidative DNA damage on the human HCC cell line. They also rule out any role of P-gp over-expression as being responsible for these observations in cells with MDR phenotype expression.

Keywords: Liver cancer; Vitamin E; Oxidative stress; DNA damage; Radio-protection; Multiple drug resistance

INTRODUCTION

The major drawback of cancer chemotherapy is the selection of a population of cancer cells with a multiple drug-resistant (MDR) phenotype, which is cross-resistant to a broad range of structurally and functionally unrelated agents.[1,2]

The presence of the MDR phenotype in cancer cells is a frequent, complex phenomenon. The development of the MDR phenotype in cancer cells has so far been attributed to the two major families of efflux pumps, P-glycoprotein (P-gp) and Multi drug Resistance-related Proteins (MRP), which belong to the ATP-binding cassette (ABC) super family. P-gp functions as an ATP-dependent efflux pump that extrudes cytotoxic drugs from the inside of the tumor cell.[3–6] P-gp is also expressed in normal tissues such as intestinal epithelia.[7] The P-gp cellular location suggests that it plays a role in the excretion of potentially toxic xenobiotics. However, the existence of P-gp has not been able to explain all types of acquired MDR. Therefore, the importance of undefined transport mechanisms other than the ABC-transporters cannot be ruled out.[8–11]

*Corresponding author. Tel.: +39-055-4296471. Fax: +39-055-4296468. E-mail: r.mazzanti@dmi.unifi.it

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Anticancer drugs and ionizing radiation share a common mechanism mediated by free radicals by which cell damage occurs. Free radicals mainly attack unsaturated fatty acids that undergo peroxidation through a chain of oxidative reactions. This mechanism is thought to play a major role in the oxygen-derived free radical toxicity.[12] During lipid peroxidation, the highly reactive hydroxyl radical ($OH^{\cdot}$) is produced. This radical induces damage of several cell components including the DNA.[13] The modified base 8-hydroxy-2′-deoxyguanosine (8-OHdG), an oxidative adduct formed from 2′-deoxyguanosine (2dG), is considered a specific marker of oxidative DNA damage. It arises from a hydroxyl radical attack at the C8 of the guanine molecule. Generally, this damage is successfully repaired by DNA reparation mechanisms.[14] However, in those cases where reparability is unsuccessful, the persistence of 8-OHdG in DNA can cause a misreading of the DNA template(s).[15]

Cells that express the MDR phenotype frequently show increased antioxidant defenses, including enzymes (e.g. glutathione peroxidase, glutathione-*S*-transferase, superoxide dismutase and catalase) and non-enzymatic moieties such as vitamin E.[16–20] Ruth *et al.* demonstrated a decrease in the radiation-induced apoptosis ratio, but could find no change in the resistance to radiation in the P-gp over-expressing cell line.[21] Previous studies by our group demonstrated that the expression of P-gp *per se*, in a human hepatocellular carcinoma (HCC) cell line, does not increase resistance to iron-stimulated lipid peroxidation. On the contrary, we demonstrated that transfection of the MDR-1 gene in NIH-3T3 cells increases susceptibility to both iron-stimulated lipid peroxidation and radiation.[18] The increased resistance to oxidative stress observed in HCC cells expressing MDR phenotype is most likely due to an increase in antioxidant defenses such as the cell content of vitamin E rather than to the over-expression of P-gp.[16] Among antioxidants, only vitamin E cell content was significantly higher in P1(0.5) in comparison with P5.[16] Moreover, treatment of cells with buthionine sulfoximine, which inhibits the synthesis of GSH and depletes cells in GSH content, did not produce changes in cell susceptibility to undergo lipid peroxidation.[16]

In an animal model, a dietary supplementation with vitamin E has been shown to result in significant chromosomal damage reduction and inhibition of hepatic tumor formation.[22] Factor *et al.* developed a transgenic mouse model in which the expression of both transgenic TGF-α and *c-myc* promoted an over-production of reactive oxygen species (ROS).[22] The authors observed that dietary vitamin E supplementation was able to decrease ROS generation, to markedly inhibit hepatocyte proliferation and to increase chromosomal and mitochondrial DNA stability in the liver.[22]

Taken together these data prompted us to investigate the role of vitamin E in the protection of the HCC cell line from free-radical induced lipid peroxidation and oxidative DNA damage. We also investigated any possible effects of vitamin E on the MDR phenotype.

MATERIALS AND METHODS

Chemicals

The following materials were used during experimentation: Dulbecco's modified Eagle's medium (DMEM), antibiotic–antimycotic solution (streptomycin, amphotericin B and penicillin), trypsin-ethylenediaminetetraacetic solution (trypsin-EDTA), phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), adenosine 5′-diphosphate (ADP), ferric chloride ($FeCl_3$), thiobarbituric acid (TBA), trichloroacetic acid (TCA), bovine serum albumin, 1,1,3,3-tetraaethoxypropan, vitamin E, α-tocopherol acetate, α-tocopherol succinate (α-TS), pyrogallol, sulforhodamine B, acetic acid and tris[hydroxymethyl]aminomethane (TRIS) base. All of them were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Hexane and methanol (both of liquid chromatography gradient grade) were purchased from Merck (Whitehouse Station, NJ). C18 reverse phase column, 3.9 mm i.d. × 30 cm, 10 μm particle size was purchased from Perkin Elmer (Wellesley, MA).

Fetal calf serum (FCS) was purchased from Gibco BRL Life Technologies (Carlsbad, CA).

Doxorubicin was purchased from Pharmacia and Upjohn (Uppsala, Sweden). RNAse, proteinase K, P1 nuclease and alkaline phosphatase were purchased from Roche Diagnostics (Basel, Switzerland). Micropure-EZ was purchased from Amicon (Bedford, MA).

Cell Lines

Experiments were performed on a human HCC cell line (PLC/PRF/5, P5).[23] Cells we cultured at 37°C in 5% CO_2 in DMEM that had been supplemented with 10% FCS, 100 IU/ml of penicillin, 10 μg/ml streptomycin and 0.25 μg/ml amphotericin B.

A drug-resistant sub-clone, P1(0.5), was developed by prolonged serial exposures of the drug-sensitive parental cell line, P5, to increasing concentrations of doxorubicin. The P1(0.5) cell line was thereafter cultured with DMEM that had been supplemented with 10% FCS, 100 IU/ml of penicillin, 10 μg/ml streptomycin and 0.25 μg/ml amphotericin B, in the presence of doxorubicin 0.5 μg/ml.

P1(0.5) cells express the MDR phenotype and appear to be up to 100-fold more resistant to doxorubicin than the parental drug-sensitive cells, P5.[3]

Experimental Protocol for α-TS Treatment

Cells were cultured for 24 or 72 h in the presence or absence of α-TS, which had been added to the cell culture media as pure compound, at the concentrations of 5, 10 or 50 μM in a 0.5% DMSO solution as the vehicle. Control samples were exposed only to the vehicle.

Extraction and Analysis of Vitamin E

Cells were trypsinized, collected, washed twice in PBS and stored at −4°C in the absence of light for 24 h, until assayed. Vitamin E was extracted from cells using hexane and dried. The residue was solved in methanol and analyzed using the high-performance liquid chromatography (HPLC) method with a diode array detector (Diode Array Detector model 235C, Perkin Elmer, Wellesley, MA). The mobile phase was 95:5 methanol–water, at a flow rate of 2 ml/min.[24]

Evaluation of Lipid Peroxidation

Cell susceptibility to free radical attack was assessed by measuring the concentration of thiobarbituric-reactive substances (TBARS), according to the method described by Ohkawa *et al.*[25] Briefly, cells in an exponential growth phase were trypsinized, collected, washed with serum-free medium, counted and diluted in serum-free medium to obtain 8×10^6 cells/ml. Aliquots containing 6×10^6 cells, were incubated with ADP-Fe^{3+} (ADP 2.5 mM plus 100 μM $FeCl_3$) solution.[26] A vehicle (deionized water) was used as the control. At 0 and 60 min of the experiments, cells were trypsinized and collected; 5% TCA was added to the cell suspension. The TBA reactivity of the cell suspensions was thereafter evaluated.[25]

Determination of Radioresistance

Cells in exponential growth were trypsinized, collected, counted and transferred into 15 ml plastic tubes which contained 2×10^4 cells. Tubes were irradiated with photons of 5 MeV nominal energy by the beam of a linear accelerator (Orion 5, General Electric Medical System, Milwaukee, WI). Each tube was then inserted into a multilayer solid polymethyl-methacrylate phantom ($25 \times 25 \times 25\,cm^3$). The dose-to-water calibration values were calculated according to the method recommended by the International Atomic Energy Agency Protocol (IAEA, 1987). Absorbed dose determination, in photon and electron beams, was calculated (Tech. Rep. Ser. N. 277, Wien, Austria). A dose rate of 2.00 Gy/min at a distance of 100 cm was used. Ionization measurements were performed using an electrometer (Electrometer Model 192X, Capintec Inc., Ramsey, NY) and a C-552 wall ionization chamber (Farmer Type, Model PR06C, Capintec Inc.). Before irradiation, both electrometer and chamber were calibrated using a primary standard (Laboratorio di Metrologia delle Radiazioni, ENEA-Casaccia, Italy). Irradiation was performed using a $20 \times 20\,cm^2$ field. An increasing series of radiation doses (5, 15, 50, 75, 100, 200, 300, 500, 750 or 1000 cGy) was used. Immediately after irradiation, cells were plated in 60 mm Petri dishes and cultured in DMEM that had been supplemented with 10% FCS. The cell response to irradiation was evaluated in clonogenic assays. Culture dishes were maintained at 37°C, in 5% CO_2 and 100% humidity for 9 days; afterwards, they were scored for the number of colonies present on the dish surface. Both plating density and time point at the ninth day were established during preliminary experiments.

Determination of 8-OHdG

Cells were trypsinized, collected, frozen and stored at −80°C. The samples were thawed at room temperature and cell DNA isolation was performed according to the method previously described by Lodovici *et al.*,[27] with a few modifications. Briefly, the cells were diluted with 1 ml of 10 mM Tris–HCl buffer pH 8.0 containing 10 mM EDTA, 10 mM NaCl, 0.5% w/v sodium dodecyl-sulfate and incubated at 37°C for 60 min in the presence of RNAse (20 μg/ml). The samples were incubated overnight at 37°C in oxygen-free conditions obtained by continuous insufflation of argon in the presence of proteinase K (100 μg/ml). At the end of the incubation period, the mixture was extracted with chloroform/isoamyl alcohol (10:2 v/v) in the presence of 0.2 volumes of 10 M ammonium acetate. The DNA was precipitated from the aqueous phase, as previously described.[27] The DNA was solubilized in 100 μl of a 20 mM acetate buffer solution (pH 5.3) and heated at 90°C for 3 min. The whole amount of extracted DNA was added to 10 μl of a solution containing 10 IU of P1 nuclease and incubated for 60 min at 37°C in oxygen-free conditions with insufflation of argon; exposure to light was avoided. The mixture was digested for 60 min at 37°C with 5 IU of alkaline phosphatase in a 0.4 M phosphate buffer pH 8.8 solution.

The hydrolyzed mixture was then filtered using a Micropure-EZ enzyme remover and 80 μl of the solution was injected into the HPLC column. The eluting solution was 50 mM KH_2PO_4, at pH 5.5,

and contained 8% methanol. The flow rate was of 1 ml/min.

8-OHdG and 2dG were detected using an electrochemical detector (Coulochem II, ESA, Chelmsford, MA) in line with a UV detector, as previously described.[27] The retention time for 2dG was 12 min; it was 17 min for 8-OHdG. The 8-OHdG levels were expressed as the ratio, 8-OHdG/2dG × 10^6 cells.

DNA Ladder Assays

Cells were trypsinized, collected and centrifuged. Genomic DNA was isolated from each cell pellet using the Wizard® Genomic DNA Purification Kit (Promega Corporation, USA) according to the instructions of the manufacturer. The DNA was resuspended in sterilized water and then analyzed by gel electrophoresis on 1.5% agarose gel stained with ethidium bromide (0.5 μg/ml).

Statistical Analysis

Statistical analysis was performed using a multifactorial ANOVA test (Tukey's test) and a multiple range test, using software for statistical analysis (Statgraphics, STSC, Inc., and Statistical Graphics Corporation, Rockville, MD). A p value of less than 0.05 was considered significant.

RESULTS

Cellular Content of Vitamin E in HCC Cell Lines

The results of this study confirm our previous data on intracellular vitamin E content in MDR cells; the vitamin E level was significantly higher in the P1(0.5) cells than in the parental P5 cells (0.426 ± 0.066 μg/10^6 cells vs 0.150 ± 0.015 μg/10^6 cells; $p < 0.01$). Moreover, the P1(0.5) cells were resistant to iron-related free radical attack, as previously demonstrated.[16]

Since increased intracellular vitamin E content in P1(0.5) cells could have been the cause of the resistance of these cells to free radical-induced cell damage, parental P5 cells were treated with α-TS (5, 10 and 50 μM) for 24 h and then incubated for 60 min with ADP-Fe^{3+} or irradiated with increasing doses of radiation.

Incubation of the P5 cells with α-TS determined a significant increase in the intracellular vitamin E content in a dose- but not in a time-dependent manner (Fig. 1A,B). Incubation with progressively

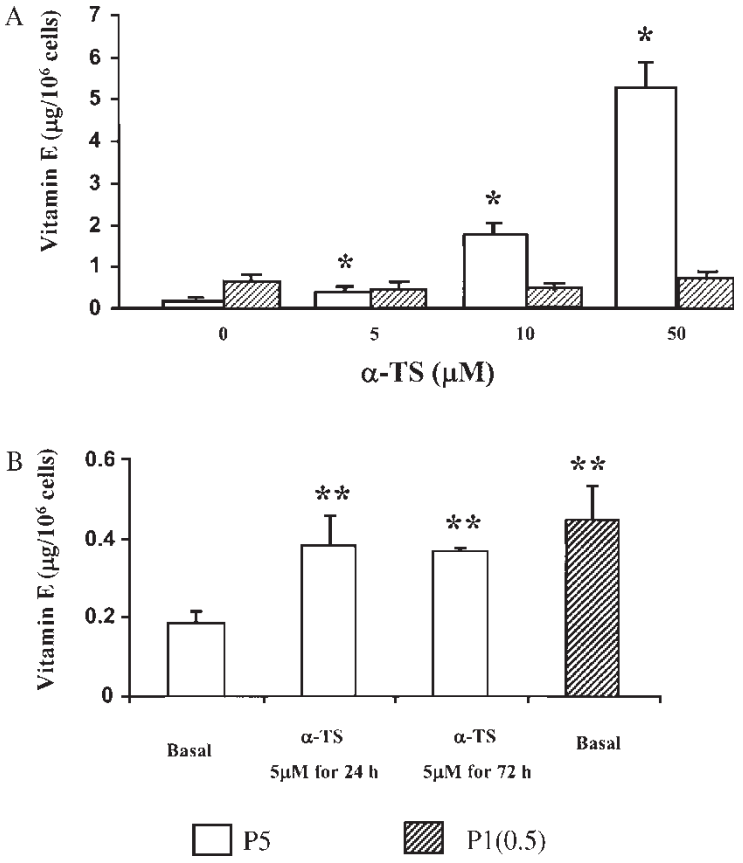

FIGURE 1 Vitamin E content of P5 and P1(0.5) cells in basal conditions and after 24 h incubation with various concentrations of α-TS (5, 10 or 50 μM) (panel A), or after incubation with α-TS 5 μM for 24 or 72 h (panel B). Data result from four separate experiments performed in triplicate. Data are presented as mean ± SEM. *$p < 0.05$ vs α-TS untreated P5 cells; **$p < 0.01$ vs P5 cells in basal conditions.

increasing concentrations of α-TS (5, 10 or 50 μM) for 24 h, determined a progressive increase in intracellular vitamin E content in comparison to untreated cells (0.393 ± 0.047, 1.750 ± 0.093 and 5.300 ± 0.220 μg/10^6 cells, respectively, vs 0.171 ± 0.011 μg/10^6 cells; $p < 0.01$) (Fig. 1A). On the contrary, prolonged exposure to α-TS (5 μM for 24 and 72 h) did not additionally affect the vitamin E content (0.380 ± 0.080 and 0.365 ± 0.012 μg/10^6 cells, respectively) (Fig. 1B). The intracellular vitamin E content of the P5 cells after 24 or 72 h of incubation with 5 μM α-TS was similar to that observed in the P1(0.5) cells at basal conditions (0.426 ± 0.066 μg/10^6 cells) (Fig. 1B). The administration of vitamin E to P1(0.5) is not effective to induce a further increase in vitamin E concentration (Fig. 1A).

The increase in the vitamin E intracellular content in the P5 cell line did not increase drug resistance to doxorubicin (data not shown).

Effects of α-TS on TBARS Formation

Sixty minutes of exposure of the P5 cells to ADP-Fe^{3+} caused a significant ($p < 0.01$) increase in the TBARS levels in the presence (0.747 ± 0.100 nmoles/10^6 cells) and absence (0.556 ± 0.072 nmoles/10^6 cells) of DMSO 0.5%, in comparison with basal conditions (0.046 ± 0.010 and 0.020 ± 0.012 nmoles/10^6 cells, respectively) (Fig. 2). TBARS production was negligible at both basal conditions and after ADP-Fe^{3+} exposure to the P1(0.5) cell line (0.060 ± 0.033 nmoles/10^6 cells and 0.199 ± 0.069 nmoles/10^6 cells, respectively), (Fig. 2). Treatment of the P5 cells with 5 μM of α-TS for 24 h abolished the TBARS increase induced by ADP-Fe^{3+} (from 0.050 ± 0.022 to 0.078 ± 0.120 nmoles/10^6 cells) (Fig. 2).

xposure of the P5 cell line to ionizing radiation caused an increase in TBARS at a dose of 5 and 500 cGy (0.598 ± 0.063 and 0.469 ± 0.056 nmoles/10^6 cells, respectively, vs 0.106 ± 0.015 nmoles/10^6 cells) (Fig. 3). The increase of TBARS induced by 5 and 500 cGy was significant as compared to basal levels. However, values were not significantly different between 5 and 500 cGy. Results were not affected by the presence of DMSO 0.5%. Treatment of the P5 cells with 5 μM of α-TS for 24 h abolished the TBARS increase induced by irradiation (Fig. 3). TBARS level did not change significantly in the P1(0.5) cells in response to irradiation, up to 500 cGy (Fig. 3).

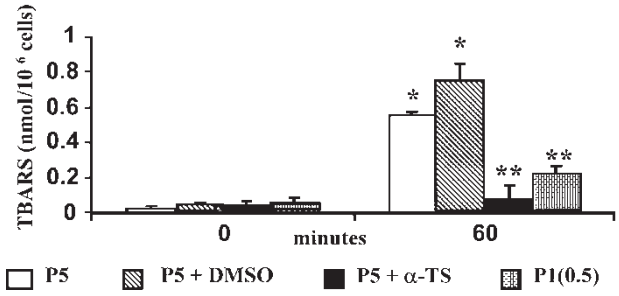

FIGURE 2 TBARS concentration in the incubation mixture at 0 and after 60 min of exposure to ADP-Fe^{3+} at 37°C in P5 cell line, in P5 cells treated with vehicle (DMSO 0.5%), in P5 cells treated with 5 μM of α-TS for 24 h and in P1(0.5) cells. Data results from four separate experiments performed in triplicate. Data are presented as mean ± SEM. *$p < 0.01$; 60 min vs time 0 of the experiment; **$p < 0.01$; P5 treated with 5 μM of α-TS and P1(0.5) vs P5 cells and P5 cells treated with vehicle (DMSO 0.5%).

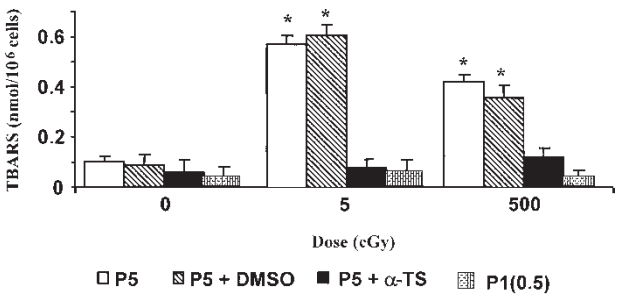

FIGURE 3 TBARS concentration in the culture medium of P5 cells; P5 cells treated with vehicle (DMSO 0.5%) and P5 cells treated with α-TS 5 μM for 24 h after exposition to different doses of radiation. Data results from four separate experiments performed in triplicate. Data are presented as mean ± SEM. *$p < 0.01$ vs basal level (no radiation).

Effects of Radiation and α-TS Exposure on Colony Forming Assay

To assess whether α-TS induced protection against lipid peroxidation was associated with changes in cell capacity to form colonies, experiments were performed using a colony forming assay.

The capacity of the P5 cells to form colonies was significantly ($p < 0.01$) inhibited by radiation at a dose of 5 cGy (57% of the controls) and at a dose of 500 cGy (60% of the controls) or higher. Intermediate doses between 5 and 500 cGy did not affect the colony forming capacity (Fig. 4). On the other hand, the P1(0.5) cells were resistant to radiation and did not show an inhibition in colony forming capacity after exposure to 5 cGy (Fig. 4).

Incubation of the P5 cells for 24 h with 5 μM α-TS abolished the 5 cGy induced inhibition of the clonogenic capacity and partially reversed inhibition when a higher dose of radiation (200, 300, 500, 750 or 1000 cGy) was administered (Fig. 4). Treatment with α-TS had no effect on the radiation-induced damage in the P1(0.5) cells (data not shown).

Effect of α-TS on 8-OHdG Formation

The effect of α-TS on oxidative damage on the DNA after cell exposure to ADP-Fe^{3+} and to radiation was studied. The level of the 8-OHdG content was not significantly ($p = 0.08$) modified in the P5 cells

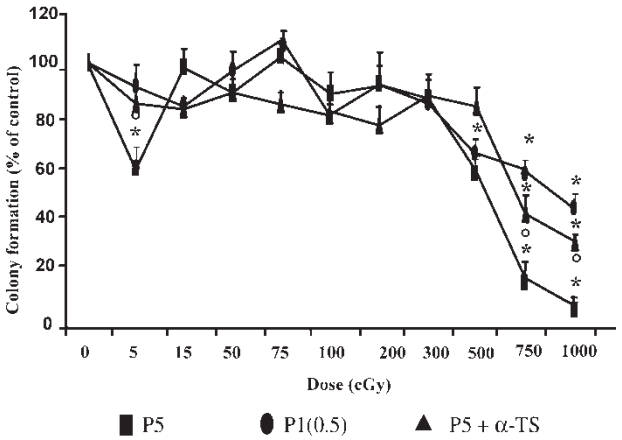

FIGURE 4 Changes in the colony forming capacity of P5 cells, P5 cells treated with α-TS (5 μM for 24 h) and P1(0.5) cells after exposure to ionizing radiation. The cells were exposed to 5, 15, 50, 75, 100, 200, 300, 500, 750 or 1000 cGy. Data results from four separate experiments performed in triplicate and are presented as mean ± SEM. *$p < 0.01$ vs α-TS untreated cells. °$p < 0.01$; P5 vs P1 (0.5) and P5 treated with α-TS.

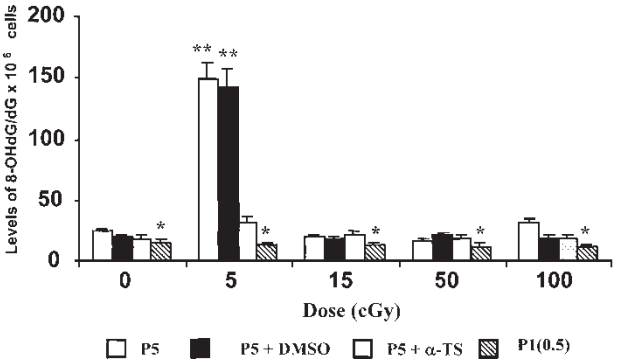

FIGURE 6 Levels of 8-OHdG in P5 cells, in P5 treated with vehicle (DMSO 0.5%) or 5 μM of α-TS for 24 h and in P1(0.5) cells exposed to different doses of radiation (5, 15, 50, 100 cGy). Each value represents the mean of four individual determinations. Data are presented as mean ± SEM. *$p < 0.01$ P1(0.5) vs P5; **$p < 0.001$ P5 and P5 treated with vehicle (DMSO 0.5%) vs P5 in basal conditions (no radiation).

when exposed to 5 μM α-TS for 24 h. Exposure to ADP-Fe^{3+} for 60 min determined a significant (3-fold, $p < 0.01$) increase in the 8-OHdG content in comparison to basal levels (Fig. 5). The effect of ADP-Fe^{3+} was abolished by pre-incubation with 5 μM α-TS (Fig. 5). The level of the 8-OHdG content was significantly ($p < 0.05$) lower in the P1(0.5) than in the P5 cells at basal conditions; exposure of the P1(0.5) cells to ADP-Fe^{3+} did not cause any increase in the 8-OHdG content (Fig. 5).

Exposure to 5 cGy determined a 7-fold increase in the 8-OHdG level in the P5 cells whereas intermediate doses, up to 500 cGy, did not exert any effect (Fig. 6). Incubation of the P5 cells with 5 μM α-TS for 24 h inhibited the 8-OHdG increase induced by 5 cGy radiation (Fig. 6). The level of 8-OHdG was not modified by radiation despite the dose in the P1(0.5) cell line, thus confirming that this clone is resistant to ionizing radiation (Fig. 6). In addition, apoptosis induction was excluded by "DNA ladder analysis" that showed no DNA fragmentation (data not shown).

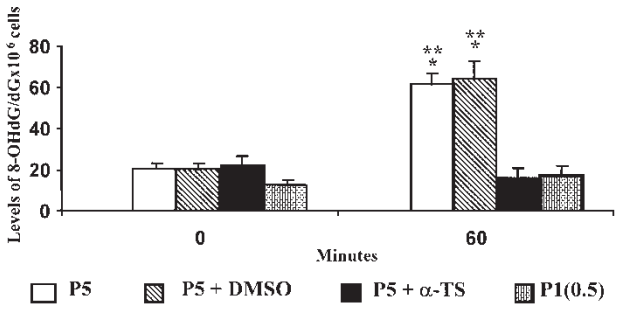

FIGURE 5 Levels of 8-OHdG in P5, P1(0.5) and in P5 cells treated with vehicle (DMSO 0.5%) or α-TS (5 μM for 24 h), exposed to ADP-Fe^{3+} at 37°C for 60 min. Data results from four separate experiments performed in triplicate and are presented as mean ± SEM. *$p < 0.05$; 60 min vs time 0 of the experiments; **$p < 0.05$; P5 and P5 treated with vehicle vs P5 treated with α-TS and P1(0.5).

DISCUSSION

Previous studies by our group have already demonstrated that the P1(0.5) cells, developed from the parental drug-sensitive cell line P5, express the MDR phenotype and are resistant to oxidative stress induced by ROS.[16] In particular, resistance to oxidative stress is associated with a high content of vitamin E.[16] A significant role of P-gp *per se* in promoting resistance of the cell membranes to lipid peroxidation seems to be excluded given that transfection of NIH-3T3 cells with the MDR-1 gene has been shown to increase sensitivity to radiation and iron-stimulated lipid peroxidation.[18] These data suggest that some other mechanisms besides P-gp over-expression could be involved in explaining the differences in resistance to lipid peroxidation between the MDR phenotype expressing cell clones and their parental drug-sensitive cells.

In the present study, we showed that vitamin E plays a protective role against iron- and radiation-induced lipid peroxidation, and against radiation cytotoxicity and oxidative DNA damage in a human HCC cell clone that expresses the MDR phenotype. Our results indicate that the administration of vitamin E to parental drug sensitive P5 cells (which does not affect resistance to doxorubicin or cell growth) reduces both the iron- and radiation-induced TBARS production and the radiation-induced inhibition of cell capacity to form colonies. Furthermore, treatment with vitamin E appears to be effective in protecting the cells against ROS-induced DNA damage.

The oxidative status plays an important role in regulating biological cell behavior.[28] Oxidative

moieties are essential to life but may also cause cell death through several mechanisms.[29,30] Recent studies have shown the key role of ROS in the development of apoptosis and in aging[31,32] and also a role of ROS in physiological conditions such as aerobic metabolism in eukaryotic cells.[33] Thus, the balance between pro- and anti-oxidant systems is crucial for cell life.

Vitamin E is known to play an essential role in balancing the oxidative status of the cell.[34] Previous studies have shown that changes in cellular vitamin E content often correlate with damage derived from oxidative stress in liver cells.[35,36] Although there are different mechanisms involved in the responses of both normal and tumoral cells that have been exposed to ADP-Fe^{3+} or to ionizing radiation, the cells may still undergo lipid peroxidation.[37,38]

Oxidative damage of proteins and DNA has a well established role in carcinogenesis, although very little is known about the role of vitamin E in tumor cell resistance to lipid peroxidation and ionizing radiation.

Vitamin E may be involved in the process of cell tumor resistance to therapeutic procedures such as chemo- and radiotherapy. Tocopherol may have not only an antioxidant function but can also act differently in the same situation according to its chemical structure.[39,40]

Increased resistance of the P1(0.5) cells to lipid peroxidation may have been due to the elevated vitamin E cell content. Therefore, we cultured the parental cells, P5, in a medium containing 5–50 μM of vitamin E to obtain an intracellular vitamin E level similar to that observed in the P1(0.5) cells in basal conditions. Thus, we showed that treatment of the P5 cells with α-TS inhibited ADP-Fe^{3+}-induced TBARS formation. This finding confirmed that vitamin E can inhibit the susceptibility of P5 cells to undergo lipid peroxidation as it does in the P1(0.5) cells.

Vitamin E abolished the inhibitory effect of radiation on the clonogenic capacity of the P5 cells; they behaved in the same manner as the P1(0.5) cells. The P5 cells also became resistant to radiation up to 500 cGy. This finding suggests that the development of the MDR phenotype and radio-resistance in the P1(0.5) cells were most probably associated with but not due to the same mechanism.

We also showed that inhibition of the clonogenic capacity of the P5 cells with 5 cGy of radiation was fully reversed by α-TS exposure and strongly reduced at higher doses (500 cGy) of radiation.

These data also demonstrate that exposure to vitamin E may protect DNA in response to ADP-Fe^{3+} exposure. The protective effect of vitamin E on DNA oxidative damage is intriguing. The observation that α-TS has some protective effect supports the hypothesis that α-TS may decrease iron and radiation induced $OH^{\cdot}$ generation, which is most likely the damaging agent of DNA.[41] However, other mechanisms involved in DNA protection cannot be ruled out.[42] Antioxidants may protect DNA from ROS-induced damage even when added to the medium within 1 h after exposure to ionizing radiation.[39] The molecular mechanism is not completely understood. Some effect on either the by-products of lipid peroxidation or the DNA repairing systems could be involved.[40]

Exposure to radiation caused a significant increase in DNA oxidative damage in only the P5 cell clone; the P1(0.5) cells did not show any increase in 8-OHdG after radiation exposure. Since the P5 α-TS treated cells showed the same radio-resistance as their MDR-positive subclone, once exposed to 5 cGy, it might be hypothesized that vitamin E can protect DNA from oxidation.[39,41,43] Considering radiation doses that were lower than 500 cGy, only a dose of 5 cGy was effective in causing any significant increase in the TBARS and the 8-OHdG levels in the P5 cells. This observation suggests that lipid peroxidation metabolites might be some of the most important mediators of oxidative damage to DNA after exposition to ionizing radiation. Thus, the colony forming capacity of the P5 cells was inhibited by exposure to 5 cGy but was not at higher doses even up to 500 cGy. These findings suggest that different molecular mechanisms of cell and DNA damage might be involved at different doses of ionizing radiation.[44] For example, DNA damage may occur after very low dose radiation exposure that is unable to trigger the DNA repair enzyme genes.[45] Vitamin E may protect DNA at low levels of radiation. At higher doses of radiation, gene coding for enzymes involved in the repair of DNA might be triggered, thus quickly removing any damaged DNA.[46]

The expression of the MDR phenotype in a clone derived from the PLC/PRF/5 cell line was associated with an increase in vitamin E cell content and with resistance to both ADP-Fe^{3+}-induced lipid peroxidation and ionizing radiation-induced cell damage. However, treatment with vitamin E seemed to increase resistance to ADP-Fe^{3+}-induced lipid peroxidation and ionizing radiation-induced DNA damage, even in the parental MDR-negative cell line. Therefore, our findings most probably rule out any role of P-gp over-expression in conferring protection from lipid peroxidation, ionizing radiation and oxidative DNA damage in the HCC cell line. Instead, these findings support the role of vitamin E in protecting DNA from oxidative damage at low doses of radiation at which cell damage is usually present.

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