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Radioprotective effect of DL-α-lipoic acid on mice skin fibroblasts

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Abstract During the course of cancer radiation treatment, normal skin invariably suffers from the cytotoxic effects of γ-radiation and reactive oxygen species (ROS), which are generated from the interaction between radiation and the water molecules in cells. The present study was designed to investigate the radioprotective role of α-lipoic acid (LA), an antioxidant on murine skin fibroblasts exposed to a single dose of 2, 4, 6, or 8Gy γ-radiation. Irradiation of fibroblasts significantly increased ROS, nitric oxide, and lipid peroxidation ($P<0.001$); all of these factors substantially decreased with 100 μM LA treatment. Hydroxyl radical ($OH^{\cdot}$) production from 8Gy irradiated fibroblasts was measured directly by electron spin resonance using spin-trapping techniques. LA was found to inhibit $OH^{\cdot}$ production at 100-μM concentrations. Dose-dependent depletion of antioxidants, such as catalase and glutathione reductase, was observed in irradiated fibroblasts ($P<0.001$), along with increased superoxide dismutase ($P<0.001$). LA treatment restored antioxidant levels. Concentration of the pro-inflammatory cytokine IL-1β was significantly

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reduced in irradiated fibroblasts when treated with LA. MTT and lactate dehydrogenase assays demonstrated that LA treatment reduced cell injury and protected cells against irradiation-induced cytotoxicity. Thus, we conclude that results are encouraging and need further experiments to demonstrate a possible benefit in cancer patients and the reduction of harmful effects of radiation therapy.

Keywords γ-Radiation · α-Lipoic acid · Radioprotection · Antioxidant · Fibroblasts · Reactive oxygen species

Introduction

Ionizing radiation has been found to produce deleterious effects on biological systems. This property of ionizing radiation has been ingeniously exploited for human welfare, especially in the treatment of various neoplastic disorders. During cancer treatment, the therapeutic efficacy of radiation is increased by delivering 20–30 fractions of 2–3Gy in each spread, over a period of 5–6 weeks (Coleman et al. 2003). Though the therapeutic gains are immense, normal skin invariably suffers from the cytotoxic effects of radiation; it produces both acute and late effects on the skin and subcutaneous tissues that have profound effects on healing (Hopewell 1990). The most dramatic consequence of radiation therapy on skin is fibrosis. Radiation-induced skin lesions establishment involves immediate free radicals production and altered antioxidant defensive system (Delanian and Lefaix 2004). Extracellular matrix deposition results from imbalance between connective tissue production and degradation and by the accumulation of specific fibroblastic cells called myofibroblasts, i.e., transiently activated fibroblasts expressing alpha-smooth muscle actin, fibrogenic growth factors (TGF-β1), and showing contractile properties (Haydont and Vozenin-Brotons 2007).

Although skin possesses an extensive and effective network of antioxidant systems, many of the free radicals produced by various agents can escape this surveillance and induce substantial damage to cutaneous constituents, especially when skin defense mechanisms are overwhelmed (Stone et al. 2003). Fibroblasts constitute a major element of bone marrow stroma, submucosal tissues, and subcutaneous tissues, where they are important for repair of tissue injury. Reactive oxygen species (ROS) may cause cellular damage by inducing oxidative stress in the skin (Darr and Fridovich 1994). Some of the general events associated with the early phase of the oxidative stress response of the skin include: depletion of endogenous intra- and intercellular antioxidants (Fuchs et al. 1989); enhancement of intracellular lipid peroxidation concentrations (Meffert et al. 1976); and the induction of specific signal transduction pathways that can modulate inflammatory, immunosuppressive, or apoptotic processes in the skin (Buttke and Sandstrom 1994). There is a general need for a safe and effective antioxidant/skin protectant to modulate the redox (antioxidant/pro-oxidant) balance in vivo. Consequently, exogenous antioxidants that scavenge ROS and restore the normal redox state may be beneficial (Katiyar et al. 2001). Use of antioxidants that protect normal tissues (like skin) might allow for the increase of radiation doses to tumors, thus increasing the probability of tumor control (Jagetia et al. 2003).

α-Lipoic acid (LA) is a naturally occurring thiol antioxidant and an essential metabolic cofactor for the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase reactions (Gueguen et al. 2000). LA is readily taken up by many cells and tissues and is reduced in mitochondria to the potent antioxidant dihydrolipoic acid (DHLA). LA and DHLA act as a redox couple which can regenerate other natural antioxidants such as glutathione, vitamin C, and vitamin E. Recent investigations have revealed potent antioxidant properties of LA, which include: scavenging of ROS, regulation of endogenous antioxidants, chelation of metals, and repair of oxidative damage (Modi and Flora 2007; Moini et al. 2002). In addition to functioning as an antioxidant, LA inhibits the expression of a variety of inflammatory proteins and adhesion molecules (Zhang and Frei 2001). LA has been shown to protect against the oxidative damage of ultraviolet (UV) radiation in rabbit conjunctiva and cornea and to maximally decrease lipid peroxidation in pig skin organ culture compared to other antioxidants (Demir et al. 2005; Fabre et al. 2003).

It is of critical importance to reduce or reverse radiation-induced oxidative damage after radiotherapy before its constitution. The aim of this study was to investigate the radioprotective role of LA against γ-radiation-induced oxidative damage in murine skin fibroblasts.

Materials and methods

Isolation and culturing of skin fibroblasts

Skin fibroblasts were isolated from biopsies taken from the skin of neonatal mice (1- to 2-day old) as described by Van Remmen et al. (2004). The fibroblasts were used for experiments within five passages. Fibroblasts were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (1:1) supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and under 5% CO_2.

Experimental protocol

Fibroblasts were divided into four groups for all experiments as follows: group 1—control (fibroblasts, sham irradiated and fibroblasts, sham irradiated and treated with 50 and 100 μM LA); group 2—fibroblasts, irradiated; group 3—fibroblasts, irradiated and treated with 50 μM LA; and group 4—fibroblasts, irradiated and treated with 100 μM LA.

Irradiation

Fibroblasts seeded in six-well plates (5×10^5cells/well) and incubated overnight were irradiated at room temperature (29±1.5°C) in a ^{60}Co source Alcyon II (GE) γ-ray irradiator at a dose rate of 0.63 Gy/min. Immediately after a single dose of 2, 4, 6, or 8 Gy irradiation, fibroblasts were treated with two different concentrations of LA (50 and 100 μM) in DMEM and were incubated in 5% CO_2 at 37°C for 3days. The culture medium was later removed and saved for the measurement of lactate dehydrogenase (LDH) activity. Cells were then washed twice with phosphate-buffered saline (PBS), scraped gently, and transferred to sterile tubes for further analysis.

Quantification of intracellular ROS

The increase in fluorescence in living cells in the presence of 2′7′-diacetyl dichlorofluorescein (DCF), a non-fluorescent probe, allows for the quantitation of reactive oxygen species such as the superoxide anion, hydrogen peroxide, and the hydroxyl radical (Scott et al. 1988). Fibroblasts in six-well plates were incubated for 15 min with 10^{-6} M 2′7′-diacetyl dichlorofluorescein in PBS, washed three times with PBS, solubilized in water, and sonicated. Fluorescence was determined at 488/525nm, normalized on a protein basis, and expressed as percentages of control.

Nitric oxide

The presence of nitrite, a stable oxidized product of nitric oxide (NO), was determined as described by Kim et al. (2001). Fibroblasts (0.1 ml) were added to each well of a 96-well microtiter plate with the reaction mixture of 0.1 ml of 1% sulfanilamide in 5% H_3PO_4 and 0.1 ml of 0.1% *N*-(1-napthyl) ethylenediamine dihydrochloride in water. The oxide dismutase (OD) was immediately measured at 550nm using an enzyme-linked immunosorbent assay (ELISA) reader (Biotek Instruments, USA). Nitrite concentration in the supernatant was determined using a calibration curve of sodium nitrite. NO was expressed as nitrite level in μM/mg of cellular protein.

Electron paramagnetic resonance spin-trapping (EPR) techniques

Fibroblasts were washed twice with 0.1 M PBS. Also, 75 mM 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was added to the culture and subsequently incubated for 1h before the cells were processed for examination by the Kochany and Bolton (1991) method. Fibroblasts were scraped from the flasks gently and transferred into 2-ml sterile tubes. Cell number was determined by counting with a hemocytometer in a separate control dish treated with trypsin/EDTA. Cell density was adjusted accordingly to 5×10^6cells/ml. EPR spectroscopy was performed on 0.5-ml samples (2.5×10^6cells) in a TM flat cell using a Bruker ESP-300 EPR spectrometer (Karlsruhe, Germany). Instrument settings were as follows: microwave power, 10 mW; klystron frequency, 9.764 GHz; modulation amplitude, 1.056 G; scan rate, 80 G/84s; and time constant, 0.16 s. Spectra resulted from five signal-averaged scans.

Lipid peroxidation

Lipid peroxidation (LPO) products were determined by thiobarbituric reactive substance (TBARS) as described by Ohkawa et al. (1979). Fibroblasts were homogenized by repeated freezing and thawing in 500 μl triple distilled water, trichloroacetic acid was

added to a final concentration of 20%, and the homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was collected and mixed with 500 μl of TBA reaction mixture (0.67% thiobarbituric acid in water with an equal volume of glacial acetic acid *w*/*v*) and heated at 95°C for 10 min, cooled on ice, and the absorbance recorded at 532 nm in a Hitachi 320 spectrophotometer. The concentration of TBARS was determined using an extinction coefficient of $1.56 \times 105\ M^{-1}\ cm^{-1}$. LPO was expressed as μM TBARS/mg of cellular protein.

Antioxidant enzyme assays

Fibroblasts were lysed in 1% Triton X-100, 0.25 M sucrose and 10 mM Tris HCl pH 7.4, and centrifuged at $1{,}000 \times g$ for 5 min at 4°C. Total superoxide dismutase (SOD) activity of the supernatants was detected based on the inhibition of the epinephrine–adrenochrome transition by the enzyme (Misra and Fridovich 1972). Catalase activity was determined by breakdown of hydrogen peroxide on addition of enzyme; the assay is performed by observing the decrease in light absorption of peroxide solution in the UV region (Beers and Sizer 1952). Total reduced glutathione (GSH) was determined by the reaction of glutathione with DTNB to give absorption at 412nm (Moron et al. 1979). The protein concentration was determined by the method of Lowry et al. (1951); 0.5mg/ml bovine serum albumin was used as the protein standard.

Analysis of IL-1β levels

Expression of IL-1β was quantified by ELISA performed on fibroblast monolayers in flat-bottom 96-well plates. After 72h of irradiation and treatment, cells were fixed in 0.1% glutaraldehyde in 0.1M PBS (pH 7.4). Plates were blocked at 37°C for 3h with 1% bovine serum albumin in PBS before incubation with a primary goat antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) at 4°C overnight. The plates were then incubated with a secondary antibody (Bangalore Genei, India) conjugated to alkaline phosphatase. The expression was quantitated by the addition of the substrate p-nitrophenyl phosphate. The absorbance of each well was measured at 405nm with a microplate spectrophotometer (Bio-Tek Instruments, Winouskie, VT, USA).

Cytotoxic studies

MTT assay

The assay was performed after 72 h by reduction of MTT (3-(4,5-dimethyethiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to a formazan pigment by living cells (Mosmann 1983). Fibroblasts were incubated for 4h in DMEM containing 0.5 mg/ml of MTT at 37°C with 5% CO_2, at the end of which isopropyl alcohol containing 0.01N HCl was added and the absorbance measured at 570/630 nm using a Hitachi 320 spectrophotometer. The absorbance of formazan formed in control fibroblasts was considered as 100% and the ratio of mitochondrial function of treated groups and control was calculated as percent change.

Lactate dehydrogenase

A cytotoxicity detection kit (Roche Diagnostics) was used for the LDH assay. Fibroblast-free culture supernatant was collected. Also, 0.1 ml of the substrate mixture containing catalyst (diaphorase/NAD^+) and dye (iodotetrazolium chloride (INT)/sodium lactate) was added to the culture supernatant. Theoretically, any LDH released into the supernatant will reduce the tetrazolium salt INT to formazan by a coupled enzymatic reaction. This leads to color change from pale yellow to red. Absorbance was measured at 500 nm in a Hitachi 320 spectrophotometer. The LDH activity in the medium was expressed as a percentage of total LDH activity present in control fibroblasts.

Statistical analysis

Data is expressed as mean±S.D. for six measurements in each group. Statistical analysis of variance (ANOVA) followed by the Tukey's test was applied to determine the significant differences among the groups. All p values less than 0.05 were considered significant.

Results

ROS

The DCF method measures intracellular generation of hydrogen peroxide, an indirect procedure for estimating ROS. In the present study, the intracellular ROS

Table 1 Effect of α-lipoic acid on the fibroblasts exposed to different doses of gamma radiation represented as mean levels±SD of ROS, NO, LPO, SOD, catalase, and GSH

	Groups	ROS	NO	LPO	SOD	Catalase	GSH	IL-1β
Control	Sham	14.71±0.91	3.53±0.29	2.32±0.15	2.32±0.15	3.32±0.21	5.22±0.49	8.32±0.6
	5 mM LA	14.76±0.77	3.72±0.22	2.12±0.21	2.34±0.21	3.41±0.14	5.37±0.52	8.17±0.7
	10 mM LA	14.29±0.89	3.83±0.25	2.24±0.18	2.32±0.22	3.42±0.29	5.43±0.58	8.05±0.6
Radiation only	2 Gy	28.28±0.99^{a1}	5.17±0.42^{a1}	3.66±0.31^{a1}	2.74±0.20^{a1}	2.66±0.17^{a1}	3.92±0.38	11.16±1.1
	4 Gy	33.72±0.65^{a1}	6.28±0.44^{a1}	4.78±0.35^{a1}	3.18±0.22^{a1}	1.78 ±.012^{a1}	2.74±0.28^{a1}	14.27±1.2^{a1}
	6 Gy	41.23±3.46^{a1}	7.18±0.51^{a1}	6.08±0.51^{a1}	3.63±0.25^{a1}	1.08±0.08^{a1}	2.18±0.22^{a1}	18.17±1.5^{a1,b1}
	8 Gy	44.72±5.67^{a1}	7.98±0.47^{a1}	7.59±0.54^{a1}	4.73±0.25^{a1}	0.59±0.03^{a1}	1.54±0.14^{a1}	19.82±1.4^{a1,b1}
Radiation+50 μM LA treated	2 Gy	21.21±0.79^{a1,b1}	4.16±0.31^{b3}	3.10±0.24^{a2}	2.44±0.18	3.10±0.24	5.05±0.48	9.16±0.8
	4 Gy	25.28±0.96^{a1,b1}	4.73±0.25^{a2,b1}	3.73±0.26^{a1,b2}	2.86±0.19^{a3}	2.73±0.21^{b2}	3.92±0.38^{a3}	10.73±0.92^{b2}
	6 Gy	29.83±0.9^{a1,b1}	5.20±0.31^{a1,b1}	4.20±0.31^{a1,b1}	3.32±0.29^{a2}	1.96±0.14^{a1,b2}	3.42±0.33^{a2,b3}	11.19±1.01^{b1}
	8 Gy	31.33±1.3 ^{a1,b1}	5.82±0.36^{a1,b1}	4.82±0.34^{a1,b1}	3.81±0.31^{a2}	1.62±0.12^{a1,b1}	2.63±0.27^{a1}	12.82±1.1^{a2,b1}
Radiation+ 100 μM LA treated	2 Gy	16.80±0.88^{a1,b3,c1}	3.81±0.28^{b2}	2.51±0.15^{b1,c3}	2.32±0.21	3.51±0.28^{b3}	5.29±0.53	8.61±0.65
	4 Gy	20.37±1.16^{a1,b1,c1}	4.17±0.31^{b1}	3.17±0.19^{a2,b1}	2.58±0.22^{b3}	3.17±0.27^{b1}	4.57±0.44^{b2}	9.17±0.70^{b1}
	6 Gy	23.79±0.96^{a1,b1,c1}	4.52±0.32^{a3,b1}	3.52±0.23^{a2,b1}	2.95±0.21^{a3,b3}	2.52±0.22^{a2,b1,c3}	4.06±0.39^{b2}	9.51±0.81^{b1}
	8 Gy	25.69±1.17^{a1,b1,c1}	5.08±0.44^{a2,b1}	4.08±0.34^{a1,b1}	3.19±0.25^{a3,b2}	1.91±0.15^{a1,b1,c1}	3.46±0.33^{a2,b2}	11.07±0.89^{b1}

[a1] $P<0.001$

[a2] $P<0.01$

[a3] $P<0.05$, compared to sham control group

[b1] $P<0.001$

[b2] $P<0.01$

[b3] $P<0.05$, compared to radiation-only group

[c1] $P<0.001$

[c2] $P<0.01$

[c3] $P<0.05$, compared to radiation+50 μM LA group

concentration was significantly higher in irradiated fibroblasts compared to the control and LA-treated groups ($P<0.001$; Table 1). Radiation dose and ROS production were positively correlated ($r = 0.917$, $P<0.001$; Table 2). LA therapy significantly decreased the intracellular ROS production in irradiated fibroblasts in a dose-dependent manner. At a radiation dose of 8Gy, ROS production was significantly lower with 100 μM LA treatment than with 50 μM LA (fluorescence intensities 25.69±1.17 and 31.33±1.37, respectively).

Nitric oxide

Nitric oxide is a short-lived cytotoxic mediator that has been implicated in the pathogenesis of irradiation (McKinney et al. 1998). NO was measured in the form of nitrite, as per milligram of cellular protein. In the present study, the NO concentration was significantly higher in the irradiated fibroblasts than in the control and LA-treated groups (Table 1). Our results demonstrate a dose-dependent positive correlation between NO concentration and radiation dose. For example, 2Gy irradiated fibroblasts had 3.81± 0.28 μM nitrite/mg of cellular protein whereas 8Gy had 7.98±0.47 μM nitrite/mg of cellular protein. LA treatment significantly reduced NO production in irradiated fibroblasts (Table 1).

EPR

Ionizing radiation causes damage to living cells through a series of molecular reactions that generate free radicals, such as the hydroxyl radical ($OH^{\cdot}$) (Stone et al. 2003). To confirm the formation of $OH^{\cdot}$ in the irradiated fibroblasts, the EPR spectra of the cell suspension-containing DMPO/$OH^{\cdot}$ was measured. EPR spectra obtained from control, irradiated, and LA-treated fibroblasts are presented in Fig. 1. Our results show that the presence of $OH^{\cdot}$ adducts of DMPO, with a typical quartet consisting of a 1:2:2:1 ratio, was observed in 8Gy radiated fibroblasts (Fig. 1b). Moreover, 100 μM LA treatment significantly reduced DMPO/$OH^{\cdot}$ signals in 8Gy radiated fibroblasts (Fig. 1c).

Table 2 Correlation between radiation dose and oxidant, antioxidant, IL-1α concentrations, and cell viability

Assays	*R*	*P*
ROS	0.917	<0.001
NO	0.964	<0.001
LPO	0.984	<0.001
SOD	0.934	<0.001
Catalase	0.991	<0.001
GSH	0.963	<0.001
IL-1β	0.969	<0.001
MTT	−0.985	<0.001
LDH	0.976	<0.001

Lipid peroxidation

Lipid peroxidation is initiated when polyunsaturated fatty acids, principally located in cellular membranes, interact with ROS (Gardner 1989). TBARS is one well-accepted index of LPO. In the present study, LPO levels were significantly higher in irradiated fibroblasts when compared to control or LA-treated fibroblasts (Table 1). A significant increase in LPO was seen in the irradiated fibroblasts which increased with radiation dosage (Table 2) and significant dose-dependent reduction of LPO was observed in 2Gy irradiated LA-treated fibroblasts ($P<0.05$; Table 1).

Antioxidant profiles

We analyzed the major antioxidants, SOD, catalase, and GSH, to determine the potential involvement of various types of ROS scavengers in irradiated fibroblasts. Our results show significant increase in SOD activity in the irradiated fibroblasts when compared to control ($P<0.001$) and 100 μM LA treatment upregulates SOD in irradiated fibroblasts (Table 1). Catalase activity is significantly decreased in the irradiated fibroblasts when compared to control ($P<0.001$). No significant difference in catalase activity is found between control and LA-treated fibroblasts which demonstrate that LA treatment protects catalase from oxidation in irradiated fibroblasts at 2 and 4Gy doses. GSH level was significantly diminished in irradiated fibroblasts when compared to control fibroblasts ($P<0.001$; Table 1). Our results also demonstrate significant depletion of GSH in a dose-dependent manner ($P<0.001$, Table 2). Also, 100 μM LA treatment significantly reduced GSH depletion in irradiated fibroblasts ($P<0.01$, Table 2).

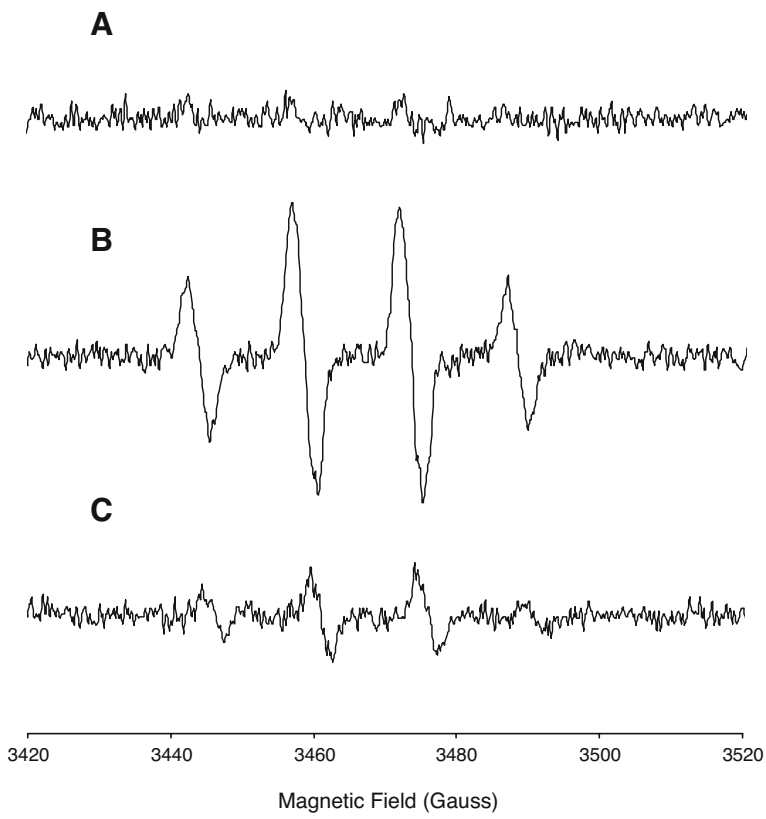

Fig. 1 EPR spectrum from five signal-averaged scans showing $OH^{\cdot}$ trapping adducts of DMPO. **a** Control sham fibroblasts. **b** 8 Gy irradiated fibroblast. **c** 8 Gy irradiated fibroblast treated with 100 μM LA decreased proportionately

IL-1β

Fibroblasts exposed to 2, 4, 6, and 8Gy doses of radiation showed increases in IL-1β levels by 34%, 71%, 118%, and 138% respectively, when compared to control fibroblasts. Treatment with 100 μM LA significantly reduced IL-1β levels in the 2, 4, 6, and 8Gy irradiated fibroblasts by 7%, 14%, 18%, and 37%, respectively. We also found that IL-1β level was significantly reduced with 100 μM LA treatment compared to 50 μM LA (Table 1).

Cytotoxic studies

We also assessed the effect of irradiation on cell viability using MTT and LDH assays. Figure 2a shows the viability obtained by the MTT assay, which detects viable cells by assessing the capability of cells to reduce MTT to a formazan product by mitochondria. Results show a significant negative correlation between radiation dose and cell viability ($r=-0.985$, $P<0.001$; Table 2, Fig. 2a). In contrast, a positive correlation was observed between radiation dose and LDH ($r=0.976$, $P<0.001$; Table 2, Fig. 2b). LDH showed a significant difference between the two doses of LA at higher radiation dose ($P<0.01$). Cell viability was restored significantly in LA-treated fibroblasts when compared to the corresponding groups of irradiated fibroblasts. Both cell viability assays taken together demonstrate that LA treatment reduces cell injury and protects cells against irradiation-induced cytotoxicity.

Discussion

Irradiation physically and chemically damages tissues, leading to cell death or neoplastic transformation. In the presence of oxygen, ionizing radiation leads to formation of ROS such as superoxide anion ($O_2^{\cdot-}$), H_2O_2, $OH^{\cdot}$, and singlet oxygen (Hall et al. 1988). Our data clearly demonstrate that radiation triggers fibroblasts to produce ROS in a dose-dependent manner. Emerging evidence indicates that ROS, at optimum concentration, may function as signaling intermediators of cellular response and stimulate the release of

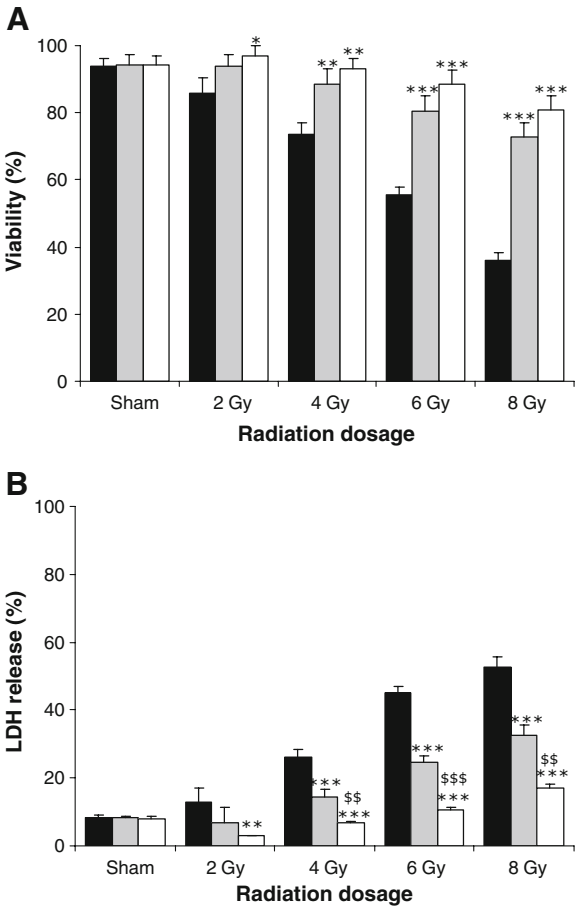

Fig. 2 Survival of murine skin fibroblasts exposed to different doses of gamma radiation and treated with α-lipoic acid. Fibroblasts seeded in six-well plates, incubated overnight, were γ-ray irradiated at a dose rate of 0.63 Gy/min. Immediately after a single dose of 2, 4, 6, or 8 Gy irradiation, fibroblasts were treated with two different concentrations of LA (50 and 100 μM) in DMEM and were incubated in 5% CO_2 at 37°C for 3 days. The culture medium was removed for measurement of LDH activity. Cells were loaded with MTT and incubated for 4 h at 37°C. Values are means±SD (n=5). ANOVA was used to test the significance of differences between groups. *P<0.05, **P<0.01, ***P<0.001, compared to radiation only; $^{\$\$}P$< 0.01, $^{\$}P$<0.001, compared to radiation+50 μM LA group. *Black columns*, radiation only; *gray columns*, radiation plus 50 μM LA treated; *white columns*, radiation plus 100 μM LA treated. **a** Cell viability determined by percentage of MTT conversion. **b** Cell viability determined by the percentage of LDH release

transforming growth factors, epidermal growth factor, and basic fibroblast growth factor (Lo and Cruz 1995). However, at higher concentrations, ROS can induce severe tissue damage and can even lead to neoplastic transformation (Cunnick et al. 1998). In this study, treatment with 100 μM LA significantly reduced ROS compared to 50 μM LA (Table 1). According to previous research, LA seem to be an ideal antioxidant because of several of its properties, including (a) the ability to scavenge reactive oxygen species, including $OH^{\bullet}$, $O_2^{\bullet -}$, alkoxy radicals, and peroxide radicals; (b) the ability to regenerate other antioxidants such as vitamins E and C and GSH from their radical or inactive forms; and (c) metal chelating activity (Fu et al. 2008; Biewenga et al. 1997). The above might be possible reasons for reduction of ROS in the LA-treated irradiated murine fibroblasts.

Lipid components in the membrane are highly susceptible to radiation damage (Gupta et al. 2003). The present study shows that LPO level in fibroblasts is directly proportional to radiation dose (Tables 1 and 2). Moreover, 100 μM LA treatment inhibited $OH^{\bullet}$ generation in 8 Gy irradiated fibroblasts (Fig. 1). This shows that LA is a potent $OH^{\bullet}$ scavenger that can protect from the oxidative effects of gamma radiation. Ionizing radiation has long been recognized to induce inflammation-like responses within irradiated tissues. The present results demonstrate that exposure of fibroblasts to irradiation resulted in an increase in IL-1β concentration. A similar irradiation-induced increase in IL-1β concentration has been observed in a number of cells and tissues (Lynch et al. 2003). Treatment with 100 μM LA significantly abrogated irradiation-induced increases in IL-1β (Table 1).

NO is a biological mediator implicated in biological processes involving nearly every organ system. However, high levels of NO produced during irradiation, in turn, form peroxynitrite by reacting with superoxide radicals; this induces the apoptotic signaling cascade in cells. Yoo et al. (2000) reported that γ-irradiation enhances NO production in murine embryonic liver cells due to formation of H_2O_2. ROS are also reported to be important in the induction of iNOS by sphingomyelinase in RAW264.7 cells (Hatanaka et al. 1998). In this study, we found that ROS and NO in irradiated fibroblasts are increased wherein ROS would have induced NO (Table 1). LA treatment reduced the radiation-induced NO levels in fibroblasts. This effect may be attributed directly to the scavenging effect of NO by the sulphydryl group of LA and indirectly to scavenging of ROS.

SOD is the initial component in the cellular defense against radiation-induced tissue damage; SOD overexpression also promotes the survival of

cells exposed to irradiation (Fridovich 1989). Our results suggest that increased cellular accumulation of SOD may be an important biological response to irradiation and may offer cells an enhanced resistance to the lethal effects of irradiation. It was previously reported that the $O_2^{\cdot}$ generating agents, such as TNF-γ, IL-1β, and ionizing radiation, could induce synthesis of MnSOD but not CuZnSOD (Hirose et al. 1993). We found a concurrent increase in both SOD and IL-1β with increase in higher radiation dose (Table 1). Overexpression of extracellular SOD in transgenic mice appears to confer protection against this radiation-induced lung injury, with a corresponding decrease in oxidative stress (Kang et al. 2003). LA treatment reduced radiation-induced SOD activity partly. A persistent increase in SOD indicates that SOD also protects against radiation-induced injury (Table 1). This might be due to the superoxide scavenging capacity and anti-inflammatory property of LA.

Catalase and glutathione peroxidase (GPx) catalyze the elimination of H_2O_2 from the system. Irradiation decreased the activities of both catalase and GPx (Table 1). Since GSH acts as the substrate for GPx, the decreased availability of GSH for the activity of GPx would result in the accumulation of H_2O_2. LA treatment restored catalase activity in irradiated fibroblasts; which may be attributed to its free radical scavenging property and its ability to increase cellular levels of NADPH and NADH (Soyöz et al. 2004); catalase requires NADPH for its regeneration from inactive form.

GSH, the main non-protein thiol, fulfills a wide variety of important functions. In particular, GSH plays a critical role in protecting cells against a varied repertoire of oxidative stress-related insults, including radiation. Our results show that radiation depletes GSH levels in fibroblasts (Table 1). LA therapy significantly restored GSH and provided protection to irradiated fibroblasts. Packer et al. (1995) suggested that LA could induce production of GSH by facilitating the transport of cystine into the cells, which is the limiting factor in GSH synthesis. LA is immediately reduced to DHLA once it is taken into the cell. The released DHLA induces a chemical reduction of extracellular cystine to cysteine. Cysteine can be taken up rapidly (10 times more) by the cells than cystine and can then be used in the biosynthesis of GSH.

In summary, this study demonstrates that γ-irradiation increases H_2O_2, $OH^{\cdot}$, NO, IL-1β, LPO, and SOD and decreases antioxidant catalase and GSH status in the fibroblast, which might contribute to the irradiation-induced cytotoxicity. Interestingly, 100 μM LA treatment was more effective than 50 μM LA treatment in diminishing irradiation-induced oxidative stress in fibroblasts. In conclusion, we suggest that results are encouraging and need further experiments, especially preclinical data to demonstrate a possible benefit in cancer patients and the reduction of harmful effects of radiation therapy.

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